INTERUNIVERSITY PROGRAMME IN WATER RESOURCES ENGINEERING

Water Quality Assessment: Determining Dioxin Potency by Means of the CALUX Bioassay

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Abstract

Dioxins and dioxin-like compounds are some of the most toxic man-made compounds known. Their hydrophobicity causes them to accumulate in river sediment and in the sludge of waste water treatment plants (WWTPs). It is important to verify that the concentration of these compounds is below an acceptable level of contamination if the sludge is to be used as fertilizers on fields. Gas chromatography in combination with high resolution mass spectrometry (GC-HRMS) analysis is the standard reference method to determine the concentration of dioxins, but it is both time consuming and expensive. GC-HRMS also does not provide information of the toxicity of the sample directly. This information is calculated by means of Toxic Equivalency Factors (TEFs) such that a Toxic Equivalent (TEQ) is estimated. To overcome these problems, a bioassay called Chemically Activated Luciferase Expression (CALUX) has been developed which makes use of genetically modified mouse hepatoma cells. It provides a measure of the toxicity of a sample as a Biological Equivalent (BEQ). In this thesis a program is developed to analyze raw CALUX data in a consistent manner after which some methodologies for improving the use of this bioassay are investigated. First, a more recently developed cell line H1L7.5c1 is validated against the already much in use cell line H1L6.1c3. It is found that both cell lines give comparable results with the same precision, but that less sample volume is necessary for the newer cell line thus effectively lowering the detection limit of the bioassay. A new sample preparation procedure is also proposed by adding an extra clean-up column that contains activated copper to remove the sulfur from the sample. This way, the same preparation procedure can be used for both CALUX and GC-HRMS analysis, facilitating the comparison between the results of both analytical methods. Lastly, two different calculation methods to calculate the BEQ from the raw data as obtained by CALUX analysis are compared to each other. It is shown that the slope ratio after Box-Cox transformation calculation provides a good alternative to the more frequently used effective concentration ratio obtained by fitting the four parameter Hill equation as less data points are necessary for calculating the BEQ while at the same time a much higher precision on the result can be obtained.

Keywords: Dioxins, sediments, GC-HRMS, CALUX, H1L6.1c3, H1L7.5c1, clean-up procedure, TEF/TEQ, BEQ, four parameter Hill equation, effective concentration ratio, Box-Cox transformation, slope ratio

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List of Abbreviations

Ah	Aryl/Aromatic hydrocarbon
AhR	Aryl/Aromatic hydrocarbon Receptor
Arnt	Aryl/Aromatic hydrocarbon receptor nuclear translocator
BEQ	Biological EQuivalent
BMD	BenchMark Dose
BMDL	BenchMark Dose Limit
BSAF	Biota Sediment Accumulation Factor
CALUX	Chemically- Activated LUciferase eXpression
CRM	Certified Reference Material
DMSO	DiMethyl SulfOxide
DRE	Dioxin Responsive Element
EC_{20}	Effective Concentration at 20% of the maximum response
EC_{50}	Effective Concentration at 50% of the maximum response
EC_{80}	Effective Concentration at 80% of the maximum response
EPA	Environmental Protection Agency
FBS	Fetal Bovine Serum
GC-HRMS	Gas Chromatography in combination with High Resolution Mass Spectrometry
MEM-α	Alpha-Minimal Essential Medium
mRNA	messenger RiboNucleic Acid
NOAEL	No Observed Adverse Effect Level
PAH	Polycyclic Aromatic Hydrocarbon
PBBs	PolyBrominated Biphenyls
PBDDs	PolyBrominated Dibenzo-*p*-Dioxins
PBDFs	PolyBrominated DibenzoFurans
PBS	Phosphate-Buffered Saline
PCBs	PolyChlorinated Biphenyls
PCDDs	PolyChlorinated Dibenzo-*p*-Dioxins
PCDFs	PolyChlorinated DibenzoFurans
PCDHs	PolyChlorinated Diaromatic Hydrocarbons
PCNs	PolyChlorinated Napthalenes
PCTs	PolyChlorinated Terphenyls
PEC	Probable Effect Concentration
PNEC	Predicted No Effect Concentration
POP	Persistent Organic Pollutant
REP	Relative (Effect) Potency
RLU	Relative Light Unit
SRM	Standard Reference Material ®
TCDD	2, 3, 7, 8-TetraChloroDibenzo-*p*-Dioxin
TEC	Threshold Effect Concentration
TEF	Toxic Equivalency Factor
TEQ	Toxic EQuivalent
WHO	World Health Organization
WWTP	Waste Water Treatment Plant
X-CARB	Proprietary carbon matrix developed by Xenobiotic Detection Systems, Inc.
XDS	Xenobiotic Detection Systems, Inc.

1 Introduction

1.1 Scientific Rationale

Dioxins and dioxin-like compounds are environmentally and biologically very stable, hydrophobic chemicals that are extremely toxic to both animals and humans (Esposito et al., 1980). Because of their hydrophobic nature they tend to accumulate in the sediments that act as a reservoir for these persistent organic pollutants. In waste water treatment plants these compounds tend to concentrate in the sludge. When this sludge is reused for other purposes, for example, fertilization of agricultural fields, it is very important to assess how many dioxins and dioxin-like compounds are present in the sludge and whether or not their concentration may pose a health risk.

Traditionally, Gas Chromatography in combination with High Resolution Mass Spectrometry (GC-HRMS) was used to determine the concentration of dioxins and dioxin-like compounds in samples, but this method is both labor-intensive and expensive. A screening method therefore needed to be developed to process many samples at low cost. For this purpose the CALUX (Chemically Activated LUciferase gene eXpression) bioassay was developed. This bioassay makes use of cells that are genetically modified to produce light after they come in contact with dioxins in a time-, dose- and chemical-specific manner. CALUX can thus provide an integrated measure of the dioxin potency. It is both a faster and cheaper method than the reference standard of GC-HRMS and can therefore be used as a screening method to process many samples (Denison et al., 1999; U.S. EPA, 2005).

The GC-HRMS method only provides information on the concentration of dioxins and dioxin-like compounds in samples. It doesn't provide any information on the toxicity of these chemicals. For this purpose Toxic Equivalency Factors (TEFs) have been developed and used to weigh dioxin concentrations to obtain a Toxic Equivalent (TEQ) which is a toxicity estimate of the mixture. There is however disagreement about how applicable and reliable the TEF concept is, namely regarding the underlying assumptions of "dose additivity" and "no interactivity between compounds" (Safe, 1993; Birnbaum & DeVito, 1995; Johansson et al., 1995). Even if one assumes that these assumptions are valid, then the values of the TEFs are still questionable since they are not absolute values but are established by expert judgment based on data obtained from both *in vivo* and *in vitro* toxicity studies with differing endpoint toxicities (Van den Berg et al., 2006). Some authors (Johansson et al., 1995; Putzrath, 1997) even argue for the use of a function instead of point estimate to express the relative toxicity of a compound to overcome the problem that the relative toxicity is dose

dependent. These issues are important factors that need to be taken into account when discussing the toxicity of compounds or a mixture of compounds.

Besides the problem of identifying which and if TEF values should be used, there are two other common problems found in literature when comparing the GC-HRMS determined toxicity to the CALUX determined potency which will both be discussed in this thesis.

The first problem is regarding the comparability of the results obtained with GC-HRMS and with CALUX. The ability to compare the dioxin toxicity obtained by multiplying the concentrations that are determined by GC-HRMS with a toxic equivalency factor, to the integrated potency measurement as determined by the relatively cheap and quick screening method CALUX, requires both analysis methods to be performed on the same extracted and prepared sample. Almost always different sample preparation procedures are used between methods leading to results that are not fully comparable. Differences may simply be attributed to the analytical method or to the sample preparation and clean-up procedure or both. It is therefore one of the objectives of this master thesis to propose a common sample preparation procedure (extraction and purification) that can be used for both the GC-HRMS analysis and the CALUX analysis. By ensuring the same sample preparation the results from both methods can be more accurately compared to one another (Carbonnelle et al., 2004; Nording et al., 2007).

The second problem is related to how the relative potency of a mixture (Biological Equivalent, BEQ) is calculated from the CALUX response. Several methods are in use (Villeneuve et al., 2000; Elskens et al., 2010) which complicates the comparison of results from different studies. Furthermore, which analysis method is used to interpret the measured data is not always properly specified (e.g. Carbonnelle et al., 2004; Han et al., 2004; Brown et al., 2007). In this work the widely used method of calculating the BEQ as the effective concentration ratio obtained by fitting the non-linear four parameter Hill equation to the experimental data points, will be compared to the use of a Box-Cox transformation to linearize the data such that the slope ratio can be calculated and used as an estimate of the BEQ. The latter calculation method requires only half of the amount of data that is necessary for the four parameter Hill equation which means that more samples can be analyzed within a given time period. It is therefore interesting to investigate if the slope ratio after Box-Cox transformation gives comparable results to the effective concentration ratio after four parameter Hill equation fit, both in absolute value of the BEQ estimate and in degree of uncertainty on this estimate.

1.2 Specific Objectives

The specific objectives of this work are:

- To develop a program that can process the raw CALUX data in a consistent manner.
- To compare the response of the new CALUX cell line H1L7.5c1 with the previously validated cell line H1L6.1c3.
- To develop a clean-up protocol that can be used for both CALUX and GC-HRMS analyses.
- To compare two different calculation methods (Hill regression and Box-Cox transformation) for estimating the sample potency.

This work was undertaken in the framework of the FERTIDIOX project involving the University of Brussels (VUB), the University of Liège (ULg) and the Scientific Institution CODA-CERVA. The project aims at evaluating the “Dioxin Risk” associated with the use of organic sediment and sludge from wastewater treatment plants in agriculture.

1.3 Thesis Overview

To start, the mechanism of toxicity of dioxins and dioxin-like compounds and their chemical structure is introduced. The issues arising from the use of toxic equivalency factors is also explored in this first chapter. To conclude this introductory chapter, the environmental risk assessment of dioxins and dioxin-like compounds is discussed. The level of concentration of dioxins in the sediments that poses a health risk is an important concern that needs to be understood in order to decide if the analysis shows a value that requires intervention (remediation). Especially in light of the discussion of whether sludge from wastewater treatment plants should be used as fertilizer in agriculture, a benchmark regarding the concentration of dioxins permitted in the sludge is necessary.

In the second chapter, the analysis method that was developed to examine the sediment samples is explained in more detailed. A distinction is made between the sample preparation that can be used for both CALUX and GC-HRMS, the sample analysis with CALUX and the data analysis procedure for the raw CALUX data.

The results that are obtained by this analysis method are provided in the next chapter together with a discussion of the results in light of the specific objectives of this work. Only the dioxin potency was determined and not the dioxin-like potency due to the time limitations inherent to a thesis project. The method should however be equally applicable to determine dioxin-like potency. This can be the subject of a subsequent study.

In the next chapter, conclusions with regard to the four specific objectives of this work are formulated. Finally recommendations for future work are provided.

2 Dioxins and Dioxin-like Compounds

2.1 Mechanism of Toxicity and its Application as a Bioassay

The exact chain of molecular events that triggers the toxic effects of dioxins and dioxin-like compounds is not well known, but many authors believe that the Aryl hydrocarbon Receptor (AhR) plays a very important role in mediating the biological effects of dioxins (Poland et al., 1976; Mason et al., 1986; Safe, 1988; Fernandez-Salguero et al., 1996; Hestermann et al., 2000). A chain of events to understand these effects has been proposed and will be discussed in the following paragraph.

On binding of the dioxin or dioxin-like compound to the intracellular AhR the receptor becomes activated and travels to the nucleus of the cell where it binds to the AhR nuclear translocator protein (Arnt). This complex then binds to specific sequences in the DNA called dioxin responsive elements (DRE). This binding of the activated receptor complexes to their specific DNA binding site in the nucleus stimulates the expression of adjacent AhR-responsive genes and thus the production of messenger RNA (mRNA) that is translated in the cytoplasm. The resulting proteins lead to the toxic and biological effects of dioxins and dioxin-like compounds (Denison & Nagy, 2003; Denison et al., 2004). This mechanism is schematically represented in Figure 1.

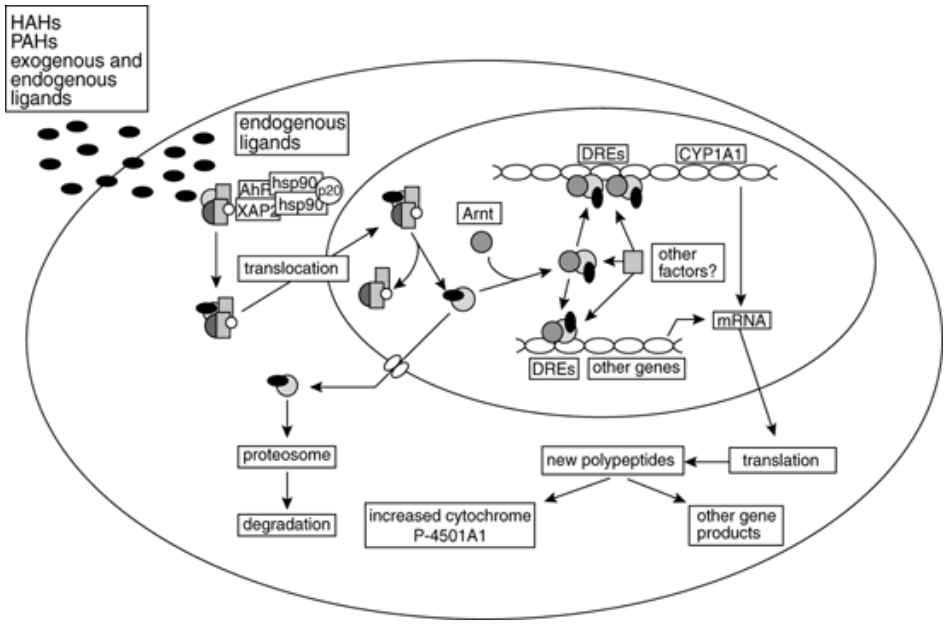

Figure 1. Mechanism of dioxin toxicity

Source: Denison & Nagy, 2003

The *in vitro* bioassay CALUX (Chemically Activated LUciferase eXpression) makes use of the mechanism by which dioxins and dioxin-like compounds cause their toxic effects to estimate the concentration of these compounds by genetically engineering cells so that they include the luciferase gene of the firefly *Photinus pyralis* under control of the AhR-dependent gene expression. These genetically modified cells thus respond to AhR agonists with the induction of luciferase in a time-, dose-, chemical- and AhR-specific manner (Garrison et al., 1996; Denison et al., 2004; Han et al., 2004). By comparing the light production of a sample to the light production of known amounts of the most toxic dioxin, this bioassay can be used to estimate a Biological Equivalent (BEQ).

2.2 Chemical Structure

Dioxins and dioxin-like compounds are some of the most toxic man-made compounds known (DeVito & Birnbaum, 1994). Toxic effects include dermal toxicity, immunotoxicity, reproductive effects and teratogenicity, endocrine disruption and carcinogenicity (WHO, 1998).

Besides being extremely toxic these compounds do not easily degrade in the environment. Typical half-life times in sediments are of the order of decades (Masunaga et al., 2001; Davis, 2004). This persistence is due to their high melting point and their stability to acids and bases resulting from the lack of reactive functional groups. The level of persistence is mostly determined by the number of chlorines present in the molecule as the carbon-chlorine bond (especially on aromatic rings) is very stable (Basu et al., 1985).

Another characteristic of dioxins and dioxin-like compounds is lipophilicity which means that they are soluble in fat. Because of this persistency and liphophilicity dioxins and dioxin-like compounds can bioaccumulate in organisms. Even very small doses in the environment can cause cumulative effects which are of great concern (Esposito et al., 1980). Therefore the Stockholm Convention on persistent organic pollutions (2001) included dioxins and dioxin-like compounds on the list of persistent organic pollutants (POPs) as they (i) possess toxic properties, (ii) resist degradation, (iii) bioaccumulate and (iv) are transported and deposited far from their place of production.

2.2.1 Dioxins

The term “dioxins” generally refers to a family of related chemical compounds that include the polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Figure 2). There are 75 different PCDDs and 135 different PCDFs, but only seven PCDDs and 10 PCDFs are considered to be of concern namely those that have a chlorine substitution at positions 2, 3, 7 and 8 (NATO/CCMS, 1988).

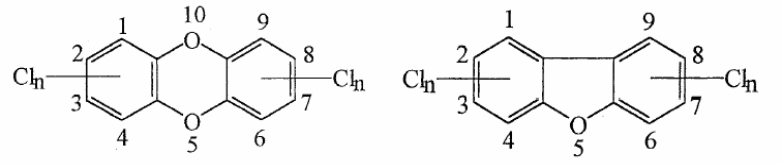

Figure 2. PCDDs (right) and PCDFs (left)

The most toxic compound in this family is 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (Figure 3, hereafter referred to as TCDD).

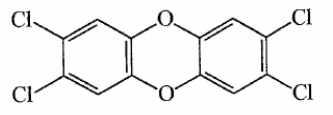

Figure 3. 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin

Dioxins have no use and are created as by-product of industrial processes and incomplete combustion, for instance: municipal and domestic waste incineration, burning fuel (wood, coal or oil), chlorine bleaching of pulp and paper and chlorinated pesticides manufacturing (Basu et al., 1985; Rappe, 1996; U.S. EPA, 2006). They can enter a river system by atmospheric deposition on the land and subsequent erosion and runoff processes. Once in a river, these compounds tend to concentrate in the sediments as they are not very soluble in water (Esposito et al., 1980).

2.2.2 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (Figure 4) are a group of compounds that can show structural similarity to dioxins. Of the 209 PCB congeners, 12 PCBs are called dioxin-like because of their ability to adopt the same stereo-configuration as dioxins. PCBs that are not substituted by chlorine at the ortho position (positions 2, 2', 6 and 6'in Figure 4, non-ortho PCBs), are free to rotate around the single carbon-carbon bond, resulting in a co-planar ('flat') configuration. PCBs that have a single ortho chlorine substitution are also able to adopt a relatively planar arrangement and are thus also dioxin-like PCBs (Safe, 1990).

Non-dioxin like PCBs are PCBs that are substituted at more than one ortho-position and are thus compounds that do not show dioxin-like activity though this does not mean that they are not of concern. Some show neurotoxic effects (Kodavanti et al., 1996) and may be present in much higher concentrations in the environment than dioxin-like PCBs. These compounds however, can not be quantified by the CALUX bioassay because of their structural dissimilarity to dioxins resulting in an inability to bind the aryl hydrocarbon receptor.

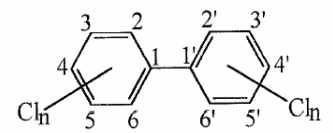

Figure 4. Polychlorinated biphenyls (PCBs)

Like dioxins, PCBs can also be formed as by-products during incomplete combustion of waste materials. In contrast to dioxins however, PCBs were also manufactured intentionally. They were manufactured in relatively large quantities for use in commercial products such as dielectrics, hydraulic fluids, plastics, coatings and paints, and although a ban has been placed on commercially producing PCBs, they continue to be released into the environment through the use and disposal of products that were made before the ban. The inadvertent production still remains a source as well (U.S. EPA, 2006).

2.2.3 Other Dioxin-like Compounds

A compound is considered as dioxin-like when it has the following characteristics (Van den Berg et al., 2006):

- Structurally similar to TCDD
- Binds to the AhR
- Elicits AhR-mediated biochemical and toxic responses
- Persistent and accumulative in the food chain

Besides PCDDs, PCDFs and PCBs there are many more compounds that have these characteristics and that are thus also considered dioxin-like. Examples of such compounds are polybrominated dibenzo-*p*-dioxins (PBDDs), polybrominated dibenzofurans (PBDFs), polybrominated biphenyls (PBBs), mixed halogenated dibenzo-*p*-dioxins, dibenzofurans and biphenyls, polychlorinated terphenyls (PCTs), polychlorinated napthalenes (PCNs) and hexachlorobenzene (Van den Berg et al., 2006). All of these compounds will elicit a response in the CALUX bioassay if present in the sample but *will not be detected by the GC-HRMS analysis unless explicitly sought*.

2.2.4 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs, Figure 5) are a family of ubiquitous environmental contaminants that consist of more than 100 chemicals of which 16 appear on the U.S. Environmental Protection Agency (EPA) list of priority pollutants[1].

[1] http://www.epa.gov/waterscience/methods/pollutants.htm

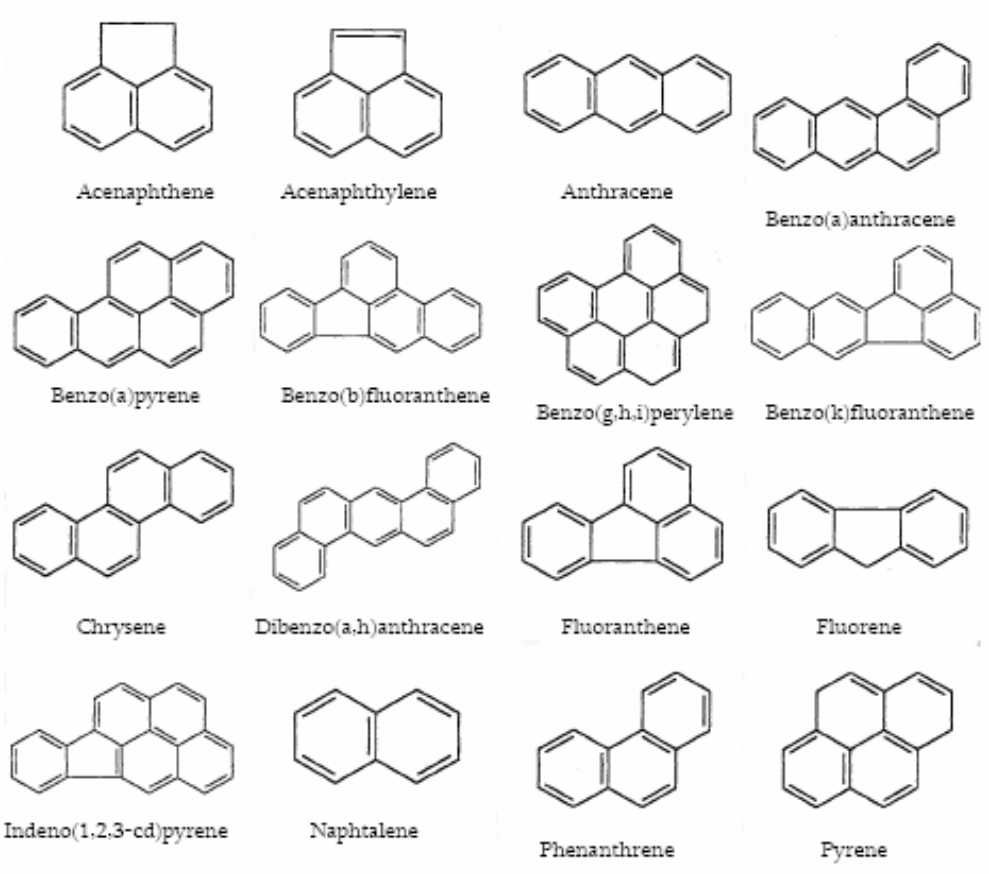

Figure 5. Structure of the 16 priority PAHs according to U.S. EPA

PAHs may also show dioxin-like activity because they are able to bind to the active site of the AhR and elicit a response. Despite this ability, they are not considered dioxin-like compounds as their toxicity is based on other mechanisms than AhR binding (Di Giulio et al., 1995) so they do not comply with the third characteristic that defines compounds as dioxin-like.

The fourth characteristic of dioxin-like compounds is also not met in the case of PAHs. PAHs are metabolically labile compounds as they undergo a biological transformation to polar products and as such cannot bioaccumulate in organisms (Ramesh et al., 2004).

Since they are not considered dioxin-like compounds and are therefore not studied in this thesis, PAHs are removed from the sample during the clean-up procedure to avoid interference effects since they are known AhR ligands.

2.3 Determining Toxicity of Compounds and Mixtures

2.3.1 Toxic Equivalent (TEQ)

PCDDs, PCDFs and PCBs are umbrella terms that encompass many different congeners. The existence of complex compositions complicates the risk evaluation for humans, thus the concept of Toxic Equivalency Factors (TEFs) was developed as an interim method in 1988 to facilitate the risk assessment and regulatory control of exposure to these mixtures (NATO/CCMS, 1988). The main concern at that time was standardization and facilitating consistency in the reporting of analytical results. Although originally conceived as an interim method until a better method to determine the toxicity was developed, the consensus is that the TEF concept is still the most plausible and feasible approach for risk assessment of halogenated aromatic hydrocarbons with dioxin-like properties (Van den Berg et al., 1998).

In the TEF/TEQ-method, the toxicity of congeners that are considered to have significant risks to human health are measured in TEFs, which weighs the toxicity of each individual congener against the most toxic and best studied compound in this family namely TCDD. The closer the ratio is to unity, the greater the toxicity of that congener.

It is important to stress that the term TEF needs to be used in a consistent way. There has been some confusion in the literature regarding the definition of this term, but TEF should be used to refer to a consensus value for the relative potency of a compound that is based on the results of several *in vivo* and *in vitro* studies. The potency of a compound to cause a particular toxic or biological effect relative to TCDD in a single study should not be referred to as a TEF, but only as a relative potency (REP) (Van den Berg et al., 1998). These REP values are method dependent as different toxic endpoints and/or different species give different values.

As TEF values are consensus values that are based on a wide range of studies, TEFs only indicate order of magnitude estimates of the toxicity of a compound relative to TCDD (Van den Berg et al., 1998). The assigned TEF values are chosen conservatively and correspond with the higher range of relative potencies (Safe, 1990).

Calculation of the total toxicity of a sample is achieved by multiplying the concentrations of the individual target compounds by their respective TEFs (see appendices1, 2 and 3) by using equation 1.

$$TEQ = \sum_{i=1}^{7}[PCDD_i \cdot TEF]_i + \sum_{i=1}^{10}[PCDF_i \cdot TEF]_i + \sum_{i=1}^{12}[PCB_i \cdot TEF_i] \quad \textbf{(1)}$$

Solving equation 1 provides a Toxic Equivalent concentration (TEQ) contributed by all dioxin-like congeners in the mixture. The key assumption behind this approach is simple dose additivity and thus the implicit assumption of no interaction between compounds. Several *in vivo* mixture studies (Gao et al., 1999; Fattore et al., 2000; Hamm et al., 2003; Walker et al., 2005) show consistent results with additivity but there are also authors that stress the importance of non-additive interactions between dioxin-like compounds (Brown et al., 1994; Safe, 1997; Schroijen et al., 2004; Windal et al., 2005; Sanctorum, 2009). This interaction can both be antagonistic, resulting in a lower response of the mixture compared to the additive response of the individual compounds, or agonistic, resulting in a higher response of the mixture compared to what is expected when the response of the single compounds are added. The latter may also be referred to as synergism (Hertzberg & MacDonell, 2002).

A second key assumption behind the TEF method is that all compounds under consideration exert their toxicity through exactly the same mechanism (cfr. Figure 1). If this is the case, all dose-response curves are parallel to each other and the same maximum induction response (efficacy) is obtained. The proposed toxicity mechanism is however a theoretical representation of how the compounds exert their toxicological effect. In reality, there might be differences between various compounds with regard to binding affinity, diffusion through the cell membrane, *in vitro* toxicokinetics and solubility. All these factors might cause nonparallel dose-response curves and may limit the magnitude of the response that can be achieved (Villeneuve et al., 2000). In that case, the toxic potency of compounds is dose dependent and thus cannot be represented by a single TEF value. To overcome the problem that the relative toxicity is dose dependent, some authors (Johansson et al., 1995; Putzrath, 1997) argue for the use of a function instead of point estimate to express the relative toxicity of a compound.

It is also important to note that there are different toxic equivalency factors for humans, fish and birds. This is due to the difference in congener half-life in living tissue of different organisms and to various metabolic rates of expulsion (Van den Berg et al., 2006). This means that the toxicity of a sample is relative and that the appropriate TEF values need to be chosen in accordance with the objectives of a specific study.

As a final remark, TEFs for dioxin-like compounds only apply to AhR-mediated responses and thus do not take into account other possible toxic impacts that occur via a different pathway (Van den Berg et al., 1998; WHO, 1998).

2.3.2 Biological Equivalent (BEQ)

Another manner of determining the dioxin potency of a sample is by using the CALUX bioassay. The mechanism through which dioxin compounds exert their toxicity in cells is genetically modified such that light is produced if these cells are exposed to dioxin compounds. CALUX can therefore provide a measure of the toxic potency directly without having to determine the concentration of the individual congeners (by GC-HRMS). The potency of the sample is expressed as a Biological Equivalent (BEQ) and can be calculated in two different ways.

The most commonly used method is to calculate the BEQ as the ratio between the concentration of the reference compound TCDD and the concentration of the sample both at the same percentage of the maximum response. These concentrations are calculated by fitting a mathematical model to the experimentally measured data points. Often the concentration at 50% of the maximum response is used (EC_{50}), but other levels of the maximum response can also be chosen. If the shape of the dose-response curve of the sample is parallel to the shape of the dose-response curve of TCDD and if the same efficacy is obtained, then the BEQ is independent from which level of the maximum response was chosen. If these conditions are not met, than different BEQs are obtained for different induction levels and it is better to provide the range of values that are obtained to indicate the degree of uncertainty on the estimate of the potency (Villeneuve et al., 2000).

Another method is to calculate the BEQ as the ratio between the slope of the sample dose response curve and the slope of the TCDD dose response curve (Elskens et al., 2010). This method is not as widely used as the effective concentration ratio but has as an advantage that one value is obtained instead of a range of values even if the dose response curves are not parallel or if the same efficacy is not obtained.

It can be concluded that the BEQ derived from CALUX experiments provides different information than the TEQ derived from GC-HRMS because CALUX (Windal et al., 2005):

- Generates an integrated signal instead of providing congener specific information.
- Is based on a response at a single toxic endpoint instead of on a toxicity assessment.
- Can also measure compounds that are not considered by the chemical analysis and/or compounds that are below the detection limit of the chemical analysis method.
- Can account for non-additive interactions whereas the chemical analysis assumes additive toxicity effects of the different congeners.

2.4 Environmental Risk Assessment

2.4.1 Distribution of Pollutants and Bioavailability

The environmental behavior of a pollutant is linked to its hydrophobicity and organophilicty which can be quantified by two numbers namely the solubility in water and the octanol-water partition coefficient (K_{ow}) respectively. This K_{ow} quantifies the distribution between the water and the organic phase. Dioxins and dioxin-like compounds have a high octanol-water partition coefficient and therefore tend to concentrate in the non-water phase in their surroundings, i.e. the organic matter in sediments and the lipid phase of biota (Hawker & Connell, 1988).

The most common theory about the concentration of organic contaminants in sediments is the physical adsorption on sediment particles or absorption in the sediment matrix (Pignatello & Xing, 1996). The amount, age and type of organic matter are the most important parameters affecting the interaction between organic pollutants and sediments (Hatzinger & Alexander, 1995; Piatt & Brusseau, 1998; Reid et al., 2000). Other parameters that influence sorption are physiochemical properties of the sediment (Yang et al., 2005), the presence of inorganic components (Mader et al., 1997), pore size and structure (Nam & Alexander, 1998), pollutant concentration (Divincenzo & Sparks, 1997), and microbial activity (Guthrie & Pfaender, 1998). Temperature and salinity also play a role as they alter the solubility of POPs in water (Tremblay et al., 2005). The release and spread of organic pollutants in the aquatic environment from the sediments is thus a very complex process. There is no simple linear relationship between the concentration of pollutants in the sediment and the concentration of pollutants in the water making it very hard to determine to which concentration of the pollutant organisms are exposed to.

The presence of a chemical in the environment does not necessarily mean that it is biologically available, as contaminants may exist in different chemical forms. Some forms may be able to enter biological systems while others may not (Wu et al., 2008). The fraction of contaminants that is available to organisms is referred to as biologically available and it is this fraction that is considered ecotoxicologically relevant. Bioavailability is however organism and species dependent which adds to the complexity of analyzing the impact of sediment contamination (Reid et al., 2000).

2.4.2 Bioaccumulation

The dioxins that are bioavailable to organisms can be taken up by them. The hydrophobic nature and resistance towards metabolism causes these chemicals to persist and bioaccumulate in fatty tissues of animals and humans. Bioaccumulation is defined as the net accumulation of a substance by an organism due to uptake by all sources of that substance in its environment and is equal to the difference between the total uptake and the degradation of the contaminant. Incorporation of dioxins and dioxin-like compounds by benthic organisms initiates the accumulation to higher trophic levels in the food web. The adverse effects often only become apparent several steps higher in the food chain where the concentration becomes high enough to cause toxic effects (Sanctorum, 2009). To model this accumulation of organic pollutants through the trophic chain, biota-sediment-accumulation factors (BSAFs) have been developed. These factors describe the ratio of the concentration in organisms (normalized to the lipid fraction) over the concentration in the sediment (normalized to the sediment organic carbon) (Thomann & Komlos, 1999).

2.4.3 Determining Threshold Concentration

Assessment of the ecological risk of dioxin toxicity requires legal reference values or scientifically sound data. However, environmental standards for PCDD/Fs or PCBs in sediments which have legal implications barely exist. The allowed concentration of these compounds in matter that will be used as fertilizer has not yet been the subject of investigation. Therefore a short introduction on how this threshold concentration could be established is given before discussing some of the limited data that is available regarding tolerable concentrations of dioxins in sediments.

For the majority of toxic chemicals it can be assumed that an exposure threshold exists, below which no adverse effect will occur (Sand et al., 2008; Wu et al., 2008). These exposure levels can thus be considered acceptable levels of contamination. What this level of contamination is can be assessed by two different methods. A traditional model for risk assessment of chemicals is the approach of the no-observed-adverse-effect-level (NOAEL), which is derived from experimental animal data and is defined as the highest experimental dose level for which the response is not significantly different compared with the response in the control group. A more recently developed model is the benchmark dose (BMD) model. This concept involves fitting a mathematical model to dose-response data and calculating a BMD or a dose causing a predefined change in the response. This benchmark response is

defined as a 1%, 5% or 10% increase in risk over background. The lower bound of a two-sided 90% confidence limit of the BMD (BMDL) is then used as a threshold concentration (Sand et al., 2008).

Both risk assessment models include a great deal of uncertainty stemming from a lack of understanding of the system. This uncertainty results from (Wu et al., 2008)

- Extrapolation of data from test animals.
- Lack of toxicological data for wildlife and humans.
- Lack of knowledge about the effects of low exposures over long time periods (most studies only study acute and not chronic toxicity).
- Modeling and predictions which involve unverifiable assumptions.

To deal with these uncertainties, an application factor of 10-100 is applied to reduce the NOAEL or BMDL. This gives a predicted no effect concentration (PNEC). The measured environmental concentration is compared to this value. If the environmental risk, defined as the ratio of the measurement over the PNEC, is greater than one, the risk is substantial. If the environmental risk is smaller than one there should be no risk (Wu et al., 2008).

Instead of using one concentration as a threshold value, some authors propose to use two values. MacDonald et al. (2000) have developed sediment quality guidelines (SQG) that include a lower limit (threshold effect concentration, TEC) at which toxicity to organisms is predicted to be unlikely and an upper limit (probable effect concentration, PEC) at which toxicity to organisms is predicted to be probable. A value between these limits may cause harmful effects, depending on species and other factors.

The New South Wales Department of Environment and Conservation in Australia has set an indicative intervention value of 1000 pg TEQ/g sediment (Birch et al., 2007). This intervention value is much higher than the recommended exposure limit of 13 pg TCDD/g sediment for birds and mammals and 200 pg TCDD/g sediment for aquatic organisms that was derived by the Health Council of the Netherlands (1996) based on measured NOELs and BSAFs. Japan has also set a sediment standard value of the same order of magnitude as the Netherlands namely 150 pg TEQ/ g sediment (Japan EPA, 2002). The Canadian government has however set much lower sediment quality guidelines, with TEC= 0.85 pg TEQ/g sediment and PEC = 21.5 pg TEQ/g sediment (Canadian Council of Ministers of the Environment, 2001).

3 Analytical Method

3.1 Sample Preparation Procedure

Figure 6 shows a schematic overview of the different steps that need to be taken to determine the potency of dioxins and dioxin-like compounds in a sample. Before the sample can be analyzed by CALUX or GC-HRMS there are three major steps that have to be undertaken: extraction, purification of the extract and separation of the different compounds. These steps are discussed individually in more detail below. More detailed information on the equipment, materials and reagents used can be found in appendices 4, 5 and 6 respectively.

To guarantee the comparability of the results obtained with GC-HRMS and CALUX analyses it is important to develop a sample preparation procedure that can be used for both analytical methods. This is difficult because each method has different requirements. There are some solvents that cannot be used for the CALUX analysis because they are lethal to the cells and sulfur has to be removed from the sample because GC-HRMS does not allow for the presence of this compound. To overcome these problems, the sample preparation protocol used in this research will be based on a CALUX sample preparation protocol as described in literature (Carbonnelle et al., 2004; Schroijen et al., 2004; U.S. EPA, 2008; Wang et al., 2009) with an additional step to remove sulfur.

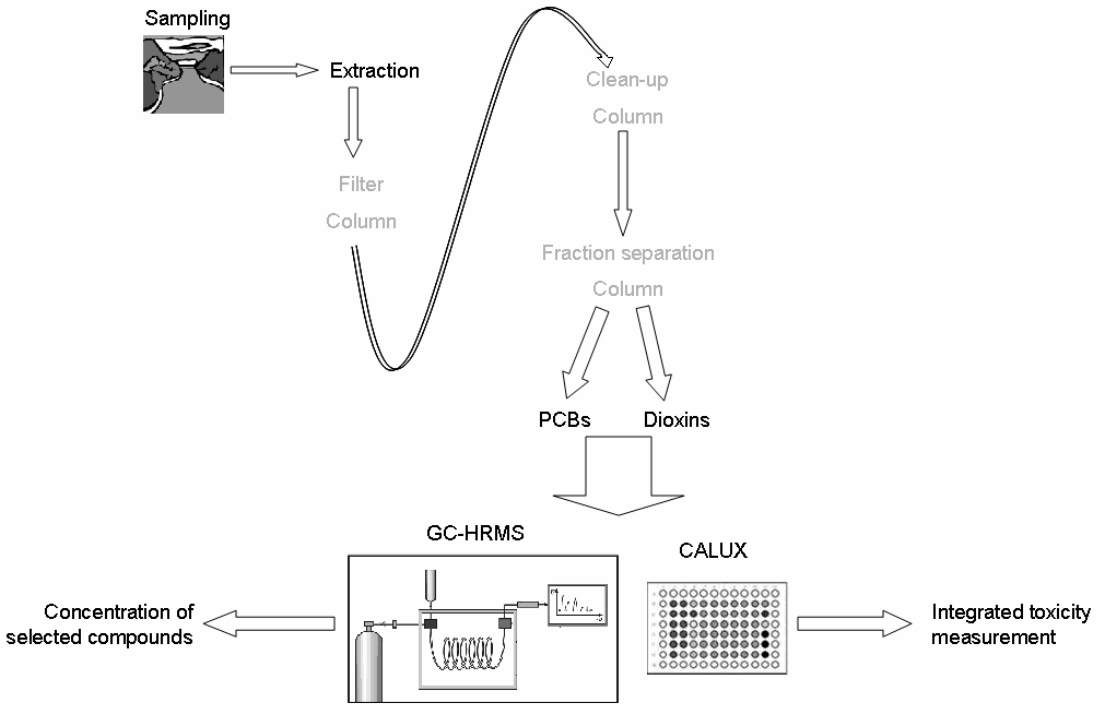

Figure 6. Schematic overview of sample processing

3.1.1 Extraction

There are a variety of extraction procedures that can be used to extract dioxins and dioxin-like compounds from the sample. The type of matrix is the most important factor in choosing an extraction method. For this thesis, sediment samples are analyzed and it is opted to use a soft extraction method such that only the bioavailable fraction of dioxins and dioxin-like compounds are extracted.

The extraction is performed by adding 10mL of 20%methanol-80%toluene solution to approximately 2g freeze-dried sediment[2] and placing this in an ultrasonic water bath for 5 minutes with periodic vortex mixing. After settling for approximately 5 minutes, the supernatant is collected and is loaded on to a filter column. The composition of this column (25mL Pyrex disposable column) is shown in Figure 7. Prior to loading the sample onto the column, the column is rinsed with 30ml of toluene which is then discarded. The sample eluate is collected in a clean baked 50mL glass centrifuge tube.

Next, 10mL of toluene is added to the same soil sample and this mixture is again sonicated for 5 minutes with periodic vortex mixing. The sample is allowed to settle and the supernatant is loaded onto the extraction column. A third aliquot of 10mL of toluene is added to the soil sample and is sonicated for 10 minutes with periodic vortex mixing. Once again the sample is allowed to settle and the supernatant is loaded onto the extraction column. The filter column is then rinsed with an additional 10mL of toluene. The pooled extracts are concentrated to near dryness in a vacuum centrifuge concentrator.

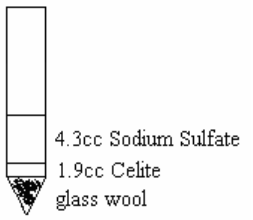

Figure 7. Filter column

[2] Samples that are analyzed for this thesis are taken from the river Scheldt and the river Zenne, both located in Belgium and from the Standard Reference Material® 1944 - New York/New Jersey Waterway Sediment (in this thesis referred to as Certified Reference Material (CRM)). Sludge from the WWTP in the North of Brussels wasn't available in time because of juridical problems.

3.1.2 Clean-up

Purification of the extract is performed to exclude additive and/or interfering effects from compounds that are co-extracted by the extraction procedure but are not under study and will interfere in the analysis (e.g. PAHs, chlorophyll, organic matter). To achieve this, the organic extract (resuspended in 5mL hexane, sonicated for five minutes with periodic vortexing) is added to a clean-up column containing acidic silica to stop or break down fat and organic matter. Above and below this layer, a sodium sulfate (Na_2SO_4) layer is added to desorb any water that might be present in the sample. This sodium sulfate is baked for four hours at 450°C prior to use to remove water and organic contaminants. The exact composition of this clean-up column (10mL Pyrex disposable column) is given in Figure 8.

After the sample has passed through the top sodium sulfate layer, the sample centrifuge tube is rinsed three times with 2mL of hexane and these rinses are also transferred on to the column. Finally this column is also rinsed three times with 5mL of hexane.

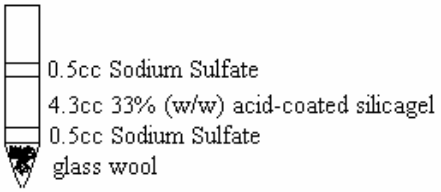

Figure 8. Clean-up column: acid-silica column

Sediment samples can be highly polluted. An additional cleaning step before putting the sample extract onto the acid-silica clean-up column can thus be needed. This pre-clean-up is achieved by adding 2.5mL concentrated sulfuric acid to the extracted sample that is resuspended in hexane and then vortexing this mixture until it is well mixed. It is very important that the different phases are allowed to separate completely and that acid is not withdrawn when taking off the hexane to put on the clean-up column (Bellar & Lichtenberg, 1981). To achieve a good separation between the two phases, the samples are placed in a centrifuge for five minutes.

Since elemental sulfur is extensively distributed in sediments and sulfur is readily dissolved in the organic solvents that are used during the extraction this can cause significant interference problems in the GC–HRMS detector (Blumer, 1957). Hence, a cleanup procedure to remove sulfur from the sample is mandatory. To achieve this, a second clean-up column is used that contains activated copper. The use of activated copper to remove sulfur from sediment samples has been well documented (Blumer, 1957; Brooks et

al., 1990; Bossi et al., 1992; Tan et al., 1993; Tong & Imagawa, 1995; U.S. EPA, 1996; Schubert, 1998; You & Lydy, 2004; Jin et al., 2007; Wang et al., 2007; Wise & Watters, 2008). The U.S. EPA (1996) also proposes the addition of tetrabutylammonium sulfite as a way of removing sulfur from the sample but this compound does not degrade organophosphorus nor organochlorine pesticides. These compounds might be present in the sediment sample and are degraded by copper, thus in this case copper is preferred over tetrabutylammonium sulfite. Silver nitrate is also often used to remove sulfur from the sample (Schubert, 1998; Ko et al., 2007; Sanctorum, 2009), but is not used here since it might contaminate the sample with nitrates as silver nitrate removes sulfur by a process of ligand exchange. The addition of metallic mercury (Goerlitz & Law, 1971) is also a possible method to remove sulfur from the sample but is not used here because of the toxicity of this compound.

The elemental copper is activated by washing it with 20% hydrochloric acid under sonication. It is stored in this solution until use to prevent oxidation which would result in deactivation of the copper. After making the copper column (glass pasture pipette, muffled, Figure 9), it is rinsed three times with 1 ml of water, acetone, toluene and hexane respectively. When the eluate from the acid-silica column goes through the activated copper column, the removal of sulfur from the sample can be clearly seen. The copper turns black as copper sulfides are being formed. As long as there is still copper present that remains its red, it can be assumed that all the sulfur was removed from the sample.

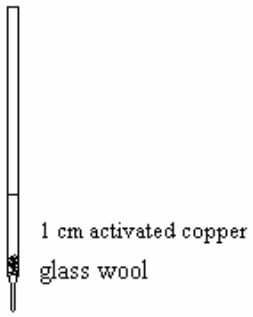

Figure 9. Clean-up column: activated copper

3.1.3 Fraction Separation

A fraction separation column is placed right after the clean-up columns, which will retain the compounds of interest until the solvent is changed. This column further cleans up the sample by retaining some non-polar compounds and allows to differentially elute the PCBs and the dioxins so that these fractions can be analyzed separately. The differential analysis of both fractions simplifies the interpretation of the results.

The composition of the fraction separation column (35cm long glass tubing with 8mm id) is shown in Figure 10. Prior to use, the column is rinsed with 5mL of acetone, 20mL of toluene and 10mL of hexane. After all the eluates from the clean-up columns have passed through the fraction separation column, the column is rinsed with 5mL of hexane.

The PCBs can then be eluted off the column by adding 5mL hexane-toluene-ethyl acetate (80-10-10) three times. These eluates are collected in a clean baked 40mL scintillation vial.

Then the column is flipped and the PCDD/Fs can be eluted by adding 5mL of toluene three times. These eluates are also collected in a clean baked 40mL scintillation vial.

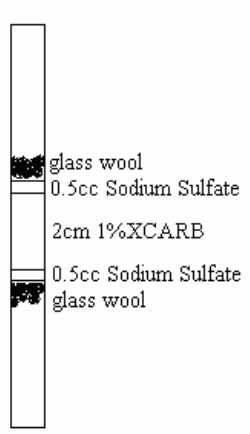

Figure 10. Fraction separation column

These eluates can be used as prepared sample to perform the GC-HRMS analysis on. Eluates that are used for the CALUX analysis are first concentrated to near dryness by using the vacuum centrifuge and then resuspended in 4mL of hexane.

3.1.4 Quality Control

For each batch of samples that is prepared, both an extraction blank and a clean-up blank are also prepared to ensure that the procedure is not introducing any contamination.

The extraction blank is processed through the complete sample preparation procedure and is thus treated in the same way as the samples. It serves as a control to measure the contribution of activity from any of the solvents or column matrices used in the sample preparation procedure. Measurement of the activity associated with this blank determines the background activity that would be expected to be present in each sample as a result of the combined effect of the extraction and clean-up process.

The clean-up blank is a control sample that is only processed through the clean-up procedure and not through the extraction procedure. Measurement of the activity associated with this blank determines the background activity that would be expected to be present in each sample as a result of the column clean-up process.

3.2 CALUX Analysis

3.2.1 Cell Line

For these experiments, the XDS-CALUX bioassay will be used. This bioassay makes uses of mouse hepatoma cells (Hepa1c1c7) that have been stably transfected with the AhR-responsive firefly luciferase reporter plasmid pGudLuc1.1 (cell line H1L6.1c3). The newer, more sensitive cell line H1L7.5c1 will also be used in conjecture with the more established cell line H1L6.1c3 to see if the same result can be obtained so that in the future only the more sensitive cell line H1L7.5c1 can be used. This would be interesting because this higher sensitivity means that lesser amounts of sample are required while at the same time yielding a higher confidence in the result.

The recombinant mouse cell line is grown at 37°C in 5% CO_2 and 85% humidity. Cells are grown in 10cm diameter plastic cell plates containing alpha-minimal essential medium (MEM-α) supplemented with 10% fetal bovine serum (FBS). No external selective pressure (i.e. Geneticin) is needed to maintain the stable integration of the DRE-reporter plasmid in the cell line. The 96-well culture plates are seeded with 100µL of cell suspension at a density of $7.5 \cdot 10^5$ cells/mL with a 12-channel multipipettor. The plates are incubated for 20-24 hours at 37°C in 5% CO_2 and 85% humidity prior to dosing.

3.2.2 TCDD Standard Curve

On each 96-well plate, a ten point TCDD standard curve is generated to quantitavely estimate the equivalent concentration of TCDD for each sample. From a supplied solution of 50µgTCDD/mL, a stock solution of $1.00 \cdot 10^{-5}$ M is prepared from which the ten standard stock solutions can be prepared. The standard stock solutions are diluted by a hundred fold before obtaining the treatment solutions that are administrated to the cells. The supplied solution contains TCDD dissolved in nonane; the dilutions made in the laboratory use the less volatile DMSO as solvent.

The concentrations of TCDD in the ten standard stock solutions are chosen such that the full range of the sigmoid curve is obtained. They are determined by first using a preliminary set of TCDD concentrations. This preliminary set of TCDD concentrations is adjusted such that the response of the new concentrations show two points in the low plateau, two points in the high plateau, and the remaining 6 points evenly distributed between these two plateaus. The values of these initial and adjusted concentrations can be found in Appendix 7.

3.2.3 Dosing of the Cells

First it is necessary to determine the approximate volume of hexane extract that is needed to start the serial dilution that will yield a full dose-response curve. Finding this proper range is done by taking 400µL of the hexane extract and serially diluting this four times by a dilution factor of 11. Then 4µL of DMSO is added and the hexane is evaporated off by a vacuum centrifuge. To each extract in DMSO 400µL of MEM-α medium is added such that the concentration of DMSO administered to the cells is only 1%.

The medium of the cell suspension is removed from each well of the incubated 96-well plate by inverting the plate on to an absorbent paper. Then 100µL of each extract in medium is added to a well (each time in triplicate). In order to have a comparison, 400µL of medium is added to 4µL of two TCDD standard solutions that are situated in the low plateau and to 4µL of two TCDD standard solutions that are situated in the high plateau. In addition DMSO/media solution and media are added to each plate in triplicate as control blanks. The set up of the 96-well plate is shown in Figure 11. It can be seen from this figure that six samples can be analyzed per plate when one wants to do a range finding analysis.

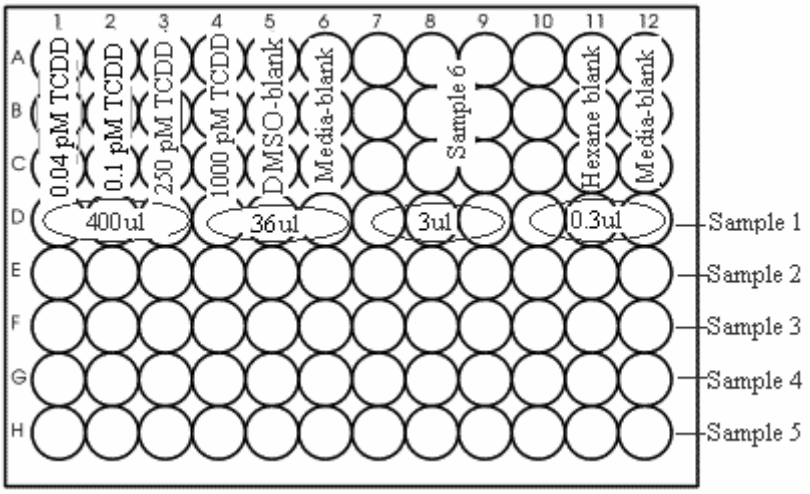

Figure 11. Set-up 96-well plate for range finding

To quantify the BEQ, the desired volume of hexane extract as determined by the range finding procedure is serially diluted nine times by a dilution factor of 3. In a tenth tube a hexane blank containing the same volume of hexane as the other tubes, but no sample extract is added as a control blank. These extracts are transferred into 4µL of DMSO using a vacuum centrifuge and 400µL of MEM-α medium is added to each extract in DMSO.

The dosing is done in a similar fashion as the range finding procedure, but this time a ten point TCDD calibration curve needs to be generated instead of only four comparison TCDD points. The set-up of the 96-well plate is shown in Figure 12. It can be seen from this figure that only 2 samples can be analyzed per plate when one wants to quantify the exact amount of equivalent TCDD per weight of the sample.

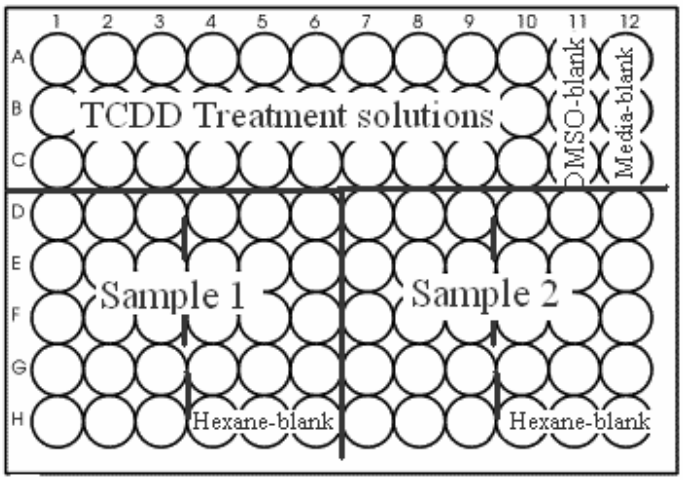

Figure 12. Set-up 96-well plate for quantifying BEQ

3.2.4 Reading of the Wells

After 24h, the 96-well plates are removed from the incubator and the media is removed by inverting the plate on to absorbent paper. Each well is rinsed with 100µL PBS buffer after which each well is inspected for cell viability, noting any damaged, morphologically changed or missing cells. White backing tape is then applied to the bottom to avoid scattering of light and 50µL of culture lysis reagent is added after which the plate is shaken for 5 minutes on a plate shaker to lyse the cells. For analysis of the luciferase activity, 50µL of luciferase substrate (i.e. luciferin) is automatically injected in each well by the microplate luminometer. For the H1L6.1c3 cell line this mixture is allowed to incubate for 4 seconds and is followed by the measurement of light produced over a 5 second time period. For the H1L7.5c1 cell line the incubation time is 5.6 seconds and the integration time is 3 seconds.

3.2.5 Quality Control

To each 96-well plate, a DMSO/media and a media blank is added in triplicate. These blanks are only used to detect contamination and to determine the experimental background level. These values are not used to correct the data because this could give rise to negative values causing problems for the statistical inference based on the curve fitting. This correction would also increase the variability of the results which is not desirable (Miller & Miller, 2005).

If a single data point from the triplicate measurement is found to cause the standard deviation of the triplicate to be more than 20% of the average, than this point is not included in the average and standard deviation calculations. This is a strong indication that something went wrong with that specific well (e.g. cell death, incorrect dosing) so therefore the measurement is discarded.

Lastly, the quality of the plate is checked by looking at the results of the standard curve. The standard curves should always have the same sigmoid shape (between certain limits) on all the plates that use the same cell line. A clear deviation of the expected shape means that the curve cannot be used for calibration and the plate must be rejected.

3.3 Data Processing: Calculating the BEQ

After the experimental work, the data is available as light production for each well, but this raw data still need to be processed in order to provide the information that we are interested in namely an estimate of the BEQ of the sample. This sample potency is calculated by comparing the dose-response curve of the sample to the dose response curve of TCDD.

Many authors (e.g. Brown et al., 2007; U.S. EPA, 2008; Wang et al., 2009) only produce a full dose-response curve for TCDD and not for the sample. Instead only one dilution point per sample is measured. The light response of this measurement point is plugged into the equation of the TCDD full dose-response curve in order to estimate the concentration of TCDD that produces the same light response as the measured concentration of sample. This method of calculation, called the inverse prediction method, will not be used here as it is not accurate for the following reasons. First of all, one is not able to determine if the point measured is located in the linear range of the sigmoid curve or if the point is situated after the maximum plateau and thus cell toxicity is being observed. Secondly, if the sample and TCDD dose-response curve are not parallel, the BEQ value is dependent on the dilution chosen for quantification (Villeneuve et al., 2000; Nording et al., 2007). When only one point is measured, it is not possible to take this deviation, and thus the uncertainty on the prediction, into account. Finally, if the sample and TCDD dose-response curve have a different efficacy (maximal induction response); this difference will go unnoticed if only one point is measured. It is however necessary that the induction response of the sample is expressed relative to the maximum sample response (Relative Light Unit, RLU) such that the effect of this non-ideal behavior is accounted for (Villeneuve et al., 2000).

To be able to calculate a corresponding concentration for a certain light production, it is necessary to fit a curve to the experimentally measured points. As the shape of this curve is sigmoid, the four parameter Hill equation is often used for this purpose because it is a versatile function to describe this shape (Van Overmeire et al., 2004; Van Wouwe et al., 2004; Brown et al., 2007; U.S. EPA, 2008; Wang et al., 2009). A disadvantage of the use of this kind of model is that at least 8 to 9 data points are necessary in order to be able to estimate the parameters of the equation correctly. To solve this, another calculation method has been developed. This makes use of the Box-Cox transformation to linearize the data such that a straight line can be fitted and only four data points per sample are sufficient for a

proper estimation of the parameters[3]. An added advantage of this method is that a higher precision can be obtained, especially at the very low ranges (Elskens et al., 2010). Both calculation methods are discussed in more detail below as they will both be used to analyze the data in order to see if the Box-Cox transformation can give comparable results to the well established four parameter Hill equation model. A short discussion on the calculation of uncertainties associated with the BEQ estimates is included as well.

3.3.1 Model Equation

3.3.1.1 Four Parameter Hill Equation

The four parameter Hill equation is typically written in the form of equation 2 (e.g. Van Overmeire et al., 2004). As the variance of Y is heteroscedastic, the parameters of this equation need to be obtained by a weighted least squares technique. The estimated variance[4] on Y ($\hat{s}_Y^2$) is used to weight each observation and will be estimated by pooling all the experimental data and determining the relationship between Y and s_Y^2 (Elskens et al., 2010).

$$Y_i = y_o + m \cdot \frac{\log X_i^n}{k^n + \log X_i^n} \qquad \textbf{(2)}$$

where Y_i - the light production for observation i normalized to the maximum light production
X_i - TCDD/sample mass for observation i
m - Limiting value of the RLU response as TCDD concentration increases
k - Dose at which the response is 50% of the maximum response
n - Parameter that determines sigmoid shape of the curve
y_0 - Intercept parameter

The log transformation of the net state variable, X, as shown in equation 2 is inspired by the graphical representation of the data where the x-axis is shown as a logarithmic scale, but this may not be a desirable operation for the regression as this transformation deteriorates the

[3] Two parameters (slope and intercept) need to be estimated so four data points measured in triplicate already gives ten degrees of freedom.

[4] The estimated variance instead of the measured variance is used because three points cannot provide an accurate estimation of the variance. The measured variance is only used as a quality control check.

estimation behavior of the model and makes the error distribution asymmetric on a linear dose scale (Elskens et al., 2010). The log transformation on the X variable is therefore avoided so that the regression simplifies to equation 3.

$$Y_i = y_o + m \cdot \frac{X_i^n}{k^n + X_i^n} \quad \textbf{(3)}$$

After determining the parameters of equation 3 by using the weighted least squares technique, the concentration for different levels of the maximum induction can be calculated with the inverse of this equation as given in equation 4.

$$\hat{X}_i = \left(\frac{(Y_i - y_0) \cdot k^n}{m - Y_i + y_0} \right)^{1/n} \quad \textbf{(4)}$$

The BEQ can be calculated as the ratio of the mass of TCDD over the mass of the sample that produces the same amount of the maximum light production. These values are termed effective concentrations (EC). For parallel dose-responses, BEQ estimates are independent of the response level (Putzrath, 1997).To take into account that the slope of the sample curve might differ from the slope of the TCDD standard curve, the BEQ is calculated for different percentages of the maximum light production i.e. EC_{20}, EC_{50} and EC_{80} will be calculated (Villeneuve et al., 2000).

3.3.1.2 Box-Cox Transformation

A transformation on Y can linearize a non-linear expression like the four parameter Hill equation. In this case, the Box-Cox transformation as given in equation 5 can be applied. A limitation to the Box-Cox transformation method is that it is impossible to fit the entire dose-response data in to one unique model. Between the decision limit (as determined later) and 75% of the maximum induction, it is possible to linearize the data with a single lambda so this range will be used as the working range for linearization.

$$Z_i = \frac{Y_i^{\lambda} - 1}{\lambda \cdot (GEOMEAN(Y))^{\lambda - 1}} \quad \textbf{(5)}$$

$$Z_i = slope \cdot X_i + intercept \quad \textbf{(6)}$$

The Box-Cox transformation is carried out to obtain a straight-line relationship (equation 6), but it is important to take into account that this transformation also modifies the shape of the variance distribution. The predicted value of the variance for the transformed variable can be approximated using the error propagation formulas based on a Taylor series expansion (equation 7).

$$\hat{S}_{Z_i}^2 = \left(\frac{Y_i^{\lambda-1}}{(GEOMEAN(Y))^{\lambda-1}} \right)^2 \cdot \hat{S}_{Y_i}^2 \quad \textbf{(7)}$$

With the variances determined, lambda, the slope and the intercept can be estimated with the method of maximum likelihood. After estimating the parameters, the BEQ can be calculated as the ratio of the slope of the sample curve over the slope of the TCDD calibration curve. This approach adjusts for the divergence in the slope and efficacy of the TCDD and sample dose-response curves and therefore allows for a more accurate estimate of the relative potency of unknown samples (Denison et al., 2004; Whyte et al., 2004).

3.3.2 Model Precision

3.3.2.1 Determining Uncertainty on Regression

The variances on the light measurement are used to calculate the variance/covariance matrix of the parameter estimates.

$$Var/Cov = \left(J^T \cdot W \cdot J \right)^{-1} \quad \textbf{(8)}$$

where J - Jacobian matrix; matrix of all first-order partial derivatives of the model with respect to the parameters

W - Weighting matrix; square diagonal matrix with $w_{i,i} = 1/\hat{S}_{Y_i}^2$ or $w_{i,i} = 1/\hat{S}_{Z_i}^2$

In the variance/covariance matrix, the square roots of the entries on the main diagonal represent the asymptotic standard error on the parameter estimates $u(p_j)$. The entries above and below the main diagonal are the covariance between parameters $u(p_j, p_l)_{j \neq l}$. With these values, the uncertainty on the regression line can be estimated by using equation 9 (Ellison et al., 2000; Elskens et al., 2010).

$$u(\hat{Y}_i) = \sqrt{\sum_{j=1,q}\left(\frac{\partial f(X_i)}{\partial p_j}\right)^2 \cdot u(p_j)^2 + \sum_{\substack{j,l=1,q \\ j\neq l}} \frac{\partial f(X_i)}{\partial p_j} \cdot \frac{\partial f(X_i)}{\partial p_l} \cdot u(p_j, p_l)} \quad \textbf{(9)}$$

3.3.2.2 Determining Uncertainty on the Predicted Mass

The uncertainty on X is related to Y rather than to X (Elskens et al., 2010). Therefore, the precision that was determined for Y is translated to a precision on X via equation 10.

$$u(\hat{X}_i) = f(Y_i + u(Y_i)) - f(Y_i) \quad \textbf{(10)}$$

3.3.2.3 Determining Uncertainty on the BEQ

After determining the uncertainty on both the TCDD and sample mass(slope) estimates, the uncertainty on the BEQ estimate is obtained by propagating the error on both estimates by using the law of propagation of uncertainty as shown in equations 11(12) (Ellison et al., 2000).

$$u(BEQ_{Hill}) = \frac{X_{TCDD}}{X_{Sample}} \sqrt{\left(\frac{u(X_{TCDD})}{X_{TCDD}}\right)^2 + \left(\frac{u(X_{Sample})}{X_{Sample}}\right)^2} \quad \textbf{(11)}$$

$$u(BEQ_{Slope}) = \frac{Slope_{Sample}}{Slope_{TCDD}} \sqrt{\left(\frac{u(Slope_{TCDD})}{Slope_{TCDD}}\right)^2 + \left(\frac{u(Slope_{Sample})}{Slope_{Sample}}\right)^2} \quad \textbf{(12)}$$

3.3.2.4 Determining Confidence Interval

The uncertainty is multiplied with a coverage factor in order to obtain the level of confidence required. This confidence interval is expected to encompass a large fraction of the distribution of values which could reasonably be attributed to the predicted value. The coverage factor is set equal to the value of the two-tailed Student's t distribution for the number of degrees of freedom (ν) associated with the model and for a 95% confidence level (Elskens et al., 2010).

$$BEQ \pm t_{0.95}(\nu_{BEQ}) \cdot u(BEQ) \quad \textbf{(13)}$$

The number of degrees of freedom for the Student's t distribution is determined by the Welch–Satterthwaite equation as given in equation 14 (Welch, 1947).

$$\nu_{BEQ} = \frac{\left(u_{TCDD}^2 / \nu_{TCDD} + u_{Sample}^2 / \nu_{Sample} \right)^2}{\frac{\left(u_{TCDD}^2 / \nu_{TCDD} \right)^2}{\nu_{TCDD} - 1} + \frac{\left(u_{Sample}^2 / \nu_{Sample} \right)^2}{\nu_{sample} - 1}} \quad \textbf{(14)}$$

It is important to stress that the 95% confidence interval reported for the BEQ results only represents the uncertainty associated with the uncertainty arising form fitting the mathematical model to the experimental data. It does not quantify other sources of uncertainty (Table 1). As the main goal is to compare two different mathematical models to estimate the BEQ, this limitation is warranted for this thesis. But, it should be investigated if other sources of uncertainty contribute significantly to the total uncertainty. If this is the case they should also be included in future results to indicate the total degree of uncertainty associated with the CALUX procedure in obtaining a BEQ.

Table 1. Typical sources of uncertainty

Sampling	Random variations between different samples.
Storage	Where test items are stored for any period prior to analysis, the storage conditions may affect the results.
Instrument effects	A temperature controller that may maintain a mean temperature which differs (within specification) from its indicated set-point.
Reagent purity	The concentration of a volumetric solution will not be known exactly even if the parent material has been assayed, since some uncertainty related to the assaying procedure remains.
Measurement conditions	Volumetric glassware may be used at an ambient temperature different from that at which it was calibrated.
Sample effects	The recovery of an analyte from a complex matrix, or an instrument response, may be affected by composition of the matrix.
Computational effects	Selection of the calibration model influences the goodness of fit and thus the uncertainty on the result.
Operator effects	The operator might read a meter or scale consistently high or low.
Random effects	Random effects contribute to the uncertainty in all determinations.

Source: Ellison et al., 2000

4 Results and Discussions

During the course of the experimental work, three batches of blanks were prepared. The first two showed only low background levels of induction, but the last batch of blanks showed that during the clean-up process (by the acid-silica column, the X-Carb column or any of the solvents used during this process), an AhR agonist was introduced. The samples that were processed together with this last batch of blanks are therefore also probably contaminated and will show higher results than previously processed samples. This is clearly a problem for the analysis of the samples as comparison between samples from the same site but prepared at different time moments is no longer possible. The total uncertainty on the BEQ that can reasonably be expected could therefore not be calculated. The comparison between different treatments will still be made under the assumption that samples that were prepared at the same time moment show the same level of contamination irrespective of the clean-up method used.

4.1 Developing Program for CALUX Data Analysis

Before starting the true analysis of the BEQ results, it is first necessary to develop a method to process the raw CALUX data from which the BEQ is calculated in a consistent manner. For this purpose, an Excel macro was written that contains the formulae used to fit the different model equations (four parameter Hill equation and Box-Cox transformation) and determine the associated expanded uncertainties. This program can be found on the CD-ROM enclosed at the back of this thesis together with both the raw and processed data.

First of all, the user of the program can indicate what the lay-out of the 96-well plate is. After indicating the number of TCDD standard points used, the number of samples analyzed, the number of points per sample that were analyzed and the number of replicates measured for each point, the “Init Sample”-button can be pressed, such that the correct number of measurements appear and can be filled in by the user. For each sample, the user needs to provide a sample label, the sample weight, the total extract volume, the extract volume that was used to start the dilution series (expressed as µL per well) and the dilution factor. For each TCDD concentration and sample dilution point, the position on the plate can then be filled out.

A maximum allowed standard deviation on the replicate measurement is applied to ensure that wells for which the measurement is not a good representation of the “true” value

are not included in the further calculations. The user of the program can change this maximum allowed standard deviation to the desired value, here 20% was chosen. After pressing the "Check %max stdev allowed"-button a color coding mechanism is applied. The background color of the wells allows mistakes in the position of the plates to become apparent at this stage. If the light production values are red and crossed-through, this means that the standard deviation of those replicate measurements is higher than the maximum allowed standard deviation. The user can then choose to remove the reference to the plate position of the point that is causing the standard deviation to be too high.

Before pressing the "Calculate BEQ"-button, the treatment volume per well and the cell-line that was used to make the measurements also need to be provided as extra parameters for the calculations.

The program calculates the optimal parameters for both mathematical models by minimizing the sum of the squared errors via the build in solver add-in in Excel. The fitting of the four parameter Hill equation is both done for the data expressed as %max TCDD and as %max sample to enable the comparison between both results for other researchers. In this work only the latter results are used as this corrects for the difference in efficacy.

It can be checked if the parameters that are obtained for the fit are meaningful by two different ways. First, a visual inspection of the graphs that are provided in the output sheet allow for an expert judgment to see if the fitted sigmoid curve follows the experimental points as expected. If from this visual inspection it is clear that there is cell death after the upper plateau has been reached, the references to the position on the plate for those concentrations on the input sheet should be removed to exclude these measurements from further calculations. Secondly, a more exact approach can be used by looking at the p-values of the model fit and the model parameters. The statistical evaluation of the goodness of fit of the model is based on a comparison between the Sum of the Squared Residuals (SSR) and the theoretical χ^2 distribution from which the SSR should be derived (Elskens et al. 2007). For a good fit the model assumptions must be satisfied so the SSR must be lower than the critical χ^2 value (upper one-tail 95%, $p > 0.05$). If the SSR > $\chi^2_{99\%}$ this indicates a substantial lack of fit or some failures of the assumptions behind the model ($p < 0.01$). For the model parameters, the standard error on the parameter estimate is assumed to follow a t-distribution such that if the p-value is lower than 0.01, the null hypotheses that the parameter wasn't determined accurately must be rejected.

If the parameters obtained by the solver add in are not satisfactory, it is advised to change the solver start values[5] as the solver could have been fixed at a local minimum. For the Box-Cox transformation, it might also be necessary to change the lower or upper bound[6] that determines which region is linearized based on expert judgment of the output graphs. If these actions cannot solve the problem, than the points are not forming a sigmoid curve/cannot be linearized and the data cannot be used for further analysis.

After making sure that the fitting of both functions has been executed correctly, the BEQ together with the expanded uncertainties on these calculated results can be found for each sample in the output sheet under the output graphs. As discussed in the data processing heading, this expanded uncertainty represents the uncertainty that could reasonably be attributed to the calculated value due to the uncertainty of the fitting of the mathematical model to the experimentally measured points. Other sources of uncertainty e.g. sampling uncertainty are not taken into account by the program.

The calculated BEQ results for each sample can now be easily used for further analysis namely for comparison between different samples, different treatments, etc., depending on the research question.

[5] The initial start values for the Solver are specified in the Visual basic code (Alt + F11) - search for "SolverStartValue".

[6] The lower and upper bound of the linearization region for the Box-Cox transformation can be changed in the input sheet after scrolling down.

4.2 Validation of the Use of the H1L7.5c1 Cell Line

4.2.1 Functional Relationship between Precision and the Mean Response Variable

Elskens et al. (2010) have already established a functional relationship between the standard deviation of the light production (sd RLU) and the light production (RLU) for the TCDD standard points measured by the H1L6.1c3 cell line. This relationship is depicted by a power law and can be used as a performance criterion. It is verified that the same precision is obtained by the author with this cell line before investigating if this relationship is also valid for the H1L7.5c1 cell line.

To determine the relationship between the standard deviation on the light production and the light produced, the $\log_{10}$ sd RLU is plotted against the $\log_{10}$ RLU. This is shown for the TCDD standard points measured with the H1L6.1c3 cell line in Figure 13. The regression appears as a straight line with a slope 0.96 (SE = 0.04, $p < 0.001$) that is significantly different from 0 which shows that the data is heteroscedastic. The coefficient of determination indicates that 76% of the variance of the sd RLU can be explained by the regression. The slope doesn't significantly differ from the estimate made by Elskens et al. (2010) on a 95% confidence level (tow-sided t-test) but the intercept is slightly higher (-0.87 compared to -1.3, $p = 0.004$). As this difference is not very large, we can still conclude that rather good performance with the H1L6.1c3 cell line was obtained.

The standard deviation on the light measurement will be estimated with equation 15 for the TCDD standard points when measured by the H1L6.1c3 cell line.

$$\hat{s}_{Y_i} = 10^{-0.87} \cdot Y_i^{0.91} \qquad \textbf{(15)}$$

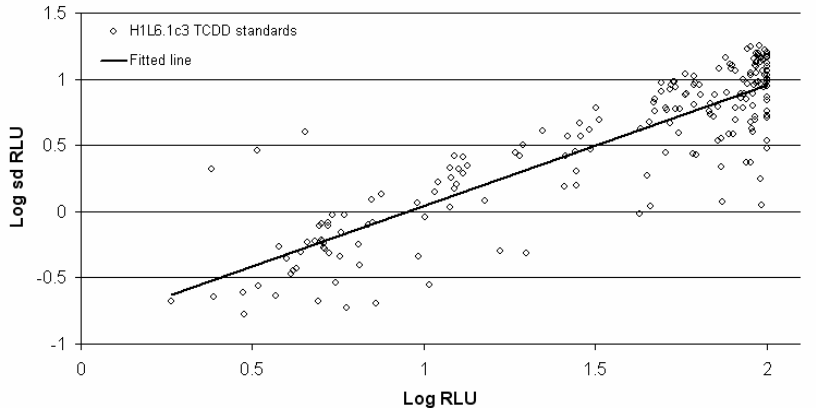

Figure 13. Linear relationship between the standard deviation and the response variable for H1L6.1c3 TCDD standard points (Log sd RLU = - 0.87 (SE = 0.06) + 0.91 (SE = 0.04) Log RLU)

It needs to be verified that the same distribution of the standard deviations can be found for the samples as for the TCDD standard points. The regression of the sd RLU of the sample points appears as a straight line with a slope 0.71 (SE = 0.04, $p < 0.001$). The variance is higher for the sample points than for the TCDD standard points as can be seen visually by comparing Figure 14 to Figure 13. This higher variance is also indicated by the much lower coefficient of determination. Only 55% of the variation can be explained by the regression as compared to 76% of the variation that could be explained for the TCDD standards.

Since both the slope ($p < 0.001$) and the intercept ($p = 0.004$) are significantly different from the slope and intercept of the TCDD standards, equation 16 is used instead of equation 15 to estimate the standard deviation on the light production for the sample points.

$$\hat{s}_{Y_i} = 10^{-0.63} \cdot Y_i^{0.71} \qquad \textbf{(16)}$$

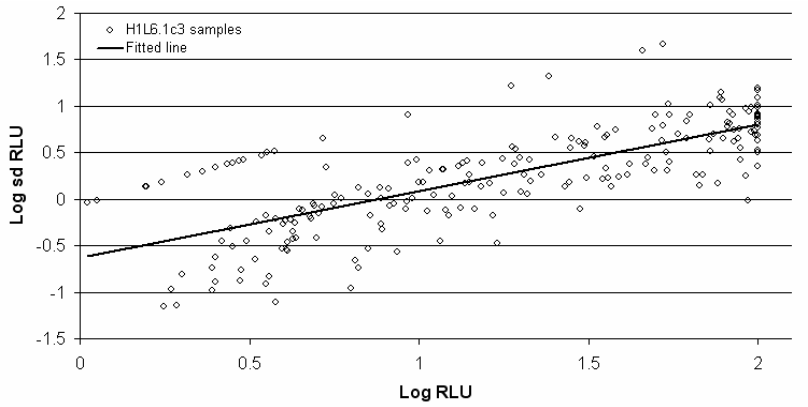

Figure 14. Linear relationship between the standard deviation and the response variable for H1L6.1c3 sample points (Log sd RLU = - 0.63 (SE = 0.06) + 0.71 (SE = 0.04) Log RLU)

Now that the relationship between the standard deviation and the light measurement has been determined for the H1L6.1c3 cell line, it needs to be checked if the same expressions hold true for the H1L7.5c1 cell line. This is first checked for the TCDD standard points (Figure 15). Again, the regression appears as a straight line with a slope 1.04 (SE = 0.04, $p < 0.001$) which is significantly different from 0. The coefficient of determination indicates that 78% of the variance of the sd RLU can be explained by the regression. The slope of the regression line doesn't significantly differ from the estimate for the H1L6.1c3 cell line (95% confidence interval for a two-sided t-test), but the intercept is significantly lower ($p < 0.001$). Therefore it is valid to use the expression as shown in equation 17 to estimate the standard deviation on the RLU for the H1L7.5c1 cell line.

$$\hat{s}_{Y_i} = 10^{-1.31} \cdot Y_i^{1.04} \qquad \textbf{(17)}$$

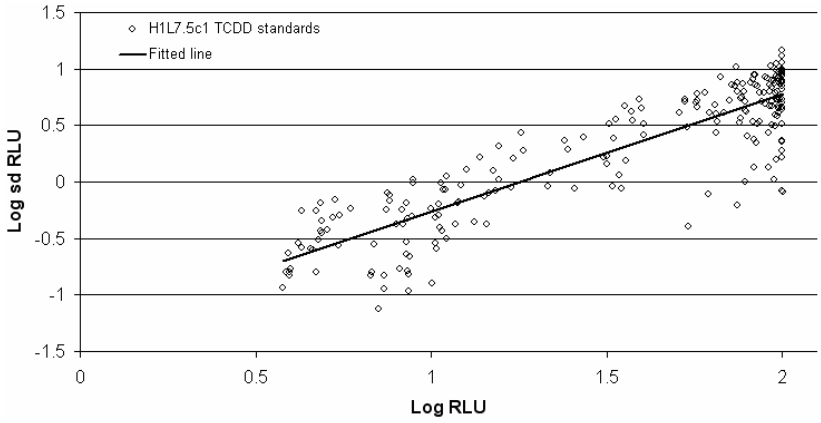

Figure 15. Linear relationship between the standard deviation and the response variable for H1L7.5c1 TCDD standard points (Log sd RLU = - 1.31 (SE = 0.06) + 1.04 (SE = 0.04) Log RLU)

Finally, it is also checked how the standard deviation can be estimated for the sample points that are measured with the H1L7.5c1 cell line. The regression of the sd RLU of the sample points (Figure 16) appears as a straight line with a slope 0.80 (SE = 0.04, $p < 0.001$). The variance is again much higher for the sample points than for the TCDD standard points as is indicated by the much lower coefficient of determination. Only 53% of the variation can be explained by the regression compared to 78% of the variation that could be explained for the TCDD standards.

The slope is not significantly different ($p = 0.19$) from the slope of the samples measured with H1L6.1c3, but the intercept is significantly lower ($p < 0.001$). Therefore equation 18 instead of equation 16 is used to estimate the standard deviation for the sample measurements with the H1L7.5c1 cell line.

$$\hat{s}_{Y_i} = 10^{-1.04} \cdot Y_i^{0.80} \qquad \textbf{(18)}$$

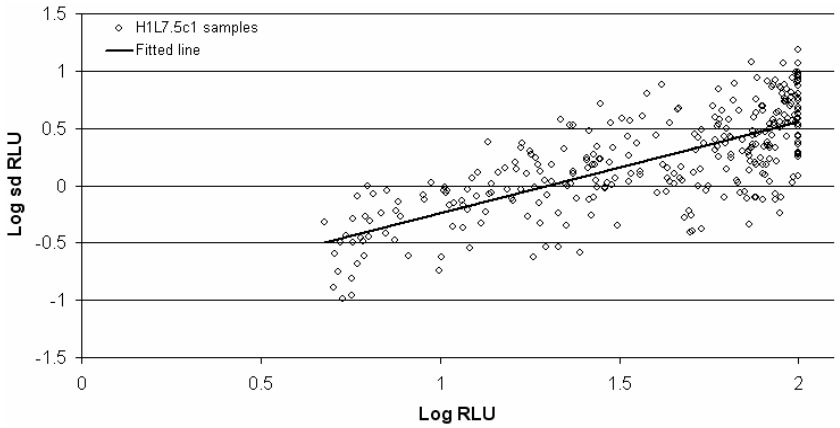

Figure 16. Linear relationship between the standard deviation and the response variable for H1L7.5c1 sample points (Log sd RLU = - 1.04 (SE = 0.07) + 0.80 (SE = 0.04) Log RLU)

4.2.2 Determining Experimental Background Level

The experimental background level is defined as the RLU level below which it is difficult to distinguish an actual response from a random noise. It is assessed from the blank measurements that are included on each 96-well plate. The decision limit and detection limit can be determined by using the IUPAC formulas (Currie, 1995) given in equations 19 and 20 and are calculated for both cell lines to see if there is a difference or not.

$$LC = \mu_{bl} + 1.645 \cdot \sigma_{bl} \quad \textbf{(19)}$$

$$LD = \mu_{bl} + 3.29 \cdot \sigma_{bl} \quad \textbf{(20)}$$

The sampling distribution of the mean DMSO blank for the H1L6.1c3 cell line (3 replicates repeated 18 times) is normal (Kolmogorov-Smirnov test, p = 0.390) with an overall mean of 7.3 and a standard deviation of 0.69 resulting in a decision limit of ~8 and a detection limit of ~10 RLUs.

The sampling distribution of the mean DMSO blank for the H1L7.5c1-cell line (3 replicates repeated 31 times) is also normal (Kolmogorov-Smirnov test, p = 0.638) with an overall mean of 10.07 and a standard deviation of 1.99 resulting in a decision limit of ~13 and a detection limit of ~17 RLUs.

Both cell lines differ significantly in the value of the overall mean of the DMSO blank (two-sided t-test, $p < 0.001$). The decision and detection limit for the H1L7.5c1 cell line are significantly higher than the decision and detection limit for the H1L6.1c3 cell line. This has the consequence that the H1L7.5c1 cell line has a smaller working range than the H1L6.1c3 cell line when linearizing the data using the Box-Cox transformation.

4.2.3 Comparison BEQ Obtained with H1L6.1c3 and H1L7.5c1

To verify that the H1L7.5c1 cell line gives the same result as the H1L6.1c3 cell line, eight sample extracts were analyzed with both cell lines and the BEQ calculated. The BEQ was calculated based on the ratio of the slopes after Box-Cox transformation. This method was chosen because for most samples it was not possible to produce a full dose-response curve. The maximum induction level was often not achieved making it impossible to fit the four parameter Hill equation with a acceptable accuracy.

After visually inspecting the BEQ for both cell lines it can be concluded that they seem to provide comparable results as the confidence interval on the BEQ results often overlap

(Figure 17). To increase the confidence in this conclusion, this hypothesis is also tested mathematically.

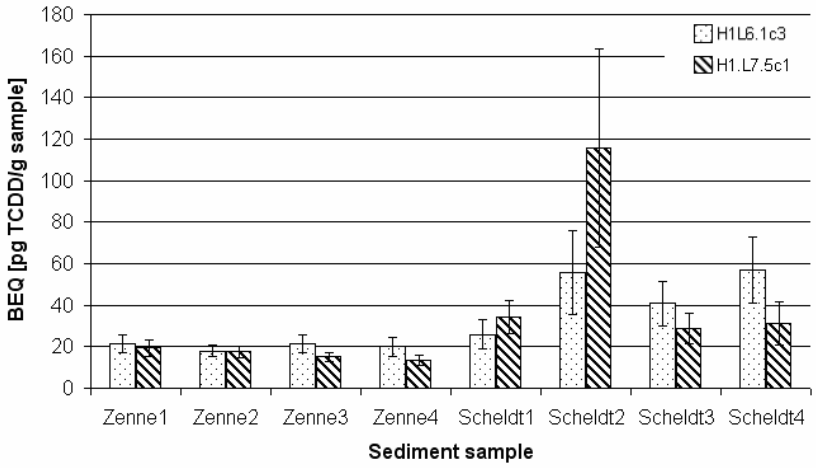

Figure 17. Comparisons of BEQ [pg TCDD/ g sample] (calculated with the slope ratio method; 95% confidence interval indicated) obtained with the H1L6.1c3 cell line and the H1L7.5c1 cell line

As different sediment samples were investigated, a simple t-test would not be sufficient to detect a difference in calculated BEQ between cell lines. As the values were not normally distributed ($p < 0.050$), it was chosen to perform a Wilcoxon Signed Rank Test instead of the normal paired t-test. The difference in result between both cell lines is not great enough to exclude the possibility that it is simply due to chance ($p = 0.547$). Based on this small random sample, it can be concluded that the same BEQ result can be obtained with the H1L6.1c3 cell line as with the H1L7.5c1 cell line. It is advisable though that this test is repeated with a much larger dataset to increase the statistical power of the experiment and thus the confidence in the conclusion made.

When the uncertainties on the estimation are expressed relative to the magnitude of the estimation, a t-test instead of a paired t-test can test if both cell lines provide the same precision on the calculated BEQ. These uncertainty values are not normally distributed ($p < 0.050$) so a Mann-Whitney Rank Sum Test was performed instead of the t-test which assumes a normal distribution. Based on this small random sample, it appears that the same precision on the calculated BEQ is obtained with both cell lines ($p = 0.442$). Again, it is recommended that this test is repeated with a much larger sample size to increase the confidence in this conclusion.

4.3 Developing Sample Preparation Protocol for CALUX and GC-HRMS

4.3.1 Comparison Different Sample Preparation Treatments

Because the investigated sediment samples are highly polluted, the acid-silica column is easily clogged. It is checked that adding concentrated sulfuric acid to the extract before putting it on the clean-up column can provide the same BEQ result as this makes the clean-up process easier. The BEQ was calculated based on the ratio of the slopes after Box-Cox transformation. This method was chosen because for most samples the maximum induction level was not reached making it impossible to fit the four parameter Hill equation.

After visually inspecting the BEQ for both the regular clean-up as described in the literature and the regular clean-up with the addition of sulfuric acid it appears that the treatment with sulfuric acid provides a higher BEQ result than the treatment without sulfuric acid (Figure 18). This difference is however not statistically significant (paired t-test, $p = 0.201$) and can probably be attributed to random sampling error. More samples should be tested to exclude the possibility that the sulfuric acid treatment removes antagonists from the sample that are not removed by the acid-silica clean-up and to demonstrate that the sulfuric acid treatment has no influence on the AhR potency of the sample. The possibility that the often higher BEQ is due to the addition of AhR agonists can be excluded because the blanks for this treatment do not show an elevated response compared to the blanks that were not treated with concentrated sulfuric acid.

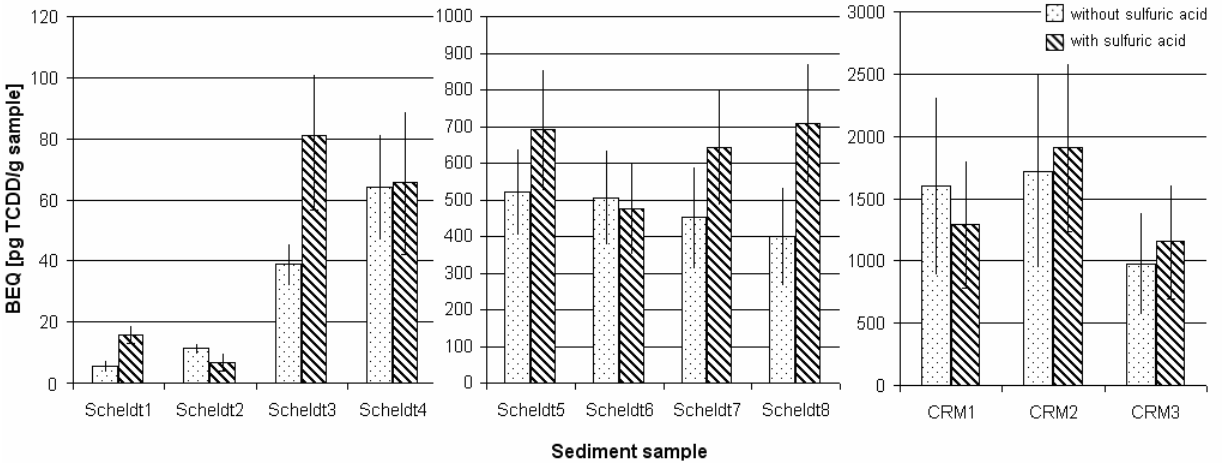

Figure 18. Comparisons of BEQ [pg TCDD/ g sample] (calculated with the slope ratio method; 95% confidence interval indicated) for clean-up with and without sulfuric acid

Now that the impact of adding the sulfuric acid is understood, it is checked if adding the copper clean-up step to remove the sulfur from the sample influences the result obtained by CALUX. This step is introduced so that the same prepared sample extract can be analyzed with GC-HRMS. This extract has been prepared, but will only be analyzed by GC-HRMS in July or August 2010 at the laboratory of the University of Liege within the framework of the FERTIDIOX project.

To ensure that any difference in results can be attributed to the difference in clean-up and not to random difference in sampling/extraction, four extracts were pooled after extraction and concentrated sulfuric acid was added. This pooled extract was then split into four equal aliquots for analysis by the different clean-up treatments (no clean-up except for sulfuric acid clean-up, regular clean-up with sulfuric acid and two times regular clean-up with sulfuric acid and activated copper, one for CALUX analysis and one for GC-HRMS analysis). The BEQ was calculated with the slope ratio method as some samples did not reach the upper plateau and others did not reach the lower plateau, making it impossible to fit the four parameter Hill equation. The calculated BEQs show no clear influence of adding activated copper (Figure 19). This is confirmed with a Wilcoxon Signed Rank Test ($p = 0.735$). There was also no significant difference in the relative uncertainty on the BEQ between the two treatment groups (two-sided t-test, $p = 0.235$).

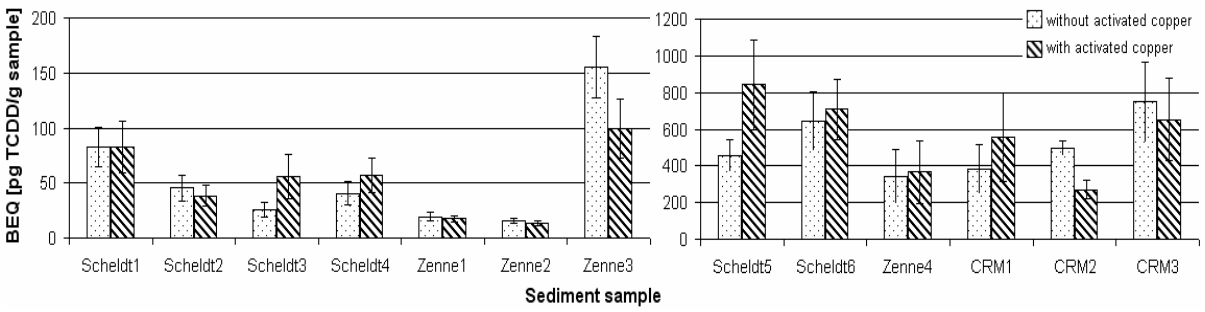

Figure 19. Comparisons of BEQ [pg TCDD/ g sample] (calculated with the slope ratio method; 95% confidence interval indicated) for clean-up with and without copper

One of the four aliquots was not subjected to further clean-up to determine the effect of the clean-up step on the AhR agonist activity in the sample. It is clear from Table 2 that not subjecting the sample to further clean-up steps results in a significantly enhanced AhR activity for the Certified Reference Material samples (on average the crude samples give a 30-fold higher potency than the cleaned-up samples) and for the Scheldt sediment samples (on average the crude samples give a 130-fold higher potency than the cleaned-up samples).

Scheldt 5 and Scheldt 6 were samples that belonged to the contaminated batch so the clean-up over the acid-silica and X-carb column introduced AhR agonists into these samples and not into the crude sample that was not processed further, thus explaining the lower increase in BEQ.

For the Zenne sediment samples however, a much lower increase in BEQ is observed when the samples are not subjected to clean-up as compared to when they are subjected to this clean-up. This Zenne sediment sample has however been stored in a plastic container for more than two years such that much of the AhR agonist may have been removed from the sediment.

Table 2. BEQ [pg TCDD/ g sample] calculated as the slope ratio after Box-Cox transformation for clean-up with and without activated copper compared to only sulfuric acid clean-up

	Sulfuric acid - acid silica X-Carb	Sulfuric acid - acid silica activated copper - X-Carb	Sulfuric acid	Factor increase
Scheldt 1	83 ± 18	83 ± 23	5173 ± 1337	63
Scheldt 2	45 ± 12	38 ± 10	4009 ± 839	96
Scheldt 3	26 ± 7	56 ± 20	5729 ± 1239	140
Scheldt 4	41 ± 11	57 ± 16	10914 ± 2909	223
Scheldt 5	456 ± 85	844 ± 244	2045 ± 529	3
Scheldt 6	644 ± 158	708 ± 166	1232 ± 380	2
Zenne 1	19 ± 4	17 ± 3	319 ± 56	17
Zenne 2	15 ± 2	13 ± 2	103 ± 27	7
Zenne 3	156 ± 28	100 ± 27	5845 ± 1406	46
Zenne 4	341 ± 147	368 ± 171	3158 ± 610	9
CRM 1	382 ± 133	555 ± 240	8920 ± 2007	19
CRM 2	494 ± 41	271 ± 49	18354 ± 5184	48
CRM 3	749 ± 219	653 ± 223	19906 ± 7292	28

To determine if the increased response of the crude extract for the Scheldt sediment and the Certified Reference Material is caused by undesired “nonclassical” AhR ligands (Nording et al., 2007) or by the presence of PCB congeners (Safe, 1990), the PCB fraction of the X-Carb eluate also needs to be analyzed by CALUX. If the total (dioxins and PCBs) BEQ can be measured by analyzing the crude extract much time can be saved as the whole clean-up procedure would not have to be undertaken. Unfortunately, the analysis of the PCB fraction was not executed in this study due to time limitations so no conclusion as to the origin of the difference in the crude result and the dioxin BEQ can be made at the moment.

4.3.2 Validation Sample Preparation Protocol

The sample preparation and analysis protocol can be validated by comparing the result from this protocol to the result obtained with an already established protocol. For this purpose, use is made of the Standard Reference Material® 1944 - New York/New Jersey Waterway Sediment (Wise & Watters, 2008).

For the Certified Reference Material, reference values for the concentrations for the 17 2, 3, 7, 8-substituted polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners obtained with GC-HRMS analysis are available. Based on these concentrations, the TEQ can be calculated by using the assigned TEF values (see appendix 1 and 2). For this sediment sample, the TEQ is relatively independent from which set of TEF values are used (Table 3) so therefore only the most recent TEQ value will be used as this contains the most up to date knowledge on the toxicity.

Table 3. Concentration of selected dibenzo-*p*-dioxin and dibenzofuran congeners in the CRM and their corresponding TEQ values

	Mass Fraction [µg/kg]	TEQ 1988	TEQ 1998	TEQ 2005
2,3,7,8-TCDD	0.133 ± 0.009	0.133 ± 0.009	0.133 ± 0.009	0.133 ± 0.009
1,2,3,7,8-PnCDD	0.019 ± 0.002	0.0095 ± 0.001	0.019 ± 0.002	0.019 ± 0.002
1,2,3,4,7,8-HxCDD	0.026 ± 0.003	0.0026 ± 0.0003	0.0026 ± 0.0003	0.0026 ± 0.0003
1,2,3,6,7,8-HxCDD	0.056 ± 0.006	0.0056 ± 0.0006	0.0056 ± 0.0006	0.0056 ± 0.0006
1,2,3,7,8,9-HxCDD	0.053 ± 0.007	0.0053 ± 0.0007	0.0053 ± 0.0007	0.0053 ± 0.0007
1,2,3,4,6,7,8-HpCDD	0.80 ± 0.07	0.008 ± 0.0007	0.008 ± 0.0007	0.008 ± 0.0007
1,2,3,4,6,7,8,9-OCDD	5.8 ± 0.7	0.006 ± 0.001	0.0006 ± 0.0001	0.0017 ± 0.0002
2,3,7,8-TCDF	0.039 ± 0.015	0.004 ± 0.002	0.004 ± 0.002	0.002 ± 0.002
1,2,3,7,8-PnCDF	0.045 ± 0.007	0.0023 ± 0.0004	0.0023 ± 0.0004	0.0014 ± 0.0002
2,3,4,7,8-PnCDF	0.045 ± 0.004	0.0225 ± 0.002	0.0225 ± 0.002	0.014 ± 0.001
1,2,3,4,7,8-HxCDF	0.22 ± 0.03	0.022 ± 0.003	0.022 ± 0.003	0.022 ± 0.003
1,2,3,6,7,8-HxCDF	0.09 ± 0.01	0.009 ± 0.001	0.009 ± 0.001	0.009 ± 0.001
1,2,3,7,8,9-HxCDF	0.019 ± 0.018	0.0019 ± 0.0018	0.0019 ± 0.0018	0.0019 ± 0.0018
2,3,4,6,7,8-HxCDF	0.054 ± 0.006	0.0054 ± 0.0006	0.0054 ± 0.0006	0.0054 ± 0.0006
1,2,3,4,6,7,8-HpCDF	1.0 ± 0.1	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001
1,2,3,4,7,8,9-HpCDF	0.040 ± 0.006	0.0004 ± 0.0001	0.0004 ± 0.0001	0.0004 ± 0.0001
1,2,3,4,6,7,8,9-OCDF	1.0 ± 0.1	0.001 ± 0.0001	0.0001 ± 0.00001	0.0003 ± 0.00003
TOTAL TEQ [pg TCDD/ g sediment]		**248 ± 10**	**252 ± 10**	**243 ± 10**

Source: NATO/CCMS, 1988; Van den Berg et al., 1998; Van den Berg et al., 2006; Wise & Watters, 2008

As discussed in the beginning of this chapter, the last batch of samples show contamination so these samples cannot be used to compare to the “known” amount. Therefore there are only a limited number of samples available to compare (6 out of the 14 samples that were

analyzed). It is recommended that the analysis is repeated for more samples in order to have a higher confidence in the conclusion.

The BEQ is calculated based on the EC_{50} ratio after fitting the four parameter Hill equation as this calculation method is the most widely used and both the lower and upper plateau is reached for all six samples. The developed bio-analytical analysis provides a reasonable estimate of the potency of the sample compared to the "true" reference value that was established for this sample (Figure 20). Thus, the same accuracy can be obtained by both methods. However, from the width of the 95% confidence intervals it is clear that the precision on the result is much lower for the bio-analytical analysis as compared to the chemical analysis.

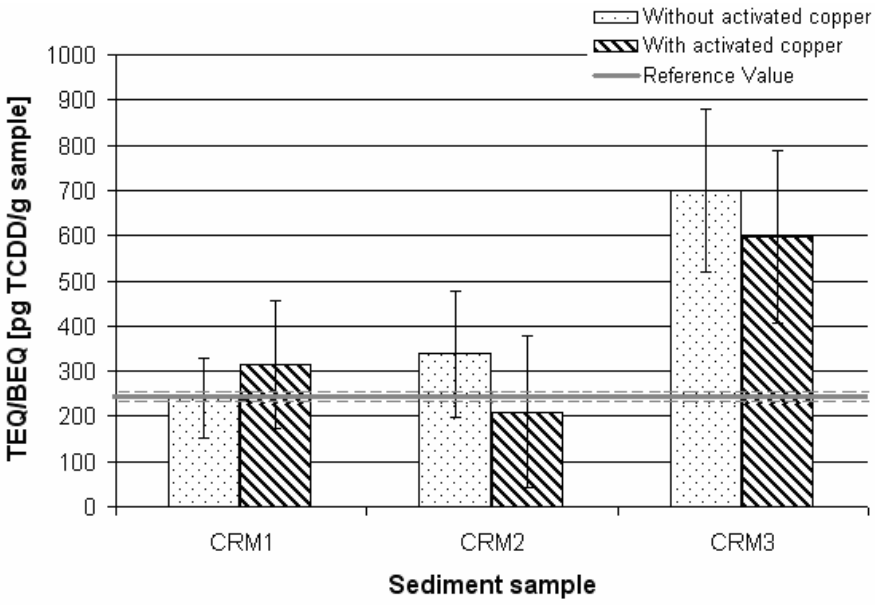

Figure 20. Comparison of measured BEQ [pg TCDD/ g sample] (calculated with the EC_{50} method; 95% confidence interval indicated) to "true" TEQ [pg TCDD/ g sample] (TEF values from Van den Berg et al., 2006; 95% confidence interval indicated)

4.4 Comparison of Different Calculation Methods BEQ

It was tried to measure complete dose-response curves for all the extracts but it is apparent from comparing the different cell lines and different clean-up treatments that this is not always possible. Sometimes there was not enough sample extract to produce the upper plateau (Figure 21); in other cases the lower plateau was not reached as the selected start volume was too high (Figure 22). This last problem is specific to this experiment as it is due to the contamination for the last batch of samples. Normally, a range finding is performed for each sample so that the correct extract volume from which the dilution series needs to start can be established. The range finding was not repeated for each prepared sample here as only three sediment samples were under investigation so the extract volume that needs to be used to start the dilution series was determined from the first batch of experiments and assumed to be fixed.

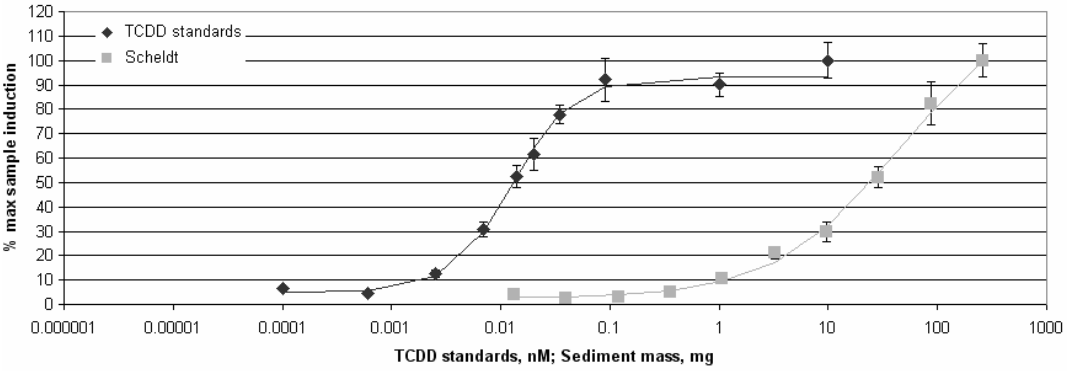

Figure 21. Dose-response curve for Scheldt sample measured with the H1L6.1c3 cell line; upper plateau was not reached making assessment of EC_{20}, EC_{50} and EC_{80} impossible

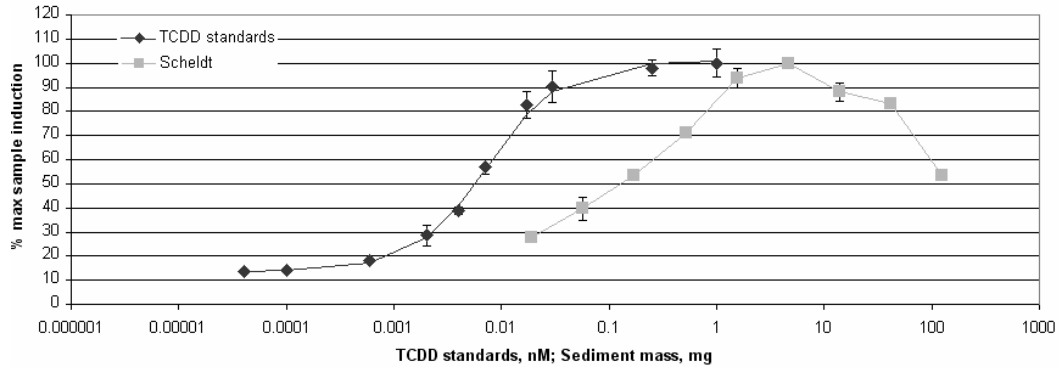

Figure 22. Dose-response curve for Scheldt sample measured with the H1L7.5c1 cell line; lower plateau was not reached making assessment of EC_{20}, EC_{50} and EC_{80} impossible

To be able to fit the four parameter Hill equation, both the lower and upper plateau of the dose-response curve are necessary. If the Box-Cox transformation is applied to linearize the data, these plateaus are not necessary. This is clearly an advantage of the slope ratio method after Box-Cox transformation. To determine if both calculation methods give the same result, it is however necessary that they can be applied on the same dose-response curve. This was the case in 29 out of the 71 dose-response curves measured so only this subset of the data will be used further.

The BEQ calculated as the slope ratio after Box-Cox transformation always lies between the range of BEQ values provided by fitting the four parameter Hill equation to the dose-response curve (Figure 23, Figure 24 and Figure 25).

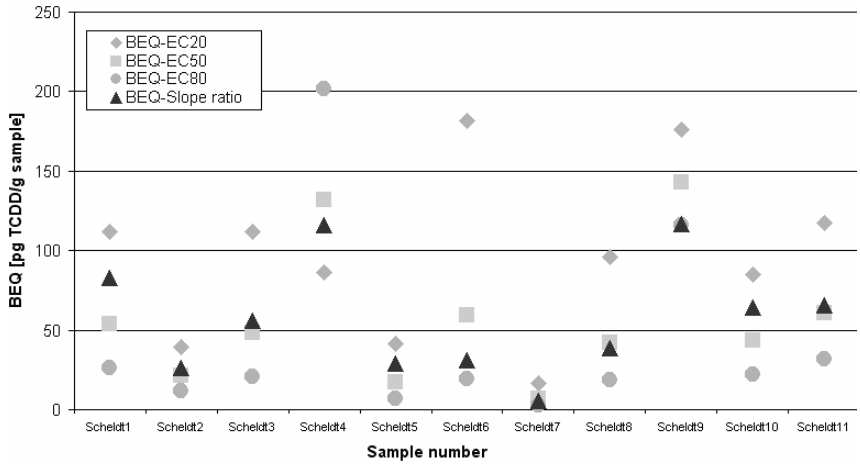

Figure 23. Comparison BEQ obtained with different calculation methods for Scheldt sediment samples

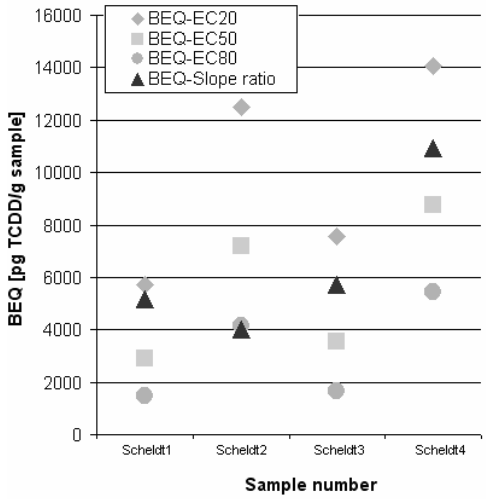

Figure 24. Comparison BEQ obtained with different calculation methods for crude Scheldt sediment samples

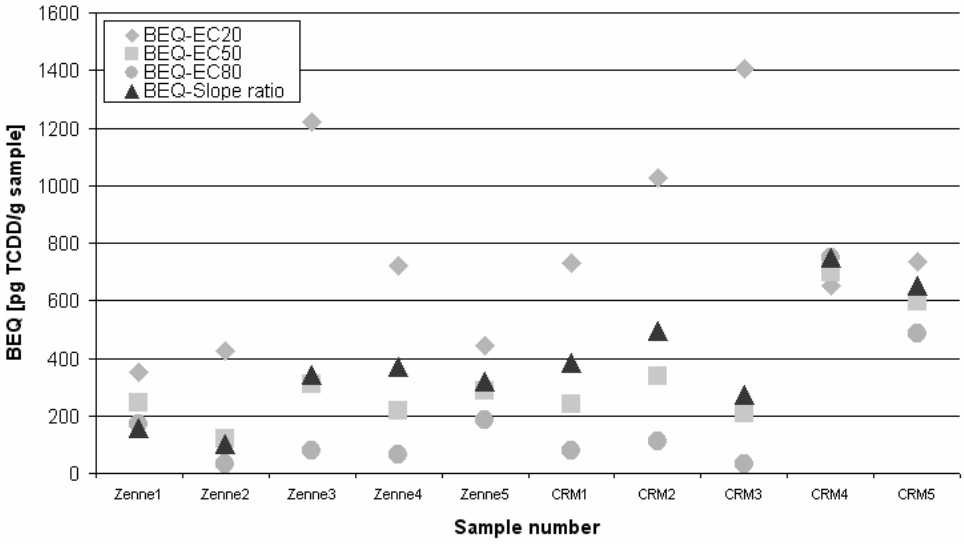

Figure 25. Comparison BEQ obtained with different calculation methods for Zenne and CRM sediment samples

The slope ratio method provides a similar result in terms of absolute value to the effective concentration ratio method, but it still needs to be determined if it also provides a similar precision on this value. The uncertainty on the BEQ associated with the mathematical model used, is expressed relative to the BEQ such that the values can be compared directly between samples with different BEQs.

The uncertainty associated with the BEQ_{EC20} estimate is not significantly different from the uncertainty associated with the BEQ_{EC50} estimate and is on average around 45%. The uncertainty associated with the BEQ_{EC80} estimate is significantly higher and is often even more than a 100%. Thus, it is very difficult to estimate the BEQ based on the ratio of the EC_{80} accurately. This is probably due to the difficulty in fitting the upper plateau to the experimental data, especially since this is never truly reached (often only the inflection point is reached after which cell-death is observed at higher concentrations).

The uncertainty associated with the BEQ estimate calculated as the slope ratio is significantly lower than the uncertainty associated with the BEQ estimates calculated as the effective concentration ratio. The average uncertainty on the BEQ_{slope} estimate is only 28%, almost half of what was obtained for the BEQ_{EC20} and BEQ_{EC50} estimates and only one fourth of the uncertainty that was obtained for the BEQ_{EC80} estimate. So it can be concluded that the Box-Cox transformation in combination with the slope ratio method is a much more robust calculation method than the more frequently used four parameter Hill equation in combination with the effective concentration method.

5 Conclusions

The hydrophobicity of dioxins and dioxin-like compounds causes sediments in rivers and sludge from WWTPs to form important sinks for these compounds. Dioxins and dioxin-like compounds are extremely toxic as even very low concentrations pose a health risk. It is therefore important that the amount of these compounds in the sediment/sludge can be tracked routinely. The standard reference method to determine the concentration of dioxins is GC-HRMS. It only provides information on the concentration of the compounds and this information is converted to a Toxic Equivalent (TEQ) by means of toxic equivalency factors (TEFs). A less expensive and faster analysis method has been developed that makes use of the toxicity mechanism of dioxins in cells. Mouse hepatoma cells are genetically engineered to contain the firefly luciferase gene under dioxin control. This measurement provides a Biological Equivalent (BEQ), an estimate of the dioxin potency of the sample. Several methodological implications that arise with the use of this bio-analytical method are investigated in this thesis in order to improve the confidence in the result that can be obtained with this method.

First, a comprehensive data analysis program has been developed allowing researchers to calculate the BEQ with an expanded uncertainty from the raw CALUX data in a consistent manner. The program calculates the BEQ by using both the four parameter Hill function in combination with the effective concentration ratio and by using the Box-Cox transformation of the y-axis in combination with the slope ratio method. The fitting of the functions can be inspected both visually by looking at the generated output graphs and mathematically by looking at the calculated p-values. It was chosen to make the program dynamic such that it can still be used even if the plate lay-out or other experimental parameters are changed. This makes the program a valuable contribution to future research.

Based on the processed results, the performance of a newer, more sensitive cell line (H1L7.5c1) is compared to the performance of an already established cell line (H1L6.1c3). It can be concluded that the same result with the same precision can be obtained but less sample extract is necessary for the analysis thus effectively improving the detection limit of the method.

To be able to compare the TEQ obtained by chemical analysis to the BEQ obtained by bio-analytical analysis it is necessary that both analyses are performed on sample extracts that are prepared the same way. Then, if a difference is still found one knows it is due to the different analysis and not due to a difference in the extraction and/or clean-up procedure. For

this purpose two additional steps are added to the current sample preparation procedure for CALUX analysis. Concentrated sulfuric acid is added to the sample extract before subjecting it to clean-up to remove pollutants that might clog the acid-silica column. This extra step does not influence the result obtained with CALUX analysis so its use is acceptable. Because sulfur must be eliminated from the sample if it needs to be analyzed by GC-HRMS, an activated copper column is added to the clean-up process. This activated copper turns black as sulfur reacts with it thus enabling the analyst to check if enough activated copper was used to ensure removal of all the sulfur from the sample. This extra step does not influence the results obtained with the CALUX analysis so it can be used for the common sample preparation protocol.

Finally, there are several different methods that are being used to calculate the BEQ based on the raw data provided by CALUX analysis. The commonly used four parameter Hill equation in combination with the ratio of the effective concentrations method is compared to the Box-Cox transformation in combination with the slope ratio method to calculate the BEQ. It is apparent that more data points are necessary for a good fit of the four parameter Hill equation (both lower and upper plateau of the dose-response curve needs to be reached) whereas less experimental data is already sufficient for a good fit when the Box-Cox transformation is used to linearize the data.

Dose-response curves that reach both the lower and upper plateau are used to compare the result of the two calculation methods to each other. It is found that the BEQ based on the slope ratio after Box-Cox transformation always lies in the range of values that are provided by the effective concentration ratios at 20, 50 and 80% of the maximum induced response obtained by fitting the four parameter Hill equation to the experimental data. The same result could thus be obtained, but with a significantly higher precision. The width of the uncertainty on the BEQ_{slope} estimate is almost half as compared to the width of the uncertainty on the BEQ_{EC20} and BEQ_{EC50} estimates and is only one fourth of the width of the uncertainty on the BEQ_{EC80} estimate.

6 Recommendations

In July or August 2010, the sediment samples that were prepared for GC-HRMS analysis will be analyzed by the University of Liege. It is recommended that a comparison is then made with the results as published in this thesis to see if a good correspondence can be obtained between the chemical (TEQ) and bio-analytical (BEQ) analysis method if both analyses are performed on identically prepared samples.

Only the dioxin fraction of the sediment samples has been investigated with CALUX. The PCB fraction has been collected as well and is ready for analysis but this was not performed in the framework of this thesis due to time limitations. It is recommended that this analysis be done in the future to see if the same conclusions can be taken with regard to the PCB fraction.

It would also be interesting to see if the accuracy on the BEQ estimate calculated as the slope ratio after Box-Cox transformation can be augmented if the dilution series is not evenly distributed on a logarithmic scale encompassing both the lower and upper plateau of the dose-response curve as was required to fit the four parameter Hill equation. Since it has been shown that the same accuracy can be obtained with both calculation methods, subsequent research should focus on improving the precision that can be obtained with the slope ratio after Box-Cox transformation method. To calculate the slope ratio after Box-Cox transformation it would be better to have the data points evenly distributed on a linear scale (now they were chosen evenly distributed on a logarithmic scale) and without encroaching on the lower and upper plateau as these data points are not being used for the fit. This recommendation applies both to the TCDD standard curve as well as to the sample dilution points.

Finally it is imperative that more toxicity studies are performed to determine the health risk associated with using sludge from WWTPs as fertilizers as they are likely to contain dioxins and dioxin-like compounds. At present only a limited amount of information is available on the levels that are acceptable for sediments in rivers and this information cannot be simply extrapolated to the scenario of using sludge as fertilizers as this influences a completely distinct set of organisms. In addition to developing the methodology to measure the toxic potency of dioxins in sludge samples - as was done in the work presented in this thesis - it is also very important that the measurements obtained can be compared to reference values on which decisions can be made regarding the use of sludge as fertilizer (FERTIDIOX project).

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8 Appendices

Appendix 1. Toxic equivalency factors for humans and mammals for different PCDDs

	NATO/CCMS 1988	WHO 1998	WHO 2005
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PnCDD	0.5	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01	0.01
1,2,3,4,6,7,8,9-OCDD	0.001	0.0001	0.0003

Source: NATO/CCMS, 1988; Van den Berg et al., 1998; Van den Berg et al., 2006

Appendix 2. Toxic equivalency factors for humans and mammals for different PCDFs

	NATO/CCMS 1988	WHO 1998	WHO 2005
2,3,7,8-TCDF	0.1	0.1	0.1
1,2,3,7,8-PnCDF	0.05	0.05	0.03
2,3,4,7,8-PnCDF	0.5	0.5	0.3
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01
1,2,3,4,6,7,8,9-OCDF	0.001	0.0001	0.0003

Source: NATO/CCMS, 1988; Van den Berg et al., 1998; Van den Berg et al., 2006

Appendix 3. Toxic equivalency factors for humans and mammals for different PCBs

		IUPAC n°	WHO 1994	WHO 1998	WHO 2005
Non-ortho-PCBs	3,3',4,4'-TCB	PCB 77	0.0005	0.0001	0.0001
	3,4,4',5-TCB	PCB 81	-	0.0001	0.0003
	3,3',4,4',5-PeCB	PCB 126	0.1	0.1	0.1
	3,3',4,4',5,5'-HxCB	PCB 169	0.01	0.01	0.03
Mono-ortho-PCBs	2,3,3',4,4'-PeCB	PCB 105	0.0001	0.0001	0.00003
	2,3,4,4',5-PeCB	PCB 114	0.0005	0.0005	0.00003
	2,3',4,4',5-PeCB	PCB 118	0.0001	0.0001	0.00003
	2',3,4,4',5-PeCB	PCB 123	0.0001	0.0001	0.00003
	2,3,3',4,4',5-HxCB	PCB 156	0.0005	0.0005	0.00003
	2,3,3',4,4',5'-HxCB	PCB 157	0.0005	0.0005	0.00003
	2,3',4,4',5,5'-HxCB	PCB 167	0.00001	0.00001	0.00003
	2,3,3',4,4',5,5'-HpCB	PCB 189	0.0001	0.0001	0.00003
Di-orhto-PCBs	2,2',3,3'4,4',5 HpCB	PCB 170	0.0001	-	-
	2,2',3,4,4',5,5'-OCB	PCB 180	0.00001	-	-

Source: Ahlborg et al., 1994; Van den Berg et al., 2006

Appendix 4. Equipment used in the experiments

	Supplier
Ultra-sonic water bath	Branson
Vortex	Thermolyne
Vacuum centrifuge	Jouan
Pipettemen (10, 20, 100, 200, 1000µL)	Eppendorf
Multhichannel pipettor (12 tip)	Transferpette® S-12 Brand
Tissue culture CO_2 incubator	Thermo Scientific
Tissue culture laminar flow hood	Thermo Scientific
Inverted tissue culture microscope	Zeiss
Shaker table for 96-well microplates	IKA® MS3 digital
Microplate luminometer	Glomax Promega
Muffle furnace	Nabertherm
Weigh balance	Sartorius

Appendix 5. Materials used in the experiments

	Supplier
Pre-cleaned scintillation vials (20mL and 40mL) with Teflon-lined caps	Packard BioScience
Stainless steel spatulas	VWR, Cole-Palmer
Graduated cylinders (muffled and stored wrapped in foil)	Pyrex (Cole-Palmer)
Pasture pipettes (5.5 inch and 9 inch, muffled and stored wrapped in foil)	Volac
Borosilicate centrifuge tubes (50mL, muffled and stored wrapped in foil)	Kimble Chase (Hellma)
Nitrile gloves	Ansell (VWR)
Drying columns (10 and 25mL)	Pyrex Corning (Cole-Palmer)
Glass wool	Sigma Aldrich
Borosilicate glass culture tubes (13 x 100mm)	Kimble Chase (Hellma)
Pipette tips (autoclave sterilized)	Eppendorf AG (VWR)
White 96-well clear bottomed sterile tissue culture plates	Perkin Elmer
Tissue culture plates (100mm)	Nunc (VWR)
Tissue culture plastic pipettes (5, 10 and 25mL, sterile)	Greiner bio-one
Neubauer hemocytometer and cover slip	Optik Labor (Carl Roth)
Sterile PP centrifuge tubes (15mL Falcon conical)	Greiner bio-one
Glass tubing (8mm i.d., 10mm o.d.)	Pierre E. bvba

Appendix 6. Reagents used in the experiments

	Supplier
2,3,7,8-Tetrachlorodibenzo-p-dioxin (Accustandard)	Da Vinci Europe Lab. Solutions
Acetone (Residue grade)	Biosolve
Methanol (Residue grade)	Sigma-Aldrich
Toluene (Residue grade)	Riedel-de Haën
n-Hexane (Residue grade)	Biosolve
X-CARB	Xenobiotic Detection Systems
Silica gel 60	Fluka
Sulfuric acid 95-97%	Merck
Dimethylsulfoxide (DMSO)	Merck
Anhydrous sodium sulfate, granular, 12-60 mesh	J.T. Baker
Celite	Merck
Ethyl acetate (Pesticide Residue grade)	Riedel-de Haën
Alpha-minimal essential medium (MEM-α)	Gibco
Phosphate-buffered saline (PBS)	Gibco
Trypsin	Gibco
Fetal bovine serum (FBS)	Gibco
Luciferase lysis reagent	Promega
Stabilized luciferin assay reagent	Promega
Luciferase assay buffer	Promega
Copper powder (particle size<63µm)	Merck

Appendix 7. TCDD standard stock solution concentrations [M]

Prepared on Used cell line	02/24/2010 H1L6.1c3, H1L7.5c1	03/30/2010 H1L6.1c3	03/30/2010 H1L7.5c1	04/20/2010 H1L7.5c1
1	1.00E-11	1.00E-11	4.00E-12	4.00E-12
2	4.02E-11	6.00E-11	1.00E-11	1.00E-11
3	1.48E-10	2.50E-10	6.00E-11	6.00E-11
4	5.45E-10	7.00E-10	2.50E-10	2.00E-10
5	2.00E-09	1.40E-09	9.00E-10	4.00E-10
6	7.38E-09	2.00E-09	1.40E-09	7.00E-10
7	2.72E-08	3.50E-09	3.50E-09	1.70E-09
8	1.00E-07	9.00E-09	7.00E-09	3.00E-09
9	3.16E-07	1.00E-07	2.50E-08	2.50E-08
10	1.00E-06	1.00E-06	1.00E-07	1.00E-07