

Introduction

- BDS developed several reporter gene assays to assess complex mixtures for a wide range of matrices, focusing on genotoxicity and endocrine disruption and developmental toxicity. Several effects have been detected in surface and waste water that have not previously been analyzed, even though extraction procedures may not have been optimal.
- BDS developed parallel several extraction and clean-up methods focusing on a wide range of compounds and matrices, including endocrine disruptors, genotoxins and pharmaceuticals. The method show good recovery for a wide range of compounds and matrices, which is important when using bioassays as there is no recovery correction (which is common in chemical analysis). These methods will be applied for the extraction of active compounds from all kinds standards and samples to be tested in the case-studies.

1. Introduction

Most of the CALUX technologies have been evaluated and applied in several European Projects such as TECHNEAU, NEW GENERIS, FIRE, FACEIT or HORIZONTAL. Please see in the following Table an overview about our current comercail available panel of CALUX technologies:

Table . Selected reporter gene assays for application in the project. Some assays have already been applied to real water samples but not in all cases effects could be detected. In some occasions (e.g. RAR), no effect was detected using the CALUX assay, but effects are described in literature.

Reporter gene assays	Pathway	Standards	Compounds e.g.	Activity in literature?
<i>Endocrine disruption</i>				
ERa CALUX/ (anti)-	ERa	Estradiol E2/ Tamoxifen	EE2, Estrone, BPA, NP, OP, BFRs, Phytoestrogens	Yes
ERb CALUX/ (anti)-	ERb	Estradiol E2/ Tamoxifen	EE2, Estrone, BPA, NP, OP, BFRs, Phytoestrogens	
AR CALUX/ (anti)-	AR	DHT/Flutamide	Nearly all anabolic steroids from the WADA	Yes
PR CALUX/ (anti)-	PR	Progesterone/RU486	Masks/SunScreens, Pharmaceuticals	Yes
GR CALUX/ (anti)-	GR	Dexamethasone/RU486	Cordisol, Pretnisolone,	Yes
TRb CALUX	TRb	T3	Tetrac, Triac, OH-PCBs	Yes
PPARy2 CALUX	PPARy2	Rosiglitason	TBT, Pharmaceuticals	No
RAR CALUX	RAR	All trans Retinoic acid	?	Yes
<i>Genotoxicity</i>				
p53 CALUX (U2OS)	p53	Actinomycin D	Pharmaceuticals (anti-cancer agents), PAHs, mutagens	No
p53 CALUX (HepG2)	p53	Actinomycin D	Pharmaceuticals (anti-cancer agents), PAHs, mutagens	No
p21 CALUX	p21	Actinomycin D	Pharmaceuticals (anti-cancer agents), PAHs, mutagens	No
Nrf2 CALUX	Nrf2	Tert-butyl hydroquinone	Oxidative stress	No
Control CALUX	-	-	Luciferase pathway	No
NFkB	NFkB	TNF α	Cytokines, free radicals, inflammatory response	No
PAH CALUX	AhR	BaP	PAHs	No
DR CALUX	AhR	2,3,7,8-TCDD/ PCB-126	PCDD/PCDF/dl-PCBs/PBDD/PBDF	Yes

In vitro assays for endocrine disruption

Reproductive toxicity refers to the adverse effects of a substance on any aspect of the reproductive cycle, including the impairment of reproductive function, the induction of adverse effects in the embryo, such as growth retardation, malformations, and death. All these events and interactions are controlled to a large extent by the body's endocrine system. However, due to the complexity of the mammalian reproductive cycle it is not possible to model the whole cycle in one *in vitro* system. However, the cycle can be broken down in its biological components that can be studied individually or in combination.

Many known endocrine disruptors, of which some can be regarded as developmental toxins, are known to target nuclear (hormone) receptors. Therefore, a panel of assay will be utilized that will focus on activity on these specific receptors. Some of these assays have already shown to be relevant for water samples and to be predictive for *in vivo* responses by measuring activity on relatively simple toxic endpoints (Sonneveld et al., 2006; Van der Burg et al., 2010a,b). For others, currently any information regarding the presence of possible ligands in surface, waste or drinking water is mostly lacking.

The following end points were selected for the analysis of endocrine disruption and developmental toxicity.

- estrogen-like activity
- androgen-like activity
- progesterone-like activity
- glucocorticoid-like activity
- thyroid hormone-like activity
- peroxisome proliferator-activated receptor-like activity
- retinoid-like activity
- dioxin-like activity
- PAH like activity

In vitro assays for genotoxic activity

The frequently applied suite of assays to detect genotoxic activity mainly utilizes bacteria, which mainly focus on mutagenic activity, while bacteria lack responses to some important classes of mammalian genotoxic compounds (e.g. topoisomerase inhibitors, clastogens). In mammalian genotoxicity assays, DNA damage is often used as end-point, but available assays are difficult to automate and generally take a long time from exposure to results. The choice for the pathways was based on the results of microarray experiments with genotoxic compounds in T47D cells (in-house unpublished data), as well as literature data. The following developed reporter gene assays have been developed by BDS so far:

- p53 activity
- Nrf2 activity
- p21 activity
- AhR activity
- NFκB activity
- Constitutive promoter activity (cytotoxicity control)

An overview of the reporter gene assays established by BDS

A.1. In vitro assays for endocrine disruption

Estrogen-like activity

Estrogens are the primary female sex hormones, and are synthesized in all vertebrates. Direct activation of the estrogen receptor (ER) is of the mechanism by which compounds alter the physiological function of endogenous steroid hormone receptors (Witorsch et al., 2000). Several developmental toxins and endocrine disruptors are known to act on the estrogen receptor, and as such estrogen receptor activity is included in the OECD test strategy on the detection of endocrine disruptors (Gelbke et al, 2004).

Estrogenic activity has been detected frequently in surface water and waste waters, both municipal and industrial (e.g. Vethaak et al, 2005). Several estrogenic compounds have been identified and range from natural and synthetic hormones to compounds previously unknown to be able to exert estrogenic responses. Some of the identified compounds have been directly linked to adverse effects on aquatic species. Especially the synthetic hormone ethinyl estradiol, used in birth control pills, has been shown to have devastating effect on fish population when exposed chronically at concentrations as low as 5 ng/L (Kidd et al, 2007).

Estrogenic activity in the water samples in this project will be measured using the ER α CALUX, A U2-OS based reporter gene assay. This reporter gene bioassay responds specifically to compounds that activate or inhibit the estrogen receptor α . Recently, this assay, which consist of an improved version of the bioassays originally described by Sonneveld et al (2005) and Van der Linden et al (2008), was shown to produce reproducible and consistent results in the detection of estrogenic developmental toxins in a blinded study (Van der Burg et al, 2010a). While most estrogenic activity measurements so far have been determined using the ER CALUX (Legler et al, 1999), the ER α CALUX is regarded to be more sensitive and is expected to give more robust results when analyzing complex matrices, e.g. sludge rich waste water samples.

Androgen-like activity

Androgens control the development and maintenance of male sex characteristics in vertebrates, by binding to the androgen receptor (AR). Activating or blocking the androgen receptor is thought to be of importance for endocrine disruption and developmental toxicity.

Androgenic activity in environmental water samples is less frequently monitored than estrogenic activity, but several studies have shown the presence of androgenic and/or anti-androgenic compounds in surface water, waste water and sediments (e.g. Van der Linden et al, 2008; Hill et al, 2010). Many known contaminants in water have been shown to exhibit androgenic or anti-androgenic activity (Kortenkamp and Faust, 2010). Androgenic activity in the water samples will be measured using the AR CALUX, a U2-OS based reporter gene assay. The reporter gene assays responds specifically to compounds that activate the AR. Recently, this assay was shown to produce reproducible and consistent results in the detection of estrogenic developmental toxins in a blinded study (Van der Burg et al, 2010b).

Progestagen-like activity

Progesterone is a steroid hormone involved in the female menstrual cycle, pregnancy and embryogenesis in vertebrates. Its function is controlled by activating the progesterone receptor (PR), a nuclear hormone receptor. Progesterone is the only natural progestagen, but synthetic progestagens like levonorgestrel, norgestrel and medroxyprogesterone are frequently used e.g. in birth control pills or in hormone replacement therapy.

Progestagenic activity of surface or waste water is not frequently analyzed, but both natural and synthetic progestins have been detected in various water samples, both by biological and chemical analysis (e.g. Van der Linden et al, 2008; Chang et al, 2009; Streck et al, 2009). Some progestins are potent preovulatory pheromones in fish (Kolodziej et al, 2003, Sorensen et al, 1990) and are known to have an effect on spawning behaviour (Besse and Garric, 2009). Progestagenic activity in the water

samples will be measured using the PR CALUX, a U2-OS based reporter gene assay that responds specifically to compounds that can activate or block the progesterone receptor (PRb). It has been shown to be able to detect progesterone-like activity in Dutch surface waters and waste waters (Van der Linden et al., 2008; Van der Linden et al, unpublished).

Glucocorticoid-like

activity

Glucocorticoids are steroid hormones that are involved in the regulation of glucose metabolism and in the immune system. Especially their ability to suppress immune system activity makes that they belong to the most frequently applied pharmaceutical worldwide (also known as corticosteroid). Glucocorticoids function via the glucocorticoid receptor (GR), which is expressed in many species.

Glucocorticoid are not commonly analyzed in water samples, however glucocorticoid-like activity has been detected in Dutch surface and waste waters (Van der Linden et al, 2008; RIWA, 2009). Several glucocorticoids have been identified (Schriks et al, 2010; Chang et al, 2008; Chang et al, 2009). As most glucocorticoid levels reported are based on surface water analysis, a screen was performed to gain more insight in waste water levels. Waste water levels can easily range up to 300 ng Dexamethasone-like equivalents per liter. Glucocorticoid-like activity in the water samples will be measured using the GR CALUX, a U2-OS based reporter gene assay that responds specifically to compounds that can activate or block the glucocorticoid receptor (GR).

Thyroid hormone-like activity

Thyroid hormones are tyrosine based hormones that are responsible for the regulation of the metabolism rate. They function via the thyroid hormone receptor (TR), which belongs to the nuclear hormone receptor super family.

Thyroid hormone levels are generally not measured in water samples. Although thyroid hormone disruption is a known effect of several environmental contaminants, not many compounds are known to act on the receptor. However, several people have reported thyroid hormone-like activity in waste water samples (e.g. Jugan et al., 2009) or known contaminants (e.g. Li et al., 2010). Thyroid hormone-like activity will be measured using the TR β CALUX, a U2-OS based reporter gene assay that responds specifically to compounds that can activate or block the thyroid receptor β (TR β).

Peroxisome proliferator-activated receptor γ 2-like activity

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors that play essential roles in the regulation of cellular differentiation, development and metabolism of higher organisms (Feige et al, 2006). More specifically, PPAR γ - which is mostly found in adipose tissue and the intestine - regulates fatty acid storage and glucose metabolism. As such, many insulin sensitizing drugs target PPAR γ , as a means to lower serum glucose.

PPAR γ ligands are generally not measured in surface water and waste water. Although the extraction of potentially active compounds has not been validated, preliminary results using a LLE extraction with ethyl acetate show that PPAR γ 2 activity can be present in waste water influents up to several hundreds of ng Rosiglitason equivalents per liter of water. Known environmental contaminants like tributyl tin and phthalates are known to exert at least part of their (endocrine) disrupting response via the PPAR γ receptor. PPAR γ ligands (together or in combination with GR ligands) are thought to be of importance for the effects of "obesogens", agents that inappropriately regulate and promote lipid accumulation and adipogenesis (Grün and Blumberg, 2009). PPAR γ 2 activity will be measured using the PPAR γ 2 CALUX, a U2-OS based reporter gene assay that responds specifically to compounds that can activate or block the PPAR γ 2 receptor.

Retinoid-like activity

Retinoids are a class of compounds that are frequently used in medicine, primarily due to their way to regulate epithelial growth. Retinoids have many functions including regulation of cells proliferation and differentiation and immune function. Systemic retinoids are contraindicated during pregnancy as they may cause CNS, cranio-facial, cardiovascular and other defects.

Retinoids are not regularly monitored in water samples. Recently the detection of such compounds has been described for Japanese surface water (Inoue et al, 2010a). Several known environmental contaminants have been shown to be able to act in these receptor(s) (Inoue et al, 2010b). Retinoid activity will be measured using the RAR CALUX, a U2-OS based reporter gene assay that responds specifically to the RAR pathway.

A.2 In vitro assays for genotoxic activity

p53

The p53 tumor-suppressor gene (sometimes called the "guardian of the genome") encodes for a pivotal protein in the response to DNA damage, mitotic spindle disruption and activation of oncogenes (Shackelford et al, 1999). Under normal (non-stress) conditions the p53 protein is a short-lived transcription factor, but activation of the G2/M checkpoint results in phosphorylation of the checkpoint kinase Chk2. Subsequently, this kinase phosphorylates p53 which prevents the binding of p53 to mdm2 that targets p53 for ubiquitylation. Targets of the activated p53 protein are pathways involved in cell-cycle arrest, DNA repair and apoptosis. The p53 transcription factor binds to a p53-responsive element in the promoter of target genes.

p53 activity will be measured using the U2-OS p53 CALUX as well as the HepG2-p53 CALUX. While both reporter gene assays are capable of responding to increasing concentrations of p53, the HepG2 cell line is capable of expressing several P450 enzymes, thereby metabolizing - and activating and/or deactivating - genotoxic compounds. The U2-OS cell line does not express any P450 enzymes thought relevant for genotoxic metabolic activation.

p21

Previous results using microarray analysis for different human cell lines and genotoxic compounds showed that the expression of p21 is one of the most sensitive markers for genotoxic activity. This protein serves as a potent cyclin dependent kinase inhibitor, and therefore functions as a regulator of cell cycle progression at G1. The expression of the protein is tightly controlled by p53. p21 activity will be measured using the U2-OS p21 CALUX.

Nrf2

An important stress pathway that protects the cell against genotoxic and cytotoxic compounds is the Nrf2 pathway (Rushmore et al, 1991). Nrf2 is a transcription factor that activates phase-II detoxifying enzymes and antioxidant-stress proteins. In an environment without oxidative stress, the Keap-1 protein binds to Nrf2 and sequesters Nrf2 in the cytoplasm. Under stress conditions, antioxidants interact with the thiol groups of Keap-1, which causes the release of Nrf2 and translocation of Nrf2 to the nucleus where it forms a heterodimer with the small Maf protein. This complex binds to the antioxidant-responsive elements, which can activate the expression of several genes. Nrf2 activity will be measured using the U2-OS Nrf2 CALUX.

AhR

The Aryl hydrocarbon receptor (AhR) is a nuclear receptor that can be activated by a wide range of environmental contaminants, including dioxins, PCBs, furanes and many PAHs. Many activators of the AhR are known non-genotoxic carcinogens or genotoxic carcinogens. The AhR activity will be detected using two different assays: one aimed at the detection of very stable compounds like PCBs and dioxines and one aimed at the detection of more liable compounds like PAHs. Both assays utilize rat liver cells (H4IIE).

NFκB

NFκB (nuclear factor kappa light chain enhancer of activated B-cells) is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, hypoxia, cytokines, free radicals, UV radiation and bacterial or viral antigens. Hence, it plays a key role in the immune response, and incorrect regulation has been linked to cancer, inflammatory and autoimmune diseases and improper immune development.

The NFκB response will be measured using the NFκB CALUX. No water samples have been analysed yet with this assay and no literature has been found regarding NFκB activity in environmental samples. However, as NFκB is a key player in the inflammatory response, it is a drug target. Also frequently used pesticides, e.g. disulfiram, olmesartan and other dithiocarbamates are known to inhibit the NFκB signaling cascade, (Cvek et al, 2007).

Luciferase expression control

Genotoxic responses are closely related to cytotoxic responses: a large amount of DNA damage is normally detected by different mechanisms in a cell, and can lead to apoptosis or programmed cell death. As stressful situation on a cellular level can change the expression of many genes, a control for luciferase expression (which is also indicative for cytotoxicity) is important in the analysis. A cell line expressing a constant amount of luciferase was developed to serve as a luciferase expression control (control CALUX). This cell line can be indicative for changes in luciferase expression and cytotoxicity, as high amount of DNA damage can lead to death of the cells.

References

- Besse J-P, Garric J (2009) Progestagens for human use, exposure and hazard assessment for the aquatic environment *Environmental Pollution* xxx (2009) 1–10
- Chang et al 2009. Determination and Source Apportionment of Five Classes of Steroid Hormones in Urban Rivers *Environ. Sci. Technol.* 2009, 43, 7691–7698
- Chang H, Hu j, Shao B (2007) Occurrence of Natural and Synthetic Glucocorticoids in Sewage Treatment Plants and Receiving River Waters *Environ. Sci. Technol.* 2007, 41, 3462-3468
- Cvek B, Dvorak Z (2007) Targeting of nuclear factor-κB and proteasome by dithiocarbamate complexes with metals". *Curr. Pharm. Des.* 13 (30): 3155–67
- Feige JN, Gelman L, Michalik L, Desvergne B and Wahli W (2006) From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Progress in Lipid Research* 45(2):120-159
- Gelbke HP, Kayser M, Poole A. OECD test strategies and methods for endocrine disruptors. *Toxicology* 2004:17–25.
- Grün F and Blumberg B (2009) Endocrine disruptors as obesogens. *Molecular and Cellular Endocrinology* 304(1-2):19-29
- Hill et al (2010) Profiles and Some Initial Identifications of (Anti)Androgenic Compounds in Fish Exposed to Wastewater Treatment Works. Effluents *Environ. Sci. Technol.* xxx, 000–000

Inoue D, Nakama K, Sawada K, Watanabe T, Takagi M, Sei K, Yang M, Hirotsuji J, Hu J, Nishikawa J, Nakanishi T, Ike M (2010) Contamination with retinoic acid receptor agonists in two rivers in the Kinki region of Japan *Water Research* 44(8): 2409-2418

Inoue D, Sei K, Ike M (2010) Disruption of retinoic acid receptor signaling by environmental pollutants. *Journal of Health Science* 56(3):221-230

Jugan ML, Oziol L, Bimbot M, Huteau V, Tamisier-Karolak S, Blondeau JP, Lévi Y (2009) In vitro assessment of thyroid and estrogenic endocrine disruptors in wastewater treatment plants, rivers and drinking water supplies in the greater Paris area (France) *Science of the Total Environment* 407 (2009) 3579–3587

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW (2007) Collapse of a fish population after exposure to a synthetic estrogen. *PNAS* 104(21):8897-8901

Kolodziej, E. P.; Gray, J. L.; Sedlak, D. L. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. *Environ. Toxicol. Chem.* 2003, 22, 2622-2629.

Kortenkamp A and Faust M (2010) Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment. *International journal of andrology* 33 (2010), 463–474

Nal I, Wang D, Meima Y and Wang Z (2010) Dibutyl Phthalate Contributes to the Thyroid Receptor Antagonistic Activity in Drinking Water Processes *Environ. Sci. Technol* (accepted for publication)

RIWA (2009) Temporal variation in multiple hormonal activities of surface waters located in the Dutch part of the Rhine basin

Rushmore TH, Morton MR, Pickett CB (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity, *J. Biol. Chem.* 266:11632–11639

Schriks M, Van Leerdam J, Van der Linden SC, Van der Burg B, Van Wezel A and De Voogt P (2010) High-Resolution Mass Spectrometric Identification and of Glucocorticoid Compounds in Various Wastewaters in The Netherlands. *Environ. Sci. Technol.* XXXX, xxx, 000–000 Quantification

Shackelford RE, Kaufmann WK, Paules RS (1999) Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environ. Health Perspect.* 107 (Suppl. 1):5–24

Sonneveld E, Jansen HJ, Riteco JAC, Brouwer A, Van der Burg B. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. *Toxicol Sci* 2005;83:136–48.

Sonneveld E, Riteco JAC, Jansen HJ, Pieterse B, Brouwer A, Schoonen WG. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. *Toxicol Sci* 2006;89:173–87.

Sorensen, P. W.; Hara, T. J.; Stacy, N. E.; Dulka, J. G. Extreme olfactory specificity of male goldfish to the preovulatory steroidal pheromone 17-R, 20- α -dihydroxy-4-pregnen-3-one. *J. Comp. Physiol., A* 1990, 166, 373-383.

Streck G. Chemical and biological analysis of estrogenic, progestagenic and androgenic steroids in the environment (2009) *Trends in Analytical Chemistry*, Vol. 28, No. 6, :635-652

Van der Burg B, Winter R, Man H-Y, Vangenechten C, Berckmans P, Weimer M, Witters H and Van der Linden S (2010) Optimization and prevalidation of the in vitro AR CALUX method to test androgenic and antiandrogenic activity of compounds. *Reproductive Toxicology* xxx (2010) xxx–xxx

Van der Burg B, Winter R, Weimer M, Berckmans P, Suzuki G, Gijsbers L, Jonas A, Van der Linden S, Witters H, Aarts J, Legler J, Kopp-Schneider A, Bremer S (2010) Optimization and prevalidation of the in vitro ER₁ CALUX method to test estrogenic and antiestrogenic activity of compounds. *Reproductive*

Toxicology xxx (2010) xxx–xxx

Van der Linden S, Heringa MB, Man HY, Sonneveld E, Puijker L, Brouwer A. Detection of multiple hormonal activities in waste water effluents and surface water, using a panel of steroid receptor CALUX bioassays. *Environ Sci Technol* 2008;42:5814–20.

Vethaak et al (2005) An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands *Chemosphere* 59(4):511-524

Witorsch et al (2000) Endocrine disruption: a critical review of environmental estrogens from a mechanistical point of view. *Toxic Subst Mech* 19:53-78.