ICES Techniques in Marine Environmental Sciences

No. 55

August 2013

Protocol for measuring dioxin-like activity in environmental samples using *in vitro* reporter gene DR-LUC assays

Cor A. Schipper • Pim E.G. Leonards • Hans J.C. Klamer

Kevin V. Thomas • Andre D. Vethaak

ICES International Council for the Exploration of the Sea

CIEM Conseil International pour l'Exploration de la Mer

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

H. C. Andersens Boulevard 44–46
DK-1553 Copenhagen V
Denmark
Telephone (+45) 33 38 67 00
Telefax (+45) 33 93 42 15
www.ices.dk
info@ices.dk

Recommended format for purposes of citation:

Schipper, C.A., Leonards, P.E.G., Klamer, H.J.C., Thomas, K.V. and Vethaak, A.D. 2013. Protocol for measuring dioxin-like activity in environmental samples using *in vitro* reporter gene DR-Luc assays. ICES Techniques in Marine Environmental Sciences No. 55. 21 pp.

Series Editor: Paul D. Keizer

This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and shown to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

ISBN 978-87-7482-131-1 https://doi.org/10.17895/ices.pub.5083

ISSN [redacted] 2707-6997

Contents

Abstract

Guidelines are given for the quantification of the dioxin-like activities of contaminants in sediment, biota and water samples using the DR-Luc reporter gene bioassay. Dioxins and dioxin-like compounds demonstrate high affinity binding to the Aryl hydrocarbon Receptor (AhR). Ah-R is a ligand-activated transcription factor and mediates most, if not all, of the toxic responses of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), coplanar polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs). The DR-Luc bioassay, or DR-CALUX® (Dioxin Response Chemical Activated LUciferase gene eXpression), utilizes a recombinant rat hepatoma H4IIE cell line with a stably integrated AhR-responsive luciferase reporter gene. Exposure of this bioassay to extracts containing dioxin-like compounds induces the enzyme luciferase in a time, dose, and chemical specific manner. Cells are cultured in the laboratory and transferred to 96-well plates. Luciferase activity is determined by measuring the light emitted, which is directly proportional to the amount of dioxin-like compounds within the test extract. Hence the DR-Luc assay is a rapid, extremely sensitive and cost-effective tool for screening sediment, biota, and water extracts for dioxins and dioxin-like compounds. The DR-Luc assay is recommended in the OSPAR JAMP guidelines as a specific biological effect method for monitoring of PCBs, polychlorinated dibenzodioxins and furans, and also as a suitable biological effect method for general biological effect monitoring. In addition, DR-Luc analysis has proven to be a very powerful tool in emission source monitoring and remediation efforts as it allows for the identification and control of the toxic compounds concerned. Critical steps, such as the extraction of sediment or biota samples and subsequent clean-up of the extracts are discussed, followed by descriptions of the DR-Luc detection technique. Emphasis is placed on analytical quality control and quality assurance.

1 Introduction

1.1 Overview of dioxins

Research on wildlife in aquatic ecosystems has shown that levels of harmful dioxins and dioxin-like compounds in the global environment have been declining for the past 30 years (Rappe, 1996; Aylward and Hays, 2002) due to reductions in emissions from anthropogenic sources. Dioxins and dioxin-like compounds from past releases will persist in the environment for many years since they are very slow to break down. Dioxins and dioxin-like compounds are a family of compounds of widely varying toxicities. Dioxin-like compounds including some polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), non-ortho and mono-ortho polychlorinated biphenyls (PCBs), and some polybrominated biphenyls (PBB's) are harmful by-products of the combustion of halogenated compounds (American Legislative Exchange Council, 2003).

It has been shown that dioxins and dioxin-like compounds cause adverse effects in a wide range of aquatic species, particularly those at high trophic levels, such as otters (Murk *et al.*, 1996), some avian species, (Bosveld *et al.*, 1995; Henshel, 1998) and harbour seals (Brouwer *et al.*, 1989). The known non-cancer effects of dioxin-like compounds in humans include endometriosis and adverse effects on reproduction (WHO, 2000; Heilier *et al.*, 2005). It has been shown that aquatic organisms can ingest dioxin-like compounds that are transported into surface water from contaminated land, providing a potential pathway into the food chain (Leonards *et al.*, 2008).

Studies of organic contaminants in the marine environment have commonly focused on persistent and bioaccumulative chemicals such as dioxins, furans, PCBs, and polycyclic aromatic hydrocarbons (PAHs), because of their non-polar nature. Of the 209 PCB congeners, only 12 are thought to share the dioxin-like toxicological mode of action with the aryl hydrocarbon receptor (AhR) in the cytoplasm, resulting in transcription of genes in the DNA. Only 7 of the 75 congeners of PCDDs and 10 of the 135 PCDFs have been identified or proposed as having dioxin-like toxicity (Aarts and Palmer, 2002; Liem and van Zorge, 2005). The most relevant biomarkers or bioassays to detect exposure to dioxin and dioxin-like compounds are considered to be the induction of cytochrome P450 monooxygenase 1A (CYP1A) mediated 7-ethoxyresorufin-O-deethylase (EROD) activity in fish liver (e.g Hylland *et al.*, 2006;) and the application of the dioxin receptor based *in vitro* test (H4IIE-luciferase cell-based Ah receptor gene assay DR-Luc or also named DR-CALUX® (Dioxin Response Chemical Activated LUciferase gene eXpression, a registered trademark of BDS, Amsterdam, The Netherlands). Alternative assays based on PCR (e.g. Procept® Rapid Dioxin Assay) and imunnoassay (e.g. Ah-Immunoassay kit) assays have been developed (e.g. Shan *et al.*, 2001).

The H4IIE DR-Luc *in vitro* bioassay was developed in the mid1990s (Aarts *et al.*, 1995; Murk *et al.*, 1996) as a screening method for dioxins and/or dioxin-like chemicals in a wide variety of aquatic environmental matrices, such as fish (Engwall *et al.*, 2003), fishoil (Hasegawa *et al.*, 2007), biota samples, (Hoogenboom *et al.*, 2006) and contaminated sediment (Murk *et al.*, 1996; Schipper and Stronkhorst, 1999;Stronkhorst *et al.*, 2003; Hurst *et al.*, 2004; Schipper *et al.*, 2009, 2010). DR-Luc can be used to measure the toxic potency, expressed as toxic equivalent quotient (TEQs), relative to the biological response in the DR-Luc bioassay arising from the most toxic compound 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD). The TEQ method uses the sum of relative toxicities of dioxin-like compounds in a mixture to express the overall toxicity of the mixture as a single TCDD toxic equivalent value. The TEQ values are calculated from the concentrations of individual congeners, as determined by gas chromatography with high resolution mass spectrometry (GC-HRMS).

A number of papers have been published describing the validation of the DR-Luc bioassay and the correlation between DR-Luc and 2,3,7,8-TCDD TEQs derived from GC-MS analyses (Van den Berg *et al.*, 1998; Stronkhorst *et al.*, 2002; Besselink *et al.*, 2004; Van Loco *et al.*, 2004). It has been shown that frequent participation in interlaboratory exercises improves performance (Besselink *et al.*, 2004), but there remains a need for Quality Assurance (QA) within international biological effects performance study programmes, such as Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM).

This guideline provides an overview of how dioxin-like activities of selected target contaminants can be quantified in environmental samples, using the DR-Luc *in vitro* bioassay. It also provides detailed steps for setting up the DR-Luc protocol in the laboratory for sediment, biota, and aqueous samples.

1.2 Role and importance of the determination of dioxins and dioxin-like compounds

The DR-Luc in-vitro bioassay with transgenic H4IIE rat hepatoma cells responds with luciferase production upon activation of the Ah receptor to quantify dioxin-like compounds (Aarts *et al.*, 1995). The sensitivity and linear working range of the rat hepatoma H4IIE cell line bioassay was slightly better than (Murk *et al.*, 1998) for the ethoxyresorufin O-deethylase (EROD) assay in H4IIE wild type (H4IIE-wt) cells (Sanderson *et al.*, 1996). The H4IIE DR-Luc assay is an improvement on the H4IIE-wt assay in that the DR-Luc assay is useful for monitoring the toxic potency of a great number of samples in a reproducible way and it is insensitive to substrate inhibition. DR-Luc is considered to be the most useful *in vitro* bioassay technique for screening for dioxins and dioxin-like compounds, although chronic *in vivo* bioassays may also be relevant (Foekema *et al.*, 2008).

The assessment of dioxins and dioxin-like compounds by DR-Luc can be used for:

a) Ecotoxicological effect assessment to determine maximum tolerable concentrations (MTCs) of chemical substances in water, sediment, and dredged material. It may compare environmental quality with sediment quality guidelines (SQG) (Stronkhorst *et al.*, 2002; Besselink *et al.*, 2004; Sanctorum *et al.*, 2007; Schipper *et al.* 2010) or assess the compliance with MTCs of dioxins in food (fish) products (legislation WHO-TEQ) (Traas *et al.*, 2001; Ross *et al.* 2004; Engwall *et al.*, 2003 and Hasegawa *et al.*, 2007). The reproducibility of *in vitro* bioassays provides a means for systematic assessment and monitoring of various matrices.

b) Monitoring the spatial and temporal trends of dioxin-like activity in sediment, suspended matter, and biota in coastal zones and estuaries (Legler *et al.*, 2002; Hurst *et al.*, 2004; Van den Brink and Kater, 2006; Houtman *et al.*, 2006; Thain *et al.*, 2006; Sanctorum *et al.*, 2007; Schipper *et al.*, 2010; Grung *et al.*, 2011). The results showed that the bioassay applied to sediment extracts for screening of potential toxicity were much more responsive than *in vivo* bioassays with invertebrates using survival as an endpoint.

c) Measure the presence of unidentified sources of toxicity. In general, when DR-Luc activity cannot be fully explained by the measured concentrations of dioxin-like compounds, it implies the presence of other sources of toxicity that can be identified using Toxicity Identification Evaluation (TIE) or similar procedures (e.g. Stronkhorst *et al.*, 2002; Müller *et al.* 2004; Balaam and Thomas, 2007; Balaam *et al.*, 2009; Grung *et al.*, 2011). The DR-Luc assay may be used in toxicity reduction evaluation (TRE), and effect-directed analysis (EDA) procedures (Burgess, 2000), as well as in sediment toxicity profiling (Hamers *et al.*, 2010). The assay shows promise for identifying emerging chemicals that contribute significantly to the toxin load on the environment. *In vitro* biotests are recognized as useful tools for identifying chemicals that can cause adverse effects at the individual and population level.

d) Studying specific dioxin-related biomarker responses on organisms and how population success can be related, as was shown in the studies of Ross *et al*. (2004), and Traas (2001) with harbour seal and otter. Population success can be related to individual animal health, to biomarkers and internal TEQs (Murk *et al.*, 1998), to TEQs in their prey (fish), and finally, to the TEQs in the local sediment (Traas *et al.*, 2001). The predicted ecotoxicological risk, preferably expressed as Potentially Affected Fraction of Species (PAF) based on Species Sensitivity Distribution (SSDs) for ecological relevant endpoints, can also be validated (Schipper *et al.*, 2010). It was observed that the actual measured bioassay response has a high ecological relevance but it is only feasible for the toxicity data which are available at the ecological level of population.

DR-Luc has been developed in combination with appropriate sample clean-ups (Table 1) that can be coupled with complementary high-resolution mass spectrometry instrumental chemical analysis (e.g. HRGCMS).

Table 1. Methods for extraction and clean-up of sediment matrices for the AhR reporter DR-Luc assay using H4IIE cells.

No	Extraction	Clean-up	References
1	soxhlet	Sulphuric acid / silica gel column	Aarts *et al.* (1995);
2	soxhlet	multilayer glass column	Stronkhorst *et al.* (2002)
3	ASE®, Gel Permeation Chromatography (GPC)	multilayer glass column	Besselink *et al.* (2004)
4	ASE®, Gel Permeation Chromatography (GPC)	Sulphuric acid / silica gel column	Klamer *et al.*, (2005a,b)

Aarts *et al.* (1995) applied the destructive clean-up procedure for which the method is validated. Stronkhorst *et al.* (2002) recommend using other cleanup procedures so that the DR-Luc system can detect a wider range of contaminants that are of environmental concern. Besselink *et al.* (2004) showed that the procedure blanks, using extraction and/or destructive cleanup, were below the limit of quantitation (LoQ). Klamer *et al.* (2005b) showed that a non-destructive clean-up procedure resulted in significantly higher DR-Luc responses than the destructive clean-up protocol of Stronkhorst *et al.* (2002). Klamer *et al.* (2005b) showed that harbour sludge 'extracts' obtained using polydimethylsiloxane (PDMS) equilibrium partitioning, produced a remarkably high response in the DR-Luc dioxin-type toxicity test, although chemical analysis of ASE® extract of the same samples showed that the typical dioxin-type compounds were not responsible for the effects.

Therefore the user has to choose between different extraction and clean-up principles either broad spectrum or target (dioxins, furans, PCBs). It is possible that clean-up and extraction methods may produce a biased profile of pollutants or introduce toxicity that is not directly related to the environmental sample.

We conclude that for extraction of freeze-dried solid samples, the ASE method is recommended (Klamer *et al.*, 2005a). For extraction of water samples, solid phase extraction using a broad spectra sorbent with high capacity in combination with a hydrophobic sorbent is recommended.

The destructive target clean-up procedure is recommended for routine use in monitoring activities (RIKZ, 2006b). However, guidelines for both the destructive and the non-destructive procedures are provided.

1.3 Determination of AH-R mediated dioxin-like activity

The DR-Luc bioassay uses rat hepatoma cells (H4IIE) that are stably transfected with the plasmid pGudLuc 1.1. This plasmid contains three Dioxin Responsive Elements (DREs) and the luciferase gene from the firefly *Photinus pyralis* as the reporter gene (Aarts *et al.*, 1995; Murk *et al.*, 1996). The bioassay is based on the common toxic mechanism of action of dioxins and dioxin related compounds. After entering cells, these ligands bind to a cytosolic receptor called the aryl hydrocarbon (Ah) receptor (Figure 1).

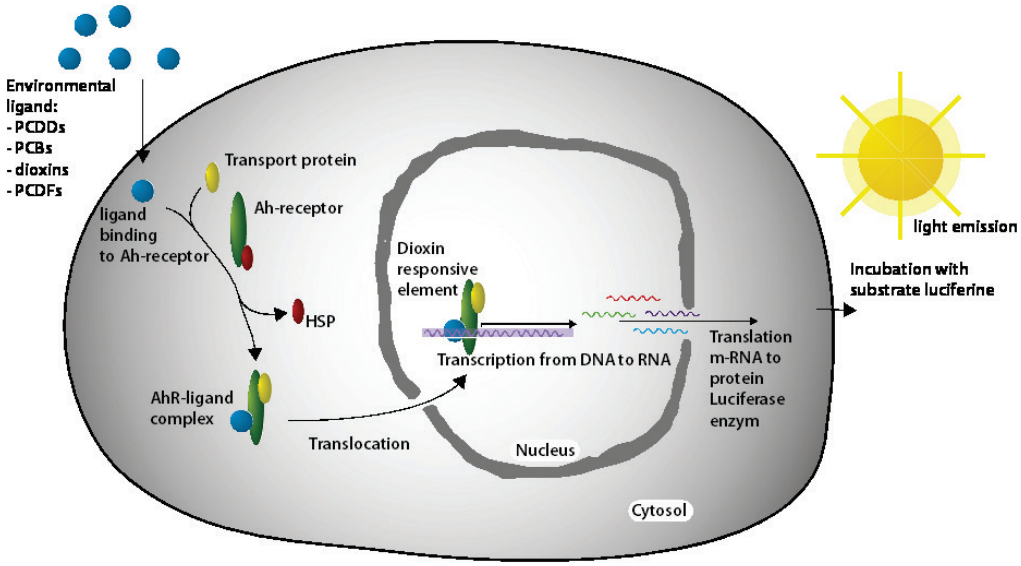

Figure 1. Activation of the Ah-receptor mediated luciferase gene in the DR-Luc bioassay (modified from RIKZ/BDS, 2006a)

Following activation of the receptor, the ligand–Ah receptor complex translocates to the nucleus of the cell, where it binds to a specific DNA sequence, the so called DREs. The binding of the ligand-Ah receptor complex to the DREs results in changes in the expression of DR-Luc associated genes (e.g. cytochrome P4501A1) and disturbance of normal cell physiology. Following exposure of the cells to dioxin or dioxin-like compounds, the enzyme luciferase is produced. Addition of the substrate luciferin to lysed cells results in the emission of light. The amount of light produced is recorded in a luminometer and is interpreted as "2,3,7,8-TCDD toxic equivalents" using a calibration curve.

The following sections provide guidance on the use of DR-Luc to quantify dioxins and dioxin-like activity in sediment, biota and water samples. Definitions of solid, biota and aqueous samples are:

- Solid samples: suspended matter, dredged material, (marine) sediments, sludge, suspended solids, and soils; (Stronkhorst *et al.*, 2002, Besselink *et al.*, 2004; Sanctorum *et al.*, 2007: Schipper *et al.* 2010)
- Biota samples: tissue, blubber, fishoil (Traas *et al.*, 2001; Ross *et al.* 2004, Engwall *et al.*, 2003 and Hasegawa *et al.*, 2007)
- Aqueous samples: seawater, surface or deep waters, production water, wastewater, sediment pore water, potable water (Sato *et al.*, 2010)

International sampling, extraction and clean-up protocols have been successfully used and alternative protocols can be used. These methods are gaining widespread use and regulatory acceptance (EC, 2002; Nakano *et al.*, 2006). The US EPA accepts several methods for the screening of dioxins in solid waste (US EPA, 2008).

The DR-Luc assay as described here may be accepted by OSPAR as a standard method for screening for dioxins in environmental samples. The proposed sampling, extraction and clean-up steps for environmental samples for DR-Luc analyses are illustrated in Figure 2. The sampling of abiotic and biotic samples may be performed according to standard operating procedures of OSPAR (OSPAR 1999, 2002), where applicable. A pretreatment, extraction and fractionation scheme should reflect the matrix tested and depends on the monitoring task being performed.

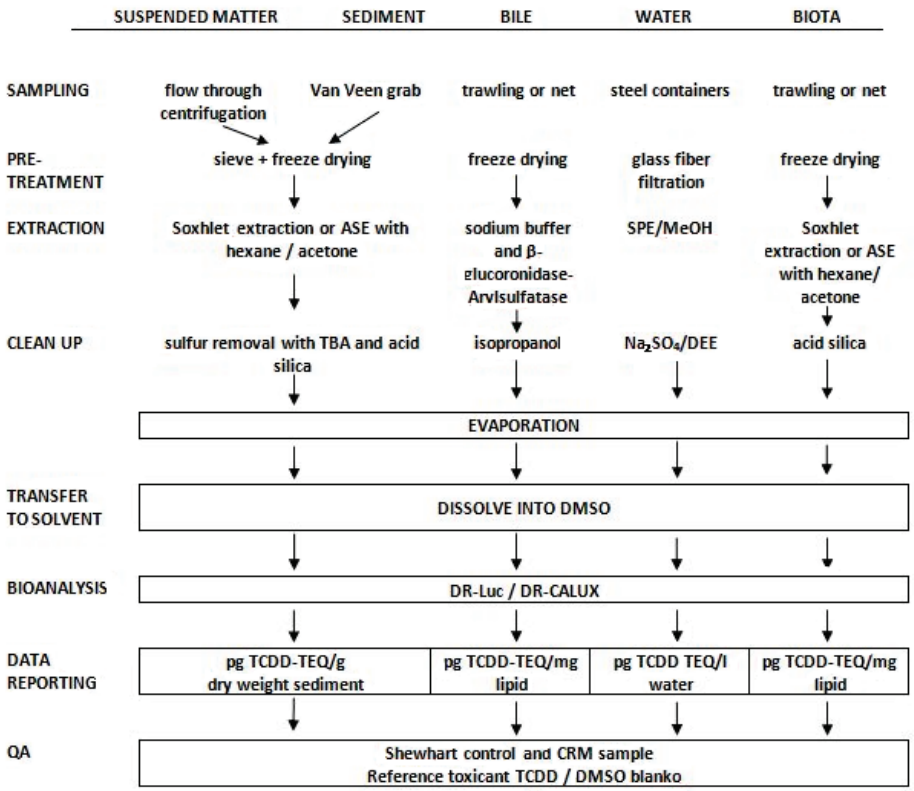

Figure 2. Sampling, extraction and clean-up scheme for field samples to be used for DR-Luc bioanalysis.

2 Field sampling and storage

2.1 Procedure sediment sampling and freeze-drying

Sediment sampling

Sampling should be performed according to the OSPAR Joint Assessment and Monitoring Programme (JAMP) guidelines for sampling sediment and suspended matter (OSPAR, 2002). In more detail, sediment samples are obtained by pooling 6 subsamples, collected by a Van Veen grab sampler from an area of about maximum 1000 m^2. All sediment samples are sieved (<63 µm) and where necessary, dried and then homogenized. Suspended matter (SPM) samples should be obtained from surface waters by flow-through centrifugation at 20 000 rpm until 200 g of material is collected. Samples should be stored in glass containers and should only be half-filled to facilitate proper homogenization of the sample prior to subsampling for analysis.

Sediment storage

Sample containers should be rinsed twice with hexane and allowed to dry in a fume cupboard. Approximately 40 g of sample is weighed out and placed in a cleaned sample container that is sealed. Solid samples should be stored frozen at -20 °C for no longer than 14 d.

Sediment freeze-drying

Samples should be freeze-dried to dryness and then ground and homogenized in a hexane-rinsed mortar or in a (ball) mill if particles greater than 0.5 mm are present.

The sample is then transferred back to its container and sealed using a new air tight lid. The sample is stored at 4°C until further processing.

2.2 Procedure biota sampling and freeze-drying

Sampling of finfish

Sampling should be performed according to the Guidelines for Monitoring Contaminants in Biota (OSPAR, 1999). Following anaesthetization 30 specimens finfish per site should be filleted, the liver or muscle tissues removed, and samples pooled. Alternatively, 25 specimens can be collected and pooled as 5 × 5 specimens. In case of sampling bile use a 1 ml syringe with needle (0.5 × 20 mm). Withdraw as much bile as possible and remove the bile bladder without contaminating the liver. Transfer the bile to an appropriate vial.

Storage of finfish samples

The collected tissues or bile should be stored at -20 °C prior to analysis.

Freeze-drying of finfish samples

Samples should be freeze-dried to dryness and then ground and homogenized in a hexane-rinsed mortar or in a (ball) mill if particles greater than 0.5 mm are present. The sample is then transferred back to its container and sealed using a new air tight lid. The sample is stored at 4°C until further processing.

2.3 Procedure aqueous sampling and filtration

Water sampling (Thomas *et al.*, 2006).

Collect 30–50 l water samples in solvent rinsed stainless steel transport containers. Transport the samples immediately to the laboratory, store at +4 °C upon arrival and process them within 24 h by filtering over a 0.45 μm glassfibre filter followed by solid phase extraction (SPE).

3 Pretreatment

3.1 Introduction to extraction techniques for determining dioxin-like activity

DR-Luc bio-analysis has been developed in combination with appropriate sample clean-ups that can be coupled with complementary high-resolution mass spectrometry instrumental chemical analysis (e.g. GCHRMS). It should be noted that although extraction techniques are powerful methods to pre-concentrate samples for bioanalytical testing, such extraction methods may produce a biased profile of pollutants and, in some cases, may also introduce toxicity that is not directly related to the environmental sample. Following extraction, the extracts are desulphurized and cleaned using an acid-silica column. Finally, the cleaned extract is transferred to and re-dissolved in DMSO.

One of the most comprehensive examples of this approach is described by Brack and Schirmer (2003). Effect-directed analysis (EDA) procedures in aquatic ecotoxicology were developed for the identification of organic pollutants, based on a combination of fractionation procedures, bioanalytical testing, and chemical analyses.

3.2 Techniques for extraction and clean-up of sediment, biota and aqueous samples

3.2.1 Sediment extraction and clean up

The literature provides extraction procedures (Klamer *et al.*, 2005a; Sato *et al.*, 2010) of sediment samples, which may be divided into two types, equilibrium or exhaustive extraction. In general, broad specificity sorbents such as silica bound long-chained alkanes (e.g. C8 and Cl8), resin based polymers (isolute ENV+, XAD) and various other sorbents such as PDMS, Tenax and blue rayon have been used to concentrate the most common waterborne pollutants (Diaz-Baez *et al.*, 2000; Ten Hulscher *et al.*, 2003; Leslie *et al.*, 2002; Qiu and Davis, 2004;). The extraction of sediment and clean-up of the extracts for analysis should be performed according to conventional clean-up procedures (Stronkhorst *et al.*, 2002). Clean-up and fractionation procedures should be applied to all crude extracts to remove interferences and to exchange the target compounds into a water-miscible, non-toxic solvent suitable for addition to the bioanalysis.

The destructive clean-up procedure is recommended for routine use in monitoring activities (RIKZ, 2006b). However, both the destructive and the non-destructive procedures are provided.

Many different pretreatment methods exist and they may all be applicable to *in vitro* bioassay testing. The sample preparation for sediments is summarized in Table 2 below. The ASE extraction is an exhaustive extraction.

Table 2. Pretreatment methods for sample preparation of sediment analysis used for DR-Luc.

Protocol steps of sediment preparation	Comment
1. Sample preparation	Sample sieved when necessary (e.g. sediment), dried and homogenized.
2. Extraction of crude sample	Accelerated Solvent Extraction (ASE®) or Soxhlet extraction. Solvents: dichloromethane (DCM) or hexane with methanol or acetone as modifiers, see for details Table 3
3. Concentration of crude extract	Automatic (e.g. Turbovap®) or manual concentration to smaller volume, typically less than 5 ml. Remove co-extracted water if necessary
4. Clean-up of crude extract	Destructive method: Normal phase chromatography with H_2SO_4. Sulphur removal may be necessary. Non-destructive method: Gel Permeation Chromatography (GPC) with DCM can be used.
5. Concentration of cleaned extract	Automatic (e.g. Turbovap) or manual concentration to smaller volume, typically less than 1 ml. Final test solvent (e.g. DMSO or methanol may be added as keeper.)

Extraction with ASE®

Extraction procedure is used with the ASE cells by adding a small layer of dried silica until the cellulose filter is no longer visible. Weigh approximately 5 g of dried sample

in the ASE cells (weighing accuracy mass ± 0.1%). Fill the ASE cells with dried silica and compact the content of the cells with the engraver pen. Close the cell and firmly twist the end-cap on the ASE cell. Extract the sample using the following ASE settings (Table 3):

Table 3. Sample extraction with ASE

Solvent	Pressure	Temp	Preheat time	Static time	Flush volume	Purge time	Static Cycles
	[psi]	[°C]	[min]	[min]	[ml]	[sec]	
Hexane/Acetone 9:1 v:v	2000	100	5	5	60	90	3
DCM or DCM/modifier**	2000	45-100*	5	5	60	90	1-3*

*** Set temperature to 45 to 50 °C and # of cycles to 3 for use with ER-CALUX and similar tests.**

**** methanol or acetone**

If water is co-extracted, dry the extract using anhydrous sodium sulphate. Rinse with solvent. Evaporate the extract to a final volume of approximately 2-5 ml in an automatic or manual system. Proceed to solvent exchange or store the crude extract at –20°C until further use.

Extraction with Soxhlet apparatus

Samples should be extracted in a Soxhlet extractor for 16 h using 200 ml hexane/acetone (3/1; v/v) and the extract concentrated to approximately 5 ml in a rotary evaporator (Besselink *et al.*, 2004).

3.2.2 Procedure for sediment extract clean-up

The extracts of the sediments samples are cleaned with an acid silica multilayer column and tetrabutylammonium (TBA) is used to remove sulphur (Besselink *et al.*, 2004; Klamer *et al.*, 2005a; RIKZ, 2006b).

Sulphur removal

To prepare the TBA solution, a 250 ml separation-funnel is washed with hexane and then filled with 100 ml HPLC-grade water. Add and dissolve 3.39 g TBA and then rinse the solution three times with 20 ml hexane. Dissolve 25 g sodium sulphite in the washed solution and store the TBA solution in a dark bottle.

Reduce the sediment extract to 1 ml hexane and then remove the sulphur as follows. Two ml TBA-sulphite solution and 2.0 ml isopropanol is added to the extract and mixed on a vortex for 1 minute. Sulphur clean-up is complete if precipitation is visible. Add an extra 100 mg sodium sulphite if no precipitation is present and mix for 1 minute on a vortex. Repeat the addition if necessary. Add 5 ml of HPLC-grade water, and mix for 1 minute on a vortex. Let the layers separate for approximately 5 minutes, and transfer the hexane layer to a clean collection vial. Add 1 ml hexane to the extract and mix for 1 min on the vortex. Allow the layers to separate and transfer the hexane layer to the clean collection vial. Repeat this step. Evaporate the hexane until approximately 1 ml is left. The concentrated, cleaned sediment extract is then concentrated to near dryness under nitrogen.

Acid silica clean-up

Prepare a solution of hexane/diethylether (97/3; v/v). Place a small piece of glass wool in a glass column. The performance of the following steps is column-dependent. Fill the column (23 cm x 2 cm) with 5 g of 33% H_2SO_4 silica and tap the column with the engraver pen. Add 5 g of 20% H_2SO_4 silica and tap the column once more. Add a small amount of dried sodium sulphate to the top of the column. Wash the column with 20 ml hexane/diethylether solution. Place the extract on the column as soon as the meniscus reaches the sodium sulphate. Wash the collection vial of the extract twice with approximately 1 ml hexane/diethylether solution. Place a clean collection vial under the column and elute the column with 38 ml hexane/diethylether. Evaporate the hexane to 1 ml. The concentrated, cleaned sediment extract is then concentrated to near dryness under nitrogen.

3.2.3 Biota extraction and clean-up

The same extraction procedure with ASE or Soxhlet as for sediment can be used for biota or fish bile samples. Tissue samples can also be extracted by pressurized liquid extraction (PLE) with hexane/acetone (Focant *et al.*, 2001). The lipids in the extract should then be removed using the acid silica clean-up as described above for sediment samples.

3.2.4 Extraction of aqueous samples

Without prior separation of particulate matter from a whole water sample, liquid phase extraction will yield the total concentration of pollutants in the sample. The extraction procedure described in Table 4 is taken from the work by Legler *et al.*, (2002). Following initial filtration through 0.45 μm glassfibre filters, an SPE cartridge is used for the extraction of the water sample (600 ml). The contaminants are then eluted with methanol. If necessary, extracts are filtered over anhydrous sodium sulphate, and eluted with diethyl ether (DEE) to remove small amounts of residual water and particles. Extracts that contain water following evaporation of the organic solvent should be extracted three times with DEE.

Table 4. Pretreatment of water samples to be used for DR-Luc.

Protocol steps extraction of water	Comment
1. Sample preparation	Thaw the sample
2. Pretreatment of crude sample	Filter through 0.45 μm glassfibre filters
3. Extraction of pretreated sample	SPE, with methanol as final eluent
4. Cleanup of crude extract	Filter over anhydrous sodium sulphate, eluate with DEE. Repeat DEE elution.
5. Concentration of extract	Extract can be concentrated by Turbovap, Kuderna-Danish, or other technique to a few millilitres
5. Concentration of to small volume	Manual concentration to dryness, solvent exchange into 50 μl DMSO.

3.2.5 Transferring to organic carrier solvents

Carrier solvents, also often referred to as co-solvents, are water miscible organic solvents that are used to add extracted organic micro-pollutants to aqueous toxicity test media. Evaluation of relevant properties of a series of solvents resulted in a list of carrier solvents that are best suited for ecotoxicity testing using *in vitro* bioassays (Bakker *et al.*, 2007). Dimethylsulfoxide (DMSO) is the most frequently used solvent as reported in the scientific literature for toxicity DR-Luc testing. It should have little or no adverse effects on test organisms and the target compounds show sufficiently high solubility in it (ICES, 2005).

Procedure of transferring sample extract to organic carrier solvents

The cleaned hexane extract is reduced in volume to approximately 1 ml and transferred to a cleaned conical vial where it is further evaporated until a small volume of hexane remains (approximately 20 µl). The vial is washed twice with at least 0.5 ml hexane/DEE solution which is transferred to the conical vial (the contents are evaporated under nitrogen between washes, taking care that the sample does not become dry). The extract is evaporated until the meniscus reaches the bottom of the conical vial and then 50 µl of DMSO is added. The sample is vortex mixed and dilutions of 3 and 10 times in DMSO are prepared.

4 Analysis

4.1 Testing field samples with the DR-Luc assay

The DR-Luc assay has yielded acceptable results for sediment, water and biota extracts. When the matrix is removed with the destructive methods for cleaning sediment extracts, DR-Luc analysis has been shown to reproducibly quantify dioxin-like compounds (Murk *et al.*, 1996, 1998; Stronkhorst *et al.*, 2002; Besselink *et al.*, 2004; Van Loco *et al.*, 2004; Legler *et al.*, 2006; Schipper *et al.*, 2010). However, it is important to note that without a thorough clean-up (destructive method with H_2SO_4-silica), this bioassay may also generate false positives as natural compounds and PAHs can induce the DR-Luc assay activity (Hamers *et al.*, 2000; Houtman *et al.*, 2004; Hurst *et al.*, 2004). The exposure period will strongly influence the response. Following 6 hours of exposure, it is mainly PAHs that induce luciferase production. But following 48 hours, persistent, dioxin-like compounds are the main cause of induction. This difference is the result of the metabolism of PAHs by the DR-Luc cells. DR-Luc activity therefore depends on the level and composition of PAHs present and the biodegradation capacity of the cells (Hamers *et al.*, 2000). As dioxin-like compounds induce a fundamentally different toxic profile than PAHs, the PAH response should not be included in the DR-Luc bioassay response for dioxin-like compounds (Schipper *et al.*, 2010).

4.2 Determination of the Ah receptor-mediated luciferase activity in the DR-Luc

The procedure for the determination of the Ah receptor-mediated luciferase activity in the DR-Luc is taken from Besselink *et al.*, 2004. Ah receptor-mediated luciferase activity in DR-Luc cells is determined by cultivating cells in optimal and controlled conditions with minimal essential medium supplemented with 10% fetal calf serum (α-MEM). Cells are cultured in a CO_2-incubator at 37°C with 5% CO_2, and 100% humidity. The DR-Luc analysis of samples in DMSO is performed in 96-well tissue culture plates. Cells seeded in 100 µl α-MEM and the tissue culture plates are incubated for 24 hours in the CO_2 incubator to reach confluence.

The dose medium is made of either

- A 2,3,7,8, TCDD standard solution,
- Environmental samples in procedure organic carrier solvents, or
- Extracts of commercially available CRM sample of sediment or biota

The cells are exposed by adding the dose medium to the cells. The maximum amount of DMSO added to the medium is 0.5%. After 24 hours the dose medium is removed. The wells are rinsed with diluted, phosphate buffer and lysed for 30 min at 4°C with a mixture containing 25 mM Tris, 2mM dithiothreitol, 2mM trans-1,2-diaminocyclohexane-N,N,N',N'-tetra acetic acid monohydrate, 10% glycerol and 1% Triton X-100 (pH 7.8). The plates should be processed the same day or else stored at –20°C for a maximum of 4 weeks. The luciferase activity is measured after adding 100 µl glowmix; 20mM trycin, 1.07 mM magnesium hydroxide carbonate pentahydrate, 2.67 mM magnesium sulfate, 0.1 mM ethelenedicaminetetraacetic acid, 33.3 mM dithiothreitol, 270 µM co-enzym A, 470 µM luciferin. The luminescence reaction is stopped after a few minutes by adding 100 µl of 0.2 M NaOH solution.

5 Data recording and reporting of DR-Luc

5.1 DR-Luc determination

Concentrations of dioxin and dioxin-like compounds are calculated from a standard calibration curve. The calibration range is typically from 0 (blank DMS0) to 20 pg TCDD per well, established in triplicate on each 96-well plate. Dose response curves are fitted using a user-defined curve fit (Fig 3), where *y*-axis is the measured response luminescence expressed in Relative Light Unit (RLU), and the *x*-axis is the concentration of the 2,3,7,8, TCDD compound.

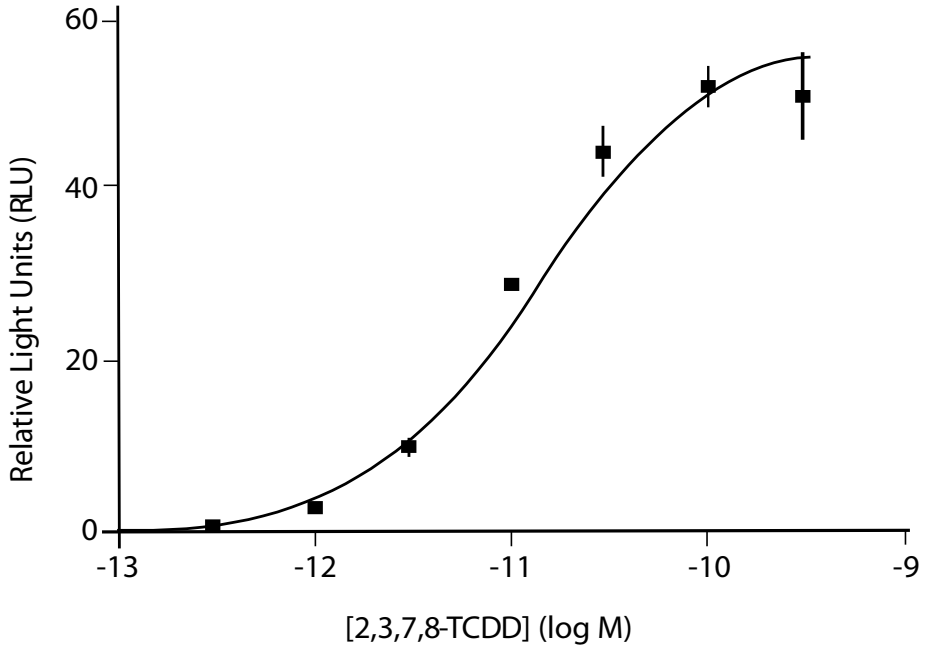

Figure 3. Example of a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8- TCDD) standard calibration curve. The relative light units have been corrected for dimethyl sulfoxide blank (modified from Besselink *et al.*, 2004).

5.2 DR-Luc data reporting

The TCDD-TEQ values are expressed of sediment in pg TCDD-TEQ/g dry weight, biota in pg TCDD-TEQ /mg lipid or surface water in pg TCDD-TEQ/L, following after 24 hour exposure. All reported data must meet the quality criteria established by QA/QC in Shewhart control cards of the internal reference material (IRM) or certified reference material (CRM) of sediment, biota or water and with the reference toxicant, TCDD.

6 Quality assurance

Interlaboratory exercises have shown that quality assurance procedures, including blanks and reference materials, improve the DR-Luc methodology and the quality of data (Besselink *et al.*, 2004). A procedural blank should be included during the extraction of each series of sediments. The procedural blank is a blank sample where all steps of the extraction have been performed. The procedural blank is included in one of the 96-well plates for each set of samples. The results of the DR-Luc analysis of the procedure blank must not be quantifiable (i.e. < 1 pM TCDD TEQ/ well). All extractions must be repeated if the procedure blank is above the limit of quantification. A sample of the DMSO used for the samples should be included on each plate during an assay. The result of the DR-Luc analysis of the DMSO blank is used for correction of the background signal of the samples.

An IRM or CRM should be included in the extraction and clean-up of sediments, biota or water samples. CRMs of sediment or biota are commercially available from SIGMA, NRC Institute for National Measurement Standards. A CRM should be included on every plate of the assay. Results for the sample dilutions should be identical. All observations of a sample from collection through the analysis and final determination should be recorded. One TCDD concentration (3 pM TCDD-TEQ/ well) from the 2,3,7,8-TCDDD calibration set must be used as reference toxicant. This concentration is included on every plate and is used to normalize the response of the DR-Luc cells as well as monitor the vitality of the DR-LUC cells. The response to the reference toxicant and CRM should be noted on a Shewhart card.

Acknowledgements

This document is based on guidelines for DR-Luc in sediments prepared by Besselink (2004), ICES (2005) and RWS-RIKZ (2006a, b). The authors thank the members of the Working Group on Biological Effects of Contaminants and Ian Davis for their input.

7 References

Aarts, J.M.M.J.G., Denison, M.S., Cox, M.A., Schalk, C.M.A, Garrison, P.A., Tullis, K., de Haan, L.H.J., Brouwer A. (1995). Species-specific antagonism of Ah receptor action by 2,2′,5,5′ tetrachloro- and 2,2′,3,3′,4,4′-hexachlorobiphenyl., Eur. J. Pharm Env. Tox. Section 293:463–474.

Aarts J.M.M.J.G. and Palmer R. (2002). Established and deduced aromatic hydrocarbon (Ah) receptor (AhR) agonists and their relative potency. Wageningen University.

American Legislative Exchange Council (2003). Tracking the Dioxin Decline, Washington, DC: March.

Aylward, L.L. and Hays, S.M. (2002). Temporal trends in human TCDD body burden: Decreases over three decades and implications for exposure levels. Journal of Exposure Analysis and Environmental Epidemiology 12, p. 319–328.

Bakker, J.F., Bulzunce-Segarra M.J., Castro R., van de Heuvel-Greve M.,. Klamer H.J.C, Brack W., Altenburger R., *et al.* (2007). Effect directed analysis and toxicity identification evaluation. In: Sediment quality and impact assessment of pollutants. Sustainable Management of Sediment Resources. Chapter 5. D. Barceló, and M. Petrovic (Eds.), Elsevier, Amsterdam, 333 pp., ISBN: 9780-444-51962-7.

Balaam, J.L. and Thomas, K.V. (2007). Bioanalytical characterisation of estrogen and arylhydrocarbon receptor agonists in transplanted blue mussels (*Mytilus edulis*): proof of concept. J. Environ. Monit. 9, 419–423.

Balaam, J.L., Chan-Man, Y., Roberts, P.H., and Thomas, K.V. (2009). Identification of nonregulated pollutants in north sea-produced water discharges. Environ. Toxicol. Chem. 28, 1159–1168.

Besselink, H.T., Schipper C.A., Klamer H.J.C., Leonards P.E.G., Verhaar H.J.M, Felzel E.C., Murk A.J., *et al.* (2004). Intra- and interlaboratory calibration of the DR -CALUX (T) bioassay for the analysis of dioxins and dioxin-like chemicals in sediments. Environ. Toxicol. Chem. 23(12), 2781–2789.

Bosveld, A.T.C., Graneder, J., Murk, A.J., Brouwer, A., Kampen, M. van, Evers, E.H.G. and Berg, M. van den (1995). Effects of PCBs, PCDDs, and PCDFs in common tern (Stern hirundo) breeding in estuarine and coastal colonies in the Netherlands and Belgium. Environ. Toxicol. Chem., 14, 99–115.

Brack, W., and Schirmer, K. (2003). Effect-directed identification of oxygen and sulfur hetero-cycles as major polycyclic aromatic cytochrome P4501 A-inducers in a contaminated sediment. Environ. Sci. Technol, 37(14), 3062–3070.

Brouwer A., Reijnders P.J.H., and Koeman J.H. (1989). Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin-A and thyroid-hormone deficiency in the common seal (*Phoca vitulina*). Aquat Toxicol 15:99–106.

Burgess R.M. (2000). Characterising and identifying toxicants in marine waters: a review of marine Toxicity Identification Evaluations (TIEs). International Journal of Environment and Pollution, 13, pp. 2–33.

Diaz-Baez, M.C., Cruz, L.E., Rodriguez, D., Perez, J., and Vargas, C.M. (2000). Evaluation of Three Water Concentration Techniques as a Prior Step to Test Acute Toxicity. Environ. Toxicology, 15:345-351.

EC (2002): Commission Directive 2002/69/EC, Official Journal of the European Communities, L 209, 5–14.

Engwall, M., Lindström, G., and Bavel, B. Van, (2003). Experiences From An International Intercalibration Of Dioxin-Like Compounds In Cod Liver Using Bioassays. Organohalogen Compounds. 60, 291-294.

Focant, J. F., Eppe, G, and De Pauw, E. (2001). Optimisation and use of tandem-in-time mass spectrometry in comparison with immunoassay and HRGC/HRMS for PCDD/F screening. Chemosphere 43(4-7):417–24.

Foekema, E.M., Deerenberg, C., and Murk, A.J., (2008). Prolonged ELS test with the marine flatfish sole (Solea solea) shows delayed toxic effects of previous exposure to PCB 126. Aquat. Toxicol. 90, 197–203.

Grung, M., Næs, K., Fogelberg, O., Nilsen, A.J., Brack, W., Lübcke-von Varel, U., and Thomas, K.V. (2011). Effects-Directed Analysis of Sediments From Polluted Marine Sites in Norway. Journal of Toxicology and Environmental Health, Part A: Current Issues, 39 –454.

Heilier J.F., F. Nackers, V. Verougstraete, R. Tonglet, D. Lison and J. Donnez, (2005). Increased dioxin-like compounds in the serum of women with peritoneal endometriosis and deep endometriotic (adenomyotic) nodules, Fertil. Steril. 84, pp. 305–312.

Hamers, T., van Schaardenburg M. D., Felzel E. C., Murk A. J. and Koeman J. H. (2000).The application of reporter gene assays for the determination of the toxic potency of airborne particulate matter. Extension of Science of the Total Environment, 262: 159–174.

Hamers T., Leonards P.E.G., Legler, J., Vethaak A. D., and Schipper C.A. (2010). Toxicity Profiling: an effect-based integrative tool for site-specific sediment quality assessment. Integrated Environmental Assessment and Management, Volume 6, 4, pp. 761–773.

Hasegawa J., Guruge K.S., Seike N., Shirai Y., Yamata T., Yamanaka N., and Miyazaki S., (2007). TEQ screening in fishoil using two ELISA kits for dioxin-like PBCs, Proc. DIOXIN 2007 International Symposium.

Henshel D.S. (1998). Developmental neurotoxic effects of dioxin and dioxin-like compounds on domestic and wild avian species. Environmental toxicology and chemistry, Vol 17, 88–98.

Hoogenboom, R., Bovee, T., Traag, W., Hoogerbrugge, R., Baumann, B., Portier, L., van de Weg, G., and de Vries, J. (2006). The use of the DR CALUX bioassay and indicator polychlorinated biphenyls for screening of elevated levels of dioxins and dioxin-like polychlorinated biphenyls in eel. Mol. Nutr. Food Res. 50(10): 945–57.

Houtman, C.J., Cenijn, P.H., Hamers, T., Lamoree, M.H., Legler, J., Murk, A.J., and Brouwer, A. (2004). Toxicological profiling of sediments using *in vitro* bioassays, with emphasis on endocrine disruption. Environ. Toxicol. Chem. 23:32–40.

Houtman, C.J., Booij, P., Jover, E., Pascual del Rio, D., Swart, K., Velzen, M. van, Vreuls, R., *et al.* (2006). Estrogenic and dioxin-like compounds in sediment from Zierikzee harbour identified with CALUX assay-directed fractionation combined with one and two dimensional chromatography analyses. Chemosphere, 65, 2244–2252.

Hurst, M.R., Balaam, J., Chan-Man, Y.L., Thain, J.E., and Thomas, K.V. (2004). Determination of dioxin and dioxin-like compounds in sediments from UK estuaries using a bio-analytical approach: chemical-activated luciferase expression (CALUX) assay Marine Pollution Bulletin Volume 49, Issues 7-8, pp. 648–658.

Hylland K., Aspholm O, Knutsen J. A., and Ruus A. (2006). Biomarkers in fish from dioxin-contaminated fjords, Biomarkers, 11, Issue 2 , pp. 97–117.

ICES. (2005). Report of the Working Group on Biological Effects of Contaminants (WGBEC). ICES Document CM 2005/MHC: 04.

Klamer, H.J.C., Thomas, K., and Tollefsen K.E. (2005a). Extraction procedures applicable to small-scale bioassays. Discussion paper, Working Group on Biological Effects of Contaminants.

Klamer, H.J.C., Leonards P.E.G., Lamoree M.H., Villerius L.A., Åkerman J.E., and Bakker J.F. (2005b). A chemical and toxicological profile of Dutch North sea surface sediments. Chemosphere 58, 1579–1587.

Legler, J., Dennekamp, M., Vethaak, A.D., Brouwer, A., Koeman, J.H., van der Burg, B., and Murk. A.J. (2002). Detection of estrogenic activity in sediment-associated compounds using *in vitro* reporter gene assays. Sci.Total Environ. 293: 69–83.

Legler J., Bulder A.S. , De Voogt P., Leonards P.E.G., and Murk T.A.J. (2006). Bioanalysis of Estrogenic and dioxin-like activity in field samples, in Estrogens and xeno-oestrogens in the aquatic environment: an integrated approach for field monitoring and effect assessment, (editors; Vethaak D., Schrap M., De Voogt P.), SETAC, ISBN-13:978-1-880611-85-2, pp. 121–149, USA.

Leonards, P.E.G., Van Hattum, B. and Leslie, H. (2008). Assessing the risks of persistent organic pollutants to top predators: a review of approaches. Integrated Environmental Assessment and Management, Vol 4, 386–398.

Leslie, H.A., Oosthoek, A.J.P., Busser, F.J.M., Kraak, M.H.S., and Hermens, J.L.M. (2002). Biomimetic solid-phase micro-extraction to predict body residues and toxicity of chemicals that act by narcosis. Environ. Toxicol. Chem., 21(2): 229–234.

Liem A. K. D. and van Zorge J. A. (1995). Dioxins and related compounds: Status and regulatory aspects Environmental Science and Pollution Research, Volume 2, Number 1 / July, pp. 46–56.

Murk A.J., Legler J., Denison M.S., Giesy J.P., Van de Guchte C. and Brouwer A. (1996). Chemical-activated luciferase gene expression (CALUX): A novel *in vitro* bioassay for Ah receptor active compounds in sediments and pore water. Fund. Appl. Toxicol., 33: 149-160.

Murk, A.J., Leonards, P.E.G., Hattum, B. van, Luit, R., Weiden, M.E.J. van der, and Smit, M. (1998). Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbonsin naturally exposed European otters (*Lutra lutra*). Environ. Toxicol. Pharm., 6(2), 91–102.

Nakano, T., Muroishi Y., Takigami H., Sakai S. and Morita M. (2006): Application of simplified analytical methods (for dioxins) that comply with Japanese regulations. Organohalogen Compd., 66, 173–176.

OSPAR (1999). JAMP Guidelines for monitoring contaminants in biota 1999-2. OSPAR.

OSPAR (2002). JAMP Guidelines for monitoring contaminants in sediment 2002-16. OSPAR.

Qiu X. and Davis J.W. (2004). Environmental bioavailability of hydrophobic organochlorines in sediments—A review. Remediation Journal, Vol. 14, Issue 2, pages 55–84.

Rappe C., (1996). Sources and environmental concentrations of dioxins and related compounds, Pure and Applied Chemistry 68 (9), pp. 1781–1789.

RIKZ (2006a). RIKZ/SPECIE-07; Determination of 2,3,7,8-TCDD Toxic Equivalents (TCDD-TEQs) in sediments with the DR-CALUX bioassay by BDS. Part of Handbook for toxicity testing of dredged material, RIKZ Technical Report ISBN 90-369-3493.

RIKZ (2006b). RIKZ/SPECIE-07E; Freeze-drying, extraction and clean-up of sediment samples for the determination of 2,3,7,8-TCDD Toxic Equivalents (TCDD TEQs) using the DR-

CALUX bioassay by BDS. Part of Handbook for toxicity testing of dredged material, RIKZ Technical Report ISBN 90-369-3493. .

Ross P.S., Jeffries S.J., Yunker M.B., Addison R.F., Ikonomou M.G. and Calambokidis J.C., (2004). Harbor seals (*Phoca Vitulina*) in British Columbia, Canada, and Washington State, USA, reveal a combination of local and global polychlorinated biphenyl, dioxin, and furan signals. Environmental Toxicology and Chemistry, Vol. 23, No. 1, pp. 157–165.

Sanderson J.T., Aarts J.M., Brouwer A., Froese K.L., Denison M.S., and Giesy J.P. (1996). Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-O-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicol Appl Pharmacol. 137(2):316-25.

Sato M., Takigami H., Hayakawa K., and Sakai S.(2010).Water-quality monitoring technique for dioxins during dredging using on-site solid phase extraction with graphitic carbon and analysis with DR-CALUX. Journal of environmental science and health. Volume: 45, Issue: 7, Pages: 867-874.

Schipper, C. and Stronkhorst, J. (Eds, 1999). National Institute for Coastal and Marine Management/RIKZ. The Hague, The Netherlands.

Sanctorum H., Windal I., Hanot V., Goeyens L., and Baeyens W. (2007). Dioxin and dioxin-like activity in sediments of the Belgian Coastal area (Southern North Sea). Archives of Env. Cont. and Tox., 52: 317–325.

Schipper C.A., Lahr, J., George, S.G., Hansen, P-D., da Silva de Assis, H.C., Oost, R. van der, Thain, J.E., *et al.* (2009). Application of Biomarkers in Flounder (*Platichthys flesus*) and Bioassays in contaminated estuarine sediments, 66, ICES Journal of Marine Science, 66 (10), pp. 2089–2105.

Schipper, C.A., Burger, R.M., Rietjens, I.M.C.M., and Murk, A.T. (2010). Application of bioassays in toxicological hazard, risk and impact assessments of dredged sediments. Marine Pollution Bulletin 60, 2026–2042.

Shan, G., Leeman, W.R., Gee, S.J., Sanborn, J.R., Jones, A.D., Chang, D.P.Y., and Hammock, B.D. (2001). Anal. Chim. Acta. 444, 169–178.

Stronkhorst J., Leonards P., and Murk A.J. (2002). Using the dioxin receptor-CALUX *in vitro* bioassay to screen marine harbour sediments for compound with a dioxin like-mode of action, Environmental Toxicology and Chemistry, Vol. 21, No. 12, 2552–2561.

Stronkhorst, J., Ariese, F., van Hattum, B., Postma, J.F., de Kluijver, M., Den Besten, P.J., Bergman, M.J.N., *et al.* (2003). Environmental impact and recovery at two dumping sites for dredged material in the North Sea. Environ. Pollut. 124: 17–31.

Ten Hulscher, T.E.M., Postma, J., den Besten, P.J., Stroomberg, G., Belfroid, A., Wegener, J.W., and van Noort, A.C.M. (2003). Tenax extraction mimics benthic and terrestrial bioavailability of organic compounds. Environ. Toxicol. Chem. 22(10): 2258–2265.

Traas, TP; Luttik, R; Klepper, O (2001). Congener-specific model for polychlorinated biphenyl effects on otter (*Lutra lutra*) and associated sediment quality criteria. Environ Toxicol Chem 20:205-212.

Thain, J.E., Hurst M.R., and Thomas K.V. (2006). Determination of dioxin-like activity in sediments from the East Shetland basin. Organohalogens. 68, 185–188.

Thomas. K.V., Hurst M.R., Reynolds W., and Thain J.E. (2006). *In vitro* bioassay testing of produced and surface water extracts. In Biological effects of contaminants in marine pelagic ecosystems (Ed. By Hyllland, Lang and Vethaak), SETAC, ISBN: 13: 978-1-880611-84-8. Belgium.

US EPA (2008): SW-846 Manual. Available at http://www.epa.gov/epaoswer/hazwaste/test/sw846.htm.

Van den Berg M., Birnbaum L., Bosveld A.T., Brunstrom B., Cook P., and Feeley M. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife Environ Health Perspect 106: 775–792.

Van den Brink P.J. and Kater B.J., (2006). Chemical and biological evaluation of sediments from the Wadden Sea, The Netherlands, Ecotoxicology 15, pp. 451–460.

Van Loco J., Van Leeuwen S.P.J., Roos P., Carbonnelle S., de Boer J., Goeyens L., and Beernaert H. (2004). The international validation of bio- and chemical- analytical screening methods for dioxins and dioxin-like PCBs: the DIFFERENCE project rounds 1 and 2 TALANTA, 63 (5), 1169–1182.

WHO (World Health Organization) (2000). Interlaboratory Quality Assessment of Levels of PCBs, PCDDs, and PCDFs in Human Milk and Blood Plasma; fourth round of WHO-coordinated study. WHO, Bilthoven.

Abbreviations and technical terminology

α-MEM	minimum essential medium
ACE	accelerated solvent extraction
AhR	aryl hydrocarbon receptor
AMD-TLC	thin-layer chromatography
BEQUALM	biological effects quality assurance in monitoring programmes
CRM	certified reference material
Cyp 1A	cytochrome P450 A1
CTT	chemical-toxicity-test
DCM	dichloromethane
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DR-CALUX®	dioxin response chemical activated luciferase gene expression
DR-LUC	dioxin response chemical activated luciferase gene expression
DRE	dioxin responsive element
EC50	effective concentration
EDA	effect-directed analysis
EROD	ethoxyresorufin-O-deethylase activity
GPC	gel permeation chromatography
H4IIE	rat hepatoma cells
HRGC	high resolution gas chromatography
HRGCMS	combination high resolution gas chromatography/mass spectrometry
IRM	internal reference material
NP-HPLC	normal-phase high-performance liquid chromatography
PAF	potentially affected fraction of species
PAH	polycyclic aromatic hydrocarbon
PBB	polybrominated biphenyl
PBDE	polybrominated diphenyl ether (brominated flame retardant)
PBT	persistent bioaccumulate toxic
PCB	coplanar polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PDMS	polydimethylsiloxane
PFC	perfluorinated compounds
PFOS	perfluorooctanesulfonic acid
PFOA	perfluorooctanoic acid
REP	relative potencies
RLU	relative light unit
SEC	size exclusion chromatography
SPE	solid phase extraction
SPM	suspended matter
SSD	species sensitivity distribution
SQG	sediment quality guidelines
TCDD	2,3,7,8,-tetrachlorodibenzo-*p*-dioxin
TEQ	international toxic equivalents
TRE	toxicity reduction evaluation
TIE	toxicity identification evaluation
QA	quality assurance
QC	quality control

Author contact information

C.A. Schipper
Deltares
Unit Marine and Coastal systems/ZKS
Rotterdamseweg 185
2629 HD Delft, The Netherlands
cor.schipper@deltares.nl

P.E.G Leonards
Institute for Environmental Studies
VU University
Amsterdam
De Boelelaan 1087
1081 HV Amsterdam, The Netherlands
pim.leonards@ivm.vu.nl

K.V. Thomas
Norwegian Institute for Water Research (NIVA)
Gaustadalléen 21
N-0349 Oslo, Norway
kvt@niva.no

A.D. Vethaak
Deltares
Unit Marine and Coastal systems/ZKS
Rotterdamseweg 185
2629 HD Delft, The Netherlands
dick.vethaak@deltares.nl