10th BIODETECTORS CONFERENCE
6-7 April 2017, Sorrento, Italy

HTPS FOR SAFETY ASSESSMENT AND COMPOUND PROFILING USING CALUX EFFECT-BASED BIOASSAYS

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Major challenge: risk assessment of complex mixtures


Toxic waste


Chemicals


Pharmaceuticals

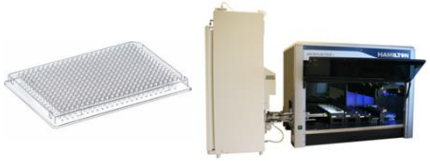

Toxins

Complex mixtures

Environmental health, Food, Human health

Monitoring complex mixtures

Analytical analysis vs biological monitoring



- Analytical analysis
- Biological monitoring: effect-based
- Measures all active chemicals, including “unknowns”
- Effect in human cells

Overview CALUX pathway-based reporter gene assays

name	basal line	species	pathway	reference compound	key reference
DR CALUX	H4IIE	rat	dioxin receptor activation	2,3,7,8-TCDD	Van Vugt 2013
PAH CALUX	H4IIE	rat	dioxin receptor activation	benzo-a-pyrene	Pieterse 2013
ER CALUX	T47D	human	estrogen receptor activation	17β-estradiol	Legler 1999

- **Sensitive quantitative assays for major hormonal systems and cell signalling pathways**
- **Adresses major types of toxicity**
  (general toxicity, genotoxicity/carcinogenicity, endocrine disruption, reproduction, developmental tox, etc)
- **More than 50 assays**
- **Agonism / antagonism**
- **Metabolism (S9, phase I & II, steroidogenesis)**
- **Robust and specific: suitable for complex mixtures**
- **Data on > 400 chemicals**

AP1 CALUX	U2OS	human	AP1 pathway activation	TPA	Piersma 2013, Van der Burg 2013
HIF1alpha CALUX	U2OS	human	Hif1alpha pathway activation	cobaltous chloride	Piersma 2013, Van der Burg 2013
ER stress CALUX	U2OS	human	ERSE activation leading to endoplasmic reticulum stress	tunicamycin	Piersma 2013, Van der Burg 2013

High-throughput screening (HTP)

SEEDING

Ready-to-seed frozen
96-well format
384-well format

EXPOSURE

Dilution series
(13/compounds)

DETECTION

Luminescence
detection

Implementation of effect-based bioassays in regulatory frameworks for safety and quality evaluation

selection bioassays

↓

development of standardized protocols

↓

formal validation

↓

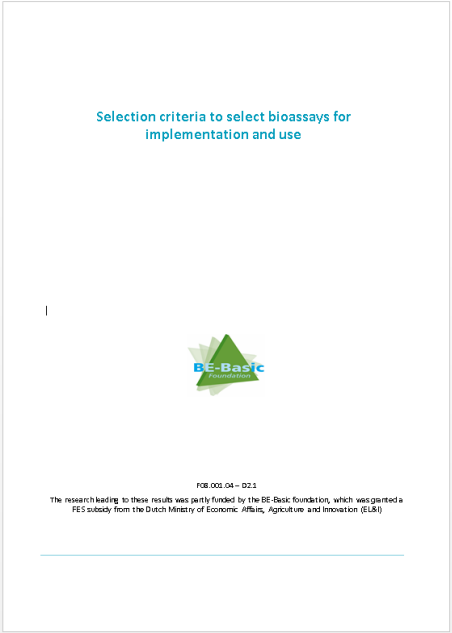
establishment of effect-based trigger values

↓

implementation in regulatory frameworks for risk assessment

Selection of bioassays

Toxicity pathway	Pertinent CALUX assay	Health effects
Xenobiotic metabolism	DR CALUX PAH CALUX PXR CALUX	Reproductive and developmental problems, interfere with hormone action and cancer
Lipid metabolism	PPARα-, PPARγ CALUX LXR CALUX	Obesity and inflammatory diseases
Hormone-mediated mode of action	(anti)ERα- and ERβ CALUX (anti)AR CALUX (anti)PR CALUX (anti)GR CALUX RAR CALUX	Tumor development Birth defects (Sexual) developmental disorders
Reactive mode of action/genotoxicity	P53-, P53 S9 CALUX	Tumor development
Adaptive stress response	Nrf2 CALUX Hif1a CALUX TCF CALUX AP1 CALUX NFκB CALUX ESRE CALUX P21-CALUX	Inflammation, organ toxicity, sensitisation and neurodegenerative diseases
Cell viability/cytotoxicity	Cytotox CALUX (10%, 50%)	General toxicity


Selection criteria to select bioassays for implementation and use

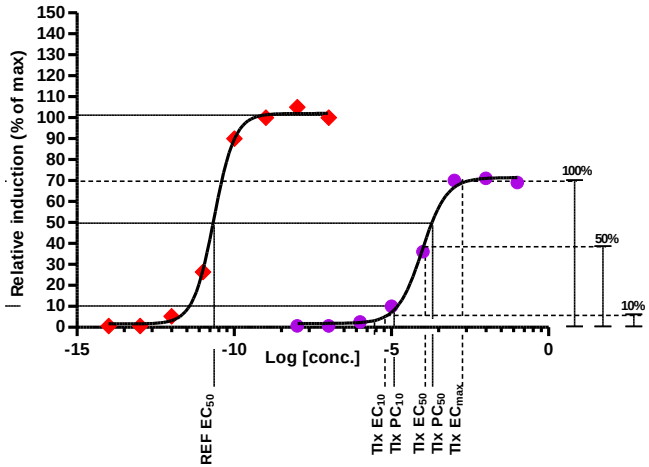
BE-Basic Foundation

FOB.001.04 – D2.1

The research leading to these results was partly funded by the BE-Basic foundation, which was granted a FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I)

WFD priority compound screening

Toxicity pathway	
Xenobiotic metabolism	DR CALUX PXR CALUX
Lipid metabolism	PPARα CALUX PPARγ CALUX LXR CALUX
Hormone-mediated MoA	ERα CALUX anti-ERα CALUX ERβ CALUX AR CALUX anti-AR CALUX PR CALUX anti-PR CALUX GR CALUX anti-GR CALUX RAR CALUX
Reactive MoA/genotoxicity	P53 CALUX (-S9) P53 CALUX (+S9) P21 CALUX
Cell-signalling pathway	Hif1a CALUX TCF CALUX
Adaptive stress response	Nrf2 CALUX AP1 CALUX NFκB CALUX ESRE CALUX
Cell viability/cytotoxicity	Cytotox CALUX (10%, 50%)



66 COMPOUNDS

HTP screening
(25 CALUX assays)

PC10/PC20
based data set

WFD priority compound screening

Priority hazardous

- Anthracene
- Benzo(b)fluoranthene
- Benzo(k)fluoranthene
- benzo[a]pyrene
- C10-13-chloroalkanes
- Cadmium chloride
- endosulfan
- Hexachlorobenzene
- Hexachlorobutadiene
- Hexachlorocyclohexane
- Indeno(1,2,3-cd)pyrene
- Mercuric chloride
- methylmercury(II)chloride
- Nonylphenol
- PBDE 100
- PBDE 47
- Pentachlorobenzene
- Tributyltin-hydride

Priority

- 1,2-Dichloroethane
- 4-n-octylphenol
- 4-tert-octylphenol
- Alachlor
- Atrazine
- Benzene
- Chlorfenvinphos
- Chloroform
- Chlorpyrifos-ethyl
- DEHP
- Dichloromethane
- Diuron
- Fluoranthene
- Isoproturon
- Lead chloride
- Naphthalene
- Nickel(II)chloride
- Pentachlorophenol
- Simazine
- Trichlorobenzenes
- Trifluralin

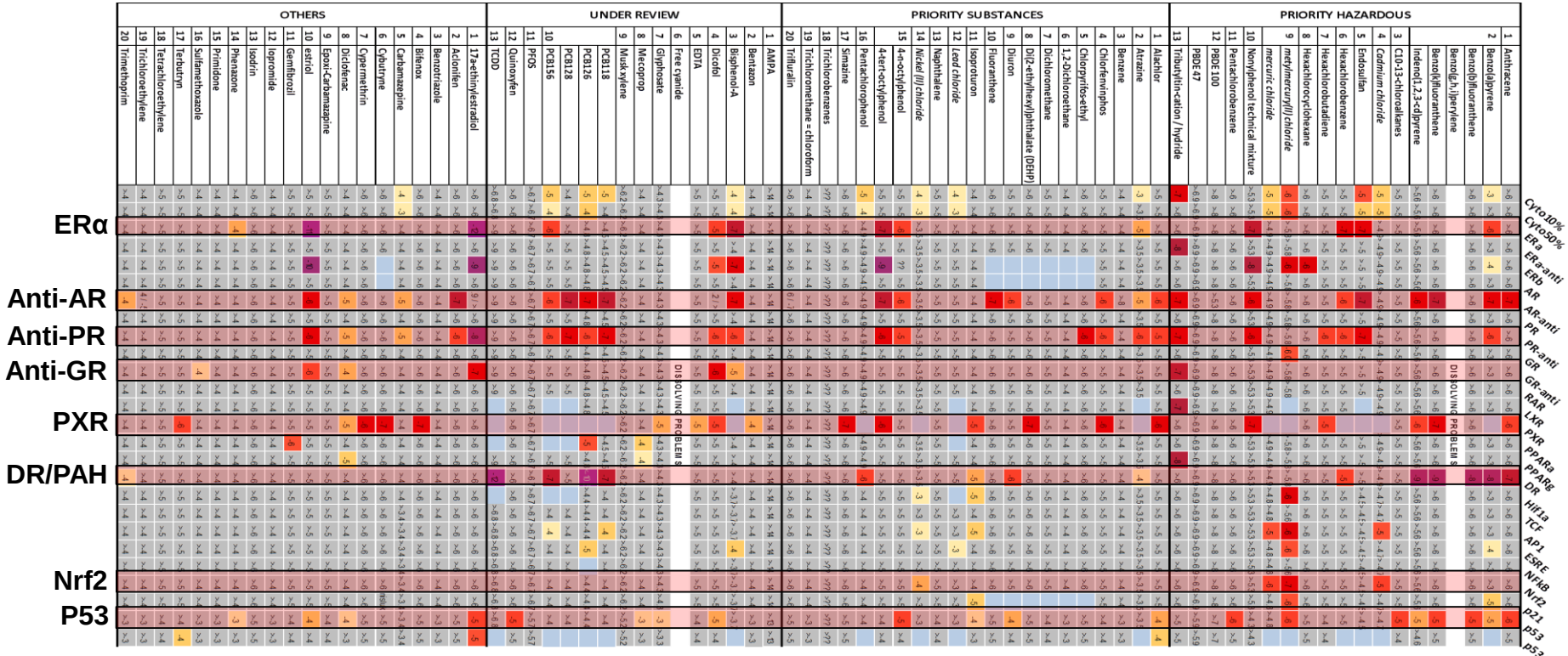
Under review

- AMPA
- Bentazon
- Bisphenol A
- Dicofol
- EDTA
- Glyphosate
- Mecoprop
- Musk xylene
- PCB118
- PCB126
- PCB128
- PCB156
- PFOS
- Quinoxyfen
- TCDD

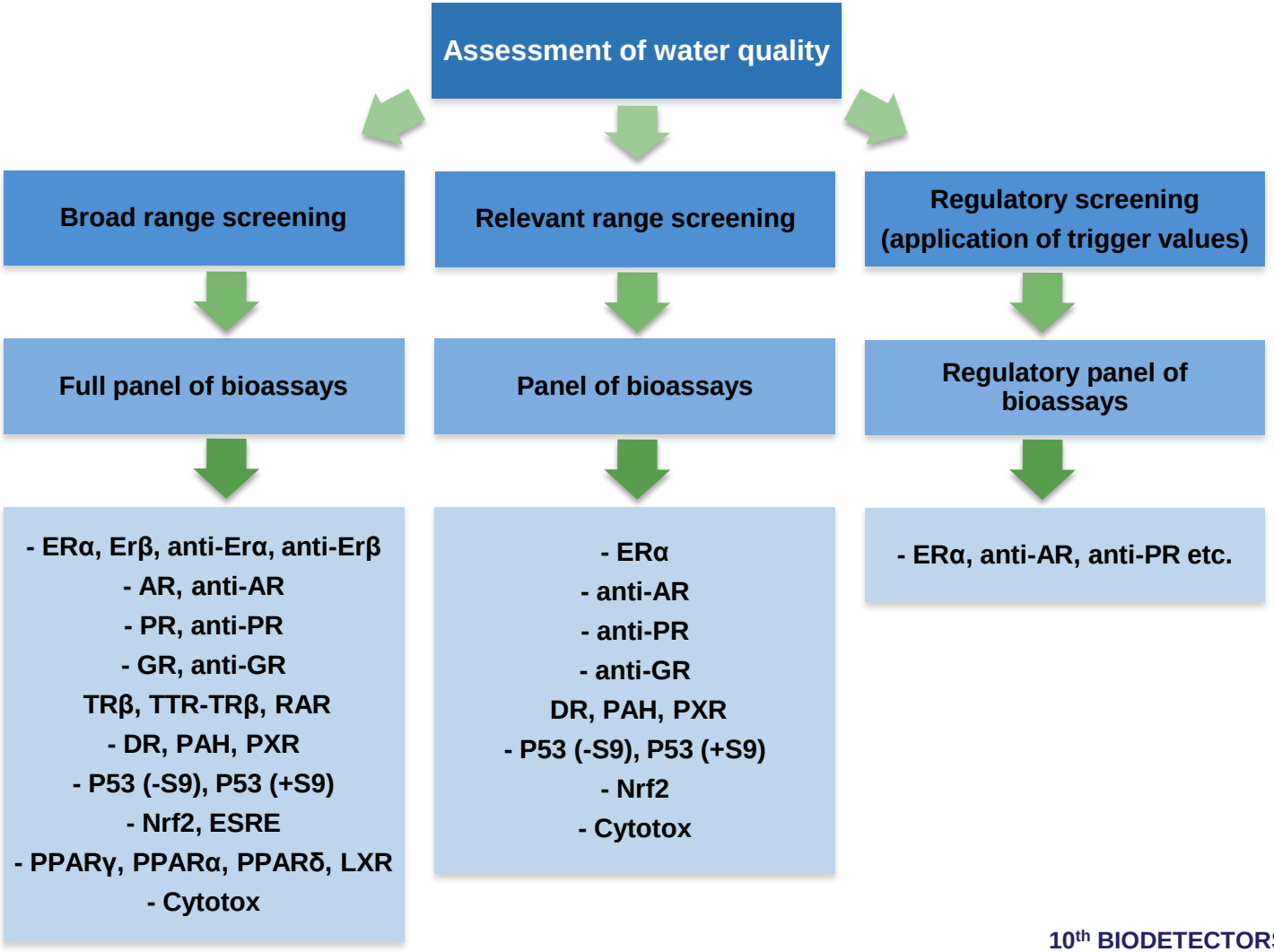
Others

- 17a-ethinylestradiol
- Aclonifen
- Aldrin
- Benzotriazole
- Bifenox
- Carbamazapine epoxide
- Carbamazepine
- Cypermethrin
- Dichlorvos
- Diclofenac
- Dieldrin
- Endrin
- Estradiol
- Gemfibrozil
- Heptachlor
- Iopromide
- Irgarol (cybutryne)
- Isodrin
- p,p'-DDT
- Phenazone
- Primidone
- Sulfamethoxazole
- Terbutryn
- Tetrachloroethylene
- Trichloroethylene
- Trimethoprim

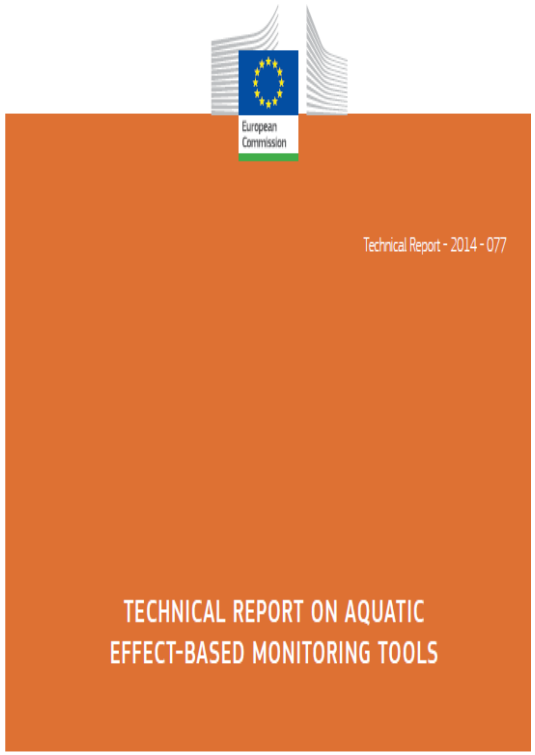
WFD priority compound screening



Selection of bioassay panel



EU technical report (2014): Aquatic Effect-based Tools



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

Development of protocols for (waste)water analysis

BioDetection Systems

Number of Pages: 50

Standard Operating Procedure

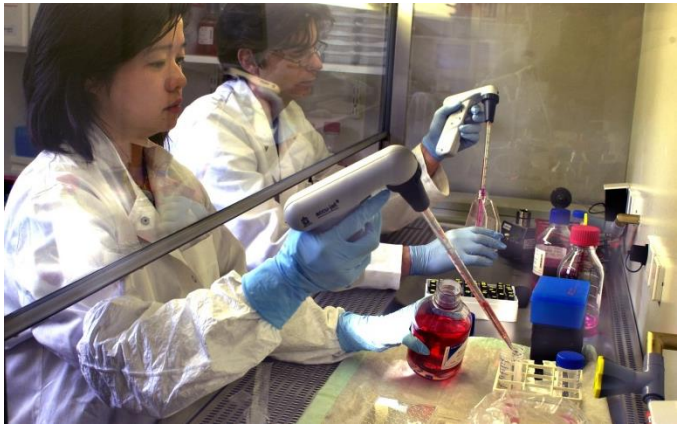
Transactivation assay for the detection of compounds with (anti)estrogenic potential using ERα CALUX® cells

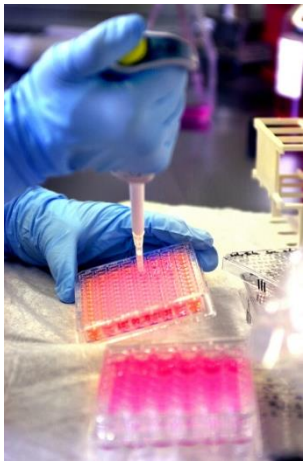
Compiled by: Harrie Besselink
Amended by: Harrie Besselink

Version No.: C
Date: 2 June 2014

Replaced version No. B
Date: 4 April 2014

Authorised by:	Approved by:	Supervisor protocol:
(Head of Quality)	(Head of Laboratory)	(Technical expert)
Emiel Felzel	Harrie Besselink	Irene Middelhof





International validation

BioDetection Systems BV

Validation of the (anti-) ERα CALUX bioassay

U2-OS cells Transcriptional ERalpha CALUX- assay for the detection of estrogenic and anti-estrogenic chemicals for inclusion in TG455

final report

Author: Dr.Ir. H.T. Besselink

Reviewed by: Prof. Dr. A. Brouwer
Dr. Z Dang
Dr T Bovee

Reportnr.: OECD ERa CALUX validation – v5

Date: 7 December 2015

OECD/OCDE **455**

Adopted:
29 July 2016

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT UPDATED TG 455: PERFORMANCE-BASED TEST GUIDELINE FOR STABLY TRANSFECTED TRANSACTIVATION *IN VITRO* ASSAYS TO DETECT ESTROGEN RECEPTOR AGONISTS AND ANTAGONISTS

GENERAL INTRODUCTION

Performance-Based Test Guideline

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

Validation of ERα CALUX for inclusion in OECD TG455

Agonism

Test chemical	CAS no.	coding pre-screen	coding comprehensive
Dimethyl sulfoxide (DMSO; solvent)	67-68-5	---	---
17β-estradiol (reference standard)	50-28-2	---	---
Norethynodrel	68-23-5	SPC-1	AEP-1
Bisphenol A	80-05-7	SPC-2	AEP-2
Ketoconazole	65277-42-1	SPC-3	AEP-3
meso-Hexestrol	84-16-2	SPC-4	AEP-4
Coumestrol	479-13-0	SPC-5	AEP-5
4-Cumylphenol	599-64-4	SPC-6	AEP-6
Butylbenzyl phtalate	85-68-7	SPC-7	AEP-7
Genistein	446-72-0	SPC-8	AEP-8
p,p'-methoxychlor	72-43-5	SPC-9	AEP-9
Diethylstilbestrol	56-53-1	SPC-10	AEP-10
Spironolactone	52-01-7	SPC-11	AEP-11
Reserpine	50-55-5	SPC-12	AEP-12
Linuron	330-55-2	SPC-13	AEP-13
Atrazine	1912-24-9	SPC-14	AEP-14
Kaempferol	520-18-3	SPC-15	AEP-15
Corticosterone	50-22-6	SPC-16	AEP-16
19-Nortestosterone	434-22-0	SPC-17	AEP-17
17a-Estradiol	57-91-0	SPC-18	AEP-18
17a-Ethinyl estradiol	57-63-6	SPC-19	AEP-19
4-tert-Octylphenol	140-66-9	SPC-20	AEP-20
Etyl paraben	120-47-8	SPC-21	AEP-21
Kepone	143-50-0	SPC-22	AEP-22

Antagonism

Test chemical	CAS no.	coding pre-screen	coding comprehensive
Dimethyl sulfoxide (DMSO; solvent)	67-68-5	---	---
Tamoxifen (reference standard)	10540-29-1	---	---
Apigenin	520-36-5	SPC-51	AEP-51
17a-Ethinyl-estradiol	57-63-6	SPC-52	AEP-52
Genistein	446-72-0	SPC-53	AEP-53
Flutamide	13311-84-7	SPC-54	AEP-54
Coumestrol	479-13-0	SPC-55	AEP-55
Raloxifen HCl	82640-04-8	SPC-56	AEP-56
Resveratrol	501-36-0	SPC-57	AEP-57
Tamoxifen	10540-29-1	SPC-58	AEP-58
4OH-tamoxifen	68047-06-3	SPC-59	AEP-59
Chrysin	480-40-0	SPC-60	AEP-60
Kaempferol	520-18-3	SPC-61	AEP-61

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
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	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
F		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
G		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
C		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
D		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
E		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
F		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
G		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
H												

Plate 1

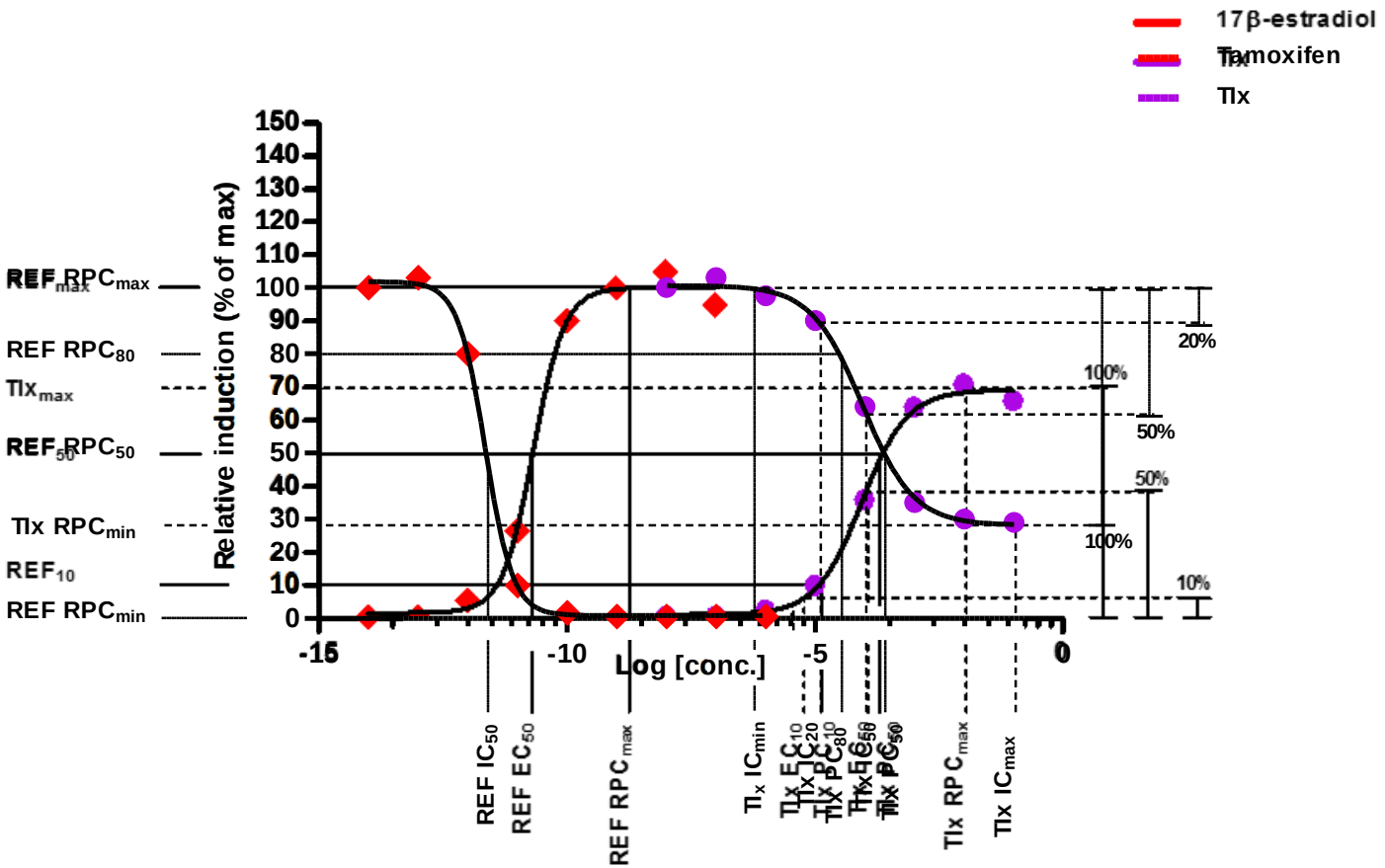
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
C		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
D		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
E		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
F		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
G		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
H												

Subsequent plates

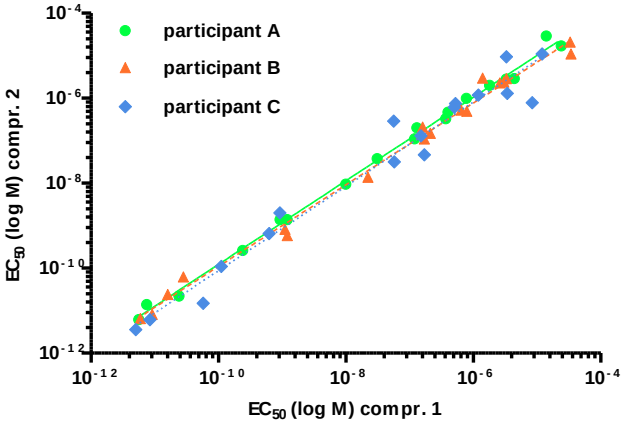
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
C		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
D		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
E		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
F		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
G		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
H												

Validation of ERα CALUX for inclusion in OECD TG455

Agonism



OECD TG455 - results



Agonism	%CV
Log[EC_{50}]	3.4
Log[PC_{10}]	3.0

Antagonism	%CV
Log[IC_{50}]	1.5
Log[PC_{80}]	2.8

Agonism	$\%VC_R$
Log[EC_{50}]	2.5
Log[PC_{10}]	2.3

Antagonism	$\%VC_R$
Log[IC_{50}]	1.3
Log[PC_{80}]	1.6

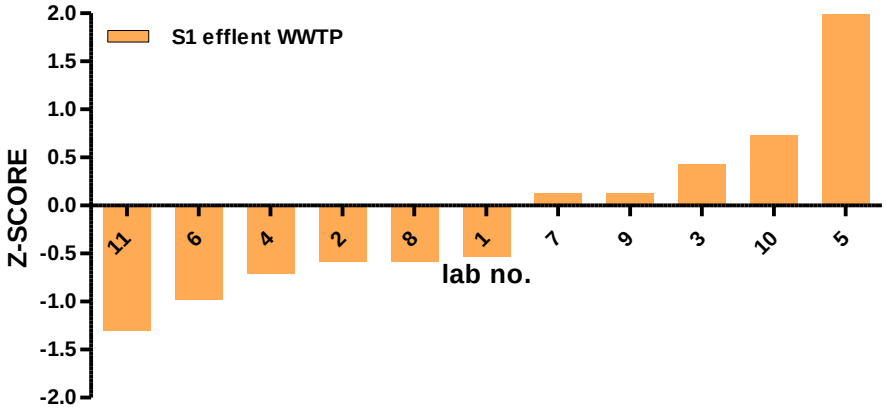
Concordance of classification (ERα CALUX vs ICCVAM)

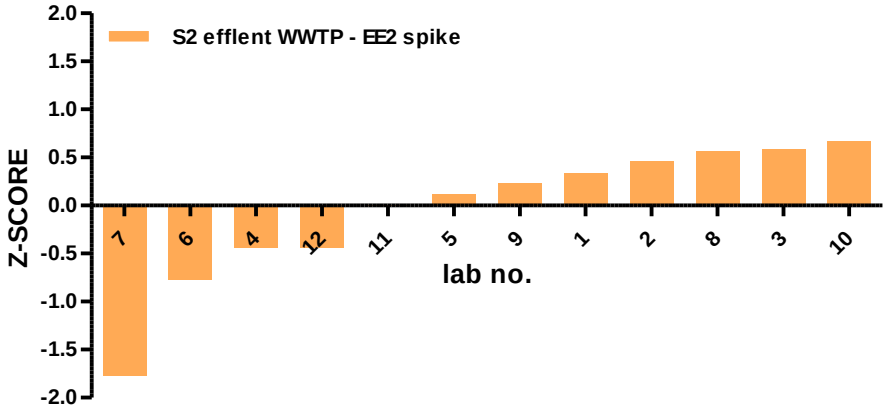
Agonism	Lab A	Lab B	Lab D
Overall accuracy (%)	96	96	100
Sensitivity (%)	100	100	100
Specificity (%)	83	83	100
False positive (%)	6	6	0
False negative (%)	0	0	0
Positive predictivity (%)	94	94	100
Negative predictivity (%)	100	100	100

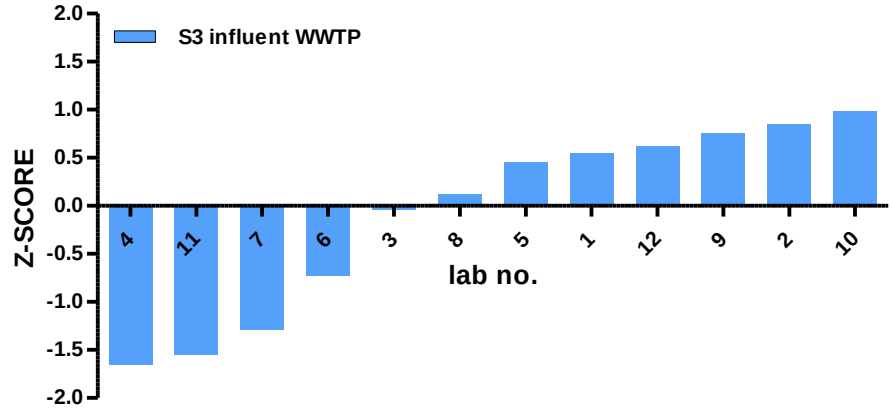
Antagonism	Lab A	Lab B	Lab D
Overall accuracy (%)	100	100	100
Sensitivity (%)	100	100	100
Specificity (%)	100	100	100
False positive (%)	0	0	0
False negative (%)	0	0	0
Positive predictivity (%)	100	100	100
Negative predictivity (%)	100	100	100

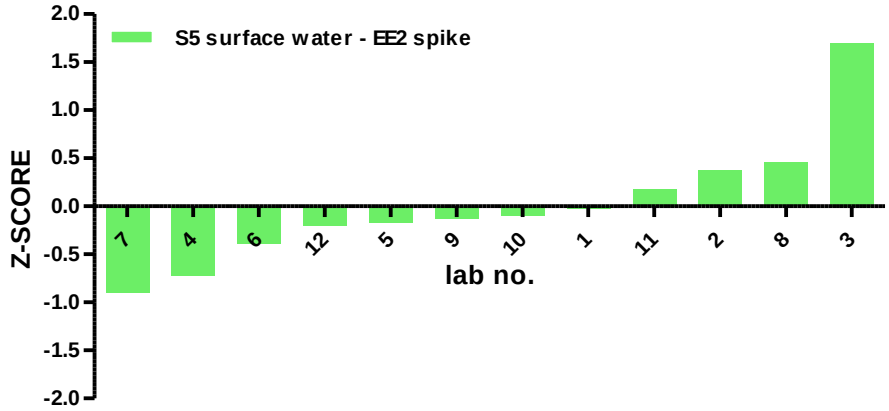
ISO/DIN 19040 - results

12 CALUX labs & 6 countries & 6 water samples









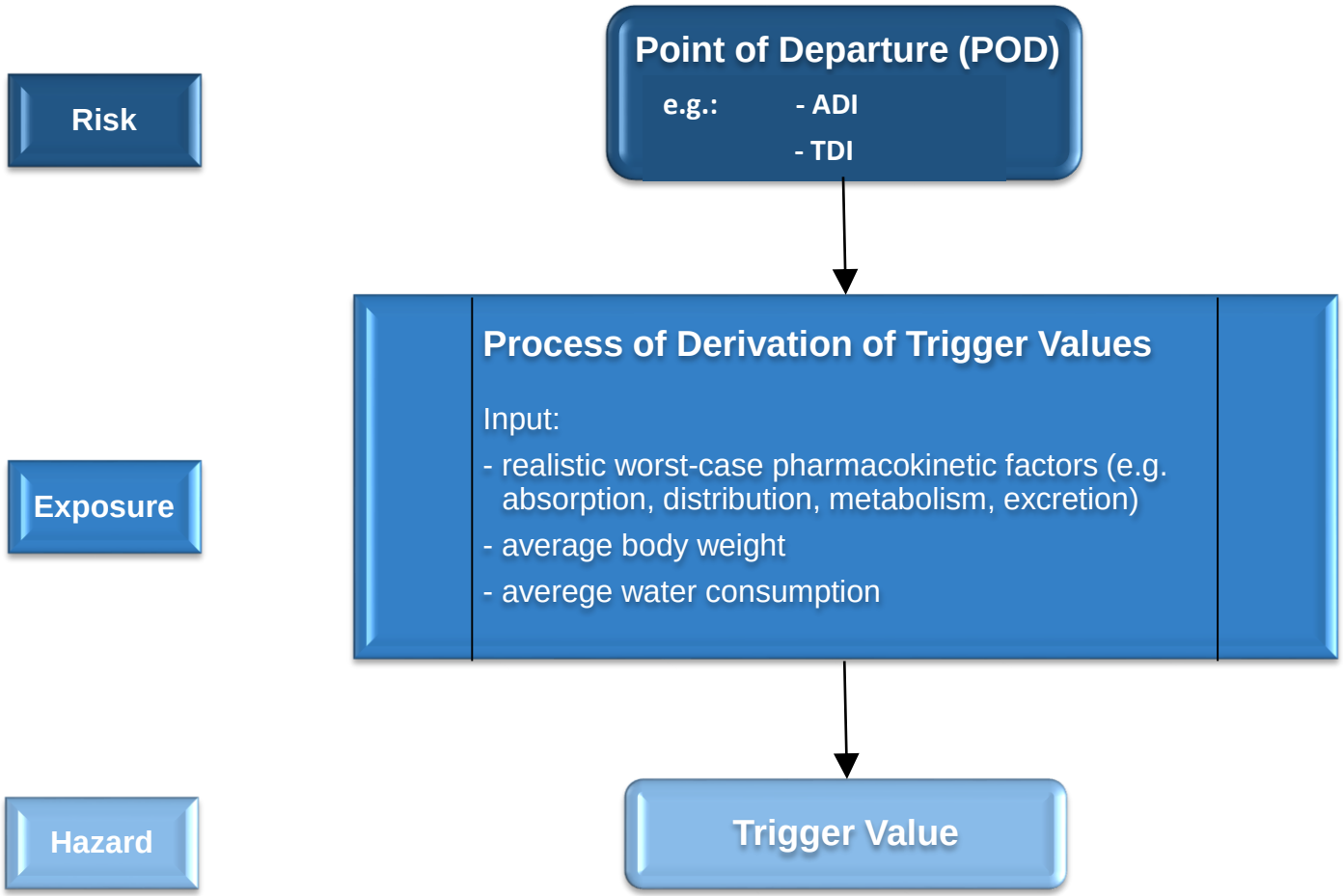
International validation

ERα CALUX bioassay according to OECD guidelines	
Purpose	To include the ERα CALUX bioassay in the OECD PBTG TG455[1]
Lead	BioDetection Systems BV, Amsterdam, the Netherlands
Study design	- 3 participating laboratories - Intra- and interlaboratory validation - Agonism and antagonism - 23 (agonism) and 12 (antagonism recommended test chemicals - Review by OECD VMG-NA
Status	Completed

AR CALUX bioassay according to OECD guidelines	
Purpose	To include the AR CALUX bioassay in a new OECD PBTG
Lead	EURL ECVAM, EU Joint Research Center (JRC), Ispra, Italy
Study design	- 3 participating laboratories (EU-NETVAL) - Intra- and interlaboratory validation - Agonism and antagonism - 30-50 (agonism) and 30-50 (antagonism) recommended test chemicals - Review by EURL ECVAM and OECD VMG-NA
Status	Ongoing

ERα CALUX bioassay according to ISO 19040-3 guideline	
Purpose	To include the ERα CALUX bioassay in ISO 19040-3 guideline[2]
Lead	DIN ISO Working Group Genotoxicity and Hormones
Study design	- 12 participating laboratories (EU-NETVAL) - Intra- and interlaboratory validation - E1, E2, EE2 - 6 water samples; direct exposure - Review by TOXRAT Solutions GmbH, Alsdorf, Germany
Status	Completed

Flow diagram for deriving bioassay trigger values (1)



Effect-based trigger values

Brand, W., de Jongh, C.M., van der Linden, S.C., Mennes, W., Puijker, L.M., van Leeuwen, C.J., van Wezel, A.P., Schriks, M., Heringa, M.B.

Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays; *Environm. Intern. 55 (2013) 109-118*

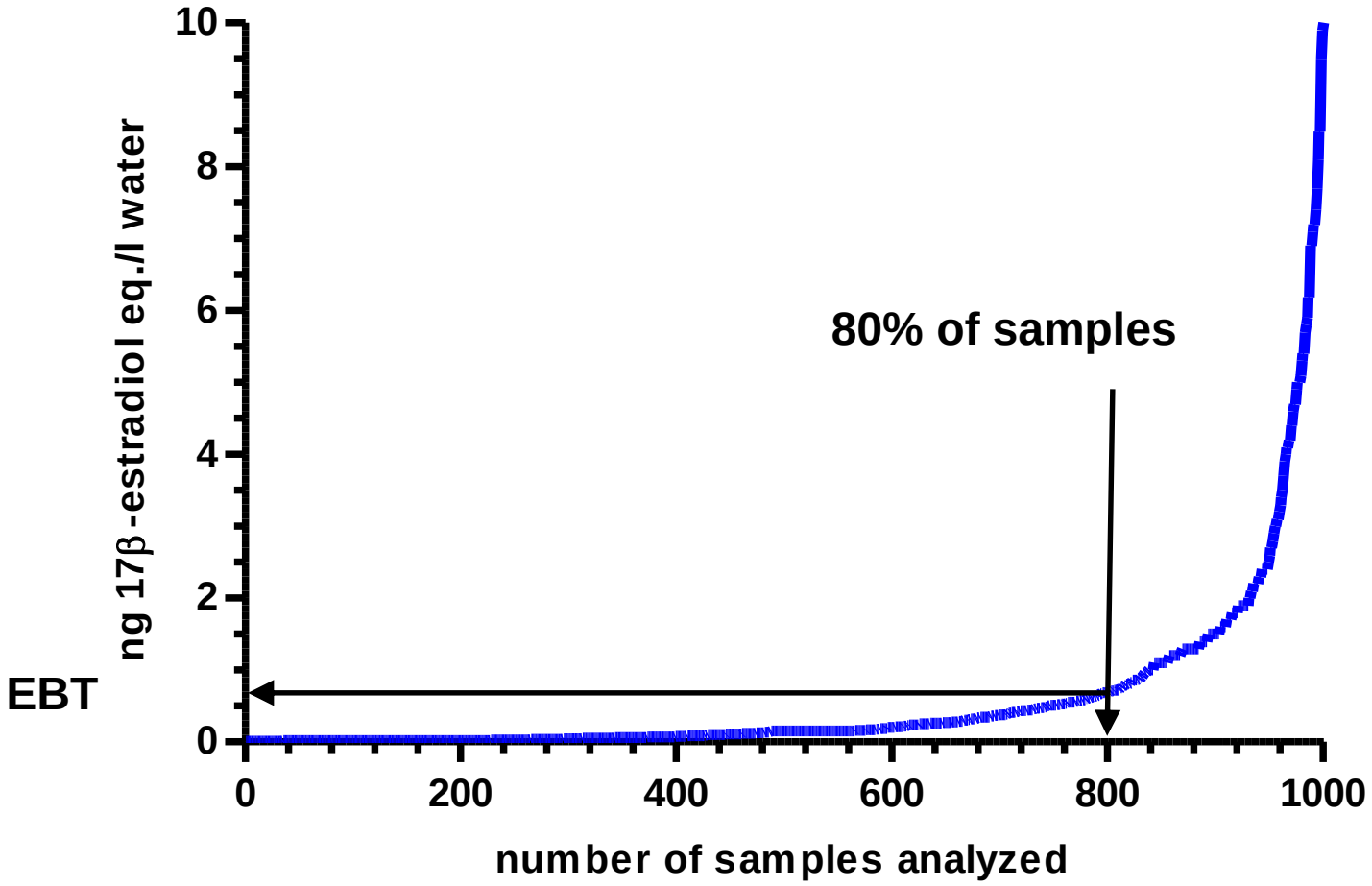
Van der Oost, R., Sileno, G., Suáres Muñoz, M., Janse, T., Nguyen, M., Besselink, H., Brouwer, A.

SIMONI (Smart Integrated Monitoring) as a novel bioanalytical strategy for water quality assessment

Part I: model design and effect-based trigger values; *Environm. Toxicol. Chem.*, accepted

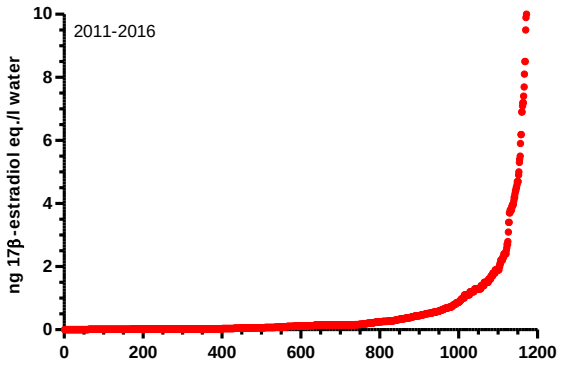
Part II. Field feasibility survey; *Environm. Toxicol. Chem.*, submitted

Deriving bioassay trigger values - practical approach

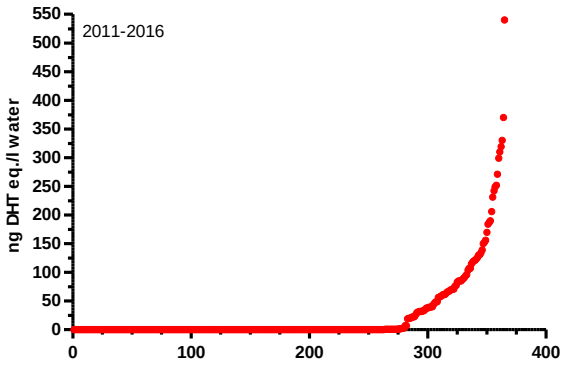


Deriving bioassay trigger values - practical approach

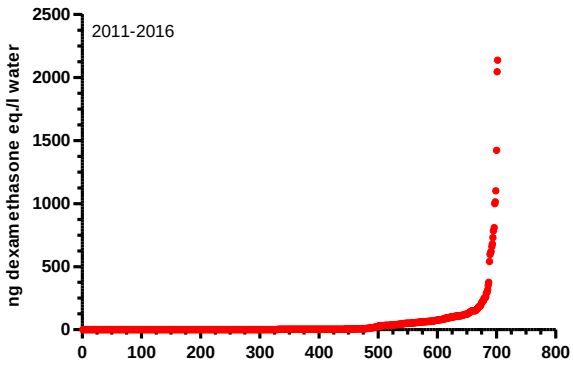
ERα CALUX (water) :Freq. dist. (histogram)



AR CALUX (water) :Freq. dist. (histogram)



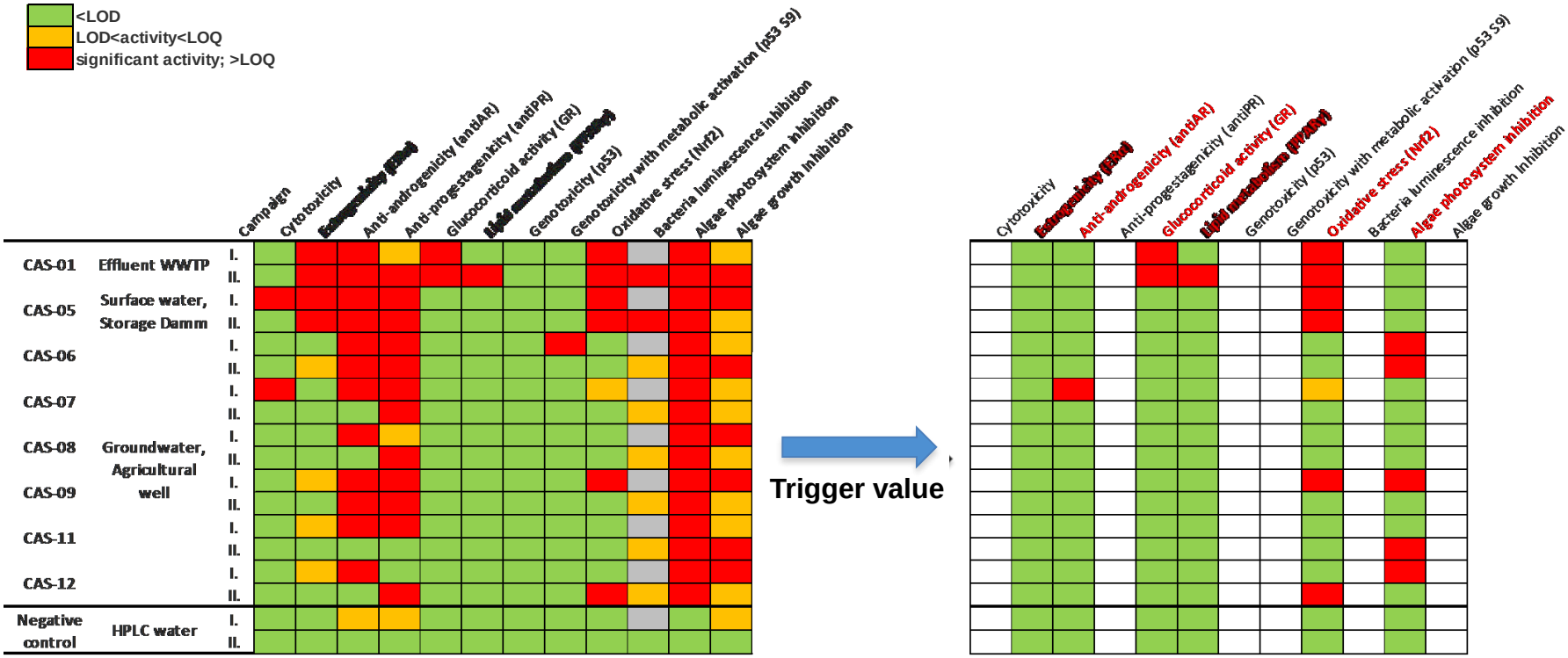
GR CALUX (water) :Freq. dist. (histogram)



	Brand	Van der Oost	BDS	Escher
Estrogens (ng 17β-estradiol eq./l water)	3.8	1.0	1.1	3.1
Androgens (ng DHT eq./l water)	11	---	32	14
Glucocorticoids (ng Dex. Eq./l water)	21	30	56	150

Brand et al. (2013) Environmental International, 55:109-118
Van der Oost et al. (2017) Environmental Toxicology and Chemistry, submitted
Escher et al (2015) Water Research 81:137-148

Application of trigger values

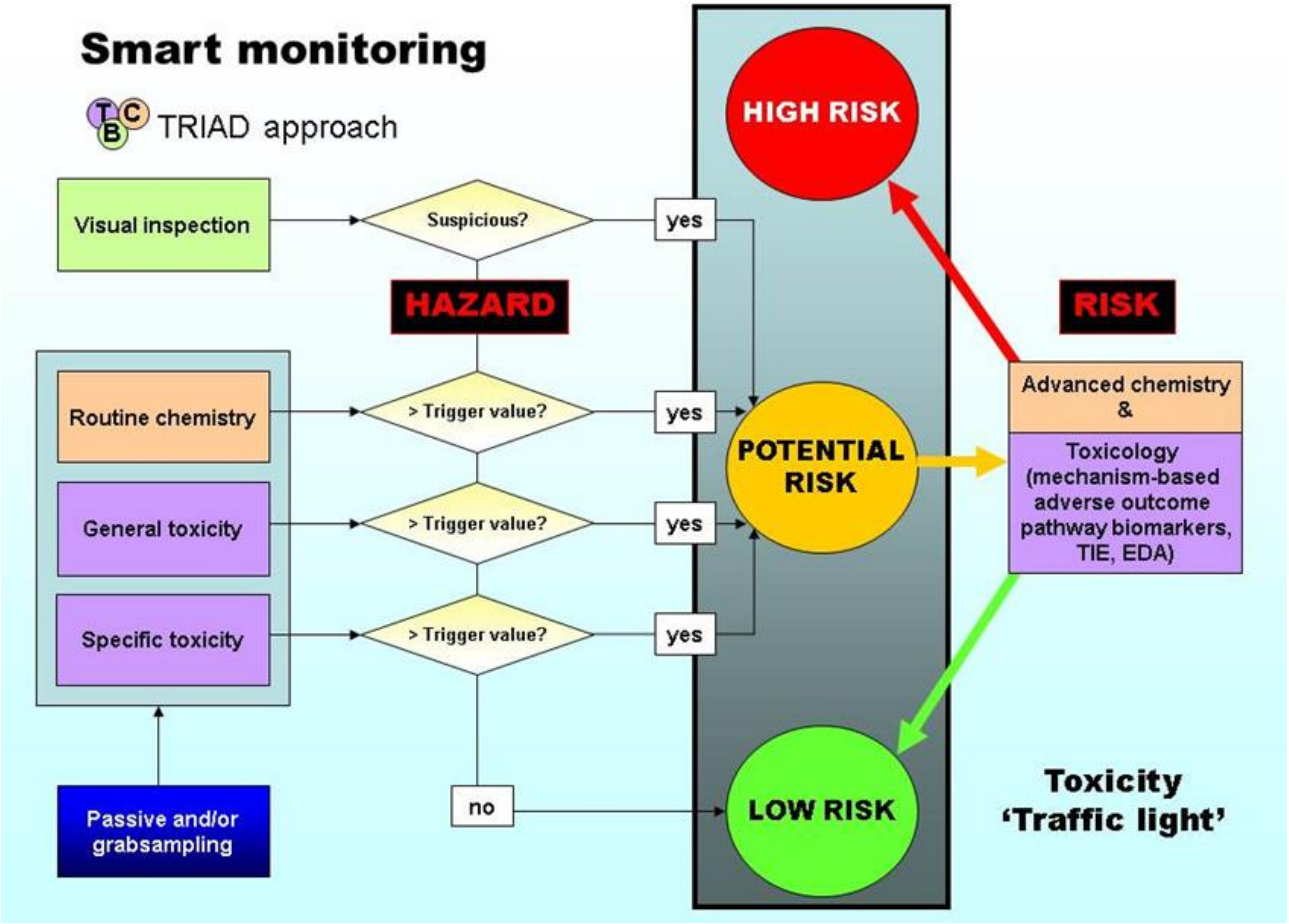


collected at two time points: **06/2014 (Campaign I)**
04/2015 (Campaign II)

Change conventional monitoring practice

Analytical chemistry alone might not cover the entire load of water pollution

Multidisciplinary quality assessment



Ron van der Oost, Waternet, NL

Development of minimum quality requirements at EU level for water reuse in agricultural irrigation and aquifer recharge

Insertion of additional minimum quality requirements for:

- Contaminants of emerging concern (CECs)
- Bioassays (ERa, AR, GR, PR CALUX)

Discussion about application of bioassays in water quality assessment

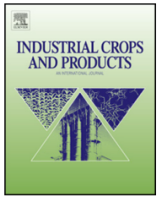
- **SOPs for sampling**
- **SOPs for sample processing**
- **Proficiency tests**
- **Trigger valus**

Summary: Validation and regulatory acceptance

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop



Validation and regulatory acceptance of bio-based approaches to assure feedstock, water & product quality in a bio-based economy

Harrie Besselink*, Bram Brouwer, Bart van der Burg

Integration of bioassays in regulatory frameworks for safety evaluation of chemicals, products, waste streams and the environment

- development/selection bioassays
  - formal validation
    - establishment of effect-based trigger values
      - inclusion in regulatory frameworks for risk assessment

HTPS water monitoring – full CALUX panel screening

EU Horizon 2020; grand agreement no, 689450

Demonstrating synergies in combined natural and engineered processes for water treatment systems

AquaNES - *Demonstrating synergies in combined natural and engineered processes for water treatment systems*

Partners & demonstration sites

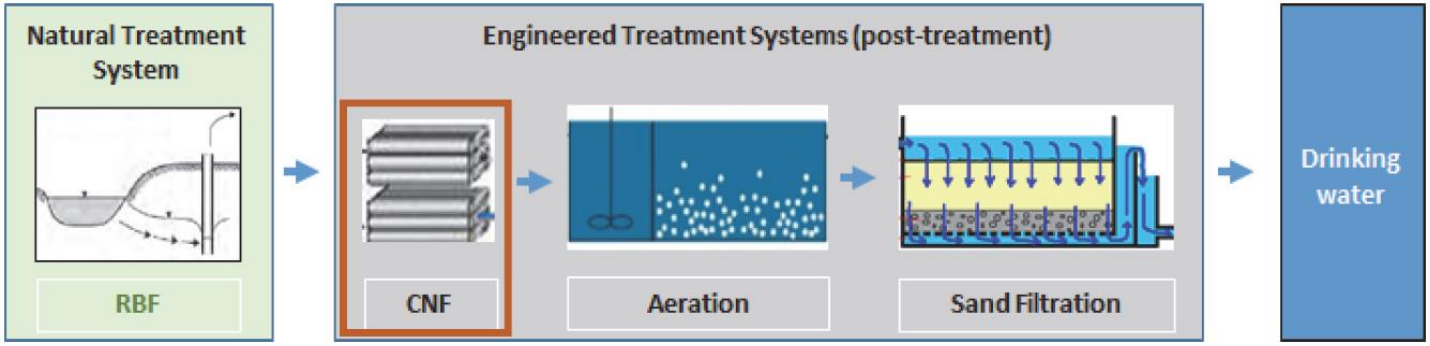
River Bank Filtration schemes for the production of drinking water
1 - Havel River, Berlin, Germany
2 - Elbe River, Dresden, Germany
3 - Danube River, Budapest, Hungary
4 - Warta River, Poznan, Poland
5 - Ganga River, Haridwar, India

Managed Aquifer Recharge & Soil Aquifer Treatment schemes for water storage & quality improvement
6 - Lange Erlen, Basel, Switzerland
7 - Shafdan WWTP, Tel Aviv, Israel
8 - Waddinxveen, Rotterdam, the Netherlands
9 - Agon-Coutainville, France

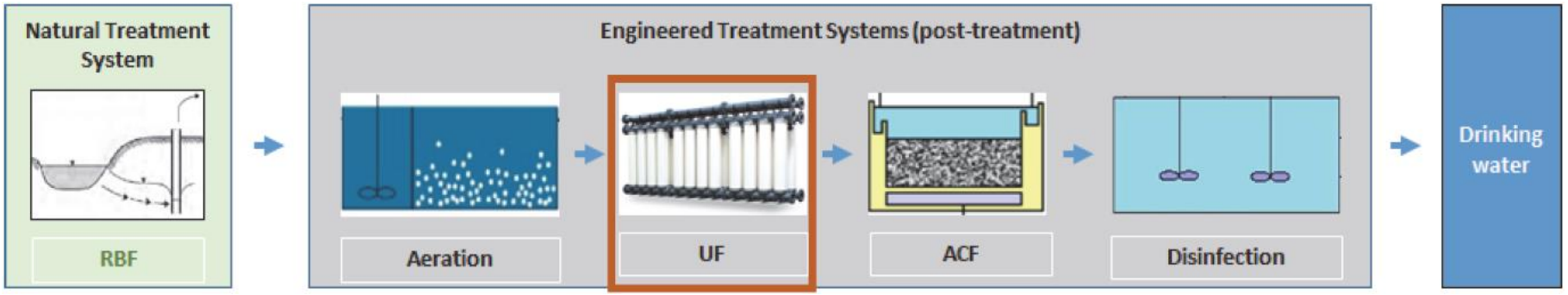
Constructed wetlands and other natural systems for improved wastewater treatment
10 - Thirasia and Antiparos Islands, Greece
11 - Erftverband, Germany
12 - Berlin, Germany
13 - Packington, UK

AquaNES - *Demonstrating synergies in combined natural and engineered processes for water treatment systems*

Demonstration site 1



Demonstration site 2

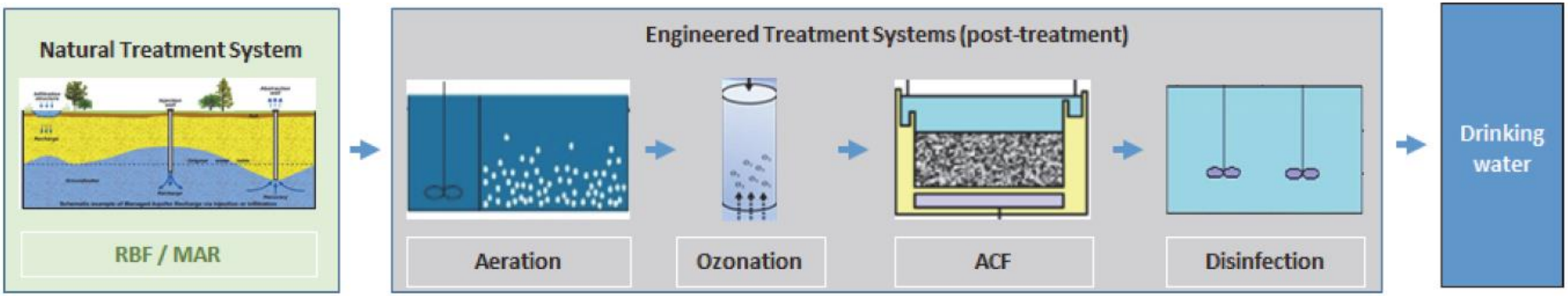


RBF - River Bank Filtration
CNF - Capillary Nanofiltration
UF - UltraFiltration
ACF - Activated Carbon Filtration

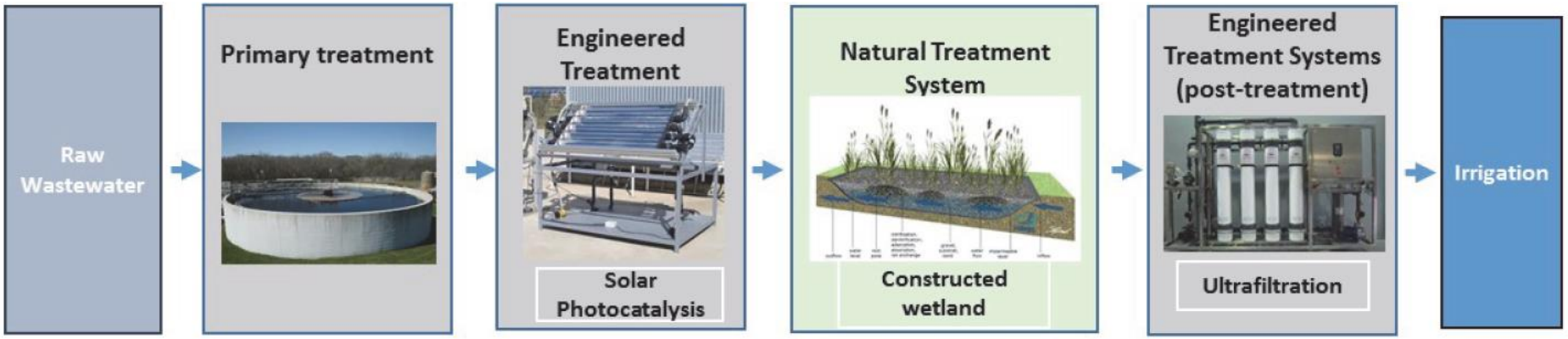
EU Horizon 2020; grand agreement no, 689450

AquaNES - *Demonstrating synergies in combined natural and engineered processes for water treatment systems*

Demonstration site 3



Demonstration site 4



MAR - **Managed Aquifer Recharge**
ACF - **Activated Carbon Filtration**

EU Horizon 2020; grand agreement no, 689450

AquaNES - *Demonstrating synergies in combined natural and engineered processes for water treatment systems*

Innovations in water and wastewater treatment processes and management through improved combinations of natural and engineered components.

Phase I

- **13 participating WWTPs**
- **Influent - effluent**
- **Full panel of CALUX bioassays**
- **Start: November 2016**
  End: Oktober 2017

Phase II

- **6 WWTPs selected**
- **Multiple samples to evaluate efficiency natural/engineered treatment processes**
- **Selection of 10 most relevant CALUX bioassays**
- **Start: December 2017**
  End: Oktober 2019

EU Horizon 2020; grand agreement no, 689450

Demo Case – Plastics additives

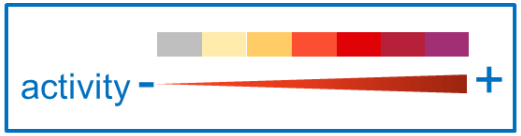
- **Plastic migration: endocrine disrupting compounds leaching from plastic packaging into food. Topic of public concern!**

Material	Special remarks	Estrogen Equivalents (mg/kg food simulant)
PET	Hard, food grade	<LOD
PET	BPA/phthalate-free; pink	5.1E-8
PET	BPA/phthalate-free; blue	8.2E-9
PET	Microwave/freezer-safe	<LOD
HD-polyethylene	Suitable as FCM	7.3E-7
LD-polyethylene	Suitable as FCM	1.8E-6
PVC	Non-food grade	<LOD
Polypropylene	Hot use	<LOD
Polypropylene	Cold use	<LOD
PolyLacticAcid	BioBased	<LOD

- Method set-up according to guidelines EC10/2011
- Leachates measured in CALUX assays
- Estrogen activity detected in several samples

CALUX toxic profile of plastic additives

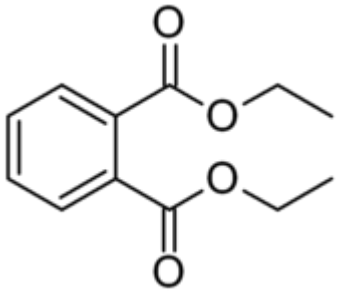
compound	Cytotox10%	ERa	ERa+S9	ERa-anti	ERb	ERb-anti	AR	AR-anti	PR	PR-anti	GR	GR-anti	TRb	RAR	LXR	PXR	PPARa	PPARg	DR	PAH	Hif1a	TCF	AP1	ESRE	NFkB	Nrf2	p21	p53 GENTOX	p53 S9 GENTOX
Di(2-ethylhexyl)phthalate		-4.0								-5.2						-6.4													
Di-n-octyl phthalate																													
monoethylhexyl phtalate	-3.5							-4.5									-5.5	-4.7											
diisodecylphthalate		-4.4			-4.2																								
diisononylphthalate																												-3.0	
Dicyclohexylphthalate	-4.5	-5.3								-5.4		-5.1				-6.7													
Diethylphthalate	-3.5	-4.3						-5.0		-4.3																			
Diisobutyl phthalate	-4.0	-5.7						-5.3		-5.5																			
Dibutylphthalate	-4.5	-5.2						-5.5		-5.5																			
Di(n-hexyl)phthalate	-3.5	-5.0						-5.0		-5.5		-4.5							-4.0				-4.2						
Butyl benzyl phthalate	-3.9	-6.4			-4.4			-5.6		-5.5									-3.7										
di(2-ethylhexyl)adipate																													
Benzophenone	-3.5	-5.2						-6.0		-4.8																			
Etyl paraben	-3.0	-5.2			-5.2			-5.0		-4.0																		-3.5	
4-tert-octylphenol	-5.5	-7.2			-8.5			-6.4		-6.1						-6.0													
4-n-octylphenol	-4.7	-6.2						-5.6		-5.3																			
Nonylphenol	-4.9	-5.1			-5.6			-6.5		-5.5														-4.6					
4-Cumylphenol	-4.2	-7.0	-6.4		-7.0			-6.7		-6.1		-4.5																	
p-(tert-pentyl)phenol	-4.0	-7.7						-6.3		-5.9																			
Diphenyl-p-phenylenediamine	-4.0	-5.5						-5.2		-5.4																			
Bisphenol A	-4.0	-7.3			-6.8			-6.8		-5.5		-4.5												-4.3					
Bisphenol A-dimethacrylate		-6.6			-6.5			-6.0		-5.5						-5.3												-4.7	
Bisphenol F		-6.6			-6.7			-5.4		-4.8						-4.3								-4.7				-4.5	-3.3



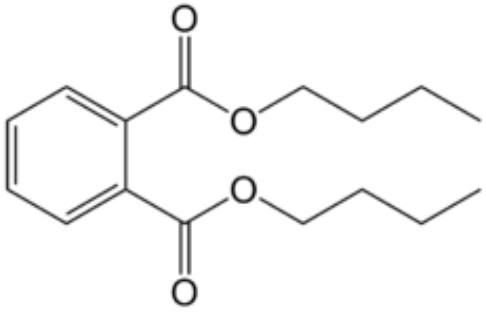
Most additives are active on the endocrine assays:
they act as estrogens, anti-androgens and anti-progestins

Searching for alternatives

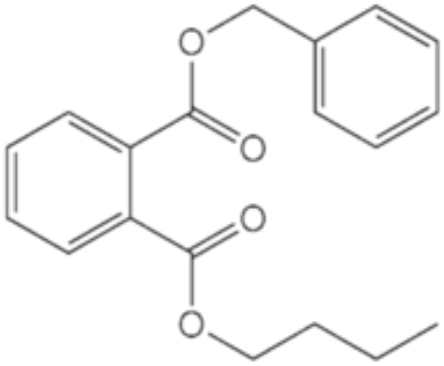
diethylphtalate

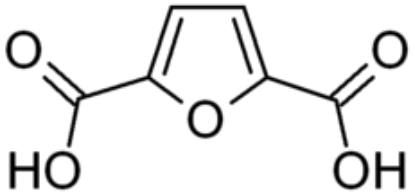


dibutylphtalate



Butyl benzyl phtalate





2,5-furaandicarbonzuur

CALUX toxic profile of plastic additives

compound	Cytotox10%	ERa	ERa+S9	ERa-anti	ERb	ERb-anti	AR	AR-anti	PR	PR-anti	GR	GR-anti	TRb	RAR	LXR	PXR	PPARa	PPARg	DR	PAH	Hif1a	TCF	AP1	ESRE	NFkB	Nrf2	p21	p53 GENTOX	p53 S9 GENTOX
Di(2-ethylhexyl)phthalate		-4.0								-5.2						-6.4													
Di-n-octyl phthalate																													
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diisodecylphthalate		-4.4			-4.2																								
diisononylphthalate																												-3.0	
Dicyclohexylphthalate	-4.5	-5.3								-5.4		-5.1				-6.7													
Diethylphthalate	-3.5	-4.3						-5.0		-4.3																			
Diisobutyl phthalate	-4.0	-5.7						-5.3		-5.5																			
Dibutylphthalate	-4.5	-5.2						-5.5		-5.5																			
Di(n-hexyl)phthalate	-3.5	-5.0						-5.0		-5.5		-4.5							-4.0				-4.2						
Butyl benzyl phthalate	-3.9	-6.4			-4.4			-5.6		-5.5									-3.7										
di(2-ethylhexyl)adipate																													
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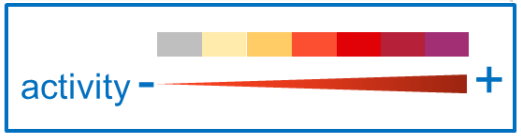
Etyl paraben	-3.0	-5.2			-5.2			-5.0		-4.0																		-3.5	
4-tert-octylphenol	-5.5	-7.2			-8.5			-6.4		-6.1						-6.0													
4-n-octylphenol	-4.7	-6.2						-5.6		-5.3																			
Nonylphenol	-4.9	-5.1			-5.6			-6.5		-5.5														-4.6					
4-Cumylphenol	-4.2	-7.0	-6.4		-7.0			-6.7		-6.1		-4.5																	
p-(tert-pentyl)phenol	-4.0	-7.7						-6.3		-5.9																			
Diphenyl-p-phenylenediamine	-4.0	-5.5						-5.2		-5.4																			
Bisphenol A	-4.0	-7.3			-6.8			-6.8		-5.5		-4.5												-4.3					
Bisphenol A-dimethacrylate		-6.6			-6.5			-6.0		-5.5						-5.3												-4.7	
Bisphenol F		-6.6			-6.7			-5.4		-4.8						-4.3								-4.7				-4.5	-3.3
FDCA																													



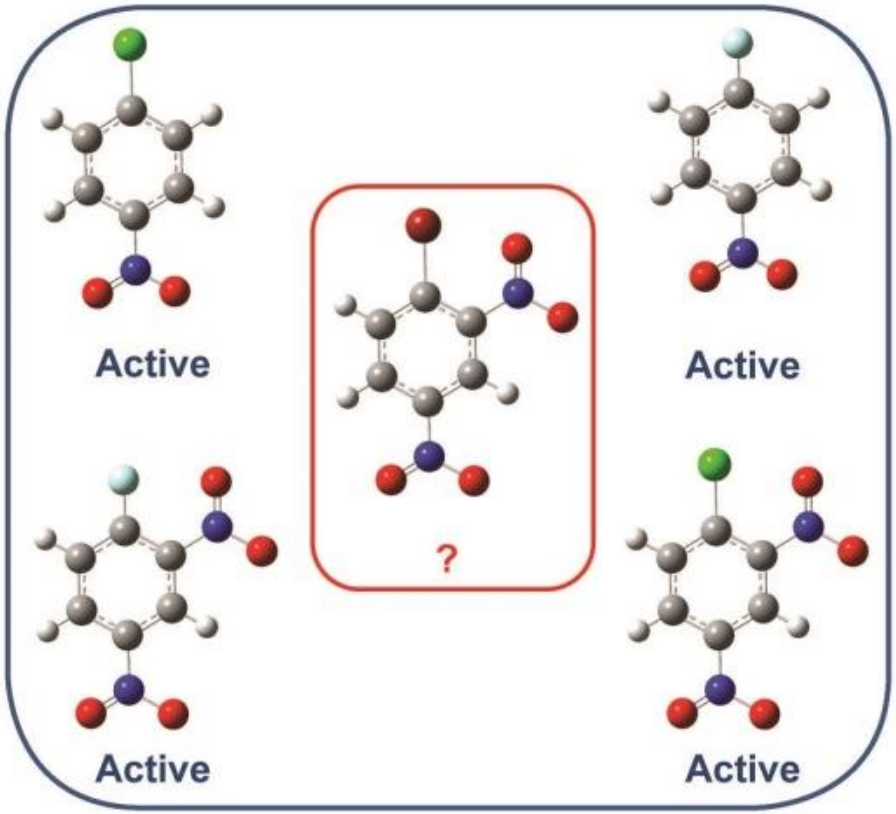
FDCA - little or no endocrine effects

- biobased building block to replace terephthalate in PET

BE-Basic

ChemScreen

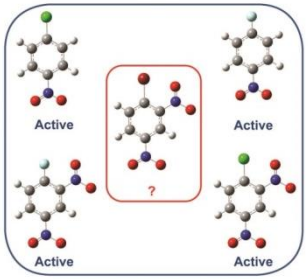
Read across



Read across

CAS	compound	CALUX Cytotox10%	CALUX Cytotox50%	ERalpha	ERalpha-anti	Erbeta	ERbeta-anti	AR	AR-anti	PR	PR-anti	GR	GR-anti	TRb	RAR	LXR	PPARa	PPARg	DR	PAH	Hif1a	AP1	ESRE	Nrf2	p21	p53	CALUX prediction
1118-46-3	Monobutyltinchloride								-4,5															-5			
683-18-1	Dibutyltindichloride	-6,5	-6,3										-7,5					-6,5				-6,7	-6,8	-6,8			
1461-22-9	Tributyltinchloride	-7,5	-7,2										-8,5			-8,7	-8,5	-8,5					-6,9	-7,2			

- ✓ **Examples with 3 chemical classes (Alkyl alkanoic acids, phthalates, organotin chlorides); all three successful**
- ✓ **Read-across used in approx. 30% reproductive tox dossiers (100-1000TPA) in REACH (ECHA 2014)**

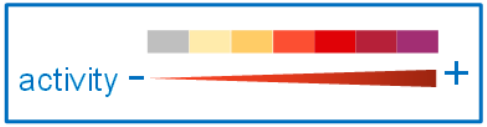


Kroese et al., Reproductive Toxicology

Conventional vs Biobased furan-ester plastic additives

Novel Biobased furan-ester plastic additives

Currently used phthalate-based analogues



compound	Cytotox	ERa	ERb	AR-anti	PR-anti	GR-anti	PPARa	PPARg	DR	AP1	ESRE	Nrf2	p53 GENTOX
Terephthalic acid													
Phthalic acid													
Isophthalic acid													
FDCA													
Dimethyl terephthalic acid		-5.9	-4.5										-4.0
Dimethyl orthopthtalate				-4.7	-3.6								
Dimethyl isophthalate		-3.3		-3.1									
Dimethyl FDCA		-3.7											
Bis(2-ethylhexyl) phthalate		-3.9											
Bis(2-ethylhexyl) furan													
Diisobutyl phthalate		-5.7		-5.6	-5.7								
Diisobutyl furan		-4.3											
Diisodecyl phthalate													
Diisodecyl furan													

The bio-based furan-based compounds are less active compared to their phthalate-based counterparts.

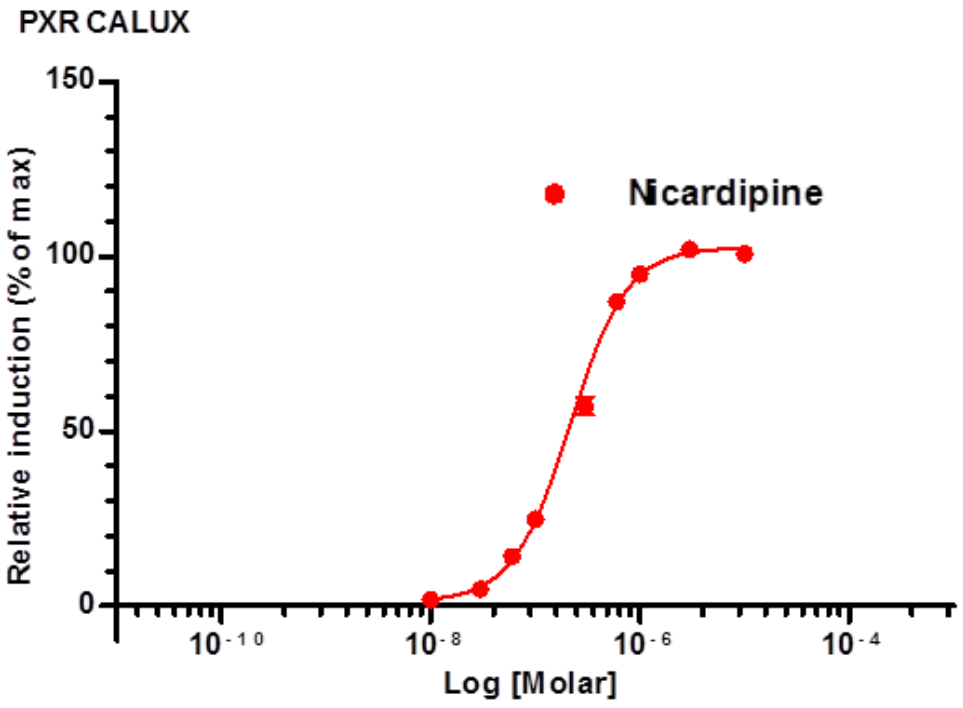
FOOD & BIOBASED RESEARCH WAGENINGEN UR

BE-Basic

ChemScreen

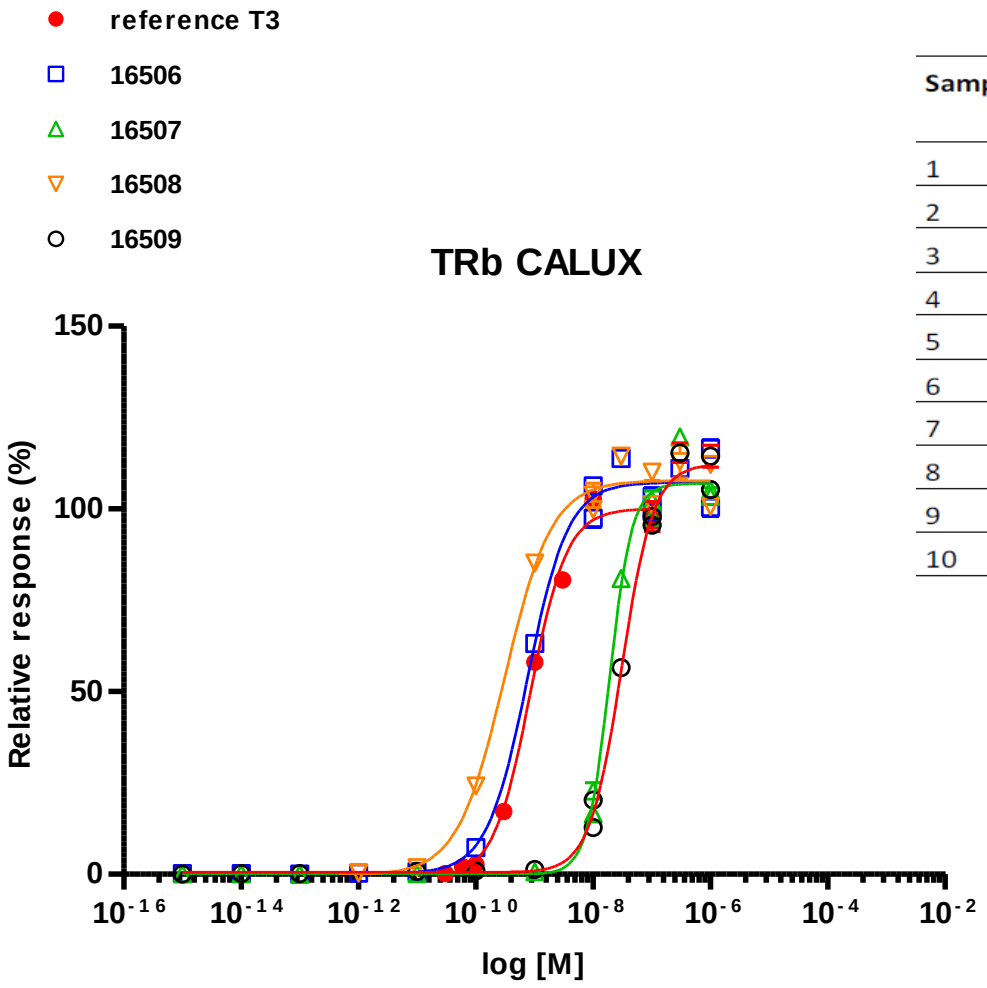
NEW developments: PXR CALUX

Pregnane X receptor (PXR) in human U2OS cell line



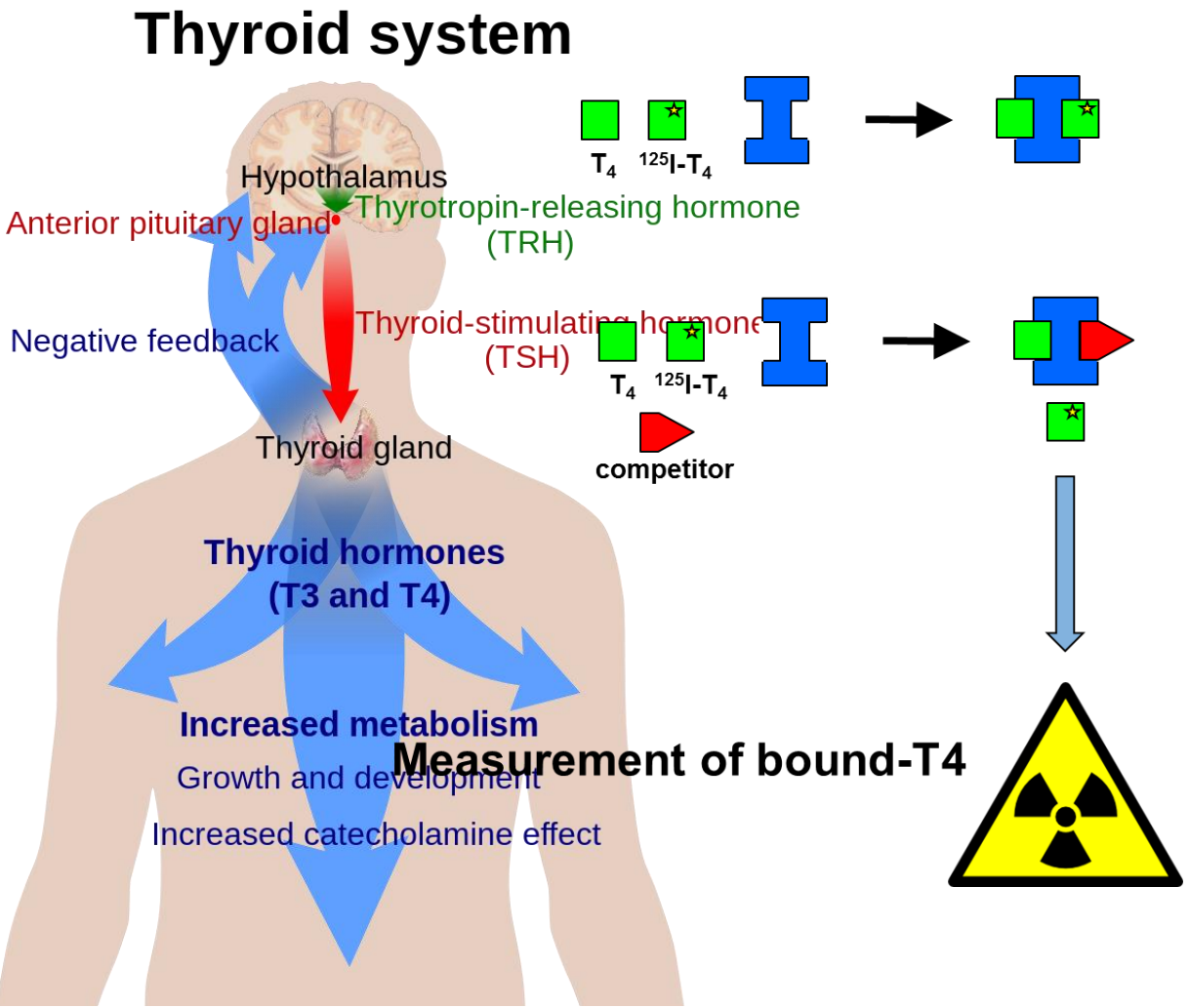
- **Nuclear receptor present in liver and intestine**
- **Senses the presence of many different xenobiotics**
- **Induces Cyp3A4 that hydroxylases those compounds to stimulate their clearance**
- **Ligands: e.g. antibiotics such as rifampicin, rifaximin, drugs like RU486, nicardipine, taxol, hyperforin, many natural- and synthetic steroids, non-coplanar polychlorinated biphenyls (PCBs).**

Thyroid hormone CALUX

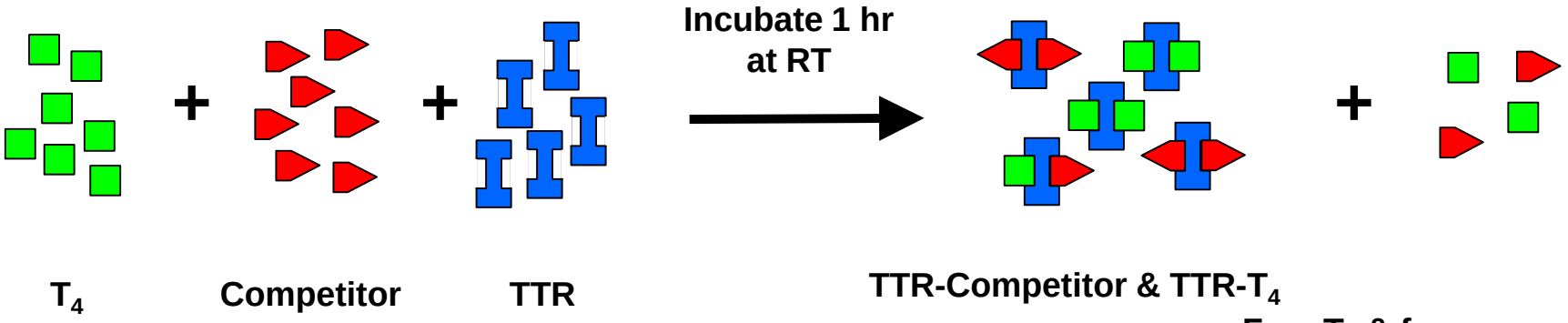


Sample ID*		Compound	CAS
1	16506	Triiiodothyronine (T3)	6893-02-03
2	16507	Thyoxine (T4)	51-48-9
3	16508	TRIAC (T3-like analogue)	51-24-1
4	16509	TETRAC (T4-like analogue)	67-30-1
5	16510	Amiodarone	19774-82-4
6	16511	Pentachlorophenol (PCP)	87-86-5
7	16512	Ethylene thiourea (ETU)	96-45-7
8	16513	2,2,4,4-tetrahydroxybenzophenone	131-55-5
9	16514	Methimazole	60-56-0
10	16515	Solvent only (negative control)	

^{125}I-T_4 – TTR binding assay



T_4 – TTR binding – TRb CALUX assay

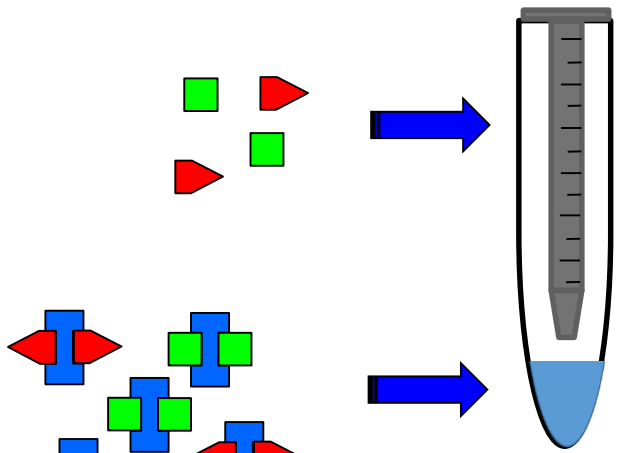


T_4 Competitor TTR

TTR-Competitor & TTR-T_4

Free T_4 & free competitor

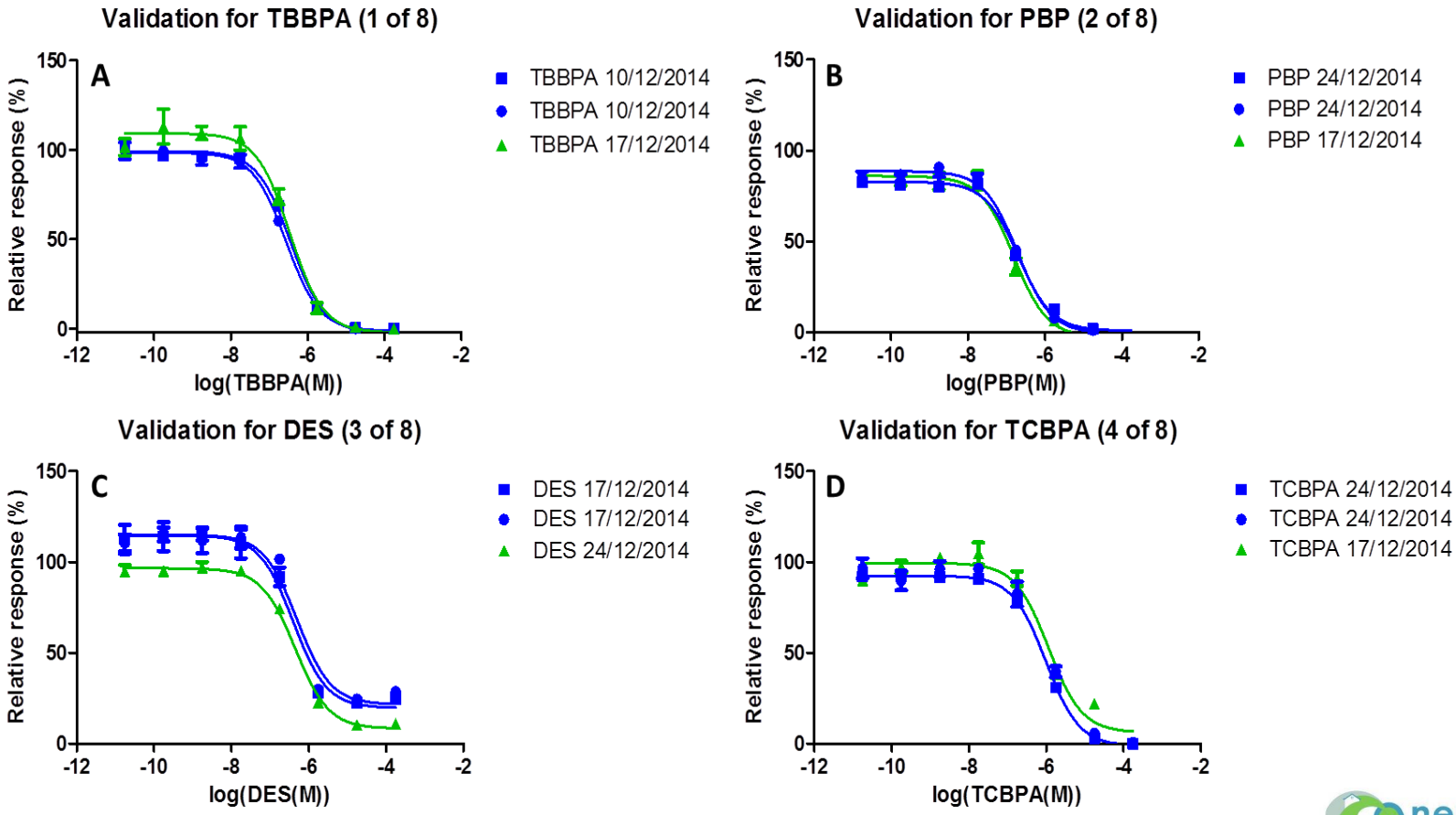
Separation of free and bound hormones



TRβ CALUX incubation (overnight) and subsequent analysis of bound T_4

- 150 µl eluate
- 500 µl exposure medium

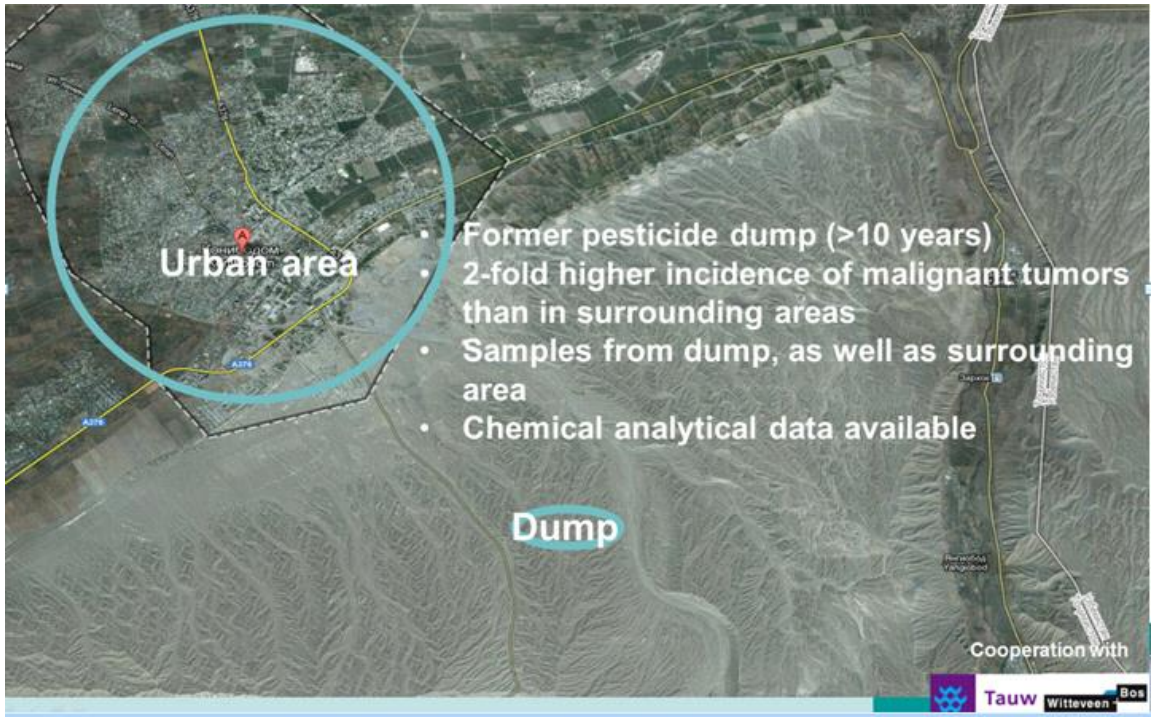
Examples of TTR-binding inhibition by halogenated phenolic compounds



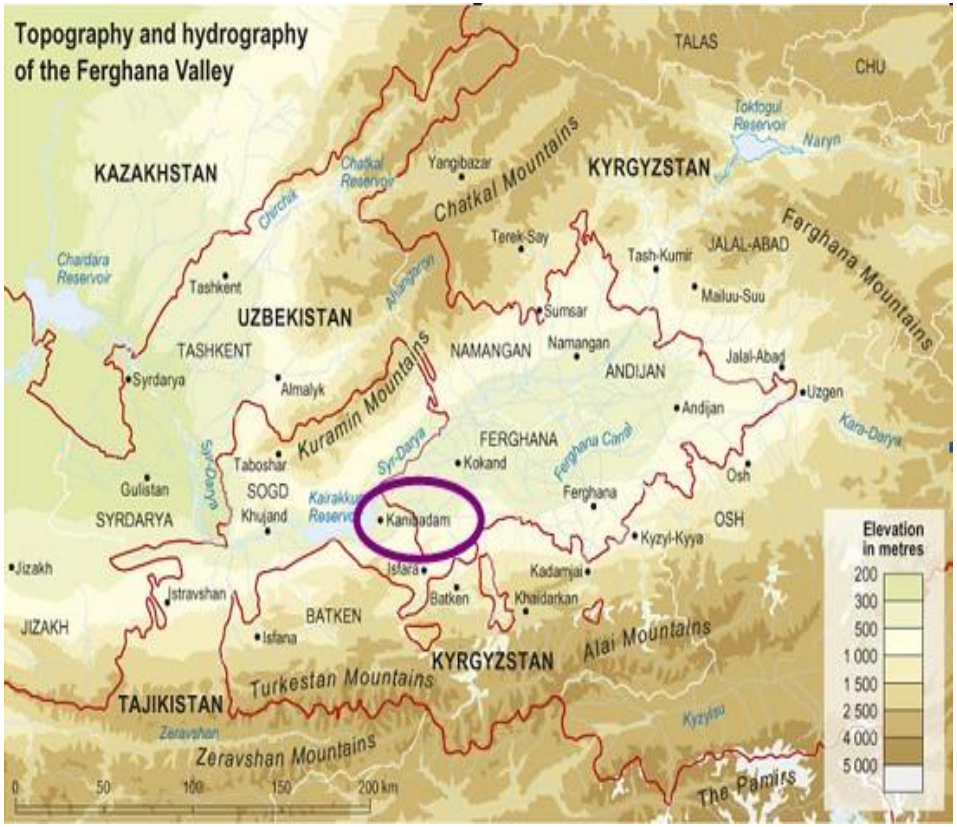
Demo Case Sandy Soil (8.2 dRISK)

- **Mixture of pesticides in sandy soil matrix: former pesticide dump in Tadzhikistan**
- **Common pesticides quantified using chemical analysis**

➡ **Does CALUX activity matches expected, chemically based activity?**

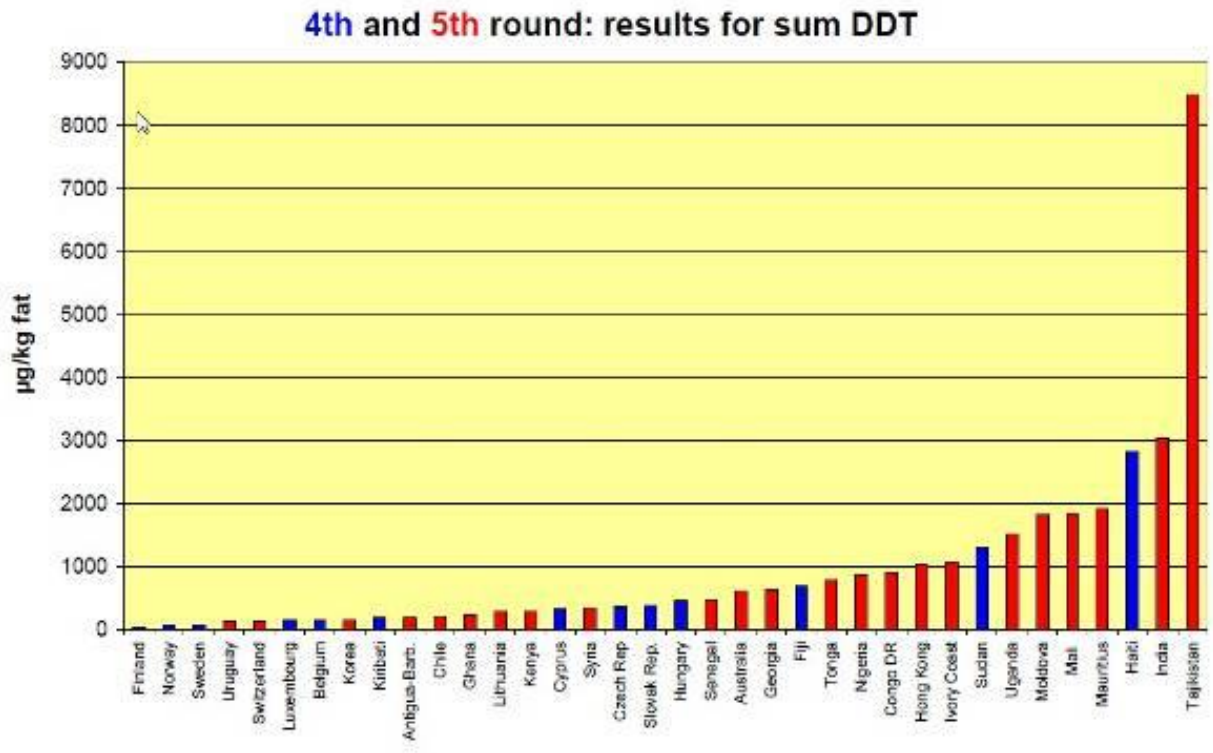


Risk assesment of a pesticide dump side in Kanibadem



	Dump 1	Dump 2
alpha-HCH	690,0	3,8
beta-HCH	120,0	13,0
gamma-HCH	8,3	570,0
delta-HCH	5,0	6,2
Aldrin	0,0	0,9
Dieldrin	1,4	0,0
Endrin	0,0	0,0
o,p-DDT	4,5	48,0
p,p-DDT	32,0	310,0
PCDDs, PCBs, PBDEs, PFTs	?????	??

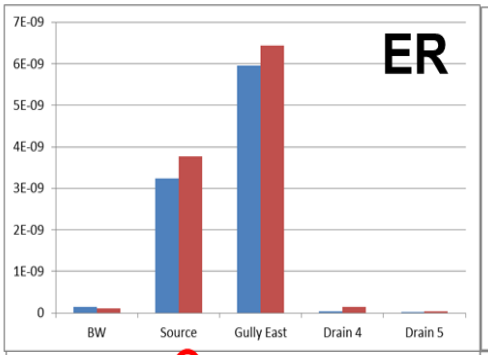
Waste cocktails from landfills

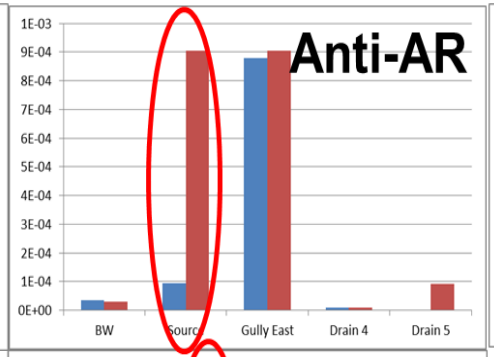


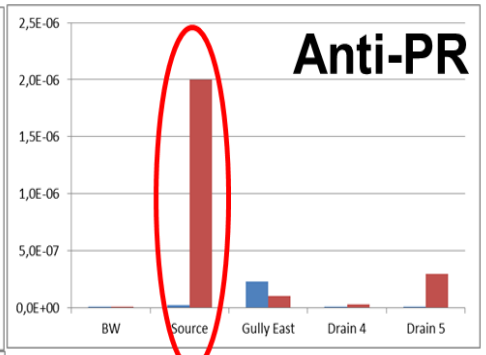
Incidence of malignant tumors 2x higher in Kanibadam than rest of Sughd region and Tajikistan.

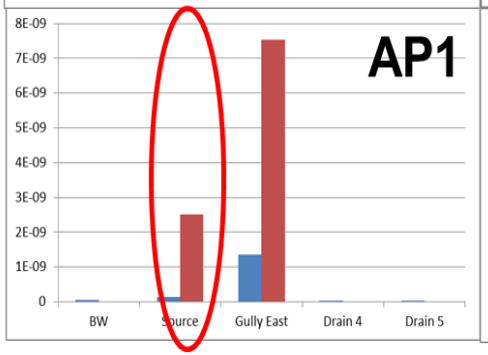
Is it only DDT or is the chemical cocktail bigger?

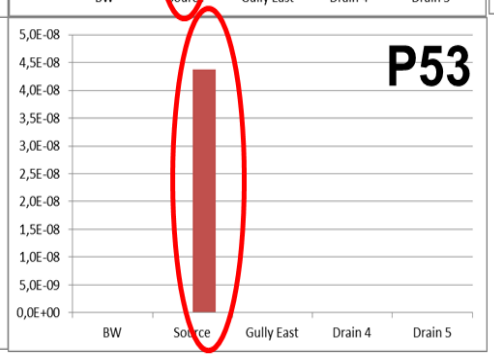
Waste cocktails from landfills











Expected

Measured

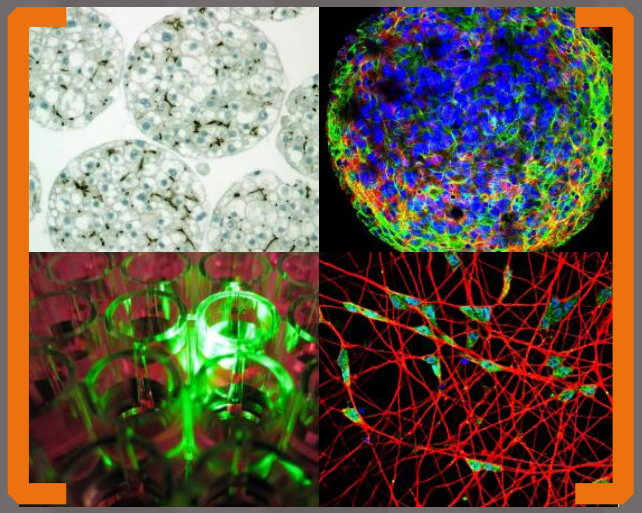
Different activity profile from what was expected for 'Source' sample

Not all pesticides included in chemical analysis!

Bart Pieterse et. al. (2015). ESPR

EUTOXRISK

An Integrated **EU**ropean 'Flagship' Program Driving Mechanism-based **Tox**icity Testing and **Risk** Assessment for the 21st Century

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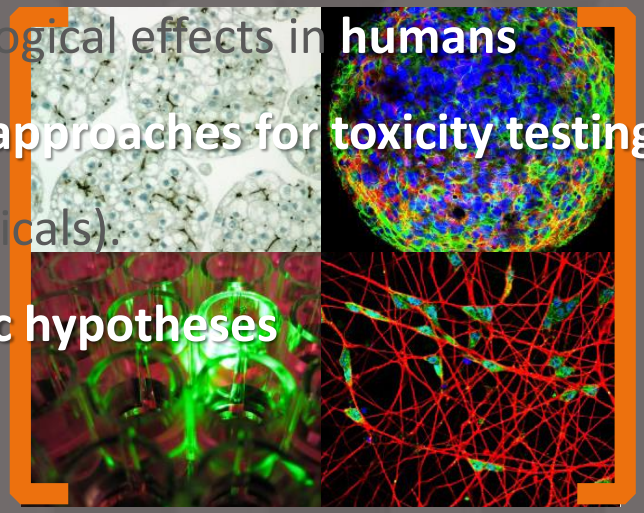
EUTOXRISK

Background of the call

- Approaches to **improve the efficiency of predictive toxicological testing** to address key areas of concern for human health
- Approaches **to meet regulatory requirements** (e.g. EU legislations on REACH, cosmetics, biocides)
- To **understand complex biological pathways of toxicological** relevance
- To **identify early markers** predictive of toxicological effects in **humans**
- To develop and validate **routine, non-animal approaches for toxicity testing** of chemical substances (excluding radio-chemicals).
- To involve confirmatory **testing of mechanistic hypotheses**



Thank you for your attention

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What is known about PCBs & PXR?

- **Non-dl-PCBs (99, 138, 153, 180, 194) are human PXR agonists, while PCB-77 not (Al-Salman et al Tox and Applid Pharm, 2012)**

- **Highly chlorinated PCBs are potent activators of rodent PXR but antagonize its human orthologue, inhibiting target gene induction. Thus, exposure to PCBs may blunt the human xenobiotic response, inhibiting the detoxification of steroids, bioactive dietary compounds, and xenobiotics normally mediated by human PXR (Tabb et al 2004, EHP, 112)**

Plaatjes PXR met ndl PCBs toevoegen?