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HORIZONTAL-ORG WP2

Report on the use of the DR-CALUX® bioassay technique on a limited number of samples of sludge, compost and amended soils

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Summary

A task of the Sampling Work Package (WP2) was to investigate how many samples of treated sewage sludge, treated biowaste or soil would be required to evaluate a specified parameter with a particular confidence level and interval.

In many cases sampling protocols can be investigated by analysing samples using microbiological/chemical methods and this has been done for metals, *E. coli*, PAH and PCB (Lambkin et al., 2004, 2006, 2007). However, it is not practical to analyse samples for all relevant organic micropollutants for reasons of cost and/or lack of well defined analytical methods. An alternative analytical approach may provide an alternative method of predicting the distribution of these compounds and consequently the number of samples to be taken. This document reports the use of the DR-CALUX® bioassay method for determining dioxins and dioxin-like PCBs in sludge-amended soil, sewage sludge and composted biowaste.

The CALUX® bioassay could be a valuable tool for rapid screening of complex pollutants in environmental samples; it could lead to relatively quick and cheap identification of samples likely to exceed specified limits for organic pollutants without the necessity of costly (chemical) analytical methods. The aim of this work was to evaluate the method for sewage sludge, composted biowaste and sludge-amended soil and answer a number of questions as set out below.

The number and combination of analyses for each sample was limited because they had been collected and analysed for different purposes. Nine samples (JRC samples) had been collected and prepared for inter-laboratory testing to provide performance validation data for draft European standards for different analytical procedures. The samples had been placed into several groups for validation of a suite of analyses. For example, Compost 1, Sludge 1 and Soil 3 had been placed in group CG1 and tested for organic pollutants. The remaining 14 samples (WP2 samples) were selected from a large number of samples that had been collected to test sampling strategies for PAHs and PCBs and had not been analysed for dioxins.

The following conclusions drawn from the data should be interpreted with caution. They are based on a limited number of data, in some cases as few as three samples, and measurements that were often close to or below the limit of quantification (LOQ), or even below the limit of detection.

The following list of conclusions is structured as a list of responses to the questions raised.

Q1. Could the DR-CALUX® method be used to screen samples for chemical analysis of dioxins? A*nswer*: Qualified YES.

Q2. Are DR-CALUX® method reliability and chemical analytical method reliability comparable? *Answer*: YES

Q3. Is TEQ measured using the DR-CALUX® method comparable to TEQ calculated using chemical analytical data and published TEF values? *Answer*: Based on the limited data, No.

Q4. Can within-field variability be differentiated from DR-CALUX® method variability? *Answer*: Based on the limited data, No.

Q5. Within a given site, are DR-CALUX® TEQ and other measured parameters spatially correlated? *Answer*: Based on the limited data, No.

Q6. Does the DR-CALUX® method fulfil the basic quality criteria requirements (e.g. LOD, false negative and false positive)? *Answer*: YES.

Since this study was based on a very restricted data set strong conclusions cannot be drawn from the results and should be regarded as illustrative. However, the following recommendations are made:

- The DR-CALUX® method fulfils the basic quality criteria requirements and shows some promise as a method for screening samples in combination with a confirmative method for samples ±25% the regulatory level. However, this conclusion is based on a very limited data set and requires additional investigation of a much larger number of samples.

- The number of chemical data was limited and most of the results were at levels well below the regulatory levels. So, although in this study it was concluded that TEQ measured by DR-CALUX® and TEQ calculated from chemical measurements and TEF/REP values are not directly comparable, this is not the conclusion arrived at by numerous other studies. It is recommended that these points are investigated further using both analytical methods, in particular the DR-CALUX® TEQ_{PCB} values.

- There are differences, sometimes large, between calculated TEQ values, depending on which set of TEF/REP values are used. It is recommended that any limits that are based on calculated TEQ should also state which TEF/REP values are to be used in the calculations. The proposed 2005 WHO-TEF values are similar to the CALUX-REP values and, therefore, a better relationship between WHO-TEQ and CALUX-TEQ could be expected. It is recommended that the differences in TEQ_{PCB} calculated using the 1998 and 2005 WHO TEF values is further investigated.

- It is recommended that an interlaboratory validation trial be organized, with several laboratories using the confirmative (chemical) and screening (bioassay) technologies, to evaluate these methods according to international guidelines for the screening/confirmative approach, such as those in Commission Regulation 2006/1883/EC. The trial should evaluate important QA/QC parameters such as CV, false positive/negative rate and measurement uncertainty and it would be essential to include samples that are above the regulatory levels (e.g. sludge above 100 ng TEQ kg^{-1} d.w. and compost above 17-20 ng TEQ kg^{-1} d.w.).

Contents

List of Tables

List of Figures

1 INTRODUCTION

1.1 General

A task of the Sampling Work Package (WP2) was to investigate how many samples of treated sewage sludge, treated biowaste or soil would be required to evaluate a specified parameter with a particular confidence level and interval.

In many cases sampling protocols can be investigated by analysing samples using microbiological/chemical methods and this has been done for metals, *E. coli*, PAH and PCB (Lambkin et al., 2004, 2006, 2007). However, it is not practical to analyse samples for all relevant organic micropollutants for reasons of cost and/or lack of well defined analytical methods. An alternative analytical approach may provide an alternative method of predicting the distribution of these compounds and consequently the number of samples to be taken. This document reports the use of the DR-CALUX® bioassay method for determining dioxins and dioxin-like PCBs in sludge-amended soil, sewage sludge and composted biowaste.

The conclusions drawn are based on analysis of a limited number of samples and most of these contained very small amounts of the compounds of interest, often below the confidence limits associated with the data. Many of the analytical data were below, or close to, the limits of quantification, or even below the limits of detection. This applies particularly to the samples collected by WP2.

1.2 The CALUX® Method

CALUX® is relatively new (developed in the early 1990s) and has been tested most widely on environmental biological (wildlife) samples, food and feedstuffs. Development of the method was driven by concerns about elevated dioxin levels in the food chain and wildlife, for example milk from cows grazing in the vicinity of waste incinerators.

Method development has been comparatively rapid. Sample clean-up methods have been developed to remove compounds that interfere antagonistically or synergistically to produce an incorrect result. Sample separation techniques have been developed to separate PCBs from PCDD/Fs. Improved calibration methods have been validated. However, interpretation of the CALUX® assay has caused some difficulty. It is a measure of cell response to exposure to **all** the dioxin-like chemicals in a sample. In contrast, chemical assays are the sum of several measured compounds: dioxin/furan congeners and dioxin-like PCBs. It is recognised that the two methods do not produce the same result, although they are usually well correlated (reported r^2 in the range 0.71-0.95) and some of the differences can be attributed to sample heterogeneity, variation between laboratories and assay reproducibility. The main differences between the methods are caused by:

(i) Compounds that contribute to CALUX® response not being considered in the chemical assay;

(ii) Compounds that contribute to CALUX® response being near or below the HRGC-MS detection limit;

(iii) TEFs are not available for all compounds that might be measured by chemical assay;

(iv) Non-additive interactions cannot be accounted for;

(v) Extraction and clean-up methods can affect the recovery.

CALUX® went through development and validation during the 1990s and tests have been commercially available since 2000/2001. Several international validation projects were initiated in 2002 and the results are now appearing in the literature. Bioassay methods, including CALUX®, have been accepted by legislators and appear in European Directives on Feed and Foodstuffs (Commission Directives 2002/69/EC,

2002/70/EC and 2006/1883/EC). Bioassay methods are also being considered in draft legislation/directives for sewage sludge and dredged material.

CALUX® is generally regarded as an easy to learn (weeks rather than months), cheap (about 30% of the chemical assay cost), rapid (days rather than weeks) and sensitive method that provides a good measure of dioxin-like toxicity. The chemical method provides more information about contamination and pollution in terms of which group of compounds are producing the dioxin-like response. CALUX® bioassay and chemical analysis are regarded as complimentary methods. CALUX® has been used as a screening tool to identify problem samples, which are then analysed chemically to identify the chemical compounds contributing to the dioxin response.

Commission Regulation 2006/1883/EC states:

> Monitoring for the presence of dioxins in foodstuffs may be performed by a strategy involving a screening method in order to select those samples with levels of dioxins and dioxin-like PCBs that are less than 25% below or exceed the maximum level. The concentration of dioxins and sum of dioxins and dioxin-like PCBs in those samples with significant levels needs to be determined/confirmed by a confirmatory method.
>
> Screening methods are methods that are used to detect the presence of dioxins and dioxin-like PCBs at the level of interest. These methods shall have a capacity for a high sample throughput and are used to sift large numbers of samples for potential positives.
>
> Confirmatory methods are methods that provide full or complementary information enabling the dioxins and dioxin-like PCBs to be identified and quantified unequivocally at the level of interest.

The CALUX screening test has been demonstrated to have false negative rates below 1% and false positive rates mostly below 5%. If it can be demonstrated that the method can be used reliably for screening sewage sludge and biowaste, similar wording might be adopted in future directives on sewage sludge and biowaste.

A fuller description of the CALUX® method is given in Annex A.

DR-CALUX® (Dioxin Responsive CALUX) is a specific CALUX® test which uses tailored cells that produce light in a dose-responsive way when exposed to dioxins and dioxin-like chemicals: The DR-CALUX® test was used in this work. Where the term is used without the 'DR' prefix it refers to the CALUX® method in general.

1.3 Aim

The DR-CALUX® bioassay could be a valuable tool for rapid screening of complex pollutants in environmental samples; it could lead to relatively quick and cheap identification of samples likely to exceed specified limits for organic pollutants without the necessity of costly (chemical) analytical methods. The aim of this work was to evaluate the method for sewage sludge, composted biowaste and sludge-amended soil and to answer the following questions:

1. Could the DR-CALUX® method be used to screen samples for chemical analysis?
2. Are DR-CALUX® method reliability and chemical analytical method reliability comparable?
3. Is TEQ measured using the DR-CALUX® method comparable to TEQ calculated using chemical analytical data and published TEF values?
4. Can within-field variability be differentiated from DR-CALUX® method variability?
5. Within a given site, are DR-CALUX® TEQ and other measured parameters spatially correlated?

6. Does the DR-CALUX method fulfil the basic quality criteria requirements (e.g. LOD, false negative and false positive)?

1.4 Scope

The primary aim of this work was to investigate the use of DR-CALUX® for sewage sludge, composted biowaste and sludge-amended soil in relation to sampling. It was not intended that it should be an in-depth investigation of whether DR-CALUX® is a good measure of TEQ.

1.5 Limitations and assumptions

Ideally a large number of samples would be analysed both by chemical methods and by DR-CALUX®. However, the number of samples tested using the DR-CALUX® method was limited to a total of 40 by the available budget. As a consequence the results can only provide a general illustration rather than an in-depth analysis.

Another constraint was that many of the samples collected by WP2 (Sampling) were below the detection limit when analysed by chemical methods (Lambkin et al., 2007). This was resolved by supplementing WP2 samples with measurable PAH and PCB concentrations with samples used as method validation materials within Project Horizontal (JRC Method Validation Group). Consequently, not all the samples had been analysed for the same parameters, or by the same laboratory, and no data were available for reference materials.

1.6 Report Layout

Chapter 2 describes sample selection and DR-CALUX® analysis, but details of the sites and collection methods are described elsewhere. Similarly, the general CALUX® method is reviewed in Annex A, but the chemical analyses are described elsewhere. The analytical results are presented.

In Chapter 3 the results are analysed and discussed. Chapter 4 summarizes the conclusions and Chapter 5 lists the recommendations.

2 Experimental materials and method

2.1 Sample selection

Within the restrictions of the budget it was possible to analyse 40 samples by DR-CALUX® bioassay. A wide range of materials were available from the Method Validation Group (JRC samples) and Sampling Work Packages (WP2 samples). Not all the JRC samples were characterised for organic compounds. They had been prepared for validation of other methods, e.g. metals and nutrients. A list of samples and available analytical data is given in Table 2.1.

All WP2 samples and three JRC samples had been characterised for PAH (EPA 16) and PCB (ICES 7), LOI and pH. Eight of the WP2 samples had also been characterised for dioxin-like PCBs. The three JRC samples had also been characterised for dioxin-like PCBs and PCDD/F. Many of the samples collected by WP2 had been below the Limit of Quantification (LOQ) or Limit of Detection (LOD) for PAH and PCB. Samples were chosen where many of the compounds were above LOQ and the raw analytical data had been provided by the laboratory. The amount of material required for each DR-CALUX® analysis was 50 g. Where sufficient material was available, sample analysis was replicated to obtain a measure of the method repeatability.

Table 2.1 Samples selected for DR-CALUX® analysis

Material	Source	Sampling Location	Replicates	Available analytical data
Compost 1	JRC	Vienna, Austria	2	PCDD/F, pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Compost 2	JRC	NRW, Germany	3	pH, LOI
Sewage Sludge 1	JRC	Essen, Germany	4	PCDD/F, pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Sewage Sludge 2	JRC	Essen, Germany	2	pH, LOI
Soil 1	JRC	Pavia, Italy	2	
Soil 2	JRC	Düsseldorf, Germany	2	
Soil 3	JRC	Barcelona, Spain	2	PCDD/F, PAH (EPA 16), PCB (ICES 7), DLP
Soil 4	JRC	Hohenheim, Germany	3	pH, LOI
Soil 5	JRC	Reading, UK	5	pH, LOI
Compost 3	WP2	UK-Site F	1	pH, LOI, PAH (EPA 16), PCB (ICES 7)
Compost 4	WP2	UK-Site F	1	pH, LOI, PAH (EPA 16), PCB (ICES 7)
Sewage sludge 3	WP2	UK-Site C	1	pH, LOI, PAH (EPA 16), PCB (ICES 7)
Sewage sludge 4	WP2	UK-Site B	1	pH, LOI, PAH (EPA 16), PCB (ICES 7)
Sewage sludge 5	WP2	UK-Site B	1	pH, LOI, PAH (EPA 16), PCB (ICES 7)
Sewage sludge 6	WP2	UK-Site B	1	pH, LOI, PAH (EPA 16), PCB (ICES 7)
Soil 6	WP2	Spain-Field 1	1	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Soil 7	WP2	Spain-Field 1	1	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Soil 8	WP2	Spain-Field 1	1	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Soil 9	WP2	Spain-Field 2	2	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Soil 10	WP2	Spain-Field 2	2	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Soil 11	WP2	Spain-Field 2	2	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Soil 12	WP2	Spain-Field 2	2	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP
Soil 13	WP2	Spain-Field 2	2	pH, LOI, PAH (EPA 16), PCB (ICES 7), DLP

DLP = Dioxin-like PCBs

The number and combination of analyses for each sample was limited because they had been collected and analysed for different purposes. The JRC samples had been collected and prepared for inter-laboratory testing to provide performance validation data for draft European standards for different analytical procedures. The samples had been placed into several groups for validation of a suite of analyses. For example, Compost 1, Sludge 1 and Soil 3 had been placed in group CG1 and tested for organic pollutants. The WP2 samples were selected from a large number of samples that had been collected to test sampling strategies for PAHs and PCBs and had not been analysed for dioxins.

2.2 DR-CALUX® analysis

The samples were delivered to BDS, Holland in amber jars with PTFE-lined (polytetrafluroethylene) caps. The samples of validation materials were supplied as air-dried material, sieved to 2 mm. The WP2 samples were supplied unprocessed. No analytical data were supplied with the samples except the type of sample matrix.

The materials were freeze-dried and the dried material was extracted using the ASE (accelerated solvent extraction: hexane/acetone) method. The extract was cleaned on an acid silica column then divided into two aliquots; one for Total TEQ determination (TEQ_{Total}) and one for fractionation and PCB determination (TEQ_{PCB}). The first aliquot was dissolved in DMSO (dimethylsulfoxide); the second aliquot was packed on a SPE-carbopack column (dispersive solid-phase extraction) and dissolved in DMSO. The diluted and cleaned extracts were exposed to cultured DR-CALUX® cells for 24 hours in 96 well plates under standardized conditions. After lysation and adding luciferin, the luciferase activity was quantified using a luminometer to determine the DR-CALUX® activity.

2.3 Results

The chemical analytical data are given in Annex C. Results of the DR-CALUX® analysis are in Table 2.2.

The results (TEQ_{Total} and TEQ_{PCB}) were reported as DR-CALUX®-TEQ (ng 2,3,7,8-TCDD TEQ/kg product). Data are quantifiable between the limit of quantification (LOQ) and the EC_{50}; only results within this range are reported. For results below the limit of quantification, an estimate is given (in parentheses). The reported measurement uncertainty was 26%.

Table 2.2 Results of DR-CALUX® analysis (ng 2,3,7,8 TCDD TEQ/kg)

	TEQ_{Total}				TEQ_{PCB}			
Material	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**
Compost 1	8.6	11			n/d	3.6		
Compost 2	17.6	26	21		n/d	3.7	5.2	
Sewage sludge 1	20	21	29.8	18	12	13	n/d	14
Sewage sludge 2	15.7	14			n/d	2.7		
Soil 1	4.4				<LOQ (1.15)			
Soil 2	7.0				<LOD			
Soil 3	7.2	6.8			<LOQ (1.05)	<LOQ (0.66)		
Soil 4	24.3	17	18		n/d	5.4	4.1	
Soil 5	2.9	3.2	2.9		n/d	0.6	<LOD	
Compost 3	43				2.9			
Compost 4	30				<LOQ (1.97)			
Sewage sludge 3	20				4.7			
Sewage sludge 4	90				3.1			
Sewage sludge 5	123				3.4			
Sewage sludge 6	132				3.3			
Soil 6	5.5				<LOQ (0.63)			

Soil 7	3.4		<LOD	
Soil 8	6.9		<LOQ (0.45)	
Soil 9	4.3		<LOQ (0.52)	
Soil 9	3.9		<LOQ (0.66)	
Soil 10	9.8	3.5	<LOQ (1.43)	2.3
Soil 11	2.5	3.3	1.8	<LOQ (1.15)
Soil 12	2.4	3.5	<LOQ (1.32)	1.6
Soil 13	5.5	5.7	1.8	<LOD

LOQ = Limit of quantification; LOD = Limit of detection; n/d = not determined

3 Discussion

3.1 DIOXINS

3.1.1 Screening for dioxins

The question is: could DR-CALUX® be used to screen samples for chemical analysis? For screening to be effective, the DR-CALUX® method would need to correctly identify samples that are substantially below a given limit so that only those that are close to, or above, the limit require further analysis by chemical methods.

To answer the question of method suitability requires calculation of dioxin-TEQ (TEQ_{Dioxin}) from TEF values and chemical analysis of samples for dioxins and DR-CALUX® analysis of the same samples. Ideally many samples with a wide range of dioxin concentrations would be analysed by both methods. However, dioxins had been measured only for three JRC samples (carried out by up to five laboratories each performing multiple replicate analyses).

The limit in German and Austrian legislation for dioxins in sludge is 100 ng I-TEQ kg^{-1} dm (± 10%) (AbfKlärV, 1992) – a limit of 100 ng TE kg^{-1} dm was also proposed in the Working Document on Sludge, 3rd draft. Guideline limits for dioxins in compost have been set in some European countries. For example, 17 ng I-TEQ kg^{-1} d.w. (±30%) is the guideline value in the Composting Decree of Baden-Württemberg (Umweltministerium Baden-Wurttemberg (HRSG), 1994) and 20 ng I-TEQ kg^{-1} d.w. has been set by the Swiss Confederation (Swiss Confederation, 2005).

Using the most widely accepted TEF values (WHO_{1998}, WHO_{2005} and $I\text{-}TEQ_{1988}$), calculated TEQ_{Dioxin} is 13.1-14.9, 4.87-5.65, and 0.56-0.62 ng TEQ kg^{-1} respectively for the JRC samples: Sewage Sludge 1, Compost 1, and Soil 3 (Table 3.2). The corresponding DR-CALUX results are 9.2, 6.2 and 6.1 ng TEQ kg^{-1} (Table 3.3). Although the resulting TEQ_{Dioxin} are not the same, both methods indicate that the samples are well below the current/proposed limits. So, the DR-CALUX® method fulfils the first requirement for a screening method, although the results have to be treated with caution since they are based on only three samples.

The DR-CALUX®-TEQ results for Sewage Sludge 4, 5 and 6 (Table 2.2) indicate that the dioxin-TEQ ($TEQ_{Dioxin} = TEQ_{Total} - TEQ_{PCB}$) at UK-Site B ranges from about 87-129 ng TEQ kg^{-1} (mean 112±22 ng TEQ kg^{-1}). Based on TEQ_{Dioxin} measured using the DR-CALUX® method and the proposed limits, sewage sludge from UK-Site B would not be compliant. The DR-CALUX®-TEQ for Composts 3 and 4 (Table 2.2) indicate TEQ_{Dioxin} ranges from about 28-40 ng TEQ kg^{-1}, which would put this compost outside both the Baden-Württemberg and the Swiss Confederation limits.

Based on the DR-CALUX® results, samples collected from these two sites are close to, or above, the limits and should be reanalysed using chemical methods. Since no chemically measured dioxin data were available for these samples, it is not possible to determine if the DR-CALUX® method has correctly identified these samples.

3.1.2 Repeatability

Replicate DR-CALUX® analysis (2, 3 or 4 replicates) was carried out on twelve samples (see Table 3.1). Four replicate chemical analyses of PCDD/F had been carried out on three of the JRC samples by a single laboratory during the sample selection phase of the inter-laboratory validation trials (see Table 3.2).

For conventional chemical analysis the CV% (coefficient of variation) ranged from 0.6-47.3%; mean 9.1% (median 6.6%), and was lowest for the sewage sludge sample and highest for the soil sample. Calculated from the chemical analysis, TEQ_{Dioxin} CV% was in the range 2.4-5.4%; mean 3.3% (median 3.2%), and was highest for the compost sample.

In contrast, for DR-CALUX® analysis the CV% for TEQ_{Total} ranged from 2.5-67.0%; mean 18.4% (median 18.4%). The variability in CV% was not related to the differences in matrices or the number of replicates.

The data show that results from conventional chemical analysis is on average more repeatable than by DR-CALUX® analysis. The reported measurement uncertainty quoted for DR-CALUX® TEQ_{Total} was 26%. The result for only one sample, Soil 10, was outside that range. Soil 10 was supplied as an unprocessed (as collected) field soil, so it is possible that the high variability between the two replicate analyses was caused by sub-sampling in the laboratory rather than the analytical procedure.

Sufficient data for calculating a CV% for TEQ_{Dioxin} by DR-CALUX® were available for only three samples (sewage Sludge 1, Compost 1, and Soil 3) and ranged from 12.8% to 34.6%.

Table 3.1 DR-CALUX® Analysis: Repeatability

	ng TEQ kg^{-1}							
	Total-TEQ				PCB-TEQ			
Material	**mean**	**stdev**	**CV%**	**n**	**mean**	**stdev**	**CV%**	**n**
Compost 1	9.8	1.70	17.3	2	3.6			1
Compost 2	21.5	4.23	19.6	3	4.5	1.06	23.8	2
Sewage Sludge 1	22.2	5.22	23.5	4	13	1.00	7.7	3
Sewage Sludge 2	14.9	1.20	8.1	3	2.7			1
Soil 3	7.0	0.28	4.0	2	0.9	0.28	32.3	2
Soil 4	19.8	3.96	20.0	3	4.8	0.92	19.4	2
Soil 5	3.0	0.17	5.8	3	0.6			1
Soil 9	4.1	0.28	6.9	2	0.6	0.10	16.8	2
Soil 10	6.7	4.45	67.0	2	1.9	0.62	33.0	2
Soil 11	2.9	0.57	19.5	2	1.5	0.46	31.2	2
Soil 12	3.0	0.78	26.4	2	1.5	0.20	13.6	2
Soil 13	5.6	0.14	2.5	2	1.8			1

Table 3.2 Chemical Analysis: Repeatability

	Sewage Sludge 1			Soil 3			Compost 1		
***n* = 4**	**mean**	**s.d.**	**CV%**	**mean**	**s.d.**	**CV%**	**mean**	**s.d.**	**CV%**
chlorinated dibenzo-p-dioxins ng kg^{-1} DW									
2,3,7,8-TCDD	0.896	0.015	1.7	<0.024			0.174	0.022	12.4
1,2,3,7,8-PeCDD	1.745	0.190	10.9	0.095	0.014	15.2	0.570	0.078	13.8
1,2,3,4,7,8-HxCDD	3.072	0.199	6.5	0.061	0.013	21.0	0.790	0.091	11.5
1,2,3,6,7,8-HxCDD	7.497	0.260	3.5	0.320	0.022	6.8	3.204	0.144	4.5
1,2,3,7,8,9-HxCDD	3.933	0.149	3.8	0.143	0.034	23.6	1.545	0.087	5.7
1,2,3,4,6,7,8-HpCDD	166.5	4.754	2.9	5.531	0.137	2.5	174.3	5.665	3.2
OCDD	1149	20.30	1.8	45.00	1.524	3.4	956.4	131.426	13.7
chlorinated dibenzofurans ng kg^{-1} DW									
2,3,7,8-TCDF	10.48	0.608	5.8	0.336	0.024	7.2	2.734	0.092	3.3
1,2,3,7,8-PeCDF	4.517	0.114	2.5	0.170	0.019	11.2	1.330	0.092	6.9
2,3,4,7,8-PeCDF	8.063	0.540	6.7	0.205	0.022	10.7	1.808	0.113	6.2
1,2,3,4,7,8-HxCDF	9.811	0.202	2.1	0.257	0.040	15.5	1.689	0.155	9.2
1,2,3,6,7,8-HxCDF	6.686	0.309	4.6	0.214	0.010	4.5	1.684	0.058	3.5
2,3,4,6,7,8-HxCDF	7.372	0.252	3.4	0.456	0.055	12.1	1.713	0.263	15.3
1,2,3,7,8,9-HxCDF	0.776	0.090	11.6	0.052	0.025	47.3	0.263	0.048	18.2
1,2,3,4,6,7,8-HpCDF	86.50	1.072	1.2	12.13	0.510	4.2	13.28	0.944	7.1
1,2,3,4,7,8,9-HpCDF	5.395	0.033	0.6	0.180	0.023	12.5	1.208	0.069	5.7
OCDF	185.4	5.617	3.0	37.09	2.586	7.0	28.58	10.444	36.5
Calculated TEQ ng kg^{-1} DW									
WHO_{1998}	14.6	0.41	2.8	0.59	0.022	3.7	5.06	0.214	4.2
WHO_{2005}	13.1	0.33	2.5	0.56	0.018	3.2	4.87	0.219	4.5
$I\text{-}TEQ_{1988}$	14.9	0.39	2.6	0.62	0.017	2.8	5.65	0.306	5.4
$CALUX_{2003}$	27.0	0.66	2.4	1.36	0.039	2.8	12.9	0.515	4.0
$CALUX_{2004}$	18.0	0.59	3.3	0.69	0.013	1.8	7.33	0.285	3.9

3.1.3 Calculated vs measured TEQ

Toxic Equivalence Factors (TEF) and Relative Potency factors (REP) are used to calculate TEQ from chemical analytical data (see Annex A). TEF/REP values published by five sources quoted in the literature are listed in Annex D. TEQ_{Dioxin} was calculated for the JRC samples using these TEF/REP values (Table 3.3 and Figure 3.1).

Table 3.3 Calculated TEQ values: PCDD/F (JRC samples)

	Dioxin-TEQ (ng 2,3,7,8-TCDD TEQ/kg)							
Material	**WHO_{1998}**	**WHO_{2005}**	**$I\text{-}TEQ_{1988}$**	**$CALUX_{2003}$**	**$CALUX_{2004}$**	**DR-CALUX® Dioxin (a-b)**	**DR-CALUX® Total (a)**	**DR-CALUX® PCB (b)**
Sewage Sludge 1	14.6	13.1	14.9	27.0	18.0	9.2	22.2	13.0
Soil 3 *	0.59	0.56	0.62	1.36	0.69	6.1	7.0	0.9
Compost 1	5.06	4.87	5.65	12.9	7.33	6.2	9.8	3.6

* Calculated using 0.5 LOD for 2,3,7,8-TCDD

Calculated TEQ based on the non-CALUX TEF values (WHO_{1998}, WHO_{2005} and $I\text{-}TEQ_{1988}$) produced similar results for all three matrices. However, TEQ calculated using $CALUX_{2003}$-REP values produced a result about twice as high as the non-CALUX methods and $CALUX_{2004}$-REP values produced a result about 30% higher (11-51%).

Scippo et al. (2004) argued that the $CALUX_{2003}$-REP values were derived from molar concentrations and weight-derived REP values are to be preferred. They used this method to obtain REP values for PCDD/Fs and dioxin-like PCBs. Their revised values are listed in Table C.1 ($CALUX_{2004}$) and used to calculate $CALUX_{2004}$ TEQ in Table 2.5. The resulting TEQ is much closer to the WHO and I-TEQ values than that calculated using $CALUX_{2003}$-REP.

Regardless of how TEQ_{Dioxin} is calculated, and taking into account the measurement error, none of the three samples exceeded the current or proposed limits.

Most of the difference in TEQ calculated using $CALUX_{2003}$–REP compared to other methods can be accounted for by high REP values for four compounds: 1,2,3,4,6,7,8-HpDD, 2,3,7,8-TCDF, 1,2,3,4,6,7,8-HpDF, and OCDF, particularly when the concentrations of these compounds in the samples was high (see Figure 3.1).

In validation studies (see A.4), in general it has been found that for the dioxin fraction, CALUX® > chemical. Whilst this is the case for Soil 3, it is not the case for Compost 1, where the results are similar, or for Sewage Sludge 1, where DR-CALUX®-TEQ is between 34-70% of the calculated values, depending on the TEF/REP used.

Regardless of how it was calculated or measured, TEQ_{Dioxin} decreased in the order sewage sludge > compost > soil, although for DR-CALUX® the difference between soil and compost was not significant.

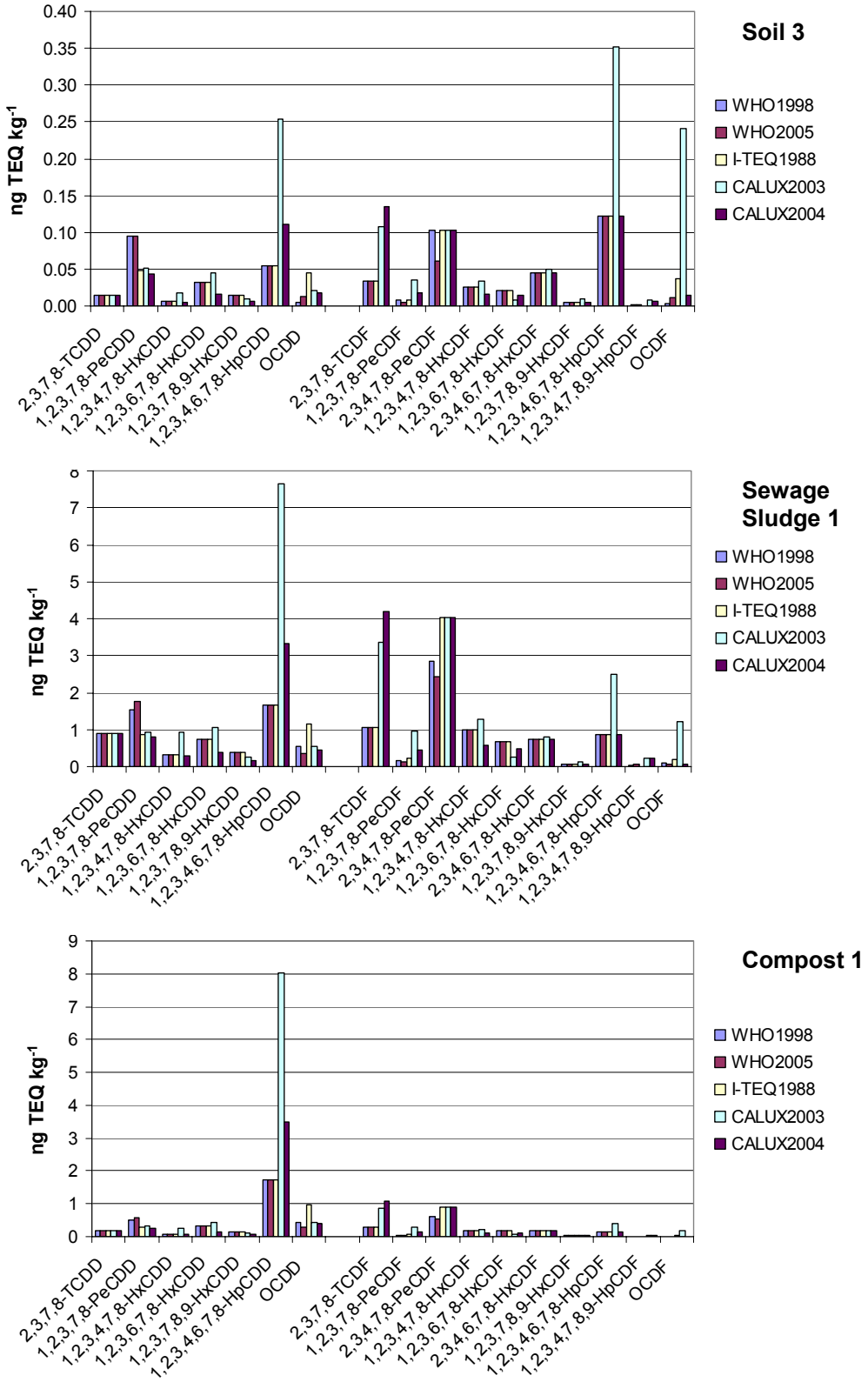

Figure 3.1 Comparison of Dioxin-TEQ calculated using five methods (JRC samples)

3.2 DIOXIN-LIKE PCBS

3.2.1 Dioxin-like PCB concentrations

Three of the JRC materials (Sewage sludge 1, Compost 1, Soil 3) had been analysed for 12 dioxin-like PCBs in an inter-laboratory validation trial. The results are summarized in Table B.2 (Annex B). Samples from two fields in Spain (WP2, Spain-Field 1 and Spain-Field 2) had also been analysed for 12 dioxin-like PCBs. The results of the chemical analyses were used to calculate TEQ for the samples, based on the four TEF values (see Table 3.4 and Table 3.5). TEF values for $CALUX_{2004}$ were set to the maximum value (e.g. 0.0001 was used for PCB 105)

It should be noted that for the JRC samples the chemical measurements are given in ng kg^{-1}, whereas for the WP2 samples the results are given in µg kg^{-1}. As a consequence, many of the WP2 analyses were reported as either <LOQ or <LOD. Also, whereas the WP2 samples were analysed once only, the JRC samples the results were supplied as mean values (and standard deviation) of many laboratory analyses and all of the results were non-zero.

To the authors' knowledge there are currently no limits for dioxin-like PCBs in sewage sludge, compost or soil within the legislation of European Member States. No limits were proposed in the Working Document on Sludge, 3rd draft, although a limit was proposed for the sum of the ICES 7: 28, 52, 101, 118, 138, 153, 180 (0.8 mg kg^{-1}).

Dioxin-like PCBs contributed between 0.02-0.25% to the total dioxin response measured by chemical analysis. By contrast, dioxin-like PCBs contributed between <2.5-77.8% to the total dioxin response measured by DR-CALUX® analysis; mean 16.3, 31.4 and 21.1 % TEQ_{Total} for compost, sewage sludge and soil respectively.

In a survey of Canadian composts, Groeneveld and Hébert (2002) concluded that dioxin-like PCBs contribute on average less that 20% to the TEQ_{Total} in a range of composts (green waste, source separated organics, municipal solid waste, paper mill biosolids). However, the contribution varied between composts from 0.7-36.7% TEQ_{Total}. Hiester et al. (2004), in a survey of 14 urban and rural soils, found that dioxin-like PCBs contribute about 15-40% to the TEQ_{Total}. The DR-CALUX® results from this study are not dissimilar to values found in the other two studies.

3.2.2 Repeatability

Replicate DR-CALUX® analysis (2 or 3 replicates) for dioxin-like PCBs was carried out on eight samples (see Table 3.1). Multiple replicate chemical analyses (n = 7-20) of PCDD/F had been carried out on three of the JRC samples (see Table B.2) as part of the inter-laboratory validation. The analyses were carried out by up to five laboratories each performing multiple replicate analyses.

For conventional chemical analysis (3 JRC samples) the CV% ranged from 9.3-254.8%; mean 43.3% (median 18.6%). There was no significant difference between matrices (P = 0.62), but there was a significant difference between compounds (P = 0.12). The CV% varied between PCBs and was consistently low (<25%) for five compounds: PCB 77, 105, 118, 156, and 157. For the other PCBs the CV% was higher, but it varied less between matrices for PCB 81 and PCB 126.

For DR-CALUX® analysis the CV% for TEQ_{PCB} ranged from 7.7-33.0%; mean 22.2% (median 21.6%). Again the variability in CV% was not related to the differences in matrices.

For calculated TEQ_{PCB} the high CV% of the chemical analyses was moderated, ranging from 17.9-163.5%, depending on the matrix and TEF values used. This is a result of the different weighting of the TEF values fro individual compounds. It is noticeable that the soil matrix has the highest CV%, mainly as a result of the very low calculated TEQ_{PCB}.

Table 3.4 Calculated TEQ values: Dioxin-like PCBs (JRC samples)

	PCB-TEQ (ng 2,3,7,8-TCDD TEQ/kg)				
Material	**WHO_{1998}**	**WHO_{2005}**	**$CALUX_{2003}$**	**$CALUX_{2004}$**	**DR-CALUX®**
Sewage sludge 1					
Mean	0.03642	0.02914	0.03028	0.02981	13.00
s.d.	0.00972	0.00923	0.00792	0.01130	1.00
CV%	26.7	31.7	26.2	37.9	7.7
Compost 1					
Mean	0.00446	0.00385	0.00292	0.00127	3.60
s.d.	0.00080	0.00070	0.00058	0.00075	
CV%	17.9	18.3	19.8	59.0	
Soil 3					
Mean	0.00044	0.00063	0.00026	0.00016	1.15
s.d.	0.00043	0.00102	0.00018	0.00013	
CV%	96.8	163.5	68.3	80.3	

Table 3.5 Calculated TEQ values: Dioxin-like PCBs (WP2 samples)

	PCB-TEQ (ng 2,3,7,8-TCDD TEQ/kg)				
Material	**WHO_{1998}**	**WHO_{2005}**	**$CALUX_{2003}$**	**$CALUX_{2004}$**	**DR-CALUX®**
Spain Field 1					
Soil 6	0.10	0.01	0.04	0.004	<LOQ (0.63)
Soil 7	0.12	0.01	0.05	0.005	<LOD
Soil 8	1.40	4.20	0.48	0.28	<LOQ (0.45)
Mean	0.54	1.40	0.19	0.096	*0.180
s.d.	0.75	2.42	0.25	0.159	*0.162
CV%	139.3	172.4	132.6	165.6	90.1
Spain Field 2					
Soil 9	2.05	6.15	0.70	0.42	0.59
Soil 10	17.51	48.85	6.09	4.12	1.9
Soil 11	3.17	8.75	1.10	0.59	1.5
Soil 12	17.68	47.91	5.77	4.27	1.5
Soil 13	25.63	66.68	8.87	6.36	1.8
Mean	13.16	35.67	4.52	3.15	1.21
s.d.	10.15	26.84	3.53	2.57	0.495
CV%	77.1	75.3	78.0	81.6	40.8

* Based on <LOQ = 0.5 x estimate and <LOD = 0

3.2.3 Calculated vs measured TEQ

Using the TEF values TEQ_{PCB} was calculated for those samples where dioxin-like PCB concentrations could be measured (Table 3.4 and 3.5). There are no published I-TEQ_{1988} values for dioxin-like PCBs so TEQ was calculated using the other four methods only.

JRC samples

For a given matrix, there was variation between calculated TEQ_{PCB}, but the four methods of calculation produced results of the same order of magnitude (see Figure 3.3). For the JRC samples TEQ_{PCB} decreased in the order sewage sludge 1 > compost 1 > soil 3 in an approximate ratio sewage sludge: compost: soil of 100: 10: 1.

TEQ_{PCB} measured using DR-CALUX® also decreased in the order sewage sludge 1 > compost 1 > soil 3. However, the results were three or four orders of magnitude greater than the calculated TEQ_{PCB}.

WP2 samples

DR-CALUX® results were closest in value to TEQ_{PCB} calculated with the CALUX-REP values. This is probably not surprising since the CALUX-REP values were derived from CALUX analyses. THE DR-CALUX analysis did not show the same within-field variability as the calculated TEQ_{PCB}, although it did identify that TEQ_{PCB} Field 2 was greater than TEQ_{PCB} in Field 1.

On average the calculated TEQ_{PCB} values decreased in the order WHO_{2005} > WHO_{1998} > $CALUX_{2003}$ > $CALUX_{2004}$ in the approximate ratio 10: 4: 1.4: 1 (r^2>0.99). However, in both fields, the main contribution to calculated TEQ_{PCB} is from PCB 169 and consequently the variability in calculated TEQ_{PCB} reflects the variability in TEF/REP values (ratio: WHO_{2005} :WHO_{1998}: $CALUX_{2003}$: $CALUX_{2004}$ = 15: 5: 1.7: 1)

Conclusions

The DR-CALUX® method correctly identifies relative differences between TEQ_{PCB}, but the results are not directly comparable with calculated TEQ_{PCB}.

The within-field variability that is identified from calculated TEQ_{PCB} is not replicated with the DR-CALUX® analysis.

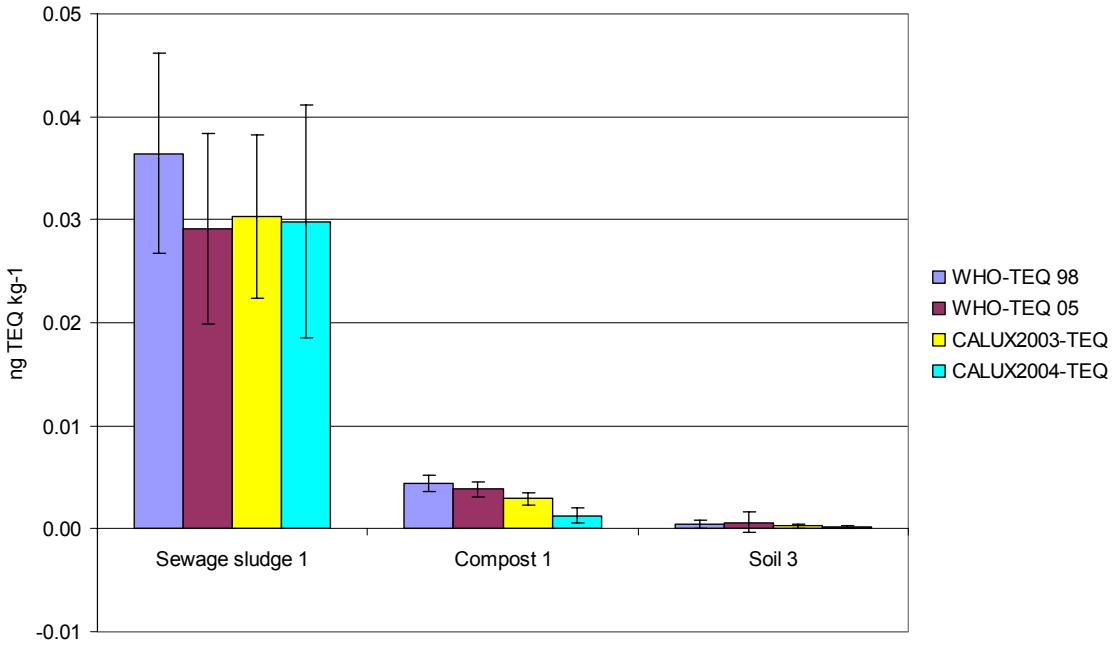

Figure 3.2 Comparison of PCB-TEQ calculated using four methods (JRC samples)

NOTE: The DR-CALUX® results are orders of magnitude larger and are not shown

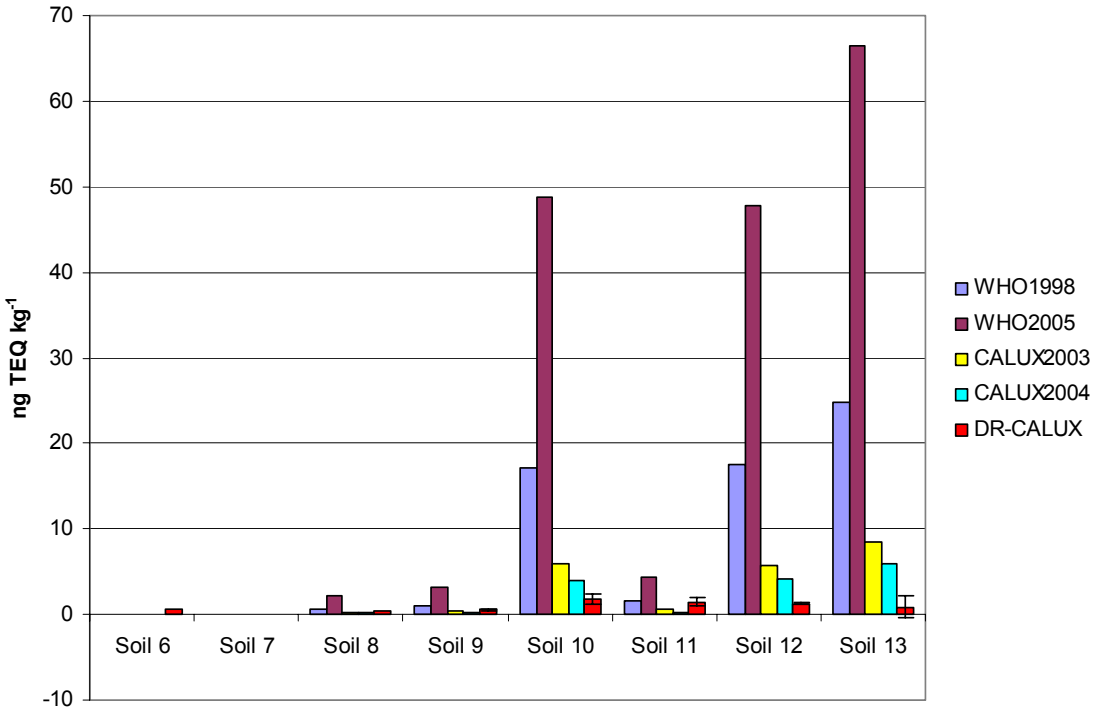

Figure 3.3 Comparison of PCB-TEQ calculated using four methods (WP2 samples)

NOTE: Based on <LOQ = 0.5 x estimate and <LOD = 0

3.3 Source of Variability

The data for Spain Field 2 (Soils 9-13) were examined using ANOVA (see Tables 3.6-3.8) to separate the between-replicate variation from the between-sample variation, i.e. to compare variability due to analysis and variability due to sampling. This data set was chosen because the DR-CALUX® results were above the LOQ. A different conclusion might be reached had more than five samples been analysed (see 1.5: Limitations and assumptions).

The results show that there is no significant difference in the between-replicates and between-sample variance, i.e. the variation due to sampling cannot be separated from the variation due to analysis. This means that there is little benefit to be gained from multiple measurements of TEQ in preference to a single sample. Also, since the sampling variance cannot be separated from the analytical variance, it is not possible to estimate how many samples would be needed to be collected and mixed to produce a composite sample.

Table 3.6 ANOVA: DR-CALUX Total-TEQ (Spain Field 2)

Source of variation	SS	df	MS	F	P-value	F crit
Between samples	21.874	4	5.4685	1.1664	0.4425	6.3882
Between replicates	2.116	1	2.1160	0.4513	0.5385	7.7086
Error	18.754	4	4.6885			
Total	42.744	9				

Table 3.7 ANOVA: DR-CALUX PCB-TEQ (Spain Field 2)

Source of variation	SS	df	MS	F	P-value	F crit
Between samples	2.061	4	0.5154	0.9705	0.5112	6.3882
Between replicates	0.135	1	0.1346	0.2534	0.6412	7.7086
Error	2.124	4	0.5310			
Total	4.320	9				

Table 3.8 ANOVA: DR-CALUX Dioxin-TEQ (Spain Field 2)

Source of variation	SS	df	MS	F	P-value	F crit
Between samples	21.863	4	5.4657	0.7793	0.5925	6.3882
Between replicates	1.183	1	1.1834	0.1687	0.7023	7.7086
Error	28.054	4	7.0136			
Total	51.101	9				

3.4 TEQ covariance with other parameters

If it was found that TEQ co-varied with other, more cheaply analysed parameters, e.g. pH or Loss on Ignition (LOI) then sampling could be targeted, possibly resulting in a reduced number of samples to be analysed to produce a result with equivalent uncertainty.

TEQ_{Total} and TEQ_{PCB} measured by DR-CALUX® were tested for correlation with four parameters: LOI, pH, ΣPAH (EPA 16) and ΣPCB (ICES 7). The results (r^2)are shown in Table 3.9.

There was no significant correlation between TEQ_{Total} or TEQ_{PCB} and LOI or pH. When matrices were considered separately there was some correlation with ΣPAH (EPA 16) or ΣPCB (ICES 7). However, in most cases, the correlation was not strong and, because the number of data was small, was prone to being heavily weighted by a single values, e.g. r^2 for sludge varied greatly depending on whether Sludge 1 was included or not.

Table 3.9 TEQ correlation matrix (r^2 values)

	All matrices n=21		Sludge n=6		Compost n=4		Soil n=11		Spain-Field 2 n=5	
	TEQ_{Total}	TEQ_{PCB}	TEQ_{Total}	TEQ_{PCB}	TEQ_{Total}	TEQ_{PCB}	TEQ_{Total}	TEQ_{PCB}	TEQ_{Total}	TEQ_{PCB}
LOI	0.4804	0.2671	0.2494	0.0201	0.0141	0.4792	0.1643	0.0087	0.1842	0.1614
pH (water)	0.5510	0.1444	0.5004	2x10-5	0.3282	0.2180	0.3408	0.2465	0.0064	0.0156
ΣPAH (EPA 16)	0.0540	0.0431	0.0498	0.6734[a]	0.5398	0.8192	0.3395	0.0294	0.3007	0.0059
ΣPCB (ICES 7)	0.0467	0.0269	0.2317[b]	0.9373[c]	0.9505	0.3285	0.0057	0.6329	0.1787	0.5584

[a]0.0723 without Sludge 1

[b]0.9921 without Sludge 1

[c]0.8186 without Sludge 1

Based on the data available it has to be concluded that there is no clear pattern of correlation between the tested parameters. If more data were available a pattern might emerge showing correlation between TEQ_{Total} or TEQ_{PCB} and ΣPAH (EPA 16) or ΣPCB (ICES 7).

3.5 Requirements for cell-based assays used for screening

The requirements for screening methods of analysis are laid out in section 7 of Commission Regulation 2006/1883/EC, which lays down methods for analysis of dioxins and dioxin-like PCBs in certain foodstuffs, and specific requirements for cell-based bioassays are laid out in section 7.3.

Based on the QA/QC information provided by the laboratory (see Annex C) and examination of the analyses in this study, the DR-CALUX® method fulfils the requirements for a screening method as described in 2006/1883/EC.

High sensitivity and low limits of detection. For dioxins and dioxin-like PCBs, TEQ detectable quantities are in the pictogram TEQ (10^{-12} g) range, which is comparable to the HRGC/HRMS chemical method. For soil and compost samples the limit of quantification reported (Table 2.2) is much lower than the regulatory limits for compost (17-20 ng TEQ kg^{-1} d.w. in German and Swiss legislation). All of the sludge samples were above the limit of detection, but TEQ in sludge samples was measured at levels less than one-fifth of the regulatory limit for sludge (100 ng TEQ kg^{-1} d.w. in German and Austrian legislation).

High selectivity (specificity). The DR-CALUX® bioassay determined TEQ values selectively as the sum of PCDDs, PCDFs and dioxin-like PCBs.

High accuracy (trueness and precision). The screening technology showed for all samples a false negative rate <1% and for almost all samples a CV <30%. The rate of false positive samples is low enough to make the use of a screening tool advantageous.

4 Conclusions

The following conclusions drawn from the data should be interpreted with caution. They are based on a limited number of data, in some cases as few as three samples, and measurements that were often close to or below the limit of quantification (LOQ), or even below the limit of detection.

The following list of conclusions is structured as a list of responses to the questions raised in section 1.3, Aims.

Q1. Could the DR-CALUX® method be used to screen samples for chemical analysis of dioxins?

Answer: Qualified YES.

For screening to be effective, the DR-CALUX® method would need to correctly identify samples that are substantially below a given limit so that only those that are close to, or above, the limit require further analysis by chemical methods.

Based on three chemical analyses of dioxins, DR-CALUX® correctly fulfilled the first requirement for screening. A number of samples from two different sites (3 sewage sludge samples from UK-Site B and 2 compost samples from UK-Site F) were identified as being close to or above the limits and would require chemical analysis to confirm the results.

For a number of reasons (budget being one) it was not possible to test this result.

Q2. Are DR-CALUX® method reliability and chemical analytical method reliability comparable?

Answer: YES

With the exception of one sample, DR-CALUX® TEQ_{Total} was within the quoted range for measurement uncertainty (26%). This measurement uncertainty was comparable to the uncertainty associated with chemical analysis of dioxins.

Measurement uncertainty for TEQ_{Dioxin} could be calculated for only three samples. Two of the three samples were within the stated range of uncertainty.

Q3. Is TEQ measured using the DR-CALUX® method comparable to TEQ calculated using chemical analytical data and published TEF values?

Answer: Based on the limited data, No.

The DR-CALUX® method could be used to differentiate between samples with higher and lower dioxin response. However, the results were not directly comparable either in the sense of equality or proportionality.

DR-CALUX® overestimated dioxin-like PCBs for the three JRC samples and underestimated dioxin-like PCBs for the 5 soils from Spain-Field 2. For Spain-Field 1 both methods produced similar results.

The results of several studies, including intra- and interlaboratory validation trials, for example RINCA, have demonstrated method correlation with $R^2 > 0.95$ for dredged sediments (e.g. Besselink et al. (2002a,b); Besselink et al. (2004)).

Q4. Can within-field variability be differentiated from DR-CALUX® method variability?

Answer: Based on the limited data, No.

Again the number of data was limited and only TEQ_{PCB} could be investigated at one site (Spain-Field 2) and the samples analysed were close to the background level for both the compared methods. No significant difference could be identified between the variability due to sampling and variability due to analysis.

Of course, this might be the same for TEQ_{Dioxin} and TEQ_{Total} and for the chemical analyses, but insufficient data were available for testing.

Q5. Within a given site, are DR-CALUX® TEQ and other measured parameters spatially correlated?

Answer: Based on the limited data, No.

Based on the limited data available it has to be concluded that there is no clear pattern of correlation between the tested parameters. If more data were available a pattern might emerge showing correlation between TEQ_{Total} or TEQ_{PCB} and ΣPAH (EPA 16) or ΣPCB (ICES 7).

Q6. Does the DR-CALUX® method fulfil the basic quality criteria requirements (e.g. LOD, false negative and false positive)?

Answer: YES.

DR-CALUX® has been accepted for use as a screening method for foodstuffs. Consequently it already complies with the requirements of Commission Regulation 2006/1883/EC, which lays down methods for analysis of dioxins and dioxin-like PCBs in certain foodstuffs. The results of this study demonstrate that the method also meets requirements for sensitivity and limits of detection for sewage sludge and composts.

5 Recommendations

This study was based on a very restricted data set; consequently strong conclusions cannot be drawn from the results. However, the following recommendations are made:

- The DR-CALUX® method fulfils the basic quality criteria requirements and shows some promise as a method for screening samples in combination with a confirmative method for samples ±25% the regulatory level. However, this conclusion is based on a very limited data set and requires additional investigation of a much larger number of samples.

- The number of chemical data was limited and most of the results were at levels well below the regulatory levels. So, although in this study it was concluded that TEQ measured by DR-CALUX® and TEQ calculated from chemical measurements and TEF/REP values are not directly comparable, this is not the conclusion arrived at by numerous other studies. It is recommended that these points are investigated further using both analytical methods, in particular the DR-CALUX® TEQ_{PCB} values.

- There are differences, sometimes large, between calculated TEQ values, depending on which set of TEF/REP values are used. It is recommended that any limits that are based on calculated TEQ should also state which TEF/REP values are to be used in the calculations. The proposed 2005 WHO-TEF values are similar to the CALUX-REP values and, therefore, a better relationship between WHO-TEQ and CALUX-TEQ could be expected. It is recommended that the differences in TEQ_{PCB} calculated using the 1998 and 2005 WHO TEF values is further investigated.

- It is recommended that an interlaboratory validation trial be organized, with several laboratories using the confirmative (chemical) and screening (bioassay) technologies, to evaluate these methods according to international guidelines for the screening/confirmative approach, such as those in

Commission Regulation 2006/1883/EC. The trial should evaluate important QA/QC parameters such as CV, false positive/negative rate and measurement uncertainty and it would be essential to include samples that are above the regulatory levels (e.g. sludge above 100 ng TEQ kg^{-1} d.w. and compost above 17-20 ng TEQ kg^{-1} d.w.).

REFERENCES

[1] AbfKlärV (2002) *Klärschlammverordunung (AbfKlärV) vom 15.04.1992*. Bundesgesetsblatt, Jahrgang 1992, Teil 1, 912-934 (Sewage Sludge Ordnance).

[2] Battelle 2005. *Technologies for monitoring and measurement of dioxin and dioxin-like compounds in soil and sediment.* Innovative Technology Verification Report EPA/540/R-05/001, Revised July 2005. Prepared for USEPA National Exposure Research Laboratory. Washington DC. Contract No. 68-C-00-185.

[3] Behnisch PA, Hosoe K and Sakai S-I (2001) Bioanalytical screening methods for dioxins and dioxin-like compounds - a review of bioassay/biomarker technology. *Environmental International* **27**, 413-439.

[4] Besselink HT. IVM (2002a) DR-CALUX® inter-laboratory validation study (RINCA) Final report. Institute for Environmental Sciences (IVM), Amsterdam, NL. Available at: http://www.zeeslib.nl/doc/RINCA%20final%20report-1.pdf

[5] Besselink HT, Schipper C, Klamer H, Leonards P, Verhaar H, Felzel E and Brouwer A (2002b) DR-CALUX interlaboratory validation study for sediments. *Organohalogen Compounds* **58**, 417-420.

[6] Besselink HT, Schipper C, Klamer H, Leonards P, Verhaar H, Felzel E, Murk AJ, Thain J, Hosoe K, Schoeters G, Legler J and Brouwer A (2004) Intra- and inter-laboratory calibration of the DR-CALUX® bioassay for the analysis of dioxins and dioxin-like chemicals in sediments. *Environmental Toxicology and Chemistry* **23**, 2781-2789.

[7] Brown DJ, Kishimoto Y, Ikeno O, Chu M, Nomura J, Murakami T and Murata H (2001) Validation study for the use of the dioxin responsive CALUX® assay for analysis of Japanese ash and soil samples. *Dioxin 2001, 21st International Symposium on Halogenated Environmental Organic Pollutants and POPs.*

[8] Clark GC, Denison MS, Morris RW, Chu M, Chu A and Brown DJ (2001) Analysis of soil samples from a hazardous waste site: Comparison of CALUX® bioassay TEQ determinations with high resolution GC/MS. *Dioxin 2001, 21st International Symposium on Halogenated Environmental Organic Pollutants and POPs.*

[9] Clark GC, Chu AC, Gordon JD, Chu MD, Brown DJ, Nakamura M, Murata H and Kayama F (2004) *Sample Processing and Validation Studies for the CALUX® Bioassay by XDS*. http://vault.thermo-bremen.com/diox/1700-1730_Fujio_Kayama_Dioxin_and_PCB_specificity.pdf

[10] Cousins I and MacKay D (2000) Correlating the physical-chemical properties of phthalate esters using the 'three solubility' approach. *Chemosphere* **41**, 1389-1399.

[11] Dearden JC (2002) Prediction of environmental toxicity and fate using quantitative structure-activity relationships (QSARs). *Journal of the Brazilian Chemical Society* **13**, 754-762.

[12] De Lima Ribeiro FA and Ferreira MMC (2003) QSPR models of boiling point, octanol-water partition coefficient and retention time index of polycyclic aromatic hydrocarbons. *Journal of Molecular Structure (Theochem)* **663**, 109-126.

[13] Dunnivant FM, Elzerman AW, Jurs PC and Hasan MN (1992) Quantitative structure-property relationships for aqueous solubilities and Henry's Law constants of polychlorinated biphenyls. *Environmental Science and Technology*, **26**, 1567-1573.

[14] European Commission (2000) *Working Document on Sludge 3rd Draft*. Unpublished, pp. 19

[15] European Commission (2002a) Commission Directive 2002/69/EC of 26 July 2002 laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBs in foodstuffs. *Official Journal of the European Communities* **L 209** (6.8.2002), 5.

[16] European Commission (2002b) Commission Directive 2002/70/EC of 26 July 2002 establishing requirements for the determination of levels of dioxins and dioxin-like PCBs in feedingstuffs. *Official Journal of the European Communities* **L 209** (6.8.2002), 15-21.

[17] European Commission (2006) Commission Directive 2006/1883/EC of 19 December 2006 laying down methods of sampling and analysis for the official control of levels of dioxins and dioxin-like PCBs in certain foodstuffs. *Official Journal of the European Communities* **L 364** (6.8.2002), 32-43.

[18] Ferreira MMC (2001) Polycyclic aromatic hydrocarbon: a QSPR study. *Chemosphere* **44**, 125-146.

[19] Fiedler H, Fürst P, Malisch R, Päpke O and Schrenk D (2000) State-of-the-Art: Dioxins. Report from 20th International Symposium on Halogenated Environmental Organic Pollutants & POPS. *Environmental Science & Pollution Research* **7**, 239-242.

[20] Frogbrook ZL and Oliver MA (2001) Comparing the spatial predictions of soil organic matter determined by two laboratory methods. *Soil Use and Management* **17**, 235-244.

[21] García MT, Compos E, Dalmau M, Ribosa I and Sánchez-Leal J (2002) Structure-activity relationships for association of linear alkylbenzene sulfonates with activated sludge. *Chemosphere* **49**, 279-286.

[22] Groeneveld, E and Hébert, M (2002?) Dioxins, furans, PCBs and PAHs in eastern Canada compost. Available at: http://www.cwwa.ca/pdf_files/Compost%20DF%20PAH%20PCB%20v2.pdf

[23] Hiester, E, Bruckmann, P, Hembrock-Heger, A, Gerlach, A, Magdt, S, Porta, M, Ristow, H, and Wasin, M (2004) Dioxin-Like PCB in the Environment - Impacts of the New WHO-TEFs on Assessment Thresholds. *Organohalogen Compounds* **66**, 3343-3349.

[24] Lambkin DC, Evans TD, Nortcliff S and White TC (2004) *Towards producing harmonise methods, with quantified precision, for sampling sludges, treated biowaste and soils in the landscape*. Project HORIZONTAL. Final Report WP2.

[25] Lambkin DC, Nortcliff S and White TC (2006) *Report on fieldwork to test statistical performance of sampling strategies for sludge and treated biowaste*. Project HORIZONTAL-HYG Report HYG WP1-2.

[26] Lambkin DC, Nortcliff S and White TC (2007) *Research report on the relationship of sampling protocols to the analytical results obtained for soils and treated biomaterials (biowastes and sludges)*. Project HORIZONTAL-ORG Report ORG WP2.6.

[27] Meylan W, Howard PH and Boethling RS (1992) Molecular topology/fragment contribution method for predicting soil sorption coefficients. *Environmental Science and Technology*, **26**, 1560-1567.

[28] Murk AJ, Jonas A and Brouwer A (1996) Application of the CALUX (chemical activated luciferase gene expression) assay for measuring TCDD-equivalents in sediment, pore water and blood plasma samples. *Organohalogen Compounds* **27**, 291-296.

[29] NATO/CCMS (1988) International Toxicity Equivalency Factor (I-TEF) method of risk assessment for complex mixtures of dioxin and related compound. Report Number 176, August 1988, North Atlantic Treaty Organization, Committee on Challenges of Modern Society.

[30] Öberg T (2001) Prediction of physical properties for PCB congeners from molecular descriptors. *Internet Journal of Chemistry* Volume **4**, Article No 11.

[31] Sakai S and Takigami H (2003) Integrated biomonitoring of dioxin-like compounds for waste management and environment. *Industrial Health* **41**, 205-214.

[32] Schroijen C, Windal I, Goeyens L and Baeyens W (2004) Study of the interference problems of dioxin-like chemicals with the bio-analytical method CALUX. *Talanta* **63**, 1261-1268.

[33] Scippo M-L, Eppe G, De Pauw E and Maghuin-Rogister G (2004) DR-CALUX screening of food samples: evaluation of the quantitative approach to measure dioxin, furans and dioxin-like PCBs. *Talanta* **63**, 1193-1202.

[34] Stronkhorst J, Leonards P and Murk AJ (2002) Using the dioxin receptor-CALUX in vitro bioassay to screen marine harbor sediments for compounds with a dioxin-like mode of action. *Environmental Toxicology and Chemistry* **21**, 2552-2561.

[35] Swiss Confederation (2005), Ordinance of 18 May 2005 on the reductions of risks linked to chemical products (ORRChem), Swiss Confederation, Bern, 2005.

[36] Tsutsumi T, Amakura Y, Nakamura M, Brown DJ, Clark GC, Sasaki K, Toyoda M and Maitani T (2003) Validation of the CALUX bioassay for the screening of PCDD/Fs and dioxin-like PCBs in retail fish. *Analyst* **128**, 486-492.

[37] Umweltministerium Baden-Wurttemberg (HRSG.) (1994) Komposterlaß. Stuttgart, AZ 48-8981.31/264 vom 30.6.1994.

[38] Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE (2006) The 2005 World Health Organization re-evaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicological Sciences* **93**, 223-241.

[39] Vanderperren H, Van Wouwe N, Behets S, Windal I, Van Overmeire I and Fontaine A (2004) TEQ-value determination of animal feed; emphasis on the CALUX bioassay validation. *Talanta* **63**, 1277-1280.

[40] Van Wouwe N, Windal I, Vanderperren H, Eppe G, Xhrouet C, Massart A-C, Debacker V, Sasse A, Baeyens W, De Pauw E, Sartor F, Van Oyen H and Goeyens L (2004a) Validation of the CALUX bioassay for PCDD/F analyses in human blood plasma and comparison with GC-HRMS. *Talanta* **63**, 1157-1167.

[41] Van Wouwe N, Windal I, Vanderperren H, Eppe G, Xhrouet C, De Pauw E, Goeyens L and Baeyens W (2004b) Importance of clean-up for comparison of TEQ-values obtained by CALUX and chemo-analysis. *Talanta* **63**, 1269-1272.

[42] Webber MD, Rogers HR, Watts CD, Boxall ABA, Davis RD and Scoffin R (1996) Monitoring and prioritisation of organic contaminants in sewage sludges using specific chemical analysis and predictive, non-analytical methods. *The Science of the Total Environment* **185**, 27-44.

[43] Windal I, Schroijen C, Van Wouwe N, Carbonelle S, Van Overmeire I, Brown DJ, Clark GC, Baeyens W and Goeyens L (2003a) Non additive interactions in CALUX. *Organohalogen Compounds* **60**, 239-242.

[44] Windal I, Van Wouwe N, Carbonelle S, Van Overmeire I, Eppe G, Xhrouet C, Debacker V, De Pauw E, Baeyens W, Joiris C and Goeyens L (2003b) Validation and discussion of CALUX analysis for marine samples. *Organohalogen Compounds* **60**, 215-218.

[45] Windal I, Denison MS, Birnbaum LS, Van Wouwe N, Baeyens W and Goeyens L (2005a) Chemically activated luciferase gene expression (CALUX) cell bioassay analysis for the estimation of dioxin-like activity: Critical parameters of the CALUX procedure that impact assay results. *Environmental Science and Technology* **39**, 7357-7364.

[46] Windal I, Van Wouwe N, Eppe G, Xhrouet C, Debacker V, Baeyens W, De Pauw E and Goeyens L (2005b) Validation and interpretation of CALUX as a tool for the estimation of dioxin-like activity in marine biological matrices. *Environmental Science and Technology* **39**, 1741-1748.

[47] Worth, AP (2003) Use of QSARs in international decision-making frameworks to predict ecologic effects and environmental fate of chemical substances. *Environmental Health Perspectives* **111**, 1376-1390.

Annex A

DR-CALUX®

A.1 The chemical method

The most toxic dioxin compound is 2,3,7,8-TCDD. Dioxin toxicity is expressed in terms of the Toxicity Equivalence Quotient (TEQ): the amount of 2,3,7,8-TCDD that would produce equivalent toxicity. The toxicity of other dioxin-like compounds relative to 2,3,7,8-TCDD is termed the Toxicity Equivalence Factor (TEF).

Calculation of the Total TEQ using the TEF calculation assumes that the TEQ of individual compounds can be summed.

To arrive at a TEQ measurement for a test sample the samples are extracted, cleaned-up and analysed by HRGC-MS (High Resolution Gas Chromatography with Mass Spectrometry detection) for a suite of dioxin-like compounds. Dioxin-like toxicity (TEQ) for each compound is calculated by multiplying the concentration by the TEF. The TEQs for all the measured compounds are summed to give the TEQ of the sample (typically expressed as pg TEQ/g or ng TEQ/kg).

In 1997, WHO published a list of TEF values for a suite of 29 compounds that produce dioxin-like toxicity with separate values given for humans/mammals, fish and birds (Stronkhorst et al., 2002). The 29 compounds represent a small number of the possible PCDD/F and PCB compounds (419 congeners), i.e. 7 of 75 PCDDs, 10 of 135 PCDFs and 12 of 209 PCBs. The WHO values were reviewed in June 2005 by a WHO-IPCS (International Programme on Chemical Safety) expert meeting (van den Berg, 2006). Previously TEFs had been assigned in increments of 0.01, 0.05, 0.1, etc, but in the re-evaluation it was decided to use half order of magnitude increments on a logarithmic scale of 0.03, 0.1, 0.3, etc (see Table C.1).

A.2 The CALUX® method

Chemically **A**ctivated **LU**ciferase gene e**X**pression (CALUX®) is a reporter gene assay that detects dioxin-like compounds based on their ability to activate the aryl hydrocarbon receptor (AhR ligands). Mammalian hepatoma cells that respond to the group of chemicals of interest are selected then genetically modified to include the firefly gene which codes for the enzyme luciferase. The gene is incorporated such that when binding to the AhR-ligand takes place the luciferase gene is activated and luciferase is produced. The amount of luciferase produced is equivalent to the amount of receptor binding and is measured by reaction with luciferin to produce a light signal which is detected by a luminometer.

The amount of light produced is proportional to the amount of ligand-AhR binding which is related to 2,3,7,8-TCDD toxic equivalents (TEQs) using a calibration curve. Calibration is carried out by analysis of TCDD standard solutions and preparation of a sigmoid calibration graph, typically modelled using a four-variable Hill equation.

It is assumed that dose-response is independent of the matrix being tested, for example test samples are complex mixtures of compounds whereas standards contain TCDD only. Most investigators recommend using the linear portion of the dose-response calibration curve and diluting test samples into that range. This reduces the matrix effect and utilises the most sensitive areas of the curve. The current detection limit for CALUX in rat hepatoma H411E recombinant cells is less than 0.06 pg TEQ/well (Sakai and Takigami, 2003). Limit of quantification depends on extraction and dilution factors.

The CALUX® method detects the total response to all dioxin-like compounds in an extract, but samples can be prepared by differentiating classes of congeners prior to cell dosing (Clark et al., 2004).

There is more than one CALUX® cell-line available. XDS (Xenobiotic Detection Systems, Inc.), an American company, has developed CALUX® methodology using the mouse hepatoma H1L6.1. BDS (BioDetection Systems B.V.) use the H411E rat hepatoma for DR-CALUX®, the **D**ioxins **R**esponsive assay. DR-CALUX® is more specific to dioxin-like contamination than other bioassays (Stronkhorst et al., 2002).

CALUX® has been used in the Netherlands as a screening tool for hot spot identification in contaminated land investigations. Based on a limit of 1000 ng TEQ/kg, samples are identified using CALUX® then high TEQ samples are analysed for PCBs and PCDD/F using chemical methods to identify specific compounds and concentrations (Stronkhorst et al., 2002).

Many parameters can be adjusted when performing CALUX® analysis. The applied procedure strongly affects the result and, consequently, the interpretation of the results. The critical parameters of the CALUX® procedure that impact assay results are examined and discussed by Windal et al. (2005a).

A.2.1 Antagonism and synergism

Because it is a biological test, CALUX® is subject to antagonistic and synergistic interferences depending on the chemical mix in the sample. Examples of these effects have been reported in several studies. PHB, PHDD/F and PAH can contribute to the CALUX® AhR response. Brominated compounds show affinities for the AhR very similar to chlorinated compounds and are co-extracted with chlorinated compounds (Scippo et al., 2004). Antagonism has been demonstrated for HCB and PCN compounds (the results were negative for PCT) but high concentrations of the antagonistic compounds were required to observe the effect. Inhibition has been observed by PCBs (52, 108, 153, 156, 159) and Aroclor 1254. Synergistic effects have been observed for corticosteroids. To avoid the problem extracted samples are passed through a cleanup procedure before CALUX® assay.

A.2.2 Extraction and cleanup

Extraction conditions determine which compounds are extracted and the %recovery. Strong extractants remove the tightly bound and soft extractants remove compounds available for bio-uptake and leaching. First, samples are passed through an acidic silica column where the PAHs are degraded. Second, samples are passed through a carbon column to separate the PCBs from dioxins. It has been found (Windal et al., 2003a) that the TEQ of the separate fractions is less than the TEQ of the unfractionated extract (using mouse H1L6.1).

For some sample matrices it is possible to analyse extracts without clean-up (Windal et al., 2005a). However, if the extract is likely to contain antagonists or chemicals that are toxic to the cell line then clean-up to isolate the chemicals of interest will be required. For example, acidic silica columns remove the fat and interfering compounds such as non-classical AhR ligands and classical ligands that are degraded under acidic conditions, e.g. PAH. A carbon column is used to separate PCDD/F from other compounds. Classical AhR ligands are defined as those having a structure that is close to TCDD, e.g. PCDD/F, PCB, PBB, PBDE, PCT, PCN, PAH. Non-classical AhR ligands bind weakly to the Ah receptor and their physical and structural properties are unlike TCDD.

A.2.3 Quality control

Most quality control investigations have been carried out using the mouse hepatoma and food, feed or biological samples. This is primarily because of concerns about bioaccumulation within the food chain and because there are two Commission Directives (European Commission, 2002a; European Commission, 2002b) that permit the use of bioassay methods for screening and specify the analytical QC. There are no equivalent Directives for sewage sludge and soils, although limit values (100 TEQ/kg dm) for PCDD/F were

proposed in the 3rd Working Document on Sludge (European Commission, 2000 [withdrawn]). In the Fourth National Policy Document on Water Management in the Netherlands bioassay methods, including DR-CALUX®, may be included for assessment of dredged material (Besselink et al., 2004).

It is not possible to use internal standards to assess recovery because bioassays do not differentiate between labelled and unlabelled compounds (Clarke et al., 2004); recovery has to be estimated using alternative methods. Quoted values for recovery are in the range 80-120%.

Table A.1. Reported Method Performance Data for CALUX®

Cell line	matrix	QC	Reference
Rat	Sediment	LOD < 1 pM; RSD ≤ 5%	Murk et al., 1996
Rat	Feed, beef fat, cod liver	DR-CALUX®; RSD <30%	Scippo et al., 2004
Rat	Dredged sediment	DR-CALUX® Inter-laboratory trial; average inter-lab repeatability ≤26%; average inter-lab reproducibility ≤28%; method variability ±10.5% for analytical samples, ±22% for sediment extracts. LOD 0.3 pM; LOQ 1.0 pM	Besselink et al., 2004
Mouse	Marine biological	Used 6 check samples – mean concentration within 15% of actual concentration, RSD of six samples <15%. Procedural blank spiked with 2,3,7,8-TCDD to check for antagonists. Labelled 2,3,7,8-TCDD used to check recovery. Repeatability 13% for dioxin, 22% for PCB	Windal et al., 2003b
Mouse	Marine sediment	LOD 0.2 ng TEQ/kg; LOQ 0.4 ng TEQ/kg, uncertainty 10% on 3 pM standard	Stronkhorst et al., 2002
Mouse	Marine biological matrices	RSD 9%; within lab repeatability 15%; LOQ 1.25 pg TEQ/g fat	Windal et al., 2005b
Mouse	Sheep feed	RSD (n=6) <30%	Vanderperren et al., 2004
Mouse	Human plasma	RSD repeatability 13%; RSD reproducibility 25%; Recovery 74.6-88.4% (n=13)	Van Wouwe et al., 2004a

A.3 Comparison of Methods

The chemical method (HRGC-MS) is regarded as the reference method against which other methods are tested to assess their correctness. The closeness of the CALUX® and HRGC-MS methods depends on a number of factors:

- The compound ratios in the samples and the matrix type;

- At low concentrations the limit of detection of HRGC-MS affects the results because undetected compounds do not contribute to the total TEQ. Some workers have tried to take this into account by assuming that where the compound concentration is below LOD a value of 0.5 LOD is used (Stronkhorst et al., 2002);

- Incubation time affects the shape and relative position of the dose-response curve; PAH and aromatic amines require a short exposure time because they are rapidly metabolized;

- The results can differ depending on which cell-line is used. This is due to differences in AhR structure and quantity and other biological factors. Antagonistic and synergistic effects are cell-line dependent and they have been shown to depend on the ratio of dioxin-like compounds in the sample (Schroijen et al., 2004);

- Chemical analysis could miss major pollutants that contribute to the overall TEQ because they have not been looked for. Some compounds with dioxin-like properties that could contribute to CALUX-TEQ have been identified and TEF values determined, e.g. Sakai and Takigami (2003), but definitive TEF values have not been agreed (Behnisch et al., 2001) and are under constant review (for example van den Berg et al., 2006);

- The result depends on which TEF values are used and the associated uncertainty (Stronkhorst et al., 2002). New sets of TEF values have been produced for many compounds (CALUX RElative Potency, CALUX-REP), based on the results of CALUX® and HRGC-MS analyses. This improves the match between the two methods;

- The results may be affected by preparation procedures, for example choice of clean-up (Behnisch et al., 2001; Van Wouwe et al., 2004b). The toxicity of solvents and extractants can significantly affect the CALUX® measurement and new methods need to be checked for contamination and toxicity;

- The TEF principal assumes the total TEQ is equal to the sum of compound TEQs. But non-additive interactions have been described and are measured (in part) by CALUX;

- Dioxin-like compounds may be unidentified, in particular chlorinated compounds (Stronkhorst et al., 2002).

A.4 Validation studies

In general it has been found that, for the dioxin fraction, CALUX > chemical and, for PCBs, CALUX < chemical. But for dioxin+PCB the results are similar.

The trueness of CALUX® is difficult to evaluate:

- It is not possible to analyse for all the dioxin-like compounds to check against chemical (reference) method;

- TEFs are not available for all compounds;

- Non-additive interactions can not be accounted for;

- Some compounds are near the limit of detection for HRGC-MS;

- The extraction and clean-up methods can affect the recovery.

It has been suggested (Stronkhorst et al., 2002) that some of the variability in DR-CALUX® activity is caused by re-sampling, variation between laboratories and assay reproducibility.

A double blind validation study comparing results from HRGC-MS and CALUX® was carried out using the mouse hepatoma H1L6.1 (US method) for soils from a hazardous material remediation area. Initial results have demonstrated a good log:log correlation (r^2=0.9815, n=18) (Clark et al., 2001).

A second study (DIFFERENCE Project), which tested methods such as CALUX® (mouse line) against HRGC-MS for fish and vegetable oils, reports that the CALUX® technique underestimated the total TEQ compared to HRGC-MS, but CALUX® repeatability is significantly higher than the other screening techniques tested.

A third study funded by the USEPA (SITE Program), is evaluating several innovative technologies for detecting dioxins in soil and sediment samples. The reports include an evaluation of the CALUX® method developed by XDS (Battelle, 2005). The CALUX® method reported data higher than certified values for PCDD/F and Total TEQ, but were generally lower than certified values for PCB.

Table A.2. CALUX® validation studies

Matrix	Outline	Reference
Marine harbour sediment	Dutch study. Used DR-CALUX® to measure PCDD/F, PCB, PBB, PBDE in 281 samples. 20 of the samples also analysed by GC-MS. For 7 samples the method difference was small; for 13 samples DR-CALUX® > GC-MS. Concluded difference due to dioxin-like compounds not measured by GC-MS, analytical uncertainty, especially close to LOD. Using CALUX-TEFs improved the correlation such that results agreed to within 10%.	Stronkhorst et al., 2002
Sediment, pore water, blood plasma	Compared relative amounts in 2 sediments. CALUX resulted in 20:1; GC-MS resulted in 17:1. Similar results for pore waters.	Murk et al., 1996
Fish	Compared GC-MS: CALUX. Correlation coefficient = 0.71 (n=16) for all dioxin-like compounds and 0.70 for PCBs. DR-CALUX® > GC-MS, by factor of 2, probably due to contribution of PCBs.	Besselink et al., 2002b
Fish	Retail Fish (n=22). Compared CALUX and HRGC-HRMS. PCDD/Fs (r= 0.89) and dioxin-like PCBs (r= 0.91). Recovery 77-117%.	Tsutsumi et al., 2003
Fly ash	Untreated fly ashes CALUX: Chemical r^2=0.92; n=6. Treated + untreated fly ash samples (n=18) reduced r^2 to 0.72.	Sakai and Takigami, 2003
Soil & Ash (Japan)	GC-MS:CALUX r^2 = 0.94. CALUX-TEQ > GC-MS-TEQ in 21 of 25 samples. Overestimation varied between samples. Suggested possible difference due to co-planar PCBs.	Fielder et al., 2000
Soil and sediment	False positive/negatives: ≤10% @ 50 pg/g TEQ for PCBs; ≤10% @ 1 pg/g TEQ for PCDD/F and Total TEQ. GC-MS and HRGC-MS vs CALUX, Method agreement: PCBs 82%; PCDD/F 69%; Total TEQ 72%.	Battelle, 2005

Annex B

Chemical sample analysis

B.1 General

The data provided by JRC are produced in Table B.1. Analytical data for WP2 samples are shown in Table B.2 and B.3.

Table B.1 Chemical analysis: JRC samples – PCDD/F

	Soil 3				Compost 1				Sewage sludge 1			
Compound	**R1**	**R2**	**R3**	**R4**	**R1**	**R2**	**R3**	**R4**	**R1**	**R2**	**R3**	**R4**
chlorinated dibenzo-p-dioxins ng kg^{-1} DW												
2,3,7,8-TCDD	<0.024	<0.042	<0.028	<0.018	0.14	0.18	0.19	0.18	0.88	0.91	0.88	0.91
1,2,3,7,8-PeCDD	0.093	0.11	0.076	0.11	0.55	0.48	0.59	0.66	1.66	1.95	1.53	1.84
1,2,3,4,7,8-HxCDD	0.050	0.056	0.080	0.058	0.77	0.84	0.88	0.67	2.87	2.97	3.32	3.13
1,2,3,6,7,8-HxCDD	0.29	0.33	0.34	0.32	3.29	3.20	3.00	3.32	7.14	7.47	7.73	7.64
1,2,3,7,8,9-HxCDD	0.19	0.11	0.14	0.14	1.53	1.49	1.49	1.67	4.04	3.81	3.80	4.08
1,2,3,4,6,7,8-HpCDD	5.63	5.33	5.61	5.55	172	172	170	183	171	171	163	162
OCDD	45.3	47.0	44.0	43.6	924	861	890	1150	1166	1167	1131	1132
chlorinated dibenzofurans ng kg^{-1} DW												
2,3,7,8-TCDF	0.34	0.31	0.37	0.33	2.85	2.67	2.65	2.76	10.4	11.3	10.5	9.77
1,2,3,7,8-PeCDF	0.15	0.18	0.16	0.19	1.26	1.29	1.31	1.47	4.62	4.45	4.39	4.61
2,3,4,7,8-PeCDF	0.21	0.22	0.17	0.22	1.75	1.76	1.75	1.98	8.38	8.52	8.04	7.31
1,2,3,4,7,8-HxCDF	0.21	0.24	0.29	0.29	1.81	1.69	1.79	1.47	9.89	9.98	9.85	9.52
1,2,3,6,7,8-HxCDF	0.23	0.22	0.21	0.21	1.76	1.67	1.62	1.69	6.51	6.69	7.12	6.43
2,3,4,6,7,8-HxCDF	0.47	0.38	0.48	0.50	1.32	1.86	1.84	1.83	7.01	7.50	7.58	7.39
1,2,3,7,8,9-HxCDF	0.041	0.030	0.087	0.051	0.27	0.26	0.20	0.32	0.87	0.69	0.84	0.70
1,2,3,4,6,7,8-HpCDF	12.6	11.9	11.5	12.6	14.0	12.2	12.8	14.1	85.2	87.5	86.1	87.4
1,2,3,4,7,8,9-HpCDF	0.21	0.15	0.18	0.18	1.16	1.15	1.22	1.30	5.43	5.36	5.41	5.38
OCDF	37.4	35.3	35.1	40.6	24.8	22.7	22.6	44.2	189	192	180	181
Calculated TEQ ng kg^{-1} DW												
WHO_{1998}	0.58	0.57	0.55	0.61	4.95	4.92	5.00	5.38	14.6	15.2	14.4	14.2
WHO_{2005}	0.55	0.54	0.53	0.58	4.77	4.71	4.81	5.19	13.1	13.6	13.0	12.9
$I\text{-}TEQ_{1988}$	0.60	0.59	0.58	0.63	5.53	5.46	5.52	6.11	14.9	15.3	14.8	14.4
$CALUX_{2003}$	1.35	1.31	1.32	1.41	12.8	12.7	12.6	13.7	27.2	27.9	26.8	26.3

Table B.2 Chemical analysis: JRC samples – Dioxin-like PCBs

	Sample											
	Sewage sludge 1				Compost 1				Soil 3			
Compound	**Mean**	**s.d.**	**CV%**	***l,n***	**Mean**	**s.d.**	**CV%**	***l,n***	**Mean**	**s.d.**	**CV%**	***l,n***
non-ortho substituted PCBs ng kg^{-1} DW												
PCB 77	6.67	0.71	10.6	5,19	0.132	0.013	10	4,16	0.0258	0.0045	17.4	3,11
PCB 81	0.524	0.244	46.6	5,19	0.019	0.0151	79.7	2,8	0.00219	0.00145	66.5	3,8
PCB 126	0.25	0.077	30.8	5,19	0.0359	0.0064	17.8	2,8	0.00244	0.00086	35.5	3,10
PCB 169	0.0415	0.0339	81.9	3,12	0.00344	0.0006	17.4	2,8	0.0122	0.0311	254.8	3,7
mono-ortho substituted PCBs ng kg^{-1} DW												
PCB 105	19.2	2.2	11.6	4,16	0.683	0.121	17.7	4,16	0.0958	0.0111	11.6	4,15
PCB 114	1.36	0.34	24.8	4,16	0.0504	0.304	60.2	2,8	0.0255	0.0393	154.1	3,10
PCB 118	33.3	3.7	11.1	4,16	1.95	0.254	13.1	4,16	0.218	0.041	18.9	5,19
PCB 123	1.94	1.72	89	5,20	0.119	0.101	84.8	2,12	0.00806	0.00305	37.8	3,8
PCB 156	7.04	0.85	12.1	4,15	0.882	0.144	16.3	4,16	0.0453	0.0057	12.6	3,12
PCB 157	0.947	0.232	24.5	4,16	0.102	0.0161	15.8	4,16	0.00788	0.00143	18.2	3,8
PCB 167	4	2.07	51.8	5,20	0.75	0.724	96.5	2,16	0.0195	0.0032	16.3	3,8
PCB 189	1.16	0.78	67.2	5,20	0.177	0.0164	9.3	4,16	0.00762	0.00112	14.7	3,8

(Standard deviation in parentheses); LOQ = Limit of quantification (estimated value in parentheses)

l,n = number of laboratories, total number of analyses

Table B.3 Chemical analysis: WP2 samples - Sewage sludge (SS) and compost

	Sample					
Compound	**Compost 3**	**Compost 4**	**SS 3**	**SS 4**	**SS 5**	**SS 6**
non-ortho substituted PCBs µg kg^{-1} DW						
PCB 77	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 81	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 126	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 169	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
mono-ortho substituted PCBs µg kg^{-1} DW						
PCB 105	<LOD	<LOD	1.56	1.35	<LOD	<LOD
PCB 114	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 118	3.66	<LOD	3.55	3.24	<LOD	<LOD
PCB 123	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 156	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 157	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 167	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 189	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
pH	8.26	8.05	7.89	5.28	6.37	6.42
LOI%	43.0	45.1	62.8	57.2	56.4	62.9

Table B.4 Chemical analysis: WP2 samples - Soils

	Sample							
Compound	**Soil 6**	**Soil 7**	**Soil 8**	**Soil 9**	**Soil 10**	**Soil 11**	**Soil 12**	**Soil 13**
non-ortho substituted PCBs µg kg^{-1}								
PCB 77	<LOD	<LOD	<LOD	<LOD	<LOQ(0.25)	<LOD	<LOD	<LOD
PCB 81	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 126	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 169	<LOD	<LOD	<LOQ(0.14)	<LOQ(0.20)	1.62	<LOQ(0.29)	1.58	2.20
mono-ortho substituted PCBs µg kg^{-1}								
PCB 105	<LOD	<LOD	<LOD	<LOD	1.97	<LOD	2.76	2.69
PCB 114	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ(0.84)
PCB 118	<LOD	<LOD	<LOD	<LOD	5.01	<LOD	6.94	6.44
PCB 123	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ(1.06)
PCB 156	<LOQ(0.19)	<LOQ(0.23)	<LOD	<LOD	<LOQ(1.11)	<LOQ(0.51)	1.62	2.36
PCB 157	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ(0.95)
PCB 167	<LOD	<LOD	<LOD	<LOQ(0.12)	<LOQ(0.39)	<LOD	<LOQ(0.48)	<LOQ(1.15)
PCB 189	<LOD	<LOD	<LOD	<LOD	<LOQ(0.49)	<LOD	<LOQ(0.49)	1.85
pH	8.07	8.16	7.90	8.17	7.96	8.31	7.88	8.31
LOI%	7.10	6.71	8.09	7.63	9.35	9.87	10.5	7.83

LOQ = Limit of quantification (estimated value in parentheses); LOD = Limit of detection

Annex C

DR-CALUX® Analysis

C.1 The analytical laboratory

The samples were analysed by BioDetection Systems B.V. (BDS), Amsterdam, Netherlands [www.biodetectionsystems.com]. Additional information about analytical QA/QC was provided by the laboratory.

C.2 Laboratory QA/QC

The laboratory is accredited to the ISO/IEC/17025:1999 standard. According to their ISO/IEC/17025:1999 accreditation they also have demonstrated the performance of their method in the range of the level of interest, e.g. 0.5x, 1x and 2x the level of interest with an acceptable variation for repeated analysis. The laboratory regularly participates in inter-laboratory studies such as the RINCA study (DR-CALUX® inter-laboratory [round robin] study).

Regular blank controls and spiking experiments or analysis control samples (preferably, if available, certified reference material) are performed as internal quality control measures. The limit of detection has been set as 3 x the standard deviation of the solvent blank or of the background response.

Every test was run with a series of reference concentrations of TCDD (full dose-response curve with R^2 > 0.95). Sample dilution was within the linear portion of the response curve. An expanded low level curve was used for samples with low concentrations.

Quality control (QC) charts for a TCDD reference concentration (about 3 x limit of quantification) and/or a reference sample on a quality control sheet have been used for the outcome of the bioassay over a constant time period. The percent standard deviation has been below 15% in a triplicate determination for each sample dilution and not above 30% between three independent experiments.

Annex D

Published TEF & CALUX-REP Values

Table C.1 Summary of published Toxic Equivalence Factors (TEF) and CALUX-REP values

Compound	WHO_{1998}	WHO_{2005} [a]	$I\text{-}TEQ_{1988}$ [b]	$CALUX_{2003}$ [c]	$CALUX_{2004}$ [e]
chlorinated dibenzo-p-dioxins					
2,3,7,8-TCDD	1	1	1	1	1
1,2,3,7,8-PeCDD	1	1	0.5	0.54	0.45
1,2,3,4,7,8-HxCDD	0.1	0.1	0.1	0.30	0.09
1,2,3,6,7,8-HxCDD	0.1	0.1	0.1	0.14	0.05
1,2,3,7,8,9-HxCDD	0.1	0.1	0.1	0.066	0.04
1,2,3,4,6,7,8-HpCDD	0.01	0.01	0.01	0.046	0.02
OCDD	0.0001	**0.0003**	0.001	$4.7x10^{-4}$	0.0004
chlorinated dibenzofurans					
2,3,7,8-TCDF	0.1	0.1	0.1	0.32	0.4
1,2,3,7,8-PeCDF	0.05	**0.03**	0.05	0.21	0.1
2,3,4,7,8-PeCDF	0.5	**0.3**	0.5	0.50	0.3
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1	0.13	0.06
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1	0.039	0.07
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1	0.11	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1	0.18	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01	0.029	0.01
1,2,3,6,7,8,9-HpCDF	0.01	0.01	0.001	0.041	0.04
OCDF	0.0001	**0.0003**	0.001	0.0065	0.004
non-ortho substituted PCBs					
PCB 77	0.0001	0.0001		0.0013	0.0003
PCB 81	0.0001	**0.0003**		0.0042	0.002
PCB 126	0.1	0.1		0.067	0.04
PCB 169	0.01	**0.03**		0.0034	0.0007
mono-ortho substituted PCBs					
PCB 105	0.0001	**0.00003**		$1.2x10^{-5}$	<0.0001
PCB 114	0.0005	**0.00003**		$4.8x10^{-5}$	0.00001
PCB 118	0.0001	**0.00003**		$4.9x10^{-6}$ (d)	<0.0001
PCB 123	0.0001	**0.00003**		$2.4x10^{-4}$	<0.0001
PCB 156	0.0005	**0.00003**		$2.1x10^{-4}$	0.00002
PCB 157	0.0005	**0.00003**		$8.0x10^{-5}$	<0.0005
PCB 167	0.00001	**0.00003**		$8.2x10^{-6}$	<0.0001
PCB 189	0.0001	**0.00003**		$6.7x10^{-6}$	<0.0001

(a) Data from Van den Berg et al. (2006) Numbers in bold indicate a change in TEF value

(b) NATO/CCMS (1988)

(c) Data from Sakai and Takigami (2003)

(d) Bovee et al. (1998) cit. in Sakai and Takigami (2003)

(e) Data from Scippo et al. (2004)

Annex E

Glossary

2,3,7,8-tetrachlorodibenzo-p-dioxin		This compound is considered the most toxic of the chemical family of dioxins and furans. It is assigned the toxicity equivalent factor (TEF) of '1', and all other dioxins and furans are assigned a TEF value less than '1'.
Dioxins		Commonly used term for polychlorinated dibenzo-p-dioxins (PCDDs), a group of chlorinated compounds with similar chemical structure. Although not strictly correct, the term "dioxins" is sometimes used to refer to all dioxins and dioxin-like compounds, including dibenzofurans (PCDFs) and PCBs.
Dioxin-like compounds		Compounds from a group of halogenated aromatic hydrocarbons that produce similar toxic effects to dioxins. Certain members of the dioxin, furan, and PCB family are termed 'dioxin-like'.
EPA 16 priority PAHs	**EPA 16**	While there are many different PAH compounds, monitoring is based primarily on a US EPA list of 16 priority PAH compounds.
International Council for the Exploration of the Sea	**ICES 7**	The following PCB congeners: 28, 52, 101, 118, 138, 153 and 180 are routinely analysed by the International Committee for the Exploration of the Sea (sometimes referred to as the 'ICES 7' congeners).
Limit of Detection	**LOD**	Lowest level of analyte that can be detected, but not necessarily quantified.
Loss on Ignition	**LOI**	The percentage weight lost on ignition provides and estimate of the organic content of a sample.
Limit of Quantification	**LOQ**	Lowest level of analyte that can be reliably measured with acceptable accuracy and precision.
Polycyclic Aromatic Hydrocarbons	**PAH**	Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 different chemicals that are formed during the incomplete burning of coal, oil and gas, waste, or other organic substances like tobacco or charbroiled meat. PAHs are usually found as a mixture containing two or more of these compounds.
Polychlorinated Biphenyls	**PCB**	Polychlorinated biphenyls are mixtures of up to 209 individual chlorinated compounds (known as congeners). There are no known natural sources of PCBs.
Polychlorinated p-dibenzo dioxins **Polychlorinated p-dibenzo furans**	**PCDD** **PCDF**	A family of 210 different by-products of mainly combustion of any organic material, whether it is chlorine containing or not; commonly called 'dioxins'.
Relative Potency	**REP**	The ratio of the potency of the congener to the standard toxicant in that specific study; a concept similar to toxic equivalency but based on a single study, species, or matrix, etc., and not averaged to obtain a general toxic equivalency value.

Toxicity Equivalence Quotient	**TEQ**	Dioxin Toxicity Equivalence Quotient or TEQ is used to express the total toxicity of a mixture of "dioxins" and "furans" in terms of the amount of 2,3,7,8-TCDD it would take to equal the combined toxic effect of all the "dioxins" and "furans" found in the mixture. Toxicity Equivalency Factors developed for each compound in the mixture are used to calculate the TEQ. (See TEF). The total TEQ is the sum of the TEQs for each of the congeners in a given mixture: $\text{Total TEQs} = \sum_{i=1}^{n} (C \times TEF_i)$
Toxic Equivalency Factor	**TEF**	Toxicity equivalency factors (TEFs) are based on congener-specific data and the assumption that the toxicity of dioxin and dioxin-like compounds is mediated by the Ah receptor and is additive. TEFs compare the potential toxicity of each dioxin-like compound present in a mixture to the well-studied and well-understood toxicity of 2,3,7,8-TCDD, the most toxic member of the group, with the TEF of 2,3,7,8-TCDD being 1. These factors or TEFs are used to calculate the toxicity equivalence or TEQ of a mixture of "dioxins," which is the amount of 2,3,7,8-TCDD it would take to equal the combined toxic effect of all the "dioxins" and "dioxin-like" compounds found in the mixture.