Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

ISBN 978-80-7514-083-8

2019

Environmental pollutants progestins: occurrence, hormonal activities and effects on fish

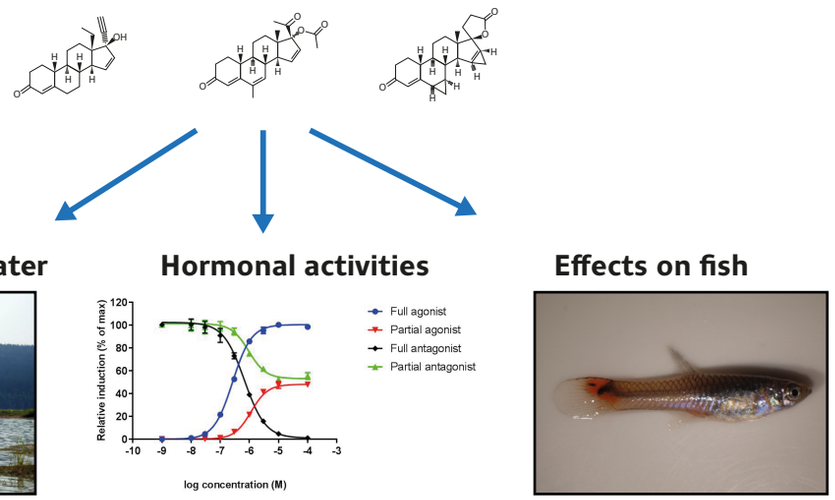
Pavel Šauer

Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Environmental pollutants progestins: occurrence, hormonal activities and effects on fish

Environmentální polutanty progestiny: výskyt, hormonální aktivity a účinky na ryby



Pavel Šauer

Czech Republic, Vodňany, 2019

Pavel Šauer

Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Environmental pollutants progestins: occurrence, hormonal activities and effects on fish

Environmentální polutanty progestiny: výskyt, hormonální aktivity a účinky na ryby

Pavel Šauer

Czech Republic, Vodňany, 2019

I, Pavel Šauer, thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

I hereby declare that, in accordance with the § 47b Act No. 111/1998 Coll., as amended, I agree with publicizing of my Ph.D thesis in full version electronically in a publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice on its web sites, with keeping my copyright to the submitted text of this Ph.D. thesis. I also agree so that the same electronic way, in accordance with above mentioned provision of the Act No. 111/1998 Coll., was used for publicizing reviews of supervisor and reviewers of the thesis as well as record about the progress and result of the thesis defence. I also agree with compering the text of my Ph.D. thesis with a database of theses "Theses.cz" operated by National Register of university theses and system for detecting of plagiarisms.

In Vodňany 21st November, 2018

Supervisor:
Hana Kocour Kroupová, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II, 389 25 Vodňany, the Czech Republic

Consultants:
Vimal Kumar Hatwal, Ph.D. and
Jitka Kolářová, DVM
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II, 389 25 Vodňany, the Czech Republic

Head of Laboratory of Aquatic Toxicology and Ichthyopathology:
Assoc. Prof. Josef Velíšek

Dean of Faculty of Fisheries and Protection of Waters:
Prof. Pavel Kozák

Board of doctorate study defence with reviewers:
Assoc. Prof. Josef Matěna – head of the board
Prof. Petr Ráb – board member
Assoc. Prof. Martin Kocour – board member
Assoc. Prof. Tomáš Policar – board member
Prof. Lukáš Kalous – board member
Assoc. Prof. Radovan Kopp – board member
Assoc. Prof. Zdeněk Adámek – board member

Dr. Ilka Lutz, Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Germany – thesis reviewer
Assoc. Prof. Klára Hilscherová, Masaryk University, Faculty of Science Research Centre for Toxic Compounds in the Environment, Czech Republic – thesis reviewer

Date and place of Ph.D. defence:
26th March 2019 in USB, FFPW, RIFCH, Vodňany, the Czech Republic

Name: Pavel Šauer

Title of thesis:
Environmental pollutants progestins: occurrence, hormonal activities and effects on fish

Environmentální polutanty progestiny: výskyt, hormonální aktivity a účinky na ryby

Ph.D. thesis, USB FFPW, RIFCH, Vodňany, Czech Republic, 2019, 114 pages, with the summary in English and Czech.

Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz

ISBN 978-80-7514-083-8

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1. General introduction

Thousands of chemicals are being daily discharged into the sewage. Many of them pass through municipal wastewater treatment plants (WWTP) and continuously contaminate surface waters. Endocrine disruptors (EDCs) are a group of environmental contaminants of special concern. They interfere with endocrine system of resident organisms and may produce adverse developmental, reproductive, neurological, and immune effects (Ankley et al., 1998; Colborn et al., 1993; Kidd et al., 2007; Leskinen et al., 2005; Sumpter, 2005). Aquatic biota face lifelong exposure to multiple EDCs and their joint actions in mixtures (Cwiertny et al., 2014; King et al., 2016), but the consequences of such an exposure are still mostly unknown. Therefore, monitoring of the environmental concentrations and hormonal activities of EDCs in surface waters and their effects on aquatic organisms came into focus of many researchers worldwide.

1.1. Detection of hormonal activities of pure compounds and in environmental samples

The presence of chemicals in water bodies can be determined by chemical analysis and then the identified chemicals can be tested for their possible adverse effects using *in vivo* toxicological tests. However, it becomes obvious that the steadily increasing number of chemicals and environmental samples to be tested in eco-toxicological research could not be processed using conventional *in vivo* tests because they are laborious, cost-demanding, and time-consuming. In addition, a high number of testing animals would be suffering. Therefore, *in vitro* bioassays might be used for rapid screening of hormonal activities in environmental samples and identification of the most harmful substances (Escher and Leusch, 2012; Rehberger et al., 2018). Unlike chemical analysis, *in vitro* bioassays take into account whole-sample specific toxicity including known as well as unknown toxicants (Escher and Leusch, 2012) because they estimate the total biological activity of all compounds present in a sample acting through the same specific mode of action (García-Reyero et al., 2001; Hilscherova et al., 2000). *In vitro* bioassays can also be employed to study the toxicity mechanisms (pathways) of tested substances (Rehberger et al., 2018). However, despite of all the advantages of *in vitro* tests, *in vivo* tests are not fully replaceable as they provide information about effects at the level of whole organism.

EDCs interfere with biological actions of endogenous hormones directly by binding to receptors or indirectly by altering synthesis and metabolism of hormones, enzymes, or receptors (Schug et al., 2011) or affect ontogeny by DNA methylation in germ cell lines (Diamanti-Kandarakis et al., 2009). Regarding receptor binding, EDCs can either activate or block them, thus, they display either agonistic or antagonistic hormonal activities, respectively.

The effects mediated via receptors can be evaluated using *in vitro* reporter gene bioassays. *In vitro* reporter gene bioassays are typically based on a tumorous cell line that in an ideal case does not contain any measurable amount of receptors. These cells are transfected with DNA sequences for receptor of interest (e.g. steroid receptors) and sequences for carrying so-called hormone response elements (HREs) along with reporter gene. Steroid receptors act as transcription factors (Evans et al., 1988; Mangelsdorf et al., 1995). Upon binding of a ligand (e.g. tested endocrine disruptor) to the receptor, this complex (ligand-receptor) binds the HREs and transactivates the reporter gene. The reporter genes inserted into these cells encode reporter proteins (e.g. luciferase or β-galactosidase) that after reaction with a substrate produce a measurable activity (e.g. emission of light or colour change) that reflects transactivation of the inserted receptor. Such a response can be quantified from these data and hormonal activity of tested sample can be calculated (Kinnberg, 2003).

1.2. Progestins

1.2.1. Chemical structure and physico-chemical properties of progestins

The term progestins is sometimes used only for synthetic chemicals but for the purpose of this thesis I will use the term progestins for both, natural and synthetic compounds, whereas the terms progestagen and progestagenic will be used for all compounds that have affinity to progesterone receptor (PR). Progestins (also called by some authors progestagens, progestogens or gestagens) are hormonally active compounds that share steroid skeleton but differ in substituents which modulate their affinities to other receptors (Africander et al., 2011; Besse and Garric, 2009). Progestins are either progesterone (pregnanes or 19-norpregnanes), testosterone (estranes and gonanes) or spironolactone (progestin drospirenone) derivatives (Africander et al., 2011; Sitruk-Ware, 2004; Table 1). Synthetic progestins are supposed to be more persistent than progesterone because they were made to have longer biological half-life (Besse and Garric 2009). Furthermore, due to the lipophilic nature of progestins with log Kow values ranging from 2.97 to 5.65 (Table 1), these compounds are anticipated to accumulate in aquatic biota (Kumar et al., 2015).

Table 1. *Classification and physico-chemical properties of progestins (adopted from Kumar et al., 2015; Liu et al., 2011a and USEPA, 2010).*

structural derivation			compound	abb	CAS number	Molar mass ($g \cdot mol^{-1}$)	ws ($mg \cdot l^{-1}$)	log K_{ow}
Progesterone derivatives			progesterone	P4	57-83-0	314.46	5.0	3.67
			dydrogesterone	DGT	152-62-5	312.46	3.7	3.45
Structurally related to progesterone	17α-OH-progesterone derivatives	Pregnanes	medroxyprogesterone	MP	520-85-4	344.49	3.0	3.5
			medroxyprogesterone acetate	MPA	71-58-9	386.52	1.2	4.09
			cyproterone acetate	CPA	427-51-0	416.94	51.7	3.1
			chlormadinone acetate	CMA	302-22-7	406.94	0.3	3.95
			melengestrol acetate	MLGA	2919-66-6	396.53	0.6	4.41
	19-norprogesterone derivatives		megestrol acetate	MGA	595-33-5	384.52	2.0	4
			nomegestrol acetate	NOMAC	58652-20-3	370.49	3.0	3.55
			ulipristal acetate	UPA	126784-99-4	475.63	0.1	5.07
17α-spirono-lactone derivative			drospirenone	DRO	67392-87-4	366.50	1.8	4.02

structural derivation			compound	abb	CAS number	Molar mass ($g \cdot mol^{-1}$)	ws ($mg \cdot l^{-1}$)	log K_{ow}
Structurally related to testosterone	19-nortestosterone derivatives	Estranes	lynestrenol	LYN	52-76-6	284.45	0.8	4.75
			norethisterone	NET	68-22-4	298.43	7.0	2.97
			norethisterone acetate	NETA	51-98-9	340.47	5.4	3.99
			dienogest	DIE	65928-58-7	311.43	57.9	2.34
		Gonanes	levonorgestrel	LNG	797-63-7	312.46	2.1	3.48
			etonogestrel	ENG	54048-10-1	324.47	NA	3.16
			gestodene	GES	60282-87-3	310.44	8.1	3.26
			norgestimate	NORG	35189-28-7	369.51	0.04	4.98
			norelgestromin	NGMN	53016-31-2	327.47	3.4	3.98
			desogestrel	DES	54024-22-5	310.48	0.3	5.65

Abbreviations and description: abb – abbreviation, ws – water solubility, log K_{ow} – octanol-water partition coefficient, NA – data not available, structures of progestins are given in chapter 2 – Golovko et al. (2018).

1.2.2. Use of progestins and their sources in the aquatic environment

Synthetic progestins (around 20 substances) are widely used in human medicine and in the contraception (State Institute for Drug Control, 2018). During the past few decades, female oral contraception has become a feature of everyday life and therefore its consumption has grown considerably. Progestins are also important components of hormone replacement therapy (Spitz and Chwalisz, 2000; Zeilinger et al., 2009) as well as they are prescribed to treat some kinds of cancer, endometriosis and dysfunctional uterine bleeding (Spitz and Chwalisz, 2000), hirsutism (Becker et al., 2001) or in paliative treatment of cancer cachexia (Maltoni et al., 2011). In human medicine, natural progestin progesterone is used to treat menstrual cycle disorders, breast cancer, uterine bleeding, to support luteal phase of the cycle in assisted reproduction techniques and to increase circulating progesterone levels in women (State Institute for Drug Control, 2018). After administration, some part of progestins is excreted by human in unchanged form and goes into the sewage. In addition, progesterone is produced and excreted naturally by human (Shore and Shemesh, 2003) and its amounts are even higher than those of natural estrogens (Scott et al., 2010). Both, the natural and synthetic progestins present in raw wastewater are not completely eliminated during wastewater treatment processing and enter the aquatic environment (Fent, 2015; Kumar et al., 2015; Orlando and Ellestad, 2014).

Progestins are also broadly used in farm animals as growth promoters (Orlando and Ellestad, 2014; Schiffer et al., 2001) or for induction, synchronization, delaying and suppression of estrus (Fent, 2015; Islam, 2011). Farm animals also naturally produce and excrete progesterone. Surface run-offs from livestock farming are therefore considered as one of the main sources of progestins for the aquatic environment. Last but no least industrial effluents from processing of pharmaceuticals can be a source of progestins for the aquatic environment (Creusot et al., 2014).

Given the broad usage of progestins, their concentration may reach relatively high levels in aquatic environment (Chang et al., 2009; Kumar et al., 2015).

1.2.3. Occurrence of progestins in effluents, surface water and groundwater

Despite that several authors attempted to detect progestins in wastewater, surface water and groundwater (Table 2), there is still insufficient knowledge on occurrence and fate of progestins in the aquatic environment compared to other classes of steroidal EDCs such as estrogens or androgens (Zeilinger et al. 2009; Fent, 2015; Kumar et al., 2015). Natural progestin progesterone and synthetic progestins levonorgestrel and norethisterone are the most frequently analysed compounds from this class (Kumar et al., 2015; Table 2). The environmental concentrations of progestins appear to be in most cases at low $ng \cdot l^{-1}$ or lower levels (Table 2).

Only a few studies have focused on determination of progestins in groundwater. Interestingly, progestins have been found in ground waters in all those studies (Table 2).

Table 2. *Occurrence of selected progestins in effluents, surface waters and ground waters.*

Compound	Water matrix	Analytical method	Country	Mean concentration ($ng \cdot l^{-1}$)[a]	Reference
progesterone	SW	GC-MS/MS	USA	<LOQ	Kolodziej et al., 2004
		LC-MS/MS	Hungary	<LOQ	Tölgyesi et al., 2010
		HPLC-MS/MS	the Czech Republic	<LOQ	Macikova et al., 2014
		UHPLC-MS/MS	China	<LOQ	Liu et al., 2015
		UHPLC-MS/MS	Japan	0.07	Chang et al., 2008
		LC-MS/MS	Spain	0.88	Kuster et al., 2008
		GC-MS/MS	Belgium	0.9	Pauwels et al., 2008
		UHPLC-MS/MS	China	1.5	Shen et al., 2018
		LC-MS/MS	France	1.6	Vulliet and Cren-Olivé, 2011
		RRLC-MS/MS	China	2.5	Liu et al., 2011b
		UHPLC-MS/MS	China	2.5	Liu et al., 2014
		LC-MS/MS	France	2.6	Vulliet et al., 2008
		LC-MS/MS	Canada	3.0	Viglino et al., 2008
		HPLC-MS/MS	Hungary	3.1	Avar et al., 2016
		HPLC-MS/MS	Switzerland	6.3	Macikova et al., 2014
		HPLC-MS/MS	USA	0.72-6.5[b]	Standley et al., 2008
		LC-MS/MS	Brazil	9.4	Kuster et al., 2009
		GC-MS	USA	9.4	Velicu and Suri, 2009
		LC-MS/MS	Brazil	9.5	Torres et al., 2015
		GC-MS	USA	110[c]	Kolpin et al., 2002
	EFF	LC/LC-MS/MS	Canada	<LOQ	Viglino et al., 2008
		HPLC-MS/MS	Switzerland	<LOQ	Macikova et al., 2014
		HPLC-MS/MS	the Czech Republic	<LOQ	Macikova et al., 2014
		GC-MS	USA	0.50	Soliman et al., 2004
		UHPLC-MS/MS	China	1.8	Shen et al., 2018
		HPLC-MS/MS	France	2.2	Vulliet et al., 2011
		GC-MS/MS	Belgium	2.5	Pauwels et al., 2008
		LC-MS	France	10	Vulliet et al., 2007
		LC-MS/MS	Hong Kong	23	Wu et al., 2017
	GW	LC-MS/MS	France	1.6	Vulliet and Cren-Olivé, 2011
		LC-MS/MS	France	3.3	Vulliet et al., 2008

Compound	Water matrix	Analytical method	Country	Mean concentration ($ng \cdot l^{-1}$)[a]	Reference
21-hydroxyproesterone	SW	HPLC-MS/MS	the Czech Republic	<LOQ	Macikova et al., 2014
		HPLC-MS/MS	Switzerland	1.3	Macikova et al., 2014
	EFF	HPLC-MS/MS	Switzerland	<LOQ	Macikova et al., 2014
		HPLC-MS/MS	the Czech Republic	2.0	Macikova et al., 2014
levonorgestrel	SW	HPLC-MS	the Czech Republic	<LOQ	Matějíček and Kubáň, 2007
		GC-MS	France	<LOQ	Labadie and Budzinski, 2005
		LC-MS/MS	Spain	<LOQ	Kuster et al., 2008
		UHPLC-MS/MS	China	<LOQ	Sun et al., 2009
		GC-MS/MS	Australia	<LOQ	Scott et al., 2014
		UHPLC-MS/MS	China	1.1[d]	Liu et al., 2014
		HPLC-MS/MS	Hungary	1.9	Avar et al., 2016
		LC-MS/MS	France	3.6	Vulliet and Cren-Olivé, 2011
		UHPLC-MS/MS	China	4.77[d]	Liu et al., 2015
		LC-MS/MS	France	6.2	Vulliet et al., 2008
		HPLC-UV-Vis	China	7.5	Qiao et al., 2009
		RRLC-MS/MS	China	22	Liu et al., 2011b
		LC-MS/MS	Malaysia	38	Al-Odaini et al., 2010
	EFF	LC-MS/MS	Malaysia	<LOQ	Al-Odaini et al., 2010
		GC-HRMS	Germany	1.0	Kuch and Ballschmiter, 2000
		LC-HRMS	Sweden	1.0	Fick et al., 2010
		HPLC-UV-Vis	China	1.1	Pu et al., 2008
		ELISA	China	1.3	Pu et al., 2008
		LC-MS	Spain	<LOQ-4.0[b]	Petrovic et al., 2002
		HPLC-MS/MS	France	3.5	Vulliet et al., 2011
		RRLC-MS/MS	China	8.0	Liu et al., 2011b
		HPLC-UV-Vis	China	8.1	Qiao et al., 2009
		LC-MS	France	13	Vulliet et al., 2007
		LC-MS/MS	Canada	30	Viglino et al., 2008
	GW	LC-MS/MS	France	4.0	Vulliet and Cren-Olivé, 2011
		LC-MS/MS	France	9.1	Vulliet et al., 2008
gestodene	SW	HPLC-MS	the Czech Republic	<LOQ	Matějíček and Kubáň, 2007
		UHPLC-MS/MS	China	1.6	Shen et al., 2018
		LC-MS/MS	Serbia	3.6	Neale et al., 2015
	EFF	UHPLC-MS/MS	China	1.8	Shen et al., 2018
norethisterone	SW	LC-MS/MS	Spain	<LOQ	Kuster et al., 2008
		UHPLC-MS/MS	China	<LOQ	Sun et al., 2009
		LC-MS/MS	Malaysia	<LOQ	Al-Odaini et al., 2010
		UHPLC-MS/MS	China	0.48	Shen et al., 2018
		LC-MS/MS	France	2	Vulliet and Cren-Olivé, 2011
		LC-MS/MS	France	2.8	Vulliet et al., 2008
		GC-MS	USA	48[c]	Kolpin et al., 2002
	EFF	UHPLC-MS/MS	China	<LOQ	Shen et al., 2018
		HPLC-MS/MS	France	2.0	Vulliet et al., 2011

Compound	Water matrix	Analytical method	Country	Mean concentration ($ng \cdot l^{-1}$)[a]	Reference
norethisterone	EFF	LC-MS/MS	Hong Kong	7.7	Wu et al., 2017
		LC-MS	Spain	8.6	Petrovic et al., 2002
		LC-MS	France	17	Vulliet et al., 2007
		LC-LC-MS/MS	Canada	53	Viglino et al., 2008
		LC-MS/MS	Malaysia	188	Al-Odaini et al., 2010
	GW	LC-MS/MS	France	1.9	Vulliet and Cren-Olivé, 2011
		LC-MS/MS	France	4.8	Vulliet et al., 2008
norethisterone acetate	EFF	GC-HRMS	Germany	<LOQ	Kuch and Ballschmiter, 2000
medroxyprogesterone	SW	UHPLC-MS/MS	China	<LOQ	Sun et al., 2009
		GC-MS/MS	USA	<LOQ	Kolodziej and Sedlak, 2007
		LC-MS/MS	Canada	<LOQ	Viglino et al., 2008
		UHPLC-MS/MS	China	<LOQ	Liu et al., 2015
			the Czech Republic	<LOQ	Macikova et al., 2014
		GC-MS/MS	USA	1.0	Kolodziej et al., 2004
		HPLC-MS/MS	Switzerland	2.7	Macikova et al., 2014
	EFF	HPLC-MS/MS	Switzerland	<LOQ	Macikova et al., 2014
		HPLC-MS/MS	the Czech Republic	2.0	Macikova et al., 2014
		GC-MS/MS	USA	<LOQ-15[b]	Kolodziej et al., 2003
medroxyprogesterone acetate	SW	UHPLC-MS/MS	China	<LOQ	Liu et al., 2014
		UHPLC-MS/MS	China	<LOQ	Liu et al., 2015
	EFF	UHPLC-MS/MS	Japan	0.23	Chang et al., 2008
		LC-MS/MS	China	0.46	Chang et al., 2011
		UHPLC-MS/MS	China	0.90	Liu et al., 2014
megestrol acetate	SW	UHPLC-MS/MS	China	0.15	Shen et al., 2018
	EFF	UHPLC-MS/MS	China	0.14	Shen et al., 2018
		LC-MS/MS	China	0.33	Chang et al., 2011
cyproterone acetate	SW	HPLC-MS	the Czech Republic	<LOQ	Matějíček and Kubáň, 2007
		HPLC-MS/MS	Switzerland	<LOQ	Zhang and Fent, 2018
		UHPLC-MS/MS	China	<LOQ	Liu et al., 2015
		UHPLC-MS/MS	China	0.36	Shen et al., 2018
	SW	LC-MS/MS	Germany	0.82	Weizel et al., 2018
	EFF	HPLC-MS/MS	Switzerland	<LOQ	Zhang and Fent, 2018
		UHPLC-MS/MS	China	0.23	Shen et al., 2018
		LC-MS/MS	Germany	2.3	Weizel et al., 2018
melengestrol acetate	SW	LC-MS/MS	USA	<LOQ	Kolok et al., 2007
	EFF	UHPLC-MS/MS	China	0.60	Liu et al., 2014
		UHPLC-MS/MS	Japan	0.35	Chang et al., 2008
		UHPLC-MS/MS	China	1.2	Liu et al., 2014
dydrogesterone	SW	HPLC-MS/MS	Switzerland	<LOQ	Zhang and Fent, 2018
		UHPLC-MS/MS	China	4.4	Shen et al., 2018
		UHPLC-MS/MS	China	3.8	Liu et al., 2015
		UHPLC-MS/MS	China	9.6	Liu et al., 2014
	EFF	HPLC-MS/MS	Switzerland	<LOQ	Zhang and Fent, 2018
		UHPLC-MS/MS	China	3.1	Shen et al., 2018
dienogest	SW	LC-MS/MS	Germany	0.64	Weizel et al., 2018
	EFF	LC-MS/MS	Germany	2.9	

Compound	Water matrix	Analytical method	Country	Mean concentration ($ng \cdot l^{-1}$)[a]	Reference
drospirenone	SW	HPLC-MS/MS	Hungary	1.9	Avar et al., 2016
desogestrel	SW	HPLC-MS	the Czech Republic	<LOQ	Matějíček and Kubáň, 2007

Abbreviations and description: SW – surface water, EFF – municipal wastewater treatment plant effluent, GW – groundwater, LOQ – limit of quantification. Only those progestins that have been found at least once in effluents, surface waters or ground waters are presented in this table. [a] – mean concentration of positive detections, [b] – range of concentrations is shown because complete data were not provided, [c] – median, [d] – norgestrel (racemic mixture of 2 optical isomers: levonorgestrel and dextronorgestrel), LC – liquid chromatography, GC – gass chromatography, MS - mass spectrometry, RR - rapid resolution liquid chromatography, HP – high performance, UHP – ultra high performance, <LOQ – concentration below limit of quantification.

1.2.4. Biological functions and hormonal activities of progestins

Progesterone is the major progestin in mammals (Ellestad et al. 2014; Chen et al., 2010; Ikeuchi et al., 2001; Nagahama, 2002), whereas 17,20-β-dihydroxypregn-4-en-3-one (DHP also known as 17,20β-P) or in certain species 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P also known as 20β-S) are considered as the major natural progestagens in most teleost fish (reviewed in Scott et al., 2010). Progesterone is a crucial hormone necessary to maintain pregnancy, regulate menstrual cycle and to successfully complete embryogenesis in humans (Clarke and Sutherland, 1990; Graham and Clarke, 1997). DHP and 17,20β,21-P are involved in final oocyte and sperm maturation in most teleost fish, and it appears that DHP has an important role in initiation of meiosis that is the first step in spermatogenesis and oogenesis (Scott et al., 2010). Progesterone and DHP bind and transactivate mammalian and fish PRs, respectively, and thus they manifest their intrinsic progestagenic activity. Nonetheless, some effects are probably mediated also through membrane PRs in fish (Thomas, 2008).

Synthetic progestins are chemicals that were designed to mimic biological action of progesterone (Stanczyk et al., 2013). In general, synthetic progestins are mostly more potent than progesterone (Besse and Garric 2009). In addition to progestagenic activity, most of them exert unwanted affinity to other nuclear receptors such as estrogen (ER), androgen (AR), glucocorticoid (GR) and mineralocorticoid (MR) receptor (Africander et al., 2011; Kumar et al., 2015; Schindler et al., 2003). Such off-target modulation of other receptors by progestins leads to side effects in users of contraception and other progestin-based hormonal preparations. Analogous endocrine-disrupting effects of progestins can be found in wildlife if exposed to these compounds (Fent, 2015; Kumar et al., 2015).

1.2.5. *In vitro* progestagenic activity of progestins

The potency of all compounds derived from *in vitro* bioassays that have an affinity to the same receptor (e.g. estrogens, androgens or progestagens) can be expressed using relative potency (REP) values. However, REPs of compounds may differ considerably between different authors (Kuhl, 1990). This is probably due to differences among various *in vitro* bioassay systems used or different incubation conditions (Kuhl, 1990; 1996). Nevertheless, the order of potency of hormonally active agents, such as synthetic progestins is mostly similar and comparable (Table 3.). It can be seen from such comparison that three gonanes (testosterone

derivatives), gestodene, etonogestrel, and levonorgestrel, have the strongest progestagenic activity of all the progestins and all of them are more potent than natural progesterone (Table 3). Their *in vitro* potencies are in agreement with observed *in vivo* progestagenic/contraceptive effects in humans (Schindler et al., 2003). However, progestins were shown not to mediate their genomic biological activity through fish PRs in *in vitro* reporter gene bioassays (Bain et al., 2015; Ellestad et al., 2014). Particularly, synthetic progestins that are 19-nortestosterone derivatives do neither transactivate Murray-Darling rainbowfish's (*Melanotaenia fluviatilis*) PR (Bain et al., 2015), nor fathead minnow's (*Pimephales promelas*) PR (Ellestad et al., 2014). A weak agonistic activity was detected just for drospirenone, a spironolactone derivative (Bain et al., 2015).

Surprisingly, no study has determined to date progestagenic potencies of all prescribed progestins using the same *in vitro* bioassay in order to provide directly comparable values. Therefore, one of the aims of this Ph.D. thesis was to determine progestagenic activities of all progestins, which are prescribed in the Czech Republic, in the same *in vitro* bioassay (chapter 3). Such information would be valuable for human and veterinary medicine as well as for eco-toxicological research.

Table 3. *Comparison of relative progestagenic potencies of selected progestins for transactivation of human progesterone receptor.*

Reference compound	Assay/tissue	Order of relative potencies	Reference
progesterone	*uterus*	MPA > P4 > NET	Kasid et al., 1978
	uterus	LNG > P4	Kuhnz et al., 1995
	SF9	GES > LNG > MPA > NET > P4	Philibert et al., 1999
	YPS	ENG > LNG > P4 > NET > MPA	McRobb et al., 2008
	YPS	GES > LNG >NET = P4 = MPA > DRO	Runnalls et al., 2013
	PR-CALUX	GES > ENG > LNG > NET > P4	Bain et al., 2015
ORG 2058	*uterus*	LNG > MPA = NET > P4	Bergink et al., 1981
	MCF-7 [a]	GES > ENG > LNG > NET	Kloosterboer et al., 1988
	MCF-7 [b]	ENG > GES > LNG > NET	Kloosterboer et al., 1988
	MCF-7 [b]	ENG > GES > MPA > LNG > NET > P4	Schoonen et al., 1995
	MCF-7	ENG > GES > LNG > NET > P4	Schoonen et al., 1998
	PR-CALUX	MPA = LNG > NET = P4	Houtman et al., 2009
	PR-CALUX	DGT = P4	Rižner et al., 2011
	PR-CALUX	ENG > MPA > LNG > NET > P4	Sonneveld et al., 2011
promegestone	*uterus*	ENG > LNG > GES > P4	Pollow et al., 1989
	uterus	LNG > ENG > GES > P4	Pollow et al., 1992
	uterus	LNG > GES > P4	Juchem et al., 1993

Abbreviations and description: YPS – yeast progesterone screen, PR-CALUX – progesterone receptor responsive chemically activated luciferase gene expression, DRO – drospirenone, DGT – dydrogesterone, ENG – etonogestrel, GES - gestodene, LNG – levonorgestrel, MPA – medroxyprogesterone acetate, NET – norethisterone, P4 – progesterone , [a] – intact cells, [b] – cytosol.

1.2.6. *In vitro* (anti-)androgenic activity of progestins

Progestins are known to exhibit either strong androgenic or anti-androgenic activities (Kuhl, 1996; Raudrant and Rabe, 2003; Schindler et al., 2003; Sitruk-Ware, 2004; Térouanne et al., 2002).

Progestins that are derived from testosterone (gonanes, estranes) exhibit mostly androgenic activities both *in vitro* and *in vivo* in human (Schindler et al., 2003). Gonanes (levonorgestrel, gestodene and etonogestrel), synthetic progestins with the strongest progestagenic activity, appear to be also the most potent androgens (Table 4). *In vitro* studies with fish ARs have shown similar results to *in vitro* studies on human ARs in this regard (Bain et al., 2015; Ellestad et al., 2014). Androgenic effects have also been observed *in vivo* in fish exposed to levonorgestrel (Hua et al., 2015; Runnalls et al. 2013; Svensson et al., 2013; 2016; Zeilinger et al., 2009), norethisterone (Paulos et al., 2010) and gestodene (Runnalls et al., 2013).

On the other hand, progesterone-derived progestins exert anti-androgenic activities (Schindler et al., 2003). Some progestins with anti-androgenic effect are even used to treat androgen-dependent disorders/diseases such as hirsutism and acne in women (Archer and Chang, 2004) or prostate cancer in men (Labrie et al., 1987). Two progesterone derivatives, chlormadinone acetate and cyproterone acetate, which have been tested for they affinity to fish ARs so far, shown also anti-androgenic activity (Siegenthaler et al., 2017a).

Table 4. *Comparison of relative potencies of selected progestins for transactivation of human androgen receptor.*

Reference compound	Assay/ tissue	Order of relative potencies	Reference
dihydrotestosterone	MCF-7[a]	LNG > GES > ENG > NET	Kloosterboer et al., 1988
	MCF-7[b]	LNG > GES > ENG > NET	Kloosterboer et al., 1988
	MCF-7[a]	LNG > ENG = MPA > NET	Bergink et al., 1983
	MCF-7[b]	MPA > LNG > GES > NET > ENG	Schoonen et al., 1995
	AR-CALUX	LNG > MPA = NET	Houtman et al., 2009
	YAS	LNG > GES = NET > MPA > DRO	Runnalls et al., 2013
	COS-1	GES > LNG > MPA > DRO > P4 > NOMAC	Louw-du-Toit et al., 2017

Abbreviations and description: DRO – drospirenone, ENG – etonogestrel, GES – gestodene, LNG – levonorgestrel, MPA – medroxyprogesterone acetate, NET – norethisterone, NOMAC – nomegestrol acetate, AR-CALUX – androgen receptor responsive chemically activated luciferase gene expression, YAS – yeast androgen screen, COS-1 – a monkey kidney cell line, [a] – intact cells, [b] – cytosol.

1.3. Detection of hormonal activities of pure compounds and in environmental samples

1.3.1. Progestagenic activities in effluents and surface waters

When considering that synthetic progestins are consumed in high amounts (Besse and Garric, 2009; Runnalls et al., 2010), municipal wastewater treatment plant discharges are supposed to be the main contributor to the progestagenic activities in the aquatic environment. However, limited amount of data is still available on the progestagenic activity of waste and surface water (Creusot et al., 2014; van der Linden et al., 2008) which might be linked to the presence of synthetic progestins. Progestagenic activities might be also found in surface waters downstream animal farming areas (e.g. cattle pastures) that are also sources of progestins as aforementioned (chapter 1.2.2.). In addition, pharmaceutical (Creusot et al., 2014) and leather (Chatterjee et al., 2008) industries were confirmed as sources of water pollution by PR agonists.

Progestagenic agonistic activities were detected in wastewater effluents as well as in surface waters in approximately 40% of studies attempting to detect them (Table 5).

A French river that received a pharmaceutical industry effluent was found out to be highly polluted by PR agonists: progesterone, levonorgestrel, spironolactone (synthetic steroidal anti-mineralocorticoid) and its metabolite canrenone (Creusot et al., 2014; Table 5). Other progestagenic substances were not analysed. LNG was found to contribute to progestagenic activities the most, namely up to 50% (Creusot et al., 2014).

To the best of my knowledge, data on progestagenic activity of environmental water samples has not been collected in the Czech Republic so far. The consequences of the presence of progestagenic activities in the aquatic environment for aquatic organisms still remain unclear. In chapter 3, we have been attempting to estimate to which extent progestins may contribute to progestagenic activities but further studies should be carried out to elucidate which level of progestagenic activity can be considered as safe for aquatic biota.

Table 5. *Occurrence of progestagenic activities in aquatic environment reported in literature.*

Water matrix	Cells/assay	Country	Range of progestagenic activity $ng \cdot l^{-1}$ ORG 2058 EQs[a]	Reference
effluent	a yeast bioassay with exogenous metabolic activation	China	ND	Li et al., 2011
	HELN-PR-B	Tunisia	ND	Mnif et al., 2010
	PR-CALUX	Australia	ND	Scott et al., 2014
	PR-CALUX, PR-GeneBLAzer	worldwide	ND[b]	Escher et al., 2014
	HELN-PR B	France	ND	Bellet et al., 2012
	PR-CALUX	Netherlands	0.78–0.86	van der Linden et al., 2008
	PR-CALUX	Australia	ND–4.6	Leusch et al., 2014
	PR-CALUX	Australia	~1.4	Bain et al., 2014
	YPS	China	ND	Rao et al., 2014
	transgenic HEK 293	India	0.33–0.58	Viswanath et al., 2008
surface water	PR-CALUX	Netherlands	4.5	van der Linden et al., 2008
	PR-CALUX, PR-GeneBLAzer	worldwide	ND[b]	Escher et al., 2014
	PR-CALUX	Australia	ND	Scott et al., 2014
	YPS	China	ND	Rao et al., 2014
	HELN-PR B	France	32.5-1556 $ng \cdot g^{-1}$ R5020 EQs[c]	Creusot et al. 2014

Abbreviations and description: YPS – yeast progesterone screen, CALUX – chemically activated luciferase gene expression, ND - not detected, R5020 – promegestone, [a] – the equivalents were normalized to ORG 2058 by relative potency values obtained in experiments from chapter 3 (used relative potencies were: ORG 2058 = 1.00, progesterone = 0.08 and levonorgestrel = 0.85), [b] – $<EC_{10}$ – only slight progestagenic activities were detected, all were below EC_{10} of the reference compound, [c] – sampled by polar organic compound integrative sampler.

1.3.2. (Anti-)androgenic activities in effluents and surface waters

The main sources of androgenic activity of anthropogenic origin in aquatic environments are municipal WWTP effluents (Kirk et al., 2002), agriculture (Soto et al., 2004) and paper mill plant effluents (Jenkins et al., 2004). It is supposed that androgenic activities are mostly

caused by natural androgens (Thomas et al., 2002) and partly also by synthetic androgens (Hashmi et al., 2018). Furthermore, there are other contaminants, such as pesticides that also possess androgenic activities (Sohoni and Sumpter, 1998; Weiss et al., 2011). Progestins were also suggested to be responsible for the androgenic activities to some extent (Hashmi et al., 2018).

Anti-androgenic activities are commonly detected in environmental samples (Chen and Chou, 2016; Macikova et al., 2014a; Urbatzka et al., 2007) and are believed to pose a greater risk for aquatic biota compared to androgenic activities (Weiss et al., 2009; Zhao et al., 2011). While androgenic activities in aquatic environment may cause masculinization of aquatic organisms, anti-androgenic activities may contribute to feminization. Therefore, feminization of fish residing downstream WWTP effluents has been linked not only to estrogenic but also to the anti-androgenic compounds (Jobling et al., 2009). AR seems to be relatively prone to blocking as many compounds out of a large tested dataset (approximately 3 000 environmental chemicals analysed for the U.S. Tox21 program) have been shown to exert anti-androgenic activities (Huang et al., 2011). These include various groups of chemicals such as progestins, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides, polychlorinated dibenzo-p-dioxins/dibenzofurans (PCDDs and PCDFs), ultraviolet filters, nonylphenols, bisphenols, fungicides, and prostate cancer treatment drugs (Eustache et al., 2009; Chatterjee et al., 2007; Kinani et al., 2010; Lee et al., 2003; Li et al., 2011; Ma et al., 2003; Schreurs et al., 2005; Sohoni and Sumpter 1998; Vandenberg et al., 2012; Vinggaard et al., 2000; Viswanath et al., 2010). However, environmental levels of PAHs, PCBs, PCDDs, PCDFs and DDT were not correlated with the total anti-androgenicity found in surface waters (Macikova et al., 2014). The effect drivers of anti-androgenic activities often remain unidentified (Kinani et al., 2010; Chen and Chou et al., 2016; Urbatzka et al., 2007). There has been only one study dealing with the determination of contribution of progestins to anti-androgenic activities. Houtman et al. (2018) found out that cyproterone acetate (CPA) is responsible for 70% of anti-androgenic activities in treated wastewater in the Netherlands.

One of the aims of this Ph.D. thesis was to determine the extent to which progestins contribute to progestagenic and (anti-)androgenic activities in aquatic environments. The share of progestins on detected (anti-)androgenic activities can then be compared to recently calculated EBT values for (anti-)androgenic activity (Escher et al., 2018; van der Oost et al., 2017) to estimate if progestins can cause (anti-)androgenic activities that could be of high risk for aquatic ecosystems.

Table 6. *Occurrence of (anti-)androgenic activities in aquatic environment reported in literature.*

Water matrix	Assay/ cells	Country	Range of activity		Reference
			Androgenic ($ng \cdot l^{-1}$ DHT EQ)	Anti-androgenic (FLU EQ)	
surface water	transgenic T47D	The Netherlands	5.7 methyltrienolone EQs	ND	Blankvoort et al., 2005
	AR-CALUX	Australia	2	ND	Leusch et al., 2014
	YAS	United Kingdom	ND-9	ND	Thomas et al., 2002
	AR-CALUX	The Netherlands	12	ND	van der Linden et al., 2008
	YAS	China	ND	22.1 $ng \cdot l^{-1}$	Rao et al., 2014
	YAS	United Kingdom	ND-143	NA	Kirk et al., 2002
	YAS	Italy	130-207	0.4-0.6 $mg \cdot l^{-1}$	Urbatzka et al., 2007
	YAS	China	ND-45	0.02-0.9 $mg \cdot l^{-1}$	Zhao et al., 2011
	MCF7-AR1	Taiwan	0.94-3.1	ND	Shue et al., 2014
effluent	AR-CALUX	The Netherlands	0.75-0.83	ND	van der Linden et al., 2008
	YAS	United Kingdom	34-635	ND	Thomas et al., 2002
	T47D	The Netherlands	8 247-178 883[a]	ND	Blankvoort et al., 2005
	YAS	China	ND	0.08-2.2 $mg \cdot l^{-1a}$	Fang et al., 2012
	T47D	The Netherlands	199	ND	Blankvoort et al., 2005
	YAS	India	0.0001-0.0012 testosterone EQs	ND	Chatterjee et al., 2007

Abbreviations and description: DHT – dihydrotestosterone, FLU – flutamide, YAS – yeast androgen screen, CALUX – chemically activated luciferase gene expression, ND – not detected, NA – not analysed, [a] – industrial wastewater effluent.

1.4. Adverse effects of progestins on aquatic organisms

Most of studies investigating effects of progestins on aquatic organisms were performed on teleost fish. Amphibians have also attracted certain research interest (Fent, 2015; Kumar et al., 2015; Orlando and Ellestad, 2014), but only little is known about effects of progestins on aquatic invertebrates (Orlando and Ellestad, 2014). I have focused on effects of progestins on fish in this Ph.D. thesis.

So far most studies focused on effects of progestins on reproduction of fish (Fent, 2015; Kumar et al., 2015; Orlando and Ellestad, 2014). Of the progestins analysed so far, testosterone-derived progestins levonorgestrel, norethisterone and gestodene pose the greatest risk to aquatic organisms due to their adverse effect on fish fecundity at low $ng \cdot l^{-1}$ levels (DeQuattro et al., 2012; Paulos et al., 2010; Runnalls et al. 2013, Zeilinger et al. 2009). Up to date, the lowest LOEC value (0.8 $ng \cdot l^{-1}$) among all tested progestins has been estimated for levonorgestrel (Table 7). Accumulating evidence indicates that testosterone-derived progestins exhibit androgenic effects *in vivo* that eventually lead to masculinization of female fish (Brockmeier et al., 2016; Frankel et al., 2016a; 2016b; Hou et al., 2018a; Hua et al., 2015; Runnalls et al., 2013) and supermasculinization of male fish (Frankel et al., 2016b). Progestins

also affect sex ratio (Hou et al., 2018a; Shi et al., 2018) and induce intersex (Fent, 2015; Hou et al., 2018a) (Table 7).

Furthermore, reproductive behaviour can be affected in fish exposed to progestins (Table 7). Male fathead minnow (*Pimephales promelas*) exposed to low $ng \cdot l^{-1}$ levels of gestodene displayed more aggressive behaviour and exhibited reduced frequency of courtship and mating behaviour, while exposed females had lower interest in courtship (Frankel et al., 2016a). A short-term exposure (8 days) of eastern mosquitofish (*Gambusia holbrooki*) to levonorgestrel (100 $ng \cdot l^{-1}$) led to decreased mating frequencies (Frankel et al., 2016b). Reduced frequency and duration of following behavior towards females was also observed in males of western mosquitofish (*Gambusia affinis*) exposed to norgestrel (racemic mixture of biologically active levonorgestrel and non-active dextronorgestrel) at ≥ 3.6 $ng \cdot l^{-1}$ level for 42 days (Hou et al., 2018b).

Progestins might also impair other physiological functions in fish (Kumar et al., 2015). These include e.g. circadian rhythm (Shi et al., 2018; Zhao et al., 2015a; 2015b; 2018), thyroid system (Liang et al., 2015b), immune system (Pietsch et al., 2009; 2011), and potentially pheromonal signalling (Besse and Garric, 2009; Frankel et al., 2016a; Scott et al., 2010). However, many studies listed above have focused just on changes at molecular level upon progestins' exposure and it still needs to be elucidated if these changes translate into physiological effects (Zhao et al., 2015b).

Table 7. *Summary of effects induced by progestins (ascending order of concentration) in fish (adopted from Kumar et al., 2015 and updated).*

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Fathead minnow (*Pimephales promelas*)	Adult	21-day	Females: ↓ egg production	0.8	LNG	Zeilinger et al., 2009
		21-day	Females: ↓ egg production	1	NET	Paulos et al., 2010
		7-day *in vivo* + 24-h *ex vivo*	*Ex vivo* ovarian production ↓ 11-KT	1	LNG	Overturf et al., 2014
		21-day	Females: development of male secondary characteristic, ↓ egg production	1	GES	Runnalls et al., 2013
Mummichog (*Fundulus heteroclitus*)	Adult (recrudescing)	14-day	Females: ↓ *in vitro* ovarian T and E2 production	1	CPA	Sharpe et al., 2004
Zebrafish (*Danio rerio*)	Embryo	6-day	↓ *ar*, ↓ *pgr*, ↓ *hsd17β3* mRNA expression	1	CPA	Siegenthaler et al., 2017a
			↓ *ar*, ↓ *pgr*, ↓ *hsd17β3* mRNA expression	1	CMA	
	Embryo	48-hour	↑ *ar* and *gr* mRNA expression	2	P4	Zucchi et al., 2012
		96-hour	↑ *pgr* and *ar* mRNA expression			
		144-hour	↑ *vtg1* mRNA expression			
		48-h	mRNA expression of ↑ *erα* and ↑ *mr*	2	NET	
		48-h	mRNA expression of ↓ *ar*	2	LNG	
		144-h	mRNA expression of ↑ *pgr*			
	Adult	14-day	Females: mRNA expression of ↓ *vtg1* in the liver, ↓ *ccnb1*, ↓ *zp3*, ↑ *arnt2*, and ↑ *gr* in the brain, and ↓ *nr1d1* in the ovary	3.5	P4	Zucchi et al., 2013
	Juvenile	60-day	↓ *cyp11a1*, ↓ *cyp11b* mRNA expression	4	LNG	Liang et al., 2015a
Western mosquitofish (*Gambusia affinis*)	Adult	42-day	↑ *arα*, ↑ *arβ*, ↓ *erα*, ↓ *erβ*, ↓ *vtgB* mRNA expression, Females: ↑ width of ray 3 in anal fin	4	P4	Hou et al., 2017
Zebrafish (*Danio rerio*)	Embryo	1-day	↑ *nis* mRNA expression	5	LNG	Liang et al., 2015b
		2-day	↑ *tg* mRNA expression			
		3-day	↑ *nis* mRNA expression			
		4-day	↓ *thrβ* mRNA expression			
		5-day	↓ *dio1* mRNA expression			
		6-day	↑ *tg*, ↑ *thr*β mRNA expression			
Zebrafish (*Danio rerio*)	Embryo	1-day	↑ *nis* mRNA expression	5	P4	Liang et al., 2015b
		3-day				
		6-day				
	Juvenile	22-day	↑ *dmrt1* mRNA expression	5	NET	Hou et al., 2018a

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Zebrafish (*Danio rerio*)	Juvenile	14-day	↓ *mhc1uea*, ↓ *mhc1ufa*, ↓ *tnfrsf19* in ovary and changes in mRNA expression of genes related to circadian rhythm in brain of females	5	DGT	Shi et al., 2018
Three-spined stickleback (*Gasterosteus aculeatus*)	Adult	21-day	Females: mRNA expression of ↓ spiggin in the kidney	5.5	LNG	Svensson et al., 2013
		45-day	Males: ↑ KEH	6.5		Svensson et al., 2014
Zebrafish (*Danio rerio*)	Larvae or juvenile	28-day	↓ *ar*, ↓ *cyp19a1a*, ↓ *nr5a1b* mRNA expression	10	LNG	Hua et al., 2015
	Larvae or juvenile	42-day	↓ *ar*, ↓ *cyp19a1a*, ↓ *cyp19a1b*, ↓ *nr5a1b* mRNA expression			
	Adult	63-day	Sex ratio: all male (males and masculinized females)			
	Adult	142-day	Sex ratio: all male (males and masculinized females)			
	Adult	14-day	Females: changes in expression of mRNA related to circadian rhythm and notch signalling pathway Males: changes in expression of mRNA related to notch signalling pathway, ↑ mature sperm	10	NGT	Shi et al., 2017
	Embryo	6-day	↓ *ar*, ↓ *pgr*, ↓ *hsd17β3* mRNA expression	10	CPA	Siegenthaler et al., 2017a
		4-day	↓ *hsd11β2* mRNA expression	10	CMA	
		6-day	↓ *ar*, ↓ *lhb*, ↓ *hsd11β2*, ↓ *hsd17β3* mRNA expression			
Fathead minnow (*Pimephales promelas*)	Adult	21-day	Females: mRNA expression of ↓ *vtg* in the liver, ↓ egg production, ↓ fertilization	10	P4	DeQuattro et al., 2012
		21-day	Females: ↓ plasma E2	10	NET	Paulos et al., 2010
		8-day	Females: ↓ egg production, reproductive behavior - ↑ aggression, ↓ courtship Males: reproductive behavior ↑ aggression, ↓ courtship and mating	10	GES	Frankel et al., 2016a
Eastern mosquitofish (*Gambusia holbrooki*)	Adult	8-day	Females: ↑ 4:6 anal fin ratio	10	LNG	Frankel et al., 2016b
Western mosquitofish (*Gambusia affinis*)	Adult	8-day	Females: ↑ anal fin 4:6 ratios	10	LNG	Frankel et al., 2018b

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Roach (*Rutilus rutilus*)	Adult	42-day	↑ levels of DJ-1 protein in brain and hepatopancreas, ↑ HSI, ↑ KSI	10 + 10 + 10	P4 + DRO + LNG	Maasz et al., 2017
Fathead minnow (*Pimephales promelas*)	Larvae	28-day	mRNA expression of ↓ *hsd3β*, ↓ *hsd20β*, ↓ *cyp17*, ↓ *ar*, ↓ *esrα*, ↓ *fshβ*	16.3	LNG	Overturf et al., 2014
Zebrafish (*Danio rerio*)	Adult	14-day	Females: ↑ egg production	25	P4	Blüthgen et al., 2013a
		21-day	Females: ↓ GSI			
		21-day	Females: mRNA expression of ↓ *cyp11b*, ↓ *hsd17β3*, ↓ *pgr* in ovary ↓ *ar* in brain			Blüthgen et al., 2013b
Japanese medaka (*Oryzias latipes*)	Adult	28-day	Females: ↓ egg production	25	NET	Paulos et al., 2010
Roach (*Rutilus rutilus*)	Pubertal	28-day	Males: ↓ plasma 11-KT and ↑ spgB (%) in the testes	31	LNG	Kroupova et al., 2014
Zebrafish (*Danio rerio*)	Larvae or juvenile	28-day	↓ *ar*, ↓ *cyp19a1a*, ↓ *nr5a1b*, ↑ *dmrt1* mRNA expression	33	LNG	Hua et al., 2015
	Larvae or juvenile	42-day	↓ *ar*, ↓ *cyp19a1a*, ↓ *cyp19a1b*, ↓ *nr5a1b*, ↑ *dmrt1* mRNA expression			
	Adult	63-day	Sex ratio: all male (males and masculinized females)			
	Adult	142-day	Sex ratio: all male (males and masculinized females)			
	Juvenile	60-day	↓ *cyp11b*, ↓ *hsd20β* mRNA expression	33	P4	Liang et al., 2015a
	Juvenile	60-day	↑ *dmrt1*, ↓ *figa*, ↓ *cyp11a1*, ↓ *cyp17*, ↓ *cyp19a1a*, ↓ *hsd3β*, ↓ *hsd17β3* mRNA expression	34	LNG	Liang et al., 2015a
	Adult	60-day exp. + 80-day in LNG-free water	Sex ratio: all male (males and masculinized females)			Liang et al., 2015a
Roach (*Rutilus rutilus*)	Adult	42-day	↑ levels of DJ-1 protein in brain and hepatopancreas, ↑ HSI, ↑ KSI	50 + 50 + 50	P4 + DRO + LNG	Maasz et al., 2017
Zebrafish (*Danio rerio*)	Juvenile	14-day	Females: ↑ *mhc1uea*, ↑ *mhc1ufa*, ↓ *il1rapl1a* in ovary and changes in expression of mRNA related to circadian rhythm in brain	50	DGT	Shi et al., 2018
	Juvenile	22-day	↑ *dmrt1*, ↓ *figa*, ↓ *foxl2*	50	NET	Hou et al., 2018a

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Zebrafish (*Danio rerio*)	Adult	45-day	Females: ↓ females, ↑ intersex, ↑*ar* mRNA expression	50	NET	Hou et al., 2018a
	Embryo	1-day	↑ *nis* mRNA expression	50	LNG	Liang et al., 2015b
		3-day	↑ *nis* mRNA expression			
		4-day	↑ *thrβ* mRNA expression			
		5-day	↓ *tg* mRNA expression			
		6-day	↓ *tg* mRNA expression			
		1-day	↑ *nis* mRNA expression		P4	
		3-day				
		5-day	↓ *dio1* mRNA expression			
		6-day	↑ *nis* mRNA expression			
	Adult	21-day	Females: ↑ *vtg1* mRNA expression, ↓ egg production Males: ↓ *erα* mRNA expression	54	CMA	Siegenthaler et al., 2017b
	Adult	14-day	Females: ↓ proportion of late VTG oocytes, mRNA expression of ↓ *vtg1* in the liver, ↓ *nr1d1* in the brain	55	DRO	Zucchi et al., 2014
	Juvenile	60-day	↓ *amh*, ↑ *figa*, ↑ *pgr*, ↑ *cyp17*, ↑ *cyp19a1a*, ↓ *cyp11b*, ↑ *hsd3β* mRNA expression	63	P4	Liang et al., 2015a
	Adult	60-day exp. + 80-day in LNG-free water	Sex ratio: shifting towards females			Liang et al., 2015a
	Adult	21-day	Females: ↓ egg production	65	CMA	Siegenthaler et al., 2017b
Three-spined stickleback (*Gasterosteus aculeatus*)	Adult	45-day	Males: ↑ KEH, ↑ NSI, inhibition of the onset of spermatogenesis, mRNA expression of ↑ spiggin and ↓ *cyp1a* in the kidney	65	LNG	Svensson et al., 2014
Zebrafish (*Danio rerio*)	Juvenile	60-day	↑ *dmrt1*, ↓ *figa*, ↑ *pgr*, ↑ *vtg1*, ↓ *cyp11a1*, ↓ *cyp17*, ↓ *cyp19a1a*, ↓ *hsd3b*, ↓ *hsd20b*, ↓ *hsd17b3*, ↑ *pomc* mRNA expression	77	LNG	Liang et al., 2015a
Zebrafish (*Danio rerio*)	Adult	60-day exp. + 80-day in LNG-free water	Sex ratio: shifting towards males (males and masculinized females)			Liang et al., 2015a

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Fathead minnow (*Pimephales promelas*)	Larvae	28-day	↓ growth, mRNA expression of ↓ *hsd3ß*, ↓ *hsd20ß*, ↓ *cyp19a1a*, ↓ *fshß*	86.9	LNG	Overturf et al., 2014
	Adult	7-day *in vivo* + 24-h *ex vivo*	Ex vivo ovarian production ↓ pregnenolone, ↓ DHP, ↓ T, ↓ 11-KT in ovaries	100		
	Adult	8-day	Females: ↓ egg production, reproductive behavior: ↑ aggression, ↓ courtship Males: reproductive behavior: ↑ aggression, ↓ courtship and mating	100	GES	Frankel et al., 2016a
	Adult	14-day	Females: mRNA expression of ↓ *mpgrß*, ↓ *erß1* in ovary, ↓ *erß1* in brain	100	NET	Petersen et al., 2015
	Adult	14-day	Females: mRNA expression of ↓ *mpgrα*, ↓ *mpgrß*, ↓ *hsd*11β, ↑ *hsd3α* in ovary	100	P4	
Eastern mosquitofish (*Gambusia holbrooki*)	Adult	8-day	Females: ↑ 4:6 anal fin ratio	100	LNG	Frankel et al., 2016b
Western mosquitofish (*Gambusia affinis*)	Adult	8-day	Females: ↑ anal fin 4:6 ratios Males: ↑ anal fin 4:6 ratios, Reproductive behavior of control males paired with exposed females: ↓ attending behavior, ↓ gonopodial thrusts	100	LNG	Frankel et al., 2018b
Zebrafish (*Danio rerio*)	Embryo	5-day	↓ tg mRNA expression	100	LNG	Liang et al., 2015b
		6-day	↑ *tg*, ↓ *dio2* mRNA expression			
	Larvae or juvenile	28-day	↓ *ar*, ↓ *cyp19a1a*, ↓ *nr5a1b*, ↑ *dmrt1* mRNA expression	100	LNG	Hua et al., 2015
	Larvae or juvenile	42-day	↓ *ar*, ↓ *cyp19a1a*, ↓ *cyp19a1b*, ↓ *nr5a1b*, ↑ *dmrt1* mRNA expression			
	Adult	63-day	Sex ratio: all male (males and masculinized females)			
Zebrafish (*Danio rerio*)	Adult	142-day	Sex ratio: all male (males and masculinized females)	100	LNG	Hua et al., 2015
	Adult	14-day	Females: changes in expression of mRNA related to circadian rhythm Males: changes in expression of mRNA related to circadian rhythm, ↑ mature sperm	100	NGT	Shi et al., 2017
	Embryo	1-day	↑ *nis* mRNA expression	100	P4	Liang et al., 2015b
		3-day	↑ *tg* mRNA expression			
		5-day	↓ *dio1* mRNA expression			

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Zebrafish (*Danio rerio*)	Embryo	6-day	↓ *ar*, ↓ *pgr*, ↓ *gr* mRNA expression	100	CPA	Siegenthaler et al., 2017a
		6-day	↑ *ar*, ↑ *pgr*, ↑ *erα*, ↑ *vtg1*, ↑ *cyp19a1b* mRNA expression	100 + 10	CPA + EE2	
		6-day	↑ *erα*, ↑ *vtg1*, ↑ *cyp19a1b* mRNA expression	100 + 100	CPA + EE2	
		4-day	↓ *hsd11α2*, ↓ *cyp11a* mRNA expression	100	CMA	
			↑ *vtg1*, ↑ *cyp19a1b*, ↑ *lhb* mRNA expression	100 + 10	CMA + EE2	
			↑ *vtg1*, ↑ *cyp19a1b*, ↑ *lhb*, ↑ *erα*, ↑ *pgr* mRNA expression	100 + 100		
		6-day	↑ *vtg1*, ↑ *cyp19a1b*, ↑ *lhb*, ↓ *fshα* mRNA expression	100 + 10		
			↑ *vtg1*, ↑ *cyp19a1b*, ↑ *erα*, ↑ *pgr*, ↑ *lhb*, ↓ *fshα* mRNA expression	100 + 100		
	Embryo (F1 generation of above fish)	120-h	mRNA expression of ↓ *gr*, ↓ *pgr*, ↓ *cyp11b*, ↓ *ar*	254	P4	Blüthgen et al., 2013b
Fathead minnow (*Pimephales promelas*)	Adult	7-day	Males: ↓ sperm motility	300	P4	Murack et al., 2011
Roach (*Rutilus rutilus*)	Pubertal	28-day	Males: ↓ plasma 11-KT, ↑ spgB (%) in the testes, and ↓ mRNA expression of *fshα*; Females: ↓ plasma E2	312	LNG	Kroupova et al., 2014
Western mosquitofish (*Gambusia affinis*)	Adult	42-day	↑ arα, ↑ arβ, ↓ erα, ↓ erβ, ↓vtgA,B,C, ↓ *cyp1a* mRNA expression, Females: ↑ width of ray 3 in anal fin, ↑ number of ray 3 segment in anal fin,↑ number of postovulatory and atretic follicles	410	P4	Hou et al., 2017
Fathead minnow (*Pimephales promelas*)	Larvae	28-day	↓ survival	462	LNG	Overturf et al., 2014
	Adult	4-hour *ex vivo*	Females: mRNA expression of ↓ *aqp8*, ↑ *hsd20α*, ↑ *foll*, ↑ *p38mapk*, and ↑ *zp3* in the ovary	500	P4	Garcia-Reyero et al., 2013
Roach (*Rutilus rutilus*)	Adult	42-day	↑ levels of DJ-1 protein in brain and hepatopancreas, ↑ GSI, ↑ KSI, ↓ serum cholesterol, ↓ LDL-cholesterol in serum, ↑ *vtg* levels in hepatopancreas of females	500 + 500 + 500	P4 + DRO + LNG	Maasz et al., 2017

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Zebrafish (*Danio rerio*)	Juvenile	22-day	Changes in expression of mRNA related to sex differentiation	500	NET	Hou et al., 2018a
	Adult	45-day	Sex ratio: ↓ females, ↑ intersex, Males: ↑ % of mature spermatocytes in testes, Females: ↑ % atretic follicles			
	Juvenile	14-day	↓ *cyp19a1a*, ↑ *dmrt1*, ↑ *casp1b*, ↑ *casp3a*, ↑ *casp9* mRNA expression	500	DGT	Shi et al., 2018
	Adult	120-day	Sex ratio: all male Males: changes in mRNA expression related to various physiological processes in testis			
Zebrafish (*Danio rerio*)	Adult	21-day	Females: ↓ egg production, ↓ E2, ↓ T in plasma Males: ↓ plasma 11-KT	666	MGA	Han et al., 2014
Fathead minnow (*Pimephales promelas*)	Egg to Larvae	28-day	↓ growth	740	NET	Overturf et al., 2012
Three-spined stickleback (*Gasterosteus aculeatus*)	Adult	45-day	Males: ↑ KEH, ↑ NSI, inhibition of the onset of spermatogenesis, mRNA expression of ↑ spiggin and ↓ *cyp1a* in the kidney, ↑ *lhα* in the pituitary	750	LNG	Svensson et al., 2014
Western mosquitofish (*Gambusia affinis*)	Adult	8-day	Males: mRNA expression of ↓ *esrα*, ↓ *esrα*, ↓ *arα*, and ↓ *mt* in the testis and ↓ *arα* in the liver	1000	P4	Huang et al., 2013
Zebrafish (*Danio rerio*)	Juvenile	22-day	Changes in expression of genes related to sex differentiation	1000	NET	Hou et al., 2018a
	Adult	45-day	Sex ratio: ↓ females, ↑ intersex, males: ↑ (%) of mature spermatocytes in testes			
	Adult	14-day	Females: changes in expression of mRNA related to circadian rhythm, ↑ atretic follicles, ↑ mature vitellogenic oocytes Males: changes in expression of mRNA related to circadian rhythm, ↑ mature sperm	1000	NGT	Shi et al., 2017
Zebrafish (*Danio rerio*)	Embryo	6-day	↓ *ar*, ↓ *pgr*, ↓ *gr* mRNA expression	1000	CPA	Siegenthaler et al., 2017a
		6-day	↑ *pgr*, ↑ *erα*, ↑ *vtg1*, ↑ *cyp19a1b* mRNA expression	1000 + 100	CPA	
		4-day	↓ *fshα*, ↓ *cyp2k7* mRNA expression	1000	CMA	
			↑ *vtg1*, ↑ *cyp19a1b*, ↑ *lhb*, ↑ *erα*, ↑ *pgr* mRNA expression	1000 + 100	CMA + EE2	
		6-day	↓ *ar*, ↓ *cyp2k7* mRNA expression	1000	CMA	
			↑ *vtg1*, ↑ *cyp19a1b*, ↑ *erα*, ↑ *pgr*, ↑ *lhb*, ↓ *fshα* mRNA expression	1000 + 100	CMA + EE2	

Species	Age class	Test	Effect	LOEC (ng · l^{-1})	Progestin	Reference
Japanese medaka (*Oryzias latipes*)	Egg to Adult	3-month	Females:↓ oogenesis; Males: ↓ spermatogenesis, ↓ mean body weight, ↓ CF	1000	CPA	Kiparissis et al., 2003
Roach (*Rutilus rutilus*)	Pubertal	28-day	Males: ↓ plasma 11-KT, mRNA expression of ↓ *fshα*, ↑ *lhα*, ↑ *vtg*, ↑ *erα*; Females: plasma ↓ E2, ↓ 11-KT, ↑ T, and mRNA expression of ↓ *fshα*, ↑ *lhα*, ↑ *vtg*, ↑ *erα*	3124	LNG	Kroupova et al., 2014
Common carp (*Cyprinus carpio*)	Juvenile	96-hour	Immunosuppression (↓ NO2- formation by the head and trunk kidney derived leukocytes stimulated *in vitro*)	3900	MPA	Pietsch et al., 2009
Fathead minnow (*Pimephales promelas*)	Adult	21-day	Females: ↓ egg production	6500	DRO	Zeilinger et al., 2009
		21-day	Females: development of male secondary characteristic, ↓ egg production, plasma ↓ E2, ↓ T, and ↓ VTG	10000	DES	Runnalls et al., 2013
Zebrafish (*Danio rerio*)	Embryo	6-day	↓ *ar* mRNA expression	10000	CPA	Siegenthaler et al., 2017a
		6-day	↑ *erα*, ↑ *vtg1*, ↑ *cyp19a1b* mRNA expression	10000 + 1000	CPA + EE2	
		4-day	↑ *vtg1*, ↑ *cyp19a1b*, ↑ *erα*, ↑ *pgr*, ↑ *lhb*, ↓ *hsd11α2*, ↑ *cyp2k7* mRNA expression	10000 + 1000	CMA + EE2	
		6-day	↑ *vtg1*, ↓ *cyp19a1a*, ↑ *cyp19a1b*, ↑ *erα*, ↑ *pgr*, ↑ *lhb*, ↓ *fshα*, ↑ *hsd17b3*, ↑ *cyp2k7* mRNA expression			
		6-day	↓ *ar*, ↓ *pgr* mRNA expression	100000	CPA	

Abbreviations and description: 11-KT – 11-ketotestosterone; *aqp8* – aquaporin 8; amh – anti-Müllerian hormone; *ar* – androgen receptor; *arα* – androgen receptor alpha; *arβ* – androgen receptor beta; *pgr* – progesterone receptor; *arnt2* – aryl hydrocarbon receptor nuclear translocator 2; casp1b – caspase 1-b; casp3a – caspase 3; casp9 – caspase 9; *ccnb1* – cyclin B1; CF – condition factor; CMA – chlormadinone acetate; CPA – cyproterone acetate; *cyp1a* – cytochrome P450, family 1, subfamily A; cyp2k7 – cytochrome P450, family 2, subfamily K, member 7; *cyp11a1* – cytochrome P450, family 11, subfamily A, member 1; *cyp11b* – 11β-hydroxylase; *cyp17* – cytochrome P450, family 17; *cyp19a1a* – gonad-type aromatase; *cyp19a1b* – brain-type aromatase; DGT – dydrogesterone; DHP – 17,20-β-dihydroxypregn-4-en-3-one; *dio1* – iodothyronine deiodinase type I; *dmrt1* – doublesex and mab-3 related transcription factor 1; DRO – drospirenone; E2 – 17β-estradiol; EE2 – ethynilestradiol; *erα* – estrogen receptor alpha; *erβ* – estrogen receptor beta; *erβ1 – estrogen receptor beta, subtype 1; figa* – folliculogenesis-specific basic helix-loop-helix; foxl2 – forkhead box L2; foll – follistatin; *fsh*β – follicle-stimulating hormone (β-subtype); GES – gestodene; GSI – gonado-somatic index; *gr* – glucocorticoid receptor; *hsd3β* – hydroxysteroid (3-β) dehydrogenase; hsd11β*2* – hydroxysteroid (11-β) dehydrogenase 2; *hsd17*β*3*

– hydroxysteroid (17-β) dehydrogenase 3; *hsd20*β – hydroxysteroid (20-β) dehydrogenase; HSI – hepatosomatic index; il1rapl1a – interleukin 1 receptor accessory protein-like 1a; KEH – kidney epithelium height; KSI – kidney somatic index; lhb – luteinizing hormone, beta polypeptide; LNG – levonorgestrel; LOEC – lowest observed effect concentration; MGA – megestrol acetate; *mpgrα* – membrane progesterone receptor, subtype α; *mpgr*β – membrane progesterone receptor, subtype β; *mhc1uea* – major histocompatibility complex class I UEA (involved in immune response); *mhc1ufa* – maor histocompatibility compley class I UFA (involved in immune response); *mr* – mineralocorticoid receptor; *mt* – metallothionein; NET – norethisterone; NGT – norgestrel; *nis* – sodium iodide symporter; *nr5a1b* – nuclear receptor subfamily 5, group A, member 1b (related to sex differentiation); T – testosterone; *nr1d1* – nuclear receptor subfamily 1, group D, member 1 (involved in circadian rhythm); NSI – nephrosomatic index; *p38mapk* – p38 mitogen-activated protein kinase; P4 – progesterone; *pomc* – proopiomelanocortin; spgB – spermatogonia B; *tg* – thyroglobulin; *thrβ* – thyroid receptor beta; *tnfrsf19* – tumor necrosis factor receptor superfamily, member 19 (involved in endothelial barrier and regulation of cell migration); *vtgA* – vitellogenin A; *vtgB* – vitellogenin B; *vtgC* – vitellogenin C; *vtg1* – vitellogenin 1; *zp3* – zona pellucida 3.

1.5. Aims of the thesis

Progestins, an emerging group of pollutants, came recently into suspicion of causing substantial risk to the aquatic environment (Fent, 2015; Kumar et al., 2015). Therefore, the overall aim of this thesis was to shed some light on their occurrence and hormonal activities in the aquatic environment as well as on their effects on fish.

The specific objectives of this Ph.D. thesis were to:

- detect progestins in the aquatic environment
- detect progestagenic and (anti-)androgenic activities in the aquatic environment
- determine relative progestagenic and (anti-)androgenic potencies of progestins prescribed in the Czech Republic
- evaluate the potential of individual progestins to cause progestagenic and (anti-) androgenic activities in the aquatic environment related to municipal WWTPs
- assess effects of etonogestrel, a synthetic progestin of the 3rd generation, on fish

Detection of progestins in aquatic environments using instrumental analysis is reported in chapters 2, 3 and 4. Monitoring of progestagenic activities is described in chapter 3, while chapter 4 presents the screening for (anti-)androgenic activities. Progestagenic and (anti-)androgenic REPs of progestins are determined in chapters 3 and 4, respectively. The effects of sub-lethal concentrations of the synthetic progestin etonogestrel on morphology, reproduction and reproductive behaviour of Endler's guppies (*Poecilia wingei*) are described in chapter 5.

1.6. References

Africander, D., Verhoog, N., Hapgood, J. P., 2011. Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. Steroids 76, 636–652.

Al-Odaini, N. A., Zakaria, M. P., Yaziz, M. I., Surif, S., 2010. Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1217, 6791–6806.

Ankley, G., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., Miller, R., Bantle, J., Campbell, P., Denslow, N., Dickerson, R., Folmar, L., Fry, M., Giesy, J., Earl Gray, L., Guiney, P., Hutchinson, T., Kennedy, S., Kramer, V., LeBlanc, G., Mayes, M., Nimrod, A., Patino, R., Peterson, R., Purdy, R., Ringer, R., Thomas, P., Touart, L., van der Kraak, G., Zacharewski, T., 1998. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environmental Toxicology and Chemistry 17, 68–87.

Archer, J. S., Chang, R. J., 2004. Hirsutism and acne in polycystic ovary syndrome. Best Practice & Research Clinical Obstetrics & Gynaecology 18, 737–754.

Avar, P., Maász, G., Takács, P., Lovas, S., Zrínyi, Z., Svigruha, R., Takátsy, A., Tóth, L. G., Pirger, Z., 2016. HPLC-MS/MS analysis of steroid hormones in environmental water samples. Drug Testing and Analysis 8, 123–127.

Bain, P. A., Williams, M., Kumar, A., 2014. Assessment of multiple hormonal activities in wastewater at different stages of treatment. Environmental Toxicology and Chemistry 33, 2297–2307.

Bain, P. A., Kumar, A., Ogino, Y., Iguchi, T., 2015. Nortestosterone-derived synthetic progestogens do not activate the progestogen receptor of Murray–Darling rainbowfish (*Melanotaenia fluviatilis*) but are potent agonists of androgen receptors alpha and beta. Aquatic Toxicology 163, 97–101.

Becker, K. L., 2001. Principles and Practice of Endocrinology and Metabolism. Lippincott Williams & Wilkins, Philadelphia, 957 pp.

Bellet, V., Hernandez-Raquet, G., Dagnino, S., Seree, L., Pardon, P., Bancon-Montiny, C., Fenet, H., Creusot, N., Aït-Aïssa, S., Cavailles, V., Budzinski, H., Antignac, J.-P., Balaguer, P., 2012. Occurrence of androgens in sewage treatment plants influents is associated with antagonist activities on other steroid receptors. Water Research 46, 1912–1922.

Bergink, E.W., Hamburger, A.D., de Jager, E., van der Vies, J., 1981. Binding of a contraceptive progestogen ORG 2969 and its metabolites to receptor proteins and human sex hormone binding globulin. Journal of Steroid Biochemistry 14, 175–183.

Bergink, E. W., Van Meel, F., Turpijn, E. W., Van der Vies, J., 1983. Binding of progestagens to receptor proteins in MCF-7 cells. Journal of Steroid Biochemistry 19, 1563–1570.

Besse, J. P., Garric, J., 2009. Progestagens for human use, exposure and hazard assessment for the aquatic environment. Environmental Pollution 157, 3485–3494.

Blankvoort, B. M., Rodenburg, R. J., Murk, A. J., Koeman, J. H., Schilt, R., Aarts, J. M., 2005. Androgenic activity in surface water samples detected using the AR-LUX assay: indications for mixture effects. Environmental Toxicology and Pharmacology 19, 263–272.

Blüthgen, N., Castiglioni, S., Sumpter, J. P., Fent, K., 2013a. Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 1. Reproductive and early developmental effects. Aquatic Toxicology 144–145, 83–95.

Blüthgen, N., Sumpter, J. P., Odermatt, A., Fent, K., 2013b. Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 2. Gene expression analysis and *in vitro* activity. Aquatic toxicology 144, 96–104.

Brockmeier, E. K., Scott, P. D., Denslow, N. D., Leusch, F. D., 2016. Transcriptomic and physiological changes in Eastern Mosquitofish (*Gambusia holbrooki*) after exposure to progestins and anti-progestagens. Aquatic Toxicology 179, 8–17.

Chang, H., Wu, S., Hu, J., Asami, M., Kunikane, S., 2008. Trace analysis of androgens and progestogens in environmental waters by ultra-performance liquid chromatography–electrospray tandem mass spectrometry. Journal of Chromatography A 1195, 44–51.

Chang, H., Wan, Y., Hu, J. Y., 2009. Determination and source apportionment of five classes of steroid hormones in urban rivers. Environmental Science & Technology 43, 7691–7698.

Chang, H., Wan, Y., Wu, S., Fan, Z., Hu, J., 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: Comparison to estrogens. Water Research 45, 732–740.

Chatterjee, S., Majumder, C. B., Roy, P., 2007. Development of a yeast-based assay to determine the (anti)androgenic contaminants from pulp and paper mill effluents in India. Environmental Toxicology and Pharmacology 24, 114–121.

Chatterjee, S., Kumar, V., Majumder, C. B., Roy, P., 2008. Screening of some anti-progestin endocrine disruptors using a recombinant yeast based *in vitro* bioassay. Toxicology in Vitro 22, 788–798.

Chen, K-Y., Chou, P-H., 2016. Detection of endocrine active substances in the aquatic environment in southern Taiwan using bioassays and LC-MS/MS. Chemosphere 152, 214–220.

Chen, S.X., Bogerd, J., García-López, Á., de Jonge, H., de Waal, P.P., Hong, W.S., Schulz, R.W., 2010. Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. Biology of Reproduction 82, 171–181.

Clarke, C. L., Sutherland, R. L., 1990. Progestin regulation of cellular proliferation. Endocrine Reviews 11, 266–301.

Colborn, T., Saal, F.S.V., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environmental Health Perspectives 101, 378–384.

Creusot, N., Aït-Aïssa, S., Tapie, N., Pardon, P., Brion, F., Sanchez, W., Thybaud, E., Porcher, J.-M., Budzinski, H., 2014. Identification of synthetic steroids in river water downstream from pharmaceutical manufacture discharges based on a bioanalytical approach and passive sampling. Environmental Science & Technology 48, 3649–3657.

Cwiertny, D. M., Snyder, S. A., Schlenk, D., Kolodziej, E. P., 2014. Environmental designer drugs: when transformation may not eliminate risk. Environmental Science & Technology 48, 11737–11745.

DeQuattro, Z. A., Peissig, E. J., Antkiewicz, D. S., Lundgren, E. J., Hedman, C. J., Hemming, J. D. C., Barry, T. P., 2012. Effects of progesterone on reproduction and embryonic development in the fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 31, 851–856.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, T. R., Gore, A. C., 2009. Endocrine-disrupting chemicals: An endocrine society scientific statement. Endocrine Reviews 30, 293–342.

Ellestad, L. E., Cardon, M., Chambers, I. G., Farmer, J. L., Hartig, P., Stevens, K., Villeneuve, D.L., Wilson, V.S., Orlando, E. F., 2014. Environmental gestagens activate fathead minnow (*Pimephales promelas*) nuclear progesterone and androgen receptors *in vitro*. Environmental Science & Technology 48, 8179–8187.

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J.Y.M., van der Burg, B., van der Linden, S.C., Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X., Leusch, F.D.L., 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. Environmental Science & Technology 48, 1940–1956.

Escher, B., Leusch, F., 2012. Bioanalytical tools in water quality assessment. IWA publishing.

Escher, B. I., Aït-Aïssa, S., Behnisch, P. A., Brack, W., Brion, F., Brouwer, A., Buchinger, S., Crawford, S. E., Du Pasquier, D., Hamers, T., Hettwer, K., Hilscherová, K., Hollert, H., Kase, R., Kienle, C., Tindall, A. J., Tuerk, J., van der Oost, R., Vermeirssen, E., Neale, P. A., 2018. Effect-based trigger values for *in vitro* and *in vivo* bioassays performed on surface water extracts supporting the environmental quality standards (EQS) of the European Water Framework Directive. Science of the Total Environment 628, 748–765.

Eustache, F., Mondon, F., Canivenc-Lavier, M. C., Lesaffre, C., Fulla, Y., Berges, R., Cravedi, J.P., Vaiman, D., Auger, J., 2009. Chronic dietary exposure to a low-dose mixture of genistein and vinclozolin modifies the reproductive axis, testis transcriptome, and fertility. Environmental Health Perspectives 117, 1272–1279.

Evans, R. M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240, 889–895.

Fang, Y.-X., Ying, G.-G., Zhao, J.-L., Chen, F., Liu, S., Zhang, L.-J., Yang, B., 2012. Assessment of hormonal activities and genotoxicity of industrial effluents using *in vitro* bioassays combined with chemical analysis. Environmental Toxicology and Chemistry 31, 1273-1282.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environment International 84, 115–130.

Fick, J., Lindberg, R. H., Parkkonen, J., Arvidsson, B., Tysklind, M., Larsson, D. J., 2010. Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. Environmental Science & Technology 44, 2661–2666.

Frankel, T. E., Meyer, M. T., Kolpin, D. W., Gillis, A. B., Alvarez, D. A., Orlando, E. F., 2016a. Exposure to the contraceptive progestin, gestodene, alters reproductive behavior, arrests egg deposition, and masculinizes development in the fathead minnow (*Pimephales promelas*). Environmental Science & Technology 50, 5991–5999.

Frankel, T. E., Meyer, M. T., Orlando, E. F., 2016b. Aqueous exposure to the progestin, levonorgestrel, alters anal fin development and reproductive behavior in the eastern mosquitofish (*Gambusia holbrooki*). General and Comparative Endocrinology, 234, 161–169.

Frankel, T., Yonkos, L., Ampy, F., Frankel, J., 2018. Exposure to levonorgestrel increases nest acquisition success and decreases sperm motility in the male fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 37, 1131–1137.

García-Reyero, N., Grau, E., Castillo, M., De Alda, M. J. L., Barceló, D., Piña, B., 2001. Monitoring of endocrine disruptors in surface waters by the yeast recombinant assay. Environmental Toxicology and Chemistry 20, 1152–1158.

Garcia-Reyero, N., Martyniuk, C. J., Kroll, K. J., Escalon, B. L., Spade, D. J., Denslow, N. D., 2013. Transcriptional signature of progesterone in the fathead minnow ovary (*Pimephales promelas*). General and Comparative Endocrinology 192, 159–169.

Golovko, O., Šauer, P., Fedorova, G., Kroupová, H. K., Grabic, R., 2018. Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS. Science of the Total Environment 621, 1066–1073.

Graham, J. D., Clarke, C. L., 1997. Physiological action of progesterone in target tissues. Endocrine Reviews 18, 502–519.

Han, J., Wang, Q., Wang, X., Li, Y., Wen, S., Liu, S., Ying, G., Guo, Y., Zhou, B., 2014. The synthetic progestin megestrol acetate adversely affects zebrafish reproduction. Aquatic Toxicology 150, 66–72.

Hashmi, M. A. K., Escher, B. I., Krauss, M., Teodorovic, I., Brack, W., 2018. Effect-directed analysis (EDA) of Danube River water sample receiving untreated municipal wastewater from Novi Sad, Serbia. Science of the Total Environment 624, 1072–1081.

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A. L., Giesy, J. P., 2000. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environmental Science and Pollution Research 7, 159–171.

Hou, L., Xu, H., Ying, G., Yang, Y., Shu, H., Zhao, J., Cheng, X., 2017. Physiological responses and gene expression changes in the western mosquitofish (*Gambusia affinis*) exposed to progesterone at environmentally relevant concentrations. Aquatic Toxicology 192, 69–77.

Hou, L. P., Chen, H., Tian, C. E., Shi, W. J., Liang, Y., Wu, R. R., Fang, X. W., Zhang, C. P., Liang, Y. Q., Xie, L., 2018a. The progestin norethindrone affects sex differentiation and alters transcriptional profiles of genes along the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes in juvenile zebrafish Danio rerio. Aquatic Toxicology 201, 31–39.

Hou, L. P., Chen, H., Tian, C. E., Liang, Y., Wu, R. R., Zhang, X. M., Fang, X. W., Zhang, C. P., Hu, J. J., Song, L. Y., Liang, Y. Q., Schlenk, D., Xie, L., 2018b. Alterations of secondary sex characteristics, reproductive histology and behaviors by norgestrel in the western mosquitofish (*Gambusia affinis*). Aquatic Toxicology 198, 224–230.

Houtman, C.J., Sterk, S.S., van de Heijning, M.P.M., Brouwer, A., Stephany, R.W., van der Burg, B., Sonneveld, E., 2009. Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays. Analytica Chimica Acta 637, 247–258.

Houtman, C. J., ten Broek, R., Brouwer, A., 2018. Steroid hormonal bioactivities, culprit natural and synthetic hormones and other emerging contaminants in waste water measured using bioassays and UPLC-tQ-MS. Science of the Total Environment 630, 1492–1501.

Hua, J., Han, J., Guo, Y., Zhou, B., 2015. The progestin levonorgestrel affects sex differentiation in zebrafish at environmentally relevant concentrations. Aquatic Toxicology 166, 1–9.

Huang, G.-Y., Ying, G.-G., Liang, Y.-Q., Liu, Y.-S., Liu, S.-S., 2013. Effects of steroid hormones on reproduction- and detoxification-related gene expression in adult male mosquitofish, *Gambusia affinis*. Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology 158, 36–43.

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., Austin, C. P., 2011. Chemical genomics profiling of environmental chemical modulation of human nuclear receptors. Environmental Health Perspectives 119, 1142–1148.

Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 2001. Two subtypes of androgen and progestogen receptors in fish testes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 129, 449–455.

Islam, R., 2011. Synchronization of estrus in cattle: a review. Veterinary World 4, 136–141.

Jenkins, R. L., Wilson, E. M., Angus, R. A., Howell, W. M., Kirk, M., Moore, R., Nance, M., Brown, A., 2004. Production of androgens by microbial transformation of progesterone *in vitro*: A model for androgen production in rivers receiving paper mill effluent. Environmental Health Perspectives 112, 1508–1511.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C. R., van Aerle, R., Santos, E., Brighty, G. (2005). Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. *Environmental health perspectives*, *114*(Suppl 1), 32–39.

Juchem, M., Pollow, K., Elger, W., Hoffmann, G., Möbus, V., 1993. Receptor binding of norgestimate – a new orally active synthetic progestational compound. Contraception 47, 283–294.

Kasid, A., Buckshee, K., Hingorani, V., 1978. Interaction of progestins with steroid receptors in human uterus. Biochemical Journal 176, 531–539.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J. M., Aït-Aïssa, S., 2010. Bioanalytical characterisation of multiple endocrine-and dioxin-like activities in sediments from reference and impacted small rivers. Environmental Pollution 158, 74–83.

Kinnberg, K., 2003. Evaluation of *in vitro* assays for determination of estrogenic activity in the environment (no. 43). Danish Environmental Protection Agency, Danish Ministry of the Environment.

King, O. C., van de Merwe, J. P., McDonald, J. A., Leusch, F. D., 2016. Concentrations of levonorgestrel and ethinylestradiol in wastewater effluents: Is the progestin also cause for concern? Environmental Toxicology and Chemistry 35, 1378–1385.

Kiparissis, Y., Metcalfe, T. L., Balch, G. C., Metcalfe, C. D., 2003. Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). Aquatic Toxicology 63, 391–403.

Kirk, L. A., Tyler, C. R., Lye, C. M., Sumpter, J. P., 2002. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. Environmental Toxicology and Chemistry 21, 972–979.

Kloosterboer, H. J., Vonk-Noordegraaf, C. A., Turpijn, E. W., 1988. Selectivity in progesterone and androgen receptor binding of progestagens used in oral contraceptives. Contraception 38, 325–332.

Kolodziej, E. P., Gray, J. L., Sedlak, D. L., 2003. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. Environmental Toxicology and Chemistry 22, 2622–2629.

Kolodziej, E. P., Harter, T., Sedlak, D. L., 2004. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. Environmental Science & Technology 38, 6377–6384.

Kolodziej, E. P., Sedlak, D. L., 2007. Rangeland grazing as a source of steroid hormones to surface waters. Environmental Science & Technology 41, 3514–3520.

Kolok, A. S., Snow, D. D., Kohno, S., Sellin, M. K., Guillette Jr, L. J., 2007. Occurrence and biological effect of exogenous steroids in the Elkhorn River, Nebraska, USA. Science of the Total Environment 388, 104–115.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., Buxton, H. T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999– 2000: A national reconnaissance. Environmental Science & Technology 36, 1202–1211.

Kroupova, H. K., Trubiroha, A., Lorenz, C., Contardo-Jara, V., Lutz, I., Grabic, R., Kocour, M., Kloas, W., 2014. The progestin levonorgestrel disrupts gonadotropin expression and sex steroid levels in pubertal roach (*Rutilus rutilus*). Aquatic Toxicology 154, 154–162.

Kuch, H. M., Ballschmiter, K., 2000. Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/L-level. Fresenius' Journal of Analytical Chemistry 366, 392–395.

Kuhl, H., 1990. Pharmacokinetics of oestrogens and progestogens. Maturitas, 12, 171–197.

Kuhl, H., 1996. Comparative pharmacology of newer progestogens. Drugs 51, 188–215.

Kuhnz, W., Fritzemeier, K. H., Hegele-Hartung, C., Krattenmacher, R., 1995. Comparative progestational activity of norgestimate, levonorgestrel-oxime and levonorgestrel in the rat and binding of these compounds to the progesterone receptor. Contraception 51, 131–139.

Kumar, V., Johnson, A.C., Trubiroha, A., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kocour Kroupová, H., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environmenta Science & Technology 49, 2625–2638.

Kuster, M., de Alda, M. J. L., Hernando, M. D., Petrovic, M., Martín-Alonso, J., Barceló, D., 2008. Analysis and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain). Journal of Hydrology 358, 112–123.

Kuster, M., Azevedo, D. D. A., De Alda, M. L., Neto, F. A., Barceló, D., 2009. Analysis of phytoestrogens, progestogens and estrogens in environmental waters from Rio de Janeiro (Brazil). Environment International 35, 997–1003.

Labadie, P., Budzinski, H., 2005. Development of an analytical procedure for determination of selected estrogens and progestagens in water samples. Analytical and Bioanalytical Chemistry 381, 1199–1205.

Labrie, C., Cusan, L., Plante, M., Lapointe, S., Labrie, F., 1987. Analysis of the androgenic activity of synthetic "progestins" currently used for the treatment of prostate cancer. Journal of Steroid Biochemistry 28, 379–384.

Lee, H. J., Chattopadhyay, S., Gong, E. Y., Ahn, R. S., Lee, K., 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicological Sciences 75, 40–46.

Leskinen, P., Michelini, E., Picard, D., Karp, M., Virta, M., 2005. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61, 259–266.

Leusch, F. D., Khan, S. J., Laingam, S., Prochazka, E., Froscio, S., Trinh, T., Chapman, H. F., Humpage, A., 2014. Assessment of the application of bioanalytical tools as surrogate measure of chemical contaminants in recycled water. Water Research 49, 300–315.

Li, J., Chen, M., Wang, Z., Ma, M., Peng, X., 2011. Analysis of environmental endocrine disrupting activities in wastewater treatment plant effluents using recombinant yeast assays incorporated with exogenous metabolic activation system. Biomedical and Environmental Sciences 24, 132–139.

Liang, Y. Q., Huang, G. Y., Liu, S. S., Zhao, J. L., Yang, Y. Y., Chen, X. W., Tian, F., Jiang, Y.X., Ying, G. G., 2015a. Long-term exposure to environmentally relevant concentrations of progesterone and norgestrel affects sex differentiation in zebrafish (*Danio rerio*). Aquatic Toxicology 160, 172–179.

Liang, Y. Q., Huang, G. Y., Ying, G. G., Liu, S. S., Jiang, Y. X., Liu, S., 2015b. Progesterone and norgestrel alter transcriptional expression of genes along the hypothalamic–pituitary–thyroid axis in zebrafish embryos-larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 167, 101–107.

Liu, S. S., Ying, G. G., Liu, S., Lai, H. J., Chen, Z. F., Pan, C. G., Zhao, J. L., Chen, J., 2014. Analysis of 21 progestagens in various matrices by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with diverse sample pretreatment. Analytical and Bioanalytical Chemistry 406, 7299–7311.

Liu, S., Chen, H., Zhou, G. J., Liu, S. S., Yue, W. Z., Yu, S., Sun, K. F., Cheng, H., Ying, G. G., Xu, X. R., 2015. Occurrence, source analysis and risk assessment of androgens, glucocorticoids and progestagens in the Hailing Bay region, South China Sea. Science of the Total Environment 536, 99–107.

Liu, Z. H., Ogejo, J. A., Pruden, A., Knowlton, K. F., 2011a. Occurrence, fate and removal of synthetic oral contraceptives (SOCs) in the natural environment: A review. Science of the Total Environment 409, 5149–5161.

Liu, S., Ying, G. G., Zhao, J. L., Chen, F., Yang, B., Zhou, L. J., Lai, H. J., 2011b. Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry. Journal of Chromatography A 1218, 1367–1378.

Louw-du Toit, R., Perkins, M. S., Hapgood, J. P., Africander, D., 2017. Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy. Biochemical and Biophysical Research Communications 491, 140–146.

Ma, R., Cotton, B., Lichtensteiger, W., Schlumpf, M., 2003. UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. Toxicological Sciences 74, 43–50.

Maasz, G., Zrinyi, Z., Takacs, P., Lovas, S., Fodor, I., Kiss, T., Pirger, Z., 2017. Complex molecular changes induced by chronic progestogens exposure in roach, *Rutilus rutilus*. Ecotoxicology and Environmental Safety 139, 9–17.

Macikova, P., Groh, K. J., Ammann, A. A., Schirmer, K., Suter, M. J. F., 2014. Endocrine Disrupting Compounds Affecting Corticosteroid Signaling Pathways in Czech and Swiss Waters: Potential Impact on Fish. Environmental Science & Technology 48, 12902–12911.

Maltoni, M., Nanni, O., Scarpi, E., Rossi, D., Serra, P., Amadori, D., 2001. High-dose progestins for the treatment of cancer anorexia–cachexia syndrome: systematic review of randomised clinical trials. Annals of Oncology 12, 289–300.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R. M., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.

Matějíček, D., Kubáň, V., 2007. High performance liquid chromatography/ion-trap mass spectrometry for separation and simultaneous determination of ethynylestradiol, gestodene, levonorgestrel, cyproterone acetate and desogestrel. Analytica Chimica Acta 588, 304–315.

McRobb, L., Handelsman, D. J., Kazlauskas, R., Wilkinson, S., McLeod, M. D., Heather, A. K., 2008. Structure–activity relationships of synthetic progestins in a yeast-based *in vitro* androgen bioassay. The Journal of Steroid Biochemistry and Molecular Biology 110, 39–47.

Mnif, W., Dagnino, S., Escande, A., Pillon, A., Fenet, H., Gomez, E., Casellas, C., Duchesne, M.-J., Hernandez-Raquet, G., Cavaillès, V., Balaguer, P., Bartegi, A., 2010. Biological analysis of endocrine-disrupting compounds in Tunisian sewage treatment plants. Archives of Environmental Contamination and Toxicology 59, 1–12.

Murack, P. J., Parrish, J., Barry, T. P., 2011. Effects of progesterone on sperm motility in fathead minnow (*Pimephales promelas*). Aquatic Toxicology 104, 121–125.

Nagahama, Y., 2002. Endocrine regulation of gametogenesis in fish. International Journal of Developmental Biology 38, 217–229.

Neale, P. A., Ait-Aissa, S., Brack, W., Creusot, N., Denison, M. S., Deutschmann, B., Hilscherova, K., Hollert, H., Krauss, M., Novák, J., Schulze, T., Seiler, T. B., Serra, H., Shao, Y., Escher, B. I., 2015. Linking *in vitro* effects and detected organic micropollutants in surface water using mixture-toxicity modeling. Environmental Science & Technology 49, 14614–14624.

Orlando, E. F., Ellestad, L. E., 2014. Sources, concentrations, and exposure effects of environmental gestagens on fish and other aquatic wildlife, with an emphasis on reproduction. General and Comparative Endocrinology 203, 241–249.

Overturf, M. D., Overturf, C. L., Baxter, D., Hala, D. N., Constantine, L., Venables, B., Huggett, D. B., 2012. Early life-stage toxicity of eight pharmaceuticals to the fathead minnow, *Pimephales promelas*. Archives of Environmental Contamination and Toxicology 62, 455–464.

Overturf, M. D., Overturf, C. L., Carty, D. R., Hala, D., Huggett, D. B., 2014. Levonorgestrel exposure to fathead minnows (*Pimephales promelas*) alters survival, growth, steroidogenic gene expression and hormone production. Aquatic Toxicology 148, 152–161.

Paulos, P., Runnalls, T. J., Nallani, G., La Point, T., Scott, A. P., Sumpter, J. P., Huggett, D. B., 2010. Reproductive responses in fathead minnow and Japanese medaka following exposure to a synthetic progestin, Norethindrone. Aquatic Toxicology 99, 256–262.

Pauwels, B., Noppe, H., De Brabander, H., Verstraete, W., 2008. Comparison of steroid hormone concentrations in domestic and hospital wastewater treatment plants. Journal of environmental engineering 134, 933–936.

Petersen, L. H., Hala, D., Carty, D., Cantu, M., Martinović, D., Huggett, D. B., 2015. Effects of progesterone and norethindrone on female fathead minnow (*Pimephales promelas*) steroidogenesis. Environmental Toxicology and Chemistry 34, 379–390.

Petrovic, M., Solé, M., López De Alda, M. J., Barceló, D., 2002. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. Environmental Toxicology and Chemistry 21, 2146–2156.

Philibert, D., Bouchoux, F., Degryse, M., Lecaque, D., Petit, F., Gaillard, M., 1999. The pharmacological profile of a novel norpregnane progestin (trimegestone). Gynecological Endocrinology 13, 316–326.

Pietsch, C., Neumann, N., Knopf, K., Wuertz, S., Kloas, W., 2009. Progestogens cause immunosuppression of stimulated carp (*Cyprinus carpio L.*) leukocytes *in vitro*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 150, 16–24.

Pietsch, C., Neumann, N., Preuer, T., Kloas, W., 2011. *In vivo* treatment with progestogens causes immunosuppression of carp *Cyprinus carpio* leucocytes by affecting nitric oxide production and arginase activity. Journal of Fish Biology 79, 53–69.

Pollow, K., Juchem, M., Grill, H. J., Elger, W., Beier, S., Henderson, D., Schmidt-Gollwitzer, K., Manz, B., 1989. Gestodene: a novel synthetic progestin—characterization of binding to receptor and serum proteins. Contraception 40, 325–341.

Pollow, K., Juchem, M., Elger, W., Jacobi, N., Hoffmann, G., Möbus, V., 1992. Dihydrospirorenone (ZK30595): A novel synthetic progestagen-characterization of binding to different receptor proteins. Contraception 46, 561–574.

Pu, C., Wu, Y.F., Yang, H., Deng, A.P., 2008. Trace analysis of contraceptive drug levonorgestrel in wastewater samples by a newly developed indirect competitive enzyme-linked immunosorbent assay (ELISA) coupled with solid phase extraction. Analytica Chimica Acta 628, 73–79.

Qiao, Y., Yang, H., Wang, B., Song, J., Deng, A., 2009. Preparation and characterization of an immunoaffinity chromatography column for the selective extraction of trace contraceptive drug levonorgestrel from water samples. Talanta 80, 98–103.

Rao, K., Li, N., Ma, M., Wang, Z., 2014. *In vitro* agonistic and antagonistic endocrine disrupting effects of organic extracts from waste water of different treatment processes. Frontiers of Environmental Science & Engineering 8, 69–78.

Raudrant, D., Rabe, T., 2003. Progestogens with antiandrogenic properties. Drugs 63, 463–492.

Rehberger, K., Kropf, C., Segner, H., 2018. *In vitro* or not *in vitro*: a short journey through a long history. Environmental Sciences Europe 30, 23.

Rižner, T. L., Brožič, P., Doucette, C., Turek-Etienne, T., Müller-Vieira, U., Sonneveld, E., van der Burg, B., Böcker, C., Husen, B., 2011. Selectivity and potency of the retroprogesterone dydrogesterone *in vitro*. Steroids 76, 607–615.

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 16, 1318–1338.

Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environmental Science & Technology 47, 2077–2084.

Schiffer, B., Daxenberger, A., Meyer, K., Meyer, H. H., 2001. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. Environmental Health Perspectives 109, 1145–1151.

Schindler, A. E., Campagnoli, C., Druckmann, R., Huber, J., Pasqualini, J. R., Schweppe, K. W., Thijssen, J. H., 2003. Classification and pharmacology of progestins. Maturitas 46S1, S7-S16.

Schoonen, W. G. E. J., Joosten, J. W. H., Kloosterboer, H. J., 1995. Effects of two classes of progestagens, pregnane and 19-nortestosterone derivatives, on cell growth of human breast tumor cells: I. MCF-7 cell lines. The Journal of Steroid Biochemistry and Molecular Biology 55, 423–437.

Schoonen, W. G. E. J., Dijkema, R., De Ries, R. J. H., Wagenaars, J. L., Joosten, J. W. H., De Gooyer, M. E., Deckers, G. H., Kloosterboer, H. J., 1998. Human progesterone receptor A and B isoforms in CHO cells. II. Comparison of binding, transactivation and ED50 values of several synthetic (anti) progestagens *in vitro* in CHO and MCF-7 cells and *in vivo* in rabbits and rats. The Journal of Steroid Biochemistry and Molecular Biology 64, 157–170.

Schreurs, R. H., Sonneveld, E., Jansen, J. H., Seinen, W., van der Burg, B., 2004. Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. Toxicological Sciences 83, 264–272.

Schug, T. T., Janesick, A., Blumberg, B., Heindel, J. J. 2011. Endocrine disrupting chemicals and disease susceptibility. The Journal of Steroid Biochemistry and Molecular Biology 127, 204–215.

Scott, A. P., Sumpter, J. P., Stacey, N., 2010. The role of the maturation-inducing steroid, 17, 20β-dihydroxypregn-4-en-3-one, in male fishes: a review. Journal of Fish Biology 76, 183-224.

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., Leusch, F.D.L., 2014. An assessment of endocrine activity in Australian rivers using chemical and *in vitro* analyses. Environmental Science and Pollution Research 21, 12951–12967.

Sharpe, R. L., MacLatchy, D. L., Courtenay, S. C., Van Der Kraak, G. J., 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (*Fundulus heteroclitus*) bioassay. Aquatic Toxicology 67, 203–215.

Shen, X., Chang, H., Sun, D., Wang, L., Wu, F., 2018. Trace analysis of 61 natural and synthetic progestins in river water and sewage effluents by ultra-high performance liquid chromatography–tandem mass spectrometry. Water Research 133, 142–152.

Shi, W. J., Zhao, J. L., Jiang, Y. X., Huang, G. Y., Liu, Y. S., Zhang, J. N., Ying, G. G., 2017. Transcriptional and histological alterations in gonad of adult zebrafish after exposure to the synthetic progestin norgestrel. Environmental Toxicology and Chemistry 36, 3267–3276.

Shi, W. J., Jiang, Y. X., Huang, G. Y., Zhao, J. L., Zhang, J. N., Liu, Y. S., Xie, L., Ying, G. G. 2018. Dydrogesterone causes male bias and accelerates sperm maturation in zebrafish (*Danio rerio*). Environmental Science & Technology 52, 8903–8911.

Shore, L. S., Shemesh, M., 2003. Naturally produced steroid hormones and their release into the environment. Pure and Applied Chemistry 75, 1859–1871.

Shue, M. F., Chen, T. C., Bellotindos, L. M., Lu, M. C., 2014. Tributyltin distribution and producing androgenic activity in water, sediment, and fish muscle. Journal of Environmental Science and Health, Part B 49, 432–438.

Siegenthaler, P. F., Bain, P., Riva, F., Fent, K., 2017a. Effects of antiandrogenic progestins, chlormadinone and cyproterone acetate, and the estrogen 17α-ethinylestradiol (EE2), and their mixtures: transactivation with human and rainbowfish hormone receptors and transcriptional effects in zebrafish (*Danio rerio*) eleuthero-embryos. Aquatic Toxicology 182, 142–162.

Siegenthaler, P. F., Zhao, Y., Zhang, K., Fent, K., 2017b. Reproductive and transcriptional effects of the antiandrogenic progestin chlormadinone acetate in zebrafish (*Danio rerio*). Environmental Pollution 223, 346–356.

Sitruk-Ware, R., 2004. Pharmacological profile of progestins. Maturitas 47, 277-283.

Sohoni, P., Sumpter, J. P., 1998. Several environmental oestrogens are also anti-androgens. Journal of Endocrinology 158, 327–339.

Soliman, M. A., Pedersen, J. A., Suffet, I. M., 2004. Rapid gas chromatography–mass spectrometry screening method for human pharmaceuticals, hormones, antioxidants and plasticizers in water. Journal of Chromatography A 1029, 223–237.

Sonneveld, E., Pieterse, B., Schoonen, W. G., van der Burg, B., 2011. Validation of *in vitro* screening models for progestagenic activities: inter-assay comparison and correlation with *in vivo* activity in rabbits. Toxicology in Vitro 25, 545–554.

Soto, A. M., Calabro, J. M., Prechtl, N. V., Yau, A. Y., Orlando, E. F., Daxenberger, A., Kolok, A. S., Guillette Jr, L. J., le Bizec, B., Lange, I. G., Sonnenschein, C., 2004. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska, USA. Environmental Health Perspectives 112, 346–352.

Spitz, I. M., Chwalisz, K., 2000. Progesterone receptor modulators and progesterone antagonists in women's health. Steroids 65, 807–815.

Stanczyk, F. Z., Hapgood, J. P., Winer, S., Mishell Jr, D. R., 2013. Progestogens used in postmenopausal hormone therapy: differences in their pharmacological properties, intracellular actions, and clinical effects. Endocrine Reviews 34, 171–208.

Standley, L. J., Rudel, R. A., Swartz, C. H., Attfield, K. R., Christian, J., Erickson, M., Brody, J. G., 2008. Wastewater-contaminated groundwater as a source of endogenous hormones and pharmaceuticals to surface water ecosystems. Environmental Toxicology and Chemistry 27, 2457–2468.

State Institue for Drug Control, 2018. URL. http://www.sukl.eu/dodavky-leciv-v-ceskerepublice-v-jednotlivych-letech?lang=2.

Sumpter, J. P., 2005. Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochimica et Hydrobiologica 33, 9–16.

Sun, L., Yong, W., Chu, X., Lin, J. M., 2009. Simultaneous determination of 15 steroidal oral contraceptives in water using solid-phase disk extraction followed by high performance liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1216, 5416–5423.

Svensson, J., Fick, J., Brandt, I., Brunström, B., 2013. The synthetic progestin levonorgestrel is a potent androgen in the three-spined stickleback (*Gasterosteus aculeatus*). Environmental Science & Technology 47, 2043–2051.

Svensson, J., Fick, J., Brandt, I., Brunström, B., 2014. Environmental concentrations of an androgenic progestin disrupts the seasonal breeding cycle in male three-spined stickleback (*Gasterosteus aculeatus*). Aquatic Toxicology 147, 84–91.

Svensson, J., Mustafa, A., Fick, J., Schmitz, M., Brunström, B., 2016. Developmental exposure to progestins causes male bias and precocious puberty in zebrafish (*Danio rerio*). Aquatic Toxicology 177, 316–323.

Térouanne, B., Paris, F., Servant, N., Georget, V., Sultan, C., 2002. Evidence that chlormadinone acetate exhibits antiandrogenic activity in androgen-dependent cell line. Molecular and Cellular Endocrinology 198, 143–147.

Thomas, K. V., Hurst, M. R., Matthiessen, P., McHugh, M., Smith, A., Waldock, M. J., 2002. An assessment of *in vitro* androgenic activity and the identification of environmental androgens in United Kingdom estuaries. Environmental Toxicology and Chemistry 21, 1456–1461.

Thomas, P., 2008. Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. Frontiers in Neuroendocrinology 29, 292–312.

Tölgyesi, Á., Verebey, Z., Sharma, V. K., Kovacsics, L., Fekete, J., 2010. Simultaneous determination of corticosteroids, androgens, and progesterone in river water by liquid chromatography–tandem mass spectrometry. Chemosphere 78, 972–979.

Torres, N. H., Aguiar, M. M., Ferreira, L. F. R., Américo, J. H. P., Machado, Â. M., Cavalcanti, E. B., Tornisielo, V. L., 2015. Detection of hormones in surface and drinking water in Brazil by LC-ESI-MS/MS and ecotoxicological assessment with *Daphnia magna*. Environmental Monitoring and Assessment 187, 379.

Urbatzka, R., Van Cauwenberge, A., Maggioni, S., Viganò, L., Mandich, A., Benfenati, E., Lutz, I., Kloas, W., 2007. Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. Chemosphere 67, 1080–1087.

USEPA, 2010. Estimation Programs Interface Suite™ for Microsoft Windows, v 4.00. United States Environmental Protection Agency, Washington, DC, USA.

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs Jr, D. R., Lee, D. H., Shioda, T., Soto, A. M., vom Saal, F. S., Welshons, W. V., Zoeller, R. T., Myers, J. P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocrine Reviews 33, 378–455.

van der Linden, S. C., Heringa, M. B., Man, H. Y., Sonneveld, E., Puijker, L. M., Brouwer, A., van der Burg, B., 2008. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environmental Science & Technology 42, 5814–5820.

Velicu, M., Suri, R., 2009. Presence of steroid hormones and antibiotics in surface water of agricultural, suburban and mixed-use areas. Environmental Monitoring and Assessment 154, 349–359.

Viglino, L., Aboulfadl, K., Prévost, M., Sauvé, S., 2008. Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS. Talanta 76, 1088–1096.

Vinggaard, A. M., Hnida, C., Breinholt, V., Larsen, J. C., 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity *in vitro*. Toxicology in Vitro 14, 227–234.

Viswanath, G., Halder, S., Divya, G., Majumder, C. B., Roy, P., 2008. Detection of potential (anti) progestagenic endocrine disruptors using a recombinant human progesterone receptor binding and transactivation assay. Molecular and Cellular Endocrinology 295, 1–9.

Viswanath, G., Chatterjee, S., Dabral, S., Nanguneri, S. R., Divya, G., Roy, P., 2010. Anti-androgenic endocrine disrupting activities of chlorpyrifos and piperophos. The Journal of steroid biochemistry and molecular biology 120, 22–29.

Vulliet, E., Baugros, J. B., Flament-Waton, M. M., Grenier-Loustalot, M. F., 2007. Analytical methods for the determination of selected steroid sex hormones and corticosteriods in wastewater. Analytical and Bioanalytical Chemistry 387, 2143–2151.

Vulliet, E., Wiest, L., Baudot, R., Grenier-Loustalot, M. F., 2008. Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. Journal of Chromatography A 1210, 84–91.

Vulliet, E., Cren-Olivé, C., 2011. Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. Environmental Pollution 159, 2929–2934.

Vulliet, E., Cren-Olivé, C., Grenier-Loustalot, M. F., 2011. Occurrence of pharmaceuticals and hormones in drinking water treated from surface waters. Environmental Chemistry Letters 9, 103–114.

Weiss, J. M., Hamers, T., Thomas, K. V., van der Linden, S., Leonards, P. E., Lamoree, M. H., 2009. Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. Analytical and Bioanalytical Chemistry 394, 1385–1397.

Weiss, J. M., Simon, E., Stroomberg, G. J., de Boer, R., de Boer, J., van der Linden, S. C., Leonards, P. E. G., Lamoree, M. H., 2011. Identification strategy for unknown pollutants using high-resolution mass spectrometry: Androgen-disrupting compounds identified through effect-directed analysis. Analytical and Bioanalytical Chemistry 400, 3141–3149.

Weizel, A., Schlüsener, M. P., Dierkes, G., Ternes, T. A., 2018. Occurrence of Glucocorticoids, Mineralocorticoids, and Progestogens in Various Treated Wastewater, Rivers, and Streams. Environmental Science & Technology 52, 5296–5307.

Wu, Q., Lam, J. C., Kwok, K. Y., Tsui, M. M., Lam, P. K., 2017. Occurrence and fate of endogenous steroid hormones, alkylphenol ethoxylates, bisphenol A and phthalates in municipal sewage treatment systems. Journal of Environmental Sciences 61, 49–58.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Lange, R., 2009. Effects of synthetic gestagens on fish reproduction. Environmental Toxicology and Chemistry 28, 2663–2670.

Zhang, K., Fent, K., 2018. Determination of two progestin metabolites (17α-hydroxypregnanolone and pregnanediol) and different classes of steroids (androgens, estrogens, corticosteroids, progestins) in rivers and wastewaters by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Science of the Total Environment 610, 1164–1172.

Zhao, J. L., Ying, G. G., Yang, B., Liu, S., Zhou, L. J., Chen, Z. F., Lai, H. J., 2011. Screening of multiple hormonal activities in surface water and sediment from the Pearl River system, South China, using effect-directed *in vitro* bioassays. Environmental Toxicology and Chemistry 30, 2208–2215.

Zhao, Y., Castiglioni, S., Fent, K., 2015a. Environmental progestins progesterone and drospirenone alter the circadian rhythm network in zebrafish (*Danio rerio*). Environmental Science & Technology 49, 10155–10164.

Zhao, Y., Castiglioni, S., Fent, K., 2015b. Synthetic progestins medroxyprogesterone acetate and dydrogesterone and their binary mixtures adversely affect reproduction and lead to histological and transcriptional alterations in zebrafish (*Danio rerio*). Environmental Science & Technology 49, 4636–4645.

Zhao, Y., Zhang, K., Fent, K., 2018. Regulation of zebrafish (*Danio rerio*) locomotor behavior and circadian rhythm network by environmental steroid hormones. Environmental Pollution 232, 422–429.

Zucchi, S., Castiglioni, S., Fent, K., 2012. Progestins and antiprogestins affect gene expression in early development in zebrafish (*Danio rerio*) at environmental concentrations. Environmental Science & Technology 46, 5183–5192.

Zucchi, S., Castiglioni, S., Fent, K., 2013. Progesterone alters global transcription profiles at environmental concentrations in brain and ovary of female zebrafish (*Danio rerio*). Environmental Science & Technology 47, 12548–12556.

Zucchi, S., Mirbahai, L., Castiglioni, S., Fent, K., 2014. Transcriptional and physiological responses induced by binary mixtures of drospirenone and progesterone in zebrafish (*Danio rerio*). Environmental Science & Technology 48, 3523–3531.

CHAPTER 2

DETERMINATION OF PROGESTINS IN SURFACE AND WASTE WATER USING SPE EXTRACTION AND LC-APCI/APPI-HRPS

Golovko, O., Šauer, P., Fedorova, G., Kocour Kroupová, H., Grabic, R., 2018. Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS. Science of the Total Environment 621, 1066–1073.

According to the publishing agreement between the authors and publisher, it is allowed to include the paper in this Ph.D. thesis

https://www.elsevier.com/about/policies/copyright/permissions

My share on this work was about 20%.

Science of the Total Environment 621 (2018) 1066–1073

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS

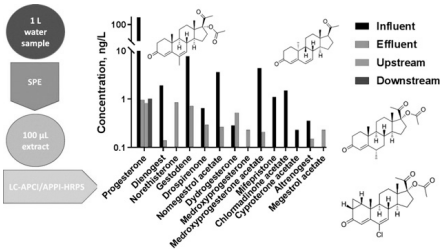
Oksana Golovko *, Pavel Šauer, Ganna Fedorova, Hana Kocour Kroupová, Roman Grabic

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czechia

HIGHLIGHTS

- 17 progestogens were analysed using SPE extraction with LC-APCI/APPI-HRPS.
- Newly: altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate
- The method is very selective and sensitive with LOQs ranging from 0.02 to 0.87 ng/L.
- In influent waste water samples, most of the progestogens were detected above LOQs.
- Megestrol acetate, medroxyprogesterone acetate, and dienogest detected most often.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 July 2017
Received in revised form 14 September 2017
Accepted 13 October 2017
Available online 6 November 2017

Editor: Thomas Kevin V

Keywords:
Endocrine disrupting compounds
Aquatic environments
Solid-phase extraction
Liquid chromatography high resolution mass spectrometry

ABSTRACT

The aim of this study was to develop a reliable analytical method for the measurement of 17 selected progestogens in waste water and surface water. Automated whole water solid phase extraction (SPE) was used for sample concentration. Liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) was applied for the analyses. The whole-method recoveries ranged from 60% to 140% for all analytes at two different spike levels (5 and 50 ng/L) in the studied matrices. The method is very sensitive with LOQs ranging from 0.02 to 0.87 ng/L. The developed method was used for the determination of progestogens in real samples of waste water from three waste water treatment plants (WWTPs) and in surface water from the corresponding recipients. Progesterone was detected in all samples with concentrations in the range of 0.82 to 1.1 ng/L in surface water and 0.11 to 110 ng/L in waste water samples. Three synthetic progestogens, namely, megestrol acetate, medroxyprogesterone acetate, and dienogest, were detected most frequently in effluents; therefore, further attention should be paid to the monitoring of these compounds.
To the best of our knowledge, this study is the first to present analysis of altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate in waste water and surface water using a solid-phase extraction method.

1. Introduction

Endocrine disrupting compounds (EDCs) have received increasing attention from the international scientific community during the last several decades (Schröder et al., 2016; Fent, 2015). Recently, synthetic

* Corresponding author.
E-mail address: ogolovko@frov.jcu.cz (O. Golovko).

http://dx.doi.org/10.1016/j.scitotenv.2017.10.120
0048-9697/

progestogens, which are widely used in human and veterinary therapies, received attention as a new EDC group of concern (Fent, 2015; Kumar et al., 2015; Liu et al., 2011). Synthetic progestogens are used primarily in contraceptive pills but also in the promoting regular menstrual cycles, treating abnormal uterine bleeding, controlling the symptoms of menopause, and preventing endometrial cancer (Apgar and Greenberg, 2000).

However, only limited data on a narrow range of progestogens have been reported in waste waters (Kolodziej et al., 2003; Jenkins et al., 2001; Fernandez et al., 2007; Chang et al., 2011) and surface waters (Jenkins et al., 2001; Chang et al., 2011; Vulliet et al., 2008).

Steroid hormones are excreted by humans and animals and subsequently reach the surface waters due to direct discharge or their incomplete removal in waste water treatment plants (WWTPs), which have been reported to be the primary source of contamination of the aquatic environments (Kumar et al., 2015). In recent studies, it has been demonstrated that synthetic progestogens can affect sexual development and reduce egg production in fish at concentrations similar to those detected in aquatic environments (Svensson et al., 2016; Zeilinger et al., 2009; Runnalls et al., 2013).

The development of fast, reliable and sensitive analytical methods for the determination of progestogens in water matrices is of crucial importance for the assessment of the concentration levels of these compounds and their related ecological risk (Kumar et al., 2015).

Due to the low concentration levels of progestogens in surface water and waste water together with the complexity of environmental matrices in which these compounds are dispersed, a pre-concentration and clean-up step (Chang et al., 2011) or large volume injection (Fayad et al., 2013) are usually performed.

For instrumental analysis, gas chromatography - mass spectrometry (GC–MS) has been applied in the determination of steroids due to its high separation and good identification capability (Kolodziej et al., 2003; Labadie and Budzinski, 2005). However, complicated cleanup and derivatization steps are required before instrumental analysis. Liquid chromatography (LC-MS) is more convenient than GC–MS because direct analysis is possible without derivatization.

Three different ionization sources, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) are available for LC-MS. ESI has demonstrated detection capabilities for steroid hormones, but its applicability is limited by selectivity (Jeannot et al., 2002; Rodriguez-Mozaz et al., 2004). In addition, co-extracted matrix compounds can cause significant suppression or enhancement of the signal and, consequently, analytical error in electrospray ionization (Schlüsener and Bester, 2005). APPI may be useful as an alternative for sensitive and selective detection of steroid hormones that are difficult to ionize by either electrospray or APCI (Schlüsener and Bester, 2005; Robb et al., 2000). In present study APCI and APPI analyses were performed to use more selective ionization in order to reduce interferences from matrix, increase selectivity and consequently increase the signal to noise ratio for target compounds in matrix-rich aqueous samples.

The most popular sample preparation technique is solid-phase extraction (SPE), and it has been widely employed for analysis of progestogens in water samples (Vulliet et al., 2008; Al-Odaini et al., 2010; Liu et al., 2014; Liu et al., 2015). A recent development in this technology is the automated SPE system, which has the following advantages: extraction of whole water samples without any pretreatment step, lower amount of solvent needed, low waste and controlling costs; increased safety by reducing exposure to solvents; better reproducibility between technicians; reliable handling of most samples and less attention required for good results.

The aim of this study was to develop a reliable analytical method to measure the selected 17 progestogens in waste water and surface water by SPE extraction followed with liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with/atmospheric pressure photoionization with a hybrid quadrupole/orbital trap system operated in high resolution product scan mode (LC-APCI/APPI-HRPS). In the present study, the SPE system works with whole water samples and sample filtration and extraction is included in one step.

The progestogens of interest were selected according to their consumption (Fig. SI 1) and concern over their possible effect on aquatic organisms (Kumar et al., 2015). Target compounds, synthetic progestogens, progesterone and selective progesterone modulators (SPRMs) were selected according to their annual consumption which was calculated from raw data freely available at website of Czech State Institute for Drug Control, Fig. SI 1 (State Institue for Drug Control, 2017). Only those compounds that are consumed in the Czech Republic were further assessed by LC-APCI/APPI-HRPS analysis. The only exception was medroxyprogesterone because it has previously been detected in the aquatic environment (Kolodziej et al., 2003; Macikova et al., 2014). Some progestogens are precursors of other progestogens; therefore, the consumption of precursors and corresponding active substances was summed up. Namely, desogestrel is a precursor of etonogestrel, and consumption of etonogestrel was calculated as the sum of consumption of desogestrel and etonogestrel. Similarly, norethisterone acetate and lynestrenol are precursors of norethisterone and norgestimate and norelgestromin are metabolized into levonorgestrel.

In our study we aimed to develop simple multi-residue method for simultaneous determination of 17 progestogens selection which was based on consumption data survey. Most studies focus only on several progestogens, such as progesterone and megestrol acetate (Chang et al., 2011; Vulliet et al., 2008; Liu et al., 2014; Sun et al., 2009), and only Liu et al. investigated the occurrence of 21 progestogens in seawater (Liu et al., 2015). Our study is the first to analyse altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate in waste water and surface water together with other 12 progestogens which have not been analysed in one analytical run. Compared to previous studies we aimed to develop rapid extraction method followed with highly selective and sensitive detection method LC-APCI/APPI-HRPS.

The applicability of the method for the analysis of environmental samples was verified on real WWTP effluents and recipient samples.

2. Materials and methods

2.1. Chemicals and reagents

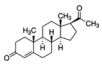
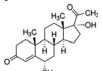
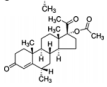
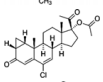
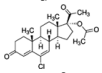
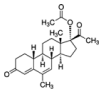
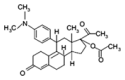
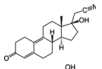
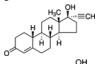
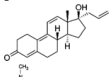
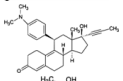
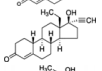
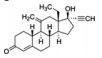
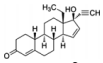
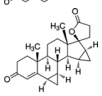
Methanol and acetonitrile (LiChrosolv® Hypergrade) were purchased from Merck (Darmstadt, Germany). Ultra-pure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). All analytical standards were of high purity (mostly 98%).

Medroxyprogesterone, progesterone, dydrogesterone, dienogest, norethisterone, gestodene, drospirenone, nomegestrol acetate, mifepristone, ulipristal acetate, altrenogest, medroxyprogesterone acetate, megestrol acetate, levonorgestrel, etonogestrel, chlormadinone acetate and cyproterone acetate were purchased from Sigma Aldrich (Steinheim, Germany). The classification and the main physical chemical properties of the selected progestogens are presented in Table 1.

The internal standards (ISs) for synthetic progestogens, Norethindrone-D6, Gestodene-D6, Drospirenone-13C6, Medroxyprogesterone-D3, Chlormadinone acetate-D6, Megestrol acetate-D3, and Progesterone-D9, were obtained from Toronto Research Chemicals, Inc. (Toronto Research Chemicals, ON, Canada). All internal standards were of analytical grade (>98% purity).

Individual stock solutions of the standards were prepared at 1 mg/mL concentration in methanol and stored at −20 °C. A spiking mixture of ISs was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg/mL for each compound. Working standard mixtures (0.01–10 μg/mL) of the native compounds were prepared monthly in methanol.

Table 1
The classification and the main physical chemical properties of the selected progestogens.

Structural derivation			Compound	Molar mass	CAS number	Purity (%)	log K_{ow}	Water solubility (mg/L)	Structure
Natural hormone			Progesterone	314.47[a]	57-83-0	99.9	3.67[a]	5.0[a]	
Progesterone derivative			Dydrogesterone	312.46[a]	152-62-5	99.5	3.45[b]	3.7[b]	
Structurally related to progesterone	17α-OH-progesterone derivatives		Medroxyprogesterone	344.50[a]	520-85-4	98.5	3.50[b]	3.0[b]	
			Medroxyprogesterone acetate	386.54[a]	71-58-9	≥97	4.09[b]	1.2[b]	
			Cyproterone acetate	416.95[a]	427-51-0	≥98	3.10[b]	51.7[b]	
			Chlormadinone acetate	404.94[a]	302-22-7	99.7	3.95[b]	0.3[b]	
	19-Norprogesterone derivatives		Megestrol acetate	384.52[a]	595-33-5	≥99	4.00[b]	2.0[b]	
			Nomegestrol acetate	370.49[a]	58652-20-3	≥98	3.55[a]	4.3[a]	
			Ulipristal acetate	475.63[a]	126784-99-4	≥98	5.07[a]	0.1[a]	
Structurally related to testosterone	19-Nortestosterone derivatives	Estranes	Dienogest	311.43[a]	65928-58-7	99.9	2.34[a]	57.9[a]	
			Norethisterone	298.43[a]	68-22-4	≥98	2.97[b]	7.0[b]	
			Altrenogest	310.44[a]	850-52-2	≥99	3.94[a]	14.8[a]	
			Mifepristone	429.61[a]	84371-65-3	≥98	5.40[b]	0.1[a]	
		Gonanes	Levonorgestrel	312.46[a]	797-63-7	≥99	3.48[b]	2.1[b]	
			Etonogestrel	324.47[a]	54048-10-1	≥98	3.16[b]	57.1[a]	
			Gestodene	310.44[a]	60282-87-3	≥98	3.26[b]	8.1[b]	
17α-Spironolactone derivative			Drospirenone	366.50[a]	67392-87-4	99.9	4.02[a]	1.8[a]	

[a] Estimation Programs Interface Suite™ for Microsoft® Windows, KOWWIN v. 1.68 estimate. United States Environmental Protection Agency, Washington, DC, USA.
[b] (Liu et al., 2011).

2.2. Sampling and sample preparation

Sampling of water was performed in November 2016 and January and February 2017. Grab waste water samples (influent and effluent) from WWTP in Vodňany (Czech Republic, 7000 inhabitants and light industry, Table 2) and surface water samples from Blanice River (Czech Republic) (7 replicates from each site) were used for method validation. Seven replicates (1 L aliquot) were spiked with ISs to achieve a concentration of 10 ng/L. Other sets of samples (seven replicates each) were also spiked with a mixture of progestogens at two concentration levels (5 and 50 ng/L for waste water and surface water) to check the recovery of target compounds.

Furthermore, to evaluate the effectiveness of the developed method, 24-hour composite samples (time proportional sampling with 15 min. intervals) were collected from influents and effluents of two WWTPs. The characteristics of the WWTPs sampled are shown in Table 2. Surface water grab samples were collected upstream and downstream of the corresponding WWTPs. The sampling region is located in the Vltava River basin. All samples were collected into amber glass bottles that were pre-cleaned with acetonitrile and distilled water.

Samples were transported to the laboratory immediately after collection, stored in darkness at 4 °C and extracted within 48 h.

2.3. Solid-phase extraction and sample evaporation

Waste and surface water samples were extracted on an SPE-DEX 4790 automated solid-phase extraction system (Horizon Technology, Salem, NH, USA).

For the extraction method, we checked two extraction solvents for the selected progestogens: acetonitrile (ACN) and methanol (MeOH). Extraction was performed as follows: the system was purged, and Atlantic C18 SPE disks (Horizon Technology, Salem, NH, USA) were conditioned with ACN and LC/MS grade water. One liter of whole water sample was loaded into the extraction system. The particles were trapped at 5 and 1 μm glass fibre filters (Horizon Technology, Salem, NH, USA). Glass fibre filtration material appears to be the best choice for extraction of progestogens because high recoveries are reached using these filters (Fayad et al., 2013). Filtered samples passed through the C18 disks, and target compounds were retained onto the sorbent. C18 disks were chosen due to their known ability to extract mid and non-polar compounds (Horizon Technology, Salem, NH, USA), such as synthetic progestogens, which are slightly polar or non-polar compounds (Kumar et al., 2015). When samples passed through the disks, the system, including sample bottle, was washed with demineralised water. Filters and C18 disks were air dried for 15 min to remove residual water. Subsequently, the sample bottle was rinsed twice with ACN to wash off residual analytes from the bottle walls. C18 disks containing adsorbed target compounds were eluted with the total volume of 10 mL of ACN into glass collection vessels. Soak time of the ACN on C18 disks was 1 min and 30 s per rinse for elution. The first rinsing was followed by 15 s of air dry, while the latter air drying after rinsing lasted 1 min. The eluates were transferred into glass vials with a conical bottom. To ensure that all target compounds were transferred, eluate collection vessels were rinsed three times with 2 mL of ACN. Combined extract was evaporated by a gentle nitrogen stream to dryness in a nitrogen sample concentrator Termovap TV10+ (ECOM, the Czech Republic). The sample residues were re-dissolved in 2 × 50 μL of ACN for further analysis. The same extraction procedure was performed using methanol as the extraction solvent for comparison purpose. The sample extract was transferred into an autosampler vial with a glass insert with a volume of 200 μL and sealed with a cap. All extracts were subsequently stored at −20 °C until analysis, which was performed within one week.

2.4. LC-MS instrumentation

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used to separate and detect target analytes. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) were used for the chromatographic separation of the target analytes.

2.5. LC-APCI/APPI-HRPS analysis

Ultra-pure water and methanol were used as the mobile phases. The LC gradient for the elution of target compounds is presented in Supplementary Materials Table SI 1. The elution conditions were programmed as follows: 350 mL/min 30% methanol in water for 1 min, isocratically followed by a gradient change to 20/80 water/methanol at a flow of 400 mL/min in 8 min and a final gradient change to 100% of methanol at a flow of 400 mL/min in 10 min. These parameters were held for 2 min and then changed to the starting conditions and held for 1.5 min to equilibrate the column for the next run.

An atmospheric pressure photoionization (APPI) in positive mode was used to ionize target compounds. The instrument was calibrated daily (mass calibration) in negative modes using a standard procedure proposed by Thermo Scientific.

The APCI/APPI parameters were set as follows: capillary temperature (300 °C), vaporizer temperature (300 °C), sheath gas pressure (40 arbitrary units), auxiliary gas (15 arbitrary units) and discharge current (4 μA in positive ionization mode). UV krypton lamp (10 eV) was used in the source.

The MS/MS conditions were optimized for each compound by infusion of individual standard solution at a concentration level of 1 μg/mL at a rate of 10 μL/min to the mobile phase stream (50/50 MeOH/water at 300 μL/min). All progestogens were detected in positive ionization mode as protonated molecules [M + H]+.

Most progestogens undergo fragmentation with many non-specific fragments. Selection of the proper fragments usually is based on intensity and selectivity of fragments not only in the standard but in the real

Table 2
Wastewater treatment plant characterization.

WWTP	Sampling technique	Water flow at influent (m^3/d)	Catchment population (capacity)	Influent (mg/L)			Effluent (mg/L)			Main producers of wastewaters (except for households)
				BOD	COD-Cr	TSS	BOD	COD-Cr	TSS	
Vodňany	Grab	2151	28,500	294[a]	516[a]	125.5[a]	3.5[a]	25.6[a]	3.45[a]	Poultry industry, research institute and instrumentation manufacturing plant
Strakonice	24-h composite	15,000	75,000	250	431	245	9	38.3	7.2	Hospital, brewery, textile and engineering industry
České Budějovice	24-h composite	37,791	375,000	247	597	250	1.9	19	<2.0	Hospitals, breweries, paper mill, dairy, heating and food manufacturing plants

Abbreviations: BOD – biochemical oxygen demand, COD-Cr – chemical oxygen demand using potassium dichromate, TSS – total suspended solids.
[a] Data taken from year 2014.

matrices, as well. The high resolution product scan (HRPS) analysis was used with the mass inclusion list and expected retention times of the target analytes with a 1 min time window. Collision energy values were optimized for all the compounds of interest and are presented in Table SI 3. General MS parameters were set up as follows: orbital trap resolution 17,500 FWHM; product scan range of 50 to 600 *m/z*; AGC target of 1e6; maximum filling time of 30 ms; and an isolation window at the quadrupole of 1 *m/z*. The advantage of HRPS is not that single transitions are monitored but that a full scan spectrum is recorded. This even allows picking up new suspicious fragment ions using old data without re-measuring. The MS/MS parameters for the Q-Exactive mass spectrometer are presented in Table SI 3.

Data analysis was performed with TraceFinder 3.3 software (Thermo Fisher Scientific).

2.6. Method validation

The performance of the method was assessed regarding linearity, limits of quantification (LOQs), trueness and repeatability. Two matrices were used for method validation: waste water (influent and effluent) and surface water.

The linearity of the calibration curve was tested in the range from 0.1 ng/L to 200 ng/L. Calibration curves were measured at the beginning and at the end of the sequence to check instrument stability. The calibration was prepared in water/MeOH (1/1). LOQs were calculated as one quarter of the lowest calibration point in the calibration curve where the relative standard deviation of the average response factor was <30% (in some cases one or two points at low concentration levels had to be removed). The peak area corresponding to this concentration was used to calculate LOQ for each individual compound in each sample.

The matrix effect was assessed for each compound. Corrections of ion suppression or enhancement were performed using matrix-matched standards for quantification if this effect exceeds 30% in a given matrix. Each matrix-matched standard was prepared from corresponding water sample extract that was spiked with ISs at concentration levels of 10 ng/L and native compounds at concentration levels of 200 ng/L. The matrix effect was evaluated as the difference between the matrix-matched standards' relative response factor and average relative response factor obtained from the calibration curve. Repeatability of the method was evaluated as the relative standard deviation (RSD) of the seven replicates at corresponding fortification levels.

In an effort to generate quality data, several quality control samples were included during sample analysis. No target analytes were detected in method blanks. The method blank was ultra-pure water. The blank was prepared and extracted in the same way as the samples.

3. Results and discussion

3.1. Efficiency of extraction procedure

The extraction solvent is one of the critical parameters influencing the extraction efficiency of the analytes and method performance. In this method, we tested two extraction solvents for the selected progestogens, ACN and MeOH. The recovery achieved by each extraction procedure for each progestogens was evaluated at a fortification level of 10 ng/L and was expressed as the ratio between the determined concentration and the nominal concentration ($n = 3$). The recovery results for progestogens and two extraction solvents are shown in Table SI 3. The most consistent results were achieved using ACN, where median recovery ranged between 62% and 130%. Extraction procedures with MeOH provided highly variable results across the selected hormones, ranging from 4% to >135%. According to the literature data, elution with ACN solvent provides better recoveries of progestogens than with MeOH, ethyl acetate or dichloromethane (Sun et al., 2009). Consequently, ACN was selected for further validation because of its consistent recoveries for most target compounds.

3.2. Method validation

The linearity, limit of quantification (LOQ), trueness and repeatability of the method were evaluated under the optimum extraction conditions for each sample matrix.

One of the analytical challenges of progestogens quantification in aquatic samples is the need to analyse very low concentration levels. Average LOQs together with linearity parameters are presented in Table 3. All progestogens showed good linear response in the range of 0.1 to 200 ng/L with R squared coefficients (R^2) higher than 0.997. The proposed analytical method resulted in very low LOQs in the range of 0.02 to 0.87 ng/L without additional purification or derivatization steps.

Varying composition of the sample matrix can suppress or enhance analyte signal and consequently may influence ion-ratio and overall method performance, as well as producing false positives, even when an IS or isotope dilution method is used (Fedorova et al., 2013). Significant matrix effect (over 30%, Fig. 1) was observed in influent waste water samples for most of the studied compounds. The dominant matrix effect in this case was ionization suppression, which varied from −33% to −101%. For the rest of the samples (surface and effluent waste water), matrix effects were between −30 and +30% for most of progestogens with the exceptions of dienogest (−31%) and dydrogesterone (−54%) in effluent samples. This finding is in accordance with our previous study, where APCI/APPI showed a lower matrix effect than ESI (Lindberg et al., 2014).

As seen in Table 4, for most target compounds, the recoveries were in the satisfactory range from 60% to 140%. Dienogest showed the lowest recoveries in all matrices (30–41% for surface water, 56–80% for waste water). This result could be attributed to the fact that dienogest is more polar compared to the rest of the studied progestogens (log Kow = 2.34, Table 1), and the C18 SPE disk might not be the best choice for extraction of this kind of compound. However, the multiresidual method is a compromise between analysing a wide range of analytes and the method performance for border compounds. Repeatability of the method for dienogest was good, and the method can also be used for this compound, at least as a semiquantitative method.

Recoveries slightly over 140% were obtained in the effluent waste water samples for levonorgestrel, norethisterone and etonogestrel. It should be mentioned that the calculation of influent recoveries for progesterone was problematic because of the presence of this hormone in these samples at high concentrations (110 ng/L). For this reason, data for progesterone in influent samples had to be omitted for method development.

Table 3
R square coefficients (R^2) and average limit of quantification (LOQ), ng/L of selected hormones measured in surface water and wastewater (influent and effluent).
Number of samples:
- surface water (Blanice River) = 7 replicates;
- wastewater Vodňany (influent and effluent) = 7 replicates of each site.

Compound	R^2	Surface water	Effluent	Influent
Dienogest	0.9992	0.10	0.08	0.04
Norethisterone	0.9989	0.04	0.11	0.05
Gestodene	0.9992	0.05	0.44	0.19
Drospirenone	0.9983	0.87	0.07	0.03
Levonorgestrel	0.9996	0.08	0.51	0.23
Etonogestrel	0.999	0.07	0.55	0.25
Nomegestrol acetate	0.9988	0.07	0.09	0.04
Dydrogesterone	0.9977	0.65	0.41	0.21
Medroxyprogesterone	0.999	0.06	0.04	0.02
Medroxyprogesterone acetate	0.9988	0.10	0.06	0.03
Mifepristone	0.9986	0.59	0.36	0.27
Ulipristal acetate	0.9996	0.6	0.37	0.28
Chlormadinone acetate	0.9983	0.44	0.32	0.24
Cyproterone acetate	0.9983	0.32	0.38	0.20
Altrenogest	0.9996	0.06	0.05	0.03
Megestrol acetate	0.9994	0.07	0.05	0.03
Progesterone	0.9991	0.15	0.06	0.03

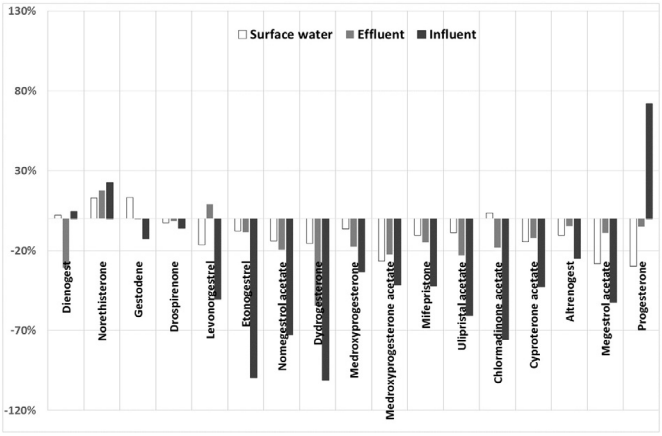


Fig. 1. Progestogens quantification MS signal suppression/enhancement in surface water from Blanice River and wastewater samples (influent and effluent) from WWTP in Vodnany (Czech Republic). Positive values correspond to ion enhancement, negative - to ion suppression.

Method repeatability was tested for both fortified and native samples, and the data are presented in Table 4. For all matrices, repeatability values were satisfactory, and the RSDs in all samples at different concentration levels were lower than 30% for most of the progestogens. Slightly higher RSDs were found for dienogest (31%), gestodene (34%), dydrogesterone (31%) and chlormadinone acetate (33%) in influent water samples at a fortification level of 5 ng/L.

3.3. Consumption of progestogens. Application of the method. Analysis of progestogens in surface and waste water samples

Data on consumption of progestogens are presented on Fig. SI 1. Six compounds (progesterone, drospirenone, megestrol acetate, cyproterone acetate, medroxyprogesterone acetate, dienogest) reached or exceeded consumption of 100 kg per year in the Czech Republic in year 2014. Progesterone was the most consumed progestogen and is also naturally produced in human body. Indeed, this compound has been detected in all studied influents (Table 5). Up to date, consumption rates of progestogens have been reported only for few times (Besse and Garric, 2009; Ji et al., 2016; Runnalls et al., 2010). Thus, the present results may provide useful addition.

The optimized method was used for the identification and determination of target hormones in real samples of waste water from three WWTPs and in samples of recipient streams taken down-stream and up-stream of the respective WWTPs.

In the waste water samples, there were higher concentrations in the influent than in the effluent samples for most of the studied compounds, as presented in Table 5. The concentration range for influent was from 0.23 ng/L (cyproterone acetate in Vodňany WWTP) to 110 ng/L (progesterone in Vodňany WWTP). Because of incomplete elimination by WWTPs, certain of the studied progestogens can be observed in effluent samples in lower concentration levels compared to influent samples:

Table 4
Trueness (% of added amount) and repeatability of selected hormones measured in surface water and wastewater (influent and effluent)-in brackets (relative standard deviation; % RSD) at different concentration levels.

Compounds	Surface water		Wastewater			
			Effluent		Influent	
	5 ng/L	50 ng/L	5 ng/L	50 ng/L	5 ng/L	50 ng/L
Dienogest	30 (16)	41 (26)	59 (15)	58 (20)	80 (31)	56 (17)
Norethisterone	66 (10)	75 (11)	125 (16)	150 (19)	106 (30)	106 (16)
Gestodene	64 (9)	70 (11)	122 (12)	121 (8)	66 (34)	81 (11)
Drospirenone	64 (13)	77 (9)	134 (11)	133 (8)	121 (16)	109 (5)
Levonorgestrel	82 (13)	85 (11)	164 (13)	152 (10)	103 (19)	88 (14)
Etonogestrel	81 (11)	91 (14)	150 (10)	132 (9)	94 (14)	90 (12)
Nomegestrol acetate	86 (9)	95 (20)	123 (12)	115 (13)	80 (24)	73 (7)
Dydrogesterone	87 (27)	85 (22)	112 (27)	109 (18)	68 (31)	73 (13)
Medroxyprogesterone	76 (9)	82 (9)	111 (11)	108 (10)	100 (14)	96 (7)
Medroxyprogesterone acetate	93 (6)	99 (11)	116 (15)	108 (10)	58 (11)	71 (6)
Mifepristone	65 (9)	78 (11)	83 (14)	92 (14)	122 (20)	106 (19)
Ulipristal acetate	68 (9)	82 (18)	78 (11)	84 (13)	114 (25)	90 (25)
Chlormadinone acetate	64 (15)	75 (10)	105 (12)	108 (7)	82 (33)	113 (30)
Cyproterone acetate	79 (20)	88 (9)	116 (12)	112 (15)	91 (10)	88 (10)
Altrenogest	63 (15)	78 (16)	107 (15)	96 (18)	111 (17)	83 (11)
Megestrol acetate	95 (8)	94 (10)	119 (8)	116 (11)	102 (11)	88 (7)
Progesterone	80 (8)	76 (8)	119 (7)	114 (11)	NA	NA

NA – not analysed.

Table 5
Average concentration ng/L (n = 3) of 17 target compounds detected in surface water, influent and effluent waste water samples from three WWTPs.

Compounds	Surface water (Blanice River)	České Budějovice				Strakonice				Vodňany	
		Upstream	Downstream	Effluent	Influent	Upstream	Downstream	Effluent	Influent	Effluent	Influent
Dienogest	<0.09	<0.08	<0.11	**1.0**	**7.0**	<0.05	<0.05	<0.05	**11**	**0.14**	**1.9**
Norethisterone	<0.04	<0.03	<0.05	<0.03	<0.02	<0.05	<0.05	<0.04	<0.17	**0.85**	<0.06
Gestodene	<0.05	<0.57	<0.78	<0.49	<0.38	<0.48	<0.36	<0.29	**5.5**	**0.71**	**7.7**
Drospirenone	<0.85	<0.71	<1.1	<0.62	<0.77	<0.24	<0.25	<0.18	<0.66	**0.29**	**0.64**
Levonorgestrel	<0.08	<0.83	<1.3	<0.83	<2.1	<0.27	<0.28	<0.22	<1.4	<0.53	<0.26
Etonogestrel	<0.07	<0.93	<1.4	<0.89	<1.4	<0.25	<0.26	<0.21	<1.1	<0.57	<0.28
Nomegestrol acetate	<0.07	<0.06	<0.09	<0.05	<0.08	<0.04	<0.04	<0.03	<0.21	**0.26**	**3.6**
Dydrogesterone	<0.63	<0.58	<0.86	<0.55	<1	<0.22	<0.24	<0.18	<1.5	**0.51**	**0.28**
Medroxyprogesterone	<0.06	<0.05	<0.08	<0.05	<0.06	<0.04	<0.04	<0.03	<0.13	**0.23**	<0.02
Medroxyprogesterone acetate	<0.1	<0.09	<0.14	<0.09	<0.15	<0.67	<0.76	**0.58**	**2.6**	**0.21**	**4.4**
Mifepristone	<0.61	<0.5	<0.81	**0.5**	**3.0**	<0.34	<0.36	<0.26	<1.3	<0.41	**1.1**
Ulipristal acetate	<0.61	<0.51	<0.84	<0.43	<1.6	<0.2	<0.21	<0.15	<0.56	<0.42	<0.27
Chlormadinone acetate	<0.45	<0.4	<0.64	<0.36	<0.63	<0.26	<0.28	<0.2	<1.3	<0.36	**1.5**
Cyproterone acetate	<0.32	<1.4	<2.2	**2.8**	**6.7**	<0.73	<0.74	<0.59	<1.8	<0.44	**0.23**
Altrenogest	<0.06	<0.05	<0.08	<0.06	<0.10	<0.05	<0.06	<0.04	<0.16	**0.15**	**0.35**
Megestrol acetate	<0.07	<0.06	<0.09	<0.06	**4.8**	<0.07	<0.07	**0.4**	**6.3**	**0.23**	<0.03
Progesterone	**1.1**	**0.82**	**1.0**	**0.59**	**47**	<0.09	<0.05	**0.11**	**14**	**0.95**	**110**

The values above LOQs are marked bold.

the concentration range for effluent was from 0.11 ng/L (progesterone in Strakonice WWTP) to 2.8 ng/L (cyproterone acetate in České Budějovice WWTP).

Eight hormones (dienogest, gestodene, drospirenone, nomegestrol acetate, dydrogesterone, altrenogest, medroxyprogesterone acetate and progesterone) were found above LOQ in both influent and effluent samples from Vodňany WWTP. Four target compounds (dienogest, mifepristone, cyproterone acetate and progesterone) were found both in influent and effluent samples from WWTP in České Budějovice, and megestrol acetate was found only in influent. Approximately the same number of hormones were found in Strakonice WWTP, but they were different compounds (progesterone, medroxyprogesterone acetate and megestrol acetate were in both waste water, but dienogest and gestodene were only in influent). The differences among cities can be assigned to presence of the regional centre for cancer treatment in České Budějovice, as well as different residence time in the sewer system. It should be mentioned that the highest positive finding in Vodňany occurred only in grab samples, which indicates that sampling technique might have an influence, as well.

Levonorgestrel, etonogestrel and ulipristal acetate were below LOQs in all studied samples, although high concentrations of levonorgestrel, 150–170 ng/L in influent and 30 ng/L in effluent, were shown in a study by Viglino et al. (Viglino et al., 2008). In a recent study by (Liu et al., 2014), the concentrations of progesterone and dydrogesterone in influent were 10.1 and 35.1 ng/L, respectively. Usually, the presence of hormones in waste water is associated with consumption rates, as well as transformation rates of conjugated to the un-conjugated form.

In our study, the concentrations of medroxyprogesterone acetate in influent samples ranged from 2.6 to 4.4 ng/L. The concentration of medroxyprogesterone acetate was reported in influent samples in two recent studies (Chang et al., 2011; Liu et al., 2014), and the concentration levels were 2.4 ng/L and 18–58 ng/L, respectively.

Despite the lowest recovery among investigated compounds, dienogest was detected in waste water, although at sub-ng/L concentrations. Medroxyprogesterone was not detected in the influent of WWTP Vodňany, but it was present in the effluent, which might be explained by biotransformation of medroxyprogesterone acetate into medroxyprogesterone.

Despite the lack of data on the biotransformation of steroids in waste water, certain progestogens (progesterone and levonorgestrel) have already been reported to rapidly undergo biotransformation to other steroids in surface waters and sediments (Ojoghoro et al., 2017; Peng et al., 2014; Sangster et al., 2016). Several compounds analysed in the present study, namely, medroxyprogesterone, norethisterone, dydrogesterone, and megestrol acetate (in WWTP Vodňany), could have undergone such a biotransformation during the waste water treatment process (Table 5) because either they were present in the effluent samples only or their concentration in the effluent samples was higher than in the influent samples.

The major point source of progestogens into the aquatic environment is WWTPs because they are not completely capable of removing progestogens during treatment processes. Therefore, treated effluents discharged into receiving water bodies (river or surface water) may still contain substantial progestogen residues (Vulliet et al., 2008; Al-Odaini et al., 2010; Liu et al., 2014; Macikova et al., 2014; Chang et al., 2008; Kuster et al., 2008; Vulliet and Cren-Olivé, 2011), see Table SI 4. Similar results were shown in work by Liu et al. (Liu et al., 2014) for progesterone in surface water samples. In a recent study by (Torres et al., 2015), the concentrations of progesterone in surface water ranged from 0.58 to 26 ng/L. For example, Liu et al. (Liu et al., 2014) detected progesterone, megestrol acetate and norethisterone in concentrations ranging from 0.6 to 1.7 ng/L, as well as up to 9.6 ng/L in the case of dydrogesterone in the river downstream of a WWTP. In our study, only progesterone was observed in surface water at concentrations ranging from 0.82 to 1.1 ng/L, and it was the most frequently detected hormone in the studied samples.

4. Conclusions

We developed a reliable analytical method for simultaneous determination of 17 progestogens in environmental samples by combining simple high sample volume SPE extraction with LC-APCI/APPI-HRPS. This method enables very low LOQs (0.02–0.87 ng/L) to be obtained without additional purification or derivatization steps. The method could be used for regular monitoring of progestogens in the aquatic environment because it uses a whole water sample.

The method has been applied for the analyses of progestogens in different environmental samples. To the best of our knowledge, this study is the first to analyse altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate in waste water and surface water.

In influent waste water samples, most of the studied progestogens were detected above the quantification limits but with high spatial variability.

The most frequently detected compound was progesterone, with its highest levels being observed in influent waste water. Three synthetic progestogens, namely, megestrol acetate, medroxyprogesterone acetate, and dienogest, were detected most frequently in effluents, and further attention to the monitoring of these compounds should therefore be paid. Only progesterone was detected in surface water samples.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects 'CENAKVA' (No. CZ.1.05/2.1.00/01.0024), 'CENAKVA II' (No. LO1205 under the NPU I program) and the project of the Czech Science Foundation No. 16-09709Y.

We thank Jiří Stara, Luděk Míka and other colleagues at CEVAK a.s. (České Budějovice, Czech Republic) and TSST s.r.o. for their help with sample collection.

We thank the Elsevier Language Editing Services for English language corrections.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.10.120.

References

Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., Surif, S., 2010. Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1217, 6791–6806.

Apgar, B.S., Greenberg, G., 2000. Using progestins in clinical practice. American Academy of Family Physicians. Vol. 62 1839–1846, 1849–1850.

Besse, J.P., Garric, J., 2009. Progestagens for human use, exposure and hazard assessment for the aquatic environment. Environ. Pollut. 157, 3485–3494.

Chang, H., Wu, S., Hu, J., Asami, M., Kunikane, S., 2008. Trace analysis of androgens and progestogens in environmental waters by ultra-performance liquid chromatography–electrospray tandem mass spectrometry. J. Chromatogr. A 1195, 44–51.

Chang, H., Wan, Y., Wu, S., Fan, Z., Hu, J., 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. Water Res. 45, 732–740.

Fayad, P.B., Prévost, M., Sauvé, S., 2013. On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry optimized for the analysis of steroid hormones in urban wastewaters. Talanta 115, 349–360.

Fedorova, G., Randak, T., Lindberg, R.H., Grabic, R., 2013. Comparison of the quantitative performance of a Q-Exactive high-resolution mass spectrometer with that of a triple quadrupole tandem mass spectrometer for the analysis of illicit drugs in wastewater. Rapid Commun. Mass Spectrom. 27, 1751–1762.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environ. Int. 84, 115–130.

Fernandez, M.P., Ikonomou, M.G., Buchanan, I., 2007. An assessment of estrogenic organic contaminants in Canadian wastewaters. Sci. Total Environ. 373, 250–269.

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, 143–159.

Jenkins, R., Angus, R.A., McNatt, H., Howell, W.M., Kemppainen, J.A., Kirk, M., Wilson, E.M., 2001. Identification of androstenedione in a river containing paper mill effluent. Environ. Toxicol. Chem. 20, 1325–1331.

Ji, K., Han, E.J., Back, S., Park, J., Ryu, J., Choi, K., 2016. Prioritizing human pharmaceuticals for ecological risks in the freshwater environment of Korea. Environ. Toxicol. Chem. 35, 1028–1036.

Kolodziej, E.P., Gray, J.L., Sedlak, D.L., 2003. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. Environ. Toxicol. Chem. 22, 2622–2629.

Kumar, V., Johnson, A.C., Trubiroha, A., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kocour Kroupová, H., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49 (5), 2625–2638.

Kuster, M., López de Alda, M.J., Hernando, M.D., Petrovic, M., Martín-Alonso, J., Barceló, D., 2008. Analysis and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain). J. Hydrol. 358, 112–123.

Labadie, P., Budzinski, H., 2005. Determination of steroidal hormone profiles along the jalle d'Eysines river (near Bordeaux, France). Environ. Sci. Technol. 39, 5113–5120.

Lindberg, R.H., Östman, M., Olofsson, U., Grabic, R., Fick, J., 2014. Occurrence and behaviour of 105 active pharmaceutical ingredients in sewage waters of a municipal sewer collection system. Water Res. 58, 221–229.

Liu, Z.-h., Ogejo, J.A., Pruden, A., Knowlton, K.F., 2011. Occurrence, fate and removal of synthetic oral contraceptives (SOCs) in the natural environment: a review. Sci. Total Environ. 409, 5149–5161.

Liu, S.-S., Ying, G.-G., Liu, S., Lai, H.-J., Chen, Z.-F., Pan, C.-G., Zhao, J.-L., Chen, J., 2014. Analysis of 21 progestagens in various matrices by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with diverse sample pretreatment. Anal. Bioanal. Chem. 406, 7299–7311.

Liu, S., Chen, H., Zhou, G.-J., Liu, S.-S., Yue, W.-Z., Yu, S., Sun, K.-F., Cheng, H., Ying, G.-G., Xu, X.-R., 2015. Occurrence, source analysis and risk assessment of androgens, glucocorticoids and progestagens in the Hailing Bay region, South China Sea. Sci. Total Environ. 536, 99–107.

Macikova, P., Groh, K.J., Ammann, A.A., Schirmer, K., Suter, M.J.-F., 2014. Endocrine disrupting compounds affecting corticosteroid signaling pathways in Czech and Swiss waters: potential impact on fish. Environ. Sci. Technol. 48, 12902–12911.

Ojoghoro, J.O., Chaudhary, A.J., Campo, P., Sumpter, J.P., Scrimshaw, M.D., 2017. Progesterone potentially degrades to potent androgens in surface waters. Sci. Total Environ. 579, 1876–1884.

Peng, F.-Q., Ying, G.-G., Yang, B., Liu, S., Lai, H.-J., Liu, Y.-S., Chen, Z.-F., Zhou, G.-J., 2014. Biotransformation of progesterone and norgestrel by two freshwater microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*): transformation kinetics and products identification. Chemosphere 95, 581–588.

Robb, D.B., Covey, T.R., Bruins, A.P., 2000. Atmospheric pressure photoionization: an ionization method for liquid chromatography—mass spectrometry. Anal. Chem. 72, 3653–3659.

Rodriguez-Mozaz, S., Lopez de Alda, M.J., Barceló, D., 2004. Picogram per liter level determination of estrogens in natural waters and waterworks by a fully automated on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry method. Anal. Chem. 76, 6998–7006.

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. Hum. Ecol. Risk. Assess. 16, 1318–1338.

Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environ. Sci. Technol. 47, 2077–2084.

Sangster, J.L., Ali, J.M., Snow, D.D., Kolok, A.S., Bartelt-Hunt, S.L., 2016. Bioavailability and fate of sediment-associated progesterone in aquatic systems. Environ. Sci. Technol. 50, 4027–4036.

Schlüsener, M.P., Bester, K., 2005. Determination of steroid hormones, hormone conjugates and macrolide antibiotics in influents and effluents of sewage treatment plants utilising high-performance liquid chromatography/tandem mass spectrometry with electrospray and atmospheric pressure chemical ionisation. Rapid Commun. Mass Spectrom. 19, 3269–3278.

Schröder, P., Helmreich, B., Škrbić, B., Carballa, M., Papa, M., Pastore, C., Emre, Z., Oehmen, A., Langenhoff, A., Molinos, M., Dvarioniene, J., Huber, C., Tsagarakis, K.P., Martinez-Lopez, E., Pagano, S.M., Vogelsang, C., Mascolo, G., 2016. Status of hormones and painkillers in wastewater effluents across several European states—considerations for the EU watch list concerning estradiols and diclofenac. Environ. Sci. Pollut. Res. 23, 12835–12866.

State Institue for Drug Control, 2017. URL. http://www.sukl.eu/dodavky-leciv-v-ceske-republice-v-jednotlivych-letech?lang=2.

Sun, L., Yong, W., Chu, X., Lin, J.-M., 2009. Simultaneous determination of 15 steroidal oral contraceptives in water using solid-phase disk extraction followed by high performance liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1216, 5416–5423.

Svensson, J., Mustafa, A., Fick, J., Schmitz, M., Brunström, B., 2016. Developmental exposure to progestins causes male bias and precocious puberty in zebrafish (*Danio rerio*). Aquat. Toxicol. 177, 316–323.

Torres, N.H., Aguiar, M.M., Ferreira, L.F.R., Américo, J.H.P., Machado, Â.M., Cavalcanti, E.B., Tornisielo, V.L., 2015. Detection of hormones in surface and drinking water in Brazil by LC-ESI-MS/MS and ecotoxicological assessment with *Daphnia magna*. Environ. Monit. Assess. 187, 379.

Viglino, L., Aboulfadl, K., Prévost, M., Sauvé, S., 2008. Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS. Talanta 76, 1088–1096.

Vulliet, E., Cren-Olivé, C., 2011. Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. Environ. Pollut. 159, 2929–2934.

Vulliet, E., Wiest, L., Baudot, R., Grenier-Loustalot, M.-F., 2008. Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr. A 1210, 84–91.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Länge, R., 2009. Effects of synthetic gestagens on fish reproduction. Environ. Toxicol. Chem. 28, 2663–2670.

CHAPTER 3

DETERMINING THE POTENTIAL OF PROGESTINS TO INDUCE PROGESTAGENIC ACTIVITIES IN THE AQUATIC ENVIRONMENT

Šauer, P., Stará, A., Golovko, O., Valentová, O., Bořík, A., Grabic, R., Kocour Kroupová, H., 2018. Two synthetic progestins and natural progesterone are responsible for most of the progestagenic activities in municipal wastewater treatment plant effluents in the Czech and Slovak republics. Water Research 137, 64–71.

According to the publishing agreement between the authors and publisher, it is allowed to include the paper in this Ph.D. thesis

https://www.elsevier.com/about/policies/copyright/permissions

My share on this work was about 35%.

Water Research 137 (2018) 64–71

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Two synthetic progestins and natural progesterone are responsible for most of the progestagenic activities in municipal wastewater treatment plant effluents in the Czech and Slovak republics

Pavel Šauer*, Alžběta Stará, Oksana Golovko, Olga Valentová, Adam Bořík, Roman Grabic, Hana Kocour Kroupová

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic

ARTICLE INFO

Article history:
Received 13 October 2017
Received in revised form
18 January 2018
Accepted 28 February 2018
Available online 2 March 2018

Keywords:
LC-APCI/APPI-HRPS
Progestogens
Pollution
PR-CALUX assay
Solid-phase extraction

ABSTRACT

Vast numbers of xenobiotics are known still to be present in treated municipal wastewater treatment plant (WWTP) effluents. Some of these possess endocrine-disrupting potency and pose risks for exposed aquatic animals. We searched for 17 potential environmental contaminants having affinity to the progesterone receptor. Relative potency values of these progesterone receptor-active chemicals were obtained. On the basis of relative potencies and measured environmental concentrations, the contribution of progestins to measured progestagenic activities was evaluated. Wastewaters (influent and effluent) and surrounding surface waters (upstream and downstream) at six municipal WWTPs were screened using instrumental chemical analysis and *in vitro* reporter gene bioassay. We showed the presence of target compounds and (anti-)progestagenic activities in municipal wastewater and surface water. Nine and seven progestins were identified in influent and effluent wastewaters, respectively. Only two compounds, progesterone and medroxyprogesterone were found in surface waters. Progestagenic agonistic activities in influents were partially masked by strong anti-progestagenic activities that were detected in all influents and ranged from 2.63 to 83 ng/L of mifepristone equivalents (EQs). Progestagenic activities were detected in all effluents and ranged from 0.06 to 0.47 ng/L of reference compound ORG 2058 EQs (a synthetic progestin equivalents), thus indicating incomplete removal of progestins during wastewater treatment processing. This activity poses a continuing risk for the aquatic environment. By contrast, anti-progestagenic activities showed better removal efficiency in WWTPs compared to progestagenic agonistic activities. Anti-progestagenic activities were found in only three of six effluents and ranged from 0.26 to 2.1 ng/L mifepristone EQs. We explained most of the progestagenic activity in municipal WWTP effluents by the presence of synthetic progestins and progesterone, which contributed 65–96% of such activity in samples where no antagonistic activity was found. The progestins medroxyprogesterone acetate, megestrol acetate and progesterone contributed most to the progestagenic activity detected in municipal effluents. Anti-progestagenic activities were found in some municipal effluents, but no causative agents were revealed because two analysed selective progesterone receptor modulators (SPRMs) with anti-progestagenic activities, mifepristone and ulipristal acetate, were not present in the effluents.

1. Introduction

It is well known that many chemicals are daily discharged into sewage and that some of them pass through municipal wastewater treatment plants (WWTPs). Therefore, WWTP effluents still contain certain amounts of xenobiotics that subsequently enter the aquatic environment. Some of these environmental contaminants may impair the endocrine systems of exposed organisms (Colborn et al., 1993; Jobling and Tyler, 2003; Lange et al., 2001; Sumpter, 1998).

One group of endocrine-disrupting pollutants, synthetic progestins, have recently come under suspicion of causing a significant

* Corresponding author.
E-mail address: psauer@frov.jcu.cz (P. Šauer).

https://doi.org/10.1016/j.watres.2018.02.065
0043-1354/

toxic burden and substantial risk to the aquatic environment (Fent, 2015; Kumar et al., 2015). Synthetic progestins are mainly used as the active ingredients in women's contraceptives, but also in other hormonal preparations (Sitruk-Ware, 2004; Zeilinger et al., 2009). As a result, they are consumed in relatively large amounts ranging from 0.34 to 9864 kg/year as reported from various European countries (Besse and Garric, 2009; Runnalls et al., 2010; Zhao et al., 2015), including the Czech Republic (Golovko et al., 2018). Therefore, municipal WWTPs might be important sources of synthetic progestins (Chang et al., 2009). The naturally occurring progestin progesterone originates from farmed animals, such as swine, cattle, and chickens, as well as from humans (Shore and Shemesh, 2003). In addition, progesterone is an active ingredient of several widely prescribed drugs (Golovko et al., 2018). Some synthetic progestins and progesterone already have been detected in WWTP effluents (Fan et al., 2011; Liu et al., 2014; Viglino et al., 2008), thereby indicating insufficient elimination of these compounds during wastewater treatment processes. Accumulating evidence indicates that municipal WWTP discharges may also contaminate surface waters (Kumar et al., 2015). Several analytical surveys conducted to date have confirmed the presence of synthetic progestins at concentrations up to tens of ng/L in aquatic environments throughout the world (Al-Odaini et al., 2010; Chang et al., 2011; Kolpin et al., 2002; Liu et al., 2011; Viglino et al., 2008; Vulliet et al., 2007) and progesterone even at concentrations as high as 199 ng/L (Kolpin et al., 2002). Nevertheless, only limited knowledge about environmental levels of synthetic progestins appears in the literature (Fent, 2015; Kumar et al., 2015).

This topic deserves greater attention, as it has been demonstrated that exposure of aquatic organisms to progestins at even low ng/L levels affects their reproduction (Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009; Zucchi et al., 2012). Namely, levonorgestrel, norethisterone, and gestodene have been shown to decrease egg production and diminish development of secondary male characteristics in fathead minnow (*Pimephales promelas*) at concentrations as low as 0.8, 1.0, and 1.0 ng/L, respectively (Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009). Furthermore, levonorgestrel was shown to negatively affect oocyte development in western clawed frog (*Xenopus tropicalis*) after sub-chronic exposure to concentration of 1.3 ng/L (Säfholm et al., 2012).

Although not all synthetic progestins are structurally similar to the natural progestin progesterone (Africander et al., 2011), they were designed to be progesterone receptor (PR) agonists and thus mimic progesterone function (Stanczyk et al., 2013). Most synthetic progestins are even more potent than natural progesterone (Besse and Garric, 2009). Surprisingly, there is a paucity of information regarding the contribution of progestins to progestagenic activity in municipal wastewaters and surface waters (Creusot et al., 2014).

Estrogenic activities have been studied very thoroughly and androgenic activities also have attracted a certain interest, but the other hormonal activities have not been given the proper research focus. Compounds exhibiting these activities, such as progestins, are nevertheless known environmental contaminants and endocrine disruptors (Kumar et al., 2015). Therefore, there exists a real need for further investigation of progestagenic activity in combination with thorough chemical analysis. To the best of our knowledge, only one study has been directed to revealing causative compounds of progestagenic activity downstream from a pharmaceutical factory (Creusot et al., 2014).

The first goals of the present study were to determine whether: 1) progestins are present in wastewaters and surface waters, 2) municipal wastewaters contain progestagenic activities, 3) progestagenic activities are removed during wastewater treatment processes, and 4) progestagenic activities emerging from the studied WWTPs affect receiving surface waters. Subsequently, we aimed to discover the extent to which detected progestins contribute to progestagenic activities in municipal WWTPs' effluents and in receiving surface waters. Because investigation of antagonistic activities should always be included into such an analysis (Ihara et al., 2014; Weiss et al., 2009), we also assessed each sampling locality in parallel for the presence of anti-progestagenic activities and for two selective progesterone receptor modulators, mifepristone and ulipristal acetate. SPRMs are synthetic compounds that block PR in certain tissues.

2. Material and methods

2.1. Chemicals

Methanol and acetonitrile (LiChrosolv® Hypergrade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

Selection of analytes was made based upon the consumption of these compounds in the Czech Republic as reported elsewhere (Golovko et al., 2018). The native standards (Table S1) were purchased from Sigma-Aldrich (Czech Republic). The chemicals for which tests were made are listed in Supplementary material (Table S1). The internal standards (ISs): Altrenogest 19,19,20,21,21-d5; Chlormadinone-d6 Acetate; Cyproterone Acetate-13C2,d3; Medroxy Progesterone-d3; 6-epi-Medroxy Progesterone-d3 17-Acetate; Megestrol Acetate-d3; Mifepristone-d3; Progesterone-d9; and Ulipristal Acetate-d3 (purity ≥98%) were obtained from Toronto Research Chemicals (Canada). All chemicals tested in the *in vitro* reporter gene bioassay were dissolved in dimethyl sulfoxide of ≥99.5% purity. Individual stock solutions of the standards were prepared for chemical analysis at 1 mg/mL concentration in methanol and stored at −20 °C. A spiking mixture of ISs was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg/mL for each compound. PR-CALUX cells, ORG 2058 standards in dimethyl sulfoxide, illuminate mix, and lysis mix were purchased from BioDetection Systems (BDS, Amsterdam, Netherlands).

2.2. Collection of samples, solid-phase extraction, and sample evaporation

Samples were taken from wastewaters and surface waters in the Czech and Slovak republics during January to June 2017. The study was performed at five Czech (Tábor-Klokoty, Strakonice, Prachatice, České Budějovice, and Brno) and one Slovak (Bratislava–Petržalka) municipal WWTPs and those watercourses receiving discharges from the WWTPs. Time proportional (15 min interval) composite wastewater samples (3–4 L) were collected from WWTP influents and effluents and cooled at 4 °C. Selected WWTPs employ mechanical-biological treatment technology with activated sludge-based secondary biological treatment in which they differ slightly as it is summarized in Supplementary material (Table S2). Grab surface water samples were collected up- and downstream from the respective WWTPs at 50 m distance from the WWTP effluent outlets at the same river side and at the same time as sampling of wastewater. Grab samples were taken close to the riverbank by submerging a 1 L amber glass bottle fastened to a stick.

Solid-phase extraction was carried out on an SPE-DEX 4790 automated solid-phase extractor (Horizon Technology, Salem, NH, USA) using a method reported by Golovko et al., 2018. Briefly, the collected samples (1 L each) were filtered through 5 and 1 μm glass fibre filters (Horizon Technology, Salem, NH, USA). Subsequently, the volume of filtered sample was passed through Atlantic C18 solid-phase extraction (SPE) discs (Horizon Technology, Salem, NH,

USA). Analytes were then retained on discs. The analytes were eluted from the discs with 10 mL of acetonitrile. SPE extracts were evaporated under gentle nitrogen stream to dryness at 37 °C on a Termovap TV10 + evaporator (ECOM, Czech Republic). After evaporation, wastewater and surface water extracts were redissolved either in 100 μL of acetonitrile or in 40 μL of dimethyl sulfoxide for chemical or biological analyses, respectively.

2.3. LC-APCI/APPI-HRPS analysis

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and HTS XT-CTC auto-sampler (CTC Analytics AG, Zwingen, Switzerland) were used to separate and detect target analytes. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) was used for chromatographic separation of the target analytes. Our analytical method for the analysis of a wide range of compounds with affinity to PR in wastewaters and surface waters using SPE followed by liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) had been validated for linearity, repeatability, quantification limit (LOQ), and recovery (Golovko et al., 2018). In the present study, the LC-APCI/APPI-HRPS method was applied to determine concentration of 15 progestins and 2 SPRMs in extracts from influent and effluent of six WWTPs and in respective up- and downstream recipient surface waters. The sample preparation is described in detail in a paper by Golovko et al., 2018. Samples from each sampling site were stored at 4 °C and analysed within 72 h. Prior to extraction for each site, a procedural blank (demineralized water) was extracted and analysed to distinguish between positive detections and potential sample contamination. Matrix matching standards corresponding to analysed matrices were used for correction of matrix effects. Briefly, the matrix standards were prepared by adding both IS and native compounds at amount of 10 ng and 200 ng into extract of corresponding matrix prepared the same way as real samples but without addition of IS. The peak area/internal standard ratio determined in non-spiked samples was subtracted from the peak area/internal standard ratio in matrix-matched standards to achieve the matrix-affected response factor. If matrix effect was lower than 20% we used response factors derived from calibration curve.

2.4. PR-CALUX and resazurin reduction assays

To detect (anti-)progestagenic activities either in water extracts or pure chemicals, PR-CALUX *in vitro* reporter gene bioassay was carried out as described elsewhere (Sonneveld et al., 2005). PR-CALUX bioassay was selected, because it is a highly sensitive *in vitro* bioassay and responds selectively to (anti-)progestagenic compounds (Sonneveld et al., 2011). The reference compound for progestagenic activity was ORG 2058, while mifepristone was the reference compound for assessing anti-progestagenic activity. All samples, including pure chemicals, were tested for cytotoxicity using resazurin reduction assay. First, PR-CALUX cells exposed to samples were visually inspected for cytotoxicity under a microscope. Subsequently, we carried out resazurin reduction assay using a resazurin-based *in vitro* toxicology assay kit (Sigma-Aldrich) to evaluate the potential effect of the sample on cell viability (O'Brien et al., 2000). The procedures of PR-CALUX assay and cytotoxicity testing are described in detail in the Supplementary material (chapters 1 and 2).

2.5. Data analysis

Total activities determined by a PR-CALUX bioassay (bio-TEQ, ng/L ORG 2058 EQs) were compared with the sum of the potencies of the individual compounds identified by chemical analysis (chem-TEQ) in order to estimate the degree to which analysed substances account for the biological activity. Chem-TEQ_i of a single compound was calculated using the following equation: chem-$TEQ_i = C_i \times REP_i$, where C_i is concentration of a compound (data from LC-APCI/APPI-HRPS chemical analysis) and REP_i is relative potency of a compound (derived from PR-CALUX). Chem-TEQ of a whole extract was calculated as the sum of chem-TEQs of individual compounds found in the sample: chem-TEQ = Σ chem-TEQ_i. Contribution of a single compound to bio-TEQ was calculated as follows: % contribution = (chem-TEQ_i/bio-TEQ) × 100. The same calculation was used for anti-progestagenic activity.

3. Results and discussion

3.1. Measured concentrations of progestins and SPRMs

Municipal WWTP influents contained nine progestins (cyproterone acetate, dienogest, drospirenone, gestodene, medroxyprogesterone, medroxyprogesterone acetate, megestrol acetate, nomegestrol acetate, and progesterone) in the range of 0.19–48 ng/L (Tables 1 and 2). Three compounds – dienogest, megestrol acetate, and progesterone – were found in all assessed influents. There was only one positive detection of an SPRM all through the sampling period and all studied types of matrices. This was mifepristone, at a concentration of 0.65 ng/L, in influent of České Budějovice's WWTP.

Seven progestins (cyproterone acetate, dienogest, drospirenone, megestrol acetate, medroxyprogesterone, medroxyprogesterone acetate, and progesterone) were detected in effluents at concentrations ranging from 0.11 to 3.2 ng/L (Tables 1 and 2). Those three compounds most widespread in influents (dienogest, megestrol acetate, and progesterone) were also those most frequently present in effluents, with four (megestrol acetate and progesterone) and three (dienogest) positive detections out of six attempts through the sampling campaign (Tables 1 and 2).

Only medroxyprogesterone and progesterone occurred even in surface waters. Progesterone was found three times both up- and downstream in concentration ranges of 0.20–0.42 ng/L (median 0.23 ng/L) and 0.17–1.2 ng/L (median 0.21 ng/L), respectively. The concentrations of progesterone are comparable with data reported from China, where progesterone was found upstream and downstream at concentrations of 0.5 and 2.5 ng/L, respectively (Liu et al., 2011). In the present study, most WWTP discharges did not significantly increase progesterone levels in surface waters. The exception was the WWTP at Bratislava–Petržalka, where progesterone was found at considerably higher concentration (Table 2) downstream (1.2 ng/L) than upstream (0.42 ng/L). Medroxyprogesterone is the only synthetic progestin found in surface water. It was detected only once (0.12 ng/L), that being in the River Svratka downstream from WWTP Brno. Occurrence of medroxyprogesterone in surface water has once been reported in the United States (Kolodziej et al., 2003) and in municipal WWTP effluent in the Czech Republic (Golovko et al., 2018). Medroxyprogesterone appears to be a specific pollutant among synthetic progestins for WWTP Brno, because this compound has already been detected in untreated wastewater from a hospital in Brno (Macikova et al., 2014). Therefore, the main source of medroxyprogesterone is likely this local hospital. Medroxyprogesterone is not prescribed in the Czech Republic and it may occur as a product of biotransformation from medroxyprogesterone acetate (Golovko

Table 1
Concentration of (anti-)progestins, chemical toxic equivalents (chem-TEQs), and biological toxic equivalents performed in agonistic and antagonistic modes (bio-TEQs and mifepristone EQs) in wastewater and receiving surface water of three wastewater treatment plants (WWTPs) with small catchment populations. "inf" = influent; "eff" = effluent; "usw" = upstream of WWTP; "dsw" = downstream of WWTP; "ND" = not determinable; "<LOQ" = results of biological method below the limit of quantification; "a" = mean of two replicates; "b" = masking effect occurred. Positive detections in chemical analysis are highlighted. Chem-TEQs were not calculated when concentrations of target compounds were below LOQ.

	Concentration (ng/L)											
	Tábor-Klokoty				Prachatice				Strakonice			
	inf	eff	usw	dsw	inf	eff	usw	dsw	inf	eff	usw	dsw
Progesterone	**9.5**	**0.63**	**0.20**	**0.17**	**48**	**0.15**	**0.23**	**0.21**	**27**	<0.04	<0.07	<0.09
Dydrogesterone	<0.18	<0.13	<0.18	<0.07	<0.29	<0.26	<0.28	<0.3	<0.49	<0.30	<0.46	<0.26
Medroxyprogesterone	<0.02	<0.01	<0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.04	<0.02	<0.04	<0.02
Medroxyprogesterone acetate	<0.20	<0.14	<0.20	<0.08	<0.83	<0.35	<0.08	<0.05	**0.88**	**0.38**	<0.02	<0.01
Cyproterone acetate	<0.14	<0.10	<0.14	<0.06	<0.91	<0.39	<0.87	<0.54	<0.29	<0.24	<0.32	<0.19
Chlormadinone acetate	<0.19	<0.15	<0.18	<0.08	<0.33	<0.27	<0.25	<0.23	<0.49	<0.28	<0.41	<0.25
Megestrol acetate	**0.52**	**0.14**	<0.02	<0.01	**8.0**	**1.0**	<0.84	<0.52	**8.0**	**0.28**	<0.07	<0.07
Nomegestrol acetate	**5.3**	<0.01	<0.02	<0.01	<0.02	<0.02	<0.02	<0.02	**10**	<0.02	<0.03	<0.02
Ulipristal acetate	<0.18	<0.15	<0.18	<0.08	<0.33	<0.27	<0.25	<0.23	<0.42	<0.25	<0.36	<0.22
Dienogest	**2.8**	**0.51**	<0.24	<0.24	**1.3**	**0.31**	<0.33	<0.39	**12**	<0.04	<0.06	<0.04
Norethisterone	<0.02	<0.02	<0.02	<0.01	<0.03	<0.03	<0.03	<0.04	<0.10	<0.05	<0.10	<0.04
Altrenogest	<0.20	<0.14	<0.20	<0.08	<0.12	<0.05	<0.11	<0.07	<0.02	<0.02	<0.02	<0.02
Mifepristone	<0.19	<0.15	<0.18	<0.08	<0.29	<0.24	<0.23	<0.20	<0.06	<0.04	<0.05	<0.03
Levonorgestrel	<0.26	<0.18	<0.21	<0.09	<0.35	<0.28	<0.31	<0.34	<0.07	<0.03	<0.05	<0.03
Etonogestrel	<0.25	<0.18	<0.20	<0.09	<0.31	<0.24	<0.27	<0.30	<0.62	<0.32	<0.46	<0.26
Gestodene	<0.41	<0.19	<0.23	<0.09	**5.0**	<0.25	<0.31	<0.34	**6.3**	<0.29	<0.52	<0.27
Drospirenone	**0.89**	<0.18	<0.20	<0.09	<0.34	<0.27	<0.30	<0.33	**3.0**	**0.11**	<0.04	<0.04
chem-TEQ (ng/L ORG 2058 EQs)	1.9	0.11	0.02	0.01	16	0.33	0.02	0.02	20	0.29	ND	ND
bio-TEQ (ng/L ORG 2058 EQs)	<LOQ	0.04	<LOQ	<LOQ	0.62	0.47	0.04	0.06	<LOQ	0.3	<LOQ	<LOQ
antagonism (ng/L mifepristone EQs)	5.6	0.77	<LOQ	<LOQ	9.7	<LOQ	<LOQ	<LOQ	83[a]	<LOQ	<LOQ	<LOQ
contribution of compounds to bio-TEQ (%)	ND[b]	>100[b]	ND	ND	>100[b]	70	45	28	ND[b]	96	ND	ND

Table 2
Concentration of (anti-)progestins, chemical toxic equivalents (chem-TEQs), and biological toxic equivalents performed in agonistic and antagonistic mode (bio-TEQs and mifepristone EQs) in wastewater and receiving surface water of three wastewater treatment plants (WWTPs) with large catchment populations. "inf" = influent; "eff" = effluent; "usw" = upstream of WWTP; "dsw" = downstream of WWTP; "ND" = not determinable; "<LOQ" = results of biological method below the limit of quantification; "a" = mean of two replicates; "b" = masking effect occurred. Positive detections in chemical analysis are highlighted. Chem-TEQs were not calculated when concentrations of target compounds were below LOQ.

	Concentration (ng/L)											
	České Budějovice				Brno				Bratislava-Petržalka			
	inf	eff	usw	dsw	inf	eff	usw	dsw	inf	eff	usw	dsw
Progesterone	**27**	<0.04	<0.07	<0.07	**4.3**	**0.31**	<0.20	<0.42	**14**	**3.2**	**0.42**	**1.2**
Dydrogesterone	<0.31	<0.26	<0.47	<0.45	<0.37	<0.31	<0.20	<0.50	<1.1	<0.81	<0.30	<0.16
Medroxyprogesterone	<0.03	<0.03	<0.04	<0.04	**0.19**	**0.95**	<0.02	**0.12**	<0.53	<0.16	<0.23	<0.09
Medroxyprogesterone acetate	<0.04	<0.04	<0.07	<0.07	**8.1**	**0.13**	<0.17	<0.40	<1.1	<0.18	<0.24	<0.10
Cyproterone acetate	**2.9**	<0.44	<0.44	<0.45	**12**	**0.50**	<0.16	<0.37	<0.07	<0.02	<0.03	<0.01
Chlormadinone acetate	<0.25	<0.23	<0.39	<0.36	<0.28	<0.14	<0.08	<0.21	<1.3	<0.40	<0.25	<0.19
Megestrol acetate	**3.4**	**0.13**	<0.06	<0.06	**13**	<0.38	<0.20	<0.48	**4.2**	<0.21	<0.29	<0.11
Nomegestrol acetate	<0.07	<0.06	<0.10	<0.10	<0.34	<0.22	<0.15	<0.36	<1.3	<0.23	<0.31	<0.11
Ulipristal acetate	<0.28	<0.26	<0.44	<0.40	<0.34	<0.24	<0.11	<0.28	<0.22	<0.07	<0.04	<0.03
Dienogest	**9.6**	**0.62**	<0.06	<0.05	**6.1**	<0.22	<0.18	<0.55	**3.9**	<4.0	<0.32	<0.72
Norethisterone	<0.04	<0.05	<0.07	<0.07	<0.36	<0.20	<0.17	<0.54	<0.91	<4.1	<0.33	<0.74
Altrenogest	<0.04	<0.04	<0.06	<0.07	<0.05	<0.03	<0.02	<0.05	<1.1	<0.25	<0.20	<0.15
Mifepristone	**0.65**	<0.26	<0.43	<0.40	<0.06	<0.03	<0.02	<0.06	<0.10	<0.08	<0.03	<0.02
Levonorgestrel	<0.36	<0.32	<0.58	<0.47	<0.32	<0.18	<0.21	<0.49	<1.2	<0.18	<0.26	<0.10
Etonogestrel	<0.38	<0.35	<0.62	<0.50	<0.38	<0.26	<0.26	<0.59	<1.2	<0.94	<0.35	<0.19
Gestodene	**7.0**	<0.25	<0.43	<0.37	<0.22	<0.15	<0.14	<0.34	<0.79	<3.5	<0.29	<0.64
Drospirenone	**1.9**	<0.07	<0.07	<0.05	**6.7**	<0.25	<0.25	<0.57	<0.91	<0.29	<0.30	<0.11
chem-TEQ (ng/L ORG 2058 EQs)	18	0.06	ND	ND	9.81	0.13	0.26	0.03	3.1	0.26	0.03	0.09
bio-TEQ (ng/L ORG 2058 EQs)	0.57	0.08	0.03	<LOQ	0.71	0.2	<LOQ	<LOQ	0.09	0.09	0.04	<LOQ
antagonism (ng/L mifepristone EQs)	8.3	<LOQ	<LOQ	<LOQ	5.4	<LOQ	<LOQ	<LOQ	2.6	0.89	<LOQ	<LOQ
contribution of compounds to bio-TEQ (%)	>100[a]	76	ND	ND	>100[a]	65	ND	ND	>100[a]	>100[a]	83	ND

et al., 2018).

3.2. (Anti-)progestagenic activity of pure compounds

It was possible to obtain complete dose-response curves for all studied progestins (Fig. 1) in order to derive EC_{50}, PC_{10}, and relative potency (REP) values (Table S3). Ulipristal acetate showed a full antagonistic dose-response curve (Fig. 2), and this was used to derive IC_{50}, PC_{20} and antagonistic REP value for the SPRM (Table S4).

Six of the 17 compounds analysed in this study (cyproterone acetate, dydrogesterone, levonorgestrel, medroxyprogesterone, mifepristone, norethisterone, and progesterone) have already been

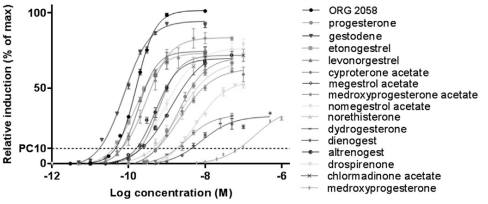


Fig. 1. Dose-response curves of synthetic progestins and progesterone in PR-CALUX cells. Maximum relative induction corresponds to 100% induction caused by reference compound ORG 2058. Dotted line indicates positive control 10 (PC_{10}) that is effect of a compound corresponding to 10% induction caused by reference compound ORG 2058. Values are expressed as means ± standard error of the mean.

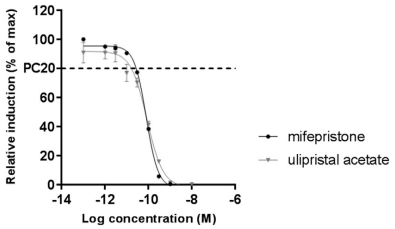


Fig. 2. Dose-response curves of two selective progesterone receptor modulators in PR-CALUX carried out in antagonistic mode. Assay medium contained EC_{50} concentration of ORG 2058. Dotted line indicates positive control 20 (PC_{20}) that is effect of a compound corresponding to 20% inhibition caused by reference compound mifepristone. Values are expressed as means ± standard error of the mean.

tested by other authors using PR-CALUX assay (Houtman et al., 2009; Riżner et al., 2011). To the best of our knowledge, this is the first systematic and comprehensive *in vitro* profiling of progestagenic activity of progestins that are relevant for the aquatic environment. In addition, medroxyprogesterone has been tested in the present study in *in vitro* reporter gene bioassay for the first time.

Ulipristal acetate was the only compound tested for anti-progestagenic activity. It has shown similar potency as a reference compound (mifepristone) for anti-progestagenic activity (Table S4). Our results together show that ulipristal acetate is the first environmentally relevant compound with anti-progestagenic activity similar to that of mifepristone.

3.3. (Anti-)progestagenic activity in wastewater and surface water

Unlike estrogenic activities, there have been only a few attempts to date to detect (anti-)progestagenic activities in aquatic environments (Table S5). In the present study, progestagenic agonistic activities were found in four of six WWTP influents in the range of 0.09–0.6 ng/L ORG 2058 EQs (median 0.38 ng/L ORG 2058 EQs). The PR-CALUX assay revealed also the presence of progestagenic activity ranging from 0.04 to 0.47 ng/L ORG 2058 EQs (median 0.15 ng/L ORG 2058 EQs) in all six WWTP effluents. That roughly corresponds to 0.5–6.1 ng/L progesterone EQs. Such contamination is still of considerable concern when bearing in mind that ORG 2058 is approximately 13 times more potent than is progesterone (Table S3; van der Linden et al., 2008). Activities found in the present study were in the same order of magnitude as those reported for effluents in the Netherlands (van der Linden et al., 2008) and India (Viswanath et al., 2008). Higher concentrations reaching to 5.4 ng/L ORG 2058 EQs have been found in effluents in Australia (Bain et al., 2014; Leusch et al., 2014). Surprisingly, no progestagenic activities were found in worldwide inter-laboratory screening (Escher et al., 2014) and at some sites in Australia (Scott et al., 2014), China (Rao et al., 2014), France (Bellet et al., 2012), and Tunisia (Mnif et al., 2010). Some non-detects, however, might be attributed to high limits of detection, such as 5 ng/L ORG 2058 EQs (Scott et al., 2014).

Progestagenic activities detected in surface water were one order of magnitude lower than those found in effluents. They were detected at three sampling points and ranged from 0.03 to 0.06 ng/L ORG 2058 EQs (median 0.04 ng/L ORG 2058), which corresponds to approximately 0.4–0.8 ng/L progesterone EQs. Surprisingly, weak progestagenic activities, at 0.03 and 0.04 ng/L ORG 2058 EQs, occurred twice in upstream but not in downstream surface waters (at the České Budějovice and Bratislava–Petržalka sampling sites). To sum up, Czech and Slovak surface waters did not seem to be seriously affected by progestagenic activity from municipal WWTP discharges in most cases. Only once did the progestagenic activity persist in surface water 50 m downstream, and then at a concentration of 0.06 ng/L ORG 2058 EQs. Such concentration is approximately equivalent to 0.8 ng/L of progesterone. The highest reported concentration of progestagenic activity has been as high as 4.5 ng/L ORG 2058 EQs in a Netherlands stream (van der Linden et al., 2008).

Anti-progestagenic activities were detected in all six influents and at three out of six investigated WWTP effluents, with concentrations ranging between 2.63 and 83 ng/L mifepristone EQs (median 6.97 ng/L mifepristone EQs) and 0.77 to 1.01 ng/L mifepristone EQs (median 0.89 ng/L mifepristone EQs), respectively. Strong anti-progestagenic activities in effluent and surface water (up to 31.5 and 121 μg/L mifepristone EQs, respectively) had previously been detected in China (Rao et al., 2014). Potent anti-progestagenic activities up to 32 μg/L mifepristone EQs were also reported in Australian surface water (Scott et al., 2014). Somewhat weaker anti-progestagenic activities were found in France with maximal concentration of 3.8 ng/L mifepristone EQs (Bellet et al., 2012). High anti-progestagenic loads detected at all six studied influents were completely or significantly removed during the treatment process. In the case of effluents at WWTP Tábor-Klokoty and WWTP Bratislava–Petržalka, anti-progestagenic activities co-occurred with progestagenic agonistic activities. No anti-progestagenic activity has been found in surface water samples (Tables 1 and 2).

3.4. Comparison of chem-TEQs and bio-TEQs in effluents and surface water

Predicted chem-TEQs of synthetic progestins and progesterone have accounted to measured bio-TEQs in effluents with 65, 70, 76 and 96% on the basis of REPs calculated from EC_{50} and 58, 64, 78 and 114% based on the latter approach with REPs calculated from PC_{10}. The unexplained portions of progestagenic activities (remaining to bio-TEQ, Table 3 and Table S6) might be attributed either to the presence of unknown compounds or to synergy between compounds (Table 3). When anti-progestagenic activities suppress the signal of agonists, the chem-TEQs are higher than the bio-TEQs (Tables 1 and 2). This phenomenon is known as masking effect (Creusot et al., 2014; Ihara et al., 2014; Weiss et al., 2009). All influent samples exhibited masking effects, while among WWTP effluents only those at Tábor-Klokoty and Bratislava–Petržalka were observed to have masking effects (Tables 1 and 2).

Additionally, contributions of the individual compounds to bio-TEQs in effluents and surface waters were estimated (Table 3). In effluents, medroxyprogesterone acetate, megestrol acetate, and

Table 3
Contribution of progesterone receptor-active compounds to measured progestagenic activity (bio-TEQs) in effluents at six municipal wastewater treatment plants on the basis of EC_{50}-derived relative potencies. Those compounds contributing most are highlighted. Values of contributing compounds were rounded to nearest whole number.

					Masking effect occurred	
Contribution to bio-TEQs (%)	Prachatice	Strakonice	České Budějovice	Brno	Tábor-Klokoty	Bratislava-Petržalka
Cyproterone acetate	0	0	0	18	0	0
Dienogest	2	0	22	0	34	0
Drospirenone	0	1	0	0	0	0
Medroxyprogesterone	0	0	0	<1	0	0
Medroxyprogesterone acetate	0	**67**	0	**34**	0	0
Megestrol acetate	**66**	29	**54**	0	108	0
Progesterone	3	0	0	12	**124**	**288**
Unknown compounds or synergy	29	3	24	36	0	0

progesterone were the main contributors to progestagenic activities (in two out of six studied WWTP effluents). Interestingly, progesterone was the greatest contributor in those effluents where masking effects were observed. It is noteworthy that the main contributors to progestagenic activity were progesterone derivatives (cyproterone acetate, medroxyprogesterone, medroxyprogesterone acetate, and megestrol acetate), while there was only one contributing testosterone (dienogest) and one spironolactone (drospirenone) derivative. To date, only one study has described a contribution of progestins to progestagenic activities. In that case, it was found that a single progestin, levonorgestrel, can contribute as much as approximately 50% to progestagenic activities in surface water (Creusot et al., 2014).

Regarding surface waters, progestins contributed as much as 83% to overall progestagenic activities. There was only one case in which progestagenic activity was detectable in surface water contaminated with progestins. The recipient of effluent from WWTP Prachatice, the Živný Brook, manifested progestagenic activities of 0.06 ng/L ORG 2058 EQs, which is equivalent to almost 1 ng/L of progesterone. This can be attributed to the fact that Živný Brook is a small stream and so the dilution is less than in the other studied recipients. Moreover, we revealed that progesterone was responsible for 45% of progestagenic activity upstream but only for 28% downstream from WWTP Prachatice. This indicates discharging of some unknown PR-active compounds in the effluent. Co-occurrence of progestagenic activity (0.04 ng/L ORG 2058 EQs) and the natural progestin progesterone (0.5 ng/L) has also been recorded upstream from WWTP Bratislava–Petržalka, and progesterone was responsible for most of the progestagenic activity (83%).

In the present study, we revealed only one substance contributing to anti-progestagenic activity of wastewater and that was mifepristone (in influent of WWTP České Budějovice). It was responsible, however, for only 7.8% of detected mifepristone EQ. As no SPRM was revealed in effluents and surface waters, the causative anti-progestagenic compounds remained unknown. We can speculate that it might originate from various industrial by-products and compounds included in personal care products, such as UV-filters or polycyclic musks, that are present in the aquatic environment and known to possess anti-progestagenic activities (Hamers et al., 2006; Schreurs et al., 2005).

3.5. Potential adverse effects in aquatic animals

Of the three compounds (dienogest, megestrol acetate, and progesterone) that occurred most frequently in effluents, effects on aquatic animals only of progesterone are reported in the literature (Kumar et al., 2015). The lowest observed effect concentration (LOEC) for progesterone in fish has been reported to be as low as 2 ng/L (Zucchi et al., 2012) which amount was exceeded in effluent of WWTP Bratislava–Petržalka and is quite close to the concentration of 1.2 ng/L detected even in surface water downstream from this WWTP. Among the compounds detected less frequently in effluents (cyproterone acetate, drospirenone, and medroxyprogesterone acetate), only the concentration of cyproterone acetate (0.5 ng/L) was close to its LOEC value, reported by Sharpe et al. (2004) to be 1 ng/L. Medroxyprogesterone acetate, which was detected twice, and drospirenone, which was found once, have LOECs (Zhao et al., 2015; Zucchi et al., 2014) much higher than the respective concentrations in effluents. If, however, the compounds occur in the water together, they may act in synergy and their LOEC values can then be significantly lower than determined under laboratory conditions. Zhao et al. (2015) describe such a synergy in the case of dydrogesterone and medroxyprogesterone acetate. Particular attention should be devoted to medroxyprogesterone, which was the only synthetic progestin detected in surface water. Unfortunately, there are no toxicological data available for this compound.

Progestins have been found to be very weak (drospirenone, gestodene, and progesterone) or non-agonists (e.g. levonorgestrel or etonogestrel) of PR in fish (Bain et al., 2015). Nevertheless, some of them do considerably transactivate fish androgen receptor (Bain et al., 2015; Ellestad et al., 2014). The mode of action in fish is likely different from what we know in humans (Kumar et al., 2015), and using PR-CALUX, which is based on human PR, may not be appropriate. Regarding aquatic vertebrates, activity detected by PR-CALUX assay seems to be a better risk indicator for amphibians than for fish, as amphibian PR is believed to have high amino acids sequence similarity (within the hormone binding domain) with PR in humans (Bayaa et al., 2000). Moreover, adverse effects of exposure to progesterone and synthetic progestins in amphibians seem to be similar to the effects in higher vertebrates, including humans (Kvarnryd et al., 2011; Säfholm et al., 2014, 2016).

4. Conclusions

Results of the present study show that synthetic progestins together with progesterone constitute the majority of causative agents of progestagenic activity in municipal WWTP effluents, with medroxyprogesterone acetate, megestrol acetate, and progesterone being the most important. Of the progestins that occurred most frequently in water, only four have been tested on aquatic vertebrates. Even in these cases, knowledge as to their possible adverse effects is very limited. Although strong anti-progestagenic activities were detected in two effluents, no causative agents were revealed. Despite that anti-progestagenic activities have been detected by various authors and were detected also in this study, *in vivo* anti-progestagenic effects on aquatic biota are practically not known and should therefore be studied.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects 'CENAKVA' (No. CZ.1.05/2.1.00/01.0024), 'CENAKVA II' (No. LO1205 under the NPU I program), project of the Czech Science Foundation (No. 16-09709Y), and GAJU (No. 012/2016/Z and No. 092/2016/Z). We would like to thank Jiří Stara (ČEVAK, a.s.), Luděk Míka (TSST Strakonice, s.r.o.), Robert Hrich (BVK, a.s.) and Tomáš Mackuľak (STU) for providing samples.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.02.065.

Abbreviations

APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
bio-TEQ	measured progestagenic activity equivalents (ng/L ORG 2058 EQs)
C	concentration
CALUX	*in vitro* bioassay called chemically activated luciferase gene expression
chem-TEQ	predicted progestagenic activity equivalents (ng/L ORG 2058 EQs)
EQ	equivalent
HRPS	high resolution product scan
LC	liquid chromatography
LOEC	lowest observed effect concentration
LOQ	limit of quantification (ng/L)
PR	progesterone receptor
REP	relative potency
SPE	solid-phase extraction
SPRM	selective progesterone receptor modulator
UV	ultraviolet light
WWTP	wastewater treatment plant

References

Africander, D., Verhoog, N., Hapgood, J.P., 2011. Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. Steroids 76 (7), 636–652.

Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., Surif, S., 2010. Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1217 (44), 6791–6806.

Bain, P.A., Kumar, A., Ogino, Y., Iguchi, T., 2015. Nortestosterone-derived synthetic progestogens do not activate the progestogen receptor of Murray-Darling rainbowfish (Melanotaenia fluviatilis) but are potent agonists of androgen receptors alpha and beta. Aquat. Toxicol. 163, 97–101.

Bain, P.A., Williams, M., Kumar, A., 2014. Assessment of multiple hormonal activities in wastewater at different stages of treatment. Environ. Toxicol. Chem. 33 (10), 2297–2307.

Bayaa, M., Booth, R.A., Sheng, Y.L., Liu, X.J., 2000. The classical progesterone receptor mediates Xenopus oocyte maturation through a nongenomic mechanism. Proc. Natl. Acad. Sci. U. S. A. 97 (23), 12607–12612.

Bellet, V., Hernandez-Raquet, G., Dagnino, S., Seree, L., Pardon, P., Bancon-Montigny, C., Fenet, H., Creusot, N., Aït-Aïssa, S., Cavailles, V., Budzinski, H., Antignac, J.P., Balaguer, P., 2012. Occurrence of androgens in sewage treatment plants influents is associated with antagonist activities on other steroid receptors. Water Res. 46 (6), 1912–1922.

Besse, J.P., Garric, J., 2009. Progestagens for human use, exposure and hazard assessment for the aquatic environment. Environ. Pollut. 157 (12), 3485–3494.

Chang, H., Wan, Y., Hu, J.Y., 2009. Determination and source apportionment of five classes of steroid hormones in urban rivers. Environ. Sci. Technol. 43 (20), 7691–7698.

Chang, H., Wan, Y., Wu, S.M., Fan, Z.L., Hu, J.Y., 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. Water Res. 45 (2), 732–740.

Colborn, T., Saal, F.S.V., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101 (5), 378–384.

Creusot, N., Aït-Aïssa, S., Tapie, N., Pardon, P., Brion, F., Sanchez, W., Thybaud, E., Porcher, J.M., Budzinski, H., 2014. Identification of synthetic steroids in river water downstream from pharmaceutical manufacture discharges based on a bioanalytical approach and passive sampling. Environ. Sci. Technol. 48 (7), 3649–3657.

Ellestad, L.E., Cardon, M., Chambers, I.G., Farmer, J.L., Hartig, P., Stevens, K., Villeneuve, D.L., Wilson, V., Orlando, E.F., 2014. Environmental gestagens activate fathead minnow (Pimephales promelas) nuclear progesterone and androgen receptors in vitro. Environ. Sci. Technol. 48 (14), 8179–8187.

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraish, F., Snyder, S., Su, G.Y., Tang, J.Y.M., van der Burg, B., van der Linden, S.C., Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X.W., Leusch, F.D.L., 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. Environ. Sci. Technol. 48 (3), 1940–1956.

Fan, Z.L., Wu, S.M., Chang, H., Hu, J.Y., 2011. Behaviors of glucocorticoids, androgens and progestogens in a municipal sewage treatment plant: comparison to estrogens. Environ. Sci. Technol. 45 (7), 2725–2733.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environ. Int. 84, 115–130.

Golovko, O., Sauer, P., Fedorova, G., Kocour Kroupová, H., Grabic, R., 2018. Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS. Sci. Total Environ. 621, 1066–1073.

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

Houtman, C.J., Sterk, S.S., van de Heijning, M.P.M., Brouwer, A., Stephany, R.W., van der Burg, B., Sonneveld, E., 2009. Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays. Anal. Chim. Acta 637 (1–2), 247–258.

Ihara, M., Ihara, M.O., Kumar, V., Narumiya, M., Hanamoto, S., Nakada, N., Yamashita, N., Miyagawa, S., Iguchi, T., Tanaka, H., 2014. Co-occurrence of estrogenic and antiestrogenic activities in wastewater: quantitative evaluation of balance by in vitro ER alpha reporter gene assay and chemical analysis. Environ. Sci. Technol. 48 (11), 6366–6373.

Jobling, S., Tyler, C.R., 2003. Endocrine disruption in wild freshwater fish. Pure Appl. Chem. 75 (11–12), 2219–2234.

Kolodziej, E.P., Gray, J.L., Sedlak, D.L., 2003. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. Environ. Toxicol. Chem. 22 (11), 2622–2629.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: a national reconnaissance. Environ. Sci. Technol. 36 (6), 1202–1211.

Kumar, V., Johnson, A.C., Trubiroha, A., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kocour Kroupová, H., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49 (5), 2625–2638.

Kvarnryd, M., Grabic, R., Brandt, I., Berg, C., 2011. Early life progestin exposure causes arrested oocyte development, oviductal agenesis and sterility in adult Xenopus tropicalis frogs. Aquat. Toxicol. 103 (1–2), 18–24.

Lange, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 20 (6), 1216–1227.

Leusch, F.D.L., Khan, S.J., Laingam, S., Prochazka, E., Froscio, S., Trinh, T., Chapman, H.F., Humpage, A., 2014. Assessment of the application of bioanalytical tools as surrogate measure of chemical contaminants in recycled water. Water Res. 49, 300–315.

Liu, S., Ying, G.G., Zhao, J.L., Chen, F., Yang, B., Zhou, L.J., Lai, H.J., 2011. Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography-electrospray ionization tandem mass spectrometry. J. Chromatogr. A 1218 (10), 1367–1378.

Liu, S.S., Ying, G.G., Liu, S., Lai, H.J., Chen, Z.F., Pan, C.G., Zhao, J.L., Chen, J., 2014. Analysis of 21 progestagens in various matrices by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with diverse sample pretreatment. Anal. Bioanal. Chem. 406 (28), 7299–7311.

Macikova, P., Groh, K.J., Ammann, A.A., Schirmer, K., Suter, M.J.F., 2014. Endocrine disrupting compounds affecting corticosteroid signaling pathways in Czech and swiss waters: potential impact on fish. Environ. Sci. Technol. 48 (21), 12902–12911.

Mnif, W., Dagnino, S., Escande, A., Pillon, A., Fenet, H., Gomez, E., Casellas, C., Duchesne, M.J., Hernandez-Raquet, G., Cavailles, V., Balaguer, P., Bartegi, A., 2010. Biological analysis of endocrine-disrupting compounds in tunisian sewage treatment plants. Arch. Environ. Contam. Toxicol. 59 (1), 1–12.

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 (17), 5421–5426.

Paulos, P., Runnalls, T.J., Nallani, G., La Point, T., Scott, A.P., Sumpter, J.P., Huggett, D.B., 2010. Reproductive responses in fathead minnow and Japanese

medaka following exposure to a synthetic progestin, Norethindrone. Aquat. Toxicol. 99 (2), 256–262.
Rao, K.F., Li, N., Ma, M., Wang, Z.J., 2014. In vitro agonistic and antagonistic endocrine disrupting effects of organic extracts from waste water of different treatment processes. Front. Environ. Sci. Eng. 8 (1), 69–78.
Rižner, T.L., Brožič, P., Doucette, C., Turek-Etienne, T., Müller-Vieira, U., Sonneveld, E., van der Burg, B., Böcker, C., Husen, B., 2011. Selectivity and potency of the retroprogesterone dydrogesterone in vitro. Steroids 76 (6), 607–615.
Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environ. Sci. Technol. 47 (4), 2077–2084.
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. Hum. Ecol. Risk Assess. 16 (6), 1318–1338.
Säfholm, M., Jansson, E., Fick, J., Berg, C., 2016. Molecular and histological endpoints for developmental reproductive toxicity in *Xenopus tropicalis*: levonorgestrel perturbs anti-Mullerian hormone and progesterone receptor expression. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 181, 9–18.
Säfholm, M., Norder, A., Fick, J., Berg, C., 2012. Disrupted oogenesis in the frog *Xenopus tropicalis* after exposure to environmental progestin concentrations. Biol. Reprod. 86 (4), 1–7.
Säfholm, M., Ribbenstedt, A., Fick, J., Berg, C., 2014. Risks of hormonally active pharmaceuticals to amphibians: a growing concern regarding progestagens. Phil. Trans. Biol. Sci. 369 (1656).
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., Leusch, F.D.L., 2014. An assessment of endocrine activity in Australian rivers using chemical and in vitro analyses. Environ. Sci. Pollut. Control Ser. 21 (22), 12951–12967.
Sharpe, R.L., MacLatchy, D.L., Courtenay, S.C., Van Der Kraak, G.J., 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (Fundulus heteroclitus) bioassay. Aquat. Toxicol. 67 (3), 203–215.
Shore, L.S., Shemesh, M., 2003. Naturally produced steroid hormones and their release into the environment. Pure Appl. Chem. 75 (11–12), 1859–1871.
Schreurs, R., Sonneveld, E., Jansen, J.H.J., Seinen, W., van der Burg, B., 2005. Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. Toxicol. Sci. 83 (2), 264–272.
Sitruk-Ware, R., 2004. Pharmacological profile of progestins. Maturitas 47 (4), 277–283.
Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., van der Burg, B., 2005. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. Toxicol. Sci. 83 (1), 136–148.
Sonneveld, E., Pieterse, B., Schoonen, W.G., van der Burg, B., 2011. Validation of in vitro screening models for progestagenic activities: inter-assay comparison and correlation with in vivo activity in rabbits. Toxicol. Vitro 25 (2), 545–554.
Stanczyk, F.Z., Hapgood, J.P., Winer, S., Mishell, D.R., 2013. Progestogens used in postmenopausal hormone therapy: differences in their pharmacological properties, intracellular actions, and clinical effects. Endocr. Rev. 34 (2), 171–208.
Sumpter, J.P., 1998. Xenoendocrine disrupters - environmental impacts. Toxicol. Lett. 103, 337–342.
van der Linden, S.C., Heringa, M.B., Man, H.Y., Sonneveld, E., Puijker, L.M., Brouwer, A., van der Burg, B., 2008. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environ. Sci. Technol. 42 (15), 5814–5820.
Viglino, L., Aboulfadl, K., Prevost, M., Sauve, S., 2008. Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS. Talanta 76 (5), 1088–1096.
Viswanath, G., Halder, S., Divya, G., Majumder, C.B., Roy, P., 2008. Detection of potential (anti)progestagenic endocrine disruptors using a recombinant human progesterone receptor binding and transactivation assay. Mol. Cell. Endocrinol. 295 (1–2), 1–9.
Vulliet, E., Baugros, J.B., Flament-Waton, M.M., Grenier-Loustalot, M.F., 2007. Analytical methods for the determination of selected steroid sex hormones and corticosteriods in wastewater. Anal. Bioanal. Chem. 387 (6), 2143–2151.
Weiss, J.M., Hamers, T., Thomas, K.V., van der Linden, S., Leonards, P.E.G., Lamoree, M.H., 2009. Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. Anal. Bioanal. Chem. 394 (5), 1385–1397.
Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Lange, R., 2009. Effects of synthetic gestagens on fish reproduction. Environ. Toxicol. Chem. 28 (12), 2663–2670.
Zhao, Y.B., Castiglioni, S., Fent, K., 2015. Synthetic progestins medroxyprogesterone acetate and dydrogesterone and their binary mixtures adversely affect reproduction and lead to histological and transcriptional alterations in zebrafish (Danio rerio). Environ. Sci. Technol. 49 (7), 4636–4645.
Zucchi, S., Castiglioni, S., Fent, K., 2012. Progestins and antiprogestins affect gene expression in early development in zebrafish (Danio rerio) at environmental concentrations. Environ. Sci. Technol. 46 (9), 5183–5192.
Zucchi, S., Mirbahai, L., Castiglioni, S., Fent, K., 2014. Transcriptional and physiological responses induced by binary mixtures of drospirenone and progesterone in zebrafish (Danio rerio). Environ. Sci. Technol. 48 (6), 3523–3531.

CHAPTER 4

DETERMINING THE POTENTIAL OF PROGESTINS TO INDUCE (ANTI-)ANDROGENIC ACTIVITIES IN THE AQUATIC ENVIRONMENT

Šauer, P., Bořík, A., Golovko, O., Grabic, R., Vojs Staňová, A., Valentová, O., Stará, A., Šandová, M., Kocour Kroupová, H., 2018. Do progestins contribute to (anti-)androgenic activities in the aquatic environment? Environmental Pollution 242, 417–425.

According to the publishing agreement between the authors and publisher, it is allowed to include the paper in this Ph.D. thesis
https://www.elsevier.com/about/policies/copyright/permissions

My share on this work was about 30%.

Environmental Pollution 242 (2018) 417–425

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Do progestins contribute to (anti-)androgenic activities in aquatic environments?☆

Pavel Šauer*, Adam Bořík, Oksana Golovko, Roman Grabic, Andrea Vojs Staňová, Olga Valentová, Alžběta Stará, Marie Šandová, Hana Kocour Kroupová

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic

ARTICLE INFO

Article history:
Received 21 May 2018
Received in revised form
28 June 2018
Accepted 30 June 2018
Available online 4 July 2018

Keywords:
Progestagens
Solid-phase extraction
Target analysis
Sample acidification

ABSTRACT

Unknown compounds with (anti-)androgenic activities enter the aquatic environment via municipal wastewater treatment plants (WWTPs). Progestins are well-known environmental contaminants capable of interfering with androgen receptor (AR) signaling pathway. The aim of the present study was to determine if 15 selected progestins have potential to contribute to (anti-)androgenic activities in municipal wastewaters and the respective recipient surface waters. AR-specific Chemically Activated LUciferase gene eXpression bioassay in agonistic (AR-CALUX) and antagonistic (anti-AR-CALUX) modes and liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) methods were used to assess (anti-)androgenic activity and to detect the target compounds, respectively. The contribution of progestins to (anti-)androgenic activities was evaluated by means of a biologically and chemically derived toxicity equivalent approach. Androgenic (0.08–59 ng/L dihydrotestosterone equivalents – DHT EQs) and anti-androgenic (2.4–26 μg/L flutamide equivalents – FLU EQs) activities and progestins (0.19–75 ng/L) were detected in selected aquatic environments. Progestins displayed androgenic potencies (0.01–0.22 fold of dihydrotestosterone) and strong anti-androgenic potencies (9–62 fold of flutamide). Although they accounted to some extent for androgenic (0.3–29%) and anti-androgenic (4.6–27%) activities in influents, the progestins' contribution to (anti-)androgenic activities was negligible (≤2.1%) in effluents and surface waters. We also tested joint effect of equimolar mixtures of target compounds and the results indicate that compounds interact in an additive manner. Even if progestins possess relatively strong (anti-)androgenic activities, when considering their low concentrations (sub-ng/L to ng/L) it seems unlikely that they would be the drivers of (anti-)androgenic effects in Czech aquatic environments.

1. Introduction

Mixtures of many chemicals are continuously discharged by wastewater treatment plants (WWTPs) into aquatic environments. Some of these compounds may adversely affect the endocrine system of exposed organisms via androgen receptor (AR)-mediated signaling pathway (Gray et al., 2001; Kelce et al., 1998; Sohoni and Sumpter, 1998). To date, natural and synthetic estrogens have drawn great eco-toxicological interest due to their ability to induce intersex and feminization in freshwater fish (Leusch et al., 2017; Sumpter, 2005). Widespread feminization of male fish living downstream from WWTPs has been revealed to be caused not only by estrogens, however, but also by anti-androgenic compounds (Jobling et al., 2009). Moreover, androgenic contaminants of surface water can cause masculinization of resident female fish (Howell et al., 1980; Parks et al., 2001). (Anti-)androgenic activities have frequently been reported in aquatic environments worldwide (Bain et al., 2014; Boehler et al., 2017; Escher et al., 2014; Kinani et al., 2010; König et al., 2017; Urbatzka et al., 2007; van der Linden et al., 2008; Zhao et al., 2011). Compounds responsible for these activities often remain unidentified (Chen and Chou, 2016; Kinani et al., 2010; Leusch et al., 2014; Urbatzka et al., 2007).

Recently, progestins have come to be one of the groups of

☆ This paper has been recommended for acceptance by Klaus Kummerer.

* Corresponding author.
E-mail address: psauer@frov.jcu.cz (P. Šauer).

https://doi.org/10.1016/j.envpol.2018.06.104
0269-7491/

emerging pollutants drawing attention (Fent, 2015; Kumar et al., 2015). The group of substances termed progestins includes not only such natural hormones as progesterone but also synthetic substances designed to have biological activity similar to that of progesterone. Progestins are contained in contraceptives and other hormonal preparations (Sitruk-Ware, 2004). Both progesterone and synthetic progestins act primarily as progesterone receptor (PR) agonists (Africander et al., 2011), but they also cause off-target modulation of other steroid receptors (Bain et al., 2015; Besse and Garric, 2009; Stanczyk, 2003). Among other activities, progestins are known to act as both potent agonists and antagonists of human AR (Bain et al., 2015; Schindler et al., 2008). Androgenicity of some progestins has recently been observed also *in vivo* (Hua et al., 2015; Runnalls et al., 2013; Svensson et al., 2013, 2016; Zeilinger et al., 2009) and *in vitro* (Bain et al., 2015; Ellestad et al., 2014) within fish. Moreover, some of these progestins have been shown to inhibit synthesis of androgens *in vivo* (Fernandes et al., 2014) and possess anti-androgenic activity *in vitro* (Siegenthaler et al., 2017) in fish.

Surprisingly, no study to date has investigated whether environmental levels of progestins reach sufficient concentrations and have relative potencies strong enough to cause a substantial part of (anti-)androgenic activity observed in aquatic environments. The aim of the present study was to discover the extent to which progestins are responsible for (anti-)androgenic activities in Czech aquatic environments associated with municipal WWTPs. In parallel, we also assessed each sampling locality for the presence of a PR antagonist mifepristone and a selective PR modulator ulipristal acetate, because these compounds are suspected to be novel environmental contaminants (Golovko et al., 2018; Liu et al., 2010; Šauer et al., 2018). (Anti-)androgenic activities of mifepristone and ulipristal acetate and their contribution to overall sample activities were determined, as well.

2. Material and methods

2.1. Chemicals and material

All progestins, mifepristone and ulipristal acetate prescribed in the Czech Republic (Golovko et al., 2018) were chosen as target compounds. In addition, medroxyprogesterone was included because it has recently been found in Czech aquatic environments (Macikova et al., 2014a; Šauer et al., 2018). All tested compounds were of high purity as follows: altrenogest (≥99%), chlormadinone acetate (99.7%), cyproterone acetate (≥98%), dienogest (99.9%), drospirenone (99.9%), dydrogesterone (99.5%), etonogestrel (≥98%), flutamide (≥99%), gestodene (≥98%), levonorgestrel (≥99%), medroxyprogesterone (98.5%), medroxyprogesterone acetate (≥97%), megestrol acetate (≥99%), mifepristone (≥98%), nomegestrol acetate (≥98%), norethisterone (≥98%), progesterone (99.9%), and ulipristal acetate (≥98%). All were purchased from Sigma-Aldrich (Czech Republic). Classification of the studied compounds and their physicochemical properties are described in more detail in the Supplementary Material (Table S1). As internal standards, Altrenogest 19,19,20,21,21-d5, Chlormadinone-d6 Acetate, Cyproterone Acetate-13C2,d3, Medroxy Progesterone-d3, 6-epi-Medroxy Progesterone-d3, 17-Acetate, Megestrol Acetate-d3, Mifepristone-d3, Progesterone-d9, and Ulipristal Acetate-d3 were purchased from Toronto Research Chemicals (Canada). Individual stock solutions of native and internal standards were prepared for chemical analysis at 1 mg/mL concentration in methanol and stored at −20 °C. A spiking mixture of internal standards was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg/mL for each compound. AR-CALUX cells, illuminate mix, lysis mix, and dihydrotestosterone standards prepared in dimethyl sulfoxide (≥99.5% purity) were purchased from BioDetection Systems (the Netherlands). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

2.2. Collection of samples, sample preparation, and solid-phase extraction

Samples were collected from wastewaters (influents and effluents) of four WWTPs located in the Czech Republic and from the receiving surface waters (upstream and downstream). The WWTPs receive domestic and industrial wastewaters and at two sites, WWTPs at Prachatice and České Budějovice, hospital wastewaters. While all the studied WWTPs are based on mechanical–biological treatment with activated sludge secondary treatment, they differ slightly in their biological treatment (Table S2). Grab or time proportional (15-minute interval) 24 h composite samples (3–4 L) were collected (Table S2). Grab surface water sampling was performed up- and downstream from the respective WWTPs at a distance of 50 m from WWTP outlets at the same side of the point of discharge. Grab samples were taken using a 2 L bottle fastened to a stick and then poured into 1 L amber glass bottles. Surface water samples were collected at the same time as were samples of effluents. The collected samples were transported to the laboratory and stored at 4 °C in darkness until extraction, which was carried out within 24 h.

In order to preconcentrate the target compounds, a recently developed protocol for solid-phase extraction (SPE) and sample evaporation was used (Golovko et al., 2018), albeit with a slight change wherein some samples were acidified prior to extraction to find out the influence of acidification on the extraction efficiency of SPE (see section 2.3.). Briefly, an SPE-DEX 4790 automated solid-phase extractor (Horizon Technology, Salem, NH, USA) was employed to extract 1 L water samples. Atlantic C18 SPE disks (Horizon Technology) were used as a sorbent and preconditioned with acetonitrile for liquid chromatography mass spectrometry (Sigma-Aldrich, Czech Republic) and demineralized water. The samples were filtered through Atlantic Fast Flow glass fiber filters of pore sizes 5 and 1 μm (Horizon Technology). After a sample had been passed through the Atlantic C18 SPE disks, the entire extraction system was rinsed with demineralized water. The Atlantic C18 SPE disks were air dried for 15 min. The retained target compounds were then eluted with total volume of 10 mL acetonitrile. The SPE extracts thus obtained were evaporated by gentle nitrogen stream until dryness at 37 °C using a Termovap TV10 + sample concentrator (ECOM, Czech Republic). The extracts were redissolved either in 2 × 50 μL of acetonitrile for chemical or in 2 × 20 μL of dimethyl sulfoxide for biological analyses.

2.3. pH test

Because sample pH is an important factor influencing extraction efficiency (Kuster et al., 2009; Vulliet et al., 2008), we tested the effect of sample acidification prior to SPE. An advantage of sample acidification prior to SPE is that it inhibits biological activity of microorganisms potentially present in samples and thereby may help in preventing biotransformation and bioconcentration of target compounds. Sample acidification also may influence the dissociation of ionizable compounds, however, and thus cause problems with retention of analytes on SPE sorbents. C18 SPE sorbents such as Atlantic disks best retain neutral forms of polar compounds, but some analytes may be affected due to sample acidification depending upon their dissociation constants (Table S3). Thus, we assessed whether sample pH adjustment had an effect on retention of progestins, mifepristone and ulipristal acetate on Atlantic C18 SPE disks. The effect was evaluated

according to the recoveries of target analytes, assessing whether recoveries were within a satisfactory range of 60–130%. Detailed description of pH test can be found in Supplementary material (see section 1).

2.4. Chemical analysis

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and HTS XT-CTC auto-sampler (CTC Analytics AG, Zwingen, Switzerland) were used for analysis of water extracts. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) was used for chromatographic separation of the target analytes. Our analytical method for the analysis of a wide range of progestins, mifepristone and ulipristal acetate in wastewaters and surface waters using SPE followed by LC-APCI/APPI-HRPS had previously been validated for linearity, repeatability, limit of quantification (LOQ), and recovery (trueness) (Golovko et al., 2018). In the present study, the LC-APCI/APPI-HRPS method was applied to determine the concentration of 15 progestins, mifepristone and ulipristal acetate in extracts from influent and effluent of the studied WWTPs and in respective up- and downstream recipient surface waters. The sample preparation is described in detail in the paper by Golovko et al. (2018). Samples from each sampling site were stored at 4 °C and analyzed within 48 h. Prior to extraction for each site, a procedural blank (demineralized water) was extracted and analyzed to distinguish between positive detections and potential sample contamination. Each sample was analyzed simultaneously with matrix matching standards for the determination of matrix effects.

Data analysis and calculation was performed using TraceFinder 3.3 software (Thermo Fisher Scientific).

2.5. In vitro reporter gene bioassays and cytotoxicity testing

AR-CALUX and anti-AR-CALUX (BioDetection Systems, the Netherlands) *in vitro* reporter gene bioassays were performed as previously described (Sonneveld et al., 2005; van der Burg et al., 2010) to detect (anti-)androgenic activities of either pure compounds or in environmental water extracts. In addition, PR- and anti-PR-CALUX bioassays were carried out using methods described elsewhere (Sonneveld et al., 2005, 2011). They were used for determining progestagenic and anti-progestagenic activities in water extracts as we wanted to find out if there is any relationship among occurrence of (anti-)androgenic and (anti-)progestagenic activities in aquatic environments (section 2.6.).

Briefly, AR- or PR-CALUX cells were seeded in a 100 μL volume of cell suspension in assay medium (phenol-red free 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F12 medium supplemented with 5% dextran coated charcoal stripped fetal calf serum, 0.2% penicillin–streptomycin solution, and 1% nonessential amino acids) into 96-well white plates with transparent bottom (Corning, the Netherlands) at density of 10^4 cells per well. Assay medium for antagonism testing contained in addition reference agonist compound at its EC_{50} value (3.5×10^{-10} M of dihydrotestosterone for anti-androgenicity or 2.0×10^{-10} M ORG 2058 for anti-progestagenicity). After 24 h of incubation at 37 °C and in a humidified atmosphere with 5% CO_2, the cells were exposed to serial dilutions of either pure compounds or environmental water extracts dissolved in either assay medium for agonism (AR- and PR-CALUX assays) or in respective assay medium for antagonism (anti-AR- and anti-PR-CALUX assays) testing and returned to the incubator. Each sample was tested at least in two independent experiments, with each calibration point performed in triplicate. A calibration row of reference agonist (dihydrotestosterone for AR-CALUX and ORG 2058 for PR-CALUX) or antagonist (flutamide for anti-AR-CALUX and mifepristone for anti-PR-CALUX) were included in each 96-well plate and tested in triplicate. Solvent control (0.1% dimethyl sulfoxide) was included at least in triplicate on each plate. Calibration rows of pure compounds and serial dilutions of water extracts were prepared in dimethyl sulfoxide of ≥99.5% purity (Sigma-Aldrich, Czech Republic). To assess joint (anti-)androgenic effects of target compounds in mixtures, we combined pure compounds at equimolar ratios. These mixtures consisted of all active compounds in the respective modes (agonism and antagonism). All the mixtures were serially diluted and tested using (anti-)AR-CALUX bioassays.

Luciferase signal was quantified using an Infinite M200 Plus luminometer (Tecan, Switzerland). Potential cytotoxic effects of samples were tested using resazurin reduction assay (O'Brien et al., 2000) and samples evaluated as cytotoxic were excluded from further analysis in CALUX assays (see section S1 in Supplementary Material and Figs. S1–S10).

2.6. Data analysis

Luciferase signal in (anti-)AR- and (anti-)PR-CALUX assays was recorded as relative light units. To normalize data, relative induction of samples was derived after subtracting a background signal (solvent control). Principal component analysis (PCA) was used to determine possible relationships among detected activities. Results below the LOQ were constrained at 0.

The data obtained from measurement of pure compounds were fitted by nonlinear regression with four parameters (robust fit) using Prism 7 software (Graph Pad, San Diego, CA, USA). The maximum response (efficacy) induced by a tested compound (at top plateau in agonism and bottom plateau in antagonism testing) was reported (= RPCmax). We determined if tested compounds possess (anti-)androgenic activity on the basis of cutoff criterion (RPCmax values reach or exceed a specific effect) as proposed by the Organisation for Economic Co-operation and Development (OECD, 2016). A compound was evaluated as active (androgenic) in AR-CALUX assay if its RPCmax value was ≥10% of relative induction in at least two out of two or two out of three experiments. A compound assigned as active (anti-androgenic) in anti-AR-CALUX assay had to have RPCmax value ≤ 80% of relative induction in at least two out of two or two out of three experiments. Estimates of potency were determined for active compounds: half-maximal effective (EC_{50}s) and inhibitory (IC_{50}s) concentrations, positive controls 10 (PC_{10}s) for agonism, and positive controls 20 (PC_{20}s) for antagonism were derived from the dose-response curves. EC_{50} is a concentration of a chemical that produces 50% stimulating efficacy in agonistic mode, while IC_{50} produces 50% inhibiting efficacy in antagonistic mode. PC*i* is the concentration of a tested chemical that produces the same effect *i* as does the reference compound. PC_{10} and PC_{20} values were calculated for agonists and antagonists of AR, respectively. Determining multiple toxicological estimates had been proposed by Villeneuve et al. (2000) to provide robust results against deviation from parallelism of curves and non-equal maximal efficacies, and thus relative potencies (REPs) of progestins were calculated using two approaches. In the first, $E(I)C_{50}$ of the reference compound was divided by $E(I)C_{50}$ of a tested compound. In the second, $PC_{10(20)}$ of the reference compound was divided by $PC_{10(20)}$ of a tested compound. The measured androgenic (DHT EQs) and anti-androgenic (FLU EQs) equivalents in extracts of water samples were derived according to the licensor's (BioDetection Systems, NL) instructions, as described elsewhere (Sonneveld et al., 2005). Predicted (anti-)androgenic activity of a

target compound was derived by multiplying its determined concentration by its relative potency based on E(I)C_{50} values.

Joint effects measured in AR-CALUX cells are mediated through the same mechanism of action (AR-mediated activities). Thus, tested individual compounds are supposed to interact in mixtures according to concentration addition model (Loewe and Muischnek, 1926), which assumes that tested compounds behave as dilution of each other. To evaluate the joint effects of individual chemicals in mixture, toxic unit method has been used as described elsewhere (Brown, 1968). Briefly, dose-response curves of single compounds and mixtures were used to calculate the concentrations of individual chemicals causing a specific effects (PC*i* values) and the concentration of individual chemicals causing the same effects in a mixture. Toxic unit value of a substance = d_A/D_A, where d_A is concentration of compound A in mixture causing specific effect (PC*i*) and D_A is concentration of the compound A needed to produce the same effect (PC*i*) on its own. Toxic units of individual compounds derived from response PC*i* were summed to derive toxic unit of a mixture at PC*i* level. We determined toxic units at four PC*i* levels (PC_{10}, PC_{20}, PC_{30} and PC_{40} for agonistic, and PC_{20}, PC_{30}, PC_{50} and PC_{70} for antagonistic mixtures, respectively) for each tested curve. If the toxic unit value equals to 1, there is additivity between compounds. Toxic unit smaller than 1 indicates synergism, while toxic unit higher than 1 indicates antagonism between compounds. Following a study by Rossier et al. (2016), we used a margin of error of ±0.5 of the toxic value for additivity to account for possible variability.

3. Results and discussion

3.1. pH test

The pH value of samples has been reduced prior to SPE in approximately 48% of those studies determining the presence of progestins in aquatic environments, albeit without testing the influence of sample pH on the recoveries of target compounds (Table S4). Recently, there have been a few reports investigating the effect of sample pH on the efficiency of extracting progestins using different solid-phase extraction sorbents (Chen et al., 2017; Huysman et al., 2017; Kuster et al., 2009; Shen et al., 2018; Vulliet et al., 2008; Table S5). Our results showed that acidification of samples resulted in slightly poorer recoveries (mean values did not lie within the satisfactory range of 60–130%) for three progestins (gestodene, levonorgestrel, and nomegestrol acetate) and PR antagonist mifepristone. In non-acidified samples, all analytes fell within this range (Fig. S11). Similarly to our observation, the results of Huysman et al. (2017), Kuster et al. (2009), and Shen et al. (2018) indicate that adjusting sample pH does not play a significant role in extraction efficiency for progestins (Table S5). In the present study, no difference was observed between detected activities in samples with and without pH acidification in (anti-)AR-CALUX assays (Fig. S12). Thus, both sample pH pretreatment (pH adjustment) and no such pH adjustment appear to be applicable for the determination of progestins in water samples using SPE that employs Atlantic C18 sorbent. Even if there were no significant differences, however, we would recommend not using sample acidification in order to better simulate real environmental conditions.

3.2. Occurrence of progestins in water samples

The results of chemical analysis are summarized in Table S6 and are comparable to those reported by other authors (reviewed in Golovko et al., 2018). Concentrations of seven progestins (cyproterone acetate, dienogest, drospirenone, gestodene, medroxyprogesterone acetate, megestrol acetate, and progesterone) in influents ranged from 1.0 to 75 ng/L. Progesterone and dienogest were found in all studied influents. PR antagonist mifepristone was detected once in the influent of the České Budějovice WWTP at concentration of 0.32 ng/L. Only two progestins (megestrol acetate and progesterone) reached effluents, and those were found in the range of 0.19–2.7 ng/L.

Progesterone was the only progestin present in surface waters at concentrations ranging from 0.47 to 1.3 ng/L. Widespread occurrence of progesterone has been observed also in other studies (Houtman et al., 2018; Liu et al., 2015; Šauer et al., 2018), and this compound has been proposed as a chemical indicator for the presence of steroids in environmental water samples (Liu et al., 2015). The lowest observed effect concentration of 2 ng/L of progesterone for fish (Zucchi et al., 2012) has been exceeded in several effluents (Fan et al., 2011; Liu et al., 2014; Šauer et al., 2018; Table S6) and even in surface waters (Kolpin et al., 2002; Liu et al., 2014; Macikova et al., 2014a; Vulliet et al., 2008). Thus, we propose to assess further the risk posed by progesterone for aquatic environments.

Some progestins that we found in the present study (Table S6) have been shown to undergo biotransformation in aquatic environments (Ojoghoro et al., 2017; Peng et al., 2014; Sangster et al., 2016). The occurrences and fates of progestin, mifepristone and ulipristal acetate metabolites deserve greater attention, because some studies have indicated that metabolites of progestins and mifepristone may be also strong activators (Schoonen et al., 2000; Houtman et al., 2009) and inhibitors (Attardi et al., 2004) of steroid receptors, respectively.

3.3. In vitro reporter gene bioassays with pure compounds

Seven progestins were active androgens and dose-dependent response curves in AR-CALUX assay could be constructed for them (Table 1; Fig. S13). Nine progestins, mifepristone and ulipristal acetate showed AR antagonism and produced full dose-response curves in the anti-AR-CALUX assay (Table 2; Fig. S14). All androgenic progestins were weaker than reference compound dihydrotestosterone (Table 1). On the contrary, all anti-androgenic progestins, mifepristone and ulipristal acetate showed stronger relative potencies than did the reference compound flutamide (Table 2).

Previous studies have tested (anti-)androgenicity for some of our target compounds using (anti-)AR-CALUX bioassays and their results have been comparable with our observations (Tables S7 and S8; Houtman et al., 2009; Sonneveld et al., 2005). We have newly identified (anti-)androgenic activity of medroxyprogesterone (Tables 1 and 2) and anti-androgenic activity of ulipristal acetate (Table 2). Particularly noteworthy is that megestrol acetate and chlormadinone acetate had similar potencies as did cyproterone acetate, a well-known strong anti-androgen (Table 2). To the best of our knowledge, this is the first study to show (anti-)androgenic potencies (EC_{50}s, IC_{50}s, PC_{10}s, and PC_{20}s) of multiple environmentally relevant progestins, mifepristone and ulipristal acetate measured in a single *in vitro* bioassay.

3.4. Joint effects of individual compounds in mixtures

Toxic units for mixture of androgenic compounds ranged from 0.93 to 1.01, while toxic units for mixture of anti-androgenic compounds ranged from 0.70 to 0.87. All these values fall within the range of 1 ± 0.5 of toxic unit which indicates that both androgenic and anti-androgenic compounds tested in the present study act in an additive manner. Up to date, *in vitro* studies have focused on joint effects of binary mixtures of progestins and observed mostly additivity (Rossier et al., 2016; Siegenthaler et al., 2017).

Table 1
Androgenic potencies of progestins in the AR-CALUX assay.

Compound (number of replicates)	Active or non-active compound	RPCmax (%)	log PC_{10} (M)	log EC_{50} (M)	REP	
					based on PC_{10}	based on EC_{50}
dihydrotestosterone (reference compound, n = 50)	active	100	−10.3	−9.6	1.00	1.00
levonorgestrel (n = 3)	active	59	−9.4	−8.9	0.14	0.22
gestodene (n = 3)	active	47	−9.2	−8.8	0.08	0.17
altrenogest (n = 3)	active	31	−8.9	−8.7	0.04	0.14
etonogestrel (n = 3)	active	55	−8.9	−8.4	0.05	0.07
medroxyprogesterone acetate (n = 3)	active	51	−8.6	−8.1	0.02	0.04
norethisterone (n = 3)	active	47	−8.3	−7.7	0.01	0.01
medroxyprogesterone (n = 3)	active	11	−6.8	−7.5	<0.01	0.01
megestrol acetate (n = 2)	non-active	9	NA	NA	NA	NA
cyproterone acetate (n = 2)	non-active	4	NA	NA	NA	NA
progesterone (n = 2)	non-active	4	NA	NA	NA	NA
dydrogesterone (n = 2)	non-active	<1	NA	NA	NA	NA

Cyproterone acetate, dydrogesterone, megestrol acetate, and progesterone did not produce response greater than PC_{10} and therefore they were designated as non-active. RPCmax = maximum response induced by the tested chemical. PC_{10} = positive control 10, the concentration of a compound eliciting effect equal to 10% induction caused by the reference compound dihydrotestosterone. EC_{50} = half-maximal effective concentration. REP = relative potency. NA = not analyzed.

Table 2
Anti-androgenic potencies of progestins, a progesterone antagonist and a selective progesterone receptor modulator in the anti-AR-CALUX assay.

Compound (number of replicates)	Active or non-active compound	RPCmax (%)	log PC_{20} (M)	log IC_{50} (M)	REP	
					based on PC_{20}	based on IC_{50}
flutamide (reference compound, n = 50)	active	4	−6.6	−6.2	1	1
megestrol acetate (n = 3)	active	24	−8.3	−8.0	52	62
chlormadinone acetate (n = 3)	active	15	−8.3	−7.9	49	48
cyproterone acetate (n = 3)	active	14	−8.3	−7.8	51	44
nomegestrol acetate (n = 3)	active	11	−8.3	−7.8	43	40
progesterone (n = 3)	active	15	−8.2	−7.7	38	34
medroxyprogesterone (n = 3)	active	19	−8.1	−7.7	30	30
ulipristal acetate (n = 3)	active	17	−7.9	−7.7	19	33
drospirenone (n = 3)	active	15	−7.7	−7.3	12	14
dydrogesterone (n = 3)	active	4	−7.7	−7.3	10	13
dienogest (n = 3)	active	19	−7.5	−7.3	8	12
mifepristone (n = 3)	active	12	−7.5	−7.2	7	9
altrenogest (n = 2)	non-active	>80	NA	NA	NA	NA

RPCmax = maximum response induced by the tested chemical. PC_{20} = positive control 20, the concentration of a compound eliciting effect equal to 20% attenuation of the signal of dihydrotestosterone by the reference compound flutamide. IC_{50} = half-maximal inhibitory concentration. REP = relative potency. NA = not analyzed.

Nevertheless, the interactions of various environmentally relevant progestins in mixtures still need to be elucidated by complementary *in vitro* and *in vivo* experiments.

3.5. In vitro reporter gene bioassays with water extracts

Androgenic and anti-androgenic activities were detected at most of our sampling localities with 75% and 85% frequency of occurrence, respectively. The greatest androgenic and anti-androgenic activities were observed in influents and reached up to 59 ng/L DHT EQs and 26 μg/L FLU EQs, respectively (Table 3). The androgenic and anti-androgenic activities determined fall within the range reported by other authors (Fang et al., 2012; Leusch et al., 2014; Roberts et al., 2015; Thomas et al., 2002; van der Linden et al., 2008; Zhao et al., 2011). Anti-androgenic activities are believed to pose even greater risk for aquatic environments than do androgenic activities (Weiss et al., 2009; Zhao et al., 2011), and they are more commonly found in environmental samples (Macikova et al., 2014b; Urbatzka et al., 2007). Recently, the effect-based trigger (EBT) value for anti-androgenic activity has been estimated at 25 μg/L flutamide EQs. Activities lying below this value are presumed to present a low risk for the environment. In our sampling campaign, the EBT for anti-androgenic activity was exceeded only in the influent of WWTP Prachatice but not in environmental samples (Table 3). In another study as one example, however, EBT was exceeded in 29% of surface water samples taken in the Netherlands (van der Oost et al., 2017).

3.6. Contribution of compounds to (anti-)androgenic activities

Androgenic activities in most of the samples were masked by anti-androgenic activities (Table 3). This phenomenon can occur in samples containing both receptor agonists and antagonists (Weiss et al., 2009) and has been reported also in other studies (Creusot et al., 2014; Ihara et al., 2014; König et al., 2017; Šauer et al., 2018; Weiss et al., 2009).

Androgenic activities in aquatic environments related to municipal wastewater discharges have been found mostly to be caused by natural steroid androgens (Jenkins et al., 2001; König et al., 2017; Thomas et al., 2002) but also with a contribution of synthetic androgenic steroids (Hashmi et al., 2018). Progestins were recently suggested to be one of the groups of compounds possibly contributing to androgenic activity in aquatic environments (Hashmi et al., 2018). In the present study, two progestins (gestodene and medroxyprogesterone acetate) accounted for 0.26–29% of androgenic activities measured within influents (Fig. 1), while neither progestins nor mifepristone and ulipristal acetate contributed to androgenic activities in effluents and surface waters (Table 3).

To date, the frequently occurring anti-androgenic activities in the aquatic environment have been explained by presence of the antimicrobial agent triclosan (Liscio et al., 2014; Ma et al., 2017;

Table 3
Predicted and measured (anti-)androgenic activities in aquatic environments and total contributions of target compounds to measured (anti-)androgenic activities.

Wastewater treatment plant	Sample	Androgenic activity (ng/L DHT EQs)		Anti-androgenic activity (μg/L FLU EQs)		Contribution to measured activities (%)	
		predicted	measured	predicted	measured	androgenic	anti-androgenic
Vodňany	inf	**3.9**	**47**	**1.4**	**5.2**	**8.3**	**27**
	eff	ND	**0.34**	**0.09**	**4.4**	ND	**2.1**
	usw	ND	**0.23**	**0.04**	**2.8**	ND	**1.6**
	dsw	ND	**0.18**	**0.04**	**3.4**	ND	**1.3**
Tábor	inf	**13**	**44**	**0.7**	**14**	**29**	**4.6**
	eff	ND	<LOQ	**0.02**	**8.9**	ND	**0.28**
	usw	ND	<LOQ	**0.03**	**5.5**	ND	**0.54**
	dsw	ND	<LOQ	**0.02**	**5.9**	ND	**0.31**
Prachatice	inf	**2.0**	**54**	**2.0**	**26**	**3.8**	**8.8**
	eff	ND	**0.42**	**0.02**	**3.5**	ND	**0.46**
	usw	ND	**0.08**	ND	<LOQ	ND	ND
	dsw	ND	**0.12**	**0.02**	**2.4**	ND	**0.77**
České Budějovice, 1st sampling campaign	inf	**3.7**	**59**	**2.0**	**18**	**6.4**	**11**
	eff	ND	**0.72**	**0.02**	**5.4**	ND	**0.40**
	usw	ND	<LOQ	**0.02**	<LOQ	ND	ND
	dsw	ND	**0.45**	**0.02**	**4.4**	ND	**0.46**
České Budějovice, 2nd sampling campaign	inf	**0.13**	**50**	**3.2**	**15**	**0.3**	**23**
	eff	ND	**0.79**	**0.04**	**4.8**	ND	**0.73**
	usw	ND	<LOQ	ND	<LOQ	ND	ND
	dsw	ND	**0.31**	**0.02**	**3.4**	ND	**0.48**

Abbreviations: ND = not determined, predicted (anti-)androgenic activities and contribution of compounds were not determined when concentrations of target compounds were below limit of quantification. LOQ = limit of quantification. DHT = dihydrotestosterone. FLU = flutamide. EQ = equivalent. inf = influent. eff = effluent. usw = upstream. dsw = downstream. Activities exceeding LOQs and estimated contributions are marked in bold; measured activities are expressed as median (n = 2–3).

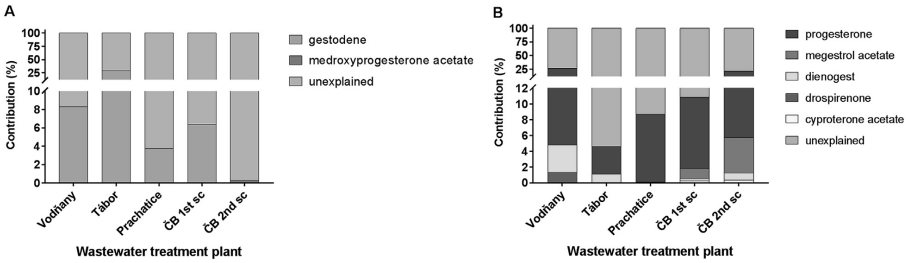


Fig. 1. Contributions of individual progestins to androgenic (A) and anti-androgenic (B) activities in influents to wastewater treatment plants. ČB = České Budějovice, sc = sampling campaign.

Rostkowski et al., 2011), dibutyl phthalate (Ma et al., 2017), phytoestrogens (König et al., 2017), and 4-methyl-7-diethylaminocoumarin (Muschket et al., 2018). Several studies have reported also co-occurrence of anti-androgenic activities and compounds with anti-androgenic potencies, such as pesticides, flame retardants, pharmaceuticals (Liscio et al., 2014), alkylphenols (Shi et al., 2016), naphthenic acids (Thomas et al., 2009), and polycyclic aromatic hydrocarbons (Weiss et al., 2011) in aquatic environments. On the other hand, no dominant causative anti-androgenic agents have been revealed in Taiwanese (Chen and Chou, 2016), Italian (Urbatzka et al., 2007), and French (Kinani et al., 2010) aquatic environments. To sum up, it appears that anti-androgenic activities are caused by various groups of chemicals in the aquatic environments. One group of possible contributors to these activities are progestins, mifepristone and ulipristal acetate, which possess considerable anti-androgenic activities (Table 2). A total of five anti-androgenic progestins (cyproterone acetate, dienogest, drospirenone, megestrol acetate, and progesterone) and a PR antagonist (mifepristone) were found in WWTP influents. They were responsible for 4.6–27% of anti-androgenic activities. Only two of these (megestrol acetate and progesterone), however, reached effluents, where they accounted for just 0.28–2.1% of anti-androgenic activities. Natural progestin progesterone was the only target compound found in surface water, contributing 0.34–1.6% of anti-androgenic activity. The contribution of individual progestins to (anti-)androgenic activities in wastewater is given in Fig. 1. Progesterone was by far the main contributor to anti-androgenic activities (up to 21%) among target substances. Of the synthetic progestins, megestrol acetate was the greatest contributor to anti-androgenic activities in influents (up to 4.5%) and the only one in effluents (0.25%). In the inter-week monitoring (1st and 2nd sampling campaigns in influent to the WWTP at České Budějovice), the extent to which compounds contributed to anti-androgenic activities changed only slightly (Fig. 1). Progestins seem to be important causative agents of anti-androgenic activities in sewage, but not in treated effluents and surface waters. This is logical considering their concentrations found there. If, however, a wastewater treatment process is ineffective in eliminating some strong androgenic (gestodene, etonogestrel, and levonorgestrel) or anti-androgenic (e.g., megestrol acetate, chlormadinone acetate or cyproterone acetate, and progesterone) progestins, and if they occur in units or tens of ng/L or

higher concentrations in effluents, then their contribution to anti-androgenic activities may be much greater due to their strong relative potencies (Tables 1 and 2). Indeed, the synthetic progestin cyproterone acetate recently found in treated wastewater in the Netherlands at a concentration of 20 ng/L was the main contributor (71%) to the detected anti-androgenic activities (Houtman et al., 2018). Moreover, metabolites of progestins might be present in an aquatic environment and also possess some anti-androgenic activity. This, too, is a topic deserving further attention.

4. Conclusions

The majority of samples in the present study exhibited relatively low (anti-)androgenic activities. Progestins occurred mostly in influents and accounted for as much as 29% of (anti-)androgenic activities. Most of them were eliminated to below LOQs during the wastewater treatment process, however, and thus did not contribute significantly to (anti-)androgenic activities in effluents and surface waters. In the light of our results, it seems unlikely that progestins are capable of inducing (anti-)androgenic activities that could pose a high risk to aquatic organisms in Czech surface waters. Our results of joint action testing indicate additive effect of individual (anti-)androgenic target compounds in mixtures. Therefore using the REPs of progestins obtained in our study, it will be possible to estimate (anti-)androgenic activity caused by progestins just on the basis of the results from chemical analysis (concentration of progestins) in future studies.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects 'CENAKVA' (No. CZ.1.05/2.1.00/01.0024), 'CENAKVA II' (No. LO1205 under the NPU I program), project of the Czech Science Foundation (No. 16-09709Y), and GAJU (No. 012/2016/Z and No. 056/2017/Z). We would like to thank Jiří Stara, Kateřina Tebichová, Jindřich Procházka, and Petr Maroš (ČEVAK, a.s.) for providing water samples, as well as Ilona Prokopová, Petra Nováková, and Jitka Kolářová for technical support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.06.104.

References

Africander, D., Verhoog, N., Hapgood, J.P., 2011. Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. Steroids 76, 636–652.

Attardi, B.J., Burgenson, J., Hild, S.A., Reel, J.R., 2004. In vitro antiprogestational/antiglucocorticoid activity and progestin and glucocorticoid receptor binding of the putative metabolites and snythetic derivatives of CDB-2914, CDB-4124, and mifepristone. J. Steroid Biochem. Mol. Biol. 88, 277–288.

Bain, P.A., Kumar, A., Ogino, Y., Iguchi, T., 2015. Nortestosterone-derived synthetic progestogens do not activate the progestogen receptor of Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) but are potent agonists of androgen receptors alpha and beta. Aquat. Toxicol. 163, 97–101.

Bain, P.A., Williams, M., Kumar, A., 2014. Assessment of multiple hormonal activities in wastewater at different stages of treatment. Environ. Toxicol. Chem. 33, 2297–2307.

Besse, J.-P., Garric, J., 2009. Progestagens for human use, exposure and hazard assessment for the aquatic environment. Environ. Pollut. 157, 3485–3494.

Boehler, S., Strecker, R., Heinrich, P., Prochazka, E., Northcott, G.L., Ataria, J.M., Leusch, F.D.L., Braunbeck, T., Tremblay, L.A., 2017. Assessment of urban stream sediment pollutants entering estuaries using chemical analysis and multiple bioassays to characterise biological activities. Sci. Total Environ. 593–594, 498–507.

Brown, V.M., 1968. The calculation of the acute toxicity of mixtures of poisons to rainbow trout. Water Res. 2, 723–733.

Chen, K.Y., Chou, P.H., 2016. Detection of endocrine active substances in the aquatic environment in southern Taiwan using bioassays and LC-MS/MS. Chemosphere 152, 214–220.

Chen, H.-X., Zhao, Y.-G., Qiu, Q.-L., Zhu, Y., Min, J.-Q., Jin, M.-C., 2017. A fast and high throughput LC-MS/MS method for the determination of 58 human and veterinary drugs in river water. Anal. Methods 9, 4228–4233.

Creusot, N., Aït-Aïssa, S., Tapie, N., Pardon, P., Brion, F., Sanchez, W., Thybaud, E., Porcher, J.-M., Budzinski, H., 2014. Identification of synthetic steroids in river water downstream from pharmaceutical manufacture discharges based on a bioanalytical approach and passive sampling. Environ. Sci. Technol. 48, 3649–3657.

Ellestad, L.E., Cardon, M., Chambers, I.G., Farmer, J.L., Hartig, P., Stevens, K., Villeneuve, D.L., Wilson, V.S., Orlando, E.F., 2014. Environmental gestagens activate fathead minnow (*Pimephales promelas*) nuclear progesterone and androgen receptors *in vitro*. Environ. Sci. Technol. 48, 8179–8187.

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J.Y.M., van der Burg, B., van der Linden, S.C., Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X., Leusch, F.D.L., 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. Environ. Sci. Technol. 48, 1940–1956.

Fan, Z.L., Wu, S.M., Chang, H., Hu, J.Y., 2011. Behaviors of glucocorticoids, androgens and progestogens in a municipal sewage treatment plant: comparison to estrogens. Environ. Sci. Technol. 45, 2725–2733.

Fang, Y.-X., Ying, G.-G., Zhao, J.-L., Chen, F., Liu, S., Zhang, L.-J., Yang, B., 2012. Assessment of hormonal activities and genotoxicity of industrial effluents using in vitro bioassays combined with chemical analysis. Environ. Toxicol. Chem. 31, 1273–1282.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environ. Int. 84, 115–130.

Fernandes, D., Pujol, S., Aceña, J., Perez, S., Barceló, D., Porte, C., 2014. The *in vitro* interference of synthetic progestogens with carp steroidogenic enzymes. Aquat. Toxicol. 155, 314–321.

Golovko, O., Šauer, P., Fedorova, G., Kocour Kroupová, H., Grabic, R., 2018. Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS. Sci. Total Environ. 621, 1066–1073.

Gray, L.E., Ostby, J., Furr, J., Wolf, C.J., Lambright, C., Parks, L., Veeramachaneni, D.N., Wilson, V., Price, M., Hotchkiss, A., Orlando, E., Guillette, L., 2001. Effects of environmental antiandrogens on reproductive development in experimental animals. Hum. Reprod. Update 7, 248–264.

Hashmi, M.A.K., Escher, B.I., Krauss, M., Teodorovic, I., Brack, W., 2018. Effect-directed analysis (EDA) of Danube River water sample receiving untreated municipal wastewater from Novi Sad, Serbia. Sci. Total Environ. 624, 1072–1081.

Houtman, C.J., Sterk, S.S., van de Heijning, M.P.M., Brouwer, A., Stephany, R.W., van der Burg, B., Sonneveld, E., 2009. Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays. Anal. Chim. Acta 637, 247–258.

Houtman, C.J., ten Broek, R., Brouwer, A., 2018. Steroid hormonal activities, culprit natural and synthetic hormones and other emerging contaminants in waste water measured using bioassays and UPLC-tQ-MS. Sci. Total Environ. 630, 1492–1501.

Howell, W.M., Black, D.A., Bortone, S.A., 1980. Abnormal expression of secondary sex characteristics in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally-induced masculinisation. Copeia 4, 676–681.

Hua, J., Han, J., Guo, Y., Zhou, B., 2015. The progestin levonorgestrel affects sex differentiation in zebrafish at environmentally relevant concentrations. Aquat. Toxicol. 166, 1–9.

Huysman, S., Van Meulebroek, L., Vanryckeghem, F., Van Langenhove, H., Demeestere, K., Vanhaecke, L., 2017. Development and validation of an ultra-high performance liquid chromatographic high resolution Q-Orbitrap mass spectrometric method for the simultaneous determination of steroidal endocrine disrupting compounds in aquatic matrices. Anal. Chim. Acta 984, 140–150.

Ihara, M., Ihara, M.O., Kumar, V., Narumiya, M., Hanamoto, S., Nakada, N., Yamashita, N., Miyagawa, S., Iguchi, T., Tanaka, H., 2014. Co-occurrence of estrogenic and antiestrogenic activities in wastewater: quantitative evaluation of balance by in vitro ERα reporter gene assay and chemical analysis. Environ. Sci. Technol. 48, 6366–6373.

Jenkins, R., Angus, R.A., McNatt, H., Howell, W.M., Kemppainen, J.A., Kirk, M., Wilson, E.M., 2001. Identification of androstenedione in a river containing paper mill effluent. Environ. Toxicol. Chem. 20, 1325–1331.

Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C., 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. Environ. Health Perspect. 117, 797–802.

Kelce, W.R., Gray, L.E., Wilson, E.M., 1998. Antiandrogens as environmental endocrine disruptors. Reprod. Fertil. Dev. 10, 105–111.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J.M., Aït-Aïssa, S., 2010. Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environ. Pollut. 158, 74–83.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater

contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.
König, M., Escher, B.I., Neale, P.A., Krauss, M., Hilscherová, K., Novák, J., Teodorović, I., Schulze, T., Seidensticker, S., Hashmi, M.A.K., Ahlheim, J., Brack, W., 2017. Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis. Environ. Pollut. 220, 1220–1230.
Kumar, V., Johnson, A.C., Trubiroha, A., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kocour Kroupová, H., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49, 2625–2638.
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, 997–1003.
Leusch, F.D.L., Khan, S.J., Laingam, S., Prochazka, E., Froscio, S., Trinh, T., Chapman, H.F., Humpage, A., 2014. Assessment of the application of bioanalytical tools as surrogate measure of chemical contaminants in recycled water. Water Res. 49, 300–315.
Leusch, F.D.L., Neale, P.A., Hebert, A., Scheurer, M., Schriks, M.C.M., 2017. Analysis of the sensitivity of *in vitro* bioassays for androgenic, progestagenic, glucocorticoid, thyroid and estrogenic activity: suitability for drinking and environmental waters. Environ. Int. 99, 120–130.
Liscio, C., Abdul-Sada, A., Al-Salhi, R., Ramsey, M.H., Hill, E.M., 2014. Methodology for profiling anti-androgen mixtures in river water using multiple passive samplers and bioassay-directed analyses. Water Res. 57, 258–269.
Liu, S., Chen, H., Zhou, G.-J., Liu, S.-S., Yue, W.-Z., Yu, S., Sun, K.-F., Cheng, H., Ying, G.-G., Xu, X.-R., 2015. Occurrence, source analysis and risk assessment of androgens, glucocorticoids and progestagens in the Hailing Bay, South China Sea. Sci. Total Environ 536, 99–107.
Liu, S.S., Ying, G.G., Liu, S., Lai, H.J., Chen, Z.F., Pan, C.G., Zhao, J.-L., Chen, J., 2014. Analysis of 21 progestagens in various matrices by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with diverse sample pretreatment. Anal. Bioanal. Chem. 406, 7299–7311.
Liu, X.J., Zhang, J., Yin, J., Duan, H.J., Wu, Y.N., Shao, B., 2010. Analysis of hormone antagonists in clinical and municipal wastewater by isotopic dilution liquid chromatography tandem mass spectrometry. Anal. Bioanal. Chem. 396, 2977–2985.
Loewe, S., Muischnek, H., 1926. Über kombinationswirkungen. Arch. Exp. Pathol. Pharmakol. 114, 313–326.
Ma, D.H., Chen, L.J., Liu, R., 2017. Removal of novel antiandrogens identified in biological effluents of domestic wastewater by activated carbon. Sci. Total Environ. 595, 702–710.
Macikova, P., Groh, K.J., Ammann, A.A., Schirmer, K., Suter, M.J.F., 2014a. Endocrine disrupting compounds affecting corticosteroid signaling pathways in Czech and Swiss waters: potential impact on fish. Environ. Sci. Technol. 48, 12902–12911.
Macikova, P., Kalabova, T., Klanova, J., Kukucka, P., Giesy, J.P., Hilscherova, K., 2014b. Longer-term and short-term variability in pollution of fluvial sediments by dioxin-like and endocrine disruptive compounds. Environ. Sci. Pollut. Res. 21, 5007–5022.
Muschket, M., Di Paolo, C., Tindall, A.J., Touak, G., Phan, A., Krauss, M., Kirchner, K., Seiler, T.B., Hollert, H., Brack, W., 2018. Identification of unknown antiandrogenic compounds in surface waters by effect-directed analysis (EDA) using a parallel fractionation approach. Environ. Sci. Technol. 52, 288–297.
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 No. 455: Performance-based Test Guideline for Stably Transfected Transactivation *in Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Available: http://www.oecd-ilibrary.org/environment/test-no-455-draft-performance-based-test-guideline-for-stably-transfected-transactivation-in-vitro-assays-to-detect-estrogen-receptor-agonists-and-antagonists_9789264243040-en. (Accessed 21 February 2018).
Ojoghoro, J.O., Chaudhary, A.J., Campo, P., Sumpter, J.P., Scrimshaw, M.D., 2017. Progesterone potentially degrades to potent androgens in surface waters. Sci. Total Environ. 579, 1876–1884.
Parks, L.G., Lambright, C.S., Orlando, E.F., Guillette Jr., L.J., Ankley, G.T., Gray Jr., L.E., 2001. Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenolloway River water is associated with androgen receptor agonist activity. Toxicol. Sci. 62, 257–267.
Peng, F.-Q., Ying, G.-G., Yang, B., Liu, S., Lai, H.-J., Liu, Y.-S., Chen, Z.-F., Zhou, G.-J., 2014. Biotransformation of progesterone and norgestrel by two freshwater microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*): transformation kinetics and products identification. Chemosphere 95, 581–588.
Roberts, J., Bain, P.A., Kumar, A., Hepplewhite, C., Ellis, D.J., Christy, A.G., Beavis, S.G., 2015. Tracking multiple modes of endocrine activity in Australia's largest inland sewage treatment plant and effluent- receiving environment using a panel of *in vitro* bioassays. Environ. Toxicol. Chem. 34, 2271–2281.
Rossier, N.M., Chew, G., Zhang, K., Riva, F., Fent, K., 2016. Activity of binary mixtures of drospirenone with progesterone and 17α-ethinylestradiol in vitro and in vivo. Aquat. Toxicol. 174, 109–122.
Rostkowski, P., Horwood, J., Shears, J.A., Lange, A., Oladapo, F.O., Besselink, H.T., Tyler, C.R., Hill, E.M., 2011. Bioassay-directed identification of novel antiandrogenic compounds in bile of fish exposed to wastewater effluents. Environ. Sci. Technol. 45, 10660–10667.
Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environ. Sci. Technol. 47, 2077–2084.
Sangster, J.L., Ali, J.M., Snow, D.D., Kolok, A.S., Bertelt-Hunt, S.L., 2016. Bioavailability and fate of sediment-associated progesterone in aquatic systems. Environ. Sci. Technol. 50, 4027–4036.
Schindler, A.E., Campagnoli, C., Druckmann, R., Huber, J., Pasqualini, J.R., Schweppe, K.W., Thijssen, J.H.H., 2008. Reprint of classification and pharmacology of progestins. Maturitas 61, 171–180.
Schoonen, W.G.E.J., Deckers, G.H., de Gooijer, M.E., de Ries, R., Kloosterboer, H.J., 2000. Hormonal properties of norethisterone, 7α-methyl-norethisterone and their derivatives. J. Steroid Biochem. Mol. Biol. 74, 213–222.
Shen, X., Chang, H., Sun, D., Wang, L., Wu, F., 2018. Trace analysis of 61 natural and synthetic progestins in river water and sewage effluents by ultra-high performance liquid chromatography-tandem mass spectrometry. Water Res. 133, 142–152.
Shi, W., Deng, D., Wang, Y., Hu, G., Guo, J., Zhang, X., Wang, X., Giesy, J.P., Yu, H., Wang, Z., 2016. Causes of endocrine disrupting potencies in surface water in East China. Chemosphere 144, 1435–1442.
Siegenthaler, P.F., Bain, P., Riva, F., Fent, K., 2017. Effects of antiandrogenic progestins, chlormadinone and cyproterone acetate, and the estrogen 17α-ethinylestradiol (EE2), and their mixtures: transactivation with human and rainbowfish hormone receptors and transcriptional effects in zebrafish (*Danio rerio*) eleutheroembryos. Aquat. Toxicol. 182, 142–162.
Sitruk-Ware, R., 2004. Pharmacological profile of progestins. Maturitas 47, 277–283.
Sohoni, P., Sumpter, J.P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158, 327–339.
Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., van der Burg, B., 2005. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. Toxicol. Sci. 83, 136–148.
Sonneveld, E., Pieterse, B., Schoonen, W.G., van der Burg, B., 2011. Validation of *in vitro* screening models for progestagenic activities: inter-assay comparison and correlation wit *in vivo* activity in rabbits. Toxicol. Vitro 24, 545–554.
Stanczyk, F.Z., 2003. All progestins are not created equal. Steroids 68, 879–890.
Sumpter, J.P., 2005. Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochim. Hydrobiol. 33, 9–16.
Svensson, J., Fick, J., Brandt, I., Brunström, B., 2013. The synthetic progestin levonorgestrel is a potent androgen in the three-spined stickleback (*Gasterosteus aculeatus*). Environ. Sci. Technol. 47, 2043–2051.
Svensson, J., Mustafa, A., Fick, J., Schmitz, M., Brunstrom, B., 2016. Developmental exposure to progestins causes male bias and precocious puberty in zebrafish (*Danio rerio*). Aquat. Toxicol. 177, 316–323.
Šauer, P., Stará, A., Golovko, O., Valentová, O., Bořík, A., Grabic, R., Kocour Kroupová, H., 2018. Two synthetic progestins and natural progesterone are responsible for most of the progestagenic activities in municipal wastewater treatment plant effluents in the Czech and Slovak republics. Water Res. 137, 64–71.
Thomas, K.V., Hurst, M.R., Matthiessen, P., McHugh, M., Smith, A., Waldock, M.J., 2002. An assessment of *in vitro* androgenic activity and the identification of environmental androgens in United Kingdom estuaries. Environ. Toxicol. Chem. 21, 1456–1461.
Thomas, K.V., Langford, K., Petersen, K., Smith, A.J., Tollefsen, K.E., 2009. Effect-directed identification of naphthenic acids as important *in vitro* xenoestrogens and anti-androgens in North Sea offshore produced water discharges. Environ. Sci. Technol. 43, 8066–8071.
Urbatzka, R., van Cauwenberge, A., Maggioni, S., Vigano, L., Mandich, A., Benfenati, E., Lutz, I., Kloas, W., 2007. Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. Chemosphere 67, 1080–1087.
van der Burg, B., Winter, R., Man, H.Y., Vangenechten, C., Berckmans, P., Weimer, M., Witters, H., van der Linden, S., 2010. Optimization and prevalidation of the *in vitro* AR CALUX method to test androgenic and antiandrogenic activity of compounds. Reprod. Toxicol. 30, 18–24.
van der Linden, S.C., Heringa, M.B., Man, H.Y., Sonneveld, E., Puijker, L.M., Brouwer, A., van der Burg, B., 2008. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environ. Sci. Technol. 42, 5814–5820.
van der Oost, R., Sileno, G., Janse, T., Nguyen, M.T., Besselink, H., Brouwer, A., 2017. SIMONI (Smart Integrated Monitoring) as a novel bioanalytical strategy for water quality assessment: part II-field feasibility survey. Environ. Toxicol. Chem. 36, 2400–2416.
Villeneuve, D.L., Blakenship, A.L., Giesy, J.P., 2000. Derivation and application of relative potency estimates based on *in vitro* bioassay results. Environ. Toxicol. Chem. 19, 2835–2843.
Vulliet, E., Wiest, L., Baudot, R., Grenier-Loustalot, M.F., 2008. Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr. A 1210, 84–91.
Weiss, J.M., Hamers, T., Thomas, K.V., van der Linden, S., Leonards, P.E.G., Lamoree, M.H., 2009. Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. Anal. Bioanal. Chem. 394, 1385–1397.
Weiss, J.M., Simon, E., Stroomberg, G.J., de Boer, R., de Boer, J., van der Linden, S.C., Leonards, P.E.G., Lamoree, M.H., 2011. Identification strategy for unknown

pollutants using high-resolution mass spectrometry: androgen-disrupting compounds identified through effect-directed analysis. Anal. Bioanal. Chem. 400, 3141–3149.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Lange, R., 2009. Effects of synthetic gestagens on fish reproduction. Environ. Toxicol. Chem. 28, 2663–2670.

Zhao, J.L., Ying, G.G., Yang, B., Liu, S., Zhou, L.J., Chen, Z.F., Lai, H.-J., 2011. Screening of multiple hormonal activities in surface water and sediment from the Pearl River system, South China, using effect-directed *in vitro* bioassays. Environ. Toxicol. Chem. 30, 2208–2215.

Zucchi, S., Castiglioni, S., Fent, K., 2012. Progestins and anti-progestins affect gene expression in early development in zebrafish (*Danio rerio*) at environmental concentrations. Environ. Sci. Technol. 46, 5183–5192.

CHAPTER 5

SYNTHETIC PROGESTIN ETONOGESTREL NEGATIVELY AFFECTS MATING BEHAVIOR AND REPRODUCTION IN ENDLER'S GUPPIES (*POECILIA WINGEI*)

Steinbach, C., Císař, P., Šauer, P., Klicnarová, J., Schmidt-Posthaus, H., Golovko, O., Kocour Kroupová, H., 2019. Synthetic progestin etonogestrel negatively affects mating behavior and reproduction in Endler's guppies (*Poecilia wingei*). Science of the Total Environment 663, 206–215.

According to the publishing agreement between the authors and publisher, it is allowed to include the paper in this Ph.D. thesis

My share on this work was about 10%.

Science of the Total Environment 663 (2019) 206–215

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Synthetic progestin etonogestrel negatively affects mating behavior and reproduction in Endler's guppies (*Poecilia wingei*)

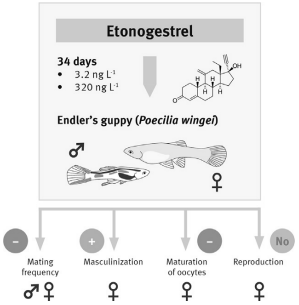
Christoph Steinbach [a,*], Petr Císař [a], Pavel Šauer [a], Jana Klicnarová [b], Heike Schmidt - Posthaus [c], Oksana Golovko [a], Hana Kocour Kroupová [a]

[a] *Faculty of Fisheries and Protection of Waters, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in České Budějovice, Zátiší 728/II, 389 25, Vodňany, Czech Republic*
[b] *Faculty of Economics, University of South Bohemia in České Budějovice, Studentská 13, 370 05 České Budějovice, Czech Republic*
[c] *Centre for Fish and Wildlife Health, Department of Infectious Diseases and Pathobiology, University of Bern, Länggass-Strasse 122, 3001 Bern, Switzerland*

HIGHLIGHTS

- This is the first report on the effects in fish of etonogestrel exposure.
- Masculinization was seen in females exposed to the highest concentration.
- All exposed females were unable to reproduce.
- Exposure reduced males' mating activity but not their reproductive success.
- Alterations in reproductive behavior appeared to be sensitive endpoints.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 November 2018
Received in revised form 14 January 2019
Accepted 21 January 2019
Available online 24 January 2019

Editor: Henner Hollert

Keywords:
Fertility
Gonads
Mating

ABSTRACT

High rates of progestins consumption in the form of active ingredients in women's oral contraceptives and other hormonal preparations may lead to their increased concentrations in aquatic environments and subsequent harmful effect on fish reproduction. The objective of the present study was to assess the effect of etonogestrel, a third-generation synthetic progestin, on the reproductive behavior, fertility, gonads histology, and secondary sexual characteristics of male and female Endler's guppies (*Poecilia wingei*). Fish were subjected for 34 days to two concentrations of etonogestrel, including one possibly environmentally relevant (3.2 ng L^{-1}) and one sublethal (320 ng L^{-1}) concentration. A mating behavior study was subsequently conducted and revealed that the treatment with etonogestrel significantly reduced mating frequency in the exposed fish compared to controls. All the exposed females were unable to reproduce. In addition, female fish exposed to the highest level of etonogestrel were masculinized, as their anal fins and body coloration showed patterns similar to those of male fish. Etonogestrel-exposed females also had fewer developed oocytes. In conclusion, the low etonogestrel

* Corresponding author.
E-mail address: steinbach@frov.jcu.cz (C. Steinbach).

https://doi.org/10.1016/j.scitotenv.2019.01.276

Sexual dimorphism
Steroid

concentration (3.2 ng L^{-1}) led to a reduction of mating activity in males without effect on their reproductive success, but it completely inhibited reproduction in females. Exposure to etonogestrel clearly has more severe consequences for females than males.

1. Introduction

Consumption of synthetic progestins as active ingredients in women's oral contraceptives and other hormonal preparations is relatively high (Kumar et al., 2015). Because these progestins are incompletely removed during wastewater treatment processes, their concentrations in aquatic environments may be as great as tens of nanograms per liter (Al-Odaini et al., 2010; Houtman et al., 2018). Such levels are of concern for inhabitants of aquatic ecosystems because they exceed concentrations that adversely affect fish and amphibians under laboratory conditions (Fent, 2015; Frankel et al., 2016a; Frankel et al., 2016b; Hoffmann and Kloas, 2012; Hou et al., 2018a; Kumar et al., 2015; Lorenz et al., 2011a; Lorenz et al., 2011b).

The synthetic progestin etonogestrel (13β-ethyl-17β-hydroxy-11-methylene-18, 19-dinor-17α-pregn-4-en-20-yn-3-one) is a testosterone derivative and the biologically active metabolite of desogestrel (Croxatto, 2002). Although etonogestrel and its precursor desogestrel have not been detected to date in waste and surface waters, there in fact have been only few attempts to analyze their levels (Golovko et al., 2018). Due to its increasing application, etonogestrel is suspected to be of environmental relevance. For instance, the use of single-rod, etonogestrel-releasing subdermal implants as a form of contraceptive increased by 50% in the U.S.A. between the years 2009 and 2012 (Kavanaugh et al., 2015; Odom et al., 2017). In the Czech Republic, the application of etonogestrel increased by approximately 45% between the years 2011 and 2015 and since that time has remained at almost the same level (State Institute for Drug Control, http://www.sukl.eu/2018) (Fig. S1). In addition, the predicted critical environmental concentration of etonogestrel is very low. A level of just 1.6 ng L^{-1} in water would result in a fish blood plasma concentration equal to the human therapeutic blood plasma level (Fick et al., 2010).

In mammals, progesterone receptor is the main target for progestins, and etonogestrel is characterized by very strong agonistic properties that exceed those of the natural ligand progesterone (Kumar et al., 2015; Sauer et al., 2018). In general, progesterone is involved in regulation of the hypothalamic–pituitary–gonadal axis and it regulates development, differentiation, and normal functioning of the female reproductive tract (Wagenfeld et al., 2016). It also triggers norepinephrine release from hypothalamus in order to mediate hormone-dependent sexual behavior in humans (Graham and Clarke, 1997). In addition to strong progestagenic activity, etonogestrel exerts androgenic, anti-estrogenic, and anti-gonadotropic activities in mammals (Kumar et al., 2015).

Several studies to date have documented adverse effects of progestins on the reproduction and development of fish and frogs (Hou et al., 2017; Kumar et al., 2015), but the exact mode of action is not fully understood (Hou et al., 2018a; Hou et al., 2018b). *In vitro* studies have demonstrated that progestins mostly do not bind to fish progesterone receptor, but some of them, mainly testosterone derivatives, including etonogestrel, possess high affinity to fish androgen receptors (Bain et al., 2015; Ellestad et al., 2014).

In ecotoxicological studies on aquatic organisms, behavioral endpoints are considered to be more sensitive than morphological, reproductive, and developmental parameters (Melvin and Wilson, 2013). Courtship and reproductive behaviors also have been identified as important endpoints for the study of endocrine disruption (Frankel et al., 2016a; Sebire et al., 2008; Zeilinger et al., 2009). Poeciliids are commonly used as model organisms in toxicological studies (OECD, 1992; Wester and Vos, 1994) and behavioral biological research (Schlupp, 2018a; Schlupp, 2018b). These viviparous fish have elaborate and well-defined courtship and mating behaviors (Pyke, 2005). A practical advantage is that they are able to breed year-round under laboratory conditions and with a short reproductive period (Baatrup and Junge, 2001). Moreover, morphological and histological responses and alterations in body color and mating behavior are readily observed in poeciliids exposed to endocrine-disrupting compounds (Angus et al., 2001; Hou et al., 2018b). In recent years, Endler's guppies (*Poecilia wingei*) increasingly have been used as an experimental animal for studying mate choice, social reproductive behavior, as well as activity and shoaling anxiety responses to new environments (Olsen et al., 2014; Sommer and Olsen, 2016). Only a few studies have thus far described the effects of synthetic progestins on the mating behavior of fish (Frankel et al., 2016b; Hou et al., 2018a; Kumar et al., 2015). Hou et al. (2018b) reported that long-term exposure of western mosquitofish (*Gambusia affinis*) to the testosterone derivative norgestrel at concentrations of 400 and 4000 ng L^{-1} led to reduced frequencies and durations of mating behavior in males and caused masculinization of females. Moreover, they observed impairment in females' reproduction. Similarly, Frankel et al. (2016b) showed reduced mating frequency in the eastern mosquitofish after eight-day exposure to 100 ng L^{-1} of levonorgestrel. Runnalls et al. (2013) reported that desogestrel, the precursor of etonogestrel, decreased egg production and caused masculinization of female fathead minnow (*Pimephales promelas*) after 21-day exposure at a high level (10,000 ng L^{-1}). Mating behavior was not evaluated in these studies, and, to the best of our knowledge, there is no report to date describing the effects of etonogestrel on fish.

The main objectives of the present study were to assess effects of sublethal etonogestrel concentrations on the different aspects of reproduction and morphology in Endler's guppies, namely (1) mate choice and mating behavior, (2) fertility, (3) secondary sexual characteristics, and (4) gonad histology.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile (LiChrosolv® Hypergrade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, South Korea). Analytical standards were of high purity (mostly 98%). Etonogestrel [(13β-ethyl-17β-hydroxy-11-methylene-18, 19-dinor-17α-pregn-4-en-20-yn-3-one; CAS no.: 54048-10-1) was purchased from Sigma Aldrich (Steinheim, Germany). The internal standard drospirenone-13C6 was obtained from Toronto Research Chemicals, Inc. (Toronto Research Chemicals, ON, Canada). Absolute ethanol (100%) and xylene (mixture of isomers) were purchased from Penta (Czech Republic). Decalcification solution 1 (formic acid solution) was obtained from VWR (Czech Republic). Ten percent neutral buffered formalin, Mayer's hematoxylin, eosin, and histological ethanol (99.7–99.9%) were obtained from Diapath (Czech Republic).

Etonogestrel was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution at a concentration of 320 mg L^{-1}.

2.2. Experimental design

Juvenile Endler's guppies were obtained from a local commercial breeder (Jindřichův Hradec, Czech Republic). Juvenile fish were kept and raised to maturity under conditions as described by Houde (1987). Briefly, the fish were acclimated to laboratory conditions for

3 months before beginning the exposure. During the acclimation period, males and females were raised separately to ensure virginity. The development of the fish inclusive of gender and virginity was checked regularly. Males were identified visually according to the developing gonopodium and body color. Pregnant females were identified based on the description of Norazmi-Lokman et al. (2016) and excluded from the experiment. Both genders were visually exposed to the opposite sex in the neighboring aquaria during the rearing. The fish were kept in dechlorinated tap water with added salt (NaCl, 1 g per 100 L) and calcium chloride (0.1 g per 100 L). These conditions were applied for all experiments. The temperature was kept constant at 25.0 ± 1.4 °C. Photoperiod (light:dark) was 14:10 h. Dissolved oxygen concentration and pH were 8.5 ± 0.5 mg L^{-1} and 7.9 ± 0.5, respectively. These parameters were measured every second day and at every experimental trial. During the acclimation and experimental periods, the fish were fed ad libitum with freshly hatched brine shrimp (*Artemia salina*) and a commercial preparation of freshly hatched brine shrimp (Artemia Sanders Premium, Sanders, USA) at 1% of their body weight per day. The fish were not fed on the sampling days.

Virgin, sexually mature fish with minimum length of 17 mm were selected (Herdman et al., 2004). Only colored males with fully developed gonopodium were used. The adult fish were randomly distributed into eighteen 100 L aquaria, each containing 22 fish. Males and females were kept separately. The fish were acclimated for 20 days to the experimental conditions. All sides of the aquaria were covered by polystyrene to prevent fish from being disturbed.

One hundred liters of the etonogestrel solution and of the water in control tanks were renewed daily. The fish were exposed to etonogestrel at environmentally relevant (E1: 3.2 ng L^{-1}) and sublethal (E2: 320 ng L^{-1}) concentrations under semi-static conditions for 34 days. DMSO with final concentration of 0.0005% was added to treatment groups to facilitate the dissolving of etonogestrel. Two control groups were included. One control group, designated C, was contained in dilution water only. The second, designated SC for solvent control, was contained in water with added dimethyl sulfoxide at the same concentration as in the treatment groups. All experimental and control trials were duplicated. The SC control was triplicated. Mortality was recorded during the acclimation and experimental periods. This study was performed in accordance with the principals of the EU-harmonized Animal Welfare Act of the Czech Republic.

2.3. Etonogestrel analysis

Individual stock solutions of the standards were prepared at 1 mg mL^{-1} concentration in methanol and stored at −20 °C. A spiking mixture of drospirenone-13C6 was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg mL^{-1} for each compound. Working standard mixtures (0.01–10 μg mL^{-1}) of the native compound were prepared in methanol.

Water samples were collected from aquaria into 1 L amber glass bottles. The concentration of etonogestrel was measured immediately after water exchange (0 h) and 24 h post-exchange to check for its concentration and stability. The collected samples were stored at +4 °C in darkness until extraction, which was carried out within 24 h. Water samples were prepared according to a protocol developed for solid-phase extraction (Golovko et al., 2018).

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used for analysis of water extracts. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) was used for the chromatographic separation of etonogestrel.

Ultrapure water and methanol were used as the mobile phases. The elution conditions were programmed as follows: 350 mL min^{-1} 30% methanol in water for 1 min, isocratically followed by a gradient change to 20/80 water/methanol at a flow of 400 mL min^{-1} for 8 min and a final gradient change to 100% of methanol at a flow of 400 mL min^{-1} for 10 min. These parameters were held for 2 min and then changed to the starting conditions and held for 1.5 min to equilibrate the column for the next run.

Atmospheric pressure photoionization (APPI) in positive mode was used to ionize target compounds. The instrument was calibrated daily (mass calibration) in positive modes using a standard procedure proposed by Thermo Scientific.

The atmospheric pressure chemical ionization/atmospheric pressure photoionization (APCI/APPI) parameters were set as follows: capillary temperature (300 °C), vaporizer temperature (300 °C), sheath gas pressure (40 arbitrary units), auxiliary gas (15 arbitrary units), and discharge current (4 μA in positive ionization mode). A UV krypton lamp (10 eV) was used in the source.

The analytical method for analyzing a wide range of progestins, including etonogestrel, in different water matrices using solid-phase extraction followed by liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) had previously been validated for linearity, repeatability, limit of quantification (LOQ), and trueness (Golovko et al., 2018).

The linearity of the calibration curve was tested in the range from 0.1 ng L^{-1} to 500 ng L^{-1}. Calibration curves were measured at the beginning and at the end of the sequence to check instrument stability. The calibration was prepared in water/MeOH (1/1).

Prior to extraction for each aquarium, a procedural blank (demineralized water) was extracted and analyzed to distinguish between positive detections and potential sample contamination. Each sample was analyzed simultaneously with matrix matching standards for the determination of matrix effects. Data analysis and calculation were performed using TraceFinder 3.3 software (Thermo Fisher Scientific).

2.4. Behavioral studies

Before and at the end of the exposure period, such activities of the fish as grouping, following, site preference, and swimming were studied (2.4.1). After 34 days of exposure, the behavioral tests, namely the choice (2.4.2) and mating tests, were conducted within 2 days. To determine fertility, females from the mating test (2.4.3) were kept in isolation for 90 days (Fig. 1).

2.4.1. Analysis of activity patterns

Behavioral parameters (grouping, following, site preference, and swimming activity) were recorded before and at the end of the exposure period. The behavior patterns were recorded over a period of 3 days with a monitoring system based on 3D cameras (Xbox One Kinect Sensor V2) placed above the aquaria. After 5 min of adaptation, the behavior was recorded for 40 min. Every aquarium was recorded 3 times and the experimental groups were recorded in parallel. The monitoring system detects the individual fish in 3D space and saves its position for later processing.

The recordings were later automatically quantified for following, grouping, and activity behaviors by software implemented in-house for fish 3D position processing and that had been developed by Saberioon and Cisar (2016). These parameters were defined based on the description of Kane et al. (2005) and with modifications as described below. Grouping of the fish (grouping behavior) was identified when >70% of the fish were present within a 40 cm radius. Following behavior was determined based on trajectories of movement, when the movement directions were not exceeding a 50° angle and fish were in a swarm-like pattern. Swimming activity is represented by the average velocity of the fish per second. This parameter was calculated frame by

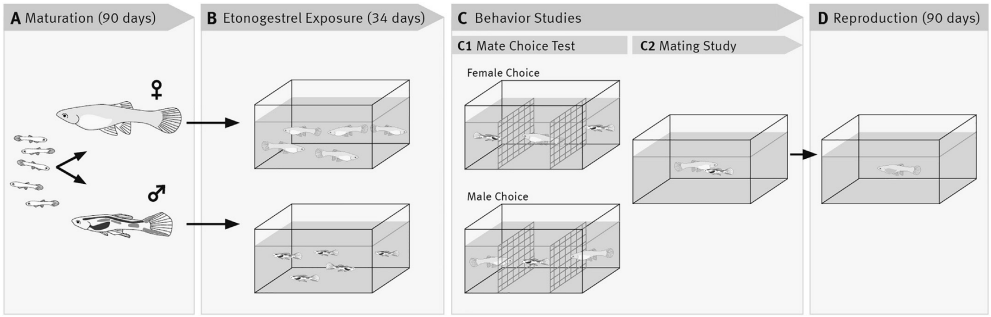


Fig. 1. Schematic workflow of different stages of the long-term reproductive study on Endler's guppies. A – Juvenile fish were raised for 3 months under laboratory conditions to mature stage. B – Exposure to etonogestrel for 34 days. C – Behavioral studies: C1, mate choice test; C2, mating study. D – Reproduction (mated females were kept in isolation for 90 days in order to determine fertility).

frame as the ratio between the length of the fish trajectory and the time of swimming, and it was then transformed into centimeters per second. The swimming activity was calculated for individual fish and averaged for the fish group. Site preference was defined as time spent in the center of the aquarium (50% of the surface) or the border area (50% of the surface). The site preference was calculated based on the time fish spent in each of the two regions.

2.4.2. Mate choice test

The mate choice test was conducted after the 34 days of exposure. This test was aimed at determining the effect of etonogestrel treatment on the preference for individuals of the opposite gender in both males and females. This binary mate choice experiment was conducted following Herdman et al. (2004) and Bierbach et al. (2011) and was carried out separately for males and females. In total, 112 naïve fish (56 males and 56 females) were tested. This test was carried out in 8 replicates for each experimental group. A 9 L aquarium (32 × 22 cm) was divided by fine mesh into three parts. To avoid disturbances, all sides of the aquaria were covered by black plastic sheeting. A naïve fish from the solvent control was placed into the middle part (16 × 22 cm), which was the test area. On the left and right sides, which were of dimensions 8 × 22 cm, exposed and unexposed fish (solvent control) of the opposite gender were placed. As an additional control, a naïve fish was placed into an aquarium with two unexposed fish from the opposite gender in the other parts. This procedure was conducted with fish from control and solvent controls (Fig. 1).

After an adaptation period of 5 min, the behavior of the fish in the middle section was recorded for 15 min. Nine aquaria were used for this experiment. The assignment of experimental trials to the aquaria was randomized. The behavior patterns were recorded with the monitoring system based on a 3D camera (Xbox One Kinect Sensor). One camera was placed above three aquaria to monitor each fish separately and save the 3D position for later processing. The experimental setup is illustrated in Fig. 1. The closeness was calculated from the recordings using software implemented in-house based on the program developed by Saberioon and Cisar (2016). Closeness was defined as spending time outside the midpoint area of the middle section located at one of the respective sides. The time of each behavior was automatically quantified by a mating processing program (Saberioon and Cisar, 2016). Attraction or avoidance was evaluated based on the presence on one of the sides. The water in the aquaria was changed after each experimental trial.

2.4.3. Mating experiment

Mating behavior was studied using an exposed male cohabitating with an unexposed female and vice versa. Moreover, male and female fish exposed to both concentrations of etonogestrel and unexposed couples were mated. In particular, combinations tested in the mating experiment were as follow: (1) male C × female C, (2) male SC × female SC, (3) male E1 × female SC, (4) male E2 × female SC, (5) male SC × female E1, (6) male SC × female E2, (7) male E1 × female E2, and (8) male E2 × female E2. A respective fish couple was transferred into a 15 L aquarium (32 × 22 cm) containing 5 L of water. The mating tests were conducted with nine replicates for each experimental group. After an adaptation period of 5 min, the mating was recorded by charge-coupled device camera (Sony, CCD-TR840E) for 15 min. The camera monitored four aquaria together from the top to record the complete trajectory and fish behavior. The videos were later analyzed for the presence of several behavior endpoints, including display of the S-shape (the S-shape is a sigmoid movement displayed by a male during the courtship), following, attending, and attempts by the male to copulate. The behavioral patterns were evaluated manually, blinded. Courtship and mating were determined as described by Baatrup and Junge (2001), Pyke (2005), Olsen et al. (2014), and Frankel et al. (2016b).

For determining fertility, female fish involved in the mating study were kept isolated in clean water for 90 days after mating. These fish were kept in isolated boxes (12 × 8 cm) under the conditions described above. The birth rate was recorded.

2.5. Secondary sexual characteristics

All fish involved were recorded for coloration analysis after the mating study. All females were photographed at the end of the mating test and 90 days post mating. During the recording, the fish were alive and placed in a special aquarium with dimensions 12 × 4 × 4 cm. A green background was selected for the recordings. The pictures were taken using an Olympus Alpha 501 camera. Blinded evaluation was made of the pictures, and alterations in body color were noted. These alterations were defined by the presence of orange or any other color different from the normal, metallic gray body color (Poeser et al., 2005).

Sixteen fish of each gender from each treatment and the water control as well as 24 fish from the solvent control were used for morphological analysis. Fish were stored in 10% neutral buffered formalin. Weight of fish was recorded after 4 months of fixation and stabilization of the relative post-fixation mass. The fish body, eye, caudal, dorsal, pelvic, pectoral fins, as well as the anal fin of the females and gonopodium of the males were photographed using an Olympus E600 camera mounted on an Olympus SZX7 stereomicroscope. All measurements were carried out using Quick Photo 2.3 software.

Measured were total (TL) and body (BL) lengths, horizontal eye diameter (ED), lengths of the first ray (DFL_{1st}) and last ray (DFL_{last}) of the dorsal fin, as well as total lengths of the caudal (CDFL), pelvic (PLFL), and pectoral (PECFL) fins. These parameters, with the exceptions

of TL and BL, were normalized to the BL. These parameters are described in detail in Wiecaszek et al. (2009).

In the cases of the anal fin and gonopodium, the thicknesses of the 3rd and 4th rays, lengths of the 4th and 6th rays, thicknesses and length ratios of 3rd:4th ray, relative length of 4th and 6th rays to BL, and the palp lengths of the gonopodium were measured as described by Angus et al. (2001). The rays were identified as described by Turner (1941). Abnormalities of the male gonopodium and the female anal fin were recorded.

2.6. Histological analysis

The in toto fixed fish (10% buffered formalin) were decalcified (for 4 h in decalcification solution 1). Male and female fish were oriented in longitudinal and sagittal directions in capsules. In the case of female fish, serial cuts were made of the area containing the ovarium. For 8 fish of every experimental group, the developmental stages of the gonads were identified (Edwards and Guillette, 2006; Golpour et al., 2016; Hou et al., 2018a). Ninety days post-exposure, possible pregnancy was checked histologically in females which had not given birth. Samples were dehydrated in an ascending series of ethanol concentrations, paraffin-embedded, cut by microtome (4 μm), then placed on slides. The sections were stained with hematoxylin and eosin (H&E), then examined by light microscopy at 10× to 1000× times magnification (Bancroft and Gamble, 2008).

2.7. Statistical analysis

The statistical analysis was performed using Statistica software version 13 (StatSoft, Czech Republic). Data were checked for normality and homoscedasticity by the Kolmogorov–Smirnov test, and by Cochran's, Hartley's and Bartlett's tests, respectively.

If these criteria were fulfilled, a one-way analysis of variance (ANOVA) was employed to detect significant differences among the experimental groups in the measured variables. Subsequently, Dunnett's multiple range test was applied to compare the control mean with the means of all treatment groups. If the conditions for ANOVA were not satisfied, nonparametric tests (Kruskal–Wallis test and Friedman ANOVA) were used. The significance level was set at $p < 0.05$.

The behavior during the experiment, namely time spent in the outer 50% of the aquarium, grouping, following, and swimming speed were analyzed by Kruskal–Wallis test and Friedman ANOVA. The mate preference of the fish was analyzed by Kruskal–Wallis test. Differences in coloration and the developmental stages of oocytes in the ovary were checked by chi-squared test. Fisher's exact test was applied for the birth rate and the presence of palp in female fish. The data are presented as mean ± standard deviation (SD).

Table 1
Concentration of etonogestrel in water in the sub-chronic toxicity test on Endler's Guppy (*Poecilia wingei*). Concentrations were measured immediately after water exchange (0 h) and 24 h post-exchange. The values are expressed as mean ± SD ($n = 4$). C = water control, SC = solvent control, LOQ = limit of quantification.

Group	Sample time (h)	Water concentration (ng L^{-1})	Stability during 24 h (%)	Min – Max
C	0	<LOQ		
	24	<LOQ		
SC	0	<LOQ		
	24	<LOQ		
3.2 ng L^{-1}	0	3.2 ± 1.8		1.6–5.1
	24	2.7 ± 1.4	88 ± 16	1.2–4.1
320 ng L^{-1}	0	483 ± 22		450–500
	24	435 ± 58	91 ± 16	360–490

3. Results

3.1. Analytical performance and water concentration of etonogestrel

Table 1 shows concentrations of etonogestrel measured in aquarium water for all treatment groups. Etonogestrel concentrations over 24 h were relatively stable (88–91%), with means corresponding to 3.0 ± 1.5 and 458 ± 48 ng L^{-1}, respectively, in treatments with nominal etonogestrel concentrations of 3.2 and 320 ng L^{-1}. In water samples from both controls, the concentrations of etonogestrel were below the LOQ (<0.35 ng L^{-1}).

3.2. Behavioral studies

3.2.1. Analysis of activity patterns

In male and female fish, the relative durations of grouping and following in all treatment groups were short, as these behaviors, respectively, were in the ranges of 1.0–1.9% and 0.3–2.2‰ of the total recorded time. Before and at the end of the exposure period, grouping and following of females and males did not differ significantly in the exposed fish compared to the controls (Friedman ANOVA, Kruskal–Wallis, and Kruskal–Wallis test applied on differences of grouping and following before and at the end of the exposure time, $p > 0.05$). Males and females exposed to nominal etonogestrel concentrations of 3.2 and 320 ng L^{-1} did not show significantly different swimming activity in comparison with the controls (ANOVA, $p > 0.05$). In all treatment groups, the fish avoided the central section and stayed preferentially in the corners of the aquaria. The site preference in fish of all treatment groups was not statistically different compared to that of the controls (Kruskal–Wallis, $p > 0.05$). The relative duration and frequency of grouping and following and the site preference are summarized in Supplementary Tables 1 and 2.

3.2.2. Mate choice test

No effect of etonogestrel on mate preference was found for treated male and female fish (Kruskal–Wallis, $p > 0.05$; Fig. S2). No site preference of the fish was found within the control groups (SC and C; Kruskal–Wallis, $p > 0.05$; Fig. S2).

3.2.3. Mating study

Both groups of exposed males (3.2 and 320 ng L^{-1}) showed significantly shorter duration and lower frequency of mating attempts compared to the controls (ANOVA, $p < 0.05$; Fig. 2), although the time for each attempt was not changed (Table S3). The courtship behaviors, namely duration and frequency of attending and following and frequency of display of the S-shape of the male fish, did not differ significantly in the etonogestrel-treated fish compared to the controls (Table S3).

3.3. Reproduction

All etonogestrel-exposed females at both concentration levels were unable to reproduce 90 days post mating (Fig. 3). In contrast, control females which mated with unexposed or 3.2 and 320 ng L^{-1} etonogestrel-exposed male fish were able to reproduce. Fisher's exact test confirms that the result is statistically significant both when we compare all control groups with all exposed groups as well as all SC groups with all exposed groups (Fisher's exact test, $p < 0.0002$).

3.4. Mortality

No mortality was recorded during the experimental period.

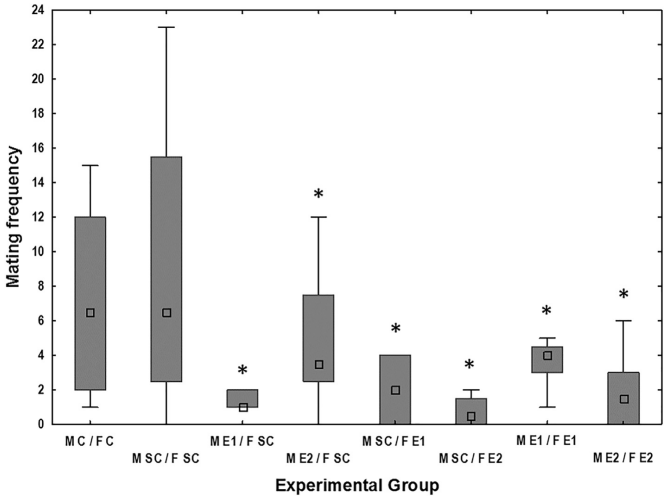


Fig. 2. Mating frequency during 15 min in Endler's guppies exposed to etonogestrel for 34 days. M = male, F = female, C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. Asterisks indicate significant difference from controls. *$p < 0.05$ (ANOVA, $n = 9$).

3.5. Secondary sexual characteristics

In female fish exposed to 320 ng L^{-1} etonogestrel, the relative length of the caudal fin (CFL/BL), absolute and relative width of the 3rd and 4th rays and length of the 4th and 6th rays of the anal fin, absolute length of the last ray of the dorsal fin (DFL_{last}), and relative length of the dorsal fin (DFL_{1st}/BL) were significantly increased compared to those of the controls (Table S5). All these parameters are close to those measured in male control fish (control and solvent control) and indicate masculinization of females exposed to the highest tested level of etonogestrel (Fig. 4). Ninety days post exposure, the length of the caudal fin and rays of anal fin remained altered (Table S6).

In males exposed to 320 ng L^{-1} etonogestrel, the ratio of DFL_{last}/BL and the absolute and relative length of the 6th ray of the gonopodium increased compared to the controls ($p < 0.05$). The ratio of the length of the 4th to the 6th fin ray in the gonopodium was significantly reduced in fish exposed to both 3.2 and 320 ng L^{-1} etonogestrel compared to the controls ($p < 0.05$, Table S4).

Other measured parameters (i.e., TL, BL, weight, FCF, ED, ED/BL, HL, HL/BL, PFL, and PFL/BL) were not significantly affected by the etonogestrel exposure either in males or females (Tables S4–6).

At the highest level of etonogestrel (320 ng L^{-1}), a significantly higher number of females displayed alterations in their body color (chi-squared test, $p < 0.05$; Fig. 5). These alterations were characterized by a yellow- to orange-colored caudal part of the body inclusive of the tail and dorsal fin. Typically, these changes started at the tip of the caudal fin. These male-like patterns indicated masculinization of fish in this experimental group (Figs. 4 and 5). This effect appeared to be reversible, as 90 days post mating only 8% of the females showed masculinization of the body color. These alterations were not present in any of the other groups.

3.6. Histology

Etonogestrel treatment affected maturation of oocytes in the exposed fish. In the control groups, mature oocytes were predominant, but the relative occurrence of mature oocytes was significantly lower in fish exposed to 3.2 and 320 ng L^{-1} etonogestrel compared to the controls (chi-squared test, $p < 0.05$; Fig. 6).

Etonogestrel did not affect the development of testes. Predominantly mature spermatocytes were present in all examined fish. Among the experimental groups, no statistically significant differences in frequency of the different developmental stages in the testes were found (chi-squared test, $p > 0.05$; Fig. S3). In none of the examined fish did the testes or ovaries show pathological alterations, and no signs of intersex were observed.

Among fish that did not give birth, no pregnancy was found 90 days post mating by the histological analysis in any of the exposed fish (3.2 and 320 ng L^{-1}) or controls.

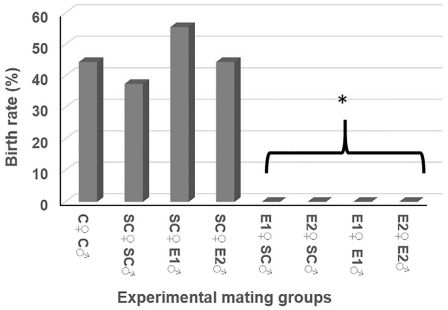


Fig. 3. Birth rate of Endler's guppies sub-chronically exposed to etonogestrel for 34 days. M = male, F = female, C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. Asterisks indicate significant differences from controls. *$p < 0.05$ (Fisher's exact test, n = 9).

4. Discussion

The main objectives of the present study were to assess effects of sublethal etonogestrel concentrations (3.2 and 320 ng L^{-1}) on Endler's

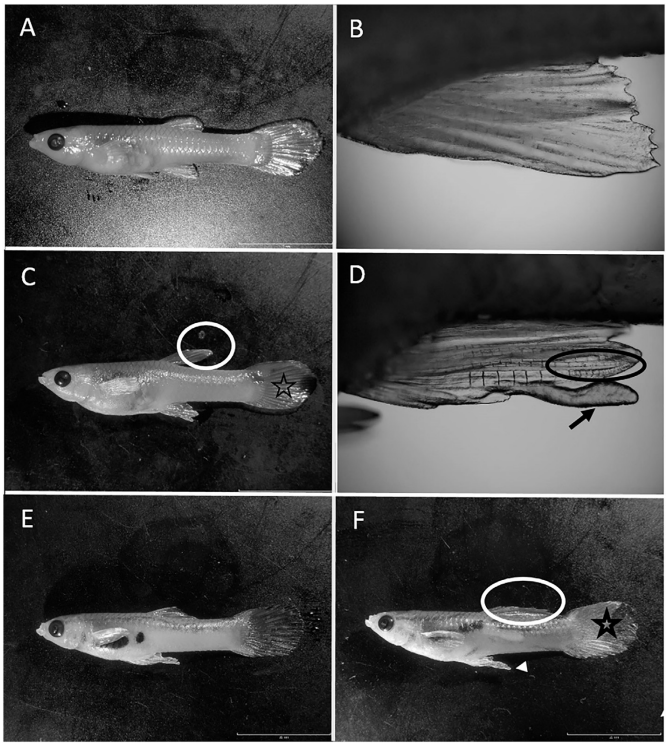


Fig. 4. Morphological changes in fins and gonopodium, of exposed Endler's guppies. (A) Whole body of an unexposed solvent control female (SC); the anal fin is shown in detail in image (B). (C) Whole body of a female exposed to 320 ng L^{-1} etonogestrel for 34 days; white circle and star indicate longer dorsal and caudal fins, respectively. (D) Detail of anal fin of etonogestrel-exposed female (from C); arrow and circle show palp and hooks, respectively. (E) Whole body of unexposed solvent control male (SC). (F) Whole body of male exposed to 320 ng L^{-1} etonogestrel for 34 days; circle and star show longer dorsal and caudal fins, respectively. Arrowhead indicates gonopodium with an altered shape.

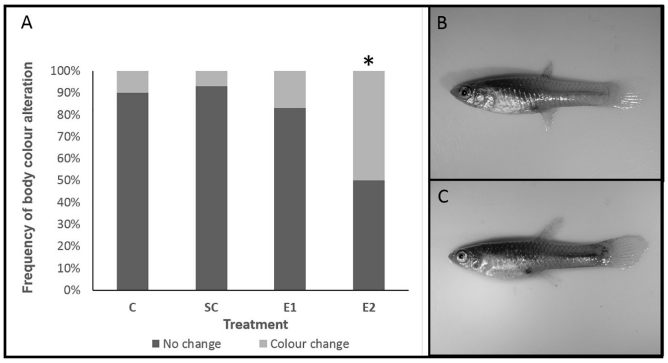


Fig. 5. (A) Percentage of color alterations in female Endler's guppies at the conclusion of 34 days of exposure to etonogestrel. C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. Asterisks indicate significant difference from controls. $^{*}p < 0.05$ (chi-squared test). $n = 9$. (B) Body color of a control female fish. (C) Altered body color of a female exposed to 320 ng L^{-1} of etonogestrel for 34 days.

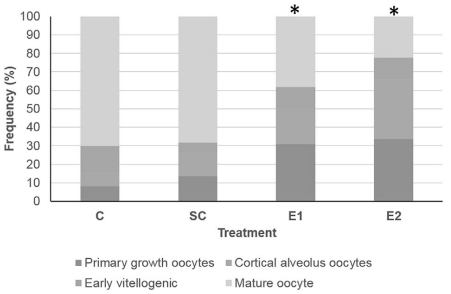


Fig. 6. Percentage of oocytes in different developmental stages within the ovaries of Endler's guppies at the conclusion of 34 days of exposure to etonogestrel. C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. $n = 8$. Asterisks indicate significant difference from controls. $^{*}p < 0.05$ (chi-squared test).

guppies. Changes in secondary sexual characteristics (morphology and coloration) are first discussed below (4.1), followed by behavioral alterations (activity patterns, mate choice and mating; 4.2), and then effects on reproduction and gonad development (4.3).

4.1. Secondary sexual characteristics

In the present study, female fish exposed to etonogestrel (320 ng L^{-1}) displayed a male-like coloration mainly at the caudal fin. In addition, the anal fin of females developed a gonopodium-like shape, including hooks and a palp, and the caudal fin was elongated. These fin alterations were still visible 90 days post exposure, thus indicating that they could be permanent. Taken together, all the changes in secondary sexual characteristics found in females exposed to etonogestrel (320 ng L^{-1}) are signs of masculinization of females and clearly show an androgenic effect of etonogestrel at high concentration. Our findings are well in line with the study by Bain et al. (2015), who reported that etonogestrel transactivated Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) androgen receptors α and β with half maximal effective concentration (EC_{50}) values of 164 and 1469 ng L^{-1}, respectively.

Similar alteration of the anal fin and/or changes in color were described in female fish exposed to androgen-active pulp and paper mill wastewaters (Deaton and Cureton, 2011; Howell et al., 1980; Parks et al., 2001) or synthetic androgenic hormones (Larsson et al., 2002; Zamora et al., 2008).

Androgenic effect has been observed also in other testosterone-derived synthetic progestins, namely (levo)norgestrel, gestodene, and desogestrel. Hou et al. (2018a) and Frankel et al. (2016b) described masculinization of anal fin in adult females of western mosquitofish exposed to ≥35.8 ng L^{-1} of norgestrel for 42 days and in eastern mosquitofish exposed to ≥10 ng L^{-1} of levonorgestrel for 8 days, respectively. Female fathead minnow developed male secondary sexual characteristics after 21 days of exposure to gestodene (1 ng L^{-1}), levonorgestrel (29.6 ng L^{-1}), and desogestrel (10 μg L^{-1}) (Runnalls et al., 2013; Zeilinger et al., 2009).

In the present study, a longer dorsal fin was observed in males exposed to the highest level of etonogestrel (320 ng L^{-1}). Worthy of note is that *Poecilia latipinna* and *Poecilia mexicana* display a sexual dimorphism of dorsal fin, where females prefer males with longer dorsal fins (MacLaren et al., 2011). In addition, exposure to etonogestrel at both concentrations caused changes in the shape of the gonopodium, namely it reduced the ratio of the 4th to 6th ray. The gonopodium in Poeciliids functions as an intromittent organ but it is also important for pre-copulatory sexual selection (Kwan et al., 2013). Therefore, any alterations of its morphology may impact reproductive success (Evans et al., 2011; Kwan et al., 2013). Contrary to the present study, an increase of the 4th:6th ray ratio was observed in males of eastern mosquitofish after 8 days of exposure to 100 ng L^{-1} levonorgestrel (Frankel et al., 2016b). It is not clear, however, whether these particular changes increase or decrease attractiveness of the affected males.

4.2. Behavioral alterations

Etonogestrel did not cause any changes in activity, following, and grouping of fish in this study, indicating that the exposed fish did not suffer from anxiety, narcosis, or disorientation. Even such behavior changes, however, probably would not strongly interfere with mating. Olsen et al. (2014) showed, for example, that although Endler's guppies exposed to high levels of the psychoactive drug citalopram displayed signs of anxiety, mating and courtship were not affected. Contrary to the present study, Zhao et al. (2018) report that locomotor activities decreased in zebrafish embryos after 2 days of exposure to progesterone and synthetic progestins (levonorgestrel and gestodene) at the level of 16 ng L^{-1}. This might be explained by differences in the experimental setup, including the tested species, life stage, exposure time, and selection of progestins.

Reproductive behavior in guppies involves association, courtship, and mating (Poeser et al., 2005; Pyke, 2005). In this study, no difference in the association and courtship (i.e., preference or attending behavioral traits; S-shape display and following) were found in etonogestrel-treated fish compared to the controls. Nevertheless, mating frequencies (mating attempts) appeared to be the most sensitive behavioral endpoint in the present study. At both etonogestrel concentrations, the mating frequencies were significantly reduced. Exposed males appeared to be affected directly, as they made a lower number of attempts to pair with both control and exposed females. Moreover, they were affected indirectly, because reduced mating frequency was also observed in unexposed males paired with exposed females. This indicates a negative effect of etonogestrel on the attractiveness of the exposed females. At the high etonogestrel level, one of the reasons for low attractiveness of females was probably their strong masculinization. In females exposed to low etonogestrel concentration, however, there were no marked changes in secondary sexual characteristics. Therefore, additional issues had to have played a role, and pheromone signaling could be one of them. Mature females release sexual pheromones into the water and thus induce mating behavior in males (Kobayashi et al., 2002; Sorensen and Stacey, 2004). Inasmuch as etonogestrel exposure negatively affected maturation of the gonads in females within the present study, this could have interfered also with pheromone signaling in females and thus reduced males' interest in mating (i.e., decreased mating frequency). In agreement with this study, Frankel et al. (2016b) and Hou et al. (2018a) found that short-term (8 days) exposure to levonorgestrel (100 ng L^{-1}) and long-term exposure (42 days) to norgestrel (3.6 ng L^{-1}) resulted in changes of reproductive behavior in western and eastern mosquitofish, respectively, but the potential adverse outcomes of these changes on the birth rate have not been studied.

4.3. Reproduction and gonad development

Females exposed to both levels of etonogestrel were unable to produce offspring after mating with either control or exposed males. On the other hand, exposed males were able to reproduce with control females even if the mating frequency was reduced. The exposure to etonogestrel clearly has more serious consequences for females than males. At present, there is no information available regarding the effect of synthetic progestins on reproduction in Poeciliids. In agreement with the present study, reproduction in fathead minnow, Japanese medaka, and zebrafish have been shown to be affected adversely by low levels of synthetic progestins, which lead to inhibited or reduced egg production (Kumar et al., 2015). In fathead minnow, for example, egg

production was reduced after 21 days of exposure to low levels of levonorgestrel (0.8 ng L^{-1}) (Zeilinger et al., 2009) and gestodene (1 ng L^{-1}) (Runnalls et al., 2013), as well as higher levels of norethindrone (100 ng L^{-1}) (Paulos et al., 2010) and desogestrel (10 μg L^{-1}) (Runnalls et al., 2013).

Reproductive failure in females exposed to both levels of etonogestrel in the present study can be explained at least in part by their markedly lower percentage of mature oocytes (22–38%) in comparison to the controls (68–70%). It is noteworthy that Hou et al. (2018a) observed a higher percentage of atretic and post-ovulatory oocytes and higher incidence of intersex in western mosquitofish exposed to norgestrel at ≥35.8 ng L^{-1}.

We found no effect of etonogestrel exposure on testes. This fact corresponds with our findings of reproductive success among exposed males paired with control females. Likewise, Frankel et al. (2016a) observed no effect of levonorgestrel on the development of testes in fathead minnow exposed to concentrations of ≥10 ng L^{-1} for 8 days. In that study, they had not examined any possible effect on reproduction.

The results showing the effect of synthetic progestins on fish reproduction are not surprising inasmuch as these pharmaceuticals were primarily designed for contraception in human medicine (Baird and Glasier, 2000; Kroupova et al., 2014). Nevertheless, synthetic progestins have a low affinity to the fish progesterone receptors (Bain et al., 2015; Ellestad et al., 2014). Therefore, this effect is likely mediated through another pathway and further studies should be undertaken to elucidate synthetic progestins' mode of action in fish.

5. Conclusions

To the best of our knowledge, this study reports for the first time the effects of etonogestrel on fish. A concentration of etonogestrel as low as 3.2 ng L^{-1} reduced mating activity in males without affecting their reproductive success but caused complete failure of reproduction in females. This shows that etonogestrel exposure had more serious consequences for females. In addition, the high etonogestrel level (320 ng L^{-1}) caused marked masculinization of females. These findings strengthen current concerns about possible detrimental effects on fish populations of synthetic progestins reaching aquatic environments.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic projects 'CENAKVA' (No. CZ.1.05/2.1.00/01.0024) and 'CENAKVA II' (No. LO1205 under the NPU I program), by projects of the Czech Science Foundation No. 16-09709Y and the Grant Agency of the University of South Bohemia No. 012/2016/Z. We are grateful for the support of Livia Baum in producing the artwork (zukunftStil Agentur für Farbdesign und Trendforschung; http://zukunftstil.de/) and to Michal Pech and Roberto Bellinvia for assistance in assessing the secondary sexual characteristics of females.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.01.276.

References

Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., Surif, S., 2010. Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1217, 6791–6806.

Angus, R.A., McNatt, H.B., Howell, W.M., Peoples, S.D., 2001. Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): a quantitative study using computer image analysis. Gen. Comp. Endocrinol. 123, 222–234.

Baatrup, E., Junge, M., 2001. Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*). Environ. Health Perspect. 109, 1063–1070.

Bain, P.A., Kumar, A., Ogino, Y., Iguchi, T., 2015. Nortestosterone-derived synthetic progestogens do not activate the progestogen receptor of Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) but are potent agonists of androgen receptors alpha and beta. Aquat. Toxicol. 163, 97–101.

Baird, D.T., Glasier, A.F., 2000. The science, medicine, and future of contraception. West. J. Med. 172, 321–324.

Bancroft, J.D., Gamble, M., 2008. Theory and Practice of Histological Techniques. Churchill Livingstone, London, pp. 73–76.

Bierbach, D., Kronmarck, C., Hennige-Schulz, C., Stadler, S., Plath, M., 2011. Sperm competition risk affects male mate choice copying. Behav. Ecol. Sociobiol. 65, 1699–1707.

Croxatto, H.B., 2002. Progestin implants for female contraception. Contraception 65, 15–19.

Deaton, R., Cureton, J.C., 2011. Female masculinization and reproductive life history in the western mosquitofish (*Gambusia affinis*). Environ. Biol. Fish 92, 551–558.

Edwards, T.M., Guillette, L.J., 2006. Seasonal reproductive variation in Gambusia holbrooki (Eastern mosquitofish) from two Florida lakes. J. Exp. Zool. A Comp. Exp. Biol. 305A, 123.

Ellestad, L.E., Cardon, M., Chambers, I.G., Farmer, J.L., Hartig, P., Stevens, K., et al., 2014. Environmental gestagens activate fathead minnow (*Pimephales promelas*) nuclear progesterone and androgen receptors in vitro. Environ. Sci. Technol. 48, 8179–8187.

Evans, J.P., Gasparini, C., Holwell, G.I., Ramnarine, I.W., Pitcher, T.E., Pilastro, A., 2011. Intraspecific evidence from guppies for correlated patterns of male and female genital trait diversification. Proc. R. Soc. B Biol. Sci. 278, 2611–2620.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environ. Int. 84, 115–130.

Fick, J., Lindberg, R.H., Parkkonen, J., Arvidsson, B., Tysklind, M., Larsson, D.G.J., 2010. Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. Environ. Sci. Technol. 44, 2661–2666.

Frankel, T.E., Meyer, M.T., Kolpin, D.W., Gillis, A.B., Alvarez, D.A., Orlando, E.F., 2016a. Exposure to the contraceptive progestin, gestodene, alters reproductive behavior, arrests egg deposition, and masculinizes development in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 50, 5991–5999.

Frankel, T.E., Meyer, M.T., Orlando, E.F., 2016b. Aqueous exposure to the progestin, levonorgestrel, alters anal fin development and reproductive behavior in the eastern mosquitofish (*Gambusia holbrooki*). Gen. Comp. Endocrinol. 234, 161–169.

Golovko, O., Sauer, P., Fedorova, G., Kroupova, H.K., Grabic, R., 2018. Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS. Sci. Total Environ. 621, 1066–1073.

Golpour, A., Psenicka, M., Niksirat, H., 2016. Subcellular localization of calcium deposits during zebrafish (*Danio rerio*) oogenesis. Micron 80, 6–13.

Graham, J.D., Clarke, C.L., 1997. Physiological action of progesterone in target tissues. Endocr. Rev. 18, 502–519.

Herdman, E.J.E., Kelly, C.D., Godin, J.G.J., 2004. Male mate choice in the guppy (Poecilia reticulata): do males prefer larger females as mates? Ethology 110, 97–111.

Hoffmann, F., Kloas, W., 2012. The synthetic progestogen, Levonorgestrel, but not natural progesterone, affects male mate calling behavior of Xenopus laevis. Gen. Comp. Endocrinol. 176, 385–390.

Hou, L.P., Xu, H.Y., Ying, G.G., Yang, Y., Shu, H., Zhao, J.L., et al., 2017. Physiological responses and gene expression changes in the western mosquitofish (*Gambusia affinis*) exposed to progesterone at environmentally relevant concentrations. Aquat. Toxicol. 192, 69–77.

Hou, L.P., Chen, H.X., Tian, C.E., Liang, Y., Wu, R.R., Zhang, X.M., et al., 2018a. Alterations of secondary sex characteristics, reproductive histology and behaviors by norgestrel in the western mosquitofish (*Gambusia affinis*). Aquat. Toxicol. 198, 224–230.

Hou, L.P., Yang, Y., Shu, H., Ying, G.G., Zhao, J.L., Fang, G.Z., et al., 2018b. Masculinization and reproductive effects in western mosquitofish (*Gambusia affinis*) after long-term exposure to androstenedione. Ecotoxicol. Environ. Saf. 147, 509–515.

Houde, A.E., 1987. Mate choice based upon naturally-occurring color-pattern variation in a guppy population. Evolution 41, 1–10.

Houtman, C.J., Ten Broek, R., Brouwer, A., 2018. Steroid hormonal bioactivities, culprit natural and synthetic hormones and other emerging contaminants in waste water measured using bioassays and UPLC-tQ-MS. Sci. Total Environ. 630, 1492–1501.

Howell, W.M., Black, D.A., Bortone, S.A., 1980. Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia-affinis-holbrooki* - evidence for environmentally-induced masculinization. Copeia 676–681.

Kane, A., Salierno, J., Brewer, S., 2005. Fish models in behavioral toxicology: automated techniques, updates and perspectives. In: Ostrander, K.G. (Ed.), Methods in Aquatic Toxicology, pp. 559–590.

Kavanaugh, M.L., Jerman, J., Finer, L.B., 2015. Changes in use of long-acting reversible contraceptive methods among US women, 2009–2012. Obstet. Gynecol. 126, 917–927.

Kobayashi, M., Sorensen, P.W., Stacey, N.E., 2002. Hormonal and pheromonal control of spawning behavior in the goldfish. Fish Physiol. Biochem. 26, 71–84.

Kroupova, H.K., Trubiroha, A., Lorenz, C., Contardo-Jara, V., Lutz, I., Grabic, R., et al., 2014. The progestin levonorgestrel affects hypothalamus-pituitary-gonad axis in pubertal roach (Rutilus rutilus). Toxicol. Lett. 229, S114–S115.

Kumar, V., Johnson, A.C., Trubiroha, A., Tumova, J., Ihara, M., Grabic, R., et al., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49, 2625–2638.

Kwan, L., Cheng, Y.Y., Rodd, F.H., Rowe, L., 2013. Sexual conflict and the function of genitalic claws in guppies (*Poecilia reticulata*). Biol. Lett. 9.

Larsson, D.G.J., Kinnberg, K., Sturve, J., Stephensen, E., Skon, M., Forlin, L., 2002. Studies of masculinization, detoxification, and oxidative stress responses in guppies (*Poecilia reticulata*) exposed to effluent from a pulp mill. Ecotoxicol. Environ. Saf. 52, 13–20.

Lorenz, C., Contardo-Jara, V., Pflugmacher, S., Wiegand, C., Nutzmann, G., Lutz, I., et al., 2011a. The synthetic gestagen levonorgestrel impairs metamorphosis in *Xenopus laevis* by disruption of the thyroid system. Toxicol. Sci. 123, 94–102.

Lorenz, C., Contardo-Jara, V., Trubiroha, A., Kruger, A., Viehmann, V., Wiegand, C., et al., 2011b. The synthetic gestagen Levonorgestrel disrupts sexual development in *Xenopus laevis* by affecting gene expression of pituitary gonadotropins and gonadal steroidogenic enzymes. Toxicol. Sci. 124, 311–319.

MacLaren, R.D., Gagnon, J., He, R., 2011. Female bias for enlarged male body and dorsal fins in *Xiphophorus variatus*. Behav. Process. 87, 197–202.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217–2223.

Norazmi-Lokman, N.H., Purser, G.J., Patil, J.G., 2016. Gravid spot predicts developmental Progress and reproductive output in a Livebearing fish, *Gambusia holbrooki*. PLoS One 11, e0147711.

Odom, E.B., Eisenberg, D.L., Fox, I.K., 2017. Difficult removal of subdermal contraceptive implants: a multidisciplinary approach involving a peripheral nerve expert. Contraception 96, 89–95.

OECD. Organization for Economic Cooperation and Development, 1992. Guidelines for the testing of chemicals, section 2. TG No. 203 Acute Toxicity Test. Paris, France.

Olsen, K.H., Ask, K., Olsen, H., Porsch-Hallstrom, I., Hallgren, S., 2014. Effects of the SSRI citalopram on behaviours connected to stress and reproduction in Endler guppy, *Poecilia wingei*. Aquat. Toxicol. 148, 113–121.

Parks, L.G., Lambright, C.S., Orlando, E.F., Guillette Jr., L.J., Ankley, G.T., Gray Jr., L.E., 2001. Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. Toxicol. Sci. 62, 257–267.

Paulos, P., Runnalls, T.J., Nallani, G., La Point, T., Scott, A.P., Sumpter, J.P., et al., 2010. Reproductive responses in fathead minnow and Japanese medaka following exposure to a synthetic progestin, Norethindrone. Aquat. Toxicol. 99, 256–262.

Poeser, F.N., Kempkes, M., Isbrucker, I.J.H., 2005. Description of poecilia (acanthophacelus) wingei n. sp from the Paria Peninsula, Venezuela, including notes on Acanthophacelus Eigenmann, 1907 and other subgenera of Poecilia Bloch and Schneider, 1801 (Teleostei, Cyprinodontiformes, Poeciliidae). Contrib. Zool. 74, 97–115.

Pyke, G.H., 2005. A review of the biology of gambusia affinis and *G-holbrooki*. Rev. Fish Biol. Fish. 15, 339–365.

Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environ. Sci. Technol. 47, 2077–2084.

Saberioon, M.M., Cisar, P., 2016. Automated multiple fish tracking in three-dimension using a structured light sensor. Comput. Electron. Agric. 121, 215–221.

Sauer, P., Stara, A., Golovko, O., Valentova, O., Borik, A., Grabic, R., et al., 2018. Two synthetic progestins and natural progesterone are responsible for most of the progestagenic activities in municipal wastewater treatment plant effluents in the Czech and Slovak republics. Water Res. 137, 64–71.

Schlupp, I., 2018a. Male mate choice in livebearing fishes: an overview. Curr. Zool. 64, 393–403.

Schlupp, I., 2018b. Male mate choice, female competition, and female ornaments as components of sexual selection. Curr. Zool. 64, 321–322.

Sebire, M., Allen, Y., Bersuder, P., Katsiadaki, I., 2008. The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. Aquat. Toxicol. 90, 37–47.

Sommer, C., Olsen, K.H., 2016. Sex odour preference in guppy (*Poecilia wingei*) males is influenced by the social environment. Behaviour 153, 1419–1434.

Sorensen, P.W., Stacey, N.E., 2004. Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. N. Z. J. Mar. Freshw. Res. 38, 399–417.

Turner, C., 1941. Morphogenesis of the gonopodium in *Gambusia affinis affinis*. J. Morphol. 69, 161–185.

Wagenfeld, A., Saunders, P.T.K., Whitaker, L., Critchley, H.O.D., 2016. Selective progesterone receptor modulators (SPRMs): progesterone receptor action, mode of action on the endometrium and treatment options in gynecological therapies. Expert Opin. Ther. Targets 20, 1045–1054.

Wester, P.W., Vos, J.G., 1994. Toxicological pathology in laboratory fish - an evaluation with 2 species and various environmental contaminants. Ecotoxicology 3, 21–44.

Wiecaszek, B., Antoszek, J., Antoszek, A., 2009. Comparative study of the biometric characters of the "golden" cod and a sample of cod of typicalpigmentation (*Gadus morhua morhua* L.) captured in the Svalbard Bank. Electron. J. Pol. Agric. Univ. 12, 1.

Zamora, H.S., Hernandez, A.A., Herrera, S.M., Pena, E.M., 2008. Anabolic and androgenic effect of steroid trenbolone acetate on guppy (*Poecilia reticulata*). Vet. Mex. 39, 269–277.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Lange, R., 2009. Effects of synthetic gestagens on fish reproduction. Environ. Toxicol. Chem. 28, 2663–2670.

Zhao, Y., Zhang, K., Fent, K., 2018. Regulation of zebrafish (*Danio rerio*) locomotor behavior and circadian rhythm network by environmental steroid hormones. Environ. Pollut. 232, 422–429.

CHAPTER 6

6. GENERAL DISCUSSION

6.1. Determination of progestins in environmental water samples

6.1.1 Selection of target compounds

One of the aims of this Ph.D. thesis was to develop a sensitive analytical method for detection of progestins in environmental water matrices. The first step was to prepare a list of progestins that could be present in Czech aquatic environment. The main criterion in selection of the analytes was their consumption in the Czech Republic.

Consumption of progestins in the Czech Republic in year 2014 has been calculated from data freely available at the website of State Institute of the Czech Republic (chapter 2; State Institute of the Czech Republic, 2017). Six compounds with the highest consumption rates (progesterone, drospirenone, megestrol acetate, cyproterone acetate, medroxyprogesterone acetate and dienogest) reached or exceeded annual consumption of 100 kg in the Czech Republic. The progestins prescribed in the Czech Republic were mostly similar to those prescribed in France, United Kingdom, and Switzerland (Table 8). However, such comparison should be treated with caution as data from different years have been compared (other data were not available) and it is possible that some new progestins were introduced there. Smaller diversity of consumed progestins has been reported from Switzerland (6 progestins) compared to United Kingdom (17 progestins), the Czech Republic (18 progestins), and France (22 progestins) (Table 8). The consumed amount of individual progestins also differed between countries. Natural progestin progesterone had the highest consumption in the Czech Republic, France and Switzerland but medroxyprogesterone acetate was the predominantly consumed progestin in United Kingdom (Table 8). The most consumed synthetic progestins were: drospirenone in the Czech Republic and Switzerland, cyproterone acetate in France and medroxyprogesterone acetate in United Kingdom (Table 8). As far as I am aware, we selected all the progestins consumed in one country (the Czech Republic in our case) as target compounds for monitoring of their presence in aquatic environment for the first time (chapter 2). Additionally, another progestin, medroxyprogesterone, was chosen as one of analytes because its presence has previously been reported in U.S. WWTP effluents (Kolodziej et al., 2003), a Czech WWTP effluent and Swiss surface waters (Macikova et al., 2014).

Table 8. *Consumption of progestins in European countries.*

	Consumption (kg · year^{-1})			
Country	**the Czech Republic**	**France**	**United Kingdom**	**Switzerland**
Year	**2014**	**2004**	**2006**	**2011**
Reference	**Chapter 2**	**Besse and Garric, 2009**	**Runnalls et al., 2010**	**Zhao et al., 2015**
progesterone	**1034.76**	**9864.25**	**141.56**	500
drospirenone	**249.77**	**149.38**	**153.19**	90
megestrol acetate	**206.65**		**146.69**[a]	
cyproterone acetate	**182.07**	**821.56**		
medroxyprogesterone acetate	**107.74**	68.50[b]	**529.70**[b]	25
dienogest	**100.32**	5.73		
norethisterone+norethisterone acetate+lynestrenol	61.81	**100.99**	**440.16** (norethisterone)	17 (norethisterone)
dydrogesterone	21.78	**744.70**	209.02	34
chlormadinone acetate	19.98	**385.17**		
gestodene	9.07	22.04	*	
etonogestrel+desogestrel	8.04	28.88	* (etonogestrel) and 13.87 (desogestrel)	
levonorgestrel+norgestimate +norelgestromin	7.94	94.51 (levonorgestrel + norgestimate)	19.59 (levonorgestrel) + 24.12 (norgestimate) + * (norelgestromin)	3 (levonorgestrel)
nomegestrol acetate	2.79	**312.10**		
medrogestone		87.73		
tibolone		52.53	64.16	
17α-hydroxyprogesterone		27.58	*	
promegestone		13.41		
norgestrel		11.92	*	
norgestrienone		2.59		
ethynodiol diacetate			13.85	
gestrienone			*	

Abbreviations and description: [a] - data in the original article referred to consumption of megestrol but it was most probably mixed up with megestrol acetate; [b] – data in the original article referred to consumption of medroxyprogesterone but it was most probably mixed up with medroxyprogesterone acetate; data on consumption reaching or exceeding 100 kg · year^{-1} are marked in bold, * - consumption of this substance was not reported separately, but as a sum of gestodene, etonogestrel, norelgestromin, 17α-hydroxyprogesterone, norgestrel, and gestrinone which is equal to 12.90 kg · year^{-1}

6.1.2. Optimization of solid-phase extraction for extracting progestins

Chapter 2 describes development and application of a chemical method (liquid chromatography coupled with tandem mass spectrometry) for analysis of progestins in wastewaters and surface waters. I have been responsible for the optimization and application of a solid-phase extraction (SPE) in the method development and therefore the SPE will be discussed more thoroughly in this chapter.

Progestins are present in the aquatic environment at trace levels (Table 2). To allow determination of compounds at trace levels in complex environmental samples by chemical analysis, it is desirable to concentrate and separate target compounds prior chemical analysis from other compounds using a sample extraction technique. SPE is advantageous due to its high recovery yield, short extraction time, high enrichment factor, and reduced use of organic solvents compared to other procedures such as liquid-liquid extraction (Ma et al., 2010).

We used a method based on automated SPE extraction system (SPE-DEX 4790, Horizon Technology, USA) that is easy to use for operators even when running high numbers of samples and it also ensures repeatability of final results as confirmed by the chemical method (chapter 2). Several factors should be considered in development of an SPE method to make it sensitive and selective towards specific group of chemicals.

Adjustment of sample pH before SPE is an important step influencing extraction efficiency (Kuster et al., 2009; Vulliet et al., 2008). The samples are being acidified to inhibit biological activities of microorganisms that could potentially be sampled together with water and thus the adjustment of pH of samples should prevent biotransformation/bioconcentration of analytes (chapter 4). Many researchers analysing progestins in water samples have automatically acidified samples, however, without testing the effect on extraction efficiency (chapter 2).

Therefore, we attempted to determine if sample acidification affects recovery of compounds (chapter 4). Recoveries of three progestins (gestodene, levonorgestrel and nomegestrol acetate) in the acidified samples were not satisfactory as they did not lie within the satisfactory range of 60–130%. However, the recoveries of all progestins in non-acidified samples fell within the optimal range. Given that acidification of samples resulted in a bit worse recoveries compared to samples without any pH adjustment, we concluded that acidification of samples prior SPE is not advantageous in extraction of progestins (chapter 4). There were also some other authors who tested the effect of sample acidification on extraction efficiency of progestins (Table 9) and similarly to our observation, their results suggest that sample pH adjustment is not a key factor influencing yield of extracted progestins. Both these techniques of sample treatment (sample acidification and no pH adjustment) are applicable methods but we recommended rather avoiding acidification of samples to better simulate real environmental conditions (chapter 4).

Table 9. *Recommendations from literature for sample pH treatment prior to solid-phase extraction for chemical analysis of progestins.*

Recommended pH of samples	Comments on the other tested pH values of samples	Used sorbent	Matrix	Reference
3.0	Compared to pH 3.0, samples with pH 5.5 and 8.0 had about 6–35% and 52–96% poorer recoveries, respectively.	Strata X	demineralized water	Vulliet et al., 2008
pH adjustment has no effect[a]	Sample pH 5.0 was recommended in the study for a wide range of chemicals but pH values of 3.0, 5.0, 7.0 and 9.0 gave similar recoveries of progestins, except for slightly higher recovery of norethisterone (ca 120%) in samples with pH 7.0.	LiChrolut RP-18	HPLC water	Kuster et al., 2009
6.0–7.0	Sample pH values 3.0, 4.0, 5.0, 6.0, 8.0 and 9.0 resulted in poorer recoveries of two tested progestins, 21αhydroxyprogesterone and norgestrel, compared to recommended sample pH.	3d-Mag-CMGO	surface water	Chen et al., 2017
pH adjustment has no effect	pH values 3.0, 5.5 and 8.0 were tested.	C18 speedisk	seawater	Huysman et al., 2017
pH adjustment has no effect	Details were not provided.	Oasis HLB	wastewater (effluent), river water	Shen et al., 2018
7.4	3.0 is also applicable.	Atlantic C18 SPE disk	wastewater (influent)	Chapter 4

Abbreviations and description: [a] - conclusion based on the results provided in the study.

Another factor influencing extraction efficiency is the choice of sorbent in SPE. C18 sorbent has been used in our SPE method (chapter 2) because slightly polar and non-polar compounds such as progestins (Table 1) are known to be retained well by C18 sorbents via hydrophobic interaction (Ma et al., 2010). In addition, the solvent used for elution of analytes is one of the key factors that have impact on extraction efficiency. We revealed that acetonitrile is a better elution solvent than methanol for extraction of progestins (chapter 2). This result is in accordance with observations made by Sun et al. (2009) who tested different solvents for extraction of progestins and found that the most optimal recoveries are yielded using acetonitrile.

6.1.3. Occurrence of progestins in aquatic environments

There have been only two studies dealing with identification of progestins in the Czech aquatic environments so far and focused only on 7 progestins (Table 2). We have been searching for the presence of progestins using a method we recently developed (chapter 2) that is based on liquid-chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS). Our papers present new results unravelling the pattern of environmental contamination by progestins in the Czech aquatic environment (chapters 2, 3 and 4).

The concentrations of individual progestins in influents ranged from 0.19 to 110 $ng \cdot l^{-1}$. Most of the target progestins were found in influents at least once. All the six progestins with annual consumption equal to or exceeding 100 kg have been detected in WWTP influents as

well as in effluents (chapters 2, 3 and 4). Progestin with the highest consumption, natural hormone progesterone, has been the most frequently detected progestin in all studied matrices (chapters 2, 3, and 4). Similarly, the progestins with consumption higher than 100 $kg \cdot year^{-1}$ in France and United Kingdom (Table 8), were found in French wastewaters (Vulliet et al., 2007; 2011) and surface waters (Vulliet et al., 2008; Vulliet and Cren-Olivé, 2011) but these include only two compounds because the others were not measured. In United Kingdom, progesterone and norethisterone (Table 8) which were consumed in relatively high quantities were detected in surface waters (Aherne et al., 1985), but other progestins were not analysed. Only the natural progestin progesterone had consumption higher than 100 $kg \cdot year^{-1}$ in Switzerland (Table 8) and it has also been found in Swiss wastewaters and surface waters (Macikova et al., 2014). Overall, it seems that the most consumed progestins can really end up in wastewaters and subsequently in surface waters. If a progestin is highly prescribed/consumed, it does not automatically mean that it will be present in aquatic environments because progestins in bodies are to some extent metabolized after their uptake by users. Then they can even be easily biotransformed in sewage during wastewater treatment processing or also in surface water. For example, synthetic progestins with the highest consumption in the Czech Republic, drospirenone, has been found only in 43% of assessed influents (chapters 2, 3, and 4). However, most of the consumed progestins have been found throughout our screenings and thus the selection of target progestins (as analytes) based on the data on consumption still appears to be relatively reliable approach.

In Czech WWTP effluents, the concentrations of individual progestins were found in the range of 0.11–3.2 $ng \cdot l^{-1}$ (chapters 2, 3, and 4). Progesterone, megestrol acetate and dienogest were the most commonly found progestins in WWTP effluents throughout our screening campaigns with the frequencies of detection 85, 50 and 35%, respectively (chapters 2, 3, and 4). Only limited attention has been paid to testing of the eco-toxicological effects of dienogest and megestrol acetate on aquatic organisms though. Megestrol acetate and progesterone occurred in Czech effluents in all of our sampling campaigns. Therefore, these compounds also deserve attention in determination of their presence in environment. The presence of altrenogest and nomegestrol acetate in WWTP effluents has been showed for the first time (chapter 2). Progestins levonorgestrel, norethisterone, medroxyprogesterone acetate and progesterone are well-known environmental contaminants that have attracted most interest in monitoring of their occurrence in the aquatic environment so far (Table 2). Nonetheless, some other progestins occur in aquatic environments of certain countries much more frequently. For example levonorgestrel has not been detected at all and norethisterone was found only once in Czech aquatic environment (Table 2, chapters 2, 3 and 4). Despite a certain increase in monitoring of progestins in last years (Table 2), such data are still scarce and much more work should be done to get closer in revealing the real contamination patterns worldwide (Fent, 2015; Kumar et al., 2015).

We observed negative removal efficiency of some compounds (chapter 2). For example, medroxyprogesterone has shown negative removal efficiency in two WWTPs (Vodňany and Brno). This phenomenon might be, in this particular case, explained by biotransformation of medroxyprogesterone acetate into medroxyprogesterone. Generally, there is a lack of data in literature on such biotransformation of progestins during wastewater treatment process. However, three recent studies reported that two progestins (progesterone and norgestrel) can be rapidly metabolized into another steroids by common water bacteria (Ojoghoro et al., 2017; Sangster et al., 2016) and microalgae (Peng et al., 2014).

In surface waters, we found progestins in up- and downstreams of WWTPs in the range of 0.20–1.3 $ng \cdot l^{-1}$ and 0.12–1.3 $ng \cdot l^{-1}$, respectively (chapters 2, 3, and 4). Only two progestins, progesterone and medroxyprogesterone have been identified in surface waters in our sampling

campaign (chapters 2, 3 and 4). Therefore, special attention should be paid to monitoring and eco-toxicological testing of these compounds.

6.2. Progestagenic and (anti-)androgenic activities of progestins

Hormonal (progestagenic, androgenic and anti-androgenic) activities of all the samples (pure compounds and water extracts) were measured using CALUX assays. CALUX assays are *in vitro* reporter gene assay based on human bone cancer U2-OS cells and transfected with plasmids for reporter protein luciferase and human receptor. They respond specifically to compounds that can activate or block the inserted receptor (Sonneveld et al., 2005). Progestagenic, androgenic, and anti-androgenic activities were measured using PR-, AR-, and anti-AR-CALUX assays, respectively.

6.2.1. Progestagenic activities of progestins

We compared progestagenic REPs of all the progestins marketed in one country (the Czech Republic) for the first time (chapter 3). Knowledge of the REP of compounds is essential for estimation of risk that they can pose to aquatic environment (Villeneuve et al., 2000).

Gestodene was by far the strongest progestagenic compound. Then, there was a cluster of three similarly potent progestins, which included etonogestrel, levonorgestrel and medroxyprogesterone acetate. Two compounds (megestrol acetate and progesterone) that were detected in all of our samplings campaigns in WWTP effluents (chapters 2, 3, and 4) are both strong progestagens. Norethisterone, a well-known environmental contaminant (Table 2) with negative effect on fish reproduction (Table 7), was equally potent as natural progestin progesterone. Drospirenone, synthetic progestin with the highest consumption in the Czech Republic (Table 8), was amongst the weakest progestins in our experiments. Dienogest had the weakest progestagenic activity out of the progestins consumed in the Czech Republic (chapter 3) but it has been frequently detected in Czech wastewaters (chapters 2, 3, and 4). In addition, medroxyprogesterone has been tested in *in vitro* reporter gene bioassay for the first time (chapter 3). Given that medroxyprogesterone was found in U.S. WWTP effluents (Kolodziej et al., 2003), Czech raw hospital wastewaters (Macikova et al., 2014), and surface waters (chapters 1 and 3), this compound deserve more attention in eco-toxicological research.

To the best of our knowledge, this is the first systematic and comprehensive *in vitro* profiling of progestagenic activity of progestins that are possibly relevant for the aquatic environment. In general, the progestagenic potencies of progestins observed in our experiments are consistent with the findings of other authors if such data were available (Table 3).

6.2.2. Androgenic activities of progestins

Seven progestins (levonorgestrel, gestodene, altrenogest, etonogestrel, medroxyprogesterone acetate, norethisterone and medroxyprogesterone) displayed androgenic activities *in vitro*. REPs of all these compounds were lower than that of reference compound which is natural androgenic hormone, dihydrotestosterone (chapter 4).

Gestodene, etonogestrel and levonorgestrel were reported by other authors as the strongest androgenic progestins (chapter 1) and they were together with altrenogest also among the four strongest androgenic progestins in our experiments (chapter 4). McRobb et al. (2008) measured androgenic activity of altrenogest using yeast androgen screen and found that it is extremely potent androgen (approximately 4 times more potent than

dihydrotestosterone). We did not observe such a high REP of altrenogest (it was 7 times less potent than dihydrotestosterone). Androgenic activity of altrenogest can be explained by its structural similarity to its precursor trenbolone that is a potent androgen. Progesterone did not display androgenic activity in our experiments (chapter 4). Some authors reported that progesterone is androgenic compound but it exerts androgenic activity only at high environmentally irrelevant levels with EC_{50} value at tens of $\mu g \cdot l^{-1}$ (McRobb et al., 2008) or higher concentrations (Louw-du-Toit et al., 2017). The other progestin, medroxyprogesterone, which has been found in effluents (chapters 2 and 3) and in surface water (chapter 3) had only weak androgenic activity with EC_{50} reaching tens of $\mu g \cdot l^{-1}$ (chapter 4).

It has been reported that some progestins can occur together in surface waters at $ng \cdot l^{-1}$ levels (Avar et al., 2016; Liu et al., 2014; Shen et al., 2018; Vulliet et al., 2008), therefore, we tested the joint effect of mixtures of androgenic progestins. Our results indicate that these progestins interact in an additive manner (chapter 4) what is in line with previous findings of Rossier et al. (2016) and Siegenthaler et al. (2017). Therefore, there is sane suspicion that the androgenic effects of progestins can add up in aquatic environments. On one hand, the progestins-induced masculinisation of fish can be a real environmental threat if multiple strong androgenic progestins are present together. On the other hand, some of the environmental contaminants including other progestins are also relatively strong anti-androgens and might attenuate or neutralize these effects.

6.2.3. Anti-androgenic activities of progestins

Nine progestins (megestrol acetate, chlormadinone acetate, cyproterone acetate, nomegestrol acetate, progesterone, medroxyprogesterone, drospirenone, dydrogesterone and dienogest) exhibited anti-androgenic activities *in vitro* (chapter 4). Four strongest progestins (megestrol acetate, chlormadinone acetate, cyproterone acetate and nomegestrol acetate) were 40 times more potent compared to flutamide. Cyproterone acetate is well-known anti-androgen but the anti-androgenic activity of the other three progestins attracted far less attention to date. To the best of my knowledge, anti-androgenic activity of medroxyprogesterone has been shown for the first time.

All these tested anti-androgenic progestins were stronger in eliciting anti-androgenic activities than reference compound flutamide (chapter 4). However, it is still not clear how strong an anti-androgen should be to pose a risk to aquatic organisms. Despite that flutamide is widely used as reference compound in *in vitro* bioassays, it is not considered as a strong anti-androgen. Nonetheless, flutamide at high concentrations (hundreds of $\mu g \cdot l^{-1}$) have partially and dose-dependently prevented male-biased sex ratio induced by low concentration of levonorgestrel (10 $ng \cdot l^{-1}$) in zebrafish (Hua et al., 2015). Given that progestins are much stronger anti-androgens than flutamide, they have a potential to neutralize androgenic effects as well.

We tested joint effect of anti-androgenic progestins and it appears that their anti-androgenic activities are additive (chapter 4). Siegenthaler et al. (2017) tested joint effect of two anti-androgenic progestins, chlormadinone acetate and cyproterone acetate in an *in vitro* bioassay based on rainbowfish AR and also found that they acted mostly additively. Considering that progestins might occur simultaneously in aquatic environments, we can expect that they contribute additively to anti-androgenic activities in aquatic environments.

6.2.4. Contribution of compounds to progestagenic and (anti-)androgenic activities in aquatic environments

Many authors including us have reported the occurrence of progestins in waste and surface waters (Table 2). Progestins are compounds that possess various hormonal activities (chapter 1) and we focused on their progestagenic and (anti-)androgenic activities (Tables 3 and 4). Progestagenic and (anti-)androgenic activities have been repeatedly detected in aquatic environments worldwide (Tables 5 and 6). However, there has not been any systematic attempt so far to determine the extent to which progestins can contribute to these activities.

Therefore, our experiments aimed at revealing the extent to which progestins contribute to progestagenic (chapter 3) and (anti-)androgenic activities (chapter 4) in Czech waste and surface waters.

The obtained REPs of progestins were applied in our experiments to determine predicted activities, using which we could estimate the contribution of progestins to measured activities. Total activities in environmental samples determined by an *in vitro* assay (measured activities) were compared with the sum of the potencies of the individual compounds identified by chemical analysis (predicted activities) in order to estimate the degree to which analysed substances account for the biological activity. Predicted activity of a single compound was calculated as follow: predicted activity of a compound *i* is equal to concentration of the compound *i* in environmental sample (data from chemical analysis) multiplied by REP of the compound *i* (derived from an *in vitro* assay). Predicted activity of whole sample was calculated as the sum of predicted activities of individual compounds found in the sample. Contribution of a single compound to measured activity was calculated as a ratio of predicted and measured activities. To the best of our knowledge, there has been only one study to date reporting the contribution of a few selected progestins to progestagenic activities so far (Creusot et al., 2014). They found that progestin, levonorgestrel, can be responsible for up to 50% of detected progestagenic activity in a French river downstream a pharmaceutical factory. Throughout our experiments, we observed overall contribution of progestins to progestagenic activities in effluents in the range of 65–96% (chapter 3). Our data indicate that one natural progestin (progesterone) and two synthetic progestins (megestrol acetate, medroxyprogesterone acetate) are responsible for majority of progestagenic activities found in municipal WWTP effluents in Czech and Slovak republics. However, it still needs to be resolved which effects can be induced in aquatic organisms when exposed to progestagenic compounds. In case of progestagenic activities, most of the developed *in vitro* bioassays employ human PR (yeast progesterone screen - YPS, PR-CALUX, HELN-PRB assay or geneBLAzer) to which progestins have intrinsic affinity (chapter 1). However, extrapolating the results obtained with an assay that is based on human PR to fish may not be appropriate because progestins were shown not to transactivate fish PRs except for some weak interaction with progesterone and drospirenone (Bain et al., 2015; Ellestad et al., 2014). The mode of action of synthetic progestins in fish is likely different from what we know in humans (Kumar et al., 2015). Nevertheless, there is a high homology between human and frog PR ligand binding domains (Bayaa et al., 2000) and thus certain harmful effects for amphibians exposed to waters exhibiting progestagenic activities measured through human PR might be expected. In addition, various semi-aquatic mammals (e.g. otters, minks or muskrats) reside in freshwater aquatic environments and for those the human PR-derived results might be relevant.

Many researchers are currently attempting to elucidate which compounds are responsible for (anti-)androgenic activities in aquatic environment. Various groups of chemicals have been revealed to contribute to (anti-)androgenic activities but the causative (anti-)androgens also have not been identified in some studies as reviewed in chapter 4. Considering that progestins

display (anti-)androgenic activities, we assessed if they have potential to contribute to (anti-) androgenic activities found in Czech aquatic environment (chapter 4).

Androgenic progestins gestodene and medroxyprogesterone acetate were found in WWTP influents and were responsible for 0.26-29% of androgenic activities there. No androgenic progestins reached WWTP effluents and have not been present in surface waters.

Five progestins with anti-androgenic properties (cyproterone acetate, dienogest, drospirenone, megestrol acetate and progesterone) were present in WWTP influents and contributed to 4.6–27% of found anti-androgenic activities. Two of them (megestrol acetate and progesterone) were not eliminated during wastewater treatment processing and were responsible for only 0.28–2.1% of anti-androgenic activities. Progesterone was the only anti-androgenic progestin present in surface waters, where it accounted for 0.34–1.6% of anti-androgenic activity. It also contributed the most out of all progestins to anti-androgenic activities in all studied matrices. In general, it appears that the potential of progestins to contribute to anti-androgenic activities found in Czech WWTP effluents and surface waters is very low.

Despite that progestins were confirmed to possess considerable (anti-)androgenic activities, they had only negligible potential to cause the (anti-)androgenic activities in WWTP effluents and surface waters, because they were simply not present or if present their concentrations were relatively low (chapter 4). This implies that some other compounds are likely responsible for (anti-)androgenic activities in Czech aquatic environments (Table 13). The degree to which progestins are responsible for anti-androgenic activities is strongly dependent on their concentrations in environment. If multiple potent progestins are present at ng · l^{-1} concentration or a few strong progestins occur at tens of ng · l^{-1}, then progestins can account considerably to anti-androgenic activities in aquatic environments. Indeed, Houtman et al. (2018) found cyproterone acetate at high concentration of 20 ng · l^{-1} in a WWTP effluent in the Netherlands and this single progestin contributed as much as 70% to anti-androgenic activities (chapter 1).

6.3. Effects of synthetic progestin etonogestrel on fish

We performed a sub-chronic test on Endler's guppy (*Poecilia wingei*) with the aim to find out whether exposure to etonogestrel at concentrations 3.2 and 320 ng · l^{-1} can negatively affect mate choice, mating behaviour, secondary sexual characteristics, gonad histology, and fertility (chapter 5). Etonogestrel is the active compound in some hormonal preparations and it is also the main metabolite of another progestin, desogestrel. Both compounds are consumed in the Czech Republic (Table 8), while the prescription of desogestrel prevails (chapter 5). Etonogestrel has not been detected in the aquatic environment yet, however, there have been only few attempts to determine its presence (chapter 2). Due to the continuous consumption of etonogestrel, it can be suspected to contaminate some surface waters. No study has focused to date on assessing the effects of progestin etonogestrel on aquatic organisms even when it is known to be biologically active compound (chapter 1).

Endler's guppies exhibit a marked sexual dimorphism in coloration and the morphology of anal fin. Males have their anal fin transformed into a copulatory organ called gonopodium, and have colourful bodies, while females are rather greyish. In our study (chapter 5), female fish exposed to etonogestrel at the high concentration (320 ng · l^{-1}) showed male-like coloration in the caudal part of bodies. In addition, they had anal fin with a gonopodium-like shape, containing hooks and a palp typical for males. These changes appeared to be irreversible even 90 days post exposure. All these effects indicate masculinization of females by high tested concentration of etonogestrel. Androgenic activity of etonogestrel has already been observed

in vitro on human ARs (chapter 1) and rainbowfish's AR (Bain et al., 2015). The elongation of anal fin following exposure to progestins considered as a sign of masculinisation has been reported for eastern mosquitofish exposed to levonorgestrel (Frankel et al., 2016a) and western mosquitofish exposed to 35.8 $ng \cdot l^{-1}$ norgestrel (Hou et al., 2018). Development of male secondary sexual characteristics in females was found in fathead minnow exposed to progestins gestodene (1 $ng \cdot l^{-1}$), levonorgestrel (30 $ng \cdot l^{-1}$), and desogestrel (10 $\mu g \cdot l^{-1}$) (Runnalls et al., 2013; Zeilinger et al., 2009). All the abovementioned progestins (chapter 6.3.) possess androgenic activity *in vitro* (chapter 4; Runnalls et al., 2013).

Reproductive behaviour in male guppies involves association, courtship, and mating (Pyke, 2005). Fish exposed to etonogestrel did not display any differences in the association and courtship (i.e., mate choice or attending behavioural traits; S-shape display and following) compared to the fish in control groups, but mating frequencies were reduced. The reduced mating frequency of fish exposed to the high concentration of etonogestrel (320 $ng \cdot l^{-1}$) can be attributed to lower attractiveness of females due to their strong masculinisation. However, fish exposed to the lower concentration of etonogestrel (3.2 $ng \cdot l^{-1}$) were not masculinized suggesting that some other effect should have been involved in reduced mating frequency. Mature female fish release pheromones into the water to induce mating behaviour in males (Kobayashi et al., 2002; Sorensen and Stacey, 2004). Given that maturation of gonads was negatively affected in females exposed to 3.2 $ng \cdot l^{-1}$, their lower mating frequency might be a result of interference with fish pheromonal signalling.

Unlike female fish in control groups, the females exposed to 3.2 and also 320 $ng \cdot l^{-1}$ of etonogestrel were not capable of reproducing 90 days post mating. In line with this finding, several studies reported that synthetic progestins could have negative impact on fish fertility by decreasing egg production (chapter 1; Table 7). We described for the first time the effect of a synthetic progestin on reproduction of a Poecilid fish which is live-bearing. Our histological analysis revealed that etonogestrel influenced maturation of oocytes (chapter 5). Fish in control group had oocytes mostly in the mature stage but there has been lower occurrence of mature oocytes in the fish exposed to 3.2 as well as 320 $ng \cdot l^{-1}$ etonogestrel. So, the reproductive failure of females exposed to both concentrations of etonogestrel in our experiment can be attributed to impaired maturation of oocytes.

Given that progestins are prescribed in human and veterinary medicine to prevent reproduction (they are used as contraceptives) or for delaying and suppression of estrus (chapter 1), respectively, the negative effect of etonogestrel on guppies' reproduction is not surprising. However, the mechanism of this effect on reproduction of fish remains unknown. Progestins do not bind fish PRs or only very weakly (chapter 1). Therefore, some other physiological pathways were likely affected. In higher vertebrates, progesterone is known to regulate gonadotropin synthesis and secretion (Christensen et al., 2012) and most of progestins, including etonogestrel, possess anti-gonadotropic activity (Kumar et al., 2015). Hence, it can be hypothesized that either synthesis or secretion of gonadotropins has been altered. Inhibition of secretion of gonadotropins can prevent follicular growth and maturation (Raudrant and Rabe, 2003). Such a decrease in secretion of gonadotropins could have inhibited the oocyte development in fish in our experiment (chapter 5).

Taken together, a concentration of etonogestrel as low as 3.2 $ng \cdot l^{-1}$ reduced mating activity in males without affecting their reproductive success, but caused complete failure of reproduction in females. The exposure to etonogestrel clearly has more serious consequences for females than males.

6.4. Conclusions

We have been searching for the presence of multiple progestins in wastewaters and surface waters. Progestins occurred at $ng \cdot l^{-1}$ and even lower concentrations in Czech WWTP effluents and surface waters. Some progestins (altrenogest and nomegestrol acetate) were detected in WWTP effluents for the first time. Results of our monitoring revealed that progestins dienogest and megestrol acetate, which have attracted only limited attention in eco-toxicological research so far, occurred frequently in treated wastewaters.

Progestins present in aquatic environments can pose a risk to aquatic organisms due to disruption of their endocrine system. To estimate progestagenic and (anti-)androgenic potencies of progestins, their REPs were determined in *in vitro* bioassays. Our results indicate that progestins have high potential to cause progestagenic activities in municipal WWTP effluents. However, the effects manifested in aquatic organisms living in surface waters exhibiting progestagenic activities are unknown.

Progestins displayed relatively strong (anti-)androgenic potencies but their contribution to (anti-)androgenic activities was negligible in Czech water bodies due to the low concentrations at which they were present. Furthermore, we tested the joint effect of mixtures of individual progestins and found that the androgenic and also anti-androgenic progestins can act in an additive manner. The contribution of progestins to (anti-)androgenic activities would be significant only if their concentrations reach or exceed tens of $ng \cdot l^{-1}$ levels or multiple strong progestins are present together at units of $ng \cdot l^{-1}$.

One of our experiments have shown that progestin etonogestrel could cause reproduction failure in female fish at low $ng \cdot l^{-1}$ level. Moreover, the females exposed to high level of etonogestrel have been masculinised. The evidence is accumulating in the literature that also other progestins can disrupt fish reproduction and cause masculinisation at environmentally relevant levels. Thus if several androgenic progestins occur simultaneously in the aquatic environment, their effects on reproduction and masculinisation of fish may be additive. Overall, this work strengthens the current concerns over the effects of progestins on aquatic organisms.

References

Aherne, G. W., English, J., Marks, V., 1985. The role of immunoassay in the analysis of microcontaminants in water samples. Ecotoxicology and Environmental Safety 9, 79–83.

Avar, P., Maász, G., Takács, P., Lovas, S., Zrínyi, Z., Svigruha, R., Takátsy, A., Tóth, L. G., Pirger, Z., 2016. HPLC-MS/MS analysis of steroid hormones in environmental water samples. Drug Testing and Analysis 8, 123–127.

Bain, P. A., Kumar, A., Ogino, Y., Iguchi, T., 2015. Nortestosterone-derived synthetic progestogens do not activate the progestogen receptor of Murray–Darling rainbowfish (*Melanotaenia fluviatilis*) but are potent agonists of androgen receptors alpha and beta. Aquatic Toxicology 163, 97–101.

Bayaa, M., Booth, R. A., Sheng, Y., Liu, X. J., 2000. The classical progesterone receptor mediates Xenopus oocyte maturation through a nongenomic mechanism. Proceedings of the National Academy of Sciences 97, 12607–12612.

Besse, J. P., Garric, J., 2009. Progestagens for human use, exposure and hazard assessment for the aquatic environment. Environmental Pollution 157, 3485–3494.

Chen, X. H., Zhao, Y. G., Qiu, Q. L., Zhu, Y., Min, J. Q., Jin, M. C., 2017. A fast and high throughput LC-MS/MS method for the determination of 58 human and veterinary drugs in river water. Analytical Methods 9, 4228–4233.

Christensen, A., Bentley, G. E., Cabrera, R., Ortega, H. H., Perfito, N., Wu, T. J., Micevych, P., 2012. Hormonal regulation of female reproduction. Hormone and Metabolic Research 44, 587–591.

Creusot, N., Aït-Aïssa, S., Tapie, N., Pardon, P., Brion, F., Sanchez, W., Thybaud, E., Porcher, J.-M., Budzinski, H., 2014. Identification of synthetic steroids in river water downstream from pharmaceutical manufacture discharges based on a bioanalytical approach and passive sampling. Environmental Science & Technology 48, 3649–3657.

Ellestad, L. E., Cardon, M., Chambers, I. G., Farmer, J. L., Hartig, P., Stevens, K., Villeneuve, D.L., Wilson, V.S., Orlando, E. F., 2014. Environmental gestagens activate fathead minnow (*Pimephales promelas*) nuclear progesterone and androgen receptors in vitro. Environmental Science & Technology 48, 8179–8187.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environment International 84, 115–130.

Frankel, T. E., Meyer, M. T., Orlando, E. F., 2016a. Aqueous exposure to the progestin, levonorgestrel, alters anal fin development and reproductive behavior in the eastern mosquitofish (*Gambusia holbrooki*). General and Comparative Endocrinology, 234, 161–169.

Frankel, T. E., Meyer, M. T., Kolpin, D. W., Gillis, A. B., Alvarez, D. A., Orlando, E. F., 2016b. Exposure to the contraceptive progestin, gestodene, alters reproductive behavior, arrests egg deposition, and masculinizes development in the fathead minnow (*Pimephales promelas*). Environmental Science & Technology 50, 5991–5999.

Grobstein, C., 1948. Optimum gonopodial morphogenesis in *Platypoecilus maculatus* with constant dosage of methyl testosterone. Journal of Experimental Zoology 109, 215–237.

Hou, L. P., Chen, H., Tian, C. E., Liang, Y., Wu, R. R., Zhang, X. M., Fang, X. W., Zhang, C. P., Hu, J. J., Song, L. Y., Liang, Y. Q., Schlenk, D., Xie, L., 2018. Alterations of secondary sex characteristics, reproductive histology and behaviors by norgestrel in the western mosquitofish (*Gambusia affinis*). Aquatic Toxicology 198, 224–230.

Houtman, C. J., ten Broek, R., Brouwer, A., 2018. Steroid hormonal bioactivities, culprit natural and synthetic hormones and other emerging contaminants in waste water measured using bioassays and UPLC-tQ-MS. Science of the Total Environment 630, 1492–1501.

Hua, J., Han, J., Guo, Y., Zhou, B., 2015. The progestin levonorgestrel affects sex differentiation in zebrafish at environmentally relevant concentrations. Aquatic Toxicology 166, 1–9.

Huysman, S., Van Meulebroek, L., Vanryckeghem, F., Van Langenhove, H., Demeestere, K., Vanhaecke, L., 2017. Development and validation of an ultra-high performance liquid chromatographic high resolution Q-Orbitrap mass spectrometric method for the simultaneous determination of steroidal endocrine disrupting compounds in aquatic matrices. Analytica Chimica Acta 984, 140–150.

Kobayashi, M., Sorensen, P. W., Stacey, N. E., 2002. Hormonal and pheromonal control of spawning behavior in the goldfish. Fish Physiology and Biochemistry 26, 71–84.

Kolodziej, E. P., Gray, J. L., Sedlak, D. L., 2003. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. Environmental Toxicology and Chemistry 22, 2622–2629.

Kumar, V., Johnson, A.C., Trubiroha, A., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kocour Kroupová, H., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environmenta Science & Technology 49, 2625–2638.

Kuster, M., Azevedo, D. D. A., De Alda, M. L., Neto, F. A., Barceló, D., 2009. Analysis of phytoestrogens, progestogens and estrogens in environmental waters from Rio de Janeiro (Brazil). Environment International 35, 997–1003.

Liu, S. S., Ying, G. G., Liu, S., Lai, H. J., Chen, Z. F., Pan, C. G., Zhao, J. L., Chen, J., 2014. Analysis of 21 progestagens in various matrices by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with diverse sample pretreatment. Analytical and Bioanalytical Chemistry 406, 7299–7311.

Louw-du Toit, R., Perkins, M. S., Hapgood, J. P., Africander, D., 2017. Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy. Biochemical and Biophysical Research Communications 491, 140–146.

Ma, J., Xiao, R., Li, J., Yu, J., Zhang, Y., Chen, L., 2010. Determination of 16 polycyclic aromatic hydrocarbons in environmental water samples by solid-phase extraction using multi-walled carbon nanotubes as adsorbent coupled with gas chromatography–mass spectrometry. Journal of Chromatography A 1217, 5462–5469.

Macikova, P., Kalabova, T., Klanova, J., Kukucka, P., Giesy, J. P., Hilscherova, K., 2014. Longer-term and short-term variability in pollution of fluvial sediments by dioxin-like and endocrine disruptive compounds. Environmental Science and Pollution Research 21, 5007–5022.

McRobb, L., Handelsman, D. J., Kazlauskas, R., Wilkinson, S., McLeod, M. D., Heather, A. K., 2008. Structure–activity relationships of synthetic progestins in a yeast-based *in vitro* androgen bioassay. The Journal of Steroid Biochemistry and Molecular Biology 110, 39–47.

Ojoghoro, J. O., Chaudhary, A. J., Campo, P., Sumpter, J. P., Scrimshaw, M. D., 2017. Progesterone potentially degrades to potent androgens in surface waters. Science of the Total Environment 579, 1876–1884.

Peng, F. Q., Ying, G. G., Yang, B., Liu, S., Lai, H. J., Liu, Y. S., Chen, Z. F., Zhou, G. J., 2014. Biotransformation of progesterone and norgestrel by two freshwater microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*): Transformation kinetics and products identification. Chemosphere 95, 581–588.

Pyke, G. H., 2005. A Review of the Biology of *Gambusia affinis* and *G. holbrooki*. Reviews in Fish Biology and Fisheries 15, 339–365.

Raudrant, D., Rabe, T., 2003. Progestogens with antiandrogenic properties. Drugs 63, 463–492.

Rossier, N. M., Chew, G., Zhang, K., Riva, F., Fent, K., 2016. Activity of binary mixtures of drospirenone with progesterone and 17α-ethinylestradiol *in vitro* and *in vivo*. Aquatic Toxicology 174, 109–122.

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 16, 1318–1338.

Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environmental Science & Technology 47, 2077–2084.

Sangster, J. L., Ali, J. M., Snow, D. D., Kolok, A. S., Bartelt-Hunt, S. L., 2016. Bioavailability and fate of sediment-associated progesterone in aquatic systems. Environmental Science & Technology 50, 4027–4036.

Shen, X., Chang, H., Sun, D., Wang, L., Wu, F., 2018. Trace analysis of 61 natural and synthetic progestins in river water and sewage effluents by ultra-high performance liquid chromatography–tandem mass spectrometry. Water Research 133, 142–152.

Siegenthaler, P. F., Bain, P., Riva, F., Fent, K., 2017. Effects of antiandrogenic progestins, chlormadinone and cyproterone acetate, and the estrogen 17α-ethinylestradiol (EE2), and their mixtures: transactivation with human and rainbowfish hormone receptors and transcriptional effects in zebrafish (*Danio rerio*) eleuthero-embryos. Aquatic Toxicology 182, 142–162.

Sorensen, P. W., Stacey, N. E., 2004. Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. New Zealand Journal of Marine and Freshwater Research 38, 399–417.

Sumpter, J. P., 2005. Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochimica et Hydrobiologica 33, 9–16.

Sun, L., Yong, W., Chu, X., Lin, J. M., 2009. Simultaneous determination of 15 steroidal oral contraceptives in water using solid-phase disk extraction followed by high performance liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1216, 5416–5423.

Villeneuve, D. L., Blankenship, A. L., Giesy, J. P., 2000. Derivation and application of relative potency estimates based on *in vitro* bioassay results. Environmental Toxicology and Chemistry 19, 2835–2843.

Vulliet, E., Baugros, J. B., Flament-Waton, M. M., Grenier-Loustalot, M. F., 2007. Analytical methods for the determination of selected steroid sex hormones and corticosteriods in wastewater. Analytical and Bioanalytical Chemistry 387, 2143–2151.

Vulliet, E., Wiest, L., Baudot, R., Grenier-Loustalot, M. F., 2008. Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. Journal of Chromatography A 1210, 84–91.

Vulliet, E., Cren-Olivé, C., 2011. Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. Environmental Pollution 159, 2929–2934.

Vulliet, E., Cren-Olivé, C., Grenier-Loustalot, M. F., 2011. Occurrence of pharmaceuticals and hormones in drinking water treated from surface waters. Environmental Chemistry Letters 9, 103–114.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Lange, R., 2009. Effects of synthetic gestagens on fish reproduction. Environmental Toxicology and Chemistry 28, 2663–2670.

Zhao, Y., Castiglioni, S., Fent, K., 2015. Synthetic progestins medroxyprogesterone acetate and dydrogesterone and their binary mixtures adversely affect reproduction and lead to histological and transcriptional alterations in zebrafish (*Danio rerio*). Environmental Science & Technology 49, 4636–4645.

ENGLISH SUMMARY

Environmental pollutants progestins: occurrence, hormonal activities and effects on fish

Pavel Šauer

Many compounds of anthropogenic origin are continuously discharged into the sewage and some of them pass through wastewater treatment plants (WWTPs), thereby contaminating surface waters. Some of these compounds may adversely affect endocrine system of exposed biota. Progestins are an emerging group of environmental pollutants with endocrine disrupting potential. Progestins are contained e.g. in contraception and other hormonal preparations and they are consumed in high amounts worldwide. Progestins possess significant affinity to the human progesterone but also other steroid receptors, e.g. androgen receptor. Progestagenic and (anti-)androgenic activities were repeatedly detected in aquatic environments. However, some compounds that cause a substantial portion of progestagenic and (anti-)androgenic activities in waste and surface waters often remain unidentified. We suspected progestins of being responsible for these activities to a considerable extent. Thus, we aimed at discovering the potential of progestins to contribute to progestagenic and (anti-)androgenic activities in selected aquatic environments associated with municipal WWTP discharges (influent and effluent to WWTPs, and upstream and downstream in receiving water bodies), where the presence of progestins is anticipated. Another objective of this Ph.D. thesis was to investigate the effect of a synthetic progestin etonogestrel on Endler's guppies' (*Poecilia wingei*) morphology, reproduction and behaviour.

Wastewater and surface water samples were extracted on an automated solid-phase extraction (SPE) system. The presence of progestins was determined by a liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) method. Progestagenic and (anti-)androgenic activities were detected using a battery of sensitive *in vitro* bioassays (CALUX). The responsibility of progestins for the detected activities was evaluated by means of biologically and chemically derived toxicity equivalent approach.

We have monitored the occurrence of all the progestins consumed in the Czech Republic and most of them were found in wastewaters including those already treated. Progestins were present in WWTP discharges mostly at $ng \cdot l^{-1}$ or lower concentrations. Progesterone, megestrol acetate and dienogest were the most frequently detected progestins in WWTP effluents. Widespread occurrence of a natural progestin (progesterone) but only one detection of synthetic progestin (medroxyprogesterone) was observed in surface waters. Progestagenic and (anti-)androgenic activities were detected in wastewaters and surface waters. We have determined progestagenic and (anti-)androgenic relative potencies of all the progestins prescribed in one country (the Czech Republic) for the first time. As expected all progestins displayed progestagenic activities, with gestodene being the strongest and medroxyprogesterone the weakest one. Some progestins displayed relatively strong androgenic activity (0.01–0.22 fold of dihydrotestosterone) and some strong anti-androgenic acitvity (9–62 fold of flutamide).

Our results indicate that progestins are significant contributors to progestagenic activities in WWTP effluents (> 50%) and also in surface waters (up to 83%). Although they accounted to some extent for androgenic (up to 29%) and anti-androgenic (up to 27%) activities in influents, the progestins' contribution to (anti-)androgenic activities was negligible (≤ 2.1%) in effluents and surface waters. Their contribution can be expected to be higher if their

concentrations reach or exceed tens of ng · l^{-1} levels or if multiple progestins co-occur and act additively.

In the experiment with Endler's guppies exposed to etonogestrel, we found that low concentration of this compound (3.2 ng · l^{-1}) can adversely affect reproduction and mating behaviour. The high tested concentration (320 ng · l^{-1}) even led to masculinisation of female fish.

Results of this work indicate that progestins have a potential to adversely affect aquatic biota due to their relatively strong progestagenic and (anti-)androgenic activities and negative effects on fish reproduction.

CZECH SUMMARY

Environmentální polutanty progestiny: výskyt, hormonální aktivity a účinky na ryby

Pavel Šauer

Mnoho látek antropogenního původu je nepřetržitě vypouštěno do odpadních vod, některé z nich prochází čistírnami odpadních vod (ČOV) a následně kontaminují povrchové vody. Některé z těchto látek mohou narušit endokrinní systém exponovaných organizmů. Představují tudíž značné riziko zejména pro reprodukci vodních organizmů. Progestiny jsou skupina nově se objevujících endokrinně aktivních environmentálních polutantů. Jsou obsaženy například v hormonální antikoncepci a jiných hormonálních preparátech a celosvětově jsou spotřebovávány ve velkém množství. Progestiny mají velkou afinitu k progesteronovému, ale také k řadě jiných steroidních receptorů, jako je například androgenní receptor. Progestagenní, androgenní a anti-androgenní aktivity byly opakovaně detekovány ve vodním prostředí. Nicméně, látky způsobující tyto progestagenní, androgenní a anti-androgenní aktivity bývá často problém identifikovat. Předpokládali jsme, že progestiny by mohly být za tyto aktivity do značné míry odpovědné. Zaměřili jsme se proto na zjištění potenciálu progestinů způsobovat progestagenní, androgenní a anti-androgenní aktivitu v komunálních odpadních vodách (na přítoku a odtoku na/z ČOV) a v recipientu nad a pod zaústěním odtoku z ČOV, kde se předpokládá výskyt progestinů. Dalším cílem této dizertační práce bylo posoudit účinky syntetického progestinu etonogestrelu na sekundární pohlavní znaky, reprodukci a chování živorodky Wingeovi (*Poecilia wingei*).

Vzorky odpadních a povrchových vod byly extrahovány automatickým extrakčním zařízením pro extrakci na pevné fázi (SPE). Přítomnost progestinů byla měřena pomocí separace progestinů na kapalinové chromatografii a následným scanem produktů ve vysokém rozlišení tandemové hmotnostní spektrometrie s chemickou ionizací a fotoionizací za atmosférického tlaku (LC-APCI/APPI-HRPS). Progestagenní, androgenní a anti-androgenní aktivity byly detekovány pomocí baterie senzitivních *in vitro* biotestů (CALUX). Podíl progestinů na detekovaných aktivitách byl odhadnut pomocí biologických a chemických ekvivalentů toxicity.

Monitorovali jsme výskyt všech progestinů, které jsou předepisovány v České republice, a většina z nich byla detekována v komunálních odpadních vodách včetně těch již vyčištěných. Progestiny byly ve vodách vypouštěných z ČOV přítomny většinou v $ng \cdot l^{-1}$ či nižších koncentracích. Progesteron, megestrol acetát a dienogest byly nejčastěji detekovanými progestiny na odtocích z ČOV. V povrchové vodě byl často detekován přírodní progestin progesteron a ze syntetických progestinů pouze medroxyprogesteron. Progestagenní, androgenní a anti-androgenní aktivity byly detekovány v odpadních i povrchových vodách. Jako první jsme v našich experimentech stanovili relativní progestagenní, androgenní a anti-androgenní účinek (potenciál) všech progestinů, které jsou předepisovány v jedné zemi (v České republice). Podle očekávání všechny progestiny vykazovaly progestagenní aktivitu a zdaleka nejsilnějším byl gestoden, naopak nejslabší byl medroxyprogesteron. Některé progestiny měly relativně silnou androgenní aktivitu (relativní síla 0,01–0,22 vůči dihydrotestosteronu) a jiné vykazovaly velmi silnou anti-androgenní aktivitu (relativní síla 9–62 v porovnání s flutamidem).

Naše výsledky indikují, že progestiny významně přispívají k progestagenním aktivitám na odtocích z ČOV (> 50 %) i v povrchových vodách (až 83 %). Progestiny mohou do jisté míry (až 29 %) přispívat k androgenním a anti-androgenním aktivitám v přítocích na ČOV. Příspěvek progestinů k (anti-)androgenním aktivitám na odtocích z ČOV a v povrchových vodách byl zanedbatelný (≤ 2.1 %). Jejich přispění k těmto aktivitám by bylo významné, pouze pokud by se vyskytovaly v desítkách $ng \cdot l^{-1}$ či vyšších koncentracích nebo pokud by se společně vyskytovalo několik progestinů, jejichž společný efekt by byl aditivní.

V pokusu s živorodkou Wingeovou exponovanou etonogestrelu bylo zjištěno, že již jeho nízká koncentrace (3.2 $ng \cdot l^{-1}$) má negativní vliv na reprodukci a reprodukční chování těchto ryb. U samic vystavených vyšší koncentraci etonogestrelu (320 $ng \cdot l^{-1}$) byla pozorována maskulinizace.

Výsledky této práce indikují, že progestiny mají vzhledem k jejich silným progestagenním, androgenním a anti-androgenním aktivitám a negativním účinkům na reprodukci potenciál negativně ovlivnit vodní organizmy.

ACKNOWLEDGEMENTS

First and foremost I want thank to my supervisor, Dr. Hana Kocour Kroupová for her example of a passionated scientist, trust and kind attitude towards me, improving my skills I needed to tackle the complex questions in research, for allowing me freedom to be creative, and teaching me to organize my thoughts. It has been an honour to be her student.

Special thanks go also to my consultants Jitka Kolářová, DVM and Dr. Vimal Kumar, for their help and introducing me into cell culture and solid-phase extraction techniques, respectively.

I deeply thank to my family, especially to my parents Milada and Jiří, wife Eliška, and my children Kryštof and Adriana for their endless love, merciful support and overall encouragement. My gratefulness to them all goes far beyond any words of appreciation I could write. I would like to dedicate my thanks also to all the other members of my family.

Notably, I greatly appreciate support by my colleagues from the Laboratory of Aquatic Toxicology and Ichtyopathology and Laboratory of Environmental Chemistry and Biochemistry who cooperated, patiently helped me, and provided such a wonderful environment to me during the time of my study.

I thank to Dr. Ilka Lutz and Assoc. Prof. Klára Hilscherová for their willingness to evaluate my Ph.D. thesis. I would also like to express great gratitude to everyone else who helped me.

I also appreciate the financial support from the following projects that funded parts of the research discussed in this dissertation:

- the Ministry of Education, Youth and Sports of the Czech Republic – projects 'CENAKVA' (No. CZ.1.05/2.1.00/01.0024), 'CENAKVA II' (No. LO1205 under the NPU I program)
- the Czech Science Foundation (project No. 16-09709Y)
- the Grant Agency of the University of South Bohemia in České Budějovice (No. 012/2016/Z, No. 092/2016/Z, and No. 056/2017/Z)

LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Steinbach, C., Císař, P., **Šauer, P.,** Klicnarová, J., Schmidt-Posthaus, H., Golovko, O., Kocour Kroupová, H., 2019. Synthetic progestin etonogestrel negatively affects mating behaviour and reproduction in Endler's guppies (*Poecilia wingei*). Science of the Total Environment 663, 206–215. (IF 2017 = 4.610)

Tumová, J., **Šauer, P.,** Golovko, O., Koba Ucun, O., Grabic, R., Máchová, J., Kocour Kroupová, H., 2019. Effect of polycyclic musk compounds on aquatic organisms: A critical literature review supplemented by own data. Science of the Total Environment 651, 2235–2246. (IF 2017 = 4.610)

Golovko, O., **Šauer, P.,** Fedorova, G., Kocour Kroupová, H., Grabic, R., 2018. Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS. Science of the Total Environment 621, 1066–1073. (IF 2017 = 4.610)

Kocour Kroupová, H., Valentová, O., Svobodová, Z., **Šauer, P.**, Máchová, J., 2018. Toxic effects of nitrite on freshwater organisms: a review. Reviews in Aquaculture 10, 525–542. (IF 2017 = 7.139)

Šauer, P., Bořík, A., Golovko, O., Grabic, R., Vojs Staňová, A., Valentová, O., Stará, A., Šandová, M., Kocour Kroupová, H., 2018. Do progestins contribute to (anti-)androgenic activities in aquatic environments? Environmental Pollution 242, 417–425. (IF 2017 = 4.358)

Šauer, P., Stará, A., Golovko, O., Valentová, O., Bořík, A., Grabic, R., Kocour Kroupová, H., 2018. Two synthetic progestins and natural progesterone are responsible for most of the progestagenic activities in municipal wastewater treatment plant effluents in the Czech and Slovak republics. Water Research 137, 64–71. (IF 2017 = 7.051)

National and international conferences

Šauer, P., Grabic, R., Golovko, O., Bořík, A., Stará, A., Valentová, O., Vojs Staňová, A., Šandová, M., Kocour Kroupová, H., 2018. Unravelling the potential of progestins to contribute to (anti-)androgenic and (anti-)progestagenic activities in aquatic environments. 11th BioDetectors International Toxicology Workshop, Aachen, Germany.

Kocour Kroupová, H., **Šauer, P.,** Tumová, J., Steinbach, C., Golovko, O., Máchová, J., Komen, H., Profant, V., Grabic, R., 2017. Simultaneous exposure to environmentally relevant concentrations of drospirenone and gestodene caused intersex in common carp, *Cyprinus carpio* L. 6th International Workshop on the Biology of Fish Gametes, September 4th–7th, 2017, Vodňany, Czech Republic, p. 90.

Steinbach, C., Císař, P., **Šauer, P.,** Prokopová, I., Máchová, J., Grabic, R., Golovko, O., Kocour Kroupová, H., 2017. Effect of synthetic progestin etonogestrel on reproduction and morphometric parameters of Endler's guppies (*Poecilia wingei*). Toxicita a biodegradabilita odpadů a látek významných ve vodním prostředí 2017, 23. – 25.8. 2017, Vodňany, Sborník abstraktů, s. 28.

Šauer, P., Stará, A., Bořík, A., Valentová, O., Šandová, M., Golovko, O., Grabic, R., Kocour Kroupová, H., 2017. Podíl syntetických progestinů na androgenní a antiandrogenní aktivitě ve vodním prostředí. Toxicita a biodegradabilita odpadů a látek významných ve vodním prostředí 2017, 23. – 25.8. 2017, Vodňany, Sborník abstraktů, s. 29.

Šauer, P., Tumová, J., Steinbach, C., Golovko, O., Máchová, J., Komen, H., Profant, V., Grabic, R., Kocour Kroupová, H., 2017. Effect of environmentally relevant concentrations of drospirenone and gestodene on sex differentiation in common carp (*Cyprinus carpio* L.). Aquaculture Europe 2017, 17–20 October, Dubrovnik, Croatia, p. 603 – 604.

Sauer, P., Stara, A., Kocour Kroupova, H., 2016. Efficiency of municipal wastewater treatment plants in removal of progestagenic activity. In: Book of abstracts from the Interdisciplinary toxicology conference TOXCON 2016, June 22–24, 2016, Stara Lesna, Slovakia, s. 59.

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Pavel Šauer
Research department	2014–2019 – Laboratory of Aquatic Toxicology and Ichthyopathology of FFPW
Supervisor	Hana Kocour Kroupová, Ph.D.
Period	29th September 2014 until 26th March 2019

Ph.D. courses	**Year**
Applied hydrobiology	2015
Basics of scientific communication	2015
Biostatistics	2015
Aquatic toxicology	2016
Ichthyology and fish systematics	2016
English language (First Certificate in English – FCE)	2017

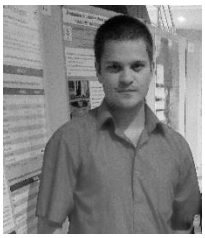
Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2015 2016 2017 2018

International conferences	**Year**
Sauer, P., Stara, A., Kocour Kroupova, H., 2016. Efficiency of municipal wastewater treatment plants in removal of progestagenic activity. In: Book of abstracts from the Interdisciplinary toxicology conference TOXCON 2016, June 22–24, 2016, Stara Lesna, Slovakia, s. 59.	2016
Šauer, P., Tumová, J., Steinbach, C., Golovko, O., Máchová, J., Komen, H., Profant, V., Grabic, R., Kocour Kroupová, H., 2017. Simultaneous exposure to environmentally relevant concentrations of drospirenone and gestodene caused intersex in common carp, *Cyprinus carpio* L. 6th International Workshop on the Biology of Fish Gametes, September 4th–7th, 2017, Vodňany, the Czech Republic, p. 90.	2017
Šauer, P., Tumová, J., Steinbach, C., Golovko, O., Máchová, J., Komen, H., Profant, V., Grabic, R., Kocour Kroupová, H., 2017. Effect of environmentally relevant concentrations of drospirenone and gestodene on sex differentiation in common carp (*Cyprinus carpio* L.). Aquaculture Europe 2017, 17–20 October, Dubrovnik, Croatia, p. 603–604.	2017

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Dr. Ilka Lutz, Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany. (8 weeks, carrying out an *in vivo* bioassay to test chronic exposure of salts on *Rutilus rutilus*)	2015
Prof. Abraham Brouwer, Prof. Peter A. Behnisch, Dr. Harrie Besselink, Dr. Emiel Felzel and Dr. Kees Swart, BioDetection Systems b.v., Amsterdam, The Netherlands. (1 week, training course for performing CALUX technology for detection of hormonal activities of pure compounds, their mixtures and in water extratcs)	2016
Dr. Selim Aït-Aïssa, Institut national de l'environment industriel et des risques (INERIS), Verneuil-en-Halatte, France. (4 weeks, determination of (anti-)estrogenic activities of synthetic progestins using *in vitro* reporter gene bioassays based on zebrafish estrogen receptors α and β2)	2018

Pedagogical activities	Year
Leading of project entitled Detection of (anti-)androgenic activities in the aquatic environment at Summer school	2017
Anouncing the project entitled Screening for hot spots of anti-androgenic pollution in surface waters at Summer school	2018
Lecturing of students of bachelor study, discipline Fishery at USB FFPW in range of 90 teaching hours	2015–2018

CURRICULUM VITAE



PERSONAL INFORMATION

Name: Pavel
Surname: Šauer
Title: Dipl.-Ing.
Born: 24th May, 1990, České Budějovice, the Czech Republic
Nationality: Czech
Languages: English (B2 level – FCE certificate), Czech (native speaker)
Contact: psauer@frov.jcu.cz

EDUCATION

2014 – present	Ph.D. student in Fisheries, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, the Czech Republic
2012–2014	Dipl.-Ing. in Fisheries, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, the Czech Republic
2009–2012	B.Sc., in Fisheries, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, the Czech Republic

COMPLETED COURSES

Aquatic toxicology, Applied hydrobiology, Basics of scientific communication, Biostatistics, English language, Ichthyology and fish systematics

TRAINING

07/03–11/03 2016	Training to carry out CALUX bioassays for detection of hormonal activity of pure compounds and water extracts

RESEARCH STAY AND COLLABORATIONS

25/10–18/12 2015	Dr. Ilka Lutz, Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Germany
1/07–28/07 2018	Dr. Selim Aït-Aïssa, Institut national de l'environment industriel et des risques (INERIS), France