



Review

In vitro bioassays for detecting dioxin-like activity – Application potentials and limits of detection, a review



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HIGHLIGHTS

- Bioassays with LODs of up to 0.1 pM 2,3,7,8-TCDD may compete with GC–MS.
- Assay applications are diverse (sediment, soil, water, tissue, food, feedstuff).
- Recombinant cell lines achieve lower LODs than their wild type counterparts.
- A bioassay LOD decides on its application (i.e. serum samples need low LODs).
- In vitro studies should list EC_x, linear working range and the LOD of an assay.

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ABSTRACT

Use of in vitro assays as screening tool to characterize contamination of a variety of environmental matrices has become an increasingly popular and powerful toolbox in the field of environmental toxicology.

While bioassays cannot entirely substitute analytical methods such as gas chromatography–mass spectrometry (GC–MS), the increasing improvement of cell lines and standardization of bioassay procedures enhance their utility as bioanalytical pre-screening tests prior to more targeted chemical analytical investigations. Dioxin-receptor-based assays provide a holistic characterization of exposure to dioxin-like compounds (DLCs) by integrating their overall toxic potential, including potentials of unknown DLCs not detectable via e.g. GC–MS. Hence, they provide important additional information with respect to environmental risk assessment of DLCs.

This review summarizes different in vitro bioassay applications for detection of DLCs and considers the comparability of bioassay and chemical analytically derived toxicity equivalents (TEQs) of different approaches and various matrices. These range from complex samples such as sediments through single reference to compound mixtures. A summary of bioassay derived detection limits (LODs) showed a number of current bioassays to be equally sensitive as chemical methodologies, but moreover revealed that most of the bioanalytical studies conducted to date did not report their LODs, which represents a limitation with regard to low potency samples.

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1. Introduction

Since the middle of the 20th century there has been an increasing concern about exposure of humans and wildlife to certain xenobiotics that were released into the environment due to diverse anthropogenic activities. One group of environmental toxicants that is of particular interest relative to potential environmental health effects are dioxin-like chemicals (DLCs). These ubiquitous compounds are hydrophobic, lipophilic and resistant to biological and chemical degradation, properties that impart persistency and a propensity to bio-accumulate and biomagnify to concentrations that can cause harmful effects. DLCs include polychlorinated dibenzo-*p*-dioxins and dibenzo furans (PCDD/Fs), dioxin-like polychlorinated biphenyls (DL-PCBs), polycyclic aromatic hydrocarbons (PAHs), as well as a multitude of other partially known and unknown compounds (Giesy et al., 2006, 1994b; Larsson et al., 2013; Poland and Knutson, 1982; Song et al., 2006; Van den Berg et al., 2006; Van der Plas et al., 2001). The in vivo behavior of these compounds depends on their uptake, distribution and metabolism (Behnisch et al., 2001a; Safe, 1986) as well as modifying factors such as species, age and reproductive status (Whyte et al., 2000). Hence, the range of biological effects across different organisms is broad. Effects may include thymic atrophy, hepatotoxicity, certain types of cancer, immunotoxicity, wasting syndrome, reproductive toxicity and the induction of monooxygenase enzymes (Brouwer et al., 1995; Denison and Heath-Pagliuso, 1998; Denison and Nagy, 2003; Giesy et al., 1994a; Poland and Knutson, 1982; Van den Berg et al., 1998).

1.1. The aryl hydrocarbon receptor (AhR)

Many studies have proven that most of these toxic effects are mediated via the aryl hydrocarbon receptor (AhR) (Bittner et al., 2006; Hankinson, 1995; Olsman et al., 2007a). More specifically, the AhR, a cytosolic receptor protein, which belongs to a subclass of helix–loop–helix-containing transcription factors (Giesy and Kannan, 1998; Goldstein and Safe, 1989), binds co-planar aromatic compounds with high affinity and translocates them into the nucleus where the complex forms a heterodimer with the AhR nuclear translocation (ARNT) protein and possibly additional factors (Hahn, 1998). The ligand–AhR–ARNT complex binds to dioxin responsive elements (DRE) in genomic sequences, which leads to transcriptional activation and synthesis of responsive genes like cytochrome P₄₅₀1A (CYP1A) (Hilscherova et al., 2000). Cytochromes represent a multigene family of heme-containing proteins, which are mainly present in the liver, but also in kidney, gastrointestinal tract, gills and other tissues of many organisms. They

possess the ability to metabolize xenobiotics via Phase-I-reactions (oxidation, hydrolysis or reduction reactions), which may lead to a detoxification or to a so-called bioactivation (toxication) (Castell et al., 1997).

1.2. CYP and reporter gene based in vitro assays

The specific and naturally occurring mechanism of CYP1A induction by DLCs has been used in in vitro bioassay techniques for the characterization of dioxin-like potentials of e.g. environmental samples (Tillitt et al., 1992, 1991a). However, as for in vivo effects, the responsiveness of in vitro systems is species or cell-line specific (Keiter et al., 2008). This is due to differing binding affinities, structures, quantities and physicochemical properties of the AhR of different cell lines (Hilscherova et al., 2001; Sanderson et al., 1996). Regarding functional AhR-based bioassays for quantification of CYP1A activity (such as the 7-ethoxyresorufin-O-deethylase assay, EROD), the dioxin-like potential of DLCs present in a certain sample is determined by quantifying the induction of the cytochrome P₄₅₀ (CYP) monooxygenase system (in the present case: the activity of the EROD enzyme) (Sanderson et al., 1996). The EROD assay has been applied using different cell lines, such as permanent fish cell line RTL-W1 (rainbow trout liver - waterloo 1) or rat hepatoma cell line H4IIE (here the assay is called “micro EROD”), which led to the title “golden standard of in vitro bioassays” (as reviewed by Behnisch et al. (2001b)).

In some cases, however, CYPs like EROD can be inhibited by their own substrates (e.g. in the presence of high concentrations of PCBs) (Sanderson and Giesy, 1998), which may lead to false-negative results. Moreover, the linear working range of EROD activity based test systems is often limited (Behnisch et al., 2001b). To overcome these issues, the process of AhR mediated activation of genes has been genetically engineered by connecting the DRE of various cell lines with certain reporter genes (Lee et al., 2013). These genes may originate from firefly (*Photinus pyralis*) or from sea pen (*Renilla reniformis*) and by activation are capable of producing the light emitting enzyme luciferase (Denison et al., 1988b, 1988a; Garrison et al., 1996; Thain et al., 2006).

Examples for those reporter gene based assays are the DR CALUX® (Dioxin Responsive–Chemical Activated LUCiferase gene eXpression) with mammalian hepatoma cell lines transfected with plasmid pGudLuc1.1, the H4IIE-luc assay using an eponymous cell line, the CALUX assay (mostly performed by using Hepa 1 mouse hepatoma cell line) (Villeneuve et al., 1999) and the P₄₅₀ reporter gene system (RGS) assay, which constitutes a related methodology by using HepG2 cells, stably transfected with a human CYP1A1 promoter sequence fused with the already mentioned firefly luciferase reporter gene (101

L cells) (Postlind et al., 1993). A further reporter gene assay is the CAFLUX (Chemical Activated FLUorescent protein eXpression) assay, which instead of luciferase utilize enhanced green fluorescent protein (EGFP), a tripeptide (fluorophore) from jellyfish (*Aequorea victoria*). Its functioning without further reagent addition lowers the assay costs (Nagy et al., 2002).

1.3. Toxicity equivalents (TEQs)

Commonly, results of in vitro bioassays are expressed in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) equivalent quotients (TEQs or bioTEQs) (Ahlborg et al., 1994; Safe, 1990). In this way, bioassay-derived results become comparable with those of instrumental analysis (chemTEQs). The bioTEQ puts the AhR inducing potential of e.g. an environmental sample in relation to the AhR induction potency of the reference compound 2,3,7,8-TCDD (Brunström et al., 1995; Engwall et al., 1996; Safe, 1998; Van den Berg et al., 1998, 2006). The x in Eq. (1) thereby represents the chosen effect level, which most often is the EC₂₅ or the EC₅₀ value.

ChemTEQs are calculated by multiplying concentrations of all single compounds (i) in an extract with their specific toxicity equivalent factor (TEF). The TEF value is a relation of the AhR inducing potential of a single compound to that of 2,3,7,8-TCDD, which has a TEF of 1. WHO-TEF values have been derived using careful scientific judgment after considering all available scientific data. This data originated from different mammalian, bird and fish studies, which were performed since 1993 with several DLCs (Van den Berg et al., 1998). Those substances, according to the WHO, met the following criteria: (a) the compound must share certain structural relationships with the PCDD/Fs; (b) it must bind to the aryl hydrocarbon receptor (AhR); (c) it must elicit AhR-mediated biochemical and toxic responses; and (d) it must be persistent and accumulate in the food chain (WHO, 1998). However, WHO TEF estimates are partially based on in vivo experiments, and thus, processes such as uptake and tissue distribution, which are negligible in cell based assays, may not be a good representation of the relative potency (REP) specific to the cell system used (Brown et al., 2001). For this reason, REPs in general possess a better alternative compared to more unspecific TEFs. This conclusion is supported by several studies (Hilscherova et al., 2001, 2003; Kannan et al., 2008). Even though recently a number of cell line specific REP values were proposed for many single DLCs, there is still a lack of REPs for cell lines that are used less extensively. An important factor for reproducibility and applicability of cell line specific REP values is their origin. Sometimes scientists do relate them to the EC₂₀ or the EC₅₀ value, which will have a significant impact on the application of REP-based chemTEQs due to the different slopes (non-parallelism) of dose–response curves of different chemicals. Therefore it is important to use the same effect levels in the bioassay as was used when determining the REPs.

By summing the calculated TEQs of all single compounds present in an environmental sample, an overall chemTEQ can be calculated as shown in Eq. (2), where i represents each single compound. In case REP values are used for chemTEQ calculation, x represents the chosen bioassay and its respective REP for each compound i .

$$\text{bioTEQ}_{[\text{pg/g}]} = \frac{\text{TCDD EC}_x [\text{pg/ml}]}{\text{extract EC}_x [\text{g/ml}]} \quad (1)$$

$$\text{chemTEQ}_x [\text{pg/g}] = \sum \text{conc}_i * \text{TEF}_i \left(\text{REP}_{i(x)} \right). \quad (2)$$

TEQ values in the case of solids are expressed as pg TEQ/g dry mass (dm) of e.g. sediment. An exemplarily value of 1 pg TEQ/g dm of sediment hence would state that 1 g dm sediment has the same effect as if it contained 1 pg of 2,3,7,8-TCDD. By interpreting TEQs, one has to keep in mind that they neither provide any specific information regarding

toxico-kinetic properties of chemicals present in a mixture (shapes/slopes of their concentration–response curves), nor about the tested species used to calculate the TEFs. Care must be taken when comparing TEQs of different studies, as the underlying effect levels (i.e. the EC₂₅) are frequently not stated.

Significant differences observed between chemTEQs and bioTEQs can be due to several reasons: In vitro bioassays integrate the overall gene activating effect of all AhR agonists and antagonists present in a mixture, while instrumental analyses focus on a selected numbers of known DLCs. Hence, non-classical and unknown AhR inducers are not taken into account. Moreover, the TEQ concept assumes additivity of single DLCs, but AhR ligands can be agonistic, antagonistic or synergistically (Windal et al., 2005). While bioassays measure one biological endpoint, instrumental results are calculated by using TEF values, which – on the contrary – are obtained from in-depth toxicity studies (Sanctorum et al., 2007). However, bioanalytical and instrumental results are most often correlated and while bioassays are well-suited screening tools for large sample numbers, which do allow for prioritization of e.g. sediment contamination, chemical analysis allows to pinpoint the actual chemicals responsible for a biological effect.

1.4. Detection limits of in vitro bioassays and chemical analysis

In most scenarios DLCs are considered trace contaminants, and are present in parts per billion down to parts per trillion quantities in environmental matrices (Rappe, 1984). Therefore, the question arises whether in vitro biotests are equal or less sensitive compared to chemical analytical investigation techniques.

In vitro bioassays have been widely accepted and used for the analysis of these contaminants. But, one of the potential limitations of in vitro bioassays for the detection of dioxin-like potentials has been their lower sensitivity (i.e., greater detection limits) compared to some chemical analytical approaches, which often resulted in their inability to meet analytical goals or regulatory guidelines (Zhao et al., 2010). Regarding foodstuff analysis, the legally permitted threshold values for DLCs are less than those for DLCs in the environment (e.g. sediments). For this reason, bioassays most often cannot replace chemical analysis in context with food safety assessments because their limits of detection (LOD) can be orders of magnitude greater than those of chemical analytical technologies (Simat, 2007). The LOD of a bioassay depends on several factors, such as chosen test conditions, including temperature, solvent and duration of exposure or the type of endpoint used (CYP measured via fluorescence/luciferase measured via luminescence). This reversely applies that it can be improved by changing or varying these factors, which will be considered in detail in the LOD chapter of this review.

2. In vitro bioassays and fields of application

A multitude of national and international studies have been conducted that focused on the determination of dioxin-like activities using in vitro bioassays with a wide variety of sample types. Here we want to distinguish two major categories of samples, including (1) *complex samples* and (2) *individual compounds and mixtures*.

- (1) *Complex samples* may include sediments, suspended particulate matter (SPM), soil, surface and ground water, domestic and industrial waste-waters, sewage sludge, industrial emissions (air particulate matter, APM) as well as human blood, animal tissue and food and feed for the examination of the potential risks DLCs pose to humans and wildlife.
- (2) To attribute the integrated overall dioxin-like potential (measured in a complex sample) to particular compounds, several studies were conducted to determine the relative potencies (REPs) of *pure reference compounds*. In addition, studies with *specific compound mixtures* were conducted to elucidate possible

interactions between chemicals and how they would influence the overall net effect compared to the typically assumed additive interaction.

2.1. Sediments

Since sediments maybe an important sink and source of DLCs, the evaluation of polluted sediments is an integral part of sediment risk assessment and a popular field of environmental science. A host of studies was conducted that investigated the dioxin-like activity of sediments of various rivers, tributaries or small streams (Behnisch et al., 2010; Chen et al., 2010; David et al., 2010; Heimann et al., 2011; Hilscherova et al., 2001, 2003; Hollert et al., 2002; Huuskonen et al., 2000; Kannan et al., 2008; Keiter et al., 2008; Kinani et al., 2010; Koh et al., 2004; Murk et al., 1996; Song et al., 2006; Soares Rocha et al., 2010; Windal et al., 2005; Wölz et al., 2010a, 2010b, 2008). Others focused on sediments from lakes (Engwall et al., 1998; Hofmaier et al., 1999; Khim et al., 1999b; Koh et al., 2005) and coastal areas (Anderson et al., 1999; Chen et al., 2010; David et al., 2010; Gale et al., 2000; Hurst et al., 2004; Kannan et al., 2008; Khim et al., 1999a; Koh et al., 2004, 2002; Sanctorum et al., 2007; Song et al., 2006; Thain et al., 2006; Wölz et al., 2009) or screened the potential of DLCs in SPM (Engwall et al., 1996, 1997; Koh et al., 2004; Kosmehl et al., 2004; Veilens et al., 1992). Soil and sediment organic matter constituents have also been investigated (Bittner et al., 2006; Larsson et al., 2013).

Most of these studies related their bioTEQs to chemical analytically determined concentrations of DLCs in the same matrix. BioTEQs and chemTEQs of acid-treated extract fractions thereby most often were in good accordance.

Different approaches have been developed to progressively enhance the comparability of biologically measured potentials and instrumentally determined quantities of DLCs. In order to distinguish between rapidly metabolized PAHs and more persistent compounds (e.g. dioxins and PCBs) that remain highly active after elongated exposure times, two studies conducted the EROD assay with PLHC-1 cells using two different exposure times (4 and 24 h, respectively) (David et al., 2010; Kinani et al., 2010). Both used benzo[a]pyrene (BaP) as standard in the 4-hour-exposure and 2,3,7,8-TCDD as standard in the 24-hour-exposure experiments. TEQs as well as BaP equivalents (BEQs) (both based on EC₂₀ values, which were proven to show smaller variability compared to EC₅₀ values) were in good accordance with chemical findings in both studies (TEQs $r = 0.84$ and 0.97 , BEQs $r_2 = 0.98$ and 0.99 , respectively). Other scientists, who used a P₄₅₀ reporter gene system (RGS assay) with the cell line 101 L first correlated their bioassay results with total PAHs ($r_2 = 0.47$), but finally found a much better correlation with BEQs ($r_2 = 0.63$) (Andersson et al., 1999). Other studies documented bioTEQs (between 3.62 and 7.92 ng TEQ/g dm sediment), which were not correlated with their respective chemTEQs, which only accounted for approximately 5% of bioassay-derived potentials (Heimann et al., 2011). Here the authors concluded that bioTEQs were dominated by PAHs and unidentified pollutants. These findings were supported by many others (Gale et al., 2000; Hilscherova et al., 2001; Hurst et al., 2004; Keiter et al., 2008; Sanctorum et al., 2007; Song et al., 2006; Soares Rocha et al., 2010), which supports the approach of BEQs with BaP as positive control as applied by David (2010) and Kinani (2010). But care has to be taken regarding the use of BaP as a control because unlike 2,3,7,8-TCDD, the potencies of BaP and other PAHs are sensitive to culture conditions, which indicates that the BEQ approach appears to be more variable compared to the TEQ approach (Bols et al., 1999).

The fact that acid labile compounds may affect the comparability between bioassay and chemical analytical results indicates additional clean-up to be necessary when investigating complex environmental samples. A need for such clean-up procedures was proven by a study that correlated chemTEQs (EC₂₀) with H4IIE-luc bioTEQs of both crude and cleaned-up extracts (Hilscherova et al., 2003). While the correlation

between chemTEQs and bioTEQs of crude extracts was moderate ($r_2 = 0.72$), a good correlation was observed among TEQs of the cleaned-up extract ($r_2 = 0.94$). Nevertheless, care has to be taken during the clean-up of extracts. A study by Villeneuve et al. (2002) indicated that following a 1 h treatment with concentrated H₂SO₄ acid-breakdown products of PAHs and other compounds were formed, which produced dioxin-like responses in vitro. This indicates that a longer acid treatment (the authors suggested a duration of 10 h or longer) followed by a water rinse should serve as an effective method to completely eliminate dioxin-like responses caused by the acid labile fraction (Villeneuve et al., 2002).

When focusing on sediments as DLC containing matrixes, many of the abovementioned studies have shown the ability of various in vitro bioassays to detect contamination sources. For instance, Hilscherova et al. (2003) could identify a 10–100-fold greater concentration of H4IIE-luc derived bioTEQs downstream the Tittabawassee River than those determined upstream. The same trend (5- to 10-fold) was observed for soils of the respective associated river banks. Both results were confirmed by instrumental analysis, which revealed that PCDD/Fs were the critical contaminants causing the dioxin-like activity observed via the H4IIE-luc. The same bioassay indicated contamination sources of sediments and floodplain soils of the Saginaw River, Michigan, USA, which exceeded the screening concentration of 50 pg TEQ/g dm soil that was suggested by the Agency for Toxic Substances and Disease Registry ATSDR (Kannan et al., 2008). Other studies using mass balance analysis also reported successful identification of causative substances using bioanalytical techniques (H4IIE-luc assay) by testing different extract fractions and comparing those with chemical analytical determined results (Koh et al., 2004; Otte et al., 2013). Thereby, PCDD/Fs were found to be responsible for the majority of the dioxin-like activity measured in sediment extracts of the Hyeongsan river, Korea, while PCBs and PAHs contributed a relatively small proportion to the overall activity (Koh et al., 2004). In the contrast, the 16 EPA PAHs explained between 47 and 118% of the H4IIE-luc assay derived bioTEQs in sediment extracts of the Elbe River, Germany (Otte et al., 2013). By comparing the H4IIE-luc assay with 3 other assays (EROD with H4IIE wild type (wt) and PHLC-1 wt and CALUX with RLT2.0), was the least variable and most sensitive biotest and lead to similar conclusions as those that would have been made based on extensive instrumental analyses (Hilscherova et al., 2001).

Extracts of coastal sediments, including samples from German (Wölz et al., 2009), Scottish (Thain et al., 2006), Belgian (Sanctorum et al., 2007), French (David et al., 2010) and UK coastal areas (Hurst et al., 2004), as well as samples from various bays alongside the USA (Anderson et al., 1999; Gale et al., 2000; Kannan et al., 2008), Korea (Khim et al., 1999a; Koh et al., 2004, 2005) and Japan (Kannan et al., 2008), revealed significant dioxin-like activity. For sediment extracts from the North Sea general low contamination levels were observed (around 0.1 pg CALUX-TEQ/g sediment) while at the mouth of two rivers – the Yser and the Scheldt – 100-fold greater concentrations were measured (10–42 pg CALUX-TEQ/g sediment) (Sanctorum et al., 2007). In a study of Baltic Sea sediment cores a combinatory approach applying bioassay (EROD with RTL-W1) and chemical analytical methods indicated a significant hazard potential at site. The authors hypothesized that benthic organisms or animals living in close contact these sediments might be at risk (Wölz et al., 2009). A different study that compared results obtained by screening both cleaned-up and whole extracts of sediments from the East Shetland basin using the DR-CALUX® determined dioxin-like potentials in some areas that were potentially harmful to organisms (Thain et al., 2006). Those areas, according to the authors, require targeted chemical analyses of a range of known potential candidate compounds to identify the causative agents. According to results obtained by the DR CALUX® in combination with a clean-up procedure the vast majority of the dioxin-like activity in the East Shetland sediments was attributable to labile compounds such as PAHs (Thain et al., 2006). Equal results were obtained

by Hurst et al. (2004) for sediments sampled along the coastal line of the United Kingdom. BioTEQs ranged between 1.0 and 106.0 pg CALUX-TEQ/g dm sediment and the majority of sediments contained levels of DLCs above concentrations that are considered to possess a low risk to aquatic organisms. Like for the previously mentioned studies, stream and inland sampling locations from Korean coastal areas were found to contain greater concentrations of DLCs than offshore sites, as was identified by using the H4IIIE-luc assay (Koh et al., 2005). When bioTEQs were compared to chemical instrumental findings it was found that bioTEQs were consistently greater than chemTEQs. On average, the known concentrations of DLCs present in the extracts accounted for only 30% of the total bioassay responses observed.

Some studies investigated extracts of typical sediment constituents to evaluate the potential interaction of these with a number of bioassays. For example, Bittner et al. (2006) used the EROD and CALUX assays with the wild type and genetically engineered H4IIIE cell line to investigate the dioxin-like potential of humic acids (HA). They reported that different treatments of HA (organic extraction, alkali solution) resulted in different dioxin-like potentials in both assays, which was unexpected due to the missing dioxin-like structure of HA. The calculated REP_{HA} was 6×10^{-8} and, thus, equates an environmental relevant concentration. These findings again illustrate the presence of numerous unknown AhR ligands in environmental samples.

In summary, the abovementioned examples show that in vitro bioassay methodologies constitute an important tool in support of environmental risk assessments. Moreover, most of the results suggest that instrumental chemical analysis alone (based on the concentrations of identified target analytes) cannot completely estimate the total dioxin-like potency of DLCs. However, care needs to be taken when using bioassays to assess dioxin-like activities of sediment extracts due to potential interactions of non-dioxin-like components with these tests.

2.2. Soils

One particular topic of interest in context with the assessment of DLCs in soils is the deposition of such contaminants on floodplains during flood events. Such studies are often closely related to those focusing on river sediments. For instance, floodplain soils from the river Rhine, Germany (Wölz et al., 2011), Saginaw and Shiawassee Rivers and Saginaw Bay, Michigan (Kannan et al., 2008) and those collected along the Tittabawassee River, Michigan, USA (Hilscherova et al., 2003) were investigated as part of environmental risk assessments of DLCs in these watersheds. All these studies revealed SPM deposited during flood events to cause contamination of inundated sites. Related studies are those focusing on agricultural soils that are in proximity to electronic waste recycling sites, such as the Taizhou area, China (Shen et al., 2009, 2007). In the case of the study of Shen et al. (2007), chemTEQs and bioTEQs correlated well ($r_2 = 0.96$) and PCBs were proven to cause 98% of the dioxin-like potential in the Taizhou area. Anderson on the other hand, who investigated clayey soils contaminated with PAHs that were collected from an old gasworks plant in Sweden with respect to a large-scale bioremediation (Andersson et al., 2009). By using the CALUX assay, they could prove an increasing dioxin-like potential in bioavailable fractions after 274 days of soil remediation (compared to day 0), which according to the authors was most likely was caused by a chemically detected release of previously sorbed PAHs.

2.3. Sewage sludge

Since tons of sewage sludge are produced worldwide every year and the capacity for incineration does not fill the demands, sludge has been used for landfilling or as fertilizer on farmland. In doing so, its release may cause a threat to the environment because a multitude of environmental contaminants can remain in the sludge after their removal from waste water (1999). Therefore, the ecotoxicological investigation of

sewage sludge is of great relevance in studies concerned with dioxin-like activities. For instance, Hofmaier et al. (1999) analyzed sewage sludge extracts originating from two waste water treatment plants (WTPs) in Selbitztal (Germany) via the micro-EROD, whereas Engwall et al. (1999) used the chicken embryo hepatocyte (CEH) bioassay to determine the dioxin-like potential of sewage sludge from different WTPs in Sweden. Both studies concluded the combination of bioassays and chemical analysis to be a well suited tool for the screening of organic residual materials. The DR CALUX and the EROD were used to investigate pharmaceutical-containing sewage sludge from Sweden. The authors could prove that an anaerobic treatment caused an increase in the levels of acid resistant AhR agonists, while an aerobic treatment did not affect the levels of these agonists (Gustavsson et al., 2007, 2004). Additionally, the uptake of DLCs in carrots, oilseed rape seeds, zucchinis and cucumbers grown in soil amended with sewage sludge from those Swedish WTPs was estimated. A sewage sludge-amendment in moderate application rates (below 10 t dm/ha) did not yield notably high carrot DLC concentrations, but the authors pointed out that a risk estimation is complicated due to a missing correlation between application rates and sludge-borne DLCs and their resulting concentrations in carrots (Engwall and Hjelm, 2000).

2.4. Water

There are many studies that have investigated dioxin-like potentials in different sample types, including ground (Schirmer et al., 2004a), waste (Kobayashi et al., 2003; Ma et al., 2005; Shen et al., 2001; Zacharewski et al., 1995), pore (Koh et al., 2004, 2002; Murk et al., 1996), stream (Shen et al., 2001; Villeneuve et al., 1997) and surface water (Rastall et al., 2004). Ground water can be used to analyze the mobility of pollutants present in soils of e.g. industrial areas and their possible transition into the ground water body. Ground water samples in the area of Zeitz, a large contaminated site in Germany where oil and lignite were refined to produce fuel, lubricants and benzene all caused EROD induction in RTL-W1 cells, which demonstrated that in vitro bioassays can be used as an early warning tool to initiate a more detailed cause-analysis and to guide subsequent chemical identification in water samples (Schirmer et al., 2004b). A study that investigated industrial and municipal wastewater-containing lake water samples (Taihu Lake, China) reported CALUX-TEQ values between 134 and 232 pg/l, which exceeded the US EPA national primary drinking water standard's maximum contaminant level of dioxin (30 pg/l) by a factor of 4.5–7.7 (Shen et al., 2001). Some studies determined the AhR inducing potential of bioavailable DLCs sampled using semipermeable membrane devices (SPMDs) serving as passive samplers for lipophilic chemicals such as DLCs in river water (Villeneuve et al., 1997). It could be demonstrated that this approach was well suited to estimate the risk posed by DLCs to fish. Sediments and pore water from several locations in the Netherlands were screened for their ability to induce AhR mediated gene expression in H4IIIE cells using the EROD and CALUX assays. The luciferase inducing potential (CALUX) of organic extracts from 450 mg sediment aliquots or 250 μ l pore water aliquots corresponded well with the instrumentally determined degree of pollution of the sediment with DLCs. The authors furthermore pointed out that the usage of pore water as a matrix in DLC studies has the advantage to be more rapid due to the need for fewer clean-up steps (Murk et al., 1996).

2.5. Human blood, food and feed

Due to their lipophilic nature and low degradability, many DLCs accumulate in animal and human tissues up to concentrations, which can cause adverse effects. Blood samples therefore widely have been evaluated using bioassays. Specifically, blood serum levels of AhR ligands in different human populations (Long et al., 2006; Olsman et al., 2007b; Pauwels et al., 2000; Schechter et al., 1999; Van Wouwe et al., 2004). Because the main exposure route to dioxins and related compounds for

humans is through the diet (Fent, 2007), the characterization of DLCs in food and feed represents an important tool for human health risks assessment of these chemicals. Many of the studies conducted to date were concerned with bioassay investigation of fish (e.g. *retail fish from local markets in China and supermarkets in Japan*, as well as *fish oil from North Sea herring and fish oil used for feed ingredients from several manufacturers in Japan* (Hasegawa et al., 2007; Nording et al., 2005; Tsutsumi et al., 2003; Wei et al., 2010)).

2.6. Air emissions and combustion products

Air emission samples originate from various sources and recently belong to the popular fields of research of DLCs (Arrieta et al., 2003; Clark et al., 1999; Franzén et al., 1988; Gierthy and Crane, 1985; Hamers et al., 2000; Hofmaier et al., 1999; Kasai et al., 2006; Klein et al., 2006; Kobayashi et al., 2003; Li et al., 1999; Mason, 1994; Till et al., 1997). Klein et al. (2006) investigated gas and particulate phases from ambient air, sampled in an urban and rural location in the Greater Toronto Area, Canada via the AhR assay with H1L6.1c1 cells. They found a distinct correlation between the AhR binding potency and the concentration of PAHs, as ascertained by other studies of APM such as traffic exhaust (Hamers et al., 2000), vehicle exhaust and urban air (Arrieta et al., 2003; Franzén et al., 1988; Hamers et al., 2000; Klein et al., 2006; Mason, 1994). Moreover, according to the authors, it was the first study in which APM was sampled between seasons over two years (Klein et al., 2006). A further interesting attempt of this topic was the investigation of AhR ligands in cigarette smoke. The results indicated that there were more AhR ligands in the smoke of one cigarette (10 mg tar) than expected. Levels of one cigarette exceeded the tolerable daily intake (TDI) of dioxin (1–4 pg/kg/d) suggested by the WHO up to 656 times (Kasai et al., 2006).

2.7. Individual compounds and mixtures

As mentioned above, the use of assay-specific REP values can enhance the comparability between chemical and bioanalytical results when assessing DLCs in environmental or human samples. A series of studies that investigated the correlation among different bioassays moreover demonstrated that they can be in good accordance when screening single reference compounds. Hence, the continuing determination of dioxin-like potencies of single compounds with various different cell lines is essential.

One compound class that already has been analyzed in this context is PAHs (Behnisch et al., 2003; Bols et al., 1999; Kennedy et al., 1996a; Machala et al., 2001; Villeneuve et al., 2002). For example, Machala et al. (2001) investigated 30 individual PAHs using the CALUX assay with two different exposure times (6 and 24 h) in order to characterize their metabolism in vitro. The authors measured the largest DLC potential after 6 h of exposure time.

The substance class of PCBs has also been explored regarding their dioxin-like potential using in vitro bioassays (Aarts et al., 1995; Behnisch et al., 2003; Brown et al., 2001; Kennedy et al., 1996a; Sanderson et al., 1996; Schneider et al., 1995; Tillitt et al., 1991b; Zeiger et al., 2001). In the process, both DL-PCBs (*mouse hepatoma cell line H1L1* (Brown et al., 2001), *human hepatoblastoma cell line HepG2* (Zeiger et al., 2001), *rat hepatoma cell line H4IIE* (Tillitt et al., 1991b), *primary cell cultures of CEH* (Kennedy et al., 1996b)) and non-dioxin like PCBs (NDL-PCBs) (*CEH* (Kennedy et al., 1996b)) were investigated, as well as several NDL-PCBs in combination with DL-PCBs in order to discover possible interactions among the two categories. In doing so, some studies could prove antagonistic effects of certain NDL-PCBs on their dioxin-like counterparts (Aarts et al., 1995; Sanderson et al., 1996).

Various congeners of PCDD/Fs (Brown et al., 2001; Garrison et al., 1996; Murk et al., 1996; Tillitt et al., 1991b; Villeneuve et al., 2000) as well as brominated and fluorinated analogs (Behnisch et al., 2003; Brown et al., 2001; Olsman et al., 2007a; Samara et al., 2009) or nitro-

(Schneider et al., 1995), methyl- and alkyl-substituted (Behnisch et al., 2003) analogs were investigated. Polychlorinated naphthalenes (PCNs) were frequently analyzed (Behnisch et al., 2003; Blankenship et al., 2000; Hanberg et al., 1991; Schneider et al., 1995; Villeneuve et al., 2000) and found to be equally active as enzyme inducers as certain DL-PCBs (Hanberg et al., 1991). Furthermore, commonly used flame retardants, the polybrominated diphenyl ethers (PBDEs) were proven to act dioxin-like (Behnisch et al., 2003; Chen et al., 2001; Hanberg et al., 1991; Schneider et al., 1995). In one study, bioTEQs of the chicken embryo hepatocyte (CEH) EROD of single tested PBDEs correlated well with those obtained by using the micro-EROD assay ($r_2 = 0.89$) (Chen et al., 2010).

Tetrachlorostilbenes, polychlorinated azobenzenes, azoxybenzenes, trans-stilbenes (Schneider et al., 1995), β -naphthoflavone (Lee et al., 1993), NSO-heterocyclic PAHs (Hinger et al., 2011), DDT metabolites (Wetterauer et al., 2012) as well as pentabromophenols (PBPs) (Behnisch et al., 2003; Schneider et al., 1995) were investigated sporadically.

3. Limit of detection

3.1. Limit of detection and limit of quantification in instrumental chemical analysis

In terms of instrumental chemical analysis, the LOD is defined as the lowest concentration of an analyte in a sample that the analytical process can reliably detect (MacDougall and Crummett, 1980), meaning that the signal of the analyte is statistically different from a blank (Bradlaw et al., 1980; Keith et al., 1983). Various methods have been described to calculate the LOD (Currie, 1968; Mandel and Stiehler, 1957). According to the IUPAC gold book, the LOD of an instrumental analysis is calculated by the mean of the blank measures plus the standard deviation of the blank measures multiplied by a numerical factor chosen according to the confidence level desired (IUPAC, 2006) (Fig. 1). The majority of studies set this numerical factor to a value of three standard deviations, but in general this value depends on the definition used. In the case that a single sample is analyzed for which there is no blank data, the LOD of chemical methods is based on the peak to peak noise measured on the base line close to the actual or expected analyte peak (MacDougall and Crummett, 1980).

The limit of quantification (LOQ) is frequently calculated by the mean of the blank plus ten times the standard deviation (Keith et al., 1983) or, in rare cases, only six times the standard deviation (Bradlaw et al., 1980). It is defined as the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated operational conditions of the used method (e.g. bioassay or high resolution (HR) GC-HRMS) (Whyte et al., 2004). Only signals above the LOQ can be quantified (Fig. 1). Signals >LOD but <LOQ are significantly detectable but not quantifiable. Signals less than the LOD hence should be reported as not detected (ND) with the limit of detection given in parentheses. Signals in the region of detection should be measured and reported as numbers with the limit of detection in parentheses (MacDougall and Crummett, 1980).

3.2. Limit of detection and limit of quantification in bioanalytical analysis

Since most in vitro bioassay studies compared their results with those obtained by instrumental chemical analysis, the bioassays LOD should also be stated.

In terms of in vitro bioassays, the definition of the LOD is very similar to that of chemical analytical methods with two exceptions: (1) the signal of an analyte equates the bioassay specific endpoint, which may be measured as fluorescence (e.g. in the EROD or CALUX assays) or luminescence (e.g. in the DR CALUX or CALUX assays); and (2) the blank to which the LOD definition refers to equates the negative control or the solvent control (e.g. dimethyl sulfoxide (DMSO), isoctane and/or

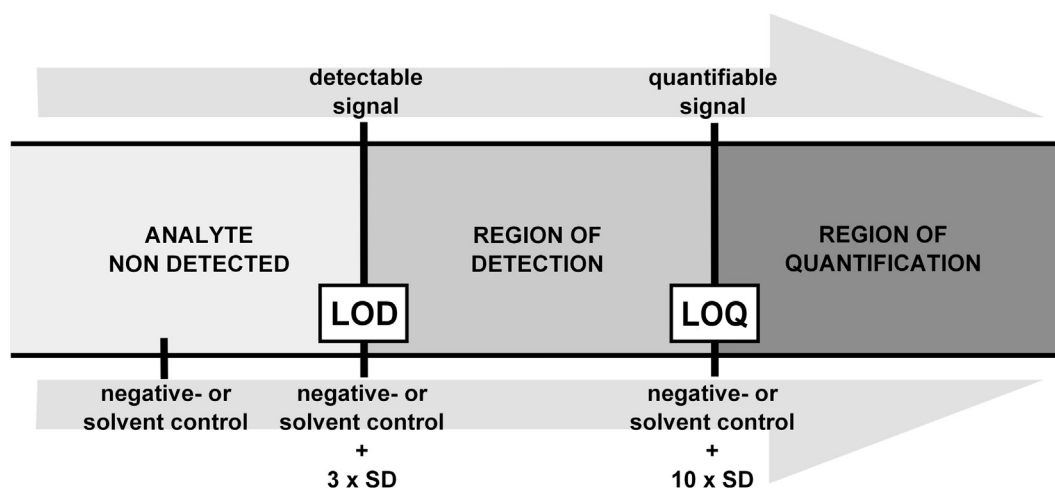


Fig. 1. Schematic diagram of limit of detection (LOD) and limit of quantification (LOQ) as well as their determination, regions of detectable, quantifiable and non-detectable analyte signals, SD = standard deviation, diagram modified according to MacDougall and Crummett (1980).

isopropanol) of the bioassay (Fig. 1). For instance, Murk and et al. (1996) who investigated sediments and pore water from the Netherlands via the CALUX assay set their blank value at the DMSO response. As a consequence, the LOD of most studies is expressed as the effect of the lowest concentration of the standard (the standard is typically 2,3,7,8-TCDD) that can be statistically separated from the effect of the control.

The LOQ of a bioassay can be defined according to chemical analysis, with two exceptions: (1) the blank again equates a negative or a solvent control; and (2) the signal level of a sample can only be related to the same signal level caused by the positive control e.g. 2,3,7,8-TCDD (Fig. 1). The respective concentration of 2,3,7,8-TCDD, which is necessary to cause this certain level is used to describe the potency of the sample. Thus, bioassay results can just be expressed as equivalents instead of actual concentrations.

According to our literature review, most of the *in vitro* bioassay studies used to quantify DLCs did not report their LOD or LOQ. However, this additional information is of critical importance as it enables scientists to decide whether the presented assay and the respective cell line reach the sensitivity goals required for e.g. the screening of samples with very low levels of DLCs (Whyte et al., 2000, 2004).

3.3. Chemically and bioassay derived LODs

Conventional GC–MS analysis has been able to achieve detection limits for 2,3,7,8-TCDD in the range of 1 pg/g (Rappe, 1984), or more recently, LODs in the parts per quadrillion range (Fernandes et al., 2004; Focant et al., 2002; Patterson et al., 1987; US-EPA, 1994). This might in parts be similar to LODs achieved by biodetection methods including the micro-EROD, the DR CALUX and the CEH assay (Behnisch et al., 2001b). For instance, the limits of quantification derived from CALUX and HRGC/HRMS in a study focusing on animal feed were approximately 0.50 pg CALUX-TEQ/g lipid and 0.25 pg chemTEQ/g lipid, respectively (as reviewed by Behnisch et al. (2001b)). This is supported by Schoeters et al. (2004), who performed the CALUX assay and reported LOD and LOQ values similar to those of chemical analysis.

The actual LOD of a compound (as measured via chemical analysis) differs from the so-called method LOD, which in the case of bioanalytical methods is, among others, influenced by matrix effects in a complex mixture (Bhavasar et al., 2007). These matrix effects can be caused by chemicals, which influence each other or chemicals such as heavy metals, which are capable of e.g. inhibiting the EROD enzyme (Oliveira et al., 2004; Viarengo et al., 1997) and thereby lessening signal strength. For this reason, bioassay detected LOQs for the positive control 2,3,7,8-TCDD and the extract, which technically should be in agreement, most

commonly differ from one another (Whyte et al., 2004). While signals that are near or less than the LOD constitute a “yes/no-decision” when using bioassays (Armbruster and Pry, 2008), the contribution of non-detected compounds in chemical analysis is often estimated by commonly presenting half their LOD (Winald et al., 1998).

3.4. LOD influencing factors and LOD enhancement

The LOD depends on several factors, including the type of cell-line, the positive control, the solvent carrier, the extract preparation and measured endpoint, the exposure time as well as multiple laboratory test conditions such as temperature.

An enhancement of the LOD of bioassays can be achieved by altering these factors in a certain manner and can significantly increase their utility as prioritization tools prior to more detailed instrumental analysis (Zhao et al., 2010).

As mentioned above, the LOD of a bioassay is species- and cell-line specific (Giesy et al., 2002; Hilscherova et al., 2000; Keiter et al., 2008). Recombinant cell lines stably transfected with vectors containing easily measurable reporter genes (i.e., luciferase or EGFP) are frequently reported to be more sensitive than bioassays performed with the respective wt cell line (Brouwer et al., 1995; Garrison et al., 1996; Sanderson et al., 1996). Additionally, the type of endpoint used determines the sensitivity of a test, and thus, the LOD. Some bioassays are based on measurement of light emission to visualize the response. For those signals the most sensitive detectors exist. For this reason and due to the additional fact that visualization systems such as the luciferase enzyme have a high turnover rate (meaning that a few enzyme molecules are sufficient to produce a detectable signal) those assays are characterized by very low detection limits (Sanderson et al., 1996; Willett et al., 1997). For instance, Sanderson et al. (1996) described a three-fold improvement in sensitivity (i.e. the minimal detection limit) of H4IIE-luc cells relative to the wt cells with detection limits of 0.2 and 0.6 fmol 2,3,7,8-TCDD/well, respectively (for further details see Table 1). This was confirmed by results of Murk et al. (1996), who reported the CALUX assay with H4IIE-luc cells to be slightly more sensitive than the micro-EROD assay with H4IIE wt cells. According to the authors this was mainly due to the missing substrate inhibition within the CALUX assay. Furthermore, the LOD of the micro-EROD described by Sanderson (1996) demonstrated a 50-fold enhancement of the LOD compared to those reported by other studies (Kennedy et al., 1993; Tillitt et al., 1991b). As reviewed by Brouwer et al. (1995) the sensitivity of such genetically engineered cell bioassays can be enhanced by increasing the number of dioxin response elements regulating the expression of the desired reporter gene and by increasing the number of copies

Table 1
List of several in vitro bioassay studies. Stated are the bioassay detection limits with 2,3,7,8-TCDD as reference substance (LOD), the EC₅₀ values for 2,3,7,8-TCDD, the cell lines, benchmarks, endpoints, solvent carriers and their final amounts in the assay, replicates (n) and coefficients of variation (CV).

Reference	Cell line (origin)	Benchmark (volume/assay)	Endpoint	Bioassay	LOD [pM]	EC ₅₀ TCDD [pM]	Solvent carrier (final amount)	n	CV [%]
Schwirzer et al. (1998)	H4IIE	96 well plate (100 µl)	EROD activity	Macro-EROD	1.9	–	DMSO/isopropanol (4:1; 0.5% (v/v))	2	25
Behnisch et al. (2002)	H4IIE	96 well plate (–)	EROD activity	Micro-EROD	0.3	5	DMSO (0.4%)	3	26
Tillitt et al. (1991)		Petri dish, 15 × 100 mm (10,000 µl)			3.1	17	Isooctane (1%)	54	3.7
Hanberg et al. (1991)		Culture plate, 20 cm ² (3000 µl)			10	47	DMSO (0.5%)	30	–
Sanderson et al. (1996)		96 well plate (–)			2.4	20	Isooctane (–)	7	10–25
Lab code 7 ^a		96 well plate (–)			3.1	6.2	DMSO/isopropanol (4:1; 50% (v/v))	–	10–15
Behnisch et al. (2002)	H4IIE-luc (H4IIE)	96 well plate (–)	Luminescence	DR CALUX	0.3	14	DMSO (0.5%)	3	20
Hurst et al. (2004)		96 well plate (–)			0.4	–	DMSO (0.4%)	3	<30
Lab code 2 ^a		96 well plate (–)			0.3	10	DMSO (0.8%)	3	15
Lab code 4 ^a		96 well plate (–)			0.3	10	DMSO (0.4%)	3	–
Lab code 5 ^a		96 well plate (–)			0.6	7.6	DMSO (0.4%)	3	12
Lab code 9 ^a	H4IIE-luc (H4IIE)	96 well plate (–)			0.3	10	DMSO (0.4%)	3	–
Lab code 23 ^a		96 well plate (–)			0.3	10	DMSO (0.4%)	–	15
Schoeters et al. (2004)	H4IIE-luc (H4IIE)	96 well plate (100 µl)	Luminescence	CALUX	0.4	–	DMSO (1%)	–	10–26
Jeong et al. (2005)	Hepa 1c1c7 (H1L1.1c2)	24 well plate (500 µl)			0.1	10	DMSO (1%)	6	5.2
Murk et al. (1996)	H4IIE-luc (H4IIE)	24 well plate (500 µl)			1	10	DMSO (0.5%)	3	–
Lab code 21 ^a	Hepa1.12cR	96 well plate (–)			2	60	DMSO (0.1%)	–	–
Lab code 28 ^a	Hepa1c1c7	96 well plate (–)			1	25	DMSO (1%)	3	–
Zhao et al. (2010)	H1G1.1c3 (Hepa1c1c7)	96 well plate (–)	GFP fluorescence	CAFLUX	1	–	DMSO (1%)	3	–
Nagy et al. (2002)	H1G1.1c3 (Hepa1c1c7)	96 well plate (100 µl)			1	18	DMSO (1%)	3	–
Lab code 20 ^a	H1G1.1c3	96 well plate (–)			1	7	DMSO (1%)	–	–
Song et al. (2006)	H4IIE-luc (H4IIE)	96 well plate (250 µl)	Luminescence	H4IIE-luc assay	1.9	31	–	3	–
Sanderson et al. (1996)		96 well plate (–)			0.8	5.6	Isooctane (–)	7	10–30
Aarts et al. (1995)	Hepa 1c1c7 (H1L1.1c7)	6 well plate (3000 µl)	Luminescence	Luciferase induction assay	0.6	–	DMSO (0.1%)	–	–
Garrison et al. (1996)	Hepa 1c1c7 (H1L1.1c2)	6 well plate (–)			0.1	20	DMSO (0.1%)	3	–
Lab code 19 ^a	101 L (HepG2)	96 well plate (–)	Luminescence	P ₄₅₀ reporter gene system	7.8	–	Isooctane (1%)	3	–
Lab code 22 ^a	RTL-W1	96 well plate (–)	EROD activity	EROD assay	1	5	DMSO (1%)	–	–
Richter et al. (1997)	RLT 2.0 (RTH-149)	96 well plate (–)	Luminescence	RLT2.0 assay	4	64	DMSO/Isooctane (0.1% (v/v))	3	–
Niwa et al. (1975)	H4IIE	Petri dish, Ø 60 mm (2500 µl)	AHH activity	AHH-enzyme assay	4	230	DMSO (0.2%)	2	–
Bradlaw et al. (1980)		Petri dish, 60 × 15 mm (4000 µl)			20	385	DMSO (0.25%)	6	–

^a Laboratories, which participated in an intra-laboratory comparison of dioxin-like compounds in food (Engwall and Van Bavel, 2004).

of the expression plasmid in the cell by amplification. Additionally, the concentration of AhR in those cells can be elevated by introducing constitutively active AhR cDNA expression vectors (1995).

Concerning the solvent carrier, it could be proven that the use of isooctane instead of DMSO, which may be cytotoxic above concentrations

of 1%, enhanced the sensitivity of the bioassay conducted with both, crude and cleaned-up extracts (Bradlaw and Casterline, 1979).

LODs are mainly linked to the available sample size (e.g. chemical analysis typically requires a sample amount of 5–10 g for solids and liquids (Harrison and Eduljee, 1999)) with greater volumes being able to

be concentrated more during extraction process, thus resulting in a decreased LOD. Applied sample extract clean-up procedures increased the sensitivity in both chemical and biological analysis by eliminating interfering substances like acid labile PAHs (Harrison and Eduljee, 1999) (see sub chapter “Sediments”).

Multiple laboratory test conditions may have various effects on a bioassays' LOD. For example, recent studies have shown that even a different temperature during the performance can decrease the LOD by increasing the level of reporter gene activity. For instance, Zhao et al. (2010) who performed the CAFLUX assay (see Table 1) observed a 2- to 3-fold greater fluorescence of EGFP at a cell incubation temperature of 33 °C compared to that measured at 37 °C.

Finally, the sensitivity of a bioassay is not only dependent on the LOD but also on the reliable production of reproducible and full concentration–response–curves and effect concentrations (Sanderson et al., 1996). From an environmental risk assessment perspective this additional information can tell us much more about the potency and efficacy of a single compound or a mixture than it would be the case for instrumental analysis alone.

3.5. Comparison of cell line and bioassay specific LODs

The aim of this section is to summarize LODs obtained by using different assays and types of cell lines (Table 1). Moreover, results from an inter-laboratory comparison study that was conducted and organized by Magnus Engwall and Bert van Bavel from the Örebro University in Sweden were discussed (Engwall and Van Bavel, 2004).

The aim of the inter-laboratory validation of bioassays was to determine: (1) the accordance of bio- and chem-TEQ values determined for 3 different types of samples; (2) the differences of TEQs among different bioassays; (3) the inter-laboratory variance and (4) the occurrence of additive effects in different bioassays. In brief, between December 2003 and April 2004 22 laboratories from Sweden, Norway, Denmark, Germany, the Netherlands, Belgium, Italy, the United Kingdom, USA, Canada, Japan, Taiwan and New Zealand participated in the above described validation study. Three types of samples were sent to all participating laboratories: sample 1 was a freeze-dried and homogenized sample of salmon muscle tissue, samples 2 and 3 were capsules containing a standard PCB and PCDD/F mixture, respectively. Participating laboratories were asked to extract the samples via their own methodologies and investigate them via their preferential *in vitro* bioassays (Table 1).

Comparing all results listed in Table 1, the data of inter-laboratory comparison collected by Engwall and Van Bavel (2004) corresponded well with the data previously reported in the literature (Behnisch et al., 2002; Hanberg et al., 1991; Hurst et al., 2004; Jeong et al., 2005; Murk et al., 1996; Nagy et al., 2002; Sanderson et al., 1996; Schoeters et al., 2004; Schwirzer et al., 1998; Zhao et al., 2010). The EROD assay with RTL-W1 as well as the related micro-EROD with H4IIE were the most TCDD-sensitive tests among the various assays with EC₅₀ values of 5 pM 2,3,7,8-TCDD (Table 1). Detection limits of all applied assays ranged between 0.1 and 20 pM 2,3,7,8-TCDD, with the CALUX and DR CALUX assays possessing the highest overall sensitivity (lowest LODs) of all listed test systems. The highest overall LOD (least sensitivity) was received by a rather obsolete method using high volume (4 ml) Petri dishes. Nevertheless, surprisingly low LODs of up to 3.1 pM TCDD could be achieved by using those larger volume approaches. In general, all luminescence-based bioassays showed the lowest LODs (Table 1). Coefficients of variance seem to be lower within these luminescence assays compared to e.g. EROD-based assays (Table 1). Some of those assays e.g. the P₄₅₀ reporter gene system appear to be less favorable since their LODs are about 10- to 20-fold greater compared to the related assays.

The intra-bioassay and intra-laboratory comparability was excellent for the DR-CALUX, the CALUX and the CAFLUX assays with mean LODs of 0.4, 0.9 and 1 pM, respectively. The relatively small data base for

each of the assays should be considered and again depicts the already mentioned difficulty of non-stated LODs of *in vitro* bioassay studies.

4. Conclusion

In recent years the use of *in vitro* bioassays for the characterization of dioxin-like activities in environmental samples and other matrices as well as for individual chemicals and mixtures has become an increasingly popular field of research. There exist a multitude of possible applications for these *in vitro* bioassays, ranging from support of environmental risk assessments to food safety. In this context, the improvement of the sensitivity of *in vitro* assay technologies is a growing field, especially in consideration of genetically engineered cell lines in so far as those are typically more sensitive compared to the respective wt cells. Regarding the sensitivity of *in vitro* bioassays they are increasingly competing with chemical analytical quantification technologies such as GC–MS with LODs of up to 0.1 pM 2,3,7,8-TCDD. While chemical investigations can give a more detailed view regarding specific quantities of different compounds present in e.g. complex environmental mixtures, bioassays are better suited to pre-screen those mixtures and hence, to identify the most dioxin-like active samples. In this context, bioassays have the distinct advantage that they can detect the overall dioxin-like potential of a sample including chemicals that cannot be analyzed by chemical analytical techniques.

We strongly recommend a standardized presentation of *in vitro* bioassay results enabling an estimate of its sensitivity. These results should include effect levels (especially in case bioTEQs are stated), the linear working range as well as the calculated LOD.

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