DETERMINATION OF STEROID ESTROGENS IN ENVIRONMENTAL SAMPLES USING CHEMICAL ANALYSIS (GC-MSD) AND AN *IN VITRO* ESTROGENICITY ASSAY (ER-CALUX®)

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Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia, April 2012

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Doctoral Dissertation

DOLOČANJE STEROIDNIH ESTROGENOV V OKOLJSKIH VZORCIH Z METODO KEMIJSKE ANALIZE (GC-MSD) IN *IN VITRO* BIOLOŠKEGA TESTA ZA DOLOČANJE ESTROGENOSTI (ER-CALUX®)

Doktorska disertacija

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Ljubljana, Slovenia, May 2012

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Abstract

The presence of steroid estrogens in the environment has been studied intensively for two decades because of their possible deleterious effects on living organisms at very low (ng/L) concentrations. To determine concentrations of steroid estrogens and the estrogenic potential of environmental samples, sophisticated and sensitive analytical techniques as well as biological methodologies are employed.

In the first part of this doctoral thesis, sample preparation and chemical analysis using gas chromatography with mass selective detection (GC-MSD) was optimised, validated and applied to environmental samples. Hospital effluent and connected wastewater treatment plant (WWTP) influent and effluent were sampled over six consecutive days to determine the levels and inter-day variations of three naturally occurring steroid estrogens: estrone (E1), 17β-estradiol (E2), estriol (E3), and synthetic 17α-ethinylestradiol (EE2). After solid phase extraction, interferences were removed with a silica gel clean-up step and the samples analyzed by GC-MSD. The determined inter-day concentrations in hospital effluent were between 8.6 and 31.3 ng/L for E1, < limit of detection (LOD) to 4.2 ng/L for E2 and 6.4 to 385.5 ng/L for E3. In the WWTP influent concentrations were 18.9 to 49.7 ng/L for E1, 2.4 to 12.7 ng/L, for E2 and <limit of quantification (LOQ) to 63.9 ng/L for E3. Reduced levels were found in the WWTP effluent: <LOD to 7.1 ng/L for E1, <LOQ for E2 and <LOD to 5.2 ng/L for E3. EE2 was detected in only one influent sample. Interestingly, the E1:E2:E3 ratio in the hospital effluent (1:0.1:9.4) was comparable to that present in the urine of pregnant women (1:0.3:20) indicating them as the most likely source of steroid estrogens. In WWTP influent the ratio was similar to that in the non-pregnant population. Calculated estradiol equivalents (EEQ) were 33.4, 22.4, 1.7 ng(EEQ)/L in the hospital effluent, WWTP influent and WWTP effluent, respectively. During wastewater treatment, 99 % of the steroid estrogens were removed from the water phase. However, there were some cases, where removal was <80 % and even <40 %. In addition, variability of removal rates (39 % up to 99 %) was observed. Results revealed E3 as being one of the most important steroid estrogens, accounting for up to 92 % of the total EEQ present in hospital samples and 37 % and 46 % in WWTP influent and effluent samples, respectively. In this study, hospitals were recognised as an important point source of steroid estrogens. Additionally, in hospital and WWTP samples, concentrations of steroid estrogens show daily variations, e.g. - from 6.4 to 385.5 ng/L for E3. Clearly, careful sampling strategies must be adopted to ensure proper risk assessment of the impact of steroid estrogens in the environment. In addition, the low potency steroid estrogens that contribute towards overall estrogenicity of the sample, e.g. E3, should be incorporated into environmental monitoring programs.

In the second part of this Thesis an *in vitro* estrogenicity ER-Calux® assay was modified and integrated with instrumental analysis (GC-MSD) to create a novel single protocol. This integrated protocol enables simultaneous determination of steroid estrogens concentrations and the total estrogenic potential of environmental samples. For integration purposes the commonly used dimethyl sulphoxide (DMSO) in the ER-Calux® assay was replaced by ethyl acetate, which is more compatible with gas chromatography and does not affect the performance of the ER-Calux® assay. The integrated protocol was

initially tested using a standard mixture of authentic estrogens. The calculated estrogenicity values of pure standards based on GC-MSD and the ER-Calux® assay showed good correlation (r^2=0.96; α=0.94). The result remained the same when spiked wastewater extracts were tested (r^2=0.92, α=1.02). When applied to "real" wastewater influent and effluent samples the results proved (r^2=0.93; α=0.99) the applicability of the method. The main advantages of this newly developed protocol are that the same sample can be analysed by both GC-MSD and ER-Calux® and method complexity together with material consumption and labour is significantly reduced. The methods can be applied as either a complete or sequential analysis where the ER-Calux® assay is used as a pre-screening method prior to chemical analysis.

However, for both, GC-MSD and ER-Calux®, time consuming sample preparation (sample extraction) is necessary. In the last part of the study the ER-Calux® assay was modified with the aim to simplify and shorten sample preparation. The ER-Calux® assay was modified in order to be able to test raw wastewater samples without the need to perform sample extraction. This method (NE-(ER-Calux®) assay) has been compared to the ER-Calux® assay and chemical analysis by GC-MSD, described above. In the case of spiked tap and wastewater samples there was no statistical difference between the results obtained by NE-(ER-Calux®) and the other two methods. The "real" influent and effluent samples from seven municipal WWTPs and receiving surface waters were tested. With some exceptions, higher estrogenic potential was determined by NE-(ER-Calux®) assay in comparison to ER-Calux® assay. The results of NE-(ER-Calux®) were 2.1-32.4 ng(EEQ)/L, 0.8-14.2 ng(EEQ)/L and 1.7-12.3 ng(EEQ)/L higher in influent, effluent and river downstream samples, respectively. The results of biological methods were in agreement with the results of chemical analysis. NE-(ER-Calux®) was also used to determine the intra-day dynamics of estrogenic potential in one WWTP. Influent and effluent samples were taken hourly over 12 h periods and analysed within 24 h. The results showed that the estrogenic potential increased in the influent from 12.9 ng(EEQ)/L reaching a peak at 40.0 ng(EEQ)/L at 16:00. The average hourly values (27.1 ng(EEQ)/L) were comparable to the estrogenic potential of the time proportional sample (25.2 ng(EEQ)/L), which was 62 % of the highest estrogenic potential measured. The reduction in the estrogenic potential was 92 % to 98 % according to hourly sampling and 96 % in the case of the time proportional sample. The results indicate the daily variability in the estrogenic potential, which should improve our understanding of the risk posed by environmental steroid estrogens in the future. Significant part in achieving these results is due to the ability of NE-(ER-Calux®) assay to test raw samples without extensive sample preparation. Since sample extraction was not necessary, sample preparation time was greatly reduced (95 %). This advantage is of particular importance in environmental studies involving large numbers of samples for screening and monitoring purposes.

Povzetek

Prisotnost steroidnih estrogenov v okolju je podrobneje preučevana v zadnjih dveh desetletjih, predvsem zaradi njihovega potencialnega negativnega vpliva na organizme že pri zelo nizkih koncentracijah (ng/L). Za določanje steroidnih estrogenov in celotnega estrogenega potenciala se uporabljajo kompleksne in občutljive kemijske in biološke metode.

V prvem delu doktorske disertacije sem optimiziral pripravo vzorca in kemijsko analizo s plinsko kromatografijo in masno selektivnim detektorjem (GC-MSD). Celotno metodo sem optimiziral, validiral in uporabil na okoljskih vzorcih. V ta namen sem iztok iz bolnišnice ter dotok in iztok iz čistilne naprave (ČN) vzorčil v šestih zaporednih dneh in v vzorcih določil koncentracije naravnih - estron (E1), 17β-estradiol (E2), estriol (E3), in umetnih - 17α-etinilestradiol (EE2) - steroidnih estrogenov. Spojine sem ekstrahiral iz vzorcev, jih čistil s silikagelom in na koncu analiziral z GC-MSD. Dnevne koncentracije, ki sem jih določil v iztoku iz bolnice, so bile od 8,6 do 31,3 ng/L za E1, pod mejo zaznave (<LOD) do 4,2 ng/L za E2 in 6,4 to 385,5 ng/L za E3. V dotoku na ČN so bile koncentracije E1 od 18,9 do 49,7 ng/L, E2 od 2,4 to 12,7 ng/L, in za E3 od pod mejo kvantifikacije (<LOQ) do 63,9 ng/L. Na iztokih sem, v primerjavi z dotoki, določil nižje vrednosti: <LOD do 7,1 ng/L za E1, <LOQ za E2 in <LOD do 5,2 ng/L za E3. EE2 sem zaznal samo v enem vzorcu. Razmerje E1:E2:E3 v vzorcih iztoka iz bolnišnice (1:0,1:9,4) je bilo primerljivo z razmerjem v urinu nosečih žensk (1:0,3:20), kar nakazuje, da bi le-te lahko bile najverjetnejši vir steroidnih estrogenov. Razmerje v vzorcih dotoka na ČN je bilo podobno razmerju, ki ga najdemo v urinu moških in žensk, ki niso noseče. Izračunal sem tudi estradiolske ekvivalente (EEQ), ki so dosegali vrednosti 33,4 ng(EEQ)/L v bolnišničnih iztokih, 22,4 ng(EEQ)/L v dotoku na ČN in 1,7 ng(EEQ)/L v iztoku iz ČN. Stopnja odstranitve steroidnih estrogenov tekom obdelave odpadne vode na ČN je bila do 99 %, v nekaterih primerih pa sem določil stopnje odstranitve tudi pod 80 %, oziroma celo pod 40 %. Prav tako sem zaznal variabilnost stopnje odstranitve ob različnih dnevih v tednu (39 % do 99 %). Pri tem je velik delež estrogenega potenciala prispeval E3, ki sem ga prepoznal kot enega najpomembnejših steroidnih estrogenov, saj je v bolnišnični odpadni vodi prispeval tudi do 92 % celotnega estrogenega potenciala, na dotokih in iztokih iz ČN pa 37 % oziroma 46 %. Povzetek rezultatov kaže, da so bolnišnice pomemben vir steroidnih estrogenov. Vendar pa koncentracije, tako v bolnišnični odpadni vodi, kot v dotoku in iztoku ČN, niso konstantne, kot npr. za E3, ki smo ga zaznali v koncentracijah od 6.4 do 385.5 ng/L. Zaradi tega je potrebna posebna pozornost pri načrtovanju vzorčenja za zagotavljanje pravilne presoje vpliva steroidnih estrogenov na okolje, hkrati pa je v raziskave potrebno vključiti manj aktivne spojine, kot je npr. E3.

V drugem delu sem v raziskavo vključil test estrogenosti ER-Calux®, ki sem ga združil z že obstoječo metodo kemijske analize (GC-MSD) v enotno metodo. Združena metoda omogoča sočasno določanje koncentracij steroidnih estrogenov in celotne estrogenosti v okoljskih vzorcih. Pri tem sem dimetil sulfoksid (DMSO), ki se običajno uporablja v ER-Calux® testu zamenjal z etil acetatom, topilom, ki je bolj primeren za uporabo v kemijski analizi in ne vpliva na delovanje ER-Calux® testa. Združeno metodo sem najprej testiral s čistimi standardi steroidnih estrogenov, kjer so rezultati GC-MSD in ER-Calux® testa

pokazali dobro ujemanje (r^2=0,96; α=0,94). Podobne rezultate sem dobil tudi, ko sem metodo testiral z mešanico standardov in ekstraktov odpadne vode (r^2=0,92, α=1,02). Uporabnost združene metode sem nato potrdil s testiranjem realnih okoljskih vzorcev dotokov in iztokov iz ČN (r^2=0,93; α=0,99). Glavna prednost, ki jo prinaša združena metoda je, da lahko vzorec na preprost način hkrati testiramo tako z GC-MSD, kot tudi z ER-Calux® testom, s čimer se zmanjšata tudi porabljen čas in material. Združeno metodo lahko uporabljamo kot celoto, ali pa v zaporedju, kjer ER-Calux® test uporabimo kot presejalni test pred kemijsko analizo.

Tako za GC-MSD, kot tudi za ER-Calux® test je vzorce potrebno pripraviti s časovno zamudnimi metodami. Zato sem se v zadnjem delu raziskave osredotočil na poenostavljenje in skrajšanje postopka priprave vzorca in posledično na potrebno prilagoditev ER-Calux® testa. Test sem prilagodil tako, da sem lahko testiral okoljske vzorce brez predhodne ekstrakcije (NE-(ER-Calux®) test). To metodo sem primerjal z ER-Calux® testom in kemijsko analizo GC-MSD. Z vsemi metodami sem testiral vodovodno in odpadno vodo z dodanimi standardi. Primerjava NE-(ER-Calux®) testa z drugima metodama je pokazala, da med odzivi pri posameznih metodah ni statistično signifikantnih razlik. Metode sem nato primerjal tudi na odpadnih vodah iz sedmih ČN (dotok in iztok), ter v rečnih vzorcih, kamor se iztekajo vode iz ČN (nad in pod mestom iztoka). Razen nekaterih izjem, so rezultati pokazali, da je estrogeni potencial vzorcev določen z NE-(ER-Calux®) testom nekoliko višji kot z ER-Calux® testom. Rezultati NE-(ER-Calux®) testa so bili na iztokih iz ČN višji za 2,1-32,4 ng(EEQ)/L, na iztokih iz ČN za 0,8-14,2 ng(EEQ)/L in 1,7-12,3 ng(EEQ)/L višji v rečnih vzorcih pod mestom iztoka. Rezultati pridobljeni z biološkima metodama so se ujemali z rezultati kemijske analize. NE-(ER-Calux®) test sem uporabil tudi za določitev variabilnosti estrogenega potenciala na ČN znotraj enega dneva. Dotoke in iztoke na ČN sem 12 h vzorčil na vsako uro in jih analiziral v roku 24 h. Rezultati so pokazali, da estrogeni potencial v dotokih na ČN narašča od jutra (12,9 ng(EEQ)/L) in doseže najvišjo vrednost ob 16:00 (40,0 ng(EEQ)/L). Povprečna vrednost vseh urnih vzorcev (27,1 ng(EEQ)/L) je bila primerljiva z estrogenim potencialom časovno povprečnega vzorca (25,2 ng(EEQ)/L), ki pa predstavlja le 62 % najvišje izmerjene vrednosti v dnevu. Zmanjšanje estrogenosti med postopkom obdelave odpadne vode je bilo 92 % do 98 %, kar se ujema s časovno povprečnim vzorcem (96 %). Z raziskavo sem dokazal variabilnost estrogenega potenciala znotraj posameznega dne. Ti rezultati lahko pripomorejo k boljšemu razumevanju kroženja in transporta steroidnih estrogenov v okolju. Pomembno vlogo pri raziskavi je igrala sposobnost NE-(ER-Calux®) testa za določanje estrogenosti neposredno v izhodnih vzorcih, brez zamudne priprave vzorca. Ker ekstrakcija vzorca ni bila potrebna, sem čas, potreben za pripravo vzorca, zmanjšal za 95 %. To je še posebej pomembno v okoljskih študijah, kjer je v kratkem času potrebno analizirali veliko število vzorcev, hkrati pa lahko ta test uporabimo kot presejalni test v nadzornih meritvah.

Abbreviations

AcON	=	acetone
APCI	=	atmospheric pressure chemical ionisation
APPI	=	atmospheric pressure photo ionisation
BDS	=	BioDetection Systems b.v.
BLYES	=	bioluminescence yeast estrogen screen
BOD	=	biological oxygen demand
BSTFA	=	N,O-Bis(trimethylsilyl)trifluoroacetamide
cEEQ	=	calculated estradiol equivalents
CI	=	chemical ionisation
COD	=	chemical oxygen demand
DCM	=	dichloromethane
DDT	=	dichlorodiphenyltrichloroethane
DMSO	=	dimethyl sulfoxide
E1	=	estrone
E2	=	17β-estradiol
αE2	=	17α-estradiol
E3	=	estriol
EC_{50}	=	median effective concentration
EDA	=	effect directed analysis
EDC	=	endocrine disrupting compounds
EDTA	=	ethylenediaminetetraacetic acid
EE2	=	17α-ethinylestradiol
EEF	=	estradiol equivalency factor
EEQ	=	estradiol equivalents
Eff	=	effluent
EI	=	electron ionisation
ELISA	=	enzyme linked immunosorbent assay
ELRA	=	enzyme linked receptor assay
ER	=	estrogenic receptor
ER-Calux®	=	estrogen responsive chemically activated luciferase expression
ERα	=	estrogen receptor α
ERβ	=	estrogen receptor β
ESI	=	electrospray ionisation
EtAc	=	ethyl acetate
EtOH	=	ethanol
FBIBT	=	2-fluoro-1-methylpyridinium p-toluenesulfonate
FBS	=	fetal bovine serum
GC	=	gas chromatography
GC-MSD	=	gas chromatography mass selective detection
GF	=	glass fiber
GMF	=	glass microfiber
HLB	=	hidrophylic-lipophylic balance copolymer sorbent
HPLC	=	high performance liquid chromatography

HRT = hydraulic retention time
ILO = international labour organization
Inf = influent
IP = identification point
IPCS = the international programme for chemical safety
IT = ion trap
LC = liquid chromatography
LOD = limit of detection
LOQ = limit of quantification
LYES = lyticase yeast estrogen screen
m/z = mass-to-charge ratio
MBR = membrane bioreactor
MBS = meta acrylate butadiene styrene
MeEE2 = mestranol
MEM = MEM non-essential amino acid solution
MeOH = methanol
MF = micro-filtration
MS = mass spectrometry
MS-MS = tandem mass spectrometry
MSTFA = N-methyl-N-(trimethylsilyl)-trifluoroacetamide
MTBSTFA = (N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide)
MTS = ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS)
NE-(ER-Calux®) = non-extraction ER-Calux® assay
NF = nano-filtration
OD = optical density
OECD = organisation for economic co-operation and development
PAH = polycyclic aromatic hydrocarbon
PBS = phosphate buffered saline
PCB = polychlorinated biphenyl
PE-HD = polyethylene – high density
PES = polietersulfone
PFBBr = pentafluorobenzyl bromide
PMS = phenazine methosulfate
POCIS = polar organic chemical integrative samplers
PTFE = polytetrafluoroethylene
PU = population units
Q = quadrupole
QqQ = triple quadrupole
Q-TOF = quadrupole time of flight hybrid
RIANA = River analyser
RLU = relative luminescence units
RO = reverse osmosis
RSD = relative standard deviation
SBSE = stir bar sorptive extraction
SCTEE = commission's scientific committee for toxicity and ecotoxicity and the environment
SD = standard deviation
SFCA = surfactant free cellulose acetate
SIM = selective ion monitoring
SPE = solid phase extraction

SPME	=	solid phase micro extraction
SRM	=	selected reaction monitoring
SRT	=	sludge retention time
TIE	=	toxicity-identification evaluation
TMCS	=	trimethylchlorosilane
TMSI	=	trimethylsilylimidazole
TOF	=	time-of-flight
TSI	=	thermospray Ionisation
U.S. EPA	=	United States Environmental protection agency
UF	=	ultra-filtration
UNEP	=	United Nations environment programme
UPLC	=	ultra performance liquid chromatography
Vtg	=	vitellogenin
WHO	=	World health organisation
WWTP	=	wastewater treatment plant
YES	=	yeast estrogen screen

1 Introduction

Environmental pollution has, for several decades, received considerable attention within the scientific community and public health bodies as one of the most important problems to be solved in order to reach a higher quality of life. There are a large number of synthetic and naturally occurring chemicals that are not regularly monitored, but could have potentially adverse effects in the environment. The release of so-called "emerging contaminants" has been occurring for a long time, but the problem was not recognized until appropriate detection methods were developed. In this doctoral thesis, we focus on steroid estrogens, pollutants that belong to a group of endocrine disrupting compounds (EDC).

1.1 The endocrine system

The endocrine system is together with the nervous and immune system the most important regulatory system in humans and animals. It is responsible for developmental, regulatory, growth and homeostatic mechanisms. The endocrine system controls reproductive structure and function, normal levels of glucose and ions in blood, general body metabolism, blood pressure, muscle and nervous system function.

Endocrine systems consist of several glands e.g., thyroid and gonads, found in various areas of the body and are responsible for production of hormones with different functions. Examples of endocrine hormones include adrenaline, testosterone and estrogen. These hormones are chemical messengers that are transported *via* the bloodstream to target cells, organs and tissues. When hormones reach their targets, they bind to a specific receptor, and such complexes can invoke a natural response. Binding is based on steric complementaries.

The natural concentration of hormones in the bloodstream is very low and to prevent functional disorders, maintaining the correct balance of hormones (homeostasis), is essential. Receptors have a very high potency for their specific hormones. The greater the potency, less substance is required to produce a given effect. Besides natural hormones, the same receptors are capable of binding to other chemical compounds. For that reason, the endocrine system is very sensitive towards disturbing influences and chemicals that can disrupt the endocrine system can cause undesirable effects (Birkett, 2003; Lintelmann et al., 2003).

1.2 Endocrine disrupting compounds

The International Programme for Chemical Safety (IPCS - which involves the WHO, UNEP and ILO) has, together with experts from Japan, USA, Canada, Europe and OECD, developed a consensus working definition for endocrine disrupters. This was adopted as a working definition by the European Community's Strategy for Endocrine Disrupters (IPCS, 2002). The definition is as follows:

"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact

organism, or its progeny, or (sub)populations."

The European Community's Strategy, as well as the WHO/IPCS definitions, also makes an effort to describe what a potential endocrine disrupter is:

"A potential endocrine disrupter is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations."

EDC encompass more than just environmental estrogens and include any agent that adversely affects any aspect of the entire endocrine system. They are usually either natural products or synthetic chemicals that can mimic a natural hormone, stimulate the formation of hormone receptors, block natural hormones, accelerate a hormone's breakdown or destroy a hormone (Birkett, 2003). Under certain circumstances, they may act as hypertrophic (stimulatory) agents and tumour promoters. Dose, body burden, timing, and duration of exposure at critical periods of life are important considerations for assessing the adverse effects of an endocrine disruptor. Effects may be reversible or irreversible, immediate (acute) or latent and not expressed for a period of time (U.S.-EPA, 1997). Table 1 gives examples of EDCs.

Table 1: Specific groups of compounds that act as endocrine disrupters.

Group	**Compound example**
Steroid hormones	17β-estradiol, 17α-estradiol, estrone, estriol
Surfactants	nonylphenol and its ethoxylates
Pesticides, herbicides, fungicides	DDT, dieldrin, tributyltin
Polyaromatic compounds	PAH's, PCB's, brominated flame retardants
Organic oxygen compounds	phthalates, bisphenol A

In the animal kingdom, the endocrine system is highly conserved. However, inter-species differences may affect sensitivity and response to EDC. These differences include qualitative and quantitative variability in endogenous hormone and receptor levels, differences in the timing and duration of critical periods of development and inter-species differences of sex-determination processes. Besides mammals, endocrine disruption can be observed in various animal species like birds, reptiles, amphibians, fish, and in invertebrates for example molluscs, annelids and arthropods (Campbell and Hutchinson, 1998; Lintelmann et al., 2003).

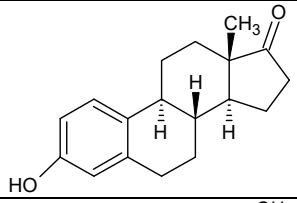
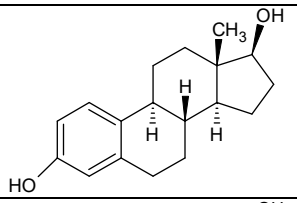
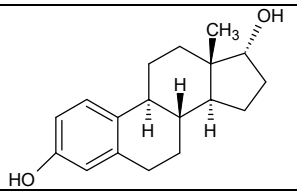
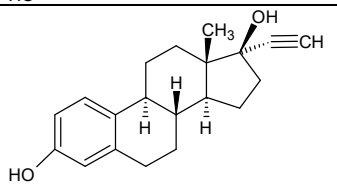
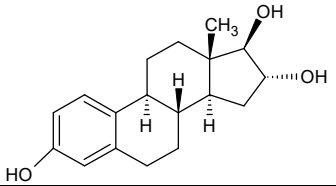
1.3 Steroid estrogens

Estrogens like 17β-estradiol (E2), 17α-estradiol (αE2), estrone (E1), estriol (E3), equilin, and equilenine are natural steroid estrogens. They are C-18 steroids synthesized from cholesterol by a series of enzymes (steroidogenic pathway) in humans and animals. Natural hormones are responsible for regulation of reproduction and female secondary sex characteristics. Specifically, E2 as the main hormone stimulates proliferation and growth in the reproductive tract organs, causes the development of the endometrium of the uterus, and influences libido. Estrogens also help maintain pregnancy and prepare the breasts for lactation. E1 and E3 are metabolites of E2. However, during pregnancy, E3 is synthesised in placental synthesis with 16α-hydroxylated steroids as precursors. This explains the high concentration of E3 in the urine of pregnant women (Fishman et al.,

1962). Synthetic compounds like 17α-ethinylestradiol (EE2) and mestranol (MeEE2) are chemical analogues of natural steroid hormones and are mainly used as contraceptives (Lintelmann et al., 2003).

Steroid estrogens are characterized by their phenolic A-ring, which is essential for biologic activity. Table 2 represents structures and physico-chemical data for five main steroid estrogens that are important for their behaviour in the environment. It can be seen that steroid estrogens are non-volatile compounds only sparingly soluble in water. However, their solubility is not a limiting factor for their presence and consequent negative effects in the environment. Their pK_a and K_{ow} values indicate that steroid estrogens are, at environmental pH, in non-ionised and moderately hydrophobic compounds with the ability to adsorb to solid organic matter.

Table 2: Structures and physic-chemical data for steroid estrogens.

Substance	Structure	Molecular weight	Water solubility (mg/L) at 20 °C)	Vapour pressure	Log K_{ow}	pK_a
Estrone (E1)		270.4	13	$2.3*10^{-10}$	3.43	10.71
17β-estradiol (E2)		272.4	13	$2.3*10^{-10}$	3.94	10.71
17α-estradiol (αE2)		272.4	-	-	4.01	-
17α-ethinylestradiol (EE2)		296.4	4.8	$4.5*10^{-11}$	4.15	10.4
Estriol (E3)		288.4	13	$6.7*10^{-13}$	2.81	10.4

Steroid estrogens bind to estrogenic receptors (ER) in tissues of humans and animals. ER is expressed in many human tissues like brain, liver, kidney, bone, lungs, mammary glands, reproductive tract as well as in the immune and cardiovascular systems (Mueller, 2004). In the human body, there are two estrogen receptors, estrogen receptor α (ERα, NR3A1, gene ESR1) and estrogen receptor β (ERβ, NR3A2, gene ESR2) (Germain et al., 2006). Endocrine disruption has been observed *via* both types of ER (Koehler et al., 2005;

Swedenborg et al., 2010).

Steroid estrogens reach target tissues *via* the bloodstream. Some free estrogenic hormones never reach their targets and are inactivated – conjugated – by the liver and kidneys (Birkett, 2003), usually in the form of sulphate or glucuronide conjugates. Conjugated forms of estrogens (Figure 1) have low potency for ER receptors, which means that steroid estrogens, that are excreted by humans, are not biologically active (Birkett, 2003; D'Ascenzo et al., 2003; Ternes and Joss, 2006; Ternes et al., 1999b).

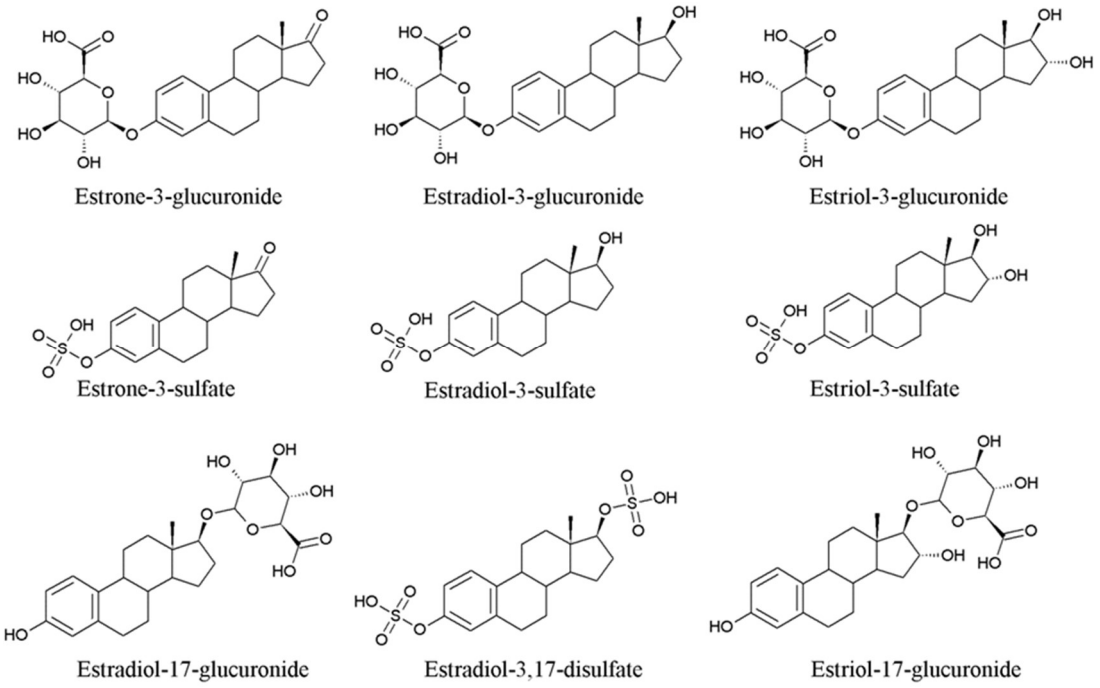


Figure 1: Conjugates of steroid estrogens (Source: "PubChem" - (Bolton et al., 2008)).

Once conjugated, steroid estrogens are excreted. Excretion rates depend on different stages of life (Liu et al., 2009). It can be seen from Table 3 that pregnant women excrete much higher concentrations of steroid estrogens than premenopausal women, postmenopausal women and men.

Table 3: Daily excretion rates of steroid estrogens (adapted from Liu et al. (2009)).

	Daily excretion rates (µg/day)			
Compound	**Premenopausal women**	**Postmenopausal women**	**Pregnant women**	**Men**
E1	10.7	5.0	1194	3.9
E2	4.7	2.8	347	1.5
E3	8.1	2.8	24078	1.5

1.4 Steroid estrogens in the environment

1.4.1 Sources of steroid estrogens

The main source of steroid estrogens is from the natural excretion by humans and animals. Steroid estrogens are also naturally present in food or are artificially used in food production (e.g., meat, fish, poultry, and dairy products) as well as for oral contraception (Birkett, 2003). Typical steroid estrogens in different sources are represented in Table 4. Once excreted and transported to the environment, steroid estrogens become environmental pollutants. Of course, it is impossible to reduce or eliminate natural excretion, which represents a constant pressure on the environment.

Table 4: Sources of steroid hormones (adapted from Birkett (2003)).

Source	**Steroid hormone**
Food – meat, fish, eggs, pork, dairy products	E1, E2
Sewage treatment work effluents	E1, E2, E3, EE2
Sewage sludge	E1, E2, E3, EE2
Oral contraception	EE2, MeEE2
Hormone replacement therapy	Conjugated estradiols
Runoff	E1, αE2
Agricultural waste	E1, αE2

Farm animals excrete 17α-estradiol (αE2) instead of E2.

1.4.2 Fate and transport of steroid estrogens in the environment

1.4.2.1 Steroid estrogens of human origin

Steroid estrogens released from humans are transported to wastewater treatment plant (WWTP) *via* the sewerage system (Figure 2). The main source of municipal wastewater are households, however, different studies include other point sources such as hospitals (Lin and Tsai, 2009; Thomas et al., 2007; Zorita et al., 2009).

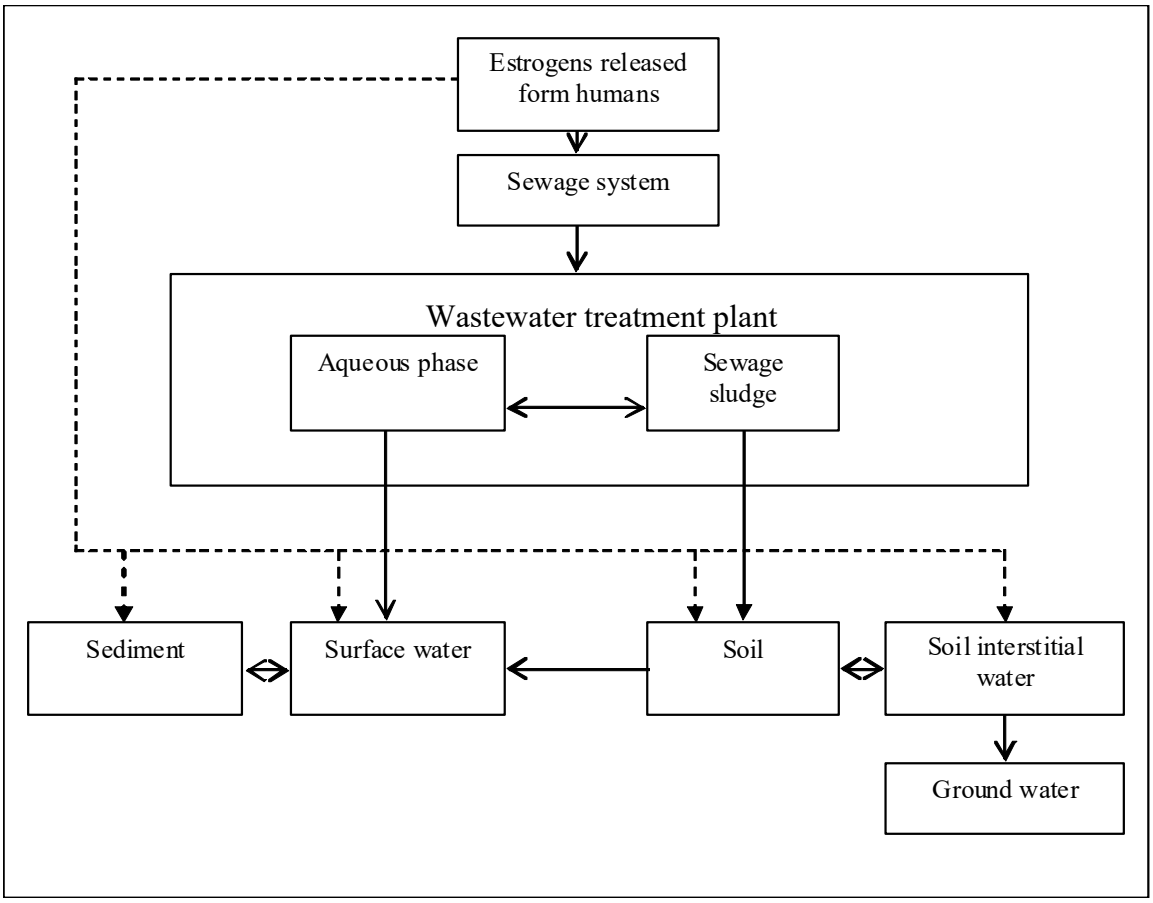


Figure 2: Conceptual diagram of compartments where estrogens from humans may occur (adapted from Ingerslev and Halling-Sørensen (2003)).

Steroid estrogens can be released to the environment directly (dashed line on Figure 2). However, in urban areas, human excreta are transported to WWTP, before reaching the environment. Therefore, water treatment process at WWTPs represents an important point, where steroid estrogens can be removed before releasing to the environment. During the sewage treatment, a part of the steroid estrogen content deconjugates and free estrogens appear in significant amounts (Atkinson et al., 2003; D'Ascenzo et al., 2003). Most steroid estrogens are removed during sewage treatment, but there are still detectable amounts of steroid estrogens in WWTP effluents (Teske and Arnold, 2008). The remaining steroid estrogens then enter surface waters: rivers, lakes, sea, and sediments. Estrogens may also adsorb to sewage sludge that is often used in agriculture and can therefore contaminate soil and groundwater. Typical concentrations of steroid estrogens in the environmental samples are presented in Table 5.

Table 5: Typical concentrations (ng/L) of steroid estrogens in the environment.

	E1	E2	E3	EE2	Reference
WWTP influent (ng/L)	2.4-670	2.5-125	0.4-660	0.4-70	Miège et al. (2009a)
WWTP effluent (ng/L)	0.6-95	0.3-30	0.4-275	0.2-5	Miège et al. (2009a)
WWTP sludge (ng/g)	<LOQ-37	<LOQ-17	<LOQ-3	<LOQ-4	Muller et al. (2010)
Surface water (ng/L)	<LOQ-4.6	<LOQ-1.2	<LOQ-1.9	<LOQ-0.33	Hohenblum et al. (2004)
Ground water (ng/L)	0.1-45	0.79-120	<LOQ-0.16	<1	Lapworth et al. (2012); Hohenblum et al. (2004)
Sediment (ng/g)	<LOQ-2	<LOQ-1.5	<LOD	<LOQ-0.9	Ternes et al. (2002); Isobe et al. (2006)

1.4.2.2 Role of wastewater treatment plants

Sewage treatment plants are the main point source of natural and steroid estrogens for the surface waters. Where a sewage pipeline exists, human wastes are transported to a WWTP. The majority of the excreted steroid estrogens are in conjugated, biologically inactive forms (D'Ascenzo et al., 2003). During transport through the sewerage system and during the wastewater treatment process, steroid estrogens can be deconjugated and converted to biologically active forms with enzymes (e.g. β-glucuronidase) produced by faecal bacteria (Andersen et al., 2003; D'Ascenzo et al., 2003; Fujii et al., 2002; Ternes et al., 1999a; Weber et al., 2005).

Miège et al. (2009a) have summarized concentrations of steroid estrogens from 117 studies. As it is evident from Table 5, typical concentrations of active compounds in influent are up to 670 ng/L. Conjugated and active steroid estrogens then pass through the secondary treatment process, where they are biodegraded (Andersen et al., 2005; Layton et al., 2000) and adsorbed to biosolids (Andersen et al., 2003; Andersen et al., 2005; Drewes et al., 2005; Layton et al., 2000). The actual removal rates during wastewater treatment reported in the literature are 55 - 99 %, 40 - 99 %, 64 - 85 % and 82 - 97 % for E1, E2, EE2 and E3, respectively (Teske and Arnold, 2008).

After water treatment, steroid estrogens leave the WWTP. The compounds that remain in the aqueous phase (WWTP effluent) are also in ng/L concentrations (Table 5). Despite these relatively low concentrations, steroid estrogens were recognised to be responsible for more than 90 % of the total estrogenic potential in municipal wastewaters. The reason is their high estrogenic activity, which is much higher compared to non-steroidal estrogenic compounds (Aerni et al., 2004; Drewes et al., 2005; Furuichi et al., 2004; Miège et al., 2009b; Salste et al., 2007).

WWTPs are originally not designed for removing steroid estrogens but are focused on removal of soluble organic matter, nitrogen and phosphorous. Two different approaches are in use for the removal of steroid estrogens and other organic micro pollutants from wastewater: the optimization of existing wastewater treatment and upgrading existing treatment with new end-of-pipe technologies (Koh et al., 2008). Removal strategies from source to the end of wastewater treatment process are listed in Table 6. It can be seen that removal of steroid estrogens can start before the wastewater reach WWTP. However, the

highest removal rates are achieved during preliminary, secondary and tertiary treatment of wastewater.

Table 6: Existing approaches to remove steroid estrogens from source to the end of water treatment process (adapted from: Koh et al. (2008)).

Water treatment process	**Approach to remove steroid estrogens**
Source separation	**Considerations:** - Urine separated from faecal waste - Separate domestic from industrial waste - Shelter for sewage treatment works to prevent dilution of sewage control of hydraulic retention time (HRT)
Preliminary treatment	**Possible options:** - Increased sludge retention time (SRT) - Introduce biofilm - Recycle from secondary treatment
Secondary treatment	**Operation optimisation:** - SRT (longer) - HRT (longer) - Constant temperature (warm 22°C-25°C) - Bioremediations using selective microbes **Process alternatives:** - Activated sludge process with nitrification - Membrane bioreactor - Enhanced biological nutrient removal
Tertiary treatment	- Adsorption and membrane processes; chlorination; advanced oxidation processes (see Table 7).

Existing treatment technologies can be optimised to get more efficient wastewater treatment and consequently better biodegradation rates. The efficiency of these processes is highly dependent on parameters such as hydraulic retention time (HRT), sludge retention time (SRT), sludge age, organic loading, redox potential and the cultivation of the right microorganisms (Drewes et al., 2005; Koh et al., 2008).

The most important operational parameters are HRT and SRT. A long HRT allows more time for adsorption and biodegradation, while a high SRT allows for a more diverse and specialized microbial culture to grow including slow growing microorganisms. If higher SRT rates are not possible, membrane bioreactors (MBR) are an alternative. Temperature and seasonal variation may affect the removal of estrogens from the sewage treatment works. Lower temperatures, i.e. in winter or in a cold climate, leads to reduced treatment efficiency as the metabolic rate of microorganisms slows down. However, the stability of the bacterial communities in activated sludge systems depends not only on operational parameters, but also on the nature of adapted microbiological populations that is an important variable in the removal of steroid estrogens (de Mes et al., 2005; Koh et

al., 2008; Ternes et al., 1999b). Andersen et al. (2003) reveal that the natural estrogens were largely degraded in the denitrifying and aerated nitrifying tanks of the activated sludge system, whereas EE2 was only degraded in the nitrifying tank.

If not biodegraded, steroid estrogens can be, due to their high K_{ow} values (Table 2), removed by sorption to excess sludge. However, in a study by Andersen et al. (2005) adsorption of E1, E2 and EE2 was estimated to be only 1.5-1.8 % of total removed estrogens, while in other research by Cirja et al. (2007) removal of radioactive labelled EE2 was mainly by sorption to the sludge and parts of the MBR reactor (app. 80 %). Silva et al. (2012) conclude that there are many conflicting studies that reveal high or low removal of steroid estrogens *via* sorption to excess sludge. The reasons might be different experimental conditions (e.g. size of suspended solids particles, total organic carbon, pH, temperature) that might affect sorption as well as desorption. However, potential sorption of steroid estrogens is an important factor for their presence in excess sludge and consequently disposal to the environment in this way.

WWTPs are important barrier, where steroid estrogens could be eliminated, especially if additional treatment processes are in use. Different possibilities of additional treatment processes and their efficiency for removal of steroid estrogens are listed in Table 7. Tertiary treatment processes can be generally separated on adsorption processes, membrane processes, chlorination and advanced oxidation processes. It can be seen from Table 7 that many processes have the ability to remove steroid estrogens and improve the quality of effluent. However, these techniques will inevitably result in large financial costs, and increased energy consumption (Koh et al., 2008; Silva et al., 2012).

Table 7: Tertiary treatment processes.

Treatment	**E1**	**E2**	**E3**	**EE2**	**Reference**
Adsorption – Granular Activated Carbon	74-98 %	77-100 %	-	53-100 %	Filby et al. (2010)
Membrane processes (RO, NF, UF, MF)	44-100 %	8-100 %	38-100 %	34-99 %	Silva et al. (2012)
Chlorination – ClO_2	96 %	90 %	-	100 %	Filby et al. (2010)
Chlorination - NaClO	-	>99 %	-	>99 %	Alum et al. (2004)
Photolysis (solar)	100 %	100 %	100 %	80 %	Fonseca et al. (2011)
$FeCl_3/NaNO_2$ photocatalysis	-	86.6-99.9 %	79.6 %	90-99 %	Wang et al. (2007)
Photo-Fenton process	-	>95 %	-	-	Zhao et al. (2008)
UV/H_2O_2	-	>95 %	-	>95 %	Rosenfeldt and Linden (2004)
Ozonation	92-100 %	93-96 %	55-77.6 %	96-100 %	Alum et al. (2004); Guedes Maniero et al. (2008); Nakada et al. (2007)
Oxidation with MnO_2	100 %	100 %	100 %	>90 %	Xu et al. (2008)
Sonification	85-98 %	95-97 %	91 %	66-87 %	Suri et al. (2008)

RO – reverse osmosis, NF – nano-filtration, UF – ultra-filtration, MF – micro-filtration

1.4.2.3 Steroid estrogens from farm animals

The excretion of natural estrogens from farm animals (i.e. cattle, pigs and chickens) is an important source of steroid estrogen contamination in the environment (Combalbert and Hernandez-Raquet, 2010; Gadd et al., 2010; Johnson et al., 2006). Figure 3 shows that after storage in a manure-tank, estrogens excreted from stabled animals may be released to the soil, when manure is used for fertilization. Estrogens may also reach soil directly from excretes of grazing animals. Once estrogens are in soil, they can reach surface waters and groundwater. Observed concentrations of steroid estrogens in manure were 28-640 ng/(g dw) (dry weight) for E1, 46-1229 ng/(g dw) for E2, 120-190 ng/(g dw) αE2. Runoff samples from soil applied with poultry litter contained 90-2520 ng/L of E2, while in spring water from a carst formation under agricultural influence 6-66 ng/L of E2 was determined (Ingerslev and Halling-Sørensen, 2003).

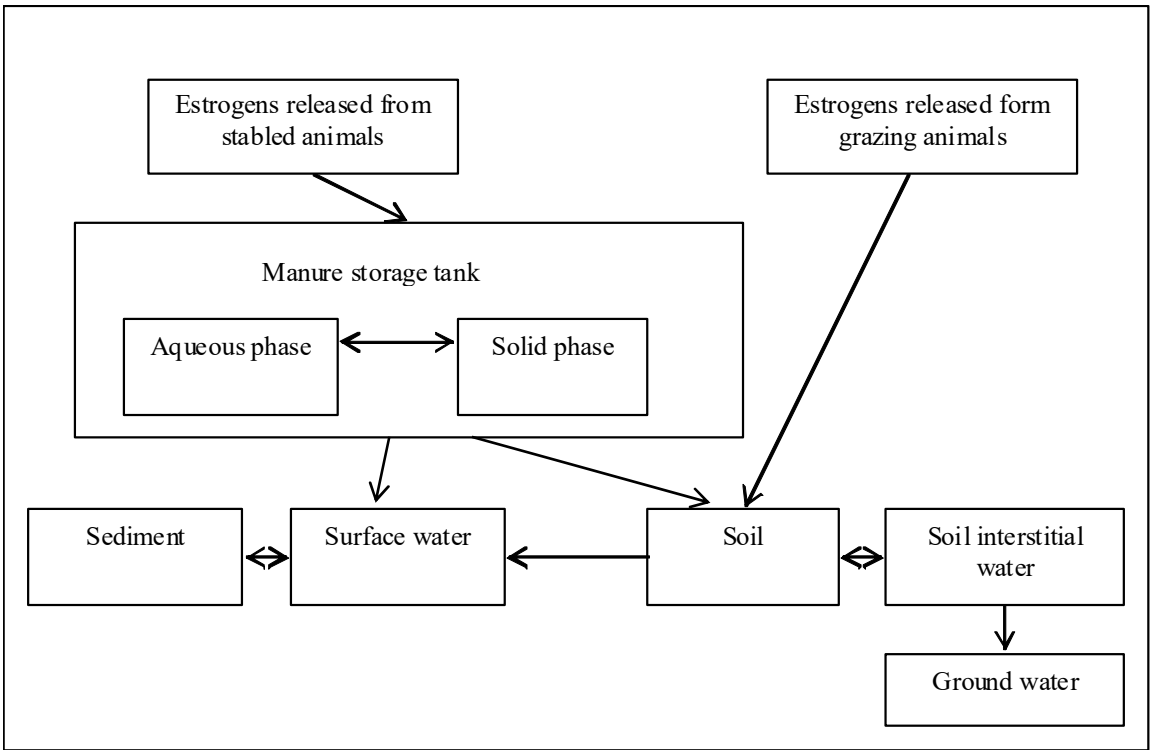


Figure 3: Conceptual diagram of compartments where estrogens from animals may occur (adapted from Ingerslev and Halling-Sørensen (2003)).

1.5 Legislation

Directive 2008/105/EC of the European Parliament and the Council on environmental quality standards in the field of water policy that amends EU Water Framework Directive (2000/60/EC) established (in Annex I) limits on concentrations of the priority substances in surface waters (33 priority substances and 8 other pollutants). However, steroid estrogens are neither on the list nor are they part of the Council Directive 98/83/EC on the quality of water intended for human consumption or Council Directive 91/271/EEC concerning urban wastewater treatment. The only directive that discusses steroid estrogens is Directive 96/22/EC that prohibits the use of substances having a hormonal action for growth promotion in farm animals and identifies precise circumstances, under which they may be administered to food producing animals for other purposes. After 14 October 2006, E2 or its ester-like derivatives may no longer be used for estrus induction

in cattle, horses, sheep or goats.

In any case, potential problems caused by endocrine disruption, and public concern has led to the European Commission establishing a legislative-based strategy for EDCs. The legislation activities began in 1996 and are still in progress. These activities are presented in the following paragraphs.

In 1999 the Commission's Scientific Committee for Toxicity and Ecotoxicity and the Environment (CSTEE) published the report "Human and wildlife health effects of endocrine disrupting chemicals with emphasis on wildlife and ecotoxicology test methods" (CSTEE, 1999). The Commission, in order to respond quickly and effectively to the concerns and recommendations highlighted in this report, adopted a strategy that was in line with the precautionary principle. This strategy (COM(1999) 706), known as the "Community strategy for endocrine disrupters", sets out the actions required to address the potential environmental and health impacts of endocrine disruption. Among the other activities, one of the important short term actions was to establish a priority list of substances for further evaluation of their role in endocrine disruption including the identification of substances for priority testing.

This document was updated in **2001** with "Community Strategy for Endocrine Disrupters - a range of substances suspected of interfering with the hormone systems of humans and wildlife" (COM(2001) 262). A study report has been produced entitled "Towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption - preparation of a candidate list as a basis for priority-setting". The COM(2001)262 document states that the priority in the short-term will be given to conducting and in depth study of 12 candidate substances, including the three synthetic/natural hormones E1, EE2 and E2. The other nine chemicals are either industrial or other substances, for which there is scientific evidence of endocrine disruption or potential endocrine disruptions and which are neither restricted nor currently being addressed under existing Community legislation. Activities concerning "Community strategy for endocrine disrupters" were reported in "Progress reports" in **2004**, in "Staff working document" SEC(2004)1372 covering years 2001-2003. In this report they conclude, that in depth evaluation of 3 natural/synthetic hormones (E1, E2 and EE2) prove that these substances evidently cause effects on fish in environmentally relevant concentrations. In the following two "progress reports" in **2007** (SEC(2007) 1635), covering period 2004-2006, and in **2011** (SEC(2011)1001, covering period from 2007 to 2011, other EDCs are focused on e.g. alkylphenols, benzophenones, bisphenols. However, despite many activities, steroid estrogens are still not part of any legislation concerning waters and the environment.

1.6 Determination of steroid estrogens in the environment

1.6.1 Sampling and sample handling

For chemical analysis and biological assays, representative samples have to be prepared. Sample collection, and sample handling are similar in both cases (Furuichi et al., 2004; Grung et al., 2007; Miège et al., 2009b; Nelson et al., 2007; Salste et al., 2007) and are crucial steps prior to detection of single compounds or estrogenic potential. Sampling and sample preparation are also time consuming. Their optimization is reflected in the enhancement of sensitivity and in the reduction of interferences in wastewater, sludge and biological samples (Tomšíková et al., 2012).

1.6.1.1 Sample collection

Sampling storage containers play an important role especially when adsorption of analytes can take place. Metallic bottles (aluminium) (Aerni et al., 2004; Hohenblum et al., 2004), glass bottles (Baronti et al., 2000; Ferguson et al., 2001; Grung et al., 2007; Murk et al., 2002; Nelson et al., 2007), amber glass bottles to prevent photodegradation (Fine et al., 2003; Hernando et al., 2004; Miège et al., 2009b; Rodriguez-Mozaz et al., 2004a) and PTFE coated bottles (Noppe et al., 2005) can be used instead of plastic to avoid plasticizers leaching from the bottles into the samples.

Concentrations of steroid estrogens vary in the environment, so in order to obtain a representative sample, different sampling techniques are in use. Composite sampling is used, if the goal is to calculate loads or mass fluxes, while grab sampling is better for determination of peak concentrations. Composite samples are average samples of some time period that can vary from 7h to 6 days (Aerni et al., 2004; Andersen et al., 2003; Baronti et al., 2000; Laganà et al., 2004; Murk et al., 2002; Nelson et al., 2007; Salste et al., 2007; Ternes et al., 1999b). Time proportional samples are collected periodically in a defined sampling period (e.g. every hour in 24 h), while flow proportional composite samples consider flow fluctuations in time. Grab samples are instant samples taken at an exact time. In many instances grab samples have been collected (Baronti et al., 2000; Cargouët et al., 2004; Ferguson et al., 2001; Furuichi et al., 2004; Gibson et al., 2007; Grung et al., 2007; Hernando et al., 2004; Jeannot et al., 2002; Ko et al., 2007; Miège et al., 2009b; Nelson et al., 2007; Quintana et al., 2004).

The low concentration of steroid estrogens requires a suitably large sample volume to be extracted and preconcentrated. The volume of sample depends on the extraction method, amount of sorbent, complexity of the matrix and sensitivity of the analytical technique used. Sampling volumes, summarised by Gabet et al. (2007), vary from <1mL to 49L. The usual sample volumes (for solid phase extraction) for surface water are 0.5 L – 2 L, for wastewater influent 0.1 L – 2 L and for wastewater effluent 0.25 L – 2 L. However, it is not advisable to extract more than 5 L with existing sample preparation methods since greater volumes create extracts with a high load of humic acids (Ingerslev and Halling-Sørensen, 2003).

Passive sampling methodology - POCIS (Polar Organic Chemical Integrative Samplers) is also used for the investigation of steroid estrogens in environmental samples (Alvarez et al., 2005; Burki et al., 2006; Liscio et al., 2009; Sellin et al., 2009). The POCIS contains a sorbent phase sandwiched between two microporous polyethersulphone membranes. Chemicals diffuse from the water over the membrane and adsorb in to the sorbent phase (e.g. Oasis HLB) and are eluted from the sampler after deployment (Liscio et al., 2009). POCIS are ideal for determining the time-weighted concentrations of hydrophilic contaminants in environments, where the concentration of the contaminants varies considerably over time (Sellin et al., 2009).

1.6.1.2 Sample pretreatment

After collection, all the samples should be treated to avoid degradation of analytes during sample handling and storage, to remove solid particles and if necessary to deconjugate target compounds. As it is seen in Table 8, different preservatives are used. However, some authors do not use chemical preservatives because preservatives might have negative impact on target compounds (Grung et al., 2007; Laganà et al., 2004; López de Alda and Barceló, 2001; Miège et al., 2009b; Murk et al., 2002; Nelson et al., 2007; Ternes et al., 1999b). Preserved or not, samples should be kept in the dark to avoid photodegradation of steroid estrogens in samples. It is also recommended that samples are stored at -18 °C or 4 °C until the extraction is performed. Preferably extraction of samples

should be performed within 48 hours (Gabet et al., 2007). If not possible, Baronti et al. (2000) showed that storage of steroid hormones, extracted on SPE cartridges, has high recoveries and is very practical, since it avoids stability issues and does not take up as much space as storing aqueous samples.

Table 8: Sample pretreatment options.

Parameter	**Option**	**Reference**
Sample preservation	Hydrochloric acid	Esperanza et al. (2007); Hohenblum et al. (2004)
	Sulphuric acid	Gibson et al. (2007)
	Formadehyde	Aerni et al. (2004); Baronti et al. (2000); Ferguson et al. (2001); Fine et al. (2003); Jeannot et al. (2002)
	Methanol	Gibson et al. (2007)
	Sodium azide	Yu et al. (2007); Hernando et al. (2004)
	No preservative	Grung et al. (2007); Laganà et al. (2004); López de Alda and Barceló (2001); Miège et al. (2009b); Murk et al. (2002); Nelson et al. (2007); Ternes et al. (1999b)
Filtration	Glass microfiber filters of 0.2-1.2 µm pore size	Gabet et al. (2007); Streck (2009)
Hydrolysis of conjugated estrogens	Enzyme β-glucuronidase or sulfatase	Atkinson et al. (2003); Belfroid et al. (1999); Miège et al. (2009b)
	Hydrochloric acid (80 °C; 20 min)	Majima et al. (2002)

Prior to extraction, most aqueous samples are filtered to remove particulate matter that might cause clogging of SPE cartridges. Most studies employ glass microfiber filters of 0.2-1.2 µm pore size (Table 8). The retention of estrogens during filtration was found to be negligible (Ingerslev and Halling-Sørensen, 2003).

The reason for a deconjugation step is that in many cases steroid estrogens are in a conjugated (biologically not active) form, unless deconjugation occurs in WWTP or in the environment. Deconjugation (hydrolysis) can be applied before or after sample extraction. It is performed by an enzyme β-glucuronidase or sulfatase, added to samples or chemically by adding hydrochloric acid. By performing deconjugation, we can determine the occurrence of conjugated estrogens or detect the total amount of steroid estrogens in samples. However, detecting conjugated estrogens directly is possible only with LC-based analysis and immunoassay techniques. Therefore, there is no need for the hydrolysis of conjugated steroid estrogens in the sample (Belfroid et al., 1999; Gabet et al., 2007).

1.6.1.3 Extraction and purification

Aqueous samples are usually processed by filtration followed by solid phase extraction (SPE). This is the preferred extraction method due to its simplicity, ease of automation, smaller consumption of organic solvents and the availability of different sorbent types with varying selectivity (Tomšíková et al., 2012). As being summarised by Gabet et al. (2007) and Tomšíková et al. (2012), most researchers use Oasis HLB with hydrophilic-lipophilic balance copolymer sorbent in SPE cartridges. Several other sorbents like graphitised carbon black (Baronti et al., 2000) and silica based reverse phase material (Aerni et al., 2004; Esperanza et al., 2007; Hohenblum et al., 2004; Jeannot et al., 2002; López de Alda and Barceló, 2001; Rodriguez-Mozaz et al., 2004a; Ternes et al., 1999b) are used.

Besides SPE cartridges, SPE discs can also be applied (Cargouët et al., 2004; Drewes et al., 2005; Hohenblum et al., 2004; Murk et al., 2002). Both SPE discs and SPE cartridges have advantages and disadvantages. The major advantage of SPE cartridges is lower price, much higher sample throughput and possibility of automation. Discs have a comparatively larger surface area for adsorbent-matrix contact, which results in higher extraction rates. They are not clogged up by the suspended matter present in the samples as easily as cartridges. If SPE disc are used, samples are free of contamination, whereas cartridges can be contaminated by plasticizers leached from the support material during elution (Ingerslev and Halling-Sørensen, 2003).

Other alternatives to SPE such as solid phase micro extraction - SPME (Carpinteiro et al., 2004), stir bar sorptive extraction - SBSE (Baltussen et al., 1999; Kawaguchi et al., 2004) and immunoaffinity extraction (Ferguson et al., 2001; Hosogi et al., 2010) are available, but were not frequently used in studies concerning steroid estrogens in environmental samples.

There are several factors besides the sorbent of choice that largely influence the performance of SPE: elution solvent, flow rate application, wash-up and elution step. Flow rates should be low enough to allow target compounds to bind to the sorbent. Flow rates are usually 1-10 mL/min (Gibson et al., 2007; Hernando et al., 2004; Jeannot et al., 2002; Liu et al., 2004), although Quintana et al. (2004) reveal that even higher rates can be applied – 15-20 mL/min.

Before elution of the analytes from the sorbents, an additional wash-up step using different diluted organic solvents can be performed to remove interferences. The examples are 5-40 % MeOH:water dilutions (Hernando et al., 2004; Jeannot et al., 2002; Laganà et al., 2004; Liu et al., 2004), AcON:water dilutions in hydrogen carbonate pH=10 (Gibson et al., 2007), acidified MeOH dilution (Baronti et al., 2000) while others can also be used.

After elution, the purification or “clean-up” step can be applied. This eliminates interferences that can seriously influence the sensitivity of the analytical method. This step is less important for the analysis of estrogens in aqueous matrices, than for solid matrices, where removal of interferences is necessary (Gabet et al., 2007; Noppe et al., 2005; Ternes et al., 1999b). The clean-up step is usually performed during the analysis of wastewater influents that contain a higher concentration of organic matter. A typical clean-up procedure uses a silica gel cartridge (Ternes et al., 2002; Ternes et al., 1999b). Another possible method involves HPLC fractionation with a “spherisorb silica” column (Esperanza et al., 2007; Fine et al., 2003; Ternes et al., 2002). One of the important advantages of biological assays is that there is no need for purification of extracts since interferences do not affect biological assays as much as chemical analysis (Aerni et al., 2004; Cargouët et al., 2004; Furuichi et al., 2004; Miège et al., 2009b; Nelson et al., 2007; Salste et al., 2007).

A promising SPE technique is on-line SPE. The main advantages are automatisation, smaller sample volume, minimal degradation and analyte loss due to evaporation steps, smaller volumes of solvent and reusable cartridges. With on-line SPE, it is possible to decrease sample preparation time, increase sample throughput and improve sensitivity. Disadvantages that make this method unattractive are higher matrix effects, limited portability, less flexibility, equipment costs and the absence of extracts for verification and further analysis (Rodriguez-Mozaz et al., 2007). An application of on-line SPE to extract steroid estrogens from wastewater influent and effluent samples was performed by Salvador et al. (2007).

1.6.1.4 Derivatization

Since steroid estrogens have low volatility, a derivatization step is necessary to increase the stability of active hydrogen and increase the volatility of steroids especially if gas chromatography (GC) is used for separation of compounds (Lerch and Zinn, 2003; Little, 1999).

If GC separation is applied, steroid estrogens are most commonly derivatised with silylation agents. Hydroxyl groups are silylated to increase thermal stability and volatility of steroid estrogens and consequently better chromatographic separation (Halket, 2003; Shareef et al., 2006). N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) are most commonly in use (Gabet et al., 2007; Streck, 2009). In a detailed study by Bowden et al. (2009) MSTFA was recognised as the best derivatization agent for steroid estrogens with the optimal reaction time and temperature of 55 °C and 30 min. Pyridine or dimethyl formamide should be added to the derivatization mixture to enable the derivatization of EE2 (Shareef et al., 2006).

A less suitable derivatization agent is N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), which is not appropriate for derivatization of EE2 (Gibson et al., 2007; Shareef et al., 2006). Sometimes additional substances such as trimethylsilylimidazole (TMSI), trimethylchlorosilane (TMCS) are used to catalyse the derivatization reaction (Esperanza et al., 2007; Ternes et al., 1999b) even though Quintana et al. (2004) showed that there was no significant difference observed when adding TMSI to the derivatization mixture. Other derivatization agents like pentafluorobenzyl bromide (PFBBr) are rarely used when chemical ionisation is applied (Fine et al., 2003; Lerch and Zinn, 2003).

Derivatization can also be applied prior to liquid chromatography (LC) to improve ionisation and enhance sensitivity. Dansyl chloride (Lien et al., 2009; Qin et al., 2008; Salvador et al., 2007), PFBBr (Singh et al., 2000) and 2-fluoro-1-methylpyridinium p-toluenesulfonate (FBIBT) (Matějíček et al., 2007) were used in studies employing LC based analysis.

1.6.2 Instrumental analysis

According to Skoog et al. (1998), “analytical chemistry deals with methods for determining the chemical composition of samples of matter. A qualitative methods yields information about the identity of atomic or molecular species or the functional groups in the sample; a quantitative method, in contrast, provides numerical information as to the relative amount of one or more of these components.” Instruments for chemical analysis of organic compounds in environmental samples usually involve two main units: a chromatograph used to separate analytes and a detector. Separation of steroid estrogens in environmental studies is achieved by either LC or GC. Compounds are afterwards introduced to mass spectrometry (MS).

1.6.2.1 Separation

The basis of LC involve partitioning of the analytes between a liquid mobile phase and a bonded solid phase, analytes that separate should be in a liquid sample, that is injected in the instrument (Skoog et al., 1998). LC is widely used analytical separation techniques for the determination of steroid estrogens (Tomšíková et al., 2012). High performance liquid chromatography (HPLC) is usually applied (Baronti et al., 2000; Ke et al., 2007; Laganà et al., 2004; López de Alda and Barceló, 2001; Pojana et al., 2007; Ren et al., 2007; Rodriguez-Mozaz et al., 2004a). The dimensions of chromatographic columns, and used stationary and mobile phases are presented in Table 9. It is evident that different dimensions as well as different stationary and mobile phases are in use. However, conventional LC has the important disadvantage of being time consuming. Therefore, promising techniques like ultra performance liquid chromatography (UPLC) that is already used in some laboratories (Farré et al., 2007; Kumar et al., 2009; Lien et al., 2009; López-Roldán et al., 2010; Sun et al., 2009) enable higher speed, greater resolution and more sensitive separation of organic pollutants in environmental samples.

Alternatively, in GC the sample is vaporized and injected onto the head of a chromatographic column. Elution is brought about by the flow of an inert gaseous mobile phase. In contrast to most other types of chromatography, the mobile phase does not interact with the analyte; its only function is to transport the analyte through the column (Skoog et al., 1998). Chromatographic columns for GC separation of steroid estrogens differ in dimensions and stationary phase (Table 9). A typical capillary column is 30 m x 0.25 mm and 0.25 μm film thickness. Steroid estrogens separate in non-polar stationary phases, while Helium is usually used as the mobile phase (Table 9).

Table 9: Column dimensions, stationary and mobile phases in liquid and gas chromatography used for environmental analysis of steroid estrogens.

	Parameter	Options	References
LC	Column dimensions	30-350 mm (length) 1-4.6 mm (diameter) 1.7-5 µm (average particle size)	Tomšíková et al. (2012)
	Stationary phase	silica based octadecyl (C-18) (reverse phase) silica based phenyl-hexyl (reverse phase)	Baronti et al. (2000); Ke et al. (2007); López de Alda and Barceló (2001); Pojana et al. (2007); Ren et al. (2007); Tomšíková et al. (2012)
	Mobile phase	water:acetonitrile water:methanol acetonitrile:methanol:water	Baronti et al. (2000); Jeannot et al. (2002); (Ke et al., 2007); Laganà et al. (2004); López de Alda and Barceló (2001); Ren et al. (2007)
GC	Column dimensions	30 m (length) 0.25 mm (diameter) 0.25 µm (film thickness)	Gabet et al. (2007); Streck (2009); Tomšíková et al. (2012)
	Stationary phase	phenyl-arlene (5 %) 5 % diphenyl- 95 % dimethylpolySiloxane 95 %-dimethyl- 5 % phenylpolysiloxane 14 %-Cyanopropyl- phenylmethylpolysiloxane methyl 5 % phenyl polysiloxane	Arroyo et al. (2007); Carpinteiro et al. (2004); Gibson et al. (2007); Hernando et al. (2004); Jeannot et al. (2002); Yu et al. (2007)
	Mobile phase	Helium	Aerni et al. (2004); Gibson et al. (2007); Hernando et al. (2004); Jeannot et al. (2002); Liu et al. (2004); Quintana et al. (2004); Ternes et al. (1999b)

From the aspect of steroid estrogens, sample preparation for LC has one important advantage compared to GC-based techniques – there is no need for derivatization of steroid estrogens prior to instrumental analysis, because the volatility of substances is not important. Also, by using LC it is possible to separate steroid estrogens from their conjugates or any other metabolites (López de Alda and Barceló, 2000).

1.6.2.2 Detection

After the analytes are separated in chromatograph, they have to be detected. For detection of steroid estrogens in environmental samples, MS is commonly applied since other detectors like fluorescence and UV detectors are only rarely used due to limited selectivity and sensitivity (high LODs) (Gabet et al., 2007; Streck, 2009; Tomšíková et al., 2012).

MS is based on the generation of gaseous ions from analyte molecules. These ions are separated according to their mass-to-charge (*m/z*) ratio and detected with mass analyser. The relative abundance of the ions produced as a function of *m/z* ratio gives a mass

spectrum of the analyte. Analyte ionisation takes place in ion source. The most common ionisation techniques are electron ionisation (EI), chemical ionisation (CI), adsorption ionisation and nebulization ion techniques like electrospray (ESI) and thermospray (TSI) ionisation (Kellner et al., 2004). The choice of ionisation technique depends on if the sample is in liquid (LC separation) or in gaseous state (GC separation).

For ionisation of steroid estrogens in liquid phase, ESI in negative mode has been mostly used (Baronti et al., 2000; Ke et al., 2007; Laganà et al., 2004; López de Alda and Barceló, 2001; Pojana et al., 2007; Ren et al., 2007; Rodriguez-Mozaz et al., 2004a). Alternatively, atmospheric pressure chemical ionisation (APCI) technique is reported (Baronti et al., 2000) as well as atmospheric pressure photo ionisation - APPI (Robb et al., 2000; Viglino et al., 2008). Baronti et al. (2000) reports two times lower signal to noise ratio using APCI in contrast to ESI.

If the steroid estrogens are vaporised during GC separation, EI is used in most cases (Gabet et al., 2007; Streck, 2009; Tomšíková et al., 2012) although CI in negative ion mode has also been reported (Drewes et al., 2005; Fine et al., 2003). A valuable feature of CI is that its selectivity can be tuned by the choice of reagent gas (Kellner et al., 2004).

After generation of ions, these are separated according to their *m/z* ratio in a mass analyser. Typical analysers are quadrupole mass filters (Q), ion trap (IT), time-of-flight (TOF) and ion-cyclotron resonance analyser. Different combination of mass analysers can be used in so-called tandem mass spectrometry (MS-MS). The most widely applied MS-MS instruments are triple quadrupole (QqQ), the ion trap (IT) and quadrupole time of flight hybrid (Q-TOF) (Kellner et al., 2004).

Single MS instruments can operate in several modes. In “full scan” mode, a series of complete mass spectra is acquired. Selective ion monitoring (SIM) mode is applied, when single MS instruments are used as highly selective and sensitive detectors for quantitative analysis. In this mode the instrument selects only one or few preselected *m/z* values. However, spectral information has to be sacrificed. With this, better signal-to-noise ratios and improved LOD can be achieved. Tandem MS-MS instruments can operate in various operational modes, since there are two mass analysers. However, for environmental analysis selected reaction monitoring (SRM) is applied to improve selectivity and signal-to-noise ratios. SRM is an operation mode in which an ion of a particular mass is selected in the first detector of a tandem mass spectrometer. The same is performed in second mass spectrometer where only preselected ion products of a fragmentation reaction are detected (Kellner et al., 2004).

The 2002/657/EC European Commission Decision set up the quality and performance criteria for the spectrometric identification and confirmation of compounds, based on the use of identification points (IPs). In GC-MS or LC-MS techniques, each monitored ion earns one IP. In GC-MS-MS and LC-MS-MS, precursor ions earn 1 IP, while daughter ions earn 1.5 IP. A minimum 4 IPs are required for banned compounds and 3 IPs for the others to confirm their identity and to fulfill the requirements of 2002/657/EC.

For steroid estrogens, the sensitivity of single LC-MS instrument can be too low for reliable detection, especially in complex environmental matrixes (Streck, 2009). However, the use of single LC-MS-instruments has been reported (López de Alda and Barceló, 2001; Pojana et al., 2007; Ren et al., 2007; Rodriguez-Mozaz et al., 2004a). On the other side, GC-MS is the most popular of all hyphenated techniques. GC-MS instruments are easy to use, small, and robust. They allow flexible operation and are reasonably priced. GC-MS provides sensitive determination of steroid estrogens with sufficient variability and selectivity for analysing environmental samples (Gabet et al., 2007; Streck, 2009). There is also no need for extraordinary clean-up efforts for reaching LOD below the sensitivity level recommended for sewage and surface waters. However,

for improved sensitivity, selected ion monitoring (SIM) has to be applied. For analysing complex matrices like wastewaters with GC-MS, rules for identification must be set up. The chromatographic peak representing the analyte should exactly match the predetermined retention time and the molecular ion of the target compound should be present in the mass spectra. Besides that, at least two additional ions should match ion ratios within 50 % (Ingerslev and Halling-Sørensen, 2003).

It can be seen form Table 10 that improved LOD can be reached with MS-MS detectors. Besides increased sensitivity, MS-MS instruments improved variability and selectivity. In this way MS-MS techniques reduce matrix interferences and confirm analyte identity with greater reliability than single MS instruments. However, MS-MS instruments are more expensive than single MS instruments (Gabet et al., 2007; Ingerslev and Halling-Sørensen, 2003; Streck, 2009). In LC-based techniques, IT, Q-TOF (Farré et al., 2006; Labadie and Hill, 2007) and QqQ detectors (Baronti et al., 2000; Ke et al., 2007; Laganà et al., 2004) are in use for detection of steroid estrogens in environmental samples (Tomšíková et al., 2012). Q-TOF and QqQ instruments has increased selectivity and sensitivity, however, Labadie and Hill (2007) and Farré et al. (2007) have shown that QqQ detector can reach up to one order of magnitude lower LOD. Moreover, the highest sensitivity in LC-ESI-MS-MS analysis of steroid estrogens is achieved when recording in SRM mode (Kellner et al., 2004; Tomšíková et al., 2012). The same improvement of selectivity and sensitivity is also reached with GC based MS-MS instruments. Ion trap MS-MS detector is usually applied after GC separation (Carpinteiro et al., 2004; Hernando et al., 2004; Jeannot et al., 2002; Noppe et al., 2005; Quintana et al., 2004; Ternes et al., 2002; Ternes et al., 1999b).

Table 10: Typical limits of detection for different analytical methods.

Instrument	**E1 (ng/L)**	**E2 (ng/L)**	**EE2 (ng/L)**	**E3 (ng/L)**
LC-MS	1-2.5	1.2-2.5	0.8-3.5	4-5
LC-MS-MS	0.1-1	0.2-1	0.3-2	0.3-3
GC-MS	0.5-2	0.7-4	0.8-5	1-10
GC-MS-MS	0.2-2	0.1-3	0.1-3	0.3-3

1.6.2.3 The use of isotope-labelled steroid estrogens

For determining fate and behaviour of steroid estrogens in environmental matrices, the use of radioactive or stable isotope-labelled chemicals can also be applied. Stable isotope-labelled steroid estrogens like E2-d_2, E1-d_4, EE2-d_4 are commonly used as internal standards (Gabet et al., 2007) and especially used to study the biosynthesis of estrogens in human body since they can be distinguished from endogenous estrogens (Wright et al., 1990). Stable isotopes can also be used to control of the illegal use of natural steroid hormones in cattle. By comparison of $^{13}C/^{12}C$ isotopic ratio of main urinary estradiol metabolite (αE2), the differentiation between endogenous and exogenous estradiol metabolites can be achieved (Buisson et al., 2005).

Radioactive isotope-labelled compounds are more suitable for studying the sorption, mineralization and production of non-extractable residues and metabolites. The precision and sensitivity of the techniques for analysis of e.g. ^{14}C-labelled substances are very high. Sample preparation for testing with radio labelled chemicals is simpler comparing to conventional techniques. The isotope-labelled substances have been therefore used to

study the persistence of steroid estrogens in soil and wastewater. The major disadvantage of these techniques are specific laboratory facilities that are needed (scintillation counters or other instruments like HPLC-MS-MS with additional radioisotope detectors) and that the cost of radioactive-labelled chemicals is high (Cirja et al., 2007; Ingerslev and Halling-Sørensen, 2003; Layton et al., 2000).

1.6.3 Validation of the analytical methods for steroid estrogens in environmental samples

Validation of the analytical method is necessary for reliable determination of steroid estrogens in the environment. The most important parameters for validation of analytical methods for detecting steroid estrogens in environmental samples are linearity, sensitivity, selectivity, trueness, precision, robustness, LOD and LOQ.

Linearity is the ability of the method to obtain test results proportional to the concentration of analyte, within a given concentration range. To confirm linearity, linear calibration model (normally least square regression model) is constructed in LOQ-150 % of the expected analyte level (Taverniers et al., 2004).

Sensitivity is a quotient of the change in an indication of a measuring system and the corresponding change in a value of a quantity being measured (JCGM 200, 2012). It is arbitrary determined by the slope of the calibration curve (Taverniers et al., 2004).

Selectivity is a property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measurands such that the values of each measurand are independent of other measurands or other quantities in the phenomenon, body, or substance being investigated (JCGM 200, 2012). There are only a few studies reporting selectivity of the methods used for the determination of steroid estrogens. Good selectivity can be achieved with clean-up steps using a combination of retention time and the optimization of MS-MS detection (Gabet et al., 2007).

Precision is closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions (JCGM 200, 2012). Precision is represented with repeatability and reproducibility which are reported as standard deviation (SD) or relative standard deviation (RSD).

Repeatability is defined as measurement precision under a set of repeatability conditions of measurement. Repeatability conditions are conditions of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time (JCGM 200, 2012).

Reproducibility is defined as measurement precision under reproducibility conditions of measurement that are defined as condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects (JCGM 200, 2012).

While analysing steroid estrogens, precision should be tested on all matrices studied in different concentration ranges. Reported precisions (RSD) for steroid estrogen determination are usually under 20 % for aqueous and solid samples (Gabet et al., 2007).

Trueness closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value (JCGM 200, 2012). Since there is no certified reference material for steroid estrogens, the trueness is expressed as a **% recovery** of known spiked amount of analyte (Taverniers et al., 2004).

Most authors reported recoveries of 75-100 % whatever the analytical technique. For determining recoveries in different matrices, some reference material would be much desired (Gabet et al., 2007).

Robustness is a measure of capacity of a method to remain unaffected by small variations in method performance parameters (Taverniers et al., 2004).

Uncertainty is non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used (JCGM 200, 2012). To the best of our knowledge uncertainty is not reported in available literature of the analysis of steroid estrogens in environmental samples.

Traceability is a property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty (JCGM 200, 2012). Up to date no reference standards are available for steroid estrogens, therefore measurement traceability to SI units is not reported in the literature.

Limit of detection (LOD) is the lowest concentration of analyte that can be reliably distinguished from zero. **Limit of quantification (LOQ)** is the lowest amount of analyte that can be determined quantitatively with an acceptable level of repeatability, precision and trueness (Taverniers et al., 2004).

LOD and LOQ can be evaluated in several ways:

- by repeated analysis of a blank solution. LOD is given as 3 times the standard deviation of the blank base-line whereas LOQ is 10 times the standard deviation (Liu et al., 2004).
- with calibration curve, where LOD and LOQ are the lowest and second lowest point in calibration curve (Noppe et al., 2005; Ternes et al., 2002).
- with signal to noise ratio (S/N) for evaluating sensitivity (Baronti et al., 2000; Gibson et al., 2007; Hernando et al., 2004).

Besides internal method validation, **interlaboratory studies** are used to validate method of interest. In interlaboratory studies different laboratories analyse the same material. Interlaboratory studies allow the assessment of the proficiency of individual laboratories, estimation of the measurement uncertainty and certification of reference material (Vander Heyden and Smeyers-Verbeke, 2007). An interlaboratory exercise concerning steroid estrogens has been performed by fourteen collaborating laboratories using either GC-MS or LC-MS/MS analysis (Heath et al., 2010). The lowest repeatability and reproducibility in this interlaboratory exercise was found while testing wastewater influent samples where interlaboratory standard deviation of GC-MS method was up to 29.8 ng/L, 7.75 ng/L and 1.52 ng/L for E1, E2 and EE2, respectively. The best results were achieved with surface water where interlaboratory standard deviation did not exceed 2.93 ng/L (E1 with GC-MS). The finding of this interlaboratory exercise was also better performance of GC-MS method in case of EE2 analysis while no clear difference was observed for E1 and E2. However, the results from this interlaboratory study reveal a high level of competence among the participating laboratories for the detection of steroid estrogens in water samples.

1.6.4 Biological methods

A range of biologically based assays are commonly available and can be applied, if objective of the work is to evaluate a particular compound or mixture of compounds displaying activity in terms of endocrine disruption. If the aim is to identify and quantify particular compounds that are present, then a chemical analysis is more appropriate.

Bioassays provide an alternative method to traditional mass-based analysis of steroid estrogens and are used mostly to identify if a compound or a mixture of compounds is estrogenic or not (Campbell et al., 2006; Gomes et al., 2003; Voulvoulis and Scrimshaw, 2003).

The use of bioassays in environmental studies has, however, some limitations. The results of bioassay may be affected by other unrelated toxic effects from complex mixtures. Especially for highly polluted samples, there have been questions about their variability, reliability, and robustness (Andersen et al., 1999). However, every environmental sample has specific chemical composition that is usually unknown. There are also mixture interactions that cannot always be inferred from the concentrations of the individual components determined by chemical analysis. Therefore, bioassays are important tools to examine the presence of EDCs and integrate their biological activity in complex mixtures (Leusch et al., 2010).

In general we can separate biological methods into *in vitro* and *in vivo*. In case of *in vivo* studies "whole organism assays" are used for evaluation of EDC impacts on the endocrine system as a whole (Gomes et al., 2003; Voulvoulis and Scrimshaw, 2003). *In vitro* assays are attractive due to their cost and time efficiency and relatively rapid screening of a large number of samples. *In vitro* assays usually employ single cell assays and non-cellular bioassays (Campbell et al., 2006).

There are many mechanisms that bioassays can use for monitoring endocrine effects. Cell proliferation estimates cell growth and reproduction in different samples. Ligand binding uses specific binding site for estrogens that can be quantified. The production of vitellogenin in male fish is an indication of endocrine disruption. Luciferase induction uses estrogen receptors and response elements to produce the protein luciferase that may be quantified by luminescence after cell lysing and addition of luciferin. Antigen-antibody interactions provide the basis for immunoassays based upon the non-covalent binding of antigen to antibodies and may use whole organisms or cellular and non-cellular assays (Campbell et al., 2006).

Biological methods employ different organisms or cells. And for that reason, different responses to the same samples are observed. To avoid false results, the use of a battery of biotests that includes both *in vivo* and *in vitro* assays especially when investigating estrogenicity of environmental samples is suggested (Gomes et al., 2003; Voulvoulis and Scrimshaw, 2003).

1.6.4.1 Whole organism assay

For monitoring the pollution with EDC in aquatic environments, measuring of endocrine disruption in amphibians, fish, birds and insect is applied (Campbell et al., 2006; Gomes et al., 2003). There are various methods for determining estrogenic response in these organism including deformity, reproductive differences, egg and offspring development and serum protein production like vitellogenin (Vtg). Some organisms are genetically engineered to give a specific response to EDCs. Transgenic zebrafish have been bioengineered with luciferase expression coordinated to Vtg production, or with expression of green fluorescence protein (Campbell et al., 2006; Kurauchi et al., 2005; Legler et al., 2002).

Whole organism assay advantages are the following (Campbell et al., 2006; Gomes et al., 2003; Kurauchi et al., 2005):

- *In vivo* assessment of true impact of estrogenicity on a target species. This species can also inhabit a range of environments and thus serve as biological indicators of EDC pollution.

- Life-cycle studies can only be performed with whole organism assays.
- There is no need to make an extrapolation to the field situation (like in chemical analysis and *in vitro* tests)
- Whole organism assay enables the screening of novel pollutants with meaningful endpoints, the integration uptake, bioconcentration, and excretion,
- Evaluation of endocrine disrupting effect with respect to toxicity requires *in vivo* biotests for estrogen like substances.

Major disadvantages of whole organism assay are:

- Lack of specificity of organism to response to various EDCs.
- Low sensitivity and the need for laborious and time-consuming work.
- Response in a biological indicator species may not identify cause and effect or point to a specific location as the source. Such indicators can provide a cumulative estrogenic response to exposure to minimal EDCs concentration in the environment.

Special attention in *in vivo* testing is paid to steroid estrogens for they have the highest affinity towards the estrogen receptor among all of the EDCs. Of course, it is not possible to observe the effects of single compounds *in vivo,* because the observed effects are always result of integrated interactions of all compounds in the sample. It is possible to apply different organisms for investigating samples, where steroid estrogens represent important part of EDCs (e.g. wastewaters). Some examples of whole organism assays to investigate estrogenic potential are presented in Table 11.

Table 11: Examples of whole organism studies as indicators of estrogenic endocrine disruption.

Species	Common name	EDC effect observed	Reference
Rana temporaria	Common frog	Gonadal abnormalities, Vtg induction	Bögi et al. (2003)
Xenopus laevis	African clawed frog	Gonadal abnormalities, Vtg induction	Bögi et al. (2003)
Chrysemis picta	Painted turtle	Vitellogenin induction	Irwin et al. (2001)
Oncorhyncus mykiss	Rainbow trout	Reproductive deficiencies, egg and offspring development, and vitellogenin induction	Aerni et al. (2004); Grung et al. (2007)
Cyprinodon variegates	Sheephead minnow	Vitellogenin induction	Folmar et al. (2002)
Danio rerio, Brachydanio rerio	Zebrafish	Gonad development, physiological development; vitellogenin induction, luminescence	Legler et al. (2002)
Oryzias latipes	Medaka fish	Gonad development; reproductive success; green fluorescence protein	Kurauchi et al. (2005)
Carrasius auratus	Goldfish	Vitellogenin induction; gonad development; histological examination	Diniz et al. (2010)
Abramis brama	Common bream	Vitellogenin induction; gonad development;	Vethaak et al. (2005)
Platichthys flesus	Flounder	Vitellogenin induction; gonad development;	Vethaak et al. (2005)
Halineetus leucocephalus	Bald eagle	Reproductive and teratogenic effect	Bowerman et al. (2000)
Gallus domesticus	Domestic chicken	Embryo development; egg shell thickness	Berg et al. (1999)

1.6.4.2 Single cell bioassay

Single cell bioassay may offer good sensitivity, but may not consistently provide a repeatable quantitative response for a specific EDC in complex environmental samples. The rapid response and low equipment requirements are an attractive alternative to chemical analyses for environmental monitoring, especially when monitoring objective is measuring relative increases in total estrogenicity (Campbell et al., 2006).

The assays often use yeast or mammalian cells that have been used as they are (Table 12), or bioengineered to stimulate estrogen response element that promotes the expression of a measurable protein (e.g. luciferase, β-galactosidase). Besides mammalian cancer cell lines, other animal cells have been used for environmental studies concerning steroid estrogens (Grung et al., 2007). Since yeasts have no indigenous estrogen receptor (ER), the receptor gene has to be added to its genome either from human, fish, frogs or other species. This is advantageous as it eliminates the multiple pathways by which cells and tissues normally respond to estrogen in organisms that have ERs (Campbell et al., 2006).

Table 12: Examples of single cell bioassays for detection of EDCs.

Common name	Cell type	e-EDC effect	Reference
E-Screen	MCF-7 breast cancer cells	Cell proliferation response	Soto et al. (1995)
Yeast estrogen screen (YES) LYES; BLYES	Various (*Saccharomyces* spp., *Cryptococcus* spp., *Candida* spp.)	Colometric and luminescent response	Aerni et al. (2004); Murk et al. (2002); Nelson et al. (2007); Routledge and Sumpter (1996); Salste et al. (2007); Sanseverino et al. (2005)
ER-luciferase assay	Human embryonic kidney (HEC)	Luminescent response	Pawlowski et al. (2003)
NA	*E. coli*	Luminescent response	Gu et al. (1999)
ER-Calux®	T47D human breast adenocarcinoma cell	Luminescent response	Legler et al. (1999)
MVLN	MCF-7 breast cancer cells	Luminescent response	Pons et al. (1990)
MELN	MCF-7 breast cancer cells	Luminescent response	Balaguer et al. (1999); Miège et al. (2009b)

1.6.4.2.1 Mammalian cell-based assays

The E-Screen assay was developed to assess the estrogenic potential of environmental chemicals using the proliferative effect of estrogens on their target cells (MCF-7). This quantitative assay compares the cell proliferation in the absence of estrogens (negative control), in the presence of E2 (positive control) and in presence of a tested sample which can be a complex mixture or single compound (Soto et al., 1995). E-Screen assay has been used in environmental studies of rivers, wastewaters and sediments (Drewes et al., 2005; Kase et al., 2008; Nelson et al., 2007; Oh et al., 2009; Tan et al., 2007).

The estrogen responsive chemically activated luciferase expression (ER-Calux®) assay is a commercially available method that uses the T47D human breast adenocarcinoma cell engineered to express the enzyme luciferase (BioDetection Systems, Amsterdam, The Netherlands). The cells are seeded on 96-well plate and exposed to the sample. Estrogenic compounds in a sample bind to estrogenic receptor and induce luciferase production. The amount of luciferase is determined with luminescence that correlates to estrogenic potential of the sample (Legler et al., 1999). Environmental application of ER-Calux® assay covers sediments or sediment extracts, wastewater end surface water (91/271/EEC, 1991; Houtman et al., 2006; Houtman et al., 2007; Legler et al., 2002; Murk et al., 2002). MELN and MVLN assay are in principal the same as ER-Calux® assay but uses MCF-7 human breast carcinoma cell line. MELN and MVLN assay has also been used in environmental studies (Cargouët et al., 2004; Furuichi et al., 2004; Miège et al., 2009b). MELN and ER-Calux® assay cells are the most sensitive *in vitro* reporter gene assays and have been selected to define a standardized test for the *in vitro* evaluation of estrogenic activity (Miège et al., 2009b). Leusch et al. (2010) studied a performance, strengths and weaknesses of three mammalian based assays and yeast estrogen screen (YES) assay (Table 13). It is revealed that each assay has advantages and limitations. However, ER-Calux® and E-Screen assays exhibit the best performance due to robust and predictable data.

Table 13: Comparison of *in vitro* cell based assay (adapted from Leusch et al. (2010)).

Assay	**E-Screen**	**ER-Calux®**	**MELN**	**YES**
Performance				
Detection limit	0.2 ng/L	0.1 ng/L	0.27 ng/L	3-5.5 ng/L
Environmental samples EEQ	++	+++	+	+
Likeness to other assays	+++	+++	-	++
Coefficient of variation	++	+++	+	+++
Method quantification limit	+++	+++	+++	-
Predicted *vs.* measured comparison	++	+++	+	+
Overall performance	++	++	+	+
Strengths and weaknesses				
Analysis of model compounds	+++	+++	++	+++
Analysis of environmental samples	+++	+++	+	-
Ease of use	+	+	+	++
Simple training	-	-	-	++
Low maintenance and consumable cost	+	-	+	+++
Free access to cell line for non-profit use	+++	-	+++	+++
Sensitivity	++	+++	++	-
Robustness	++	++	++	-
Reproducibility	++	+++	+	++
Maturity (widespread use)	+++	++	+	+++
High-throughput screening	+++	+++	+++	+++
Quick results	-	++	++	++

"-" below average; "+" fair; "++", good; "+++" excellent

1.6.4.2.2 Yeast based assays

The YES assay was developed in the Genetics department of GlaxoSmithKline and later on used by Routledge and Sumpter (1996). The DNA sequence of the human estrogen receptor (hER) was integrated into the yeast genome, which also contained expression plasmids carrying estrogen-responsive sequences (ERE) controlling the expression of the reporter gene lac-Z (encoding the enzyme β-galactosidase). Thus, in the presence of estrogens, β-galactosidase is synthesized and secreted into the medium, where it causes a colour change from yellow to red. In comparison to cell-based assays (see also Table 13), yeast based assays are easier to use, but lack complex estrogenic interactions (Routledge and Sumpter, 1996), are less sensitive than a cell-based assay (Leusch et al., 2010). YES assay is used in many published studies, where the presence of an estrogenic effect in environmental samples was determined. The YES assay was used to determine the presence of estrogenicity in wastewater (Aerni et al., 2004; Desbrow et al., 1998; Murk et al., 2002; Nelson et al., 2007; Rehmann et al., 1999; Salste et al., 2007), surface water (Garcia-Reyero et al., 2001; Matthiessen et al., 2006; Murk et al., 2002; Pawlowski et al., 2004) estuarine surface and sediment pore waters (Thomas et al., 2001) and coastal surface waters (Beck et al., 2006).

Lyticase Yeast Estrogen Screen - LYES (Schultis and Metzger, 2004) and BioLuminescent Yeast Estrogen Screen - BLYES (Sanseverino et al., 2005) are the variances of YES assay. BLYES has bioluminescent strain of *Saccharomyces cerevisiae*. With such assay, rapid, high throughput screening of estrogenic compounds can be

achieved. This assay has the ability to be used for remote, near-real-time monitoring of EDC in environment. BLYES has the same or better sensitivity to the test chemicals as the YES assay (Sanseverino et al., 2005). LYES is improved YES assay assisted by enzymatic digestion with lyticase. LYES has a good sensitivity, it is cheap and much faster than other assays, but has not, yet, been applied to the environmental samples (Schultis and Metzger, 2004).

1.6.4.3 Non-cellular assays

Non cellular assays are assays that do not use organisms or single cells, but their response is caused by biological – in most cases – antigen/antibody interaction. Tests like enzyme linked immunosorbent assay (ELISA) and enzyme linked receptor assay (ELRA) do not directly apply any living organism, but they follow biological principles. Moreover, assays that do not require whole cells can avoid some of the difficulties related to membrane permeability, cell function, organism life stages, and toxicity responses to a given sample. ELISA assays are available as commercial kits for many of environmental relevant compounds including steroid estrogens. ELISA as well as ELRA have been successfully applied to environmental samples. They also form the basis for biosensors that were developed for detecting EDCs (Campbell et al., 2006).

ELISA test is based on recognition of steroid estrogens (E1, E2, E3) by specific monoclonal antibodies. ELISA assays for steroid estrogens have been used in investigations of aquatic environment, surface and wastewaters (Farré et al., 2006; Farré et al., 2007; Hintemann et al., 2006; Li et al., 2004; Mauricio et al., 2006). ELISA provides a relatively simple and practical method of assessing the fate of estrogenic hormones in the environment (Huang and Sedlak, 2001). Farré et al. (2006) compare ELISA with chemical analysis. They conclude that ELISA as a screening technique has several advantages since it is possible to measure 40 samples simultaneously together with the calibration curve and the blanks; required time is only 2.5 h. Compared to LC-MS-MS, for the same number of samples approximately 90 h is required. LC-MS-MS analysis is also 50 times more expensive. Monoclonal antibodies show good sensitivity and high reproducibility. Anyway, they suggest that immunological methods of analysis can only be accepted, if they have been adequately validated and there is an independent confirmatory method like LC-MS-MS available.

ELRA represent a bioeffect-related approach to the analysis of xenoendocrine disrupters. The receptor assay employs the same principles as competitive immunoassays based on ligand protein interactions. However, receptor binding implicates a biological effect, either agonistic or antagonistic (Seifert, 2004; Seifert et al., 1999). ELRA was applied for testing sediment associated samples. It was concluded that ELRA is a fast and reproducible method that can be used for high-throughput screening in a microplate format at low cost. It is robust to microbial contamination, and is less susceptible to cytotoxic interferences than cell culture methods (Kase et al., 2008).

1.6.4.4 Biosensors

A biosensor is a self-contained (all parts being packaged together), usually small, integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information. The biological recognition element is retained in direct spatial contact with a transduction element, which converts the biological recognition event into a useable output signal. Biosensors should be distinguished from a bioassay or a bioanalytical system, which require additional processing steps, such as reagent addition

and where the assay design is permanently fixed in the construction of the device. Biosensors are able to provide information about biological effects (toxicity, endocrine disruption) of a sample or a certain compound, and can also distinguish the bioavailable fraction of pollutants from the total sample content, what is of high environmental interest. Biosensors usually allow the detection of single analyte and not of several compounds simultaneously. Biosensors demand minimum sample preparation, are simple and user friendly, portable, use minimum organic solvents are cost effective and give fast results. Limitations of biosensors are low biological material stability and single analyte determination. They are also available only as prototypes (Rodriguez-Mozaz et al., 2004b).

An example of a developed biosensor for E1 is RIver ANAlyser (RIANA) system that has a potential to be made field portable for environmental monitoring (Rodriguez-Mozaz et al., 2004b; Tschmelak et al., 2005). This is fully automated immunoassay for E1 with a LOD below 0.20 ng/L and a LOQ below 1.40 ng/L. In contrast to common analytical methods, such as GC–MS or LC–MS, the biosensor requires no sample pre-treatment and pre-concentration. The other example is the ENDOTECT^{TM} biosensor. However, it is designed for use in medicine, not environmental monitoring (Wittliff et al., 2008).

1.6.5 Combination of chemical and biological methods

Biological and chemical methods have different advantages and disadvantages, listed in Figure 4. In general, analytical techniques provide excellent sensitivity and precision for determining steroid estrogens in environmental samples. However, a measurement of concentrations is necessary in determining fate and transport of these compounds. Analytical techniques do not provide data on estrogenic effects or synergistic and agonistic and antagonistic influences from multiple estrogenic compounds. They also measure specific steroid estrogens individually, so the target compound must have been already identified as to have estrogenic properties (Campbell et al., 2006; Gomes et al., 2003; Voulvoulis and Scrimshaw, 2003). Biological tools are needed to measure effects, which are integrated responses to all estrogenic chemicals present, including those, that might be missed by chemical analyses (Aerni et al., 2004).

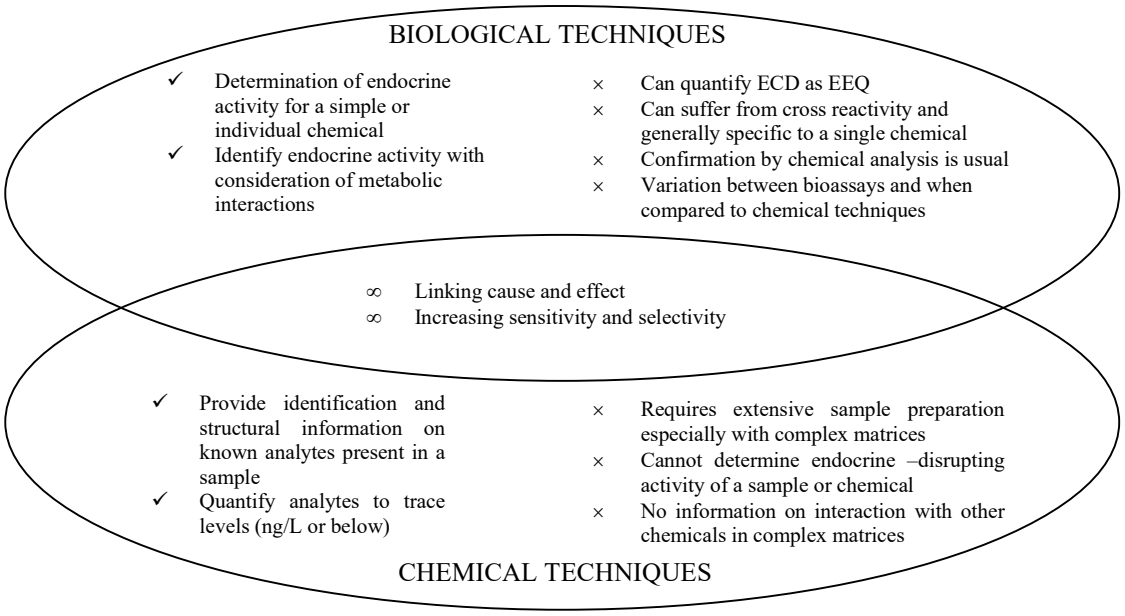


Figure 4: Advantages and disadvantages of biological and chemical determination of EDCs (adapted from Gomes et al. (2003)).

Environmental fate of steroid estrogens (and other steroid hormones) in WWTP is very complex and there is not a universally accepted bioassay or chemical technique for quantification in the aquatic environment. Chemical analyses are sensitive and specific, but limited in that only target substances are analysed. *In vitro* bioassays have comparable sensitivity (Table 14) and can be very useful and cost effective in determining the total estrogenic activity in the mixture. It is clear from Figure 4, that chemical analysis and biological assays provide different type of information about the sample. Therefore, the use of a suite of bioassays and chemical analysis appears to be the best strategy in analysing WWTP samples for estrogens (Nelson et al., 2007).

Table 14: Limits of detection of different methods for determining steroid estrogens (E2) (adapted from Campbell et al. (2006)).

Method	**Detection limit for E2 (ng/L)**
E-Screen	0.27
ER-Calux®	0.14
YES	0.3-30
ELISA	20-40
LC-MS	1.2-2.5
LC-MS-MS	0.2-1.0
GC-MS	0.7-4.0
GC-MS-MS	0.1-3.0

Combination of chemical and biological analysis can be used in different ways. The most frequently used are surveys based on target analysis of pre-selected compounds. In this approach, individual compounds are pre-selected, so that optimized and validated methods for chemical analysis can be used. The results of chemical analysis are checked for correlation with findings of the biological analysis. Due to a high number of different bioassays and chemical analyses, many different combinations can be found in the literature. Direct comparisons of different studies are difficult. However, it can be seen, that in some surveys similar results of chemical and biological approach were observed (Farré et al., 2006; Huang and Sedlak, 2001; Kurauchi et al., 2005; Miège et al., 2009b), while in other studies, bioassay derived results were generally higher (Bicchi et al., 2009; Murk et al., 2002; Nelson et al., 2007; Pawlowski et al., 2004; Tan et al., 2007; Wang et al., 2011), lower (Aerni et al., 2004; Brix et al., 2010; Lee et al., 2008; Liscio et al., 2009; Rutishauser et al., 2004) or both, higher and lower, depending on the tested sample (Balsiger et al., 2010; Beck et al., 2006; Cargouët et al., 2004). There are many reasons for different results of chemical and biological analysis. They are usually explained by the presence of estrogenic agonists, antagonists and the presence of unknown compounds, which albeit relevant (Körner et al., 1999), remain unverified.

With an aim to identify chemical stressors without targeting specific compounds, schemes such as toxicity-identification evaluation (TIE) and effect directed analysis (EDA) are used. In the TIE process (Mount and Anderson-Carnahan, 1988), toxicity (estrogenicity) is characterised and afterwards chemical stressors identified and confirmed. To characterise toxicity (estrogenicity), sample is physically or chemically manipulated in order to isolate or to change the toxicants (estrogens) that might be present, and these compounds are determined by applying *in vitro* or *in vivo* bioassays. After collecting information on the kind of toxicants (estrogens), they are identified by target-analysis or chemical identification methods. The confirmation step checks whether

the detected compounds can explain the effects observed in bioassays (Streck, 2009). The examples of TIE like approach that studied steroid estrogens in environmental matrices are Desbrow et al. (1998), Thomas et al., (2001) and Fernandez et al. (2007).

In the EDA process that was developed in late 1980s (Schuetzle and Lewtas, 1986), repeated fractionation steps systematically reduce the complexity of a sample by isolating groups of toxicants (estrogens) into individual fractions. With bioassays, active fractions are identified and non-active fractions excluded from further processing. Active fractions are afterwards analysed with chemical-identification techniques that reveal compounds responsible for the adverse effects quantified by the biological analysis. Finally, confirmation steps, as in TIE, validate the findings (Brack, 2003; Streck, 2009). EDA has proved to be a powerful tool for identifying unknown toxicants (estrogenic compounds) and linking effects observed in bioassays to individual compounds (Furuichi et al., 2004; Grung et al., 2007; Houtman et al., 2006; Peck et al., 2004; Salste et al., 2007; Streck, 2009; Thomas et al., 2004; Viganò et al., 2008).

2 Aims and Hypothesis

Because of low concentrations, determination of steroid estrogens in complex environmental samples requires selective and sensitive methods. For this, chemical and biological methods can be applied. However, the chemical analysis only provides discrete information about sample composition and concentrations of individual compounds and does not account for potential mixture interactions. The presence of non-target compounds is also not considered despite their potential effect on the overall estrogenic activity. The alternative is to measure the estrogenicity of the samples for which *in vivo* and *in vitro* assays are employed. For an adequate analysis and risk assessment, a combination of both chemical and biological methods is recommended. Moreover, this type of environmental monitoring program requires the analysis of a large number of samples involving lengthy and costly sample preparation.

This thesis is based on the hypothesis that it is feasible to integrate chemical analysis and a biological assay into a single protocol for simultaneous determination of the concentrations of steroid estrogens and the total estrogenic potential of environmental samples. Hypothesized was also that the sample extraction step in ER-Calux® assay can be omitted.

The aims of doctoral thesis were to:

- develop an analytical method for determining of four most potent steroid estrogens in environmental samples
- integrate the analytical method and the biological assay (ER-Calux®) into a single protocol for determining steroid estrogen concentrations and total estrogenic potential of a sample
- modify ER-Calux® assay to be able to determine total estrogenic potential in environmental samples without extensive sample preparation and compare it to the existing analytical method and conventional ER-Calux® assay
- apply developed methods on real samples

3 Materials and Methods

3.1 Chemical analysis with GC-MSD

3.1.1 Standards

Authentic standards estrone (E1; min 99 %), 17β-estradiol (E2; min 98 %), 17α-ethinylestradiol (EE2; min 98 % (HPLC)), estriol (E3; min 99 %) and a deuterated internal standard (bisphenol A)-d_{16} (98 atom% D) were purchased from Sigma (Steinheim, Germany). To prepare a stock solution, an appropriate amount of the standards, in powder form, was accurately weighed out using an analytical balance (Mettler Toledo AT201; Greifensee, Switzerland) and diluted in methanol (MeOH). The stock solution was then further diluted to give a series of working standards. These solutions were used for the preparation of the spiked water samples and directly for the optimization of the chemical analysis. When spiking the samples, the concentration of MeOH did not exceed 0.5 vol% in the water sample. Standards were prepared at least once per month.

3.1.1.1 Internal standard

The internal standard (bisphenol A)-d_{16} was used in all samples. To each sample 10 ng of internal standard (50 ng/L; 200 mL) was added and, unless specified otherwise, the internal standard was added to the sample prior to extraction.

3.1.2 Chemicals

The organic solvents MeOH, ethyl acetate (EtAc) and acetone (AcON) were of "Baker ultra resi-analysed®" grade, and acetonitrile (AcN) of "Baker HPLC analysed" and were purchased from J.T. Baker (Deventer, the Netherlands). Pyridine (max 0.01 % H_2O) was purchased from Merck (Darmstadt, Germany). The derivatizing agent N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA, derivatization grade) was purchased from Sigma (Steinheim, Germany).

3.1.3 Sample pretreatment

All glassware was cleaned using dichloromethane (DCM) and MeOH and then heated at 400 °C to remove any organic residues. Prior to extraction, the samples were defrosted, if necessary. Afterwards particular matter that might cause clogging of the SPE cartridges has to be removed. With that purpose, samples were filtered with glass fibre pre-filter and nylon filter. Pre-filter (GF/C, Whatman, Maidstone, England) that removes particles larger than 1.2 µm. was used to prevent clogging of a nylon filter with a defined pore size of 0.45 µm (Supelco; Bellefonte, ZDA). The samples were then extracted as described in the following section (3.1.4).

3.1.4 Solid phase extraction

Each sample was extracted (200 mL) using a SPE cartridge containing Oasis HLB reversed-phase sorbent; 3 cc/60 mg (Waters; Milford, ZDA). The sorbent was preconditioned with EtAc (3 mL), MeOH (3 mL) and MilliQ water acidified with HCl to pH ~ 2.5 (3 mL). The sample flow rate was maintained between 3-5 mL/min. After extraction, the cartridges were air dried. If the analytes could not be eluted on the same day, cartridges were stored at -20 °C. Elution was performed with EtAc (3 mL). The eluents were evaporated under a stream of nitrogen to *cca* 0.5 mL.

3.1.5 Clean-up step

A clean-up step using silica gel was used to remove any potential interferences present in the wastewater samples. ISOLUTE cartridges filled with 500 mg of silica gel (Biotage, Uppsala, Sweden) were conditioned with 4 mL of EtAc. Afterwards, the sample extracts were added to the cartridge. The analytes were then eluted from the silica gel using 10 mL of EtAc/AcON (2 % v/v). The purified samples were reduced to dryness (N_2). If the samples were not derivatised on the same day, evaporation was stopped at 0.5 mL and the samples were stored at 4 °C for a maximum of 24 h.

3.1.6 Derivatization

After sample clean-up, the solvent was completely removed prior to derivatization. The dried extracts were reconstituted with 100 µL MSTFA and pyridine (1:1, v/v). The samples were heated at 60 °C for 30 min before being cooled in a refrigerator and finally transferred to vials with 100 µL inserts. To avoid decomposition, analyses were performed on the same day as derivatization.

3.1.7 GC-MSD analysis

Chemical analysis was performed using GC-MSD (HP 6890 Series, Hewlet-Packard, Waldbron, Germany). Samples were injected (1 µL) in the splitless mode. The injector and the interface temperature were maintained at 270 °C and 280 °C, respectively. Analytes were separated on a 30 m × 0.25 mm × 0.25 µm, HP5-MS capillary column with Helium as the carrier gas (1 mL/min). The GC temperature program was initially set at 100 °C and then ramped to 230 °C at 20 °C/min (held for 2 min) and to 280 °C at 15 °C/min (held for 5 min). EI mode (70 eV) was used. SIM mode with the following characteristic ions was used: E1 (*m/z*: **342**, 257, 218), E2 (*m/z*: **416**, 285), EE2 (*m/z*: 425, **285**), E3 (*m/z*: 505, **504**, 311), bisphenol A-d_{16} (*m/z*: **368**, 386) (quantification ions are in bold). SCAN mode was performed at *m/z* ratios from 50 to 500.

3.1.8 Method validation

The analytical method (Figure 5) was evaluated in terms of linearity, repeatability, recovery, LOD and LOQ. Tap water and wastewater effluent were used as a matrix for calibration samples. For wastewater effluent, calibration samples were blank corrected, since target compounds were present in the blanks.

The linearity of the method was determined from a series of calibration curves. For E1 and E2, six point calibration curves (2, 10, 50, 100, 300, 600 ng/L) were prepared, while the same was made for EE2 and E3 with five points between 10-600 ng/L. Intra- and inter-day repeatability and recoveries were checked at two concentration levels: 5 ng/L

for E1 and E2 (10 ng/L for EE2 and E3) and 50 ng/L for all four compounds. Five parallel measurements were made at each concentration level. LOD and LOQ were calculated as 3 and 10-times the standard deviation of the base line of five blank samples, respectively.

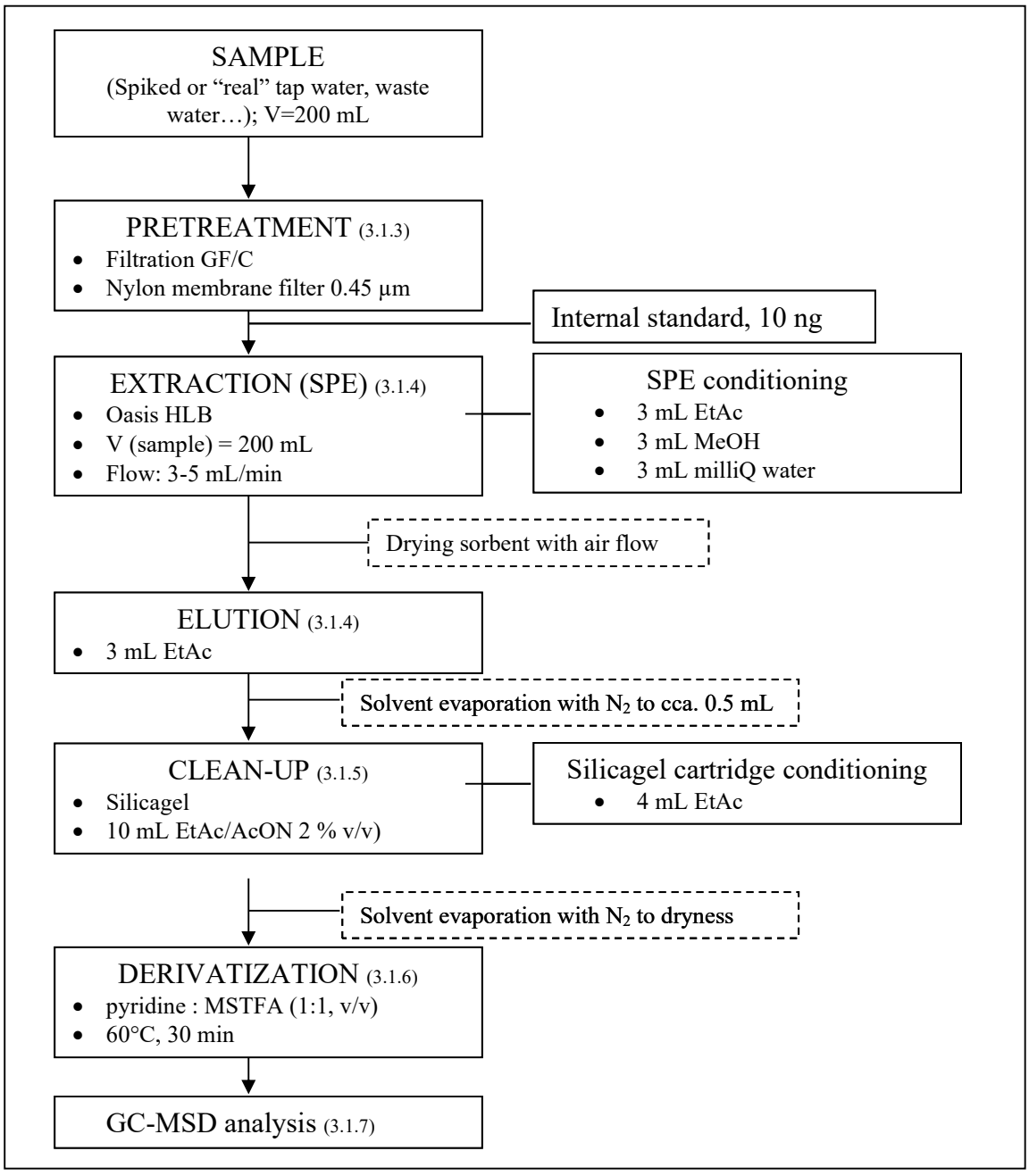


Figure 5: Scheme of sample preparation for GC-MSD analysis.

3.1.9 Application of chemical analysis - monitoring of a hospital and wastewater treatment plant

3.1.9.1 Sampling and sample preparation

Samples were collected from a hospital effluent and receiving WWTP (WWTP6, Table 21), where both the influent and effluent were sampled. Effluent from the hospital (9628 patients hospitalized in 2007) derives from the main building, where the diagnostic

laboratory, departments of internal ward, and surgery are located. The hospital's wastewater (80 000 m^3/year in 2007) enters the sewerage network, that terminates at the WWTP. The WWTP incorporates mechanical and biological treatment processes with anaerobic stabilization of the waste sludge. In 2007 approximately 5 400 000 m^3 of wastewater passed through this plant.

To assess the inter-day variation, hospital effluent, WWTP influent and effluent samples were collected daily over a 6 day period (Monday to Saturday; January, 2007). Additional samples were taken on a Monday during two separate weeks in December 2006. The samples were collected in polyethylene (PE-HD) bottles (1 L), immediately acidified with HCl to pH 2.5 to prevent biodegradation and frozen prior to analysis. The hospital samples were time proportional (24 h), while the WWTP samples were flow proportional. Transportation times from the hospital to the WWTP (24 h) and hydraulic retention times at the WWTP (24 h) were taken into account during sampling and data analysis (Table 15). All the samples were prepared, extracted, cleaned-up and analysed with the validated method as described in the previous sections (Figure 5).

Table 15: Sampling dates of hospital effluent, WWTP influent and effluent.

	Date of sample		
Sample name	**hospital**	**WWTP influent**	**WWTP effluent**
Mon	08.01.2007	09.01.2007	10.01.2007
Tue	09.01.2007	10.01.2007	11.01.2007
Wed	10.01.2007	11.01.2007	12.01.2007
Thr	11.01.2007	12.01.2007	13.01.2007
Fri	12.01.2007	13.01.2007	14.01.2007
Sat	13.01.2007	14.01.2007	15.01.2007
Mon2	11.12.2006	12.12.2006	13.12.2006
Mon3	18.12.2006	19.12.2006	20.12.2006

3.1.9.2 Calculated estradiol equivalents

To predict the estrogenic potential of the four analysed compounds and to compare the results of chemical analysis with biological assays, estradiol equivalents were calculated using the following equation:

$$cEEQ = \Sigma(EEF_i * c(i)), \quad (1)$$

where cEEQ represents the total calculated estradiol equivalents, EEF_i is the estradiol equivalency factor of a specific compound and c(i) its concentration.

The EEFs are usually determined for each compound using bioassays. Initially, the EEFs were taken from the literature (Campbell et al., 2006). To estimate the maximum estrogenic potential of determined steroid estrogens in samples, the highest EEFs for each compound (Table 16) were taken.

Table 16: Estradiol equivalency factors (EEF) published by Campbell et al. (2006).

Compound	EEF
E1	0.1
E2	1
E3	0.08
EE2	1.9

3.1.9.3 Analysis of the results

The presence of E1, E2, E3 and EE2 was determined in hospital effluent and WWTP influent and effluent. The results were analysed as follows:

a) Ratio of E1:E2:E3 was calculated using the following equation:

$$E1:E2:E3 = 1 : c(E2)/c(E1) : c(E3)/c(E1), \quad (2)$$

where c(E1), c(E2) and c(E3) represents concentration of E1, E2 and E3 in the sample.

b) cEEQs were calculated as described in Section 3.1.9.2. The contribution of E3 towards total estrogenicity was calculated as follows:

$$Contribution(E3) = cEEQ(E3)/cEEQ(tot), \quad (3)$$

where cEEQ(E3) and cEEQ(tot) represents E3-derived and total calculated estradiol equivalents, respectively.

c) Removal of steroid estrogens by the WWTP was determined using the equation:

$$Removal(i) = 1- (c(i)_{eff}/c(i)_{inf}), \quad (4)$$

where $c(i)_{eff}$ and $c(i)_{inf}$ represents the concentration of separate compounds in the effluent and influent, respectively.

3.2 ER-Calux® assay

3.2.1 Standards

In the ER-Calux® assay, the same standards as for chemical analysis were used (Section 3.1.1). Standards were prepared in EtAc in seven concentrations as used for the calibration curve (E2) and estimation of the EC_{50} values (Section 3.3.1). No internal standard was used.

3.2.2 Growth media and chemicals for the ER-Calux® cells

Media Gibco® D-MEM/F-12 with GlutaMAX™ (with phenol red), Gibco® D-MEM/F-12 with L-Glutamine (without phenol red) and Stripped FBS (Fetal Bovine Serum) were purchased from Invitrogen (Paisley, UK). EDTA, non-essential amino acids (MEM

100x), penicillin/streptomycin, dimethyl sulphoxide (DMSO, 99.9 %) and absolute ethanol (EtOH) were purchased from Sigma (Steinheim, Germany). Both fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from PAA (Pasching, Austria), while Difco trypsin was obtained from Becton Dickinsen (Heidelberg, Germany).

For maintenance of the ER-Calux® cells, "Growth medium" and "Test medium" must be prepared prior to use. "Growth" and "Test media" were prepared fresh weekly. The "Growth medium" D-MEM/F12 with GlutaMAX™ containing phenol red as pH indicator was supplemented with 7.5 % FBS, 1 % non-essential amino acids (MEM 100x) and a 0.2 % penicillin/streptomycin solution (5000 U/mL penicillin and 5000 U/mL streptomycin).

For test purposes, "Test medium" was prepared. The D-MEM/F12 medium with L-glutamine without phenol red was supplemented with 5 % stripped FBS, 1 % non-essential amino acids (MEM 100x) and 0.2 % penicillin/streptomycin solution (5000 U/mL penicillin and 5000 U/mL streptomycin).

3.2.3 Cell preparation and sample exposure

The T47D-EREtata-Luc cells used in the ER-Calux® assay were provided and licensed by BioDetection Systems b.v. (BDS), Amsterdam, Netherlands. The assay was performed according to the BDS protocol but with certain modifications (Figure 6).

The "Growth medium" was used for growth, maintenance and subculturing of the ER-Calux® cells. Cells were grown at 37 °C in 5 % CO_2. Subculturing was performed every 3-4 days, depending on cell density. For assay purposes the "Growth medium" was replaced with the "Test medium". The cells that were suspended in the "Test medium" were seeded at a density of 10 000 cells/well into 96-well white microtiter plates (Nunc, Roskilde, Denmark). After 24 h of incubation at 37 °C the medium was replaced with fresh "Test medium" and incubated for an additional 24 h. Subsequently, the cells were exposed to 100 µL of the "Test medium" supplemented with the test sample, its dilution or the calibration standard (final solvent concentration 0.1 vol%).

Test samples were diluted 1:1, 1:3, 1:10, 1:30 and 1:100 prior to the test. A solvent control (0.1 vol% of solvent) was also included to exclude possible solvent effects. For calibration purposes, the E2 standard solution in a concentration range from 0.6 to 30 pM was included in every microtiter plate. Calibration curve standards and test sample dilutions were tested in triplicate.

After 24 h of exposure, 100 µL of the luminescence kit "Steadylite plus™" (Perkin Elmer, Shelton, USA) was added to the medium and gently shaken for 15 min at room temperature. Luminescence was measured using a luminometer with a set integration time of 4000 ms and a gain of 200 (Tecan Genios, Maennedorf, Switzerland).

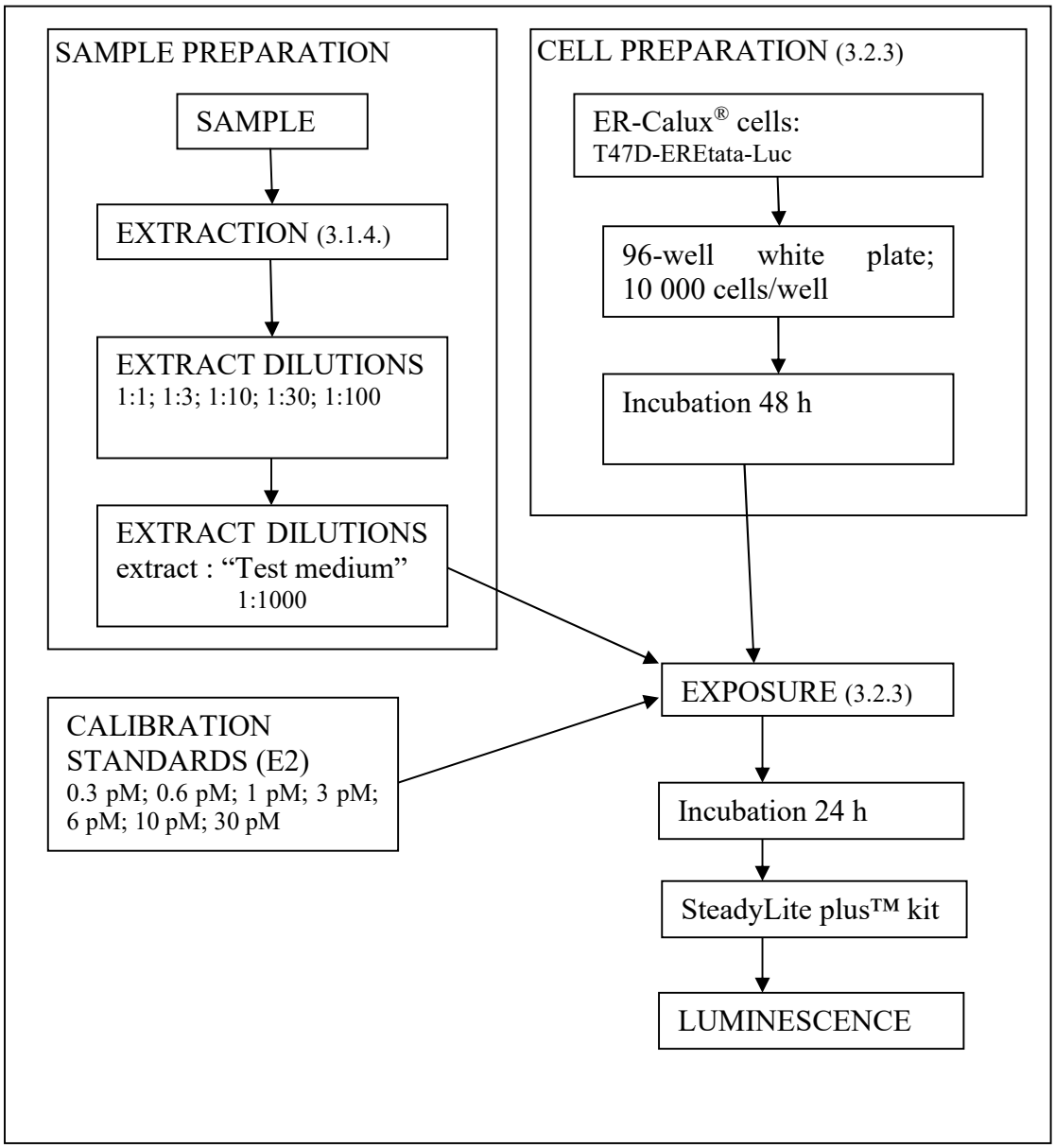


Figure 6: Scheme of ER-Calux® assay procedure.

3.2.4 Analysis of the results

Results expressed in relative luminescent units (RLU) were processed using a Sigmoidal calibration curve constructed using a Microsoft Excel template (provided by BDS) together with an add-in "Solver". The curve fitting equation is as follows:

$$y=A/(1+(x/B)^C), \quad (5)$$

where y represents the response in RLU (corrected for solvent); x represents the concentration in pM(E2)/well, A, B and C represents maximum response, the EC_{50} (median effective concentration) of the curve and the slope of the curve, respectively.

The E2 equivalents (EEQ) were calculated from Equation 5. For quality control purposes, only the results that reached the predefined parameters of a r^2 of calibration

curve ≥ 0.99, a concentration between the LOQ and EC_{50} (median effective concentration) and a relative standard deviation < 15 %, were used.

3.2.5 Validation of ER-Calux® assay

Limits of detection (LOD) and quantification (LOQ) ER-Calux® assay were provided by BDS – 0.5 pM/well and 1.5 pM/well for LOD and LOQ, respectively. The values bases on their long-term experiences with ER-Calux® assay. However, LODs and LOQ are calculated from the calibration curve performed on every microtiter plate and therefore determined in each experiment separately. Recovery of SPE-ER-Calux® assay and repeatability were determined with tap water spiked with E2 (4.1 ng/L; 15 pM) in five parallels.

3.3 Integration of ER-Calux® assay and chemical analysis with GC-MSD

Chemical analysis (Figure 5) and ER-Calux® (Figure 6) were integrated into a single protocol, where the same sample is analysed by both methods (Figure 7). Spiked or "real" samples were prepared and extracted as usual. After the clean-up step, extracts were dried and reconstituted in 1 mL EtAc. A total of 50 µL of this extract was used for ER-Calux® assay, while the remaining 950 µL was used for chemical analysis. An internal standard (10 ng) was added prior to derivatization (comparable to 50 ng/L in water samples before extraction, 200 mL). The solvent was completely removed using a gentle steam of N_2. The sample was then derivatised (Section 3.1.6) and analysed using GC-MSD (Section 3.1.7). The results of the chemical analysis were corrected according to the amount of sample remaining for chemical analysis after it was used for the ER-Calux® assay (95 %). For the integration purposes, a solvent, compatible with both methods, was chosen (Section 3.3.2). The performance of the integrated protocol was then checked using spiked and "real" samples (Section 3.3.3).

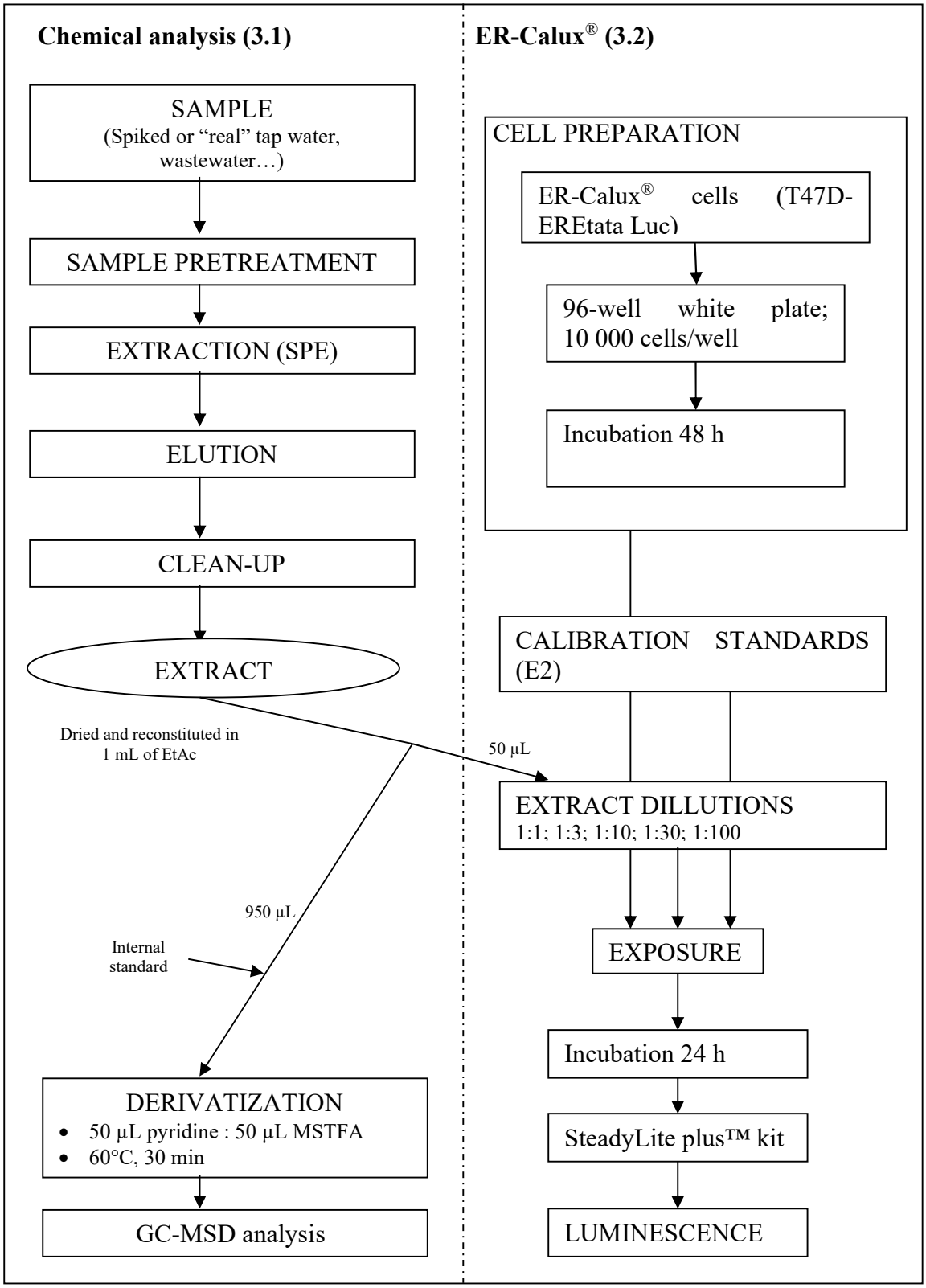


Figure 7: Scheme of the integrated protocol.

3.3.1 Calculated estradiol equivalents

To compare the results of chemical analysis with ER-Calux® assay, estradiol equivalents were calculated as described in Section 3.1.9.2. The difference here was that the EEFs were not taken from the literature, but were determined using the ER-Calux® assay.

To calculate EEFs, EC_{50} values have to be determined. The EC_{50} is calculated from the graded dose response curve and represents the concentration of a compound where 50 % of its maximal effect is observed. The dose response curve was obtained using the ER-Calux® assay for each tested compound. Used concentrations of E2, E1, E3 and EE2 standards are evident from Table 17. For each compound, an EEF was calculated using the equation:

$$EEF_i = EC_{50\,E2} / EC_{50\,i} \quad (6)$$

where EEF_i is the estradiol equivalency factor of a tested compound; EC_{50i} and $EC_{50\,E2}$ are the median effective concentrations of the tested compound and E2, respectively.

Table 17: Concentrations used for the determination of EC_{50} values of E1, E2, E3 and EE2.

	Concentration in well (pM)			
Concentration #	**E2**	**E1**	**E3**	**EE2**
0	0	0	0	0
1	0.3	0.4	1	0.1
2	0.6	1	2	0.3
3	1	2	4	0.6
4	3	4	10	1
5	6	10	20	3
6	10	20	40	6
7	30	40	100	10

3.3.2 Solvent compatibility

3.3.2.1 Test of different solvents

DMSO, EtAc, AcON, MeOH and EtOH in concentrations of 0.1 %; 0.2 % and 0.5 % were studied for their effect on the ER-Calux® cells. Solvents in the "Test medium" (100 µL) were exposed to the cells and examined using the MTS assay (Section 3.3.2.1.1).

Survival (viability) was determined by comparing the optical density (OD) values of the solvent exposed cells with those of unexposed cells and presented as % of cell viability. The cytotoxicity was measured in five replicates per treatment point. A Student's t-test was used to compare cell survival between unexposed cells and solvent exposed cells; $p < 0.05$ was considered as statistically significant.

3.3.2.1.1 MTS assay

For the MTS assay (Cell Titer 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega, Madison, USA), a mixture of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) in a ratio of 20:1 was prepared.

Cells were cultivated in microtiter clear plates (Nunc, Roskilde, Denmark) in the same way as for the ER-Calux® assay (Section 3.2.3) and exposed to the sample. After a 24 h exposure time, 20 µL of freshly prepared MTS:PMS reagent was added instead of the luminescence kit. The MTS:PMS reagent was added directly to 100 µL of medium in the culture wells. After a further 3 h of incubation (37 °C; 5 % CO_2 atmosphere), absorbance (λ=490 nm) was measured using a Tecan Genios spectrofluorimeter (Maennedorf, Switzerland).

3.3.2.2 Dose response

To determine the different effects of EtAc and DMSO on the performance of the ER-Calux®, standard solutions of E2 (0.6 to 30 pM) were prepared in EtAc and DMSO and tested using the ER-Calux® assay. Calibration curves were constructed and compared.

3.3.2.3 "Real" samples

Influents and effluents from WWTP1, WWTP2 and WWTP3 (see Table 21) were sampled on the same day and extracted as soon as possible. Extraction was performed in parallel as described in Figure 5. After extraction, one extract from each WWTP was reconstituted in 1 mL EtAc, and the other in DMSO. All samples were afterwards tested with the ER-Calux® assay, together with the E2 standard solution prepared in the same solvent as the samples. The results were compared and checked for the different influences affecting the solvent that was used.

3.3.3 Performance of integrated protocol

3.3.3.1 Standards and spiked wastewater extracts

Standard mixtures of E1, E2, E3 and EE2 were prepared. Standard mixtures were added to glass vials as "Standards without wastewater extract" (Table 18). For each mixture, the MeOH was evaporated and the residue reconstituted with 1 mL of EtAc. From each sample a small aliquot of reconstituted sample (50 µl) was used for the ER-Calux® assay and the remainder (950 µl) for chemical analysis (Figure 7).

Mixtures of standards were also added to wastewater extracts as follows: wastewater extracts from WWTP3 (see Table 18) including both influent and effluent were prepared. Standards were transferred to glass vials, already containing dried wastewater influent and effluent extracts. Samples with influent and effluent extracts were processed in the same way as the "standards without wastewater extracts". Quantities of steroid estrogens (Table 18) were chosen randomly with the aim to equally distribute the predicted E2 equivalents in the sample mixtures over the desired concentration range. The concentrations were in the range of actual environmental concentrations.

Table 18: Concentrations of steroid estrogens used to prepare standards mixtures and spiked wastewater extracts.

	sample name	ng/L			
		E1	E2	EE2	E3
Standards without wastewater extract	St1	0	30.0	0	50.0
	St2	0	20.0	0	50.0
	St3	10.0	20.0	0	50.0
	St4	20.0	30.0	0	50.0
	St5	20.0	20.0	0	50.0
	St6	30.0	30.0	0	50.0
	St7	30.0	20.0	0	50.0
	St8	15.0	10.0	0	50.0
	St9	15.0	10.0	0	50.0
	St10	15.0	10.0	0	50.0
Standards with influent extracts	Inf1	10.0	10.0	10.0	10.0
	Inf2	24.0	24.0	24.0	24.0
	Inf3	15.0	10.0	0	50.0
	Inf4	15.0	10.0	0	50.0
	Inf5	15.0	10.0	0	50.0
Standards with effluent extracts	Eff0	0	0	0	0
	Eff1	3.0	3.0	3.0	3.0
	Eff2	10.0	10.0	10.0	10.0
	Eff3	17.0	17.0	17.0	17.0
	Eff4	24.0	24.0	24.0	24.0
	Eff5	30.0	30.0	30.0	30.0
	Eff6	40.0	0	0	40.0
	Eff7	27.0	7.0	0	54.0
	Eff8	20.0	14.0	7.0	50.0

The concentrations in Table 18 are presented as if the compounds were extracted in the same way as the "real" water samples (from 200 mL of water sample). This allows the values to be compared with "real" sample data, e.g. – concentration 1 μg/L of pure standard in EtAc (1 mL) is equivalent to 5 ng/L in water sample (200 mL).

3.3.3.2 "Real" samples

Grab samples (1 L) of wastewater influents and effluents from three municipal WWTPs with conventional wastewater treatment were collected: WWTP1, WWTP2 and WWTP3 (see Table 21). Two influent and effluent samples were taken at each WWTP, in WWTP3, effluent was sampled three times. Samples were extracted within 4 h after sampling and analysed by both the ER-Calux® assay and chemical analysis using the integrated protocol (Figure 7).

3.4 NE-(ER-Calux®)

The difference between a NE-(ER-Calux®) assay and an ER-Calux® assay is sample preparation and exposure (Figure 8), while the preparation of the cells and test incubation parameters and detection of luminescence remain the same (Section 3.2.3).

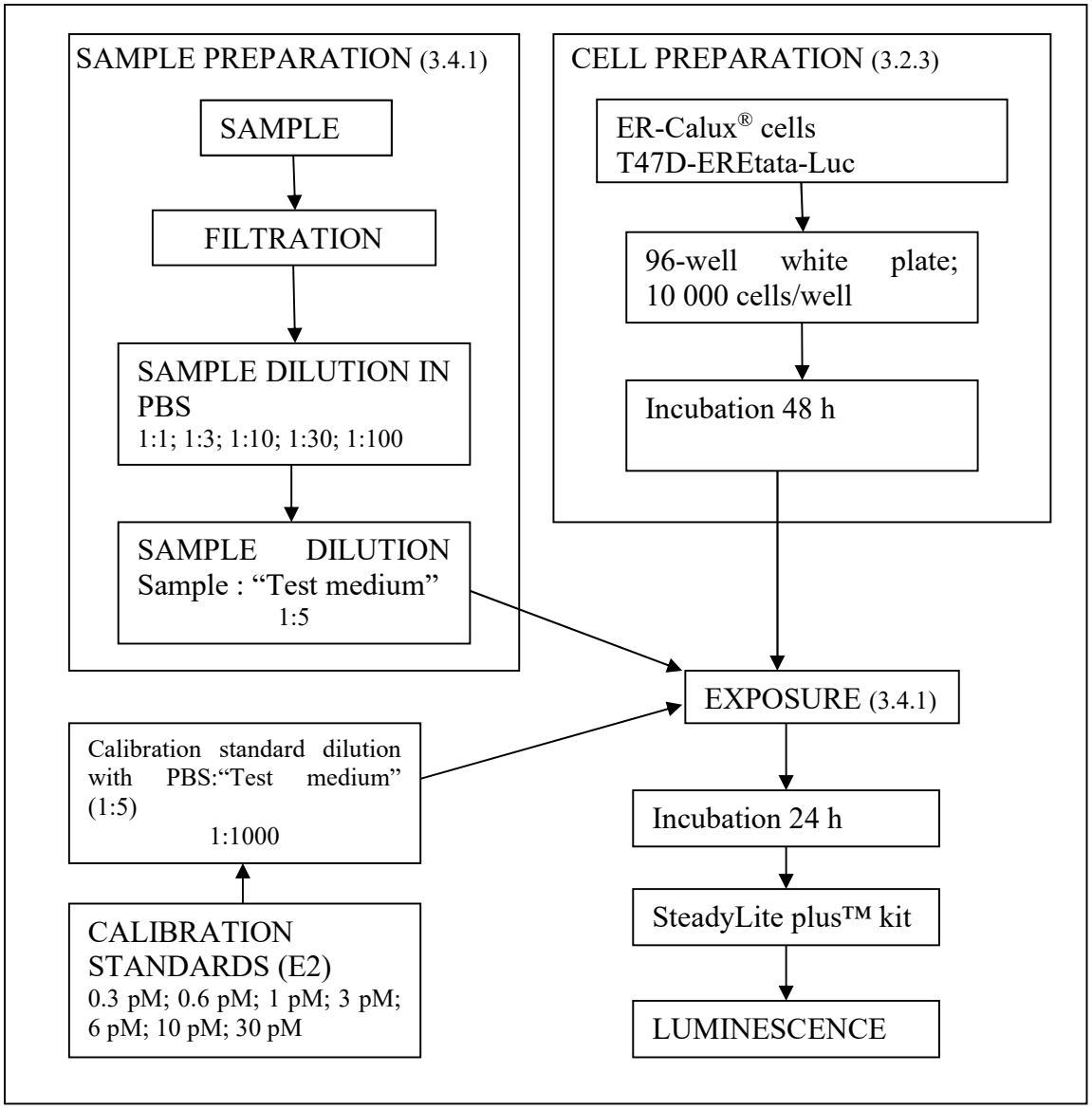


Figure 8: Scheme of NE-(ER-Calux®) procedure.

3.4.1 Sample preparation

The frozen raw samples (10 mL) were thawed and immediately sterilised by filtration (Whatman, ANOTOP25, pore size 0.2 μm). Samples were diluted 1:1, 1:3, 1:10, 1:30 and 1:100 with PBS. Each dilution was mixed with "Test medium" (1:5) and exposed to cells prepared as for conventional ER-Calux® assay (Figure 6).

For calibration purposes, the PBS was mixed with "Test medium" (1:5) and standard dilutions of E2 in EtAc (0.3; 0.6; 1; 3; 6; 10; 30 pM/well) were added (the final concentration of solvent – 0.1 %) and exposed to the cells. After 24 h exposure, luminescence was measured as described in Section 3.2.3.

3.4.2 Optimization of NE-(ER-Calux)® assay

3.4.2.1 Medium dilution

Serial dilutions of 0 %, 5 %, 10 %, 20 %, 30 % and 40 % PBS in the "Test medium" were prepared and exposed to ER-Calux® cells. Cell viability was tested using the MTT assay (3.5). The response of the cells was compared with the viability of non-diluted medium. Each dilution was tested in five parallels.

3.4.2.2 Sterilization

In order to choose optimal sterilization of the samples different syringe filters were tested (Table 19). The experiment was performed using sterile PBS and wastewater effluent.

Table 19: Syringe filters used for testing sterilization of the sample.

Filter #	Commercial name	Pore size (µm)	Material (filter)	Material (housing)	Manufacturer
1	Syringe filter 25 mm GD/X	0.2	Nylon w/GMF	pp	Whatman
2	GD/X CA filter	0.2	Celulose acetate	pp	Whatman
3	25mm GD/X (sterile, non-pyrogenic)	0.2	PES	pp	Whatman
4	Anotop25	0.2	Alumina based anopore membrane	pp	Whatman
5	Corning syringe filter	0.2	PES	pp	Corning
6	minisart CA	0.45	SFCA	MBS	Sartorius
7	Mini uniprep	0.2	Nylon	pp	Whatman
8	Mini uniprep	0.45	GF	pp	Whatman

GMF – glass microfiber; PES - polietersulfone; SFCA –surfactant free cellulose acetate; GF - glass fiber; MBS - meta acrylate butadiene styrene

3.4.2.2.1 Filtration of sterile PBS

Forty mL of sterile PBS was spiked with E2 (20.2 pM in sample; 4.05 pM/well). A sterile polypropylene syringe was filled with spiked PBS. Different filters (Table 19) were used with the same syringe (same sample, without refilling of the syringe). Before using the first filter, 1 mL of sample was dispensed without filtration. For "Mini uniprep" filters, the sample was added to the filter from the syringe. After the last sample was filtered, 1 ml of the sample was taken from the syringe (without filtration) to check if the concentration of E2 remains the same during the process. Samples were then analysed with the NE-(ER-Calux®) assay. Recoveries from each filter were then determined.

3.4.2.2.2 Filtration of Wastewater

Forty mL of wastewater effluent (WWTP2; Table 21) was spiked with E2 (c=14.7 pM in sample; 2.9 pM/well). In parallel, 40 mL of sterile PBS was spiked with the same concentration. Sterile polypropylene syringe was filled with spiked wastewater. Different filters (Table 19) were used with the same syringe (same sample, without refilling of the syringe). For "Mini uniprep" filters, the sample was added to the filter from the syringe. The samples were analysed with NE-(ER-Calux®) assay. Recoveries of each filter were determined and compared with the unfiltered spiked PBS.

3.4.2.3 Validation of NE-(ER-Calux®) assay

LOD and LOQ of NE-(ER-Calux®) assay were calculated from calibration curve, performed at each microtiter plate and compared to conventional ER-Calux® assay. Recovery of NE-(ER-Calux®) assay and repeatability were determined with tap water spiked with E2 (4.1 ng/L; 15 pM) in five parallels.

3.4.3 Comparison of NE-(ER-Calux®), ER-Calux® and GC-MSD

NE-(ER-Calux®), ER-Calux® and GC-MSD were compared by testing spiked and "real" samples. Spiked tap and wastewater effluent samples were used as well as "real" WWTP influent, effluent and surface water samples. All samples were tested with all three methods as shown in Figure 9. Cell preparation was the same for NE-(ER-Calux®) and ER-Calux®, both assays were performed at the same time.

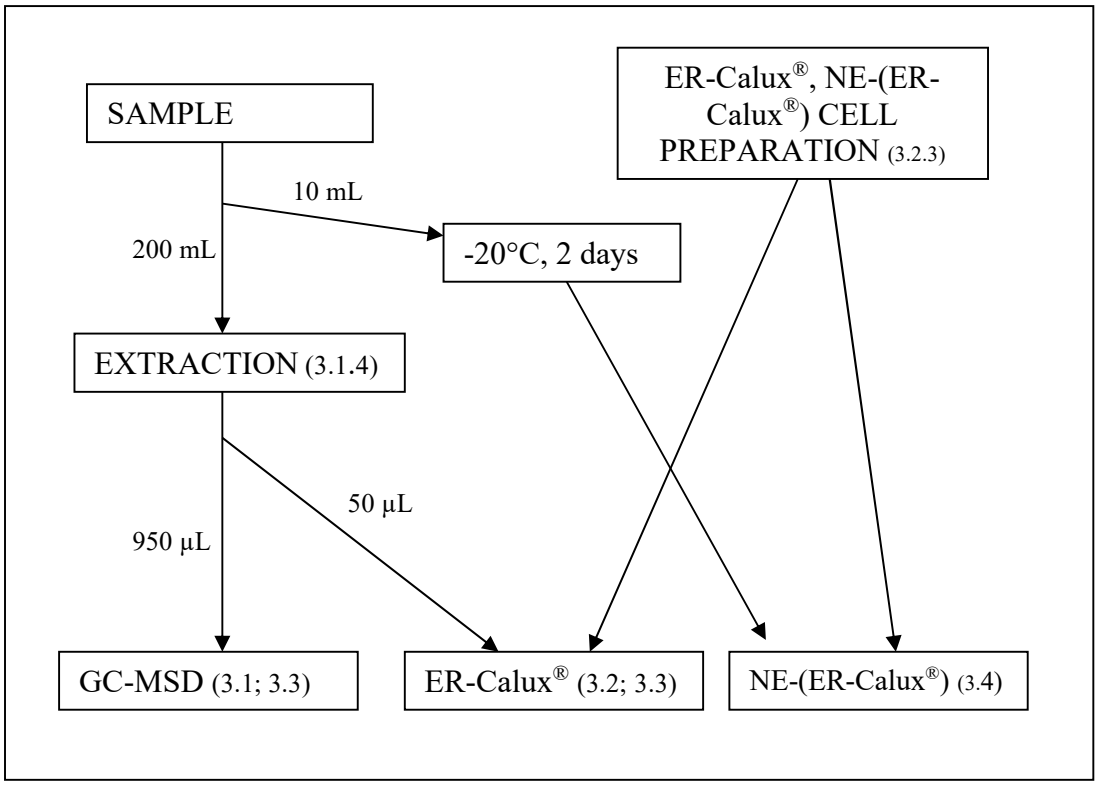


Figure 9: Scheme of testing the sample with all three methods.

3.4.3.1 Spiked samples

Tap water from the laboratory and grab wastewater effluent sample from WWTP2 (Table 20) were spiked with E1, E2, EE2 and E3 in environmentally relevant concentrations (Table 20). Spiking levels were chosen randomly within the desired EEQ range. After spiking, samples were homogenised by shaking at 300 rpm for 30 min. For the NE-(ER-Calux®) assay, 10 mL of each sample was stored at -20 °C, while 200 mL was immediately used for SPE (Section 3.1.4). Samples and their extracts were then analysed by ER-Calux®, GC-MSD, and NE-(ER-Calux®) as seen from Figure 9. The results of GC-MSD were used for calculation of cEEQ (Section 3.3.1) and compared to the other two methods. In parallel WWTP effluent sample was checked for cytotoxic potential with MTT assay (Section 3.5).

Table 20: Spiking levels of standards in tap and wastewater samples.

		GC-MSD analysis (ng/L)			
		E1	**E2**	**EE2**	**E3**
Spiked tap water	TW1*	0	0	0	0
	TW2	3	3	0	0
	TW3	5	5	5	5
	TW4	10	10	10	10
	TW5	10	15	10	10
	TW6	15	15	15	15
	TW7	20	20	20	20
	TW8	25	25	25	25
	TW9	30	30	25	25
	TW10	30	30	30	30
	TW11*	0	0	0	0
	TW12	2	2	0	10
	TW13	2	3	0	20
	TW14	5	3	0	30
	TW15	5	5	0	20
	TW16	10	3	0	30
	TW17	10	5	5	30
	TW18	15	7	10	30
	TW19	15	7	15	30
	TW20	10	7	15	30
Spiked wastewater	WW1*	0	0	0	0
	WW2	3	2	0	10
	WW3	3	5	0	30
	WW4	5	2	0	30
	WW5	5	5	0	30
	WW6	5	2	5	30
	WW7	10	2	5	30
	WW8	15	5	10	30
	WW9	15	5	15	30
	WW10	15	5	20	40

*blank sample

3.4.3.2 “Real” wastewater and surface water samples

Two hundred and fifty mL of grab WWTP influent and effluent samples and surface river water samples (upstream and downstream of the effluent site) were collected from seven different WWTPs. For each WWTP, basic design parameters (treatment technology, capacity, flow), properties of wastewater (chemical oxygen demand (COD) and biological oxygen demand (BOD)) as well as operational parameters (HRT, SRT) are described in Table 21, together with estimated flow of surface water that receives WWTP effluent.

Samples at each WWTP were collected during four consecutive weeks (two WWTP each week), on a Monday morning. To assure the same sample storage and preparation time for all samples, effluent and river samples were taken at the same time without considering HRT at each WWTP. Samples were immediately transported (T=0 °C) to the

laboratory. For NE-(ER-Calux®) 10 mL of each sample was stored at -20 °C, while 200 mL was used for extraction and for analysis by both the ER-Calux® assay and GC-MSD (Figure 9). To avoid sample degradation, extraction was performed within 4 h after sampling. In parallel with the ER-Calux® and NE-(ER-Calux®) analyses, the cytotoxic potential of the samples was determined using the MTT assay (Section 3.5).

Table 21: Wastewater treatment plants.

		capacity (PU)		Flow/year	mean influent COD (BOD)	mean effluent COD (BOD)	HRT	SRT	estimated surface water flow
name	**treatment**	**design**	**actual**	**m^3**	**mg/L**	**mg/L**	**h**	**days**	**m^3/s**
WWTP1	biofiltration with P and N removal,	50000	45000	6161222	400 (196)	39.7 (8.2)	2.5	NA*	2
WWTP2	activated sludge, nitrification; no P removal	200000	143623	7303085	576 (66)	294 (15)	18	15-32	7
WWTP3	activated sludge; nitrification; no P removal	360000	420000	29928900	590 (312)	43 (<10)	19	8	20
WWTP4	activated sludge; nitrification; no P removal	100000	85000	5500000	800 (300)	80 (15)	22	20	110
WWTP5	activated sludge, anoxic zones, P removal	250000	160000	10000000	740 (400)	26 (6)	29	12	20
WWTP6	activated sludge, no P removal	68000	110805	4795963	1013 (506)	128 (28)	22	4-10	0,5
WWTP7	activated sludge, nitrification, P removal	70000	85000	8486259	429 (214)	17 (4)	22.5	20-24	35

*sludge age as determined for suspended biomass is not relevant in water treatment with biofiltration

3.4.4 Application of NE-(ER-Calux®) on environmental samples

3.4.4.1 Sampling and sample preparation

Samples (10 mL) were collected at WWTP1 (Table 22). Hourly samples and time proportional samples were collected at the influent and effluent site. Grab influent samples were taken every hour from 7:00 to 19:00. Appropriate effluent samples were taken within a 2.5 hour shift (9:30 to 21:30) according to the HRT at WWTP1 (Table 21). Samples were immediately stored at -20 °C. Time proportional samples for the whole sampling interval (sampling on 15 min) were collected from the influent (7:00-19:00) and effluent (9:30-21:30) and stored at -20 °C. On the next day all samples were analysed at the same time with NE-(ER-Calux®). In parallel all the samples were checked for

cytotoxicity with MTT assay (3.5).

Table 22: Sampling regime for hourly and time proportional samples at WWTP1.

Sample	Influent	Effluent
1	7:00	9:30
2	8:00	10:30
3	9:00	11:30
4	10:00	11:30
5	11:00	12:30
6	12:00	13:30
7	13:00	14:30
8	14:00	15:30
9	15:00	16:30
10	16:00	17:30
11	17:00	18:30
12	18:00	19:30
13	19:00	20:30
Time proportional sample	7:00-19:00 (every 15 min)	9:30-20:30 (every 15 min)

3.4.4.2 Performance of WWTP during the experiment

Monitoring of several characteristic parameters in the effluent was also performed in order to check the stability of the water treatment system. Temperature, pH, COD (sensor name: UVAS), dissolved oxygen - O_2 (LDO), ammonium - NH_4 (AMTAX), phosphates - PO_4 (PHOSPHAX), nitrates - NO_3 (NITRAX) were measured with HACH LANGE controller system (Düsseldorf, Germany) routinely used in WWTP1.

3.5 MTT assay

An MTT assay was performed on the ER-Calux® cells according to Mosmann (1983) with minor modifications (Žegura et al., 2009). The MTT reagent (1-(4,5 dimethyltiazol-2-yl)-2,5 diphenyl tetrazolium bromide) was prepared in 5 mg/mL concentration. The cells were prepared as in Section 3.2.3. The samples were prepared and exposed to the cells in the same way as for ER-Calux® (Figure 7) or NE-ER-Calux® assay (Figure 8). Instead of the luminescence kit, the MTT reagent (final concentration 0.5 mg/mL) was added after 21 h of exposure time. After 3 h incubation, the medium was carefully removed and 100 µL of DMSO added to the wells. The difference in optical density at 570 and 690 nm was measured (Tecan Genios, Maennedorf, Switzerland). The results were compared to the control (no sample exposure).

Survival (viability) was determined by comparing the OD values of the sample exposed cells with those of unexposed cells and presented as % of cell viability. The cytotoxicity was measured in five replicates per treatment point. A Student's t-test was used to compare cell survival between unexposed cells and solvent exposed cells; $p < 0.05$ was considered as statistically significant.

4 Results

4.1 Chemical analysis with GC-MSD

4.1.1 Clean-up step

A clean-up step was used to remove interferences from wastewater extracts. Figure 10 shows the chromatograms that compares the use of the clean-up step. It can be seen that removal of interferences enables easier identification of E1 and EE2 chromatographic peaks.

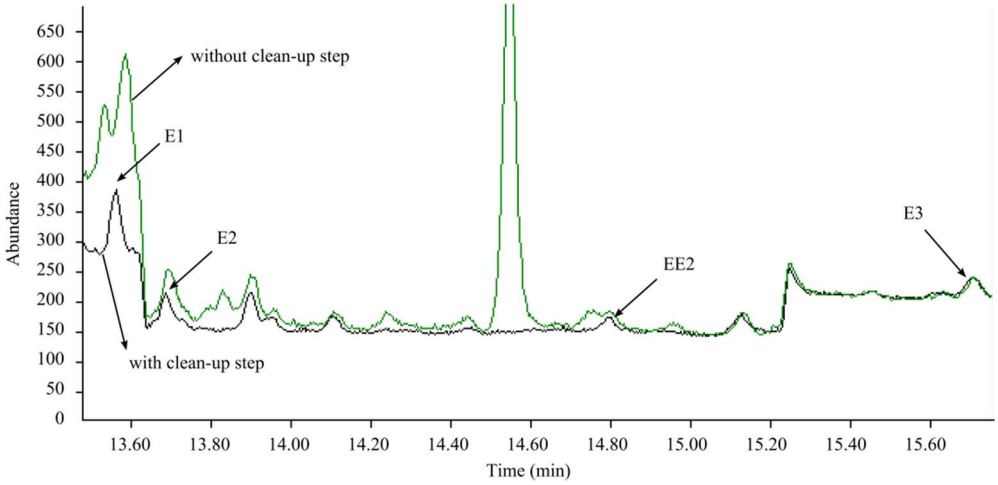


Figure 10: Comparison of spiked (10 ng/L) wastewater effluent sample chromatograms (SIM mode) with and without a clean-up step.

4.1.2 Method validation

Chemical analysis was validated using tap and wastewater as a matrix. The results are presented in Tables 23 and 24.

Table 23: Results of validation parameters for tap water.

		Tap water			
	Compound	**E1**	**E2**	**EE2**	**E3**
Low concentration (5 ng/L for E1 and E2; 10 ng/L for EE2 and E3)	Recovery (n=5) (%)	98	96	94	99
	Inter-day repeatability (n=5) (RSD %)	4	6	9	9
	Intra-day repeatability (n=5) (RSD %)	4	4	13	9
High concentration level, 50 ng/L	Recovery (n=5) (%)	101	94	97	98
	Inter-day repeatability (n=5) (RSD %)	2	3	11	7
	Intra-day repeatability (n=5) (RSD %)	4	4	13	8
	LOD (ng/L)	0.2	0.4	1.1	1.1
	LOQ (ng/L)	0.7	1.2	3.7	3.5
	r^2 (calibration curve)	0.9966	0.9991	0.9967	0.9974
	Equation (calibration curve)	y = 0.3601x + 0.0098	y = 0.1776x - 0.0011	y = 0.1026x - 0.0029	y = 0.0347x - 0.0008

RSD – relative standard deviation, LOD – limit of detection, LOQ – limit of quantification, r^2 – correlation factor

Table 24: Results of validation parameters for wastewater.

		Wastewater			
	Compound	**E1**	**E2**	**EE2**	**E3**
Low concentration (5 ng/L for E1 and E2; 10 ng/L for EE2 and E3)	Recovery (n=5) (%)	83	94	87	92
	Inter-day repeatability (n=5) (RSD %)	4	4	10	9
	Intra-day repeatability (n=5) (RSD %)	6	6	10	12
High concentration level, 50 ng/L	Recovery (n=5) (%)	81	91	87	94
	Inter-day repeatability (n=5) (RSD %)	2	3	8	9
	Intra-day repeatability (n=5) (RSD %)	5	6	11	11
	LOD (ng/L)	0.2	0.4	2.0	1.4
	LOQ (ng/L)	0.7	1.2	6.5	4.5
	r^2 (calibration curve)	0.9986	0.9980	0.9963	0.9988
	Equation (calibration curve)	y = 0.3952x + 0.0211	y = 0.1727x - 0.0336	y = 0.1383x - 0.0371	y = 0.0326x - 0.0005

RSD – relative standard deviation, LOD – limit of detection, LOQ – limit of quantification, r^2 – correlation factor

4.1.3 Application of chemical analysis - monitoring of hospitals and wastewater treatment plant

4.1.3.1 Presence of steroid estrogens

In hospital effluent, the three natural steroid estrogens, E1, E2 and E3 were present in all the samples analysed (Table 25). From Monday to Friday, concentrations were between 14.0 and 31.3 ng/L for E1, <LOQ and 2.8 ng/L for E2 and 152 and 385.5 ng/L for E3, while on Saturday, concentrations were lower, especially for E3 (6.4 ng/L). Fluctuations between three samples taken on Monday were also in the same range as Monday to Friday variations i.e., 10.9 - 31.3 ng/L, <LOD to 4.2 ng/L, 39.1 - 254.0 ng/L for E1, E2 and E3, respectively. Synthetic EE2 was below LOD in all samples.

Table 25: Steroid estrogen concentrations in hospital and WWTP samples.

sample	E1 (ng/L)			E2 (ng/L)			EE2 (ng/L)			E3 (ng/L)		
	H	WWinf	WWeff	H	WWinf	WWeff	H	WWinf	WWeff	H	WWinf	WWeff
Mon	31.3	24.2	<LOQ	1.4	4.8	<LOD	<LOD	<LOD	<LOD	254.0	37.7	<LOD
Tue	14.0	19.7	<LOQ	<LOQ	4.0	<LOQ	<LOD	<LOD	<LOD	385.5	24.7	5.2
Wed	22.3	49.7	<LOD	2.8	3.5	<LOD	<LOD	<LOD	<LOD	276.2	60.8	<LOQ
Thr	15.5	31	0.8	<LOQ	9.9	<LOQ	<LOD	<LOQ	<LOD	152.0	25.6	<LOQ
Fri	20.9	20.7	<LOD	<LOQ	5.7	<LOD	<LOD	<LOD	<LOD	175.2	7.3	<LOQ
Sat	8.6	46	<LOD	<LOQ	12.7	<LOQ	<LOD	<LOD	<LOD	6.4	63.9	<LOQ
Mon2	10.9	23.8	7.1	<LOD	2.5	<LOD	<LOD	<LOD	<LOD	39.1	<LOQ	4.6
Mon3	17.5	18.9	0.8	4.2	2.4	<LOQ	<LOD	<LOD	<LOD	86.7	9.1	<LOQ

H – Hospital; WWinf – wastewater influent; WWeff – wastewater effluent;

4.1.3.2 Ratio of E1:E2:E3

The observed concentrations of steroid estrogens from the hospital and the WWTP were used to calculate the ratio of E1:E2:E3 at different sampling sites. These results were compared with the ratio found in the urine of men and women during different life stages determined by Liu et al. (2009). The ratio E1:E2:E3 in hospital effluent was 1:0.1:9.4. On a Saturday the hospital effluent sample ratio (1:0.2:0.75) was similar to that observed in the WWTP influent 1:0.2:0.9 (Table 26). Since most effluent concentrations were <LOD or <LOQ, the effluent ratio was not calculated.

Table 26: Steroid estrogen ratio in environmental samples and excreted urine.

Sample	E1:E2:E3 ratio	Source for calculation
Hospital effluent – average	1 : 0.1 : 9.4	this study
Hospital effluent - Saturday	1 : 0.2 : 0.75	this study
WWTP influent – average	1 : 0.2 : 0.9	this study
Premenopausal women urine	1 : 0.4 : 0.8	Liu et al. (2009)
Postmenopausal women urine	1 : 0.6 : 0.6	Liu et al. (2009)
Pregnant women urine	1 : 0.3 : 20.2	Liu et al. (2009)
Men urine	1 : 0.4 : 0.4	Liu et al. (2009)

4.1.3.3 Theoretical estrogenic potential and contribution of E3

Estrogenicity was expressed in total estradiol equivalents (EEQ) using published estrogen equivalency factors (Campbell et al., 2006). In hospital effluent, the total cEEQs of the daily samples was 2.6 to 33.4 ng(cEEQ)/L in which the contribution of E3 varied from 20-92 % (Table 27). In the WWTP influent, cEEQs vary from 5.0 to 22.4 ng(cEEQ)/L and the contribution of E3 is as high as 37 %. In the effluent, 0.6-1.7 ng(cEEQ)/L was calculated with the contribution made by E3 accounting for up to 46 %.

Table 27: Calculated total cEEQs in hospital and WWTP samples and contribution of E3.

Sample	**Total cEEQ (ng(cEEQ)/L)**			**E3 contribution**		
	H	**WWinf**	**WWeff**	**H**	**WWinf**	**WWeff**
Mon	24.9	10.3	0.6	82 %	29 %	19 %
Tue	33.4	8.0	1.7	92 %	25 %	25 %
Wed	27.1	13.3	0.8	82 %	37 %	46 %
Thr	14.9	15.1	1.6	82 %	14 %	22 %
Fri	17.3	8.3	0.8	81 %	7 %	46 %
Sat	2.6	22.4	1.6	20 %	23 %	23 %
Mon2	4.6	5.2	1.5	68 %	7 %	25 %
Mon3	12.9	5.0	1.6	54 %	15 %	22 %

H – Hospital; WWinf – wastewater influent; WWeff – wastewater effluent

4.1.3.4 Removal of steroid estrogens and total estrogenicity

Table 28 summarises the removal rates of four steroid estrogens and cEEQs from the water phase on different days. Removal rates in six consecutive days (Mon 08.01.2007-Sat 13.01. 2007) were from >96 % to >99 %, >70 % to >92 % and >39 % to >93 % for E1, E2 and E3 respectively. Removal rates determined on three “Mondays” were 70 % to >97 % for E1, >50 % to >92 % for E2 and >-2 % to >96 % for E3. Removal of cEEQ from Monday to Saturday varied from 78 % to 94 %, which is similar while comparing three “Mondays” (68 % - 94 %).

Table 28: Removal rates of E1, E2, E3 and total estrogenic potential at WWTP6.

Sample	**E1**	**E2**	**E3**	**Total estrogenic potential (cEEQ)**
Mon	>97 %	>92 %	>96 %	94 %
Tue	>96 %	>70 %	79 %	78 %
Wed	>99 %	>88 %	>93 %	94 %
Thr	97 %	>88 %	>82 %	89 %
Fri	>99 %	>93 %	>39 %	90 %
Sat	>99 %	>91 %	>93 %	93 %
Mon2	70 %	>84 %	>-2 %	71 %
Mon3	96 %	>50 %	>51 %	68 %

“>” Indicates worst case scenario, since LOD or LOQ concentrations were used in calculations.

4.2 Integration of ER-Calux® assay and GC-MSD

4.2.1 Calculated estradiol equivalents (cEEQ)

E1, E2, E3 and EE2 were evaluated for their activity in the ER-Calux® assay by defining their estradiol equivalency factors (EEF) from the observed EC_{50} concentrations of each compound (Table 29). The EEFs determined were used in further calculations as described in Section 3.3.1.

Table 29: Steroid estrogen EC_{50} and EEF factors in ER-Calux® assay.

Compound	EC_{50} (pM)	EEF
17β-estradiol (E2)	2.64	1.0
Estrone (E1)	6.64	0.4
Estriol (E3)	18.21	0.14
17α-ethinylestradiol (EE2)	1.57	1.68

4.2.2 Solvent compatibility

4.2.2.1 Solvent, dose response and "real" samples

The results of the MTS assay revealed that none of the solvents tested (Figure 11) reduced the viability of the ER-Calux® cells for more than 18 % (n=5), compared to the control. Statistical analysis of the results shows that EtAc has no significant effect on cell viability. There was also no difference between dose responses obtained for the E2 standard solutions in ER-Calux® assay using either EtAc or DMSO (Figure 12).

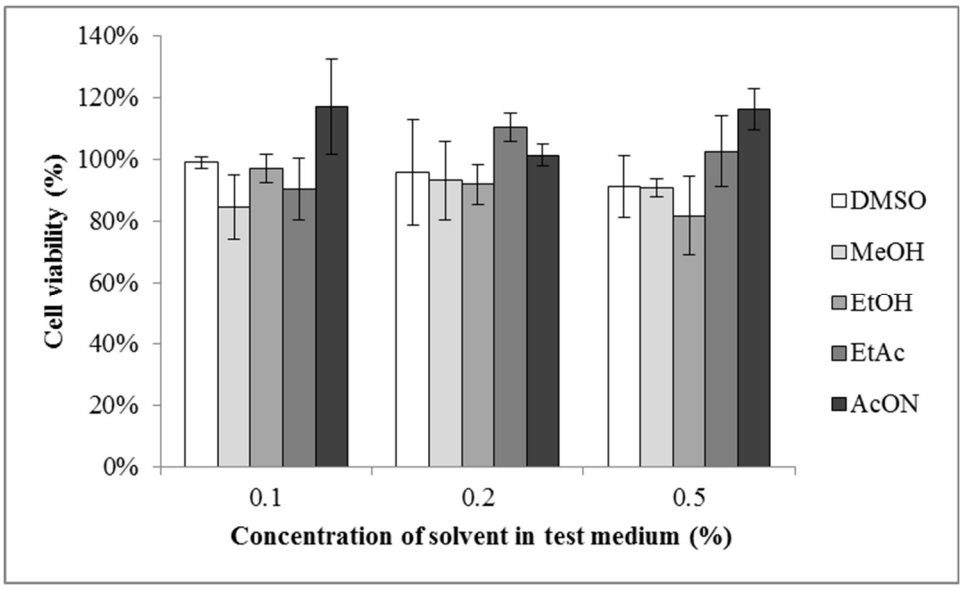


Figure 11: The effect of different solvents on ER Calux® cells. The results are presented as relative cell viability (%) ± SD compared to control (unexposed cells) calculated from five parallels.

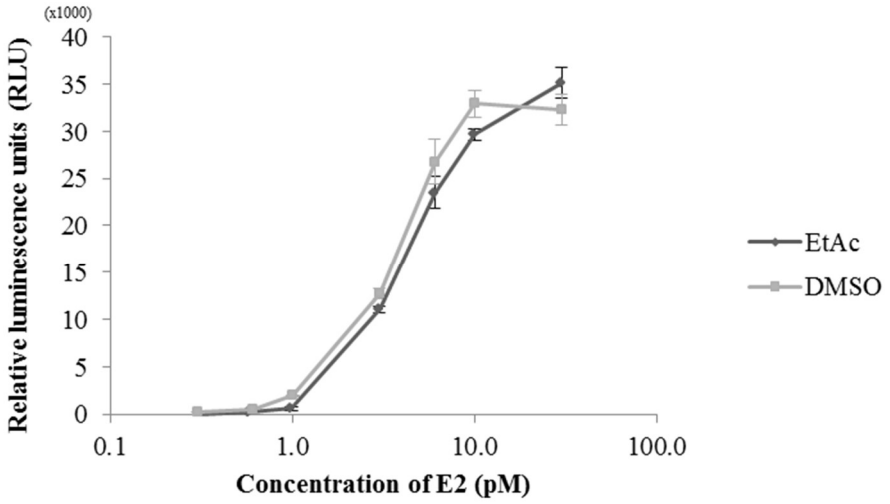


Figure 12: ER-Calux® results of E2 standard solution in DMSO and EtAc. The results are presented as mean ± SD calculated from three parallels.

"Real" samples were extracted in parallel and reconstituted in DMSO and EtAc. The results (Figure 13) show that the use of EtAc or DMSO for "real" samples shows no significant differences regarding the performance of ER-Calux® assay.

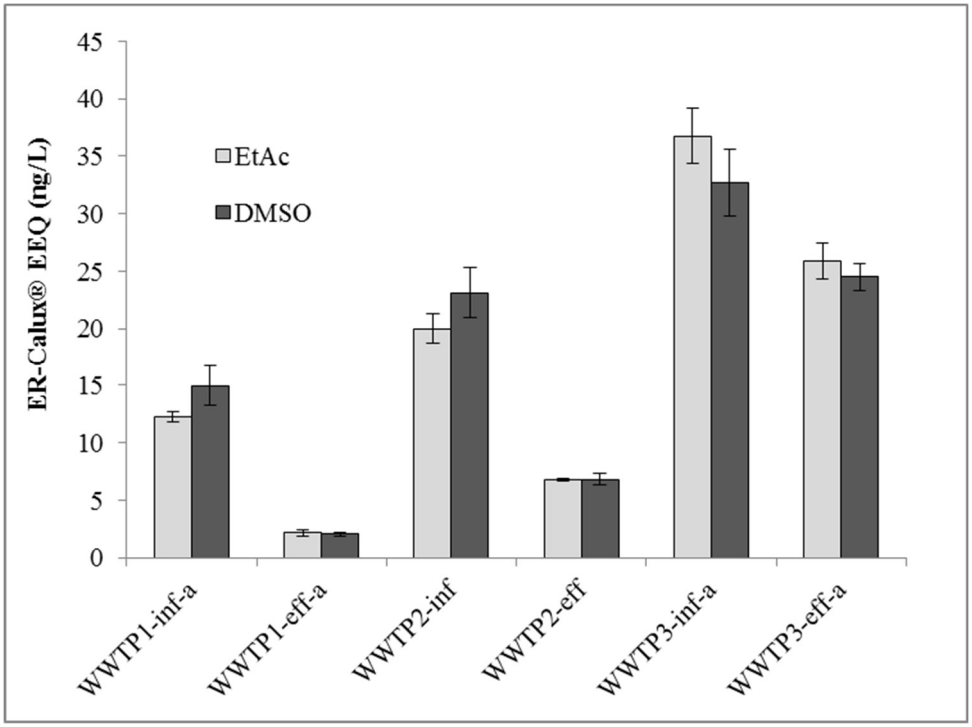


Figure 13: ER-Calux® results of "real" samples dissolved in DMSO and EtAc. The results are presented as mean ± SD calculated from three parallels.

4.2.3 Performance of integrated protocol

4.2.3.1 Validation of ER-Calux® assay

LOD and LOQ of the SPE - ER-Calux® assay were taken as recommended by BDS - 0.68 ng(EEQ)/L and 2.05 ng(EEQ)/L, respectively. The LODs and LOQs that were determined experimentally in each microtiter plate with ER-Calux® assay, were always lower than the recommended values. Recovery (97 %), and repeatability (4 %) were determined with tap water spiked with E2 (4.1 ng/L; 15 pM) in five parallels.

4.2.3.2 Spiked samples

The results of ER-Calux® and cEEQ values based on the results of chemical analysis with GC-MSD (Table 30) were compared.

Table 30: The ER-Calux® and GC-MSD results of standards and spiked samples.

		ER-Calux®	GC-MSD	GC-MSD analysis (ng/L)			
		ng(EEQ)/L	ng(cEEQ)/L	E1	E2	EE2	E3
Standards without wastewater extract	St1	35.4	40.4	<LOD	32.2	<LOD	58.9
	St2	24.5	27.5	<LOD	20.1	<LOD	52.8
	St3	28.6	31.1	8.4	20.8	<LOD	49.7
	St4	43.6	46.5	19.3	30.6	<LOD	58.4
	St5	36.8	36.3	16.3	22.1	<LOD	54.9
	St6	44.9	47.1	27.9	30.0	<LOD	42.5
	St7	39.5	43.8	29.3	24.1	<LOD	57.2
	St8	23.2	22.7	11.0	11.1	<LOD	51.9
	St9	23.2	22.3	12.1	10.8	<LOD	47.6
	St10	23.2	23.5	10.8	10.5	<LOD	61.6
Standards with influent extracts	Inf1	53.1	44.4	25.8	16.2	7.0	44.1
	Inf2	102.1	95.4	29.3	29.0	28.0	54.6
	Inf3	44.9	39.8	32.4	13.7	<LOD	93.7
	Inf4	44.9	39.6	34.8	12.7	<LOD	93.0
	Inf5	47.7	43.0	34.6	13.6	<LOD	111.5
Standards with effluent extracts	Eff0*	3.1	2.6	5.2	<LOQ	<LOD	<LOD
	Eff1	10.9	5.4	9.4	1.6	<LOD	<LOD
	Eff2	28.6	28.7	12.2	10.6	6.8	13.0
	Eff3	57.2	50.4	21.8	15.4	14.1	18.9
	Eff4	40.9	49.1	18.4	15.8	14.0	18.0
	Eff5	85.8	98.7	36.0	29.9	29.9	30.4
	Eff6	24.5	26.2	44.4	<LOD	<LOD	60.2
	Eff7	32.7	33.1	36.5	5.6	<LOD	91.7
	Eff8	44.9	31.4	22.7	13.2	<LOD	65.1

*blank sample

The results obtained from standard mixtures without wastewater extracts (Figure 14) show good correlation (r^2=0.96) and a slope close to 1 (α=0.94). The same situation (r^2=0.92, α=1.02) was observed, where standard mixtures were added to wastewater extracts (Figure 15). Statistical analysis using a paired t-test confirmed that ER-Calux® values are statistically the same as theoretical estrogenicity calculated from the results of

GC-MSD. At the 0.05 level, the difference in the population means was not significantly different with the test difference (0).

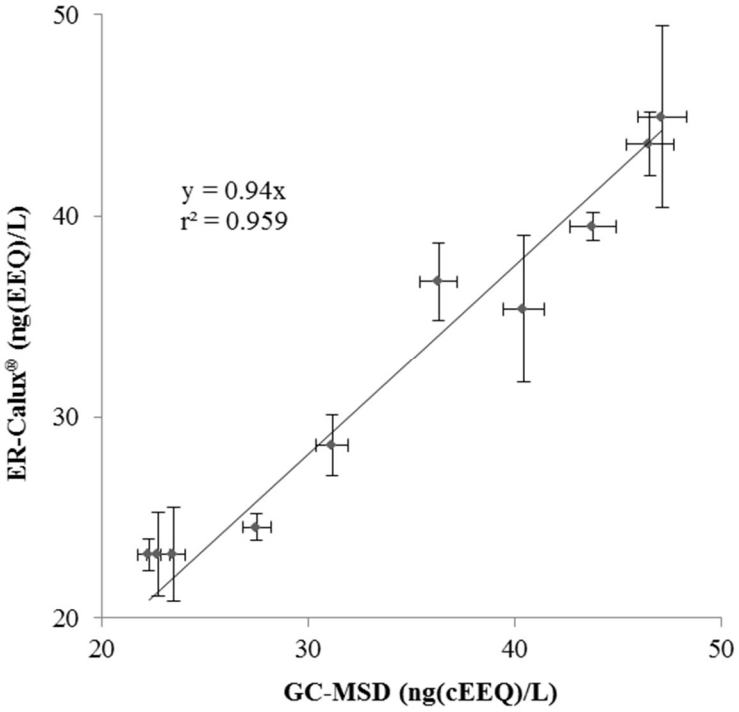


Figure 14: Comparison of GC-MSD cEEQ and ER-Calux® EEQ for standard mixtures without wastewater extract. The results are presented as the mean ± SD calculated from three parallels.

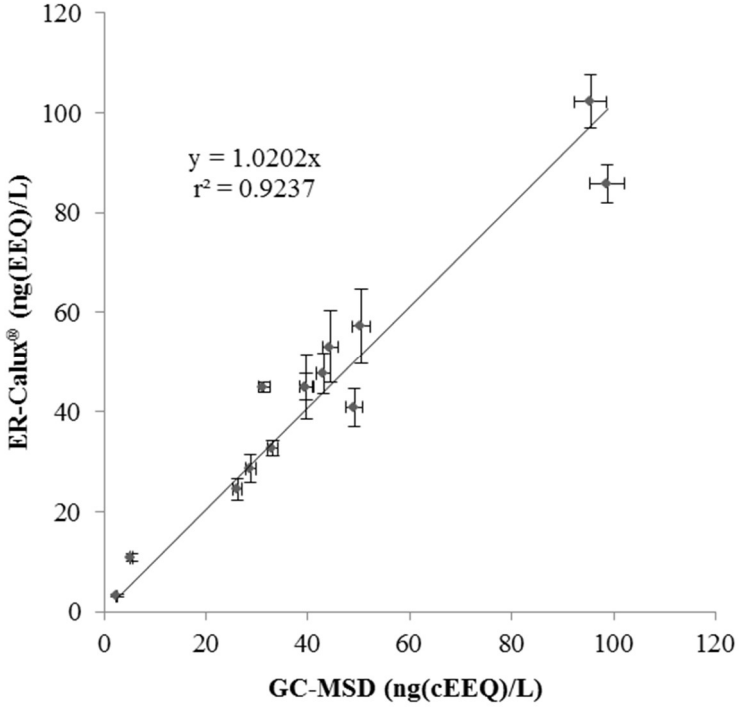


Figure 15: Comparison of GC-MSD cEEQ and ER-Calux® EEQ for spiked wastewaters. The results are presented as mean ± SD calculated from three parallels.

4.2.3.3 "Real" samples

"Real" samples from WWTP1, WWTP2 and WWTP3 (influents and effluents) were tested. Table 31 shows the concentrations of steroid estrogens in "real" waste influent samples. Concentrations of separate compounds were 1.7-61.8 ng/L for E1, 1.5-8.1 ng/L for E2 and <LOD-107.9 for E3. EE2 was not detected in any of the samples. From the determined concentrations, the cEEQs were calculated and compared to the results of the ER-Calux® assay (Figure 16). It is evident, that all the samples gave similar results for both chemical analysis and biological testing. An overall comparison of the "real" sample data analysed by GC-MSD and the ER-Calux® assay shows a good correlation (r^2=0.93; α=0.99).

Table 31: The ER-Calux® and GC-MSD results of "real" samples.

	ER-Calux®	**GC-MSD**	**GC-MSD analysis (ng/L)**			
	ng(EEQ)/L	**ng(cEEQ)/L**	**E1**	**E2**	**EE2**	**E3**
WWTP1-inf-a	12.3	17.9	11.3	4.2	<LOD	65.8
WWTP1-eff-a	2.2	4.9	4.0	1.5	<LOD	12.5
WWTP2-inf	20.0	23.4	13.4	4.4	<LOD	97.2
WWTP2-eff	6.8	8.7	16.5	2.1	<LOD	<LOD
WWTP3-inf-a	36.8	29.8	25.2	4.6	<LOD	107.9
WWTP3-eff-a	25.9	32.8	61.8	8.1	<LOD	<LOD
WWTP1-inf-b	19.1	21.8	16.4	3.7	<LOD	82.4
WWTP1-eff-b	4.1	6.2	1.7	2.9	<LOD	18.4
WWTP3-inf-b	49.0	48.3	82.8	10.1	<LOD	37.0
WWTP3-eff-b	42.2	35.8	51.1	9.0	<LOD	45.7
WWTP3-eff-c	3.1	2.9	6.0	0.5	<LOD	<LOD

Inf – influent; eff – effluent; a,b,c – parallels from the same WWTP

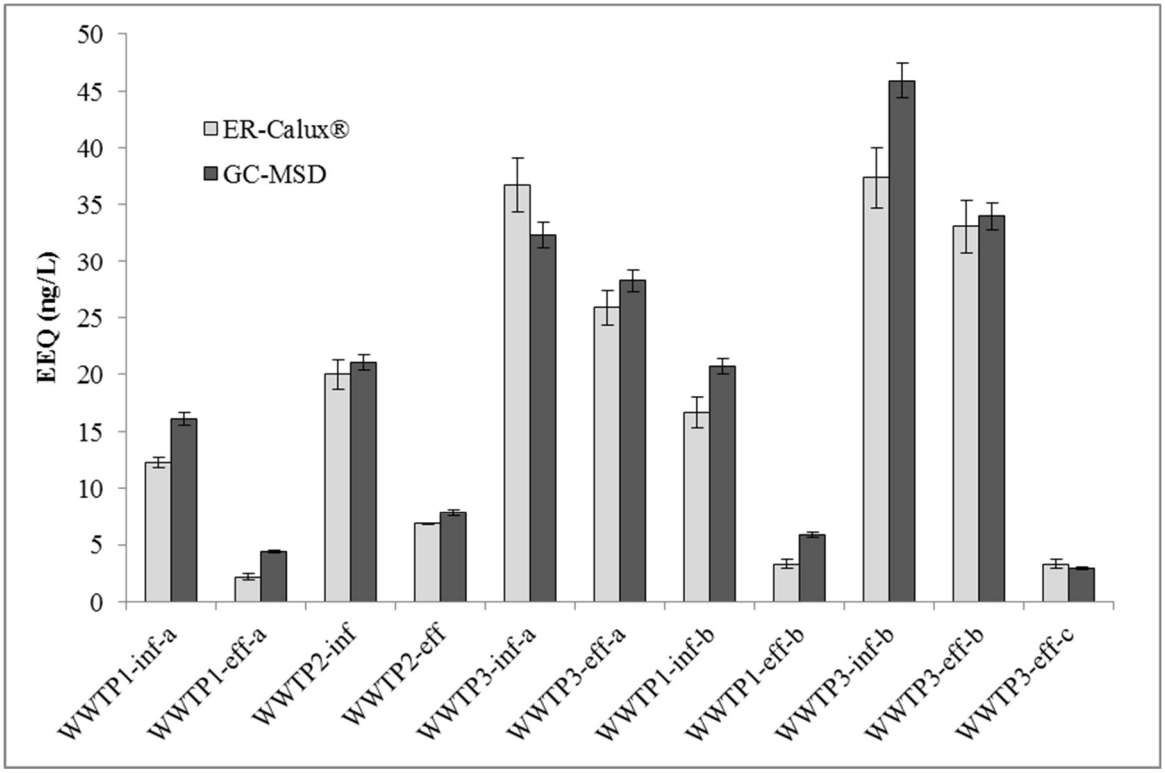


Figure 16: GC-MSD and ER-Calux® results of "real" wastewater samples. The results of ER-Calux® assay are presented as the mean ± SD calculated from three parallels. The results of GC-MSD are presented as the determined concentrations of one measurement ± RSD of the measurements made by GC-MSD.

4.3 NE-(ER-Calux®)

4.3.1 Optimization of NE-(ER-Calux®) assay

4.3.1.1 Medium dilution

As seen from Figure 17, the extent to which the "Test medium" can be diluted without significantly influencing the cells was investigated. With an increase in the amount of PBS in the "Test medium", lower cell viability was observed. However, a 20 % addition of PBS (sample) to the "Test medium" was chosen for further experiments, since the cell viability remains above 90 %.

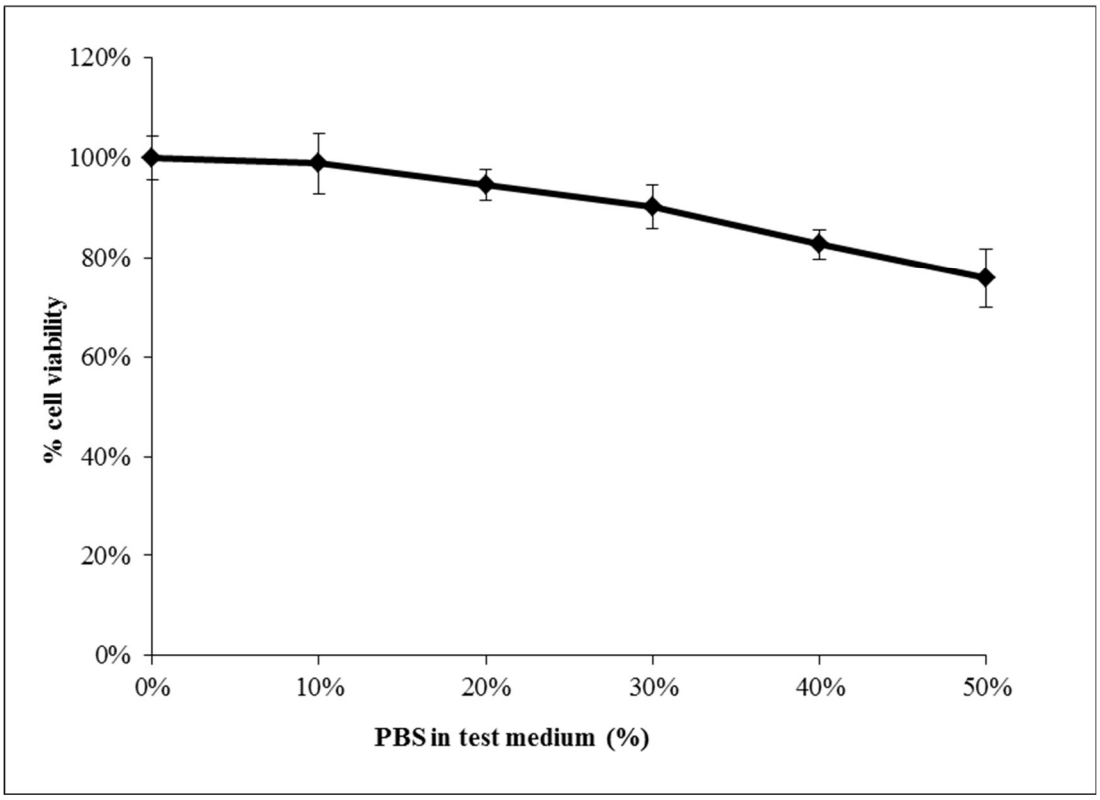


Figure 17: Influence of different dilutions of "Test medium" with PBS on cell viability of ER-Calux® cells.

4.3.1.2 Sterilization

Several filters were tested with sterile spiked PBS. The results show that most of the filters retain the target compounds (E1, E2, E3 and EE2). Only one filter (Anotop25 – alumina based anopore membrane) gave the same results (100 % recovery) as non-filtered PBS (Figure 18). The same results were obtained while filtering wastewater from WWTP2. Only "alumina based anopore" membrane gave a similar estrogenic potential than non-filtered PBS (Figure 19). However, recovery above 100 % (124 %) compared to sterile PBS can be explained by the presence of steroid estrogens in wastewater. Recovery values obtained with the other investigated filters were below 50 %. On the basis of these results Anotop25 syringe filters were used for sterilization.

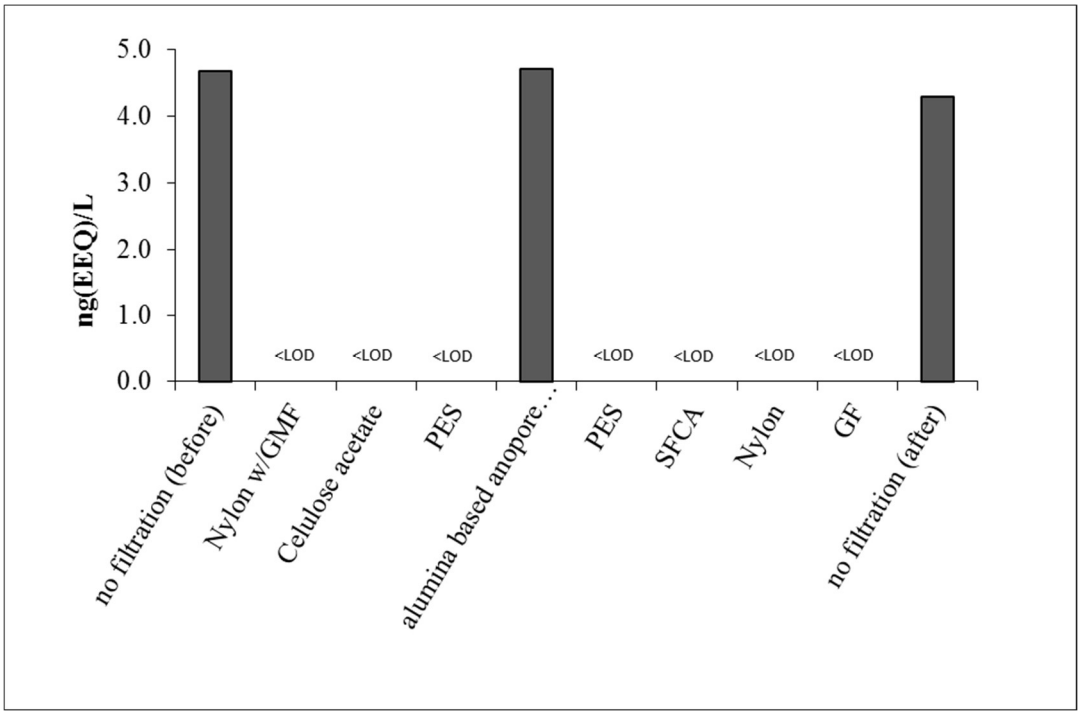


Figure 18: Performance of different filters with spiked sterile PBS.

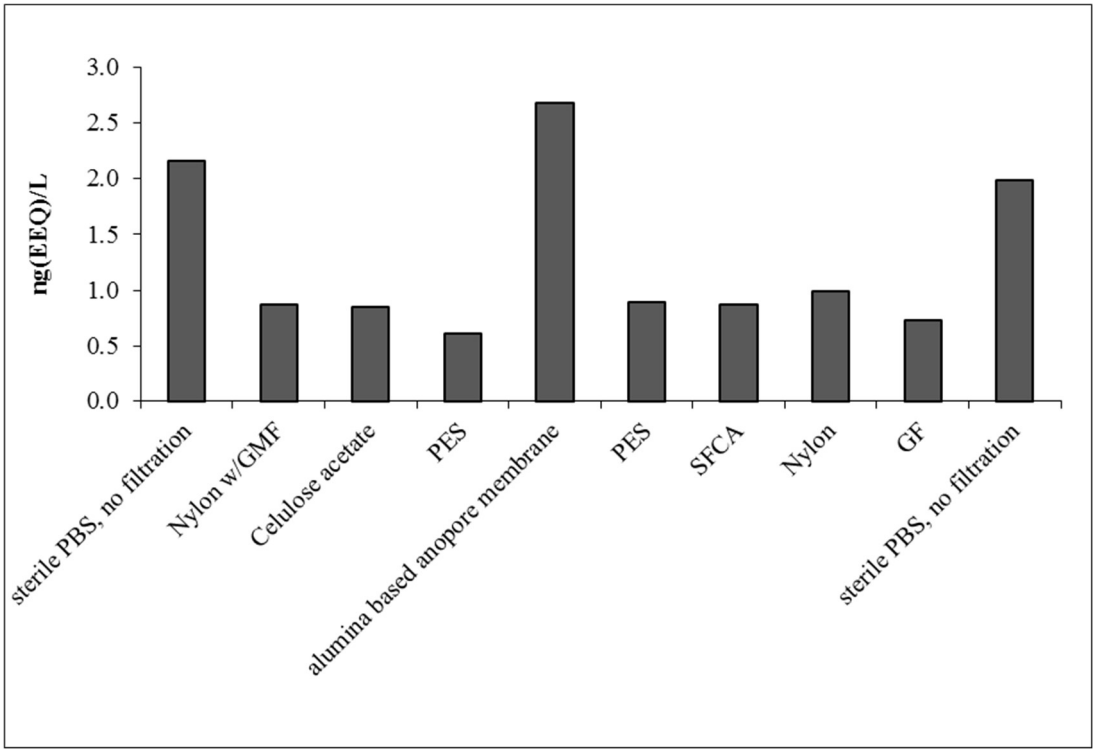


Figure 19: Performance of different filters with spiked wastewater in comparison with spiked sterile PBS.

4.3.1.3 Validation of NE-(ER-Calux®) assay

LOD and LOQ of NE-(ER-Calux®) assay were calculated from calibration curve, performed at each microtiter plate and compared to ER-Calux® assay. Since there were no observed differences in LODs and LOQ compared to the ER-Calux® assay, the same LODs (0.68 ng (EEQ)/L) and LOQ (2.05 ng (EEQ)/L) values were used as in ER-Calux® assay. The LODs and LOQs that were determined experimentally in each microtiter plate using NE-(ER-Calux®) assay, were always lower than the stated values. Recovery of NE–(ER Calux)® assay (92 %) and the repeatability (5 %), was determined with tap water (n=5), spiked with E2 (4.1 ng/L; 15 pM).

4.3.2 Comparison of NE-(ER-Calux®), ER-Calux® and GC-MSD

4.3.2.1 Spiked samples

The results of all three methods are evident from Table 32. Correlation between ER-Calux® and NE-(ER-Calux®) and the slopes of the regression line were r^2=0.98; α=0.96 and r^2=0.96; α=0.93 in tap and wastewater, respectively. Comparison of NE-(ER-Calux®) and GC-MSD reveals a r^2=0.98; α=1.01 in tap and r^2=0.97; α=0.89 in spiked wastewater (Figure 20). A paired t-test was performed to determine if the NE-(ER-Calux®) values are statistically the same as those obtained by ER-Calux® and the theoretical estrogenicity calculated from the results of GC-MSD (Table 32). At the 0.05 level, the difference in the population means was not significantly different with the test difference (0) in any tested combination (NE-(ER-Calux®):ER-Calux®; NE-(ER-Calux®):GC-MSD; tap or wastewater).

Table 32: The NE-(ER-Calux®), ER-Calux® and GC-MSD results of spiked samples.

Water sample		estrogenicity			GC-MSD analysis			
		NE-(ER-Calux®)	ER-Calux®	GC-MSD	E1	E2	EE2	E3
		ng(EEQ)/L	ng(EEQ)/L	ng(cEEQ)/L	ng/L	ng/L	ng/L	ng/L
Spiked Tap water	TW1*	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	TW2	11.8	9.3	4.2	2.8	3.1	<LOD	<LOD
	TW3	14.8	22.5	16.3	6.5	6.5	3.9	5.0
	TW4	22.9	33.7	31.5	11.0	10.6	9.1	8.5
	TW5	37.7	40.0	40.1	11.8	14.5	11.6	10.6
	TW6	51.1	50.5	58.4	18.5	19.3	17.6	16.6
	TW7	74.0	63.2	67.0	22.1	21.6	20.1	21.3
	TW8	66.6	73.7	80.6	25.6	25.9	24.6	24.4
	TW9	96.2	88.5	84.8	26.9	26.7	26.4	23.3
	TW10	91.0	91.3	93.6	29.9	30.3	28.1	31.3
	TW11*	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	TW12	4.1	4.8	3.4	1.5	1.8	<LOD	7.4
	TW13	7.0	7.1	7.4	2.3	3.1	<LOD	24.1
	TW14	7.8	7.2	8.9	5.1	2.6	<LOD	30.5
	TW15	9.7	8.4	9.9	4.7	4.8	<LOD	23.0
	TW16	15.2	11.3	13.7	6.2	3.3	2.0	32.9
	TW17	17.8	14.3	21.2	8.8	5.3	4.8	31.2
	TW18	29.8	22.7	35.2	13.4	6.3	11.2	34.0
	TW19	40.2	36.2	39.9	14.7	6.9	13.4	33.2
	TW20	42.9	34.5	41.3	11.3	5.8	15.5	36.6
Spiked wastewater	WW1*	11.1	6.4	5.4	<LOD	4.7	<LOD	5.1
	WW2	10.5	12.8	9.4	4.5	5.2	<LOD	16.8
	WW3	5.6	12.5	12.8	4.1	7.3	<LOD	28.0
	WW4	16.7	18.0	14.0	6.2	6.3	<LOD	37.1
	WW5	20.4	14.6	16.6	5.8	8.9	<LOD	38.5
	WW6	24.1	25.8	21.5	6.7	7.2	4.1	33.9
	WW7	25.9	29.2	25.8	10.2	6.3	6.6	31.6
	WW8	38.9	46.1	40.4	15.6	9.7	11.6	36.2
	WW9	50.0	50.5	48.7	17.0	10.1	16.1	35.7
	WW10	66.6	50.5	52.0	14.7	8.8	18.8	42.7

*Blank sample

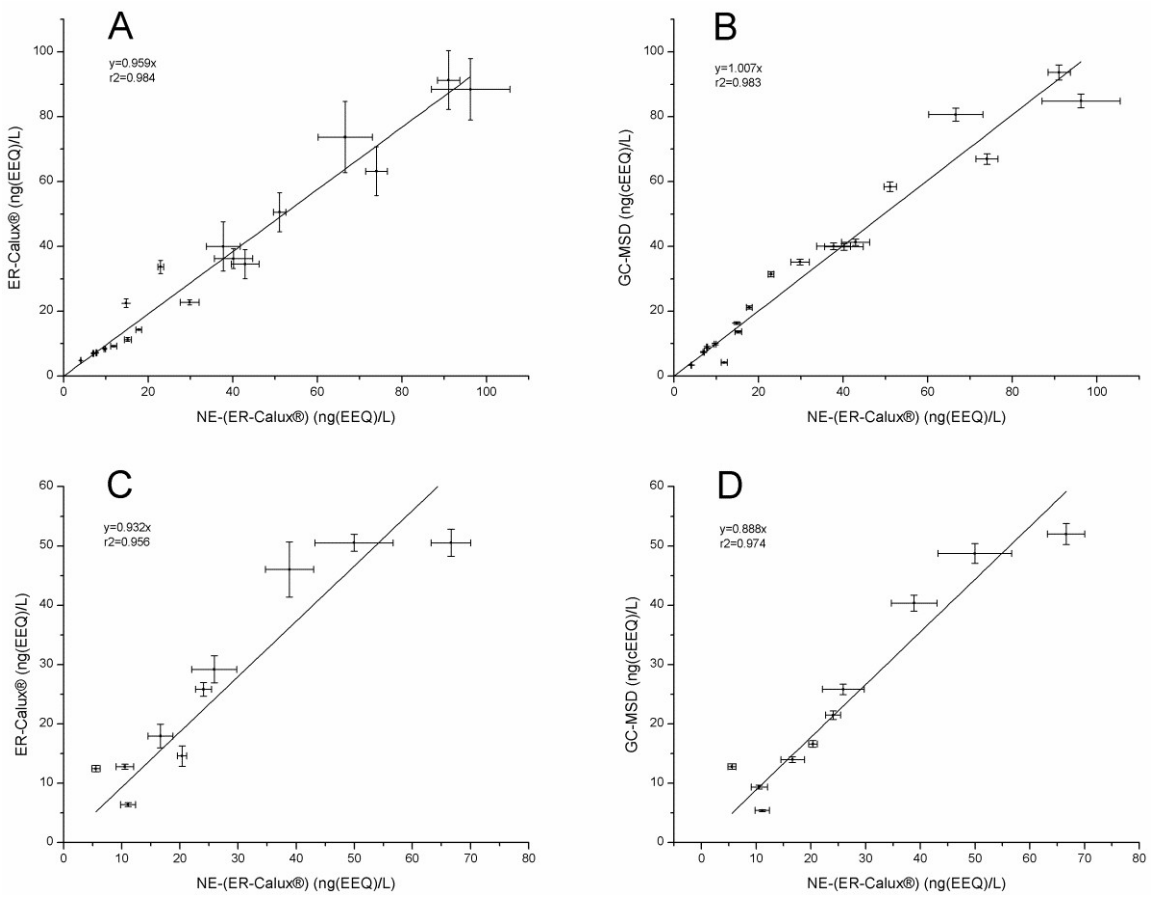


Figure 20: Comparison of ER-Calux® and GC-MSD with NE-(ER-Calux®) on spiked tap (A, B) and wastewater (C, D). Three results of NE-(ER-Calux®) and ER-Calux® assay are presented as means ± SD of three parallels, while the results of GC-MSD are represented as mean ± RSD of measurement by GC-MSD.

4.3.2.2 "Real" wastewater and surface water samples

The concentrations of E1, E2, EE2 and E3 in "real" samples obtained by GC-MSD (Table 33) were as high as 119 ng/L (E3) in influent, 51.1 ng/L (E1) in effluent, 7.4 ng/L river downstream and 2.0 ng/L river upstream. EE2 was below LOD in all tested samples. The calculated EEQs determined in the influent samples were 13.0-48.3 ng(cEEQ)/L and 0.9-35.8 ng(cEEQ)/L, <LOD-17.0 ng(cEEQ)/L and <LOD-2.4 ng(cEEQ)/L in effluent, river downstream and river upstream, respectively.

Table 33: The NE-(ER-Calux®), ER-Calux® and GC-MSD results of "real" samples.

Samples			Estrogenicity			GC-MSD analysis			
WWTP	**code**	**sample type**	**NE-(ER-Calux®)**	**ER-Calux®**	**GC-MSD**	**E1**	**E2**	**EE2**	**E3**
			ng(EEQ)/L	**ng(EEQ)/L**	**ng(cEEQ)/L**	**ng/L**	**ng/L**	**ng/L**	**ng/L**
WWTP1	WWTP1-i	influent	26.6	19.7	21.8	16.4	3.7	<LOD	82.3
	WWTP1-e	effluent	5.3	4.2	6.2	1.7	2.9	<LOD	18.4
	WWTP1-rd	river downstream	5.8	7.2	7.5	3.7	3.1	<LOD	20.5
	WWTP1-ru	river upstream	2.1	5.1	2.4	2.0	1.6	<LOD	<LOD
WWTP2	WWTP2-i	influent	56.3	50.5	48.3	82.7	10.1	<LOD	37.0
	WWTP2-e	effluent	57.7	43.5	35.8	51.1	9.0	<LOD	45.7
	WWTP2-rd	river downstream	29.6	17.3	17.0	7.4	2.9	<LOD	79.8
	WWTP2-ru	river upstream	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
WWTP3	WWTP3-i	influent	40.0	37.9	25.8	14.7	9.5	<LOD	74.5
	WWTP3-e	effluent	16.4	11.8	12.5	18.5	5.1	<LOD	<LOD
	WWTP3-rd	river downstream	4.3	2.5	1.8	4.5	<LOD	<LOD	<LOD
	WWTP3-ru	river upstream	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
WWTP4	WWTP4-i	influent	38.5	32.3	40.7	44.1	9.2	<LOD	99.0
	WWTP4-e	effluent	5.3	15.2	7.4	5.8	3.7	<LOD	9.8
	WWTP4-rd	river downstream	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	WWTP4-ru	river upstream	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
WWTP5	WWTP5-i	influent	57.7	25.3	18.8	9.6	6.2	<LOD	62.6
	WWTP5-e	effluent	5.6	3.8	6.4	4.9	1.5	<LOD	21.0
	WWTP5-rd	river downstream	<LOQ	<LOQ	0.8	2.1	<LOD	<LOD	<LOD
	WWTP5-ru	river upstream	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
WWTP6	WWTP6-i	influent	20.4	9.8	13.0	6.2	1.3	<LOD	65.3
	WWTP6-e	effluent	5.9	5.1	8.7	3.3	2.8	<LOD	32.3
	WWTP6-rd	river downstream	5.5	3.8	2.4	2.1	1.6	<LOD	<LOD
	WWTP6-ru	river upstream	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
WWTP7	WWTP7-i	influent	29.6	25.3	31.4	29.7	2.9	<LOD	119.0
	WWTP7-e	effluent	<LOQ	<LOD	0.9	2.2	<LOD	<LOD	<LOD
	WWTP7-rd	river downstream	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	WWTP7-ru	river upstream	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

i-influent, e-effluent, rd-river downstream, ru-river upstream

The estrogenic potential determined by ER-Calux® assay in the influent samples was 9.8-50.5 ng(EEQ)/L in WWTP influent, <LOD-43.5 in WWTP effluent, <LOD-17.3 ng(EEQ)/L in river downstream and <LOD-5.2 in river upstream samples. The estrogenic potential determined by NE-(ER-Calux®) was 20.4-57.7 ng(EEQ)/L, <LOQ-57.7 ng(EEQ)/L, <LOD-29.6 ng(EEQ)/L and <LOD-2.1 ng(EEQ)/L in influent, effluent, river downstream and river upstream samples, respectively (Figure 21).

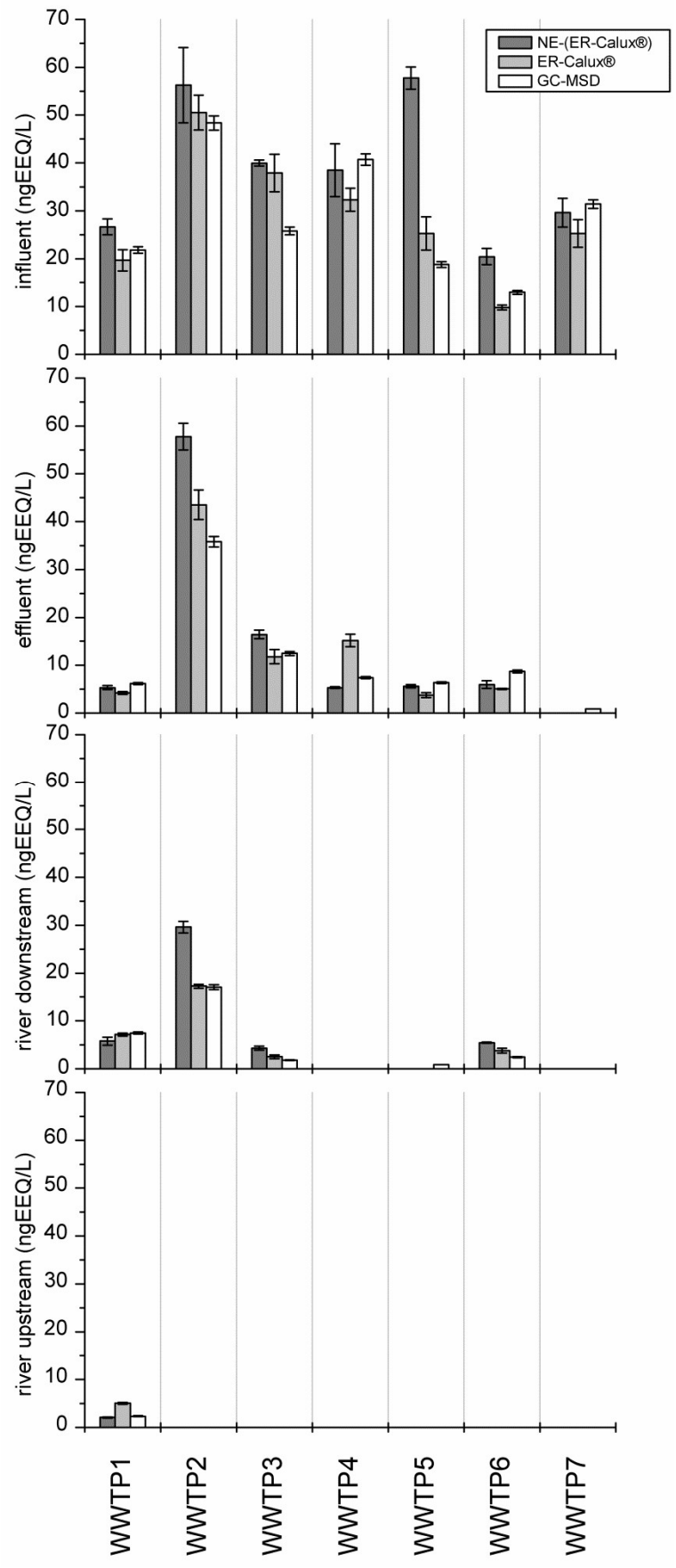


Figure 21: Estrogenic potential of "real" samples from WWTPs, tested with NE-(ER-Calux®), ER-Calux® and GC-MSD. Error bars represent standard deviation of three parallels in NE-(ER-Calux®) and ER-Calux® and RSD of measurement with GC-MSD.

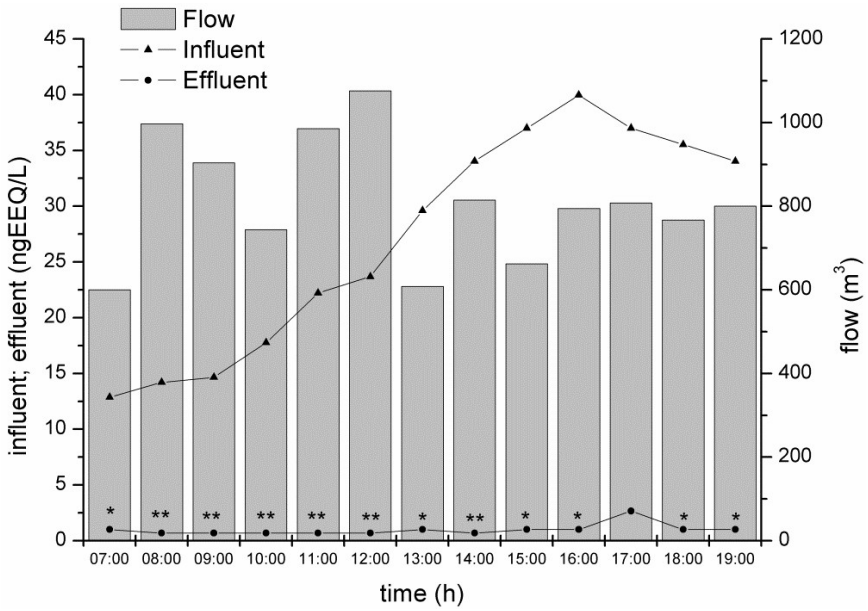
A comparison of ER-Calux® and NE-(ER-Calux®) shows that the results were generally higher in non-extracted samples. In the influent samples (Figure 21) the estrogenic potential determined using NE-(ER-Calux®) exceeds that obtained using ER-Calux® for 2.1-6.9 ng(EEQ)/L. A higher variability was observed in case of WWTP5 and WWTP6, where 32.4 ng(EEQ)/L and 10.6 ng(EEQ)/L higher NE-(ER-Calux®) values were determined. Effluent samples from WWTP1, 3, 5 and 6 show higher estrogenic potential (0.8-4.6 ng(EEQ)/L) for non-extracted samples. In WWTP2 effluent sample the difference was 14.2 ng(EEQ)/L. The opposite was observed in WWTP4 effluent where the NE-(ER-Calux®) values were 9.9 ng(EEQ)/L lower than that determined after sample extraction. In WWTP 7 effluent, estrogenic potential was below the LOQ of both methods.

In WWTP2, WWTP3 and WWTP6 river downstream samples, NE-(ER-Calux®) values were 12.3, 1.7 and 1.8 ng(EEQ)/L higher than those obtained using ER-Calux®, respectively. The opposite was observed in the WWTP1 river downstream sample, where NE-(ER-Calux®) concentration was 1.4 ng(EEQ)/L lower. Other river downstream samples were below the LOD or LOQ.

Similar values of the estimated biological activity by GC-MSD (cEEQ) and the results of NE-(ER-Calux®) and ER-Calux® were observed (Figure 21). However, in the case of WWTP6 effluent sample, theoretical estrogenic potential was 32 % and 42 % higher than NE-(ER-Calux®) and ER-Calux® assay, respectively. In WWTP3 and WWTP5 influent and WWTP2 effluent samples the GC-MSD result was lower than the value measured by the biological assays. River upstream samples from the river exhibited no measureable or lower estrogenic potential (WWTP1) compared to downstream samples. All samples were checked for potential toxicity using the MTT assay. None of the samples exhibit any cytotoxic potential for T47D cells (data not shown).

4.3.3 Application of NE-(ER-Calux®)

It is evident from Figure 22 that the estrogenic potential of the influent varies during the day increasing from 12.9 ng(EEQ)/L at 7:00 to 40.0 ng (EEQ)/L at 16:00. Alternatively, the estrogenic potential of the effluent (with 2.5 h delay in sampling) was consistently below the LOQ (or LOD) in all cases except at 17:00. The average estrogenic potential of the hourly samples (27.1 ng (EEQ)/L) were similar to the result of the time proportional samples (25.2 ng(EEQ)/L). An average of all effluent hourly samples and time proportional sample was <LOQ. The time proportional influent sample exhibits only 62 % of the sample with the highest estrogenic potential (40.0 ng(EEQ)/L at 16:00).



*<LOQ (value set to 1 ng(EEQ)/l); **<LOD (value set to 0.68 ng(EEQ)/L)

Figure 22: Intra-day variations of estrogenicity in WWTP influent and effluent.

For each hour, the removal of the estrogenic potential was calculated and the results reveal variability of a removal rate from 92 % in the morning to 98 % at 16:00. The average removal was 96 %, a value similar to that shown by time proportional data. All hourly samples were also checked for potential toxicity with MTT assay. None of the samples exhibit a cytotoxic potential for T47D cells (data not shown). The performance of WWTP1 was checked during the experiment. During sampling time, the WWTP performance was stable (Figure 23). All parameters represented typical performance of the WWTP during the sampling time.

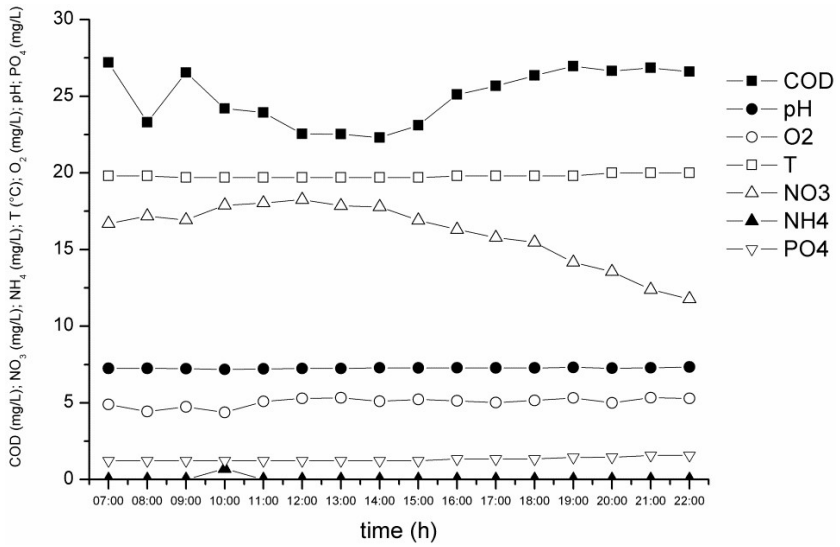


Figure 23: WWTP performance – online measurements in effluent.

5 Discussion

5.1 Chemical analysis of hospital effluent and WWTP samples

Sophisticated and sensitive analytical methods are necessary to determine low concentrations of steroid estrogens in the environment, especially in complex matrices like wastewater. In the first part of this doctoral thesis, sample preparation and chemical analysis of four most potent steroid estrogens with GC-MSD was optimised, validated (Table 23, 24) and applied to wastewaters and receiving surface waters.

Optimization included the addition of a sample clean-up step to remove interferences. The importance of this step is evident from Figure 10, which shows a clear improvement in the determination of E1 and EE2 when introduced. With its introduction a more reliable determination of the steroid estrogens in hospital effluents and WWTP6 samples is possible.

5.1.1 Steroid estrogens in hospital effluent and WWTP samples

Since steroid estrogens are commonly present in wastewater influent and effluents, their presence in WWTP6 influent and effluent was expected. The results (Table 25) are comparable to the published data of steroid estrogen concentrations in WWTPs summarised by Miège et al. (2009a). EE2 was detected only in one influent sample, but the concentration was <LOQ. EE2 is an important steroid estrogen, since its high estrogenic activity can add significantly to total estrogenicity. However, the LOD for EE2 (2.0 ng/L) was higher than the median concentration reported in the literature - 1.9 ng/L in influent and 0.5 ng/L in effluent (Miège et al., 2009a). This suggests that EE2 is probably present in concentrations <LOD. In the WWTP samples, the variability in concentration between weekdays (Table 25) is the same as for five consecutive days (Monday to Friday) and for three Mondays.

The WWTP receives wastewaters mainly from domestic sources; however, institutions like hospitals are important sources of estrogenicity. In hospital effluent, three natural steroid estrogens were detected in concentrations up to 31.3 ng/L, 2.8 ng/L and 385 ng/L for E1, E2 and E3, respectively (Table 25). The results are comparable to published data (Lin and Tsai, 2009; Thomas et al., 2007; Zorita et al., 2009). A high inter-day variation between concentration levels was observed, e.g. 6.4–385.5 ng/L for E3. The same variations were also observed between Monday to Friday and between three Mondays. However, a significant difference was shown in the concentration of steroid estrogens in hospital effluent between working days and Saturday, especially in the case of E3. The reason is most likely due to working practices, since at the weekend the hospital is limited to accident and emergency. This effect is expected to be greater in samples taken on a Sunday. Unfortunately, it was not possible to collect samples on this day.

The main difference between hospital and WWTP samples is in the high concentrations of E3 in hospital effluent, while other steroid estrogens are in similar concentrations (Table 25). The source of high E3 concentrations in hospital effluent during the week is likely in pregnant women that excrete up to 3000-times more E3 and 100-times more E1 and E2 compared to non-pregnant women and men (Fishman et al.,

1962; Liu et al., 2009). However, since hospital effluent contains wastewater from other hospital sources than human excreta, it is difficult to compare the absolute concentrations of steroid estrogens in hospital effluent to the amounts excreted by pregnant women.

For this reason, the E1:E2:E3 ratio was calculated (Table 26) and compared to published data for excreted steroid estrogens by women during different stages of life (Liu et al., 2009). The ratio in hospital effluent was similar to that found in pregnant women's urine (1:0.3:20.2), especially regarding the contribution made by E3. The most likely source is the diagnostic laboratory, where urine samples from patients including pregnant women are analysed and discarded directly to the sewerage system. On Saturday, when the laboratory is open only for emergency cases, the ratio (1:0.2:0.75) is similar to that found in the non-pregnant population. The likely source in this case could be the excreta of employees and hospitalised patients, where pregnant women represent only a minority. This also explains the low Saturday concentrations of E3. In contrast to hospital effluent, the ratio in the WWTP influent samples (1:0.2:0.9) is comparable to the ratio for men and non-pregnant women (1:0.4:0.8), which is expected for wastewater that is predominantly from domestic sources. Clearly, the contribution of estrogens from the hospital effluent to the total amount in the WWTP influent is negligible due to the high dilution factor (1:70) of the hospital effluent at the inflow of the WWTP.

5.1.2 Estrogenic potential

Beside the absolute concentrations of the target compounds and their source, total estrogenicity (EEQ) is of great importance. Since the estrogenicity assay was not introduced to our laboratory at that time, the estrogenicity (expressed in total estradiol equivalents -EEQ) was estimated using published (Campbell et al., 2006) estrogen equivalency factors (Table 27). The results are most likely underestimated since other compounds with estrogenic activity can be present in the hospital effluent (Fatta-Kasinos et al., 2011) and WWTP influent and effluent samples (Campbell et al., 2006; Salste et al., 2007). The calculated total EEQs (Table 27) of the daily samples indicates that hospital effluent should be considered as an important source of estrogenic pollution.

The contribution of E3 – a pregnancy hormone – was studied. The results show that E3, despite its lower estrogenic potential, accounts for the majority (up to 92 %) of the total estrogenic potential of the hospital effluent and up to 46 % in the WWTP effluent analysed. The results are in agreement with a study by Drewes et al. (2005), where E3 was found to attribute to the majority of estrogenic potential in WWTP samples. E3 should therefore be considered as an important estrogenic pollutant and should be investigated, particularly when hospital wastewaters are treated in municipal WWTP.

5.1.3 Removal of steroid estrogens at WWTP

Removal rates determined in this study (Table 28) are comparable to those found in several WWTPs reported in the literature, where removal rates were 55–99 %, 40–99 %, 64–85 % and 82–97 % for E1, E2, EE2 and E3, respectively (Teske and Arnold, 2008). Up to 99 % of the steroid estrogens are eliminated from the water phase. However, there are some cases where the removal was <80 % and even <40 %. Only one sample (Monday, 11.11.2006) actually exhibited a higher concentration of E3 in the effluent than in the influent. Reasons for these lower removal rates or even higher concentrations at the effluent sites are likely to be the following: the poor removal efficiency of the treatment process (due to temperature, HRT, sludge age etc.), adsorption and desorption processes, the deconjugation of steroid estrogens during treatment that increases the concentration of active estrogens in the effluent and/or the presence of free steroid estrogens that were not

detected in the influent samples (Andersen et al., 2003; D'Ascenzo et al., 2003; Teske and Arnold, 2008). In addition, removal of E1, E2 and E3 is not constant during the week and varies from 39 % up to 99 %. Such periodical lower removal rates of steroid estrogens in WWTP lead to a higher amount of pollution in receiving waters. Besides understanding the fate and transport of steroid estrogens inside water treatment system (Andersen et al., 2003; Drewes et al., 2005), understanding the time-dependent concentration variations are of great importance. Steroid estrogens have high biological activity and therefore, for environmental organisms, episodes, where removal rates are much lower and consequently the higher concentrations reach surface waters, are significant. Again, such frequency can be understood only by obtaining detailed information about the dynamics of estrogen concentrations at the WWTP influent and effluent site. In order to make any definite conclusions, an extended sampling campaign, including inter week and seasonal variations, should be performed. Alternatively, more efficient wastewater treatment technologies, such as those presented in Table 7, that are able to remove up to 100 % of steroid estrogens should be considered when designing or upgrading an existing wastewater treatment.

5.2 Integration of ER-Calux® assay and chemical analysis with GC-MSD

The chemical analysis described in the first part of this thesis only provides discrete information about sample composition and concentrations of individual compounds and does not account for additive and synergistic effects of mixtures and the presence of unknown compounds despite their possible contribution to the overall estrogenic activity. The alternative is to measure the estrogenicity of the samples for which *in vivo* and *in vitro* assays are employed (Campbell et al., 2006). In the second part of the doctoral thesis, the introduction of the ER-Calux® assay and its integration with the developed chemical analysis was performed. The aim was to develop a protocol for fast, simple and in particular the simultaneous determination of steroid estrogen concentrations and the total estrogenicity of the sample.

Combined chemical and biological approaches for the investigation of steroid estrogens and other EDC are increasingly being employed (Section 1.6.5). Despite this, published studies are difficult to compare since they incorporate different analytical methods and biological assays, sample handling procedures and approaches to method integration. In addition, values and ratios of estradiol equivalents derived from chemical analysis (cEEQ) and estrogenicity assay (EEQ) vary. In those studies, where detailed comparisons of chemical and biological methods are made (Aerni et al., 2004; Bicchi et al., 2009; Liscio et al., 2009; Miège et al., 2009b; Nelson et al., 2007; Tan et al., 2007; Wang et al., 2011), correlation factors up to r^2=0.85 are reported. This is proof that chemical analysis and estrogenicity assays are closely related, but the fact that the slopes of the regression lines are usually not optimal (α=1) means that differences exist. A possible explanation is the presence of agonists, antagonists, and unknown compounds, which albeit relevant (Körner et al., 1999), remain unverified. By using additional fractionation, Salste et al. (2007) report the presence of antagonists, while other authors did not confirm their presence (Furuichi et al., 2004; Heisterkamp et al., 2004).

A summary of the literature data reveals that most studies use different sample handling procedures for chemical analysis and estrogenicity assays. In many cases they use completely separate protocols for each method including separate sampling and sample preparation, extraction and different solvents (Aerni et al., 2004; Brix et al., 2010; Cargouët et al., 2004; Miège et al., 2009b; Rutishauser et al., 2004; Tan et al., 2007). The aim of our study was not to characterise sample handling error, but to develop a simple

integrated protocol that could be used in both chemical and biological analysis.

5.2.1 Optimization of integrated protocol

DMSO is the most commonly used solvent in cell-based bioassays. However, it is not suitable for chemical analysis, due to its low volatility. Results show that EtAc, which is already used in sample preparation ("Clean-up" step) in chemical analysis, can also be used in the ER-Calux® assay. EtAc did not exhibit any significant difference in cell viability (Figure 11), dose response of E2 standard solutions (Figure 12) and "real" sample data (Figure 13) compared to DMSO. Using the same solvent means that no additional sample preparation steps are required for the bioassay since the ER-Calux® assay requires only several µL of sample. Only 5 % of the sample, which was initially prepared for chemical analysis, is required for the ER-Calux® assay. Alternatively, 95 % of the sample that is available for chemical analysis is sufficient to obtain the required LOD and LOQ for analysing environmental samples. Simple sample preparation means that uncertainties derived from different sample handling procedures are reduced.

It is evident from the results of testing mixture of standards (Figure 14), that besides the good correlation (r^2>0.92), EEQs determined by GC-MSD, were comparable to those deriving from the ER-Calux® assay, since the regression line slopes were close to optimal i.e., α=0.94. A similar result (Figure 15) was obtained with spiked wastewater extracts (α=1.02), although a lower correlation was expected due to the complexity and consequent possible mixture effects caused by the presence of agonists or antagonists (Murk et al., 2002; Salste et al., 2007; Viganò et al., 2008).

5.2.2 "Real" samples

After method optimization, the integrated protocol was applied to actual "real" WWTP influent and effluent samples (Figure 16). Concentrations of steroid estrogens in wastewater samples (Table 31) are comparable to concentrations reported by Miège et al. (2009a). The concentrations were used to calculate cEEQ and closely correlate to the results of the ER-Calux® assay (r^2=0.93), which is about 10 % better than published values. Moreover, the estrogenicity detected with ER-Calux® assay can be explained by concentrations of E1, E2, E3 and EE2, determined by GC-MSD. This is in agreement with several studies, where these four compounds were responsible for more than 90 % of the total estrogenic potential in municipal wastewaters (Aerni et al., 2004; Furuichi et al., 2004; Miège et al., 2009b; Salste et al., 2007). This means that the estrogenicity of municipal wastewater samples can be, for rapid screening purposes, estimated by determining the four most potent steroid estrogens.

The integrated protocol is applicable for every sample, where extracts are prepared for analysis. Moreover, with optimization of the extraction solvent, the ER-Calux® assay can be integrated into any existing chemical analysis. At the same time the ER-Calux® assay can be used as a pre-screening tool prior to chemical analysis, since the extracts that are used in the estrogenicity assay, can be temporarily stored and analysed at a later time by GC-MSD, if necessary. While the ER-Calux® assay is sensitive and allows for a much higher sample throughput, chemical analysis, which is more costly, can be used to analyse only critical samples with high responses in ER-Calux® assay. For GC-MSD and ER-Calux® time consuming sample preparation is necessary.

5.3 NE-(ER-Calux®)

Time consuming sample preparation methods are commonly used for determining

estrogenic potential in surface and wastewater samples. The same is true for GC-MSD and ER-Calux® assay. In the final part of this thesis sample preparation is simplified by modifying the ER-Calux® assay to determine estrogenic potential directly.

5.3.1 Optimization of sample preparation

One of the main guidelines for modifying the ER-Calux® assay was to keep the existing LOD and LOQ, that are relevant to the concentrations in environmental samples (below ng/L). This was achieved by increasing the sample volume exposed to the cells. However, higher volumes of the sample in the "Test medium" might affect cell viability since the nutrients in the medium are diluted. The results show that the cells are not influenced if the "Test medium" is diluted by 10-30 % with PBS (Figure 17). In this study, a 20 % dilution of the "Test medium" was used. If a test sample is introduced in place of PBS, the cells are exposed to a 1:5 dilution. The sample dilution factor in NE-(ER-Calux®) therefore remains the same as in ER-Calux® assay, where the sample is concentrated using SPE (1:200) and afterwards diluted in the "Test medium" (1:1000). With this, the same LODs and LOQs values were achieved as for conventional ER-Calux® assay.

The other challenge for testing non-extracted samples is sterilization. Various filters were tested (Figure 18, 19) since commonly used filters exhibit poor recoveries for steroid estrogens. Only "alumina based anopore membrane" gave satisfactory recoveries close to 100 %, while other filters exhibit poor recoveries (below 50 %).

5.3.2 Spiked samples

A comparison of the methods GC-MSD, ER-Calux® and NE-(ER-Calux®) reveals a good correlation and optimal slope of the regression line for tap and wastewater spiked samples (Figure 20). In tap water, the presence of compounds that might interfere with biological assay is not expected. Alternatively, several authors prove that complexity of the matrix i.e., the presence of impurities, might affect biological assay (Murk et al., 2002; Salste et al., 2007; Viganò et al., 2008). Therefore, differences were expected (but not observed) in case of spiked wastewater samples (Figure 20 – C, D). It is possible, that in that exact sample, no agonists or antagonists were present. In general, the results prove, that within this concentration range, the NE-(ER-Calux®) method is comparable to the other two methods and can be used to analyse environmental samples.

5.3.3 "Real" samples

Wastewaters have different compositions that can influence the results. For this reason, all three methods were checked for responses on seven different influent, effluent, river upstream and downstream samples. The observed results of chemical analysis (Table 33) are in agreement with published results (Miège et al., 2009a). The estrogenic potential determined by ER-Calux® assay are comparable to other studies employing ER-Calux® (Murk et al., 2002), MELN (Cargouët et al., 2004) and recombinant yeast assay (Salste et al., 2007).

To the best of my knowledge, there exists no published data for the determination of estrogenic potential in wastewaters without sample extraction using cell-based assays. The NE-(ER-Calux®) results can only be compared to a few studies employing recombinant yeast assay. Salste et al. (2007) analysed freeze dried samples from two WWTP samples with recombinant yeast assay. Their EEQ values from WWTP effluent were approximately 5 ng(EEQ)/L which is in agreement with the NE-(ER-Calux®) results, where values in the effluent range from <LOQ – 57.7 ng(EEQ)/L, however four

WWTPs effluent samples were from 5.3 to 5.9 ng(EEQ)/L. Results are also in the same range as those determined by Colosi and Kney (2011), where samples from four WWTPs were analysed by the YES assay with no sample extraction. Similar results were obtained by Balsiger et al. (2010), who developed a four hour yeast assay using neither sample extraction nor sterilisation.

When comparing the responses of NE-(ER-Calux®) and ER-Calux® assay, the highest variability was expected in influent samples, due to the complexity of the matrix. In effluent and river downstream samples, measured variability in estrogenic potential was lower with the exception of the WWTP2, where a slightly higher estrogenic potential was observed in the non-extracted samples. The results suggest that unidentified water soluble agonists as well as antagonist may be present in the samples. Such compounds cannot be extracted using non-polar organic solvents and are therefore undetectable with the ER-Calux® assay.

The results of river upstream samples indicate that the estrogenic potential derives from the wastewater effluent. This was observed in the case, where the effluent is discharged to smaller surface waters (WWTP 1, 2 and 6). The estrogenic potential in these river downstream samples exceed 50 % of estrogenic potential that was detected in the effluent sample.

As discussed in Section 5.2, the highest portion of the total estrogenic potential in municipal wastewaters can be explained by four steroid estrogens. The same was observed in this study since similar values of estimated biological activity by GC-MSD (cEEQ) (Table 33) and the results of NE-(ER-Calux®) and ER-Calux® were observed (Figure 21). Exceptions were WWTP3 and WWTP5 influent and WWTP6 and WWTP2 effluent samples, where GC-MSD results were significantly higher or lower in comparison to biological assays. This might be due to the presence of antagonists or agonists in the wastewater sample.

Other compounds present in environmental samples can also exhibit toxic effects that could interfere with the determination of estrogenic potential. For that reason all wastewater and river samples were tested for their cytotoxic activity using the MTT assay (data not shown) in parallel with the NE-(ER-Calux®) and ER-Calux® assays. Negative MTT results for all samples exclude cytotoxicity affecting the T47D cells and consequently measurement of the estrogenic potential.

5.3.4 Intra-day wastewater samples

NE-(ER-Calux®) was used to study the intra-day variability of estrogenic potential in one WWTP. The method confirms the variability (12.9-40.0 ng(EEQ)/L) of the total estrogenic potential in wastewater influent (Figure 22). The average of all hourly samples was similar to the results of the time proportional sample. The same variability was observed for the removal rates (92 %-98 %) where the average removal of hourly samples was the same as the removal of the time proportional sample (96 %). These removal rates agree with other studies (Teske and Arnold, 2008).

However, comparison of the highest hourly value and time proportional sample confirms that time proportional samples give a comparable average value, but can miss peak values that might be of important for influencing the ecosystem. Consequently, sampling strategies should be carefully chosen. The results indicate the variability in the estrogenic potential over a day and should improve our understanding of the risk to the environment posed by the presence of steroid estrogens.

To assure that the performance of the WWTP1 was optimal during the experiment, several parameters were measured on-line. During sampling time, the WWTP

performance was stable (Figure 23) and all parameters reflect the normal performance of the WWTP during sampling.

5.3.5 Advantages of NE-(ER-Calux®) assay

Sample preparation is an important advantage that distinguishes NE-(ER-Calux®) from the other two methods described (Sections 5.1, 5.2). Since there is no sample extraction, water soluble agonists or antagonist can be detected. With this, NE-(ER-Calux®) gives us more accurate information of estrogenic potential, to which environmental organisms are exposed.

Since low sample volumes are necessary, sample storage is made easier and freezing and thawing faster. An important aspect of NE-(ER-Calux®) assay is analysis time, which without extraction is reduced by approximately 95 %. In addition, less than five minutes are necessary for the sample to be ready for the test. Moreover, uncertainties such as analytes losses (and contaminations) derived from sample handling procedures e.g., sample storage, extraction and evaporation can be avoided. Advantages of the NE-(ER-Calux®) method have been exploited in a study where intra-day (hourly) dynamics of estrogenic potential in a WWTP was investigated.

Besides a simpler sample preparation and reduced analysis time, the method is more appropriate as a pre-screening tool, than conventional ER-Calux® assay, where extensive sample preparation is necessary. However, all the samples that are used in the NE-(ER-Calux®) assay can be temporarily stored and later extracted and analysed by GC-MSD if necessary. A simpler sample preparation procedure and shorter preparation time is an important advantage in environmental analysis that cannot be overlooked, as most often a large number of samples need to be analysed within a short time.

5.3.6 Further work

Combination of NE-(ER-Calux®), ER-Calux® and chemical analysis with GC-MSD was successfully applied. In the future, this combination should be tested on additional wastewater and surface water samples to check the performance on a larger number of samples. The combined method can then be used in a comprehensive investigation of the presence of steroid estrogens in wastewaters during different time intervals (daily, weekly, monthly, yearly, seasonal etc.) and for a detail study of the fate and transport of steroid estrogens during the water treatment process. For this, a combination of biological and chemical approach should be applied on solid samples since wastewater sludge should be investigated as well.

With the introduction of a sample fractionation step, combination of three methods can be used for identification of new estrogenic compounds using either TIE or EDA process (Section 1.6.5), to identify antagonists and agonists in complex samples that will hopefully resolve the observed differences between biological assays and chemical analysis.

6 Conclusions

The ability to determine steroid estrogens in the environment is crucial to defining their potential impact on environmental organisms. In this work, chemical and biological methods were combined into a single protocol, where the same sample can be tested using both methods simultaneously. Moreover, attention was paid to the simplification of sample preparation, which significantly reduces analysis time and material consumption and makes it suitable in those cases, where a large number of samples are required for monitoring.

Chemical analysis was used to study the presence of steroid estrogens in a hospital effluent and a receiving WWTP's influent and effluent. After sampling over six consecutive days to study the inter-day variation of steroid estrogens, especially high levels of E3 (up to 385.5 ng/L), were revealed in all the hospital effluent samples. The remaining steroid estrogens in the hospital effluent were present in similar concentrations to that in the WWTP influent and effluent. The presence of synthetic EE2 was detected only in one influent sample. Concentrations in both, hospital and WWTP samples were comparable to those published in the literature.

Slight day to day variations between the concentration levels of all three natural steroid estrogens in the hospital effluent and WWTP influent and effluent was observed. The same variations were observed between Monday to Friday and between three Mondays. There was also a significant difference in the concentration of steroid estrogens in hospital effluent between working days and Saturdays. The impact of hospital effluent on wastewater in WWTP was not recognised, mostly due to the high dilution factor. Hospitals are an important source of pollution concerning steroid estrogens. In the hospital effluent the E1:E2:E3 ratio was similar to that in pregnant women's urine, while in WWTP influent and effluent, the ratio is comparable to that excreted by non-pregnant women and men.

The variation in wastewater treatment efficiencies was 38 % to 99 %. This discrepancy will require further investigation to avoid either over or underestimating estrogen pollution. The less potent estrogen (E3) must also be incorporated into future environmental monitoring programs and risk assessments since E3 contributes up to 92% of the total estrogenicity of hospital effluent and up to 37 % and 46 % in influent and effluent. Clearly, E3 should be considered as an important pollutant.

Integration of the ER-Calux® assay into the existing analytical methodology enables simultaneous determination of individual steroid estrogen concentrations and an assessment of total estrogenicity. Integration was achieved by exchanging DMSO with EtAc, a solvent compatible with both GC-MSD and the ER-Calux® assay. A comparison of GC-MSD and ER-Calux® assay using mixtures of authentic compounds and spiked wastewater extracts show good correlation with the regression line slope close to optimal; $\alpha=1$.

The integrated protocol was afterwards applied and successfully verified on actual wastewater samples. The integrated protocol enables easy, fast and reliable determination of steroid estrogens and total estrogenic potential in environmental samples. The results

prove that integrated GC-MSD and ER-Calux® enables concurrent determination of both parameters, while reducing material consumption and labour. However, both GC-MSD and ER-Calux® requires time consuming sample preparation.

In the final part of this thesis sample preparation is simplified by modifying the ER-Calux® assay to determine estrogenic potential directly without sample extraction. The reduced time consumption (95 % time saving) of NE-(ER-Calux®) method, low sample volume and simple sample preparation are important advantages and useful for multi-sample studies and environmental monitoring.

The modified method NE-(ER-Calux®) was comparable to conventional ER-Calux® assay and theoretical estrogenic potential (cEEQ) obtained by chemical analysis with GC-MSD as confirmed by using spiked samples. The analysis of "real" waste and surface water samples revealed that generally higher results were achieved with NE-(ER-Calux®) method, possibly due to the ability of the method to detect water soluble estrogenic potential.

The NE-(ER-Calux®) method was also applied to study intra-day variations of estrogenic potential. Estrogenic potential on the day of sampling gradually increased until reaching a peak in the afternoon. An average sample was comparable to a time proportional sample but represents only 62 % of the highest observed estrogenic potential. This is the first evidence of intra-day dynamics of estrogenic potential and this knowledge should improve risk assessment strategies for the presence of steroid estrogens in the environment.

7 Acknowledgements

I would like to thank to everybody that formally or informally help in formation of this doctoral thesis.

Special thanks go to my family for all the support. Thank you for your patience and love.

I would like to thank my mentor ass. prof. dr. Ester Heath for her support in research, writing research papers, and in the preparation of this doctoral thesis.

Thanks also to all colleagues in the laboratory (Silva, Tina, Mojca, Kristina, Marjeta, Mitja, Simona, Tina, Breda, Anže, Tea, Eva, Marko, Darja) for scientific and unscientific debate and the beautiful moments that I spent in the laboratory.

I would like to thank the staff at the National Institute of Biology, especially dr. Bojana Žegura for enormous support and time at the "biological part" of my work.

I also thank the Slovenian Research Agency for young researcher grant.

****

Rad bi se zahvalil vsem, ki so kakorkoli pripomogli k nastajanju te doktorske naloge.

Posebna zahvala gre moji družini za vso podporo, ki sem je bil deležen. Hvala vam za vašo potrpežljivost in ljubezen.

Rad bi se zahvalil tudi mentorici izr. prof. dr. Ester Heath za njeno podporo pri raziskovalnem delu, pisanju raziskovalnih člankov in pri pripravi te doktorske naloge.

Hvala tudi vsem kolegom v laboratoriju (Silva, Tina, Mojca, Kristina, Marjeta, Mitja, Simona, Tina, Breda, Anže, Tea, Tadeja, Eva, Marko, Darja) za znanstveno in neznanstveno razpravo in za lepe trenutke, ki sem jih preživel v laboratoriju.

Zahvalil bi se rad tudi osebju na Nacionalnem Inštitutu za Biologijo, še posebej pa dr. Bojani Žegura za izdatno pomoč pri “biološkem delu” doktorata.

Zahvaljujem se tudi “Agenciji RS za raziskovalno dejavnost” za finančno podporo.

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Journal of Environmental Monitoring

Cite this: *J. Environ. Monit.*, 2011, **13**, 2221

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PAPER

Dynamics of steroid estrogen daily concentrations in hospital effluent and connected waste water treatment plant

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Received 15th February 2011, Accepted 2nd June 2011
DOI: 10.1039/c1em10147a

Hospital effluent and connected waste water treatment plant (WWTP) influent and effluent were sampled daily to determine the levels and inter-day variations of three naturally occurring steroid estrogens: estrone, 17β-estradiol, estriol, and synthetic 17α-ethinylestradiol. After solid phase extraction, interferences were removed with a silica gel clean-up step and the samples analysed using gas chromatography with mass selective detection (GC-MSD). The determined inter-day concentrations in hospital effluent were between 8.6 to 31.3 ng L^{-1} for estrone, <LOD (limit of detection) to 4.2 ng L^{-1} for 17β-estradiol and 6.4 to 385.5 ng L^{-1} for estriol. In the WWTP influent concentrations were 18.9 to 49.7 ng L^{-1} for estrone, 2.4 to 12.7 ng L^{-1}, for 17β-estradiol and <LOQ (limit of quantitation) to 63.9 ng L^{-1} for estriol. Reduced levels were found in the WWTP effluent: <7.1 ng L^{-1} for estrone, <LOQ for 17β-estradiol and <5.2 ng L^{-1} for estriol. 17α-ethinylestradiol was detected in only one influent sample. Calculated estradiol equivalents (EEQ) were 33.4, 22.4, 1.7 ng (EEQ) L^{-1} in the hospital effluent, WWTP influent and WWTP effluent, respectively. Interestingly, the estrone : 17β-estradiol : estriol ratio in the hospital effluent (1 : 0.1 : 9.4) is comparable to that found in the urine of pregnant women (1 : 0.3 : 20) indicating the most likely source of steroid estrogens. In WWTP influent the ratio was similar to that found in the non-pregnant population. Our result recognise estriol as being one of the most important steroid estrogens, accounting for up to 92% of the total EEQ present in hospital samples and 37% and 46% in WWTP influent and effluent samples, respectively. The study reveals how concentrations of steroid estrogens vary on a daily basis and concludes that careful sampling strategies must be adopted when making a risk assessment. In addition, the low potency steroid estrogens that contribute towards overall estrogenicity of the sample, *e.g.* estriol, should be incorporated into environmental monitoring programs.

Introduction

Steroid estrogens are organic pollutants known to be present in the environment for more than 15 years.[1,2] They belong to a group known as endocrine disrupting compounds (EDC), have the highest affinity for the estrogenic receptor[3,4] and can have adverse effects on organisms even at sub ng L^{-1} levels.[1]

Steroid estrogens, albeit naturally synthesized in the human body, can enter the body *via* the use of oral contraceptives and other hormonal therapies where synthetic and natural hormones are used as the active ingredients. Both types are excreted either in the urine and or faeces in a conjugated (inactive) form. Deconjugation, leading to their reactivation, can be initialised by extracellular bacterial enzymes present in the sewerage system and in biological waste water treatment processes.[2,5–7]

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Environmental impact

More and more contaminants with estrogenic potential are known to be present in the environment. Among them, steroid estrogens represent the majority of the estrogenic potential in municipal waste waters. They are of natural source, but through the sewerage system are concentrated at waste water treatment plants, which increases their potential to cause negative effects in the environment. In this study we have investigated hospital effluent that has been revealed as an important source of steroid estrogens for the connected waste water treatment plant (WWTP). Inter-day dynamics of steroid estrogen concentration in hospital effluent and WWTP influent and effluent were studied as well. Altogether this provides important information for understanding the transport and fate of steroid estrogens in the environment.

Domestic wastewater containing excreted steroid estrogens usually arrives at a wastewater treatment plant (WWTP) before being released to receiving surface waters. During treatment approximately 99% are removed by either degradation or adsorption onto solid particles, however, the remaining 1% still poses a risk in the environment.[1,2,8–14] The majority of published investigations concerning waste waters and receiving surface waters focus on the naturally occurring steroid estrogens like estrone (E1), 17β-estradiol (E2), estriol (E3) and on the synthetic 17α-ethinylestradiol (EE2). The data show individual concentrations of 660 ng L^{-1} and 250 ng L^{-1} at influent and effluent sites, respectively.[15]

Domestic discharges are the main source of waste water reaching municipal WWTP but institutions like hospitals and health centres are also important sources. Hospital effluent has been recognized as a source of pollutants including pharmaceuticals, microorganisms, X-ray contrast agents, disinfectants and antibiotics,[16–18] yet only a few publications investigate the presence of both natural and synthetic steroid estrogens. A study of two Norwegian hospitals[19] revealed high levels of E3 (>500 ng L^{-1}) and moderate concentrations of E1 and E2 in effluent. Zorita *et al.* found 150 ng L^{-1} of E1 and 17 ng L^{-1} of E2 in hospital effluent in Sweden,[20] while in Taiwan up to 415 ng L^{-1} (E1), 230 ng L^{-1} (E2), and 432 ng L^{-1} (EE2) were present in six hospital effluent.[21]

Estriol (E3), the natural hormone, excreted by pregnant women in high concentrations[22,23] is often omitted and its contribution towards total estrogenicity neglected. Results from different *in vivo* and *in vitro* tests show that estriol has, compared to E2, a lower affinity (0.01–0.08) to the estrogen receptor.[4,24,25] Despite this, estriol is recognized as the most important endocrine disrupting substance in turtles, having either an equivalent or a higher potency than E2.[26] In addition, estriol is not researched as frequently as the other steroid estrogens in environmental studies. Miège *et al.*[15] compare 117 case studies where estriol was determined in only 36 (33) of the case-studies involving steroid hormones in influent (effluent). The other steroid estrogens—E1, E2 and EE2—were analysed in 109 (79), 108 (63) and 70 (33) case-studies, respectively.

The aims of our research were to investigate the presence and possible sources of both natural (E1, E2 and E3) and synthetic steroid estrogens (EE2) in a hospital effluent and in the connected waste water treatment plant influent and effluent. Sampling was performed daily to observe inter-day variations of concentrations. Special attention was given to estriol, on account of its high excretion levels in pregnant women and its potential contribution towards total estrogenicity. To the authors' knowledge, this is the first attempt to provide important information concerning the fluctuation of steroid estrogens in hospital waste waters and WWTP.

Materials and methods

Chemicals

Estrone (E1; CAS 53-16-7, min 99%), 17β-estradiol (E2; CAS 50-28-2, min 98%), 17α-ethinylestradiol (EE2; CAS 57-63-6, min 98% (HPLC)), estriol (E3; CAS 50-27-1, min 99%), the deuterated internal standard bisphenol A-d_{16} (CAS 96210-87-6, 98 atom% D), derivatization agent *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA; CAS 24589-78-4, derivatization grade) and HCl (CAS 7647-01-0, min 37%) were purchased from Sigma-Aldrich (Steinheim, Germany). Pyridine (max 0.01% H_2O) was purchased from Merck (Darmstadt, Germany), while methanol (CAS 67-56-1), ethyl acetate (CAS 141-78-6) and acetone (CAS 67-64-1) "Baker ultra resi-analysed®" grade, were purchased from J.T. Baker (Deventer, the Netherlands).

Sampling sites and sampling

Samples were collected from hospital effluent and the connected WWTP, where both the influent and effluent were sampled. Effluent from the hospital (9628 patients hospitalized in 2007) derives from the main building, where the diagnostic laboratory, internal medicine wards, and surgery are located. The hospital's waste water (80 000 m^3 $year^{-1}$ in 2007) enters the sewerage system that terminates at the WWTP (105 000 PU). Distance from the hospital to the WWTP is approximately 1.5 km. The WWTP incorporates mechanical and biological treatment processes with anaerobic stabilization of the waste sludge. In 2007 approximately 5 400 000 m^3 of waste water passed through the WWTP.

Hospital effluent, WWTP influent and effluent samples were sampled daily to assess the inter-day variation (January 2007) over a 6 day period (Monday to Saturday). The samples were collected in polyethylene (PE-HD) bottles (1 L), immediately acidified with HCl to pH 2.5 and frozen prior to analysis. The hospital samples were time proportional (24 h) while the WWTP samples were flow proportional. Additional samples were taken on a Monday during two separate weeks in December 2006. Transportation times from the hospital to the WWTP (24 h) and hydraulic retention times at WWTP (24 h) were taken into account during sampling and data analysis.

Sample handling

All glassware was cleaned with organic solvents prior to use and heated at 400 °C to remove organic contamination. Prior to extraction, samples were defrosted and filtered through a glass fibre filter (GF/C, Whatman, Maidstone, England) and a nylon filter with a pore size of 0.45 μm (Supelco; Bellefonte, ZDA). 50 μL of internal standard (bisphenol A-d_{16}, $c = 0.2$ mg L^{-1}) was then added to 200 mL of each sample—the final concentration was 50 ng L^{-1}. The samples were extracted (200 mL) using solid phase extraction (SPE) cartridges containing an Oasis HLB reversed-phase sorbent; 3 cc/60 mg (Waters; Milford, ZDA). The sorbent was preconditioned with 3 mL ethyl acetate, 3 mL methanol and 3 mL MilliQ water acidified to pH ~ 2.5. The sample flow rate was maintained between 3–5 mL min^{-1}. After extraction, the cartridges were dried (air) and stored at −20 °C.

Elution was performed with 3 mL of ethyl acetate. The eluents were evaporated under a stream of nitrogen to *ca.* 0.5 mL. A clean-up step using silica gel was introduced to remove any potential interferences arising from the waste water samples. ISOLUTE cartridges filled with 500 mg of silica gel (Biotage, Uppsala, Sweden) were conditioned with 4 mL of ethyl acetate and 0.5 mL of each sample was added to the cartridge. The analytes were eluted from the silica gel using ethyl acetate/acetone (2% *v*/*v*). The purified samples were reduced to dryness

(N_2). If the samples were not derivatised on the same day, evaporation was stopped at 0.5 mL and the samples were stored at 4 °C for a maximum of 24 h. The solvent was completely removed prior to derivatization with 50 μL MSTFA : 50 μL pyridine mix. The samples were heated at 60 °C for 30 min before being cooled in a refrigerator and finally transferred to vials with 100 μL inserts. Analyses were always performed on the same day as derivatization to avoid decomposition.

Analysis

The derivatised sample extracts were analysed using gas chromatography with mass selective detection (HP 6890 Series, Hewlet-Packard, Waldbron, Germany). Separation was achieved using a 30 m × 0.25 mm × 0.25 μm, HP5-MS capillary column with Helium as the carrier gas (1 mL min^{-1}). The injector temperature was maintained at 270 °C and the interface at 280 °C; 1 μL of sample was injected in splitless mode. Electron ionisation mode (70 eV) was used. The GC temperature program was initially set at 100 °C and then ramped to 230 °C at 20 °C min^{-1} (held for 2 min) and again ramped to 280 °C at 15 °C min^{-1} (held for 5 min). SIM mode with the following characteristic ions was used for quantification: E1 (*m/z*: **342**, 257, 218), E2 (*m/z*: **416**, 285), EE2 (*m/z*: 425, **285**), E3 (*m/z*: 505, **504**, 311), bisphenol A-d_{16} (*m/z*: **368**, 386) (quantification ions are in bold). SCAN mode was performed at an *m/z* ratio from 50 to 500.

Method validation

The method was evaluated in terms of linearity, extraction efficiency, repeatability, limits of detection and limits of quantification for each compound of interest (Table 1). A method blank was also prepared. To account for any possible matrix effects, method validation was performed using spiked WWTP effluent. Quantification was achieved using bisphenol A-d_{16}, an internal standard, the relative response factor of which had been checked previously (data not shown). Six point calibration curves between 2–610 ng L^{-1}, were constructed and the method showed good linearity for all four compounds (Table 1).

Both extraction efficiency and repeatability were checked using 50 ng L^{-1} of each compound. Even though acetone gives the best extraction efficiency, ethyl acetate was chosen since it is compatible with the clean-up solvent and eliminates the need for two additional evaporation steps.

Limits of detection and quantification were calculated as 3 times and 10 times the standard deviation of the blank samples, respectively. All the calibration samples were blank corrected *e.g.* present target compound was deducted under the condition that there was a satisfactory regression factor ($R^2 > 0.99$).

17β-estradiol equivalents (EEQ)

The estrogenic potency of different steroid estrogens depends on the type of *in vitro* and *in vivo* test. As a "worst case scenario", we chose the highest reported potencies (estrogenic equivalency factors—EEF) as reported in the review by Campbell *et al.*[25] The LOD and LOQ values were taken in cases where the concentrations were lower than the LOD or LOQ. Since EE2 was below the LOD in all the samples, the contribution of EE2 would be the same in all samples and thus provides no valuable information to the calculated results except that the total EEQ of all the samples would increase by 3.8 ng (EEQ) L^{-1}. For this reason, EE2 was excluded from the calculation of the total EEQ.

Total 17β-estradiol equivalents (EEQ) were calculated using the following equation:

$$\text{total EEQ (ng L}^{-1}) = c(\text{E1}) \times \text{EEF(E1)} + c(\text{E2}) \times \text{EEF(E2)} + c(\text{E3}) \times \text{EEF(E3)} \quad (1)$$

Where the values of EEF(E1) = 0.1, EEF(E2) = 1.0, EEF(E3) = 0.08, were taken from the literature.[25] The concentrations *c*(E1), *c*(E2) and *c*(E3) were taken from Table 2.

Results and discussion

Low concentrations of steroid estrogens in the environment, especially in complex matrices like waste water, demand sophisticated and sensitive analytical methods. In our study, in addition to GC-MSD (SIM mode), also a clean-up step was applied to remove interferences. The importance of clean-up step is evident in Fig. 1, which shows clear improvement in the determination of E1 and EE2 when the clean-up step was used. By introducing this additional step more reliable determination of the steroid estrogens in hospital effluents and waste water samples was achieved.

In hospital effluent, the three natural steroid estrogens, E1, E2 and E3 were present in all the samples we analysed (Table 2). From Monday to Friday, concentrations were between 14.0 and 31.3 ng L^{-1} for E1, <LOQ and 2.8 ng L^{-1} for E2 and 152 and 385.5 ng L^{-1} for E3, while on Saturday, concentrations were lower, especially for E3 (6.4 ng L^{-1}). Fluctuations between three samples taken on Monday (Table 2) were also in the same range as Monday to Friday variations, 10.9–31.3 ng L^{-1}, <LOD to 4.2 ng L^{-1}, 39.1–254.0 ng L^{-1} for E1, E2 and E3, respectively. Synthetic EE2 was below the detection limits in all samples. The

Table 1 Results of validation parameters[a]

	E1	E2	EE2	E3
Extraction efficiency (at 50 ng L^{-1}) (%)	81	91	87	94
Linearity range (ng L^{-1})	2–610	2–610	2–610	2–610
R^2 (calibration curve)	0.9986	0.9980	0.9963	0.9988
Interday repeatability (RSD %)	2	3	8	9
LOD (ng L^{-1})	0.2	0.4	2.0	1.4
LOQ (ng L^{-1})	0.7	1.2	6.5	4.5

[a] RSD – relative standard deviation; LOD – limit of detection; LOQ – limit of quantitation.

Table 2 Steroid estrogen concentrations in hospital and WWTP samples[a]

		E1 (ng L^{-1})			E2 (ng L^{-1})			EE2 (ng L^{-1})			E3 (ng L^{-1})		
Date	Day	H	WWinf	WWeff	H	WWinf	WWeff	H	WWinf	WWeff	H	WWinf	WWeff
11.12.06	Mon	10.9	23.8	7.1	<LOD	2.5	<LOD	<LOD	<LOD	<LOD	39.1	<LOQ	4.6
18.12.06	Mon	17.5	18.9	0.8	4.2	2.4	<LOQ	<LOD	<LOD	<LOD	86.7	9.1	<LOQ
08.01.07	Mon	31.3	24.2	<LOQ	1.4	4.8	<LOD	<LOD	<LOD	<LOD	254.0	37.7	<LOD
09.01.07	Tue	14.0	19.7	<LOQ	<LOQ	4.0	<LOQ	<LOD	<LOD	<LOD	385.5	24.7	5.2
10.01.07	Wed	22.3	49.7	<LOD	2.8	3.5	<LOD	<LOD	<LOD	<LOD	276.2	60.8	<LOQ
11.01.07	Thr	15.5	31.0	0.8	<LOQ	9.9	<LOQ	<LOD	<LOQ	<LOD	152.0	25.6	<LOQ
12.01.07	Fri	20.9	20.7	<LOD	<LOQ	5.7	<LOD	<LOD	<LOD	<LOD	175.2	7.3	<LOQ
13.01.07	Sat	8.6	46.0	<LOD	<LOQ	12.7	<LOQ	<LOD	<LOD	<LOD	6.4	63.9	<LOQ

[a] H – Hospital; WWinf – waste water influent; WWeff – waste water effluent.

above concentrations, including high E3 concentrations, are comparable to published data.[19-21] The reason for the lower Saturday concentrations, especially in the case of estriol, is most likely due to working practices, since at the weekend the hospital is limited to accident and emergency. This effect is expected to be expressed if Sunday samples were available; however, due to technical difficulties they were not available.

The source of high E3 concentrations in hospital effluent from Monday to Friday is likely in pregnant women who are known to excrete 3000 times more E3 and 100 times more E1 and E2 compared to non-pregnant women and men.[23,27] Since hospital effluent contains waste water from other hospital sources other than human excreta, it is difficult to compare the absolute concentrations of steroid estrogens in waste water to the amounts excreted by pregnant women. For this reason, we have calculated the ratio of E1 : E2 : E3 using our results and compared it to the available data for excreted steroid estrogen concentrations by women during different stages of life.[23] The resultant ratio can then be used to define possible sources of steroid estrogens (Table 3). The approximate ratio E1 : E2 : E3 in hospital effluent was about 1 : 0.1 : 10, a value similar to that found in pregnant women's urine (1 : 0.3 : 20.2), especially when regarding the contribution made by E3. The most likely source is therefore the diagnostic laboratory, where urine samples from patients including pregnant women are analysed and discarded to sewerage system. On Saturday, when the laboratory is open only for emergency cases, the E1 : E2 : E3 ratio (1 : 0.2 : 0.75) is similar to that found in men or premenopausal women (1 : 0.4 : 0.8). The likely source in this case is from excreta of employees and hospitalised patients where pregnant women represent a minority over weekend since they could not be hospitalised in the observed hospital, which explains the low Saturday concentrations of E3 as well.

Beside the absolute concentrations of the target compounds and their source, total estrogenicity (estradiol equivalents, EEQ) is of great importance. Since we could not perform an estrogenicity assay, we estimated the estrogenicity (expressed in total estradiol equivalents—EEQ) using published[25] estrogen equivalency factors. However, our results are most likely underestimated since other compounds with estrogenic activity might be present in hospital effluent[28] and WWTP influent and effluent samples.[25] The calculated total EEQs of the daily samples was 2.6 to 33.4 ng (EEQ) L^{-1} in which the contribution of E3 varied from 20–92% (Table 4). The high total EEQ indicates that hospital effluent should be considered as an important source of estrogenic pollution. Furthermore, estriol should also, due to its high concentrations in hospital effluent, be considered as an important pollutant despite its lower potency at the estrogenic receptor.

The reported data of steroid estrogen concentrations in WWTPs summarised by Miège *et al*[15] are comparable to those reported in this paper, *i.e.*, from 18.9–49.7 ng L^{-1}, 2.4–12.7 ng

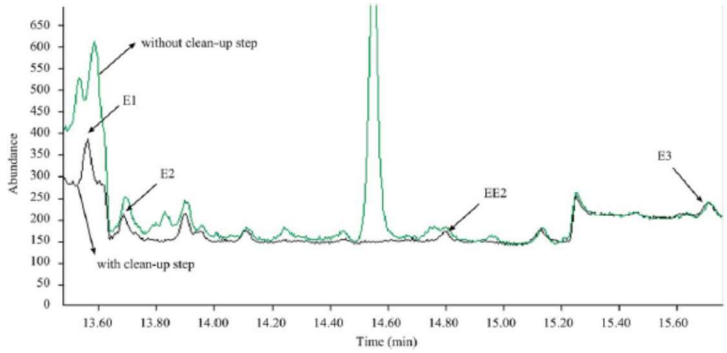

Fig. 1 Comparison of spiked (c = 10 ng L^{-1}) waste water effluent sample chromatograms (SIM mode) with and without clean-up step.

Table 3 Ratio of steroid estrogens in environmental samples and excreted urine

Sample	E1 : E2 : E3	Source for calculation
Hospital effluent—average	1 : 0.1 : 9.4	our results
Hospital effluent—Saturday	1 : 0.2 : 0.75	our results
WWTP influent—average	1 : 0.2 : 0.9	our results
Premenopausal women urine	1 : 0.4 : 0.8	Liu *et al.*[23]
Postmenopausal women urine	1 : 0.6 : 0.6	Liu *et al.*[23]
Pregnant women urine	1 : 0.3 : 20.2	Liu *et al.*[23]
Men urine	1 : 0.4 : 0.4	Liu *et al.*[23]

Table 4 Calculated total EEQs for the sampling sites and the contribution of E3[a]

		ng (EEQ) L^{-1}			E3 contribution		
Date	Day	H	WWinf	WWeff	H	WWinf	WWeff
11.12.2006	Mon	4.6	5.2	1.5	68%	7%	25%
18.12.2006	Mon	12.9	5.0	1.6	54%	15%	22%
08.01.2007	Mon	24.9	10.3	0.6	82%	29%	19%
09.01.2007	Tue	33.4	8.0	1.7	92%	25%	25%
10.01.2007	Wed	27.1	13.3	0.8	82%	37%	46%
11.01.2007	Thr	14.9	15.1	1.6	82%	14%	22%
12.01.2007	Fri	17.3	8.3	0.8	81%	7%	46%
13.01.2007	Sat	2.6	22.4	1.6	20%	23%	23%

[a] H – Hospital; WWinf – waste water influent; WWeff – waste water effluent.

L^{-1}, <LOQ–63.9 ng L^{-1} for E1, E2 and E3, respectively. The effluent concentrations were ≤7.1 ng L^{-1} for E1, <LOQ for E2 and ≤5.2 ng L^{-1} for E3. Despite expecting that EE2 would be present in hospital and waste waters, it was detected only in one influent sample, where the concentration was still below the LOQ. EE2 is an important steroid estrogen with high activity and might make an important contribution to the total estrogenicity of a sample, however our LOD for EE2 (2.0 ng L^{-1}) was higher than the median concentration found in the database (1.9 ng L^{-1} in influent and 0.5 ng L^{-1} in effluent).[15] This suggests that EE2 is probably present in concentrations below our LOD. In comparison to hospital effluent, much higher concentrations of E3 in the hospital effluent than in the inflow of the municipal WWTP were observed, while other steroid estrogens were in similar concentrations (Table 2). In contrast to hospital effluent, the ratio of E1 : E2 : E3 in the influent samples (1 : 0.2 : 0.9) was comparable to the ratio for men and non-pregnant women (1 : 0.4 : 0.8), which is expected for waste water that is predominantly from domestic sources. This suggests that the contribution of estrogens from the hospital effluent to the total amount in the WWTP influent is negligible due to the high dilution factor of hospital effluent at the inflow of the WWTP (×70).

Table 5 summarises the removal rates of steroid estrogens from the water phase on different days. Up to 99% of the steroid estrogens are eliminated from the water phase, but there are samples where the removal was <80% and even <40%. Only one sample (Monday, 11.11.2006) actually exhibited higher concentrations of estriol in the effluent than in the influent. Removal rates are comparable to those found in several WWTPs reported in the literature, where removal rates were 55–99%, 40–99%, 64–85% and 82–97% for E1, E2, EE2 and E3, respectively.[29] Reasons for the observed lower removal rates or even higher concentrations at the effluent sites are likely to be the following: the poor removal efficiency of the treatment process, adsorption and desorption processes, the deconjugation of steroid estrogens during treatment that increases the concentration of active estrogens in the effluent and/or the presence of free steroid estrogens that were not detected in the influent samples.[6,11]

Table 5 Removal rates at the WWTP[a]

Date	Day	E1 (ng L^{-1})	E2 (ng L^{-1})	E3 (ng L^{-1})
11.12.2006	Mon	70%	>84%	>−2%
18.12.2006	Mon	96%	>50%	>51%
08.01.2007	Mon	>97%	>92%	>96%
09.01.2007	Tue	>96%	>70%	79%
10.1.2007	Wed	>99%	>88%	>93%
11.1.2007	Thr	97%	>88%	>82%
12.1.2007	Fri	>99%	>93%	>39%
13.1.2007	Sat	>99%	>91%	>93%

[a] >Indicates worst case scenario, since LOD or LOQ concentrations were used in calculations.

Calculated EEQs (Table 4) vary from 5.2–22.4 ng (EEQ) L^{-1} in the influent samples and from 0.6–1.7 ng (EEQ) L^{-1} in the effluent samples of which E3 contributes up to 37% in the influent and 46% in the effluent. The removal of EEQ at WWTP was from 72–94%. Results show, that the estrogenic potential of hospital effluent was equivalent to or higher than the inflow at the municipal WWTP except on Saturday. Hospital effluent estrogenic potential is however diluted 70 times at the WWTP, but these results suggest that hospital effluent is an important source of steroid estrogens and estrogenic potential.

In the WWTP samples, the concentration variability between weekdays is the same as for five consecutive days (Monday to Friday) and for three consecutive Mondays. Nevertheless, concentrations on Saturday are higher, without any reasonable explanation. The concentration trend in the WWTP influent does not follow that observed in the hospital effluent. In addition, waste water treatment removal of steroid estrogens varies from 50% up to 99%.

Periodical lower removal rates of steroid estrogens in WWTP lead to a higher amount of pollution in receiving waters and understanding the daily or even more frequent concentration variations is of great importance. Steroid estrogens have high biological activity and therefore, for environmental organisms, episodes, where removal rates are much lower and consequently higher concentrations reach surface waters, are significant. Again, such frequency can be understood only by obtaining detailed information about the dynamics of estrogen concentrations at the WWTP influent and effluent site. In order to draw some definite conclusions, an extended sampling campaign, including inter week and seasonal variations, should be performed. Alternatively, more efficient waste water treatment technologies with regard to estrogen compound removal should be considered when designing waste water treatment.

Special attention should also be given to estriol, which is often ignored in the literature. Our results show that estriol, despite its low estrogenicity, accounts for the majority (up to 92%) of the total estrogenic potential of hospital effluent and up to 46% in

the WWTP effluent analysed. Concentrations up to 385 ng L^{-1} seem extremely high, but are in agreement with those reported by Thomas *et al.*[19] in Norwegian hospitals and as revealed in the database of Miège *et al.*[15] This shows that our research is not only of local interest but that hospital effluent and particularly the "pregnancy hormone"—estriol—should be investigated more carefully particularly when hospital waste waters are treated in municipal WWTPs.

Conclusions

In this paper hospital effluent and connected WWTP were investigated for the presence of steroid estrogens. Sampling was performed over six following days to study inter-day variations in concentrations. Hospitals were recognised as an important source of pollution concerning steroid estrogens. This study shows that steroid estrogens, especially high levels of estriol (up to 385.5 ng L^{-1}) were present in all the hospital effluent samples analysed while the concentration of steroid estrogens in the WWTP samples were comparable to those published in the literature. The presence of synthetic EE2 was detected only in one influent sample. The E1 : E2 : E3 ratios were calculated to recognise the possible source of steroid estrogens. Hospital effluent ratio exhibit similarities with ratios in pregnant women urine, while in WWTP influent and effluent, E1 : E2 : E3 is comparable to non-pregnant women and men. Slight day to day variations between concentration levels of all three natural steroid estrogens in the hospital effluent (*e.g.* 6.4–385.5 ng L^{-1} for E3) and WWTP influent (7.3–63.9 ng L^{-1}) and effluent (<LOD–5.2 ng L^{-1}) were observed. The same variations were observed between Monday to Friday and between three Mondays. However, as expected a significant difference was shown in the concentration of steroid estrogens in hospital effluent between working days and Saturdays. The impact of hospital effluent on waste water in WWTP was not recognised, mostly due to a high dilution factor. Importantly, variations also exist in waste water treatment efficiencies (50 to 99%) a fact that requires further investigation when planning any future sampling campaign to avoid either over or underestimation of estrogen pollution. Finally, the less potent estrogen (E3) must be incorporated into future environmental monitoring programs and risk assessments since estriol in our research contributes to 20–92% of the total estrogenicity of hospital effluent and up to 37% and 46% in influent and effluent. Clearly, estriol should be considered as an important pollutant, not only in hospital effluent but also in WWTP samples.

Acknowledgements

Financial support of the Ministry of Higher Education, Science and Technology and EU Structural Funds for supporting the Centre of Excellence Environmental Technologies (Contract No. 3211-05-000184), Slovenian Research Agency Program group P1-0143, Projects L1-6552 and J1-0005 and young researcher grant to Miha Avberšek, are acknowledged.

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Science of the Total Environment 409 (2011) 5069–5075

Contents lists available at SciVerse ScienceDirect

Science of the Total Environment

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

Integration of GC-MSD and ER-Calux® assay into a single protocol for determining steroid estrogens in environmental samples

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ARTICLE INFO

Article history:
Received 9 June 2011
Received in revised form 3 August 2011
Accepted 14 August 2011
Available online 15 September 2011

Keywords:
Steroid estrogens
ER-Calux® assay
Gas chromatography-mass selective detection
Waste water

ABSTRACT

There are many published studies that use either chemical or biological methods to investigate steroid estrogens in the aquatic environment, but rarer are those that combine both. In this study, gas chromatography with mass selective detection (GC-MSD) and the ER-Calux® estrogenicity assay were integrated into a single protocol for simultaneous determination of natural (estrone – E1, 17β-estradiol – E2, estriol – E3) and synthetic (17α-ethinylestradiol – EE2) steroid estrogens concentrations and the total estrogenic potential of environmental samples. For integration purposes, several solvents were investigated and the commonly used dimethyl sulphoxide (DMSO) in the ER-Calux® assay was replaced by ethyl acetate, which is more compatible with gas chromatography and enables the same sample to be analysed by both GC-MSD and the ER-Calux® assay. The integrated protocol was initially tested using a standard mixture of estrogens. The results for pure standards showed that the estrogenicity calculated on the basis of GC-MSD and the ER-Calux® assay exhibited good correlation ($r^2 = 0.96$; $\alpha = 0.94$). The result remained the same when spiked waste water extracts were tested ($r^2 = 0.92$, $\alpha = 1.02$). When applied to real waste water influent and effluent samples the results proved ($r^2 = 0.93$; $\alpha = 0.99$) the applicability of the protocol. The main advantages of this newly developed protocol are simple sample handling for both methods, and reduced material consumption and labour. In addition, it can be applied as either a complete or sequential analysis where the ER-Calux® assay is used as a pre-screening method prior to the chemical analysis.

1. Introduction

The presence of steroid estrogens in the environment has garnered much attention due to their adverse effects on living organisms at low (ng/L) concentrations (Sumpter and Jobling, 1995). The principal pathway by which natural (estrone – E1, 17β-estradiol – E2, estriol – E3) and synthetic (17α-ethinylestradiol – EE2) steroid estrogens enter the environment is through human excreta. Estrogenic compounds, originally in a non-active conjugated form, are transported *via* the sewerage system to municipal waste water treatment plants (WWTP) where they are re-activated (deconjugated) during biological treatment processes (D'Ascenzo et al., 2003). However, their removal during water treatment is variable (40–99%) (Teske and Arnold, 2008), and the residuals are released to the receiving surface waters (Ternes et al., 1999; D'Ascenzo et al., 2003). A database of 117 case studies created by Miege et al. (2009a) reveals that concentrations of active steroid estrogens may be as high as 670 ng/L with a median concentration of 69 ng/L and 285 ng/L (median 10 ng/L) in WWTP influent and effluent, respectively. As well as being ubiquitous, steroid estrogens pose very high activity when compared to other (xeno-)estrogens and therefore account for >90% of the total estrogens activity of municipal waste water samples (Aerni et al., 2004; Furuichi et al., 2004; Salste et al., 2007; Miège et al., 2009b).

The detection of steroid estrogens in environmental matrices is based on either chemical analysis or biological assays. The most commonly used analytical method includes extraction, separation using either gas or liquid chromatography and detection with mass spectrometry (Gabet et al., 2007). However, the results only provide discrete information about sample composition and concentrations of individual compounds and does not account for potential mixture interactions. The presence of unknown compounds is also not considered despite their potential effect on the overall estrogenic activity. The alternative is to measure the estrogenicity of the samples for which *in vivo* and *in vitro* assays are employed (Campbell et al., 2006). *In vitro* assays are capable of estimating the total estrogenic potential with the added advantage of being low cost and labour saving (Routledge and Sumpter, 1996; Legler et al., 1999). Commonly used *in vitro* assays include the Yeast Estrogen Screen – YES (Routledge and Sumpter, 1996; Murk et al., 2002; Aerni et al., 2004; Nelson et al., 2007), other recombinant yeast assays (Sanseverino et al., 2005; Salste et al., 2007) and the cell-based assays: E-Screen (Soto et al., 1995), MVLN (Pons et al., 1990), MELN (Balaguer et al., 1999; Miège et al., 2009b), and ER-Calux® (Legler et al., 1999). For the purposes of this

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doi:10.1016/j.scitotenv.2011.08.020

study we have applied ER-Calux® assay, which is a sensitive cell-based estrogenicity assay originally used to determine endocrine disrupting compounds in sediments (Legler et al., 2002; Houtman et al., 2006, 2007) and in waste and surface waters (Murk et al., 2002). Since chemical analysis and bioassays provide different, but compatible information about steroid estrogens and estrogenicity, their integrated application is desirable, resulting in fast and qualitative identification and characterization of estrogenicity in environmental samples (Furuichi et al., 2004; Nelson et al., 2007; Streck, 2009).

A number of studies are published employing different combinations of chemical analysis and *in vitro* assays (Legler et al., 2002; Murk et al., 2002; Aerni et al., 2004; Furuichi et al., 2004; Rutishauser et al., 2004; Houtman et al., 2006; Nelson et al., 2007; Salste et al., 2007; Tan et al., 2007; Lee et al., 2008; Bicchi et al., 2009; Liscio et al., 2009; Miège et al., 2009b; Oh et al., 2009; Balsiger et al., 2010; Brix et al., 2010; Wang et al., 2011). In most studies, when comparing the results of both methods, estradiol equivalents were calculated from concentration data (cEEQ) and compared to estradiol equivalents determined by an estrogenicity assay (EEQ). In those studies where detailed comparisons of calculated and bioassay derived EEQs are available, correlation coefficients of $r^2 \leq 0.85$ are reported (Aerni et al., 2004; Nelson et al., 2007; Tan et al., 2007; Bicchi et al., 2009; Liscio et al., 2009; Miège et al., 2009b; Wang et al., 2011). Additionally only two of them report a slope of the regression line, a factor that defines agreement of the method results, close to optimal ($\alpha = 1$) (Brix et al., 2010; Wang et al., 2011). The observed disagreements between chemical analysis and bioassays in these studies are explained by the presence of unknown compounds or by synergistic and antagonistic effects.

The aim of this study was to integrate chemical analysis with ER-Calux® assay to create a novel single protocol to investigate the presence and impact of estrogenic compounds in natural and waste waters. To achieve this, the protocol was optimised using standard mixtures of steroid estrogens E1, E2, E3 and EE2 in environmentally relevant concentrations. The method was then applied to real waste water influent and effluent samples.

2. Materials and methods

2.1. Standards and chemicals

Estrone (E1; CAS 53-16-7, min 99%), 17β-estradiol (E2; CAS 50-28-2, min 98%), 17α-ethinylestradiol (EE2; CAS 57-63-6, min 98% (HPLC)), estriol (E3; CAS 50-27-1, min 99%), a deuterated internal standard (bisphenol A)-d_{16} (CAS 96210-87-6, 98 at.% D), the derivatizing agent N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA; CAS 24589-78-4, derivatization grade), dimethyl sulphoxide (DMSO, 99.9%, CAS 67-68-5) and absolute ethanol (CAS 64-17-5) were purchased from Sigma (Steinheim, Germany). Pyridine (max 0.01% H_2O) was purchased from Merck (Darmstadt, Germany). Methanol (CAS 67-56-1), ethyl acetate (CAS 141-78-6) and acetone (CAS 67-64-1) "Baker ultra resi-analysed®" grade, and acetonitrile (CAS: 75-05-8) in "Baker HPLC analysed" were purchased from J.T. Baker (Deventer, the Netherlands). All standards were prepared freshly in methanol. Standards for the ER-Calux® assay were prepared separately in ethyl acetate or DMSO and were used for bioassay purposes only.

2.2. Growth media and chemicals for the ER-Calux® assay

Stripped FBS (Foetal Bovine Serum), media Gibco® D-MEM/F-12 with GlutaMAX™ and phenol red, Gibco® D-MEM/F-12 with L-Glutamine and without phenol red were purchased from Invitrogen (Paisley, UK). FBS and PBS (Phosphate Buffered Saline) were purchased from PAA (Pasching, Austria), non-essential amino acids (MEM 100×), EDTA (CAS 6381-92-6), and penicillin/streptomycin were purchased from Sigma (Steinheim, Germany), Difco trypsin was purchased from Becton Dickinsen (Heidelberg, Germany).

2.3. Sample extraction

Grab samples (1 L) of waste water influents and effluents from three municipal WWTPs with conventional waste water treatment were collected: WWTP1 (50 000 population units — PU, 6 100 000 m^3/year in 2009), WWTP2 (360 000 PU, 30 000 000 m^3/year in 2009) and WWTP3 (100 000 PU, 7 300 000 m^3/year in 2009). Samples were extracted by solid phase extraction (SPE) and afterwards analysed using the integrated protocol. Samples from WWTP3 were also used for optimization and were spiked after the extraction.

Solid phase extraction (SPE) and the subsequent clean-up step were performed as described elsewhere (Avberšek et al., 2011). Briefly, 200 mL of each sample was extracted with Oasis HLB cartridge (Waters, Milford, USA) and further cleaned-up with 500 mg silica gel ISOLUTE cartridge (Biotage, Uppsala, Sweden). The extracts were then transferred to glass vials, dried and stored at −20 °C prior to analysis (see Section 2.4).

2.4. Test sample preparation

Samples for method optimization were prepared as a mixture of E1, E2, E3 and EE2 standard solutions and spiked waste water extracts from WWTP3: influent and effluent (Table 1). In the case of the latter, standards were added to glass vials, containing dried waste water influent and effluent extracts (as described in Section 2.3). For each spiked sample, the methanol was evaporated and the residue reconstituted with 1 mL of ethyl acetate. For solvent compatibility testing (see Section 2.5), dried waste water sample extracts were reconstituted with 1 mL of ethyl acetate or DMSO. From each sample a small aliquot of reconstituted extract (50 μL) was used for the ER-Calux® assay and the remainder for chemical analysis.

Table 1 gives the spiked concentration of each compound. Quantities of steroid estrogens were chosen randomly with the aim to equally distribute predicted E2 equivalents in the sample mixtures over the

Table 1
Concentrations of steroid estrogens in standard mixtures and spiked waste water influent and effluent extracts.

	Sample name	ng/L			
		E1	E2	EE2	E3
Standards without waste water extract	St1	0	30.0	0	50.0
	St2	0	20.0	0	50.0
	St3	10.0	20.0	0	50.0
	St4	20.0	30.0	0	50.0
	St5	20.0	20.0	0	50.0
	St6	30.0	30.0	0	50.0
	St7	30.0	20.0	0	50.0
	St8	15.0	10.0	0	50.0
	St9	15.0	10.0	0	50.0
	St10	15.0	10.0	0	50.0
Standards with effluent extracts	Eff0	0	0	0	0
	Eff1	3.0	3.0	3.0	3.0
	Eff2	10.0	10.0	10.0	10.0
	Eff3	17.0	17.0	17.0	17.0
	Eff4	24.0	24.0	24.0	24.0
	Eff5	30.0	30.0	30.0	30.0
	Eff6	40.0	0	0	40.0
	Eff7	27.0	7.0	0	54.0
	Eff8	20.0	14.0	7.0	50.0
Standards with influent extracts	Inf1	10.0	10.0	10.0	10.0
	Inf2	24.0	24.0	24.0	24.0
	Inf3	15.0	10.0	0	50.0
	Inf4	15.0	10.0	0	50.0
	Inf5	15.0	10.0	0	50.0

Concentrations are presented as mentioned in the end of Section 2.4.

desired concentration range. The concentrations were also in the range of actual environmental concentrations.

The pure standards and spiked waste water samples were not extracted. However, the concentrations in Table 1 are presented as if they were extracted in the same way as the real water samples (from 200 mL of water sample). This allows the values to be compared with real sample data, *e.g.* — concentration 1 μg/L of pure standard in ethyl acetate (V = 1 mL) is equivalent to 5 ng/L in water sample (V = 200 mL).

2.5. Solvent compatibility

Solvent compatibility was evaluated by studying cell viability using the MTS assay. To test the influence of solvent on the ER-Calux® assay, dose response of standards in ethyl acetate or DMSO and real sample extracts, reconstituted with ethyl acetate or DMSO were tested.

a) MTS assay

Various solvents including DMSO, ethyl acetate, acetone, methanol and ethanol in 0.1%; 0.2% and 0.5% concentrations in the test medium were examined using the MTS assay to study their influence on the ER-Calux® cells. For the MTS assay (Cell Titer 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega, Madison, USA), a mixture of MTS tetrazolium compound ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and PMS (phenazine methosulfate) solutions (20:1) were prepared. Cells were cultivated in microtiter clear plates (Nunc, Roskilde, Denmark) in the same way as for the ER-Calux® assay and exposed to the test solvents. After a 24 h exposure time, 20 μL of freshly prepared MTS:PMS reagent was added directly to the 100 μL of medium in the culture wells. After a further 3 h of incubation (37 °C; 5% CO_2 atmosphere), absorbance (λ = 490 nm) was measured using a Tecan Genios spectrofluorimeter (Maennedorf, Switzerland).

Survival (viability) was determined by comparing the OD values of the solvent exposed cells with those of unexposed cells and presented as % of cell viability ± SD. The cytotoxicity was measured in five replicates per treatment point. A Student's *t*-test was used to compare cell survival between unexposed cells and solvent exposed cells; $p<0.05$ was considered as statistically significant.

b) Dose response

To determine the different effects of ethyl acetate and DMSO on ER-Calux® performance, standard solutions of E2 (0.6 to 30 pM) were prepared using each solvent and tested with the ER-Calux® assay (see Section 2.6).

c) Real samples

Influents and effluents from WWTP1, WWTP2 and WWTP3 were extracted in parallel (see Section 2.3). After extraction one sample was reconstituted in 1 mL ethyl acetate, and the other in DMSO. All samples were afterwards tested with the ER-Calux® assay (see Section 2.6), together with the E2 standard solution in the same solvent as the samples.

2.6. ER-Calux® assay

The T47D-EREtata-Luc cells used in the ER-Calux® assay were provided and licenced by BioDetection Systems b.v. (BDS), Amsterdam, Netherlands. The assay was performed according to the BDS protocol but with certain modifications. Briefly, cells were routinely maintained in the "Growth medium" D-MEM/F12 with GlutaMAX™ containing a phenol red as pH indicator and supplemented with 7.5% FBS, 1% non-essential amino acids (MEM) and a 0.2% penicillin/streptomycin solution (5000 U/mL penicillin and 5000 U/mL streptomycin). Cells were grown at 37 °C in 5% CO_2.

For the assay purposes the "Growth medium" was replaced with the "Test medium" containing D-MEM/F12 medium with L-glutamine without phenol red and supplemented with 5% stripped FBS, 1% non-essential amino acids (MEM) and 0.2% penicillin/streptomycin solution (5000 U/mL penicillin and 5000 U/mL streptomycin). The cells in the "Test medium" were seeded at a density of 10 000 cells/well into 96-well white microtiter plates (Nunc, Roskilde, Denmark). After 24 h incubation at 37 °C the medium was replaced with fresh "Test medium" and incubated for an additional 24 h. Subsequently, the cells were exposed to 100 μL of the "Test medium" supplemented with either the diluted test samples or the calibration standard (final solvent concentration 0.1%). After 24 h of exposure, 100 μL of the luminescence kit "Steadylite plus" (Perkin Elmer, Shelton, USA) was added to the medium and gently shaken for 15 min at room temperature. Luminescence was measured using a luminometer with a set integration time of 4000 ms and a gain of 200 (Tecan Genios, Maennedorf, Switzerland).

For calibration purposes, the E2 standard solution in a concentration range from 0.6 to 30 pM was included in every microtiter plate. Test samples were diluted by a factor of 1:1, 1:3, 1:10, 1:30 and 1:100 prior to the test. A solvent control (0.1% v/v of solvent) was also included to exclude possible solvent effects. Calibration curve standards and test sample dilutions were tested in triplicate. For each spiked sample, three independent analyses were performed, while real samples were analysed only once.

Results expressed in relative luminescent units (RLU) were processed using a Sigmoidal calibration curve constructed using a MS Excel template (provided by BDS) together with an add-in "Solver". The curve fitting equation is as follows:

$$y = A/\left(1 + (x/B)^C\right) \quad (1)$$

where y represents response in RLU (corrected for solvent); x represents the concentration in pM(E2)/well, A, B and C represents maximum responses, the EC_{50} (median effective concentration) of the curve and the slope of the curve, respectively.

The E2 equivalents (EEQ) were calculated from Eq. 1. For quality control purposes, only the results that reached the predefined parameters of r^2 of calibration curve ≥0.99, a concentration between the LOQ and EC_{50} (median effective concentration) and a relative standard deviation <15%, were used.

Limits of detection (LOD) and quantification (LOQ) of SPE–ER-Calux® assay were 0.68 ng(EEQ)/L and 2.05 ng(EEQ)/L, respectively. Recovery (97%), and repeatability (4%) were determined with tap water (n = 5), spiked with E2 (c = 15 ng/L).

2.7. Chemical analysis

The remaining 950 μL of the sample was used for chemical analysis. An internal standard (bisphenol A)-d_{16} was added to give a final concentration of 10 μg/L (comparable to 50 ng/L in water samples before extraction, V = 200 mL). The solvent was then removed using a gentle steam of N_2. Sample was afterwards derivatised with MSTFA and analysed with GC-MSD as described in Avberšek et al. (2011), where sample chromatogram can also be found. The results of chemical analysis were corrected according to the amount of the sample that remained for chemical analysis after it was used for ER-Calux® assay.

2.7.1. GC-MSD method validation

The method was evaluated in terms of linearity, repeatability, recovery, limits of detection and limits of quantification for each test compound (Table 2). Tap water and waste water effluent were used as a matrix for calibration samples. For E1 and E2, six point calibration curves (2, 10, 50, 100, 300, 600 ng/L) were prepared under the same conditions as for the test samples. The same was done for EE2 and E3 with five concentrations between 10 and 600 ng/L. In case of waste

Table 2
Results of validation parameters (RSD — relative standard deviation, LOD — limit of detection, LOQ — limit of quantitation, r^2 — correlation factor).

Matrix for validation	Tap water				Waste water effluent			
Compound	E1	E2	EE2	E3	E1	E2	EE2	E3
Recovery (n=5) (c=50 ng/L) (%)	101	94	97	98	81	91	87	94
Inter-day repeatability (c=50 ng/L) (n=5) (RSD%)	2	3	11	7	2	3	8	9
LOD (ng/L)	0.2	0.4	1.1	1.1	0.2	0.4	2.0	1.4
LOQ (ng/L)	0.7	1.2	3.7	3.5	0.7	1.2	6.5	4.5
r^2 (calibration curve)	0.997	0.999	0.997	0.997	0.999	0.998	0.996	0.999

water effluent, calibration samples were blank corrected since target compounds were present in blank samples. The method showed good linearity for all tested compounds. Recoveries and repeatability were checked at a concentration of 50 ng/L. Limits of detection and quantification were calculated as 3 and 10-times the standard deviation of the base line of five blank samples.

2.8. Calculated estradiol equivalents (cEEQ)

For each compound, the median effective concentration (EC_{50}) was determined from the ER-Calux® assay. From EC_{50}, estradiol equivalent factors (EEF) were calculated using Eq. 2:

$$EEF_i = EC_{50\,i}/EC_{50\,E2}, \quad (2)$$

where EEF_i is the estradiol equivalent factor of a test compound; EC_{50i} and EC_{50E2} are the median effective concentrations of the test compound and E2, respectively.

The EEF_i were included in the calculation (see Eq. 3) of "Calculated estradiol equivalents" (cEEQ) as follows:

$$cEEQ = \sum(EEF_i{*}c(i)), \quad (3)$$

where cEEQ is the total calculated estradiol equivalents, EEF_i is the estradiol equivalent factor of a specific compound and c(i) its concentration determined by chemical analysis.

3. Results

The results of MTS assay showed that none of the solvents tested (Fig. 1) reduced the viability of the ER-Calux® cells for more than 18%, compared to the control. The results for ethyl acetate showed no significant effect on the cell viability. This solvent was therefore chosen as the most suitable due to its compatibility with gas chromatography. In addition, there was no difference between dose responses obtained for the E2 standard solutions in ER-Calux® assay using either ethyl acetate or DMSO (Fig. 2). Comparison of ethyl acetate and DMSO used on real samples reveal no significant differences regarding the performance of ER-Calux® assay (Fig. 2).

E1, E2, E3 and EE2 were evaluated for their activity in the ER-Calux® assay by defining their estradiol equivalent factors (EEF) from the observed EC_{50} concentrations of each compound (Table 3). The EEFs were calculated using Eq. 2. The results were also compared to those determined by Houtman et al. (2004) who studied the same compounds also using the ER-Calux® assay. Table 3 shows that E2 and E3 have similar EEF values compared to those published by Houtman et al. (2004) while E1 and EE2 are higher in this study. To give a reasonable explanation for this, an extended study of EEFs would be needed. However the aim of this study was only an "in house" determination of EEF factors to be used for calculating cEEQ values.

For each spiked (standards and waste water extracts) and each real sample calculated estradiol equivalents (cEEQs) were obtained. They were calculated from Eq. 3 where EEF factors and the concentrations of the separate compounds — c(i) (determined by GC-MSD, Table 4) were used.

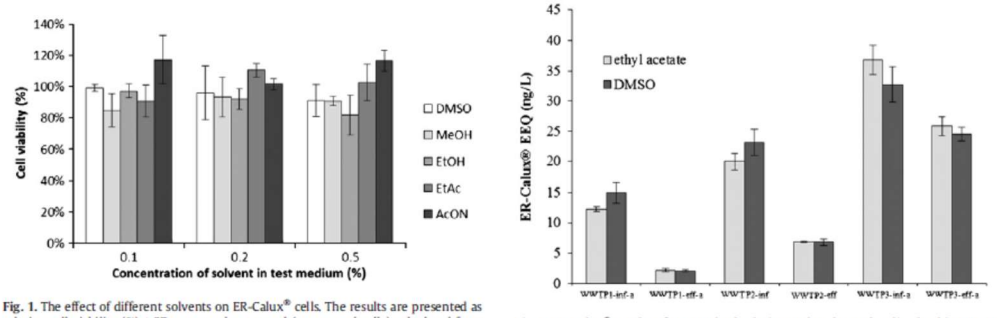

Fig. 1. The effect of different solvents on ER-Calux® cells. The results are presented as relative cell viability (%) ± SD compared to control (unexposed cells) calculated from five parallels. DMSO — dimethyl sulphoxide; MeOH — methanol; EtOH — ethanol, EtAc — ethyl acetate; and AcON — acetone.

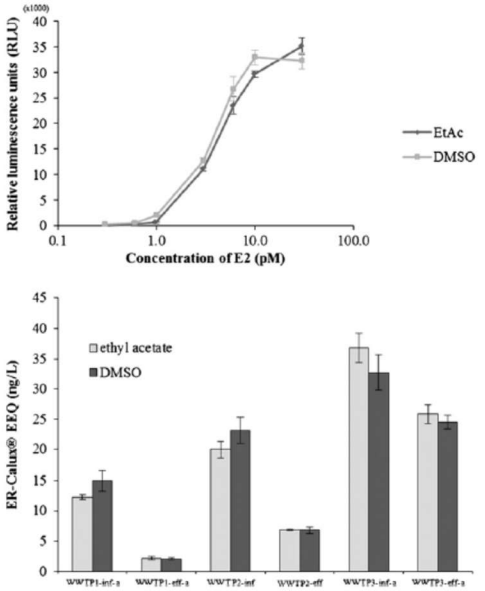

Fig. 2. ER-Calux® results of E2 standard solution and real samples dissolved in DMSO and ethyl acetate. The results are presented as mean ± SD calculated from three parallels.

Table 3
Steroid estrogen EC_{50} and EEF factors.

Compound	EC_{50} (pM)	EEF (this paper)	EEF (Houtman et al., 2004)
17β-Estradiol (E2)	2.64	1	1
Estrone (E1)	6.64	0.4	0.12
Estriol (E3)	18.21	0.14	0.13
17α-Ethinylestradiol (EE2)	1.57	1.68	1.12

The results of pure standards show good correlation ($r^2 = 0.96$) and a slope close to 1 ($\alpha = 0.94$), indicating good agreement between the GC-MSD cEEQs and the ER-Calux® assay EEQs (Fig. 3). The same situation ($r^2 = 0.92$, $\alpha = 1.02$) was observed where standard mixtures were added to the waste water extracts (Fig. 3).

Real samples from three WWTPs were tested. The concentrations of steroid estrogens in real waste influent samples are presented in Table 5. From concentrations cEEQs were calculated and compared to the results of ER-Calux® assay (Fig. 4). It is evident from Fig. 4, that all the samples gave similar results in both, the chemical analysis and the biological testing. Overall comparison of real sample data analysed with GC-MSD and ER-Calux® assay exhibit good correlation ($r^2 = 0.93$; $\alpha = 0.99$).

4. Discussion

In this study two stand-alone methods for determining steroid estrogens and estrogenicity were successfully integrated into a single protocol and verified by testing real samples from a waste water treatment plant. The integration of GC-MSD and the ER-Calux® estrogenicity assay enables fast and simple simultaneous determination of steroid estrogen concentrations and assessment of the total estrogenicity of the sample.

Combined chemical and biological approaches for the investigation of steroid estrogens and other endocrine disrupting compound are increasingly being employed. However, published studies are difficult to compare, since they incorporate various chemical analyses and biological assays, different sample handling procedures and different approaches of method integration. In addition, values and ratios of estradiol equivalents derived from chemical analysis (cEEQ) and estrogenicity assay (EEQ) vary. In those studies, where detailed comparisons of chemical and biological approaches are available, correlation factors up to $r^2 = 0.85$ are reported. This proves that chemical analysis and

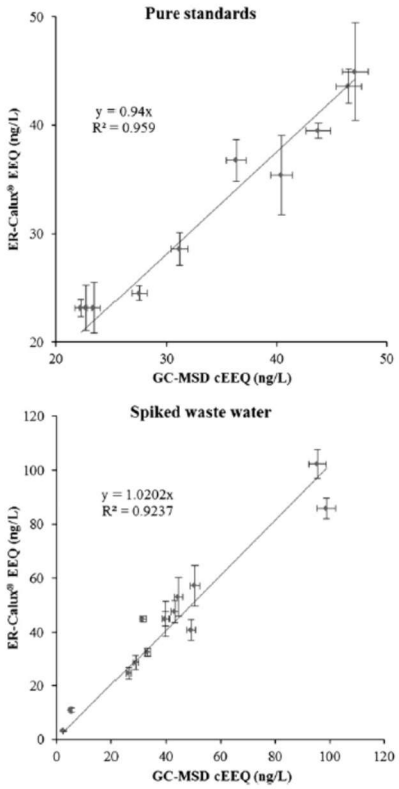

Fig. 3. Comparison of GC-MSD cEEQ and ER-Calux® EEQ for pure standards and spiked waste waters. The results are presented as mean ± SD calculated from three parallels.

Table 4
The results of GC-MSD analysis of spiked samples.

		GC-MSD analysis (ng/L)			
	Sample name	E1 ng/L	E2 ng/L	EE2 ng/L	E3 ng/L
Pure standards	St1	<LOD	32.2	<LOD	58.9
	St2	<LOD	20.1	<LOD	52.8
	St3	8.4	20.8	<LOD	49.7
	St4	19.3	30.6	<LOD	58.4
	St5	16.3	22.1	<LOD	54.9
	St6	27.9	30.0	<LOD	42.5
	St7	29.3	24.1	<LOD	57.2
	St8	11.0	11.1	<LOD	51.9
	St9	12.1	10.8	<LOD	47.6
	St10	10.8	10.5	<LOD	61.6
Standards with effluent extracts	Eff0[a]	5.2	<LOQ	<LOD	<LOD
	Eff1	9.4	1.6	<LOD	<LOD
	Eff2	12.2	10.6	6.8	13.0
	Eff3	21.8	15.4	14.1	18.9
	Eff4	18.4	15.8	14.0	18.0
	Eff5	36.0	29.9	29.9	30.4
	Eff6	44.4	<LOD	<LOD	60.2
	Eff7	36.5	5.6	<LOD	91.7
	Eff8	22.7	13.2	<LOD	65.1
Standards with influent extracts	Inf1	25.8	16.2	7.0	44.1
	Inf2	29.3	29.0	28.0	54.6
	Inf3	32.4	13.7	<LOD	93.7
	Inf4	34.8	12.7	<LOD	93.0
	Inf5	34.6	13.6	<LOD	111.5

Concentrations are presented as mentioned in Table 1.
[a] Blank sample.

Table 5
Results of GC-MSD analysis of real samples.

	ng/L			
Sample name	E1	E2	EE2	E3
WWTP1-inf-a	11.3	4.2	<LOD	65.8
WWTP1-eff-a	4.0	1.5	<LOD	12.5
WWTP2-inf	13.4	4.4	<LOD	97.2
WWTP2-eff	16.5	2.1	<LOD	<LOD
WWTP3-inf-a	25.2	4.6	<LOD	107.9
WWTP3-eff-a	61.8	8.1	<LOD	<LOD
WWTP1-inf-b	16.4	3.7	<LOD	82.4
WWTP1-eff-b	1.7	2.9	<LOD	18.4
WWTP3-inf-b	82.8	10.1	<LOD	37.0
WWTP3-eff-b	51.1	9.0	<LOD	45.7
WWTP3-eff-c	5.2	<LOQ	<LOD	<LOD

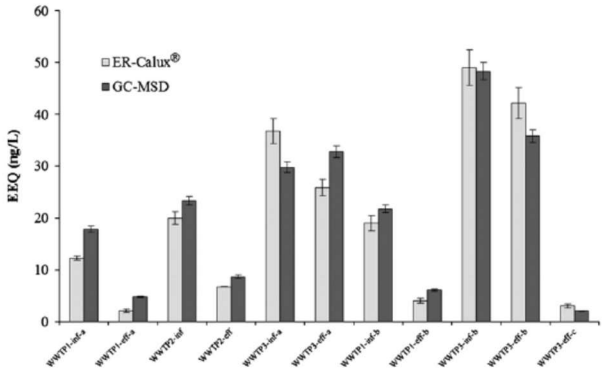

Fig. 4. GC-MSD and ER-Calux® results of real waste water samples. The results of ER-Calux® assay are presented as mean ± SD calculated from three parallels. The results of GC-MSD are presented as determined concentrations of one measurement ± relative standard deviation of measurement by GC-MSD.

estrogenicity assays are closely related, but the fact that the slopes of the regression lines are usually not optimal ($\alpha = 1$) means that differences exist, which are usually explained by the presence of agonists, antagonists, and unknown compounds, which albeit relevant (Körner et al., 1999), remain unverified. However, by additional fractionation the presence of antagonists was confirmed by Salste et al. (2007), while other authors report the opposite (Furuichi et al., 2004; Heisterkamp et al., 2004).

A summary of the literature data reveals that most studies use different sample handling procedures for chemical analysis and estrogenicity assays. In many cases they use completely separate protocols for each method including separate sampling and sample preparation, extraction and different solvents (Aerni et al., 2004; Cargouët et al., 2004; Rutishauser et al., 2004; Tan et al., 2007; Miège et al., 2009b; Brix et al., 2010). The aim of our study was not to characterise sample handling error, but to develop a simple integrated protocol with special attention on finding the optimum solvent that could be used in both chemical and biological analyses. In cell-based bioassays, DMSO is the most commonly used solvent. However, it is not suitable for chemical analysis, due to its low volatility. Our results showed (Figs. 1 and 2) that ethyl acetate, which is routinely used in chemical analysis, can also be used in the ER-Calux® assay since cell viability, dose response of E2 standard solutions and real sample data did not show significant differences compared to DMSO. Using the same solvent avoids uncertainties derived from different sample handling procedures. It also means that no additional sample preparation steps are required for the bioassay since the ER-Calux® assay requires only several μL of a sample. Only 5% of the sample that was initially prepared for chemical analysis is required for the ER-Calux® assay. Alternatively, 95% of the sample that is available for chemical analysis is sufficient to obtain the required low limits of detection (LOD) and quantification (LOQ) for analysing trace levels of steroid estrogens in environmental samples.

It is evident from our results of testing pure standards (Fig. 3) that besides the good correlation ($r^2 > 0.92$), EEQs determined by GC-MSD were comparable to those deriving from the ER-Calux® assay, since the regression line slopes were close to optimal *i.e.*, $\alpha = 0.94$. A similar result was obtained with spiked waste water extracts ($\alpha = 1.02$), although we expected lower agreement due to the complexity and consequent possible mixture effects caused by agonists or antagonists present in waste water.

The integrated protocol was then applied to real WWTP influent and effluent samples. Concentrations of steroid estrogens in waste water samples are comparable to concentrations summarised in a database of steroid estrogens in waste water influents and effluents (Miège et al., 2009a). The concentrations were used for cEEQ calculation and were closely correlated with the results of ER-Calux® assay ($r^2 = 0.93$), which is about 10% better than that reported in the available literature data. Moreover, the estrogenicity detected with ER-Calux® assay can be explained by concentrations of E1, E2, E3 and EE2, determined by GC-MSD. This is in agreement with several studies where these four compounds, were recognised to be responsible for more than 90% of the total estrogenic potential in municipal waste waters (Aerni et al., 2004; Furuichi et al., 2004; Salste et al., 2007; Miège et al., 2009a). This means that the estrogenicity of municipal waste water samples can be, for rapid screening purposes, estimated by determining the four most potent steroid estrogens.

The integrated protocol is applicable on every environmental sample where extracts are prepared for analysis. At the same time the ER-Calux® assay can be used as pre-screening prior to the chemical analysis, since the extracts that are used in the estrogenicity assay can be temporarily stored and analysed at a later time by GC-MSD, if necessary. While the ER-Calux® assay is sensitive and allows for a much higher sample throughput, chemical analysis, which is more costly and time consuming, can be used to analyse only critical samples. In environmental monitoring this is an important advantage that cannot be overlooked, as most often a lot of samples have to be analysed within a short time.

5. Conclusion

Increased application of combined chemical analysis and estrogenicity assays is evident from the literature. However, detailed comparisons of both methods are rarely made. In this study two stand-alone methods addressing chemical (quantitative and qualitative determination of steroid estrogens with GC-MSD) and estrogenicity (ER-Calux® assay) characterisation of waste water samples were successfully integrated into a single protocol. Integration enables simultaneous determination of steroid estrogen concentrations and assessment of total estrogenicity of the sample. The integration of both methods and its optimization were achieved by introducing a suitable solvent (ethyl acetate) that is compatible with the gas chromatography and the ER-Calux® assay. This enables the same sample to be tested by both methods, GC-MSD and ER-Calux® assay. The comparison of GC-MSD and ER-Calux® assay performed on pure standards and spiked waste water extracts showed that the methods gave similar results (regression line slope close to optimal; $\alpha = 1$) producing a good correlation ($r^2 > 0.92$). The integrated protocol was afterwards applied and successfully verified on real waste water samples. Simple sample handling and the applicability of the protocol

is the basis for easy, fast and reliable risk assessment of steroid estrogens in environmental samples. Our method enables concurrent determination of both parameters while reducing material consumption and labour.

Acknowledgement

The authors would like to acknowledge the financial support given by the Slovenian Research Agency (Programme groups P1-0143, P1-0245, Project J1-0005 and young researcher grant to Miha Avberšek) and EU Structural Funds to the Centre of Excellence in Environmental Technologies (Contract No.: 3211-05-000184).

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