Bratislava, 6-9-2018

BIOASSAY MONITORING OF WATER AND BIOTA IN THE DANUBE RIVER BASIN

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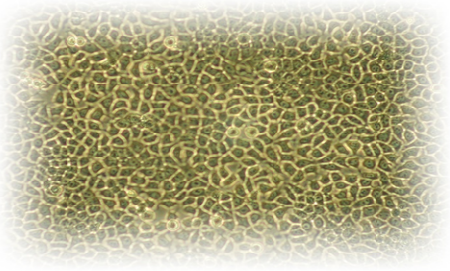
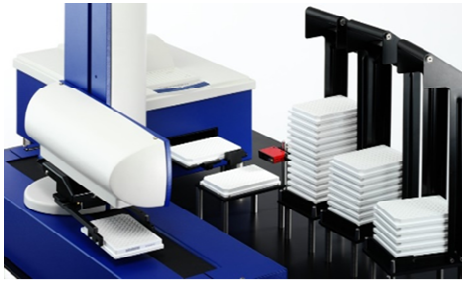
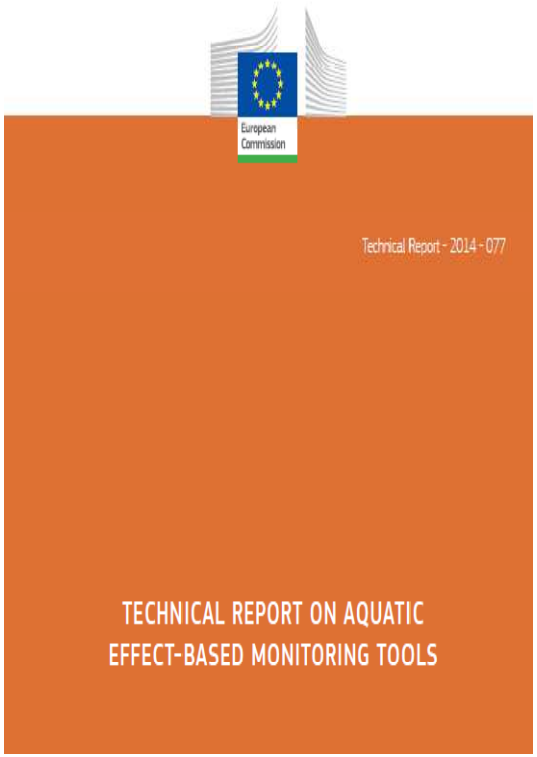




Overview about presentation

- Techniques for JDS4
- Goals of our organization
- Planned achievements
- Suggestions of Topics for Norman Joint Network

EU technical report (2014): Aquatic Effect-based Tools – the way forward to evaluate mixtures in a non-animal testing way



DR CALUX

The Dioxin Responsive (DR) CALUX® comprises rat hepatoma cell lines (H4IIE), incorporating the firefly luciferase gene coupled to Dioxin Responsive Elements (DREs) as a reporter gene for the presence of dioxins (PCDDs) and dioxin-like compounds (e.g. furans (PCDFs) and dioxin-like PCBs (dlPCBs)). Following binding of dioxins and/or dioxin-like compounds to the cytosolic Arylhydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. Cells that are exposed to dioxins or dioxin-like compounds not only express proteins that are under normal circumstances associated to DRE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds (2,3,7,8-TCDD). DR CALUX bioassays report total 2,3,7,8-TCDD TEQs for environmental matrices and total BEQs for food/feed matrices.

- **What is analysed (endpoint; unit):** ng 2,3,7,8-TCDD equivalents/kg sample processed
- **Test duration:** 24h
- **Method used**: Marine Quality Assurance procedures available in the future through between particular independent laboratories (Davies & Vethaak 2012)
- **Positive control used**: 2,3,7,8-TCDD
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample, but the substances that the assay responds to are in the aquatic environment primarily found accumulated in e.g. sediments and biota (tissues).
- **Cells examined:** Rat liver cell line
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below).
- **What /type of/ substances does the assay respond to:** Ah receptor active compounds, e.g. Polyhalogenated dioxins/furans, dioxin like PCBs, and if using other pretreatment of samples also PAHs (see PAH CALUX).
- **Sensitivity (LOD/Q):** . The bioassays' LOQ is 1 pg 2,3,7,8-TCDD equivalents per amount of material processed. For example, if 5 grams of dried soil/sediment or 1 liter of water is processed, an LOQ of 0.2 ng 2,3,7,8-TCDD equivalents per gram of soil/sediment or 1 ng 2,3,7,8-TCDD equivalents per liter of water is obtained respectively.
- **Variability (e.g. CV for single substance tests) if known:** <20%
- **Influence by cytotoxicity/risk of false positives/negatives:** As the sample is cleaned up by a sulphuric acid treatment and afterwards with an additional step to separate dl-PCBs from PCDD/Fs, cytotoxicity is rarely occurring. In case of false positive/false negative guided levels has to be established to compare it with. In case of the EC project HORIZONTAL no false positive or false negative samples occurred. For such methods usually a false positive and negative ratio of 5% is reasonable.
- **Complexity/learning period:** 2 weeks of training
- **Costs:** Low[56], especially compared to chemical analysis of dioxins and dioxin-like compounds. Generally not depending on matrix studied.
- **Commercial availability:** Commercial ISO 17025 accredited performers are available

ERα CALUX (agonistic/antagonistic)

The ERα Responsive (ERα) CALUX® comprises a human bone marrow cell line (U2OS), incorporating the firefly luciferase gene coupled to Estrogen Responsive Elements (EREs) as a reporter gene for the presence of estrogens and/or estrogen-like compounds. Following binding of estrogens or estrogen-like compounds to the cytosolic estrogen receptor, the ligand-receptor complex binds the ERE. Cells that are exposed to estrogens and/or estrogen-like compounds not only express proteins that are under normal circumstances associated to ERE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds 17β-estradiol. ERα CALUX bioassays report total 17β-estradiol equivalents for environmental matrices.

- **What is analysed (endpoint; unit):** pg 17β-estradiol equivalents/g sample processed
- **Test duration:** 24h
- **Method used**: Dutch Rijkswaterstaat RIKZ-Specie-08 guideline; Australian Water Commission; Ongoing evaluations at the ISO-TC 147 standardisation group led by BFG-Germany; EPA California; China National Water Quality Monitoring in Jinan.
- **Positive control used**: 17β-estradiol (E-2)
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample.
- **Cells examined:** Human bone marrow cell line
- **Sample volume or mass needed for different matrices:** Depending on type of material analysed and required Limit of Quantitation (LOQ) (see below).
- **What /type of/ substances does the assay respond to** Binding to the Estrogen receptor (alpha and beta for original ER CALUX and only alpha for ERalpha CALUX)
- **Sensitivity (LOD/Q):** The bioassays' LOQ is 35 pg 17β-estradiol equivalents per amount of material processed. For example, if 5 grams of dried soil/sediment or 1 liter of water is processed an LOQ of 7 pg 17β-estradiol equivalents per gram of soil/sediment or 35 pg 17β-estradiol equivalents per liter of water is obtained respectively. Original ER CALUX: 0.1 ng EEQ/l water (see e.g. Leusch, 2008).
- **Variability (e.g. CV for single substance tests) if known:** <20%
- **Influence by cytotoxicity/risk of false positives/negatives:** Depending on the SPE extraction/clean-up as well as type of water matrix.
- **Complexity/learning period:** 1 week of training
- **Costs:** Low[58]. Costs are generally not depending on matrix studied.
- **Commercial availability:** Commercial ISO 17025 accredited performers available
- **WFD relevance:** This bioassay analysis is more sensitive than most chemical analyses (lowest LOD reported by Loos 2012 is e.g. 0.1 ng/l for a chemical analysis of EE-2 and E-2, if using USEPA method 1698; in practice the LOQ that is possible to reach by regular laboratories is generally higher). The assay could therefore be very valuable on a screening level to identify water bodies at risk due to the combined exposure to a large number of estrogenic substances that could constitute RBSPs (see case studies "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach") and to lower the frequency of analytical high end monitoring in water bodies for E2. EE2 and E2 are also suggested to be included in 2008/105/EC. Because EE2 is significantly (about 10-25 times) more potent *in vivo* than E2, but only 3 times more potent in ER CALUX, this should be taken into account if evaluating data in an absolute manner (comparison with EQS), when considering the need for additional studies. In vivo studies of oestrogenic response, or using precautionary EE2 equivalents can be considered, if the presence of EE2 is likely, e.g. via high ratio of municipial waste water. The EU-EQS proposal for E2 is based on a SSD approach of the most sensitive aquatic organisms, and concludes that an

Techniques for JDS4: New ISO and OECD Standards for estrogen analysed by ER CALUX

INTERNATIONAL STANDARD

ISO 19040-3

First edition
2018-07

Water quality — Determination of the estrogenic potential of water and waste water —

Part 3: In vitro human cell-based reporter gene assay

Qualité de l'eau — Détermination du potentiel oestrogène de l'eau et des eaux résiduaires —

Partie 3: Essai in vitro sur cellules humaines avec gène rapporteur

Reference number
ISO 19040-3:2018(E)

OECD/OCDE 455

ANNEX 4

Stably Transfected Human Estrogen Receptor-α Transactivation Assay for Detection of Estrogenic Agonist and Antagonist Activity of Chemicals using the ERα CALUX cell line

INITIAL CONSIDERATIONS AND LIMITATIONS (See also GENERAL INTRODUCTION, page 1)

1. The ERα CALUX transactivation assay uses the human U2OS cell line to detect estrogenic agonist and antagonist activity mediated through human estrogen receptor alpha (hERα). The validation study of the stably transfected ERα CALUX bioassay by BioDetection Systems BV (Amsterdam, the Netherlands) using the human U2OS cell line to detect estrogenic agonist and antagonist activity mediated through human estrogen receptor alpha (hERα) demonstrated the relevance and reliability of the assay for its intended purpose (1). The ERα CALUX cell line expresses stably transfected human ERα only (2) (3).

2. This test method is specifically designed to detect hERα-mediated transactivation by measuri chemiluminescence as the endpoint. The use of chemiluminescence is commonly used in bioassays becau of the high signal-to-noise ratio (4).

3. Phytoestrogen concentrations higher than 1 µM have been reported to over-activate the lucifer reporter gene, resulting in non-receptor-mediated luminescence (5) (6). Therefore, higher concentrations phytoestrogens or other similar compounds that can over activate the luciferase expression, have to examined carefully in stably transfected ER transactivation assays (see Appendix 2).

4. The "GENERAL INTRODUCTION" and "ER TA TEST METHOD COMPONENTS" (pag 1-14) should be read before using this test method for regulatory purposes. Definitions and abbreviations used in this TG are described in Annex 1.

PRINCIPLE OF THE TEST METHOD (See also GENERAL INTRODUCTION, page 1)

5. The bioassay is used to assess ER ligand binding and subsequent translocation of the receptor-ligand complex to the nucleus. In the nucleus, the receptor-ligand complex binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Following the addition of the luciferase substrate luciferine, the luciferine is transformed into a bioluminescent product. The light produced can easily be detected and quantified using a luminometer.

6. The test system utilises stably transfected ERα CALUX cells. ERα CALUX cells originated from the human osteoblastic osteosarcoma U2-OS cell line. Human U2-OS cells were stably transfected with 3xHRE-TATA-Luc and pSG5-neo-hERα using the calcium phosphate co-precipitation method. The U2-OS cell line was selected as the best candidate to serve as the estrogen- (and other steroid hormone) responsive reporter cell line, based on the observation that the U2-OS cell line showed little or no endogenous receptor activity using reporter plasmids only. Furthermore, this cell line supported strong hormone-mediated responses when cognate receptors were transiently introduced (2) (3) (7).

COMMISSION REGULATION (EU) 2017/644

of 5 April 2017

laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014

(Text with EEA relevance)

1.4. 'Bioanalytical methods' means methods based on the use of biological principles such as cell-based assays, receptor-assays or immunoassays. They do not give results at the congener level but merely an indication (1) of the TEQ level, expressed in Bioanalytical Equivalents (BEQ) to acknowledge the fact that not all compounds present in a sample extract that produce a response in the test may meet all requirements of the TEQ-principle.

Bioanalytical screening methods

— The result of the screening shall be expressed as compliant or suspected to be non-compliant ('suspected').

— In addition, an indicative result for PCDD/F and/or dioxin-like PCBs expressed in BEQ (not TEQ) may be given (see point 1). Samples with a response below the reporting limit shall be expressed as lower than the reporting limit. Samples with a response above the working range shall be reported as exceeding the working range and the level corresponding to the upper end of the working range shall be given in BEQ.

— For each type of sample matrix, the report shall mention the maximum or action level on which the evaluation is based.

— The report shall mention the type of test applied, the basic test principle and kind of calibration.

EC 605 (2018): Endocrine disrupting chemical = endocrine mode of action

COMMISSION REGULATION (EU) 2018/605

of 19 April 2018

amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties

(Text with EEA relevance)

'From 20 October 2018, an active substance, safener or synergist shall be considered as having endocrine disrupting properties that may cause adverse effect in humans if, based on points (1) to (4) of the sixth paragraph, it is a substance that meets all of the following criteria, unless there is evidence demonstrating that the adverse effects identified are not relevant to humans:

(1) it shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;

(2) it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system;

(3) the adverse effect is a consequence of the endocrine mode of action.

First new EU guidance for endocrine disrupters in case of biocides and pesticides (2018)

ADOPTED (ECHA): 05/06/2018

ADOPTED (EFSA): 05/06/2018

doi:

Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009

European Chemicals Agency (ECHA) and European Food Safety Authority (EFSA) with support from the Joint Research Centre (JRC).

Abstract

This Guidance describes how to perform hazard identification for endocrine-disrupting properties by following the scientific criteria which are outlined in Commission Delegated Regulation (EU) 2017/2100 and Commission Regulation (EU) 2018/605 for biocidal products and plant protection products, respectively.

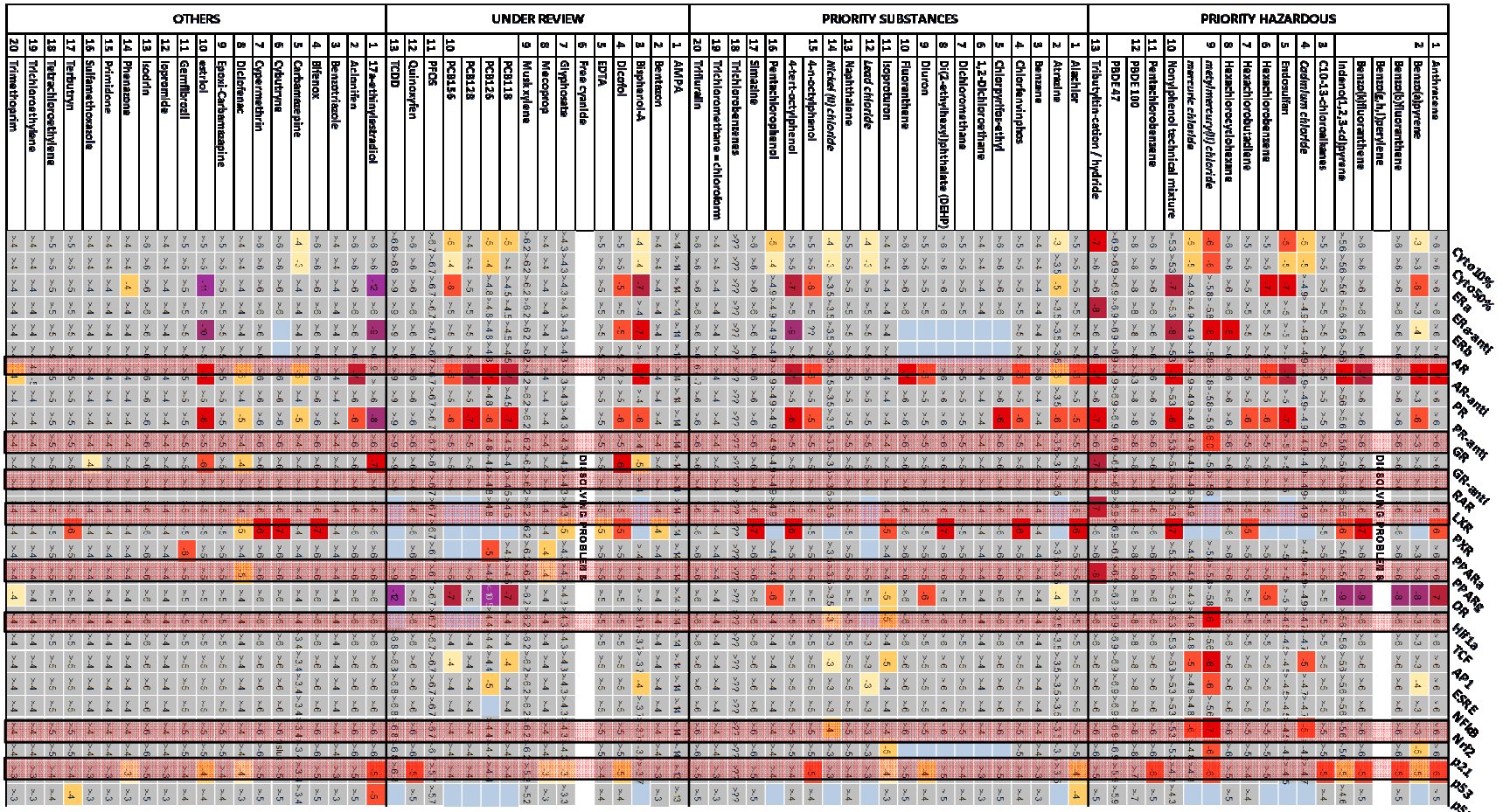
Key words: biocidal product, plant protection product, endocrine disruptor, guidance, hazard identification

Table 12: Parameters in OECD CF Level 2 'in vitro mechanistic', for which guidance is provided in OECD GD 150.

Test guideline	OECD TG	455	493		458		456
	US EPA OPPTS		890.1250	890.1150		890.1200	
Species / *in vitro* test system		ER TA (human) cells expressing ERα	Binding to rat (EPA) or human (OECD) estrogen receptor	Binding to rat androgen receptor	AR TA (human AR-EcoScreen™) cell line	Human recombinant microsomes	Human H295R cells
Indicative of: [a]		E	E	A	A	S	S
Androgen receptor binding/transactivation				X	X		
Aromatase						X	
Estrogen receptor binding/transactivation		X	X				
Steroidogenesis (estradiol and/or testosterone synthesis)							X

[a] Based on OECD GD 150, indicative of: the (E)strogen-; (A)ndrogen-; (S)teroidogenesis-; or (T)hyroid- modalities.

EU project DEMEAU: Water Framework relevant compounds & Bioassay Multiple Mode of Actions Screening



Most relevant Modes of Action in water:
ER, anti-AR, anti-PR, anti-GR, PXR, AhR, Nrf2, p53 genotoxicity

Proposed techniques and target effect-based trigger (EBT) values

Mode of Action	Reference Compound	International Standard	Method e.g.	Effect-based triggervalue	1 to 3-times EBT level	3- to 10-times EBT level	Above 10-times EBT level	Above 100-times EBT level
Estrogenicity	ng eq E2/l	ISO 19040 OECD TG 455	Human or Yeast TA cell assay	0,3	0,9	3,0	30	>30
Inhibition Androgenicity	µg eq Flutamide/l	OECD TG 458-like	Human or Yeast TA cell assay	3,3	9,9	33	330	>330
AhR receptor activation	ng eq B(a)P/l		Rat liver cells H4IIE TA assay	6,2	18,6	62	620	>620
Adaptive Stress (Nrf2)	µg eq dichlorvos/l	ISO 19040-like OECD TG 455 like	Human TA cell assay	26	78	260	2600	>2600
Early warning chemicals: Activation pregnane x receptor (PXR)	µg eq DEHP/l	ISO 19040-like OECD TG 455-like	Human TA cell assay	272	816	2720	27200	>27200

Goals of our organisation

- Use state of the art high-through-put robotics BIOlogical screening tools for many important Mode of Actions for water and biota
- Use biodiagnostic tools to monitor and improve water and biota
- Use international accepted bioassays for e.g.
  - water (for estrogens: ISO 19040-3) and
  - biota such as fish (for dioxins/dl-PCBsEC/771/2017;
  for anti-AR such as marker PCBs and PBDEs OECD TG458)
- in a ISO 17025 laboratory QA/QC system
- as sensitive and reliable as chemical analysis,
- but as a safety umbrella covering more relevant chemicals/pharmaceutcials
- Building a bridge between chemical and animal testing
- Finding new toxic relevant chemicals
- Covering effects of “142 Mio CAS chemicals” rather than a “few historical selected chemicals” in the current EU framework

Planned achievements: Confirmation of first results of CALUX® panel results and suggested action plan for operators of WWTPs from the Danube River Basin

	ERα CALUX	anti-AR CALUX	GR CALUX	PPARγ CALUX	PAH CALUX	Nrf2 CALUX	PXR CALUX
1 - Varazdin	5	5.7	<19	640	72	41	8.2
2 - Amstetten	1.1	22	<20	<520	122	46	13
3 - Cluj	<0,06	31	34	<420	52	<50	8.4
4 - Augsburg	1	10	72	<410	72	46	13
5 - Vipap	0.65	32	<25	<460	242	74	48
6 - Budapest	0.56	11	<23	<430	62	46	14
7 - Ljubljana	6.6	8.4	120	<350	62	50	9.4
8 - Bucurest	7.4	5.7	38	<340	82	130	10
9 - Zilina	2.2	8.9	78	<480	72	60	5.2
10 - Sabac	1.1	14	<41	<490	72	46	4
11 - Brno	0.54	13	47	<1100	122	80	17
12 - Zagreb	0.8	6	<42	<1100	52	<17	17

Suggestions of Topics for Norman Joint Network

- Add bioassay testing in current EU guidelines (minimal the ISO and OECD approved bioassays) for water and biota
- Improve and training of urgent need for such bioassay testing for at minimal the endocrine disrupting chemicals
- Confirmation of the first results of Danube River bioassay activities
- Verification and recommendation of Safe EBTs here suggested
- Recommendation for panel of effect-based Mode of Action testing
- Evaluation ratio of known chemical vs. total toxicity bioassay (in TEQs)
- Find most relevant drivers for found endocrine disrupting chemicals activities (ER, anti-AR and anti-PR CALUX® were above the chosen safe EBTs)
- Evaluate need of glucocorticoid testing in elderly homes/hospital effluents (In 50% of the water samples significant GR CALUX® results were measured but only one measurement excided the in the Netherlands routinely used EBT of 100 ng Dex-eq/l)
- Further investigations and water monitoring campaigns are recommended by using such a panel of CALUX® bioassays