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In situ active sampling of steroid hormones in water using a novel TIMFIE device: Validation and applicability

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ABSTRACT

Monitoring trace levels of endocrine-disrupting steroid hormones in water is essential in environmental assessment, necessitating development of appropriate sampling techniques. Grab/passive sampling methods are commonly used, but use of time-integrated microflow *in-situ* extraction (TIMFIE) for sampling steroid hormones remains unexplored. This study aimed to evaluate the feasibility of TIMFIE samplers equipped with a hydrophilic-lipophilic balanced sorbent for monitoring seven different steroid hormones in different water matrices. Method validation demonstrated good reproducibility and accuracy of the TIMFIE samplers for extracting almost all target hormones in surface water and effluent wastewater, but not influent wastewater. Method quantification limit (0.4 ng L^{-1}) of TIMFIE samplers for estrone (E1) in surface water and effluent wastewater met EU Water Framework Directive requirements. Comparison of TIMFIE samples and the parallel flow-based composite samples confirmed the consistency of E1, estradiol (E2), 17α-ethinylestradiol, and dienogest measurements in effluent wastewater. With TIMFIE samplers deployed in surface water, the time-weighted average concentrations of E1 (0.5–1.5 ng L^{-1}) and E2 (0.3–0.4 ng L^{-1}) were found below the predicted no-effect concentrations, indicating low risk to aquatic organisms. Given the challenges in assessing trace levels of steroid hormones in waters, TIMFIE as active, time-integrated samplers is a promising, green sampling tool for efficient, resource-conscious in situ extraction of steroid hormones, allowing a sustainable monitoring of these chemicals in the environment, including conditions under frozen-surfaced water bodies.

Introduction

Steroid hormones are a well-known class of endocrine-disrupting chemicals (EDCs) that can adversely affect aquatic organisms at trace levels [63]. Most EDCs released into the aquatic environment are synthetic compounds developed for human and animal consumption, but some are of endogenous origin, e.g. estrone (E1), estradiol (E2), and estriol (E3), which are produced and excreted during the menstrual cycle and pregnancy. Discharges of treated wastewater and runoff water from farmland and animal husbandry are the main sources of steroid hormones in the aquatic environment [31,65,68]. Water pollution with hormones has been linked to various ecological health issues, such as changes in the sex ratio of fish due to feminisation [4,17,37]. These chemicals can exert harmful effects at very low concentrations, e.g. progesterone (PGR) at 2 ng L^{-1} affects gene expression in zebrafish embryos [78], while 17α-ethinyl estradiol (EE2) at 2 ng L^{-1} induces vitellogenesis [59]. Despite the adverse impact of steroid hormones on ecosystems [22,25,56], there are no regulations limiting their release into the environment. However, E1, E2 and EE2 were previously on the EU Watch List for surface water quality monitoring across Europe and are under evaluation as priority substances in surface water in the proposed EU directive on water policy [16].

Choosing an appropriate sampling method from among different active and passive approaches is crucial to reduce uncertainty when assessing hydrophobic organic micropollutants, such as steroid hormones, that are present at low levels in the aquatic environment. For active sampling, most previous studies have relied on manual grab (spot) samples, aiming to obtain snapshot (time-discrete) information on steroid hormones in water [30,31,43,50,74]. The main advantage of grab sampling is simplicity, while the main drawback is inability to

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assess average concentrations of target compounds that show significant fluctuations in environmental concentrations over time, which is often of interest. Alternatively, active time-integrated and flow-proportional sampling can be performed using auto-samplers, to assess relevant average concentrations of pollutants in the aquatic environment [10,11,60]. However, auto-samplers require an electrical power supply or batteries and continuous service and maintenance, are expensive and are prone to be stolen or destroyed.

Another approach for time-integrated measurement is passive sampling. Different techniques have been evaluated and employed, such as the polar organic chemical integrative sampler (POCIS) with Oasis hydrophilic-lipophilic balanced (HLB) sorbent, the Chemcatcher sampler and other enhanced passive sampling systems with Empore SDB-RPS discs [6,52,66]. When deployed over a certain period in water, passive samplers allow *in-situ* extraction of the dissolved fraction of micropollutants (i.e. not bound to suspended particles) and determination of time-integrated concentrations of chemicals, where the water volume extracted can be calculated indirectly. However, before successful field application, calibration and preliminary studies are needed to determine sampling rate constants and uptake profiles of target chemicals. These vary with environmental conditions, e.g. water temperature, flow, pH, salinity and fouling [36,76]. Hence, use of passive sampling can be laborious and the results can be uncertain when knowledge on relevant field conditions is lacking.

Improving the utility of active sampling for time-integrated measurements would be highly beneficial for micropollutant monitoring, especially of steroid hormones in water. The recently developed time-integrated microflow *in-situ* extraction (TIMFIE) sampler represents significant progress towards more environmentally friendly monitoring techniques. It operates as a low-tech (non-electrical) and cost-effective time-integrated active sampler [28], while also incorporating principles of green analytical chemistry [3,19,72]. The TIMFIE sampler consists of a syringe that is set under negative pressure, a flow restrictor, a syringe filter, and one or several solid phase extraction (SPE) cartridges. The eco-conscious design combines active sampling and in-field extraction, by actively pumping whole water through the SPE cartridge into a syringe, where final sample volume is measured for quantitative determination [28]. TIMFIE samplers have been validated and applied for studying pesticides and their transformation products in surface waters [9,27,46], but their capacity to measure steroid hormones in water accurately and precisely has not yet been investigated. In fact, studies on steroid hormones in Swedish waters are rather scarce [1,18,23,40,77] and, so far, mainly based on grab sampling [1,18,77]. Wastewater collection in studies on steroid hormones often uses flow-proportional sampling [23,40], but TIMFIE samplers could be used in wastewater channels between the treatment processes at wastewater treatment plants (WWTPs).

The main aim of the present work was to study the feasibility of using TIMFIE samplers in evaluation of steroid hormone levels in water. Specific objectives were to: (a) assess the applicability of TIMFIE samplers with an HLB sorbent for sampling analytes in different water matrices; (b) compare steroid hormone levels in effluent wastewater measured using TIMFIE samplers and parallel flow-proportional composite samples at the WWTP; and (c) determine time-average hormone levels in wastewater streams and surface waters using TIMFIE samplers.

Material and methods

Selection of steroid hormones

The target compounds ($n = 7$) comprised natural estrogens, synthetic estrogens and progestins (Table 1). E1, E2 and EE2 were selected due to their relevance in previous EU Watch Lists ([15], 840) and current evaluation as priority substances in surface water in the 2022 proposal for the EU Water Directive. The compounds E3, etonogestrel (ETO), dienogest (DIE) and PGR were included because of their common use in hormone therapies and birth control medications. They are also either under discussion or in clinical trials as male contraceptives [13,34,42,45,47,51,55,61].

Table 1
Summary of the target compounds.

Name (abbreviation)	Log K_{ow}	Group	Origin/Use
Estrone (E1)	3.1*	Estrogen	Endogenous
17α-Estradiol (E2)	4.0*	Estrogen	Endogenous
Estriol (E3)	2.6*	Estrogen	Endogenous
17α-Ethinylestradiol (EE2)	3.7*	Estrogen	Birth control pills
Etonogestrel (ETO)	3.3ϖ	Progestin	Birth control implant
Dienogest (DIE)	3.0ϕ	Progestin	Birth control pills
Progesterone (PGR)	3.9*	Progestin	Endogenous

* Schäfer, A.I.; Akanyeti, I.; Semiao, A.J.C. (2011) Advances in Colloid and Interface Science 164, 100–117.
ϖ He, M.; Yang, G.; Zhang, S.; Zhao, X.; Gao, Y. (2018) Journal of Pharmaceutical Sciences 107, 1037–1045.
ϕ Fekete, S.; Fekete, Jeno, Molnar, I.; Ganzler, K. (2009) Journal of Chromatography A 1216, 7816–7823.

Chemicals and materials

Analytical reference standards of the native compounds (purity >99 %) were purchased from Sigma-Aldrich. Individual solid standards were dissolved in methanol (1000 μg mL^{-1}). Two ^{13}C-labelled internal standards (IS) with purity >99 %, E3–^{13}C3 in methanol at 100 μg mL^{-1} and PGR-^{13}C3 in acetonitrile at 100 μg mL^{-1}, were purchased from Cambridge Isotope Laboratories with purity >99 %. Each TIMFIE sampler consisted of a polypropylene syringe (100 mL) with a Luer lock connection (JMS, Hiroshima, Japan), a syringe filter (Titan3, Nylon, 17 mm, 0.45 μm, Thermo Scientific, Waltham, USA), polyether ether ketone (PEEK) tubing (1/16″, inner diameter 0.075 mm, 40 cm) (VICI Jour, Schenkon, Switzerland) and HLB SPE cartridges (No. 731,921, size small, Macherey-Nagel, Düren, Germany).

TIMFIE sampler preparation

TIMFIE samplers were prepared as described in Jonsson et al. [28]. In brief, the 100 mL polypropylene syringe was connected to narrow-bore PEEK tubing, which in turn was connected to the syringe filter (to protect the flow restrictor from clogging by particles) and the SPE cartridge. The syringe piston was pulled and locked by inserting a pin through a hole drilled in the piston, thus creating negative pressure in the barrel. The PEEK tubing served as a flow restrictor and its resistance to flow was determined by pumping methanol at a flow of 0.5 mL min^{-1} using a high-performance liquid chromatography (HPLC) pump before mounting the TIMFIE. Back pressure in the flow restrictors used in this study was in the range 24–30 bar, which allowed for constant sample flow rate over seven days (~12 mL day^{-1}) resulting in an average sample volume of about 80 mL (RSD 11 %). The HLB cartridges were conditioned using methanol (3 mL) followed by MilliQ water (10 mL) and then attached between the syringe filter and an inlet tube (4 cm, 1/16″ PTFE, inner diameter 0.5 mm). The inlet tube was closed with a needle after preparation and was removed when deploying the samplers. The filter and cartridge were wrapped with aluminium foil to prevent chemical degradation by light over the sampling period.

TIMFIE deployment and retrieval

The TIMFIE samplers ($n = 3$) (Figure S1) were deployed in the effluent channel of a municipal WWTP in Uppsala, Sweden. The sampling point was located indoors in a side-stream of the outlet channel, allowing the samplers to be placed with wastewater continuously flowing in and out. The samplers were deployed for a week, followed by retrieval, and this was repeated twice, i.e., one sample per week over

three consecutive weeks (February-March 2019). In parallel, daily composite wastewater samples were collected using flow-proportional sampling across one of the weeks. At the laboratory, the seven individual daily composite samples were pooled based on wastewater daily flows to give a weekly composite sample. This sample can then serve as a 'golden reference' for the TIMFIE samplers deployed, enabling us to better understand whether TIMFIE's ability to capture the average concentrations of these chemicals in effluent wastewater streams is independent of wastewater flow rates. Furthermore, TIMFIE samplers ($n = 3$) were also deployed weekly at different points in the aquatic environment downstream of the WWTP, i.e., in the river Fyrisån (59.831697 N, 17.661369E), river Sävjaån (59.832475 N, 17.681107E) and lake Ekoln (59.781956 N, 17.627326E), over three consecutive weeks (July 2019). Grab water samples were collected on the retrieval days of the TIMFIE samplers. This helps evaluate and illustrate the feasibility of TIMFIE samplers in capturing these chemicals at trace levels in the surface water environment than snapshot (grab) sampling. Sample extraction volumes by the TIMFIE samplers in the field deployments were ~50–74 mL for the effluent wastewater and ~57–97 mL for the aquatic environment. Upon retrieval of the samplers, each HLB cartridge was wrapped in aluminium foil on-site and stored maximum 24 h at −20 °C until extraction.

Extraction method validation

Method performance with HLB SPE cartridges was evaluated using tap water, surface water, effluent wastewater and influent wastewater. Within-day and between-day precision (as relative standard deviation (RSD, %) and accuracy (% bias from the nominal value)) were assessed using tap water spiked at two levels, 1 and 5 ng L^{-1}. RSD values below 25 % and bias within ± 25 % were considered satisfactory. Between-day precision and accuracy were also evaluated, using surface water at 5 ng L^{-1}. Three replicate samples (each 60 mL) per level were included in each validation batch, and the validation was performed across two days. As blank effluent and influent wastewater were not available, between-day precision was examined using the actual analyte concentrations in the matrix, with three replicates per batch and across two days.

Extraction recovery of analytes was determined based on the response (peak area) of analytes in water samples (surface water, effluent wastewater and influent wastewater) spiked with the analytes before (pre-spike) and after (post-spike) the extraction process. Matrix effects of analytes were estimated through comparing post-spike samples to standards at the same concentration level. Spiking level was 30 ng L^{-1} for influent and effluent wastewater and 5 ng L^{-1} for surface water. Method detection and quantification limits (MDL and MQL) of the analytes were estimated at a signal-to-noise ratio of 3 and 10, respectively, using spiked surface water and wastewater with low analyte concentrations. These also applied to estimating instrumental detection and quantification limits (LOD and LOQ) using the lowest calibration standard. Instrumental linearity was evaluated based on calibration standards over nine levels (0.1–500 ng mL^{-1}, equivalent to 1–5000 pg on column) analysed in triplicate.

Chemical elution and instrumental analysis

After sample extraction, the cartridges were rinsed with MilliQ water (5 mL) and dried using centrifugation. Internal standard (10 ng mL^{-1} in MilliQ water) was then added to the cartridges, at a rate of 0.1 % of the extraction volume, followed by loading with MilliQ water (5 mL). The chemicals were eluted using acetonitrile (6 mL). The samples were preconcentrated under nitrogen in a water bath at 40 °C to dryness and reconstituted with methanol/MilliQ water (20:80, v/v) solution for the final extracts. A concentration factor of 500 was applied for wastewater and 1000 for surface water (eq. (1)). In every batch of TIMFIE preparation and extraction, matrix blanks and MilliQ water blank samples were included as the procedure blank/quality measures to verify that there was no contamination throughout the process.

$$\text{Final extract volume} = \frac{\text{Initial extraction volume}}{\text{concentration factor}} \quad (1)$$

Final extracts, together with nine-point calibration standards, were analysed using ultra-HPLC (UltiMate 3000, Thermo Scientific) with tandem mass spectrometry (TSQ Quantiva, Thermo Scientific) (UHPLC-MS/MS). Injection volume was 10 μL. Chemical separation was performed on a C18 column (Waters Acquity BEH—C18, 100 × 2.1 mm, 1.7 μm particle size) and mobile phases consisted of MilliQ Water (A) and methanol (B), each with 0.03 % ammonia, running in a LC gradient programme of: 0–1.05 min, 20 % B, 0.5 mL min^{-1}; 1.05–3 min, 40 % B, 0.55 mL min^{-1}; 3–6 min, 80 % B, 0.6 mL min^{-1}; 7–10 min, 100 % B, 0.6 mL min^{-1}; 10–13 min, 20 % B, 0.5 mL min^{-1}. Column oven temperature was 40 °C. The MS source parameters were set for spray voltage 3500 V (heated electrospray ionisation), vaporizer 400 °C, capillary 325 °C, sheath gas 50, auxiliary gas 15 and sweep gas 2. Data acquisition was conducted using selected reaction monitoring with two transitions of each analyte (Table S1) and the data were evaluated using TraceFinder software (version 3.3, ThermoFisher Scientific). Quantification was performed considering correction with responses of IS compounds that are in the same analogue as the native analytes, in which E1, E2, E3 and EE2 are coupled with E3–^{13}C while ETO, DIE and PGR are coupled with PGR-^{13}C (Table S1). Linearity was in 0.9967–0.9990 for the target analytes (Table S1). Chromatography peaks of the native and IS analytes in the lowest and highest calibration standards (Table S2) and in the selected samples (Table S3) are provided in the supporting information.

Results and discussion

Extraction method performance

The extraction performance with HLB SPE cartridges (Macherey-Nagel) for the target analytes was examined based on within-day and between-day precision (RSD, %) and accuracy (% bias) in tap water and other water matrices (Table 2). The precision in tap water was generally satisfactory. Within-day precision ranged from 7.1 % to 20 % at the lower spiking level and 7.3 % to 27 % at the higher level, while the corresponding range for between-day precision was 8.8–26 % and 11–28 %, respectively. Between-day precision for E1 and DIE was slightly higher than 25 %, as was within-day precision for EE2. In surface water, between-day precision was satisfactory for all target analytes (RSD 4.1–16 %.) In wastewater, between-day precision in effluent was satisfactory overall for all analytes, with RSD in the range 16–23 % (except for EE2, RSD 34 %). In influent wastewater, most of the analytes showed high between-day variation, but especially E1 (32 %), ETO (63 %) and DIE (30 %), whereas the precision for E2, E3, EE2 and PGR was relatively better (RSD 17–26 %). Overall, between-day precision for the target analytes was satisfactory for tap water, surface water and effluent wastewater, but less so for influent wastewater (Figure S2A). The method gave reproducible results across different water matrices, as the variation within one water matrix was similar to that in all other water matrices except influent wastewater.

The accuracy of the tap water measurements for E1, E2, EE2, ETO and DIE was satisfactory (Table 2). Within-day bias ranged from 5 % to −28 % at the lower spiking level and from 16 % to −25 % at the higher level, while between-day bias ranged from 10 % to −17 % at the lower level and from 0.013 % to −23 % at the higher level. Again, E1 and EE2 showed bias that was slightly higher (−25 %). At both spiking levels, the accuracy for E3 and PGR was outside the satisfactory range for tap water, with up to −50 % bias. For surface water, the results were satisfactory for almost all target analytes, with bias ranging from 11 % to −13 % for all compounds except E3 (−45 % bias). The reduced accuracy for E3 appeared to be independent of water type, whereas PGR measurements showed lower accuracy in tap water, but not in surface water.

Table 2
Performance of the extraction method validation.

	Tap water								Surface water			Effluent wastewater		Influent wastewater	
	Precision (RSD %)				Accuracy (bias %)				Precision (RSD %)	Accuracy (bias %)	MQL	Precision (RSD %)	MQL	Precision (RSD %)	MQL
	within-day		day-to-day		within-day		day-to-day		day-to-day	day-to-day		day-to-day		day-to-day	
Analyte*	L1	L2	L1	L2	L1	L2	L1	L2	L2	L2					
E1	14	16	26	11	−26	−25	−9.6	−6.7	16	11	0.4	18	0.4	32	0.8
E2	14	24	15	21	−13	−12	−2.9	−20	11	−8.2	1.0	16	1.0	26	2.0
E3	7.1	20	12	23	−50	−37	−51	−46	4.1	−45	1.0	17	1.0	23	2.0
EE2	20	27	19	19	−28	−19	−17	−23	14	−13	2.5	34	2.5	17	5.0
ETO	12	7.3	8.8	14	5.5	11	9.7	0.013	7.0	3.8	1.0	23	1.0	63	1.5
DIE	9.5	12	13	28	−22	16	−13	−6.3	10	−6.5	0.1	17	0.2	30	0.5
PGR	7.9	11	25	17	−51	−33	−37	−40	11	−13	0.1	15	0.2	24	1.0

L1: 1 ng L^{-1}; L2: 5 ng L^{-1}; $n = 3$ for within-day; day-to-day, 2 days; RSD: relative standard deviation; MQL: method quantification limit in ng L^{-1}. *Estimated instrumental quantification limit (LOQ): 0.138 ng mL^{-1} (1.38 pg on column) for E1; 0.091 ng mL^{-1} (0.91 pg) for E2; 0.111 ng mL^{-1} (1.11 pg) for E3; 0.1 ng mL^{-1} (1 pg) for EE2; 0.185 ng mL^{-1} (1.85 pg) for ETO; 0.075 ng mL^{-1} (0.75 pg) for DIE; 0.08 ng mL^{-1} (0.80 pg) for PGR. For analyte's abbreviations, see Table 1.

Presence of organic matter and other particulates in surface water, but not in tap water, could have facilitated PGR retention. A similar explanation may apply to the other analytes, since the values generally showed more variation in between-day accuracy in tap water than in surface water (Figure S2B). Further refinement of our analytical approach could benefit from additional IS compounds corresponding to the native analytes in the future. The lower bias observed for E3 than for the other compounds analysed is consistent with previous findings [70]. It is likely attributable to the higher polarity of E3. It has also been demonstrated that urinary estriol undergoes acid hydrolysis [64], a process that is influenced by the co-presence of other polar compounds. This potentially represents an additional pathway through which a consistent reduction in E3 concentration could occur in acidic waters. Overall, the TIMFIE extraction method was reproducible and accurate for all target analytes in the surface water matrix except E3, for which accuracy was consistently low. Method performance was also satisfactory with tap water and effluent matrices for all target analytes except E3 and PGR (consistently low accuracy) and EE2 (high variation).

Estimated MQLs were within the range 0.1–2.5 ng L^{-1} for surface water, 0.2–2.5 ng L^{-1} for effluent wastewater and 0.5–5.0 ng L^{-1} for influent wastewater (Table 2). For E1, the MLQ in surface water and effluent wastewater complied with the level (0.4 ng L^{-1}) set in the environmental quality standards of the EU Water Framework Directive. The target analytes have LOQs estimated in a range of 0.0075–0.185 ng mL^{-1}, equivalent to 0.75–1.85 pg on column.

Extraction efficiency was determined based on absolute recovery in different water matrices (Fig. 1 and Table S4). In surface water, most of the analytes showed good recovery, in the range 80–120 %, but E3 and EE2 were slightly outside this range (72 % and 130 %, respectively) (Table S4). On average, extraction with HLB SPE cartridges resulted in approximately 100 % absolute recovery (Fig. 1). Similar results were obtained for most of the analytes in effluent wastewater, with 92–120 % absolute recovery (mean 110 %) (Fig. 1). In influent wastewater, the recoveries differed between the analytes (Fig. 1). E1, E2 and ETO showed acceptable recoveries (Table S4), while PGR was slightly below the acceptable range (70 % recovery) (Table S4). The recovery of E3, EE2 and DIE in influent wastewater was higher than 120 %.

Ion suppression was the most common matrix effect observed for the analytes (Table S4). In surface water, the matrix effect was similar among the analytes and ranged from −66 % to −77 %. In effluent

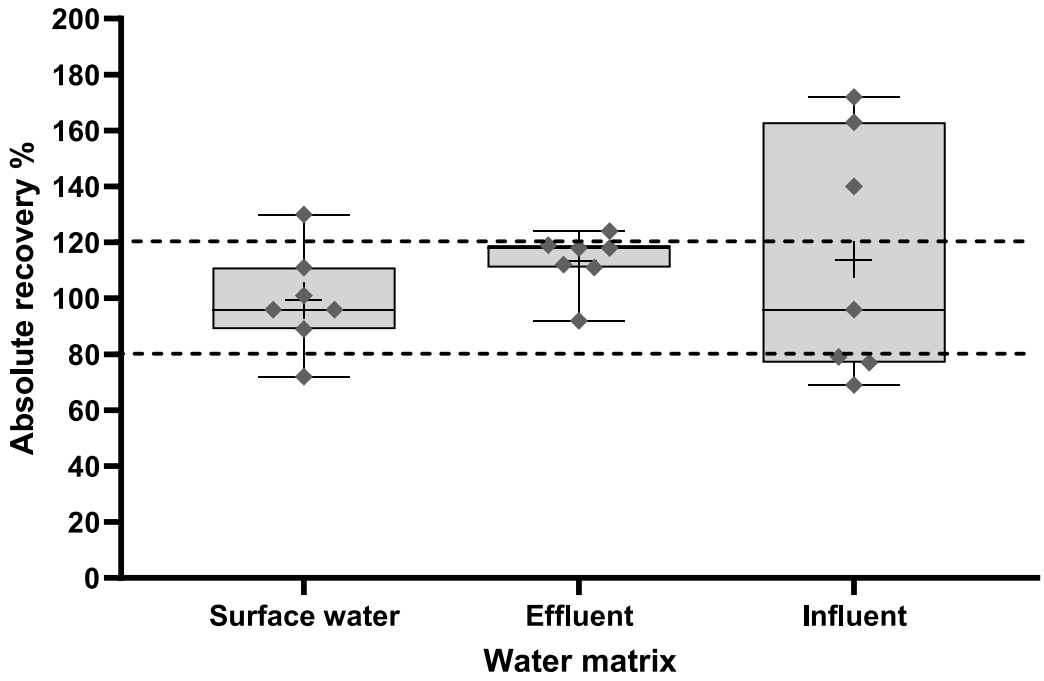

Fig. 1. Box-plot of the absolute recovery (%) of the analytes in different water matrices. "+" represents the average. Diamond represents each data point.

wastewater, similar values were found for E3, ETO, DIE and PGR, with matrix effects ranging from −8 % to −100 %, but ion enhancement was observed for E1, E2 and EE2, with matrix effects of 11 %, 7 % and 0.11 %, respectively. In influent wastewater, ion suppression was generally observed for all analytes, ranging from −43 % to −99 % except for E3 (64 % matrix effect). Generally, the matrix effect results appeared reasonable, as the suppression values for most compounds in surface water were similar and less varied compared to the more complex matrix of wastewaters which exhibited higher variability in the matrix effects. To reduce the matrix effects, potential approaches include applying a solvent washing step before elusion or using alternative elusion solvents. These, however, could influence the recovery of the chemicals, and therefore require further investigation.

Overall, use of TIMFIE samplers equipped with a HLB sorbent produced robust and reliable results for steroid hormones in surface water and effluent wastewater, but the method encountered challenges with influent wastewater.

Comparison of TIMFIE with composite samples

The *in-situ* extraction performance of TIMFIE samplers was examined by comparing the analyte concentrations in effluent wastewater samples obtained using the TIMFIE samplers, deployed over one week, with the corresponding concentrations in weekly flow-based composite samples (Fig. 2). Consistent results were obtained with the TIMFIE samplers and composite wastewater samples for E1 and E2, while EE2 and DIE were not quantifiable with these two sampling methods. Discrepancies in measured concentrations were observed for E3, ETO and PGR. The levels of E3 and PGR were lower in TIMFIE samples than in composite samples, whereas ETO showed higher levels in TIMFIE samples than in composite samples. While they captured weekly average concentrations, TIMFIE samplers appeared unable to capture high flow (loads) of E3 and PGR. Both E3 and PGR are excreted in conjugates (e.g. estriol-3-sulphate, estriol-16-glucosiduronate 20α-dihydroprogesterone, 5α-dihydroprogesterone) [12,38,48,49], so de-conjugation during storage of the seven daily samples used to create weekly composite samples could have increased the levels of free E3 and PGR in the final samples compared with *in-situ* extraction with TIMFIE samplers. ETO, a long-acting reversible contraceptive, has been shown to be excreted continuously [57,58] and thus periods with lower total flow volume could have resulted in higher concentrations being measured with the time-integrated method compared with flow-proportional sampling, although actual mass load was higher in the flow-proportional method.

TIMFIE applications

Of the seven steroid hormones analysed, five (E1, E2, E3, ETO and PGR) were quantified in effluent wastewater sampled with the TIMFIE samplers, with consistent occurrence rates over the three consecutive weeks of monitoring (Table 3). EE2 and DIE were not found in any sample. The mass load in wastewater effluent was generally highest for ETO (mean 2700 mg day^{-1}), followed by the estrogens E3 (200 mg day^{-1}), E2 (200 mg day^{-1}) and E1 (120 mg day^{-1}), and then PGR (40 mg day^{-1}). Hormone-based long-acting reversible contraceptives are widely used in the Nordic countries [24,41], and ETO is often the main ingredient (National Institutes of Health [[54] and National Library of Medicine (NLM], 2023b, [53]). In the study region (Uppsala county), there has been an increase in the volume of ETO-containing contraceptives prescribed over the past 15 years, with a peak in 2019 (the time of this study) [67]. ETO is also a known human metabolite of desogestrel [33,44,75], but further research is required on potential transformation of other progestins into ETO, e.g. during WWTP processes. Given that ETO has been found to adversely impact the mating behaviour of aquatic

Table 3
Target analytes in effluent wastewater using TIMFIE ($n = 3$) deployed weekly.

	Concentration (ng/L)			Mass load* (mg/day)		
Analytes	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
E1	1.9 ± 0.5	1.3 ± 0.3	2.2 ± 1.0	120 ± 30	90 ± 20	170 ± 80
E2	3.3 ± 1.0	3.0 ± 0.9	2.6 ± 0.1	200 ± 60	200 ± 60	200 ± 6
E3	4.0 ± 1.0	2.3 ± 0.4	2.4 ± 0.8	250 ± 60	160 ± 30	190 ± 70
EE2	<2.5	<2.5	<2.5	<150	<170	<200
ETO	50 ± 1.0	31 ± 9.0	36 ± 0.7	3000 ± 100	2100 ± 600	2800 ± 60
DIE	<0.2	<0.2	<0.2	<10	<10	<12
PGR	0.5 ± 0.1	0.35 ± 0.2	0.77 ± 0.3	30 ± 5	24 ± 10	61 ± 20

* average effluent flow: 61,500 m^3 (week 1), 67,400 m^3 (week 2) and 79,200 m^3 (week 3). For analyte's abbreviations, see Table 1.

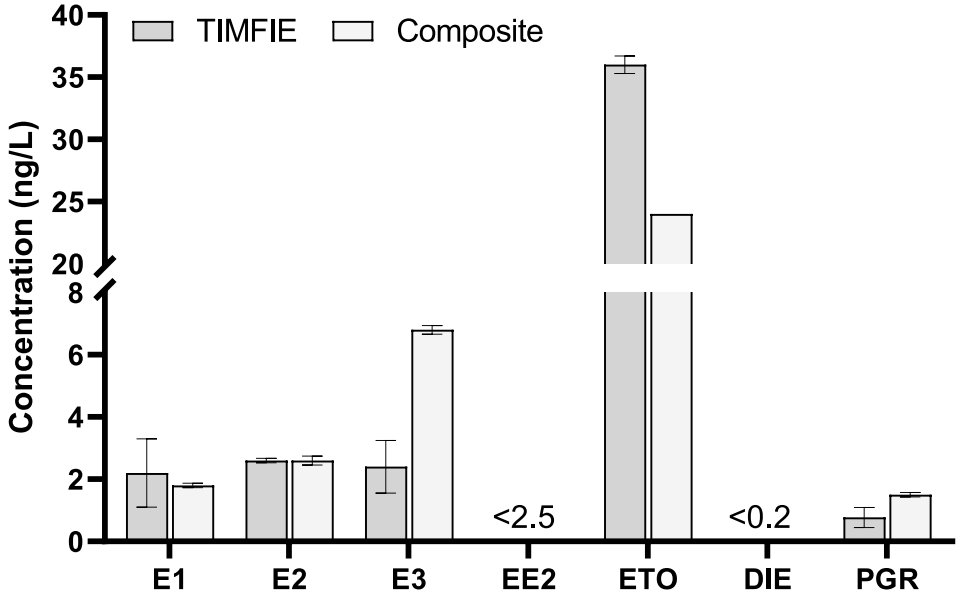

Fig. 2. Comparison of analyte concentrations (ng L^{-1}) between TIMFIE samplers and flow-based composite samples in the effluent wastewater stream. For analyte's abbreviations, see Table 1.

organisms [69], even at concentrations one-tenth of those quantified in effluent wastewater in this study, our findings are highly concerning. E1 is the result of accumulated biotransformation of E2, suggesting that concentrations or mass loads of E1 might be higher than those of E2. However, E1 is more photosensitive than other estrogens, so higher mass loads of E1 in outdoor environments are less probable [26]. In the present study, E1 and E2 were found to be present at similar levels, with slightly higher levels seen occasionally for E2. Further transformation of E1 to E3 has been reported [14], as has direct human biotransformation of E1 and E2 to E3 via the cytochrome P450 family [35,71,73]. In this study, the concentrations of E1, E2 and E3 in effluent wastewater were within a similar range of magnitude, and no discernible pattern or trend was observed throughout the three-week sampling period. This indicates the possibility of time-sensitive trends that require higher temporal resolution for clarification.

At sampling points in the downstream surface water environment, only two steroid hormones, E1 and E2, were occasionally detectable (Table 4). E1 was detected using TIMFIE samplers in the rivers Fyrisån and Sävjaån, at concentrations of 1.5 and 0.47 ng L^{-1}, respectively, during the first week of monitoring. Occurrence of E1was not captured by the grab water samples collected on the retrieval day. E2 was detected by TIMFIE samplers in both rivers and in lake Ekoln, at 0.3–0.4 ng L^{-1}, during the third sampling week and similar results were obtained for the grab water samples. The river Fyrisån receives effluent wastewater from the municipal WWTP in Uppsala, so higher concentrations of the target analytes than in the river Sävjaån were expected. One source of steroid hormones in the Sävjaån could be effluent discharge from on-site sewage facilities [8,21]. The steroid hormones found in effluent wastewater were not always detected in downstream water bodies over the sampling period, probably due to the dilution effect, potential degradation and sorption onto particulate matter. For E2, Caldwell et al. (2012) estimated a chronic exposure predicted no-effect concentration (PNEC) of 2 ng L^{-1} for aquatic organisms, based on species-sensitivity distribution. For E1, due to insufficient data those authors used values from an *in-vivo* vitellogenin induction study to derive an aquatic PNEC of 6 ng L^{-1}. All concentrations of E1 and E2 in the present study were below the respective PNEC, indicating that these compounds currently do not pose a risk to aquatic life. E1 was detected in the first sampling week in both rivers, at concentrations one order of magnitude lower than its PNEC, while E2 was detected in the third week at all three sites, at concentrations just above the MQL.

Table 4
Concentrations (ng L^{-1}) of E1 and E2 detected in surface water.

	E1		E2	
	TIMFIE (n = 3)	Grab water (n = 2)	TIMFIE (n = 3)	Grab water (n = 2)
Week 1				
Fyrisån River	1.5 ± 0.3	<0.2	<0.3	<0.3
Sävjaån River	0.47 ± 0.2	<0.2	<0.3	<0.3
Ekoln Lake	NA	NA	NA	NA
Week 2				
Fyrisån River	<0.2	<0.2	<0.3	<0.3
Sävjaån River	<0.2	<0.2	<0.3	<0.3
Ekoln Lake	<0.2	<0.2	<0.3	<0.3
Week 3				
Fyrisån River	<0.2	<0.2	0.33 ± 0.1*	0.31 ± 0.03
Sävjaån River	<0.2	<0.2	0.30 ± 0.1	0.38 ± 0.06
Ekoln Lake	<0.2	<0.2	0.43 ± 0.05*	0.32 ± 0.02

NA: not available;.
* n = 2 due to technical issues; method detection limits: <0.2 (E1) and <0.3 (E2). For analyte abbreviations, see Table 1.

Environmental implications and study limitations

This work demonstrated good potential of TIMFIE samplers for use in monitoring steroid hormones in the whole water phase. It also provided a good example of green sampling and sample preparation [2,62]. Being able to perform active sampling without using electrical power minimises energy consumption and avoids the need for more high-tech, resource-consuming equipment. In addition, the small, lightweight properties of TIMFIE samplers make sample transport and storage easier than with other techniques where large volumes of water must be collected in flasks, transported and stored. *In-situ* sample preparation, which minimises the amount of sampling materials required, is a key characteristic of the TIMFIE device as a green sampler. Moreover, it is built with reusable materials, except the filters and SPE cartridges, which is more economical and lowers waste generation. Unlike the power-free active osmotic pump time-integrated sampler [39], the TIMFIE sampler is temperature-independent and can be used all year round without additional validation experiments [32]. For instance, even at sub-freezing outdoor temperatures, sampling can still be conducted below the ice cover, which can be particularly useful for monitoring studies in the Nordic and Arctic regions. This study was the first to use active time-integrated sampling to investigate Swedish waters for steroid hormones, especially E1 and E2, with quantification limits lower than the PNEC values. A previous study performed in Sweden used a method with higher quantification limits (12 ng L^{-1} for E1 and 5.5 for E2) than the respective PNEC values [77], although Andersson et al. (2005) used a method with MQLs (0.3 ng L^{-1} for E2) below the respective PNEC values. However, most MQLs applied in those studies were at least one order of magnitude higher than the concentrations detected in Swedish surface waters in the present study. Thus, the TIMFIE samplers proved to be efficient and applicable for determination and assessment of steroid hormone concentrations in Swedish surface waters.

While our study illustrates an alternative active sampling device for in situ water extraction of steroid hormones, a few limitations are noteworthy for appropriate interpretation of the applications. As an extraction process, molecular interactions between the target analytes and HLB sorbent occur via different chemical binding forces. This generally makes the analytes more stable on SPE sorbents than when dissolved in water samples [5,7,20,29]. However, the influence of the water temperature at 8–18 °C on the in-cartridge stability of the steroid hormones over time still cannot be excluded. Additionally, it is not possible to disregard the potential of chemical degradation or transformation influenced by microbial activities taking place within the cartridge during the deployment period. Furthermore, varying amounts of particles taken up between replicate TIMFIE samplers could lead to more variation (relative standard deviations) in the average concentration of a given chemical, especially when it comes to monitoring rather hydrophobic chemicals, such as those in our study, which could have a higher tendency to bind with particles in the particulate phase than in the aqueous phase. Such potential intake of varying amounts of particles into the cartridges is not specific to TIMFIE sampling alone. It can also occur in various sampling techniques (e.g. passive sampling, filtration methods) and is dependent on the turbidity or content of suspended matter in water bodies. Fifth, accurate knowledge of the dynamics of analytes in surface water bodies is crucial when using TIMFIE samplers to calculate mass transport, as the results obtained are in the form of time-integrated average concentrations. This can be particularly relevant when analytes show high concentration fluctuations over the seven-day deployment period, e.g., during hydrological events and/or changes in patterns of discharge from sources, since TIMFIE samplers cannot capture the analytes in a flow-proportional or event-driven manner.

Conclusions

This study showed that TIMFIE sampling with HLB sorbent is an appropriate and robust method for time-integrated weekly sampling for analysis of steroid hormone concentrations in different water matrices. Satisfactory performance was observed for E1, E2, ETO, DIE and PGR in surface water and effluent wastewater matrices. E1 and E2 concentrations in effluent wastewater were similar in TIMFIE samples and in parallel flow-based composite samples. While most of the target analytes were captured by TIMFIE in effluent wastewater, their occurrences and concentrations at downstream surface water sampling points were reduced, likely due to dilution effects and potential degradation and sorption onto suspended particles, followed by sedimentation. Future studies should examine chemical stability within the HLB SPE cartridge under different environmental conditions. Our results indicate that TIMFIE samplers can be useful for screening and monitoring programmes by producing time-integrated, whole water concentration data on (prioritised) steroid hormones in wastewater and surface water in a cost- and resource-efficient manner. The low-tech design and small size of TIMFIE samplers also meet the principles of green sample preparation, for more sustainable monitoring studies.

CRediT authorship contribution statement

Paul Löffler: Writing – original draft, Writing – review & editing, Methodology, Visualization. **Ove Jonsson:** Funding acquisition, Formal analysis, Conceptualization, Methodology, Supervision, Writing – review & editing. **Annika S. Niemeyer:** Methodology, Writing – review & editing. **Anna-Karin Dahlberg:** Methodology, Supervision, Writing – review & editing. **Oksana Golovko:** Methodology, Supervision, Writing – review & editing. **Oscar Götlind:** Methodology, Writing – review & editing. **Inga Haalck:** Formal analysis, Methodology, Writing – review & editing. **Lutz Ahrens:** Writing – review & editing. **Karin Wiberg:** Writing – review & editing. **Foon Yin Lai:** Funding acquisition, Supervision, Conceptualization, Methodology, Writing – review & editing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2024.100143.

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