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Establishment of Analytical Methods to Assess Endocrine Disrupting Compounds in a Swedish Wastewater Treatment Plant: Water Sampling using the Novel TIMFIE Device.

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Establishment of Analytical Methods to Assess Endocrine Disrupting Compounds in a Swedish Wastewater Treatment Plant: Water Sampling using the Novel TIMFIE Device.

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Popular Science Summary

Steroid hormones – curse and blessing

Imagine one drop of food colouring in 70 million litres of water. This low concentration equals one nanogram per litre, which is the level most steroid hormones are effective at. Steroids are produced and excreted by our bodies and are in charge of our hormonal processes, for example the development of sex organs, menstrual cycle and sperm production. But they are also produced synthetically and commonly used as an effective growth indicator for aquaculture and agriculture or hormonal contraception such as contraceptive pills, IUDs and implants. When excreted by our bodies, these steroids end up in wastewater. Unfortunately, many studies have shown that common wastewater treatment plants fail to fully remove these chemicals, and they are potentially released to rivers and lakes. There, they interfere in hormonal processes of fish, amphibian and mammal species and thus pose a big threat to ecosystems. Worst of all, even unimaginably low concentrations, which are difficult to detect, can lead to these effects.

This study developed a sensitive measurement technique, which can detect and quantify such low concentrations. With this method, levels of eleven natural and synthetic steroids were measured in the inlet and outlet of a Swedish wastewater treatment plant. All out of the tested steroids were found in the inlet water, while five steroids were found in the outlet water. This study indicates an insufficient removal of most tested steroid hormones and stresses the need of a steroid screening in Swedish freshwaters.

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Table of contents

List of figures

List of tables

List of abbreviations

ACN	Acetonitrile
AS	Auto Sampler
CI	Chemical Ionization
CV	Coefficient of variation
DIE	Dienogest
E1	Estrone
E2	17β - Estradiol
E3	Estriol
EDC	Endocrine Disrupting Compound
EE2	17α - Ethinylestradiol
EW	Effluent Water
EI	Electron Impact Ionization
EPA	Environmental Research Institute
ESI	Electrospray Ionization
ETO	Etonogestrel
GC	Gas Chromatography
GC-MS/MS	Gas Chromatography – Tandem Mass Spectrometry
GES	Gestodene
He	Helium
HESI	Heated electrospray ionization
IDL	Instrumental Detection Limit
IQL	Instrumental Quantification Limit
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry

LNGT	Levonorgestrel
LOEC	Lowest observed effect concentration
MeOH	Methanol
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MRM	Multiple reaction monitoring
NGT	Norgestrel
NOR	Norethindrone
PCOS	Poly Cystic Ovarian Syndrome
PGR	Progesterone
PI	Photoionization
POCIS	Polar Organic Chemical Integrative Sampler
SD	Standard Deviation
S/N	Signal to Noise Ratio
SPE	Solid Phase Extraction
SPE-LC-MS/MS	Solid Phase Extraction coupled with Liquid Chromatography Tandem Mass Spectrometry
SRM	Selected reaction monitoring
SW	Surface Water
TIMFIE	Time-integrated, MicroFlow, In-line Extraction
TTR	Testosterone
WWTP	Wastewater Treatment Plant

1 Introduction

1.1. Endocrine disrupting compounds

Water is the most valuable resource on this planet, crucial for biochemical processes in all living organisms and indispensable for human activities in domestic households, agriculture or industry. While a high quality of water is essential for ecosystems and humans, pollution of water through contaminants can result in devastating ecological and health related effects. The increasing use and disposal of chemicals from agriculture, industry, medicine and households leads to contamination of fresh water with organic substances (Carlsson et al., 2006; Kolpin et al., 2002), such as micropollutants (Ribeiro et al., 2015). These micropollutants can be found at levels between ng/L and µg/L (Jurado et al., 2012), and leak out into freshwater through agriculture, livestock and aquaculture runoff and industrial, domestic and hospital effluents (Luo et al., 2014; Mompelat et al., 2009).

Part of the many micropollutants are steroid hormones (Jurado et al. 2012), some of which are considered endocrine disrupting compounds (EDCs). EDCs can be divided into natural substances, such as phytoestrogens, and human or industrial products, including agricultural chemicals, plastics and plasticizers and pharmaceutical hormones that stem from contraceptives (Hampl et al., 2016).

Due to their resemblance to natural hormones, EDCs can have hazardous effects on the human body (Gibson and Saunders, 2014; Giulivo et al., 2016; Sousa et al., 2018), and intervene in biochemical processes which require hormones, such as hormone biosynthesis, cell transport, metabolism and gene expression (Hampl et al., 2016; Vandenberg et al., 2012). Main issues include adverse effects on reproduction systems (Gallo et al., 2016; Sheikh et al., 2016) such as on the menstrual cycle and fertility (Bloom et al., 2016; Gallo et al., 2016) and possibly a decrease in semen quality (Joffe, 2001; Toft et al., 2012). Further, endocrine disruptors have been associated with polycystic ovarian syndrome (PCOS) (Tarantino et al., 2013), breast cancer (Soto and Sonnenschein, 2015) as well as testicular and prostate cancer (Daston et al., 1997).

Besides health related effects, EDCs contribute to several environmental problems such as the dysfunction of sexual organs in mammals and feminization of male embryos in several mammal, bird and fish species (reviewed by Lintelmann et al., 2003).

1.2. Steroid hormones

One group of EDCs are steroid hormones. There are more than 1500 different steroid hormones that have either been isolated from biological sources or produced synthetically (Andersson, 2008). Three of the main groups are estrogens (female sex steroids), androgens (male sex steroids) and progestins, which all derive from cholesterol (Norman, 2003). Since they all stem from the same molecule and might differ only in the position of the hydroxyl group, they are particularly challenging to analyze (French, 2017). Progestins play a crucial role in reproduction, estrogens are involved in the development of female secondary sexual characteristics, and androgens induce male secondary sexual characteristics (Norman 2003). Most common in wastewater are the natural estrogens Estrone (E1), 17β-estradiol (E2) and Estriol (E3), and the synthetic Estrogen 17α-Ethynylestradiol (EE2), which is commonly used as an oral contraceptive (Racz and Goel, 2010).

Sources of steroid estrogens are discharge of manure and urine from livestock (Soto et al., 2004), aquaculture effluents (Kolodziej et al., 2004), and discharge of treated wastewater from wastewater treatment plants (WWTPs), since traces of steroids can still be present in treated water (Silva et al., 2012). All these effluent wastewaters lead to steroids accumulating in ecosystems, threatening the environment and human health. For instance, approximately 17% of women in western countries use EE2 as a contraceptive measure. Around 6 μg of EE2 per day per person are excreted into the wastewater system through urine and feces (Johnson and Williams, 2004), which adds up to a total discharge of 4.4. kg of EE2 per one million inhabitants within one year (Combalbert and Hernandez-Raquet, 2010).

In order to identify ways of assessing and removing micropollutants such as steroid hormones, it is crucial to understand their physical-chemical properties such as water solubility (S_w), sorption coefficient (K_d) and the octanol/water partition coefficient (K_{ow}). In general, all four estrogens show a low S_w value, low volatility, a high K_{ow} value and low biodegradability (Silva et al., 2012). Substances with high K_{ow} tend to be more hydrophobic and usually have a high sorption potential (K_d).

1.3. Environmental assessment

1.3.1. Current policy on EDCs and measurement standards

Despite the potentially hazardous effects on the human body and ecosystems, there are no legal treaties within the EU to limit the discharge of most steroid hormones. Monitoring guidelines are only provided for some directives. The European Union Directive 2013/39/EU recommends to include EE2 and E2 on the watch list for European monitoring (Directive 2013/39/EU). The Commission Implementing Decision (EU) 2018/840 of 5 June 2018, amending Decision 2015/495/EU, added the steroid hormone E1 to the watch list as well (Decision (EU) 2018/840) and recommends a maximum acceptable method detection limit of 0.035 ng/L and 0.4 ng/L for EE2 and E1/E2 respectively (Decision (EU) 2018/840).

Since low levels of steriod hormones can exhibit endocrine malfunctions of aquatic organisms, it is crucial to assess trace amounts of these substances in water bodies The European Commission recommends liquid chromatography tandem mass spectrometry (LC-MS/MS) for the assessment of E1, E2 and EE2 (Decision (EU) 2018/840), and the Joint Research Center conducted a study to assess these estrogens in surface water (SW) with SPE-LC-MS/MS. The results indicate that this method was eligible to assess low levels of these steroid hormones (Tavazzi et al., 2016). However, according to some studies, current chemical analytical methods are not sufficient to quantify E2 and EE2 at environmental concentrations (Könemann et al., 2018). So far, there is no universal, standardized analytical method to analyze at the same time a broad range of progestins, androgens and estrogens

commonly used in Sweden. The next section will give an overview of the most recent studies (2004 - 2018), which aimed to develop analytical methods for assessing steroid hormones. Steroid hormones have usually been analyzed through immunoassays, Gas Chromatography (GC) paired with Mass Spectrometry (MS) or Liquid Chromatography (LC) paired with MS (French, 2017). Due to the numerous drawbacks of immunoassays such as a high cross-reactivity, GC and LC paired with MS or Tandem Mass Spectrometry (MS/MS) are becoming more popular to measure E2 and EE2 in biological matrices (Barreiros et al., 2016). Both GC-MS/MS and LC-MS/MS provide a high sensitivity and selectivity to analyze multiple compounds at once for biological samples (F. Zhang et al., 2011). However, when assessing compounds, sample matrix can result in a major impact on measurement results (Gabet et al., 2007). This is one of the important factors when determining levels of steroid hormones in environmental matrices such as SW or wastewater, posing analytical challenges.

1.3.2. Sampling and extraction

There are different advantages and limitations in active and passive water sampling methods. While active grab sampling represents a low-cost assessment method, it may be insufficient to take into account variations in water flow and precipitation events (Xing et al., 2013). Passive sampling is beneficial due to low costs and no need of electricity (reviewed by Lai et al., 2019). The passive Polar Organic Chemical Integrative Sampler (POCIS) has proven to be particularly suitable to accumulate highly hydrophobic substances such as steroid hormones (Morin et al., 2013). With POCIS, pre-concentration can be conducted simultaneously, and since it samples water over a long period of time, contaminants, which otherwise would be undetectable, can now be accumulated and measured (Alvarez et al., 2004). In POCIS, water samples need to pass five compartments: [1] an external water boundary layer, [2] a layer adjacent to the bulk water environment, [3] a membrane, which is permeable for specific substances, [4] a boundary layer and [5] a sorbent phase. The sorbent phase can concentrate hydrophobic substances continuously over a long period of time,

which allows temporal concentration fluctuations to be taken into account and delivers the time-weighted average concentration of a substance (Alvarez et al., 2004; Morin et al., 2013). The way in which the specific target compounds pass all the compartments and the sorbent phase gives hints about the rate at which they accumulate in biological matrices (Bartelt-Hunt et al., 2011). When sampling passively, the water temperature can influence the sampling rate (Vrana et al., 2005).

Besides many advantages of passive samplers in contrast to active grab sampling, the exact volume of the water sample taken must be calculated indirectly. The newly developed low cost and power-independent active sampling method TIMFIE solves this drawback. The Time-Integrated, MicroFlow, In-line Extraction (TIMFIE) extracts a wide range of pesticides continuously under field conditions (Jonsson et al., 2019). It combines active sampling and in-field extraction, by actively pumping whole water through an SPE cartridge into a syringe where the final sample volume is measured for a quantitative determination (Jonsson et al., 2019).

1.3.3. Liquid and gas chromatography sequenced with mass spectrometry

Chromatography is a technique of separation of components into the stationary phase and the mobile phase, which moves in a defined direction (IUPAC, 2014). In GC, separation of the analytes occurs while the compounds carried in a gaseous phase (helium or nitrogen) interact with a stationary phase at the inside of the column. The separation is based on the analytes boiling point and vapor pressure, which depends on the compounds polarity and molecular weight (Bachmann and French, 2017; French, 2017; Stauffer et al., 2008). In order to minimize matrix effects, samples must be extracted and cleaned up before undergoing GC. This may for example include Solid Phase Extraction (SPE) (Bachmann and French, 2017). Since hormones have high polarity and low volatility, hormones need to be derivatized (Barreiros et al., 2016) in order to obtain chromatographic separation and

sensitivity of the analysis using GC-MS/MS (Díaz-Cruz et al., 2003). Agents used for derivatization for GC can for example be TMCS (Ronderos-Lara et al., 2018), BSTFA, MTBSTFA, MSTFA, TMSI, DTE, heptafluorobutyric anhydride, pentafluoropropionic acid, and acetic anhydride (reviewed by Alda and Barceló, 2001).

Since derivatization is time consuming and might lead to analyte loss due to insufficient derivatization (Fayad et al., 2013), LC-MS has slowly become the most common detection tool for hormones (Barreiros et al., 2016; Díaz-Cruz et al., 2003). LC separates compounds in a liquid mixture based on the polarity and the interaction of the compounds with the chromatography column (French, 2017). Compared to GC it provides the possibility to assess a wider range of analytes, including polar and nonvolatile substances. Further, preparation of samples is less extensive, since derivatization is not necessarily required and the analytes do not need to be volatile (Bachmann and French, 2017). Sensitivity of this analytical method depends on the quality of sample preparation, the efficiency of chromatography, and sensitivity of the mass spectrometer (Bachmann and French, 2017). LC coupled with MS/MS is the most common method used to analyze steroid hormones in water matrices, however, due to lower costs and wider availability, GC-MS has also been commonly used (reviewed by Z. Liu et al., 2011) (see Table 1).

1.3.4. Mass spectrometry and tandem mass spectrometry

Mass Spectrometry is a useful analytical tool because it does not only give quantitative information about a sample, but provides qualitative data about structure and composition of chemical compounds (Stauffer et al., 2008). The analysis consists of four basic steps, including [1] electron ionization, [2] ion separation according to mass-to-charge ratio, [3] ion detection and measurement of their abundance and [4] procession of electronic signals (Hoffmann and Stroobant, 2007). Through ionization, compound molecules become charged. The method through which ions are produced is crucial, because different approaches can affect the mass spectrum. Common ionization techniques used by GC-MS is

electron impact ionization (EI), and chemical ionization (CI). For LC-MS electrospray ionization (ESI) is commonly used (Lifshitz and Märk, 2017). During ESI, gas-phase ions are produced from highly charged droplets, which are generated through a liquid atomization process (Tang et al., 2017). The sensitivity of ESI-MS depends on ionization efficiency, which is the efficiency in generating gas-phase ions from the droplets, and ion transmission efficiency, which is the transport from gas-phase ions to the MS analyzer (Tang et al. 2017). After ionization, the analytes are evaluated and detected in the Mass Spectrometer according to their mass-to-charge ratio (m/z) and their abundance (%) (Hoffmann and Stroobant, 2007), which is usually presented in form of a mass spectrum (Stauffer et al., 2008). Ionized molecules can undergo a fragmentation process, the different fragments can give information about nature and structure of their precursor molecule (Hoffmann and Stroobant, 2007). Detection of ions takes place through a deflection by an electric and magnetic field. The weight and charge of the fragment determines how much it will be deflected (Hoffmann and Stroobant, 2007). In order to validate MS, parameters matrix effect and extraction recovery have to be determined (French, 2017). MS/MS in contrast to MS provides a more selective detection and analysis of compounds, because two mass analyses are coupled sequentially with a collision cell in between. After the first MS, so called 'precursor ions' are selected and fragmented. The ions formed during this fragmentation are called 'product ions'. Product ions are then measured by the detector (Lynch, 2017).

1.4. Occurrence of steroid hormones in wastewater and surface water

Many studies that assess levels of steroid hormones in SW, IW and EW have been conducted in the past 20 years, some of which are listed in Table 1. Most prominent method used is LC-MS/MS, however, even in relatively recent studies, GC-MS or GC-MS/MS has been used as an analytical technique. The measurement values differ immensely. For instance, one and the same study by Labadie and Budzinski, 2005 found average levels E1 in

effluents of 30 ng/L in one WWTP and an average of 1.9 for the same compound in a different WWTP.

Table 1: List of Studies that assessed hormones (i.e. E1, E2, EE2, E3, ETO, GES, DIE, NGT, NOR, PGR, TTR) in influent water, effluent water and surface water.

Hormone	Matrix	Analytical Method	Country	Mean Concentration [ng/L]	Study
E1	Influent	LC-ESI-MS/MS	France	23	Salvador et al., 2007
	Influent	UPLC-ESI-MS/MS	Spain	20 - 40	Guedes-Alonso et al. 2013
	Influent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Influent	LC-MS/MS	Italy	35	Laganà et al., 2004
	Influent	LC-MS/MS	China	80	Cui et al., 2006
	Influent	GC-MS	Canada	30	Lishman et al., 2006
	Influent	HPLC-MS/MS	Sweden	3.0–70	Zorita et al. 2009
	Influent	GC-MS	China	10–35	Z. Zhang et al., 2011
	Influent	HPLC–MS/MS	Korea	47	Behera et al. 2011
	Influent	HPLC-MS/MS	Korea	29	Sim et al. 2011
	Influent	HPLC-MS/MS	Spain	–	Martin et al. 2012
	Influent	LC-ESI-MS/MS	China	6.5–19	Chang et al., 2010
	Influent	GC-MS	Australia	13	Tan et al. 2007
	Influent	GC-MS	Canada	11–370	Atkinson et al. 2012
	Influent	ELISA	South Africa	84	Manickum and John 2014
	Influent	GC-MS	Iran	11	Mohagheghian et al. 2014
	Influent	GC-MS/MS	USA	41	Esperanza et al. 2007
	Influent	GC-MS	UK	20–60	Zhang and Zhou 2008
	Influent	LC-ESI-MS/MS	Turkey	31	Muz et al. 2012
	Influent	RIA	South Africa	48	Surujlal-Naicker and Bux, 2013
	Influent	GC-MS	Brazil	570	Pessoa et al. 2014
	Influent	LC-MS/MS	Japan	29	Hashimoto et al. 2007
	Influent	GC-MS	China	42–110	Ye et al. 2012
	Influent	GC-MS	China	130	Huang et al., 2014
	Influent	YES	Canada	49	Servos et al. 2005
	Influent	UPLC–MS–MS	UK	51	Kumar et al. 2011

	Influent	LC-MS/MS	China	8.7 ± 7.5	Chang et al., 2010
	Influent	RRLC-MS/MS	China	22 ± 0.7	(S. Liu et al., 2011a)
E1	Effluent	LC-MS/MS	China	8.6	Chang et al., 2010
	Effluent	GC-MS	France	30; 1.9	Labadie and Budzinski, 2005
	Effluent	LC-ESI-MS/MS	France	9.7; 8.8	Salvador et al. 2007
	Effluent	LC-ESI-MS/MS	Taiwan	26	Chen et al. 2007
	Effluent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Effluent	RRLC-MS/MS	China	8.5 ± 0.4	Liu et al. 2011a
E1	Surface Water	GC-MS	France	10; 2.0	Labadie and Budzinski, 2005
	Surface Water	LC-APPI-MS/MS	Japan	Not readable	Yamamoto et al. 2006
	Surface Water	LC-ESI-MS/MS	Taiwan	35	Chen et al. 2007
	DWTP	LC-ESI-MS/MS	Spain	–	Rodriguez-Mozaz et al., 2004
	Influent	LC-ESI-MS/MS	France	5.5	Salvador et al. 2007
	Influent	UPLC-ESI-MS/MS	Spain	20 - 40	Guedes-Alonso et al. 2013
	Influent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Influent	LC-MS/MS	Italy	25	Lagana et al. 2004
	Influent	LC-MS/MS	China	85	Cui et al. 2006
	Influent	GC-MS	Canada	8.3	Lishman et al. 2006
	Influent	HPLC-MS/MS	Sweden	2.5–9.2	Zorita et al. 2009
	Influent	GC-MS	UK	5	Koh et al. 2007
	Influent	HPLC–MS/MS	China	47–93	Z. Zhang et al., 2011
E2	Influent	HPLC-MS/MS	Korea	4	Behera et al. 2011
	Influent	HPLC-MS/MS	Korea	–	Sim et al. 2011
	Influent	LC-ESI-MS/MS	Spain	–	Martin et al. 2012
	Influent	GC-MS	China	0.9–3.8	Chang et al., 2010
	Influent	GC-MS	Australia	17	Tan et al. 2007
	Influent	ELISA	Canada	–	Atkinson et al. 2012
	Influent	GC-MS	South Africa	120	Manickum and John 2014
	Influent	GC-MS/MS	Iran	3	Mohagheghian et al. 2014
	Influent	GC-MS	USA	41	Esperanza et al. 2007
	Influent	LC-ESI-MS/MS	UK	26–51	Zhang and Zhou 2008

	Influent	RIA	Turkey	–	Muz et al. (2012)
	Influent	GC-MS	South Africa	43	Surujlal-Naicker and Bux 2013
	Influent	LC-MS/MS	Brazil	140	Pessoa et al. 2014
	Influent	GC-MS	Japan	12	Hashimoto et al. 2007
	Influent	GC-MS	China	7.4–33	Ye et al. 2012
	Influent	YES	China	31	Huang et al. 2014
	Influent	UPLC–MS–MS	Canada	16	Servos et al. 2005
	Influent	RRLC-MS/MS	China	–	Liu et al. 2011a
	Effluent	GC-MS	France	1.2	Labadie and Budzinski, 2005
	Effluent	LC-ESI-MS/MS	France	0.5	Salvador et al. 2007
	Effluent	LC-ESI-MS/MS	Taiwan	23	Chen et al. 2007
E2	Effluent	UPLC-ESI-MS/MS	Spain	–	Guedes-Alonso et al. 2013
	Effluent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Effluent	RRLC-MS/MS	China	–	Liu et al. 2011a
	Surface Water	GC-MS	France	1.1	Labadie and Budzinski, 2005
	Surface Water	LC-APPI-MS/MS	Japan	Not readable	Yamamoto et al. 2006
E2	Surface Water	LC-ESI-MS/MS	Taiwan	14	Chen et al. 2007
	Surface Water	LC-MS/MS	Hungary	N.d.–5.2	Avar et al. 2016
	Influent	UPLC-ESI-MS/MS	Spain	100 - 140	Guedes-Alonso et al. 2013
	Influent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Influent	LC-MS/MS	Italy	31	Lagana et al. 2004
	Influent	LC-MS/MS	China	73	Cui et al. 2006
	Influent	GC-MS	Canada	–	Lishman et al. 2006
	Influent	HPLC-MS/MS	Sweden	–	Zorita et al. 2009
E3	Influent	GC-MS	UK	50	Koh et al. 2007
	Influent	HPLC–MS/MS	China	50 - 120	Z. Zhang et al., 2011
	Influent	HPLC-MS/MS	Korea	420	Behera et al. 2011
	Influent	HPLC-MS/MS	Korea	380	Sim et al. 2011
	Influent	LC-ESI-MS/MS	Spain	830	Martin et al. 2012
	Influent	GC-MS	China	–	Chang et al., 2010
	Influent	GC-MS	Australia	110	Tan et al. 2007

	Influent	ELISA	Canada	–	Atkinson et al. 2012
	Influent	GC-MS	South Africa	5	Manickum and John 2014
	Influent	GC-MS/MS	Iran	–	Mohagheghian et al. 2014
	Influent	GC-MS	USA	14	Esperanza et al. 2007
	Influent	LC-ESI-MS/MS	UK	–	Zhang and Zhou 2008
	Influent	RIA	Turkey	–	Muz et al. 2012
	Influent	GC-MS	South Africa	0.1	Surujlal-Naicker and Bux 2013
	Influent	LC-MS/MS	Brazil	–	Pessoa et al. 2014
	Influent	GC-MS	Japan	160	Hashimoto et al. 2007
	Influent	GC-MS	China	110 - 850	Ye et al. 2012
	Influent	YES	China	49	Huang et al. 2014
	Influent	UPLC–MS–MS	Canada	–	Servos et al. 2005
	Effluent	GC-MS	France	1	Labadie and Budzinski, 2005
	Effluent	LC-ESI-MS/MS	Taiwan	45	Chen et al. 2007
E3	Effluent	UPLC-ESI-MS/MS	Spain	–	Guedes-Alonso et al. 2013
	Effluent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Surface Water	GC-MS	France	1.5	Labadie and Budzinski, 2005
	Surface Water	LC-APPI-MS/MS	Japan	Not readable	Yamamoto et al. 2006
E3	Surface Water	LC-ESI-MS/MS	Taiwan	19	Chen et al. 2007
	DWTP	LC-ESI-MS/MS	Spain	–	Rodriguez-Mozaz & Alda 2004
	Influent	LC-ESI-MS/MS	France	1.5	Salvador et al. 2007
	Influent	UPLC-ESI-MS/MS	Spain	20 - 40	Guedes-Alonso et al. 2013
	Influent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Influent	LC-MS/MS	Italy	–	Lagana et al. 2004
	Influent	LC-MS/MS	China	160	Cui et al. 2006
EE2	Influent	GC-MS	Canada	–	Lishman et al. 2006
	Influent	HPLC-MS/MS	Sweden	–	Zorita et al. 2009
	Influent	GC-MS	UK	1.2	Koh et al. 2007
	Influent	HPLC–MS/MS	China	–	Z. Zhang et al., 2011
	Influent	HPLC-MS/MS	Korea	–	Behera et al. 2011
	Influent	HPLC-MS/MS	Korea	–	Sim et al. 2011

	Influent	LC-ESI-MS/MS	Spain	150	Martin et al. 2012
	Influent	GC-MS	China	–	Chang et al., 2010
	Influent	GC-MS	Australia	–	Tan et al. 2007
	Influent	ELISA	Canada	–	Atkinson et al. 2012
	Influent	GC-MS	South Africa	30	Manickum and John 2014
	Influent	GC-MS/MS	Iran	6.2	Mohagheghian et al. 2014
	Influent	GC-MS	USA	39	Esperanza et al. 2007
	Influent	LC-ESI-MS/MS	UK	0.8–10	Zhang and Zhou 2008
	Influent	RIA	Turkey	–	Muz et al. 2012
	Influent	GC-MS	South Africa	–	Surujlal-Naicker and Bux 2013
	Influent	LC-MS/MS	Brazil	420	Pessoa et al. 2014
	Influent	GC-MS	Japan	–	Hashimoto et al. 2007
	Influent	GC-MS	China	8.6–45	Ye et al. 2012
	Influent	YES	China	13	Huang et al. 2014
	Influent	UPLC–MS–MS	Canada	–	Servos et al. 2005
	Effluent	GC-MS	France	< 3.0	Labadie and Budzinski, 2005
	Effluent	LC-ESI-MS/MS	France	0.9	Salvador et al. 2007
EE2	Effluent	LC-ESI-MS/MS	Taiwan	15	Chen et al. 2007
	Effluent	UPLC-ESI-MS/MS	Spain	–	Guedes-Alonso et al. 2013
	Effluent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Surface Water	GC-MS	France	<1.8	Labadie and Budzinski, 2005
	Surface Water	LC-APPI-MS/MS	Japan	Not readable	Yamamoto et al. 2006
EE2	Surface Water	LC-ESI-MS/MS	Taiwan	23	Chen et al. 2007
	Surface Water	LC-ESI-MS/MS	Czech Republic		Matejícek & Kubán 2007
	Surface Water	LC-MS/MS	Hungary	n.d.–0.68	Avar et al. 2016
	DWTP	LC-ESI-MS/MS	Spain	–	Rodriguez-Mozaz & Alda 2004
NOR	Influent	LC-APCI/APPI-HRPS	Czech Republic	0.85	Golovko et al. 2018
NOR	Effluent	LC-APCI/APPI-HRPS	Czech Republic	<0.06	Golovko et al. 2018
	Effluent	GC-MS	France	<5.0	Labadie and Budzinski, 2005
NOR	Surface Water	GC-MS	France	<2.5	Labadie and Budzinski, 2005

	Surface Water	UPLC-MS/MS	China	0.11 - 0.78	Shen et al. 2018
PGR	Influent	LC-MS/MS	China	66 ± 36	Chang et al., 2010
	Influent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Influent	LC-APCI/APPI-HRPS	Czech Republic	110	Golovko et al. 2018
	Influent	RRLC-MS/MS	China	5.4 ± 0.6	Liu et al. 2011a
	Effluent	GC-MS	France	<5.0	Labadie and Budzinski, 2005
	Effluent	LC-MS/MS	China	2.3	Chang et al., 2010
PGR	Effluent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Effluent	LC-APCI/APPI-HRPS	Czech Republic	0.95	Golovko et al. 2018
	Effluent	RRLC-MS/MS	China	–	Liu et al. 2011a
	Surface Water	GC-MS	France	<2.5	Labadie and Budzinski, 2005
PGR	Surface Water	UPLC-MS/MS	China	0.14 - 4.5	Shen et al. 2018
DIE	Influent	LC-APCI/APPI-HRPS	Czech Republic	1.9	Golovko et al. 2018
DIE	Effluent	LC-APCI/APPI-HRPS	Czech Republic	0.14	Golovko et al. 2018
GES	Influent	LC-APCI/APPI-HRPS	Czech Republic	7.7	Golovko et al. 2018
GES	Effluent	LC-APCI/APPI-HRPS	Czech Republic	1.7	Golovko et al. 2018
	Surface Water	LC-ESI-MS/MS	Czech Republic		Matějíček and Kubáň, 2007
GES	Surface Water	UPLC-MS/MS	China	0.61 - 8.3	Shen et al., 2018
NGT	Influent	UPLC-ESI-MS/MS	Spain	20 - 40	Guedes-Alonso et al. 2013
	Influent	RRLC-MS/MS	China	29 ± 3.7	Liu et al. 2011a
	Effluent	GC-MS	France	<4.5	Labadie and Budzinski, 2005
NGT	Effluent	UPLC-ESI-MS/MS	Spain	–	Guedes-Alonso et al. 2013
	Effluent	RRLC-MS/MS	China	9.2 ± 1.0	Liu et al. 2011a
	Surface Water	GC-MS	France	<4.0	Labadie and Budzinski, 2005
NGT	Surface Water	UPLC-MS/MS	China	3.0 - 23	Shen et al. 2018

ETO	Influent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Influent	LC-APCI/APPI-HRPS	Czech Republic	<0.28	Golovko et al. 2018
ETO	Effluent	LC-HESI-MS/MS	Sweden	–	Lindberg et al., 2014
	Effluent	LC-APCI/APPI-HRPS	Czech Republic	<0.57	Golovko et al. 2018
TTR	Influent	UPLC-ESI-MS/MS	Spain	35 - 300	Guedes-Alonso et al. 2013
	Influent	LC-MS/MS	China	34 ± 23	Chang et al., 2010
	Influent	RRLC-MS/MS	China	5.4 ± 0.4	Liu et al. 2011a
TTR	Effluent	UPLC-ESI-MS/MS	Spain	1.2 - 10	Guedes-Alonso et al. 2013
	Effluent	LC-MS/MS	China	0.2	Chang et al., 2010
	Effluent	RRLC-MS/MS	China	<LOQ	Liu et al. 2011a
TTR	Surface Water	LC-APPI-MS/MS	Japan	Not readable	Yamamoto et al. 2006

1.5. Scope and importance of the study

As steroid hormones pose a threat to human health and the environment even in very low concentrations, continuous monitoring and assessment of water bodies must be conducted. Further, three estrogenic steroid hormones (namely EE2, E1 and E2) are included in the EU watch list, which gives each member state the responsibility to assess whole water samples (Decision (EU) 2018/840). However, in recent years, only limited studies, which assessed levels of steroid hormones, have been conducted in Sweden. Most recent Swedish studies include Fick et al., 2011 and Lindberg et al., 2014. Neither of those studies succeeded in reaching low enough Limits of quantification (LOQs) for estrogens, thus estrogen levels in these studies remain unknown. The only successful Swedish study that assessed a variety of steroid hormones was conducted by the Swedish Environmental Research Institute (EPA) (Andersson et al., 2005). While the EPA study provided some basic data on these compounds in the environment, their occurrence and levels are important to be updated in the future. The lack of knowledge about levels of steroid hormones in Swedish waters are of concern and

national screening programs including a broad range of steroid hormones should be conducted in several different water matrices.

1.6. Study aims

This study aims to develop an instrumental method of steroid hormones using either GC-MS/MS or LC-MS/MS. It further aims to evaluate steroid hormones in surface water (SW), effluent wastewater (EW) and influent wastewater (IW) using the novel TIMFIE sampling device.

Further, the study provides an assessment for 11 different steroid hormones including synthetic estrogens, progestins and androgens (Table 2) in Swedish SW, EW and IW. Studied Compounds include: E1, 17βE2, E3, EE2, Etonogestrel (ETO), Dienogest (DIE), Gestodene (GES), Norgestrel (NGT), Norethindrone (NOR), Progesterone (PGR) and Testosterone (TTR).

2 Material and methods

2.1. Target analytes

Natural estrogens, synthetic estrogens and progestins as well as one natural androgen were chosen as target compounds (Table 2).

Table 2: Target compound names and abbreviations, their respective steroid group and use/origin. Use from www.pubchem.ncbi.nlm.nih.gov

Abbreviation	Name	Steroid Group	Use
E1	Estrone	Estrogen	Natural
E2	17β-Estradiol	Estrogen	Natural
E3	Estriol	Estrogen	Natural
EE2	17α-Ethynylestradiol	Estrogen	Birth Control Pill
ETO	Etonogestrel	Progestin	Birth Control Implant
DIE	Dienogest	Progestin	Birth Control Pill, Treatment for endometriosis
GES	Gestodene	Progestin	Birth Control Pill, Menopause Control
NGT	Norgestrel	Progestin	Birth Control Pill, Menopause Control
NOR	Norethindrone	Progestin	Birth Control Pill, Menopause Control
PGR	Progesterone	Progestin	Natural
TTR	Testosterone	Androgen	Natural

2.2. Preparation of Samplers

2.2.1. TIMFIE sampler

Conditioning of cartridges

HLB cartridges (Chromafix HR-P, Macherey-Nagel, Düren, Germany) used for sampling, method optimization and method validation were conditioned by attaching them to a flow distributor with 10 openings to attach the cartridges (see Appendix XII). Each cartridge was automatically flushed with 3 mL of MeOH followed by 10 mL of MilliQ water using a quaternary LC-pump (Jonsson et al., 2019).

Preparation of TIMFIE materials and construction of samplers

Polypropylene syringes with a volume of 120 mL were prepared by pulling the plunger to the 115 mL mark and drilling a hole into the plunger. An eyebolt was screwed into the end of the plunger. Flow restrictors (1/16" polyether ether ketone (PEEK) capillary flow restrictor, inner diameter = 0.075 mm (Vici-Jour, Schenkon, Switzerland)) were cut down to 40 cm, and inner pressure was measured by means of a HPLC pump set at a 0.5 mL/min flow of 100% MeOH. Pressures of flow restrictors ranged from 24 – 30 bar. Luer Lock connections (Plastikpak, BD, Franklin Lakes, USA) were attached to both ends of the flow restrictor, which was then attached to the Luer Lock connection of the syringe. The conditioned cartridges were attached between flow connector and a 4 cm inlet tube (Vici-Jour, inner diameter = 0.5 mm). The inlet tube was closed for storage and transportation with a long pin which was inserted (Jonsson et al., 2019).

2.2.2. POCIS sampler

POCIS sampler were composed according to Ahrens et al., 2016. 200 mg of Waters Oasis® HLB as a sorbent was placed between two PES membranes, which were framed by two stainless steel disks (inner diameter = 5.4 cm). Two passive samplers were attached onto a stainless-steel construct, which was then placed into a metal cage. In order to keep the metal cage balanced, one disk without membranes and sorbent was placed opposite of the two other samplers.

2.3. Sampling

2.3.1. Sampling locations

Sampling was conducted at two influents and the final effluent of the Uppsala WWTP, which is located south of the Uppsala city center, east of the Fyrisån. It receives

household wastewater from the Uppsala city center as well as the area surrounding Kungsängen, Ultuna and Sävja. Figure 1 shows a schematic overview of the WWTP. The cleaning steps are divided into mechanical, biological and chemical cleaning. Two active samplers (AS) are located at influents A+B, which contains wastewater from the Uppsala city center and influent C, which receives the wastewater of the domestic area surrounding Ultuna and Sävja. A third AS is located at the final effluent, which is discharged into the Fyrisån.

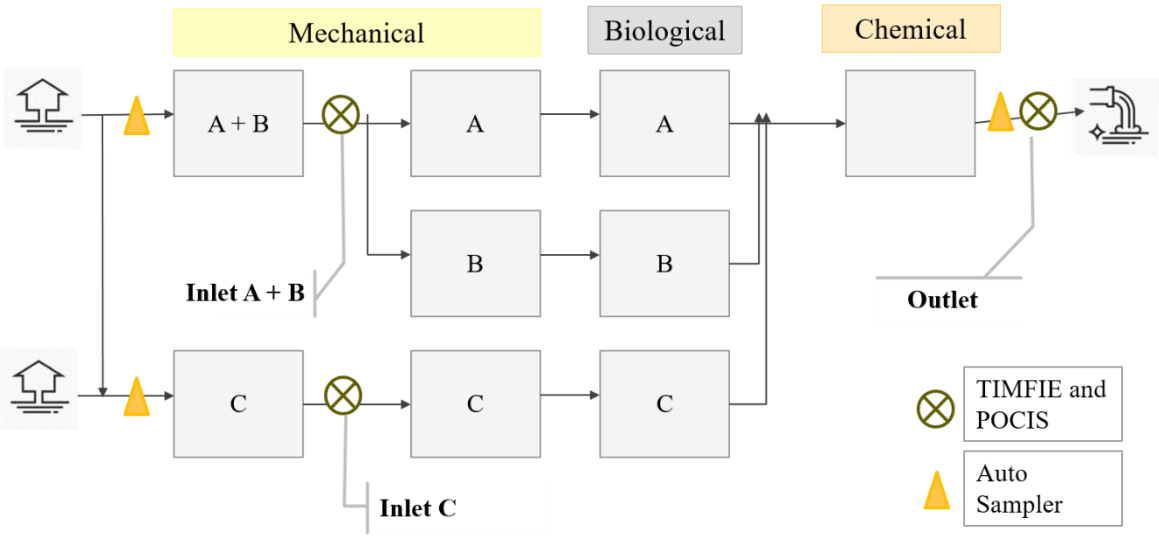

Figure 1: Schematic overview of the Uppsala WWTP with sampling points on inlet A+B and C and the final outlet.

2.3.2. *Active sampling*

TIMFIE samplers

The setup of TIMFIE samplers was conducted similar to Jonsson et al., 2018 and adjusted to the indoor environment at the WWTP. Figure 2 shows the schematic and on-site set-up of the TIMFIE samplers. On site, a looped rope was attached to the eyebolt at the end of the syringe plunger. By placing one foot in the rope loop and pulling the syringe, the plunger was pulled out. A needle was inserted into the hole in the plunger. Then, pins were removed from the inlet tube, which was then placed into the sink. Flow restrictors were attached to a cardboard. Inlet tubes were positioned as far from the sink inlet and outlet as possible, in order to minimize the amount of air and particles penetrating tubing and cartridges.

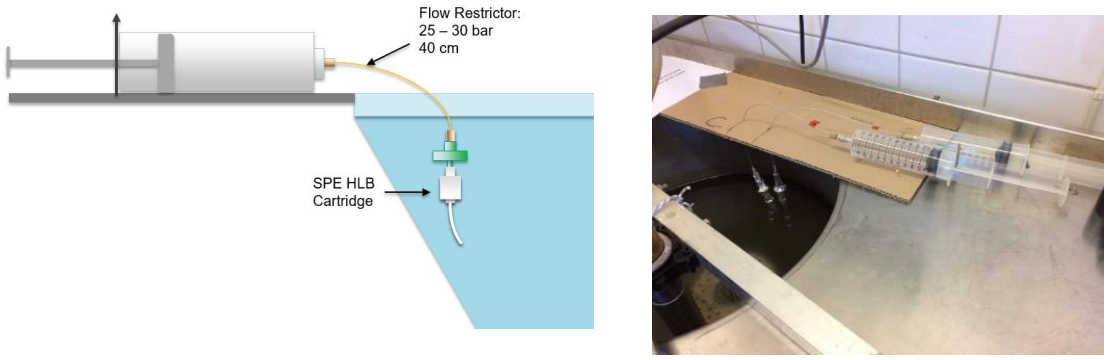

Figure 2: Schematic (left) and on-site (right) set-up of the TIMFIE samplers at the WWTP.

Auto sampler

In order to capture daily fluctuations, instead of grab samples, active samples were conducted by an AS, which was located at the main stream pipe in the WWTP. Proportional to the fluctuations in flow, the AS took more or less volume from the main flow, with a frequency of every 10 minutes. This resulted in a daily flow proportional composite sample that was stored refrigerated during the course of the 1-week sampling period.

2.3.3. *Passive sampling – POCIS*

Figure 3 shows the schematic set-up of the integrative sampling. Metal cages prepared in the lab were attached to a short rope and a carabiner, which was attached to a metal handle on top of the sampling sinks. The sampler was placed 5 cm below water surface.

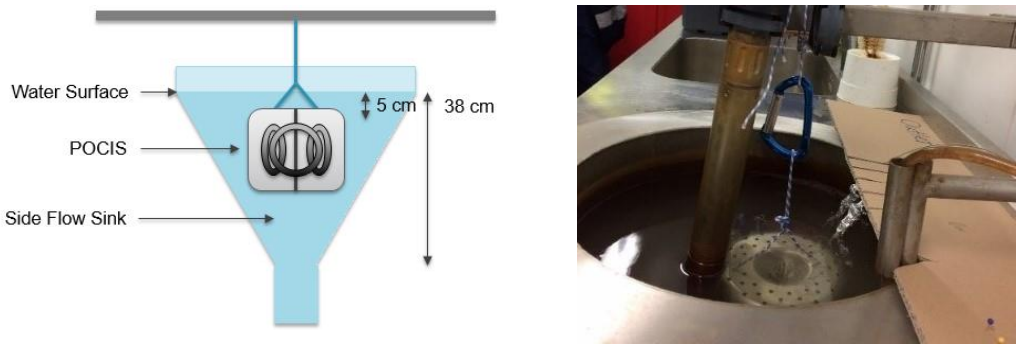

Figure 3: Schematic (left) and on-site (right) set-up of the POCIS at the WWTP

2.4. Sample extraction

2.4.1. *Active samples*

TIMFIE samplers

Back in the lab, cartridges were rinsed by pushing 5 mL of MilliQ water through a 5 mL syringe attached to the cartridge. Cartridges were then placed with the ending into the opening of a glass vial. Both were then placed into a falcon tube and centrifuged at 3000 g for 5 minutes. The remaining liquid, accumulated in the attached glass vial, was disposed. All columns were put in the freezer overnight, pending elution. Before elution, internal standard (IS) was added according to the total volume of extracted water, meaning that a sample with an amount of 60 mL in total was spiked with 60 µL of the same [10 ng/mL] spiking solution used for the method validation, in order to reach the desired IS level (10 ng/L) in samples. IS was added with a microtiter pipette to the opening of the cartridges. After adding IS, the cartridges were again flushed with 5 mL of MilliQ water.

Elution was conducted after first drying the columns to constant weight under a gentle nitrogen stream. This was done by attaching them onto a Nitrogen gas distributor (Appendix XII). 6 mL of ACN was chosen as elution solvent. Cartridges were attached to a flow distributor similar to the one described in subsection 2.2.1. Falcon tubes were placed underneath the cartridges. Eluates were then dried in the Falcon tubes under a gentle Nitrogen stream in a water bath at 40 degrees. The final extracts were dissolved in 120 µL of a 20% MeOH/-MilliQ solution. All samples were transferred to 2 mL glass vials with 200 µL inserts and put into the freezer, pending analysis.

Auto Sampler

In order to get a weekly composite sample, daily flow proportional samples for each stream over a week (week 3) were mixed in relation to the respective flow, in duplicates. Table 3 shows all flow data for both inlets and the outlet for the week of sampling.

Table 3: Flow [m³] of both influents (A+B and C) and the effluent of the WWTP for four days.

	Inlet A+B [m³]	Inlet C [m³]	Outlet [m³]
19-03-12	27000	31000	65000
19-03-14	29000	35000	70000
19-03-16	31000	41000	78000
19-03-18	31000	60000	100000

These composite samples were then extracted as closely to TIMFIE on-site conditions as possible. A conditioned HLB SPE cartridge was on one end connected to a 10 cm inlet tube (Vici-Jour, inner diameter = 0.5 mm) and on the other side to a flow restrictor (1/16" polyether ether ketone (PEEK) capillary flow restrictor, inner diameter = 0.25 mm (Vici-Jour,

Schenkon, Switzerland)). The flow restrictor was connected via to a 60 ml plastic syringe, which had a stop-bar at the 60 mL mark and an eyebolt at the end of the piston.

A weight of 3 kilograms attached to the eyebolt led to a low flow of approximately 1 drop per second, from the water sample bottle through the cartridge into the syringe (see Appendix XII). To prevent particles from accumulating on the bottom of the bottle, a stirring magnet was used, set at a speed of 250 rpm. The extraction of 60 mL took approximately 40 minutes for IW, EW and SW, and approximately 30 minutes for MilliQ water.

2.4.2. Passive sampling – POCIS

Preparation of SPE cartridges

SPE cartridges, PP tubes and stop-cock were rinsed three times with MeOH and dried by air. One clean frit was inserted into the SPE cartridge, The HLB powder between the membranes was transferred into the cartridge via a glass funnel, which was then rinsed with MilliQ water. Then, the cartridges were dried via vacuum for approximately 10 minutes and sealed with the second frit.

Elution

The PP tube, SPE manifold and stop-cock was rinsed three times with methanol and dried by air. The cartridges were connected to the SPE manifold and a clean PP-tube was placed underneath the outlet. IS was added to the upper surface of the second frit (10 µL from [0.1 µg/mL] to 120 uL in vial for an 8.3 ng/mL concentration). Elution was conducted similar to TIMFIE elution procedure (see subsection 'TIMFIE samplers').

Evaporation of extracts

The eluates were dried under a gentle nitrogen stream and a water bath at 40°C to complete dryness. The residues were then reconstituted in 120 µL of MeOH:MilliQ water 20:80 (v/v) and transferred to 2 mL glass vials with 200 µL inserts, vortexed in order to dissolve air on the bottom, and put in the freezer, pending analysis.

2.5. Method Optimization

2.5.1. Spiking for absolute recovery estimations

All target hormones spiking levels for the different samples and matrices are shown in Table 4.

Table 4: Spiking level concentrations of native compounds and internal standards for SW, EW and IW into prespike and postspike samples.

Matrix	Native Compounds				Internal Standard
	Prespike Concentration		Postspike Concentration		
	in vial [ng/mL]	in sample [ng/L]	in vial [ng/mL]	in sample [ng/L]	in sample [ng/L]
SW	5	10	5	10	10
EW	15	30	15	30	10
IW	15	30	15	30	10

Prespike samples were spiked into the matrix sample with 60 µL of a [10 ng/mL] spiking solution in 60 mL of sample for SW and 180 µL of the spiking solution for EW and IW. Postspike samples were spiked by adding 20 µL of a [30 ng/mL] spiking solution to the extracts for SW, and 60 µL of the spiking solution for EW and IW samples. Matrix blanks were not spiked. After extraction, elution and spiking, 60 µL of a [10 ng/mL] internal standard (IS) solution was added to all samples, including postspike, prespike and matrix

blanks. By comparing the LC-MS/MS results from the prespike samples with the postspike samples, following background subtraction of the blank samples, the absolute recoveries for each substance in the different water matrices were determined.

2.5.2. Elution

Elution was conducted as described in section 2.4. In order to test whether MeOH or ACN is more suitable for the elution of the target compounds in dirty matrices, both solvents were used to elute the columns. Additionally, in order to investigate the amount of solvent needed to quantifiably elute the compounds, after having eluted each sample with 4 mL of MeOH and ACN respectively, the columns were further eluted with 2 more mL of each solvent. After elution, all samples were processed the same way as described in subsection 2.4.1.

2.5.3. Experimental set-up

In order to test for the most suitable analytical method and sample preparation, grab samples of IW, EW and SW from Fyrisån were taken. For each matrix, postspike, matrix blank and prespike samples were extracted. Recovery and matrix effect were calculated accordingly:

$$Absolute\ Recovery\ [\%] \ = \ \left(\frac{Prespiked\ Samples}{Postspiked\ Samples} \right) \cdot \ 100$$

$$Matrix\ Effect\ [\%] \ = \ \left(\left(\frac{Postspiked\ Sample - Matrix\ Blank}{Spiked\ Solvent} \right) - 1 \right) \cdot \ 100$$

Hormone recoveries relative to IS was investigated at spiking level 10 ng/L for SW and spiking level 30 ng/L for EW and IW. Relative Recoveries have been calculated as follows:

$$Relative\ Recovery\ (\%) = \left(\frac{Prespiked\ Samples\ \left[\frac{ng}{L}\right] - Matrix\ B\ [\frac{ng}{L}]}{Nominal\ Concentration} \right) \cdot\ 100$$

2.6. TIMFIE method validation

2.6.1. Experimental set-up

Method validation was conducted by extracting triplicates of four different matrices including MilliQ water, SW, EW and IW. The different water matrices were spiked with a low native hormone concentration (1 ng/L) and a medium native hormone concentration (5 ng/L). The procedure was repeated after two weeks, in order to evaluate between day variation. Spiking was conducted by adding 125 µL for the medium concentration and 25 µL for the low concentration of a 10 ng/mL spiking solution in MilliQ water to 250 mL of sample. The spiked water was allowed to equilibrate under stirring for at least 15 minutes before extraction started. Spiked water matrices were extracted in triplicates from these 250 mL. In addition, the different matrices were extracted in duplicate without spiking of native compounds in order to determine background levels.

2.6.2. Sample preparation

Extraction was conducted according to subsection 2.4. However, IS was added to the small volume of water standing in the cartridge void on the inlet side and mixed ten times with the pipette. Then, 5 mL of MilliQ water was run through the cartridge at an initially low flow rate, using a 5 mL syringe, to load the IS and to wash off any non-extractable matrix components. Elution was from thereon conducted following the procedure described in subsection 'TIMFIE samplers'.

2.7. Instrumental analysis

2.7.1. Gas chromatography – mass spectrometry

Calibration curve

The calibration curve was prepared in MeOH:MilliQ water 20:80 (v/v) and ranged from 0.5 to 500 ng/L.

Derivatization

Samples were gently evaporated to dryness under a gentle stream of nitrogen gas. The dried substance was dissolved in 50 µL Pyridine. BSTFA was used as a derivatization (silylation) agent. The derivatization mechanism is shown in Figure 4 after SUPELCO product specification. After adding 50 µL of BSTFA, the mixture was vortexed for 30 seconds, and kept in a water bath for one hour at approximately 60°C. The derivatization method was based on Ronderos-Lara et al., 2018.

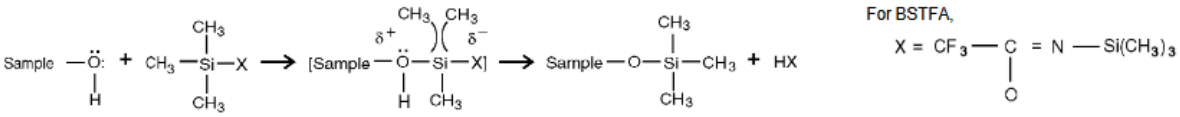

Figure 4: Derivatisation mechanism (silylation) of a sample sompound with derivatization agent BSTFA. Adapted graphic from SUPELCO product specifications.

System set-up

All measurements were conducted on a gas chromatograph (GC 7890A, Agilent Technologies) coupled to a triple quadrupole mass spectrometer (7010 GC-MS/MS Triple Quad, Agilent Technologies). The injection was performed in splitless mode, injection volume 2 µL and the temperature 300°C. The column used was an Agilent DB-5 (30 m, 0.25 mm, film thickness 0.25 µm) and Helium (He) was used as a carrier gas (flow rate 1mL/min).

The temperature program of the GC started at 150°C (held for 2 minutes), thereafter the temperature was increased 15°C/min until 250°C, 5 °C/min until 280°C and 20°C/min until 300°C was reached (held for 2 min). The MS/MS was operated using electron impact (EI) ionization at 70eV. The ion source temperature was set at 230°C and the quadrupole temperature at 150°C. The instrument was operated in multiple reaction monitoring (MRM) mode using the precursor and product ions settings presented elsewhere (U.S. Geological Survey, 2012).

2.7.2. Liquid chromatography – mass spectrometry

Calibration curve

The calibration curve was prepared in 20% MeOH MilliQ solvent and ranged from 0.01 to 100 ng/L.

System set-up

The samples were analyzed using LC-MS/ MS with an LC system from Thermo Fisher Scientific, San Jose, CA, USA and a triple-stage quadrupole MS/MS TSQ Quantiva (Thermo Fisher Scientific). An Acquity UPLC BEH-C18 column (100mmx 2.1 i.d., 1.7 µm particle size, Waters Corporation, Manchester, UK) was used as an analytical column. Injection volume was 10 µL for all samples. A heated electrospray ionization (HESI) was used to ionize the target compounds. The spray voltage was set to static: positive ion (V) 3500.00. Nitrogen (purity >99.999%) was used as a sheath gas (50 arbitrary units), auxiliary gas (15 arbitrary units) and sweep gas (2 arbitrary units). The vaporizer was heated to 400 ^{0}C and the capillary to 325 ^{0}C. Two selected reaction monitoring (SRM) transitions were monitored for all analytes (Table 6). Data were evaluated using TraceFinder™ 3.3 software (Thermo Fisher).

Table 5: Multi gradient program for UPLC-MS/MS Analysis

Retention Time [min]	Flow [mL/min]	% MeOH
0	0.5	20
0	0.5	20
1.05	0.5	20
3	0.55	40
6	0.6	80
7	0.6	100
10	0.6	100
10	0.5	20
13	0.5	20

MilliQ water with 0.03% Ammonia and methanol with 0.03% Ammonia were used as the mobile phases. All samples were dissolved in a 20% MeOH MilliQ mixture. Table 5 shows the Multi-Step Gradient program for the liquid chromatographic separation. SRM settings for LC-MS/MS analysis are shown in Table 6 and Appendix I for all compounds.

Table 6: LC-MS/MS scan parameters for all estrogens, progestins and androgens with their respective retention time [min], polarity, precursor and product ion [m/z] as well as the collision energy [V], dwell time [min] and RF lens [V]

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	Min Dwell Time (ms)	RF Lens (V)
Estrogens								
E1	5.26	2	-	269	143.04	54.7	24.091	78.94
E2	5.4	2	-	271.12	145.04	40.29	24.091	83.15
E3	3.75	2	-	287.2	143.04	51.56	25.107	86.11
EE2	5.32	2	-	295	145.04	40.94	24.091	81.17
Progestins								
ETO	5.73	2	+	325	147.111	79	24.091	49.28
DIE	4.75	2	+	312.2	135.04	29.21	24.091	63.37
GES	5.36	2	+	311	109.04	25.42	24.091	48.29
NOR	5.32	2	+	299.2	109.04	26.23	24.091	49.53
NGT	5.65	2	+	313.2	159.04	25.83	24.091	56.2
PGR	6.03	2	+	315.25	97.04	22.08	24.091	47
Androgens								
TTR	5.48	2	+	289	97.04	22.34	24.091	45.08
Carbon Labeled Hormones								
E2-C13	3.75	2	-	290.2	146.054	55	25.107	176.34
EE2-C13	5.9	2	-	295	145.04	36.04	24.091	127.89
NOR-C13	5.32	2	+	301.2	109.04	26.69	24.091	86.61
PGR-C13	6.03	2	+	318.25	100.111	22.34	24.091	82.4

3 Results and discussion

3.1. Comparison of LC-MS/MS and GC-MS/MS

In order to decide for the most sensitive and precise analytical method, analysis of all 11 target compounds was compared between GC-MS/MS and UPLC-MS/MS. A means of comparison was the instrumental quantification limit (IQL) on column [pg], which takes into account the different injection volumes, which are used by GC-MS/MS (2 µL) and UPLC-MS/MS (10 µL).

Figure 5 compares IQL on column for UPLC-MS/MS and GC-MS/MS for all targeted compounds. Most apparent is that only E1, E2, EE2, E3 and TTR were quantifiable by GC-MS/MS analysis, thus leading to the suggestion that GC-MS/MS may not be a suitable instrument to detect estrogens, progestins and androgens simultaneously.

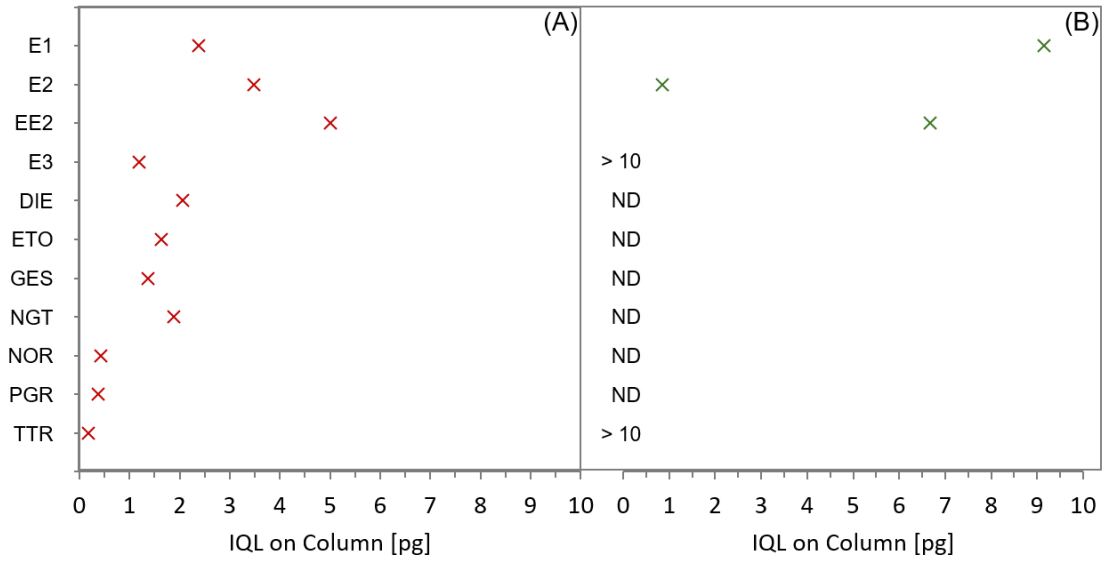

Figure 5: Instrumental quantification limit (IQL) on column [pg] on the respective analytical method, taking into account different injection volumes (10 µL for LC-MS/MS and 2 µL for GC-MS/MS) for all target compounds. (A) = LC-MS/MS; (B) = GC-MS/MS; >10 = IQL above 10 pg; ND = not detected.

However, for E2, the IQL on column is lower for GC-MS/MS compared to LC-MS/MS, with a limit of 0.85 pg and 3.5 pg respectively (see Appendix III). Thus, if analyzing a single target compound E2, GC-MS/MS could provide a higher sensitivity and detect levels way below the detection limit of LC-MS/MS.

For a wide range of hormones however, LC-MS/MS provides the better analytical method, since all tested hormones are quantifiable below a level of 5 pg, and compounds of all three tested steroid groups are quantifiable.

Potential in optimization of the used GC-MS/MS method lies in the derivatization process. A higher sensitivity and a higher number of quantifiable hormones may have been reached through adapting and improving the derivatization process. The used derivatization agent BSTFA might not have been the most suitable agent for analysis of hormones, and different agents could be tested for their applicability. Further, the amount of agent as well as the time samples were kept in a water bath for the derivatization reaction could have been adapted. However, these potential adjustments can be subject to further studies, and are not within the scope of this thesis. Also, it has to be mentioned that the calculated IQL on column is for samples in pure solvent.

Since in this case, UPLC-MS/MS is more sensitive as well as applicable to the selected range of steroid hormones from different groups, for the further analysis and method validation, UPLC-MS/MS was selected.

3.2. TIMFIE method validation

3.2.1. Optimization of clean-up and elution

Figure 6 shows the percentage of total amount of hormones in sample that has been eluted with 4 mL ACN followed by 2 mL ACN in a separate sample. The results clearly show that elution with 4 mL of solvent is not sufficient to fully elute the complete amount of hormones present in a sample. Especially for E2, E3 and EE2, elution with 6 mL ACN is crucial, since it makes up for up to 42% of the total eluted amount (see Appendix V and VI). For compounds TTR, NGT, PGR and DIE, it does not make a significant difference if the compounds are being eluted with 4 or 6 mL, since the additional eluted amount only adds up to maximal 10%.

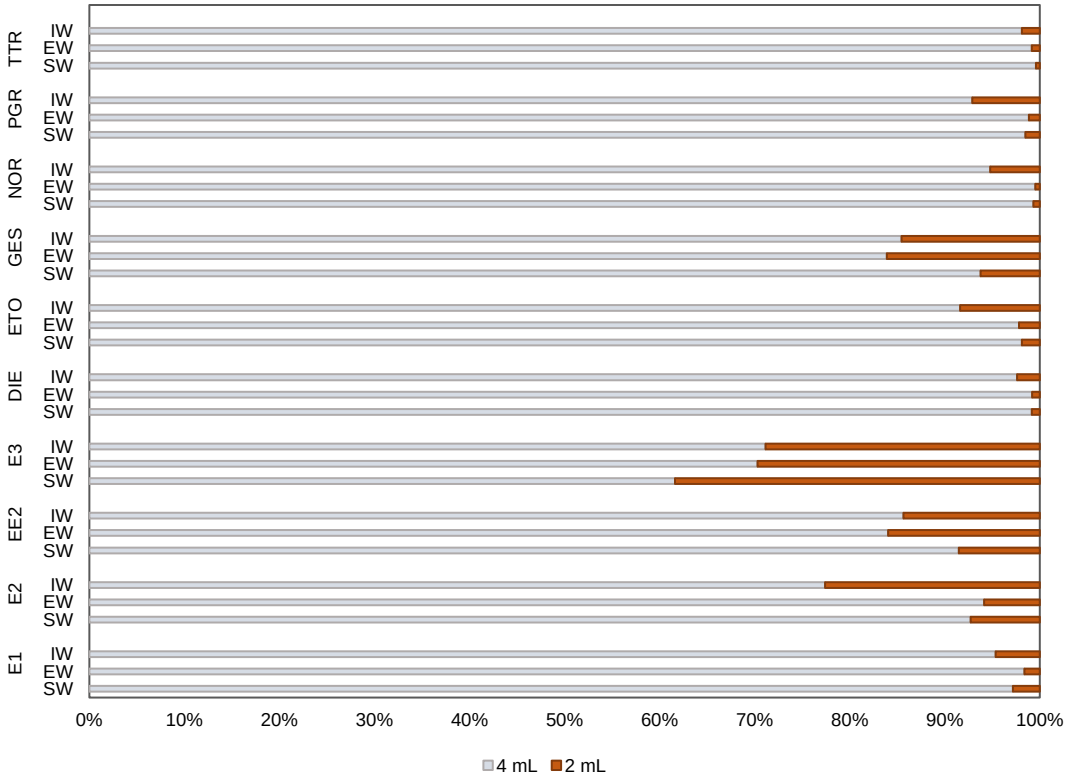

Figure 6: Percentage of hormones eluted with 4 mL of ACN following 2 mL of ACN for SW, EW and IW; 100 % equals the amount eluted with 4 mL ACN plus the amount eluted with 2 more mL ACN. Samples were spiked with 30 ng/L in EW (n = 2) and IW (n = 2) and with 10 ng/L in SW (n = 1). Extraction of 60 mL and elution procedure was conducted according to TIMFIE protocol.

In order to decide for the most efficient and suitable solvent, influent samples were eluted with MeOH as well as ACN. In the following, absolute recovery and matrix effect are compared between the different elution techniques.

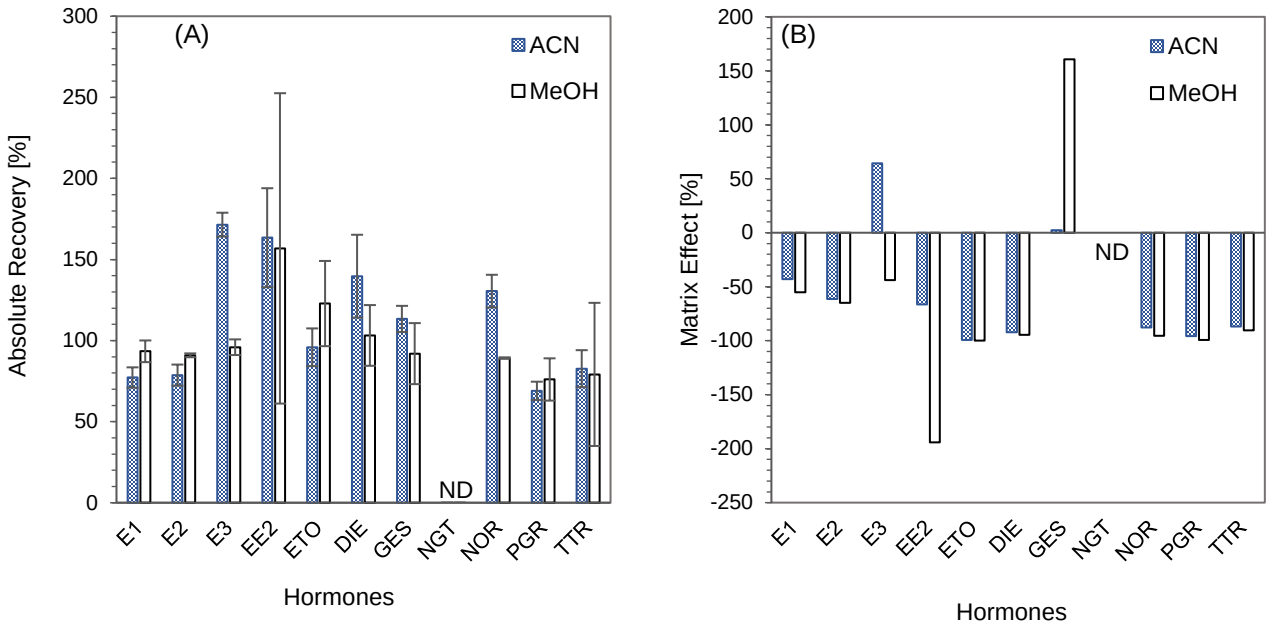

Figure 7: (A) = Absolute recovery [%] of influent water samples eluted with ACN (n = 2) or MeOH (n = 2). (B) Matrix effect [%] of influent water samples eluted with ACN or MeOH. ND = Not detected. Samples were spiked with 30 ng/L in EW and IW and with 10 ng/L in SW. Extraction of 60 mL and elution with 6 mL ACN was conducted according to TIMFIE protocol.

Absolute recoveries of IW samples eluted with MeOH or ACN do not differ significantly. Only E3 shows a much higher recovery (170%) when eluted with ACN compared to the samples eluted with MeOH (95%) (see Figure 7, Appendix XI and Appendix IV). Although the absolute recoveries of samples eluted with MeOH show recoveries closer to 100%, for further experiments and the real water samples ACN was used as solvent. Firstly, this was decided due to the high variability of EE2 and TTR in recovery, with a coefficient of variation (CV) of 61% and 55% respectively, while CV values for elution with ACN reached maximum values of 20%. Secondly, ACN was chosen because of the higher matrix effect for

elution with MeOH in influent water for compounds EE2 and GES, which can be seen in Figure 7.

Similar tests have been conducted by previous studies. Liu et al., 2014 tested four different elution solvents for the extraction of steroid hormones: EtOAc, MeOH, DCM, and MeOH/DCM (7:5, v/v). This study found that all tested elution solvents yielded similar recoveries within a range of 70 – 120%. EtOAc was chosen as the final elution solvent, since it minimized the matrix effect and is the less toxic solvent out of the four tested ones.

Pedrouzo et al., 2009 found that adding 5% of ACN to MeOH improved the recovery for some steroid hormones. Another study by Chang et al., 2018 used Hexane as elution solvent, as it reduced the extraction of hydrophilic interference due to its low polarity.

Similar findings as in this study have been reported by Golovko et al., 2018, who tested both ACN and MeOH as elution solvent for the elution of numerous progestins. As this study, Golovko et al., 2018 found a high variability within the recoveries obtained by the elution with MeOH (ranging from 4-135%), whereas the elution with ACN yielded recoveries between 62–130%. This supports the suggestion, that MeOH does elute all tested hormones; however, it varies significantly and therefore does not serve as an applicable elution solvent for the analysis of a wide range of steroid hormones.

3.2.2. Absolute recovery and matrix effect

Figure 8 shows the absolute recoveries for all compounds in SW, EW and IW with their respective standard deviation.

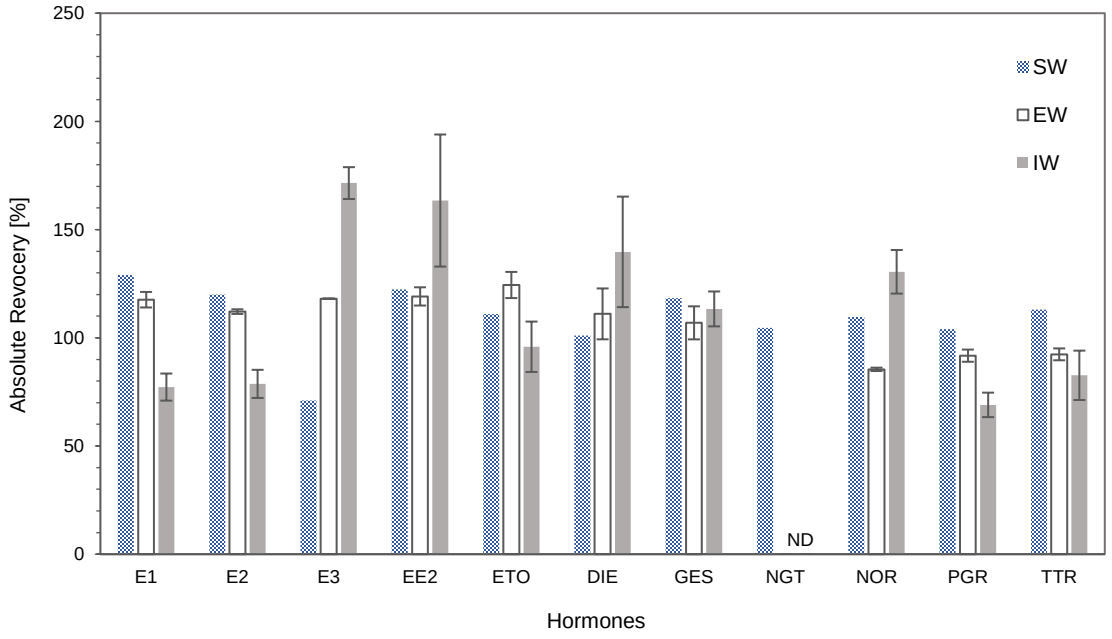

Figure 8: Absolute recovery [%] of all target compounds extraxted from SW (n = 1), EW (n = 2) and IW (n = 2) with the respective standard deviation. Samples were spiked with 30 ng/L in EW and IW and with 10 ng/L in SW. Extraction of 60 mL and elution with 6 mL ACN was conducted according to TIMFIE protocol. ND = Not detected

Absolute recoveries from SW range from 70% to 130%. EW shows relatively constant recoveries from 85–120% with low variation between samples (CV = 0.1–10 %). Recoveries that are out of the range of 80–120% are apparent in IW samples, with high values for E3, EE2, DIE and NOR of 170, 160, 140 and 130% respectively. Further, the variation within IW sample recoveries are relatively low (CV = 4.3–18 %) but higher compared to SW and IW sample variations. Those high absolute recoveries as well as the higher variation between samples are most likely due to a high matrix effect, which might increase the peak signal. However, for SW and EW, the used extraction, elution and analytical method leads to very

good absolute recovery values close to 100% with CV below 25%. The Matrix effect for SW ranges between a suppression of 60–80 % for all compounds. Matrix effect for EW has a higher variation. For E1 and E2, it leads to a small signal enhancement of 10–15%, whereas for the other compounds, the matrix effect is minimal or ranges between 45–100%.

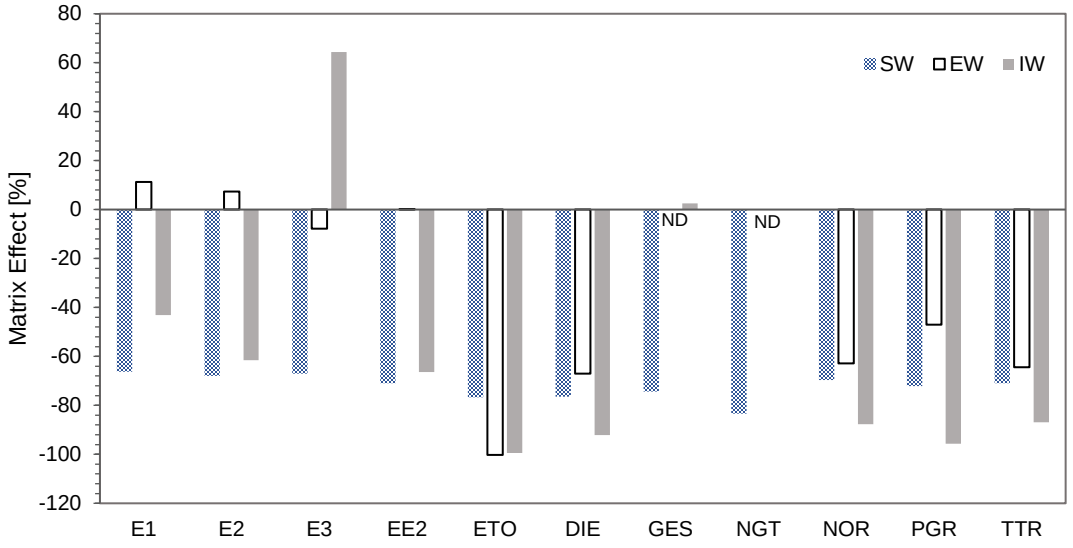

Figure 9: Matrix effect [%] of SW, EW and IW for all tested compounds. Samples were spiked with 30 ng/L in EW and IW and with 10 ng/L in SW. Extraction of 60 mL and elution with 6 mL ACN was conducted according to TIMFIE protocol. ND = Not detected

Extraction from 10 ng/L spiked SW yields relative recoveries of 110–120% for all compounds except GES, which was recovered to 2100%. In SW, EW and IW, NGT fails to be extracted completely, and GES reaches values of 710 ± 47- 650 ± 64 %. Since GES and NGT do not perform well enough in relative recovery, due to too high background levels of the compounds as compared to the spiking levels, both compounds are excluded from further validation and are not quantified in the method application. For all other compounds, relative recoveries from all matrices lie within the acceptable range (50–150 %). Appendix II lists relative recoveries of similar studies such as Zhang and Fent, 2018, Shen et al., 2018 and

Koh et al., 2007. While this study fails to reach reasonable relative recoveries for GES with a spiking level of 10 ng/L, Koh et al., 2007 reaches a relative recovery of 97 ± 16 % for GES in EW, however, with a spiking level of 15 ng/L. Higher spiking levels up to 50 ng/L for IW have also been applied by S. Liu et al., 2011a. These comparisons suggest that future method validation of EW and IW assessment methods should use higher spiking levels, for the added concentration to be distinguishable from background levels. However, none of the studies above mentioned assessed such a broad range of steroids, making this study applicable to higher number of different steroids.

Table 7: Relative recoveries [%] of all compounds extracted from spiked SW (n = 1), EW (n = 2) and IW (n = 2). Samples were spiked with 30 ng/L in EW and IW and with 10 ng/L in SW. Extraction of 60 mL and elution with 6 mL ACN was conducted according to TIMFIE protocol.

Spiking Level	10 ng/L	30 ng/L	30 ng/L
Matrix	SW	EW	IW
Compounds			
E1	120	120 ± 3	67 ± 7
E2	110	110 ± 1	56 ± 5
E3	110	110 ± 0.9	120 ± 3
EE2	110	36 ± 0.2	72 ± 14
ETO	120	150 ± 9	68 ± 17
DIE	110	140 ± 13	100 ± 5
GES	2100	710 ± 47	650 ± 64
NGT	110	0	0
NOR	110	100 ± 1	89 ± 2
PGR	110	110 ± 2	54 ± 3
TTR	110	110 ± 4	59 ± 8

Accuracy is reported as the percent recovery of the known, added amount of hormones. Relative recoveries of all compounds extracted from MilliQ are given in Table 8.

Extraction from 5 ng/L spiked MilliQ water yielded recoveries close to 100% for E3, EE2 and TTR and between 60 and 75% for E1, E2, ETO, NOR and PGR. Only DIE has not been recovered sufficiently. Since DIE has shown good recovery from SW, EW and IW (see Appendix II), it will still be included in further analysis. These findings suggest an excellent accuracy for E3, EE2 and TTR and a good accuracy for E1, ETO, NOR and PGR. As stated above, additional cleaning could be investigated in further studies, which could yield better accuracy for some of the compounds.

Table 8: Relative recoveries [%] of all compounds extracted from 5 ng/L spiked MilliQ Water (n = 3). Extraction of 60 mL and elution with 6 mL ACN was conducted according to TIMFIE protocol.

Compounds	E1	E2	E3	EE2	ETO	DIE	NOR	PGR	TTR
Relative Recovery [%]	72 ± 11	73 ± 9	96 ± 14	93 ± 2	65 ± 23	0	60 ± 11	72 ± 8	84 ± 8

3.2.3. Precision of the analytical method: repeatability and reproducibility

Repeatability

In order to evaluate if the used method is precise, repeatability has to be analyzed. Figure 10 shows the detected concentrations for each compound at spiking level 5ng/L in MilliQ, SW and EW (n=3). Since the hormone concentrations in IW are high above the calibration range (0.01 – 100 ng/L) (see Appendix VII), measured concentrations cannot be plotted in the same way. Instead, the variation can be seen in Table 9.

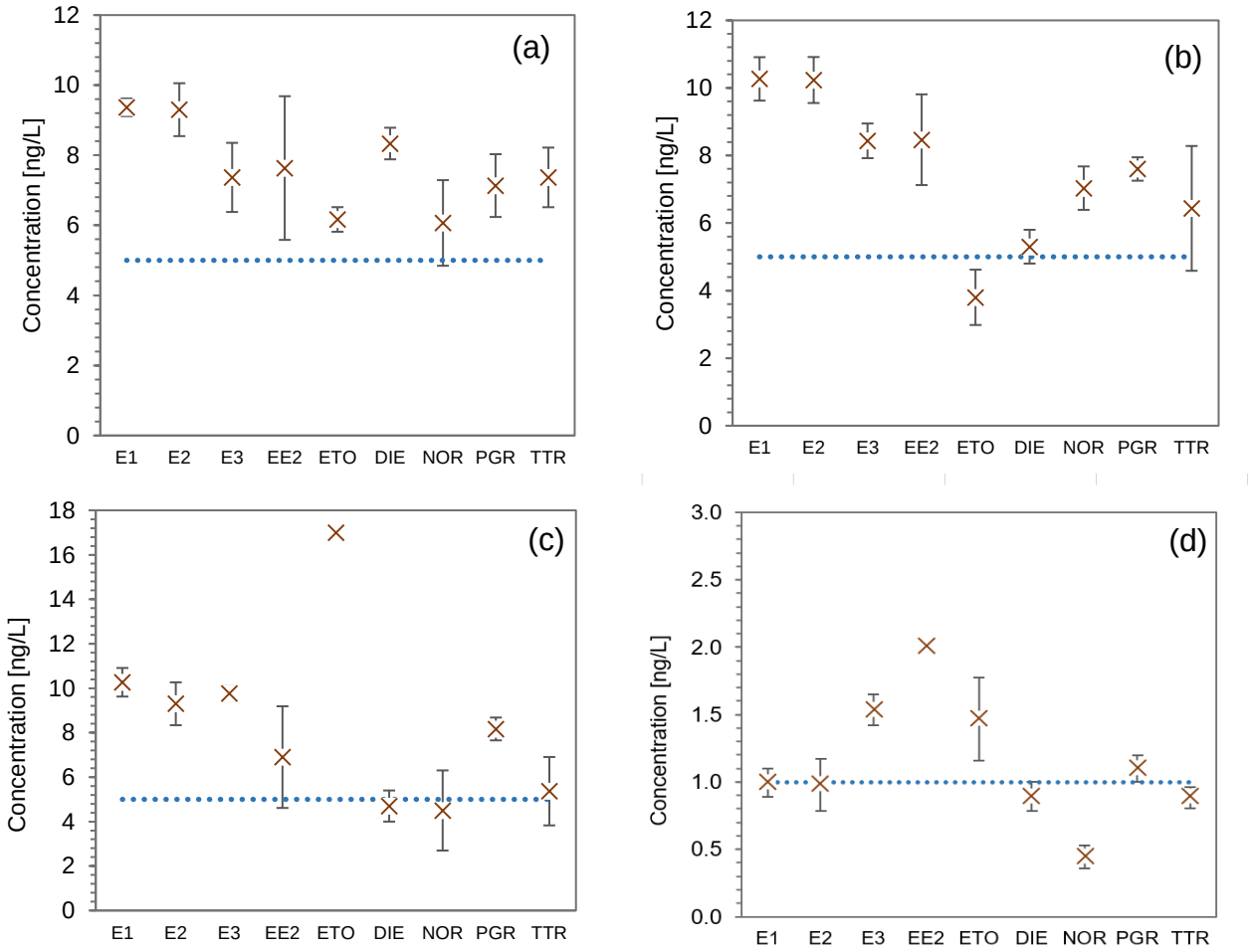

Figure 10: Measurement levels (n = 3) of spiked matrices with their respective standard deviation between triplicates within a day. Spiked with 5 ng/L (a) MilliQ, (b) SW, (c) EW. Spiked with 1 ng/L: (d) MilliQ extraction of 60 mL and elution with 6 mL ACN was conducted according to TIMFIE protocol. Data points were not corrected by background level.

Most significant is the fact, that even in the clean matrices MilliQ water and SW, the measured concentrations reach above the 5 ng/L spiking level (see Appendix IX and X), which could indicate signal enhancement especially for the compounds E1 and E2. This can also be seen in the CV values listed in Table 9. The variation in measured concentration of the spiked matrices remains below the 25% tolerance limit for E1, E2, E3, ETO and PGR. EE2 shows variation from the mean of more than 25% for MilliQ water, EW and IW. For TTR,

CVs are higher than 25% in SW and EW, while DIE has a higher than tolerable variation in IW. GES and EE2 show a very high variation.

Table 9: CV's for concentration measurements (n=3) of the 5ng/L spiked matrices MilliQ, SW, EW and IW. Values above the 25% tolerance limit are kept in bolt. Extraction of 60 mL and elution with 6 mL ACN was conducted according to TIMFIE protocol. Concentrations used for CV calculation were not corrected by background level.

	MilliQ	SW	EW	IW
E1	2.7	6.3	6.3	25
E2	8.1	6.7	10	13
E3	13	6.1	3.3	14
EE2	**27**	16	**33**	**56**
ETO	5.7	22	0	16
DIE	5.4	9.4	15	**28**
NOR	20	9.1	**40**	24
PGR	13	4.6	6.3	24
TTR	12	**29**	**29**	18

EE2 and GES show CV above the 25% tolerance limit. For EE2, these high variations only occur in the dirty matrices EW and IW, whereas GES measurements show more variation in the clean matrices MilliQ and SW. TTR shows high variation for SW and EW. However, the remaining CV values remain below the 25% limit, and with the exception of EE2 and GES, the method proves to be repeatable in all tested matrices. Spiking level of 1 ng/L proved problematically low for dirty matrices SW, EW and IW and measurement results for this spiking level has been excluded from further analysis. When working with dirty matrices such as IW and EW, high measurement variations can be common. Thus, next studies should use higher spiking levels, include more cleaning steps and use a calibration range that exceeds 100 ng/L. With a more accurate measurement of concentrations above

100 ng/L, subtraction of the matrix blank might yield more accurate results, and the spiked concentration might be determined more precisely.

Reproducibility

In order to test the reproducibility of the used analytical method, the spiking experiment has been repeated on a second day two weeks after the first spiking experiment. The following evaluates the variation between the measurement results of two different days. As for the day-to-day variation, only the medium spiking level of 5ng/L has been repeated. CVs are listed in Table 10.

Table 10: Coefficients of variation [%] of measured concentrations in 5 ng/L spiked samples for day-to-day variation in SW, EW and IW. Values above the 25% tolerance are kept in bolt. Extraction of 60 mL and Elution with 6 mL ACN was conducted according to TIMFIE protocol. Concentrations used for CV calculation were not corrected by background level.

	SW	EW	IW
Compounds	CV [%]	CV [%]	CV [%]
E1	3.8	19	**53**
E2	7.5	10	6
E3	8	19	11
EE2	5.4	21	19
ETO	0.62	**28**	**75**
DIE	4.1	3.1	26
NOR	21	2.1	16
PGR	1.9	6	1.3
TTR	19	3	**98**

For SW the variation stays below the 25% tolerance limit. For EW however, ETO and GES show a variation above 25% from the mean. This is the case in IW for TTR, NGT, ETO and E1, where TTR shows a variation of 98% and ETO a variation of 75%. The high variation

in IW can on one hand be explained by the high level of hormones in the matrix itself. If the concentration lies around 300 ng/L, then it is difficult to detect a spiking by 5 ng/L. On the other hand, measured concentrations might not be entirely correct since most of the compounds exceeded the 100 ng/mL calibration limit, and therefore calculated CVs might not reflect the true variation. For future studies and further validation, higher spiking levels should be used in EW and IW, and the calibration limit should be increased up to 1 µg/L.

3.2.4. Sensitivity of the instrumental method: IDL and IQL

IDL was calculated by taking the lowest concentration of the 0.01 ng/L – 100 ng/L calibration range where a peak was still visible with a signal to noise ratio (S/N) higher than 3. For the IQL, S/N was set to 10. S/N was calculated by dividing peak height by average noise height. IDLs reached in this study are comparable to a similar study conducted by Yamamoto et al., 2006 (see Table 11). For almost all compounds, this study reached a twice as low IDL compared to Yamamoto et al., 2006. Lowest IDL has been reached for TTR (0.035 ng/mL), while NGT has the highest IDL with 0.38 ng/L. However, IDL for all compounds lies below 0.5 ng/mL, and 1.3 ng/L. Compared to Yamamoto et al., 2006, this study investigates 11 instead of 9 compounds, including ETO and GES. Even though IDLs in this study reach very low values, optimization could still be reached. Further studies could investigate the use of different mobile phases, further adapt gradient programs or testing sensitivity of APPI and APCI compared to HESI.

Table 11: IDL and IQL in ng/L of all analyzed compounds of this study and in comparison with other studies.

	This Study LC-MS/MS Pure Solvent		Yamamoto et al., 2006 LC-MS/MS Surface Water
	IDL [ng/L]	IDL [ng/mL]	IDL [ng/mL]
E1	0.48	0.24	0.51
E2	0.7	0.35	0.41
E3	0.24	0.12	0.72
EE2	1	0.5	1.2
ETO	0.33	0.33	-
DIE	0.41	0.21	-
GES	0.91	0.27	0.54
NGT	1.3	0.38	0.76
NOR	0.28	0.085	0.17
PGR	0.25	0.075	0.15
TTR	0.12	0.035	0.07

3.2.5. Sensitivity of the analytical method: MQL or LOQ

The method detection limit (MDL, also often referred to as LOD) and method quantitation limit (MQL, also often referred to as LOQ) of each compound were calculated based on the signal-to-noise ratio (S/N) where the noise was estimated from the chromatogram close to the target peak. The MDL was defined as three times of S/N under the lowest spiked concentration of all three matrices, while MQL is ten times of S/N. MQLs in all four tested matrices for all compounds are listed in Table 12.

Reference values for MQL's are listed in Table 13. The MQL's in this study lie significantly below MQL's reached by other comparable studies. For instance, a comparable Swedish study by Lindberg et al., 2014, which reached a MQL of 40 ng/L for E1 in IW and EW, while this study reached a MQL of 0.75 ng/L in IW and 0.41 ng/L in EW for E1. Most

similar to findings of this study are MQLs reached by Zhang and Fent, 2018 in Switzerland. They reached values of 0.09 ng/L for E1 in IW samples, while this study reached an MQL of 0.75 ng/L in the same conditions (Zhang and Fent, 2018). In this study, before drying of cartridges, an additional cleaning step with a MeOH MilliQ mix was conducted. Another comparable study was conducted by Zuehlke et al., 2005 in Germany, with an MQL of 0.2 ng/L for E1 in EW. This study as well added a cleaning step before elution of cartridges. Cleaning was conducted by a silica-gel extraction column with a clean-up solvent (n-hexane/acetone) (Zuehlke et al., 2005). Since both studies, which include sample clean-up in the preparation process, reach lower MQLs for several compounds, additional cleaning for IW and EW could be considered as another optimization step for further studies.

Table 12: MQL [ng/L] of all target compounds in MilliQ, SW, EW and IW, extracted in the lab through the TIMFIE extraction technique. Elution conducted as all TIMFIE samples. Of each sample, 60 mL were extracted.

MQL [ng/L]	MilliQ Water	SW	EW	IW
E1	0.37	0.41	0.37	0.75
E2	0.88	0.97	0.90	1.8
E3	0.87	0.94	0.86	1.8
EE2	2.4	2.7	2.4	4.9
ETO	0.77	0.84	0.77	1.6
DIE	0.081	0.072	0.12	0.41
NOR	0.48	0.43	0.73	2.4
PGR	0.16	0.14	0.24	0.81
TTR	0.14	0.12	0.21	0.71

Table 13: MQL's of comparable studies of the 11 target compounds in SW, EW and IW.

	Study	Country	Analytical Method	MQL [ng/L]
Influent				
E1	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	40
	Zhang and Fent, 2018	Switzerland	HPLC–MS–MS	0.09
	Liu et al. 2011a	China	RRLC-MS/MS	0.68
E2	Zhang and Fent, 2018	Switzerland	HPLC–MS–MS	0.8
	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	30
	Liu et al. 2011a	China	RRLC-MS/MS	4.8
E3	Zhang and Fent, 2018	Switzerland	HPLC–MS–MS	0.4
	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	40
	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	20
EE2	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	30
	Liu et al. 2011a	China	RRLC-MS/MS	2.4
NGT	Liu et al. 2011a	China	RRLC-MS/MS	1.0
NOR	Liu et al. 2011a	China	RRLC-MS/MS	1.4
PGR	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	20
	Liu et al. 2011a	China	RRLC-MS/MS	0.29
TTR	Liu et al. 2011a	China	RRLC-MS/MS	0.88
Effluent				
E1	Kolodziej et al., 2003	USA	GC–MS/MS	0.4
	Guedes-Alonso et al., 2013	Spain	UPLC-MS/MS	8.7
	Carballa et al., 2004	Spain	GC–MS/MS	1
	Gabet-Giraud et al., 2014	France	LC-MS/MS	0.3–2.7
	Zuehlke et al. 2004	Germany	LC-MS/MS	0.2
	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	40
	Liu et al. 2011a	China	RRLC-MS/MS	0.17
E2	Guedes-Alonso et al. 2013	Spain	UPLC-MS/MS	8.5
	Carballa et al., 2004	Spain	GC–MS/MS	1

	Kolodziej et al., 2003	USA	GC–MS/MS	0.3
	Gabet-Giraud et al., 2014	France	LC-MS/MS	0.3–2.7
	Zuehlke et al. 2004	Germany	LC-MS/MS	0.4
	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	30
	Liu et al. 2011a	China	RRLC-MS/MS	0.95
E3	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	20
	Guedes-Alonso et al. 2013	Spain	UPLC-MS/MS	31
EE2	Guedes-Alonso et al. 2013	Spain	UPLC-MS/MS	1.7
	Carballa et al., 2004	Spain	GC–MS/MS	1
	Gabet-Giraud et al., 2014	France	LC-MS/MS	0.3–9.0
	Zuehlke et al. 2004	Germany	LC-MS/MS	0.4
	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	30
	Liu et al. 2011a	China	RRLC-MS/MS	1.6
NGT	Guedes-Alonso et al. 2013	Spain	UPLC-MS/MS	7.0
	Liu et al. 2011a	China	RRLC-MS/MS	0.1
NOR	Liu et al. 2011a	China	RRLC-MS/MS	0.71
PGR	Lindberg et al. 2014	Sweden	LC-HESI-MS/MS	20
	Liu et al. 2011a	China	RRLC-MS/MS	0.27
TTR	Guedes-Alonso et al. 2013	Spain	UPLC-MS/MS	5.0
	Liu et al. 2011a	China	RRLC-MS/MS	0.37
Surface Water				
E1	Liu et al. 2011a	China	RRLC-MS/MS	0.3
	Yamamoto et al., 2006	Japan	LC-MS/MS	0.7
E2	Liu et al., 2011a	China	RRLC-MS/MS	0.8
	Yamamoto et al., 2006	Japan	LC-MS/MS	0.7
E3	Yamamoto et al., 2006	Japan	LC-MS/MS	1.5
EE2	Liu et al. 2011a	China	RRLC-MS/MS	0.64
	Yamamoto et al., 2006	Japan	LC-MS/MS	0.9
NGT	Liu et al. 2011a	China	RRLC-MS/MS	0.12
NOR	Liu et al. 2011a	China	RRLC-MS/MS	0.08

PGR	Liu et al. 2011a	China	RRLC-MS/MS	0.17
TTR	Liu et al. 2011a	China	RRLC-MS/MS	0.18
TTR	Yamamoto et al., 2006	Japan	LC-MS/MS	0.06

3.2.6. *Linearity of the calibration curve*

This method has a linear calibration range of 0.01 ng/L – 100 ng/L with correlation coefficients close to 1 (0.96 – 0.99) for all estrogens, TTR, PGR, NGT and ETO, and a medium correlation coefficient for DIE, GES and NOR (0.86 – 0.89) (see Table 14)

Table 14: Coefficient of correlation (R^2) of all compounds with a calibration range of 0.01 ng/L to 100 ng/L for LC-MS/MS.

Compound	R^2
E1	0.97
E2	0.96
E3	0.99
EE2	0.96
ETO	0.97
DIE	0.86
GES	0.88
NGT	0.97
NOR	0.89
PGR	0.98
TTR	0.96

Even though these R^2s suggest a straight-line relationship between the nominal concentration and the measured peak area ratios (relative to IS area), these values cannot be proof enough for the linear relationship between input (x) and output (y), since no statistical analysis was conducted.

3.2.7. *Potential of the analytical method*

The tested analytical method had been optimized concerning its sample preparation and shows good linearity, sensitivity below achieved MQL's and IDL's of comparable studies and absolute recovery between the tolerance range for all compounds in all three matrices. When eluted with 6 mL of ACN, matrix effect stays relatively consistent for all compounds. For the application in SW, the current analytical method has been optimized for SW with

relative recoveries between 110–120% for all compounds except GES. However, relative recoveries for EW and IW could be improved by higher concentration levels of the calibration curve as well as spiking levels, since concentrations in these matrices are higher than previously expected. The method shows good accuracy for all compounds except EE2 and is reproducible and repeatable for all compounds in MilliQ, for all compounds except TTR in SW and for most compounds in EW and IW. To further analyze repeatability and reproducibility, experiments should be repeated with higher spiking levels and a wider calibration range.

3.3. Method application: hormone detection at the WWTP

3.3.1. Cumulative concentrations of hormones in IW and EW measured with TIMFIE

Influent samples

All compounds except EE2 and DIE in week 1 and week 2 have been detected at levels above 5 ng/L. NOR and E3 have been detected at levels above 100 ng/L (Table 15). Since these measurements are higher than the upper calibration limit, it cannot be said with certainty, which concentration has been measured. All measurements that are above 100 ng/L are stated as >100 ng/L.

Table 15: Concentration levels in ng/L for all compounds during three weeks. Concentrations measured in both inlets have been summed up. >100 = concentration higher than 100 ng/L. Samples have been conducted by means of TIMFIE. Exact values for replicates, mean and SD are given in Appendix XIV.

	E1	E2	E3	EE2	ETO	DIE	NOR	PGR	TTR
Week 1	15	9.0	> 100	<4.9	5.0	<1.0	> 100	12	43
Week 2	63	22	> 100	<4.9	18	7.0	> 100	31	98
Week 3	47	21	> 100	<4.9	19	<1.0	> 100	49	87

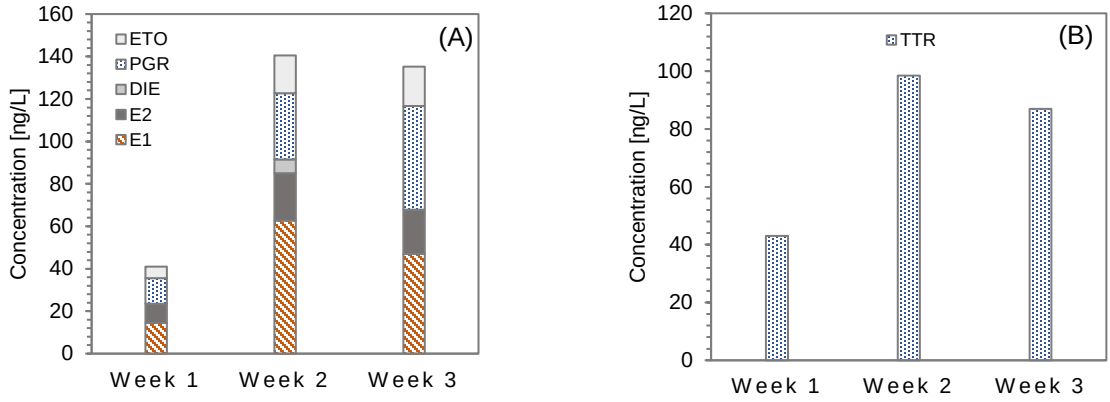

Figure 11: Cumulative concentrations of low-level hormones (A) and high-level hormones (B) in ng/L. Since levels of E3 and NOR lie above 100 ng/L, they are not included in high-level-hormone graph b. Three weeks in comparison. Samples have been conducted by means of TIMFIE.

Within the low-level hormones, E1 and PGR account for the largest proportion of hormone concentration in IW. Concentrations reach average values for week 1, week 2 and week 3 of 15, 63, 47 ng/L and 12, 31, 49 ng/L of E1 and PGR respectively. Low levels up to 7 ng/L of DIE only occur in week 2. There is an overall trend that concerning low-level-hormones, the total cumulative concentration in both influents reaches values of around 40 ng/L in week 1, while week 2 and 3 reaches concentrations of 140 and 135 ng/L respectively. This can be due to changes in wastewater flow over the weeks and an improvement in sampling. Since numerous samplers in week 1 have been clogged, the positioning of TIMFIE samplers has been adapted as well as the measures against clogging been taken. Low-level-hormones do not exceed concentrations above 70 ng/L, while high-level hormones such as TTR, NOR, NGT, GES and E3 reach levels above 100 ng/L. High concentrations of E3 can be explained by the decomposition of E1, E2 and EE2 to their common metabolite E3 reviewed by Adeel et al., 2016.

E3 has been measured to levels between 31 – 830 ng/L in Europe (Guedes-Alonso et al., 2013; Koh et al., 2007; Laganà et al., 2004; Martín et al., 2012) and to levels between 5 –

414 ng/L outside of Europe (Esperanza et al., 2007; Manickum et al., 2016; Peng et al., 2008; Sim et al., 2011; Ye et al., 2012; Z. Zhang et al., 2011). In this study, E3 exceeds 100 ng/L in IW, which is compatible with the concentration range from the other studies mentioned above.

E1 concentrations range between 3 – 70 ng/L in Sweden (Zorita et al., 2009) and 20 – 51 ng/L in the rest of Europe (Guedes-Alonso et al., 2013; Kumar et al., 2011; Laganà et al., 2004; Salvador et al., 2007). Outside of Europe, the measured concentrations of E1 lay between 6.5 – 566 ng/L (Atkinson et al., 2012; Chang et al., 2010; Esperanza et al., 2007; Hashimoto et al., 2007; Lishman et al., 2006; S. Liu et al., 2011a; Manickum et al., 2016; Mohagheghian et al., 2014; Muz et al., 2012; Pessoa et al., 2014; Servos et al., 2005; Sim et al., 2011; Surujlal-Naicker and Bux, 2013; Ye et al., 2012; Z. Zhang et al., 2011).

Comparison with literature shows that concentrations of hormones highly vary from country to country and from study to study. Differences lie in the different use of analytical and sampling method, different approaches in sample preparation, different hormone consumption and excretion by the population and lastly different WWTP cleaning procedures.

Effluent samples

Concentration levels of EW samples can be seen in Table 16 and Figure 11. E1, E2, E3, PGR and ETO have been detected above the respective quantification limit with concentrations between 0.99 – 2.1 ng/L, 2.5 – 3.6 ng/L, 3.0 – 4.6 ng/L, 0.43 – 0.55 ng/L and 40 – 50 ng/L respectively. All remaining progestins, EE2 and TTR were not quantifiable.

Table 16: Concentration levels [ng/L] of EW samples collected with TIMFIE for all three sampling weeks.

	E1	E2	E3	EE2	ETO	DIE	NOR	PGR	TTR
Week 1	2.1	3.5	3.6	<2.4	50	<0.12	< 0.74	0.55	< 0.21
Week 2	1.9	3.6	4.6	<2.4	50	<0.12	< 0.74	0.43	< 0.21
Week 3	0.99	2.5	3.0	<2.4	40	<0.12	< 0.74	0.43	< 0.21

Measured concentrations of E1 are comparable with other studied within Europe with concentrations ranging from 1.9 to 30 ng/L (Labadie and Budzinski, 2005; Salvador et al., 2007). Studies outside Europe (i.e. China and Taiwan) measured values between 8.5 – 26 ng/L (Chang et al., 2010; Chen et al., 2007; S. Liu et al., 2011a).

In European studies (i.e. Spain and France), E2 and E3 were in some countries undetected (Guedes-Alonso et al., 2013; Lindberg et al., 2014), or measured in levels between 0.5 – 1.2 ng/L and 1.0 ng/L respectively (Guedes-Alonso et al., 2013; Labadie and Budzinski, 2005; Lindberg et al., 2014; Salvador et al., 2007). E2 and E3 were either undetected or reached a value of 23 ng/L and 44.5 ng/L respectively in studies outside of Europe (Chen et al., 2007).

While this study was not able to quantify EE2 in EW, the French study by Salvador et al. 2007 detected EE2 at a level of 0.9 ng/L, which is much below the LOQ of 2.4 ng/L for EE2. Another Swedish study by Lindberg et al., 2014 failed to quantify EE2 in EW. In Taiwan, EE2 levels reach concentrations up to 15.3 ng/L (Chen et al., 2007).

PGR and ETO have not yet been detected in Sweden (Fick et al., 2011; Lindberg et al., 2014), making this study the first ever to detect and quantify PGR and ETO in EW. Within Europe, PGR has been quantified in the Czech Republic with a concentration level of 0.95 ng/L (Golovko et al., 2018) and outside of Europe in a concentration of 2.3 ng/L (Chang et al., 2010). This study did not quantify DIE, GES, NOR, NGT and TTR in EW. Levels of DIE, GES, NGT and TTR however have been quantified by other studies with values of 0.14 ng/L,

1.71 ng/L, 9.2 ng/L and 0.2 – 9.95 ng/L respectively (Chang et al., 2010; Golovko et al., 2018; Guedes-Alonso et al., 2013; S.-S. Liu et al., 2014).

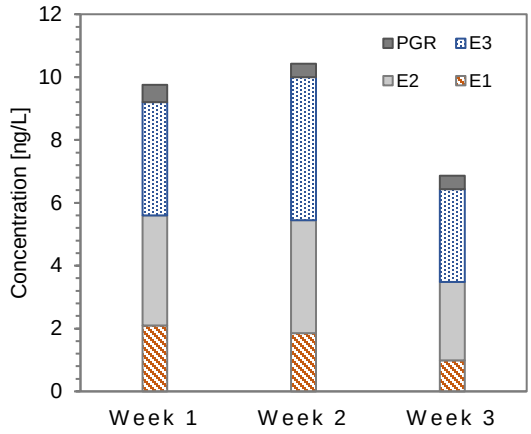

Figure 12: Cumulative concentration Levels [ng/L] of EW samples collected with TIMFIE for all three sampling weeks

It is well established, that seasonal variations can affect the removal of natural and synthetic estrogens. Lower temperatures lead to a reduction in removal efficiency due to decreased bacterial activity and thus higher concentrations in EW of WWTP (Nakada et al., 2006; Ternes et al., 1999). Since sampling has been conducted in winter-spring months (February and March), concentrations might differ if the same approach is conducted during spring and summer months. Implications of these measured concentrations will be discussed in subsection 3.4.

3.3.2. Removal efficiency

Removal efficiency has been calculated by comparing the EW concentrations with cumulative IW concentrations. As shown in Table 17, 100% of TTR, NOR and DIE is achieved by the Uppsala WWTP. Concentrations of E1, E2, E3 and PGR are not fully removed with, respectively, 3.9, 18, 3.7 and 1.5% remaining from the incoming concentration

(Table 17). The concentration of ETO increases by more than 300%. Studies have shown, that in the human metabolism, ETO is built as a degradation product of Desogestrel (Madden et al., 1990; Viinikka, 1979). Further studies should evaluate degradation processes of the included target analytes, in order to assess if high concentrations of ETO could also be caused by a degradation of other progestins such as Desogestrel.

Table 17: Average IW and EW hormone concentration [ng/L] and the removal efficiency of incoming concentration

	IW concentration [ng/L]	EW concentration [ng/L]	Removal efficiency [%]
E1	41	1.6	96
E2	17	3.2	82
E3	>100	3.7	96
EE2	0	0	-
ETO	14	46	-230
DIE	2.2	0	100
NOR	>100	0	100
PGR	31	0.47	99
TTR	76	0	100

A German study by Zuehlke et al. 2004 showed consistent results with a removal efficiency of 93.3, 92.9, and 80.4 % for E1, E2 and EE2 respectively. Other studies (see Table 18) showed very varying removal efficiencies. These differences may be due to different treatment techniques of the WWTPs. Clearly, as this study and numerous other studies show, many steroids are not removed sufficiently from IW. In order to identify optimization potential in wastewater cleaning steps, concentration changes before and in between the separate cleaning steps should be further investigated.

Zhang and Zhou, 2008 found a higher removal efficiency for E2 from wastewater when treated with UV radiation compared to degradation by sunlight, due to the high UV

absorbance by EDCs. Even though cleaning steps as UV radiation might lead to a degradation of incoming steroids, formation of toxic metabolites through degradation processes should not be neglected. Thus, when speaking about removal efficiency, in reality, endocrine disrupting degradation products might be persistent in quantifiable levels in EW.

Table 18: List of reference studies that calculated removing efficiency [%] of estrogens by WWTPs.

	E1	E2	E3	EE2
Zorita et al., 2009	78	–	–	–
Koh et al., 2007	80	86	98	17
Ye et al., 2012	50	70	95	–
Zhang and Zhou, 2008	78–92	69–90	–	77–100
Zuehlke et al., 2004	93	93	–	80

3.3.3. Comparison between TIMFIE and Composite Samples

In order to evaluate the TIMFIE sampling device for the assessment of steroid hormones in EW and IW, measured concentrations from TIMFIE were compared with those from weekly composite AS samples, which have been conducted in the same week. Concentrations of both inlets and outlet in TIMFIE and composite samples are given in figure 13 and 14 for IW and figure 15 for EW.

Hormone concentrations in TIMFIE samples and in composite samples are within the same range for most compounds in both EW and IW.

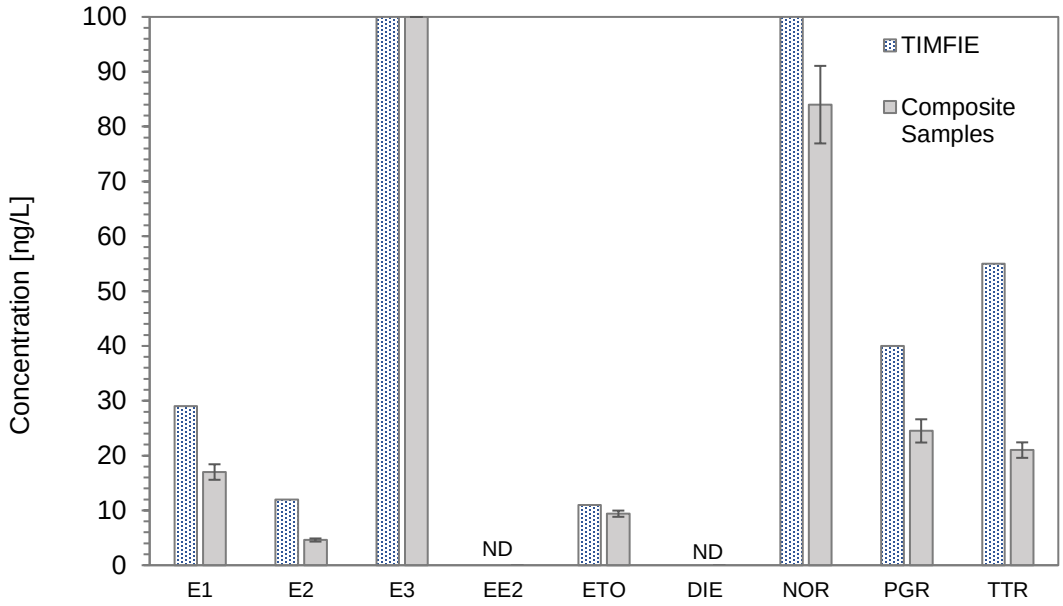

Figure 13: Concentrations [ng/L] of hormones with respective standard deviation for IW samples at Inlet A+B, sampled by TIMFIE and composite samples in sampling week 3. ND = Not detected. Graph cuts data at 100 ng/L, since concentrations above calibration range (0.01 – 100 ng/L) can not be quantified exactly.

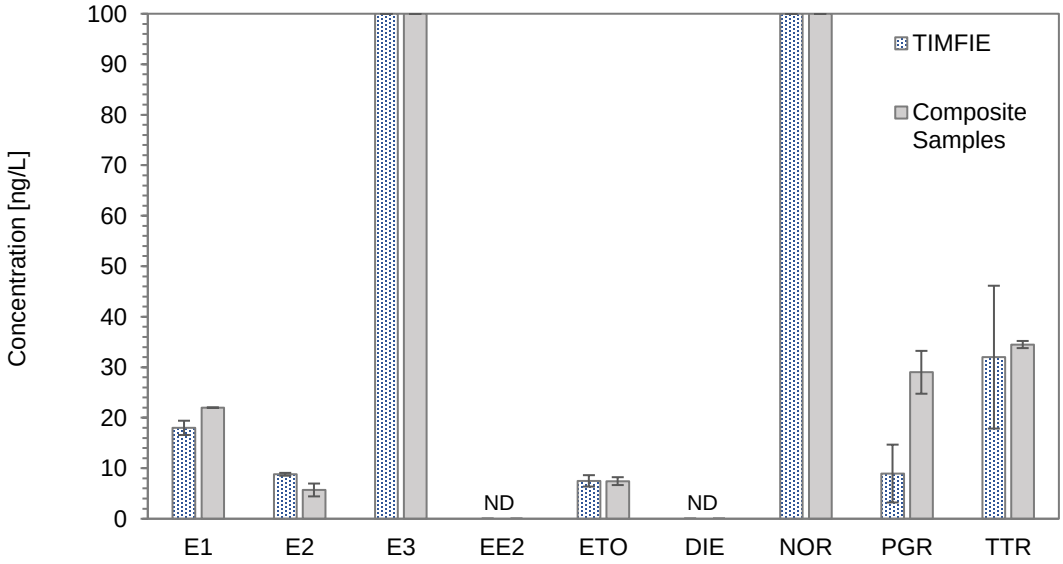

Figure 14: Concentrations [ng/L] of hormones with respective standard deviation for IW samples at Inlet C, sampled by TIMFIE and composite samples in sampling week 3. ND = Not detected. Graph cuts data at 100 ng/L, since concentrations above calibration range (0.01 – 100 ng/L) can not be quantified exactly.

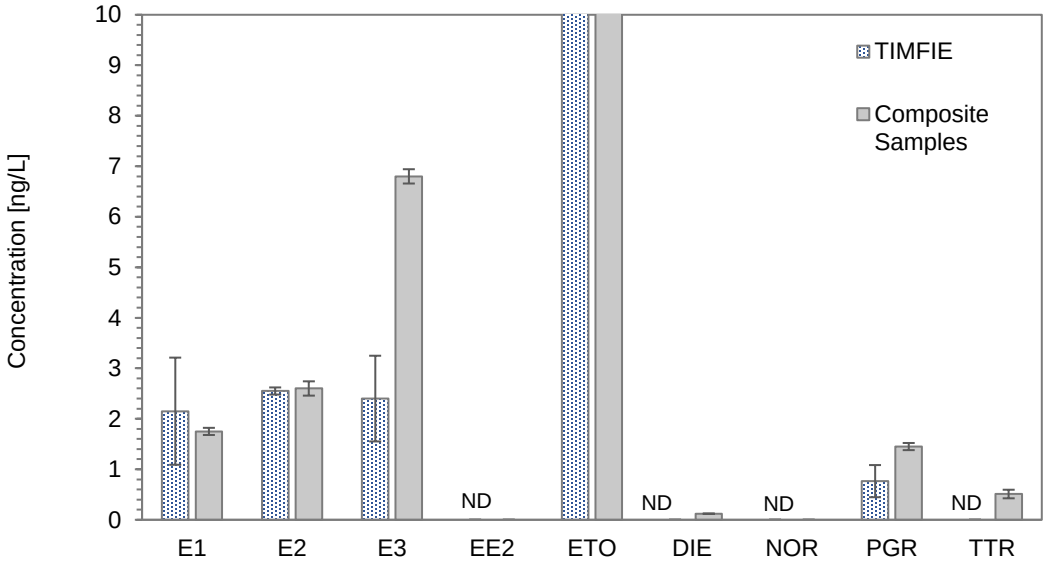

Figure 15: Concentrations [ng/L] of hormones with respective standard deviation for EW samples sampled by TIMFIE and composite samples in sampling week 3. ND = Not detected. Graph cuts data at 100 ng/L, since concentrations above calibration range (0.01 – 100 ng/L) can not be quantified exactly.

Small variations could be due to the fact that for the composite samples, only every second day was chosen, neglecting Wednesday, Friday and Sunday. Including these days might have changed measured concentrations of the composite samples.

The high variation could also be explained through the limited volume and extraction of TIMFIE samplers. Particle mass in the sampling sink might have caused a variation in extraction duration. Partly clogged TIMFIE samplers might have taken full 7 days in order to extract 90 mL of water, while for some samplers, full 90 mL might have been extracted after only 4–5 days.

3.3.4. POCIS results

In order to assess IW and EW qualitatively, POCIS sampler have been deployed at inlet and outlet for the entire sampling period of three weeks. If hormones have not been

quantified in TIMFIE and composite samples, the POCIS sampler might still have detected traces of those compounds. In this way, POCIS serves as a supportive qualitative sampler.

Table 19: Concentrations [ng/L] measured by means of TIMFIES and composite samples in influent A+B and influent C. Qualitative POCIS data for each influent is given as either D = detected or ND = undetected. Ex. = Excluded from analysis due to bad performance in TIMFIE method validation.

	Influent A+B			Influent C		
	TIMFIE (n = 1)	Composite samples (n = 2)	POCIS (n = 2)	TIMFIE (n = 3)	Composite samples (n = 2)	POCIS (n = 2)
E1	29	22 ± 0	D	18 ± 1.4	17 ± 1.4	D
E2	12	5.7 ± 1.3	D	8.8 ± 0.28	4.6 ± 0.28	D
E3	>100	>100	D	>100	>100	D
EE2	<4.9	<4.9	D	<4.9	<4.9	D
ETO	11	7.5 ± 0.78	D	7.5 ± 1.1	9.4 ± 0.57	D
DIE	<0.41	<0.41	ND	<0.41	<0.41	ND
GES	Ex.	Ex.	D	Ex.	Ex.	D
NGT	Ex.	Ex.	D	Ex.	Ex.	ND
NOR	>100	>100	ND	>100	84 ± 7.1	ND
PGR	40	29 ± 4.2	D	9.0 ± 5.7	25 ± 2.1	D
TTR	55	35 ± 0.71	D	32 ± 14	21 ± 1.4	D

All target hormones except DIE and NOR have been detected in influent A+B, while DIE, NGT and NOR have not been detected in influent C (see Table 19). Interestingly, NOR was not detected in POCIS samples, however, it was detected in quantifiable levels in TIMFIE and AS samples in both inlets.

In EW, E1, E2, E3, ETO, GES and PGR have been detected in POCIS samples (see Table 20). TTR and DIE have been detected in low levels (0.51 ng/L and 0.12 ng/L respectively) in AS samples, while it remained undetected in POCIS and TIMFIE samples. For TIMFIE procedure, NGT and GES have been excluded, since neither of those compounds

performed well enough in TIMFIE method validation. Analysis of POCIS samples can now give a hint about levels of GES and NGT in EW. GES was found in POCIS samples; thus, it can be concluded, that this compound is present in EW in detectable levels.

Table 20: Concentrations [ng/L] measured in TIMFIES and composite samples in EW. Qualitative POCIS data for each influent is given as either D = detected or ND = undetected. Ex. = Excluded from analysis due to bad performance in TIMFIE method validation.

	Effluent		
	TIMFIE (n = 2)	Composite samples (n = 2)	POCIS (n = 2)
E1	2.2 ± 1.1	1.8 ± 0.071	D
E2	2.6 ± 0.071	2.6 ± 0.14	D
E3	2.4 ± 0.85	6.8 ± 0.14	D
EE2	<2.4	<2.4	D
ETO	36 ± 0.71	24 ± 0	D
DIE	<0.12	0.12 ± 0	ND
GES	Ex.	Ex.	D
NGT	Ex.	Ex.	ND
NOR	<0.73	<0.73	ND
PGR	0.77 ± 0.32	1.5 ± 0.071	D
TTR	<0.21	0.51 ± 0.085	ND

EE2 has a relatively high MQL (2.4 ng/L) for EW, thus concentrations below this threshold were not quantifiable. However, EE2 was found in POCIS samples and is hence present in EW in detectable levels and not removed sufficiently by the WWTP.

POCIS results are consistent with TIMFIE and composite samples results for all compounds except NOR in both matrices and TTR in EW. Thus, it can be concluded that TIMFIE is a valid sampling device and can be used for the assessment of steroid hormones

in wastewater. Variations between TIMFIE and POCIS can be due to the clogging of the POCIS metal cage, which after three weeks, was partly covered in big particles. Increasing coverage of the metal cage might have blocked some compounds or prevented higher incoming flows to be caught. Further investigation of EE2, GES, DIE, NGT and NOR should be subject to future studies, since those compounds prove to be problematic for POCIS, TIMFIE and composite samples.

3.4. Implications for environment and health related risks

The findings of this study clearly show that all natural estrogens E1, E2 and E3 as well as the natural progestin PGR are present in EW in quantifiable levels, indicating that these compounds are not sufficiently removed by the Uppsala WWTP. Further, sampling by means of POCIS revealed that 7 out of the 11 tested steroid hormones are still present in EW in detectable levels. Since EW water of the Uppsala WWTP is discharged into the Fyrisån, these compounds are discharged into the SW of Fyrisån and may accumulate in the environment.

As for synthetic progestins, fate in the environment is barely studied (Fent, 2015). The fate of E1, E2, E3, TTR and PGR in the environment has been studied by Zhang et al., 2014. In this study, it was found that 73% of discharged hormones ended up in sediments, 7% in soils and 20% of the total discharged mass ended up in water bodies.

Since even low levels of steroids can cause severe damage in several mollusk, amphibians, fish and mammal species (Lintelmann et al., 2003), the measured concentrations released might deteriorate ecological processes in Swedish waters. As for progestins, due to their low K_{ow} , these compounds often accumulate in biological plasma in aquatic environments (Fent, 2015; Kumar et al., 2015). When accumulated in mammals, progestins negatively affect reproductive organ development and cause feminization in fish (Fent, 2015). Estrogens were shown to cause change in population sex ratios (Lange et al., 2008) and to induce ovotestis (Jobling et al., 2006). Likewise do androgens impair sex development in zebrafish (Ankley et al., 2003; Fenske and Segner, 2004).

These effects can already occur even if very low concentrations are accumulated in the organs. For instance, a study by Zucchi et al., 2012 showed that the lowest observed effect concentration (LOEC) for PGR to adversely affect gene expression in embryo zebrafish was 2 ng/L (see Table 21). The synthetic Progestin NGT impairs reproduction and decreases fecundity decrease in fathead minnows at a lowest observed effect concentration of 1 ng/L (Table 21). So far, the lowest LOEC for steroid hormones in Fathead minnows was found by Brian et al., 2005, where a EE2 concentration of 0.036 ng/L induced vitellogenesis in males.

This concentration lies below the in this study achieved MQL of 2.4 ng/L, and concentrations below could have been present, but not quantified.

According to an estimation by Kumar et al., 2015b, the LOEC for ETO lies at 29 ng/L. The current study found levels of approximately 50 ng/L in EW. Even though these concentrations might be further diluted in SW and partly accumulate in sediment and soil, discharges from different WWTPs adhering the Fyrisån, hospital, agriculture and aquaculture might add up in the SW and accumulate in environmental and biological matrices. Thus, one could expect ETO to be present in SW adhering the Uppsala WWTP in LOECs. However, in order to draw an informed conclusion between the findings of this study and implications for ecosystems, it is crucial to further assess SW of the Fyrisån and adjacent lakes, sediments and soil. This will help to fully understand the distribution and effect of steroid hormones in Swedish waters surrounding Uppsala.

Further, since recent studies have found estrogens in drinking water and rural groundwater (Adeel et al., 2016; Gee et al., 2015), these findings are a reason enough for concern about health related effects. If concentrations detected in EW accumulate in groundwater sources or pass through drinking water treatment plants without being removed completely, they might cause diseases such as reproduction and fertility disorders, PSOC, breast cancer, testicle and prostate cancer (Bloom et al., 2016; Daston et al., 1997; Gallo et al., 2016; Joffe, 2001; Sheikh et al., 2016; Soto and Sonnenschein, 2015; Tarantino et al., 2013; Toft et al., 2012)

Table 21: List of Lowest Observed Effect Concentrations (LOEC) [ng/L] for estrogens E1, E2 and EE2 and progestins ETO, GES, NGT, Levonorgestrel (LNGT), NOR and PGR. [a]Theoretical calculation by using therapeuthical levels for human use, concentration in fish plasma and bioaccumulation factor.

	Fish species	LOEC [ng/L]	Sex, life stage	Exposure Duration	Effects	References
E1	Rainbow Trout	3.3	Female Juvenile	14 days	Induced Vitellogenesis	Thorpe et al., 2003
E2	Fathead minnows	1	Males	15 days	Induced Vitellogenesis	Brian et al., 2005
	Zebrafish	1	Males	9 days	Induced Vitellogenesis	Rose et al., 2002
	Rainbow Trout	14	Female Juvenile	14 days	Induced Vitellogenesis	Thorpe et al., 2003
EE2	Fathead minnows	0.036	Males	14 days	Induced Vitellogenesis	Brian et al., 2005
	Zebrafish	0.06	Males	8 days	Induced Vitellogenesis	Rose et al., 2002
	Zebrafish	1.10	Males	20 days	Fertility	Schäfers et al., 2007
	Rainbow Trout	1	Female Juvenile	14 days	Induced Vitellogenesis	Thorpe et al., 2003
ETO	-	29[a]	-	-	Impairment of reproduction	Kumar et al., 2015b
GES	Fathead minnows	1	Females	21 days	Fecundity decrease, reproductive	Runnalls et al., 2013
	Fathead minnows	100	Females	21 days	Steroid levels	Runnalls et al., 2013
NGT	Zebrafish	5	Embryos	144 h	Transcripts	Liang et al., 2015
LNGT	Fathead minnows	0.8	Females	21 days	Fecundity decrease, reproduction	Zeilinger et al., 2009
	Zebrafish	2	Embryos	48–144 h	Transcripts	Zucchi et al., 2012
	Three-spined stickleback	65	Males	45 days	Transcripts, spiggin, spermatogenesis	Svensson et al., 2014
NOR	Fathead minnows	1	Females	21 days	Fecundity decrease, reproductive	Paulos et al., 2010
	Fathead minnows	10	Females	21 days	Steroid hormones	Paulos et al., 2010
	Zebrafish	2	Embryos	48–144 h	Transcripts	Zucchi et al., 2012
PGR	Fathead minnows	100	Females	21 days	Fecundity, reproduction	DeQuattro et al., 2012
	Fathead minnows	10	Females	21 days	Vitellogenin mRNA decrease	DeQuattro et al., 2012
	Zebrafish	2	Embryos	48–144 h	Transcripts	Zucchi et al., 2012
	Zebrafish	63	Embryos	40 days	Sex ratio, transcripts	Liang et al., 2015

4 Conclusion

This study aimed to establish an analytical method for measurement of steroid hormones in water samples. It further aimed to evaluate steroid hormones in waters using the novel TIMFIE sampling device, and to finally assess steroid levels of 4 estrogens, 6 progestins and one androgen in influent and effluent water of the Uppsala WWTP.

It was found that for the analysis of a broad range of steroids, LC-MS/MS is the more sensitive and applicable instrumental method. Sensitivity of the analytical method was in the range of lowest observed effect concentrations in mammals and fish. It was further proven to be reproducible, repeatable and accurate for 10 out of the 11 tested target compounds. Extraction by TIMFIE yielded very good absolute and relative recoveries in all tested matrices. Thus, it can be concluded that TIMFIE is a suitable and effective sampling device for the assessment of steroid hormones in surface water, effluent water and influent water.

All target hormones except EE2 and DIE have been quantified in influent water of the WWTP by means of TIMFIE, while all compounds have been detected by means of POCIS. In effluent water, E1, E2, EE2, ETO and PGR have been quantified while all target compounds except DIE, NGT, NOR and TTR have been detected. Thus, 5 out of 11 target compounds are still persistent in effluent water in quantifiable levels while 7 out of 11 steroids occur at detectable concentration levels.

This study has been the first ever study to successfully assess a broad range of steroid hormones in a Swedish WWTP. It further set the foundation for future assessments of steroids in surface waters with the novel TIMFIE sampling device followed by LC-MS/MS determination. Future studies should assess a broader range of steroid hormones and their pathological and environmental effects, their levels in environmental matrices as well as treatment approaches, which are able to completely remove all traces of steroids.

5 Appendix

Appendix I

TableA1: SRM Parameters for LC-MS/MS Analysis.

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	Min Dwell Time (ms)	RF Lens (V)
Estrogens								
E1	5.26	2	-	269	143.04	54.7	24.091	78.94
E2	5.4	2	-	271.12	145.04	40.29	24.091	83.15
E3	3.75	2	-	287.2	143.04	51.56	25.107	86.11
EE2	5.32	2	-	295	145.04	40.94	24.091	81.17
Progestins								
ETO	5.73	2	+	325	147.111	79	24.091	49.28
DIE	4.75	2	+	312.2	135.04	29.21	24.091	63.37
GES	5.36	2	+	311	109.04	25.42	24.091	48.29
NOR	5.32	2	+	299.2	109.04	26.23	24.091	49.53
NGT	5.65	2	+	313.2	159.04	25.83	24.091	56.2
PGR	6.03	2	+	315.25	97.04	22.08	24.091	47
Androgens								
TTR	5.48	2	+	289	97.04	22.34	24.091	45.08
Carbon Labeled Hormones								
E2-C13	3.75	2	-	290.2	146.054	55	25.107	176.34
EE2-C13	5.9	2	-	295	145.04	36.04	24.091	127.89
NOR-C13	5.32	2	+	301.2	109.04	26.69	24.091	86.61
PGR-C13	6.03	2	+	318.25	100.111	22.34	24.091	82.4

Appendix II

TableA2: Relative Recoveries [%] of this study for MilliQ water, SW, EW and IW in comparison with studies that used similar or comparable sample preparation approaches and LC-MS/MS.

Spiking Level																	
Study	Zhang 2017	This Study		Zhang 2017	Shen 2018	This Study	Koh 2007	Zhang 2017	Shen 2018	Liu 2011a	This Study	Liu 2011a	Zhang 2017	This study
Matrix	MilliQ			SW			EW				IW			
	N.S.	1 ng/L	5 ng/L	N.S.	10 ng/L	10 ng/L	15 ng/L	N.S.	10 ng/L	**20 ng/L**	30 ng/L	50 ng/L	N.S.	30 ng/L
E1	106 ± 5	0	72 ± 11	94 ± 6	–	120	88 ± 3	90 ± 7	–	111 ± 3.8	120 ± 3	123 ± 15	102 ± 8	67 ± 7
E2	113 ± 3	75 ± 5	73 ± 9	119 ± 6	–	110	88 ± 4	99 ± 9	–	106 ± 1.5	110 ± 1	123 ± 14	106 ± 4	56 ± 5
E3	96 ± 7	260 ± 60	96 ± 14	82 ± 11	–	110	86 ± 6	96 ± 5	–	–	110 ± 0.9	–	126 ± 9	120 ± 3
EE2	114 ± 2	50 ± 5	93 ± 2	112 ± 9	–	110	83 ± 5	96 ± 8	–	142 ± 0.1	36 ± 0.2	89 ± 7	119 ± 6	72 ± 14
ETO	–	26 ± 17	65 ± 23	–	82 ± 13	120	–	–	94 ± 17	–	150 ± 9	–	–	68 ± 17
DIE	107 ± 5	0	0	101 ± 5	–	110	–	124 ± 7	–	–	140 ± 13	–	114 ± 5	100 ± 5
GES	101 ± 5	45 ± 5	84 ± 4	111 ± 5	90 ± 16	2100	–	97 ± 16	96 ± 14	–	710 ± 47	–	103 ± 12	650 ± 64
NGT	–	23 ± 5	53 ± 23	–	87 ± 13	110	–	–	91 ± 15	101 ± 4.6	0	111 ± 5	–	0
NOR	110 ± 4	23 ± 4	60 ± 11	114 ± 4	88 ± 9	110	–	110 ± 7	100 ± 12	121 ± 10	100 ± 1	94 ± 6	114 ± 9	89 ± 2
PGR	107 ± 11	45 ± 4	72 ± 8	105 ± 5	101 ± 11	110	–	108 ± 6	102 ± 12	74.2 ± 4.8	110 ± 2	104 ± 1	108 ± 8	54 ± 3
TTR	112 ± 3	0	84 ± 8	116 ± 5	–	110	–	118 ± 7	–	95.4 ± 1.6	110 ± 4	98 ± 1	120 ± 3	59 ± 8

Appendix III

TableA3: LOQ [ng/L], IDL [ng/L] and IQL on column [pg] for all compounds in LC-MS/MS and GC-MS/MS. IQL on column takes into consideration the different injection volumes for both instruments, such that the limit is given in pg, without volume. ND = Not detected

	UPLC-MS/MS				GC-MS/MS			
	S/N	LOQ [ng/L]	IDL [ng/L]	IQL on Column [pg]	S/N	IDL [ng/L]	LOQ [ng/L]	IQL on Column [pg]
E1	6.3	1.6	0.48	2.4	3.3	4.6	15	9.1
E2	4.3	2.3	0.7	3.5	3.5	0.43	1.4	0.85
E3	13	0.8	0.24	1.2	3	10	33	20
EE2	3	3.3	1	5	4.5	3.3	11	6.7
ETO	9.1	1.1	0.33	1.6	ND	ND	ND	ND
DIE	7.3	1.4	0.41	2.1	ND	ND	ND	ND
GES	11	0.91	0.27	1.4	ND	ND	ND	ND
NGT	8	1.3	0.38	1.9	ND	ND	ND	ND
NOR	35	0.28	0.085	0.43	ND	ND	ND	ND
PGR	40	0.25	0.075	0.38	ND	ND	ND	ND
TTR	85	0.12	0.035	0.18	89	25	2.8	50

Appendix IV

TableA4: Method optimization data with area, calculated absolute recoveries, mean, standard deviation, cv and matrix effect for samples eluted with 6 mL of ACN. Samples extracted according to TIMFIE procedure.

	Area			Recovery [%]		Mean	SD	cv [%]	Matrix Effect [%]
	Prespike	Prespike	Postspike	1	2				
E1	85000	96000	98000	89	98	93	6.7	7.2	-55
E2	20000	22000	24000	90	92	91	1.2	1.3	-65
E3	230000	230000	250000	99	92	96	4.8	5	-44
EE2	280000	310000	140000	89	220	160	96	61	-190
ETO	47000	45000	32000	100	140	120	26	21	-100
DIE	88000	97000	84000	90	120	100	19	18	-95
GES	6200000	5900000	7500000	110	79	92	19	20	160
NGT	0	0	0	0	0	0	0	0	0
NOR	42000	47000	53000	89	90	89	0.44	0.49	-95
PGR	38000	44000	66000	85	67	76	13	17	-99
TTR	170000	150000	320000	110	48	79	44	56	-90

Appendix V

TableA5: Results of Method Optimization Experiment with area for all IW samples, eluted with 4 mL and 2 mL in comparison.

	4 mL	2 mL	4 mL	2 mL	Av. 4 mL	Av. 2 mL	STDV	CV
	Influent						Influent	
E1	82000	4100	92000	4300	87000	4200	7300	8.3
E2	14000	4000	16000	4800	15000	4400	1000	6.9
EE2	33000	4600	24000	4900	28000	4800	6500	23
E3	380000	130000	330000	160000	360000	150000	36000	10
DIE	200000	3800	150000	4700	170000	4200	33000	19
ETO	36000	3800	44000	3500	40000	3700	5500	14
GES	$65 * 10^5$	$11* 10^5$	$72 * 10^5$	$12* 10^5$	$68* 10^5$	$12* 10^5$	490000	7.2
NOR	190000	0	150000	19000	170000	9600	28000	16
PGR	73000	5800	65000	4800	69000	5300	5400	7.8
TTR	350000	8100	430000	7000	390000	7600	56000	14

Appendix VI

TableA6: Results of Method Optimization Experiment with area for all SW and EW samples, eluted with 4 mL and 2 mL in comparison.

	Surface Water		Effluent		Av. 4 mL	Av. 2 mL	STDV	CV
	4 mL	2 mL	4 mL	2 mL				
E1	64000	1800	190000	3200	190000	3100	6000	3.2
E2	24000	1800	67000	4200	67000	4200	750	1.1
EE2	12000	1100	39000	6500	39000	7400	260	0.66
E3	14000	8900	47000	19000	48000	20000	1700	3.5
DIE	370000	3100	520000	4200	560000	4700	61000	11
ETO	100000	2000	370000	8000	360000	8100	18000	4.9
GES	210000	14000	$15 * 10^6$	$26 * 10^5$	$15 * 10^6$	$28 * 10^5$	980000	6.7
NOR	380000	2600	360000	0	360000	1800	810	0.22
PGR	590000	9200	940000	11000	960000	11000	30000	3.1
TTR	90000	3500	90000	7400	89000	7600	26000	3

Appendix VII

TableA7: Concentration levels [ng/L] for all IW method validation samples

	E1	E2	E3	EE2	ETO	DIE	GES	NGT	NOR	PGR	TTR
Influent Spiked 5 ng/L Day 2	22	37	>100	6	19	4.9	>100	>100	18	17	4.1
	22	27	>100	5.9	13	3.9	>100	>100	19	13	3.1
	38	48	>100	6.6	22	8.4	>100	>100	15	23	5
Influent Spiked 5 ng/L	64	42	>100	4.3	60	7.7	>100	>100	23	16	24
	44	35	>100	6.8	48	6.4	>100	>100	26	14	18
	73	45	>100	13	67	11	>100	>100	16	22	26
Influent spiked 1 ng/L	57	36	>100	20	23	3.7	>100	<12	18	17	11
	53	35	>100	17	36	2.6	>100	<9.6	16	14	9.1
	61	36	>100	15	61	2.9	>100	<12	24	19	12
Influent Blank Dav 2	32	41	>100	23	27	<0.41	>100	<9.5	39	33	10
	55	28	>100	16	12	<0.55	>100	<13	18	15	4.7
Influent Blank Day 1	55	34	>100	16	15	<0.52	>100	<12	13	14	4.2
	48	16	>100	7.7	27	<0.21	>100	<4.9	<1.2	33	18
	57	21	>100	9.7	29	<0.37	>100	<8.6	<2.2	51	24

Appendix VIII

TableA8: Concentration levels [ng/L] for all EW method validation samples. Extraction and elution conducted according to TIMFIE protocol.

	E1	E2	E3	EE2	ETO	DIE	GES	NGT	NOR	PGR	TTR
	9.3	9.3	8.2	5	12	5.2	>100	<3.3	3.1	8.9	4.4
Effluent Spiked 5 ng/L day 2	6.7	6.3	6	3.5	10	3.4	>100	<3.1	3.5	5.7	7.2
	7.5	8.6	8.3	6.8	12	4.9	>100	<4.2	6.5	7.9	5.2
	11	10	10	9.5	17	5.4	>100	<4.1	3	8.6	3.6
Effluent Spiked 5 ng/L	10	9.7	9.4	5.2	17	4.7	>100	<3.8	4	8.3	6.1
	9.8	8.2	9.9	6	17	4	>100	<3.8	6.5	7.6	6.4
	4.6	3.1	7.3	3.5	33	1.5	>100	<4.4	<1.1	2.5	1.5
Effluent Spiked 1 ng/L	3.6	3	3.9	<2	20	1.2	>100	<3	<0.77	1.9	1
	3.5	2.4	2.7	<2.1	25	1.2	>100	<3.5	<0.88	2.2	1.1
Effluent Blank Day 2	0.76	1.8	1.8	<2.1	25	<0.12	>100	<2.7	<0.69	<0.23	0.89
	0.88	1.9	1.4	<2.3	28	<0.12	>100	<2.7	<0.7	<0.23	<0.2
Effluent Blank	1.4	2.9	3.5	<2.9	37	<0.13	>100	<3.1	<0.79	<0.27	<0.23

Appendix IX

TableA9: Concentration levels [ng/L] for all SW method validation samples

	E1	E2	E3	EE2	ETO	DIE	GES	NGT	NOR	PGR	TTR
Surface Water spiked with 5 ng/L Day 2	10	7.9	7.5	10	4.1	5	4.3	<2.1	6.9	7.5	5.6
	10	10	8.1	9.6	3.7	5	6.3	<2	4.6	7.7	4.7
	9.2	9.7	7	7.8	3.7	5	3.7	<2	4.2	7	4.5
Surface Water spiked with 5 ng/L	11	11	9	7.9	4.5	5.8	2	<2.4	7.5	7.8	7.5
	9.8	10	8	7.5	2.9	5.3	2.4	<2.3	7.3	7.8	7.5
	10	9.7	8.3	10	4	4.8	5.1	<2	6.3	7.2	4.3
Surface Water spiked with 1 ng/L	2.6	1.4	<0.98	<2.7	<0.86	0.74	22	<2.3	<0.58	1.6	0.66
	1.7	1.9	<0.77	<2.1	<0.67	0.48	25	<1.3	<0.33	1.4	0.58
	1.5	1.4	<0.75	<2.1	<0.66	0.48	26	<1.2	<0.29	1.5	0.68
Surface Water Blank	0.84	2	<0.88	<2.5	<0.78	<0.077	30	<1.8	<0.45	<0.15	<0.13
	0.82	2.1	<0.63	<1.8	<0.55	<0.054	27	<1.3	<0.32	<0.11	<0.093
Surface Water Blank	<0.48	3.4	<1.1	<3.2	<0.99	<0.077	37	<1.8	<0.45	0.28	<0.13
	<0.44	2.7	<1	<2.9	<0.92	<0.072	42	<1.7	<0.43	0.3	<0.13
	<0.46	2.7	<1.1	<3	<0.94	<0.082	46	<1.9	<0.49	<0.16	<0.14

Appendix X

TableA10: Concentration levels [ng/L] for all MilliQ water method validation samples

	E1	E2	E3	EE2	ETO	DIE	GES	NGT	NOR	PGR	TTR
MilliQ Spiked with 5 ng/L	9.6	10	8.5	10	6.2	8.8	7.6	9.1	5	7.6	6.5
	9.1	9.4	6.9	6.4	6.5	7.9	3	8.4	5.8	7.7	7.4
	9.4	8.5	6.7	6.5	5.8	8.3	5.1	7.8	7.4	6.1	8.2
MilliQ Spiked with 1 ng/L	1	1.2	1.6	2	1.4	0.78	0.42	<1	0.47	1	0.8
	0.89	0.86	1.6	<1.8	1.8	0.99	0.58	<1.2	0.51	1.2	0.9
	1.1	0.87	1.4	<1.8	1.2	0.91	0.37	<1.3	0.35	1.1	0.95
MilliQ Blank	<0.4	2.3	<0.94	<2.6	9.7	<0.072	<0.4	<1.7	<0.43	<0.14	<0.13
	<0.36	1.7	<0.84	<2.3	9.7	<0.085	<0.47	<2	<0.5	<0.17	<0.15
	<0.36	4.7	<0.84	2.3	<0.74	<0.085	<0.47	<2	<0.5	<0.17	<0.15

Appendix XI

TableA11: Absolute Recoveries [%] of all compounds extracted from SW, EW and IW, eluted with 4 mL or 6 mL of ACN

	Eluted with 4 mL ACN Absolute Recovery [%]			Eluted with 6 mL ACN Absolute Recovery [%]		
	SW	EW	IW	SW	EW	IW
E1	129	118	77	129	118	77
E2	120	112	79	120	112	79
E3	71	118	172	71	118	172
EE2	122	119	163	122	119	163
ETO	111	124	96	111	124	96
DIE	101	111	140	101	111	140
GES	118	107	113	118	107	113
NGT	105	0	0	105	0	0
NOR	110	85	130	110	85	130
PGR	104	92	69	104	92	69
TTR	113	92	83	113	92	83

.

TableA12: Absolute Recovery [%] and Matrix Effect [%] of IW Samples eluted with 4 mL of MeOH

	Sample A Recovery [%]	Sample B Recovery [%]	Mean Recovery [%]	Standard Deviation	CV [%]	Matrix Effect [%]
E1	89	98	93	6.7	7.2	-55
E2	90	92	91	1.2	1.3	-65
E3	99	92	96	4.8	5.0	-44
EE2	89	225	157	96	61	-194
ETO	104	141	123	26	21	-100
DIE	90	116	103	19	18	-95
GES	105	79	92	19	20	161
NGT	0	0	0	0	0	0
NOR	89	90	89	0.44	0.49	-95
PGR	85	67	76	13	17	-99
TTR	110	48	79	44	56	-90

Appendix XII

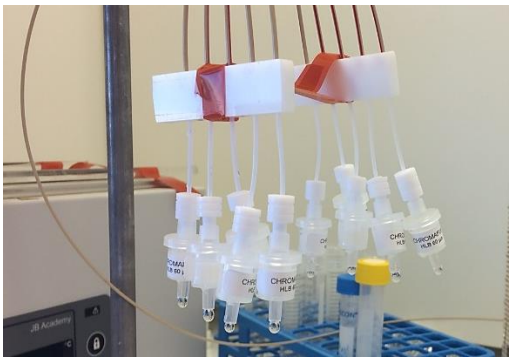

Figure A1: Simultaneous conditioning of HLB cartridges on a flow distributor.

Figure A2: Nitrogen flow distributor used to gently dry attached TIMFIE SPE cartridges.

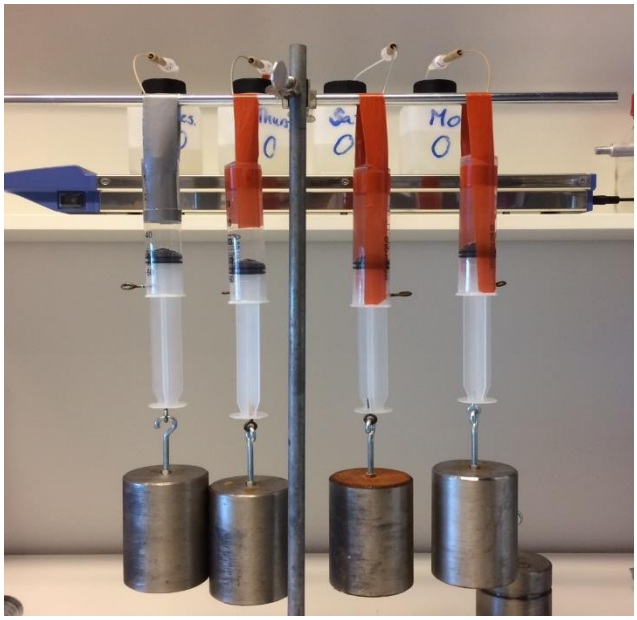

Figure A3: Extraction of water samples in the lab through a TIMFIE set-up with a vacuum created through weight.

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