FINAL PUBLISHABLE REPORT

Grant Agreement number	18NRM01
Project short name	EDC-WFD
Project full title	Metrology for monitoring endocrine disrupting compounds under the Water Framework Directive

Project start date and duration:		1 September 2019, 42 months
Coordinator: Béatrice LALERE, LNE Tel:+33140433810 E-mail: beatrice.lalere@lne.fr Project website address: http://projects.lne.eu/jrp-edc-wfd/		
Chief Stakeholder Organisation: CEN/ TC 230 “Water Analysis”		Chief Stakeholder Contact: Ulrich Borchers, (Chair TC 230)
Internal Funded Partners: 1. LNE, France 2. BAM, Germany 3. NIC, Slovenia 4. SYKE, Finland 5. TUBITAK, Türkiye	External Funded Partners: 6. ISPRA, Italy 7. JSI, Slovenia 8. UBX, France	Unfunded Partners:
Linked Third Parties: 1. CNRS, France (linked to UBX)		
RMG: -		

TABLE OF CONTENTS

1 Overview

Natural and pharmaceutical estrogens are key Endocrine Disrupting Chemicals (EDC) which are monitored differently depending on the country, and for which standardised reference methods were not yet available. The overall objective of the project was to develop reliable and harmonised measurement methods for estrogens, to comply with the Water Framework Directive requirements (Directive 2013/39EC, Commission Directive 2009/90/EC and Commission Implementation Decision (EU) 2018/840). The outcomes of this project, in particular the validated mass spectrometry (MS) based reference methods, were disseminated to CEN/ TC 230 and ISO/ TC 147 to be fed into the documentary standards they develop. Accredited analysis laboratories will be able to implement the standard and provide accurate and reliable data. And thus, the project will enable public authorities to provide data with high level of confidence, to ensure an efficient and comparable implementation of the WFD between Member States, and to inform European citizens who have clearly demonstrated their concern about EDC.

2 Need

It is known that estrogens end up in surface waters *via* wastewater, and due to their physicochemical properties, they can partition in the different compartments (water and suspended particulate matter (SPM)) of water systems. Despite occurring at ultra-trace levels (below ng/L), it is believed that they are, due to their estrogenic potency, contributing to the feminisation of fish and other endocrine disruptive effects. Moreover, they may be a factor in biodiversity loss. Therefore, appropriate measurement methods are needed allowing determination and monitoring of estrogen levels below the environmental quality standard (EQS) and in order, even more importantly, to show if a water body is at risk.

- The objectives of this project were derived from the need expressed by different communities. For example: CEN/ TC 230 “Water analysis” has agreed that there is a lack of standardised analytical methods to monitor three relevant estrogenic substances (17-beta-estradiol, 17-alpha-ethinylestradiol and estrone) in conditions that meet the requirements of the WFD and its derivatives. There were no methods yet available to guarantee the integrity of samples between sampling and analysis, nor quality control tools to ensure reliability. In addition, there were no CEN or ISO standards available to address the measurement of EDCs by conventional chemical analysis, and reference materials for validating in-house methods and establishing quality assurance and control measures are not available. Some promising effect-based methods for EDC were under development at ISO but they just complemented the classical approach of quantitative methods.
- ASLAE (ASsociation des directeurs et cadres des Laboratoires publics Agréés pour les analyses d'Eau), an association representing 50 testing laboratories in the field of water analysis, has highlighted that many of its members face difficulties in developing and validating estrogens measurement capabilities and have failed to achieve the very low limits of quantification that are required by WFD.
- In addition, for a substance to be added to a regulatory monitoring list, the French Ministry of the environment requires a reliable reference method to be available.

3 Objectives

The overall objective of this project was 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) have been included to demonstrate the reliability of the developed methods and to support the requirements of Directive 2013/39/EC, Directive 2009/90/EC and Commission Implementation Decision (EU) 2018/840, hence improving the comparability and compatibility of measurement results within Europe.

The specific objectives of the project were to:

1. Optimise and validate traceable aqueous reference mass spectrometry-based methods for the analysis of 5 estrogenic compounds prioritising 3 selected estrogenic compounds 17-beta-estradiol, 17-alpha-ethinylestradiol, and estrone, 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. Evaluate the capability of developed methods to address the different fractions of matrix (whole water and dissolved concentrations of estrogens).
3. 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.
4. Improve the comparability of estrogen measurements with selected Effect-Based Methods (EBM) in whole water samples at EQS level. Methods that have been correctly calibrated and information on uncertainty were provided.
5. Organise and perform an interlaboratory comparison (ILC) to demonstrate the performance of the developed methods using the reference material (RM) for the selected estrogen substances.
6. Contribute to the work of key European and international standardisation organisations e.g. CEN TC 230 and ISO TC 147 ensuring that the outputs of the project were aligned with needs, communicated quickly to those developing the standards and to those who will use them to support the implementation of directives, and in a form that can be incorporated into the standards at the earliest opportunity.

4 Results

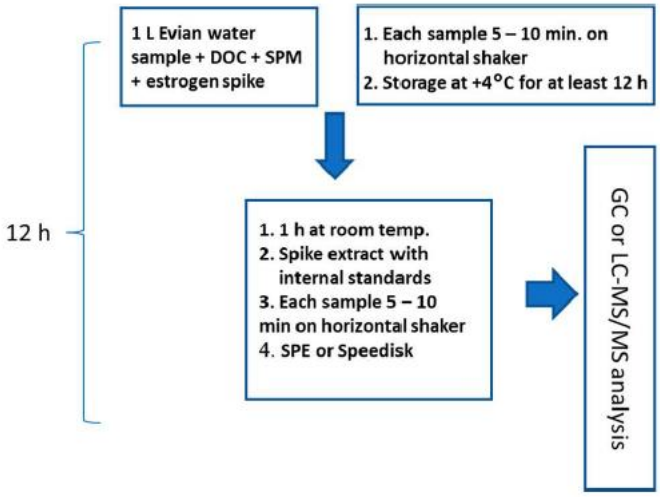
4.1 Fully validated MS-based reference methods for the detection of estrogens (Objective 1):

It is mandatory for quantifying the five target estrogens (17βE2, 17αEE2, E1, 17αE2 and E3) under the requirements of the EU-WFD in whole water samples to apply a pre-concentration either as Solid phase extraction (SPE) disk for a solid particulate matter (SPM) load higher than 50 mg L^{-1} or an SPE approach for an SPM load lower than 50 mg L^{-1}. A purification step of the obtained organic extracts from pre-concentration is strongly recommended for LC-MS(/MS) methods to avoid ion suppression effects in the ESI source (e.g. by removing dissolved organic compounds (DOC), namely humic substances) from the extracts. This leads to a better performance in sense of sensitivity. Mi-SPE (Molecular imprinted polymers) or a common SPE purification column (e.g., LC-NH2 SPE) were positively evaluated and validated. The GC-MS/MS methods re, compared to LC-MS/MS, more robust in term of matrix effect, however, the purification step is also recommended. It is mandatory to apply isotope dilution calibration in both chromatographic techniques using ^{13}C- or deuterated species of the individual target estrogens. GC-MS/MS allows reaching the former EQS levels for all the analytes, whereas LC-MS/MS allows to reach the former EQS levels for all target estrogens and is also ready to reach the new EQS levels, especially for EE2. These results are only valid for the instruments tested and used within the project.

In order to guarantee the effective recognition of the results of the project by standardisation, partners agreed that the validation of the methods would have been performed following the guidance of the CEN/TS 16800 and ISO 21253-1 standards. Furthermore, all the partners have evaluated uncertainty according to ISO 11352, additionally NMI have applied GUM approach. To support the demonstration, experimental designs have been discussed and agreed among partners:

- On the basis of an overview European database, the selection of 3 representative water samples (synthetic real matrix water) with distinct amounts of SPM (solid particulate matter) and DOC (dissolved organic carbon) has been discussed and agreed. Partners agreed that the validation of the methods would have also been completed by addressing three matrix of their choice.
- The methods performance characteristics were assessed through ad-hoc experiments on six matrices (i.e., three synthetic waters and three natural waters) at three different concentrations level each (i.e., LOQ-value medium and high concentration).

The figure 1 shows the preparation of the sample and selected levels for method validation.



Matrix	EDC levels of concentration tested (ng/L)
In-house reference materials containing EVIAN+DOC 1 mg/L as matrix. Three levels, each spiked with different amount of estrogens	LOQ-V
	3LOQ-V
	10LOQ-V
In-house reference materials containing EVIAN+DOC 7 mg/L as matrix. Three levels, each spiked with different amount of estrogens	LOQ-V
	3LOQ-V
	10LOQ-V
In-house reference materials containing EVIAN+DOC 7 mg/L + SPM 50 mg/L as matrix. Three levels, each spiked with different amount of estrogens	LOQ-V
	3LOQ-V
	10LOQ-V
1st Natural sample spiked at three different levels of concentration	LOQ-V
	3LOQ-V
	10LOQ-V
2nd Natural sample spiked at three different levels of concentration	LOQ-V
	3LOQ-V
	10LOQ-V
3rd Natural sample spiked at three different levels of concentration	LOQ-V
	3LOQ-V
	10LOQ-V

Figure 1: Design of method validation.

A common spreadsheet was also used by all partners (LNE, BAM, NIC, SKYE, TUBITAK, ISPRA, JSI, UXB, CNRS) to evaluate the characteristics of the methods. All the results of within laboratory validation are summarised in the table 1.

	Compound	17β-estradiol			17α-estradiol			17α-ethinyl estradiol			Estrone			Estriol		
	Concentration level	LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ
Laboratory 1/ Method 1	C (ng/L)	0.123	0.275	0.958	0.111	0.247	0.859	0.008	0.017	0.060	0.134	0.298	1.037	0.112	0.250	0.869
	Bias (%)	15	8	10	17	13	10	31	26	20	13	10	14	69	33	12
	Precision (%)	20	11	10	11	17	12	20	15	12	14	12	11	22	8	10
	U (k=2) (%)	50	26	29	41	43	32	74	60	47	37	31	35	140	68	32
Laboratory 2/ Method 2	C (ng/L)	0.117	0.303	1.011	0.124	0.320	1.070	0.132	0.342	1.141	0.153	0.396	1.321	0.126	0.326	1.088
	Bias (%)	15	13	13	13	7	5	15	7	6	24	19	17	25	8	6
	Precision (%)	6	3	2	13	2	0	5	2	1	7	1	1	12	4	2
	U (k=2) (%)	32	26	26	37	14	9	33	14	13	51	38	34	56	19	12
Laboratory 3/Method 3	C (ng/L)	0.099	0.274	0.931	0.094	0.267	0.909	0.849	2.455	8.349	0.096	0.280	0.951	0.948	2.764	9.397
	Bias (%)	23	17	18	22	31	28	28	30	32	23	24	20	20	15	16
	Precision (%)	13	5	13	7	10	7	9	6	3	9	5	5	9	2	4
	U (k=2) (%)	53	35	44	47	65	57	58	61	63	48	49	41	43	31	34
Laboratory 4/Method 4	C (ng/L)	0.056	0.163	0.560	0.057	0.165	0.567	0.016	0.047	0.161	0.114	0.332	1.139	0.113	0.331	1.133
	Bias (%)	18	22	29	8	13	14	39	14	22	25	31	31	10	9	8
	Precision (%)	23	16	15	9	4	6	21	15	17	9	6	7	9	6	7
	U (k=2) (%)	59	55	65	24	28	31	89	41	56	53	62	64	27	22	20
Laboratory 5/method 5	C (ng/L)	0.120	0.364	1.331	0.122	0.370	1.353	0.087	0.265	0.969	0.128	0.387	1.414	0.183	0.553	2.023
	Bias (%)	40	15	6	26	11	12	11	11	5	95	86	14	109	29	6
	Precision (%)	40	8	3	18	6	3	6	3	3	30	30	3	96	18	3
	U (k=2) (%)	110	34	14	63	26	25	24	22	11	200	180	29	290	69	14

Table1: Characteristics and performances of the partners method

These results show that the majority of laboratories can reach the WFD limits of quantification with uncertainties of less than 60%, and this for very low concentrations like the 17αEE2.
For all other compounds, the LQs and associated uncertainties are of the same order of magnitude regardless of the laboratory or method.

As a result of this work, the deliverable D3 describes the MS-based methods for the measurement of 3 selected estrogens in whole water sample compatible with the requirements of the QA/QC directive, and presents the results of the validation. This objective has been fully and successfully completed.

4.2 Evaluation of the capability of developed methods to address the different fractions of matrix (whole water and dissolved concentrations of estrogens) (objective 2):

This paragraph presents the optimisation and comparison of a wide range of sample preparation techniques focusing on extraction and enrichment. All the selected sample preparation techniques are available within the testing laboratories and are already standardised. To make the whole set of experiments more comparable within the project consortium, synthetic real water matrix was used as simulation of representative inland surface water. Synthetic water matrix can be setup independently by each laboratory using Evian mineral water (or equivalent) as a major constituent and a defined composition of inorganic anions and cations. This matrix was spiked with a concentrated solution containing a dissolved organic carbon simulant which consists of a commercially available humic acid in a distinct concentration dissolved in Evian water. The pH value was maintained to 7.3. In a second approach, this complex water matrix was expanded into a whole water sample by adding suspended solids in terms of suspended particulate matter to this matrix. Here, a common load of 50 mg of SPM to the matrix was added representing common surface water. These representative waters were spiked with the desired amounts of the five selected estrogens to evaluate and validate the most promising and robust sample preparation procedure and preconcentration method.
It has been demonstrated that an effect of deconjugation could be observed during storage and sample preparation. Calculated on the average molar mass of these three conjugates approximately 0.16% of the used model conjugates was deconjugated during the whole analytical procedure. It was considered as negligible with respect to inland waters monitoring.

Concerning the behaviour of the internal standard (labelled estrogens), an equilibrium time of at least 12 h is suggested. No difference is observed between different types of internal standards (deuterated, ^{13}C). In consequence, the samples must be spiked with the isotopically labelled standards at the end of a working day to store them overnight (12 h) at +4±3°C.
Concerning the sample preparation:

- For low complex matrix containing water samples like mineral, tap or demineralised water solid phase extraction and SPE disk are the procedures of choice. Here, the variety of sample volume (e.g., between 100 and 1000 mL) has no effect on the absolute recovery rates of the target analytes. Together with the use of isotopically labelled standards, the relative recovery rates are in an acceptable range between 83 and 102% with a sufficient standard deviation depending on the analytical method. Both chromatographic approaches, liquid and gas chromatography are able to separate all five targeted estrogens especially aE2 and bE2. The more specific MiSPE is limited with regards to the maximum sample volume of 100 mL, while for achieving the mentioned EQS for all selected estrogens a larger volume is necessary.
- For high complex matrix like surface or ground water with a load of suspended particulate matter, solid phase extraction and SPE disk are the procedures of choice. Here, the variety of sample volume (e.g., between 100 and 1000 mL) has no effect on the absolute recovery rates of the target analytes but the SPE is limited with respect to the SPM load. To avoid clogging of the cartridges, only a restricted SPM load is tolerable depending on the type of cartridge. Due to the implementation of isotopically labelled standard compounds, the relative recovery rates are in a acceptable range between 76 and 116% with an acceptable standard deviation depending on the analytical method. The more specific MiSPE is limited with regards to the sample volume of 100 mL in maximum and the load of SPM. To achieve the mentioned EQS for all selected estrogens, a larger volume is necessary. Both chromatographic approaches, liquid and gas chromatography, are able to separate all five targeted estrogens especially aE2 and bE2.

In conclusion, SPE and SPE disk are suggested for the analysis of whole water samples with individual sample volume. The common SPE is limited by the SPM load and must be evaluated before use. A further purification of the extract from the preconcentration procedure is recommended by the project partners (BAM, LNE, UBx Université de Bordeaux, SYKE, TUBITAK – UME, JSI).

As a result of this work, the deliverable D1 describes the most appropriate methods preparation for the measurement of 3 selected estrogens in whole water sample compatible with the requirements of the QA/QC directive. This objective has been fully and successfully completed.

4.3 Reference materials preparation (objective 3):

Two types of reference materials have been developed during the project:

- pure compounds to assure the traceability of the measurements;
- aqueous reference materials (RM), which are as close as possible to real water samples.

Pure Compounds:

TUBITAK have produced CRMs of five analytes listed in the project proposal (17βE2, 17αEE2, E1, 17αE2 and E3) according to ISO 17034. The CRMs have been dispatched to project partners (BAM, ISPRA, JSI, LNE, UBx Université de Bordeaux, SYKE) after certification. TUBITAK has made the selection list of the raw material after been discussed and agreed with all partners. The five hormones materials have been purchased. Stability (i.e., short term stability study and long term stability study 6 months) and homogeneity (i.e. analyses on 10 units) studies were performed.

The CRM developed were presented in the figure 2 and table 2 below:

Figure 2: Reference materials :pure compounds

Standard	Structure	CAS#	Unit Produced	Amount in Unit	UME CRM Code	Purity Estimation Method
17-beta-Estradiol		50-28-2	550	250 mg	UME CRM 1330	qNMR, HPLC-UV
Ethinylestradiol		57-63-6	600	250 mg	UME CRM 1331	qNMR, HPLC-UV
Estrone		53-16-7	780	250 mg	UME CRM 1332	qNMR, HPLC-UV
17-alpha-Estradiol		57-91-0	500	250 mg	UME CRM 1333	qNMR, HPLC-UV
Estriol		50-27-1	550	250 mg	UME CRM 1334	qNMR, HPLC-UV

Table 2: CRM main characteristics

Aqueous reference materials

The reference material candidate is provided as a kit that consists of Evian water, a DOC-spiking solution, SPM, and an estrogen spiking solution at the desired concentration level (Figure 3).

Figure 3: Aqueous reference materials

The determination of the stability and the interbottle homogeneity of the RM candidate with respect to the five selected estrogens (17βE2, 17αEE2, E1, 17αE2 and E3).

Within a typical storage time of 14 days at +4±3°C, the prepared reference material is stable with regards to a possible loss or degradation of the five target analytes. Moreover, the study shows that also the storage of sample extracts at -20°C for 7 days has no effect on the recovery rates of the spiked estrogens.

As a final goal of the study, a SOP is given to the customer or the project partners to set up the reference material under comparable conditions and to provide recommendations for participants to the ILC:

- *Samples should be prepared in amber glass bottles (1000 mL are recommended). A fixed volume of the prepared DOC spiking solution with known DOC concentration (actual 1100 mg·L^{-1}) using a 0,45 µm syringe filter (PTFE syringe filters are recommended) giving a final DOC of 7 mg·L^{-1} must be added to the Evian water. Additionally, an aliquot of at least 100 µL of an estrogen spiking solution (e.g., 10 ng·mL^{-1} of* 17βE2, 17αEE2, E1, 17αE2 and E3*in acetonitrile or methanol) must be added to the DOC containing Evian water. Finally, the desired amount (50 mg·L^{-1}) of suspended particulate matter is added. All steps must be controlled gravimetrically. The resulting reference material sample solutions are homogenised on a horizontal shaker or equivalent for at least 10 minutes. Subsequently, an appropriate internal standard mix is used with the needed concentrations (e.g., 1 ng·L^{-1} for each isotopically labelled estrogen). After spiking the samples with the internal standards, they are homogenised again on a horizontal shaker or equivalent for at least 5 to 10 minutes. Samples were stored for at least for 12 h at +4±3°C to ensure the equilibrium time. This final RM can be stored for a maximum of two weeks at +4±3 °C. When analysing the stored whole water samples allow them to stand at room temperature (+20 °C) for at least one hour.*
- *The procedure can be modified by setting up a batch of DOC-containing Evian water and preparing individual subsamples which can be spiked with the estrogen spiking solution and the desired amount of SPM.*

As a result, two reference materials have been produced and the stability of the substances studied. This objective has been fully and successfully completed.

4.4 Well characterised bioassays methods (objective 4):

An experimental design to validate the EBMs (i.e., sample preparation procedure + A-YES and ERα-CALUX bioassay) optimised within the Project was agreed among the partners. All the partners validated their methods in accordance with CEN/TS 16800:2020. This standard provides a guideline for the validation of physico-chemical analytical methods, the chosen approach was nevertheless considered fit for purpose for EBMs validation. In fact, doing so, the EBMs validation resulted aligned to MS methods validation improving the comparability of estrogen measurements in whole water samples at the former EQS levels.
The methods performance characteristics were assessed through ad-hoc experiments on six matrices (i.e. three synthetic waters and three natural waters) at three different concentrations level each (i.e. LOQ-V, medium and high concentration) similarly to MS methods. The addressed performance characteristics were the following: calibration, application range, verified limit of quantification, selectivity, precision, trueness and measurement uncertainty.

Selectivity was considered a key point within the validation. In fact, as highlighted in CEN/TS 16800 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. Since the EEQ determination in real samples by means of bioassays could be influenced by the presence of other matrix components, within the method validation, three different effects were investigated: 1) matrix effect; 2) interferents effects and 3) DOC effect by analysing 3 different levels of DOC concentrations (1-7-14 mg/L) spiked at high concentration of EEQ. Calibration was established by using proper dilutions of CRMs produced within the Project, whereas trueness and precision studies were carried out according to CEN/TS 16800. Finally, the uncertainty for both the optimised procedures was estimated in accordance with the ISO 11352 and applying a common spreadsheet.

In Table 3 the outcomes of the validation study for both the optimized EBMs are reported.

	Precision Component	Bias Component	Total Combined Std Uncertainty

EBM	EEQ_{bio} Concentration Level (ng/L)	%	%	%	Rounded Relative Expanded Uncertainty, k=2 (%)
A-YES	0,13-0,40	43,4	35,9	56,3	110
	0,40-1,3	6,6	5,7	8,7	17
	>1,3	3,2	7,4	8,1	16
ERα-CALUX	0,12-0,38	18,0	24,8	30,6	61
	0,38-1,2	15,8	29,7	33,6	67
	>1,2	24,0	26,0	35,4	71

Table 3: Characteristics and performances of the optimised EBMs

As additional outcomes of the validation study the following recommendations are given:

- the analysis as sample of a βE2 concentration-response curve independently prepared from the calibration curve is helpful in the assessment of the calibration curve validity over batches.
- the preparation of more than a reference plate decreases the risk of discharging all the other samples plates in case the reference curve does not fulfil the acceptance criteria.
- sensitivity to different compounds is not always stable over the time, thus it is recommended that the laboratory should determine relative potencies and periodically check them.

In conclusion, the applied Validation Experimental Design has proven to be fit for purpose for EBMs validation and their validation resulted more aligned to MS based Methods validation. Matrix, interferents and DOC do not impact on the samples analyses in terms of results and concentration-response curves when the implemented procedure is applied as preparation procedure of the samples. Therefore, both methods have been correctly calibrated and the uncertainty estimation was carried out in accordance with ISO 11352.

Finally, at concentrations close to the former EQS, CALUX bioassay showed better results in terms of precision and bias component, whereas A-YES provided the lowest uncertainties when higher concentrations were considered.

The bioassays (CALUX and A-YES) methods have been developed and characterized (performances and uncertainties). This objective has been fully and successfully completed.

4.5 standardisation and intercomparison as knowledge transfer to end-users (objective 5):

The ILC demonstrated the performance of the developed methods using the reference material (RM) for the selected estrogen substances.
The objective of the ILC was to demonstrate the fitness for purpose of optimised and validated methods for estrogens measurements by MS-based and effect-based methods (e.g. ERα-Calux and A-YES), defining performance characteristics of the methods in terms of repeatability within laboratories and reproducibility.

The ILC demonstrated the performance of the developed methods using the reference material (RM) for the selected estrogen substances.
The objective of the ILC was to demonstrate the fitness for purpose of optimised and validated methods for estrogens measurements by MS-based and effect-based methods (e.g. ERα-Calux and A-YES), defining performance characteristics of the methods in terms of repeatability within laboratories and reproducibility.

By 2nd In November 2022, the test materials were sent to each involved laboratory and. the involved laboratories provided the results by 30th November 2022.
The laboratories received separate kits for chemical measurements and EBMs respectively. In particular, each kit included:
- 4 bottles of commercially available mineral water (2 bottles for each test material, 1L each);
- Ampoules of SPM (suspended particulate matter, estrogen-free, and heat sterilized) and of a simulated dissolved organic carbon-DOC solution (commercially available humic acid);- 2 Vials (EDC-WFD_C1 and EDC-WFD_C2, for chemical measurements; EDC-WFD_B1 and EDC-WFD_B2, for EBMs) containing, at different concentration, 3 mL of the standard of the following targeted substances in methanol:
    - o 17-beta-estradiol (17βE2),
    - o 17-alpha-ethinylestradiol (17αEE2),
    - o estrone (E1),
    - o 17-alpha-estradiol (17αE2),
    - o estriol (E3).

With reference to the internal quality control, the laboratory received, together with the kit, 1 extra unit (bottle) of water (blank) to be measured for the same targeted substances. In addition, for chemical measurements, a vial containing 3 mL of a target estrogens mixture in methanol was provided to verify the laboratory calibration (EDC-WFD_QC).
Each unit was reconstituted by the laboratory participating in the ILC, following the specific protocol provided to the laboratories by the organizers

The characteristics of the final materials are given in Tables 4, 5 and 6.

EDC-WFD C1	Low Concentration Range ng/L,	SPM mg/L	DOC mg/L	**EDC-WFD C2**	Medium Concentration Range ng/L	SPM mg/L	DOC mg/L
17βE2	0,1-0,5			17βE2	0,5-2,5		
17αEE2	0,02-0,1			17αEE2	0,1-0,5		
E1	0,2-1,0	10	2	E1	0,5-2,5	50	5
17αE2	0,1-0,5			17αE2	0,5-2,5		
E3	0,2-1,0			E3	0,5-2,5		

Table 4: RMs composition – Chemical methods

EDC-WFD B1	Low EEQ Range ng/L βE2 eq	SPM mg/L	DOC mg/L	**EDC-WFD B2**	Medium EEQ Range ng/L βE2 eq	SPM mg/L
Cumulative effect	0,1-1,0	10	2	Cumulative effect	0,5 -5,0	50

Table 5: RMs composition – Effect based methods

EDC-WFD C1/B1	Low Concentration ng/L	EDC-WFD C2/B2	Medium Concentration ng/L
17βE2	0,22	17βE2	1,01
17αEE2	0,035	17αEE2	0,210
E1	0.40	E1	1,01
17αE2	0,20	17αE2	1,02
E3	0,41	E3	1,01

Table 6: Reference estrogens concentration - gravimetric

For ***chemical measurements*** 20 laboratories received the kit containing the RMs to be analysed. Some laboratories applied more instrumental techniques for the quantification of the estrogens (GC-MS/MS and LC-MS/MS), so that the overall number of potential reporting templates was 22; 17 reporting templated were returned, from 15 laboratories.
For ***EBMs measurements*** 10 laboratories received the kit containing the RMs to be analysed; 9 reporting templates were filled and returned.

The overall data sets were analysed and for the final statistical evaluation were not considered:
- values less than LOQ (Limit of Quantification);
- single measurement result (instead of duplicate measurements results as requested).

On the revised Data Base ***h* and *k* Statistics (Mandel),** according to ISO 5725-2:2019, were used to check the consistency of the data (graphical technique):
- the first (*h*) evaluates between-laboratory variability
- the second (*k*) evaluates within-laboratory variability

Examination of the *h* and *k* plots can indicate that specific laboratories exhibit patterns of results that are markedly different from the others. The critical values of the *h* statistic depend on the number of laboratories participating in the ILC while those of the *k* statistic depend on the number of laboratories and the number of replicates performed by each laboratory.
Grubbs's test was applied (iterative procedure) to identify *"straggler"* values (for test statistic > 5% or ≤ 1% critical value) and *"outliers"* (for test statistic > 1% critical value). Outlier values were evaluated considering the statistic outcomes. **Cochran's test** was also applied for verifying the homogeneity of variance within laboratories.

The performance characteristics of the measurement methods were calculated following ISO 5725-2:2019. **In Tables 6** are summarized the performance characteristics for Chemical measurements. For each reference material the mean value is calculated based on the laboratories' measurement results; between *brackets* the number of laboratories and associated valid data considered for each parameter (m/*n*).
Considering the number of valid data for EBMs, not sufficient to give reliable **repeatability-Sr** and **reproducibility-SR** values, two different evaluation approach were carried out:
- all the measurement results for each reference material, obtained by different bioassays (ISO 19040-1, 19040-2, 19040-3 and p-YES) were pooled, providing an information on repeatability and reproducibility for the overall EBMs (Table 7);
- the measurement results were grouped by the two main different bioassays, providing only indicative repeatability and reproducibility values if statistically possible (Table 8**)**.

EDC-WFD C1	Reproducibility S_R (%)	Repeatability S_r (%)	Mean value^ ng/L	EDC-WFD C2	Reproducibility S_R (%)	Repeatability S_r (%)	Mean value* ng/L
17βE2	13,9	7,5	0,25 *(12-24)*	17βE2	14,8	11,8	1,16 *(15-30)*
17αEE2	36,9	10,5	0,034 *(12-24)*	17αEE2	12,9	5,9	0,21 *(13-26)*
E1	16,1	7,2	0,39 *(14-28)*	E1	18,3	5,7	0,92 *(15-30)*
17αE2	18,6	7,9	0,23 *(10-20)*	17αE2	16,0	7,9	1,02 *(10-20)*
E3	14,8	7,7	0,44 *(8-16)*	E3	8,6	4,7	1,01 *(8-16)*

(*) between *brackets* the number of laboratories n and associated valid data considered for each parameter m (n/*m*)

Table 7: Performance characteristics for chemical measurements

		Reproducibility S_R (%)	Repeatability S_r (%)	Mean value (*) ng/L βE2 eq
EDC-WFD B1	Cumulative effect	56,4	19,4	0,31 *(7-14)*
EDC-WFD B2		60,8	10,8	1,17 *(8-16)*

(*) between *brackets* the number of laboratories n and associated valid data considered for each parameter m (n/*m*)

Table 7: Performance characteristics of EBM bioassays

	Bioassay		Indicative Reproducibility (%)	Indicative Repeatability (%)	Mean value (*) ng/L βE2 eq
EDC-WFD B1	**ERα-Calux**	Cumulative effect	61,8	11,1	0,33 *(3-6)*
	A-YES		29,1	29,0	0,21 *(3-6)*
EDC-WFD B2	**ERα-Calux**		74,2	7,3	1,29 (4-*8*)
	A-YES		37,8 (CV%)**	16,0 (CV%)**	0,95 (2-*4*)

(*) between *brackets* the number of laboratories n and associated valid data considered for each parameter m (m/*n*).
(**) CV% was calculated because only results from two laboratories are available.

Table 8: Indicative repeatability and reproducibility for ERα-Calux and A-YES

The main outcomes of the EDC-WFD ILC show good application and performance characteristics of the tested methods. The general accurate application of RMs reconstitution procedure by the laboratories, positively influenced the very good agreement of the measurements results with the reference concentration of the RMs, leading to acceptable repeatability and reproducibility values, especially for the chemical measurements. In this context, the laboratories, by using a set of extraction and purification methods, with associated LC-MS/MS and GC-MS/MS quantification techniques, had to face with low concentration of the selected estrogens, close to their LOQ, with variable content of the interferents.
More questionable, however, the results on the EBMs, for which – nevertheless - in the future an intense effort for their implementation in Europe is foreseen. The low, and in some case insufficient, number of laboratories and valid measurements results, suggest the need for carrying out new interlaboratory trials. These should test challenging samples with low concentration of estrogenic substances and should be based on increased number of laboratories (public and private), coming from different European countries. The EDC-WFD ILC adds, despite some bounds, useful information in the application of the Effective Based Methods, in a future perspective of implementation within the framework of environmental control and monitoring of waters together with chemical methods.

The EIL provided data on the repeatability and reproducibility of the analytical methods for the three hormones for the draft standard (deliverable D6). This objective has been fully and successfully completed.

4.6 Contribution to European and international standardisation work

The project EDC_WFD partners developed several methods to support both the implementation of the WFD and standardisation.

Each of the eight partners has optimised their methods to try to reach the target LOQ (limit of quantification). After methods development, all the partners agreed to validate five MS methods (GC/MSMS, LC/MSMS and HRMS methods with IDMS technique).

In order to guarantee the effective recognition of the results of the project by standardisation, partners agreed that the validation of the methods will have been performed following the guidance of the CEN/ TS 16800 and ISO 21253-1 standards. Furthermore, all the partners will evaluated uncertainty according to ISO 11352, additionally the National Metrology Institutes will applied GUM approach. To support the demonstration, experimental plans have been discussed and agreed between partners.

The data validation show that for all other compounds, the LQs and associated uncertainties are of the same order of magnitude regardless of the laboratory or method.

Based on the methods and aqueous reference materials (RM) developed during this project, an inter laboratory comparison has been organised. The general accurate application of RMs reconstitution procedure by the laboratories positively influenced the very good agreement of the measurements results with the reference concentration of the RMs. Moreover, the results show good methods reproducibility, particularly for low levels: from 14 % for 17βE2 (0,25 ng/L) to 37 % for 17αEE2 (0,034 ng/L) for MS based methods.

All the five methods were described in the draft standard as well the ILC results (repeatability and reproducibility).

In parallel with these works, a working group was created: ISO/TC 147/SC 2/WG 84 "Estrogens using MS based methods" whose coordinator is Mrs Lardy-Fontan (ANSES) and whose secretariat is provided by AFNOR (Arnaud Gaudrier). The project ISO 13646 was initiated 'Water quality — Determination of selected oestrogens in whole water samples — Method using solid phase extraction (SPE) followed by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) detection'. The takeover by CEN TC 230 WG1 of the ISO 13646 project is currently being validated (consultation in progress until April 14, 2023). The consultation validated this recovery, the project will become an international, European and French project standard; NF EN ISO 13646.

5 Impact

The website of the project has been created. http://projects.lne.eu/jrp-edc-wfd/

Nine presentations of the project have been realised at Eurachem Workshop - Uncertainty from sampling and analysis for accredited laboratories (Berlin, 19-20 November 2019); ICRAPHE 2nd International conference on risk assessment of pharmaceuticals in the environment (Barcelona, 28/-29 November 2019); SETAC (Dublin, 3-7 May 2020); Goldschmidt 2021 (Lyon; 4-9 July 2021); CIM 2021 (Lyon, 6-9 September 2021), EuChemS 2022 (Lisbon, 28 August-1 September 2022); IMEKO TC11&TC24 (Croatia, 17-19 October 2022); Maastricht IMSC22 (Netherlands, 27 August-2 September 2022) and CIM 2023 (Lyon 7-10 march 2023).

One published open access papers present the evaluation, comparison and combination of molecularly imprinted polymer solid phase extraction and classical solid phase extraction for the preconcentration of endocrine disrupting chemicals from representative whole water samples.

Two interactive online training courses for members' consortium about method validation were organised (November 2020 and February 2021).

An online workshop for external audience on "Solutions to tackle WFD requirements for estrogen determination in water" was organised on the (7-9 September 2022).

A final meeting was organised face to face for members' consortium (21 February 2023) and on line for user community (22 February 2023).

An advisory group composed by: Ulrich BORCHERS (IWW water center CHIEF STAKHOLDER of the project, TC CHAIR CEN/TC230 « Water Quality»), Mario CARERE (National Institute of Health ISS, researcher, expert for WG Chemical EC), Pierre François STAUB (French Office for Biodiversity OFB; head project water pollution and metrology), Olivier PERCEVAL (French Office for Biodiversity OFB; head project ecotoxicology), Marina RICCI (Join Research Center) has been implemented and was kept informed on the project regularly. Their recommendations from the first meeting 8th October 2019, second meeting 29th November 2021 and 31st January 2022 have been discussed, amended and implemented by the project partners. Some of them have participated in the final meeting.

Impact on industrial and other user communities

This project enabled harmonised monitoring of endocrine disrupting compounds in water in response to European water policies. Regulatory acceptance of emerging technologies is a slow process, and currently hampers the use of such modern bioassays for compliance testing and regulatory purposes. The outcomes of this project will facilitate the adoption of such technologies. Testing (accredited) analytical laboratories will be targeted to benefit from this project, therefore supporting the provision of services. This has been fostered by the webinar training that was organised in September 2022 and at the final meeting in February 2023.

Impact on the metrology and scientific communities

This project will supported the metrology community in handling the long-standing scientific problem of environmental monitoring and risk assessment. This project have direct impact on different metrology committees, especially the EURAMET Technical Committee of Metrology in Chemistry (TC-MC) and the Organic Analysis Working Group (OAWG) of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) of the BIPM. CCQM-OAWG is responsible for the CMC (Calibration and Measurement Capabilities) entries related to chemistry. It contributes to the visibility of EURAMET and its leadership in chemical metrology for environment to the wider metrology and scientific communities. The interlaboratory comparison that was organised within the project has been included in the OAWG strategic document 2021-2027 as Track C comparison. The results of the project will form the basis for developing calibration and measuring capabilities (CMC) entries related to estrogens and comparable substances in water.

The project is a positive answer of the EURAMET 2030 strategy and a demonstrator to support the European Metrology network Pollution Monitoring (EMN POLMO).

The project communicates the results and scientific knowledge gained in the project to the scientific environmental analytical community via open access publications in peer-reviewed journals, workshops and training. It increases the awareness of a wider community on quality assurance and quality control (QA/QC) issues as well as metrology concepts that are often misunderstood or misapplied. As measurements are often instrumental for research in diverse scientific fields, the methods developed provides scientists with guidance to make their measurements metrologically sound.

Impact on relevant standardisation bodies

Dialogue with **Standardization Organisations** AFNOR, SFS, DIN, CEN and ISO has been strengthened and lead to the establishment of formal liaisons. In its annual meeting on the 27th of October 2021, ISO TC 147 SC2 in its Resolution 419 (WebEx-20) states "*SC 2 appreciates the presentation of Sophie Lardy-Fontan and agrees to register a Preliminary Work Item on "Monitoring estrogens" as soon as the working draft is forwarded to the secretariat.*

Once the methods were validated, the former project coordinator, Sophie LARDY-FONTAN, proposed a draft standard at the European Standardisation Technical Committee on water analysis (CEN TC 230). In parallel, the German standardisation group also prepared a draft standard. The two delegations decided therefore to submit a single project to ISO TC 147 "Water quality - SC2 "Physical, chemical and biochemical methods".

A working group was then created: ISO/TC 147/SC 2/WG 84 "Estrogens using MS based methods" whose coordinator is Mrs Lardy-Fontan (ANSES) and whose secretariat is provided by AFNOR (Arnaud Gaudrier). The project ISO 13646 was initiated 'Water quality — Determination of selected oestrogens in whole water samples — Method using solid phase extraction (SPE) followed by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) detection'

This working group met on 26 September 2022 to discuss the the JRP outcome document, first draft of ISO 13646. 4 countries were present: Finland, France, Germany and United Kingdom.

It was agreed that:

- The drafting committee consists of M Gaudrier, Mme Lardy-Fontan and M Türk.

- ISO/CD 13646 prepared the CD version before the end of October 2022, based on the discussion on the comments of the meeting. This new version circulated – for checking – about October 15, 2022 during about one week. As there was no disapproval, the project was submitted to the ISO TC 147 SC2 secretariat for the launch of the CD consultation (Committee Draft). This consultation took place between October 28 and December 23, 2022. The results of the ISO CD ballot vote were :
  - ✓ 10 countries did not comment
  - ✓ 7 countries submitted a comment form
  - ✓ 14 abstained
- WG 84 members decided to propose to register the project in the work program of CEN TC 230 "Water Analysis" (WG 1 – “Physical and biochemical methods”) and to activate the Vienna agreements (ISO lead). The secretary of WG 84 will communicate this decision to the secretary of ISO TC 147 SC2 as well as to the secretariat of CEN TC 230 "Water Analysis" to start the procedure. The takeover by CEN TC 230 WG1 of the ISO 13646 project is currently being validated (consultation in progress until April 14, 2023). If the consultation validates this recovery, the project will become an international, European and French project standard; NF EN ISO 13646.

The coordinator and the WG 84 working group reviewed the document for consideration at the WG 84 on March 14 in a hybrid meeting (Afnor/Zoom) and during the meeting at the ISO week meeting of TC 147; on 17th April 2023:

The project will become an international, European and French project standard; NF EN ISO 13646.

Longer-term economic, social and environmental impacts

The outcomes of this project will improve the assessment of human and environmental risks related to the occurrence of endocrine disrupting chemicals in the environment through more accurate and reliable measurement data. The project will enable public authorities to provide data with high level of confidence, to ensure an efficient and comparable implementation of the WFD between Member States, and to inform European citizens who have clearly demonstrated their concern about EDC. By providing measurement results with full uncertainty budgets at very low level of concentration, the project will contribute to better decision making by European policy makers and, as a consequence, to a better protection of human health, aquatic environment and biodiversity. Furthermore, the comparability of data will enable an indirect financial impact by reducing the costs of monitoring and prevention of incorrect decision-making.

With the implementation of the methods developed in this JRP and summarised and validated in the draft project ISO 13646 'Water quality — Determination of selected oestrogens in whole water samples — Method using solid phase extraction (SPE) followed by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) detection'. Testing laboratories will be able to provide reliable results with full uncertainty budgets at very low level of concentration. Public authorities will thus be able to ensure an efficient and comparable implementation of the WFD.

6 List of publications

1) Evaluation, comparison and combination of molecularly imprinted polymer solid phase extraction and classical solid phase extraction for the preconcentration of endocrine disrupting chemicals from representative whole water samples, L.B.E. Steinhaeuser, T. Westphalen, K. Kaminski, C. Piechotta; Talanta Open Volume 6, December 2022, 100163; https://doi.org/DOI:10.1016/j.talo.2022.100163

This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/

7 Contact details

n/a