EDC WFD

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

7-9september 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

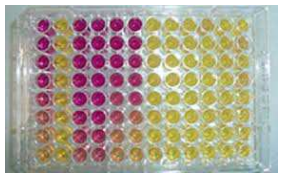
10:30 - 10:45: Break

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

About Session 1

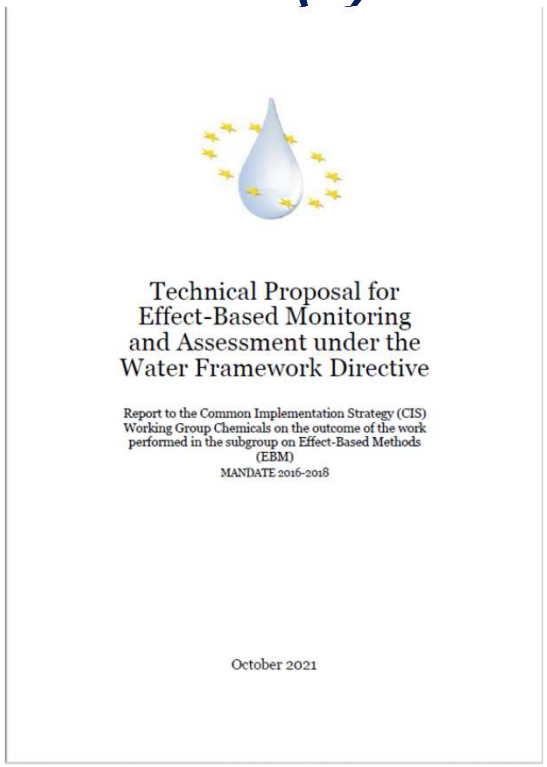
EDC WFD

Context and EBMs (Effect Based Methods) presentation

7-9 September 2022

EDC WFD

Effect Based Methods state of art (1)



EBMs state of art (2)

Main uses of the EBMs in a WFD context

- As screening tools, as part of the pressures and impact assessment
- To consider the effects from mixtures of pollutants not routinely analyzed
- To provide additional support in water and sediment quality assessment

EBMs state of art (3)

They can be categorised into three main groups depending on the type of the selected monitoring approach:

1. **Bioassay, *in vitro* and *in vivo***, which measures the toxicity of samples on cellular and individuals levels respectively
2. **Biomarkers**, which provide biological responses at individual level or lower organisational levels
3. **Ecological indicators**, that measures variations observed at higher organisation levels

EBMs state of art (4)

More than 100 assays are listed in the technical report inventory

EBMs Mode of action:

- Endocrine disruption of sex
- Endocrine disruption of glucocorticoids
- Endocrine disruption of thyroid hormones)
- Genotoxicity and mutagenicity
- Immune response
- Activation of metabolic enzymes
- Oxidative stress
- Internal regulation
- Hemoglobin synthesis
- Lysosomal membrane stability (biomarker)
- Inhibition of photosynthesis
- Neurotoxicity
- Cytotoxicity (cell death)
- Embryotoxicity (in vivo)
- Spermiotoxicity (in vivo)
- Development (in vivo)
- Histopathological changes
- Malformation (in vivo)
- Behaviour (in vivo)
- Reproduction (in vivo)

EBMs state of art (5)

In vitro reporter gene assays have been used to determine the total estrogen receptor (ER) mediated estrogenicity of an environmental sample

ER activation leads to the expression of the reporter gene product causing a quantifiable signal such as fluorescence, light emission or color change.

The resulting overall estrogenic activity of a sample is reported as "biological equivalent concentrations" (BEQ).

EBMs state of art (6)

Table I.2: Recommended modes of action (MoA) for inclusion in WFD monitoring.
EBM=Effect-Based-Method, SOP=Standard Operating Procedure, EBT=Effect-Based-Trigger-value, SW= Surface Water, WW=Waste Water, DW=Drinking Water

MoA with proven relevance	Protection aim/ reasoning	Effect based method (EBM)	Reference compound	Standardised SOP	Defined effect based trigger value (EBT) to reference compound[40]	Known applicability
Relevant MoAs with developed EBMs for potential implementation in the WFD						
Activation of estrogen receptor (ER)	Aquatic wildlife (fish) Is the most investigated MoA of endocrine disruption relevant for aquatic and human health; currently, mixture effects are not assessed. Well-developed *in vitro* EBMs capturing additive effects of ER-agonists are available.	ERα-CALUX	17-beta-estradiol	ISO 19040-3	0.283 ng/l E2-equivalence	SW, WW, DW, sediments
		T47D				
		A-YES		ISO 19040-2	0.400 ng/L	
		ER GeneBLAzer		Validity for ISO 19040-3 to be demonstrated	0.242 ng/l E2-equivalence	
		Hela 9903		Validity for ISO 19040-3 to be demonstrated	0.182 ng/l E2-equivalence	
		MELN		Validity for ISO 19040-3 to be demonstrated	0.557 ng/l E2-equivalence	
		p-YES		No standard	0.500 ng/l	

Stardardised SOP according to OECD guideline n.455

EBMs state of art (7)

The mentioned standardised SOPs (i.e. ISO standards and OECD guidelines) are developed for real water samples without providing clear indication on the sample preparation to be applied.

None have been validated in accordance with QA/QC directive requirements.

Added value

- Determination of the estrogenic potential of water vs the concrete possibility to determine the estrogen content when a selective sample prep is associated
- Method validation (sample preparation + bioassay) in accordance with QA/QC directive requirements

Effect Based Methods in general (1)

The estrogenic potential of a sample is normally expressed as **EEQ** (i.e. **E2 equivalent concentration**)

A guideline to calculate BEQ from experimental data has recently been published by ISO (**ISO 23196: Water quality – Calculation of biological equivalence concentrations (BEQ)**)

Effect Based Methods in general (2)

The **EEQ** concentration corresponds to the E2 concentration that elicits the same effect as the combined activity of all the compounds (i.e. agonist and antagonists) present in the sample.

Two main models for joint actions of mixtures: **concentration addition and independent action**. **The concept of concentration addition is based on the idea of a similar action of mixture components**.

Relative potencies assessment (1)

- Different responses among estrogens (e.g. EE2, NP or BPA)
- Not similar behaviour between the chosen reference compound, mixtures or the sample

Data interpretation issue

- Determination of relative potencies associated to the contaminants present in samples

Relative potencies assessment (2)

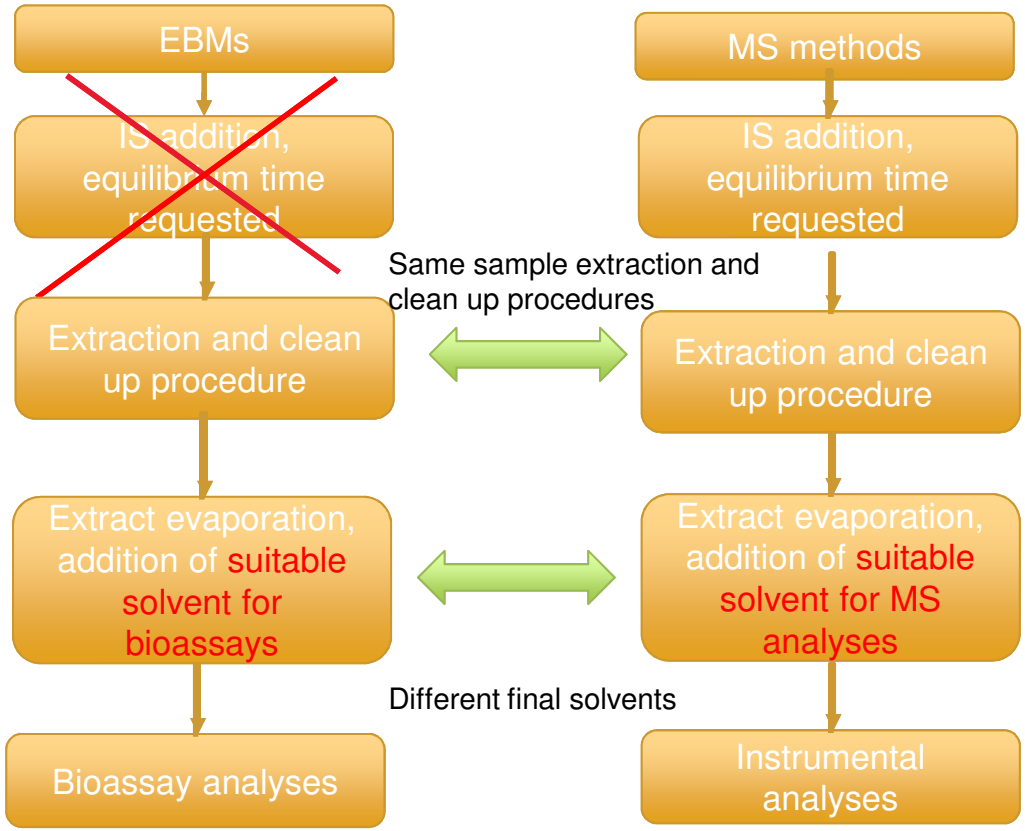
- Relative potencies (REPs) are specific to each estrogen and bioassay
- **REPs can be used to bridge chemical analyses to bioassay**
- Assumption: **similar behaviour for dose-response curves.**

$$\mathbf{EEQchem} = \sum_{\mathbf{i=1}}^{\boldsymbol{n}} \mathbf{REPi} \times \mathbf{Ci} = \sum_{\mathbf{i=1}}^{\boldsymbol{n}} \frac{\mathbf{EC}\boldsymbol{x}(\boldsymbol{R})}{\mathbf{EC}\boldsymbol{x}(\boldsymbol{i})} \times \mathbf{Ci}$$

Ci: is the detected concentration of the estrogenic compound "i" by chemical analyses
REPi : is the potency of a compound C relative to a reference compound R
EC_x (R): is the effective concentration at x level (%) of the reference compound
EC_x (i): is the effective concentration at x level (%) of the target compound.

EEQchem *VS* EEQbio

EBMs vs MS based Methods (1)



EBMs vs MS based Methods (2)

EBMs

Detect the overall estrogenic activity in samples

Interaction of target compounds which can give toxicological effects different from the expected ones

Toxic effects can be due to unknown contaminants which are not addressed by WFD

MS based methods

Measurement of single target compounds

Aligned with WFD chemical status evaluation of surface waters

EBMs vs MS based Methods (3)

EBMs

No internal standard can be added to the sample

it will be detected in the overall activity. Identification of contaminants is not feasible. Absolute recoveries can be assessed, whereas relative recoveries cannot be determined.

MS based methods

Sample preparation includes internal standard addition

Identification of contaminants is possible. Moreover, both absolute and relative recoveries can be achieved (more robust recoveries)

Sample preparation is more expensive due to the addition of IS (i.e. labelled compounds).

EBMs vs MS based Methods (4)

EBMs

Promising low LOQ achievable with appropriate sample prep

MS based methods

Low LOQ are challenging.

EBMs as evaluable prescreening tool to support chemical analysis in chemical status evaluation under WFD

EBMs vs MS based Methods (5)

- Good agreement between EEQ_{bio} and nominal EEQ_{Chem} → a few potent estrogens determine the overall estrogenic activity
- Model criteria not fulfilled in samples where significant discrepancies in mass balances are observed. Presence of unknowns or interaction between components could have an impact on the model.

→ In the context of the WFD, EBMs can be used as link between chemical and ecological assessments.

Tested EBMs within The Project

- Within the Project, two whole procedures (i.e. sample prep + either A-YES or ERα-CALUX) will be fully validated
- A-YES and ERα-CALUX are the selected EBMs: 1) standardized EBMs 2) easily available in the market
- Speed disk + MiSPE as sample prep
- The validation is ongoing and at the end of the process, results will be compared


QUESTIONS?
COMMENTS?
CONCERNS?

THANKS FOR YOUR ATTENTION!

7-9 September 2022

EDC WFD

EDC WFD

Effect based methods: Sample preparation

7-9 September 2022

EDC WFD

EBM – Sample preparation

Sample preparation strategy:

- To avoid cross reactivity from whole water matrix components it is essential to have a specific sample preparation before applying effect based methods like CALUX® or A-YES®
- A two-stage preconcentration and purification procedure is suggested by the consortium which is also suitable for MS based techniques
- The procedure was also applied in both validation studies either for MS based methods or for EBMs
- But: the use of isotopically labeled internal standard is not possible but also not necessary

EBM – Sample preparation

Preconcentration step: SPE disk (combination of filtration and SPE)

- Good compromise of filtration and enrichment
- Atlantic® HLB-L disks or analogues with 47 mm are the discs of choice

EBM – Sample preparation

Preconcentration step: SPE disk (combination of filtration and SPE)

- Conditioning: 20 mL ACN, 10 mL MilliQ water (MQ) , 20 mL MQ
- Sampling: 1 L whole water sample without ISTD in approx. 30 min
- Wash steps: 10 mL MQ – rinse sample bottle with MQ to transfer SPM completely
- Drying of SPE disk by applying vacuum for 5 min
- Elution: 5 x with portion of 10 ml ACN
- Organic extract (yellowish) is concentrated with N_2 to 1 mL (Turbovap)
- Residue is reconstituted with 75 mL MQ

EBM – Sample preparation

Purification step: MiSPE

AFFINIMIP® SPE

Estrogens

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

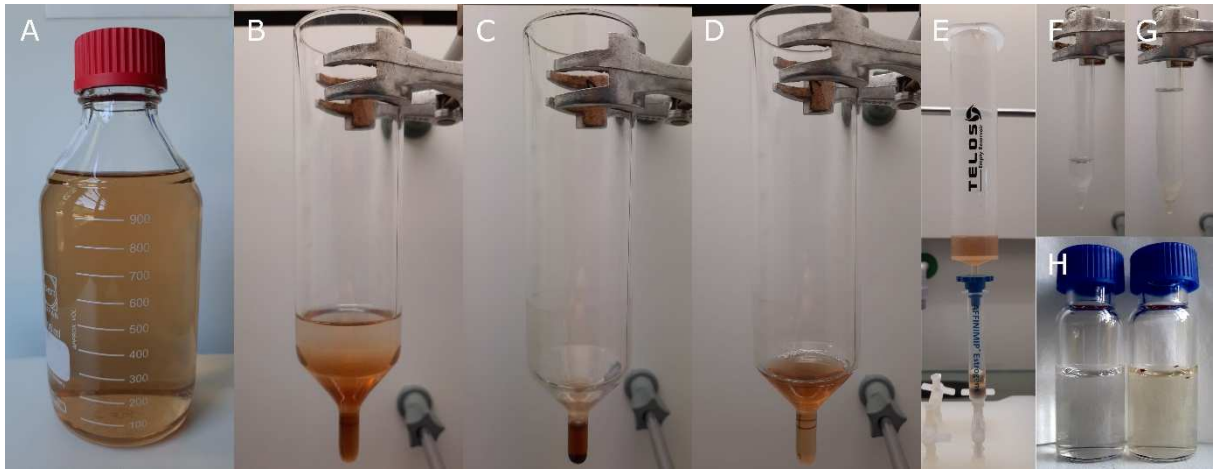
Loading solution from sample preparation

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

Elution (E)
3mL Methanol

- Standard protocol for MiPs-SPE is applied
- Sample volume is 75 ml from the previous SPE disk preconcentration
- Sample loading 1 mL min^{-1}!
- 3 mL methanol extract is concentrated with N_2 to dryness (Turbovap)
- The residue can be reconstituted as given by the individual EBM protocol and is ready for EBM analysis

EBM – Sample preparation



(A) 1 L of a high complex matrix water sample
(B) 50 mL of SPE disk eluate
(C) SPE disk eluate after evaporation to 1 mL
(D) SPE disk eluate diluted in 10 mL of H_2O
(E) Diluted SPE disk eluate applied to MiSPE column.
(F) 3 mL of MiSPE eluate
(G) HLB-SPE eluate of a 1 L sample of synthetic whole water matrix
(H) 1 mL final sample after MiSPE (left) and HLB-SPE (right)

EBM – Sample preparation

EDC WFD

Effect based methods: A-YES assay

7-9 September 2022

EDC WFD

EBM – The A-YES assay

- A-YES - Arxula - Yeast Estrogen Screen: Detection of estrogenic effects

- Commercially available test provided exclusively by New Diagnostics, Germany

- The A-YES (Arxula-Yeast Estrogen Screen) is an effect-based 96 well assay for the detection of estrogenic activity in various types of water samples. The A-YES uses the non-conventional and recombinant yeast *Arxula adeninivorans* as an estrogen responsive biosensor.

- It is applicable to ultrapure, drinking and mineral water, surface water, ground water and well water, sewage, seawater and brackish water, aqueous extracts and for substance testing

EBM – The A-YES assay

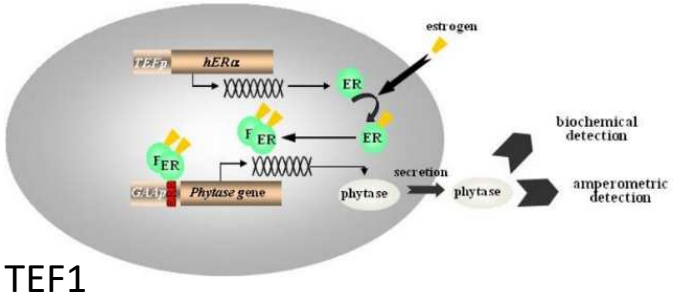
- The A-YES is a robust assay for analyzing EEQ (17β-Estradiol Equivalents) and LID (Lowest Ineffective Dilution) of samples especially with high matrix load or with high salt content. No sterile conditions are required for the whole test procedure.

- The A-YES is a standardized method according to ISO 19040-2:2018. An international interlaboratory trial was performed for method validation.

- The reference standard for calibration and quantification is 17β-Estradiol (E2). The dose-response curve corresponds to a non-linear model.

EBM – The A-YES assay

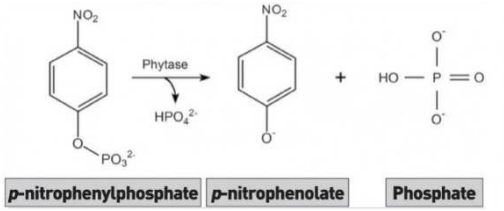
new_diagnostics

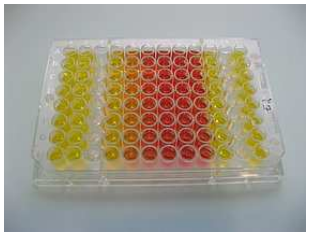


- A-YES yeast cells containing a receptor expression cassette with a constitutive TEF1 promoter and the gene for the human estrogen receptor (hERa)

- Second expression cassette is a reporter expression cassette with a GAA promoter with a recognition sequence for the estrogen receptor equipped with the gene for the enzyme phytase (reporter gene)

- Phythase catalyzes a dephosphorylation reaction to a specific dye (photometric at 405 nm)





EDC WFD

EBM – The A-YES assay

Temperature controlled orbital shaker

Deepwell plate reader (405 and 630 nm)

What is needed?

Centrifuge with rotor for deepwell plates

Single- and multichannel pipettes

EBM – The A-YES assay

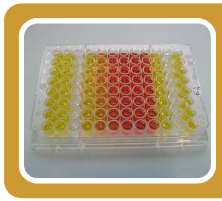
Typical procedure

Yeast activation

- Activation of freeze dried yeast by washing and dissolving in medium – 1 h incubation at 30°C and 350 rpm at orbital shaker
- Stock solution of activated yeast cells in 9.5 mL medium

Incubation with samples

- Filling cavities of the DWP with 100 µL sample solution, calibration levels and negative control
- Adding 100 µL of yeast solution to each cavity
- 20 h incubation at 30°C and 350 rpm at orbital shaker



Evaluation of estrogen activity

- Centrifuge DWPs at 3700 rpm for 25 min
- Pipitting of 40 µL of supernatant for each cavity to a new DWP
- Add p-nitrophenylphosphate solution to each cavity and incubate for 1h at 37°C
- Reading the DWP in a deepwell plate reader at 405 and 630 nm

EBM – The A-YES assay

Pipetting scheme

		1	2	3	4	5	6	7	8	9	10	11	12
Type	A	NC	S1	S1	S1	S3	S3	S5	S5	S5	S5	S7	NC
	B	CS	S1	S1	S1	S3	S3	S5	S5	S5	S5	S7	CS
	C	CS	S1	S1	S1	S3	S3	S5	S5	S5	S5	S7	CS
	D	CS	S1	S1	S1	S3	S3	S5	S5	S5	S5	S7	CS
	E	CS	S2	S2	S2	S4	S4	S6	S6	S6	S6	S7	CS
	F	CS	S2	S2	S2	S4	S4	S6	S6	S6	S6	S7	CS
	G	CS	S2	S2	S2	S4	S4	S6	S6	S6	S6	S7	CS
	H	CS	S2	S2	S2	S4	S4	S6	S6	S6	S6	S7	CS
Spike [ng/L]		1	2	3	4	5	6	7	8	9	10	11	12
	A	0	0	0	0	0	0	0	0	0	0	0	0
	B	1	0	0	0	0	0	0	0	0	0	0	1
	C	2	0	0	0	0	0	0	0	0	0	0	2
	D	4	0	0	0	0	0	0	0	0	0	0	4
	E	8	0	0	0	0	0	0	0	0	0	0	8
	F	20	0	0	0	0	0	0	0	0	0	0	20
	G	40	0	0	0	0	0	0	0	0	0	0	40
	H	80	0	0	0	0	0	0	0	0	0	0	80

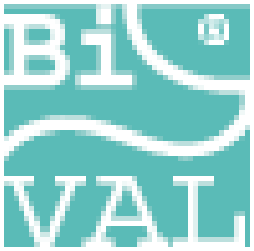
EBM – The A-YES assay

Pipetting scheme and raw data

Sample Dilution	1	2	3	4	5	6	7	8	9	10	11	12
A	0	10	10	10	10	10	25	25	25	25	200	0
B	0	20	20	20	20	20	50	50	50	50	400	0
C	0	50	50	50	50	50	75	75	75	75	800	0
D	0	100	100	100	100	100	100	100	100	100	1000	0
E	0	10	10	10	5	5	25	25	25	25	200	0
F	0	20	20	20	10	10	50	50	50	50	400	0
G	0	50	50	50	20	20	75	75	75	75	800	0
H	0	100	100	100	50	50	100	100	100	100	1000	0

OD Phytase	1	2	3	4	5	6	7	8	9	10	11	12
A	0.322	1.848	1.873	1.827	2.792	2.722	0.373	0.421	0.395	0.361	0.362	0.313
B	0.334	1.255	1.215	1.241	1.686	1.643	0.376	0.369	0.396	0.378	0.354	0.323
C	0.329	0.685	0.664	0.653	0.711	0.701	0.366	0.400	0.375	0.376	0.383	0.325
D	0.398	0.448	0.452	0.433	0.456	0.430	0.363	0.370	0.369	0.392	0.350	0.376
E	0.634	2.148	2.133	2.097	1.232	1.207	0.366	0.364	0.362	0.364	0.357	0.643
F	1.928	1.625	1.596	1.589	0.815	0.790	0.363	0.359	0.363	0.349	0.364	1.950
G	3.257	0.876	0.869	0.877	0.546	0.511	0.372	0.363	0.356	0.368	0.353	3.354
H	3.661	0.566	0.563	0.518	0.424	0.345	0.366	0.358	0.366	0.376	0.367	3.635

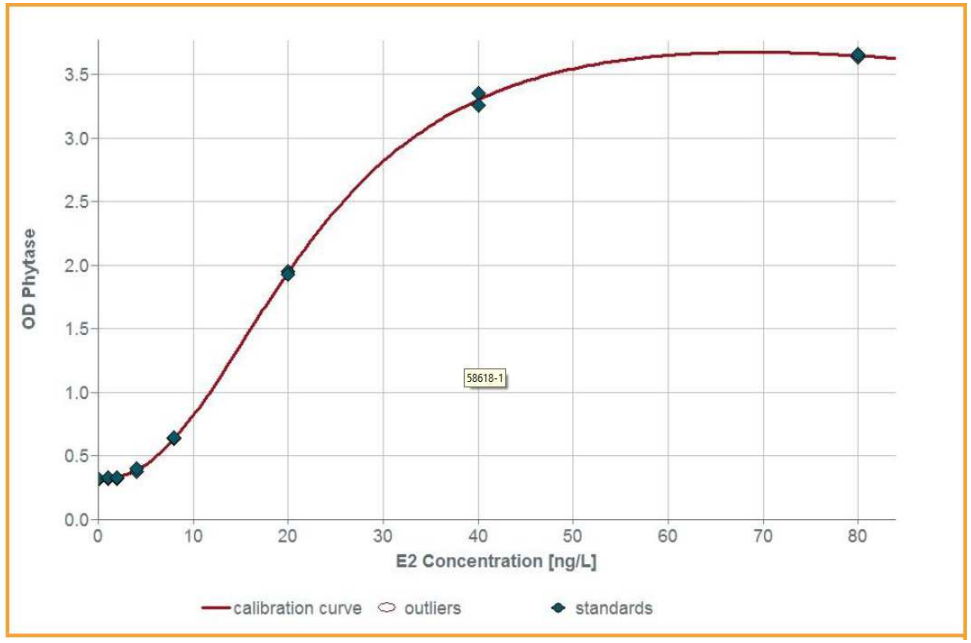
EBM – The A-YES assay



- Data evaluation will be carried out by a web tool with free access
- Simple and intuitive use
- Statistical assessment of results
- Comprehensive report with graphics
- Customized report available

EBM – The A-YES assay

Calibration curve



EBM – The A-YES assay

Calibration curve

Characterization of Calibration Curve

Expected Optical Density for the Blank Value	A	0.319	OK
Slope Parameter of the Curve	B	2.588	OK
Center of the Test [ng/L]	C	20.493	OK
Maximum Optical Density	D	3.676	OK
Variability of Residuals		2.13 %	OK
Sensitivity Range Characteristic		11.5	OK
Coverage of Calibration Range		100.0 %	OK
Check of Homogeneity of Relative Variances			OK

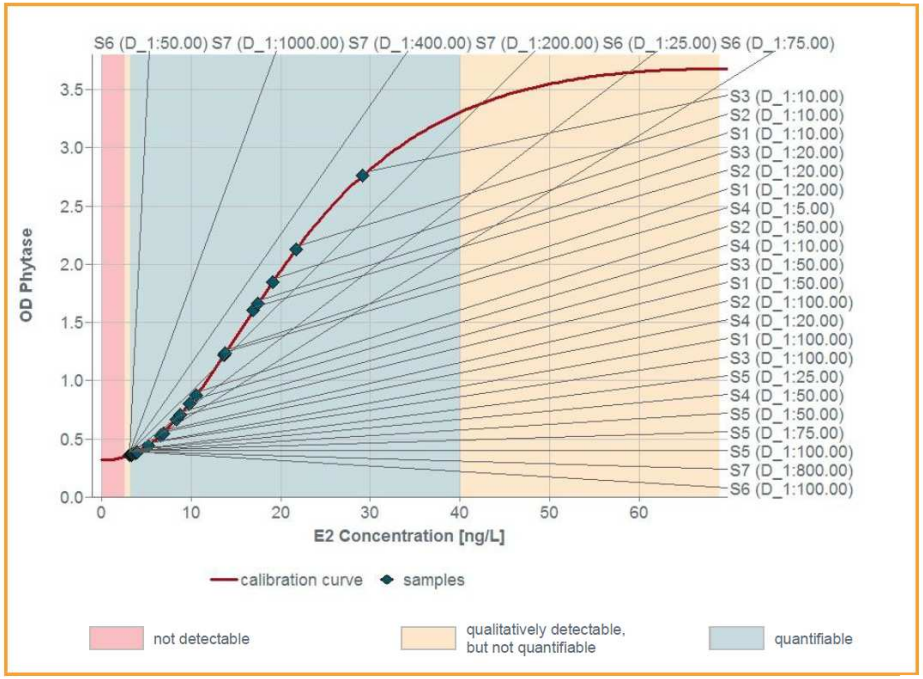
Quality Control Parameters of Calibration Curve

Limit of Detection		2.57 ng/L
Limits of Quantification	Lower Limit	3.19 ng/L
	Upper Limit	40.02 ng/L

A-YES

EBM – The A-YES assay

Calibration curve and data evaluation



EBM – The A-YES assay

Summary results of sample analysis

Sample	Sample Dilution	Spiked E2 Concen-tration [ng/L]	Diluted and Spiked Sample		Undiluted and Unspiked Sample	
			Qualitatively Detected	Quantifiable	E2 Equivalents (EEQ) [ng/L]	Measurement Uncertainty
S1	1:100.00	0.0	yes	yes	521.9	7.2 %
S1	1:50.00	0.0	yes	yes	418.9	4.4 %
S1	1:20.00	0.0	yes	yes	276.5	3.7 %
S1	1:10.00	0.0	yes	yes	191.3	4.1 %
S2	1:100.00	0.0	yes	yes	688.9	5.2 %
S2	1:50.00	0.0	yes	yes	527.3	3.8 %
S2	1:20.00	0.0	yes	yes	339.2	3.9 %
S2	1:10.00	0.0	yes	yes	217.4	4.5 %
S3	1:100.00	0.0	yes	yes	519.4	7.2 %
S3	1:50.00	0.0	yes	yes	440.8	4.2 %
S3	1:20.00	0.0	yes	yes	349.8	4.0 %
S3	1:10.00	0.0	yes	yes	291.4	6.7 %

EBM – The A-YES assay

Summary results of sample analysis

Sample	Sample Dilution	Spiked E2 Concentration [ng/L]	OD Phytase		E2 Equivalents (EEQ) in ng/L for		Measurement Uncertainty for Undiluted and Unspiked Sample
			Mean Value *)	Relative S. D. *)	Diluted and Spiked Sample	Undiluted and Unspiked Sample	
S1	1:100.00	0.0	0.444	2.3 %	5.2	521.9	7.2 %
S1	1:50.00	0.0	0.667	2.4 %	8.4	418.9	4.4 %
S1	1:20.00	0.0	1.237	1.6 %	13.8	276.5	3.7 %
S1	1:10.00	0.0	1.849	1.2 %	19.1	191.3	4.1 %
S2	1:100.00	0.0	0.549	4.9 %	6.9	688.9	5.2 %
S2	1:50.00	0.0	0.874	0.5 %	10.5	527.3	3.8 %
S2	1:20.00	0.0	1.603	1.2 %	17.0	339.2	3.9 %
S2	1:10.00	0.0	2.126	1.2 %	21.7	217.4	4.5 %
S3	1:100.00	0.0	0.443	4.2 %	5.2	519.4	7.2 %
S3	1:50.00	0.0	0.706	1.0 %	8.8	440.8	4.2 %
S3	1:20.00	0.0	1.664	1.8 %	17.5	349.8	4.0 %
S3	1:10.00	0.0	2.757	1.8 %	29.1	291.4	6.7 %

All participants in the audience who are familiar with the A-YES assay are invited to give further detailed information or additional comments and hints

EDC WFD

ERα-CALUX protocol

7-9 September 2022

EDC WFD

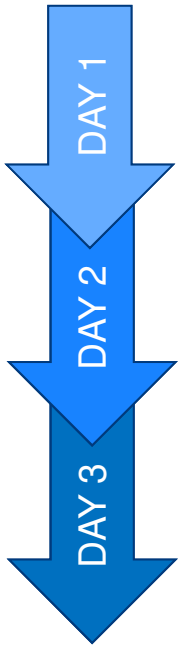
Analysis of extracts with ERα-CALUX® bioassay (1)

U2-OS CALUX cells are human osteoblastic cells (U2-OS) that are genetically engineered to produce the enzyme luciferase in response to a defined pathway that is activated.

The amount of luciferase produced by the samples is related to known concentrations of reference compound and the final results are therefore expressed as reference compound equivalents.

Analysis of the extracts takes 3 days.

Analysis of extracts with ERα-CALUX® bioassay (2)



- Seeding the cells in microtiter plate

- Preparing the extract dose medium
- Preparing the reference compound dose medium
- Exposure of cells

- Measuring the response

Day 1 – Seeding the cells in microtiter plate (1)

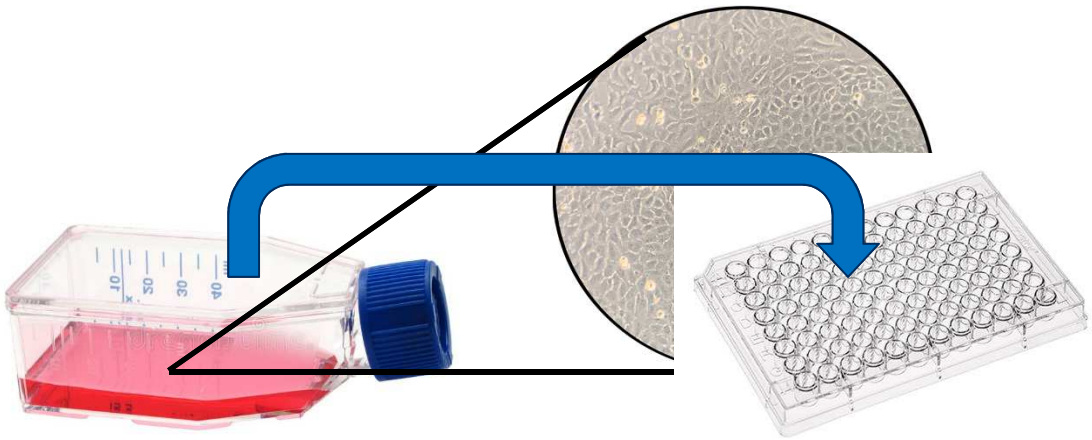
It is **important** to:

- Chose the first day of analysis. E.g. You can start on Monday and measure the luciferase activity on Wednesday.

- Verify that the confluence of the cells is between 85%-95%. From one flask can be prepared 2-3 microtiter plates.

Day 1 – Seeding the cells in microtiter plate (2)

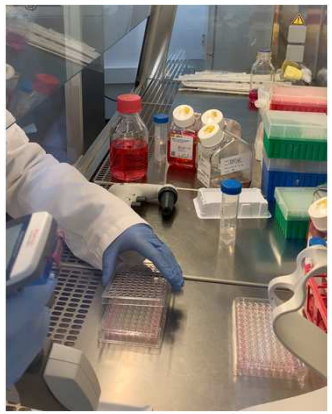
- ✓ If the confluence of the cells is between 85% - 95%, the cells can be trypsinate and cultivated in 96-well microtiter plates.



Day 1 – Seeding the cells in microtiter plate (3)

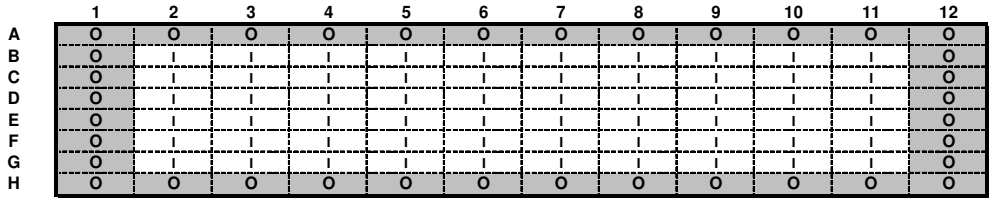
In this step the cells are counted with a counting chamber (e.g. Bürker-Türk) in order to calculate the dilution factor to obtain a cell suspension of 100.000 cells per ml (10.000 cells/well)

Then cell suspension can be added in the 60 inner wells:



Day 1 – Seeding the cells in microtiter plate (4)

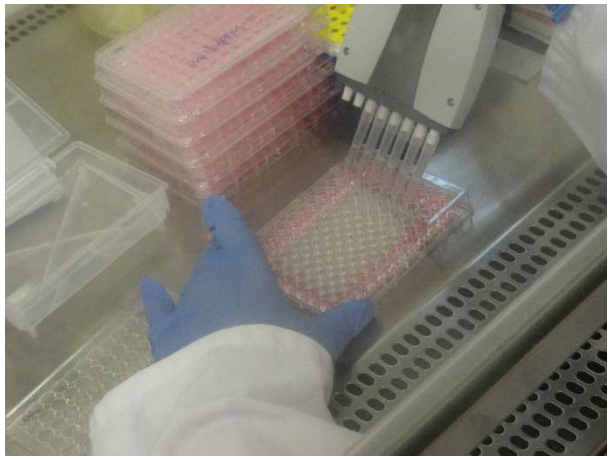
Schematic representation of a 96-well microtiter plate after seeding. The outer wells (grey) are filled with 200 µl PBS and the inner wells (clear) with 100 µl cell-suspension.



After seeding, incubate the plates for 16-30 hours in the CO_2 incubator (37°C, 5% CO2, 95% humidity)

Day 1 – Seeding the cells in microtiter plate (5)

MAKE SURE YOU WORK ALWAYS UNDER STERILITY CONDITION!



Day 2 – Making dose medium for chemical standard and extracts (1)

Reference compound (17β-estradiol) and extracts are both in DMSO.

Cells can tolerate a percentage of DMSO between 0,1% and 1%.

A dilution of the solutions is required

Day 2 – Making dose medium for chemical standard and extracts (2)

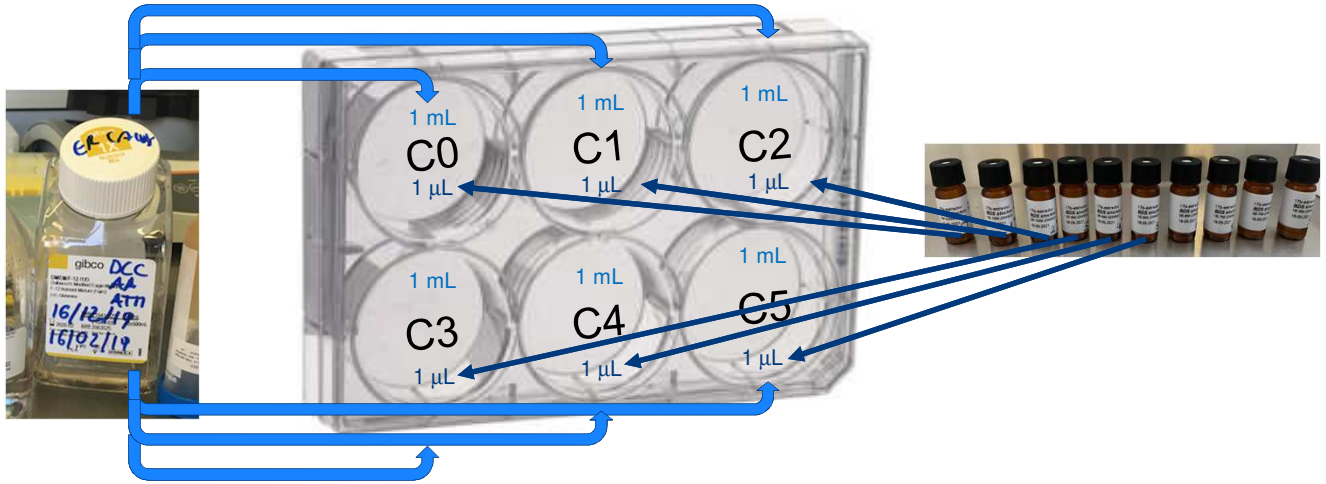
We chose an exposure with 0.1% DMSO implying a 1:1000 dilution.

Each concentration level is diluted in the assay medium (DMEM/F12) using a 6-well plate.

1 µL of extract/reference compound in 1 mL of assay medium

Day 2 – Making dose medium for chemical standard and extracts (3)

Example: reference compound calibration levels from C0 to C5

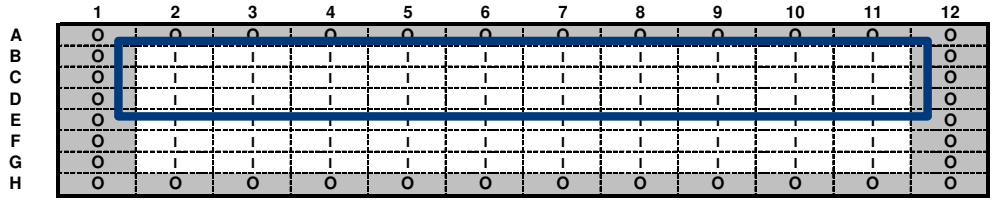


Day 2 – Exposure of cells (1)

At this points the cells can be exposed to the prepared exposure medium.

Each concentration level is analysed in triplicate.

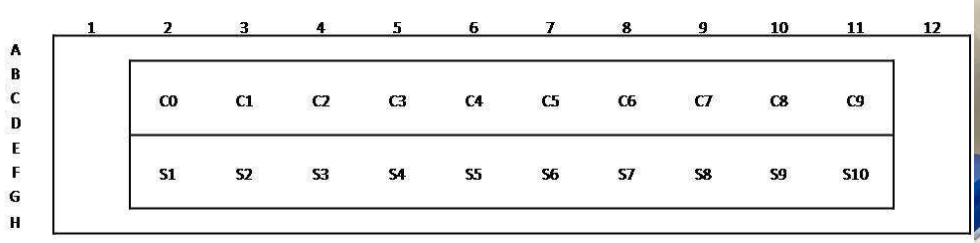
1. Carefully remove the medium from the top 30 wells



In these three rows the reference compound dose medium is added

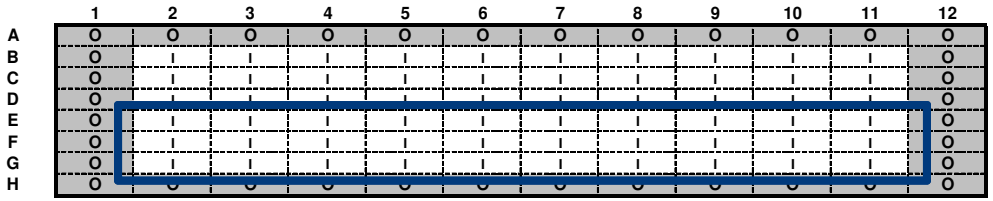
Day 2 – Exposure of cells (2)

2. Add 200 µl of the lowest concentration of the reference compound dose medium (C0) to three wells according to the scheme. Continue with the lowest chemical standard concentration (C1) until all the standard chemical concentrations are transferred to the microtiter plate.



Day 2 – Exposure of cells (3)

3. For the extract dose medium the procedure is the same, but the exposure medium is added to the bottom 30 wells:



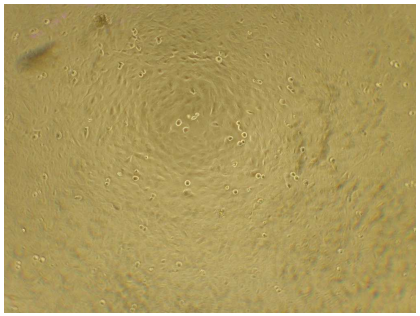
4. After the exposure place the microtiter plate in the CO_2-incubator and incubate for 24 hours.

Day 2 – Exposure of cells (4)

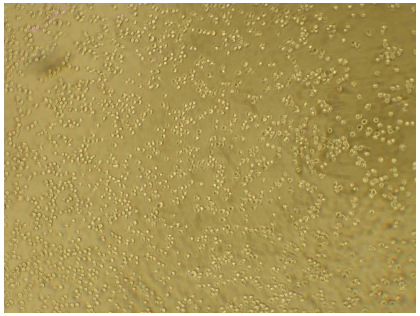
MAKE SURE YOU WORK ALWAYS UNDER STERILITY CONDITION!

Day 3 – Measuring the response (1)

- ✓ Check the cells for signs of **cytotoxicity** and/or contamination under a microscope before measuring the response.
- ✓ If cytotoxicity is observed in some wells, those wells **cannot be considered** in the analysis



Normal monolayer cells

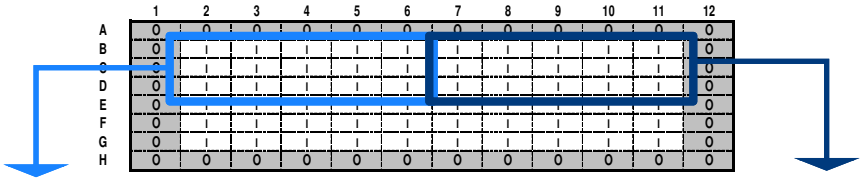


Monolayer destroyed by cytotoxicity

Day 3 – Measuring the response (2)

Cytotoxicity can occur due to high concentration of toxic substances to cells but also for the incorrect or slow exposure execution

To limit the latter cause we suggest to divide the exposure in two steps:



1° → removal of the medium and exposure of the first rows

2° → removal of the medium and exposure of the last rows

Day 3 – Measuring the response (3)

After the check of cytotoxicity it is possible to proceed with the plates reading:

- The medium is removed from the cells and the lysis solution is added
- The amount of luciferase can be read by a luminometer.
- In this step sterility conditions are not required.

Chemiluminometer

TECAN Infinite 200 Pro is in use in our laboratory.

It is important that the luminometer is equipped with **two injectors:**

Injector A: "illuminate Mix" → containing the substrate (luciferin) necessary to make the luminescence reaction possible.

Injector B: 0.25% Acetic acid solution → used for quenching the luminescence reaction to prevent cross contamination between wells.

ERα-CALUX® bioassay

The ERα-CALUX® bioassay is under license.

if any laboratory were interested, we could provide information on the company selling the license


QUESTIONS?
COMMENTS?
CONCERNS?

THANKS FOR YOU ATTENTION!

EDC WFD

EDC WFD

EBMs Data Treatment

7-9 September 2022

EDC WFD

Data Analysis (1)

EEQ concentrations can be affected by the chosen method for data treatment.

Reference dose-response curves are fitted applying functions commonly studied in biological fields (i.e. functions sigmoidal in shape).

Dose-response curves for the reference compound may have a different behaviour (e.g. different slope, different asymptotes) from the one observed with the sample and the interpolation of the sample can be affected.

Data Analysis (2)

Quantification of the samples by means of a calibration curve (i.e. standard curve)

↓

Standard curve is an approximating function of the "*true curve*" (infinite levels of concentrations each replicated infinite times).

↓

Choice of the curve model to be applied within the fitting process → **SOURCE OF ERROR**

EDC WFD

Data Analysis (3)

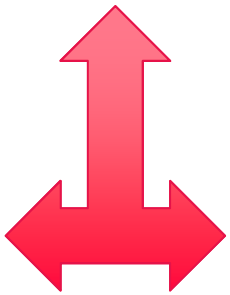
One example of **S-curve** is the four parameters logistic function described below:

$$y = Bottom + \frac{Top - Bottom}{1 + \left(\frac{EC50}{C}\right)^{Hill's\ slope}}$$

Bottom = lower limit related to blank signal
Top = upper limit related to the highest effect
Hill's slope = slope of the curve which gives the idea of its steepness
EC50 = dose corresponding to the 50% response and to the inflection point
C= tested dose
Generally plotted using a logarithmic scale

Data Analysis (4)

Fit errors identification to improve quality of the fit



Lack of fit error due to model function: it is independent from the number of replicates

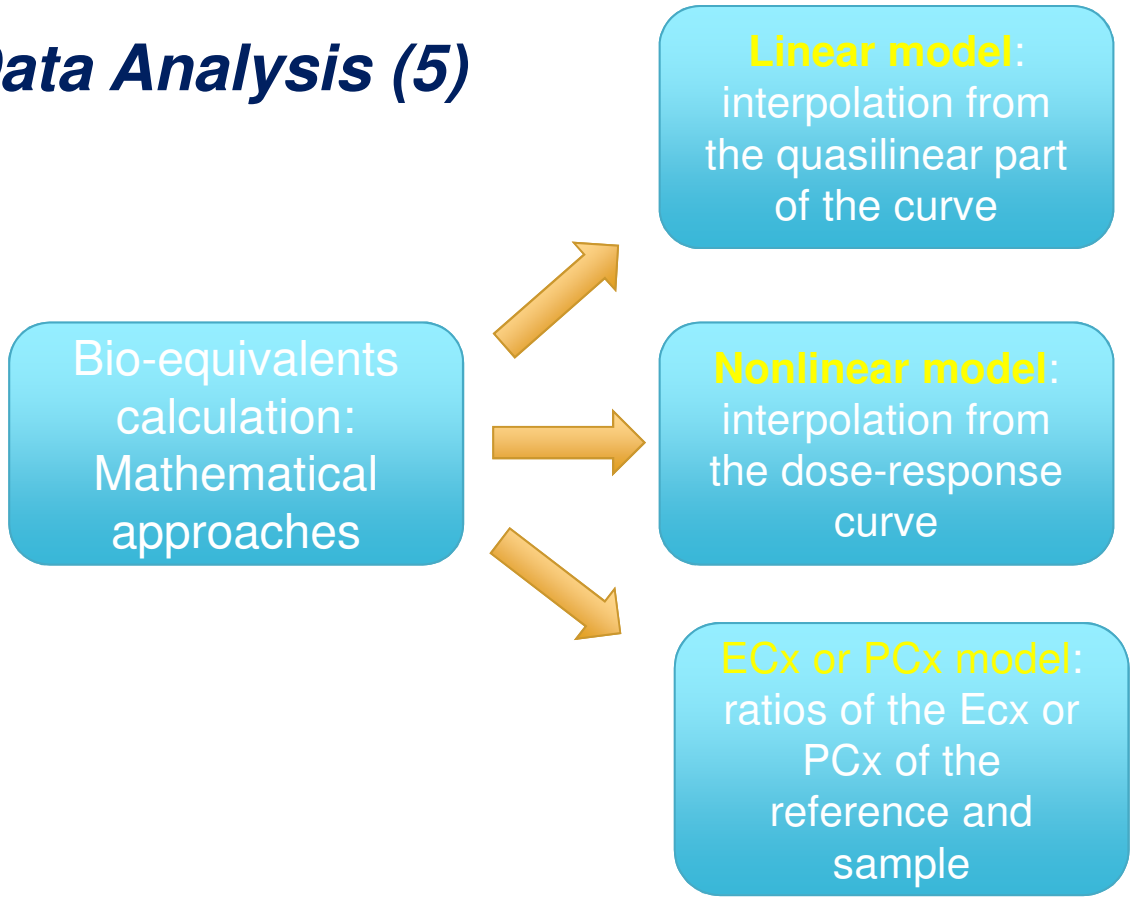
Bioassay data have sigmoidal shape: non linear regression for the whole range is required

Pure Error due to random variation in the data: it decreases with the increase of the number of replicates

Number of replicates optimisation

EDC WFD

Data Analysis (5)



EDC WFD

PCx model (1)

The fundamental assumption of this approach is that dilution series of samples behave like dilution series of the reference compound.

- Parallelism and same maxima of dose-response curves are prerequisites
- PCx definition: Concentration of a reference compound at which a specific level expressed in x-percentage is observed.

PCx model (2)

$$Concentration\ Factor\ Sample\ Prep = \frac{V_{sample}}{V_{Extract}}$$

$$Dilution\ Factor\ Bioassay = \frac{Volume\ of\ extract\ added\ to\ bioassay}{Volume\ in\ total\ of\ the\ bioassay}$$

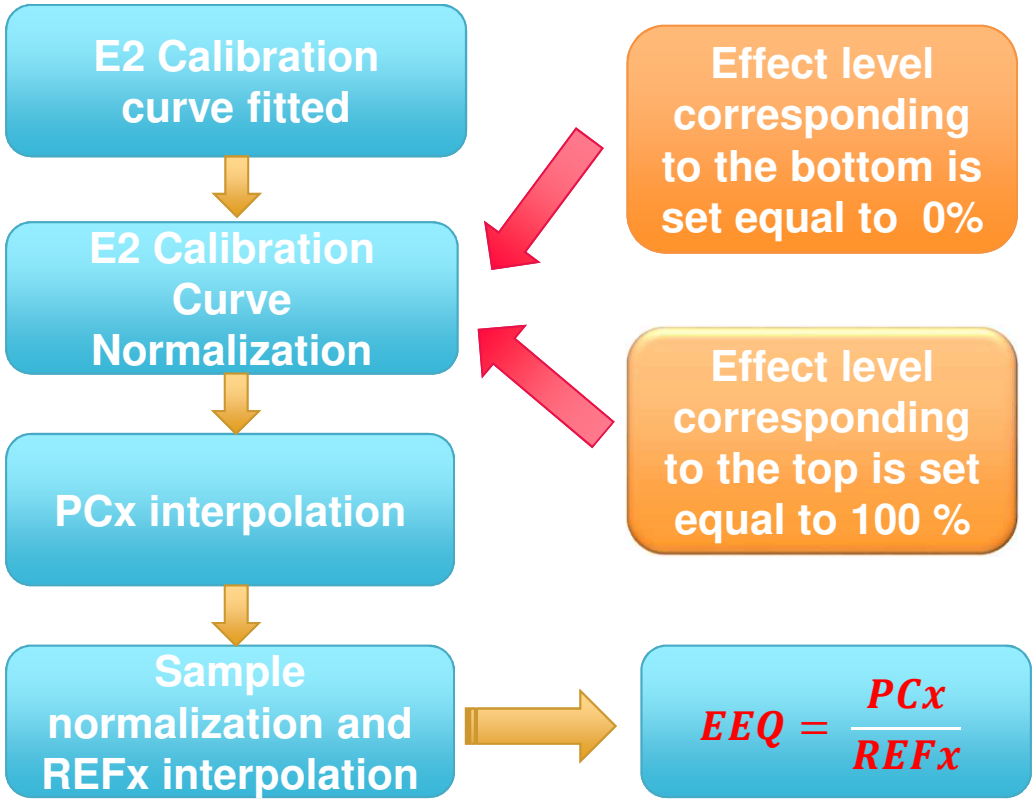
$$REF = Concentration\ Factor\ Sample\ Prep\ X\ Dilution\ Factor\ Bioassay$$

Enrichment or dilution of the original water sample

REF is dimensioless number

EDC WFD

PCx model (3)



EDC WFD

PCx Example (1)

Exposure information	
Name technician:	XXXX
Cell type:	Era-CALUX
Passage cells:	22
Plate type:	96 well Falcon
Plate and filename:	
Date seeding:	02/03/2022
Date exposure:	03/03/2022
Exposure-time (hr):	23
Date measurement:	04/03/2022
DMSO concentration (%)	0.1
Luminometer:	TECAN

Compound/sample information					
ID code	**Compound**	**Solvent**	**Sample Volume (mL)**	**Extract volume (mL)**	**Concentration factor**
REFERENCE (C1-C8)	17ß-estradiol	DMSO			
PC	17α-methyltestosterone	DMSO			
NC	corticosterone	DMSO			
TI1	BRM1 BC		500	0.100	5000
TI2	BRM2 BC		500	0.100	5000
TI3	BRM3 BC		500	0.100	5000

EDC WFD

PCx example (2)

E2 Calibration curve fitted

Plate 1										
	2	3	4	5	6	7	8	9	10	11
B	C0	C1	C2	C3	C4	C5	C6	C7	C8	PC
C	C0	C1	C2	C3	C4	C5	C6	C7	C8	PC
D	C0	C1	C2	C3	C4	C5	C6	C7	C8	PC
E	SC	TI1-1	TI1-2	TI1-3	TI1-4	TI1-5	TI1-6	TI1-7	TI1-8	NC
F	SC	TI1-1	TI1-2	TI1-3	TI1-4	TI1-5	TI1-6	TI1-7	TI1-8	NC
G	SC	TI1-1	TI1-2	TI1-3	TI1-4	TI1-5	TI1-6	TI1-7	TI1-8	NC

Plate 1										
	2	3	4	5	6	7	8	9	10	11
B	54	241	304	402	1377	3451	6417	6654	6421	6421
C	62	230	245	360	1411	3901	5965	6313	6425	6425
D	74	240	219	403	1191	4201	6610	6078	6496	6496
E	122	135	96	116	188	548			5968	104
F	130	71	68	130	214	492	2072	4420	5213	136
G	105	101	87	121	123	455	1508	3979	4338	121

EDC WFD

PCx example (3)

E2 Calibration curve fitted

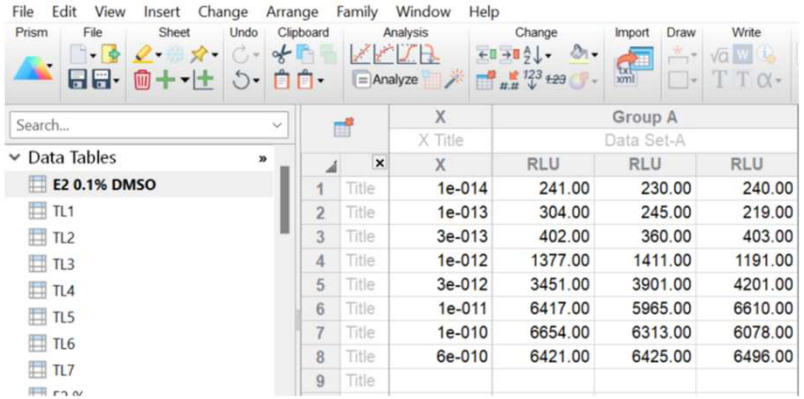
Plate 2										
	2	3	4	5	6	7	8	9	10	11
B	SC	TI2-1	TI2-2	TI2-3	TI2-4	TI2-5	TI2-6	TI2-7	TI2-8	C7-max
C	SC	TI2-1	TI2-2	TI2-3	TI2-4	TI2-5	TI2-6	TI2-7	TI2-8	C7-max
D	SC	TI2-1	TI2-2	TI2-3	TI2-4	TI2-5	TI2-6	TI2-7	TI2-8	C7-max
E	SC	TI3-1	TI3-2	TI3-3	TI3-4	TI3-5	TI3-6	TI3-7	TI3-8	C4 (EC50)
F	SC	TI3-1	TI3-2	TI3-3	TI3-4	TI3-5	TI3-6	TI3-7	TI3-8	C4 (EC50)
G	SC	TI3-1	TI3-2	TI3-3	TI3-4	TI3-5	TI3-6	TI3-7	TI3-8	C4 (EC50)

Plate 2										
	2	3	4	5	6	7	8	9	10	11
B	191	134	102	111	188	660	2533	4838	5673	6421
C	102	132	81	95	163	435	2427	4632	5401	6425
D	95	97	89	97	175	612	2030	4619	5202	6496
E	61	89	98	203	247	512	2134	4726	5674	4723
F	66	113	58	133	248	545	2169	3935	5510	4899
G	91	109	72	138	230	475	1582	3732	4852	5051

EDC WFD

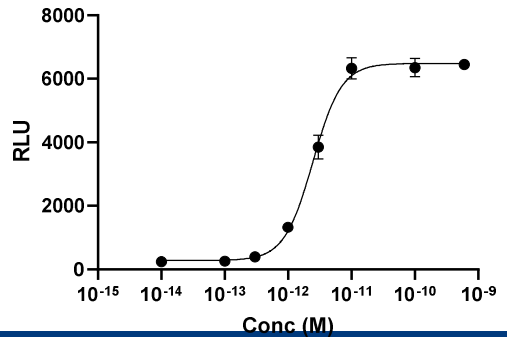
PCx example (4)

E2 Calibration curve fitted



RLU	Reference
Best-fit values	
Bottom	275.70
Top	6484
EC50 (M)	2.5E-12
HillSlope	1.93
R2	0.9943

E2 RLU CA 25-03-2022



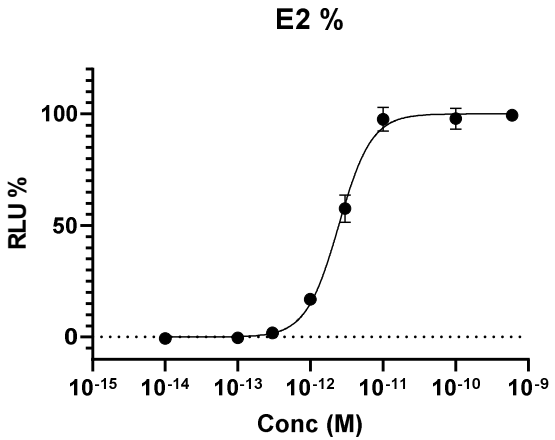
EDC WFD

PCx example (5)

E2 Calibration curve normalization

$$Rel.Ind\ (\%) = 100 * \frac{y_{std}(RLU) - bottom}{Top - bottom}$$

RELATIVE INDUCTION (%)				
rel.ind.-1	**rel.ind.-2**	**rel.ind.-3**	**avg**	**SD**
-0.6	-0.7	-0.6	-0.6	0.1
0.5	-0.5	-0.9	-0.3	0.7
2.0	1.4	2.1	1.8	0.4
17.7	18.3	14.7	16.9	1.9
51.1	58.4	63.2	57.6	6.1
98.9	91.6	102.0	97.5	5.3
102.7	97.2	93.5	97.8	4.7
99.0	99.0	100.2	99.4	0.7



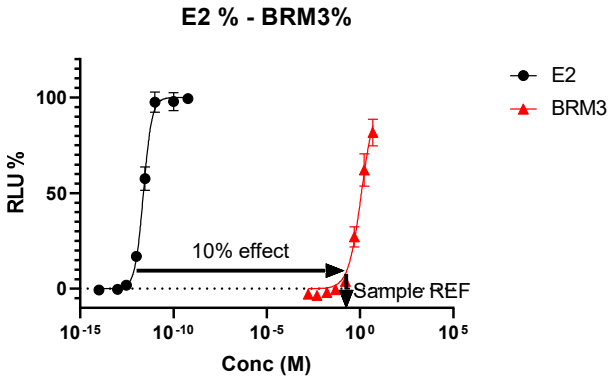
EDC WFD

PCx example (6)

PCx and REFx interpolation

$$Rel.Ind\ (\%) = 100 * \frac{y_{sample}\ (RLU) - bottom}{Top - bottom}$$

RELATIVE INDUCTION (%)				
rel.ind.-1	**rel.ind.-2**	**rel.ind.-3**	**avg**	**SD**
-3.0	-2.6	-2.7	-2.8	0.2
-3.5	-3.5	-3.3	-3.4	0.1
-1.2	-2.3	-2.2	-1.9	0.6
-0.5	-0.4	-0.7	-0.5	0.2
3.8	4.3	3.2	3.8	0.6
29.9	30.5	21.0	27.2	5.3
71.7	58.9	55.7	62.1	8.5
87.0	84.3	73.7	81.7	7.0



$$EEQ = \frac{PCx}{REFx}$$

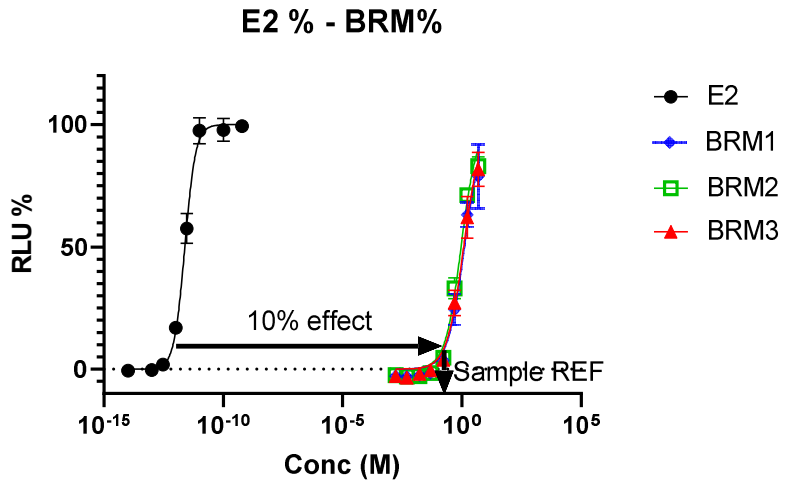
Concentration (M)

Adimensional

EDC WFD

PCx example (7)

PCx and REFx interpolation



	Approach	Effect level (%)	Sample	EEQ (M)	Average (M)	RSD (%)	Reference Value (M)	Recovery (%)
TI1	PCx	10	BRM1 BC	3.58E-12	3.98E-12	12	3.40E-12	117
TI2			BRM2 BC	4.50E-12				
TI3			BRM3 BC	3.85E-12				

EDC WFD

QA/QC procedures (1)

Within a bioassay batch, responses may change due to either nonspecific reasons or to decreasing luciferase levels due to the increasing passages of the cells, thus as a good practice, several quality controls should be used

EDC WFD

QA/QC procedures (2)

	CALUX values
Relative Standard Deviation (RSD %) of replicate wells	→ 15-30 % Depending on the concentration
Ratio of the reference standard solvent control (plate 1) and solvents on other plates	→ 0.5 - 2.0
Ratio of the appr. EC50 and highest reference standard concentration on plate 1 and the corresponding on the other plates.	→ 0.70 - 1.30

QA/QC procedures (3)

Calux values

Sigmoidal Curve of Reference Standard Regression	→	R squared > 0.98
Observed EC50 range	→	4*E-12 – 4*E-11 M, but also depending on DMSO concentration
PC Relative Induction (%)	→	17α-methyltestosterone> 30%
NC Relative Induction (%)	→	corticosterone< 10%

EDC WFD


QUESTIONS?
COMMENTS?
CONCERNS?

THANKS FOR YOUR ATTENTION!

7-9 September 2022

EDC WFD

Linear Model

- Bioequivalents are obtained by linear interpolation from the reference compound
- Possible contradiction with the nonlinear nature of the dose-response function in bioassays
- Crucial point: choice of the linear range within the curves
- Possible linear range: from 10% up to 30 % of effect depending on slopes values.
- Samples extracts serial dilutions are recommended

Non Linear Model

- Bioequivalents are obtained by direct interpolation from the reference compound dose-response curve.
- Accurate estimation depending on the fitting quality and model.
- Measurements on the samples extracts with or without their serial dilutions

EDC WFD

Next step: Towards Interlaboratory Comparison and standardisation

7-9 September 2022

EDC WFD

- The aim of this ILC is to demonstrate the fitness for purpose of the optimised and validated methods in the project:
  - MS-based methods (GC or LC coupled to MS (TQD or HRMS)
  - Effect-based methods (ER Calux and A-YES)

- This ILC will allow to define performance characteristics of the methods in terms of repeatability within laboratories and reproducibility of the validated methods and support standardization process

Time schedule

by 30 th September 2022 The Organizer sends the Protocol to the laboratories
by 2nd November 2022 The test materials will be sent to each involved laboratory
by 23rd November 2022 The involved laboratories will provide the results to ilc-empir@isprambiente.it
by 20th December 2022 A Preliminary statistical evaluation will be sent to the involved laboratories
By 15th February 2023 The Final Report will be sent to the involved laboratories. Plenary meeting for the presentation and discussion of the results

MATERIALS

- 2 materials representative of natural waters
- 1 QC as blank water
- Delivered as kits to be reconstituted by each laboratory
- Kit will be constituted of 4*1l water + SPM + DOC solution+ spike solution
- A written protocol + video will be delivered ⇒ the receipt

In order to formalize your pre-registration for this ILC, we kindly ask you to fill the following pre-registration form
https://forms.gle/dBYjMCDShkHJLfks7

ILC contact persons

Paolo de Zorzi (ISPRA): paolo.dezorzi@isprambiente.it
Teemu Näykki (SYKE): teemu.naykki@syke.fi
Sabrina Barbizzi (ISPRA): sabrina.barbizzi@isprambiente.it

ISO/TC 147

Sustain, contribute to the revision of ISO 19040 series

The standards will become CEN standards