

JULIANA POLLONI SILVA

**"Aspects of endocrine disruptors remediation using in vitro and
in vivo ecotoxicological assays"**

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JULIANA POLLONI SILVA

**"Aspects of endocrine disruptors remediation using in vitro and
in vivo ecotoxicological assays"**

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Orientadora: Profa. Dra. Renata Fracácio Francisco

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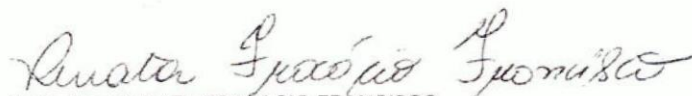
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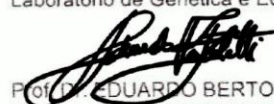
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“O preço de qualquer coisa é a quantidade de vida que você troca por isso.”
Henry David Thoreau

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RESUMO

Produtos químicos capazes de causar desregulação endócrina em vertebrados e invertebrados têm sido detectados a baixas concentrações em ambientes aquáticos do mundo. Desta maneira, tornou-se de interesse mundial a busca por metodologias de remoção mesmos nos ambientes aquáticos. Por isso pesquisa teve como objetivo investigar três materiais (carvão ativado em pó – PAC, zeólitas naturais em pó – ZP e substâncias húmicas aquáticas – AHS) na remediação química e ecotoxicológica de 17 β -estradiol (E2) e 17 α -etinilestradiol (EE2) em água mediante ensaios químicos e biológicos *in vitro* e *in vivo*. Uma concentração ambientalmente relevante de hormônios (30 ng.L⁻¹) foi utilizada durante os ensaios em laboratório. Os resultados obtidos por meio de uma extensa relação de parâmetros morfológicos, reprodutivos e histológicos adotados permitiram concluir o significativo impacto dos HSFs sobre o desenvolvimento e manutenção de peixes expostos. Comprovou-se a superior eficiência do PAC em relação aos demais substratos na remoção de interferentes endócrinos (IEs) em água. Não obstante, suas propriedades não garantiram o mesmo desempenho com relação a amostras ambientais e, tampouco possibilitou a recuperação das injúrias biológicas monitoradas após período de depuração investigado. Um estudo adicional também permitiu a elaboração de um protocolo histoquímico capaz de identificar a produção de vitelogenina (VTG) incitada pela exposição ao esteróide sintético EE2.

Palavras-chave: Hormônios sexuais, Peixe-zebra., Peixe - Efeito da poluição da água, Desreguladores endócrinos, Remediação.

POLLONI-SILVA, J. Ecotoxicological aspects of endocrine disrupters remediation in water by in vitro and in vivo assays. 2017. 174 p. Thesis (PhD's degree in Environmental Sciences) – Campus de Sorocaba, UNESP - Universidade Estadual Paulista, Sorocaba, 2017.

ABSTRACT

Chemicals with potential to cause endocrine disruption in vertebrates and invertebrates have been detected at low concentrations in the world's aquatic environments. Therefore, the search for removal methodologies in aquatic environments became a worldwide interest. The aim of this research was to investigate three materials (powered activated carbon - PAC, powered natural zeolites - ZP and aquatic humic substances - AHS) in chemical and ecotoxicological remediation of 17β -estradiol (E2) and 17α -ethinylestradiol (EE2) in water through chemical and biological tests *in vitro* and *in vivo*. An environmentally relevant concentration of hormones (30 ng.L^{-1}) was used during laboratory tests. The results obtained through an extensive list of morphological, reproductive and histological parameters showed the significant impact of HSFs on the development and maintenance of exposed fish. The superior efficiency of PAC was verified in relation to the other substrates in the endocrine disrupting chemicals (EDCs) removal in water. Nevertheless, their properties did not guarantee the same performance to the environmental samples, neither allow biological injuries monitored to be recovered after the period of depuration investigated. An additional study also allowed the development of a histochemical protocol capable of identifying the production of vitellogenin (VTG) prompted by exposure to the synthetic steroid EE2.

Keywords: Sex hormones, Zebrafish., Fish - Effect of water pollution, Endocrine disrupters, Remediation.

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Apresentação

Nas duas últimas décadas, com o aprimoramento das técnicas analíticas que permitem a detecção e a quantificação de substâncias em níveis traço, tornou-se possível a análise e o estudo de uma diversidade de novos compostos orgânicos em ambientes aquáticos, sobretudo aqueles ainda não integrados à legislação, como os denominados Interferentes Endócrinos (IEs).

IEs podem ser definidos como agentes exógenos capazes de modificar a síntese, transporte, recepção, ação, ou eliminação dos hormônios naturais de um organismo e abrangem uma grande faixa de classes de estruturas distintas (Bila e Dezotti, 2007). Devido a essas características, as funções reprodutivas, o desenvolvimento, o comportamento, a imunidade e a sobrevivência em diferentes níveis tróficos aquáticos passam a ser comprometidos na presença dos mesmos.

É sabido também que tais substâncias, mesmo presentes a baixas concentrações, a longo prazo possuem capacidade para desencadear efeitos crônicos à biota exposta (Bolong et al., 2009). Nesse sentido, Diamond et al. (2011) realizaram um levantamento literário para detectar quais os grupos químicos de IEs deveriam ser priorizados em avaliações de riscos ecológicos, e definiram três critérios: I) a capacidade de detecção dos compostos orgânicos por métodos químicos; II) a disponibilidade de trabalhos com dados sobre esses grupos químicos detectados em diferentes ambientes e III) a existência de dados sobre a ecotoxicidade na fauna aquática em determinadas concentrações. Os autores concluíram que os hormônios naturais e sintéticos, tal como os hormônios sexuais femininos (HSFs), deveriam ser priorizados em estudos ambientais para que se obtenham concentrações seguras, em função do seu maior conhecimento e presença nos ambientes aquáticos.

Os HSFs, também chamados de esteroides estrogênicos, são substâncias de elevado e contínuo aporte ao meio aquático em função da sua produção natural (17 β -estradiol, estriol

e estrona) e artificial, como é o caso do 17α -etinilestradiol presente em pílulas anticoncepcionais usadas no controle populacional, reposição hormonal, dentre outros tratamentos. Esses hormônios são excretados naturalmente e atingem o meio ambiente por meio do esgoto doméstico.

Em ambientes de água doce, a maior quantidade de estudos ecotoxicológicos com HSFs são com os peixes pela ampla quantidade de dados sobre a biologia dos mesmos, o que facilita a interpretação das respostas obtidas em testes de toxicidade (Jobling e Tyler, 2006). As alterações mais comuns descritas incluem: diminuição da fertilidade e da produção de ovos pelas fêmeas; redução no tamanho das gônadas de peixes machos e fêmeas; feminizarão de machos e defeminização das fêmeas; indivíduos intersexos; aumento na concentração de vitelogenina nos machos (lipoglicoproteína comum em fêmeas por ser precursora do vitelo); diminuição da imunidade; aumento na mortalidade de embriões e aumento de ocorrência de deformidades corporais e histopatológicas (Arcand-hoy e Benson, 1998; Kirby et al. 2004; Reis et al, 2006; Reis, 2008). No entanto, poucos são os trabalhos que avaliam a capacidade de recuperação biológica destes organismos, diante da melhoria das condições ambientais.

De acordo com Saha et al. (2010) é de interesse mundial a remoção nos ambientes aquáticos dos compostos que influenciam a dinâmica hormonal nos sistemas biológicos. Para tanto, ainda há a necessidade de estudos que avaliem a eficiência de tratamentos de água e efluentes, já existentes, bem como o desenvolvimento de novas estratégias para a remoção, minimização ou inativação dos mesmos.

Diante de toda a temática exposta, a presente pesquisa buscou responder de maneira geral questões relevantes, como: 1) Materiais com capacidade de remediar a ação de micropoluentes, como as substâncias húmicas aquáticas, os carvões ativados e as zeólitas, são eficientes na redução de HSFs disponíveis em água utilizando-se de uma concentração

ambientalmente relevante?; 2) As concentrações resultantes destes tratamentos são suficientes para reduzir ou recuperar os possíveis efeitos adquiridos pelos organismos após período crônico de exposição a HSFs?; 3) Essa recuperação biológica pode ser observada apenas sobre a geração exposta ou também influencia na primeira geração (F1)?

A fim de facilitar o entendimento da pesquisa e dos resultados obtidos, esta tese foi estruturada na forma de capítulos, como apresentado a seguir:

Capítulo 1 - ***“Female sex hormones in surface water: Why safe exposure limits are not yet defined?”***: O primeiro capítulo, apresentado em forma de um artigo de revisão apresentado em língua inglesa, teve como pretensão responder às seguintes perguntas com base na compilação e análise dos dados comunicados pelos estudos químicos e ecotoxicológicos que investigaram a exposição a esteróides estrogênicos ao longo dos últimos 20 anos: I) Os dados existentes sobre as respostas biológicas observadas com maior frequência após a exposição a HSFs já são suficientes para servir como base para estabelecer os limites de exposição seguros para proteger a vida aquática? e II) Quais são as principais dificuldades enfrentadas ao usar esses dados para definir os limites de exposição seguros?

Capítulo 2 - ***“Remediation efficiency of three treatments on water polluted with endocrine disruptors: Assessment by means of in vitro techniques”***: O segundo capítulo, apresentado em forma de artigo científico em língua inglesa, teve como objetivo investigar três métodos simples de remediação, utilizando-se para isso de tecnologia de ensaios ecotoxicológicos *in vitro* e buscando responder questões, tais como: I) Materiais com capacidade complexante/adsortiva para micropoluentes, como as substâncias húmicas aquáticas, os carvões ativados e as zeólitas, são eficientes na redução/eliminação da resposta endócrina observada em linhas celulares específicas para análises de atividades estrogênicas e androgênicas à níveis traços? e II) Os resultados obtidos seriam igualmente observados

quando aplicados ao tratamento de amostras contaminadas em laboratório e amostras ambientais?

Capítulo 3 - “***Remediation of 17 β -estradiol (E2) and 17 α -ethinyl estradiol (EE2) in water: chemical and ecotoxicological approach with emphasis on the biological reversibility of gonadal tissues of *Danio rerio* (Cypriniformes, Cyprinidae)***”: O terceiro capítulo, novamente apresentado na forma de artigo científico em língua inglesa, buscou investigar três métodos simples de remediação utilizando-se desta vez de análises químicas e ensaios ecotoxicológicos *in vivo*, de modo a responder as seguintes questões: 1) Qual a interferência endócrina dos esteróides femininos 17 β -estradiol (E2) e 17 α -etinilestradiol (EE2), em uma concentração representativa de águas superficiais naturais, sobre os aspectos reprodutivos e a histologia dos tecidos gonadais de peixes expostos a ensaios ecotoxicológicos crônicos?; 2) Qual a eficiência de três materiais de fácil acesso e baixo custo (carvão ativado em pó, zeólitas em pó e substâncias húmicas aquáticas) quando aplicados como tratamento visando a redução de E2 e EE2 disponíveis em água? e 3) É possível alcançar a remediação, considerada neste trabalho como a reversão biológica dos efeitos subletais observados em peixes expostos cronicamente aos estrógenos E2 e EE2, mediante subsequentes ensaios de depuração empregando-se soluções-teste tratadas com carvão ativado em pó, zeólitas em pó ou substâncias húmicas aquáticas?

Capítulo 4 – “***Histoquímica como ferramenta de baixo custo aliada a análise de vitelogenina em tecidos hepáticos de zebrafish***”: O quarto capítulo desta tese, apresentado na forma de um resumo expandido, retrata dados obtidos após análise dos tecidos hepáticos de organismos-teste expostos de maneira crônica aos hormônios sexuais femininos E2 e EE2, utilizando-se de técnicas histológica e histoquímica de coloração. A análise dos dados buscou responder questões, tais como: I) Qual a interferência endócrina de E2 e EE2 em uma concentração ambiental sobre a saúde hepática dos organismos expostos, segundo

análise histológica? e II) Através do uso da técnica histoquímica de coloração, seria possível observar e indicar a presença de uma maior produção de vitelogenina (VTG) no fígado de organismos machos como consequência da crônica exposição à estrógenos?

Capítulo 5 - ***Considerações finais e perspectivas futuras***: O quinto capítulo da tese, pontua de maneira sucinta os resultados obtidos e apresentados no decorrer dos quatro capítulos anteriores e também apresenta as considerações finais e perspectivas futuras segundo a opinião da autora em relação a pesquisa e ao atual estado da arte no que concerne as áreas da Ecotoxicologia e Interferência Endócrina.

Referências

- Bila, D.M., Dezotti, M. 2007. Desreguladores endócrinos no meio ambiente: efeitos e consequências. **Quím. Nova**, 30 (3), 651-666.
- Bolong, N., Ismail, A.F., Salim, M.R., Matsuura, T. 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. **Desalination**, 239 (1-3), 229-246.
- Diamond, J.M., Latimer, H.A., Munkittrick, K.R., Thornton, K.W., Bartell, S.M., Kidd, K.A. 2011. Prioritizing contaminants of emerging concern for ecological screening assessments. **Environ. Toxicol. Chem.**, 30, (11), 2385-2394.
- Jobling, S.; Tyler, C.R. 2006. Introduction: The Relevance of Chemically Induced Endocrine Disruption in Wildlife. **Environmental Health Perspectives**, 14, supplement 1, 7-8.
- Saha, B.; Karounou, E.; Streat, M. 2010. Removal of 17 β -oestradiol and 17 α -ethinylestradiol from water by activated carbons and hypercrosslinked polymeric phases. **Reactive & Functional Polymers**, 70, 531-544.

Female sex hormones in surface water: Why safe exposure limits are not yet defined?

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Abstract

The presence of emerging contaminants in aquatic systems has raised the interest of the scientific community, particularly with regard to endocrine disruptors like FSH (female sex hormone). The known FSHs (estrone, 17 β -estradiol, estriol, and 17 α -ethinylestradiol) have been the most investigated for endocrine disruption, but no safe limits has been legislated for them. The objective of this study was to search whether data in the literature are adequate to establish safe FSHs exposure concentrations for aquatic biota, such as PNEC (predicted no effect concentration). A wide range in detected FSH concentrations could be observed in several countries, but some of concern, warning actual problems in certain surface waters. From experimental studies surveyed on FSHs, the measured effects in ecotoxicological tests showed frequency of 47% related to reproductive functions (such as reduction of reproductive success, feminization of males, changes in the sex ratio), which are essentials to extrapolate results to aquatic population maintenance. Analyzing the aspects covered in the review, it was found that there is sufficient information to establish PNECs for FSHs, even rudimentary. The lack of consensus among researchers has postponed the choice of two PNECs established in recent years. Considering the unquestionable impact of FSHs on aquatic environments, it is urgent the adoption of any of PNECs available as standard in national/international legislation, viewing to protecting aquatic life.

Keywords: Endocrine disruptors, Female sex hormones, Estrogens, Ecotoxicological tests, PNEC, Water quality standards.

1. Introduction

Over the past two decades, improvements in analytical techniques to detect and quantify substances at trace levels have made it possible to analyze and study a variety of new organic compounds in aquatic environments. These compounds include different classes of micropollutants, referred to as "emerging contaminants" for which, in general, little is known regarding their impact on the environment and human health (Deblonde et al., 2011).

The presence of these emerging contaminants in water has encouraged scientific studies, particularly those regarding contaminants that potentially interfere with the endocrine system of organisms, called endocrine disruptors (EDs). Currently, EDs are probably the most in depth studied group of environmental contaminants.

Endocrine disruptors are defined as exogenous agents that can affect the synthesis, transport, reception, action, or elimination of natural hormones of an organism and encompass a wide range of different structural classes (Bila and Dezotti, 2007). Due to such characteristics, the reproductive functions, development, behavior, immunity, and survival of organisms pertaining to different aquatic trophic levels are compromised in the presence of EDs.

These EDs, even at low concentrations, are sufficient to trigger long-term chronic effects on exposed biota (Bolong et al., 2009; Machado et al., 2014). Diamond et al. (2011) performed a literature review to identify which ED chemical groups should be prioritized in ecological risk assessments and defined three criteria: I) the ability to detect organic compounds using chemical methods; II) the availability of studies with data on these chemical groups found in different environments, and III) the availability of ecotoxicity data on aquatic fauna at certain concentrations. The authors concluded that natural and synthetic hormones, such as female sex hormones (FSHs), should be prioritized in environmental

studies to determine safe concentrations based on additional knowledge on these compounds and their presence in aquatic environments.

FSHs, which are properly called estrogenic steroids, are substances that, in large amounts, continuously enter aquatic environments because they are naturally (17β -estradiol, estriol, and estrone) and artificially produced (e.g., 17α -ethinylestradiol present in birth control pills, which is used for population control and hormone replacement and is found in other medications). These hormones are naturally excreted and reach the environment via wastewater treatment plants.

This review intends to answer the following questions based on the compilation and analysis of data reported by chemical and ecotoxicological studies that have investigated exposure to estrogenic steroids over the past 20 years: 1) Are the existing data on the most frequently observed biological responses to FSH exposure sufficient to serve as a basis to establish safe exposure limits to protect aquatic life? 2) What are the primary difficulties faced when using these data to define safe exposure limits?

2. Materials and methods

A literature review was conducted to obtain chemical and ecotoxicological data concerning the primary FSHs detected in aquatic environments. The review was conducted by consulting the PubMed and ScienceDirect databases and considering articles published over the last two decades. The keywords used in the literature search were *endocrine disruptors*, *surface water*, and *ecotoxicological test*. Only articles reporting actual concentrations of estrogenic FSHs in surface waters were considered.

3. Characteristics of FSHs

Natural estrogens 17 β -estradiol (E2) and estrone (E1), its metabolite estriol (E3), and the synthetic hormone 17 α -ethinylestradiol (EE2) constitute the FSH group that is most frequently cited in the literature in relation to aquatic pollution caused by EDs for the following reasons: high potential to cause endocrine disruption, continuous input into and detection in the environment, and high consumption of conjugated estrogens and oral contraceptives (Huang et al., 2010; Shin et al., 2011).

According to the available literature, the daily excretion of estrogenic steroids in humans, such as FSHs, varies according to gender and the hormone cycle of women, as shown in Table 1. Despite having a relatively short half-life when compared with other organic compounds that exhibit endocrine disruption action, natural and synthetic estrogens are introduced daily into aquatic environments and are thus characterized as being persistent (Reis Filho et al., 2006). To give an idea of the potential amount that can reach water resources, a study conducted in the United Kingdom in 2010 estimated that 2,193 tons of hormones in annual prescriptions reached water bodies (Runnalls et al., 2010). Aris et al. (2014) mentioned studies indicating that the amount of estrogens excreted by livestock was of the same order of magnitude as by humans. Therefore, currently, FSHs represent the most concerning ED group due to their chronic effect on exposed aquatic organisms (Machado et al., 2014).

Table 1. Daily excretion of FSHs by humans (µg/individual).

Type	Reference	E1	E2	E3	EE2
Males	Johnson and Williams (2004)	2.6	1.8	-	-
	Liu et al. (2009)	3.9	1.5	1.5	-
	Kostich et al. (2013)	3.51	1.83	3.21	-
	Johnson et al. (2000)	3.9	1.6	15	-
Premenopausal women	Johnson and Williams (2004)	11.7	3.2	-	-
	Liu et al. (2009)	10.73	4.71	8.12	-
	Kostich et al. (2013)	9.32	6.14	17.4	-
	Johnson et al. (2000)	8	3.5	4.8	-
Postmenopausal women	Johnson and Williams (2004)	1.8	1.0	-	-
	Liu et al. (2009)	5	2.78	2.78	-
	Kostich et al. (2013)	2.93	1.49	3.9	-
	Johnson et al. (2000)	4.0	2.3	1.0	-
Pregnant women	Johnson and Williams (2004)	550	393	-	-
	Liu et al. (2009)	1194	347	24078	-
	Kostich et al. (2013)	787	277	9850	-
	Johnson et al., (2000)	600	259	6000	-
Women using birth control pills	Johnson et al. (2000)	-	-	-	35

Legend: E1 – Estrone, E2 – 17β-Estradiol, E3 – Estriol, EE2 – 17α-Ethinylestradiol.

From a biological point of view, FSH molecules are extremely active and difficult to remove from water by wastewater treatment plant (WWTPs), whose effluents show frequently high endocrine activity (Jeannot et al., 2002; Körner et al., 2000). In addition, the absence of WWTPs in many municipalities in certain developing countries further aggravates the scenario of possible contamination of surface waters by EDs (Moreira, 2008).

Thus, FSHs represent a significant risk to various forms of life because these compounds persist in the environment, show evidences of accumulation in fish (Länge et al., 2001; Al-Ansari et al., 2013) and benthic invertebrates (Al-Ansari et al., 2013), and accumulate in sediments and soils (Campbell et al., 2006).

3.1. Quantification of FSHs in surface waters

In terms of water quality assessment to aquatic life, it is essential to know the concentration of FSHs expected in the receiving environment, viewing the comparison with the concentrations of adverse effect. Thus, the Table 2 shows a selection of the results of the survey conducted in this study, showing the occurrence of FSHs around the world with special focus on surface freshwaters, and chemical analysis used to detect them as well. A wide range in detected FSH concentrations can be observed.

Table 2. Concentration (min. – max.) of FSHs detected in surface freshwaters in several locations (in descending order of maximum concentration), and chemical methods used.

SFHs	Country, Region	Method	[] Min. (ng.L ⁻¹)	[] Max. (ng.L ⁻¹)	Reference
E1	Brasil, Paraná	HPLC-UV	120	1040	Machado et al. (2014)
	Brasil, São Paulo	HPLC-UV	nd	600	Lopes et al. (2010)
	China, Qingdao	GC-MS	14	180	Zhou et al. (2011)
	China, Guangdong	GC-MS	-	180	Yang et al. (2006)
	Brasil, São Paulo	LC-MS-MS	2.2	39	Sodré et al. (2010)
	United States, California	GC-MS-MS	-	38	Kolodziej and Sedlak (2007)
	Spain, Barcelona	LC-MS	<2.5	21.7	Rodriguez-Mozaz et al., (2004)
	United States, Nebraska	LC-MS-MS	1.4±0.7	12.9±2.6	Kolok et al. (2007)
	Italy, Roma	LC-MS-MS	5	12	Laganà et al. (2004)
	United Kingdom, West Sussex	GC-MS	-	9.9	Zhang et al. (2008)
	Japan, Tokyo	LC-MS-MS	3.4	6.6	Isobe et al. (2003)
	Turquia, Istambul	LC-MS-MS	1.4	5.74	Aydin and Talini (2013)
	United States, South Florida	GC-MS	0.88	5.2	Singh et al. (2010)
	France, Paris	GC-MS	1.1±0.3	3.0±0.9	Cargouet et al. (2004)
	Korea	GC-MS	nd	1.03	Shin et al. (2011)
	China, Beijing	LC-MS	-	1.1	Hu et al. (2005)
	Mexico, Mexico DF	GC-MS	0.16	0.17	Gibson et al. (2007)

SFHs	Country, Region	Method	[] Min. (ng.L ⁻¹)	[] Max. (ng.L ⁻¹)	Reference
E2	Spain	UPLC-QTOF-MS	bdl	bdl	Farré et al. (2007)
	United States, Lousiana	GC-MS	nd	nd	Boyd et al. (2003)
	Brasil, Paraná	HPLC-UV	170	13450	Machado et al. (2014)
	Brasil, São Paulo	HPLC-UV-DAD	106	6806	Montagner and Jardim (2011)
	Brasil, São Paulo	HPLC-UV-DAD	38	2510	Sodré et al. (2007)
	China, Qingdao	GC-MS	nd	134	Zhou et al. (2011)
	China, Guangdong	GC-MS	-	100	Yang et al. (2006)
	Brasil, São Paulo	HPLC-UV	6.8	30.6	Lopes et al. (2010)
	United Kingdom, East and West Sussex	GC-MS	14	17	Liu et al. (2004)
	United Kingdom, West Sussex	GC-MS	-	11.8	Zhang et al. (2008)
	United States, Nebraska	LC-MS-MS	1.7±0.3	8.8±3.4	Kolok et al. (2007)
	Brasil, São Paulo	LC-MS-MS	<0.6	7.3	Sodré et al. (2010)
	Italy, Roma	LC-MS-MS	2	6	Laganà et al. (2004)
	Turquia, Istambul	LC-MS-MS	1.1	5.39	Aydin and Talini (2013)
	Czech Republic , Praga	GC-MS	1.3	3.8	Morteani et al. (2006)
	France, Paris	GC-MS	1.4±0.6	3.2±0.3	Cargouet et al. (2004)
	United States, South Florida	GC-MS	nd	1.8	Singh et al. (2010)
	United States, California	GC-MS-MS	-	1.7	Kolodziej and Sedlak (2007)
	Japan, Tokyo	LC-MS-MS	0.6	1	Isobe et al. (2003)
	Korea	GC-MS	nd	0.34	Shin et al. (2011)
	Mexico, Mexico DF	GC-MS	0.01	0.02	Gibson et al. (2007)
	United States, Lousiana	GC-MS	nd	nd	Boyd et al. (2003)
	China, Beijing	LC-MS	nd	0.2	Hu et al. (2005)
	Spain	UPLC-QTOF-MS	bdl	bld	Farré et al. (2007)
	Spain, Barcelona	LC-MS	nd	nd	Rodriguez-Mozaz et al. (2004)
	China, Qingdao	GC-MS	4	94	Zhou et al. (2011)
	Brasil, São Paulo	LC-MS	1.48	7.7	Jardim et al. (2012)
	Brasil, Rio de Janeiro	LC-MS	1	7.27	Kuster et al. (2009)
	Turquia, Istambul	LC-MS-MS	2.15	5.2	Aydin and Talini (2013)
	Italy, Roma	LC-MS-MS	2	5	Laganà et al. (2004)
	United States, Nebraska	LC-MS-MS	2.1±1.3	3.3±3.3	Kolok et al. (2007)
	France, Paris	GC-MS	1.0±0.4	2.5±0.6	Cargouet et al. (2004)
	Brasil, São Paulo	LC-MS-MS	<0.6	2.3	Sodré et al. (2010)
	Spain	UPLC-QTOF-MS	bdl	bdl	Farré et al. (2007)
	Korea	GC-MS	nd	nd	Shin et al. (2011)
	Spain, Barcelona	LC-MS	nd	nd	Rodriguez-Mozaz et al. (2004)
E3	China, Qingdao	GC-MS	4	94	Zhou et al. (2011)
	Brasil, São Paulo	LC-MS	1.48	7.7	Jardim et al. (2012)
	Brasil, Rio de Janeiro	LC-MS	1	7.27	Kuster et al. (2009)
	Turquia, Istambul	LC-MS-MS	2.15	5.2	Aydin and Talini (2013)
	Italy, Roma	LC-MS-MS	2	5	Laganà et al. (2004)
	United States, Nebraska	LC-MS-MS	2.1±1.3	3.3±3.3	Kolok et al. (2007)
	France, Paris	GC-MS	1.0±0.4	2.5±0.6	Cargouet et al. (2004)
	Brasil, São Paulo	LC-MS-MS	<0.6	2.3	Sodré et al. (2010)
EE2	Spain	UPLC-QTOF-MS	bdl	bdl	Farré et al. (2007)
	Korea	GC-MS	nd	nd	Shin et al. (2011)
	Spain, Barcelona	LC-MS	nd	nd	Rodriguez-Mozaz et al. (2004)
	Brasil, Paraná	HPLC-UV	160	4530	Machado et al. (2014)
	Brasil, São Paulo	HPLC-UV-DAD	6	310	Sodré et al. (2007)
	Brasil, São Paulo	LC-MS-MS	ND	25	Sodré et al. (2010)
	China, Qingdao	GC-MS	7	24	Zhou et al. (2011)
	Turquia, Istambul	LC-MS-MS	11.7	14	Aydin and Talini (2013)
EE2	Czech Republic, Praga	GC-MS	1.4	4.6	Morteani et al. (2006)
	France, Paris	GC-MS	1.1±0.1	2.9±0.6	Cargouet et al. (2004)

SFHs	Country, Region	Method	[] Min. (ng.L ⁻¹)	[] Max. (ng.L ⁻¹)	Reference
	Italy, Roma	LC-MS-MS	nd	1	Laganà et al. (2004)
	Mexico, Mexico DF	GC-MS	bdl	0.06	Gibson et al. (2007)
	Brasil, Rio de Janeiro	LC-MS	nd	nd	Kuster et al. (2009)
	Korea	GC-MS	nd	nd	Shin et al. (2011)
	Spain, Barcelona	LC-MS	nd	nd	Rodriguez-Mozaz et al. (2004)
	Spain	UPLC-QTOF-MS	bdl	bdl	Farré et al. (2007)

Legend: - - Not reported; nd - Not detected; bdl - Below detection limit; HPLC-UV - High-pressure liquid chromatography with UV detection; LC-MS - Liquid chromatography with mass spec detection; LC-MS-MS - Liquid chromatography-tandem mass spectrometry; GC-MS - Gas chromatography with mass spectrometry; GC-MS-MS – Gas chromatography-tandem mass spectrometry; HPLC-UV-DAD - Liquid chromatography with ultraviolet and diode array detection; UPLC-QTOF-MS - Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry; UPLC-ESI-MS-MS - Ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry.

According to Table 2, very high concentrations were detected in Brazil in the states of Paraná and São Paulo, where to date, the largest number of studies conducted in this country is concentrated in the latter. It is important to emphasize that these studies were restricted to only 3 of the 26 Brazilian states, which limits the knowledge on environmental FSH concentrations to the states of São Paulo, Rio de Janeiro, and Paraná despite the large Brazilian territory and the large portion of South America it encompasses.

Differences observed among the FSH concentrations (Table 2) may not only be associated with normal differences among regions, but also with the use of different analytical techniques applied to quantify trace levels of chemical compounds. The reviewed articles contained no criticisms of the analytical procedures, which, until now, continue to be used without any international standardization. Validation data are typically required for these chemical methods but were often not presented in the articles. This fact, which is associated with the variety of methodological adaptations, equipment, and detectors

available on the market today, may influence the detection (DL) and quantification (QL) limits obtained and, moreover, the results reported in each scientific study.

Another point to consider is the losses experienced by the analyzed river basins as a result of the human population density of the urban centers at their particular location. The environmental impact experienced by each basin may likewise increase exposure to FSHs in aquatic environments and reduce system resilience after ecological imbalance. Considering the information on Table 2, it can be seen enough data on the occurrence of FSHs in surface waters.

4. Ecotoxicological aspects

In addition to the FSH concentration in aquatic environments determined by chemical analyses, the knowledge derived from ecotoxicological analyses is essential to understand whether such concentrations have the potential to adversely affect living organisms. Thus, the results of ecotoxicological tests are key to demonstrate the effects of endocrine disruption according to pollutant concentration, exposure time, and/or life stage of the tested organisms. Therefore, based on integrated chemical and ecotoxicological data, the extrapolation of environmentally safe FSH limits for aquatic biota is possible.

The effects triggered by FSHs found in aquatic environments may spread from microinvertebrates to organisms occupying the top of the food chain, such as fish, and therefore must be considered an issue of global scope (IPCS, 2002). According to Manahan (2003), the primary ecotoxicological concern with these substances is their undeniable capacity to affect the reproduction of species and interfere with the healthy development of offspring.

In this review, the studies were grouped according to the most frequent biological responses reported *in vivo* assays after chronic exposure (total or partial) to the estrogens E1,

E2, and EE2. The data obtained are shown in Table 3, with their frequency showed in Figure 1. This research was directed to the experimental results with fish, which are proven (Segner et al., 2003; Caldwell et al., 2008) to be most sensitive to the effects of FSHs. Besides that, such results are originated from analytical methods that allow to perform risk assessments for these substances (USEPA, 2008a; EFSA, 2013).

Table 3. Chronic ecotoxicological effects determined in fish exposed to FSHs, in laboratory.

FSH type	Test organism	Life stage	Nominal concentration (ng.L ⁻¹)	Exposure time (days)	Observed effect	Depuration time (days)	Reversal of observed effects	Reference
E1	<i>Pimephales promelas</i> *	Adult	32, 100, 320 and 1000	21	Induced VTG synthesis	N.I.	N.I.	Thorpe et al. (2007)
			320 and 1000		Reduced reproductive success			
		Adult	10, 50 and 100	21	Reduced GSI	N.I.	N.I.	Dammann et al. (2011)
			10		Induced VTG synthesis			
E2	<i>Pimephales promelas</i> *	Adult	10, 32 and 100	14	Increased reproductive success	N.I.	N.I.	Dammann et al. (2011)
			50 and 100		Reduced reproductive success			
		Adult	9, 20 and 44	21	Induced VTG synthesis	13	Irreversible	Shappell et al. (2010)
			10, 32 and 100		Induced VTG synthesis			
		Adult	5, 25 and 50	21	Induced VTG synthesis	N.I.	N.I.	Dammann et al. (2011)
			5		Increased reproductive success			
		Adult	100	10	Reduced reproductive success	N.I.	N.I.	Wolf et al. (2004)
			2780		Sperm necrosis			
		Adult	30, 60 and 120	21	Follicular atresia	21	Irreversible	Panter et al. (2000)
			10 and 30		Induced VTG synthesis			
		Juvenile	10 and 30	21	N.E.D.	N.I.	N.I.	Jorgenson et al. (2015)
			10		Change in sex ratio			
	<i>Hybognathus amarus</i>	Juvenile	10	21	Induced VTG synthesis	N.I.	N.I.	Jorgenson et al. (2015)
	<i>Lepomis macrochirus</i>	Juvenile	10	21	Change in sex ratio	N.I.	N.I.	Jorgenson et al. (2015)
	<i>Danio rerio</i> *	Embryo/larva	5 and 25	21	N.E.D.	21	N.I.	Brion et al. (2004)
			100		Induced VTG synthesis		Reversible	

FSH type	Test organism	Life stage	Nominal concentration (ng.L ⁻¹)	Exposure time (days)	Observed effect	Depuration time (days)	Reversal of observed effects	Reference
		Juvenile	5 and 25		N.E.D.		N.I.	
			100		Induced VTG synthesis		Reversible	
		Adult	5, 25 and 100		Change in sex ratio		Irreversible	
			25, 100		Induced VTG synthesis		N.I.	
					Reduced reproductive success		N.I.	
		Adult	10, 20, 40, 70, 100, 200 and 500	8	Induced VTG synthesis	N.I.	N.I.	Rose et al. (2002)
		Full life cycle	100	91	Feminization of males	N.I.	N.I.	Knörr and Braunbeck (2002)
		<i>Oryzias latipes</i> *	9,5 ^a	168	N.E.D.	N.I.	N.I.	Kang et al. (2002)
			16, 68, 159 and 243 ^a		Reduced reproductive success			
			68, 159 and 243 ^a		Induced VTG synthesis			
			159 and 243 ^a		Feminization of males			
	<i>Cyprinodon variegatus</i>	Adult	20	16	N.E.D.	N.I.	N.I.	Folmar et al. (2000)
	<i>Oncorhynchus mykiss</i> *	Adult	0,5	50	N.E.D.	N.I.	N.I.	Lahnsteiner et al. (2006)
			1 and 2.5		Decreased semen volume and quality			
	<i>Thymallus thymallus</i>	Adult	0.5	50	N.E.D.	N.I.	N.I.	Lahnsteiner et al. (2006)
			1		Decreased semen volume and quality			
E3	<i>Oryzias latipes</i> *	Full life cycle	5	90	N.E.D.	N.I.	N.I.	Lei et al. (2014)
EE2	<i>Danio rerio</i> *	Full life cycle	500 and 5000	120	Reduced GSI	224	Partially reversible	Larsen et al. (2009)
			5000		Reduced reproductive success		N.I.	
					Feminization of males		N.I.	
		Adult	1, 2, 4, 8, 12, 16, 20, 30 and 100		Induced VTG synthesis		N.I.	
					Induced VTG synthesis		N.I.	
					Change in sex ratio		N.I.	
		Embryo/larva to juvenile	15.4 ± 1.4 ^a	60	Feminization of males	N.I.	N.I.	Andersen et al. (2003)
							N.I.	
							N.I.	

FSH type	Test organism	Life stage	Nominal concentration (ng.L ⁻¹)	Exposure time (days)	Observed effect	Depuration time (days)	Reversal of observed effects	Reference
		Embryo/larva to juvenile	0.1, 1, 10 and 25	90	Change in sex ratio	150	Reversible	Van den Belt et al. (2003)
			1, 10 and 25		Induced VTG synthesis		N.I.	
			10 and 25		Reduced reproductive success		Irreversible	
		Full life cycle	0.05	124	Feminization of males	N.I.	N.I.	Larsen et al. (2008)
			0.5		Change in sex ratio			
			5		Change in courtship behavior			
		Adult	5	14	Increased reproductive success	N.I.	N.I.	Reyhanian et al. (2011)
			25		Reduced reproductive success			
			5 and 25		Induced VTG synthesis			
		Full life cycle	3	177	Change in courtship behavior	N.I.	N.I.	Baatrup and Henriksen (2015)
	<i>Cyprinodon variegatus</i>	Adult	20	16	N.E.D.	N.I.	N.I.	Folmar et al. (2000)
			100, 200, 500 and 1000		Induced VTG synthesis			
		Adult	50	35	Induced VTG synthesis	N.I.	Reversible	Schmid et al. (2002)
	<i>Pimephales promelas</i> *	Adult	0.1 and 1	21	Increased reproductive success	N.I.	N.I.	Pawlowski et al. (2004)
			3, 10 and 100		Reduced reproductive success			
			10 and 100		Reduced GSI			
		Full life cycle	0.32 ^a	150	Change in sex ratio	N.I.	N.I.	Parrott and Blunt. (2005)
			0.32 and 0.96 ^a		Reduced reproductive success			
			3.5, 9.6 and 23 ^a		Feminization of males			
		Adult	0.25	21	Reduced GSI	N.I.	N.I.	Armstrong et al. (2015)
			1.54 and 3.92 ^a		Induced VTG synthesis			
		Adult	0.47, 1.54 and 3.92 ^a	21	Reduced reproductive success	N.I.	N.I.	Armstrong et al. (2016)
	<i>Oncorhynchus mykiss</i> *	Juvenile	1 and 10	14	N.E.D.	N.I.	N.I.	Verslycke et al. (2002)
			100		Induced VTG synthesis			
	<i>Gobiocypris rarus</i>	Adult	4	21	Reduced reproductive success	N.I.	N.I.	Zha et al. (2008)
					Feminization of males			
					Induced VTG synthesis			

FSH type	Test organism	Life stage	Nominal concentration (ng.L ⁻¹)	Exposure time (days)	Observed effect	Depuration time (days)	Reversal of observed effects	Reference
	<i>Gulf pipefish</i>	Adult	1 and 100	10	Feminization of males Change in courtship behavior	N.I.	N.I.	Partridge et al. (2010)
	<i>Pomatoschistus minutus</i>	Adult	11	28	Induced VTG synthesis Change in courtship behavior	N.I.	N.I.	Saaristo et al. (2010)

Legend: N.I. – Not investigated; N.E.D. – No effect detected; * Test organisms suggested in international protocols for ecotoxicological tests, ^a Effective concentrations.

Figure 1. Frequency of biological effects observed in studies conducted with fish.

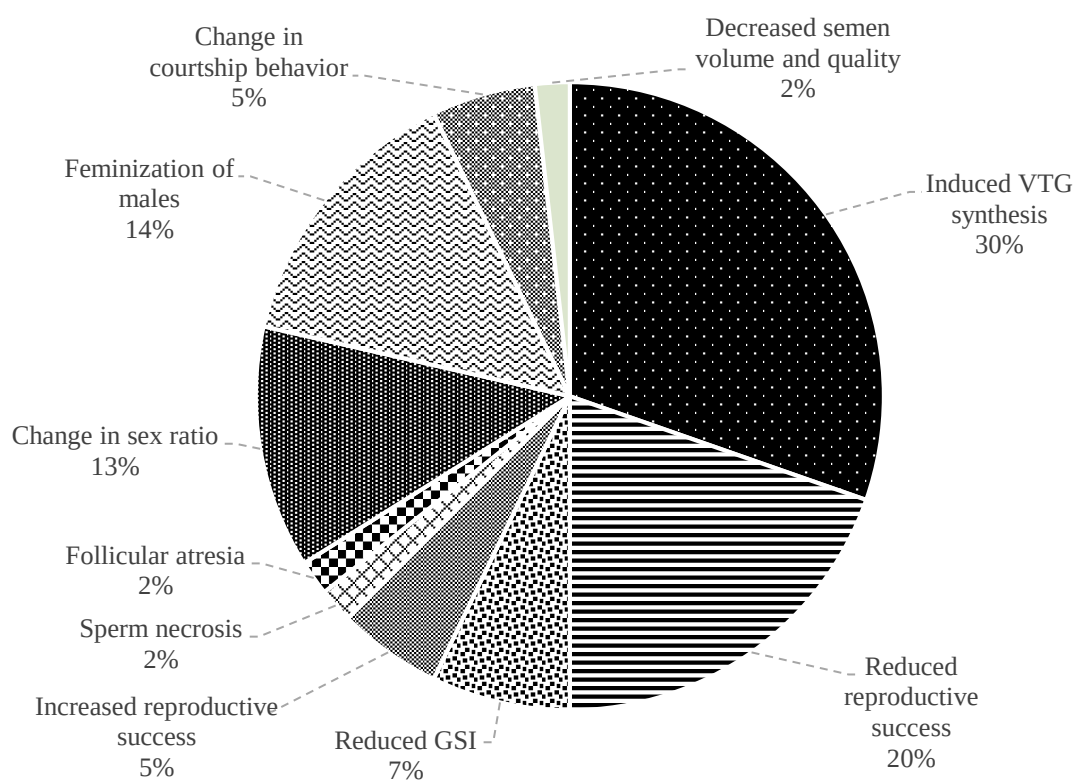


Figure 1 shows that 47% of the effects observed is related to events of biological relevance, such as reduced reproductive success (20%), feminization of males (14%) and changes in sex ratio (13%). These endpoints are essential to establish safe levels of FSHs, since those biological responses are appropriate to estimate the adverse effects at the population level (USEPA, 2008a). Thus, considering that fishes are more sensitive to the

effects of FSHs (Segner et al. 2003; Caldwell et al., 2012), those observed effects can be used to establish water quality standards for aquatic life.

As can be seen in Figure 1, the highest percentage of effects (30%) involved the induction of VTG synthesis (the phosphoglycoprotein, normally present in plasma of females, precursor of egg yolk). The VTG induction is a biomarker of exposure to estrogens (such as FSHs) which demonstrates the mode of action of these substances. Thus, when fishes (male or female) are exposed to the amount of endogenous estrogens VTG induction increases beyond the normal (especially in males) indicating the endocrine disruption effect. Although the representativeness of this biomarker, Hutchinson et al. (2006) cited that many scientists believe that small VTG increases can not represent direct effects on fish reproduction. These authors also state that, due to other uncertainties involved, this biomarker should be used as a screening test for directing the appropriate tests for establishing secure concentrations of FSHs in water.

According to Hutchinson et al. (2006), the same limitation of use of a biomarker applies to GSI (gonadosomatic index), which accounted for 7% of the toxicity studies with fishes (Figure 1). The frequency of effects on behavior (5%) is too minor (Figure 1), especially due to the intense work on the test, subjectivity in the interpretation of this effect, no specific behavioral changes to estrogens, and difficult to extrapolate the results to other effects such as survival, growth and reproduction of fish (USEPA, 2008a).

Table 4 presents the results of assays with FSHS and invertebrates, which are commonly used in risk assessments. The studies described in Table 4 show effects at high concentrations, with small environmental relevance, which are in general greater than those for fish (Table 3). The reduced sensitivity to other invertebrates was also noted by Segner, et al. (2003), demonstrating that these organisms respond to toxic effects instead of endocrine disrupting effects of FSHs, as suggested by these authors. For these reasons,

invertebrates do not have appropriate sensitivity for risk assessment studies of the FSHs in aquatic environments.

Table 4. Chronic ecotoxicological effects determined in aquatic invertebrates used as bioindicators of endocrine disruption in the water.

FSH type	Test organism	Life stage	Concentration (ng L ⁻¹)	Exposure time (days)	Observed effect	Reference
E2	<i>Acartia tonsa</i>	Full life cycle	23000	12	Increased egg production	Andersen et al. (1999)
	<i>Balanus amphitrite</i>	Nauplius	1000	6	Induced VTG synthesis	Billinghurst et al. (2000)
EE2	<i>Hyalella azteca</i> *	Multi-generation	320, 3200, 10000	14	Change in sex ratio	Vandenbergh et al. (2003)
	<i>Ceriodaphnia reticulata</i> *	Multi-generation	100, 320, 3200, 10000	32	Hermaphroditism	Jaser et al. (2003)
			10000, 50000, 80000, 100000		N.E.D.	
			200000, 300000		Reduced reproductive phase	
			20000, 50000, 100000		N.E.D.	
			200000, 350000, 500000		Juvenile mortality (F1)	
	<i>Sida crystallina</i>					

Legend: N.E.D. – No effect detected; * – Test organism suggested in international protocols for ecotoxicological tests.

Important endocrine disruptions ranging from 0,25 to 25 ng.L⁻¹ were observed in ecotoxicological studies (Table 3), which are near those detected in the environment (Table 2). These effects, reported at such low concentrations, are fundamental to the definition of exposure limits based on threshold effect concentrations, i.e., concentrations that do not exhibit disruptive effects. However, at first glance, one can assume that this variation of the disrupting effect concentrations in fish is due to exposure time dependence in the experiments. For example, a wide variation in exposure time (Table 3) ranged from 8 (Rose et al., 2002) to 168 days (Kang et al., 2002). This temporal variation would seem inappropriate for getting comparable data, from standardized methods, to the traditional assessment risk for chemicals. However, such temporal variation is representative for FSHs, considering that for these substances the primordial effects are endocrine, and further toxic

effects. Thus, the intrinsic importance of time exposure justify certain changes observed in interpretation of traditional ecotoxicological tests with FSHs.

Some consequences related to time exposure reflect directly on two aspects: reversibility and multigenerational effects. The reversibility of the disrupting effects of FSHs, after an absence of exposure, has been demonstrated in a few studies in this review (Table 3). While VTG induction presents greater number of events, reproductive endpoints (sex ratio, feminization, reduced reproductive success) were restricted to limited data. However, other studies confirm the irreversibility of EE2 effects to VTG induction at 14 ng.L⁻¹ (Van den Belt et al., 2002), and reduced reproductive success at 3 ng.L⁻¹ (Maack and Segner, 2004), 5 ng.L⁻¹ (Nash et al., 2004) and 10 ng.L⁻¹ (Baumann et al., 2014). Moreover, reversible effect was also described in the same EE2 concentration range, as VTG induction at 10 ng.L⁻¹ (Baumann et al., 2014) and reduced reproductive success endpoints ranging from 3 to 14 ng.L⁻¹ EE2 (Van den Belt et al., 2002; Maack and Segner, 2004; Baumann et al., 2014).

Based on all the studies mentioned, it is reasonable agree with Schäfers et al. (2007), which cited that the reversibility of the effects of FSHs vary according to the concentration of exposure, exposure duration, the timing (specific periods of exposure) and the species of fish used. The knowledge of this characteristic of FSHs allows select the appropriate endpoints for the establishment of safe concentrations in the aquatic environment.

Regarding the multigenerational effects of FSHs, which are related to continuous or intermittent exposures of fish in different generations, seven studies may be considered instructive (Länge et al., 2001; Wenzel et al., 2001; Nash et al., 2004; Schäfers et al., 2007; Kidd et al., 2007; Zha et al., 2008; Schwindt et al., 2014). In these studies it was found that the LOECs (lowest observed effect concentrations) ranged from 0.2 to 6.0 ng.L⁻¹, and the NOECs (no observed effect concentrations) averaged six times smaller (ranging from 0.2 to

0.5 ng .L⁻¹). According to the concepts of Schwindt (2015) also transgenerational effects may occur, which are adverse effects observed in generation that was not directly exposed to the pollutant (or exposed as germ cell). Some studies (Zha et al., 2008; Schwindt et al., 2014) suggest the occurrence of transgenerational EE2 effects on fish at concentrations ranging from 0.2 to 3.2 ng.L⁻¹.

Information on the reversibility of the disrupting effects of FSHs, as well as aspects relating to its trans/ multigenerational effects are important for the establishment of safe concentrations. Although there is a need for NOEC to transgenerational effects on FSHs, information available is useful to assess the impact that fish populations are subject, viewing of the fluctuating concentrations of FSHs expected in the environment, especially due to the variability of wastewater treatment plants.

A trend in ecotoxicological studies toward the use of alternative methods (e.g., *in vitro* or *in silico* techniques) was observed during the review process. However, such tests were not considered in the present review because there is no consensus among the scientific community as to whether the responses obtained using these alternative procedures can be extrapolated to reflect long exposure periods to low concentrations of FSHs (Scholz et al., 2013).

4.1. Safe exposures limits and water quality standards for FSH in surface water

It is interesting to note that despite two decades of studies with the FSHs there are no water quality standards for these endocrine disruptors, particularly viewing the protection of aquatic life. Usually these standards are established based on risk assessment procedures that are described in USEPA (2010) and European Commission (2011). Although the procedure adopted by the USEPA (2010) was produced for other substances, USEPA (2008a) indicates that such procedure is also appropriate for the risk assessment of estrogenic steroids. In spite

of all the information available on FSHs, as demonstrated in previous sections of this review, the lack of water quality standards suggests that there are difficulties in establishing the PNECs (Predicted No Effect Concentrations), which are generated in the corresponding risk assessments of most chemicals. So, it is convenient to explore some issues on the PNECs for FSHs with the goal to establish quality standards for protection of aquatic life.

A brief review of the literature (Hutchinson et al, 2006; USEPA, 2008a,b; European Commission, 2011; EFSA, 2013) indicates that some conditions are sufficient to estimate PNECs for the FSHs in surface waters, such as: a) use of a series of results originating from chronic ecotoxicological tests (or surrogates) with various fish species, where recognized effects with potential damaging populations are measured (e.g, survival, reproduction, fertility or growth); b) calculation of PNEC derived from statistical extrapolation of the results available, using the methods sensitive species distribution (SSD).

Only three proposals for PNECs were found in the literature (Table 5). In order to facilitate discussion, these values were called single-PNEC, full-PNECs and EU-PNEC.

Table 5. PNECs (Predicted no effect concentrations) established to estrogenic steroids.

Type of estimate	FSH Type (ng.L ⁻¹)			
	EE2	E1	E2	E3
Single-PNEC (Williams et al., 2008)	0.1	3.0	1.0	-
Full-PNEC (Caldwell et al., 2012)	0.1	6.0	2.0	60.0
EU-PNEC (European Commission, 2012)	0.035	-	0.4	-

Legend: EE2 - 17 α -ethinylestradiol; E1- estrone; E2 - 17 β -estradiol; E3 – estriol.

It is possible to note (Table 5) a numerical similarity between single-PNEC and full-PNEC to EE2, although they were calculated in different mode. The single-PNEC for EE2 and E2 were calculated by Williams et al. (2008) using the result of only one fish

ecotoxicological test, and applying an assessment factor for both test results. Thus, the single-PNEC for EE2 and E2 did not meet one of the basic conditions for derivation of this value, as mentioned earlier. The single-PNEC for E1 had a simplest derivation, it means the obtained value was based on studies that showed E1 being three fold less potent than E2.

The full-PNECs calculated for EE2 and E2 (Caldwell et al., 2012) met the conditions recommended for the establishment of these values. However, due to the lack of results of reproductive effects on fish, the authors calculated the full-PNEC for E1 and E3 based on studies on the induction of vitellogenin in vivo (considered of insufficient ecological relevance in literature.). Thus, in view of the potency of these steroids in relation to the E2 and using assessment factor (ten) for E3, the full-PNECs were established 6 and 60 ng.L^{-1} to E1 and E3 respectively (Table 5).

The suggested EU-PNECs for EE2 (European Commission, 2012) was estimated with a database similar to the full-PNEC for these estrogens (SCHER, 2011a). Thus, EU-PNEC for EE2 was calculated at 0.07 ng.L^{-1} , but with an additional assessment factor = 2, resulting in the EU-PNEC = 0.035 ng.L^{-1} (Table 5). The assessment factor equal 2 was considered relevant (SCHER, 2011a) due to the potential for increasing toxic effect of EE2 over generations. This potential to affect fish generations was demonstrated by Kidd et al. (2007) and Zha et al. (2008) studies, which have recently been confirmed by Schwindt et al. (2014). The EU-PNEC for E2 (0.4 ng.L^{-1}) is also quite different from full-PNEC (2 ng.L^{-1}). The lower value of the EU-PNEC for E2 is due to the inclusion of different analytical result in the calculations, and also the application of assessment factor = 2 because of lack of more data and similar mode of action to EE2 (SCHER, 2011b). For the reasons mentioned the EU-PNECs become lower than the full-PNECs, with values of 2.8 and 5 times lower for E2 and EE2 respectively (Table 5).

As discussed before, regarding E1 (estrone) and E3 (estriol) it is clear that the PNECs proposed are inaccurate and need further studies to its derivation. At the moment, the establishment of standards for both of these estrogens can be delayed, since their amounts and effects in surface waters are not of concern. Opposite situation is occurring for the EE2 (17 α -ethinylestradiol) and E2 (17 β -estradiol), where it was sufficiently demonstrated the relevance of established PNECs as well as their relevant environmental effects to aquatic life. Thus, due to the lack of standards for EE2 and E2 the PNECs presented (Table 5) have been used as guidelines in several studies. Two purposes are predominant in these studies, such as, ecological risk evaluation of FSHs into surface waters (Williams et al., 2008; Williams et al., 2009; Anderson et al., 2012; Aukidy et al., 2014; Laurenson et al., 2014; Scott et al., 2014; Chou et al., 2015; Keller et al., 2015), and to model the expected concentrations of the FSHs in the aquatic environment (Anderson et al., 2012; Johnson et al., 2013; Klasmeier et al., 2015). It is important to highlight that the use of mathematical models for estimating the FSHs in surface water has been justified by difficulties on chemical analysis monitoring. These difficulties lie over the lack of equipment that have sufficient limits of detection/quantification to determine the amounts of FSHs smaller than 0.1 ng.L⁻¹ (Johnson et al., 2013; Klasmeier et al., 2015; Kunz et al., 2015; Manickum and John, 2015). The problem is even greater when one considers that, to European Union members, the equipment must have limit of quantification 30% lower than standard water quality established for FSHs (Loos, 2012), for example 0.03 ng.L⁻¹ (based on full-PNEC) or 0.01 ng.L⁻¹ (based on EU-PNEC) to EE2.

According to documents of SCHER (2011a, b) it is clear that there is a lack of consensus, in the European community, on the adoption of full-PNECs or EU-PNECs (Table 5) for FSHs. This absence of consensus has remained since 2010, when a group of experts concluded the works on the proposal of environmental quality standards (European

Commission, 2012). Apparently, the European Union will adopt the EU-PNEC based on values published in the watch list (European Commission, 2015). However, the adoption of full-PNECs values may be an option to other continents/countries, considering that Caldwell et al. (2012) have good justification for these values. These authors have included the precise dimension of the uncertainties involved in the estimates, which has not accurately occurring with the values of EU-PNEC. Therefore, while the quality standards for FSHs are not defined legally, particularly to EE2 and E2, the full-PNEC or EU-PNEC values should be used as guidelines, especially for the protection of aquatic life.

5. Conclusion

According to this review can be seen that there are information, chemical and ecotoxicological, sufficient for the establishment of safe levels of FSHs to aquatic organisms. However, other transgenerational studies are desirable to define more NOEC values, in order to improve the current estimate. The lack of consensus among researchers seems to be the cause of the delay in the adoption of safe levels of FSHs exposure (expressed as PNEC). The European Union certainly will adopt the EU-PNEC for establishing water quality standards, however, the full-PNEC can be used with the same purpose by other countries. The choice of safe limits of exposure to aquatic organisms (full-PNEC or EU-PNEC) will depend on the required safety level, and the available instrumental to chemical monitoring of FSHs. At the moment, the important thing is not to worry about the details of implied uncertainty of each of the PNECs, but use either of them for the protection of aquatic life.

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References

- Al-Ansari, A.M., Atkinson, S.K., Doyle, J.R., Trudeau, V.L., Blais, J.M. 2013. Dynamics of uptake and elimination of 17 α -ethinylestradiol in male goldfish (*Carassius auratus*). **Aqua. Toxicol.** 132-133: 134-140.
- Andersen, H.R., Halling-Sørensen, B., Kusk, K.O. 1999. A parameter for detecting estrogenic exposure in the copepod *Acartia tonsa*. **Ecotoxicol. Environ. Saf.** 44, (1), 56-61.
- Andersen, L., Holbech, H., Gessbo, A., Norrgren, L., Petersen, G.I. 2003. Effects of exposure to 17 α -ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). **Comp. Biochem. Physiol. C Toxicol. Pharmacol.** 134 (3), 365-374.
- Anderson, P.D., Johnson, A.C., Pfeiffer, D., Caldwell, D.J., Hannah, R., Mastrocco, F., Sumpter, J.P., Williams, R.J. 2012. Endocrine disruption due to estrogens derived from humans predicted to be low in the majority of U.S. surface waters. **Environ. Toxicol. Chem.** 31(6): 1407-1415.
- Aris, A.Z., Shamsuddin, A.S., Praveena, S.M. 2014. Occurrence of 17 α -ethinylestradiol (EE2) in the environment and effect on exposed biota: a review. **Environ. Int.** 69: 104-119.
- Aukidy, M.A., Verlicchi, P., Voulvoulis, N. 2014. A framework for the assessment of the environmental risk posed by pharmaceuticals originating from hospital effluents. **Sci. Total. Environ.** 493: 54-64.
- Baumann, L., Knörr, S., Keiter, S., Rehberger, K., Volz, S., Schiller, V., Fenske, M., Holbech, H., Segner, H., Braunbeck, T. 2014. Reversibility of endocrine disruption in zebrafish (*Danio rerio*) after discontinued exposure to the estrogen 17 α -ethinylestradiol. **Toxicol. Appl. Pharmacol.** 278 (3), 230-237.
- Bila, D.M., Dezotti, M. 2007. Desreguladores endócrinos no meio ambiente: efeitos e consequências. **Quím. Nova** 30 (3), 651-666.
- Billinghurst, Z., Clare, A.S., Matsumura, K., Depledge, M.H. 2000. Induction of cypris major protein in barnacle larvae by exposure to 4-n-nonylphenol and 17 β -oestradiol. **Aquat. Toxicol.** 47 (3-4), 203-212.
- Bolong, N., Ismail, A.F., Salim, M.R., Matsuura, T. 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. **Desalination** 239 (1-3), 229-246.
- Boyd, G.R., Reemtsma, H., Grimm, D.A., Mitra, S. 2003. Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada. **Sci. Total Environ.** 311 (1-3), 135-149.

Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J., Flammarion, P. 2004. Impacts of 17 β -estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). **Aquat. Toxicol.** 68 (3), 193-217.

Caldwell, D.J., Mastrocco, F., Anderson, P.D., Länge, R., Sumpter, J.P. 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17 β -estradiol, estriol, and 17 α -ethinylestradiol. **Environ. Toxicol. Chem.** 31(6): 1396-1406.

Caldwell, D.J., Mastrocco, F., Hutchinson, T.H., Länge, B., Heijerick, D., Janssen, C., Anderson, P.D., Sumpter, J.P. 2008. Derivation of an aquatic predicted no-effect concentration for the synthetic hormone, 17 α -ethinylestradiol. **Environ. Sci. Technol.** 42 (19): 7046-7054.

Campbell, C.G., Borglin, S.E., Green, F.B., Grayson, A., Wozel, E., Stringfellow, W.T. 2006. Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: a review. **Chemosphere** 65: 1265-1280.

Cargouët, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S., Levi, Y. 2004. Assessment of river contamination by estrogenic compounds in Paris area (France). **Sci. Total Environ.** 324 (1-3), 55-66.

Chou, P., Lin, Y., Liu, T., Chen, K. 2015. Exploring potential contributors to endocrine disrupting activities in Taiwan's surface waters using yeast assays and chemical analysis. **Chemosphere** 138: 814-820.

Dammann, A.A., Shappell, N.W., Bartell, S.E., Schoenfuss, H.L. 2011. Comparing biological effects and potencies of estrone and 17 β -estradiol in mature fathead minnows, *Pimephales promelas*. **Aquat. Toxicol.** 105 (3-4), October, 559-568.

Deblonde, T., Cossu-Leguille, C., Hartemann, P. 2011. Emerging pollutants in wastewater: a review of the literature. *Int. J. Hyg.* **Environ. Health** 214 (6), 442-448.

Diamond, J.M., Latimer, H.A., Munkittrick, K.R., Thornton, K.W., Bartell, S.M., Kidd, K.A. 2011. Prioritizing contaminants of emerging concern for ecological screening assessments. **Environ. Toxicol. Chem.** 30, (11), 2385-2394.

EFSA (European Food Safety Authority). 2013. Scientific Committee. Scientific Opinion on the hazard assessment of endocrine disruptors: scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment. **EFSA Journal** 11(3):3132, 84 p.

European Commission. 2011. **Technical Guidance for Deriving Environmental Quality Standards. Guidance Document n° 27**. Technical Report 2011-055, 203 p.

European Commission. 2012. **Proposal for a Directive of the European Parliament and of the Council. Amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.** COM(2011) 876 final. Brussels. 31.1.2012, 35 p.

European Commission. 2015. **Commission Implementing Decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council.** Off. J.Eur.Union, 24.3.2015, L 78/40.

Farré, M., Kuster, M., Brix, R., Rubio, F., López de Alda, M.J., Barceló, D. 2007. Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography-tandem mass spectrometry, and ultra performance liquid chromatography-quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples. **J. Chromatogr. A** 1160 (1–2), 166-175.

Folmar, L.C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., Denslow, ND. 2000. Comparative estrogenicity of estradiol, ethynylestradiol and diethylstilbestrol in an *in vivo*, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. **Aquat. Toxicol.** 49 (1-2), 77-88.

Gibson, R., Becerril-Bravo, E., Silva-Castro, V., Jiménez, B. 2007. Determination of acidic pharmaceuticals and potential endocrine disrupting compounds in wastewaters and spring waters by selective elution and analysis by gas chromatography-mass spectrometry. **J. Chromatogr. A** 1169 (1–2), 31-39.

Hu, J., Zhang, H., Chang, H. 2005. Improved method for analyzing estrogens in water by liquid chromatography-electrospray mass spectrometry. **J. Chromatogr. A** 1070 (1–2), 221-224.

Huang, B., Pan, X., Liu, J., Fang, K., Wang, Y., Gao, J. 2010. New discoveries of heating effect on Trimethylsilyl derivatization for simultaneous determination of steroid endocrine disrupting chemicals by GC–MS. **Chromatographia** 71 (1–2), 149-153.

Hutchinson, T.H., Ankley, G.T., Segner, H., Tyler, C.R. 2006. Screenig and testing for endocrine disruption in fish – Biomarkers as “signposts,” not “traffic lights” in risk assessment. **Environ. Health Perspect.** 114 (suppl, 1):106-114.

IPCS (Institute of Peace and Conflict Studies), 2002. **International Programe on chemical safety.** Global assessment of the state-of-science of endocrine disruptors, Geneva.

Isobe, T., Shiraishi, H., Yasuda, M., Shinoda, A., Suzuki, H., Morita, M. 2003. Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry. **J. Chromatogr. A** 984 (2), 195-202.

Jardim, W.F., Montagner, C.C., Pescara, I.C., Umbuzeiro, G.A., Di Dea Bergamasco, A.M., Eldridge, M.L. 2012. An integrated approach to evaluate emerging contaminants in drinking water. **Sep. Purif. Technol.** 84, 3-8.

Jaser, W., Severin, G.F., Jütting, U., Jüttner, I., Schramm, K.W., Kettrup, A. 2003. Effects of 17 α -ethinylestradiol on the reproduction of the cladoceran species *Ceriodaphnia reticulata* and *Sida crystallina*. **Environ. Int.** 28 (7), 633-638.

Jeannot, R., Sabik, H., Sauvard, E., Dagnac, T., Dohrendorf, K. 2002. Determination of endocrine-disrupting compounds in environmental samples using gas and liquid chromatography with mass spectrometry. **J. Chromatogr. A** 974 (1–2), 143-159.

Johnson, A.C., 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 (2–3), 163-173.

Johnson, A.C., Dumont, E., Williams, R.J., Oldenkamp, R., Cisowska, I., Sumpter, J.P. 2013. Do concentrations of ethinylestradiol, estradiol, and diclofenac in European rivers exceed proposed EU environmental quality standards? **Environ. Sci. Technol.** 47: 12297-12304.

Johnson, A.C., Williams, R.J. 2004. A model to estimate influent and effluent concentrations of estradiol, estrone, and Ethinylestradiol at sewage treatment works. **Environ. Sci. Technol.** 38 (13), 3649-3658.

Kang, I.J., Yokota, H., Oshima, Y., Tsuruda, Y., Yamaguchi, T., Maeda, M., Imada, N., Tadokoro, H., Honjo, T. 2002. Effect of 17 β -estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). **Chemosphere** 47, 71-80.

Keller, V.D.J., Lloyd, P., Terry, J.A., Williams, R.J. 2015. Impact of climate change and population growth on a risk assessment for endocrine disruption in fish due to steroid estrogens in England and Wales. **Environ. Pollut.** 197: 262-268.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W. 2007. Collapse of a fish population after exposure to a synthetic estrogen. **PNAS** 104 (21): 8897-8901.

Klasmeier, J., Kehrein, H., Berlekamp, J. 2015. Ethinylestradiol in surface waters – Analysis of consequences of the proposed environmental quality standard (EQS). In: Fuchs, S., Eyckmanns-Wolters (Eds.). **River Basins 2015**. Monitoring, modelling & management of pollutants. KIT. Karlsruhe/DE. pp.75-80.

Knörr, S., Braunbeck, T. 2002. Decline in reproductive success, sex reversal, and developmental alterations in Japanese medaka (*Oryzias latipes*) after continuous exposure to Octylphenol. **Ecotoxicol. Environ. Saf.** 51 (3), 187-196.

Kolodziej, E.P., Sedlak, D.L. 2007. Rangeland grazing as a source of steroid hormones to surface waters. **Environ. Sci. Technol.** 41 (10), 3514-3520.

Kolok, A.S., Snow, D.D., Kohno, S., Sellin, M.K., Guillette, L.J. 2007. Occurrence and biological effect of exogenous steroids in the Elkhorn river, Nebraska, USA. **Sci. Total Environ.** 388 (1–3), 104-115.

Körner, W., Bolz, U., Süssmuth, W., Hiller, G., Schuller, W., Hanf, V., Hagenmaier, H. 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. **Chemosphere** 40 (9–11), 1131-1142.

Kostich, M., Flick, R., Martinson, J. 2013. Comparing predicted estrogen concentrations with measurements in US waters. **Environ. Pollut.** 178, 271-277.

Kuster, M., Azevedo, D.A., López de Alda, M.J., Aquino Neto, F.R., Barceló, D. 2009. Analysis of phytoestrogens, progestogens and estrogens in environmental waters from Rio de Janeiro (Brazil). **Environ. Int.** 35 (7), 997-1003.

Kunz, P.Y., Kienle, C., Carere, M., Homozava, N., Kase, R. 2015. In vitro bioassays to screen for endocrine active pharmaceuticals in surface and waste waters. **Jour. Pharm. Bio. An.** 106: 107-115.

Laganà, A., Bacaloni, A., De Leva, I., Faberi, A., Fago, G., Marino, A. 2004. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. **Anal. Chim. Acta** 501 (1), 79-88.

Länge, 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*). **Environ. Toxicol. Chem.** 20(6): 1216-1227.

Larsen, M.G., Bilberg, K., Baatrup, E. 2009. Reversibility of estrogenic sex changes in zebrafish (*Danio rerio*). **Environ. Toxicol. Chem.** 28 (8), 1783–1785.

Larsen, M.G., Hansen, K.B., Henriksen, P.G., Baatrup, E. 2008. Male zebrafish (*Danio rerio*) courtship behaviour resists the feminising effects of 17 α -ethinylestradiol-morphological sexual characteristics do not. **Aquat. Toxicol.** 87 (4), 234-244.

Laurenson, J.P., Bloom, R.A., Page, S., Sadrieh, N. 2014. Ethinyl estradiol and other human pharmaceutical estrogens in the aquatic environment: A review of recent risk assessment data. **Amer. As. Pharm. Sci.** 16(2): 299-310.

Liu, R., Zhou, J.L., Wilding, A. 2004. Simultaneous determination of endocrine disrupting phenolic compounds and steroids in water by solid-phase extraction-gas chromatography-mass spectrometry. **J. Chromatogr. A** 1022 (1–2), 179-189.

Liu, Z.H., Kanjo, Y., Mizutani, S. 2009. Urinary excretion rates of natural estrogens and androgens from humans, and their occurrence and fate in the environment: a review. **Sci. Total Environ.** 407 (18), 4975-4985.

Loos, R. 2012. **Analytical methods relevant to the European Commission's 2012 proposal on priority substances under the Water Framework Directive.** European Commission. JCR/IES. 70 p.

Lopes, L.G., Marchi, M.R.R., Souza, J.B.G., Moura, J.A., Lorenzon, C.S., Cruz, C., Amaral, L.A. 2010. Estrogênios em águas naturais e tratadas da região de Jaboticabal – São Paulo. **Quim. Nova.** 33 (3), 639-643.

Maack, G., Segner, H. 2004. Life-stage-dependent sensitivity of zebrafish (*Danio rerio*) to estrogen exposure. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 139 (1–3), 47-55.

Machado, K.S., Cardoso, F.D., Azevedo, J.C.R., Braga, C.B. 2014. Occurrence of female sexual hormones in the Iguazu river basin, Curitiba, Paraná State, Brazil. **Acta Sci. Technol.** 36 (3), 421-427.

Manahan, S.E. 2003. **Toxicological Chemistry and Biochemistry.** Lewis Publishers, Boca Raton.

Manickum, T., John, W. 2015. The current preference for the immune-analytical Elisa method for quantitation of steroid hormones (endocrine disruptor compounds) in wastewater in South Africa. **Anal. Bioanal. Chem.** 407: 4949-4970.

Montagner, C.C., Jardim, W.F. 2011. Spatial and Seasonal Variations of Pharmaceuticals and endocrine disruptors in the Atibaia river, São Paulo State (Brazil). **J. Braz. Chem. Soc.** 22 (8), 1452-1462.

Moreira, D.S. 2008. **Desenvolvimento de metodologia analítica por cromatografia/espectrometria de Massas para avaliação da ocorrência de perturbadores endócrinos em mananciais de abastecimento da região metropolitana de Belo Horizonte.** Dissertação (Mestrado em Engenharia Ambiental). Universidade Federal de Ouro Preto, Ouro Preto, p. 124 f.

Morteani, G., Möller, P., Fuganti, A., Paces, T. 2006. Input and fate of anthropogenic estrogens and gadolinium in surface water and sewage plants in the hydrological basin of Prague (Czech Republic). **Environ. Geochem. Health** 28 (3), 257-264.

Nash, J.P., Kime, D.E., Ven, L.T.M.V., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R. 2004. **Envir. Health. Persp.** 112 (17): 1725-1733.

Panter, G.H., Thompson, R.S., Sumpter, J.P. 2000. Intermittent exposure of fish to estradiol. **Environ. Sci. Technol.** 34 (13), 2756-2760.

Parrott, J.L., Blunt, B.R. 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. **Environ. Toxicol.** 20 (2), 131-141.

Reis Filho, R.W., Araújo, J.C.D, Vieira, E.M. 2006. Hormônios sexuais estrógenos: contaminantes bioativos. **Quim. Nova** 29 (4), 817-822.

Rodriguez-Mozaz, S., de Alda, M.J., Barceló, D. 2004. Monitoring of estrogens, pesticides and bisphenol A in natural waters and drinking water treatment plants by solid-phase extraction-liquid chromatography-mass spectrometry. **J. Chromatogr.** 1045 (1–2), 85-92.

Rose, J., Holbech, H., Lindholst, C., Nørum, U., Povlsen, A., Korsgaard, B., Bjerregard, P. 2002. Vitellogenin induction by 17 beta-estradiol and 17 alpha-ethinylestradiol in male zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. Ctoxicol. Pharmacol.* 131 (4), 531-539.

Runnalls, T.J., Margiotta-Casaluci, L., Kugathas, S., Sumpter, J.P. 2010. Pharmaceuticals in the aquatic environment: steroids and anti-steroids as high priorities for research. Human and ecological risk assessment. **Human and Ecological Risk Assessment** 16 (6), 1318-1338.

Schäfers, c., Teigeler, m., Wenzel, a., Maack, g., Fenske, m., Segner, H. 2007. Concentration- and time-dependent effects of the synthetic estrogen, 17 α -ethinylestradiol, on reproductive capabilities of the zebrafish, *Danio rerio*. **J. Toxicol. Environ. Health A** 70 (9), 768-779.

SCHER (Scientific Committee on Health and Environmental Risks). 2011a. **Opinion on draft environmental quality standards under the Water Framework Directive – Ethinylestradiol**, 30 March 2011. 9 p.

SCHER (Scientific Committee on Health and Environmental Risks). 2011b. **Opinion on draft environmental quality standards under the Water Framework Directive – 17 β -estradiol**, 30 March 2011. 10 p.

Schmid, T., Gonzalez-Valero, J., Rufli, H., Dietrich, D.R. 2002. Determination of vitellogenin kinetics in male fathead minnows (*Pimephales promelas*). **Toxicol. Lett.** 131 (1–2), 65-74.

Scholz S, Sela E, Blaha L, Braunbeck T, Galay-Burgos M, García-Franco M, Guinea J, Klüver N, Schirmer K, Tanneberger K, Tobor-Kapłon M, Witters H, Belanger S, Benfenati E, Creton S, Cronin MTD, Eggen RIL, Embry M, Ekman D, Gourmelon A, Halder M, Hardy B, Hartung T, Hubesch B, Jungmann D, Lampi MA, Lee L, Léonard M, Küster E, Lillicrap A, Luckenbach T, Murk AJ, Navas JM, Peijnenburg W, Repetto G, Salinas E, Schüürmann G, Spielmann H, Tollefsen KE, Walter-Rohde S, Whale G, Wheeler JR, Winter MJ. 2013. A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. **Regul. Toxicol. Pharmacol.** 67 (3), 506-530.

Schwindt, A.R. 2015. Parental effects of endocrine disrupting compounds in aquatic wildlife: Is there evidence of transgenerational inheritance? **Gen. Comp. Endoc.** 219: 152-164.

Schwindt, A.R., Winkelman, D.L., Keteles, K., Murphy, M., Vadja, A.M. 2014. An environmental oestrogen disrupts fish population dynamics through direct and transgenerational effects on survival and fecundity. **Journ. Appl. Ecol.** 51: 582-591.

Scott, P.D., Bartkow, M., Blockwell, S.J., Coleman, H.M., Khan, S.J., Lim, R., McDonald, J.A., Nice, H., Nugegoda, D., Pettigrove, V., Tremblay, L.A., Warne, M.S.J., Leush, F.D.L. 2014. An assessment of endocrine activity in Australian rivers using chemical and in vitro analyses. **Environ. Sci. Pollut. Res.** 21: 12951-12967.

Segner, H., Carroll, K., Fenske, M., Janssen, C.R., Maack, G., Pascoe, D., Schäfers, C., Vandenberg, G.F., Watts, M., Wenzel, A. 2003. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. **Ecotox. Env. Saf.** 54: 302-314

Shappell, N.W., Hyndman, K.M., Bartell, S.E., Schoenfuss, H.L. 2010. Comparative biological effects and potency of 17 α - and 17 β -estradiol in fathead minnows. **Aquat. Toxicol.** 100 (1), 1-8.

Shin, H., Oh, J., Shin, S. 2011. Sensitive determination of natural and synthetic steroidal hormones of a free and conjugated form in surface water by gas chromatography-mass spectrometry. **Bull. Korean Chem. Soc.** 32 (3), 809-814.

Sodré, F.F., Montagner, C.C., Locatelli, M.A.F., Jardim, W.F., 2007. Ocorrência de interferentes endócrinos e produtos farmacêuticos em águas superficiais da região de Campinas (SP, Brasil). **J. Braz. Soc. Ecotoxicol.** 2 (2), 187-196.

Sodré, F.F., Pescara, I.C., Montagner, C.C., Jardim, W.F. 2010. Assessing selected estrogens and xenoestrogens in Brazilian surface waters by liquid chromatography – tandem mass spectrometry. **Microchem. J.** 96 (1), 92-98.

Thorpe, K.L., Benstead, R., Hutchinson, T.H., Tyler, C.R. 2007. Associations between altered vitellogenin concentrations and adverse health effects in fathead minnow (*Pimephales promelas*). **Aquat. Toxicol.** 85 (3), 176-183.

USEPA (U.S. Environmental Protection Agency). 2008a. **White paper – Aquatic Life Criteria for contaminants of emerging concern. Part I – General challenges and recommendations.** Draft document. OW/ORD Emerging Contaminants Workgroup. 36p.

USEPA (U.S. Environmental Protection Agency). 2008b. **White paper – Aquatic Life Criteria for contaminants of emerging concern. Part II – Illustration of recommendations using data for 17 α -ethynylestradiol (EE2).** Draft document. OW/ORD Emerging Contaminants Workgroup. 46 p.

USEPA (U.S. Environmental Protection Agency), 2010. **Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses**. Office of Research and Development (electronic version). PB85-227049.

Van den Belt, K., Verheyen, R., Witters, H. 2003. Effects of 17 α -ethynylestradiol in a partial life-cycle test with zebrafish (*Danio rerio*): effects on growth, gonads and female reproductive success. **Sci. Total Environ.** 309 (1–3), 127-137.

Van den Belt, K., Wester, P.W., Ven, L.T.M., Verheyen, R., Witters, H. 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): Time dependency and reversibility. **Environ. Toxicol. Chem.** 21(4): 767-775.

Vandenbergh, G.F., Adriaens, D., Verslycke, T., Janssen, C.R. 2003. Effects of 17 α -ethynylestradiol on sexual development of the amphipod *Hyaella Azteca*. **Ecotoxicol. Environ. Saf.** 54 (2), 216-222.

Verslycke, T., Vandenbergh, G.F., Versonnen, B., Arijs, K., Janssen, C.R. 2002. Induction of vitellogenesis in 17 α -ethynylestradiol-exposed rainbow trout (*Oncorhynchus mykiss*): a method comparison. **Comp. Biochem. Physiol. C Toxicol. Pharmacol.** 132 (4), 483-492.

Wenzel, A., Schäfers, C., Vollmer, G., Michna, H., Diel, P. 2001. **Research efforts towards the development and validation of a test method for the identification of endocrine disrupting chemicals**. Fraunhofer Institute für Umweltchemie und Ökotoxikologie. Germany. Final Report. Contract B6-7920/98/000015. <http://www.researchgate.net/publication/256741999>.

Williams, R.J., Jonson, A.C., Keller, V., Wells, C., Holmes, M.G.R., Young, A.R. 2008. **Catchment risk assessment of steroid oestrogens from sewage treatment works**. Science report SC030275/SR3. Environment Agency. Bristol. UK. 167 p.

Williams, R.J., Keller, V.D.J., Johnson, A.C., Young, A.R., Holmes, M.G.R., Wells, C., Gross-Sorokin, M., Benstead, R. 2009. A national risk assessment for intersex in fish arising from steroid estrogens. **Environ. Toxicol. Chem.** 28(1): 220-230.

Wolf, J.C., Dietrich, D.R., Friederich, U., Caunter, J., Brown, A.R. 2004. Qualitative and quantitative histomorphologic assessment of fathead minnow *Pimephales promelas* gonads as an endpoint for evaluating endocrine-active compounds: a pilot methodology study. **Toxicol. Pathol.** 32 (5), 600-612.

Yang, L., Luan, T., Lan, C. 2006. Solid-phase microextraction with on-fiber silylation for simultaneous determinations of endocrine disrupting chemicals and steroid hormones by gas chromatography-mass spectrometry. **J. Chromatogr. A** 1104 (1–2), 23-32.

Zha, J., Sun, L., Zhou, Y., Spear, P.A., Ma, M., Wang, Z. 2008. Assessment of 17 α -ethynylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). **Toxicol. Appl. Pharmacol.** 226(3): 298-308.

Zhang, Z., Hibberd, A., Zhou, J.L. 2008. Analysis of emerging contaminants in sewage effluent and river water: comparison between spot and passive sampling. **Anal. Chim. Acta** 607 (1), 37-44.

***Remediation efficiency of three treatments on water polluted with endocrine disruptors:
Assessment by means of in vitro techniques***

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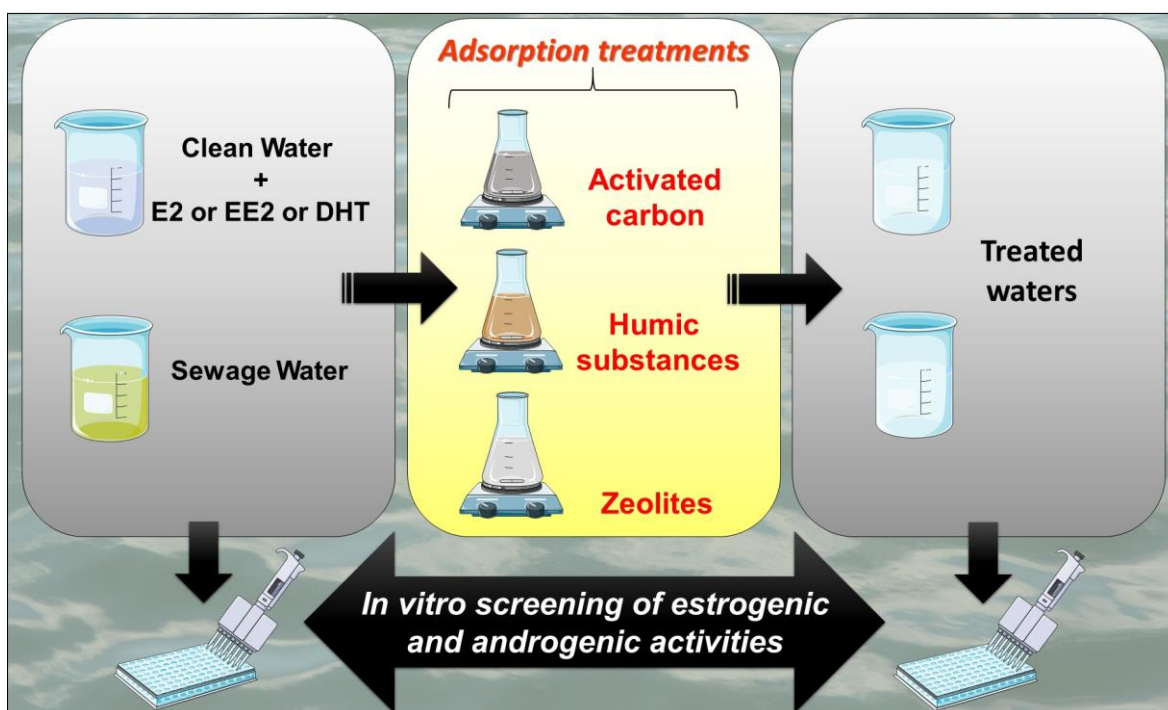
- Different substrates were used to remove endocrine disrupters (EDs) from waters.
- Activated carbon (AC) retired efficiently EDs from laboratory prepared waters.
- AC did not show a good performance in the treatment of effluent samples.
- Zeolite (ZEO) was assayed as a substrate for the removal of EDs.
- ZEO allowed the reduction of androgenic activity in actual wastewaters.

Abstract: Chemical substances with potential to disrupt endocrine systems have been detected in aquatic environments worldwide, making necessary the investigation about water treatments able to inhibit such potential. The present work aimed to assess the efficiency for removing endocrine disruptors (with estrogenic and androgenic activity) of three simple and inexpensive substrates that could be potentially used in sectors or regions with limited resources: powdered activated carbon (PAC), powdered natural zeolite (ZEO) (both at a concentration of 500 mg L^{-1}) and natural aquatic humic substances (AHS) (at 30 mg L^{-1}). MilliQ-water and mature water from fish facilities (aquarium water, AW), were artificially spiked with 17β -estradiol (E2), 17α -ethinylestradiol and dihydrotestosterone. Moreover, effluent samples from waste water treatment plants (WWTP) were also submitted to the remediation treatments. Estrogenic and androgenic activities were assessed with two cell lines permanently transfected with luciferase as reporter gene under the control of hormone receptors: AR-EcoScreen containing the human androgen receptor and HER-LUC transfected with the sea bass estrogen receptor. PAC was efficiently removing the estrogenic and androgenic compounds added to milliQ and AW. However, androgenic activity detected in WWTP effluents was only reduced after treatment with ZEO. The higher surface area of PAC could have facilitated the removal of spiked hormones in clean waters. However, it is possible that the substances responsible of the hormonal activity in WWTP have adsorbed to micro and nanoparticles present in suspension that would have been retained with higher efficiency by ZEO that show pores of several microns in size.

Keywords: Androgenicity; Estrogenicity; In vitro assays; Remediation; Water treatment.

Abbreviations: AHS, aquatic humic substances; AW, aquarium water; DHT, dihydrotestosterone; DHT-Eq, DHT equivalents; DMEM, Dulbecco's minimal essential medium; E2, 17 β -estradiol; ED, endocrine disrupter; EDCs, endocrine disrupting chemicals; EDTA, ethylene diamine tetraacetic acid; EE2, 17 α -ethinylestradiol; FBS, fetal bovine serum; PAC, powdered activated carbon; PAH, polyaromatic hydrocarbon; PCB, polybrominated biphenyl; SDS, sodium dodecyl sulfate; SPE, solid-phase extraction; WWTP, waste water treatment plant; ZEO, zeolite

Graphical abstract:



1. Introduction

The adverse effects of endocrine disrupting chemicals (EDCs) on aquatic organisms are well documented, and are a focal point of current environmental research worldwide (Grover et al., 2011). EDCs include natural substances such as reproductive hormones (e.g. estrogens, androgens and progestogens), thyroid hormones, and corticosteroids (Scholz et al., 2013), as well as a wide range of chemicals including synthetic hormones, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, furans, alkylphenols, pharmaceuticals, and pesticides (Andersen et al., 1999 and Combalbert et al., 2012).

It is widely accepted that the most prominent source of EDCs into the aquatic environment is via sewage treatment work effluents (Grover et al., 2011, Snyder et al., 2007, Stasinakis et al., 2008, Zhang and Zhou, 2008 and Zhou et al., 2009). Residual waters of domestic origin transport a number of natural and artificial hormones (Ternes et al., 1999, Baronti et al., 2000 and Ying et al., 2002) that are not completely removed by conventional treatment processes (D'Ascenzo et al., 2003 and Auriol et al., 2006) reaching surface waters and affecting organisms present there, including fish. According to Dias et al. (2015) such a problem is more acute in developing countries due to the lack of appropriate treatment of waste waters.

Many advanced treatment technologies have been tested for their efficiency in further removing EDCs following secondary treatment (Grover et al., 2011). These include photodegradation (Zhang et al., 2007), chemical oxidation (Jiang et al., 2005), membrane filtration, or activated carbon (AC) adsorption (Choi et al., 2005 and Snyder et al., 2007). When evaluating a range of adsorbents for EDCs, AC was found to be the most effective (Zhang and Zhou, 2005). Furthermore, AC (both powdered and granular) is effective not only for estrogenic compounds (Yoon et al., 2003, Choi et al., 2005, Zhang and Zhou, 2005

and Redding et al., 2009), but also for many other types of organic pollutants, e.g. pharmaceuticals (Snyder et al., 2007). Hence it is widely promoted as a standard tertiary treatment for EDCs and other emerging pollutants. However, AC presents some disadvantages, such as the high cost of the disposal of contaminated sewage sludge. Therefore, inorganic adsorbents (e.g. zeolite) with high surface specific areas have been used as alternatives to AC (Ali and Gupta, 2006; Tsai et al., 2009).

The use of zeolites as adsorbent treatment are gaining popularity due to the low-cost and availability of raw material (i.e. fly ash) and also due to the presence of well-defined molecular and porous structure, high thermal stability, ion selectivity, ion exchange capacity, and high surface specific area (Koshy and Singh, 2016). Nevertheless, the major scientific studies in this field focus primarily on treatment of heavy metals (Petrus and Warchoř, 2005 and Nascimento et al., 2009), leaving open the issue of its efficiency with respect to organic molecules like most EDCs.

Once in water systems, organic molecules, including EDCs, can dissolve but they will also interact with other substances or with particles in suspension. Aquatic humic substances (AHS) constitute the most important class of complexation agents present in the natural aquatic environment and they play a key role in the transport and behavior of EDCs (Sun et al., 2007). In a study conducted by Botero et al. (2011), it was observed that the availability of endocrine disruptors can be directly influenced by the presence of humic substances in aquatic systems. Therefore, studies about the interaction between AHS and endocrine disruptors are vital for a better understanding of the transport and availability of EDCs in the environment.

Taking all this into account, the main goal of the present work was to assess the efficiency of three simple and inexpensive treatments to remove estrogenic and androgenic activities from waste waters. Polluted waters (prepared in the laboratory or collected in the

field) were allowed to interact with different substrates (powdered activated carbon – PAC, powdered natural zeolite – ZEO, and AHS) that could potentially be used in sectors or regions with limited resources. In vitro assays based on the use of cells stably transfected with hormonal receptors and reporter genes have been used to determine these hormonal activities in water before and after treatment. These assays can serve as a rapid, sensitive and relatively inexpensive integrative screening method to evaluate estrogenic and androgenic activities in Waste Water Treatment Plant (WWTP) effluents and receiving water courses (Jarošová et al., 2014).

2. Material and Methods

2.1. Chemicals

17 β -estradiol (E2 $\geq$ 98% purity), 17 α -ethinylestradiol (EE2 $\geq$ 98% purity), dihydrotestosterone (DHT $\geq$ 98% purity), ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS) and methanol ($\geq$ 99.9% purity) were purchased from Sigma-Aldrich (Madrid, Spain). Fetal bovine serum (FBS), penicillin–streptomycin (10,000 U mL⁻¹), hygromycin, trypsin–EDTA, geneticin, ultraglutamine, and cell culture Dulbecco's minimal essential medium (DMEM) were obtained from Lonza (Barcelona, Spain). Coenzyme A hydrate (CoA) and luciferin were purchased from Sigma-Aldrich. Phenol red-free DMEM was from Pan-Biotech (Zaragoza, Spain). The stock solutions of E2, EE2, and DHT were prepared in dimethyl sulfoxide (DMSO) from Sigma-Aldrich.

2.2. Water samples

In this study three different kinds of water samples were used; milliQ-water; mature aquarium water (AW) collected from the INIA animal facilities housing different fish species (rainbow trout, *Oncorhynchus mykiss*, gold-fish, *Carassius auratus*); and lastly

waste water treatment plant (WWTP) effluents. MilliQ-water and AW were spiked with E2 or EE2 at 100 μM , or with DHT at 500 nM in order to observe the efficiency of the treatments to remove these substances. The applied concentrations of hormones were those that allowed an appropriate detection by the transfected cells used in this work that exhibited different sensitivities. WWTP effluent waters were collected in August 2015 from four WWTP processing waste water from different areas of Madrid city. Water was collected in 1 L amber glass bottles, and transported immediately to the laboratory. Once in the laboratory, water samples were filtered through filter paper and stored at 4 °C. Extraction for the initial assessment of endocrine activity or application of treatments (see below) for removing such activity were performed within 48 h in order to avoid any degradation.

2.3. Adsorption treatments

Water samples were treated with three different substrates. One PAC of mineral origin (Synth, Brazil) that was applied to waters at 500 mg L⁻¹. This AC has been characterized previously (de Costa et al., 2015) and exhibits a BET surface area of 542 m² g⁻¹. AHS previously extracted from the Brazilian Iguape and Itapanhaú rivers were used at 30 mg L⁻¹. Finally, a clinoptilolite ZEO (from Celta Brasil, Cotia, Brazil) that was added to waters at 500 mg L⁻¹. For treatment, each adsorbent was added to different water samples (milliQ or AW contaminated in laboratory with E2, EE2, or WWTP samples) and maintained for 2 h in constant agitation and light deprivation. Before extracting EDCs from waters the substrates were retired. In the case of PAC and AHS waters were filtered through simple laboratory filter paper. ZEO was retired by centrifugation for 10 min at 1250 g using a Gyrozen 1248R centrifuge (Gyrozen, Korea).

2.4. Extraction of EDCs from water

Endocrine disrupting chemicals present in waters were extracted by solid-phase extraction (SPE) following a methodology already described (Valdehita et al., 2014). These extracts were obtained from original MilliQ, AW, and WWTP effluents, from MilliQ waters and AW to which hormones were added, and from all these waters after application of treatments for the removal of endocrine activities. Briefly, the pH of all samples were adjusted to pH 8 with NaOH. Thereafter, SPE commercial Oasis HLB cartridges from Waters (Mildford, MA, USA) were preconditioned with 6 mL of methanol and 5 mL of HPLC-grade deionized water (pH 8) at a flow rate of 1 mL min⁻¹. After the conditioning step, 100 mL of water passed through the cartridges at a flow rate of 10 mL min⁻¹. Thereafter the cartridges were rinsed with 5 mL of deionized water pH 8 and dried under an air stream to remove the excess of water. Finally, the retained analytes were eluted with 2 × 4 mL of methanol at 1 mL min⁻¹. The extracts were evaporated until dryness using a vacuum dry system (miVac Duo concentrator, Genevac, Spain). The samples were then reconstituted with 300 µL of methanol and stored at -20 °C until analysis in the in vitro assays (maximal 24 h thereafter). Recovery tests were designed to evaluate the efficiency of the extraction method. For that, 300 µL of a solution of 100 µM E2, 100 µM EE2, and 500 nM DHT in methanol was added to 100 mL of water samples (milliQ, AW, and WWTP water) allowing the interaction of the hormones with the matrix for 30 min, and extracted as above. The resulting extracts were named water+E2, water+EE2 or water+DHT.

2.5. Cell culture and exposure to water extracts

The AR-EcoScreen cell line served to assess the androgenic activity in water extracts. This cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank. The cells are derived from a Chinese hamster ovary cell line and are stably transfected

with a plasmid containing an androgen receptor response element (derived from the rat prostate C3 gene) fused to a luciferase gene, along with a plasmid encoding the androgen receptor cDNA sequence (Kojima et al., 2003). AR-EcoScreen cells were maintained in DMEM/F-12 supplemented with 10% FBS, penicillin (100 U mL^{-1}), streptomycin (100 U mL^{-1}), hygromycin ($25 \text{ } \mu\text{g mL}^{-1}$), and zeocin ($50 \text{ } \mu\text{g mL}^{-1}$). HER-LUC cells derived from HEK-293 stably expressing sbEra and luciferase as reporter gene (Muriach et al., 2008) served to assess estrogenic activity. This cell line was maintained in DMEM supplemented with 10% FBS, 1% antibiotic mixture (penicillin/streptomycin) and 2% ultraglutamine. Both cell lines were grown in 75-cm² flasks under a 5% CO₂ humidified atmosphere at 37 °C. Cells were split weekly after detaching them with 0.5% trypsin:0.02% EDTA. Before exposure to water extracts cells were plated in 96-well microtiter plates using 100 μL of cell suspension per well at a concentration of $10^5 \text{ cells mL}^{-1}$ (AR-EcoScreen) or $2.5 \times 10^5 \text{ cells mL}^{-1}$ (HER-LUC). The cells were maintained in incubation for 24 h at 37 °C and 5% CO₂ allowing them to attach to the bottom of the wells. Thereafter, culture medium was substituted with new cell culture medium containing water extracts and cells were incubated for 24 h. To calculate the total water concentrations to which cells were exposed to, the total water volume used in the extraction process was referred to the volume of cell culture medium used in the exposure experiments (100 μL). The water concentrations used in the exposure experiments ranged from 0.013 to 3.3 $\mu\text{L of water } \mu\text{L}^{-1}$ medium (i.e. since each well received 100 μL medium, it can be assumed that cells were effectively exposed to amounts of water ranging from 1.3 to 330 μL). Positive (1 μM E₂, 1 μM EE₂ or 5 nM DHT) and negative (methanol 1%) controls (PC, NC, respectively) were included. Controls and each sample concentration were assayed in triplicate in each plate.

2.6. Screening of estrogenic and androgenic activities

After 24 h treatment, cell viability was determined by measuring the cellular metabolic activity by means of the resazurin method (O'Brien et al., 2000). Given that resazurin does not interfere with the luminescence assay, cell viability assays were performed prior to luciferase assays in the same plate (Valdehita et al., 2014). 5 μ L of the resazurin dye solution (ToxKit8, Sigma-Aldrich, Madrid, Spain) was added to each well, and plates were incubated at 37°C and 5% CO₂ for 90 min. Fluorescence was then read with a Microplate-Reader (Tecan Genios Pro, Männedorf, Switzerland) using 530 nm and 590 nm as excitation and emission wavelengths, respectively. As a positive control of cytotoxicity, 1 mM SDS was included. Luciferase activity was then measured using appropriate substrates, as previously described by Quesada-García et al. (2012). Bioluminescence was measured in the culture plates using a luminometer (Tecan Genios Pro, Männedorf, Switzerland).

2.7. Data analysis

The results are presented as mean $\pm$ standard error of the mean (SEM) of three independent experiments. Each experiment consisted in the application of adsorbents to water samples. Three independent extracts were obtained from water samples of these experiments and analyzed using the in vitro approaches as previously explained. Results of the reporter gene assay are expressed as percentage of the maximal luciferase response induced by the PCs. Luminescence data were treated as explained in the OECD TG 458 (OECD, 2016). The luminescence value (luminescence counts per second, LCPS) generated by the control wells that received only vehicle (methanol) was subtracted from the luminescence outputs from PCs and experimental samples. The estimation of the

concentration–response function and the calculation of the EC50 (effective concentration 50, i.e. the concentration causing 50% of the maximal effect) were done by fitting the luminescence results to a regression equation for a four parameter logistic sigmoidal curve: $y = \text{max}/[1 + (x/\text{EC50})^b] + \text{min}$, (where max is the maximal response observed, b is the slope of the curve, and min is the minimal response) using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). In order to assess the efficiency of the extraction in the spiking experiments, the signal generated by the EC50 of the pure hormone was compared with this generated by the same concentration after extraction from the matrix. The results are expressed as percentage of the signal generated of the hormone EC50. Hormone equivalents (DHT-Eq) for WWTP sample extract were calculated for each dilution of the extract by interpolating the corresponding luminescence output (light counts per second) values in the appropriate curve. Final DHT-Eqs in WWTP were calculated as the mean of DHT-Eqs detected in the different dilutions of the sample.

3. Results

3.1. Evaluation of the efficiency of steroids extraction from different water types by means of the reporter gene assays

Data of hormonal (estrogenic and androgenic) activity of extracts obtained from milliQ and aquaria waters spiked with E2, EE2, or DHT are shown in Fig. 1 and in Table 1. The comparison of the induction of estrogenicity caused by these extracts with the estrogenic activity induced by E2 allowed us to assess the efficiency of the hormone extraction. The potency of the estrogenic response induced by extracts from milliQ and AW spiked with E2 (Fig. 1A, Table 1) was close to that produced by pure E2. Since the dose response curves were parallel we can assess the extraction efficiency by comparing the amplitude of the curve for the highest concentration of hormone used. It is observed that efficiency of extraction

was also close to 100% for milliQ and AW respectively. As expected, EE2 presented the same recovery behavior, with EC50 values of the EE2 spiked water extracts similar to EC50 of the pure EE2 (Fig. 1B, Table 1). In the AR-EcoScreen cell line, extracts of waters (milliQ and AW) spiked with DHT induced a response dependent on the applied dose. The highest difference between the EC50 of DHT and these of water extracts corresponded to AW, that was three times that of the former (Table 1). The efficiency of the extraction was maximal in all cases (Fig. 1C, Table 1).

Figure 1. Luminescence induced by serial concentrations of hormones and by the extracts of waters (milliQ and AW) spiked with the corresponding hormones. (A) E2 and extracts of waters spiked with E2. (B) EE2 and extracts of waters spiked with EE2. (C) DHT and waters spiked with DHT. Values are mean $\pm$ SEM (n = 3).

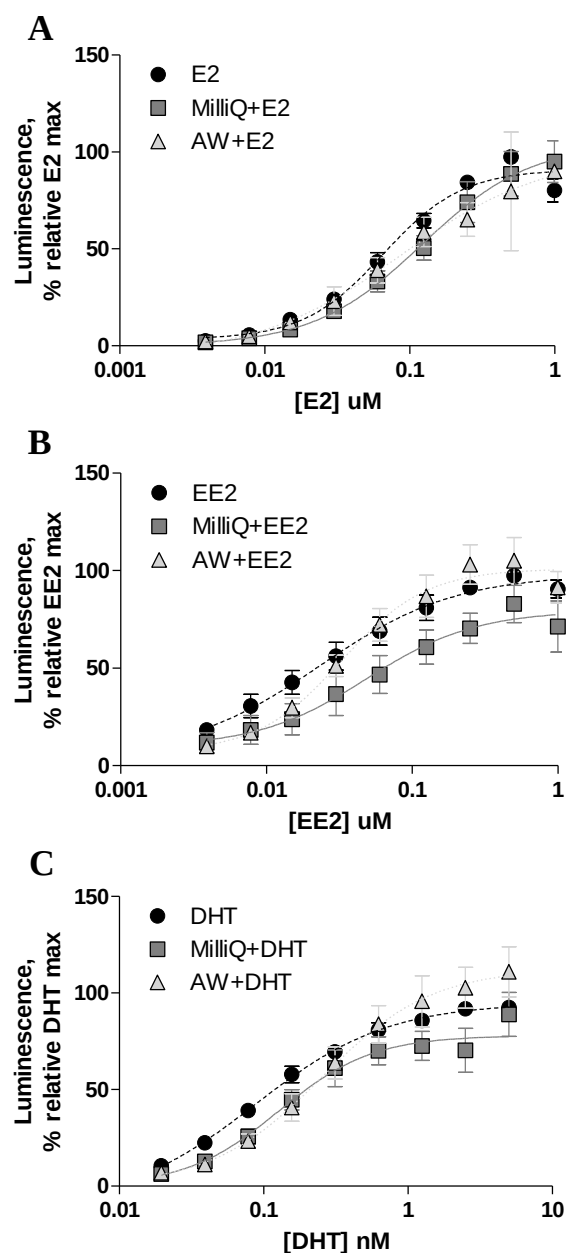


Table 1. Potency (EC_{50}) and recovery percentage of pure E2, EE2 and DHT and of extracts of waters spiked with these hormones. Values represent the mean $\pm$ SEM (n = 3).

	Estrogenicity (HER-LUC)			Androgenicity (AR-EcoScreen)	
	EC_{50} (μ M)	% Recovery		EC_{50} (nM)	% Recovery
<i>E2 pure</i>	0.065	100	<i>DHT pure</i>	0.087	100
<i>MilliQ+E2</i>	0.121	97.5	<i>MilliQ+DHT</i>	0.127	96.1
<i>AW+E2</i>	0.086	92.3	<i>AW+DHT</i>	0.254	119.9
<i>EE2 pure</i>	0.023	100			
<i>MilliQ+EE2</i>	0.048	90.2			
<i>AW+EE2</i>	0.032	107.7			

3.2. Evaluation of the efficiency of PAC, AHS and ZEO treatments on the steroids removal from waters

The efficiency of the three substrates to remove estrogenic and androgenic activities was assessed in the first place by applying them to milliQ and AW spiked with E2, EE2, or DHT. Results are shown in Fig. 2 and Table 2. Only PAC was able to remove almost completely the hormonal activities with water, leading to increases in the values of EC_{50} . The amplitude of the curves for the maximal concentrations of the hormones was compared to estimate in the worst case scenario the percentage of removal that varied for estrogenic activities between 86.4% (for milliQ waters spiked with EE2) and 99.8% (for AW spiked with E2), and for androgenic activities between 67.7% (for milliQ water spiked with DHT) and 92.1% (for AW spiked with DHT). However, low removal efficiencies were observed for the other treatments, thus invalidating its application as a complementary water treatment under the established conditions.

Figure 2. Effect of the treatment with PAC (500 mg L⁻¹), AHS (30 mg L⁻¹) and ZEO (500 mg L⁻¹) in two water kinds (milliQ and AQ) spiked with hormones. (A) 17 β -estradiol (E2) applied to MilliQ water. (B) E2 applied to aquarium water (AW). (C) 17 α -ethinylestradiol (EE2) applied to MilliQ water. (D) EE2 applied to AW. (E) Dihydrotestosterone (DHT) applied to MilliQ water. (F) DHT applied to AW. Values represent the mean $\pm$ SEM (n = 3).

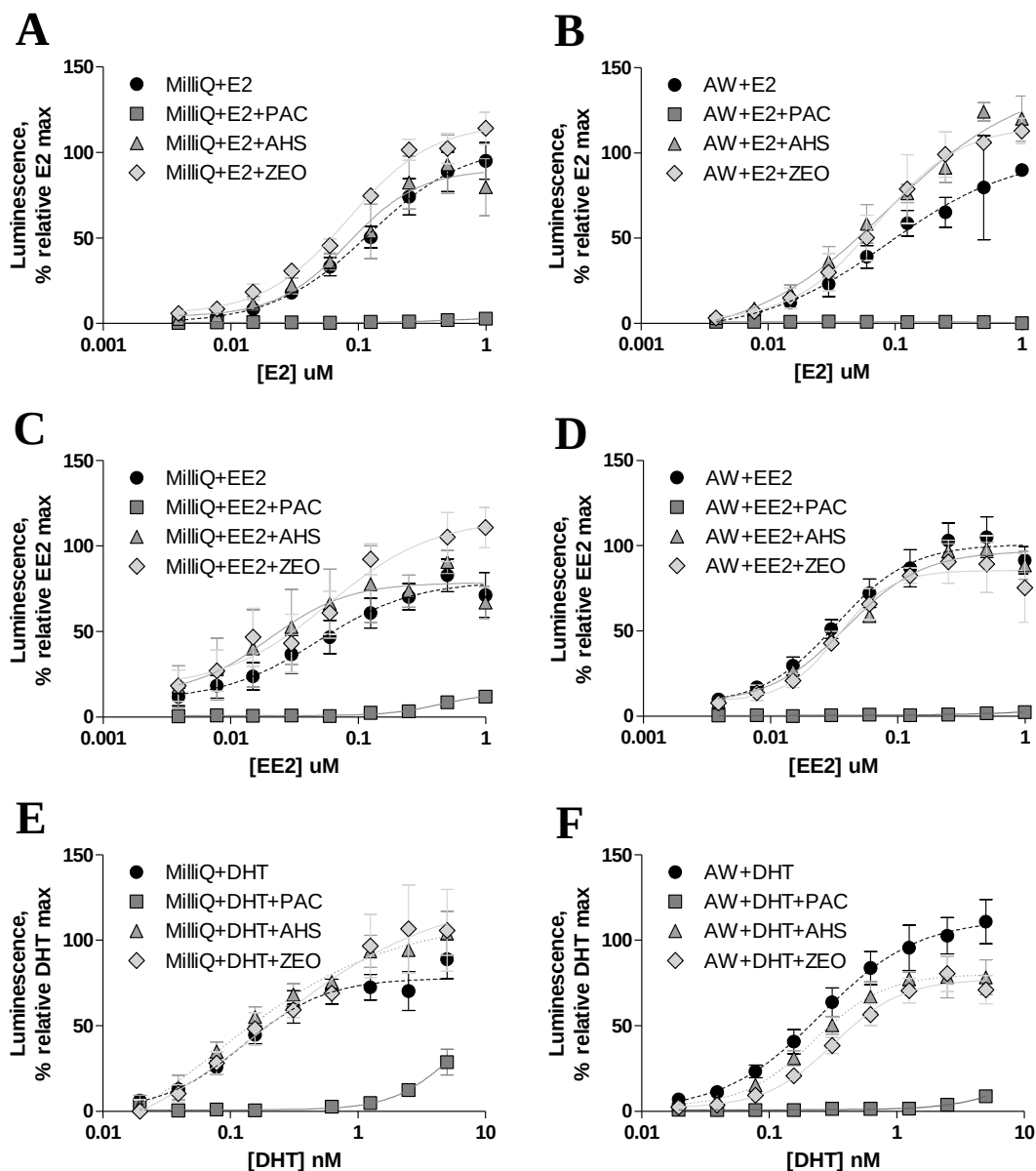


Table 2. EC₅₀ and recovery percentage calculated for extracts obtained from waters spiked with E2, EE2 and DHT and treated with PAC (500 mg L⁻¹), AHS (40 mg L⁻¹) and ZEO (500 mg L⁻¹). Values represent the mean ± SEM (n = 3).

	Estrogenicity (HER-LUC)				Androgenicity (AR-EcoScreen)		
	EC ₅₀ (μM)	Recovery (%)	Removal efficiency (%)		EC ₅₀ (nM)	Recovery (%)	Removal efficiency (%)
<i>MilliQ+E2</i>	0.121	100		<i>MilliQ+DHT</i>	0.127	100	
<i>MilliQ+E2+PAC</i>	0.595	2.9	97.0	<i>MilliQ+DHT+PAC</i>	-	32.3	67.7
<i>MilliQ+E2+AHS</i>	0.082	97.8	2.1	<i>MilliQ+DHT+AHS</i>	0.073	117.1	-
<i>MilliQ+E2+ZEO</i>	0.083	120.0	-	<i>MilliQ+DHT+ZEO</i>	0.185	119.9	-
<i>AW+E2</i>	0.086	100		<i>AW+DHT</i>	0.254	100	
<i>AW+E2+PAC</i>	-	0.1	99.8	<i>AW+DHT+PAC</i>	-	7.9	92.1
<i>AW+E2+AHS</i>	0.088	133.5	-	<i>AW+DHT+AHS</i>	0.224	70.8	29.1
<i>AW+E2+ZEO</i>	0.072	125.4	-	<i>AW+DHT+ZEO</i>	0.329	72.8	27.1
<i>MilliQ+EE2</i>	0.048	100					
<i>MilliQ+EE2+PAC</i>	0.436	13.5	86.4				
<i>MilliQ+EE2+AHS</i>	0.020	103.0	-				
<i>MilliQ+EE2+ZEO</i>	0.059	119.5	-				
<i>AW+EE2</i>	0.032	100					
<i>AW+EE2+PAC</i>	-	2.3	97.6				
<i>AW+EE2+AHS</i>	0.039	93.3	6.6				
<i>AW+EE2+ZEO</i>	0.033	85	15				

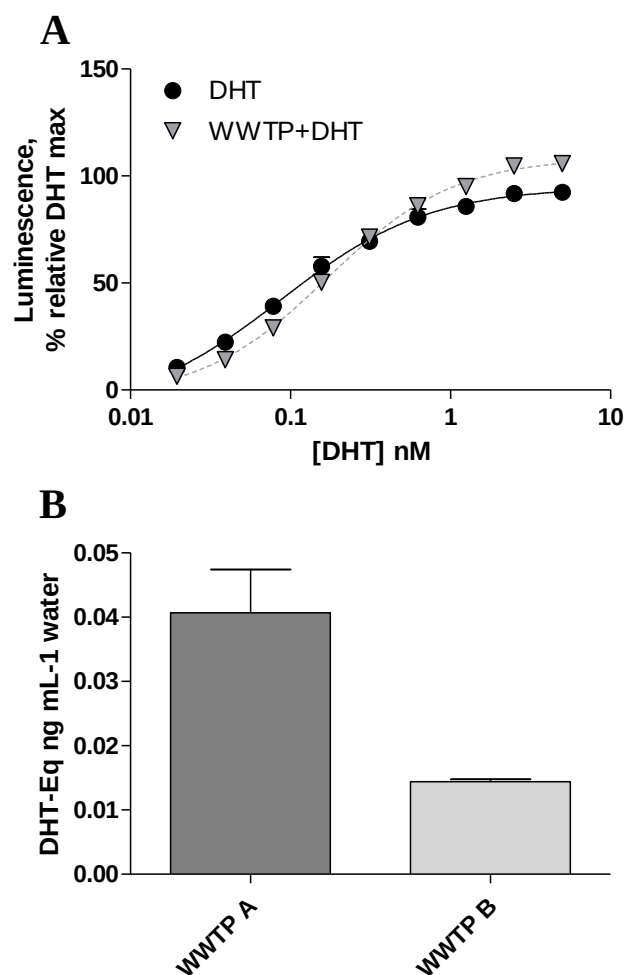
3.3. Estrogenic and androgenic activity in water samples from WWTP

In addition to the laboratory treatments described before, the capacity of the different substrates to remove hormonal activities was assessed in real WWTP effluent samples. From the four WWTP effluents collected, two of them showed androgenic activities but no

estrogenic activity was observed. Since our interest was in evaluating the capacity of removal of hormone activities by the substrates, and taking into account the structural similarities between androgens and estrogens, we decided to continue concentrating our efforts on androgen activities assuming that the obtained results could be applicable in general to estrogenicity and androgenicity.

First of all, WWTP waters not showing any androgenic activity were spiked with DHT and subjected to the extraction process as described. Results are shown in Fig. 3A. The EC_{50} of the spiked water extracts was approximately twice ($EC_{50} = 0.170$ nM) this of the pure DHT ($EC_{50} = 0.087$ nM). The efficiency of the extraction was maximal when comparing the amplitude of the curves for the maximal concentration of DHT. Therefore, we considered that the extraction androgenic activity from WWPT effluents was appropriate to assess the efficiency of the treatments. Androgenic activity in WWTP water effluents (designated as WWTP A and B) before application of treatments appears in Fig. 3B. Here we show the maximal androgenic activity observed at the maximal concentration of effluent in medium (0.33 mL effluent/ mL medium) expressed as DHT-Eq (Table 3).

Figure 3. (A) Luminiscence induced by dihydrotestosterone (DHT) and by extracts of WWTP water not showing originally any androgenic activity spiked with DHT. (B) Global androgenicity induced by extracts of two WWTP (WWTP A and B) effluents. Values are mean $\pm$ SEM (n = 3).



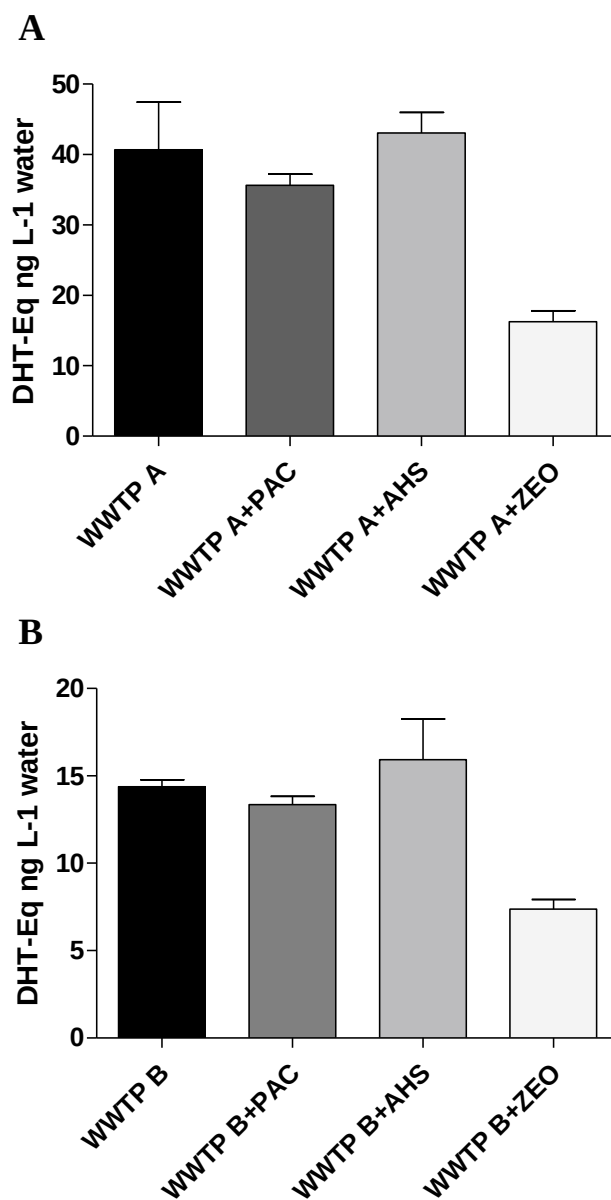
Androgenic activity in WWTP water effluents (designated as WWTP A and B) before application of treatments appears in Fig. 3B. Here we show the maximal androgenic activity observed at the maximal concentration of effluent in medium (3.3 mL effluent mL⁻¹ medium) expressed as DHT-Eq (Table 3).

Table 3. DHT-Eq present in WWTP A and B effluents, before and after treatment with PAC, AHS and ZEO. Values are mean $\pm$ SEM (n = 3).

	Androgenicity (AR-EcoScreen)	
	DHT-Eq (ng/nL)	Removal efficiency (%)
<i>WWTP A</i>	0.040	
<i>WWTP A+PAC</i>	0.035	12.3
<i>WWTP A+AHS</i>	0.043	-
<i>WWTP A+ZEO</i>	0.016	60
<i>WWTP B</i>	0.014	-
<i>WWTP B+PAC</i>	0.013	6.9
<i>WWTP B+AHS</i>	0.015	-
<i>WWTP B+ZEO</i>	0.007	48.9

The efficacy of the three treatments for reducing the androgenic activity present in two waste water samples is shown in Fig. 4 and Table 3. In contrast to the results obtained from water samples spiked in laboratory, the removal efficiency of PAC was only 12.3% and 6.9% for WWTP A and B, respectively. However, satisfactory results were revealed by treatment with ZEO showing relevant removal efficiencies of 60% and 48.9% for WWTP A and B, respectively (Table 3).

Figure 4. Androgenicity induced by the original extracts of two WWTP (WWTP A and B) effluents and by extracts of the same waters after treatment with PAC (500 mg L⁻¹), AHS (40 mg L⁻¹), and ZEO (500 mg L⁻¹). Values are mean $\pm$ SEM (n = 3).



4. Discussion and conclusion

Several approaches exist to monitor the presence of EDCs in surface waters. Traditional assessment of water contamination has been based on chemicals analyses, identifying and quantifying individual compounds, but this approach has some limitations

(Caldwell et al., 2012), related with the fact that chemical analyses are not able to evidence the presence of chemicals below the limit of detection neither to reflect the interactions (antagonistic, agonistic, additive) among the plethora of substances present in environmental samples (Valdehita et al., 2016). Therefore, the exclusive use of chemical analyses in evaluating the global toxicity or hormonal activity of environmental samples can lead to important misinterpretation of the results. Bioassays, particularly those based on *in vitro* techniques, such as those applied here, have the enormous advantage of assessing global endocrine activity, thus any contribution from any substance, even if present at trace levels, is recognized, and all the interactions are reflected in the final readout (Valdehita et al., 2014 and Valdehita et al., 2016). In the present work, *in vitro* approaches based on the use of cell lines stably transfected with the genes of interest and with reporter genes were used to detect estrogenic and androgenic activities and, hence, to evaluate the removal efficiency of hormonal activities by PAC, AHS and ZEO treatments.

According to Chang et al. (2009) the on-going EDCs research trends can be classified into three major categories, such as the identification and determination of the effects of EDCs, the development and improvement of analytical methods, and the application and modification of water treatment options for the removal of EDCs. In this sense, the investigation to find simple and inexpensive treatments that could be potentially applied in sectors or regions with limited resources should be further encouraged.

It has been reported that PAC is able to remove through adsorption processes a number of EDCs, including E1, E2, EE2, E3 and bisphenol A (Joseph et al., 2013, Schenck et al., 2012, Westerhoff et al., 2005 and Yoon et al., 2003). In the past few years, many researchers have demonstrated that AC also has a strong capability of removing a broad range of representative EDCs for artificial and real waste water in the laboratory and pilot and full-scale plants (Choi et al., 2005, Fukuhara et al., 2006, Tsai et al., 2006 and Snyder et

al., 2007). In this paper, the significant PAC removal efficiency of endocrine disruptors (E2, EE2 and DHT) from different types of waters artificially contaminated was also confirmed. However, the same treatment did not show the same effectiveness when applied to actual WWTP effluent samples, in which PAC treatment only provoked a slight decrease in androgenic activity as measured by *in vitro* bioassays. However, in accordance with Conley et al. (2016), the adsorption efficiency can vary widely depending on a number of parameters including water quality. Endocrine activity detected in effluents cannot be assigned to a single hormone (as it was the case in the clean waters use in the present work) but to a number of substances that show additive, agonistic and antagonistic interactions. Normally, these hormones exhibit some hydrophobicity and tend to interact with particles in suspension (Aris et al., 2014) and to accumulate in sediments. It is highly possible that effluents used here had a high number of micro and nanoparticles that sequestered the substances with hormonal activity avoiding their accommodation in PAC porous. In addition to this, other chemicals present in waste waters could also have interacted with the endocrine active substances contributing to their inability to enter the carbon pores.

Strikingly, natural ZEO that showed a limited efficacy in removing endocrine activities from artificially contaminated waters, were able to provoke an important reduction of androgenic activity in WWTP effluents. Commercial zeolites with different surface and pore properties have been increasingly studied for the adsorption of dissolved pollutants in water and/or waste water (Metes et al., 2004). However, such kind of applications have concentrated so far on the removal of metals (Koshy and Singh, 2016) and dyes (Tsai et al., 2009). To the extent of our knowledge, this is the first report on its effectiveness in reducing endocrine disruption in waste waters. ZEO exhibited a higher efficacy than PAC in removing hormonal activities from effluents. As indicated previously, it is possible that substances

with endocrine activity could be complexed with microparticles present in waste water and that these particles were retained by ZEO.

Regarding the possible complexation of endocrine disruptors with AHS, previously demonstrated by Botero et al. (2011), in the present work this substrate did not show any influence on the reduction of estrogenic or androgenic activities. Instead, a maximization effect on estrogenic activity was observed for a few tests. The possibility that humic substances can act or enhance endocrine disruption activity was also reported in other studies (Ren et al., 2016 and Silva et al., 2016).

In conclusion, we have observed a good efficiency of PAC in the removal of specific hormones from different kinds of waters artificially contaminated, a fact that did not ensure its good performance in the treatment of actual effluent samples. ZEO was investigated for the first time as a possible treatment for the removal of EDCs, and although it had a limited efficiency in the removal of E2, EE2, and DHT added to waters, however it has achieved significant reduction of androgenic activity in actual waste waters. AHS did not show to be effective in any of the tests. Probably, real effluents contain a plethora of substances with mixed molecular structures that will interact among them preventing or favoring the retention of endocrine active substances by the matrices used in this study. The possibility that the complexation of hormones with other molecules could prevent an appropriate activity of the applied treatments must also be explored.

Acknowledgments

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References

- Ali, I., Gupta, V.K., 2006. Advances in water treatment by adsorption technology. **Nat. Protoc.** 1 (6), 2661-2667.
- Andersen, H.R., Anderson, A.M., Arnold, S.F., Autru, H., Barfoed, M., Beresford, N.A., et al., 1999. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. **Environ. Health Persp.** 107, 89-108.
- Aris, A.Z., Shamsuddin, A.S., Praveena, S.M., 2014. Occurrence of 17 α -ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. **Environ. Intern.** 69, 104-119.
- Auriol, M., Filali-Mehnassi, Y., Tyagi, R.D., Adams, C.D., Surampalli, R.Y., 2006. Endocrine disrupting compounds removal from wastewater a new challenge. **Process Biochem.** 41, 525-539.
- Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., Samperi, R., 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. **Environ. Sci. Technol.** 34 (24), 5059-5066.
- Botero, W.G., Oliveira, L.C., de Cunha, B.B., Oliveira, L.K., de Goveia, D., Rocha, J.C., Fraceto, L.F., Rosa, A.H., 2011. Characterization of the interactions between endocrine disruptors and aquatic humic substances from tropical rivers. **J. Braz. Chem. Soc.** 22 (6), 1103-1110.
- Caldwell, D.J., Mastrocco, F., Anderson, P.D., Lange, R., Sumpter, J.P., 2012. Predicted no-effect concentrations for the steroid estrogens estrone, 17 β -estradiol, estriol, and 17 α -ethynylestradiol. **Environ. Toxicol. Chem.** 31 (6), 1396-1406.
- Chang, H., Choo, K., Lee, B., Choi, S., 2009. The methods of identification, analysis, and removal of endocrine disrupting compounds (EDCs) in water. **J. Hazard. Mater.** 172, 1-12.
- Choi, K.J., Kim, S.G., Kim, C.W., Kim, S.H., 2005. Effects of activated carbon types and service life on removal of endocrine disrupting chemicals: amitrol, nonylphenol, and bisphenol A. **Chemosphere** 58, 1535-1545.
- Combalbert, S., Bellet, V., Dabert, P., Bernet, N., Balaguer, P., Hernandez-Raquet, G., 2012. Fate of steroid hormones and endocrine activities in swine manure disposal and treatment facilities. **Water Res.** 46, 895-906.
- Conley, J.M., Evans, N., Mash, H., Rosenblum, L., Schenck, K., Glassmeyer, S., Furlong, E.T., Kolpin, D.W., Wilson, V.S., 2016. Comparison of in vitro estrogenic activity and estrogen concentrations in source and treated waters from 25 U.S. drinking water treatment plants. **Sci. Total Environ.** <http://dx.doi.org/10.1016/j.scitotenv.2016.02.093>. ISSN 0048-9697.

D'Ascenzo, G., DiCorcia, A., Gentili, A., et al., 2003. Fate of estrogen conjugates in municipal sewage transport and facilities. **Sci. Total Environ.** 302, 199-209.

de Costa, P.D., Furmanski, L.M., Domingui, L., 2015. Produção, caracterização e aplicação de carvão ativado de casca de nozes para adsorção de azul de metileno. **Ver. Virtual Quim.** 7, 1272-1285.

Dias, A.C.V., Gomes, F.W., Bila, D.M., Sant'Anna Jr., G.L., Dezotti, M., 2015. Analysis of estrogenic activity in environmental waters in Rio de Janeiro state (Brazil) using the yeast estrogen screen. **Ecotox. Environ. Safe.** 120, 41-47.

Fukuhara, T., Iwasaki, S., Kawashima, M., Shinohara, O., Abe, I., 2006. Adsorbability of estrone and 17 β -estradiol in water onto activated carbon. **Water Res.** 40 (2), 241-248.

Grover, D.P., Balaam, J., Pacitto, S., Readman, J.W., White, S., Zhou, J.L., 2011. Endocrine disrupting activities in sewage effluent and river water determined by chemical analysis and in vitro assay in the context of granular activated carbon upgrade. **Chemosphere** 84 (10), 1512-1520.

Jarosova, B., Blaha, L., Giesy, J.P., Hilscherova, K., 2014. What level of estrogenic activity determined by in vitro assays in municipal wastewaters can be considered as safe? **Environ. Inter.** 64, 98-109.

Jiang, J.Q., Yin, Q., Zhou, J.L., Pearce, P., 2005. Occurrence and treatment trials of endocrine disrupting chemicals (EDCs) in wastewaters. **Chemosphere** 61, 544-550.

Joseph, L., Boateng, L.K., Flora, J.R.V., Park, Y., Son, A., Badawy, M., Yoon, Y., 2013. Removal of bisphenol A and 17 α -ethinylestradiol by combined coagulation and adsorption using carbon nanomaterials and powdered activated carbon. **Sep. Purif. Technol.** 107, 37-47.

Kojima, H., Iida, M., Katsura, E., Kanetoshi, A., Hori, Y., Kobayashi, K., 2003. Effects of a diphenyl ether-type herbicide, chlornitrofen, and its amino derivative on androgen and estrogen receptoractivities. **Environ. Health Perspect** 111, 497-502.

Koshy, N., Singh, D.N., 2016. Fly ash zeolites for water treatment applications. **J. Environ. Chem. Eng.** 4 (2), 1460-1472.

Metes, A., Kovacevic, D., Vujevic, D., Papic, S., 2004. The role of zeolites in wastewater treatment of printing inks. **Water Res.** 38 (14e15), 3373-3381.

Muriach, B., Cerda-Reverter, J.M., Gomez, A., Zanu, S., Carrillo, M., 2008. Molecular characterizationand central distribution of the estradiol receptor alpha (ER α) in the sea bass (*Dicentrarchus labrax*). **J. Chem. Neuroanat** 35, 33-48.

Nascimento, M., Soares, P.S.M., Souza, V.P.D., 2009. Adsorption of heavy metal cations using coal fly ash modified by hydrothermal method. **Fuel** 88 (9), 1714-1719.

O'Brien, J., Wilson, I., Orton, T., Pognan, F., 2000. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. **Eur. J. Biochem** 267, 5421-5426.

OECD, 2016. **Test Guide for testing of chemicals No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals.**

Petrus, R., Warchoř, J.K., 2005. Heavy metal removal by clinoptilolite. An equilibrium study in multi-component systems. **Water Res.** 39 (5), 819-830.

Quesada-García, A., Valdehita, A., Fernández-Cruz, M.L., Leal, E., Sanchez, E., Martín-Belinchon, M., Cerda-Reverter, J.M., Navas, J.M., 2012. Assessment of estrogenic and thyrogenic activities in fish feeds. **Aquaculture** 338e341, 172-180.

Redding, A.M., Cannon, F.S., Snyder, S.A., Vanderford, B.J., 2009. A QSAR-like analysis of the adsorption of endocrine disrupting compounds, pharmaceuticals, and personal care products on modified activated carbons. **Water Res.** 43, 3849-3861.

Ren, D., Huang, B., Xiong, D., He, H., Meng, X., Pan, X., 2016. Photosensitized degradation of 17 β -estradiol and 17 α -ethinylestradiol: role of humic substances Fractions. **J. Environ. Qual.** 45 (2), 693-700.

Schenck, K., Rosenblum, L., Wiese, T., Wymer, L., Dugan, N., Williams, D., Mash, H., Merriman, B., Speth, T., 2012. Removal of estrogens and estrogenicity through drinking water treatment. **J. Water Health** 10 (1), 43-55.

Scholz, S., Renner, P., Belanger, S.E., Busquet, F., Davi, R., Demeneix, B.A., et al., 2013. Alternatives to in vivo tests to detect endocrine disrupting chemicals (EDCs) in fish and amphibians - screening for estrogen, androgen and thyroid hormone disruption. **Crit. Rev. Toxicol.** 43, 45-47.

Silva, C.P., Lima, D.L.D., Groth, M.B., Otero, M., Esteves, V.I., 2016. Effect of natural aquatic humic substances on the photodegradation of estrone. **Chemosphere** 145, 249-255.

Snyder, S.A., Adham, S., Redding, A.M., Cannon, F.S., DeCarolis, J., Oppenheimer, J., Wert, E.C., Yoon, Y., 2007. Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals. **Desalination** 202, 156-181.

Stasinakis, A.S., Gatidou, G., Mamais, D., Thomaidis, N.S., Lekkas, T.D., 2008. Occurrence and fate of endocrine disruptors in Greek sewage treatment plants. *Water Res.* 42, 1796-1804. Sun, W.L., Ni, J.R., Xu, N., Sun, L.Y., 2007. Fluorescence of sediment humic substance and its effect on the sorption of selected endocrine disruptors. **Chemosphere** 66 (4), 700-707.

Ternes, T.A., Stumph, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M., 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants. I. Investigations in Germany. **Can. Braz. Sci. Total Environ.** 225, 81-90.

Tsai, W.T., Hsu, H.C., Su, T.Y., Lin, K.Y., Lin, C.M., 2006. Adsorption characteristics of bisphenol-A in aqueous solutions onto hydrophobic zeolite. **J. Colloid Interf. Sci.** 299, 513-519.

Tsai, W., Hsien, K., Hsu, H., 2009. Adsorption of organic compounds from aqueous solution onto the synthesized zeolite. **J. Hazard. Mater.** 166 (2-3), 635-641.

Valdehita, A., Quesada-García, A., Delgado, M.M., Martín, J.V., García-Gonzalez, M.C., Fernández-Cruz, M.L., Navas, J.M., 2014. In vitro assessment of thyroidal and estrogenic activities in poultry and broiler manure. **Sci. Total Environ.** 472, 630-641.

Valdehita, A., Fernández-Cruz, M.L., Gonzalez-Gullon, M., Becerra-Neira, E., Delgado, M.M., García-Gonzalez, M.C., Navas, J.M., 2016. Androgens and androgenic activity in broiler manure assessed by means of chemical analyses and in vitro bioassays. **Environ. Toxicol. Chem.** 9999 (9999), 1-9.

Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water processes. **Environ. Sci. Technol.** 39, 6649-6663.

Ying, G., Kookana, R.S., Ru, Y., 2002. Occurrence and fate of hormone steroids in the environment. **Environ. Inter.** 28 (6), 545-551.

Yoon, Y., Westerhoff, P., Snyder, S.A., Esparza, M., 2003. HPLC-fluorescence detection and adsorption of bisphenol A, 17b-estradiol, and 17a-ethynylestradiol on powdered activated carbon. **Water Res.** 37, 3530-3537.

Zhang, Y., Zhou, J.L., 2005. Removal of estrone and 17b-estradiol from water by adsorption. **Water Res.** 39, 3991-4003.

Zhang, Y., Zhou, J.L., 2008. Occurrence and removal of endocrine disrupting chemicals in wastewater. **Chemosphere** 73, 848-853.

Zhang, Y., Zhou, J.L., Ning, B., 2007. Photodegradation of estrone and 17b-estradiol in water. **Water Res.** 41, 19-26.

Zhou, J.L., Zhang, Z.L., Banks, E., Grover, D., Jiang, J.Q., 2009. Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water. **J. Hazard. Mater.** 166, 655-661.

Remediation of 17 β -estradiol (E2) and 17 α -ethinylestradiol (E2) in water: chemical and ecotoxicological approach with emphasis on the biological reversibility of Danio rerio (Cypriniformes, Cyprinidae) gonadal tissues

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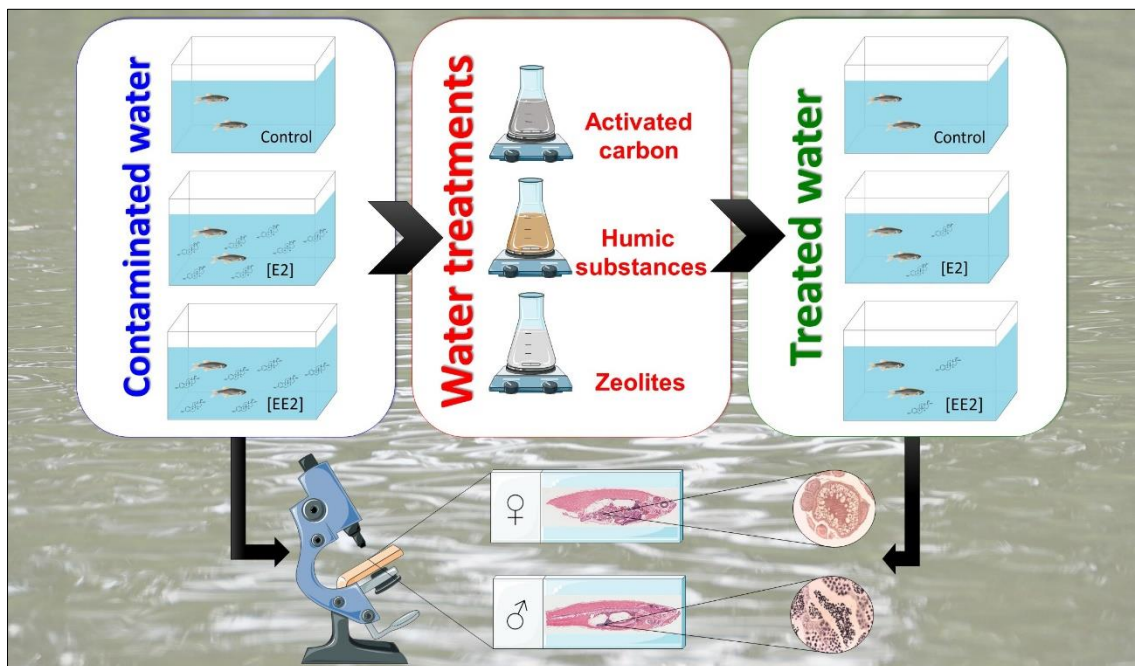
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Highlights:

- Different substrates were used to remove female sex hormones (FSHs) from water.
- Powdered activated carbon (PAC) efficiently reduced E2 and EE2 in water.
- Delayed spermatogenesis and oogenesis of zebrafish exposed to EE2.
- Minimal severity grade in female gonads exposed to E2 and EE2.
- Mild severity grade in male gonads exposed to EE2.

Abstract: The removal from the aquatic environment of compounds that influence the hormonal dynamics of biological systems is a global concern. The aim of this research was to investigate the remediation of artificially enriched 17β -estradiol (E2) and 17α -ethinylestradiol (EE2) in water by three materials (powdered activated carbon – PAC; powdered natural zeolites – ZP; and aquatic humic substances - AHS) through chemical analyses and ecotoxicological *in vivo* assays. Adult zebrafish were chronically exposed (21 days) to a relevant concentration (30 ng.L^{-1}) of each hormone in the laboratory. Water enriched with estrogens was treated with PAC, ZP or AHS for 2 h, promoting clearance of the available medium over a 7-day interval. Morphological, reproductive and histopathological effects were monitored after each experiment. PAC demonstrated adequate chemical efficiency for the removal of both hormones. Decreased embryo production and hatching rate and F1 sex ratio alterations (favoring females) were related to female exposure to E2. Spermatogenesis and oogenesis were delayed in animals exposed to EE2 in association with impaired reproduction. None of the treatments reversed the sub-lethal effects after the clearance periods. Therefore, the clearance test interval should be extended in future research.

Keywords: Remediation, Female sex hormones, Zebrafish, Histopathology of gonads.

Graphical abstract:

1. Introduction

According to Saha et al. (2010), the removal from aquatic environments of elements that influence the hormonal dynamics of biological systems is of worldwide interest. Therefore, there is a need for studies that evaluate the efficiency of existing water and effluent treatments; develop new strategies; and expand our understanding of natural processes that contribute to environmental removal, minimization or inactivation.

It is widely accepted in the scientific literature that conventional techniques for urban effluent treatment are not efficient for the complete reduction of available endocrine-disrupting chemicals (EDCs), especially estrogenic compounds. The most recently developed studies in the field confirm that such techniques (both biological and physico-chemical) remain incapable of removing these compounds, resulting in their persistence in the environment (Muller, 2008; Coleman, 2000 and Tan, 2007).

Once in the environment, the environmental components capable of influencing the activity of endocrine disrupters in the aquatic environment are aquatic humic substances (AHS), the main complexing agents that are naturally present in water, sediments, and soils (Rosa, 2008; Rocha and Rosa, 2003). However, few studies have demonstrated an association between the influence of SHA with EDC transport and bioavailability in an aquatic system (Botero et al., 2011, Sun et al., 2007), especially by verifying the toxicological influence of EDC on the biota. Characterization of the interactions of EDC with AHS may provide important data related to the availability of EDC to aquatic biota by verifying the formation and determining the stability of possible EDC-AHS complexes (Rosa, 2008).

Other materials that are recognized as efficient for the removal of organic and inorganic pollutants in water treatment systems are activated carbon and zeolites. Zhang and Zhou (2005) showed that these materials have the ability to absorb organic

micropollutants present in water and may be alternatives for reducing the toxicity of EDCs in the aquatic biota. As reported by Ahmaruzzaman (2010), natural zeolites have shown good selectivity for organic substances and, therefore, have become important materials for reducing aquatic pollutants. Activated carbon is characterized by its enormous internal surface area developed during the production process by oxidation. The adsorption capacity of a sample of carbon may depend on its content and the type of material (organic and inorganic), polarity and morphology. Yoon et al. (2003) reported that the adsorption process associated with activated carbon with a small particle size can be widely used to remove organic micropollutants from water. Nonetheless, the authors note the need for studies demonstrating the efficiencies of these materials for adsorption of minimum concentrations of pollutants as observed in natural environments.

Although there is chemical evidence of the efficacy of activated carbon for the removal of EDCs, which can range from 30% to 90% (Veras, 2006; Saha et al., 2010), it is unknown whether the concentrations remaining in the environment after adsorption trigger effects during periods of chronic exposure of the biota. This situation must be understood to generate a database capable of detecting safe concentrations for the legislative protection of aquatic life, as well as to design appropriate treatments to achieve maximum permissible concentrations of EDC pollutants.

The aim of this research was to investigate three simple methods for the remediation of IE in water using both chemical analyses and *in vivo* ecotoxicological tests to answer the following questions: 1) What is the endocrine interference of 17 β -estradiol (E2) and 17 α -ethynylestradiol (EE2) female steroids, using a representative concentration of surface water, on the reproductive characteristics and gonadal tissue histology of fish exposed to chronic ecotoxicological tests?; 2) What is the efficiency of three easily accessible and low-cost materials (powder activated carbon – PAC; powdered natural

zeolites – ZP; and aquatic humic substances - AHS) when applied as treatments to reduce the availability of E2 and EE2 in water?; 3) Can remediation be achieved by purification experiments using test solutions treated with PAC, ZP or AHS and its biologically reversing the sublethal effects observed in fish chronically exposed to E2 and EE2?

2. Methods

2.1. Testing organisms and maintenance

Adult test organisms of the *Dario rerio* species (~3 months old) were maintained according to the OECD (2013) and ABNT (2015) recommendations and guidelines in a pre-established culture at the Laboratory of Toxicology of Environmental Contaminants and Histology (LATHIS, UNESP - Sorocaba Campus, Brazil) in culture water obtained through a public water network and previously treated using an activated carbon filter (Hydro Filter POU 7" BR Carbon, Pentair). The water was filtered with a PVC (polyvinyl chloride) plankton separation vessel (dimensions of 10 x 10 cm, 25- μ m mesh opening, Liminotec, São Carlos-SP, Brazil) and dechlorinated by intense aeration (for 24 h) with a controlled pH (7.2-7.8), hardness (44 mg.L⁻¹ CaCO₃), temperature (26 $\pm$ 1°C), dissolved oxygen (>5 mg.L⁻¹) and photoperiod (12h dark:12h light). Numerous individual fish were distributed in glass aquariums with a capacity of 20-L each, preserving proportions of up to 1 gram of fish per liter of water with partial culture water replacement (25%) once per week by siphoning. Tetramin® commercial feed (47% crude protein, Águia de Ouro, Sorocaba, SP, Brazil) was administered *ad libitum* twice daily (8 a.m. and 16 p.m.) as regular feeding. Newly hatched *Artemia nauplii* (Maramar Pet, Arraial do Cabo, RJ, Brazil) were also fed *ad libitum* on a daily basis (12 h) as incorporated supplementary feed during the week prior to the mating tests.

2.2. Chemicals

17 β -estradiol (E2 $\geq$ 98% pure) and 17 α -ethinylestradiol (EE2 $\geq$ 98% pure) were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). Stock solutions of each hormone were individually prepared at a concentration of 1 g.L⁻¹ using methanol as a solvent (GC grade, $\geq$ 99% pure, Sigma-Aldrich) and maintained at -20°C during the experiment. In the absence of scientific consensus, test solutions at an environmentally relevant concentration defined of 30 ng.L⁻¹ were prepared from stock solutions diluted in culture water, and these solutions were prepared prior to each renewal of the medium during the ecotoxicological tests, avoiding the degradation of molecules over the duration of preparation or storage. Hydrochloric acid (ACS, 37%), ethyl acetate (GC Grade, $\geq$ 99.8% pure), methanol (GC grade, $\geq$ 99.8% pure), isooctane (GC grade) and activated MSTFA III obtained from Sigma-Aldrich (São Paulo, SP, Brazil) were used during the sample preparation, solid phase extraction (SPE) and derivatization of the extracts during the course of the preliminary stages of the chromatographic analysis. In addition, absolute ethyl alcohol PA ($\geq$ 99.5% pure, Synth), xylene (Solven), hydrochloric acid (PA, Synth), Bouin's solution (Biofix), Entellan® (Merck), yellow dye eosin (Suetam), Harris hematoxylin (Suetam) and histological paraffin (melting point 62°C, Solven) acquired from O Patologista (Grarulhos, SP, Brazil) were used to process and perform histological analyses. All the chemical residues generated during the experiments were sent to the university's waste committee for treatment and final disposal.

2.3. Treatments

PA grade powdered activated carbon (PAC) obtained from Synth (Diadema, SP, Brazil), powdered natural zeolites (ZP) obtained from Celta Brasil (Cotia, SP, Brazil), and natural humic substances previously extracted from the Brazilian rivers Iguape and

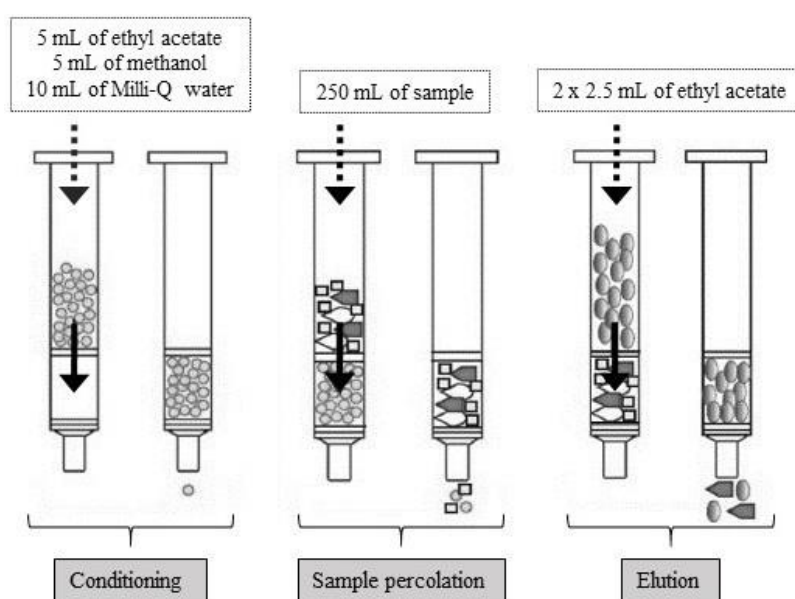
Itapanhaú (Fracácio, 2010) were the three materials investigated in this study as optional treatments for the remediation of two female sex hormones (FSHs), E2 and EE2, in water.

2.4. FSH remediation in water

Culture water previously enriched in a laboratory with FSHs (E2 and EE2, separately) was treated with three different substrates (PAC, ZP and AHS) according to the method previously described by Polloni-Silva et al. (2017). The substrates were applied at specific concentrations defined (PAC and ZP at 500 mg.L⁻¹ and AHS at 30 mg.L⁻¹) to the water samples, followed by constant agitation with light deprivation for 0 (aliquots collected after 5 min of stirring), 2 and 24 h. To determine the chemical efficiency of each treatment for the removal of the selected FSHs, SPE and chromatographic analyses were performed according to the methods described by Dallegrave (2012). These procedures were conducted at the Institute of Chemistry (IQ) of Federal University of Rio Grande do Sul (UFRGS). Briefly, 250-mL samples (aliquots in triplicate) were filtered through glass fiber microfilters (1.2-micron pore aperture) in a Millipore glass system (47 mm, Liminotec, São Carlos, SP, Brazil) and adjusted to pH 3 with HCl (1 N). Then, by operating a manifold from Agilent Technologies (Santa Clara, CA, USA), SPE Bond Elut cartridges (C18, 500 mg, Agilent Technologies) were conditioned with 5 mL of ethyl acetate followed by 5 mL of methanol and 10 mL of Milli-Q water (pH 3) at a flow rate of 1 mL/min. After conditioning, the samples were percolated through the cartridges at a flow rate of 10 mL/min for 15 min. Thereafter, the cartridges were dried under a stream of air to remove excess water for 15 min. Finally, the retained analytes were eluted with 2 x 2.5 mL of ethyl acetate at 1 mL/min to obtain their respective extracts, which were packed in amber glass vials (Figure 1). Derivatization procedures were performed by adding 100 µL of activated MSTFA III

(~5% imidazole) to each vial, followed by continuous agitation for 30 sec in a vortex. Another 100 μL of isocyanate was then added, and a Thermo Scientific TM Reacti-Therm TM Heating and Stirring Module (Thermo Fisher Scientific, São Paulo, SP, Brazil) heating system was applied for 30 min at 55°C. Subsequently, the extracts were diluted to 1 mL in isooctane and finally analyzed using a gas chromatograph (GC-MS, model QP-2010 SE, Shimadzu, Kyoto, Japan) equipped with a Restek Rtx-5MS column (30 m x 0.25 mm x 0.25 μm).

Figure 1. General diagram of SPE demonstrating the steps of cartridge conditioning, sample percolation and elution of analytes (modified from Dallegrave, 2012).



2.5. Ecotoxicological tests

2.5.1. Chronic toxicity test

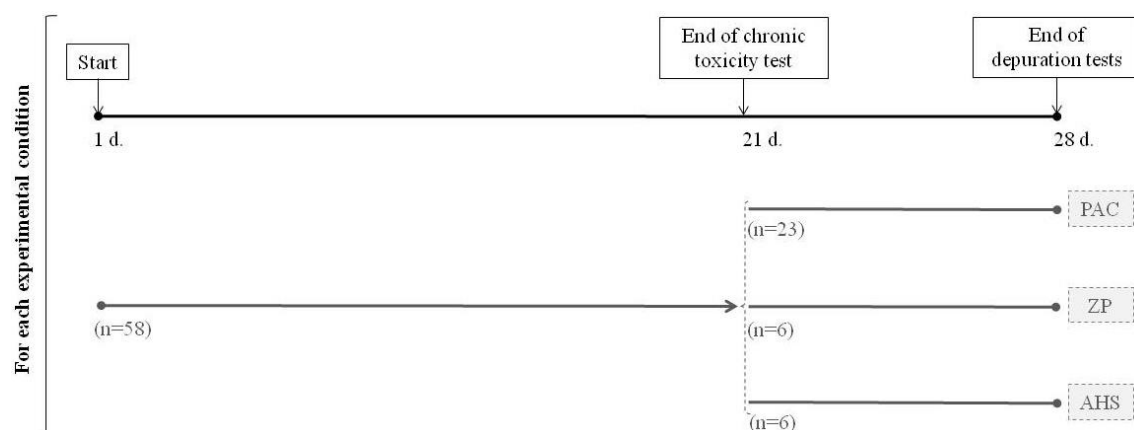
Preliminary tests performed with the maximum solvent concentration used to dilute the test solutions (methanol at 25 mL.L^{-1} , positive control) were performed by Fracácio (2010), and they showed that this concentration of solvent had no effect on the evaluated morphological, reproductive or histological parameters. Consequently, the

chronic toxicity test was prepared by exposing the test organisms to only three experimental conditions: negative control (culture water), E2 (culture water contaminated with E2) and EE2 (culture water contaminated with EE2), with both FSHs enriched at a concentration of 30 ng.L⁻¹. The semi-static laboratory test (total renewal of the test solutions every 48 h) lasted for 21 days. An equal proportion of test organisms (n=58, 32 males and 26 females) was exposed to each environmental condition in glass aquariums with a 2-L capacity, accommodating two fish of the same gender in each. At the end of the experiment, randomly selected subjects (n=14, 7 males and 7 females) within each experimental condition were subjected to euthanasia (ice submersion for 15 min) for consecutive analysis of the following morphological parameters: standard length, total mass and female gonadosomatic index (GSI) ($GSI = W_g / W_t \cdot 100$, according to Vazzoler, 1996). Among the individuals, three females and three males were randomly selected for removal and isolation of the gonads followed by immediate tissue fixation in Bouin solution (18 h), washing under running water (12 h) and dehydration in 70% ethyl alcohol, remaining in this last stage until the beginning of the histological analysis protocol. Regarding the individuals that underwent PAC treatment, 9 other organisms (6 males and 3 females, again randomly chosen) were subjected to the mating protocol performed in triplicate (3 independent experiments, ratio of 2 males/1 female), which preceded analyses of the following reproductive parameters: average embryo production per female, viability of the embryos produced and rate and hatching time of the larvae until the 7th day post-fertilization (DPF). The established F1 up to 160 DPF also analyzed the following parameters: standard length, total weight (n=7) and sex ratio (n=30). The remaining fish in this stage proceeded without interruption to the purification tests (Figure 2).

2.5.2. Depuration tests

The remaining fish ($n=35$, 19 males and 16 females) from the chronic toxicity test were subjected to 7-day clearance tests (28 experimentation days), in which the test solutions for each experimental condition (E2, EE2 and negative control) received three optional treatments for the remediation of endocrine activity (PAC, ZP and AHS), according to the method described by Polloni-Silva et al. (2017). The treatments were performed every 48 h, during the 2 h preceding each renewal of the medium. At the end of the final day of testing, for the subjects undergoing PAC treatment, the same amount and proportion of randomly selected fish at the end of the chronic toxicity test were sacrificed to repeat the previously described analyses of morphological, reproductive and histological parameters. Concerning the individuals subjected to ZP and AHS treatments, only histological analyses of gonadal tissue were performed, excluding, in these two cases, evaluations of morphological and reproductive features.

Figure 2. Timeline between the chronic toxicity and clearance tests performed in the 28-day experiment.



2.6. Mating protocol

Supplementary feeding provided by newly hatched *Artemia nauplii* was incorporated into the routine maintenance of test organisms during the final experimental week, preceding the beginning of the mating protocol performed according to OECD (2013) recommendations by exactly 7 days. To achieve this goal, glass tanks with specific measurements (45x25x20) and a capacity of 22.5 L were used, in which nylon mesh (4 mm aperture) was suspended 3 cm from the bottom, allowing the passage and fertilization of eggs and preventing their communication with parental individuals. These aquariums also sustained a higher temperature ($29^{\circ}\text{C} \pm 1$) maintained by heaters with an automatic thermostat. At the end of the day preceding the 12-h light deprivation interval pre-established according to the nocturnal photoperiod of the assay, 1 female accompanied by 2 males were introduced into each aquarium. On the following day, the incinerated lighting of the aquariums was intensified during the first 30 min of the daytime photoperiod, which functioned to initiate the mating process, with the production of eggs by the female and fertilization of eggs by the males. Finally, the produced eggs were transferred to Petri dishes, in which the total number of eggs produced per female was counted and subsequently qualified as viable (observed until the 7th day) or inviable (discarded and sacrificed on ice for 15 min). The obtained larvae were also kept in the laboratory until the establishment of the F1 generation at 160 DPF.

2.7. Histological protocol

Gonadal tissues that were previously preserved in ethyl alcohol (70%) from both sexes (triplicate) exposed to each experimental condition were processed with an automatic tissue processor (model San Pietro, O Patologista, Guarulhos, SP, Brazil) through a sequence of controlled submersions (1 h each) using the following reagents:

absolute ethyl alcohol (7 submergences) followed by xylene (3 submergences) and histological paraffin at 65°C (2 submergences), retaining reagent renewal for each subsequent submersion. Paraffin blocks were manually prepared using steel molds according to the support provided by the semi-automatic rotary microtome (MR 2014, O Patologista, Guarulhos, SP, Brazil) used to obtain the sections. Serial sections (4 µm thick) segmented by low-profile stainless steel razors from Leica Biosystems (The Pathologist, Guarulhos, SP, Brazil) were then placed on glass slides and subsequently stained with hematoxylin-eosin (H&E) using the following manual submergences: xylene (4 submergences, 3 min each), absolute ethyl alcohol (4 x 10 submergences, ~1 sec each), running water (10 submergences, ~1 sec each), hematoxylin (1 submersion, 2 min), running water (10 submergences, ~1 sec each), reactive differentiator (98% ethyl alcohol prepared at 70% + 2% hydrochloric acid, 3 submersions, ~1 sec each), running water (1 submergence, 3 min) and eosin (1 submergence, 3 min), maintaining continuous renewal of the reagents used during the procedures detailed at each stage. A PowerShot A620 digital camera (Canon, New York, USA) coupled to an Axio Scope.A1 light microscope (Zeiss, Oberkochen, Germany) was used to obtain images, which were analyzed and classified according to the OECD guide (2010) for diagnosis based on the histopathology of fish gonads following exposure to EDCs. Changes identified according to these guidelines were also compared between the investigated FSHs and after the purification tests with PAC, ZP and AHS.

2.8. Data analysis

Statistical analyses of the data obtained from the investigated morphological and reproductive parameters were performed using BioEstat version 5.3 software (Ayres et al., 2007). The Kruskal-Wallis (ANOVA) statistical test followed by the Dunn post-test

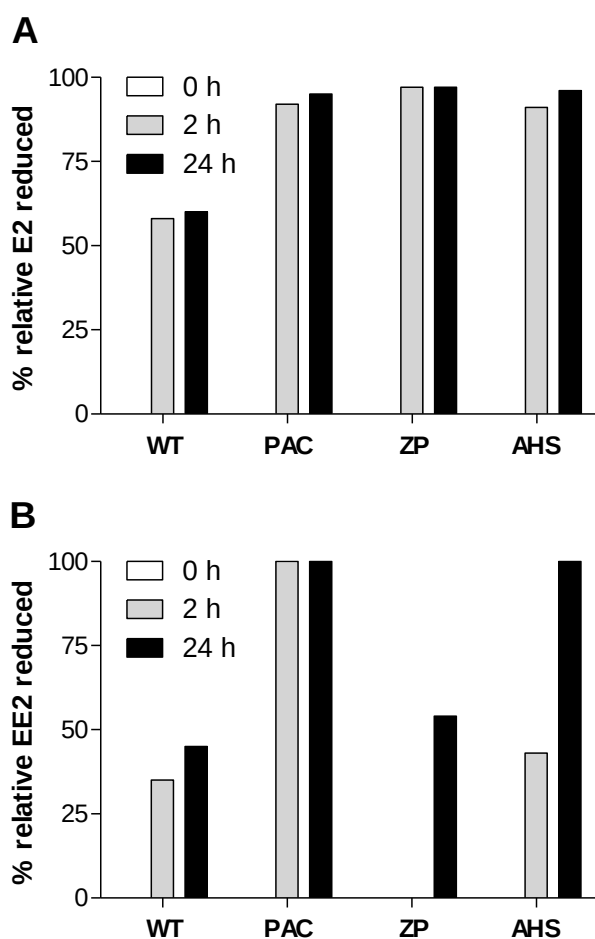
were selected to evaluate the significance of the morphological results (standard length, total mass and GSI), and the Student-Newman-Keuls post-test was used to evaluate the average egg production per female. Chi-square (X^2) tests were conducted to establish the significance of the results of hatching embryos and sex ratio rate. GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) was used to plot the graphs related to these analyses. The Carl Zeiss AxioVision Imaging System, version 4.8 (Zeiss, Oberkochen, Germany), was used for the qualitative analysis of the images established after the histological protocol utilized for female gonad assembly and marking of the figures.

3. Results

3.1. Chemical reduction

Considering the nominal concentration of 30 ng.L⁻¹ of E2 and EE2 used for the enrichment of culture waters applied to the ecotoxicological tests, chemical analyses were performed to verify the efficiency between the investigated materials (PAC, ZP and AHS) for reducing the original concentration over time (0, 2 and 24 h). The results, which were obtained by integrating the areas of the chromatographic peaks established during sample reading (triplicate) by gas chromatography, were then represented by their respective percentages of reduction achieved (Figure 3), which were separately calculated according to each hormone and treatment used.

Figure 3. Percentage reduction over time of the initial concentration of E2 (A) and EE2 (B) without treatment (WT) and after treatments with PAC, ZP or AHS.



No reductions were noted during analysis of the investigated samples from 0 h, when the aliquots were collected, to only 5 min after initiating the test.

A natural reduction of the original concentrations of E2 and EE2 was also investigated without any premeditated treatment and calculated in the same way during all studied periods. Thus, availability reductions of 58 and 60% for E2 and 35 and 45% for EE2 in water maintained under conditions of constant agitation and deprivation of light were calculated after 2 and 24 h, respectively.

The adsorbent material, ZP, presented satisfactory results during E2 treatment, achieving 97% removal of the available hormone after both 2 and 24 h. Nevertheless, the same treatment provided a poorer result when applied in the presence of the synthetic molecule EE2, presenting an efficiency of only 54%.

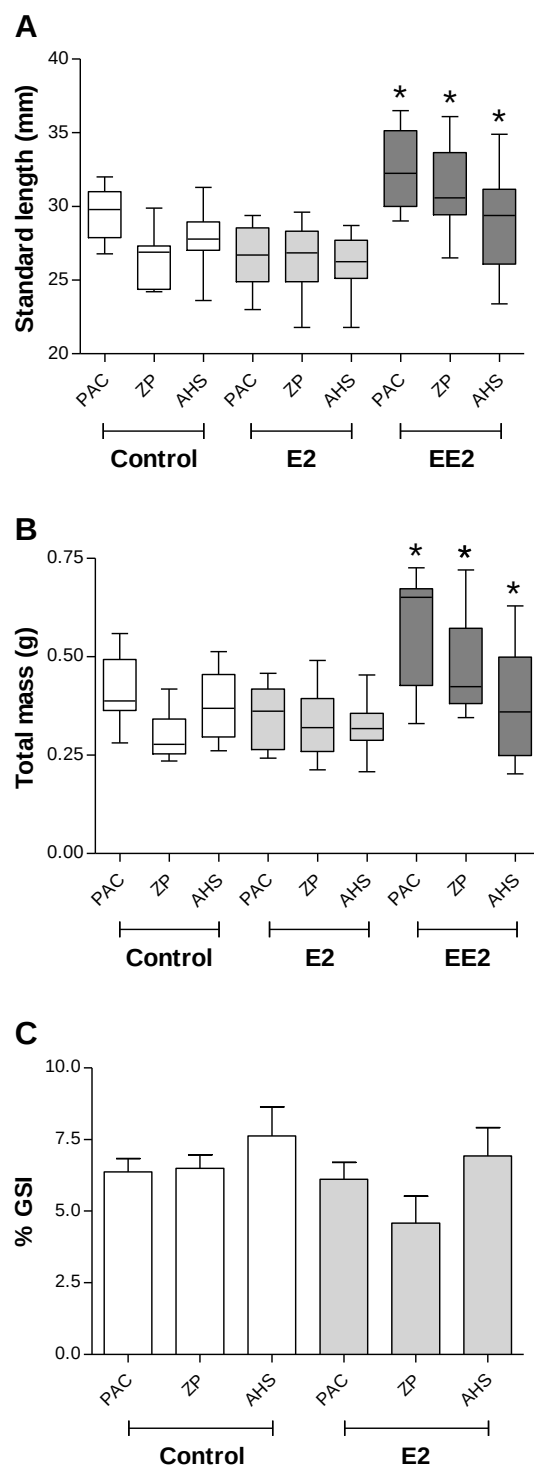
The formation of complexes in the presence of both FSHs and AHS in water also revealed interesting results. The samples treated with AHS showed 91 and 96% removal efficiency of E2 and 43 and 100% of EE2 after 2 and 24 h, respectively.

3.2. Morphological features

Significant differences ($p \leq 0.05$) in the means of standard length and total mass were identified between the group of organisms experimentally exposed to EE2 and the other groups (control and E2) (Figure 4A and B). No statistically significant differences were observed in the variations observed before and after the treatments in the context of each experimental condition.

Despite the morphological differences exhibited by the organisms exposed to EE2, there were technical difficulties during the section and isolation procedures of female gonads of individuals from the same group, which prevented the gathering of sufficient replicates for statistical analysis. Regarding the other experimental conditions, control and E2, despite the observed variations, no significant differences ($p \leq 0.05$) were observed between the statistically evaluated GSIs before and after any of the treatments (Figure 4C). The results are presented as the means $\pm$ standard error of the mean (SEM) by a minimum number of analyzed individuals ($n=7$).

Figure 4. Effects of exposure to E2, EE2 and the ability of the investigated treatments (PAC, ZP and AHS) to reduce the same HFSs in water based on the following morphological parameters: (A) standard length, (B) total mass and (C) female GSI. The values represent the means $\pm$ SEM (n=7).

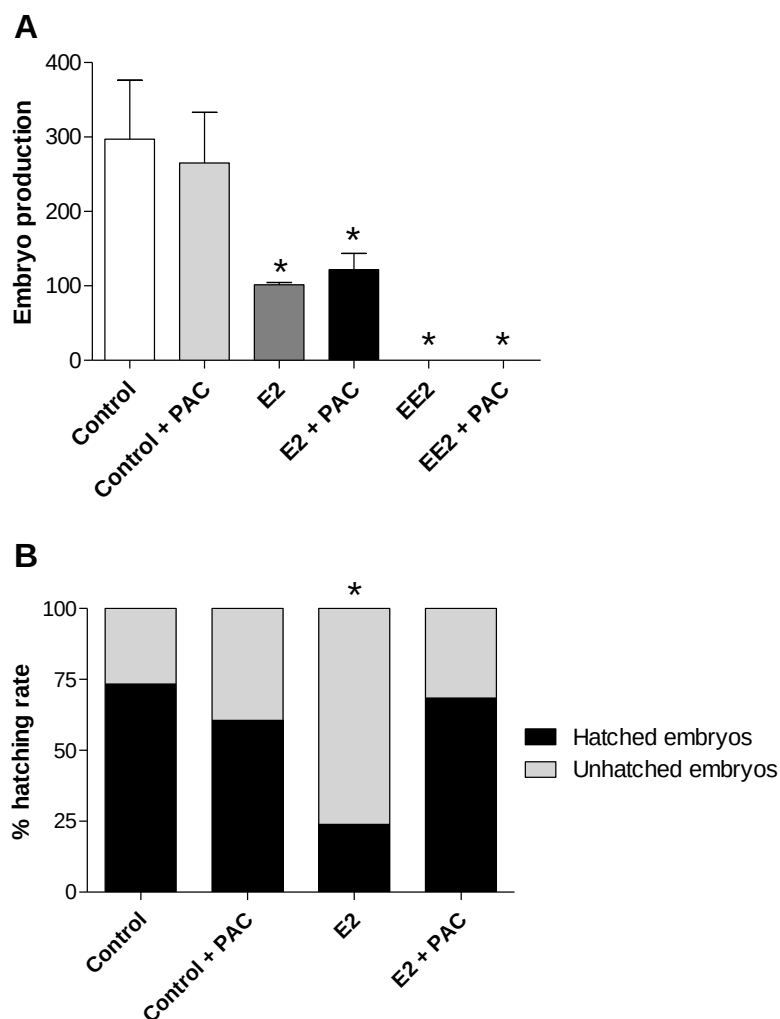


3.3. Reproductive features and F1

The average number of embryos produced by females was investigated in each of the three experimental exposure conditions (control, E2 and EE2). Individuals treated with FSH EE2, exposed to the mating protocol previously described, did not produce embryos in any of the prepared replicates. Parental controls (297 ± 79.09) and controls treated with PAC (265 ± 68.12) presented significant differences ($p \leq 0.05$) regarding the number of embryos produced after parental mating from EE2 (zero) and also from E2 (101.66 ± 2.84) and E2 treated with PAC (121.66 ± 21.78). The results are presented in Figure 5A as the means $\pm$ SEM of three independent experiments.

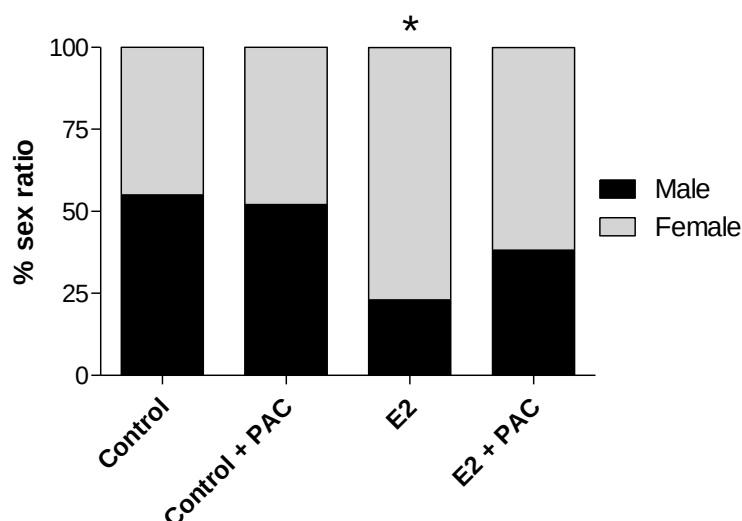
The percentage of hatched embryos was also verified, revealing a significant difference in rate ($p \leq 0.05$) between the controls (73.4%) and E2 (23.93%). However, this difference could be overcome with the application of PAC to the test solutions, resulting in an E2 hatching rate (68.49%) approximating that of the control after receiving the same treatment (60.57%) (Figure 5B).

Figure 5. Effects of exposure to E2 and application of PAC as a treatment to reduce E2 levels in water on the following reproductive parameters: (A) average number of embryos produced by females, (B) embryo hatching rate and (C) embryo hatching time. The values are shown as the means $\pm$ SEM of three independent experiments.



After establishing the F1 generation at the end of 160 DPF, have been observed the sex ratio ($\text{♂}:\text{♀}$) revealed larger trends were observed for the significant differentiation ($p \leq 0.05$) of females exposure to E2 (Figure 6).

Figure 6. Effects of exposure to E2 and application of PAC to water as a hormone-reducing treatment on the following parameters in the established F1 generation at 160 DPF: (A) standard length, (B) total mass and (C) sex ratio. The values represent the means $\pm$ SEM (n=7) related to A and B.



3.4. Histopathological features

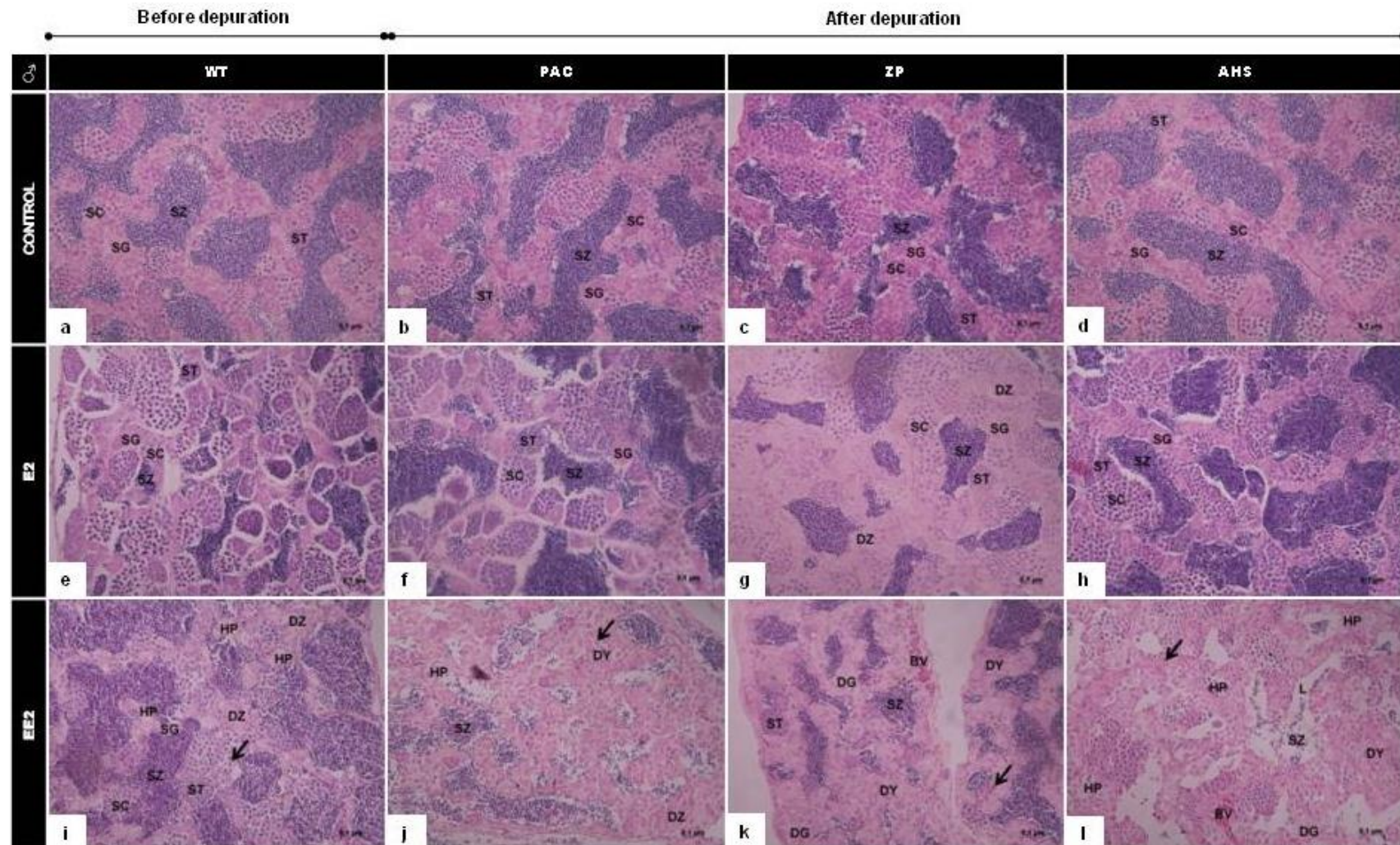
Gonadal tissues from male and female organisms were analyzed for all experimental conditions investigated and were classified according to the guidelines described by the OECD (2010) for the diagnosis of histopathologies in fish gonads associated with exposure to potential EDCs.

Considering the analysis of male individuals, images representing the obtained results are shown in Figure 7. Organisms from the control condition (before and after purification tests) demonstrated normal spermatogenesis, with asynchronous development typical of the spawning cycle of *Danio rerio* species in the adult phase. Figures 7a to d show well-organized cell nests at different stages of development (spermatogonia, spermatocytes, spermatids and spermatozoa).

Regarding exposure of the organisms to the E2 hormone (Figure 7e) and their respective treatments with PAC (Figure 7f) and AHS (Figure 7h), well-developed gonads were again observed, as presented by the control organisms. However, although developing nests were present, those organisms that were exposed to E2 and subsequently treated with ZP (Figure 7g) displayed zones indicating the beginning of a cellular degeneration process.

In tissues subjected to chronic exposure to EE2 hormone (Figure 7i), areas of cell degeneration, cell hyperplasia and morphological alterations in the nucleus were identified; these changes were distinct from the features observed in the control organisms. Despite the verified chemical reduction of the three treatments used, there was no evidence of recovery from the damage to the gonadal tissue after seven days of purification. In addition, after clearance completion, the sublethal effects became even more severe in all treatments (Figure 7j, k and l), revealing areas of cell dysplasia and indefinite spermatogenesis maturation stages, features that are usually easily determined and organized in healthy organisms.

Figure 7. Histological sections of zebrafish male gonads (H&E, 400x).



Legend: SG - spermatogonia, SC - spermatocytes, ST - spermatids, SZ - spermatozoa, BV - blood vessel, L - lumen, DZ - cell degeneration zone, DY - cell dysplasia zone, HP - cell hypertrophy and → - morphological changes in the nucleus.

Table 1 classifies the maturation stages of gonads from the male organisms analyzed in this study following the guidelines presented in the OECD guide (2010). Applying this type of qualification for the results, it was possible to verify a delay in gonadal development (spermatogenesis) presented by individuals exposed to EE2 estrogen.

Table 1. Classification of testicle stages in zebrafish male gonads according to the OECD (2010).

♂		Stages						
Treatment		Juvenile	0	1	2	3	4	5
Control	WT				✓			
	PAC				✓			
	ZP				✓			
	AHS				✓			
E2	WT				✓			
	PAC				✓			
	ZP				✓			
	AHS				✓			
EE2	WT			✓				
	PAC	✓						
	ZP	✓						
	AHS	✓						

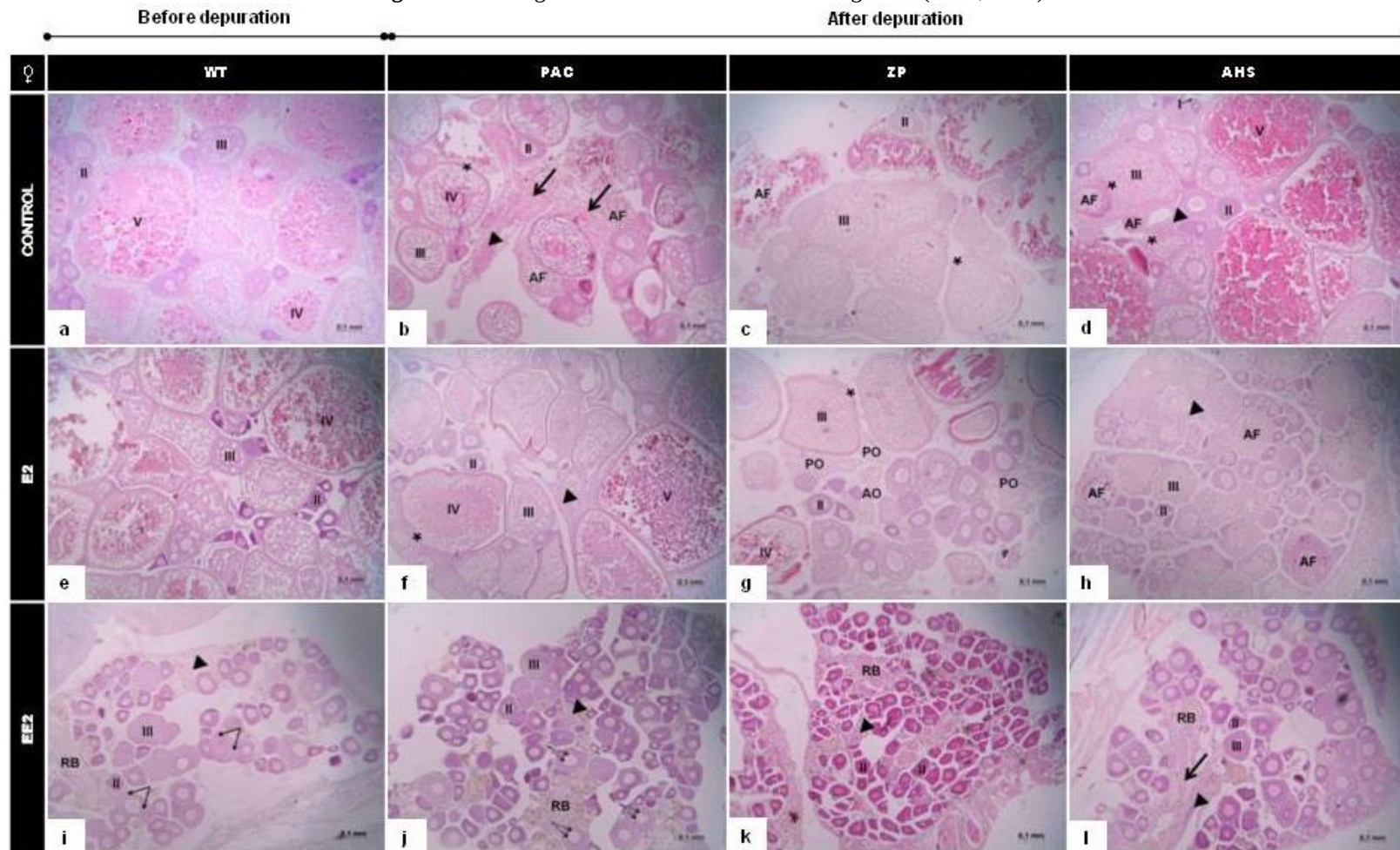
Similar to male organisms, female control gonads showed an expected asynchronous development in adult species, presenting cells at different stages of oogenesis (I, II, III, IV and V) according to the classification originally described by Vazzoler (1996). Control organisms maintained several phases after treatment with PAC (Figure 8b). However, higher frequencies of atretic follicles, perifollicular cell hyperplasia and interstitial fibrosis were revealed along with the beginning of an inflammatory process in germ cells. These same sub-lethal effects could essentially be repeated in the AHS-treated control (Figure 8d), in which granulomatous inflammation, increased atretic follicles and perifollicular hyperplasia were again observed. For the ZP-

treated control (Figure 8c), only perifollicular hyperplasia of the oocytes and an increased number of atretic follicles were observed.

Female gonads that were subjected to the hormone E2 (Figure 8e) showed normal development of the organisms, with similar results to those observed in the control. Nonetheless, inflammation between germ cells and hyperplasia of superficial epithelia were identified after treatment with PAC (Figure 8f). Regarding females exposed to E2 and treated with AHS (Figure 8h), increased frequencies of atretic follicles and granulomatous inflammation were observed. ZP application as a treatment revealed a different condition in which a larger presence of post-ovulatory follicles was noticed, despite the previously described perifollicular hyperplasia.

The results of exposure to the artificial hormone EE2 (Figure 8i al) were the most discrepant from those of the other experimental conditions. Germ cell maturation was delayed, and the conditioned organisms differed mainly because they exhibited only the initial cells in the oocyte development process, a common situation for young organisms, contradicting the expected asynchronism in oogenesis of adult fish from the evaluated species. In addition, residual bodies appeared more frequently, and an inflammatory process was observed in the germ cells in all animals that were exposed to this condition before and after the clearance assays.

The peripheral granules in the cortical region of the cytoplasm of cells presented greater dye staining, as observed in the images of organisms from the chronic EE2 assay (Figure 8i). Another additional diagnosis related to alteration of the nucleolus in the peripheral zone of the cell nucleus was observed after depuration with PAC (Figure 8j). The presence of increased fibrous connective tissue (collagenous fibers and fibrocytes or fibroblasts) within the ovarian interstitium was, at that time, only observed in the subjects treated with AHS (Figure 8l).

Figure 8. Histological sections of zebrafish female gonads (H&E, 100x).

Legend: I - young germ cells, II - oocyte from reserve stock, III - oocyte with lipidic vitellogenesis, IV - oocyte with lipid and proteinic vitellogenesis, V - oocyte with complete vitellogenesis, AF - atretic follicle, PO – post-ovulatory follicle, RB - residual body, → - interstitial fibrosis, ► - granulomatous inflammation, ★ - perfollicular cell hyperplasia, ⇔ (continuous double arrow) - cortical cytoplasmic granulation and ⇔ (segmented double arrow) - changes in the nucleolus.

Table 2 shows a classification of the maturation stages of the female gonads analyzed according to the OECD guidelines (2010). A delay in gonadal development (oogenesis) was observed in individuals that were chronically exposed to EE2.

Table 2. Classification of the ovary stages in zebrafish female gonads according to the OECD (2010).

♀		Stages						
Treatment		Juvenile	0	1	2	3	4	5
Control	WT				✓			
	PAC				✓			
	ZP				✓			
	AHS				✓			
E2	WT				✓			
	PAC				✓			
	ZP				✓			
	AHS				✓			
EE2	WT			✓				
	PAC		✓					
	ZP	✓						
	AHS		✓					

As previously described in the results regarding the histological aspects investigated in this study, some additional diagnoses were used that were not previously mentioned by the OECD guide (2010), which was considered the basis for the analysis and classifications performed. Thus, the complete list of utilized diagnoses is shown in Table 3 and 4, identifying those specific to this research. Two of the complementary diagnoses among the sub-lethal effects listed below are further detailed in Figure 9.

Table 3. Primary and secondary diagnoses with histopathological changes in male and female zebrafish after the observation of potential EDCs in this study (adapted from OECD, 2010).

	♂ OECD (2010)	This study	♀ OECD (2010)	This study
Primary Diagnoses	Increased proportion of spermatogonia	-	Increased oocyte atresia	+
	Presence of testis-ova	-	Perifollicular cell hyperplasia/hypertrophy	+
	Increased testicular degeneration	+	Decreased vitellogenesis	+
	Interstitial (Leydig) cell hyperplasia/hypertrophy	-	Changes in gonadal staging	+
Secondary Diagnoses	Decreased proportion of spermatogonia	+	Interstitial fibrosis	+
	Increased vascular/interstitial proteinaceous fluid	-	Egg debris in the oviduct	-
	Asynchronous gonad development within a single spermatocyte	-	Granulomatous inflammation	+
	Altered proportions of spermatozoa/spermatocytes	-	Decreased post-ovulatory follicles	+
	Gonadal staging	-		
	Granulomatous inflammation	-		

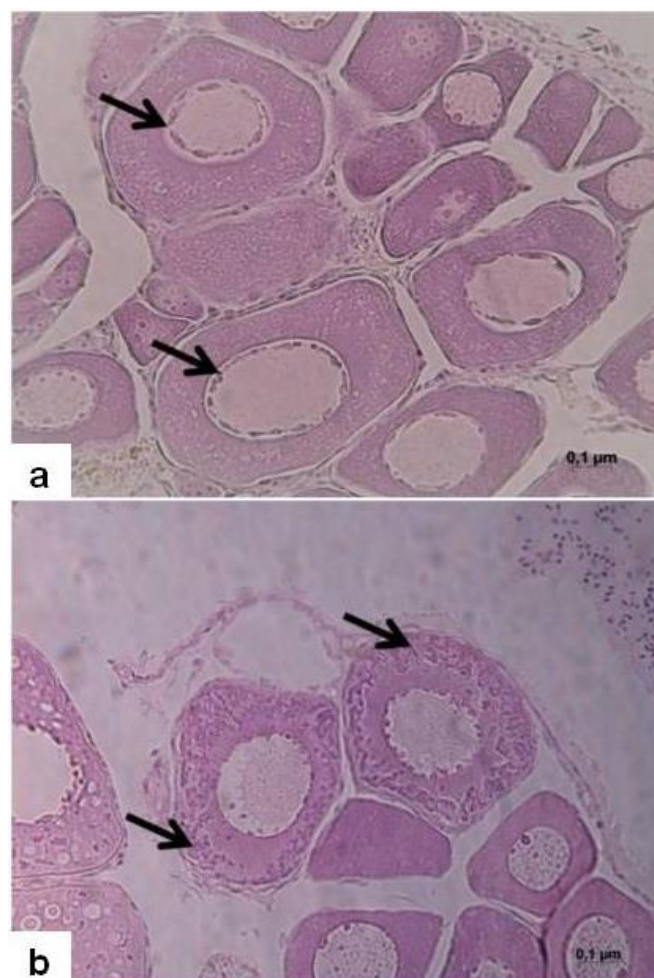
Legend: - – absent, + – present.

Table 4. Additional diagnoses with histopathological changes in male and female zebrafish after the observation of potential EDCs in this study.

♂ Additional diagnoses*	This study	♀ Additional diagnoses*	This study
Hyperplasia of testicular cells	+	Changes in the structure of the nucleolus	+
Morphological changes in the nucleus	+	Cortical cytoplasmic granulation	+
Indefinition of the spermatogenesis stages	+		

Legend: - – absent, + – present, * – observed in this study but not described in the OECD guidelines (2010).

Figure 9. Two additional diagnoses observed in this study that were not referenced in the OECD guidelines (2010): (a) changes in the structure of the nucleolus and (b) cortical cytoplasmic granulation (H&E, 400x).



In toxicological pathology, compounds may exert subtle effects on tissues that are not adequately represented by simple binary (positive or negative) responses. Therefore, the purpose of severity grading is to provide an efficient, semi-objective mechanism for comparing changes (including potential compound-related effects) among animals, treatment groups and studies (OECD, 2010).

The results of this study were also classified according to their degree of severity, and the results are presented in Table 4 with reference to the scale developed by the same OECD guide (2010) that is briefly described in Chart 1.

Chart 1. General severity grading scale according to the OECD (2010).

Grade	Description
Not remarkable	This grade is used if there are no findings associated with a particular diagnostic criterion.
1	For discrete changes, grade 1 is used when there are less than 2 occurrences per microscopic field or 1-2 occurrences per section. For multifocal or diffusely distributed alterations, this grade is used for processes involving $\leq 20\%$ of the tissue in the section.
2	For discrete changes, grade 2 is used when there are 3-5 occurrences per microscopic field or per tissue section. For multi-focal or diffusely distributed alterations, this grid is used for processes involving 20-50% of the tissue in the section.
3	For discrete changes, grade 3 is used when there are 6-8 occurrences per microscopic field or per tissue section. For multifocal or diffusely distributed alterations, this grade is used for processes involving 50-80% of the tissue in the section.
4	An overwhelming feature of the tissue. For discrete changes, grade 4 is used when there are more than 9 occurrences per microscopic field or per tissue section. For multi-focal or diffusely distributed alterations, this grade is used for processes involving $>80\%$ of the tissue in the section.

Table 4. General severity grading scale in zebrafish male and female gonads observed in this study according to the OECD (2010).

		Severity scale				
Treatment		Not remarkable	Grade 1 (Minimal)	Grade 2 (Mild)	Grade 3 (Moderate)	Grade 4 (Severe)
Control ♂	WT	✓				
	PAC	✓				
	ZP	✓				
	AHS	✓				
E2	WT	✓				
	PAC	✓				
	ZP		✓			
	AHS	✓				
EE2	WT			✓		
	PAC			✓		
	ZP			✓		
	AHS			✓		
Control ♀	WT	✓				
	PAC			✓		
	ZP		✓			
	AHS			✓		
E2	WT	✓				
	PAC		✓			
	ZP		✓			
	AHS		✓			
EE2	WT		✓			
	PAC		✓			
	ZP		✓			
	AHS		✓			

4. Discussion

Traditional assessments of water contamination have been based on chemical analyses and the identification and quantification of individual compounds; however, this approach has some limitations (Caldwell et al., 2012) because chemical analyses do not reveal the presence of chemicals below the limit of detection, nor do they reflect the interactions (antagonistic, agonistic, or additive) among the plethora of substances present in environmental samples (Valdehita et al., 2016). Therefore, exclusive use of chemical

analyses for evaluating the global toxicity or hormonal activity of environmental samples can lead to important misinterpretations of results (Polloni-Silva et al., 2017).

In this research, chemical analyses and *in vivo* approaches based on morphological, reproductive and histopathological parameters of zebrafish were conducted to evaluate the efficiency of PAC, ZP and AHS in reducing the available E2, EE2 and FSHs in water and for remediating biological injuries manifested after chronic exposure.

4.1. Discussion of chemical results

PAC is known for its ability to remove a large number of EDCs, including E2 and EE2, through chemical adsorption processes (Joseph et al., 2013; Westerhoff et al., 2005 and Yoon et al., 2003). In a previous paper, the significant efficiency of PAC for the removal of the EDCs E2, EE2 and DHT in different types of artificially contaminated waters was also confirmed using results obtained *in vitro* (Polloni-Silva et al., 2017).

Natural ZP, despite also having adsorbent properties, has not been well assessed in terms of its applicability for EDC removal. Studies on its use for the adsorption of pollutants in water have been, to date, mainly focused on the removal of metals (Koshy and Singh, 2016) and dyes (Tsai et al., 2009). The research developed by Polloni-Silva et al. (2017), although the first and only associated with this subject, did not reveal satisfactory results for ZP removal of E2 and EE2 in water that was artificially contaminated in the laboratory.

The complexation of EDCs with AHS, as previously demonstrated by Botero et al. (2011), was investigated in the present research as a possible treatment for the removal of available E2 and EE2 molecules from water. However, during a review of the scientific literature, the possibility of maximizing the effect of estrogenic activity in some

biological tests was noticed (Polloni-Silva et al., 2017). The probability that AHS could increase endocrine activity has been reported in other studies (Ren et al., 2016, Silva et al., 2016).

Considering the chemical results obtained herein for the 2 h treatment period (range used during the ecotoxicological tests), a satisfactory efficiency (above 90%) was established for all ZP, PAC and AHS materials investigated in relation to E2 (achieving reductions of 97, 92 and 91%, respectively). However, these results were not equally satisfactory when associated with the artificial steroid EE2, considering that only CAP was able to offer adequate treatment for the hormone (100%) but ZP and AHS treatments were not (54 and 43%).

These results corroborate a number of other studies available in the literature currently considering PAC as the most widely used adsorbent in wastewater treatment (Silva et al., 2012; Rivera-Utrilla et al., 2011; Babel and Kurniawan, 2003). Other authors have also reported considerable removal of FSHs using activated carbon adsorption compared with the removal rate achieved by conventional treatments in water treatment plants (ETAs) (Peres, 2011; Westerhoff et al., 2005; Fernandes, 2007; Yu et al. 2008; Rossner et al., 2009).

4.2. Discussion of morphological, reproductive and F1 results

The previously described chemical scenario (item 4.1) incorporated purification treatment of test organisms that had been previously chronically exposed to three experimental conditions (control, E2 and EE2). After this period, relevant results for biological damage and remediation were collected.

The morphological features were the first results to be observed at the end of each test. Despite the significant differences in standard length and total mass of the organisms

exposed to EE2, all fish used during the study were obtained from the same batch of animals. This occurrence was probably related to coincidence during the random selection of subjects submitted to each experimental condition.

Although the test organisms from the EE2 group were larger, considerable technical difficulty during the isolation of gonads was observed, precluding the collection of sufficient replicates to analyze GSIs. Considering only the GSI values obtained for the control and E2, no damage to the health of the evaluated organs was detected. Such data contradict the findings of Brion et al. (2004), who also investigated the exposure of zebrafish to E2 (100 ng.L⁻¹) and observed a significant reduction of the same index.

According to OECD (2013), *Danio rerio* females produce an average of 50 to 80 embryos per day per female during spawning, of which approximately 70 to 90% are viable. Therefore, the values obtained for the control in the present study can be classified within the range described by the standard.

Reproduction inhibition in the organisms subjected to EE2 hormone, even after exposure to purification tests, corroborated the results obtained by Schäfers et al. (2007), who examined chronic exposure of zebrafish to EE2 (9.3 ng.L⁻¹) that resulted in a total blockade of reproduction. This phenomenon was irreversible even after a 3-month period of clearance in clean water. Likewise, Fenske et al. (2005) observed an inhibition of embryo production when fish were exposed to only 3 ng.L⁻¹ from the first day to 180 DPF.

Similar results were reported in experiments performed with the same organism by Segner et al. (2003), who examined exposure to EE2 from fertilization to adulthood; they observed a decrease in the number of embryos per female and successful fertilization at concentrations ≥ 1.67 ng.L⁻¹. Larsen et al. (2008) observed a decreasing number of fertilized *Danio rerio* embryos in the presence of only 0.05 and 0.5 ng.L⁻¹ of EE2 from 1

to 124 DPF. These results suggest a particular influence of the synthetic hormone on the reproductive function of the species.

Still, significant differences in embryo production were observed between E2 exposure and the control, which showed reduced egg production as well as a lower hatching rate. However, this result was reversible after the clearance period upon treatment with PAC.

The sex ratio was also noticeably affected by exposure to E2, tending to differentiate females across the F1 generation from parental organisms exposed to the hormone. Brion et al. (2004) also verified such a change in the sex ratio of *Danio rerio* embryos exposed from fertilization to 42 days post-hatching (DPH) at 100 ng.L⁻¹ of E2. Other studies using low concentrations of EE2 also revealed inversions in proportions between genders of adult zebrafish (increasing the number of females in the F1 generation) during surveys using 0.05 and 0.5 ng (Larsen et al., 2008) and 15.4 ± 1.4 ng.L⁻¹ (Andersen et al., 2003). The decreased number of male offspring should be considered as a warning and may have irreversible, long term effects on the preservation and survival of the species in its natural environment.

4.3. Discussion of histopatological results

According to Connolly et al. (2014), *Danio rerio* adults display characteristics of asynchronous maturation, with continuous spawning according to the maturation of germinative cells and sequential replacement. The predominance of early stages of maturation in organisms exposed to EE2 indicated a delay in gonadal maturation in both genders, resulting in serious consequences for their reproductive success. The inhibition of reproduction in EE2-exposed parents was confirmed after analysis of gonadal tissues

from these organisms, revealing representative gonads of young individuals with restricted gonadal development caused by the influence of the synthetic hormone.

Results reported by Silva et al. (2012) reaffirmed this condition using a concentration of 100 ng.L^{-1} of EE2, which was responsible for suppressing ovarian maturation and inducing a redistribution of the gonadal maturation stage (GMS) composed mainly of developmental cells.

The delayed maturation process associated with EE2 exposure to *Danio rerio* adults had also been previously described by Lin and Janz (2006), who subjected test organisms to even lower hormone concentrations (1 and 10 ng.L^{-1}) from 2 h to 60 DP. They similarly observed a delay in gonadal development in both genders, depending on both the concentration and the exposure duration to EE2 in water. In experiments performed by Örn et al. (2003) at nominal EE2 concentrations of 1, 2, 5, 10 and 25 ng.L^{-1} , exposure of *Danio rerio* from 20 to 60 DPH revealed a predominance of immature gonads in practically all histological analyses performed for both sexes.

In a more recent work, Örn et al. (2016) reiterated that EE2, again at low concentrations, may inhibit female sexual maturation. These authors used lower concentrations (2 and 5 ng.L^{-1}) to expose *Danio rerio* juveniles from 20 to 60 DPH to EE2 and concluded that estrogen affects the differentiation of gonads in this species.

Hill and Janz (2003) also demonstrated that *Danio rerio* exposure to 10 ng.L^{-1} of EE2 from 2 h to 60 DPH resulted in a majority of fish with no gonadal development based on histological observation, suggesting that sexual differentiation and gametogenesis were delayed in both sexes at this level of exposure. The same research verified the possibility of reversal of the delayed sexual maturation after a period of purification in clean water (between 60 and 160 DPH). The results showed total reversibility of the estrogenic effects on the sexual differentiation and gonadal development of the species.

During the biological recovery assays conducted in the present study, no improvement of the sublethal damage to male and female gonadal tissues caused by EE2 was observed, resulting in the maintenance of juvenile characteristics sustained by delayed spermatogenesis and oogenesis, even after clearance tests. These results suggest the need to broaden the time interval applied for the clearance of water enriched with hormones beyond 7 days in future studies.

Considering the effects caused by the natural hormone E2 on the analyzed gonadal tissues, compared to the control, no changes in the level of maturation and distribution of GMS were identified, compatible with the reproductive age of the species.

Other specific changes were identified according to the OECD classification guidelines (2010) for primary and secondary diagnoses of histopathology regarding exposure to EDCs. Additional diagnoses were considered according to the observed injuries depending on the gonadal tissue analyses. This criterion also allowed the classification of results according to the severity scale proposed by the same guide. For males, an important severity in the classified results (grade 2) was reported in all organisms exposed to EE2. However, females exhibiting injuries that were classified into higher scales, even in the control group, after treatment with PAC and AHS (grade 2) as well as ZP (grade 1). All tissues that were chronically exposed to E2 and subsequently treated with PAC, ZP and AHS again demonstrated grade 1 severity. In contrast, all tissues from the gonads of females exposed to EE2 presented grade 1 severity prior and subsequent to the clearance tests.

The classification proposed by the OECD (2010) aims to facilitate the recognition of the severity of the exposure of fish to EDCs based on their impact on the health of gonadal tissues. The entire list of histopathological features described in these guidelines was considered alongside other alterations (3 for males and 2 for females) that were

described in this study and added to the final diagnosis. The OECD guide (2010) proved to be of great value for qualitative analyses of the obtained results. Despite its availability, most studies do not use this tool, which could facilitate histopathological diagnosis by specialists and prevent biased comparisons (false negatives or positives) using results from different laboratories. Histopathology is a descriptive and interpretive science and, therefore, somewhat subjective. Consequently, the results described in this study are also presented as an example to encourage the use of the tools provided by the OECD (2010) in future research.

5. Conclusion

In conclusion, the most suitable efficiency of PAC for the removal of E2 and EE2 hormones was observed in artificially enriched waters for ecotoxicological tests.

The inhibition of reproduction in parental organisms subjected to EE2 was observed by histopathological analysis of gonadal tissues, which revealed delayed spermatogenesis and oogenesis in these individuals; this was caused by complete interference of the synthetic steroid opposing the maintenance of the species in the environment. This condition was sustained after the respective clearance tests with PAC, ZP and AHS, suggesting a need to extend the assay interval to more than seven days in future research.

Decreased embryo production and hatching rates and alterations of the sex ratio in the F1 generation (tending toward females) were related to female exposure to E2.

Female gonads exposed to E2 and EE2 demonstrated minimal severity regardless of the clearance treatment applied. Evaluations of male gonads exposed to EE2 revealed mild severity, which again was not remedied after any clearance test.

The classification of tissues according to the OECD (2010) has been confirmed to be convenient and should therefore be encouraged in other scientific studies to facilitate comparisons between results and to avoid false negative/positive results.

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References

- ABNT (Associação Brasileira de Normas Técnicas), 2015. **Ecotoxicologia Aquática – Toxicidade crônica de curta duração – método de ensaios com peixes**. Norma ABNT-NBR 15499. Rio de Janeiro. 23p.
- Adu, R.O. and Thonsen, J.P. 2011. Improving production of zebrafish embryos in the lab. **Journal of Environmental Protection**, 2: 1360-1363.
- Ahmaruzzaman, M. 2010. A review on the utilization of fly ash. **Progress in Energy and Combustion Science**, v. 36, issue 3, 327-363.
- Andersen, L., Holbech, H., Gessbo, A., Norrgren, L.; Petersen, G.I. 2003. Effects of exposure to 17 α -ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). **Comp Biochem Physiol C Toxicol Pharmacol** 134: 365-74.
- Ayres, M.; Ayres, JR.; M.; Ayres, D.L.; Santos, A. A.. **Bio Estat 5.3**. Computacional Program (2007).
- Babel, S.; Kurniawan, T.A. 2003. Low-cost adsorbents for heavy metals uptake from contaminated water: a review. **Journal of Hazardous Materials**, Volume 97, Issues 1–3, Pages 219-243.
- Botero, W.G; Oliveira, L.C. de; Cunha, B.B; Oliveira, L.K. de; Goveia, D; Rocha, J.C; Fraceto, L.F; Rosa, A.H. 2011. Characterization of the interactions between endocrine disruptors and aquatic humic substances from tropical rivers. **Journal of the Brazilian Chemical Society**, 22(6), 1103-1110.
- Brion, F.; Tyler, C.R.; Palazzi, X.; Laillet, B.; Porcher, J.M.; Garric, J.; Flammarion, P. Impacts of 17 β -estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). **Aquatic Toxicology**, Volume 68, Issue 3, 24 June 2004, Pages 193-217.
- Caldwell, D.J., Mastrocco, F., Anderson, P.D., Länge, R., Sumpter, J.P. 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17 β -estradiol, estriol, and 17 α -ethinylestradiol. **Environ. Toxicol. Chem.** 31(6): 1396 – 1406. doi: 10.1002/etc.1825.
- Coleman, Heather M; Eggins, Brian R.; Byrne, J.Anthony; Palmer, Fiona L.; King, Emma; 2000. Photocatalytic degradation of 17- β -oestradiol on immobilised TiO₂. **Applied Catalysis B: Environmental**. 24(1),L1-L5.
- Connolly, Michelle H.; Dutkosky, Rachel M.; Heah, Tze P.; Sayler, Gary S. and Henry, Theodore B. Temporal dynamics of oocyte growth and vitellogenin gene expression in Zebrafish (*Danio Rerio*). **Zebrafish**. April 2014, 11(2): 107-114.
- Dallegrave, A. **Determinação de hormônios estrógenos e progestágenos em amostras ambientais por GC-MS**. 2012. 112f. Dissertação (Mestrado em Química), Universidade Federal do Rio Grande do Sul, Porto Alegre, 2012.

Fenske, M.; Maack, G.; Schäfers, C.; Segner, H. An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*. **Environ. Toxicol. Chem.**, 24 (2005), pp. 1088–1098.

Fernandes, R. **Estudos de Remoção de 17 α -Ethinilestradiol de Águas para Abastecimento, utilizando Dióxido de Cloro, Hipoclorito de Sódio, Carvão Ativado em Pó (CAP) e Tratamento Físico-químico**. Dissertação (Mestrado). Faculdade de Engenharia Civil, Arquitetura e Urbanismo, UNICAMP, 2007.

Fracácio, R. **Avaliação do potencial ecotoxicológico de interferentes endócrinos em amostras de água e sedimento na presença de substâncias húmicas: formulação e aplicação de sedimentos artificiais em testes ecotoxicológicos**. São Paulo: FAPESP, 2010. (Processo FAPESP 10/11537-7).

Hill, JR.; Robert, L.; Janz, D.M. 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*): I. Effects on sex ratio and breeding success. **Aquatic Toxicology**. 63(4), 417-429.

Joseph, L.; Boateng, L.K.; Flora, J.R.V.; Park, Y.; Son, A.; Badawy, M.; Yoon, Y. 2013. Removal of bisphenol A and 17 α -ethinylestradiol by combined coagulation and adsorption using carbon nanomaterials and powdered activated carbon. **Separation and Purification Technology**, 107, 37-47.

Koshy, N.; Singh, D.N. 2016. Fly ash zeolites for water treatment applications. **Journal of Environmental Chemical Engineering**, 4, 2, 1460-1472.

Larsen, M.G.; Hansen, K.B.; Henriksen, P.G.; Baatrup, E. 2008. Male zebrafish (*Danio rerio*) courtship behaviour resists the feminising effects of 17 α -ethinylestradiol morphological sexual characteristics do not. **Aquatic Toxicology**, Volume 87, Issue 4, Pages 234-244.

Lin, L.L.; Janz, D.M. Effects of binary mixtures of xenoestrogens on gonadal development and reproduction in zebrafish. **Aquatic Toxicology**, Volume 80, Issue 4, 30 December 2006, Pages 382-395.

Muller, M., Rabenoelina, F., Balaguer, P., Patureau, D., Lemenach, K., Budzinski, H., Barceló, D., López De Alda, M., Kuster, M., Delgenès, J.P. and Hernandez-Raquet, G. (2008). Chemical and biological analysis of endocrine-disrupting hormones and estrogenic activity in an advanced sewage treatment plant. **Environmental Toxicology and Chemistry**, 27: 1649–1658.

OECD (Organisation for Economic Co-operation and Development), 2010. **Guidance document on the diagnosis of endocrine-related histopathology in fish gonads**. Series on Testing and Assessment: Testing for Endocrine Disrupters No 123. OECD Publishing, Paris.

OECD, 2013. **Test No. 236: Fish Embryo Acute Toxicity (FET) Test**. OECD Publishing, Paris. DOI: <http://dx.doi.org/10.1787/9789264203709-en>

Örn, S; Holbech, H; Madsen, T.H; Norrgren, L; Petersen, G.I. Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. **Aquat Toxicol**. 2003 Dec 10;65(4):397-411.

Örn, Stefan; Holbech, Henrik; Norrgren, Leif. Sexual disruption in zebrafish (*Danio rerio*) exposed to mixtures of 17 α -ethinylestradiol and 17 β -trenbolone. **Environmental Toxicology and Pharmacology**, Vol. 41, January 2016, 225-231.

Peres, M.R. **Remoção dos interferentes endócrinos 17 α -etinilestradiol, 17 β -estradiol e 4-nonilfenol por carvão ativado em pó em água de abastecimento público**. 2011. Dissertação (Mestrado em Engenharia Civil) – Faculdade de Engenharia Civil, Arquitetura e Urbanismo, Universidade Estadual de Campinas, 2011.

Polloni-Silva, J.; Valdehita, A.; Fracácio, R.; Navas, J.M. 2017. Remediation efficiency of three treatments on water polluted with endocrine disruptors: Assessment by means of *in vitro* techniques. **Chemosphere**, 173, 267-274.

Ren, D; Huang, B; Xiong, D; He, H; Meng, X; Pan, X. 2016. Photodegradation of 17 α -ethinylestradiol in dissolved humic substances solution: Kinetics, mechanism and estrogenicity variation. **Journal of Environmental Sciences**. ISSN 1001-0742. doi: <http://dx.doi.org/10.1016/j.jes.2016.03.002>.

Rivera-Utrilla, J.; Sánchez-Polo, M.; Gómez-Serrano, Álvarez, P.M.; Alvim-Ferraz, M.C.M.; Dias, J.M. 2011. Activated carbon modifications to enhance its water treatment applications. An overview. **Journal of Hazardous Materials**, Volume 187, Issues 1-3, 15, Pages 1-23.

Rocha, J. C.; Rosa, A. H. **Substâncias húmicas aquáticas: interações com espécies metálicas**. São Paulo: Editora

Rosa, A. H. **Interferentes endócrinos em sistemas aquáticos: interações com substâncias húmicas e perspectivas de tratamento utilizando-se turfas**. São Paulo: FAPESP, 2008. (Processo FAPESP 08/09682-9).

Rossner, A.; Snyder, S.A. E Knappe, D.R.U. Removal of emerging contaminants of concern by alternative adsorbents. **Water Research**, v.43, p.3787-3796, 2009.

Saha, B.; Karounou, E.; Streat, M. Removal of 17 β -oestradiol and 17 α -ethinyl oestradiol from water by activated carbons and hypercrosslinked polymeric phases. **Reactive & Functional Polymers**, 70, pp. 531-544, 2010.

Schäfers, C.; Teigeler, M.; Wenzel, A.; Maack, G.; Fenske, M.; Segner, H. Concentration- and Time-dependent Effects of the Synthetic Estrogen, 17 α -ethinylestradiol, on Reproductive Capabilities of the Zebrafish, *Danio rerio*. **Journal of Toxicology and Environmental Health, Part A**, Vol. 70, Iss. 9, 2007

Segner H, Navas JM, Schafers C, Wenzel A. 2003. Potencies of estrogenic compounds in in vitro screening assays and in life cycle tests with zebrafish *in vivo*. **Ecotoxicol Environ Saf**, 54:315–322.

Silva, C.P.; Lima, D.L.D.; Groth, M.B.; Otero, M.; Esteves, V.I. 2016. Effect of natural aquatic humic substances on the photodegradation of estrone. **Chemosphere**, Volume 145, Pages 249-255.

Silva, P.; Rocha, M.J.; Cruzeiro, C.; Malhão, F.; Reis, B.; Urbatzka, R.; Monteiro, R.A.F.; Rocha, E. Testing the effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of fish gonads—A stereological study using the zebrafish (*Danio rerio*) as model. **Aquatic Toxicology**, Volumes 124–125, 15 November 2012, Pages 1-10,

Sun, W. L.; Ni, J. R.; Xu, N.; Sun, L. Y. Fluorescence of sediment humic substances and its effect on the sorption of selected endocrine disruptors. **Chemosphere**, v. 66, p. 700-707, 2007.

Tan, Benjamin L.L.; Hawker, Darryl W.; Muller, Jochen F.; Leusch, Frédéric D.L.; Tremblay, Louis A.; Chapman, Heather F. 2007. Comprehensive study of endocrine disrupting compounds using grab and passive sampling at selected wastewater treatment plants in South East Queensland. **Australia. Environment International**. 33 (5), July 2007, 654-669.

Tsai, W; Hsien, K; Hsu, H. 2009. Adsorption of organic compounds from aqueous solution onto the synthesized zeolite. **Journal of Hazardous Materials**, 166, 2–3, 635-641. ISSN 0304-3894.

Valdehita, A; Fernández-Cruz, M.L; González-Gullon, M; Becerra-Neira, E; Delgado M.M; García-González, M.C; Navas, J.M. 2016. Androgens and androgenic activity in broiler manure assessed by means of chemical analyses and *in vitro* bioassays. **Environ. Toxicol. Chem**, In press, 1552-8618. doi: 10.1002/etc.3495

Vazzoler, A.E.A.M. 1996. **Biologia da reprodução de peixes teleósteos: teoria e prática**. Maringá, EDUEM; São Paulo, SBI. 169p, 1996.

Veras, D.F. **Remoção dos perturbadores endócrinos 17β-estradiol e P-nonilfenol por diferentes tipos de carvão ativado em pó (CAP) produzidos no Brasil - avaliação em escala de bancada**. Dissertação (Mestrado) – Universidade de Brasília, Brasília, 2006.

Westerhoff, P., Yoon, Y., Snyder, S. E Wert, E. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. **Environmental Science & Technology**, v. 39, p.6649-6663, 2005.

Westerhoff, P.; Yoon, Y.; Snyder, S.; Wert, E. 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. **Environ Sci Technol**. Sep 1;39(17):6649-63.

Yoon, Y., Westerhoff, P., Snyder, S. A.; Esparza, M.: 2003. HPLC-fluorescence detection and adsorption of bisphenol A, 17β-estradiol, and 17α-ethynyl estradiol on powdered activated carbon. **Water Research**. 37, 3530–3537.

Yoon, Y.; Westerhoff, P.; Snyder, S.A. Adsorption of 3H-Labeled 17- β Estradiol on Powdered Activated Carbon. **Water, Air and Soil Pollution**, vol 66, No. 1-4, pp 343-351, 2005.

Yu, Z., Peldszus, S. E Huck, P. M. Adsorption characteristics of selected pharmaceuticals and an endocrine disrupting compound - Naproxen, carbamazepine and nonylphenol - on activated carbon. **Water Research**, v. 42, p. 2873-2882, 2008.

Zhang, Y.; Zhou, J.L. 2005. Removal of estrone and 17 β -estradiol from water by adsorption. **Water Res**, 39, 3991–4003.

Histoquímica como ferramenta de baixo custo aliada a análise de vitelogenina em tecidos hepáticos de Danio rerio (Cypriniformes, Cyprinidae)

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1. Introdução

A vitelogenina (VTG) é reconhecida por ser sintetizada pelo fígado de fêmeas ovíparas em resposta ao hormônio endógeno 17 β -estradiol (Funkestein et al. 2000). Não obstante, indivíduos machos (adultos e juvenis) também apresentam o receptor nuclear de estrógeno, no entanto em condições normais não costumam possuir concentrações circulantes deste hormônio suficientes para ativar seu receptor. A presença de estrógenos no ambiente, ou de compostos que mimetizem o mesmo, promove a ativação do receptor culminando com o processo de vitelogênese em machos adultos e juvenis. O fato de a VTG ser uma proteína específica de fêmeas, podendo ser expressa por machos frente a desreguladores endócrinos (DEs) estrogênicos torna esta molécula um biomarcador apropriado para revelar disfunções do sistema endócrino (Marin et al., 2004).

Atualmente o método de detecção mais amplamente utilizado para análise de VTG é a imuno-histoquímica (ELISA ou western blot). Entretanto, a quantificação da VTG pode ainda ser alcançada por meio de outras metodologias como hibridização “*in situ*”, cromatografia e reação em cadeia da polimerase em tempo real (PCR em tempo real).

Uma outra opção é a análise qualitativa da mesma por meio de uma coloração histoquímica específica.

A histoquímica associada a detecção de VTG em tecidos hepáticos pode ainda tornar-se a alternativa mais viável por apresentar baixo custo relativo as demais técnicas difundidas no meio científico e também devido a sua fácil excussão e reprodutibilidade em laboratório. Além disso, permite a observação de alterações morfológicas e histológicas associadas as análises dos tecidos, possibilitando a associação de um maior número de *endpoints* à análise. Circunstância esta inexecutável no decorrer do desenvolvimento de técnicas consideradas mais avançadas.

Neste sentido, o presente estudo teve como objetivo investigar a aplicabilidade de um protocolo histoquímico desenvolvido para identificar VTG em tecidos hepáticos de peixes expostos a EDCs em concentração ambientalmente relevante. *Danio rerio* foi dentro desta pesquisa selecionado como organismo-teste modelo utilizado durante os ensaios crônicos realizados, submetendo-se os mesmos aos hormônios sexuais femininos (HSFs) 17 β -estradiol (E2) e 17 α -etinilestradiol (EE2).

2. Metodologia

2.1. Organismo-teste e manutenção

Organismos-teste adultos da espécie *Danio rerio* (~ 3 meses de idade) estiveram mantidos em cultura pré-estabelecida no Laboratório de Toxicologia de Contaminantes Ambientais e Histologia (LATHIS, UNESP – Campus de Sorocaba, Brasil) em água de cultivo obtida via rede pública de abastecimento, previamente tratada em um filtro de carvão ativado (Hidro Filter POU 7" BR Carbon, Pentair) adquirido via Leroy Merlin (Sorocaba, SP, Brasil), filtrada em um copo de separação de plâncton em PVC (dimensões 10 x 10 cm, abertura de malha de 25 μ m, Liminotec, São Carlos SP, Brasil), desclorada

por meio de aeração intensa (durante 24 h) e ajustada para as condições controladas de pH (7,2 - 7,8); dureza (44 mg.L⁻¹ CaCO₃), temperatura (26 ± 1°C), oxigênio dissolvido (>5 mg.L⁻¹) e fotoperíodo (12h:12h, claro:escuro), segundo recomendações dos guias OECD (2013) e ABNT (2015). Lotes de indivíduos estiveram distribuídos em aquários de vidro com capacidade para 20 L, preservando-se a proporção de até 1 grama de peixe por litro de água, com trocas parciais da água de cultivo (25%) uma vez por semana por sifonamento. Ração comercial Tetramin® (47% de proteína bruta, Águia de Ouro, Sorocaba, SP, Brasil) foi ministrada “*ad libitum*” duas vezes ao dia (8 e 16 h) como alimentação regular.

2.2. Reagentes químicos

17β-estradiol (E2 ≥ 98% de pureza) e 17α-etinilestradiol (EE2 ≥ 98% de pureza) foram adquiridos a partir de Sigma-Aldrich (São Paulo, SP, Brasil). Soluções-estoque de cada hormônio foram preparadas individualmente a uma concentração de 1 g.L⁻¹, utilizando-se como solvente metanol (grau GC, ≥ 99% de pureza, Sigma-Aldrich) e mantidas a -20°C durante o experimento. Soluções de trabalho (soluções-teste) a uma concentração ambientalmente relevante de 30 ng.L⁻¹ foram realizadas a partir das solução-estoque diluídas em água de cultivo, sendo estas soluções preparadas precedendo cada renovação do meio no decorrer dos ensaios ecotoxicológicos, de forma a evitar a degradação das moléculas ao longo do tempo de preparo ou estocagem. Álcool etílico absoluto P.A. (≥99,5% de pureza, Synth), xileno (Solven), ácido clorídrico (P.A., Synth), cloreto de ferro (III) hexahidratado (P.A., Synth), ferrocianeto de potássio (P.A., Cinética), ácido acético glacial comercial (100%, Synth), solução de Bouin (Biofix), Entellan® (Merck), os corantes eosina amarela (Suetam), hematoxilina de Harris (Suetam) e vermelho neutro (65%, INLAB) e parafina histológica (ponto de fusão à 62°C,

Solven) adquiridos via O Patologista (Guarulhos, SP, Brasil) estiveram empregados no processamento e obtenção das análises histológicas e histoquímicas realizadas. Todos os resíduos químicos gerados no decurso dos experimentos foram ao final encaminhados à comissão de resíduos da universidade para tratamento e correta destinação final.

2.3. Ensaio ecotoxicológico

Ensaio preliminares realizados com a máxima concentração de solvente utilizada nas diluições das soluções-teste (metanol a 25 mL.L^{-1} , controle positivo) foram realizados por Fracácio (2012), concluindo-se não haver influência desta concentração de solvente sobre os mesmos parâmetros histológicos e histoquímicos avaliados nesta pesquisa. Desta maneira, um ensaio de toxicidade crônica foi preparado expondo-se organismos-teste a somente três condições experimentais: controle negativo (água de cultivo), E2 (água de cultivo contaminada com E2) e EE2 (água de cultivo contaminada com EE2), sendo ambos HSFs enriquecidos a concentração relevante de 30 ng.L^{-1} . Tal ensaio realizado em laboratório sob regime semi-estático (renovação total das soluções-teste a cada 48 h), teve duração de 21 dias expondo-se exclusivamente organismos-teste do gênero masculino ($n=6$) à cada condição ambiental em aquários de vidro com capacidade para 2 L, acomodando 2 peixes cada. Ao final do experimento, os mesmos indivíduos foram sujeitos a eutanásia (submersão em gelo por 15 min) para subsequente fixação dos tecidos em solução de Bouin (18 h), lavagem em água corrente (12 h) e desidratação em álcool etílico 70%, permanecendo nesta última etapa até o início dos protocolos de coloração histológico ($n=3$) e histoquímico ($n=3$).

2.4. Protocolos de coloração

Organismos-teste, provenientes de cada condição experimental, previamente reservados em álcool etílico (70%), foram processados por meio de um processador de tecidos automático (modelo San Pietro, O Patologista, Guarulhos, SP, Brasil) através de uma sequência de submersões controladas (1 h cada) aos seguintes reagentes: álcool etílico absoluto (7 submersões), seguido por xileno (3 submersões) e parafina histológica à 65°C (2 submersões), conservando-se a renovação do reativo a cada submersão subsequente. À continuação, prepararam-se manualmente blocos de parafina em moldes de aço, conforme suporte oferecido pelo micrótomo semiautomático rotativo (MR 2014, O Patologista, Guarulhos, SP, Brasil) utilizado para obtenção dos cortes. Cortes seriados (4µm de espessura) segmentados através de navalhas de aço inoxidável de perfil baixo da Leica Biosystems (O Patologista, Guarulhos, SP, Brasil) foram em seguida dispostos sobre lâminas de vidro que, posteriormente, encaminharam-se às colorações com hematoxilina-eosina (H.E.) (protocolo histológico) ou vermelho neutro (protocolo histoquímico). Para a conclusão do protocolo histológico realizaram-se sucessivas submersões manuais, resumidamente descritas a seguir: xileno (4 submersões, 3 min cada), álcool etílico absoluto (4 x 10 submersões, ~ 1 seg. cada), água corrente (10 submersões, ~ 1 seg. cada), hematoxilina (1 submersão, 2 min), água corrente (10 submersões, ~ 1 seg. cada), reativo diferenciador (98% de álcool etílico preparado a 70% + 2% ácido clorídrico) (3 submersões, ~ 1 seg. cada), água corrente (1 submersão, 3 min), eosina (1 submersão, 3 min), mantendo-se a renovação contínua dos reativos utilizados no decorrer dos procedimentos detalhados a cada etapa. Para a execução do protocolo histoquímico, uma sequência distinta de submersões manuais foi realizada conforme descrição a seguir: xileno (2 submersões, 2 min cada), álcool etílico absoluto (2 x 10 submersões, ~ 1 seg. cada), solução de cloreto de ferro hexahidratado (10 mM.L) (1

submersão, 60 min), água corrente (15 submersões, ~ 1 seg. cada), solução de Prussian Blue (ferrocianeto de potássio 2% + ácido clorídrico 2%) (1 submersão, 10 min), água corrente (10 submersões, ~ 3 min cada). Solução de vermelho neutro (75%) (1 g preparado em 100 mL de água destilada e 1 mL de ácido acético glacial) (1 submersão, 1 min), água corrente (1 submersão, ~ 1 min), álcool etílico absoluto (15 submersões, ~ 1 seg. cada) e xileno (15 submersões, ~ 1 seg. cada), mantendo-se a renovação contínua dos reativos utilizados no decorrer dos procedimentos detalhados a cada etapa. Ao final das etapas de coloração, lâminas foram montadas de forma permanente através da inclusão de Entellan® sob lamínulas de vidro. Uma câmera fotográfica digital PowerShot A620 (Canon, New York, EUA) acoplada a um microscópio de luz Axio Scope.A1 (Zeiss, Oberkochen, Alemanha) foram utilizados na obtenção das imagens.

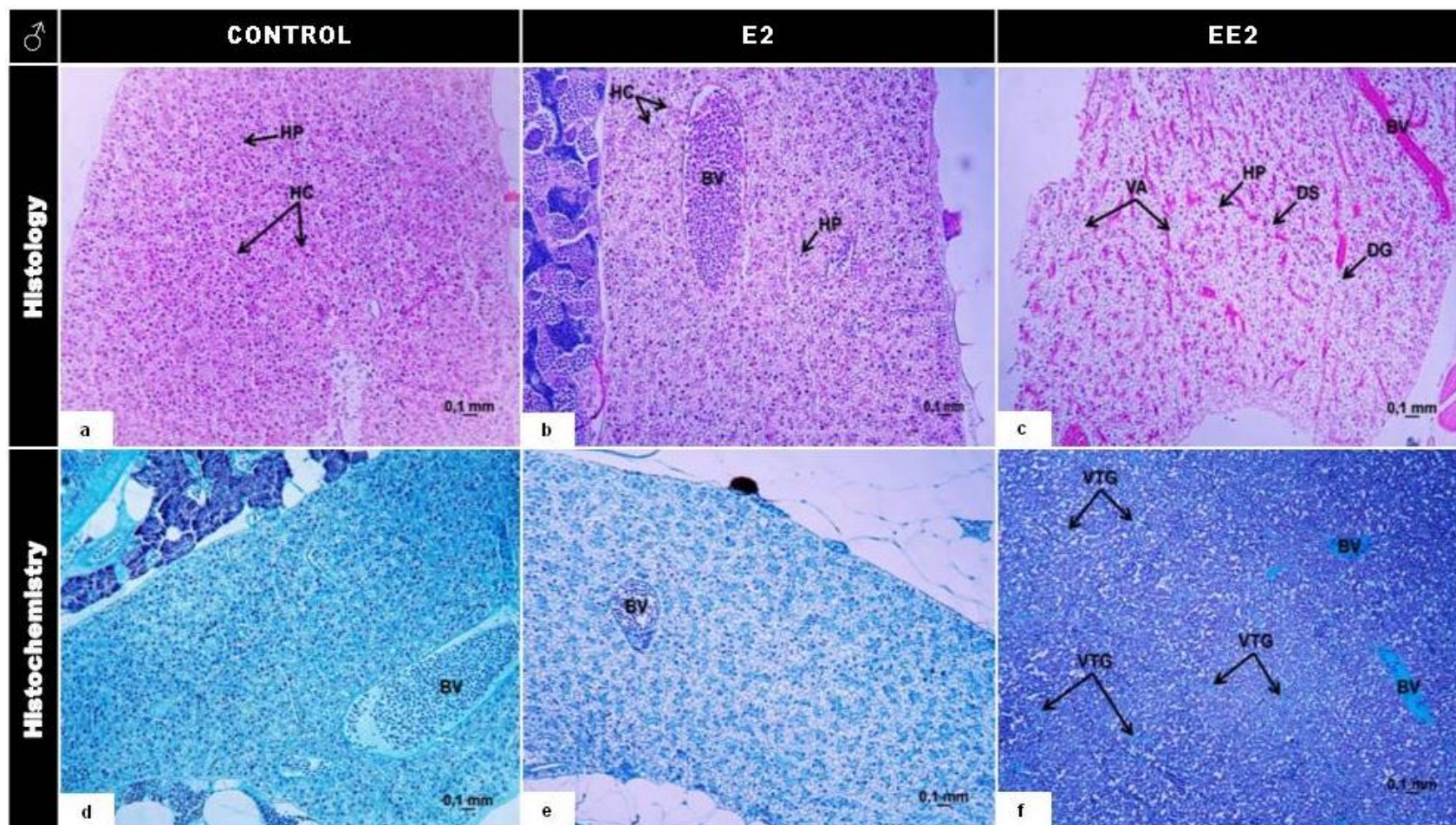
2.5. Análise dos resultados

Para a análise qualitativa das imagens estabelecidas após protocolo histológico e histoquímico de gônadas masculinas, montagem e marcação da prancha, foi empregado o microscope software Carl Zeiss AxioVision Imaging System, versão 4.8 (Zeiss, Oberkochen, Alemanha).

3. Resultados e discussão

Realizaram-se para este estudo análises histológicas (Figura 1a até c) e histoquímicas (Figura 1d até f) de tecidos hepáticos de machos adultos da espécie *D. rerio* expostos às condições: controle, E2 e EE2. Os resultados apresentam-se reunidos na Figura 1, onde foram evidenciadas as imagens mais representativas e sinalizas as alterações determinadas dentro de cada cenário de exposição.

Figura 1. Cortes histológicos (400x) de fígados masculinos de zebrafish corados com H&E (a - c) e vermelho neutro (d - f).



Legenda: HP – hepatócito, HC – cordões hepáticos, BV – vaso sanguíneo, VA – vacuolização, DS – desarranjo dos cordões hepáticos, DG – zona de degeneração e VTG – vitelogenina. Fonte: Elaborado pelo autor.

Considerando-se a análise histológica dos tecidos hepáticos de organismos controle (Figura 1a) e de organismos expostos ao hormônio natural E2 (Figura 1b), foram apresentadas células hepáticas bem organizadas em seus respectivos cordões, representando o desenvolvimento normal e saudável do órgão em questão. Em contrapartida, a exposição ao estrógeno sintético EE2 ocasionou danos teciduais significativos, revelando zonas de degeneração celular e vacuolização celular entre os hepatócitos, além de o início de um processo de desarranjo dos cordões hepáticos (Figura 1c).

A aplicação do protocolo histoquímico de coloração aos tecidos gonadais de peixes submetidos as mesmas condições experimentais, demonstrou efeitos mais severos relacionados àqueles indivíduos expostos ao hormônio EE2. Sendo nestes organismos identificada a presença de VTG entre as células hepáticas ao longo de todos os órgãos avaliados dentro da mesma condição. No entanto, machos expostos a igual concentração de E2 não revelaram a existência de VTG, fato que discorda de resultados obtidos por Van der Ven et al. (2003) que comprovou anteriormente em sua pesquisa a eficiência do método de coloração na identificação de interferência endócrina promovida pelo hormônio E2 disponível em água.

Segundo os mesmos autores, as vantagens dos métodos histológicos e histoquímicos são que eles podem ser realizados utilizando-se de materiais de rotina em laboratório, oferecendo ainda custo-benefício e tempo de análise eficazes. A especificidade, sensibilidade e validade destes métodos também são consideradas satisfatórias (Van der Vem et al., 2003).

4. Conclusão

Conclui-se, portanto, que o protocolo histoquímico apresentado neste estudo demonstrou adequada aplicabilidade para a identificação de VTG produzida em associação

a exposição de zebrafish machos à uma concentração ambientalmente relevante de EE2. O uso de técnicas histoquímicas deve ser melhor encorajado dentro para pesquisas científicas, somando-se neste sentido vantagens associadas ao baixo custo, tempo reduzido e satisfatória eficiência na identificação de potencial interferência endócrina no ambiente.

Palavras-chave: Histoquímica, Histologia, Vitelogenina, Zebrafish, Hormônios sexuais femininos.

Referências

- Funkenstein , B.; Bowman , C. J.; , Denslow, N. D.; Cardinali, M.; Carnevali, O. 2000. Contrasting effects of estrogen on transthyretin and vitellogenin expression in males of the marine fish, *Sparus aurata*. **Mol. Cell. Endocrinol.** 167, 33-41.
- Marin, M. G.; Matozzo, V. 2004. Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments. **Mar. Poll. Bul.** 48, 835-839.
- Van der Ven, L. T. M. et al. 2003. Vitellogenin expression in zebrafish *Danio rerio*: evaluation by histochemistry, immunohistochemistry, and in situ mRNA hybridisation. **Aquatic Toxicology.** 65 (1), 1-11.

Considerações finais e perspectivas futuras

Todos os resultados apresentados nesta tese permitem concluir o significativo impacto causado pelos HSFs estudados sobre o desenvolvimento e manutenção de espécies expostas a concentrações ambientais. Além disso, ainda durante revisão realizada, constatou-se haver informações químicas e ecotoxicológicas suficientes para o estabelecimento de níveis seguros de HSFs para organismos aquáticos. No entanto, uma falta de consenso entre os investigadores parece ser a causa do atraso na adoção de níveis seguros de exposição (expressa como PNEC).

Três tratamentos para remediação dos hormônios E2 e EE2 foram extensivamente estudados por meio de ensaio químicos, *in vitro* e *in vivo*. Comprovou-se, desta maneira, a elevada eficiência do carvão ativado na remoção de EDCs em água. Não obstante, suas propriedades não garantiram o mesmo desempenho com relação a amostras ambientais. Neste caso, zeólitas naturais em pó demonstraram eficiência superior. É importante lembrar que as pesquisas apresentadas nesta tese foram as primeiras a utilizarem as zeólitas como recurso para a redução de EDCs em água.

Uma concentração ambiental para HSFs foi empregada aos ensaios ecotoxicológicos e revelaram danos biológicos expressivos. Como o atraso nos processos de espermatogênese e oogênese de peixes expostos a EE2 que ademais resultou na incapacidade absoluta de procriação dos mesmos. Efeitos sobre parâmetros reprodutivos, como a diminuição na produção de embriões, taxa de eclosão e o destoamento da razão de sexo apresentada pela geração F1 estiveram relacionados a exposição a E2. Lamentavelmente, tais condições alteradas não foram recuperadas após o período de depuração realizado mediante tratamentos com qualquer dos substratos aplicados com esta finalidade.

Estudos adicionais também permitiram determinar a elaboração de um protocolo histoquímico de coloração capaz de identificar a produção de VTG incitada pela exposição ao esteróide sintético EE2.

As contruibuições científicas geradas por esta tese possibilitam ainda concluir a necessidade de haver continuidade nos estudos em busca de tratamentos eficientes na remediação não somente química, mas também biológica de EDCs. Considerando-se os materiais utilizados durante os experimentos desta tese, notou-se a conveniência por se estender o intervalo de ensaio de depuração para mais de sete dias, tendo como perspectiva pesquisas futuras na área de estudo.

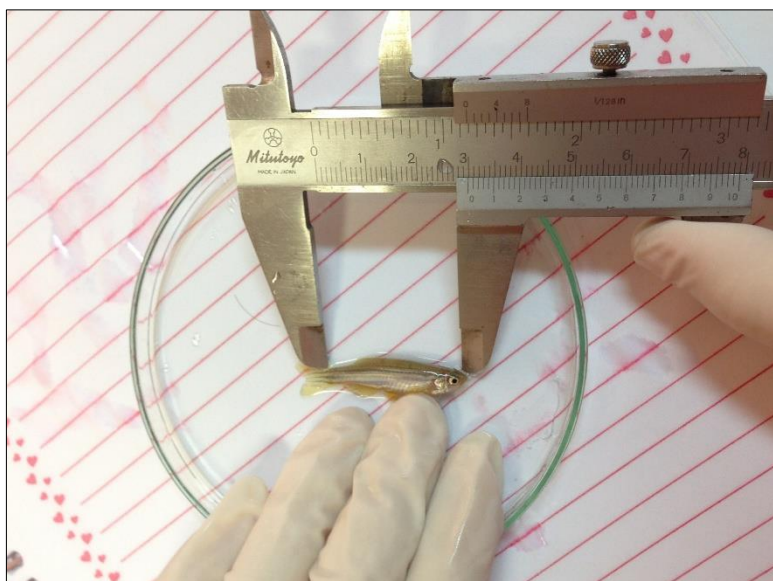
O guia para diagnóstico de histopatologias OECD (2010), amplamente utilizado na análise dos resultados histológicos obtidos, deveria ser mais bem difundido dentro para pesquisas científicas, simplificando comparações entre trabalhos efetuados e reprimir a adução de falsos negativos / positivos.

Do mesmo modo, careceria ser incentivado o maior uso de protocolos histoquímicos associados ao biomarcador VTG na detecção de potencial exposição a interferência endócrina, tendo em consideração o baixo custo, fácil acesso, tempo reduzido e satisfatória eficiência comparado a metodologias atualmente consideradas mais avançadas.

Apêndice A: Testes de sensibilidade e obtenção da carta-control

Maack e Segner (2003) consideram que o tamanho dos peixes da espécie *Danio rerio* é diretamente proporcional a idade (em semanas), a diferenciação e desenvolvimento gonadal dos mesmos. Posto que, ainda segundo os autores, peixes com tamanho médio $\geq 2,7$ cm podem ser considerados indivíduos adultos e com idade acima de 11 semanas de vida. A partir destas informações foi confirmada a idade do lote de organismos utilizados nos experimentos preparados ao longo desta pesquisa de doutorado e durante a obtenção da carta-control apresentada a seguir (Figura 1).

Figura 1. Medição do tamanho corporal de zebrafish adultos segundo Maack e Segner (2003).



Fonte: Elaborado pelo autor.

Testes de sensibilidade são realizados para atribuir maior confiabilidade aos testes de ecotoxicidade, de maneira a padronizar o nível de sensibilidade dos organismos-teste empregados durante uma pesquisa científica.

Com esse intuito, cloreto de sódio (NaCl) da marca Synth obtido via Solara Analítica (Sorocaba, SP, Brasil) foi determinado como substância de referência utilizada nos testes de sensibilidade para cultura de *Danio rerio* no Laboratório de Toxicologia de Contaminantes Ambientais e Histologia (LATHIS, UNESP – Campus de Sorocaba, Brasil). A escolha deu-se em função da menor toxicidade atribuída a esta substância no meio ambiente, pelo fato de dispensar tratamento específico para seu descarte e como uma forma de padronizar os testes de sensibilidade realizados entre os demais organismos cultivados no laboratório (*Danio rerio*, *Hyallela azteca* e *Ceriodaphnia dubia*).

Dois testes de sensibilidade foram realizados considerando o mesmo lote de peixes adultos, de forma que as concentrações de NaCl de exposição foram estabelecidas em: 0 (controle); 5; 7,5; 11,25; 16,875 e 25,3105 g.L⁻¹ (mediante fator de diluição: 1,5).

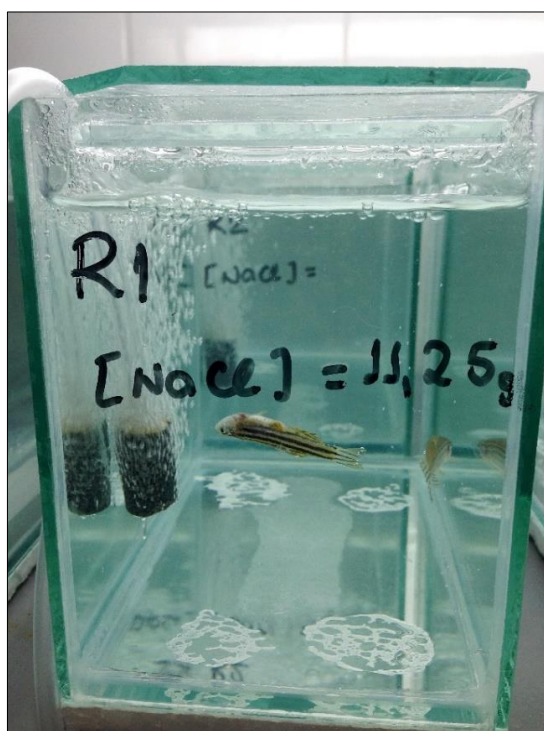
Para cada concentração foram preparadas duas réplicas em aquários de vidro de 2 L, contendo 2 peixes por aquário (Figuras 2 e 3). Os testes foram mantidos em condições controladas de pH (7,2 - 7,8); dureza (44 mg.L⁻¹ CaCO₃), temperatura (26 ± 1°C), oxigênio dissolvido (>5 mg.L⁻¹) e fotoperíodo (12h:12h, claro:escuro) segundo recomendações dos guías OECD (2010) e ABNT (2015). Recebendo alimentação “*ad libitum*” 2 vezes ao dia e aeração constante por um período de 48 horas.

Figura 2. Disposição de um teste de sensibilidade de *Danio rerio* ao NaCl.



Fonte: Elaborado pelo autor.

Figura 3. Morte de um organismo-teste verificada após 24 horas de exposição a NaCl em ensaio de sensibilidade.

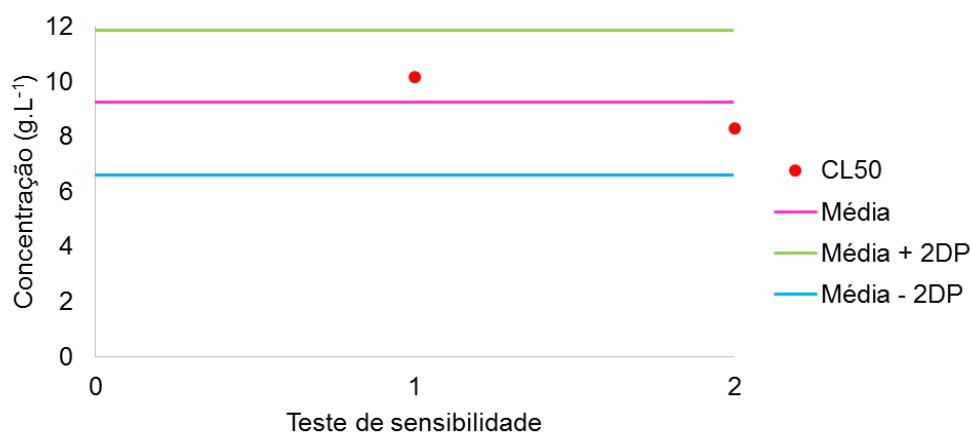


Fonte: Elaborado pelo autor.

Ao final de cada teste, foram calculadas as CL_{50-48h} (concentração letal) média através do software Trimmed Spearman–Karber (Hamilton, M.A.; Russo, R.C. e Tusrtun, R.V., 1977).

A CL_{50-48h} média estimada aos testes de sensibilidade com a substância de referência NaCl foi de $9,235 \text{ g.L}^{-1}$, com limite superior (LS) de $11,87 \text{ g.L}^{-1}$ e inferior (LI) de $6,595 \text{ g.L}^{-1}$ (Figura 4). Os limites foram obtidos com a adição ou subtração de duas vezes o desvio padrão (DP) da média encontrada entre as CL_{50-48h} obtidas ao final de ambos os ensaios.

Figura 4. Carta-controlre relacionada a sensibilidade da cultura de *Danio rerio* a NaCl.



Fonte: Elaborado pelo autor.

Valores próximos foram determinados por Santos (2009), onde o autor por meio de dez testes de sensibilidade obteve em sua carta-controlre a CL_{50-48h} média de $9,79 \text{ g.L}^{-1}$, com LS de $11,53 \text{ g.L}^{-1}$ e LI de $8,06 \text{ g.L}^{-1}$.

Referências

- ABNT (Associação Brasileira de Normas Técnicas), 2015. **Ecotoxicologia Aquática – Toxicidade crônica de curta duração – método de ensaios com peixes**. Norma ABNT-NBR 15499. Rio de Janeiro. 23p.
- Hamilton, M.A.; Russo, R.C.; Tusrtun, R.V. 1977. **Trimed Spearman-Kärber Method for estimating median lethal concentrations in toxicity bioassays**. Environmental Science and Technology, 11, 714-719.
- Maack, G.; Segner, H. 2003. Morphological development of the gonads in zebrafish. **Journal of Fish Biology**. 62 (4), 895-906, 2003.
- OECD (Organisation for Economic Co-operation and Development). 2013. **Test No. 236: Fish Embryo Acute Toxicity (FET) Test**. OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris.
- Santos, P. I. M. D. 2009. **Avaliação da toxicidade aguda do inseticida metomil e o seu efeito sobre a atividade da acetilcolinesterase do peixe *Danio rerio***. Dissertação (Mestrado), 64 p. Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal.

Apêndice B: Memória fotográfica da tese

Figura 1. Aquários utilizados para o preparo e estocagem de água reconstituída (água de cultivo).



Fonte: Elaborado pelo autor.

Figura 2. Cultura de *Danio rerio* mantida pela doutoranda no laboratório.



Fonte: Elaborado pelo autor.

Figura 3. Equipamentos utilizados para a medição dos parâmetros físico-químicos.



Fonte: Elaborado pelo autor.

Figura 4. Exemplares de réplicas do controle preparadas durante os ensaios *in vivo*.



Fonte: Elaborado pelo autor.

Figura 5. Disposição das réplicas de aquários durante o ensaio ecotoxicológico crônico.



Fonte: Elaborado pelo autor.

Figura 6. Procedimento de secção dos peixes para a remoção e isolamento das gônadas.



Fonte: Elaborado pelo autor.

Figura 7. Exemplos de gônadas femininas de zebrafish isoladas após eutanásia para avaliação do índice gonadosomático.



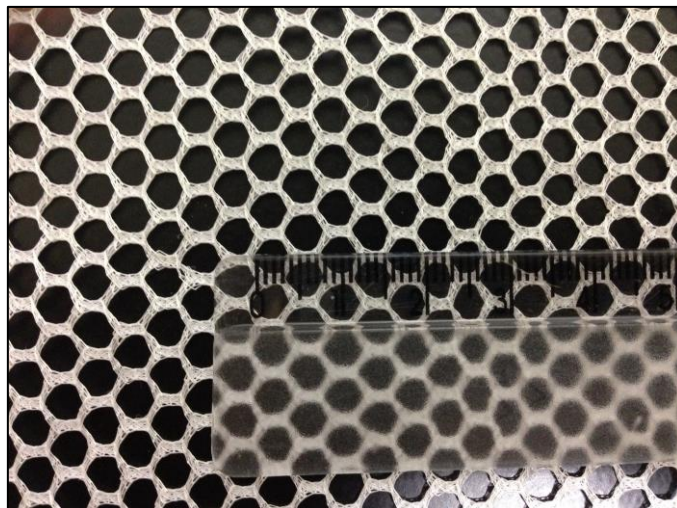
Fonte: Elaborado pelo autor.

Figura 8. Dimorfismo sexual observado entre os organismos-teste.



Fonte: Elaborado pelo autor.

Figura 9. Abertura (4 mm) da malha empregada na confecção dos aquários utilizados durante os ensaios de acasalamento.



Fonte: Elaborado pelo autor.

Figura 10. Malha suspensa (~ 3 cm do fundo) no aquário preparado para o ensaio de acasalamento.



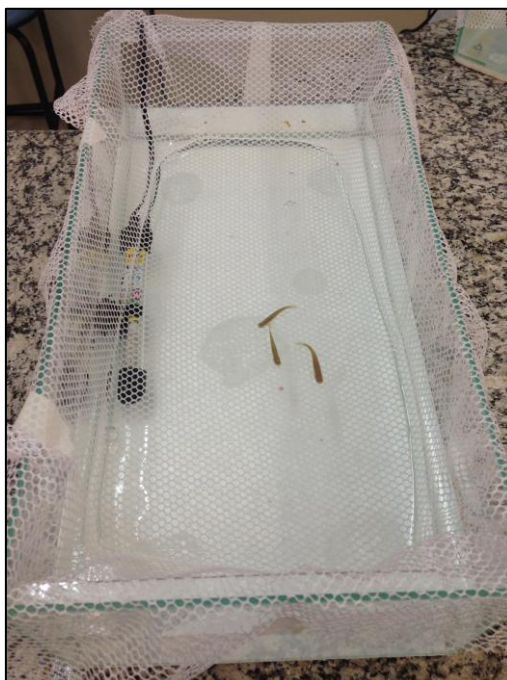
Fonte: Elaborado pelo autor.

Figura 11. Réplicas de aquários preparados para os ensaios de acasalamento.



Fonte: Elaborado pelo autor.

Figura 12. Exemplar das réplicas de aquários confeccionados para ensaios de acasalamento.



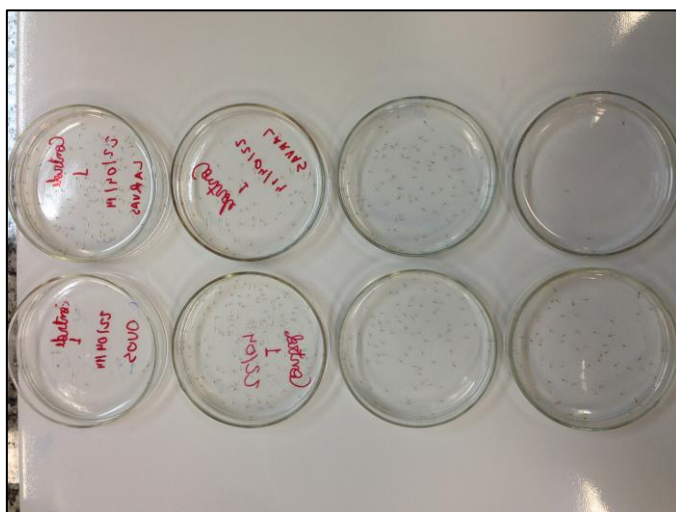
Fonte: Elaborado pelo autor.

Figura 13. Larvas recém eclodidas (geração F1).



Fonte: Elaborado pelo autor.

Figura 14. Disposição utilizada para a manutenção das larvas durante período de monitoramento (7 dias).



Fonte: Elaborado pelo autor.

Figura 15. Equipe “Zebrafish team”.



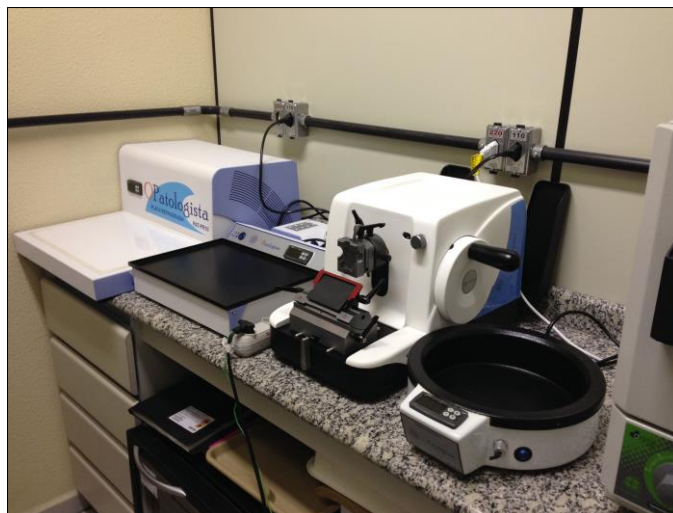
Fonte: Elaborado pelo autor.

Figura 16. “Zebrafish team” trabalhando no preparo de ensaios ecotoxicológicos.



Fonte: Elaborado pelo autor.

Figura 17. Equipamentos utilizados durante o protocolo histológico (da esq. para a dir.): placa refrigerada, placa aquecida, micrótomo semi-automático manual e banho histológico.



Fonte: Elaborado pelo autor.

Figura 18. Processador automático utilizado durante o processo inclusão de parafina nos tecidos histológicos.



Fonte: Elaborado pelo autor.

Figura 19. Processo de preparo dos blocos de parafina (parte do protocolo histológico).



Fonte: Elaborado pelo autor.

Figura 20. Bancada da capela preparada com a sequência de soluções utilizadas no protocolo de coloração histológica com H&E.



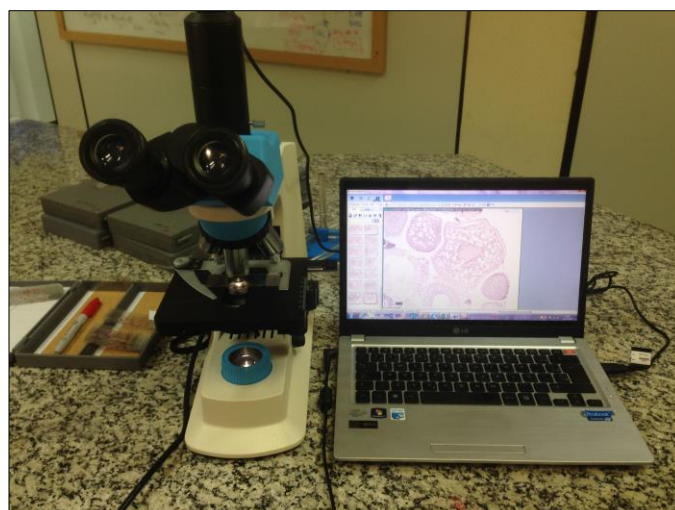
Fonte: Elaborado pelo autor.

Figura 21. Bancada da capela preparada com a sequência de soluções utilizadas no protocolo de coloração histoquímica com vermelho neutro.



Fonte: Elaborado pelo autor.

Figura 22. Bancada utilizada pela doutoranda para análise das lâminas histológicas.



Fonte: Elaborado pelo autor.

Figura 23. Profa. Dra. Renata Fracácio (orientadora) e Dr. Rhaul de Oliveira com a doutoranda na UNESP.



Fonte: Elaborado pelo autor.

Figura 24. Dr. Eduardo Bertoletti ao lado da doutoranda e demais alunos da pós-graduação da UNESP.



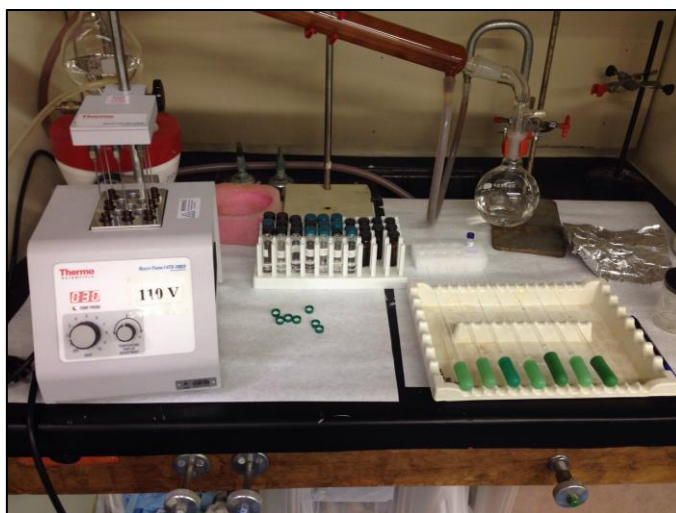
Fonte: Elaborado pelo autor.

Figura 25. Profa. Dra. Tânia Mara Pizzolato e o Dr. Alessandro Dallegrave com a doutoranda na UFRGS.



Fonte: Elaborado pelo autor.

Figura 26. Bancada de trabalho da doutoranda durante o procedimento de derivatização das amostras encaminhadas para análise cromatográfica.



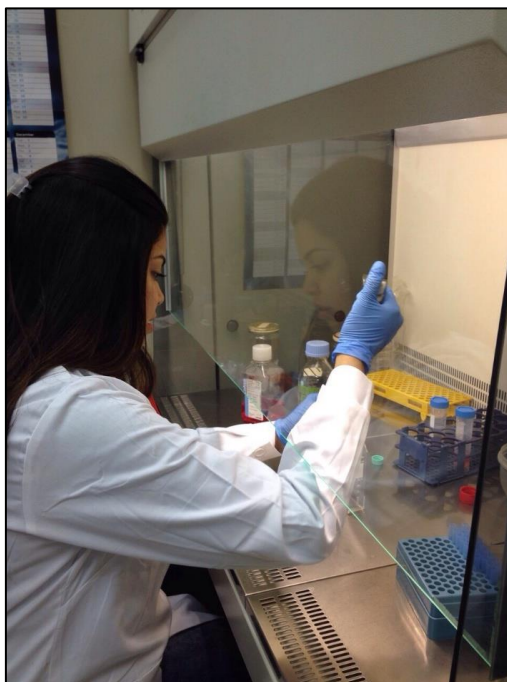
Fonte: Elaborado pelo autor.

Figura 27. Cromatógrafo a gás (GC-MS) utilizado para a leitura das análises químicas realizadas na UFRGS.



Fonte: Elaborado pelo autor.

Figura 28. Doutoranda trabalhando no exterior durante o preparo de um ensaio *in vitro* no *Laboratorio de Disrupción Endocrina y Toxicidad de Contaminantes* do INIA.



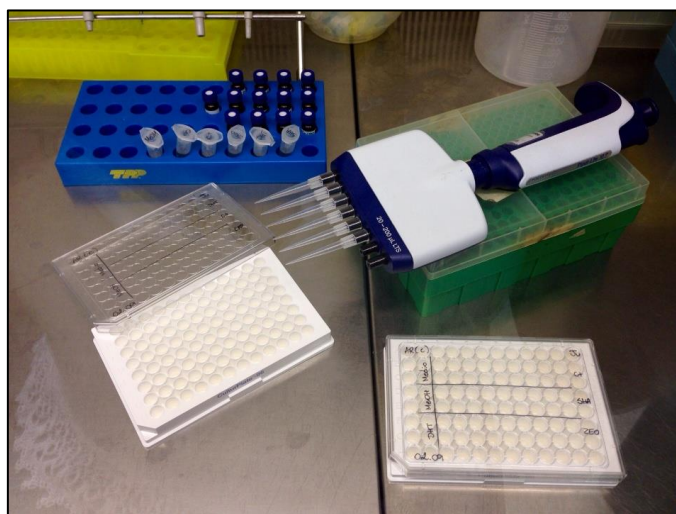
Fonte: Elaborado pelo autor.

Figura 29. Doutoranda trabalhando no exterior com as linhas celulares mantidas no *Laboratorio de Disrupción Endocrina y Toxicidad de Contaminantes* do INIA.



Fonte: Elaborado pelo autor.

Figura 30. Bancada de trabalho da capela de fluxo laminar utilizada pela doutoranda durante o preparo de um ensaio *in vitro*.



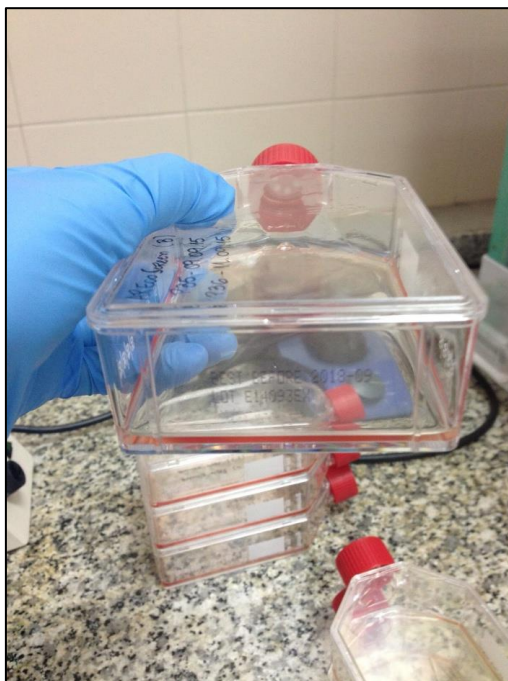
Fonte: Elaborado pelo autor.

Figura 31. Câmara de cultivo com as linhas celulares mantidas e utilizadas pela doutoranda durante seus experimentos no INIA.



Fonte: Elaborado pelo autor.

Figura 32. Exemplar de uma linha celular mantida no INIA.



Fonte: Elaborado pelo autor.

Figura 33. Bancada de trabalho para preparação de amostras/soluções pela doutoranda no *Laboratorio de Disrupción Endocrina y Toxicidad de Contaminantes* do INIA.



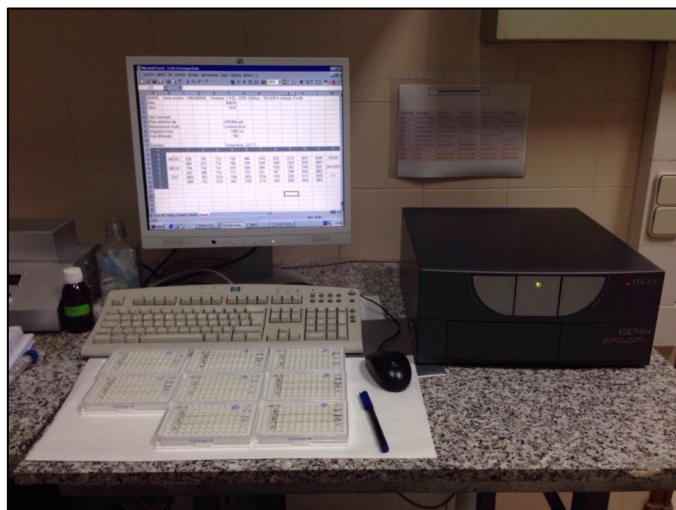
Fonte: Elaborado pelo autor.

Figura 34. Bancada de trabalho da doutoranda em capela utilizada durante a realização do procedimento de SPE aplicado à amostras de água no INIA.



Fonte: Elaborado pelo autor.

Figura 35. Equipamento utilizado para a leitura da luminescência produzida pelas células após ensaios *in vitro* no INIA.



Fonte: Elaborado pelo autor.

Figura 36. Dr. José María Navas (orientador no exterior) com a doutoranda.



Fonte: Elaborado pelo autor.

Figura 37. Doutoranda completando a equipe de pesquisadores do grupo de pesquisa em *Disrupción endocrina y Toxicidad de Contaminantes* do INIA em 2015.



Fonte: Elaborado pelo autor.