EDC WFD

Training workshop: Solutions to tackle WFD requirements for estrogen determination in water

7-9 September 2022

EDC WFD

BEFORE TO START

THIS TRAINING WILL BE REGISTERED

DOES ANYONE HAVE AN OBJECTION?

- This **Training/Workshop** aims:
  - to present the **knowledge gained from the EDC-WFD** project whose objective is to develop reliable and harmonized measurement methods for estrogens, which are key Endocrine Disrupting Chemicals (EDC), to comply with Water Framework Directive requirements
  - to **accelerate the transfer of the most promising measurement methods and methodologies to interested parties**: laboratories, PT providers, researchers
- The training workshop will cover **all aspects of measurements** from sampling to final method validation and will address both Mass spectrometry based methods as well as incoming Effect Based Methods (in vitro bioessays)

7th of September
Session 1

09:00 - 09:10: Welcome address

09:10 - 09:50: Presentation of the project and context

09:50 - 10:20: Issues and challenges related to estrogen analysis in relation to the WFD

10:20 - 11h00: Challenges related to sampling

11:00 - 11:15: Break

11:15 - 11:35: Overview of quantification strategy

11:35 - 12:15: Sample preparation

8th of September
Session 2

09:00 - 09:30: Discussion forum / debriefing from day 1

09:30 - 10:30: Mass spectrometry methods - Instrumental developments

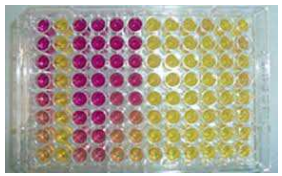
10:30 - 10:45: Break

10:45 - 11:45: Achievements of Mass spectrometry based methods _ method performances and measurement reliability

11:45 - 12:00: Concluding remarks

12:00 - 12:15: Next step _ Towards Interlaboratory Comparison

9th of september
Session 3 dedicated to Effect Based Methods (EBM)



09:00 - 09:10 : Welcome address

09:10 - 09:40 : Presentation of the project and context

09:40 - 10:05 : Context and presentation of EBM methods versus MS based methods

10:05 - 10:40 : EBM protocols

10:40 - 11:15 : EBM data treatments

11:15 - 11:30 : Break

11:30 - 11:45 : Concluding remarks

11:45 - 12:00 : Next step : Towards Interlaboratory Comparison


QUESTIONS?
COMMENTS?
CONCERNS?

EURAMET is...

- The gateway to Europe's integrated metrology infrastructure
- It facilitates access to European measurement expertise, and underpins the delivery of globally competitive, high impact metrology
- It ensures Europe maintains its global reputation for excellence in measurement science
- It raises awareness of the value of measurement by demonstrating the impact of metrology on society's grand challenges

European Association of National Metrology Institutes

EURAMET

Members:

38 European NMIs

28 of them are participating in EMPIR

Associates:

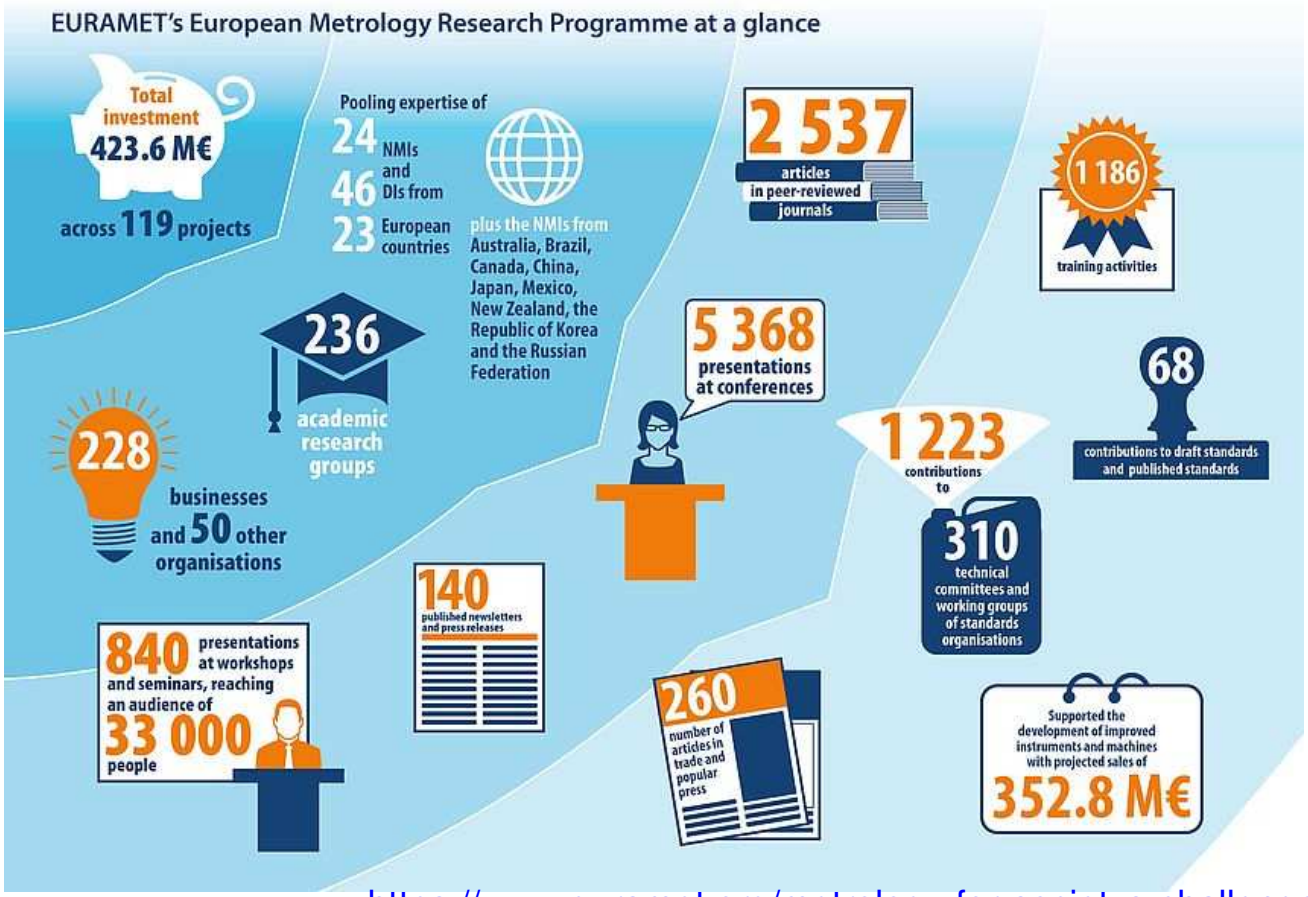
78 DIs (Designated Institutes)

metrologists:

NMIs: ~ 5000

DIs: ~ 1500

https://www.euramet.org/about-euramet/



https://www.euramet.org/metrology-for-societys-challenges/

What is EMPIR?

- It is about improving measurement to drive innovation and competitiveness.
- It enables European metrology institutes, industrial organisations and academia to collaborate on joint research projects.
- It is implemented by EURAMET (European Association of National Metrology Institutes).
- It is based on Article 185 of the Lisbon Treaty.
- It is jointly funded by the EMPIR participating countries and the European Union and has a budget of approximately 600 M€ over seven years.

https://www.euramet.org/research-innovation/research-empir/about-empir/

Objectives for EMPIR

Challenge /problem: increasing demands on metrology infrastructure alongside static or reducing national budgets. This requires strategically planned metrology R&D (reducing fragmentation and duplication to achieve critical mass) to ensure delivery of future metrology capabilities / services to meet European needs

EMPIR

High-level objectives	**European growth and jobs** **Respond to societal challenges** **Create an integrated European Research Area**
Specific objectives	• **Boost industrial uptake of metrology research** supporting development of **new & improved products and services** • **Improve standardisation / regulation** • Underpin a coherent, sustainable and **integrated European metrology landscape**
Operational objectives	• Develop common research agenda • Support European collaboration • Fund projects that support innovation, regulation, societal challenges • Capacity building across Europe • Efficient & effective programme management

EMPIR objectives

Boost industrial uptake and improve standardisation

- At least 400 M€ of European turnover from new or significantly improved products and services that can be attributed to the research activities of EMPIR and its predecessors.

  346 M€ identified so far.

- At least 60% of CEN/CENELEC /ISO/IEC Technical Committees and equivalent standardisation bodies with potential to benefit directly from EMPIR projects to engage with the programme.

  Contributions to 17 published and 103 draft standards.

EMPIR objectives

Underpin a coherent, sustainable and integrated European metrology landscape to fully exploit the EU potential

- Maintain a level of at least 50 % of dedicated national metrology research investments in Europe being coordinated or influenced via the programme. **Level maintained**
- All European NMIs and their designated institutes to interact with the programme. **Cyprus, Malta**
- European leadership in at least 20 % of international metrology committees. **50 %**

EUROPEAN PARTNERSHIP ON METROLOGY

- The EPM will bring together the measurement science community and stakeholders to deliver on global challenges including health and climate, support the European Green Deal, and underpin innovation in industry through collaborative research.

- The EPM aims to **support accelerating the transition towards a green, climate neutral and digital Europe**, as well as strengthening the resilience, competitiveness, and economic growth of the European industry.

- European Partnerships are a key implementation tool of the European Commission's Horizon Europe; an ambitious research and innovation programme, running from 2021 to 2027.

EUROPEAN PARTNERSHIP ON METROLOGY

- The EPM is co-funded by the Member States and the European Union with an expected budget of over **650 million euro**. Its expected impact is manifold, as it will support a wide range of policies, commerce and advancement of key European challenges.

- It builds on the progress achieved under the previous European Metrology Research Programme, and aims to break new ground by contributing to the development of self-sustaining, coordinated metrology infrastructures, with the capacity to continue joint research and innovation after 2030.

https://www.euramet.org/research-innovation/metrology-partnership/

OVERVIEW OF THE PROJECT

THE CONSORTIUM

- 8 partners / 6 European countries
- 5 NMI/DI, one DI operating outside of its designation, one academic research laboratory, one research institute

- Consortium brings together **scientific excellence** in research institutes and experience in **ultra-trace measurements of micropollutants**

- **Balance of expertise**: development and certification of RM, proficiency tests / interlaboratory comparison design, method development and validation, standardisation

Start date: 1st September 2019
Duration: 36+6 months
Budget: 800K€

S. LARDY-FONTAN, V. LE DIOURON, C. FALLOT, B. LALERE & our LC/MS^2 (critical member of the team)

K. LE MENACH, P. PARDON, H. BUDZINSKI

C. PIECHOTTA, L. STEINHAÜSER, T. WESTPHALEN, U.-A. KLYK-SEITZ, K. KAMINSKI, S. KLUGE

T. NÄYKKI, J. VIIDANOJA

E. HEATH, A. KOVACIC

T. GÖKCEN, I. UN

P. De ZORZI, M. POTALIVO, C. ASCENZI, E. CALABRETTA, S. BARBIZZI, G. MOLTEDO

THE PROJECT

AIMS:

- ❖ Address the standardization lack for harmonised measurement methods for estrogens in whole water
- ❖ Ensure that measurements of estrogens are traceable, well defined, meet the requirements of the WFD, and thus are comparable across Europe (and worldwide)

- ➢ Chief Stakeholder: Ulrich BORCHERS Chairman of CEN/TC 230
- ➢ Chief Stakeholder and DIN secretary (Andreas Paetz) are kept inform of the progress

- ➢ Support from AFNOR (Arnaud Gaudrier: Secretary of SC «Water Quality»

CONTEXT

- **Estrogens:** group of chemicals of similar structure mainly responsible for female sexual development and reproduction.
- In water ecosystem:
  - **Pseudo-ubiquitous** and occur at **ultra-trace level** (<<ng L^{-1} to tens ng L^{-1})
  - Level at which they can have effects in natural species
    - **Threat for biodiversity**

→ ***Included in the First Watch List of the Water Framework Directive WFD*** → ***Under review to become priority substances (PS) of the WFD***

- **No EN or ISO standard for MS-based methods** currently available or in progress
- (Accredited) testing laboratories **develop and validate in-house methods** according to internal criteria
- Most of (accredited) testing laboratories **failed to achieve the very low LOQ** to enable monitoring of estrogens at relevant level
- **Metrological endpoints** have been highlighted of particular importance if effect-based method (EBM) results are to be used in a regulatory context
- **Lack/absence of reference materials and ad'hoc proficiency tests**

AIM

Natural and pharmaceutical estrogens are key Endocrine Disrupting Chemicals (EDC) which are monitored differently depending on the country, and for which standardised reference methods are currently not available.

⇒ Main Objective: Develop reliable and harmonized measurement methods for estrogens, to comply with the WFD Directive requirements

⇒ **Outcomes: to be disseminated to CEN/ TC 230 and ISO/ TC 147 to be fed into the documentary standards they develop**

OBJECTIVES

- The overall objective of this project is to **develop traceable measurement methods** for endocrine disrupting chemicals, with a specific focus **on three estrogens of the first watch list: 17-beta-estradiol (17βE2), 17 alpha ethinylestradiol (17αEE2), and estrone (E1)**)

- Estrogens 17-alpha-estradiol (17αE2) and estriol (E3) will be included to demonstrate the reliability of the developed methods

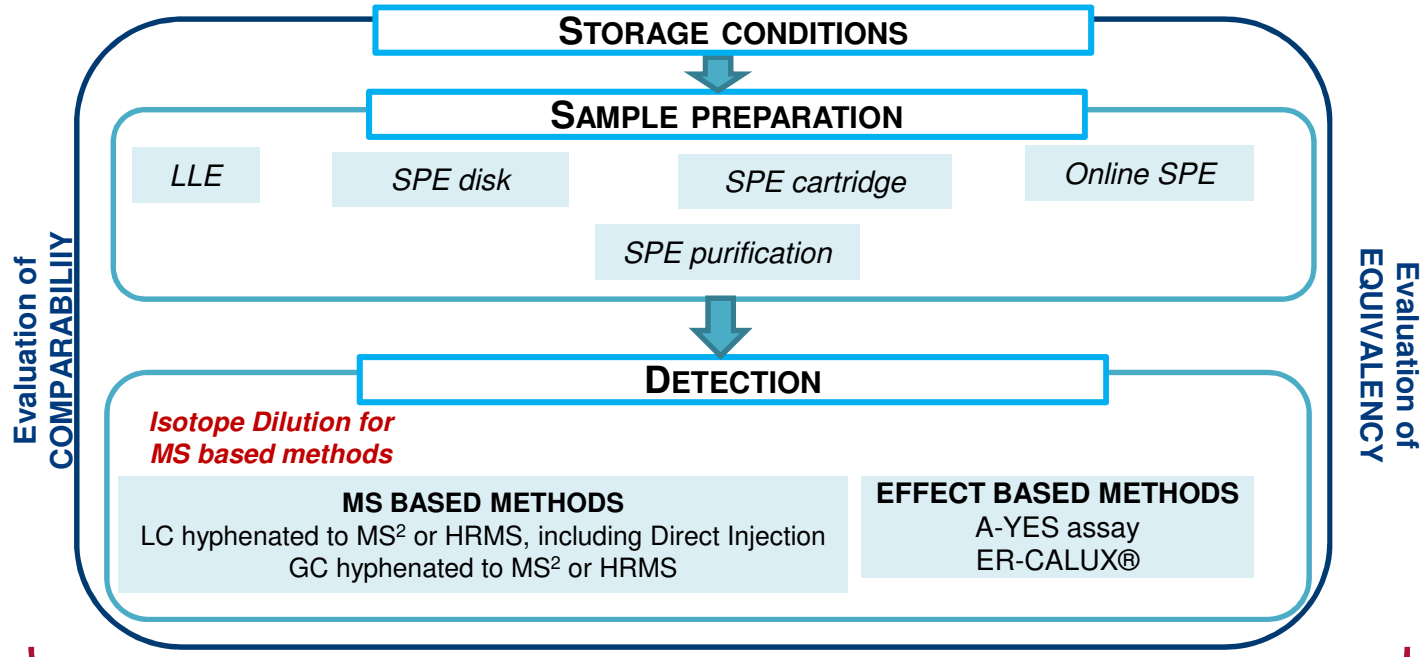
  ⇒ **to support the requirements of Directive 2013/39/EC, Directive 2009/90/EC and Commission Implementation Decision (EU) 2018/840,**

  ⇒ **improve the comparability and compatibility of measurement results within Europe**

The specific objectives of the project are to:

1. **Optimize and validate traceable aqueous reference Mass Spectrometry-based methods for the analysis of 5 estrogenic** compounds prioritizing 17βE2, 17αEE2, and E1 in **whole water samples** at environmental quality standard (EQS) levels. **Methods will have limit of quantification (LOQ) not exceeding 30% EQS with a measurement uncertainty of ≤50 % at EQS**
2. **Develop production methods for aqueous reference materials (RM),** which are as close as possible to real water samples, with proven homogeneity, short- and long-term stability
3. **Improve the comparability of estrogen measurements with selected Effect-Based Methods (EBM)** in whole water samples at EQS level. Methods will have been correctly calibrated and information on uncertainty will be provided
4. **Organize and perform an interlaboratory comparison (ILC)** to demonstrate the performance of the developed methods using the reference material (RM) for the selected estrogen substances
5. **Contribute to the work of key European and international standardization organizations e.g. CEN TC 230 and ISO TC 147**

METHOD OPTIMIZATION



Comparison of sample preparation techniques for estrogens in whole water & recommendations on the most appropriate ones

Most promising MS-based method(s) for the measurement of selected estrogens in whole water samples compatible with the requirements of the QA/QC Directive (LOQ≤ 30% EQS with U(k=2) ≤50% at EQS)

Recommendations to improve the comparability of estrogens measurements with the set of selected EBM in whole water samples at EQS levels


QUESTIONS?
COMMENTS?
CONCERNS?

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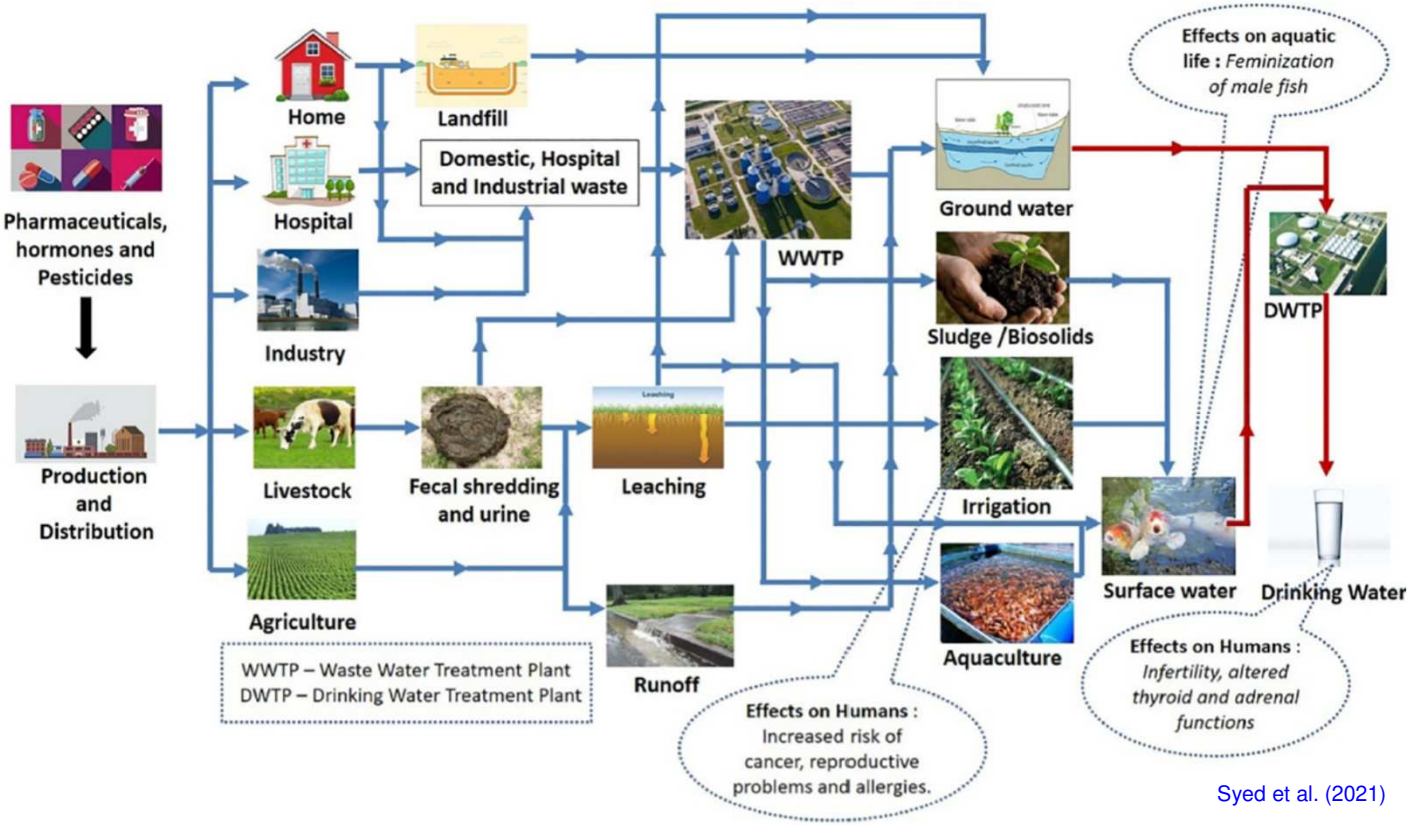
EDC WFD

GENERALITIES

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE



Syed et al. (2021)

To learn more (Review Aris et al., 2014; Adeel et al., 2017)

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

	17βE2	E1	17αEE2	E3
Water solubility ($mg\ L^{-1}$)	3.6	760 (20°C) 30 (25°C)	11.3 at 27°C 4.7 at 20°C 19 at 20°C	27.34 at 25 °C
Volatilisation	According to vapor pressure and Henry constant values, the substance is not likely to volatilise from water phase.			
Organic carbon – water partition coefficient (K_{OC})	K_{OC} = 791.7 log Koc = 2.9	log Koc= 3.019 log Koc= 4.375	Log Koc = 2.92 – 4.68 Koc = 192 – 2 955 log Koc = 3.21 – 5.44 Koc = 1 622 – 275 423	Koc = 1200
Sediment – water partition coefficient ($K_{susp\text{-}water}$)	251		25 – 34 429	log Kd = 1.33
Octanol-water partition coefficient (Log Kow)	4.01	3.13 3.43	3.67 4.2, 25°C, pH 7	2.45

- Estrogens typically have both low water solubility and mid Kow ⇒ hydrophobic + high potential to bind to sediments/ SPM
- Photolysis, biodegradation, and sorption are the likely leading attenuation pathways controlling the fate of estrogens in water ecosystem

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

Sorption

- Yu et al. (2004) and Brett et al. (2014) observed that in one day, 80-90% of E1 and EE2 can be sorbed to suspended solids and reach steady-state within ten days
- Estrogens can also sorb to humic acids (HA) in water (Chowdhury et al., 2011)
- The presence of saline compounds such as NaCl induces flocculation and aggregation, which can promote sorption processes (Lai et al., 2000; De Mes et al., 2005).
- Estrogen sorption capacity is also positively related to total organic carbon (TOC) content since the sorption occurs through hydrogen bonding reactions between organic carbon and estrogen compounds (Lai et al., 2000; Nghiem et al., 2004; D'Alessio et al., 2014).
- Yu et al. (2004) reported a strong sorption competition effect between one estrogen compound and other estrogens or hydrophobic compounds for sorption sites. The competition is most significant when the concentration of the primary sorbate is low and the concentration of the competitive sorbate is high (Lai et al., 2000; Yu et al., 2004)

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

Sorption

- The isomer-specific sorption of E2 isomers is dominated by H-bonding and aromatic interaction which 17β-E2 sorption was preferential than 17α-E2 (Qiao et al. 2011). The orientation of the –OH group at the C17 position in the D-ring plane may favor the sorption of 17β-E2 while that of 17α-E2 is outside the plane.

- The sorption process of estrogens is associated with organic compounds, colloids, composition of clay minerals, specific surface area (SSA), cation exchange capacity (CEC), and pH value in the environment (Yu et al., 2019)

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

Photodegradation

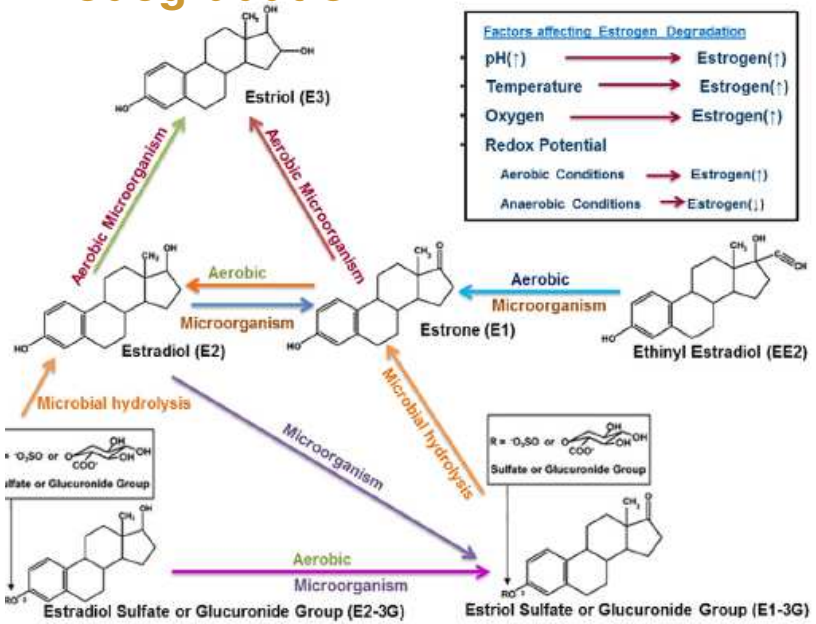
- Estrogens such as E2 and EE2 in aquatic environments are also susceptible to breakdown by photocatalysis and photolysis. The extent of degradation by photolysis and photocatalysis depends upon an estrogen's chemical structure.

- The photolysis process of estrogens is affected by humic substances under simulated sunlight

- Zuo et al., 2013: EE2 degradation was studied in a lake site in the USA. Under aerobic conditions, half-life was estimated as 108 days. However, under natural sunlight, photo degradation accounted for a much shorter half-life of 23 h

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

Biodegradation



- Estrone (E1), estradiol (E2), and estriol (E3) lie on interconnecting metabolic pathways
- Microorganisms living in aerobic and anaerobic conditions can convert one estrogen to another
- Even EE2 can be converted to E1

Table 5
Half-life (in days) of steroidal estrogens from different aquatic sources.

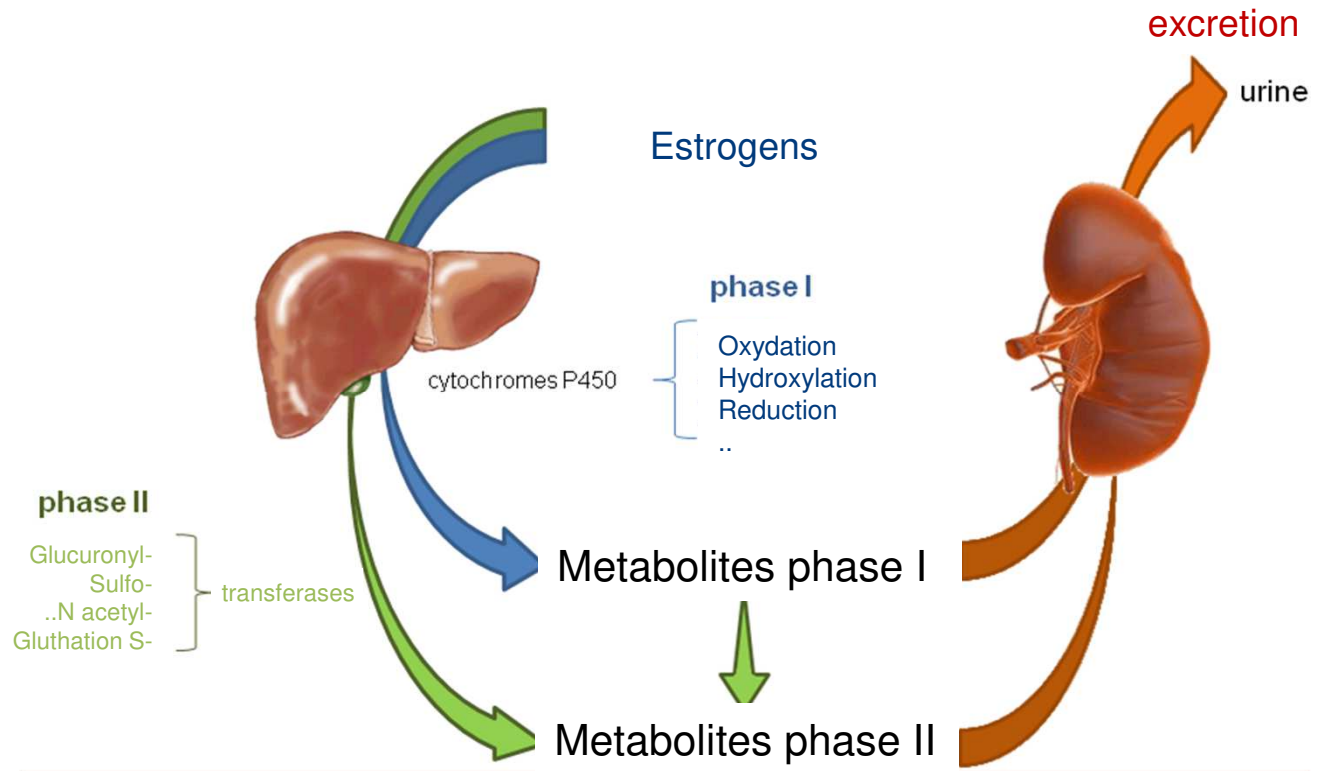
Medium	Oxygen status	E1	17β-E2	E3	EE2	Reference
Natural soil	Aerobic	2.8–4.9	0.8–1.1	0.7–1.7	NDA	Biswas et al., 2013
Aire river water	Aerobic	2.4	2.3	NDA	NDA	Jürgens et al., 2002
Calder river water	Aerobic	2.2	2.7	NDA	NDA	
Thames river water	Aerobic	3.1	4.1	NDA	NDA	
River water	NDA	2–3	2–3	NDA	4–6	Ying et al., 2002
Sandy-loam	Anaerobic	NDA	NDA	NDA	NDA	Ying and Kookana, 2005
Aquifer materials	Aerobic	NDA	NDA	NDA	81	Ying et al., 2003
Natural water	Aerobic	NDA	NDA	NDA	1.5	Zheng et al., 2011

Adeel 2017

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

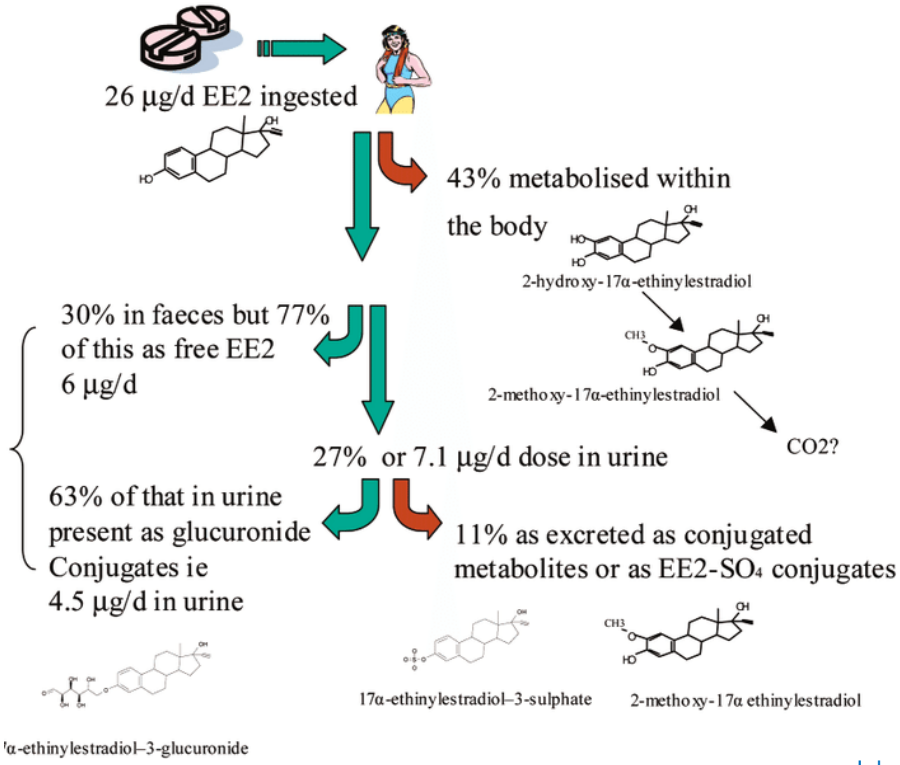
Metabolisation

Excretion by humans and animals in complex mixtures of free and conjugated forms



BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

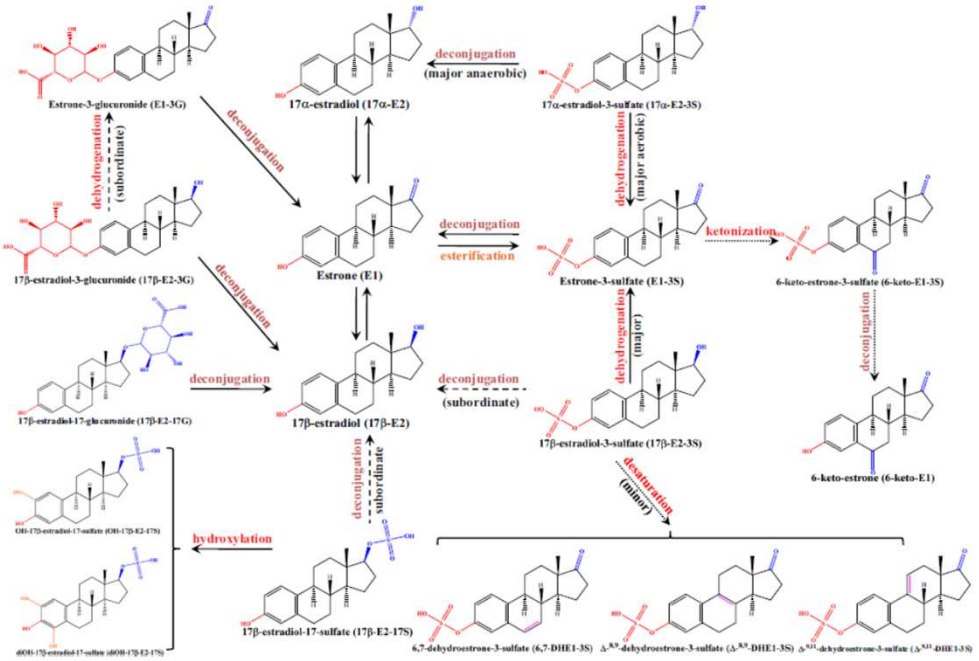
Metabolisation



Johnson et Richard (2004)

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

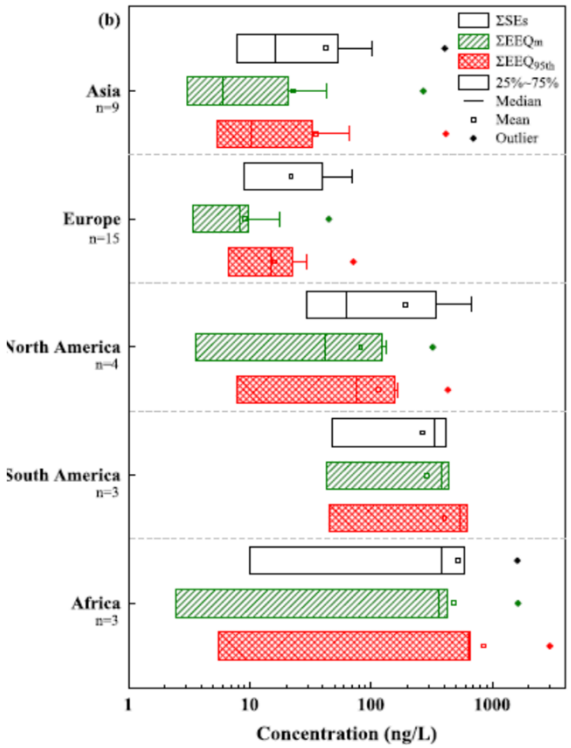
Metabolisation



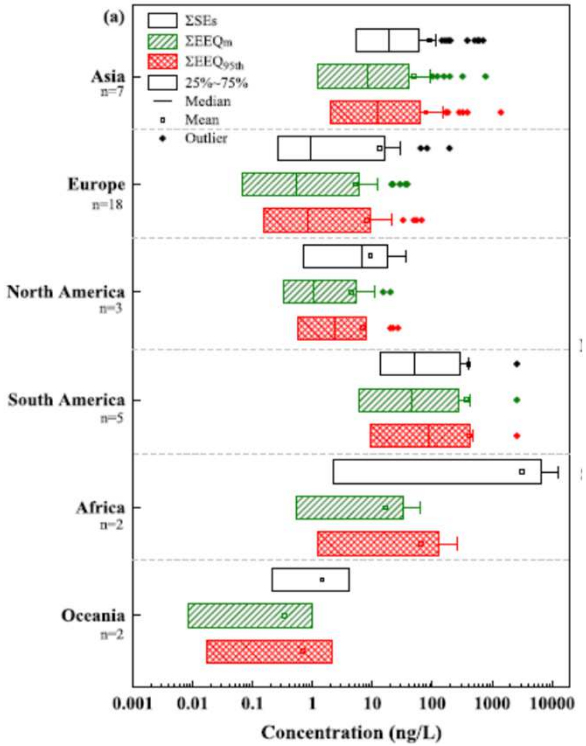
- Chronic release to the environnement
- Conjugation is reversible and could lead to the formation of free conjugated forms or new transformation products (eg Ma et al 2018, Yu 2019)

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

Wastewater treatment plants effluent



Natural water body

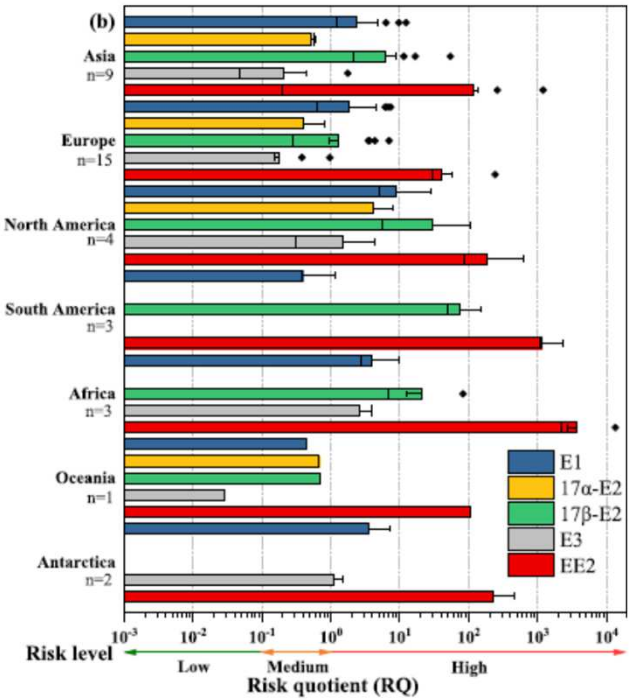


⇒ **Worldwide contamination**

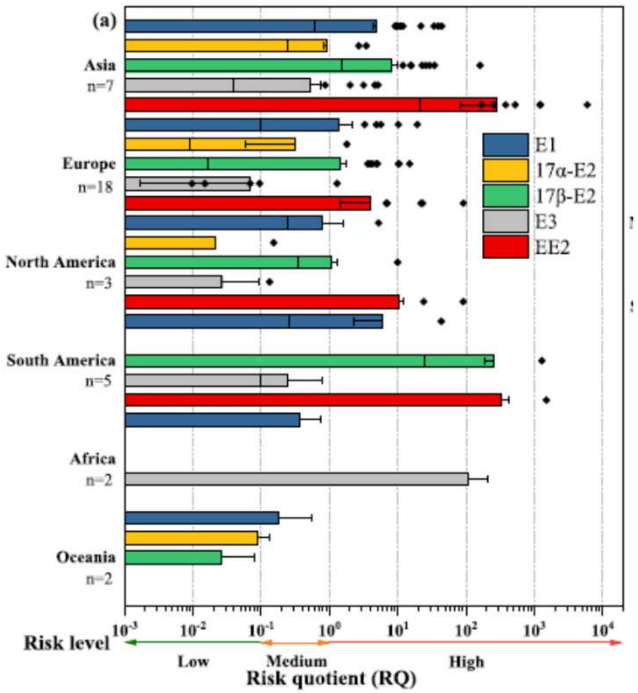
Du et al. 2020

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

Wastewater treatment plants effluent



Natural water body

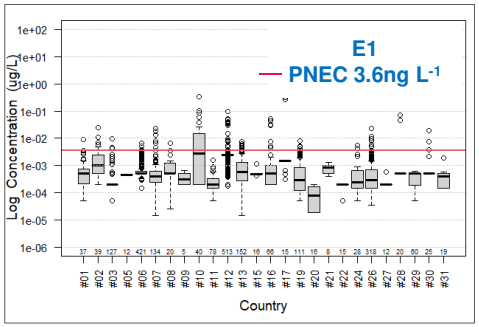


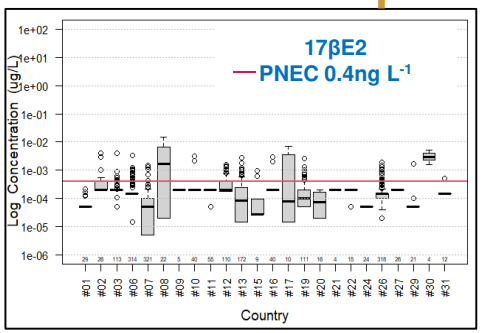
⇒ **Water bodies at risks**

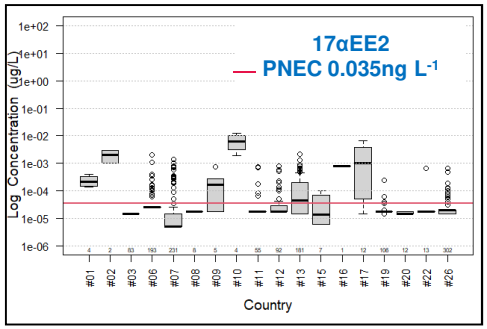
Du et al. 2020

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

Focus on Europa







Statistics for MEC according to different choices (<LOQ replaced by 50% LOQ as setted by Directive QA/QC)

Concentration (µg L^{-1})	E1	17βE2	17αEE2
Min	1.50E-05	5.00E-06	0,000005
Mean	2.39E-03	2.54E-04	9.81E-05
StDev	1.61E-02	7.53E-04	5.51E-04
Median	5.00E-04	1.50E-04	1.75E-05
P90	0.0025	3.20E-04	0.00013
P95	0.005	7.92E-04	0.0003
P99	0.03289	3.08E-03	0.00139
Max	0.481	0.015	0.0125

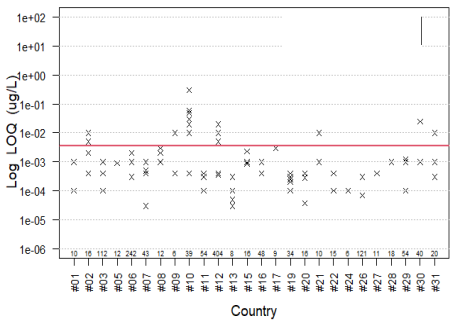
EQS suggestion EE2--JRC-2021-DRAFT; EQS E2 & E1-JRC-2021-FINAL DRAFT

BIOGEOCHEMICAL CYCLE: FATE AND OCCURRENCE

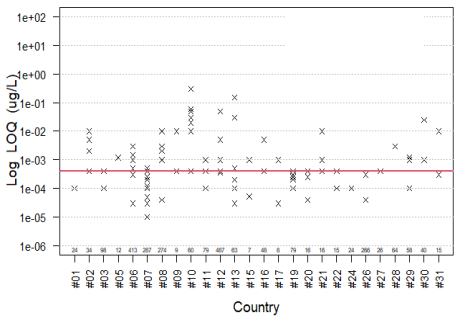
Focus on Europa

Range of LOQs for non-quantified samples in combined dataset per country.

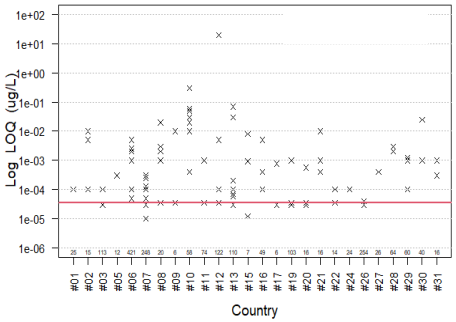
E1
PNEC 3.6 ng L^{-1}



17βE2
PNEC 0.4 ng L^{-1}



17αEE2
PNEC 0.035 ng L^{-1}



- EU-wide comprehensive assessment of the WL compounds E1, E2 and especially EE2 is not feasible in the current situation

EQS suggestion EE2--JRC-2021-DRAFT; EQS E2 & E1-JRC-2021-FINAL DRAFT

REGULATORY CONTEXT

REGULATORY CONTEXT

- **Directive 2000/60/EC** of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy“ ⇒ precise timetable, with 2015 as the deadline for getting all European waters into good condition

- **Directive 2008/105/EC** of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy ⇒ lays down environmental quality standards (EQS) for priority substances and certain other pollutants

- **Directive 2009/90/EC “QA/QC”** ⇒ lays down technical specifications for chemical analysis and monitoring of water status

- **Directive 2013/39/EU** amending the Environmental Quality Standards Directive 2008/105/EC ⇒ introduces the “Watch List” monitoring mechanism in order to collect high-quality Union-wide monitoring data for the purpose of supporting future prioritisation exercises.

REGULATORY CONTEXT

- **COMMISSION IMPLEMENTING DECISION (EU) 2015/495** of 20 March 2015 ⇒ establishes a watch list of substances for Union-wide monitoring in the field of water policy

ANNEX

Watch list of substances for Union-wide monitoring as set out in Article 8b of Directive 2008/105/EC

Name of substance/group of substances	CAS number (1)	EU number (2)	Indicative analytical method (3) (4) (5)	Maximum acceptable method detection limit (ng/l)
17-Alpha-ethinylestradiol (EE2)	57-63-6	200-342-2	Large-volume SPE — LC-MS-MS	0,035
17-Beta-estradiol (E2), Estrone (E1)	50-28-2, 53-16-7	200-023-8	SPE — LC-MS-MS	0,4

- **COMMISSION IMPLEMENTING DECISION (EU) 2018/840** of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495 ⇒ Maintain in the list because of insufficient data quality

REGULATORY CONTEXT

➢ **DECISION TO UPDATE THE LISTS OF PRIORITY ENDORSED BY COMMISSION** ⇒ Shall be finalized by end of 2022

Inland waters	**Method Detection Limit Watch List (µg L^{-1}) ≈ PNEC**	**EQS December 2021 scientific opinion by SCHEER (µg L^{-1})***
17αEE2	0.000035	0.000017
17β-E2	0.0004	0.00018
E1	3.6	0,00036

Salted waters	**Method Detection Limit Watch List (µg L^{-1}) ≈ PNEC**	**EQS December 2021 scientific opinion by SCHEER (µg L^{-1})***
17αEE2	No specification	0.0000016
17β-E2		0.000009
E1		0,000018

Scientific Opinion on "Draft Environmental Quality Standards for Priority Substances under the WFD"-17-Alpha-Ethinylestradiol (EE2), Beta-Estradiol (E2) and Estrone (E1) https://ec.europa.eu/health/publications/scientific-opinion-draft-environmental-quality-standards-priority-substances-under-wfd-17-alpha_en

REGULATORY CONTEXT

➢ **DECISION TO UPDATE THE LISTS OF PRIORITY ENDORSED BY COMMISSION** ⇒ Shall be finalized by end of 2022

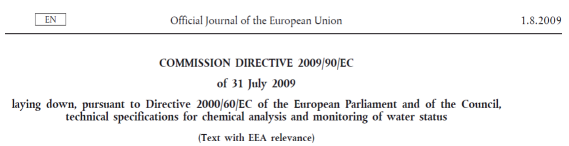
- EU-wide comprehensive assessment of the WL compounds E1, E2 and especially EE2 is not feasible in the current situation.
- However, when employing *in vitro* EBMs together with respective substance specific Effect-Based Trigger (EBT) values, it is possible to discriminate between a sufficient and insufficient chemical status, as defined by the suggested EQSs for E1, E2 and EE2, with sensitivities and specificities near 90%.
- Moreover, EDC-WFD project has been launched in 2018. Within the project four *in vitro* EBMs will be validated according to QA/QC Directive requirements and the comparability of estrogen measurements with the selected EBMs in whole water samples at EQS level will be assessed and improved. The assessment of metrological references will demonstrate their accuracy sustaining the *In vitro* EBMs suitability for the detection of estrogenicity for trend monitoring, status assessments, prioritisation of water bodies, identification of sources and investigative monitoring.

REGULATORY CONTEXT

➢ **DECISION TO UPDATE THE LISTS OF PRIORITY ENDORSED BY COMMISSION** ⇒ Shall be finalized by end of 2022

- *In vitro* EBMs for the detection of the ER activation cover a relevant Mode of Action.
- SOPs for this type of bioassays are available and three assays are even ISO standardised. Further validation and interlaboratory studies for other bioassays evaluating effects of estrogenic compounds would provide a wider choice of methods.
- **Annex VIII** of the WFD was developed to also target the substances that these EBMs respond to. In the short term (2019 WFD review), it could be considered to rephrase the Annex **allowing the use of *in vitro* EBMs to assess the presence of substances causing effects on endocrine-related functions.**
- However, field studies should be performed to evaluate the potential of these EBMs to identify sources of emission as a basis for subsequent measures for improvement. *In vitro* EBMs evaluated with suitable EBT values can screen, with a high level of safety, water samples for further chemical analyses. **Medium-term (next mandate) *in vitro* EBMs for the detection of ER activation might be included in future WL program after the development of guidance documents and a comparison of suitable bioassays.**

DIRECTIVE QA/QC



EN Official Journal of the European Union 1.8.2009

COMMISSION DIRECTIVE 2009/90/EC

of 31 July 2009

laying down, pursuant to Directive 2000/60/EC of the European Parliament and of the Council, technical specifications for chemical analysis and monitoring of water status

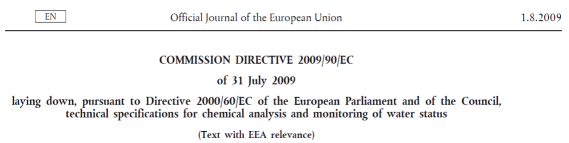
(Text with EEA relevance)

➢ Establishes **minimum performance criteria for methods of analysis** to be applied by Member States when monitoring water status, sediment and biota, as well as **rules for demonstrating the quality of analytical results**.

➢ Article 2 definitions

- **limit of quantification**' means a stated multiple of the limit of detection at a concentration of the determinand that can reasonably be determined with an acceptable level of **accuracy (trueness) and precision**. The limit of quantification can be calculated using an appropriate standard or sample, and may be obtained from the lowest calibration point on the calibration curve, excluding the blank
- **uncertainty of measurement**' means a non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used

DIRECTIVE QA/QC



EN Official Journal of the European Union 1.8.2009

COMMISSION DIRECTIVE 2009/90/EC

of 31 July 2009

laying down, pursuant to Directive 2000/60/EC of the European Parliament and of the Council, technical specifications for chemical analysis and monitoring of water status

(Text with EEA relevance)

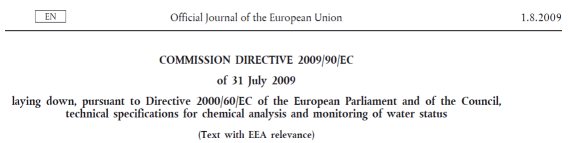
- Article 3 Methods of analysis
- All methods of analysis, are validated and documented in accordance with EN ISO/IEC-17025 standard or other equivalent standards accepted at international level.

- Article 4 Minimum performance criteria for methods of analysis

- the minimum performance criteria for all methods of analysis applied are based on
  - an uncertainty of measurement of 50 % or below (k= 2) estimated at the level of relevant EQS and
  - a limit of quantification equal or below a value of 30 % of the relevant EQS

- In the absence of relevant EQS for a given parameter, or in the absence of method of analysis meeting the minimum performance criteria monitoring is carried out using best available techniques not entailing excessive costs.

DIRECTIVE QA/QC



EN Official Journal of the European Union 1.8.2009

COMMISSION DIRECTIVE 2009/90/EC

of 31 July 2009

laying down, pursuant to Directive 2000/60/EC of the European Parliament and of the Council, technical specifications for chemical analysis and monitoring of water status

(Text with EEA relevance)

- Article 6 Quality assurance and control
- laboratories or parties contracted by laboratories apply quality management system practices in accordance with **EN ISO/IEC-17025 or other equivalent** standards accepted at international level

- laboratories or parties contracted by laboratories demonstrate their competences in analysing relevant physico-chemical or chemical measurands by:
  - participation in **proficiency testing programmes** at levels of **concentrations that are representative** of chemical monitoring programmes
  - analysis of available reference materials that are representative of collected samples + appropriate levels of concentrations

  - No requirements on traceability to SI

23.12.2020 EN Official Journal of the European Union L 435/1

I

(Legislative acts)

DIRECTIVES

DIRECTIVE (EU) 2020/2184 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL
of 16 December 2020
on the quality of water intended for human consumption
(recast)

(Text with EEA relevance)

Brussels, 19.1.2022
C(2022) 142 final

COMMISSION IMPLEMENTING DECISION

of 19.1.2022

establishing a watch list of substances and compounds of concern for water intended for human consumption as provided for in Directive (EU) 2020/2184 of the European Parliament and of the Council

ANNEX

WATCH LIST OF SUBSTANCES AND COMPOUNDS OF CONCERN FOR WATER INTENDED FOR HUMAN CONSUMPTION

Name of substance/group of substances or compound/group of compounds	CAS number	EU number	Guidance value (ng/L)	Limit of Quantification[1] (ng/L)	Possible method of analysis
17-beta-estradiol	50-28-2	200-023-8	1	≤ 1	-
nonylphenol[2]	84852-15-3	284-325-5	300	≤ 300	EN ISO 18857-2


QUESTIONS?
COMMENTS?
CONCERNS?

WHAT DO WE NEED TO MEASURE?
WHAT ARE WE MEASURING?

WHOLE WATER MEASUREMENTS WHAT ARE WE TALKING ABOUT ?

Definitions

- **Whole water** is synonym for the original water sample and shall mean the water sample when solid matter and the liquid phase have not been separated [Guidance CIRCA 19]

- **Liquid (dissolved) fraction s**hall mean an operationally defined fraction of whole water from which suspended particulate matter has been removed by an appropriate methodology. [Guidance CIRCA 19]

- **Suspended particulate matter** shall mean the particulate matter fraction of the whole water sample after separation with an appropriate methodology. [Guidance CIRCA 19]

WHOLE WATER MEASUREMENTS WHAT ARE WE TALKING ABOUT ?

Definitions

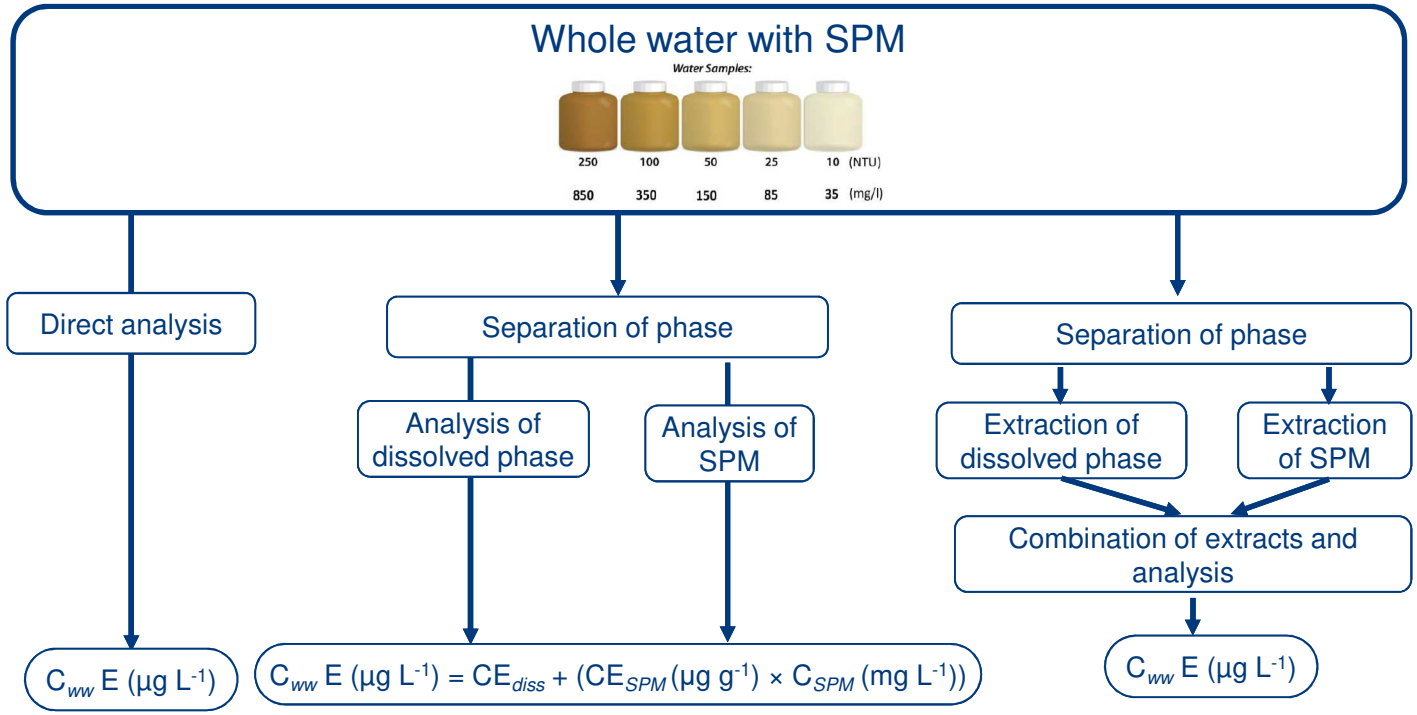
- **Total concentration of the analyte** shall mean the total concentration of the analyte in the whole water sample, reflecting both dissolved and particle bound concentrations of the analyte. [Guidance CIRCA 19]

- **Dissolved concentration of the analyte** shall mean the concentration of the analyte in the liquid (dissolved) fraction of a whole water sample. [Guidance CIRCA 19]

- **Particle bound concentration of the analyte** shall mean the concentration of the analyte bound to SPM. [Guidance CIRCA 19]

WHOLE WATER MEASUREMENTS

How to deal with Whole Water measurements ?



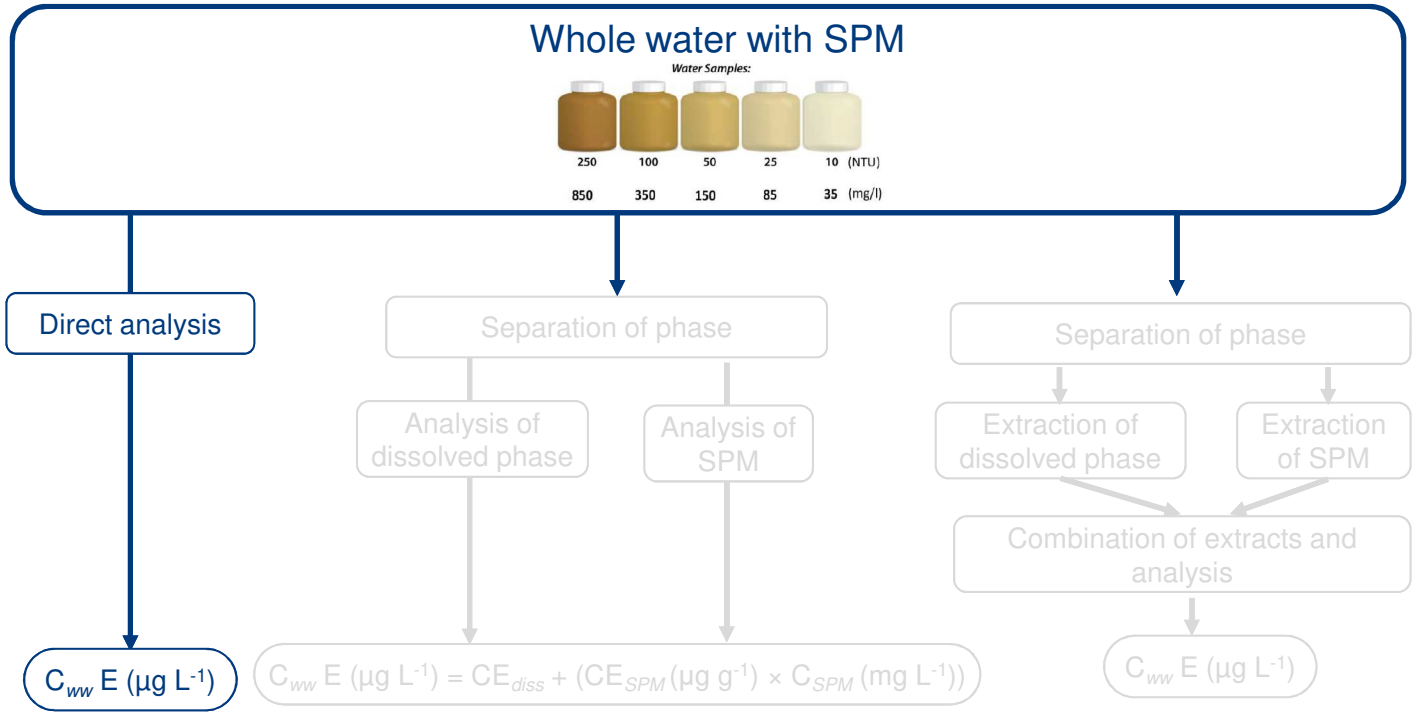
WHOLE WATER MEASUREMENTS

How to deal with Whole Water measurements

	Direct analysis	Separate analysis of SPM and Dissolved phase	Separate extraction of SPM and Dissolved phase and combined analysis
Advantages	▪ Simple to implement ▪ Limitations of intermediate steps in sample preparation to minimize risks of sorption and contamination	▪ Knowlegde of the partitionning ▪ Compatible with automated sample preparation approach	▪ Compatible with automated sample preparation approach ▪ Overpass issue of calculation
Limitations / Drawbacks	▪ Absence of knowlegde of the partitionning ▪ Limitation on the choice of sample preparation approach	▪ Sufficient quantity of SPM, large volume sampling ▪ Issue of calculation (impact of C< LOQ) ▪ Loss by sorption during the filtration ▪ Risk of cross contamination during the filtration and multiple sample preparation approach	▪ Absence of knowlegde of the partitionning ▪ Loss by sorption during the filtration ▪ Risk of cross contamination during the filtration and multiple sample preparation approach ▪ Imply ad'hoc quantification approach

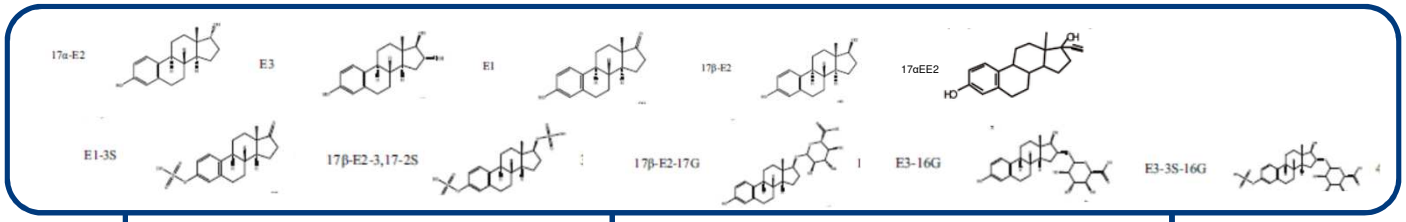
WHOLE WATER MEASUREMENTS

How to deal with Whole Water measurements



WHOLE WATER ESTROGENS MEASUREMENTS

How to deal with free/conjugates estrogens measurements



Direct analysis

Separation of forms (extraction)

Deconjugation

Analysis of free form

Analysis of conjugated forms

Analysis of total E as E free

(Opt Deconjugation) if GC_MS

C_{ww} E Free (µg L^{-1}) and/or C_{ww} E conjugated (µg L^{-1}) (if LC-MS)

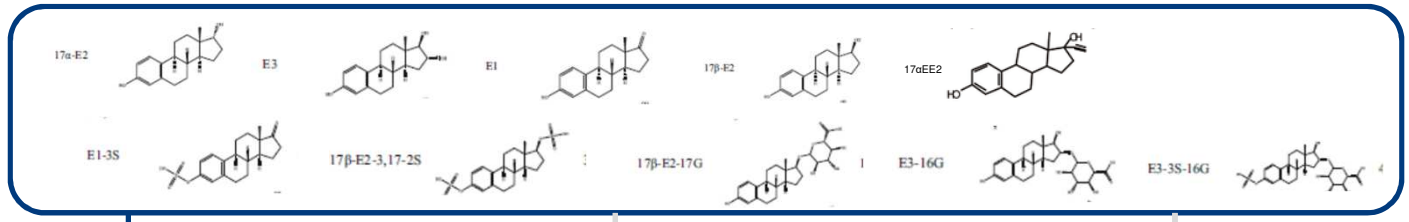
C_{ww} E Free (µg L^{-1})

C_{ww} E conjugated (µg L^{-1}) or as Free equivalent

C_{ww} E total (µg L^{-1}) as E free

WHOLE WATER ESTROGENS MEASUREMENTS

How to deal with free/conjugates estrogens measurements



Direct analysis

C_{ww} E Free (µg L^{-1}) and/or C_{ww} E conjugated (µg L^{-1}) (if LC-MS)

Separation of forms (extraction)

Analysis of free form

Analysis of conjugated forms

(Opt Deconjugation) if GC_MS

C_{ww} E Free (µg L^{-1})

C_{ww} E conjugated (µg L^{-1}) or as Free equivalent

Deconjugation

Analysis of total E as E free

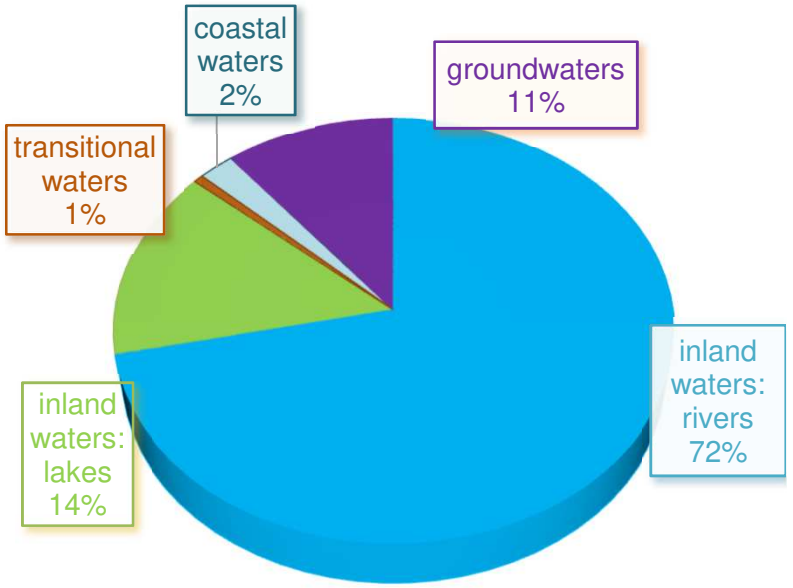
C_{ww} E total (µg L^{-1}) as E free

EUROPEAN WATER BODIES

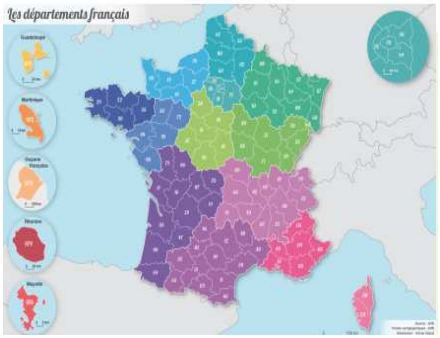
Europe (25)

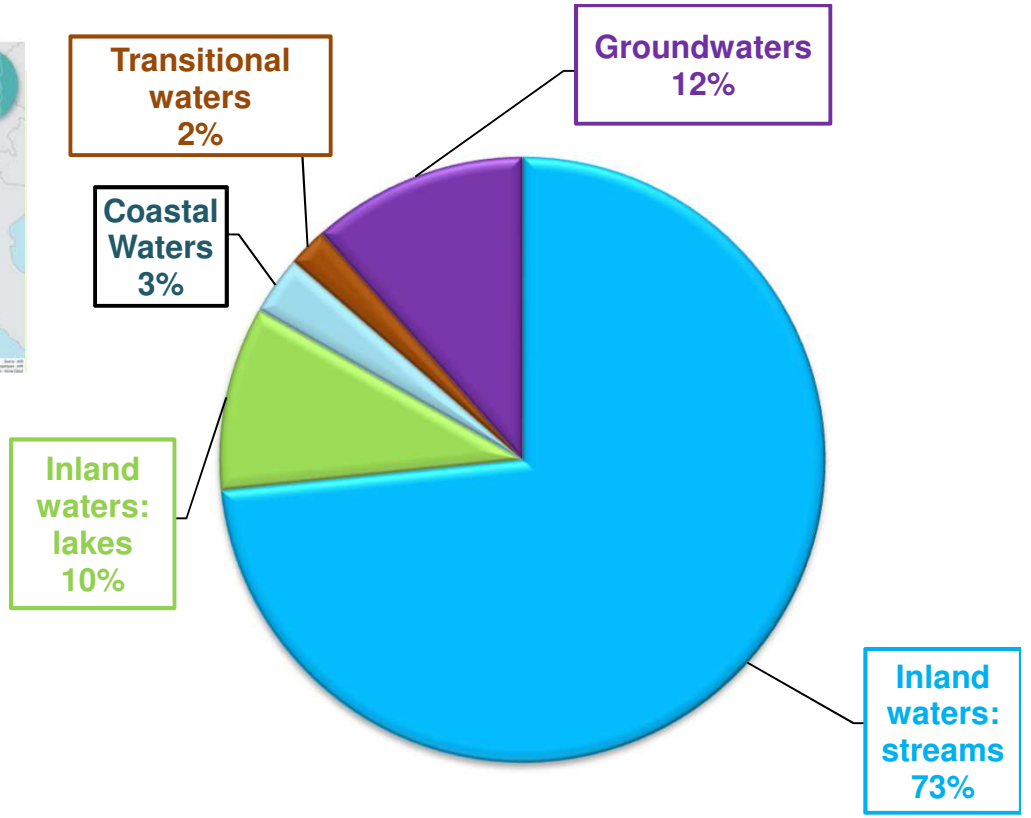
Source:
https://www.eea.europa.eu/themes/water/european-waters

TYPOLOGY OF WATER BODIES



FRENCH WATER BODIES





Source FNE

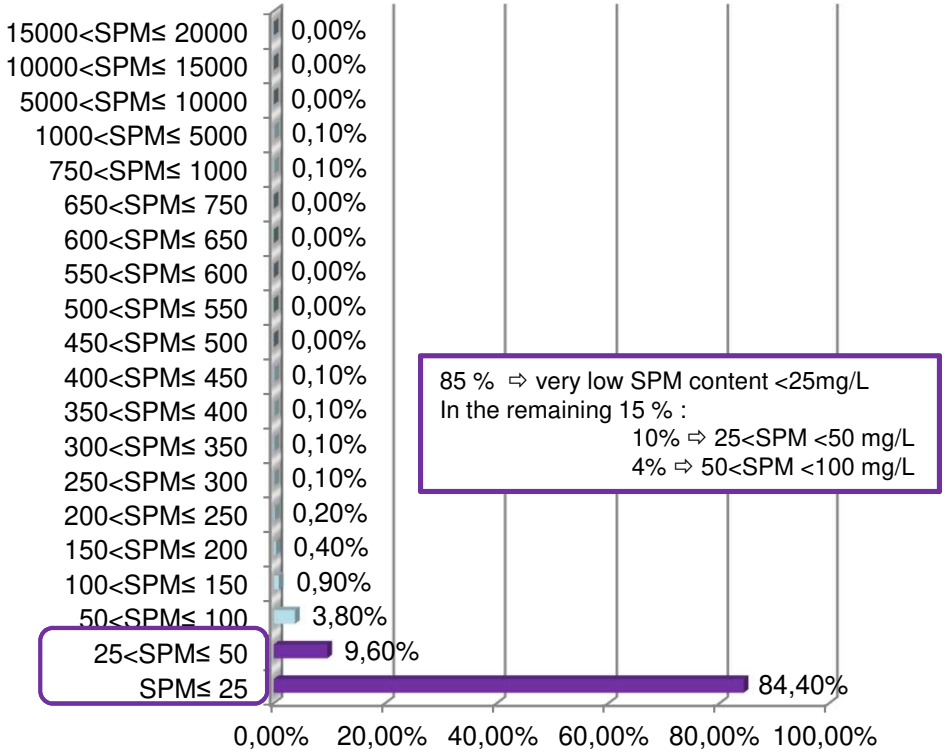
Suspended Particulate Matter

Inland surface waters

Min	0.06 mg/L
Max	16, 000 mg/L
Average	21.7 mg/L
Median	9 mg/L
Quartile 25	4.8 mg/L
Quartile 75	17 mg/L

Number of data = 188 283.according to EN 872 with few exceptions

Period : 03/01/2011 01/01/2018



Source :
naiades.eaufrance.fr

➢ Total Organic Carbon

Min	0.09 mg C/L	Median	3.1 mg C/L
Max	190 mg C/L	Quartile 25	2.04 mg C/L
Average	3.80 mg C/L	Quartile 75	4.6 mg C/L

➢ Conductivity

Min	0.131 µS/cm	Median	381 µS/cm
Max	60,500 µS/cm	Quartile 25	181 µS/cm
Average	490 µS/cm	Quartile 75	602 µS/cm

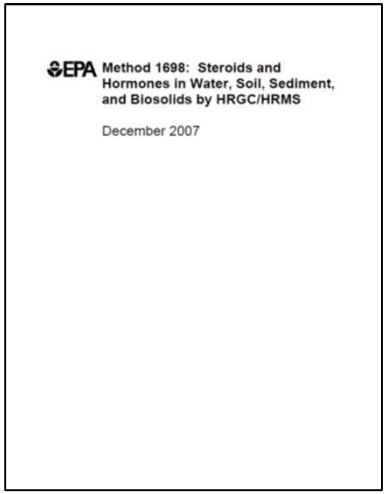
➢ pH

Min	5.3	Median	7.9
Max	12.96	Quartile 25	7.5
Average	7.88	Quartile 75	8.2

Source :
naiades.eaufrance.fr

AVAILABLE REFERENCES

MS based methods


EPA Method 1698: Steroids and Hormones in Water, Soil, Sediment, and Biosolids by HRGC/HRMS

December 2007

- Only one available CRM (17βE2) as primary calibrant to establish traceability to SI
- No matrix matched Certified Reference Material
- Some PT but material not representative (see eptis database)

AVAILABLE REFERENCES

EBM

ISO 19040-1
Water quality — Determination of the estrogenic potential of water and waste water — Part 1: Yeast estrogen screen (Saccharomyces cerevisiae)

ISO 19040-2
Water quality — Determination of the estrogenic potential of water and waste water — Part 2: Yeast estrogen screen (A-YES, Arxula adeninivorans)

ISO 19040-3
Water quality — Determination of the estrogenic potential of water and waste water — Part 3: In vitro human cell-based reporter gene assay

- Only one available CRM (17β E2) as primary calibrant to establish traceability to SI
- No matrix matched Certified Reference Material
- Some PT (see eptis database) but design is not totally appropriate


QUESTIONS?
COMMENTS?
CONCERNS?

EDC WFD

Training workshop: Solutions to tackle WFD requirements for estrogen determination in water

7-9 september 2022

EDC WFD

SAMPLING

SAMPLING

- Comply with general principles of ISO 5667 guidances
- Implement general principles for ultratraces analysis
- No specific risk of contamination on the field identified: environment, operator, …

- Recommandations:

  - use glass bottle + prevent from photodegradation (amber, green bottles, alumina foil, …)
  - avoid intermediate material to prevent from losses due to sorption and risks of cross contamination
  - cleaning procedure should be implemented: eg calcination of glassware, rincing, …

Avoid plastics especially if EBM have to be implemented

SAMPLING

Illustration of sorption phenomena

Table 2. Relative mass lost over 24 h and first-order sorption rates for selected r

Material	%E2†	%EE2	%E1
Type 304 stainless steel	24.9 ± 5.5	53.4 ± 2.0	52.7 ± 2.4
Type 316 stainless steel	30.5 ± 1.8	56.1 ± 3.7	54.3 ± 3.6
Glass (culture tubes)	1.0 ± 0.7	0.7 ± 0.6	0.6 ± 0.4
PolyCarbonate	8.7 ± 5.1	51.2 ± 8.3	44.1 ± 6.8
PVC‡	4.5 ± 2.5	5.0 ± 2.1	7.7 ± 4.5
Teflon	2.3 ± 1.3	4.2 ± 0.6	2.2 ± 0.8
Autoclaved			
Type 304 stainless steel	2.3 ± 7.0	33.7 ± 7.6	28.4 ± 6.1
Type 316 stainless steel	6.8 ± 17.7	33.2 ± 6.2	20.9 ± 5.8
Glass (culture tubes)	−4.9 ± 0.6	−0.2 ± 1.2	0.5 ± 0.8

- Minimum losses with glass container and materials

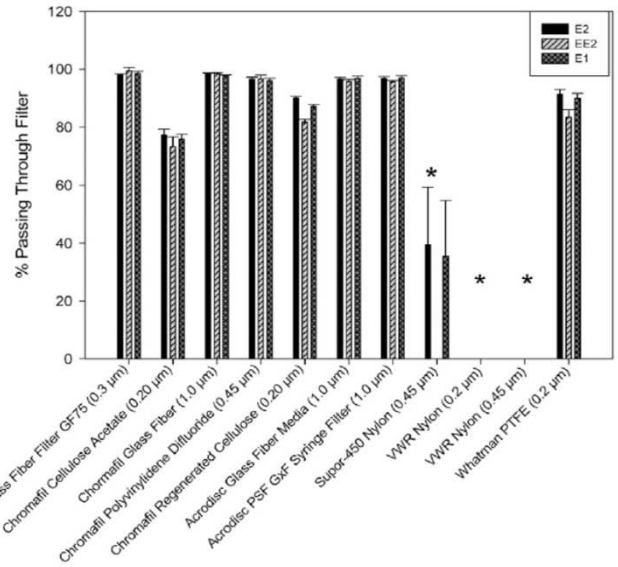


Fig. 3. Percent passing through different filter types. *Significant differences ($p < 0.05$) between the percent passing and the initial concentration. Error bars represent 1 SD of the mean for triplicate samples. E1, estrone; E2, 17β-estradiol; EE2, 17α-ethynylestradiol.

Charles W. Walker* and John E. Watson, 2010


QUESTIONS?
COMMENTS?
CONCERNS?

STABILITY/ INTEGRITY

Stability

Main drivers

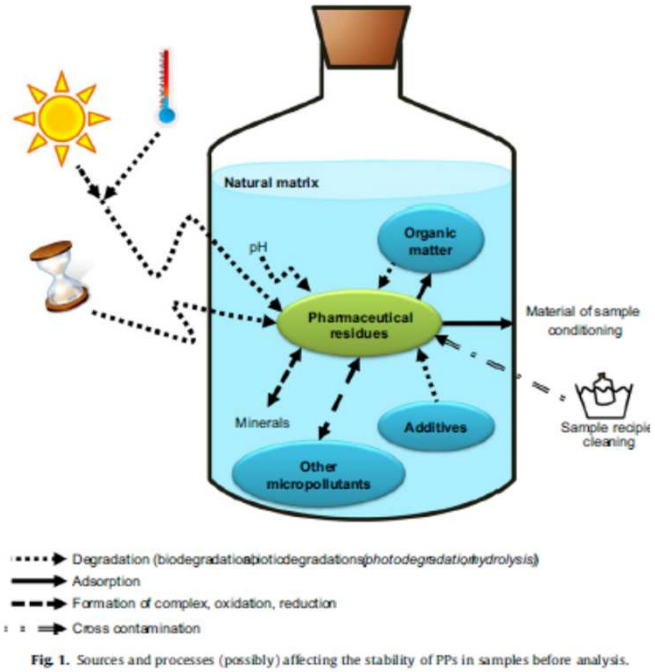


Fig. 1. Sources and processes (possibly) affecting the stability of PPs in samples before analysis.

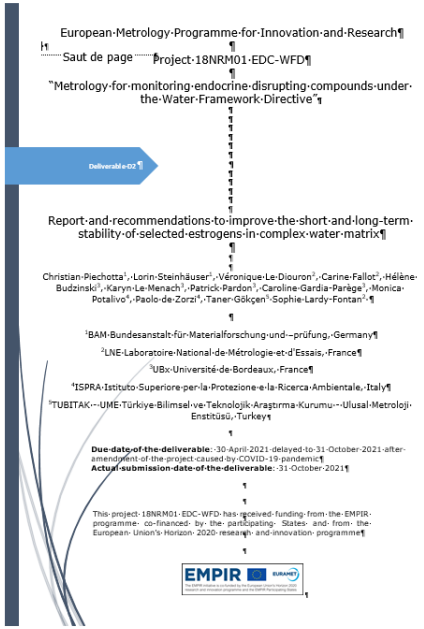
Mompelat et al. (2013)

Numerous review and publications

⇒ Not all in agreement

- Methodological strategy
- Acceptance criteria
- Missing key information

Stability


European Metrology Programme for Innovation and Research

Project 18NRM01 EDC-WFD

"Metrology for monitoring endocrine disrupting compounds under the Water Framework Directive"

Deliverable D2

Report and recommendations to improve the short and long term stability of selected estrogens in complex water matrix

Christian Piechotta[1], Lorin Steinhäuser[1], Véronique Le Diouron[2], Carine Fallot[2], Hélène Budzinski[2], Karyn Le Menach[2], Patrick Pardon[2], Caroline Gardia Parège[2], Monica Potalivo[3], Paolo de Zorzi[3], Taner Gökçen[4], Sophie Lardy Fontan[4]

[1]BAM Bundesanstalt für Materialforschung und prüfung, Germany

[2]LNE Laboratoire National de Métrologie et d'Essais, France

[2]UBx Université de Bordeaux, France

[3]ISPRA Istituto Superiore per la Protezione e la Ricerca Ambientale, Italy

[4]TUBITAK UME Türkiye Bilimsel ve Teknolojik Araştırma Kurumu Ulusal Metroloji Enstitüsü, Turkey

Due date of the deliverable: 30 April 2021 delayed to 31 October 2021 after amendment of the project caused by COVID 19 pandemic

Actual submission date of the deliverable: 31 October 2021

This project 18NRM01 EDC WFD has received funding from the EMPIR programme co financed by the participating States and from the European Union's Horizon 2020 research and innovation programme

EMPIR

Following

- ISO GUIDE 35:2017 Reference materials — Guidance for characterization and assessment of homogeneity and stability
  ⇒ develop candidate reference material(s)

- ISO TS 5667-25 Water quality — Sampling — Part 25: Guideline on the validation of the preservation time of water samples
  ⇒ recommandations on maximum acceptable delay before analysis (MaxADs)

Stability study design 1

Stability water sample from sampling until analysis

- Five selected estrogens E1, 17αE2, 17βE2, E3 and 17αEE2 in water matrices
- Representative synthetic real water matrix: Evian water, DOC of 7 mg/L, pH 7.3 without SPM
- Concentration level of 10 ng/L for each species.
- Container: glassware+ protection from sunlight
- Sample preservation: : none, 0.1% methanol, 0.1% ascorbic acid
- Storage conditions: +4°C and room temperature
- Duration: 14 days
- Time laps : Day1, Day2, Day3, Day4, Day5, Day14
- Isochronous study : experimental study of “reference” material stability in which units exposed to different storage conditions and times are measured in a short period of time

  - ➢ Unstability at room temperature
  - ➢ Stability at 4°C up to 14 days
  - ➢ With Ascorbic acid ↗ SD%

Stability study design 2

Stability of a whole water sample from sampling until analysis

- five selected estrogens E1, 17α-E2, 17βE2, E3 and 17α-EE2
- Representative synthetic real water matrix: Evian water, DOC of 7 mg/L, pH 7.3 and SPM 50 mg/L
- Concentration level of 10 ng/L for each species.
- sample preservation: none, 0,1% methanol, (0,1% ascorbic acid)
- Container: glassware+ protection from sunlight
- Storage conditions: +4°C Duration: 14 days
- Time laps : Day1, Day 5, Day 14
- Isochronous study

  - ➢ without stabilising reagent or with 1% methanol show no significant trend with regards to degradation or loss of the analytes during storage time
  - ➢ With Ascorbic acid ↗ SD%

Stability study design 3

Stability of a whole water sample from sampling until analysis

- five selected estrogens E1, 17α-E2, 17βE2, E3 and 17α-EE2 in water matrices
- Representative synthetic real water matrix: Evian water, DOC of 7 mg/L, pH 7.3 and SPM 50 mg/L iron and manganese oxidizing bacteria (*Sphingomonas spec. and Sphaerotilus spec.*)
- Concentration level of 10 ng/L for each species.
- Container: glassware+ protection from sunlight
- Sample preservation: none, 0,1% ascorbic acid
- Storage conditions: +4°C
- Duration: 14 days
- Time laps : Day1, Day 5, Day 14
- Isochronous study

➢ impact of microbial activity showed only a small influence on the decrease of the estrogen concentration within a period of 14 days

Stability

- Recommandations:

- Material in contact with water samples whould be cleaned cautiously
- Glassware (coloured) shall be prefered to any other type of material if LOQ <<0,1ng/L targeted to minimize the risks of sorption/contamina tion (interferences)
- Storage at 4°C is OK for 7 days if low complex matrix.
- For complex matrix storage time shall be shorten. Addition of 1% MeOH is recommended to avoid biodegradation
- Storage at -20°C is possible but could be at risks of clogging if SPE cartridge are implemented. Some impacts on ME% (↗) have also been observed

Plastic container shall be avoided if LOQ <<0,1ng/L targeted


QUESTIONS?
COMMENTS?
CONCERNS?

EDC WFD

Quantification strategies for estrogens with regards to the EU-WFD

7-9 September 2022

EDC WFD

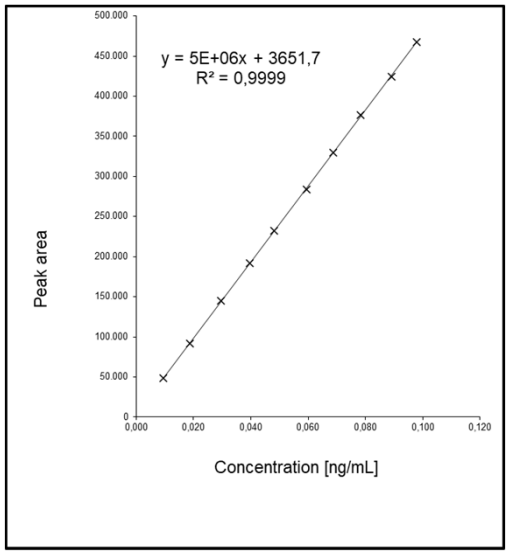
Overview on the quantification of estrogens

Selectivity – Identification of target estrogens in chromatograms with mass spec detection

- It is necessary to demonstrate that the identification of the target analytes is properly achieved and, moreover, that the target analytes signals are not influenced by the presence of chemically or physically interferents.
- Target analytes shall be identified in accordance with ISO 21253-1: Define target m/z ratios for each analyte (if possible a quantifier and at least two qualifier ions with distinct intensity ratios)
- Using tandem mass spec: implementation of transitions for the target molecules to specific fragments
- Using high res mass spec: define specific exact m/z ratios for the target molecules or of specific fragments

Overview on the quantification of estrogens

- External calibration using unlabeled target estrogens:



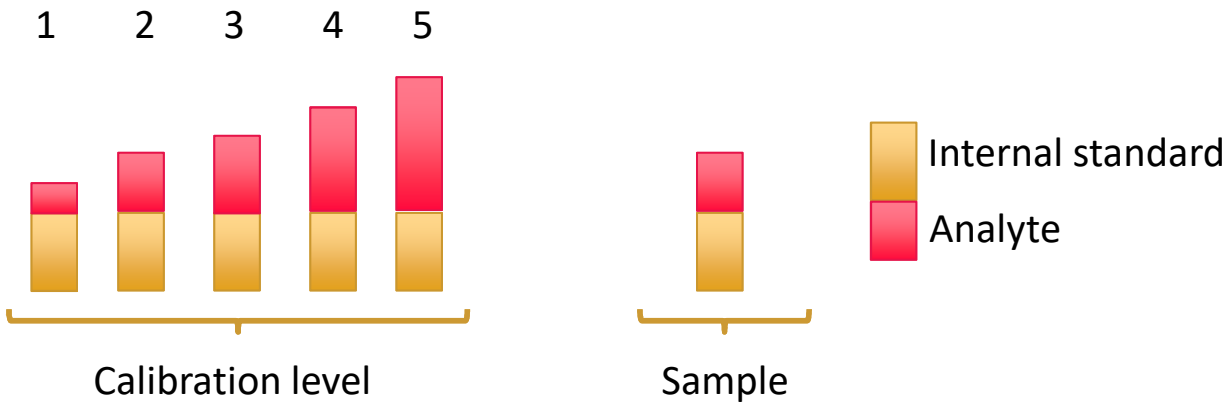
- After regression calculation of estrogen concentration by using the regression equation and the area of the sample extract obtained by LC- or GC-MS(/MS)

Overview on the quantification of estrogens

- Loss of analyte during preconcentration and clean up steps is not considered
- Influence of matrix on the measurement can enhance or reduce the signal intensity compared to non-matrix containing calibration level
- Internal standard is needed to correct the loss of analyte during sample preparation or sample inlet to chromatography with mass spec detection
- Internal standard is a compound that is similar to chemical species of interest in the samples
- Physicochemical properties should be identically

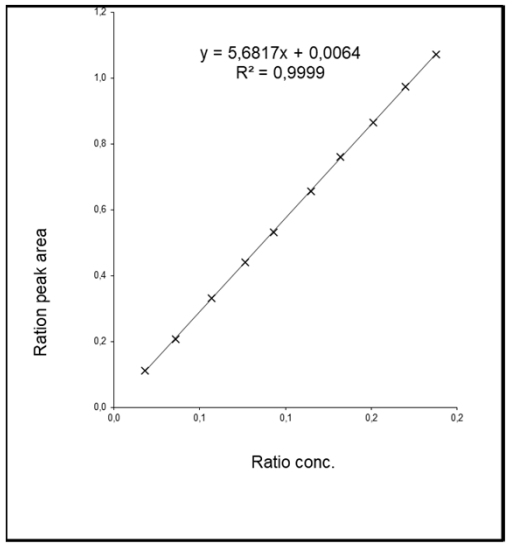
Overview on the quantification of estrogens

- Internal standard of choice: stable isotopically labeled target analytes (e.g., deuterated, ^{13}C- or ^{18}O-labeled species)
- Identical boiling points, polarities, and fragmentation patterns in EI-MS
- Internal standard and analyte get the same impact by matrix, sample prep., purification and analysis



Overview on the quantification of estrogens

	Concentration in ng L^{-1}		ratio conc. Analyt/ INT	peak area (counts)		Ratio peak area Analyt/ INT
	Analyt	INT-STD		Analyt	INT	
Cal 1	0,098	0,521	0,19	467955	436874	1,07
Cal 2	0,089	0,524	0,17	424045	435361	0,97
Cal 3	0,078	0,517	0,15	376758	435804	0,86
Cal 4	0,069	0,522	0,13	330095	434732	0,76
Cal 5	0,060	0,519	0,11	283936	432834	0,66
Cal 6	0,048	0,516	0,09	231994	436468	0,53
Cal 7	0,040	0,521	0,08	191640	435065	0,44
Cal 8	0,029	0,520	0,06	145134	437598	0,33
Cal 9	0,019	0,524	0,04	91929	443525	0,21
Cal 10	0,010	0,524	0,02	48572	438664	0,11



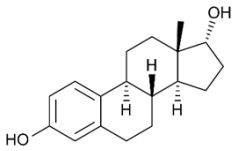
Solved equation from the linear regression:

$$\beta_A = \frac{\left(\frac{y_A}{y_{IS}} - b\right)}{a} * \beta_{IS}$$

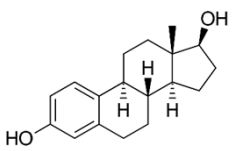
- Typical ten point calibration curve of estriol. Internal standard concentration at mid concentration level of the analyte

- Concentration ratio vs. area ratio obtained from the analysis

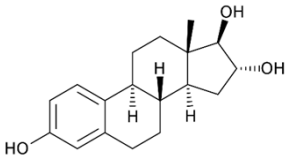
Overview on the quantification of estrogens



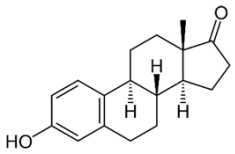
17-alpha-estradiol



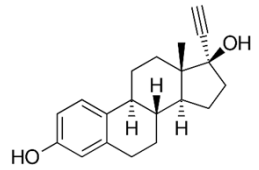
17-beta-estradiol



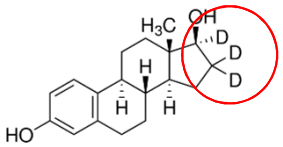
Estriol

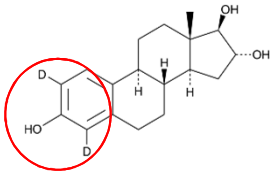


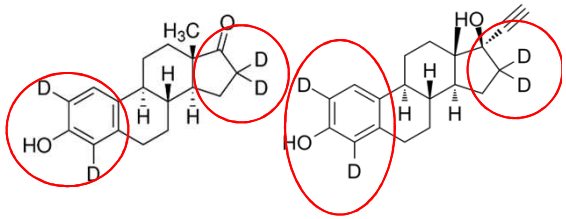
Estrone



17-alpha-ethinylestradiol





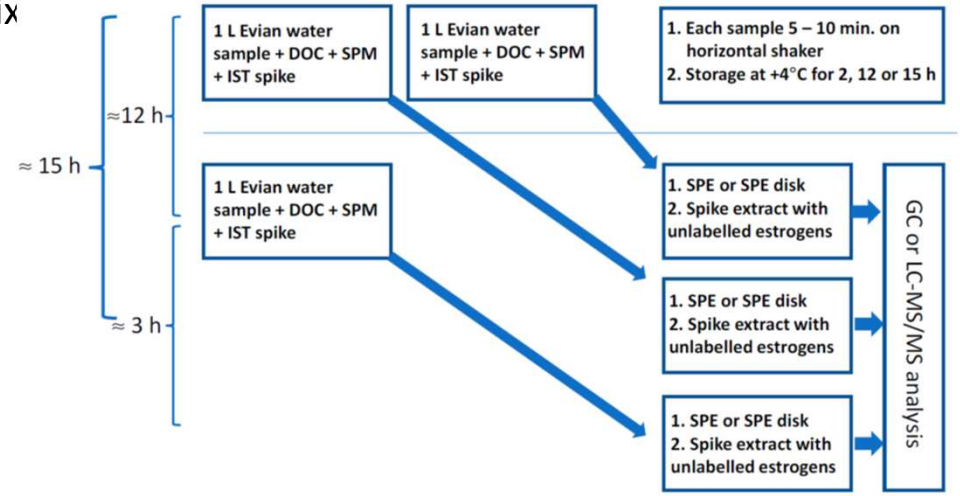


- In general ^{13}C-labeled species are more stable than deuterated once (deuterium-hydrogene exchange, depending on the position of the labeling)
- It could be demonstrated that deuterated estrogens are stable and can comparable be used as ^{13}C-labeled species (costs for routine labs)
- IDMS-technique – organic isotope dilution calibration

Overview on the quantification of estrogens

Equilibrium time for internal standards

- The presence of humic acid and SPM reduces the extraction efficiency down to 10 and 75% and the recovery of the internal standards depending on the equilibrium time. To obtain reliable estimates it is essential to allow a certain time for equilibration between the isotope spike and the aqueous matrix



Overview on the quantification of estrogens

Equilibrium time for internal standards

For the different labeled estrogens, an equilibrium time of at least 15 h is suggested. No difference is observed between the different types of internal standards (deuterated, ^{13}C). In consequence, the **samples** must be **spiked** with the **isotopically labeled standards** at the **end of a working day** to store them **overnight (15 h)** at **+4°C**.

Overview on the quantification of estrogens

- Within a typical method validation the method recovery (i.e. absolute and relative recovery) will be assessed so see the performance or capability of the analytical procedure.

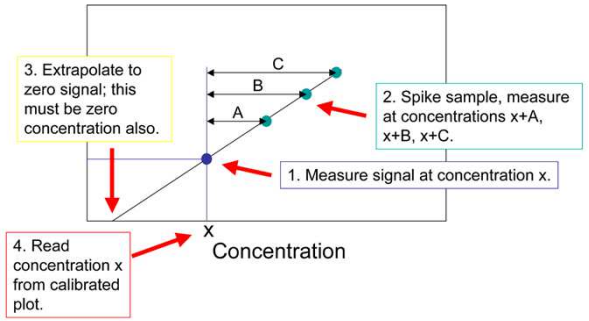
- Absolute recovery will be determined in each matrix by comparing the peak areas of spiked samples prior and post extraction.

- Relative recoveries will be calculated in each matrix by comparing the ratios of the peak areas of the target analytes to the corresponding peak areas of the internal standards in spiked samples prior extraction and post extraction.

Overview on the quantification of estrogens

Alternative quantification strategies:

- Standard addition: a type of quantitative analysis approach whereby the standard is added directly to the analyzed sample. It is used where the sample matrix also contributes to the analytical signal, a situation known as the matrix effect, thus making it impossible to compare the analytical signal between sample and standard using the traditional calibration curve approach



- Use of non-isotopically labeled internal standard: Here, structural analogous were used e.g. 3-*O*-methyl estrone

Overview on the quantification of estrogens

Conclusion:

- Analyzing estrogens in complex water matrix suitable internal standards are needed
- To respect matrix effects and the loss of analytes during sample prep. and purification stable isotopically labeled species of the target analytes should be used
- Organic isotope dilution calibration with mass spectrometric detection is a powerful tool to increase the relative recovery of the target analytes within the analytical procedure


QUESTIONS?
COMMENTS?
CONCERNS?

EDC WFD

Sample preparation strategies for estrogens with regards to the EU-WFD

7-9 September 2022

EDC WFD

Overview on preconcentration procedures

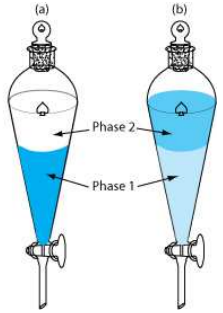
Needs for sample preparation

In general a compromise is necessary which addresses the whole water sample, a reasonable preconcentration factor, and the robustness of the method:

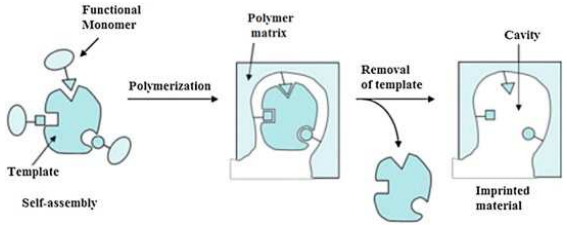
- Preconcentration of target analytes due to low EQS given by the EU-WFD
- Separation of matrix components from target analytes to prevent matrix effects on the chromatographic separation e.g., decreasing separation efficiency of the chromatographic column, enhancing or decreasing signal intensity (ion suppression in ESI source)
- Avoiding contamination of sample inlets (GC liner contamination by matrix components)

A preselection of feasible and applicable techniques was done by all project partner due to their practical knowledge

Overview on preconcentration procedures



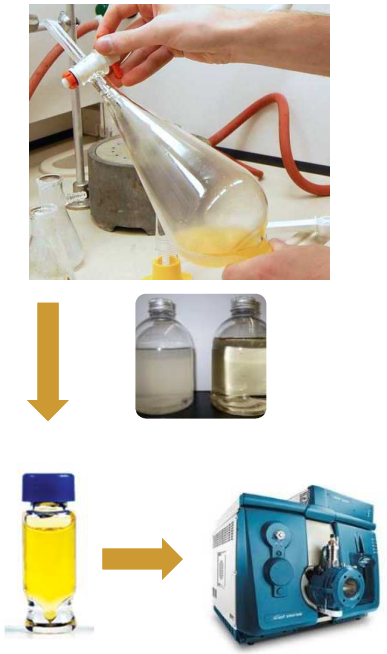
- Liquid-liquid extraction (LLE)
- Solid phase extraction (SPE) columns (off- and online)
- SPE disks
- Mi-SPE (molecular imprinted polymers)



Overview on preconcentration procedures

Liquid-liquid extraction (LLE)

- Use of high volumes of organic extraction solvents (commonly 30 – 50 mL; green chemistry)
- Only non-miscible organic solvents can be used for extraction (limited selectivity)
- Only “batch extraction”, must be repeated with portions of fresh extractions solvent
- Physical stressful for whom how do the extraction – it is not automatable – no extractions in parallel (not feasible for routine labs)
- Batch extracts must be combined and evaporated



Not applicable in routine lab!

Overview on preconcentration procedures

Liquid-liquid extraction (LLE)

Typical procedure:

500 mL water sample

- 3 x extraction with 30 mL DCM
- Extraction time 1 min

Combining organic layers

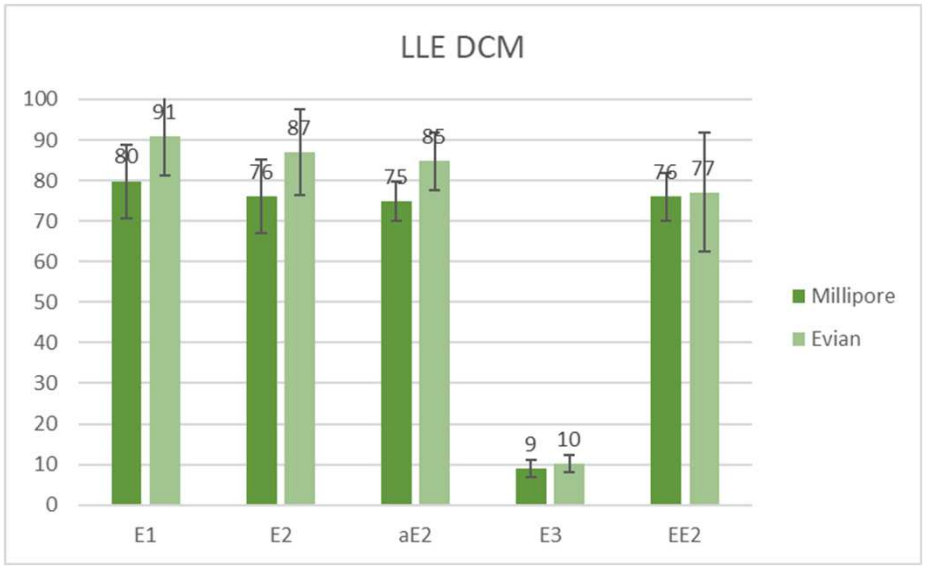
- Evaporation to dryness
- Reconstitution with 1 mL ACN

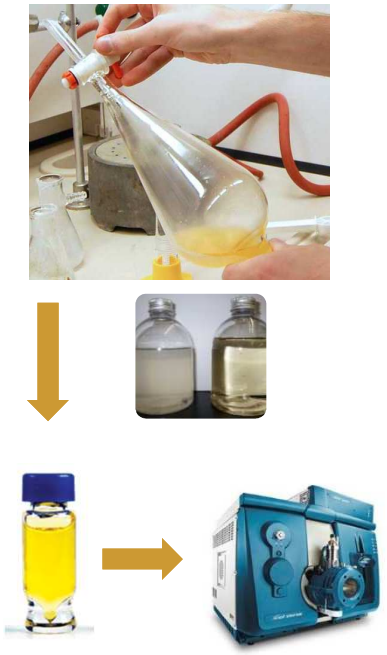
Analysis

- The ACN extract is ready for analysis

Overview on preconcentration procedures

Liquid-liquid extraction (LLE)





- Sufficient absolute recoveries for E1, E2, alphaE2 and EE2 but poor for E3 independently from the type of water matrix

Overview on preconcentration procedures

Solid phase extraction (SPE) off- or online

- E.g., HLB (hydrophilic-lipophilic balanced) 6 or 3 mL / filled with 100 or 60 mg sorbent material. Other materials are also feasible (C_{18}, C_{18} eq, …)
- With a content of less than 50 mg L^{-1} SPM no clogging can be observed (it strongly depends on zhe ration of SPM to sample volume)
- Preconcentration not only of the target analytes but also enrichment of DOC content (was evaluated by measuring the DOC of the sample extract)
- Problems of ion suppression when doing LC-MS/MS (in most cases decrease of the signal intensity, known effect in the ESI source)

Overview on preconcentration procedures

A typical procedure

1000 mL water sample

- Spiked with IS-mix at desired level
- 15 min on horizontal shaker
- Oasis HLB 3 mL/100mg

Conditioning / sample load

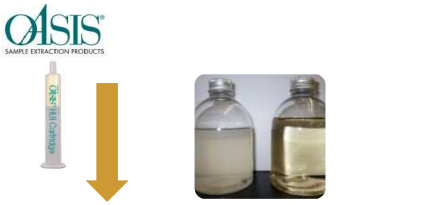
- Conditioning with 10 mL ACN
- Conditioning with 15 mL H_2O
- Load 1000 mL water sample 20 mL/min

Washing step and elution

- Washing cartridge with 10 mL H_2O
- Dry cartridge with N_2 for 1 min
- Collect 10 mL fraction into sample tube using ACN

Concentration and analysis

- Concentrate organic fraction to 1 mL (TurboVap)
- Extract is ready for analysis by LC-MS/MS



Overview on preconcentration procedures

Solid phase extraction (SPE) off- or online

- Experiments were carried out by all project partners
- Different matrix compositions were used:
  - Low complex matrix (Evian water with low DOC and without SPM)
  - High complex matrix (Evian water with moderate DOC and with SPM)
- Relative and absolute recovery rates were determined to compare the different experiments with regards to their performance (applicability in routine analysis)

Overview on preconcentration procedures

Solid phase extraction (SPE) off- or online

Low complex matrix (Evian water with low DOC and without SPM)

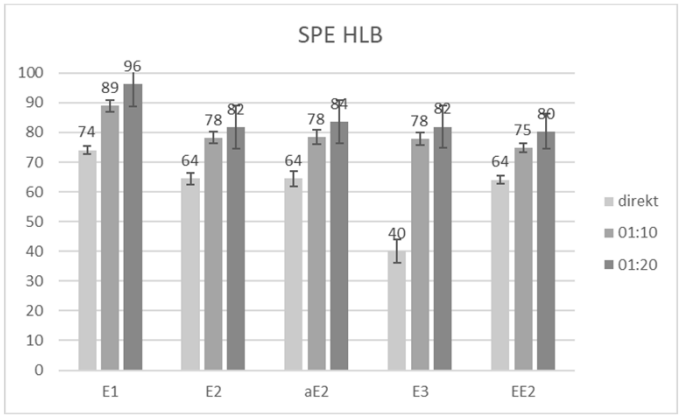
Extraction method / Parameter	Waters Prime HLB size 3cc/60mg, Evian water (500ml) + DOC (7 mg/L) and estrogens at 5 ng/L level			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	111,8	5,0	101,3	0,6
17α-Estradiol (aE2)	132,8	5,7	98,5	6,5
17β-Ethinylestradiol (bE2)	110,5	6,3	97,9	0,6
Estriol (E3)	118,4	6,9	101,0	5,9
17α-Ethinylestradiol (EE2)	110,9	4,6	98,3	4,1

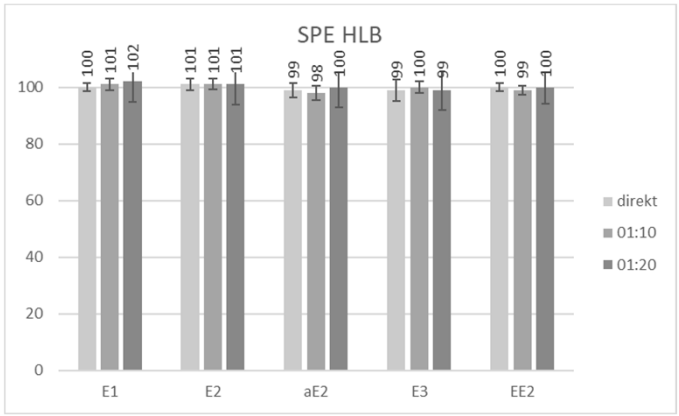
- Sufficient absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Ideal relative recoveries by isotope dilution calibration
- But: no SPM in the water matrix which can cause clogging of the cartridge

Overview on preconcentration procedures

Solid phase extraction (SPE) off- or online

High complex matrix (Evian water with moderate DOC and with SPM)





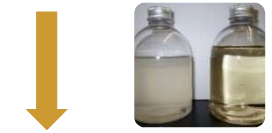
- Sufficient absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Matrix will also be preconcentrated; increasing signals by diluting the sample extracts
- Ideal relative recoveries by isotope dilution calibration

EDC WFD

Overview on preconcentration procedures

SPE disk (combination of filtration and SPE)

- Good compromise of filtration and enrichment
- E.g., HLB, DVB or C_{18} disks with 47 mm diameter are feasible (capacity is only defined as L, H and M – not correlation to distinct amounts of SPE sorbents possible)
- Need of special manifold suitable for SPE disks
- Sufficient absolute recovery rates within 80 to 90% in complex water matrix (DOC, SPM an inorganic content)
- Applicable for SPM content higher than 50 m L^{-1} g e.g., 500 mg L^{-1}
- Preconcentration of DOC content comparable to common SPE



Overview on preconcentration procedures

A typical procedure

1000 mL water sample

- Spiked with IS-mix at desired level
- 15 min on horizontal shaker
- SPE disk e.g., Atlantic HLB M/L or H

Conditioning / sample load

- Conditioning with 20 mL ACN
- Conditioning with 3 x 10 mL H_2O
- Load 1000 mL water sample in approx. 30 min

Washing step and elution

- Washing cartridge with 10 mL H_2O
- Drying of SPE disk by applying vacuum for 5 min
- Collect 5 x 10 mL fractions using ACN

Concentration and analysis

- Concentrate organic fraction to 1 mL (TurboVap)
- Yellowish extract is ready for analysis by LC-MS/MS

Overview on preconcentration procedures

SPE disk (combination of filtration and SPE)

- Experiments were carried out by all project partners
- Different matrix compositions were used:
  - Low complex matrix (Evian water with low DOC and without SPM)
  - High complex matrix (Evian water with moderate DOC and with SPM)
- Relative and absolute recovery rates were determined to compare the different experiments with regards to their performance (applicability in routine analysis)

Overview on preconcentration procedures

SPE disk (combination of filtration and SPE)

Low complex matrix (Evian water with low DOC and without SPM)

Extraction method / Parameter	Atlantic® C-18 Disks, 47 mm, Evian without additives (1000 mL) and estrogens at 10 ng/L level; no sample clean-up or derivatization			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	77,2	12,6	90,0	0,3
17α-Estradiol (aE2)	NA	NA	86,8	0,4
17β-Ethinylestradiol (bE2)	88,1	16,3	84,5	10,0
Estriol (E3)	82,8	9,8	88,1	1,4
17α-Ethinylestradiol (EE2)	72,6	10,2	93,8	5,6

- Sufficient absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Ideal relative recoveries by isotope dilution calibration

Overview on preconcentration procedures

SPE disk (combination of filtration and SPE)

High complex matrix (Evian water with moderate DOC and with SPM)

Extraction method / Parameter	Atlantic® DVB Disks, 47 mm , Evian water (500ml) + 25 mg SPM + DOC (7 mg/L) and estrogens at 5 ng/L level			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	92,6	4,0	95,0	7,5
17α-Estradiol (aE2)	92,4	8,8	88,1	6,5
17β-Ethinylestradiol (bE2)	90,8	9,7	88,1	1,4
Estriol (E3)	99,6	4,4	92,5	4,2
17α-Ethinylestradiol (EE2)	97,1	10,7	99,0	2,6

- Sufficient absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Ideal relative recoveries by isotope dilution calibration

Overview on preconcentration procedures

SPE disk (combination of filtration and SPE)

High complex matrix (Evian water with moderate DOC and with SPM)

Extraction method / Parameter	Atlantic® C-18 Disks, 47 mm, unfiltered surface water (1000 mL) and estrogens at 10 ng/L level; no sample clean-up or derivatization			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	25,3	6,0	90,8	4,3
17α-Estradiol (aE2)	NA	NA	78,4	9,7
17β-Ethinylestradiol (bE2)	29,4	4,8	90,5	5,0
Estriol (E3)	23,4	10,4	87,8	0,6
17α-Ethinylestradiol (EE2)	20,7	4,4	104,5	2,6

- Poor absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Ideal relative recoveries by isotope dilution calibration

Overview on preconcentration procedures

AFFINIMIP® SPE

Estrogens

Mi-SPE (molecular imprinted polymers)

PROTOCOL OF PURIFICATION

Sample preparation

100mL of tap water spiked with 17ß-E2-d_3 to a final concentration of 75ng/L was the loading solution.

Purification with a 3mL/100mg AFFINIMIP® SPE Estrogens cartridge

Equilibration
- 3mL Acetonitrile
- 3mL Water

Loading solution from sample preparation

Washing of interferents
- 3mL water
- 3mL Water/Acetonitrile (60/40)

Elution (E)

3mL Methanol

Publications

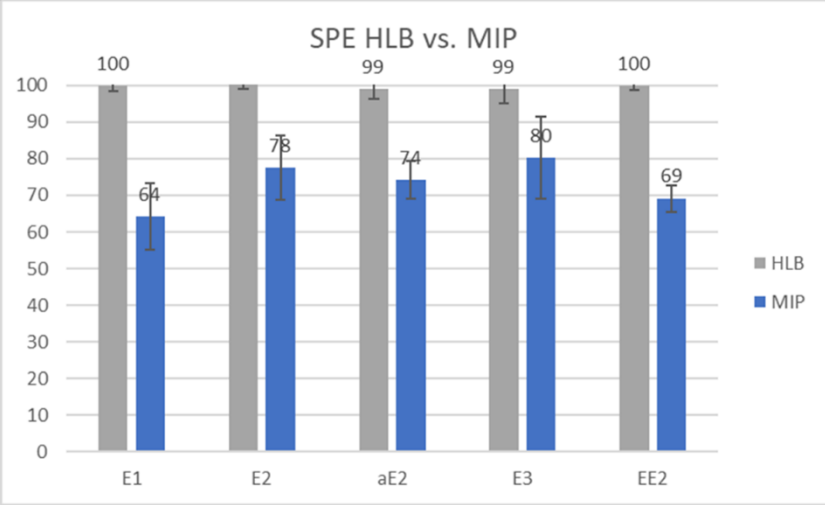
Data extracted from **Determination of steroidal oestrogens in tap water samples using solid-phase extraction on a molecularly imprinted polymer sorbent and quantification with gas chromatography-mass spectrometry (GC-MS)**, D. Zacs, I. Perkons, V. Bartkevics, *Environ Monit Assess* 188, 433, 2016.

- Given protocol by the cartridge provider – limited sample volume of 100 mL – not suitable for estrogens with regards to EU-WFD
- Advantage: specific preconcentration of estrogens – key lock mode of action
- Ideal procedure to separate matrix (e.g., DOC, inorganics, ….) from estrogens
- Possible implementation as purification procedure for extracts obtained from preconcentration steps

Overview on preconcentration procedures

Mi-SPE (molecular imprinted polymers)

Low complex matrix (Evian water with low DOC and without SPM)



- Moderate relative recoveries for E1, E2, alphaE2, EE2 and E3 in comparison to common SPE; no high complex matrix experiments possible due to clogging of the cartridge

Conclusions on preconcentration procedures

Overview of preconcentration methods including limitations and restrictions

(O: fulfilling , +: good, ++: excellent, -: poor, --: unsatisfactory)

Extraction method / Parameter	DI	SPE		SPE disk	LLE	Mi-SPE
		off-	online			
Preconcentration	--	++	++	++	O	--
Clean-up	--	+	+	+	O	++
Compatible to common solvents	--	+	+	+	-	O
Selectivity	--	++	++	++	O	++
Time / efficiency	++	+	++	++	+	O

Conclusions on preconcentration procedures

Extraction method (Typical preconc. factor) / Parameter	SPE (1:1000)		SPE disk (1:1000)		MiSPE (1:100)	
	1	2	1	2	1	2
Estrone (E1)						
17α-Estradiol (aE2)						
17β-Ethinylestradiol (bE2)						
Estriol (E3)						
17α-Ethinylestradiol (EE2)						

- Focusing only on the solid phase extraction techniques SPE is applicable for whole water samples with a SPM load less then 50 mg L^{-1} to avoid clogging of the cartridge
- For higher SPM load the SPE disk is favored from all project partners as the method of choice

Overview on purification/ clean up procedures

Mi-SPE (molecular imprinted polymers)

PROTOCOL OF PURIFICATION
Sample preparation
100mL of tap water spiked with 17ß-E2-d_3 to a final concentration of 75ng/L was the loading solution.
Purification with a 3mL/100mg AFFINIMIP® SPE Estrogens **cartridge**

Equilibration
- 3mL Acetonitrile
- 3mL Water

Loading solution from sample preparation
Washing of interferents
- 3mL water
- 3mL Water/Acetonitrile (60/40)

Elution (E)
3mL Methanol

- Extract from preconcentration step can be evaporated to at least 1 mL and can be diluted using MilliQ water up to 100 mL
- The volume is compatible to the max volume given by the provider SOP
- Advantage: specific preconcentration of estrogens – key lock mode of action
- Ideal procedure to separate matrix (e.g., DOC, inorganics, ….) from estrogens
- DOC in the Mi-SPE is decreased to a minimum (color changed from yellowish to a complete clear and colorless extract (evaluated by complementary DOC measurements) with sufficient recoveries

Overview on purification/ clean up procedures

Other SPE cartridges for purification of sample extracts

- For an alternative clean up of the sample extracts a Supelclean™ LC-NH2 SPE (500 mg, 6 mL) cartridge (Merck, Darmstadt, Germany can be used
- Silica gel based material with amino bounding functional groups
- Increase of matrix components but not specific as MiSPE

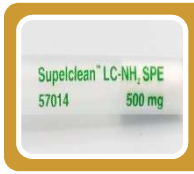
- Also mixed phase cartridges containing e.g., C_{18} and aminopropyl functionality are available (e.g., Chromabond NH_2/C_{18}). Due to limited capacity not sufficient as a two step procedure

Overview on purification/ clean up procedures

Other SPE cartridges for purification of sample extracts

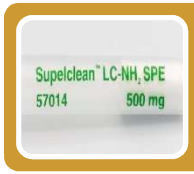
1 mL sample extract

- SPE e.g., Supelclean LC-NH2 (500 mg/6 mL)



Conditioning / sample load

- Conditioning with 4 mL MeOH
- Load sample extract



Washing step and elution

- Collect 2 mL fraction using MeOh

Concentration and analysis

- Concentrate organic fraction to desired volume (TurboVap)
- Clear extract is ready for analysis by LC-MS/MS

Conclusions

- Appropriate techniques either for preconcentration or for purification of the extracts could be evaluated and established within the project by the partners
- Using e.g., HLB sorbent materials (SPE or SPE disk) the DOC will be co-preconcentrated
- Only 50 to 70% of the initial DOC can be removed by the preconcentration
- This can interfere the analytical method e.g., in case of LC-ESI-MS ion suppression can occur
- The result: loss of sensitivity
- A further purification of the extract from the preconcentration procedure is recommended by the project consortium

Two-step procedure preconcentration/purification

- Experiments were carried out by all project partners
- High complex matrix (Evian water with moderate DOC and with SPM)
- Relative and absolute recovery rates were determined to compare the different experiments with regards to their performance (applicability in routine analysis)

Two-step procedure preconcentration/purification

SPE preconcentration + SPE purification

High complex matrix (Evian water with moderate DOC and with SPM)

SPE – LC-NH_2 SPE purification

Extraction method / Parameter	SPE extraction OASIS **HLB 500mg/6cc** + SPE purification **LC-NH2 500mg/3cc**, **200 mL** Evian water + SPM (50mg/L) + DOC (7 mg/L) and estrogens at **0,4 ng/L level excepted 17α-ethynylestradiol at 0,035ng/L**). Sample acidification at pH = 5, 1% MeOH added.			
	absolute recovery in %	RSD in %	Isotope dilution recovery in %	RSD in %
Estrone (E1)			98	2
17α-Estradiol (aE2)			98	15
17β-Estradiol (bE2)			108	11
Estriol (E3)			116	7
17α-Ethinylestradiol (EE2)			109	10
Estrone-13C3 (E1-13C3)	83	7		
17α-Estradiol-d2 (aE2-d2)	74	4		
17β-Estradiol-13C3 (bE2-13C3)	81	6		
Estriol-d2 (E3-d2)	80	8		
17α-Ethinylestradiol-d4 (EE2-d4)	79	5		

Two-step procedure preconcentration/purification

SPE disk preconcentration + SPE purification

High complex matrix (Evian water with moderate DOC and with SPM)

SPE disk – LC-NH_2 SPE purification

Estrogen	Evian® + SPM (50 mg L^{-1})	Evian® + DOC (5 mg L^{-1})	Evian® + DOC (5 mg L^{-1}) + SPM (150 mg L^{-1})
EE2	68 ± 22%	67 ± 3%	88 ± 1%
E3	64 ± 14%	62 ± 6%	76 ± 4%
aE2	68 ± 20%	70 ± 2%	85 ± 2%
bE2	67 ± 19%	69 ± 9%	90 ± 2%
E1	68 ± 22%	66 ± 1%	91 ± 4%

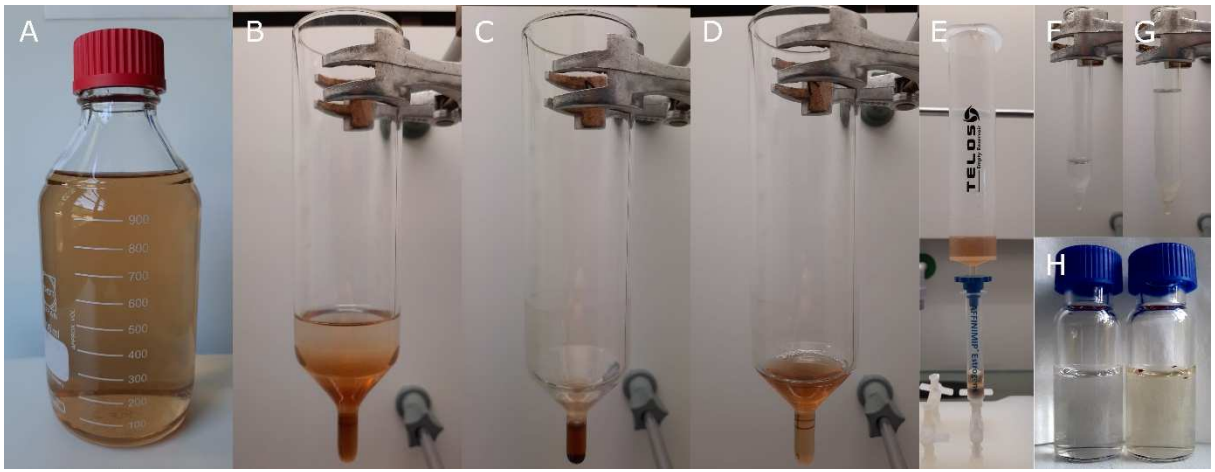
Estrogen	Evian® + SPM (50 mg L^{-1}) (%)		Evian® + DOC (5 mg L^{-1}) (%)		Evian® +DOC (5 mg L^{-1}) + SPM (150 mg L^{-1}) (%)	
EE2	109	5	93	5	84	1
E3	97	8	90	9	86	6
aE2	98	1	99	4	98	0,40
bE2	96	2	93	4	92	4
E1	105	1	98	2	96	0,50

Two-step procedure preconcentration/purification

SPE disk preconcentration + SPE purification

High complex matrix (Evian water with moderate DOC and with SPM)

SPE disk – MiSPE purification



- DOC content of the preconcentrated and purified sample is less then 1% then the initial on (picture H left)

Two-step procedure preconcentration/purification

SPE disk preconcentration + SPE purification

High complex matrix (Evian water with moderate DOC and with SPM)

SPE disk – MiSPE purification

1 L Evian + 7 mg L^{-1} DOC and 50 mg SPM spiked with estrogens at 0.1 ng L^{-1}

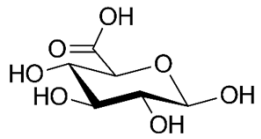
	HLB disk		MiSPE		HLB disk + MiSPE	
	Relative recovery [%]	Standard deviation [%]	Relative recovery [%]	Standard deviation [%]	Relative recovery [%]	Standard deviation [%]
Estrone	97.00	0.25	92.54	0.105	96.20	0.30
Estradiol	100.02	0.01	100.02	0.01	100.01	0.01
17α-Estradiol	100.09	0.02	100.07	0.04	100.14	0.06
Estriol	99.65	0.09	99.16	0.21	99.60	0.07
Ethinylestradiol	100.17	0.05	100.21	0.06	100.12	0.04

Conjugates in whole water samples

Conjugated target estrogens (optional)

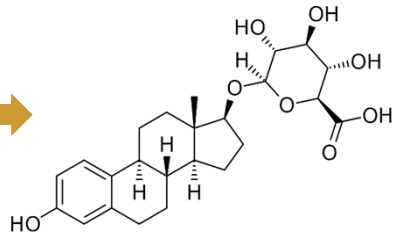
- Further phase-II-metabolism of E1, E2 and E3 leading to conjugated species
- Conjugates can be reconverted back into estrogens as a kind of a circulating reservoir for estrogens

Possible conjugates



e.g. glucuronic acid

e.g. E2



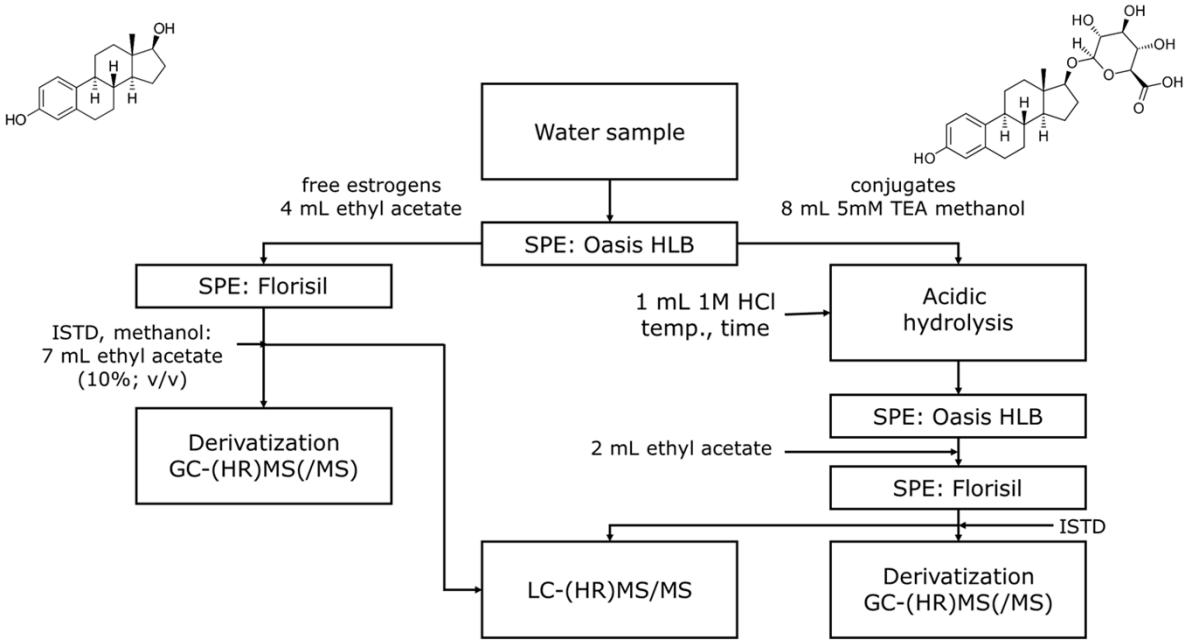
estradiol 17β-D-glucuronide

Other types: sulphates, phosphates, glycine, etc.

- To be known: Influence of the sampling, sample storage, and sample preparation on the conjugates stability

Conjugates in whole water samples

Conjugated target estrogens (optional)



According to Liu, Z.-h., et al. (2010). "Deconjugation characteristics of natural estrogen conjugates by acid-catalyzed solvolysis and its application for wastewater samples." Journal of Environmental Monitoring **12(8): 1594-1600.**


QUESTIONS?
COMMENTS?
CONCERNS?