New developments in oestrogen and Endocrine Disrupting Compound (EDC) monitoring: towards regulatory options for water quality management

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PROJECT DESCRIPTION

With the publication of the European Commission Implementing Decision EU 2015/495, three steroidal oestrogens, namely 17-α-ethinylestradiol (EE2), 17β-estradiol (E2) and estrone (E1), have been included in the so-called "watch list" of the Water Framework Directive (WFD) involving the monitoring of these hormones at representative sampling locations in European surface waters. The acquisition of high-quality exposure data for E1, E2 and EE2 is needed for the possible implementation of measures at European level. However, the monitoring of these substances within the watch list mechanism of the WFD and national monitoring programmes may in general be difficult because of the important gap between the detection limits of the majority of the available routine analytical methods and the very low target EQS values (notably for E2 and EE2) defined for the protection of aquatic ecosystems.

Before applying more demanding and expensive chemical analytical methods to monitor these substances, we recommend the screening of environmental samples [1] for the presence of oestrogenic activity. *In vitro* bioassays, among the different possible applications, are able to detect oestrogenic activity of environmental mixtures in a cost-effective way. In the context of the Working Group "Chemicals" and as a follow-up to the Science-to-Policy Interface activity [2] an international project has been approved which aims at:

- Promoting reliable screening methods for the monitoring of endocrine disrupting compounds (EDCs) in wastewater and surface waters;
- Harmonising monitoring strategies for EDCs across Europe as well as data interpretation methods;
- Implementing cost-effective and reliable effect-based tools in regulatory monitoring.

The project includes several reporting lines which are intended to address needs identified by the Science-Policy-Interface and the Chemical Monitoring of Emerging Pollutants activities of the Common Implementation Strategy of the WFD. The project results are additionally intended to support the main task of the WG "Chemicals" about effect-based tools, mixtures and links between chemical and ecological status for the period 2016-2018.

PROJECT PLAN

The project started in September 2014. Currently 25 research organisations and environmental agencies from 12 different countries are involved. Around 16 surface water samples and 17 wastewater samples have been collected across Europe (Figure 1).

The following chemical analytical and effect-based methods are being applied:

- High-end chemical HPLC MS-MS analysis (JRC, BfG, Swiss Centre for Applied Ecotoxicology)
- ER-Calux (BDS, Bio Detection Systems B.V.)
- MELN (INERIS)
- ER-GeneBLAzer (UFZ)
- Hela 9903 (RECETOX)
- Planar Yeast Estrogen Screen assay pYES (BfG)

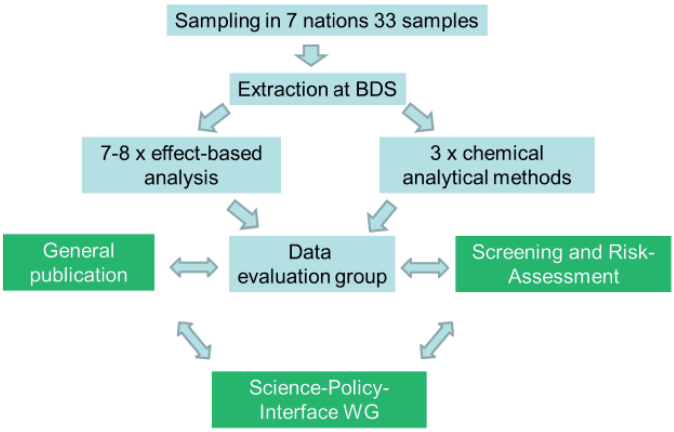

Figure 1. Oestrogen monitoring reporting activities 2015-2017.

As a complement to these methods, zebrafish-based *in vivo* reporter gene assays (INERIS) and non-target analysis (Environmental Institute, SK) will be applied for 5 samples.

The 3rd meeting of the project took place at the end of February 2016 at the French National Agency for Water and Aquatic Environments (ONEMA) in Vincennes (France), where first results of wastewater analysis were presented and discussed by around 30 project partners. In addition to the above mentioned activities, a wastewater oestrogenicity assessment group was launched at this 3rd project meeting with the collaboration of pharmaceutical industries.

The final results of the project are expected to be delivered in the first half of 2017.

PRELIMINARY RESULTS

First results for wastewater assessment with ER-CALUX bioassays show that effect-based methods can effectively quantify chemical pressures and mixture risks.

Cumulative risk quotients for chemical measurements of E1, E2 and EE2 were calculated according to the equation in Figure 2 for each wastewater sample.

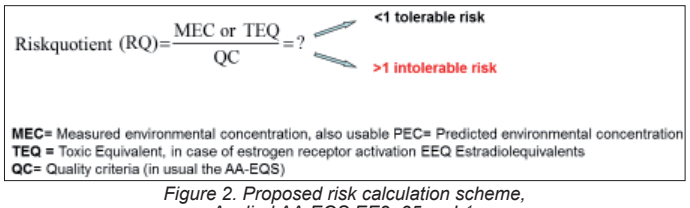

Figure 2. Proposed risk calculation scheme, Applied AA-EQS EE2=35 pg.L^{-1}, AA-EQS E2=400 pg.L^{-1}, AA-EQS E1=3600 pg.L^{-1}

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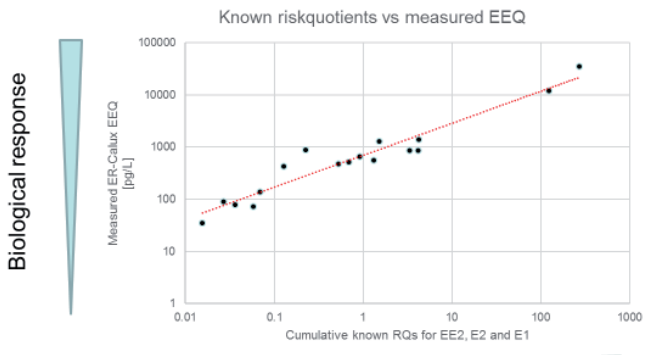
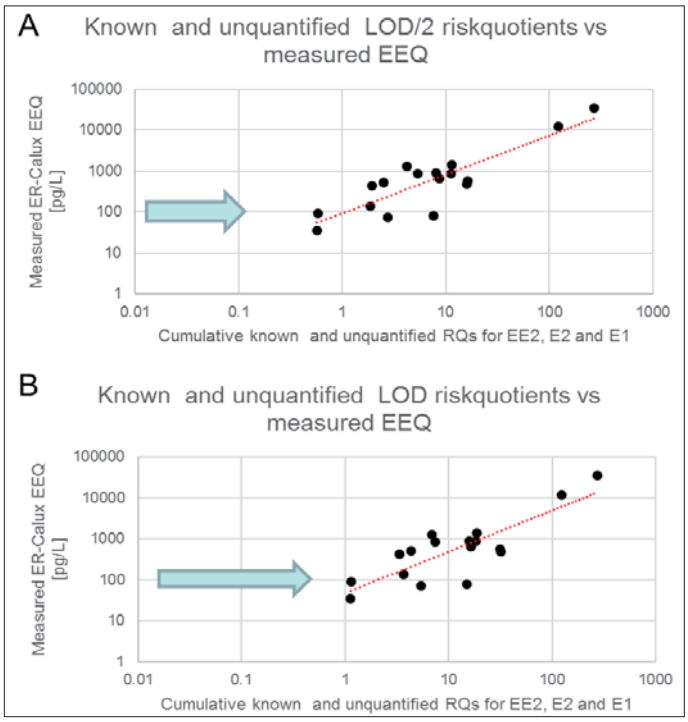
The first results show that the sum of risk quotients ("mixture risk of steroidal oestrogens") derived from chemical measurements were highly correlated with the measured ER-CALUX EEQs in the respective samples (Figure 3+4AB).



Figures 3+4AB (3). Known mixture risks of steroidal oestrogens and biological ER-Calux response for 17 international waste water extracts (4AB) Known mixture + unquantified LOD/2 (4A) and LOD (4B) risks of steroidal oestrogens and biological ER-Calux responses for 17 international waste water extracts



Given that the EQS values of EE2 and E2 are based on population-relevant long-term effect-data (the EQS were derived from Species Sensitivity Distribution based on data from 9-11 fish species), the mixture risk can be considered as directly indicative for population-relevant effects in fish species and, as a consequence, the (receptor activation-based) biological response measured with ER-CALUX can also be considered as directly correlated to the risk for aquatic species.

Overall, EEQ measured with ER-CALUX (expressed as estradiol equivalent concentration) correctly assigned the "chemical status" of wastewater samples as determined by the sum of the risk quotients for E1, E2 and EE2, with highest EEQ signals detected at sites where EE2 was quantified.

Moreover, the mixture analysis and the identified correlation between cumulative risk measured by chemical analysis and biological response provide a justification for the application of trigger value approaches described in the literature [3,4,5] to identify "known" and "unknown" mixture risks for steroidal oestrogens.

A trigger value is a "threshold value" quite similar to the RQ (Figure 1) which is used to differentiate between tolerable and intolerable risks. The application of an effect–based trigger value of 0.4 ng.L^{-1} (corresponding to the AA-EQS proposal of E2) was tested. The first results show that such a trigger value of 0.4 ng.L^{-1} is effective to distinguish with high accuracy polluted wastewater sites (with risk quotients above 1) from non-polluted sites. Specific effect-based tools can indicate known and unquantifiable risks in water samples for EE2, E2 and E1 with a high risk indication accuracy. Effect-based methods should therefore be applied as screening tools to identify polluted water bodies, because they are the only tools to address unknown mixture risks.

From the results in Table 1 it is possible to observe that when the sum of the E1, E2, EE2 population relevant risk quotients (derived from quantified data) was above 1 (samples N° 2, 9, 12, 13, 20, 23 and 33 highlighted in red) the bioanalytically measured EEQ with ER-CALUX was also above the trigger value of 0.4 ng.L^{-1}, showing that the response of the bioassay was able to indicate an analytically measured positive risk in every case.

An EEQ above the trigger value was also observed for samples N° 5, 14, 16, 19 and 21 for which the cumulative risk quotients value, calculated using quantified data, was below 1. This can be caused by other not measured weaker receptor activating substances, such as BPA or nonlyphenols, or more likely this can be caused by analytical detection problems and an underestimation of the concentration of steroidal oestrogens due to matrix effects.

It is interesting to observe that a cumulative risk quotient above 1 could be obtained when replacing the non-quantified data by LOD/2 (Limit Of Detection) in the calculation of the risk quotient. This can be a way to estimate the influence of unknown (non-quantified) steroidal oestrogens.

In turn, for 3 samples (N° 4, 17 and 31), the cumulative risk measured by chemical analysis showed a risk quotient above 1 (when replacing the non-quantified data by LOD/2), whereas the measured biological EEQ response was below the tested trigger value. It remains unclear if the actual concentration of E1, E2 and EE2 in these three samples was below or above LOD/2. Nevertheless, a good correlation of chemical analytical and bioanalytical results was found (Figure 4 AB).

Table 1. Comparison of measured chemical analytical risk quotients (RQ) with bioanalytical results of 17 wastewater samples. The risk indication was calculated for samples with an RQ >1 (highlighted in red) and exceedance of the trigger value of 0.4 ng.L-1 measured by ER-CALUX. A positive risk indication by trigger value exceedance was labelled as 'yes'.

[a]Example for 'known or LOD/2' scenario: The ER-CALUX was able to identify the risk with the exception of 3 out of 17 samples (indicated as 'no'), or also an 'accurate risk indication' was possible for 14 out of 17 (=82%) samples.

	Cumulative RQ				Trigger value 400 pg/L indicates risk		
Sample	known	known or LOD/2	known or LOD	measured EEQ ER-Calux [pg/l]	known	known or LOD/2	known or LOD
2	3.33	5.39	7.44	850	yes	yes	yes
4	0.06	2.74	5.42	72		no	no
5	0.53	16.06	31.60	480		yes	yes
9	1.31	16.22	31.13	560	yes	yes	yes
12	4.13	11.27	18.42	870	yes	yes	yes
13	1.53	4.21	6.88	1300	yes	yes	yes
14	0.23	7.99	15.76	880		yes	yes
16	0.92	8.68	16.45	649		yes	yes
17	0.07	1.87	3.68	140		no	no
19	0.69	2.50	4.30	520		yes	yes
20	122.29	122.29	122.29	12000	yes	yes	yes
21	0.13	1.93	3.36	430		yes	yes
23	271.12	271.12	271.12	35000	yes	yes	yes
26	0.02	0.57	1.12	35			no
29	0.03	0.58	1.13	91			no
31	0.04	7.55	15.07	79		no	no
33	4.21	11.35	18.49	1400	yes	yes	yes
				Risk indication accuracy[a]:	100%	82 %	70%

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In conclusion, it is possible to affirm that for the tested wastewater samples the trigger value approach allowed 100% risk indication accuracy when considering quantified chemical analytical results for steroidal oestrogens E1, E2 and EE2 , 82% when considering quantified results or LOD/2 (for non-quantified results) and 70% when considering quantified results or LOD (for non-quantified results).

These findings confirm recently published approaches to screen for endocrine active pharmaceuticals [6] and other receptor activating substances.

Finally, regarding the use of *in vitro* bioassays as screening tools, it could be argued that the response of oestrogen-receptor activation assays, such as those applied in this study is not limited to receptor activation caused by steroidal oestrogens (i.e. an EEQ greater than 1 is indicative of the presence of a wider range of (xeno)oestrogens than E1, E2, EE2), but the good correlation between analytical results and biological response (Fig. 3) shows that it is very likely that the biological response is mainly caused by steroidal oestrogens in the investigated wastewater samples. This finding is in accordance with the results of Jarosova et al. 2014, which found in 78 European wastewaters high levels of steroidal oestrogens and which estimated that around 90% oestrogen receptor mediated oestrogenicity is caused by steroidal oestrogens in municipal wastewater.

The combination of these results demonstrates that water quality assessment can progress from a purely analytical approach to effect-based monitoring, from single substance to known and unknown mixture assessment, and from *in vitro* screening to population-relevant risk assessment. Moreover, in the project we will compare additional water results and intend to evaluate the comparability of results among 8 effect-based and three chemical analytical methods.

FIRST COMPARISON OF EFFECT-BASED WASTE WATER AND SURFACE WATER RESULTS

The first ER-CALUX results indicate that 6 out of 16 surface water samples were above the trigger value and 12 of 17 waste water samples were above the trigger value without applying any dilution factor (Figure 5).

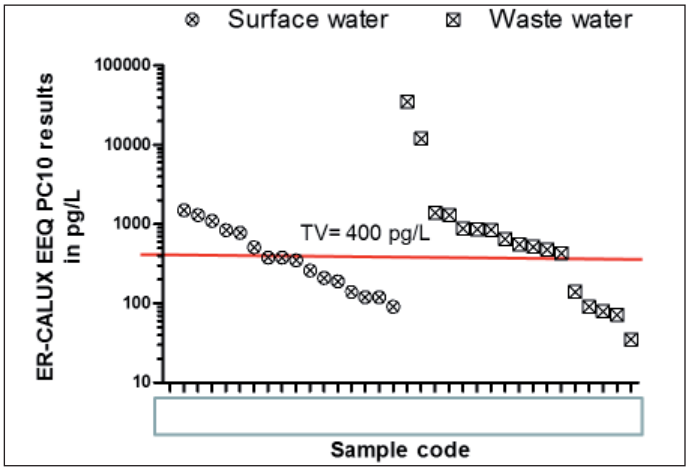

Figure 5. ER-Calux PC10 results in pg.L^{-1} EEQ for 16 surface waters and 17 waste-waters. Sampling codes were removed to avoid an unnecessary influence to ongoing effect-based measurements.

For surface- and wastewater assessments the trigger value approach offers good differentiation between more and less polluted samples. Taking into account that in this project mainly potentially polluted samples were investigated, it can be expected that with effect-based methods the chemical analytical monitoring load can be efficiently reduced if they are applied as screening methods.

EXPECTED FOLLOW-UP

- Discussion of a roadmap on how to assess the risks of steroidal oestrogens in future monitoring programmes;
- Discussion on how to improve monitoring efficiency, thereby reducing monitoring costs;
- Recommendations of methods for the characterisation of surface water and municipal wastewater quality;
- Answering the question: How to bridge the gap between conventional analytical and an effect-based monitoring for steroidal oestrogens?

As mentioned earlier the project results will also represent a contribution to the recently approved work programme of the Common Implementation Strategy (CIS) of the WFD for the period 2016-2018, in which it is foreseen to work on the best available methods to detect and evaluate the mixtures of pollutants and to link the WFD chemical and ecological status classification of water bodies across Europe. The results of the project will also contribute to the European Community Strategy on Endocrine Disruptors that highlights the needs for action on endocrine disrupting chemicals.

PROJECT INFORMATION

More information on aquatic effect-based monitoring tools is available in Wernersson et al. [7,8]. More project-info is available at: http://www.ecotoxcentre.ch/projects/aquatic-ecotoxicology/monitoring-of-steroidal-estrogens/.

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CRED: Criteria for Reporting and Evaluating ecotoxicity Data

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THE CRED PROJECT

Predicted No Effect Concentrations (PNECs) or Environmental Quality Standards (EQSs), are derived in a large number of legal frameworks worldwide. When deriving these safe concentrations, it is necessary to evaluate the reliability and relevance of ecotoxicity studies. This evaluation is often subject to expert judgment, which may introduce bias and decrease consistency when risk assessors evaluate the same study.

The CRED project, short for Criteria for Reporting and Evaluating ecotoxicity Data, is a collaboration between the Dutch RIVM, the Swiss Centre for Applied Ecotoxicology, EAWAG, and Stockholm University. It aims at improving the reproducibility, consistency and transparency of reliability and relevance evaluations of ecotoxicity studies, both within and between regulatory frameworks, countries, institutes and individual assessors. To this end, the CRED evaluation method was developed. In addition, to improve the reporting of ecotoxicity studies, a set of recommendations for reporting methodological details and results were established. The CRED project addresses aquatic ecotoxicity studies, but can be adapted to other type of ecotoxicity studies. The CRED evaluation method and the CRED reporting recommendations are available in an open access publication [1].

RELIABILITY AND RELEVANCE CRITERIA

The CRED evaluation method contains 20 reliability and 13 relevance criteria (examples of the criteria can be found in table 1). Each criterion is accompanied by extensive guidance that helps evaluators navigate throughout the assessment.

Reliability concerns the intrinsic quality of a study, regardless of the purpose for which it is assessed. It is determined by an assessment of the design, performance and analysis of the experiment. For example, a study may be considered less reliable because of an inadequate experimental design (e.g. too few replicates), poor performance (e.g. too high mortality in the controls) or insufficient data analysis (e.g. inadequate statistics).

The relevance of a study depends on the purpose of the assessment or the regulatory framework for which it is evaluated. Thus, a reliable study can be very relevant for one assessment but not relevant for another. For instance, a sediment toxicity study can be irrelevant for aquatic EQS or PNEC derivation, but very relevant for risk assessment for sediment.

The CRED evaluation method is accompanied by an Excel spreadsheet which can be used to document whether or not a criterion is met, including a rationale for this choice. This is essential since the method aims to document the choices made by the individual assessor. Thus, the CRED evaluation method offers extensive guidance and a structured assessment scheme, while at the same time acknowledging expert judgement.

REPORTING RECOMMENDATIONS

The CRED reporting recommendations contain 50 specific criteria. Researchers performing aquatic ecotoxicity studies are recommended to go through the reporting recommendations at an early stage of designing their experiments to make sure that all aspects connected to reliability are considered. Some of the recommendations are critical for the reliability of a particular study, others will be of less importance. Often this will depend on test organism, test duration, and/or test substance. When reporting ecotoxicity studies, authors are encouraged to include as much information as reasonably possible in a structured manner, if necessary using the supplemental data. When no information can be provided for one or several of the reporting recommendations, it is suggested that authors transparently explain why the information was not reported. In this way, anyone evaluating the study can get a clear picture of the experimental design, results, and the possible limitations of a particular study. The possibility that a study is under-reported and essential information is missing is likely to decrease if the CRED reporting recommendations are applied.

We conclude after having performed a ring test with 75 risk assessors that the CRED evaluation method is a suitable replacement for the Klimisch method, and that its use may contribute to an improved harmonization of hazard and risk assessments of chemicals across different regulatory frameworks [2].

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Table 1: Examples of reliability and relevance criteria from the CRED evaluation method (Moermond et al. 2015).

Reliability criteria (examples)
Are appropriate controls performed (e.g. solvent control, negative and positive control)?
Is the test substance identified with name or CAS-number? Are test results reported for the appropriate compound?
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?
Are chemical analyses adequate to verify concentrations of the substance over the duration of the study?
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?
Relevance criteria (examples)
Are the reported endpoints appropriate for the regulatory purpose?
Are appropriate life-stages studied?
Are the experimental conditions relevant for the tested species?
Is the exposure duration relevant and appropriate for the studied endpoints and species?
Is the tested exposure scenario relevant for the substance?

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Polarity-extended chromatographic separations: a novel view on trace organic compounds in environmental samples

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INTRODUCTION

Monitoring of trace organic compounds is the foundation stone of most national strategies aiming to improve surface water quality and to maintain drinking water safety. These compounds originate from human activities and enter the aquatic environment through various pathways, urban or agricultural run-off or wastewater treatment plants. Adverse effects of trace organic compounds on the aquatic environment cannot be ruled out. In order to provide information about the occurrence and concentration of trace organic compounds in water bodies, monitoring and screening programmes have been implemented [1,2]. Therefore detection techniques, namely gas and liquid chromatography (LC), both coupled to highly sensitive mass spectrometry (MS) are well established worldwide. While the use of gas chromatography is restricted to volatile compounds, which represent only a minor fraction of the compounds detectable in environmental water samples, LC-MS techniques are now recognised as the leading techniques in water monitoring. For the separation of compounds in LC-MS, reversed phase chromatography (RPLC) is mainly applied. RPLC is likely to be the best established, most robust and best understood separation technique in this field. Most known trace organic compounds of interest can be separated and detected by RPLC-MS. But interestingly, a huge number of compounds from water samples show little or no retention in RPLC and can therefore hardly be detected by MS. Based on its characteristics, RPLC is suitable for the separation of medium polar to nonpolar (i.e. hydrophobic) compounds [3]. Since retention increases in RPLC with increasing hydrophobicity, low retention indicates high compound polarity. In order to access this compound polarity range, RP stationary phases are continuously modified. Although polar-endcapping and polar-embedded RP phases have improved the polarity range of reversed phases, very polar compounds still cannot be separated by RP.

For the detection of very polar compounds normal phase (NP), ion-exchange chromatography (IEC) or hydrophilic interaction liquid chromatography (HILIC) need to be applied. Since RP and NP/IEC/HILIC are orthogonal technologies, the application of one of the three technologies will provide a view of only one side of the 'chemical universe' of a sample. Regarding the polar nature of water and the origin of trace organic compounds it is very likely that a huge number of very polar compounds might be present in environmental water samples. So, one of the future challenges in water analyses will certainly be the extension of screening programmes to very polar compounds. For this task, RPLC-MS alone will no longer be sufficient and new techniques and couplings are required.

ADVANCED CHROMATOGRAPHY FOR EXTENDED POLARITY

It is indispensable to monitor the broadest possible polarity range to provide a comprehensive view on the chemical universe contained in water bodies. For that purpose, existing techniques can be coupled or novel techniques may be implemented. Regardless of the chosen technique, levels of performance comparable to RPLC (in terms of robustness and reproducibility of separations) must be guaranteed.

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