European Union Reference Laboratory
for Dioxins and PCBs in Feed and Food

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CEN Ring Trial 2010 - Animal Feed

Ring trial on determination of PCDD/Fs and PCBs in animal feed, fat/oil

Report

PCDD/Fs and PCBs

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European Committee for Standardization (CEN), RIKILT - Institute of Food Safety, EU-RL for Dioxins and PCBs in Feed and Food

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1. Introduction

At the request of the European Committee for Standardization (CEN) and in close cooperation of the RIKILT - Institute of Food Safety, Wageningen, Netherlands, the Food and Environment Research Agency, York, UK, and the European Union Reference Laboratory (EU-RL) for Dioxins and PCBs in Feed and Food in Freiburg a standard for the determination of PCDD/Fs and PCBs in animal feed was described. In order to validate this new CEN method a ring trial was organized to be accomplished within the EU-RL/NRL network as well as within the CEN network between November 2009 and April 2010. The objective is to assess the comparability of results of all relevant parameters (17 PCDD/F, 12 dioxin-like PCBs, 6 indicator PCBs) obtained by the new draft CEN standard in four samples of animal feed and in a standard solution.

The new draft CEN standard is split in four modules each describing a part of the whole procedure:

- Module A: Description of standards that might be used
- Module B: Description of extraction procedures
  - B I : Automated method using PLE /ASE
  - B II : Soxhlet method
- Module C: Description of clean-up procedures
  - C I : Automated method described by RIKILT
  - C II : Manual method described by FERA
  - C III : Manual / semi automated method described by EU-RL
- Module D: GC/HRMS determination

Each module describes a part of the whole method as well as, when applicable, equivalent alternatives. Each module has to be regarded as an example. Combining modules and/or alternatives gives a highly flexible procedure which is “performance based“. It is permitted to modify the method provided that all performance criteria laid down in Commission Regulation (EC) No 152/2009 are met.

NRLs for Dioxins and PCBs were requested to participate as part of their work program for 2009. NRLs were requested to use at least one combination of modules or to use the in-house method. NRLs are invited to encourage the participation of official laboratories (OFLs) from EU member states as part of their duties following Article 33 of Council Regulation 882/2004.

In addition to the validation of the CEN method described above, the available data was evaluated for checking of the analytical performance of participating laboratories, in particular NRLs and OFLs in a proficiency test (PT) on the determination of PCDD/Fs and PCBs in animal feeding stuff.

2. Design of the study

2.1 Samples and coding

The four samples have been prepared from regular market feed with or without fortification with the analytes of interest.

- Sample 1 : Mineral clay
- Sample 2 : Bovine compound feed
- Sample 3 : Fish oil
- Sample 4 : Fish meal

Each participant received about 100 g of each animal feed sample (1, 2 and 4) and 20 gram of fish oil (3). The samples were shipped at room temperature in polypropylene jars (1, 2 and 4) or a glass bottle (3).

The WHO-PCDD/F-TEQ and WHO-PCB-TEQ concentrations in the samples were in the range of action to maximum levels defined for animal feed in Commission Regulation No 2006/13/EC of 3 February 2006.

Next to the four above mentioned samples an ampoule containing PCDD/Fs provided by Wellington Laboratories, was also supplied. The ampoule contained ca. 0.5 ml of a solution of unlabeled PCDD/Fs (solvent n-nonane) with a concentration ranging from around 5 to 250 pg/µl. Exact amounts in pg/µl should be calculated using the laboratory calibration curve.

2.2 Analytes

Participants were requested to determine the following analytes and sum parameters:

- 17 2,3,7,8-substituted PCDD/Fs
- WHO-PCDD/F-TEQ (using WHO-TEF 1998)
- 12 dioxin-like PCBs
- WHO-PCB-TEQ (using WHO-TEF 1998)
- Six indicator PCBs: PCB #28, 52, 101, 138, 153, 180

2.3 Coding of laboratories and confidentiality

The laboratory code of the participating laboratories will be kept confidential and will not be revealed to other participants.

For NRLs, the “Protocol for management of underperformance in comparative testing and/or lack of collaboration of National Reference Laboratories (NRLs) with Community reference laboratories (CRLs) activities” will be observed.

The confidentiality of NRLs will be kept according to this protocol. The identity of OFLs will be kept confidential, unless a Member State initiated a cooperation between the NRL, OFLs and the EU-RL to use this PT simultaneously as national PT with OFLs.

3. Analysis and Reporting

3.1 Animal feed samples

Laboratories should:

- use their own reference standards for identification and quantification of all congeners
- analyze all four animal feed samples in duplicate
- report results for each analyte and test material,
- report the limit of quantification (LOQ),
- report upper, middle and lower bound results for WHO-PCDD/F-TEQ and WHO-PCB-TEQ using 1998-WHO-TEFs (upper bound concentrations are calculated assuming that all values of the different congeners less than the limit of quantification are equal to the limit of quantification. Lower bound: LOQ = 0; Middle bound: ½ LOQ)
- report moisture content
- give information on any deviation of the CEN standard

TEQ-based results must be calculated using the WHO-TEFs (1998) (Van den Berg et al., 1998. Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 106, 775 - 792).

3.2 PCDD/F standard solution

Laboratories should:

- analyze the aliquot of the PCDD/F solution in duplicate using their own reference standards for identification and quantification of congeners
- report results for each analyte

3.3 Reporting

All results for the animal feed samples had to be reported on product basis in ng/kg. TEQ-based results have to be calculated using the WHO-TEFs (1998) (Van den Berg et al., 1998. Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 106, 775 - 792). Laboratories using bioanalytical screening methods were asked to report an indication of PCDD/F-TEQ, PCB-TEQ and/or PCDD/F-PCB-TEQ, expressed in BEQ.

Results for the PCDD/F solution had to be reported in pg/µl. Participants were requested to submit the reports electronically to avoid possible transcription errors.

4. Statistical evaluation

4.1 General

The main objective of this ring trial is the validation of the new CEN method for the determination of PCDD/Fs and PCBs in animal feed. In addition, the results of all participating laboratories, using the new CEN method or any other method, were evaluated in a proficiency test.

4.2 Participating laboratories and applied methods

This ring trial/proficiency test was open for participation of:

- NRLs of EU member states and official laboratories (OFLs) and private laboratories for PCDD/Fs and PCBs in feed (from the EU-RL/NRL and CEN network) applying GC/MS-methods and/or bioassays for PCDD/Fs and dioxin-like PCBs and any kind of method for PCBs.

As outlined in the introduction, participation in this ring trial was mandatory for NRLs and open for official laboratories (OFLs) and private laboratories

In total 43 laboratories (NRLs, OFLs, private laboratories and the EU-RL for Dioxins and PCBs) participated in the ring trial/proficiency test. 9 laboratories reported results using more than one method, either different CEN methods, in-house validated GC/MS methods or bioanalytical methods.

32 results were reported using the CEN method (28 results were included in the evaluation of the CEN ring trial), 19 results were reported using an in-house validated method (12 using HRMS, 7 using MS/MS, LRMS or ECD methods) and 3 using a bioanalytical method.

42 to 44 results were reported for PCDD/Fs in the four samples using physico-chemical methods, 40 to 43 for DL-PCBs and 42 to 44 for indicator PCBs.

National Reference Laboratories:

23 NRLs representing 25 EU member states participated using HRMS, LRMS, ECD and/or bioanalytical methods. 6 NRLs reported results using different physical-chemical or bioanalytical methods.

28 results for PCDD/F, DL-PCB and/or NDL-PCB were reported using physical-chemical methods, 3 results using bioanalytical methods. 4 results were reported using LRMS, ECD and MS/MS methods.

The EU-RL for Dioxins and PCBs participated in the ring trial using two different combinations of modules described in the CEN method.

4.3 Test for sufficient homogeneity

The test for sufficient homogeneity was performed by the RIKILT - Institute of Food Safety according to “The International harmonized protocol for the proficiency testing of analytical chemistry laboratories” (Pure Appl. Chem, Vol. 78, No. 1, pp-145-196, 2006).

Therefore, 10 portions of each test material were analyzed in duplicate. The test for sufficient homogeneity was performed for PCB 153. The test material showed sufficient homogeneity for this ring trial/proficiency test.

4.4 Determination of the assigned value

The following calculations were performed before the evaluation of the data:

- Calculation of the average of duplicate results for congeners (including LOQ) and WHO-TEQ
- Calculation of WHO-TEQ from reported congener results and comparison with reported TEQ
- Calculation of sum of six indicator PCBs from individual congeners

The determination of the assigned value was performed according to “The international harmonized protocol for the proficiency testing of analytical chemistry laboratories" (Pure Appl. Chem, Vol. 78, No. 1, pp.145-196, 2006) by estimating the assigned value as the consensus of all participants’ results (using only GC/MS and GC/ECD results)[1].

For the determination of the assigned value the median of all average values was calculated, values outside the range of ± 50 % of the median of all reported results removed and congeners excluded, if more than 15 % of the results were removed. Additionally

[1] Due to late reporting the results of the laboratories with the laboratory codes 1, 11, 14, 18, 33, 35, 39, 46 and 54 were not included in the calculation of the consensus value.

congeners were excluded, if the distribution of the results in a histogram was not symmetric, too broad or without one clear maximum (depending on the distribution of the data some congeners with more than 15 % of the results removed were included).

The Huber robust mean (= consensus value) was calculated for all individual PCDD/F and PCB congeners and sum parameters (WHO-PCDD/F-PCB-TEQ, WHO-PCDD/F-TEQ, WHO-PCB-TEQ, the sum of six indicator PCBs). For excluded congeners the robust mean was only calculated for comparison, not for evaluation.

- TEQ-based results were additionally re-calculated using the WHO-TEFs (1998) (Van den Berg et al., 1998. Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 106, 775 - 792). Only re-calculated TEQ-based results were used for evaluation of the results.
- For individual congeners the limit of quantification (LOQ) was taken for evaluation, if only the LOQ was reported or the reported level for the corresponding congener was below the LOQ.

The following table summarizes the total number of assigned values (for sum parameters and individual congeners) per sample including all reported results. The percentage of the total number of parameters is given in brackets.

	Sample 1	Sample 2	Sample 3	Sample 4
PCDD/F[1]	12 (60%)	20 (100%)	11 (55%)	8 (40%)
DL-PCB[2]	8 (53%)	15 (100%)	12 (80%)	13 (65%)
NDL-PCB[3]	7 (88%)	8 (100%)	7 (88%)	7 (88%)

[1] including WHO-PCDD/F-TEQ upper bound, middle bound, lower bound
[2] including WHO-PCB-TEQ upper bound, middle bound, lower bound
[3] including sum of six indicator PCBs upper bound, lower bound

In total for all four matrices for 128 of 178 parameters (= 74 %) an assigned value could be calculated.

4.5 Conversion of participants' results into z-scores

The z-scores were calculated on the basis of the average results using the robust mean of all values.

$$z = (x - x_a) / \sigma_p$$

x_a: assigned value

x: participants result

σ_p: target deviation (fitness-for-purpose-based "standard deviation for proficiency assessment")

For the results for WHO-PCDD/F-TEQ, WHO-PCB-TEQ and WHO-PCDD/F-PCB-TEQ, the target deviation σ_p is defined as 10 %, for the sum of six indicator PCBs (PCB #28, 52, 101, 138, 153, 180) as 15 % and for evaluated individual PCDD/F and PCB congeners as 20 %.

Acceptable z-scores for TEQ and congener results are between - 2 and + 2. Not acceptable are z-scores outside the range of - 3 to + 3.

The comparison of the z-scores for WHO-PCDD/F-TEQ, WHO-PCB-TEQ and sum of six indicator PCBs showed a rather symmetric distribution with more than 2/3 of z-scores within the range of +/- 2 z-scores for most sample/analyte combinations. Only for PCBs in sample 1 (mineral clay) considerably more z-scores outside the range of +/- 3 were observed, probably due to the extreme low concentrations of PCBs in this sample. The distribution for all participants is shown in figure 1.

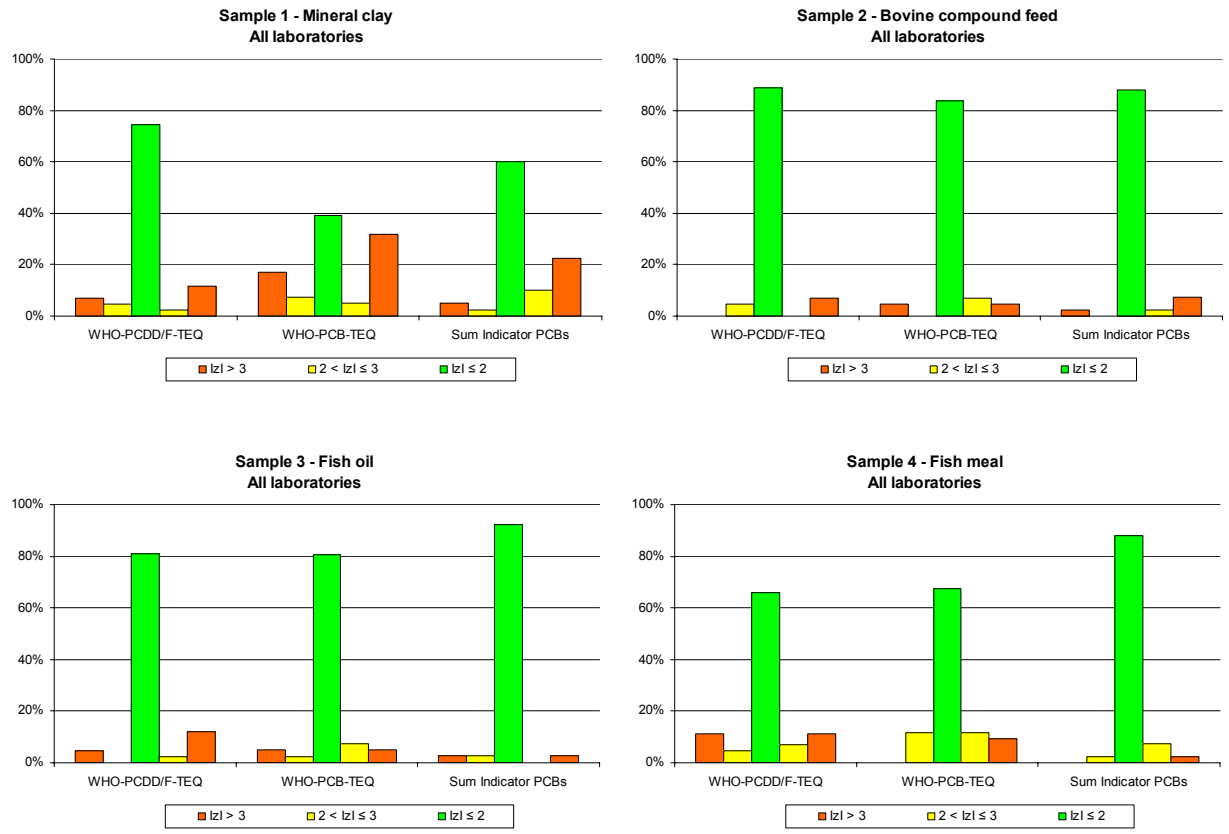


Figure 1: Distribution of z-scores for samples 1 - 4 (all participating laboratories)

The distribution of z-scores for WHO-PCDD/F-TEQ, WHO-PCB-TEQ and sum indicator PCBs were comparable for all participants (including NRLs) and NRLs only. Except for sample 1 (mineral clay) more than 70 % of z-scores were between -2 and +2. The distribution of z-scores of NRL results is shown in figure 2.

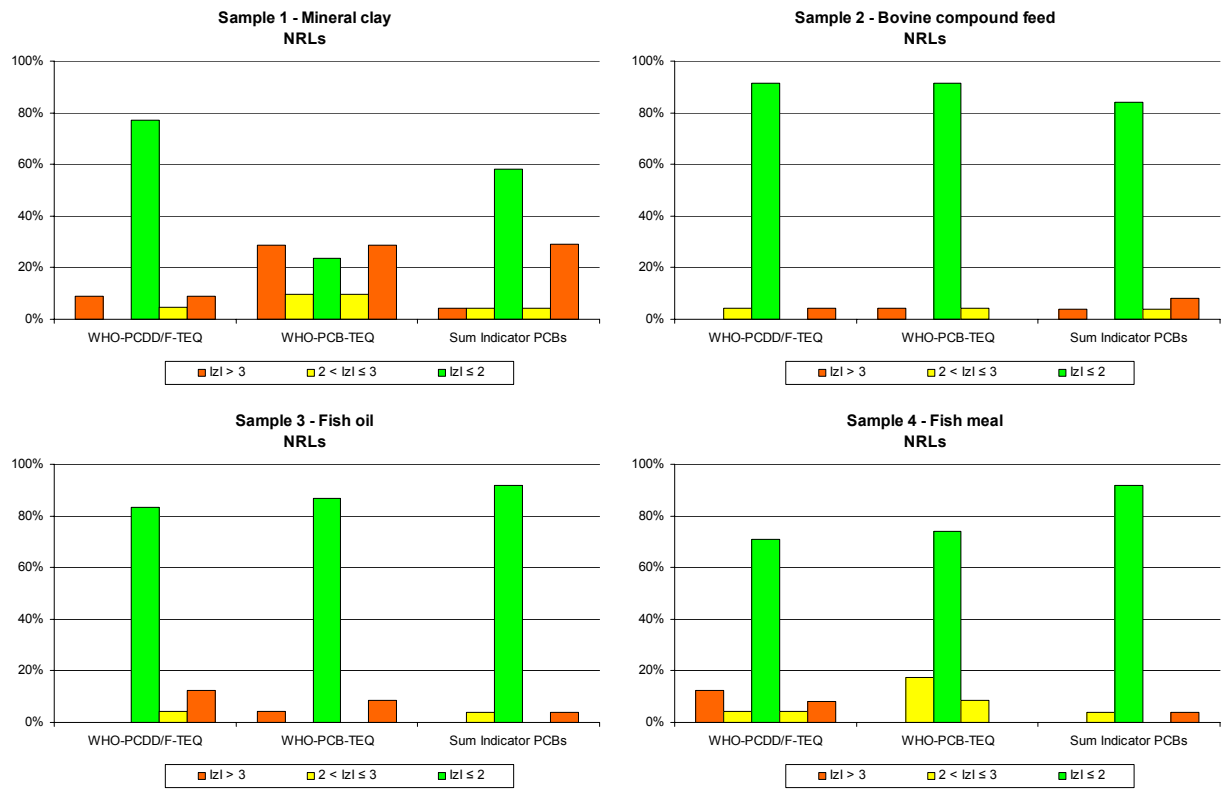


Figure 2: Distribution of z-scores for samples 1 - 4 (participating NRLs)

4.6 Congener pattern and contribution to WHO-TEQ

PCDD/F:

The samples 3 and 4 (fish oil and fish meal) showed comparable congener patterns and contribution of individual congeners to the WHO-TEQ, most abundant 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF. Sample 1 (mineral clay) showed a higher contribution of PCDD than PCDF to the WHO-PCDD/F-TEQ. Sample 2 (bovine compound feed) was spiked with PCDD/F standards at three different concentration levels for TCDD/F, PeCDD/F and HxCDD/F, and OCDD/F.

The congener patterns and contributions to the WHO-TEQ are shown in figure 3.

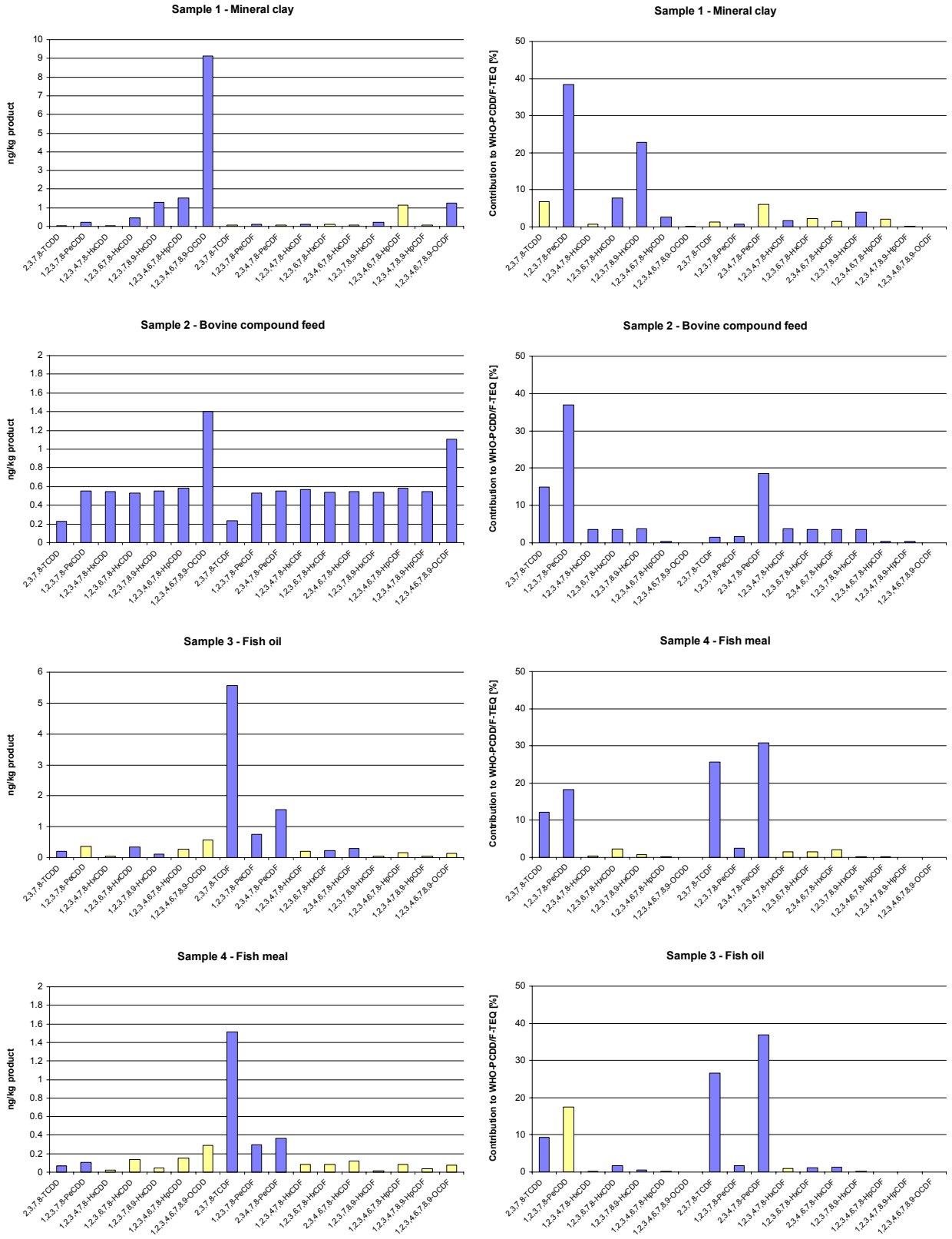
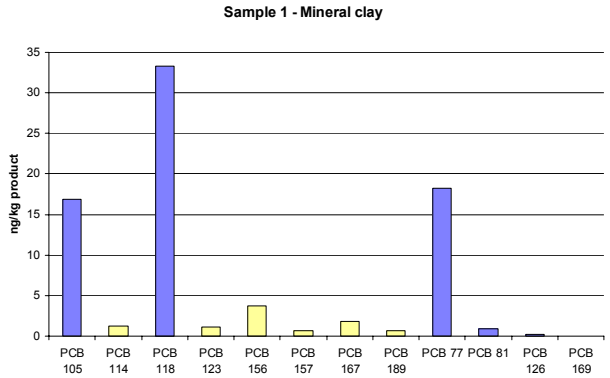
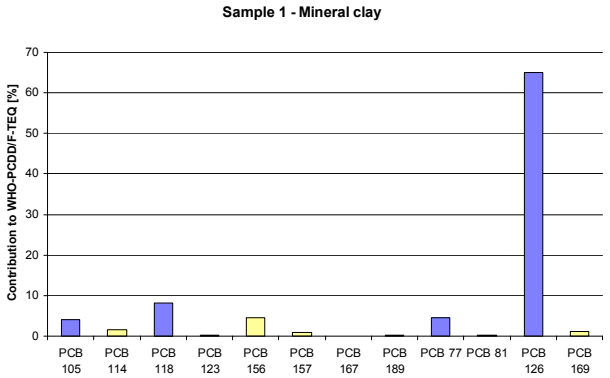


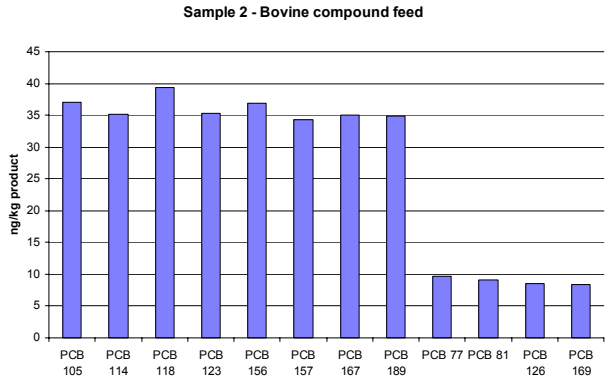
Figure 3: PCDD/F congener pattern and contribution of congeners to WHO-PCDD/F-TEQ in samples 1 - 4 (congeners with consensus value in blue, congeners with only robust mean for comparison in yellow)

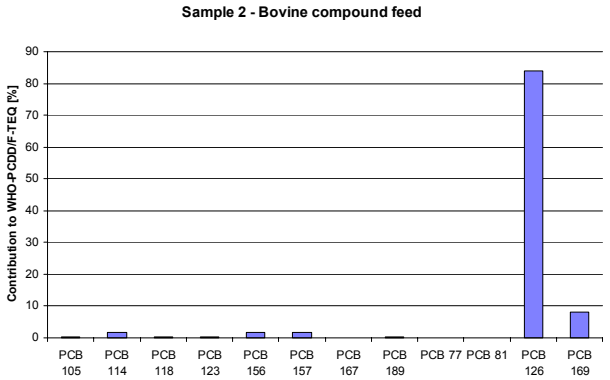
DL-PCB:

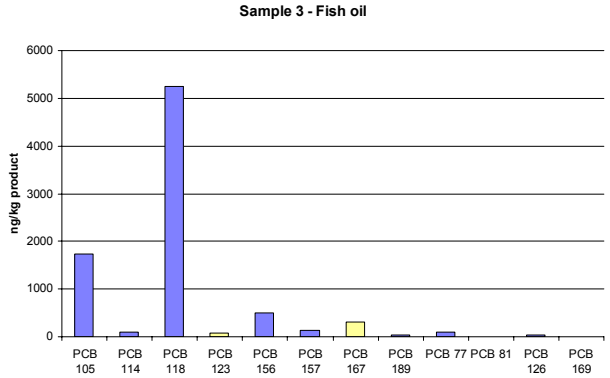
For samples 1, 3 and 4 PCB 105 and PCB 118 (in sample 1 also PCB 77) were the most abundant congeners. Sample 2 was spiked with DL-PCBs at two different concentrations levels (for mono- and non-ortho-PCBs). For all four samples PCB 126 contributed most to the WHO-PCB-TEQ. Congener pattern and contribution to the WHO-PCB-TEQ are shown in figure 4.

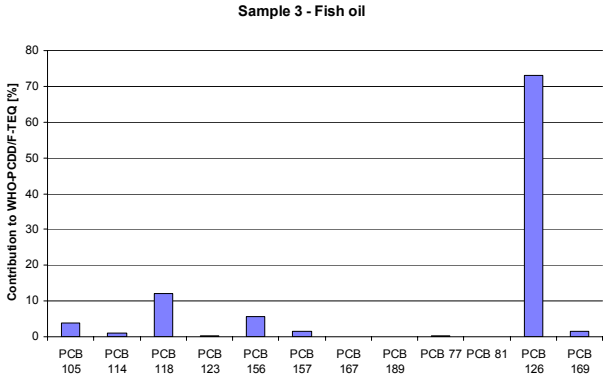












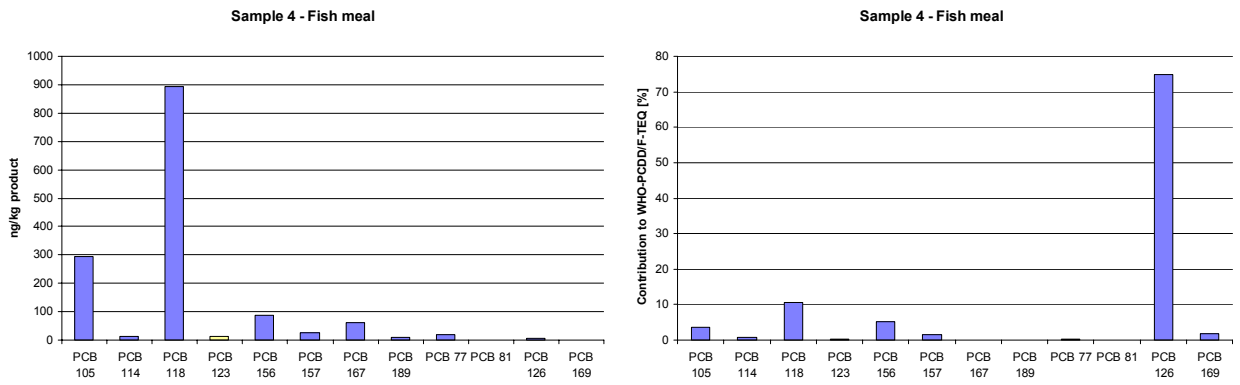


Figure 4: DL-PCB congener pattern (left) and contribution of congeners to WHO-PCB-TEQ in samples 1 - 4 (congeners with consensus value in blue, robust mean in yellow)

NDL-PCB:

Sample 3 and 4 had comparable congener patterns with PCB 138 and 153 as predominant congeners. Sample 1 (mineral clay) showed a considerably higher contribution of the lower chlorinated PCBs (#28 and 52) compared to the higher chlorinated congeners. Sample 2 was spiked with NDL-PCB congeners at the same concentration level. The NDL-PCB congener pattern is shown in figure 5.

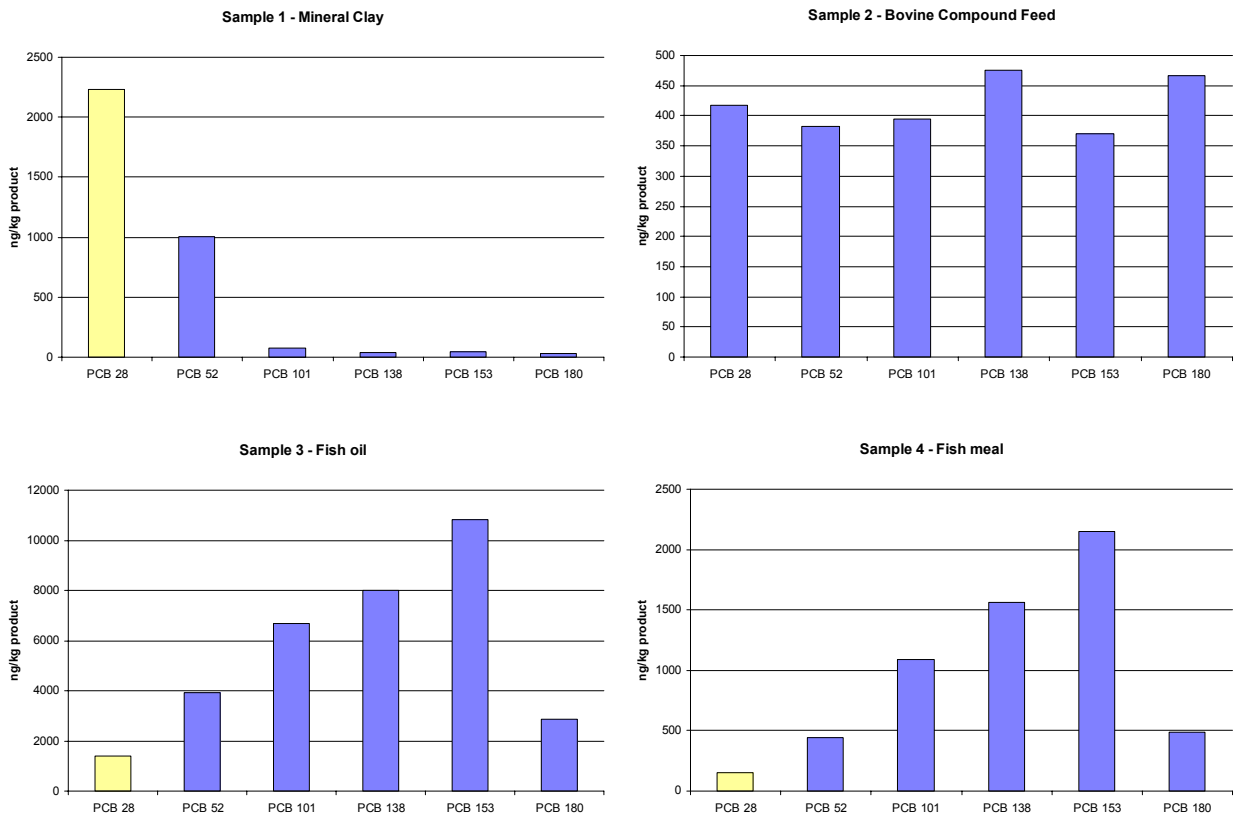


Figure 5: NDL-PCB congener pattern of samples 1-4 (congeners with consensus value in blue, robust mean in yellow)

4.7 Standard solution

The concentration of PCDD/F congeners in the standard solution ranged between 9 pg/µl (2,3,7,8-TCDD) and 65 pg/µl (OCDD). The consensus and target values for the PCDD/F standard solution are compared in table 1.

Table 1: Comparison of target and consensus concentrations of PCDD/F congeners in standard solution (provided by Wellington Laboratories)

Analyte	Levels pg/ul	Consensus value	Target value
2,3,7,8-TCDD		9.1	9.0
1,2,3,7,8-PeCDD		29.4	30.0
1,2,3,4,7,8-HxCDD		30.2	30.0
1,2,3,6,7,8-HxCDD		31.1	30.0
1,2,3,7,8,9-HxCDD		29.7	30.0
1,2,3,4,6,7,8-HpCDD		41.0	40.0
1,2,3,4,6,7,8,9-OCDD		64.8	60.0
2,3,7,8-TCDF		9.8	9.0
1,2,3,7,8-PeCDF		31.2	30.0
2,3,4,7,8-PeCDF		30.9	30.0
1,2,3,4,7,8-HxCDF		30.5	30.0
1,2,3,6,7,8-HxCDF		30.0	30.0
2,3,4,6,7,8-HxCDF		30.8	30.0
1,2,3,7,8,9-HxCDF		31.0	30.0
1,2,3,4,6,7,8-HpCDF		41.2	40.0
1,2,3,4,7,8,9-HpCDF		41.1	40.0
1,2,3,4,6,7,8,9-OCDF		64.6	60.0

The deviations of the consensus value of the target value (provided by Wellington Laboratories) were in all cases below 10 %. For 14 of the 17 congeners the consensus value was greater than the target value. For OCDD and 1,2,3,7,8-PeCDF a statistically significant difference between target value and consensus value could be observed (one-sided t-test, 99% significance). The deviations of the consensus values of the target values are shown in figure 6.

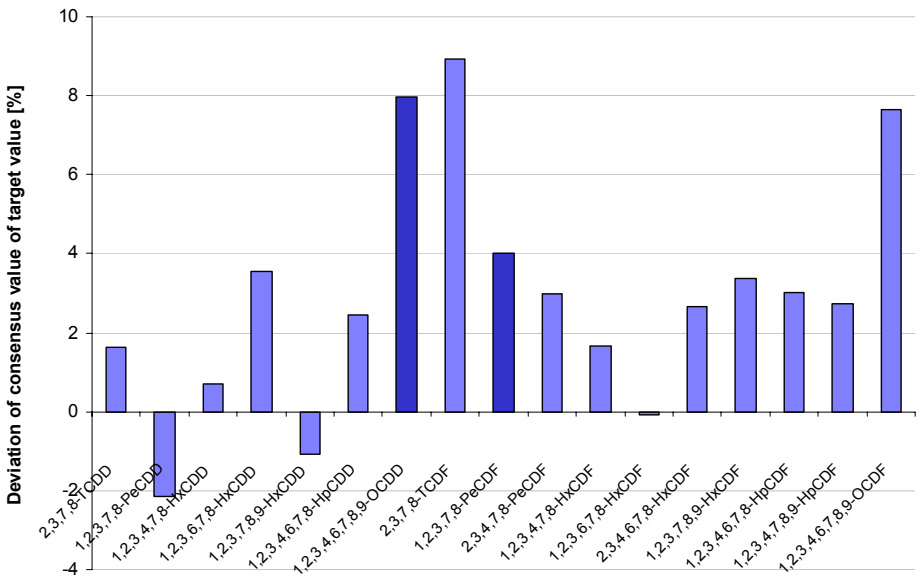


Figure 6: Deviations of consensus value of target value, statistically significant differences between target and consensus value for OCDD and 1,2,3,7,8-PeCDF are marked in dark blue.

94 % of laboratories results for the standard solution were inside the range of +/- 2 z-scores. The distribution of z-scores is shown in figure 7.

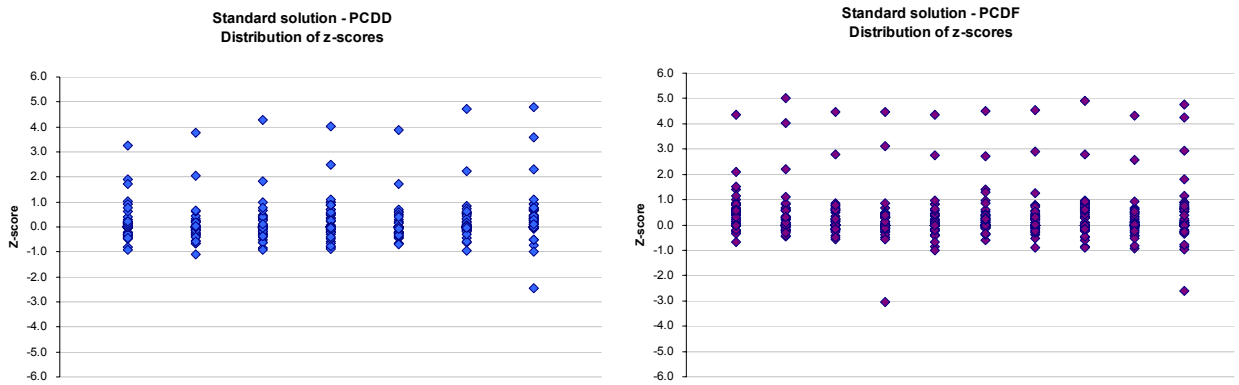


Figure 7: Distribution of z-scores for PCDD/F standard solution (all participants results)

4.8 Evaluation of results of bioanalytical screening methods

Three participating laboratories reporting results for Total-BEQ and partly also PCDD/F-BEQ and PCB-BEQ using DR-CALUX and XDS-CALUX. A further evaluation of the results of bioanalytical screening methods could not be performed due to the limited number of data sets available. The bioassay results and z-scores are shown in figure 8.

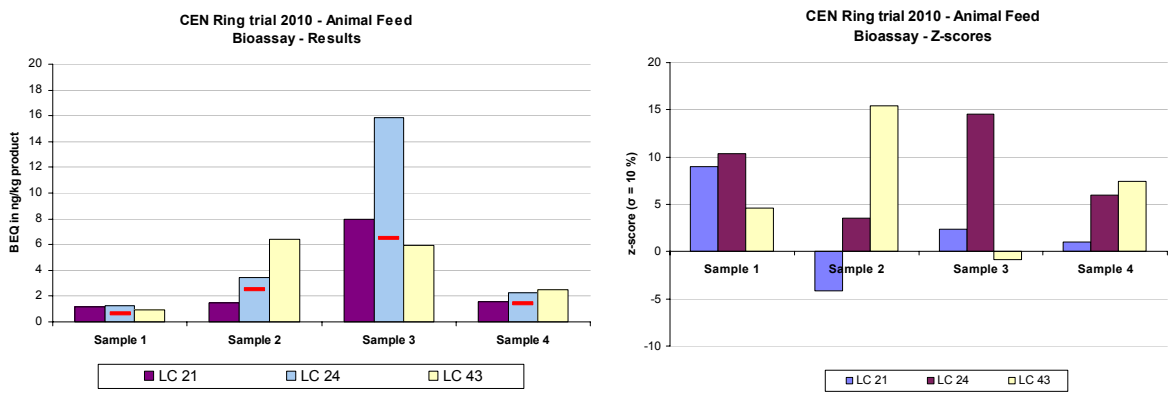


Figure 8: Comparison of bioassay results (Total-BEQ) with consensus value derived from GC/MS-analysis (marked in red) and z-scores of Total-BEQ

4.9 Evaluation of data of the CEN ring trail

32 results were reported using the CEN method, 28 results were included in the evaluation of the CEN ring trial. The following number of reported values were included in the CEN evaluation:

	Sample 1		Sample 2		Sample 3		Sample 4	
Modules	**PCDD/F**	**DL-PCB**	**PCDD/F**	**DL-PCB**	**PCDD/F**	**DL-PCB**	**PCDD/F**	**DL-PCB**
BI CI	11	11	11	11	11	11	11	11
BI CIII	3	2	3	2	2	1	3	2
BII CI	3	3	3	3	3	3	3	3
BII CII	2	2	2	2	2	2	2	2
BII CIII	7	7	8	8	8	8	8	8
BI	14	13	14	13	13	12	14	13
BII	13	13	14	14	14	14	14	14
BI + BII	27	26	28	27	27	26	28	27

4.9.1 Comparison of robust mean for different groups and modules

Besides the calculation of the assigned value for all reported results the robust mean values were calculated in the same way also for the different groups and modules and compared:

- Total CEN+NRL: Consensus values for all reported results
- Total CEN: Consensus values for results using the CEN method (all combinations of the available modules)
- CEN BI: Consensus values for results using the CEN method with extraction module BI and clean-up modules CI, CII or CIII
- CEN BII: Consensus values for results using the CEN method with extraction module BII and clean-up modules CI, CII or CIII
- CEN BI CI: Consensus values for results using the CEN method with modules BI and CI
- CEN BII CIII: Consensus values for results using the CEN method with modules BII and CIII
- Low resolution methods: Consensus value for results obtained by low resolution GC/MS or GC/ECD methods (only for PCB)

For 50 to 74 % of all evaluated congeners and sum parameters (in total 172 for four matrices) an assigned value could be calculated for the different modules.
The comparison of the consensus values calculated for the different groups and modules showed no significant differences for WHO-PCDD/F-TEQ and WHO-PCB-TEQ (see figure 9).

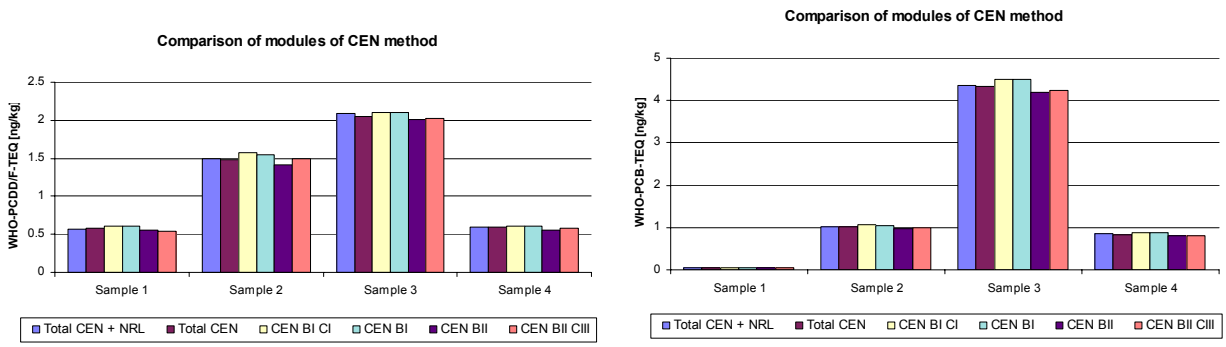


Figure 9: Comparison of consensus values of different modules of the CEN method, also with the consensus values of all participants (Total CEN+NRL, Total CEN, CEN BI, CEN BII, CEN BICI and CEN BIICIII)

4.9.2 Calculation of Horrat-Values

The Horwitz ratio (Horrat) is a normalized performance parameter indicating the acceptability of methods of analysis with respect to among-laboratory precision. It is the ratio of the observed relative standard deviation among laboratories calculated from the actual performance data to the corresponding predicted relative standard deviation calculated from the Horwitz equation (Journal of AOAC International, Vol. 89, No. 4, pp. 1095-1109, 2006). Due to the low concentration ranges in this ring trail (considerably below 100 ppb), instead of the predictions of the Horwitz function, the constant standard deviation of 22 % proposed by Thompson (Analyst, Vol. 125, pp. 385-386, 2000) was applied. The acceptable range for the Horrat value is < 2.0.

For the calculation of the Horrat values the data was checked for outliers using the Grubbs' test on a 95 % significance level. After the elimination of the outliers the coefficient of variation (CV) was calculated of the remaining results.

The Horrat values for different groups and modules were calculated using a standard deviation of 22 % (Thompson, Analyst, Vol. 125, pp. 385-386, 2000).

The assigned values (robust mean values) were compared with the Horrat values for the different groups and modules. Other congeners without a consensus value (only robust mean) were not included in the evaluation. For PCBs additionally the low resolution MS and ECD results were excluded from the evaluation of the results for Total CEN+NRL.

The number of evaluated congeners and sum parameters with a Horrat value > 2.0 are summarized in table 2.

Modules	Sample 1		Sample 2		Sample 3		Sample 4		Total	Percentage
	PCDD/F	PCB	PCDD/F	PCB	PCDD/F	PCB	PCDD/F	PCB		of all evaluated congeners
Total CEN + NRL	0	6	0	6	1	2	0	4	**19**	15
Total CEN + NRL (without LRMS)	0	1	0	0	1	0	0	0	**2**	2
Total CEN	1	0	0	0	0	0	1	2	**4**	3
BI	0	0	0	0	0	0	0	0	**0**	0
BII	4	1	0	0	0	0	0	0	**5**	6
BI CI	0	0	0	0	0	0	0	0	**0**	0
BII CIII	0	1	0	0	0	0	0	0	**1**	1

Table 2: Number of evaluated congeners and sum parameters with a Horrat value > 2.0 for all four matrices, the total number and the percentage of these parameters of the maximum number of evaluated congeners/sum parameters

5. Results

An overview of the PCDD/F and PCB results for the four feed samples and the evaluation of the results is given in the following tables and diagrams. Laboratories are coded according to the laboratory codes sent after registration.

Annex:

(Please click on the pdf-icons to open the annexes.)

1. CEN Ring trial 2010 - Assigned values	
2. CEN Ring trial 2010 - Z-score limits	
3. CEN Ring trial 2010 - Participants' results PCDD/F	
4. CEN Ring trial 2010 - Participants' results PCB	
5. CEN Ring trial 2010 - Participants' z-scores PCDD/F (table)	
6. CEN Ring trial 2010 - Participants' z-scores PCB (table)	
7. CEN Ring trial 2010 - Participants' z-scores PCDD/F (graph)	
8. CEN Ring trial 2010 - Participants' z-scores PCB (graph)	
9. CEN Ring trial 2010 - Participants' results, z-scores Bioassay	
10. CEN Ring trial 2010 - Consensus values of modules	
11. CEN Ring trial 2010 - Horrat values	