Technical Report - 2014 - 077

TECHNICAL REPORT ON AQUATIC EFFECT-BASED MONITORING TOOLS

Annex

Environment

BIOASSAY FACT SHEETS

DR CALUX/DR Luc assay

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 compound (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**: Method developed originally by Wageningen University (Aarts et al., 1995; Murk et al., 1996). TIMES protocol available. QA procedures are in place and interlaboratory performance studies are organized frequently. 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).Good correlations were usually observed between bioassay results studying marine biological matrices and results from advanced chemical methods (Windal et al., 2002; Hoogenboom, 2002).
- **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[57], especially compared to chemical analysis of dioxins and dioxin-like compounds. Cost effectiveness increases even more if comparing to the price of analysing also other compounds with the same mode of action (such as brominated dioxins and furans). By applying several in vitro tools in a battery (such as PAH CALUX and ER-Luc/ER CALUX; see separate fact sheets) can further increase cost effectiveness. Generally not depending on matrix studied.
- **Commercial availability:** Commercial ISO 17025 accredited performers are available
- **WFD relevance:** Dioxins and planar PCBs included in 2008/105/EC. The bioassay analysis is significantly cheaper than chemical, and therefore valuable to include on a screening basis (see case study "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach" in section 1). Valuable to identify water bodies at risk of combined exposure to compounds that are also normally not analysed chemically but that could constitute potential RBSPs, having a relevant mode of action (Ah receptor binding).

[57] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample

PAH CALUX

The PAH Responsive (PAH) 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 poly aromatic hydrocarbons (PAHs). Following binding of PAHs to the cytosolic Arylhydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. Cells that are exposed to PAHs 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 benzo(a)pyrene (B(a)P). PAH CALUX bioassays report total B(a)P equivalents for environmental and food/feed matrices.

- **What is analysed (endpoint; unit):** pg B(a)P equivalents/g sample processed
- **Test duration:** 6h
- **Method used**: See DR CALUX fact sheet, but different pretreatment procedures.
- **Positive control used**: Benzo(a)pyrene
- **Matrices (sediment, water, tissue etc) that can be investigated:** Any type of sample
- **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. Benzo(a)pyrene like compounds. Especially the higher aromatic PAHs have high activity in the PAH CALUX.
- **Sensitivity (LOD/Q):**The bioassays' LOQ is 0.45 ng B(a)P equivalents per amount of material processed. For example, 5 grams of dried soil/sediment or 1 liter of water is processed resulting in a LOQ of 0.09 ng B(a)P equivalents per gram of soil/sediment or 0.45 ng B(a)P equivalents per liter of water respectively.
- **Variability (e.g. CV for single substance tests) if known:**<20%
- **Influence by cytotoxicity/risk of false positives/negatives:** depending on the clean-up systems and separation technology (HPLC-SPE)
- **Complexity/learning period:** 1 week of training
- **Costs:** Low[58]. Costs are generally not depending on matrix studied.
- **Commercial availability:** Commercial performers available
- **WFD relevance:**The assay could be very valuable on screening level to identify water bodies at risk of exposure to the combined exposure to a large number of relevant PAHs that are normally not analysed chemically but that could constitute potential RBSPs (see case study "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach" in section 1)

[58] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample

ERα CALUX/ER-Luc (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. ERαCALUX 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[59]. 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.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

[59] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample

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 HC5 of 800 pg/L and an AF of 2 is sufficient, resulting in an AA-EQS of 0.4 ng/L. In most cases, the commonly used TEQ approach would most likely allow a direct comparison of the EEQ value with the AA-EQS of E2 to decide where enough oestrogen-receptor binding potential is available to cause population relevant effects on the most sensitive aquatic species.

AR CALUX (agonistic/antagonistic)

The AR Responsive (AR) CALUX® comprises a human bone marrow cell line (U2OS), incorporating the firefly luciferase gene coupled to Androgenic Responsive Elements (AREs) as a reporter gene for the presence of androgens and/or androgen-like compounds (such as Bisphenol A). Following binding of androgens or androgen-like compounds to the cytosolic estrogen receptor, the ligand-receptor complex binds the ARE. Cells that are exposed to androgens or androgen-like compounds not only express proteins that are under normal circumstances associated to ARE, 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 di-hydro-testosterone (DHT). AR CALUX bioassays report total DHT equivalents for environmental matrices.

- **What is analysed (endpoint; unit):** pg DHT 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**: DHT
- **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 Androgen receptor
- **Sensitivity (LOD/Q):**The bioassays' LOQ is ca. 350 pg DHT equivalents per amount of material processed. For example, 5 grams of dried soil/sediment or 1 liter of water is processed resulting in a LOQ of ca. 70 pg DHT equivalents per gram of soil/sediment or 350 pg DHT equivalents per liter of water respectively.Original AR CALUX: ca.1 ng EEQ/l water
- **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 which kinds of water matrixes.
- **Complexity/learning period:** 1 week of training
- **Costs:** Low[60]. Costs are generally not depending on matrix studied.
- **Commercial availability:** Commercial accredited performers available
- **WFD relevance:**The assay could be valuable on screening level to identify water bodies at risk of exposure to the combined exposure to androgenic compounds that are normally not analysed chemically but that could constitute potential RBSPs (see case study "Laxsjön – investigating sediment contamination, using chemical and in vitro bioassay approach" in section 1).

[60] Laboratory equipment about 40 kEuro; material costs for one batch: depending on material costs in the location or country around 20-25 Euro/sample