Mol. Nutr. Food Res. 2006, *50*, 945 – 957 DOI 10.1002/mnfr.200600061

The use of the DR CALUX® bioassay and indicator polychlorinated biphenyls for screening of elevated levels of dioxins and dioxin-like polychlorinated biphenyls in eel

Ron Hoogenboom[1], Toine Bovee[1], Win Traag[1], Ronald Hoogerbrugge[2], Bert Baumann[2], Liza Portier[1], Guido van de Weg[1] and Jaap de Vries[3]

[1] RIKILT, Institute of Food Safety, Wageningen, The Netherlands
[2] RIVM, Bilthoven, The Netherlands
[3] VWA, Zutphen, The Netherlands

The DR CALUX® bioassay is a very suitable screening method for dioxins and dioxin-like-PCBs in feed and food. This was, *e.g.* demonstrated in a survey in the Netherlands to control the dioxin levels in eel. The DR CALUX® assay, but also indicator polychlorinated biphenyls (PCB) were evaluated as a screening method. Based on the limit for polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDD/F) [at that time 8 pg toxic equivalents (TEQ)/g eel], and the relation between PCDD/F and dioxin-like-PCB, a decision limit of 30 pg TEQ/g eel was used for screening of 153 field samples. Suspected samples (21) and part of the higher contaminated negative samples (35) were analyzed by GC/MS for dioxins, non-ortho, mono-ortho and indicator PCB, revealing 13 samples exceeding the action limit of 30 pg TEQ/g eel. Only one sample slightly exceeded the dioxin level of 8 pg TEQ/g eel. The relatively low sensitivity for mono-ortho PCB was overcome by the use of reference samples, as shown by the correlation of 0.93 between GC/MS and CALUX determined total TEQ levels. The present data show that the DR CALUX® assay can be used for screening of total TEQ levels in eel. The use for dioxins only requires a safe, and therefore relatively low, decision limit. The indicator PCB also showed a good correlation with total TEQ levels, mainly due to the large contribution of the mono-ortho PCB at higher concentrations. The relation with dioxins was very poor and as such indicator PCB seem less suitable than the DR CALUX® assay for screening for dioxins only. The present study clearly shows that part of the wild eel samples contains high total TEQ levels and will exceed the future European Union limit of 12 pg TEQ/g eel for dioxins and dioxin-like PCB. Especially at high TEQ levels, dioxin-like PCB contribute most to the total TEQ. In practice, wild eel presents only a minor part of the eel consumed.

Keywords: Dioxins / DR CALUX® bioassay / Eel / Polychlorinated biphenyls

Received: April 24, 2006; accepted: April 28, 2006

1 Introduction

In order to deal with the emerging problems with dioxins [polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDD/F)] and dioxin-like polychlorinated biphenyls (PCB) in food at the end of the 1980s, The Dutch Ministry of Agriculture supported the development of a rapid screening method by a consortium of the University of Wageningen, the RIKILT-Institute of Food Safety and the Michigan State University. This resulted in the development of a bioassay which was called the Chemical Activated LUciferase Gene Expression assay or CALUX [1, 2], later changed into DR CALUX®, following the development of a similar assay, the ER-CALUX for estrogens. Both mouse (hepa1c1c7) and rat (H4IIE) hepatoma cells were transfected with a reporter gene construct such that the exposure to dioxin-like compounds resulted in the increased production of luciferase in a dose-dependant manner. Following exposure, the luciferase concentration could easily be measured by releasing the enzyme from the cells, followed by the addition of a reac-

Correspondence: Dr. Ron Hoogenboom, Safety and Health, RIKILT-Institute of Food Safety, P.O. Box 230, Wageningen, 6700 AE, Netherlands
E-mail: ron.hoogenboom@wur.nl
Fax:+31 317 417717

Abbreviations: EU, European Union; **PCB**, polychlorinated biphenyls; **PCDD/F**, polychlorinated dibenzo-*p*-dioxins/dibenzofurans; **TCDD**, tetrachlorodibenzo-*p*-dioxin; **TEF**, toxic equivalent factors; **TEQ**, toxic equivalents

tion mixture with luciferin and ATP and the quantification in a luminometer of the amount of light produced. Initial studies were performed with tetrachlorodibenzo-*p*-dioxin (TCDD) but other dioxins and dioxin-like PCB also showed a response proportionally to the toxic equivalent factors (TEF) values assigned by the WHO (reviewed in [3]). It should, however, be mentioned that in both cell lines the mono-ortho PCB showed a relatively poor response and that samples containing high levels of these compounds might be seriously underestimated by the assay. PCB 118, in terms of contribution to the TEQ level one of the more important mono-ortho congeners, showed, *e.g.* a relative potency (REP) around $1-5 \times 10^{-6}$ as compared to a TEF value of 1×10^{-4} [4, 5]. Following the development of the cells and the improvement of the actual cell assay by using, *e.g.* 48- or 96-well plates, relatively simple methods were developed for the extraction of environmental and food samples. In the case of milk fat, *e.g.* a clean up over an acid-silica column was shown to be enough to test the samples in the assay. This method not only removed the fat, but apparently also a number of less stable Ah-receptor agonists (like PAH and natural plant ingredients), which could have interfered with the use of the assay for detection of dioxins and dioxin-like PCB. This method was subsequently validated for milk fat [5], and allowed the official application of the assay at a period that dioxins in the food chain appeared to have been disappeared. This changed in 1998 when dioxins were discovered in citrus pulp from Brazil and especially in 1999 during the Belgian dioxin crisis [6]. In both incidents, RIKILT was allowed to apply the assay in order to select samples that required confirmation by high-resolution (HR)GC/HRMS and especially to release non-contaminated samples without further confirmation. This was also the case during more recent incidents like the contaminated bakery offal from Germany in 2003 [7], and the recent incident with kaolinic clay in potato peels used as animal feed. In 1999, the assay was brought under official accreditation by RIKILT and used for monitoring of feed and food. Only more recently, the use of the assay by other laboratories allowed international validation studies, like the study organized by JRC [3], and the studies within the European Union (EU) DIFFERENCE project [8]. Based on these and previous studies, the value of screening methods like DR CALUX® was recognized by the European Commission (EC) and performance criteria were included in the guidelines on analytical methods [9], on the basis of an internationally harmonized approach [10].

The JRC and DIFFERENCE studies showed that the DR CALUX® assay is very well capable of showing a dose-related response with both standards and incurred samples. However, the actual quantification of the levels in. *e.g.* feed and food samples, including the setting of decision limits to discriminate between negative and suspected samples, clearly requires fine-tuning. This is due to factors like background contamination of solvents, recovery during extraction and clean up, curve-fitting of calibration curves and the use of DMSO as a keeper during the evaporation of the organic solvents [3, 11], and in particular the deviations between the relative potencies (REP) of the different congeners and the TEF values. An approach to deal with these aspects is demonstrated by the use of the assay during a special survey in the Netherlands on the presence of dioxins and dioxin-like-PCB in eel. A study in fish sampled at the Dutch market, revealed that in particular wild eel may contain high levels of dioxins and dioxin-like PCB (Leonards, P. E. G., Lohman, M., de Wit, M. M., Booy, G. *et al*., 2000, RIVO-report C0034/00). The average level of PCDD/F and dioxin-like-PCB in five samples of wild fresh-water eel was 19.0 pg TEQ/g fish (range 8.7–36.7), as compared to 7.5 pg TEQ/g fish (range 3.9–10.7) for four samples of farmed eel. A risk evaluation resulted in a consumption advice to eat wild eel no more than once a week, followed by a temporary limit for PCDD/F only of 8 pg TEQ/g fish (more recently changed to 4 pg TEQ/g by the new EU-regulations). Although used in the risk evaluation, dioxin-like PCB were not included in this limit regarding a lack of validated and accreditated methods. In the study mentioned above (Leonards, P. E. G., Lohman, M., de Wit, M. M., Booy, G. *et al*., 2000, RIVO-report C0034/00) the average ratio between total TEQ and PCDD/F TEQ was 5.5, which was used to derive the virtual limit of 44 pg total TEQ/g described below.

Following the establishment of the official residue limit, a control program for eel was started. To allow the control of a large number of samples, it was decided to use the DR CALUX® bioassay for selection of samples which may exceed the limit for PCDD/F of 8 pg TEQ/g. The fact that the limit was set for dioxins only, posed some problems, in particular since non-ortho and mono-ortho dioxin-like PCB contributed much more to the total TEQ level. Furthermore, as mentioned above the DR CALUX® bioassay has been shown to be relatively insensitive for mono-ortho PCB [5].Moreover, it was decided to keep the amount of oil used for clean up constant, rather than the amount of fish. Therefore, fish oil extracted from the samples was tested and the total TEQ level estimated by comparison with a set of fish oil samples spiked at total TEQ-levels of 30, 60, 120 or 200 pg TEQ/g fat with a mixture of dioxins, non-ortho PCB and PCB 118 and 156. Relative contribution of dioxins, non-ortho and mono-ortho PCB to the total TEQ level in these samples was based on the study by Leonards *et al.* (Leonards, P. E. G., Lohman, M., de Wit, M. M., Booy, G. *et al*., 2000, RIVO-report C0034/00), being respectively 15, 40 and 45%. Oil levels were subsequently converted to fish levels and compared to a decision limit. Regarding preliminary uncertainties, a relatively low decision limit of 30 pg TEQ/g eel was chosen, as compared to the virtual limit of 44 pg TEQ/g. To evaluate the possible use of the

seven indicator PCB for screening, samples were also analyzed for these compounds.

2 Materials and methods

2.1 Materials

The p-GudLuc-transfected H4IIE cells were obtained from Wageningen University and are the same as those sold by Biodetection Systems (BDS, Amsterdam, The Netherlands). Dioxin and PCB standards were purchased from CN Schmidt (Amsterdam, The Netherlands).

2.2 Sampling, extraction of eel and determination of fat

Eel samples were collected at local stores, fish farms and fishermen and about 25 eels were mixed into one sample. Part of the homogenized samples were send to the RIKILT, where the oil was extracted and quantitatively determined using the method developed by Bligh and Dyer [12].

2.3 Preparation of reference and pool samples

A relatively clean sample of fish oil was selected. Dioxins and dioxin-like-PCB were removed by treatment with activated carbon as described previously for butter fat [5]. Part of the cleaned oil was subsequently spiked with a mixture of equal amounts of the 17 PCDD/F congeners, a mixture of the non-ortho PCB 77, 126 and 169, and stock solutions of PCB 118 and PCB 156. Final concentrations for dioxins, non-ortho PCB and PCB 118 and 156 were, respectively, 29, 81, 44 and 49 (total 203) pg TEQ/g oil. This oil was subsequently diluted to samples containing approximately 120, 60 and 30 pg TEQ/g. Levels were confirmed by HRGC/HRMS analysis.

Pool samples of eel fat were prepared from individual eel samples based on the DR CALUX determined TEQ levels using selection windows of 10–20 (I, n = 14), 20–30 (II, n = 22), 30–40 (III, n = 22), 40–50 (IV, n = 16), 50–60 (V, n = 24), 60–70 (VI, n = 11), 70–80 (VII, n = 11), 80–102 (VIII, n = 6), 120–180 (IX, n = 10), 180–230 (X, n = 9) and 230–260 pg TEQ/g fat (XI, n = 5), *i.e.* all 14 samples with a CALUX level between 10 and 20 pg TEQ/g fat were combined to pool sample number I, etc.

2.4 DR CALUX® bioassay

Samples of 0.5-g oil were purified on columns containing 10 g acid silica (33% H_2SO_4), as described by Bovee *et al.* [5]. In each series of up to 21 samples the blanc fish oil and the 4 references were included. Before total evaporation of the hexane/diethylether extract in a SpeedVac, 200 µL of DMSO was added as a keeper. An aliquot of 20 µL was added to 2 mL incubation medium and 250 µL added in triplicate to three different wells of a 48-well plate containing p-GudLuc-transfected H4IIE cells. After 24 h, the medium was aspirated, the cells washed and lysed and an aliquot used for determining the luciferase content in a Luminoskan (Labsystems). Total TEQ sample in the oil was estimated from a calibration curve of the reference samples whose response was fitted with an exponential curve fit. The level in oil was subsequently translated to the level in eel using the fraction of oil in the fish samples.

2.5 Dioxin and dioxin-like PCB analysis

Levels of dioxins, non-ortho and mono-ortho PCB were determined by HRGC/MS, basically as described by Tuinstra *et al.* [13]. Prior to extraction, ^{13}C-labeled dioxins and dioxin-like PCB were added to the eel fat samples. Separation between dioxins and fat was carried out using gel permeation chromatography. The system consisted of an HPLC pump (Gilson, model 305), an autosampler (Gilson, model 231) equipped to inject 12.5 mL of sample solution, and a fraction collector (Gilson, model 202) adapted to collect 300-mL fractions using 500-mL glass collection flasks. The glass GPC column (Spectrum) (60 cm × 2.5 cm) was packed with Biobeads SX 3. After an additional clean up with activated Al_2O_3, separation between planar compounds (dioxins) and non-planar compounds, *e.g.* chlorobiphenyls, was carried out with porous graphitized carbon. This resulted in a fraction containing the PCDD/F and non-ortho PCB, and a second fraction containing the mono-ortho PCB. The alumina (basic) clean up was performed with an automatic sample preparation system using solid phase extraction columns (ASPEC, Gilson). The columns were packed with 1.00 g deactivated Al_2O_3 (7% water) shortly before use. Porous graphitized carbon clean up was performed using an HPLC system consisting of an HPLC pump (Gilson model 205), a column switching device (Gilson, valvemate), a solvent switching device (Gilson, valvemate), an autosampler (Gilson model 231), equipped with a 5.0-mL loop and a fraction collector (Gilson model 202) adapted to collect 100-mL fraction. The used column is Hypercarb (100 × 4.6 mm) (Shandon). The final extracts were concentrated to 10 µL and analyzed with HRGC/MS (Autospec, Micromass). The MS method to determine the tetra through octa dioxins is based on United States Environmental Protection Agency protocols. Included in the analysis is a standard QA program, *e.g.* determination of recovery of internal standards, accuracy of spiked samples and blanks. Absolute levels were transfered to TEQ levels using the TEF values described by van den Berg *et al.* [14].

2.6 Indicator PCB analysis

Levels of the seven indicator PCB were determined by a newly developed GC/MS method: Fish oil is dissolved in iso-octane, PCB are isolated from the oil by straight phase HPLC. The PCB fraction is transferred on-line to a GC-MS equipped with a large volume injector and an early vapour exit.

3 Results and discussion

3.1 General

At the beginning of this study, the major question was whether it is possible to use the DR CALUX® assay or the indicator PCB as a screening method for selection of eel samples with high levels of dioxins and dioxin-like PCB, and more in particular with dioxin levels above the tolerance limit. This is of major importance regarding the costs, duration and relatively low sample-throughput of the HRGC/MS reference method. Preliminary data had shown that the test is suitable for estimating levels of dioxins and non-dioxin-like PCB in fish products [6], and the test was later also validated for fish by Tsutsumi *et al.* [15]. However, comparitive data of screening and confirmatory methods on large sets of field samples are still scarce. Furthermore, in particular in eel the mono-ortho PCB contribute significantly to the total TEQ level (Leonards, P. E. G., Lohman, M., de Wit, M. M., Booy, G. *et al.*, 2000, RIVO-report C0034/00), and previous studies have shown that the DR CALUX® assay is relatively insensitive to compounds with lower TEF values like the mono-ortho PCB [5]. The relative potency of PCB 105, 118 and 156 in the test was calculated to be respectively 0.02×10^{-4}, 0.05×10^{-4} and 0.4×10^{-4} as compared to WHO TEF values of 1×10^{-4}, 1×10^{-4} and 5×10^{-4}. Therefore, a specific test strategy was developed that should reliably select suspected eel samples without too many false-positives. The strategy was based on the use of reference samples in combination with a safe action limit.

3.2 Validation study

Prior to the analysis of eel samples, a validation study was carried out with samples of fish oil, spiked at 30, 60, 120 and 200 pg TEQ/g oil, with a relative contribution of dioxins, non-ortho and mono-ortho PCB to the TEQ level of, respectively 15, 40 and 45%. In this validation study, the levels were determined based on the TCDD calibration curve, which is the procedure recommended by the companies selling the assay. Testing of the samples in sixfold in one extraction and test series, and calculations based on the TCDD calibration curve followed by correction for the blank, revealed TEQ-levels of, respectively, 13, 37, 81 and 151 pg TEQ/g with a %CV of, respectively, 17, 14, 17 and 10% (Table 1). When tested in single in five independent extraction and test series, the %CV was, respectively, 26, 19, 19 and 14% (Table 2). This validation study confirmed that under the current conditions the assay could easily detect fat levels of 30 pg TEQ/g fat. At fat contents of, *e. g.* 20 or 10%, this would correspond to fish levels of, respectively, 6 or 3 pg TEQ/g fish, being around the EU-limit of 4 pg TEQ/g fish. Variation coefficients for repeatability were within the requirement of 30% set by the EU [9]. However, it is evident that the use of a TCDD calibration curve resulted in a clear underestimation of the TEQ levels, and as such could result in erroneous decisions and false-negative results. Therefore, it was decided to use the spiked fish oil samples to estimate the total TEQ levels in eel samples. Other criteria presented in the EC guidelines [9], like the lower than 15% variability between the triplicate wells for one sample extract, the inclusion of reference samples, and

Table 1. Repeatability of the DR CALUX® determined TEQ-levels in spiked fish oil samples, measured in sixfold. Cleaned fish oil was spiked with dioxins, non-ortho and mono-ortho PCB, contributing respectively for 15, 40 and 45% to the total TEQ level. Levels were calculated by comparison with a TCDD calibration curve. Spiked concentrations were checked by the GC/MS reference method

Concentration (pg TEQ/g oil)	DR CALUX® determined level (pg TCDDeq/g oil)				Recovery (%)
	Mean	SD	CV (%)	Corrected for blank	
0	7	1	20	0	
30	20	3	17	13	43
60	44	6	14	37	62
120	87	15	17	81	68
200	157	16	10	151	76

Table 2. DR CALUX® determined TEQ levels in spiked fish oil samples as determined in five different test series. Levels were determined based on the TCDD calibration curve. Spiked concentrations were checked by the GC/MS reference method

Series	DR CALUX® determined level (pg TCDDeq/g oil)				
	0	30	60	120	200
1	7	20	44	87	157
2	5	20	38	86	216
3	5	30	57	114	174
4	7	17	55	131	207
5	7	17	39	118	170
Mean ± SD	6 ± 1	21 ± 5	47 ± 9	107 ± 20	185 ± 25
%CV	24	26	19	19	14

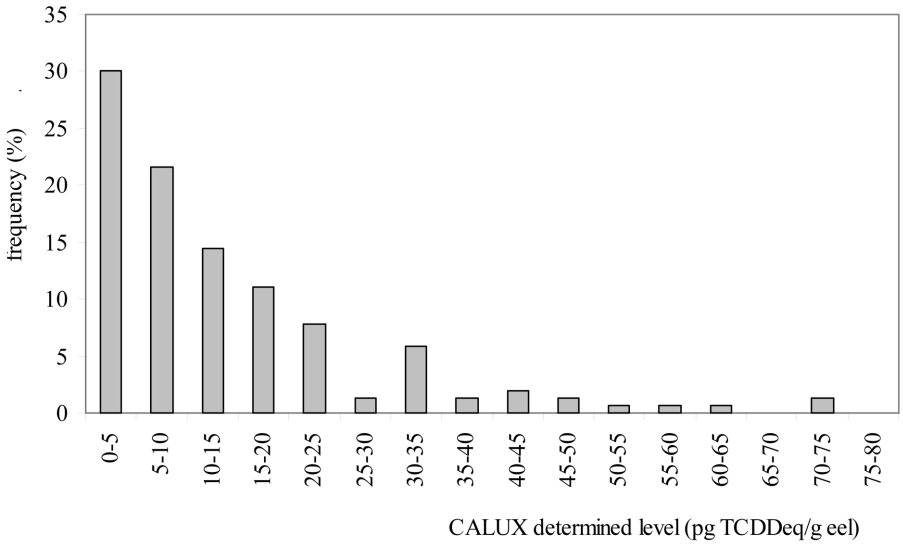

Figure 1. DR CALUX® estimated total TEQ levels in 153 eel samples. The decision limit used was 30 pg TEQ/g, as compared to a virtual residue limit of 44 pg TEQ/g.

the GC/MS confirmation of suspected samples were all met in the current project.

3.3 Screening of individual samples by DR CALUX®

The assay was subsequently used for screening of 153 field samples of eel, collected by the Food Inspection Service. Levels were estimated by comparison of the response of the sample extract with that of the spiked fish oil samples and converted to product base using the fat content of the individual eel samples. This resulted in the distribution curve of total TEQ levels as shown in Fig. 1. The average DR CALUX® determined content was 14 pg TEQ/g eel, the median level 10 pg TEQ/g eel. Twenty-one samples (14%) were estimated to contain total TEQ levels above the decision limit of 30 pg TEQ/g fish. Eight (5%) of these samples even exceeded the virtual limit of 44 pg TEQ/g, indicating dioxin levels above the former Dutch limit for dioxins of 8 pg TEQ/g eel. From the 35 samples of farmed eel, two (6%) exceeded the decision limit in this study of 30 pg TEQ/g fish, as compared to 19 of the 118 samples of wild eel (including all 8 samples above 44 pg TEQ/g eel). The average DR CALUX® determined levels in farmed and wild eel were estimated to be 15 and 14 pg TEQ/g eel, respectively.

3.4 GC/MS analysis of pooled eel fat samples

As an additional step to control the performance of the bioassay for this particular matrix, the 153 eel fat samples were pooled, based on the DR CALUX® determined fat levels. This resulted in 11 fat samples with increasing DR CALUX® based total TEQ levels. The latter was confirmed by re-analysis of the pooled samples with both the bioassay and with the HRGC/MS reference method (Table 3). Table 4 shows the individual levels of the different dioxin congeners, as well as the non-ortho, mono-ortho and indicator PCB in these pooled samples as determined by GC/MS. The TEQ levels of dioxins, non-ortho and mono-otho PCB are shown in Table 3. Based on these levels, the number of samples in each pool, and the average fraction of fat in each pool, it can be calculated that the average eel sample contained 11 pg TEQ/g eel, with individual contributions for dioxins, non-ortho PCB and mono-ortho PCB of 2.6, 4.2 and 4.2 pg TEQ/g, respectively. In terms of relative importance, 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDD and 2,3,7,8-TCDD contributed most to the dioxin-related TEQ-level, with an increasing role for the latter compound at higher concentrations (Table 4). This was also reflected in the ratio PCDD/PCDF, showing an increase at higher TEQ levels (Table 3). In the case of the non-ortho PCB, PCB 126 was the only relevant congener, in the case of the mono-ortho PCB, the TEQ level was primarily determined by PCB 118 and 156, with a minor role for PCB 105 and 157. As a class, the relative contribution of the dioxins decreased at higher TEQ levels from an initial 31 to 15%. As a result the total TEQ/dioxin TEQ ratio increased from 3.2 to 6. 7.

Figure 2A shows a comparison of GC/MS and DR CALUX® determined TEQ levels in the pooled samples. In the case of the DR CALUX® assay, the levels were either calculated from a TCDD calibration curve (lower graph) or from the spiked fish oil samples. This demonstrates that the latter results in an overestimation of the levels, whereas the TCDD-based calculation again results in a clear underesti-

Table 3. Levels of dioxins, non-ortho and mono-ortho PCB in pooled eel fat samples as determined by GC/MS

Sample	DR CALUX®[a] (pg TCDDeq/g)	HRGC/HRMS						GC/MS Indicator PCB (ng/g)
		PCDD/PCDF	Non-ortho PCB	Mono-ortho PCB	Sum	PCDD/PCDF (%)	PCDD/PCDF[b] ratio	
		(pg TEQ/g)						
I	18	3.3	4.3	3.1	10.6	31	1.8	124
II	21	4.1	6.1	3.5	13.7	30	1.3	151
III	35	5.3	8.9	5.9	20.1	26	1.1	243
IV	46	9.2	11.4	9.3	29.9	31	1.8	430
V	58	12.0	12.8	6.8	31.6	38	1.7	306
VI	69	14.1	15.5	10.1	39.7	36	1.5	423
VII	74	12.0	19.0	14.7	45.7	26	1.1	692
VIII	111	15.5	23.9	28.2	67.6	23	1.9	1585
IX	150	18.7	39.9	51.2	109.8	17	2.3	2716
X	201	24.9	58.7	93.7	177.3	14	2.5	5199
XI	216	27.3	65.1	92.7	185.1	15	2.9	5346

a) DR CALUX® determined TEQ-level based on fish oil reference samples.
b) Ratio of dioxins and furans based on their contribution to the TEQ level.

mation, which can be explained by the relatively low response of the mono-ortho PCB. The data show that the problem can be overcome by the use of a set of fish oil samples spiked with dioxins, non-ortho PCB and mono-ortho PCB, assuming that a slight overestimation is more acceptable for a screening assay than an underestimation. Furthermore, it should be stressed that such an overestimation may be caused by the use of the spiked samples but also by other Ah-receptor agonists present in the samples.

Tables 3 and 4 include the levels of the seven indicator PCB in the pooled samples, as determined by GC/MS. The average level of indicator PCB was calculated to be 216 ng/g eel, with PCB 153 and 138 being the most important congeners. A comparison between the GC/MS determined levels of total TEQ and either the indicator PCB, or PCB 153 is shown in Fig. 2B, showing a good correlation between the two parameters, supporting previous data by De Boer *et al.* [16]. This indicates that not only the DR CALUX® assay but also indicator PCB are a good method for estimating the total TEQ level in eel samples. This should be confirmed by results from individual samples.

3.5 GC/MS analysis of individual samples

Twenty-one eel samples exceeding the decision limit of 30 pg TEQ/g eel were analyzed by GC/MS, as well as 12 of the 14 samples in the range 20–30 and 6 in the range 8–20 pg TEQ/g eel. As shown in Fig. 3A, this revealed a good correlation (r^2 = 0.93) between DR CALUX® estimated levels and total GC/MS determined TEQ-levels of dioxins and dioxin-like PCB. Levels estimated by DR CALUX® and based on the use of the reference samples, were always equal or higher than those determined by GC/MS. All 18 samples estimated to be lower than the decision limit, were confirmed to be below 30 pg TEQ/g fish by GC/MS. In the set of 21 samples higher than this decision limit, 13 (65%) were confirmed to contain levels above 30 pg TEQ/g eel. Five (63%) of the eight samples exceeding the virtual limit of 44 pg TEQ/g eel were confirmed to do so by GC/MS. The two farmed eel samples showed total TEQ levels of 17 and 39 pg TEQ/g eel, the 19 samples of wild eel showed levels between 22 and 57 pg TEQ/g eel.

As shown in Fig. 3B, the relation between the DR CALUX® response and dioxins for individual samples was relatively weak (r^2 = 0.31). Only one sample actually exceeded the former Dutch limit of 8 pg TEQ/g eel for dioxins, showing a total GC/MS determined level of 55 pg TEQ/g eel. In general, the correlation between dioxins and total TEQ, as determined by GC/MS is weak and PCB were far more important than dioxins especially at higher total TEQ levels. The relation between the GC/MS determined total TEQ level and the ratio total TEQ/dioxin TEQ in these individual samples is shown in Fig. 4, demonstrating an increased ratio at higher TEQ levels (r^2 = 0.73). All samples with a level less than 20 pg TEQ/g eel showed a ratio below the 5.5 used for evaluating the consequences of the dioxin limit of 8 pg TEQ/g eel. All samples exceeding 30 pg TEQ/g eel showed a higher ratio. As an example, the five samples exceeding the virtual limit of 44 pg TEQ/g eel showed ratios of 6.8, 8.7, 9.6, 14.8 and 16. 3. Overall, the data show that the DR CALUX® assay, using the approach with the spiked reference samples, is very suitable for predicting the total TEQ level and for selection of samples requiring GC/MS analysis.

Table 4. Concentrations of the different dioxin (pg/g), non-ortho (pg/g) and mono-ortho (ng/g), and indicator (ng/g) PCB congeners in pooled samples of eel fat. Data are expressed per gram of fat

	Pooled sample										
Congener	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Dioxins											
2,3,7,8-TCDF	0.24	0.53	0.71	0.65	1.46	1.28	0.98	0.78	0.79	1.03	0.62
1,2,3,7,8-PeCDF	a)	a)	a)	a)	a)	a)	a)	1.12	a)	6.77	a)
2,3,4,7,8-PeCDF	**2.02**[c)]	**3.10**	**4.44**	**5.91**	**8.12**	**10.40**	**10.20**	**9.12**	**9.68**	**10.70**	**11.60**
1,2,3,4,7,8-HxCDF	0.74	0.83	0.90	1.39	1.07	1.25	1.86	3.51	4.35	7.09	5.79
1,2,3,6,7,8-HxCDF	0.54	0.49	0.71	0.80	0.85	1.06	1.18	1.57	1.74	2.46	2.20
2,3,4,6,7,8-HxCDF	b)	0.58	0.76	0.92	0.90	1.21	1.30	1.66	1.86	2.58	2.43
1,2,3,7,8,9-HxCDF	0.15	0.16	0.15	0.13	0.12	0.15	<0.10	0.18	0.22	0.26	<0.10
1,2,3,4,6,7,8-HpCDF	0.87	0.63	1.05	1.26	0.93	0.86	1.13	2.13	2.72	2.98	2.71
1,2,3,4,7,8,9-HpCDF	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	0.26	0.34	0.43
OCDF	1.52	1.23	2.28	9.81	2.84	a)	1.15	1.31	2.90	2.39	4.76
2,3,7,8-TCDD	**0.41**	**0.58**	**1.13**	**1.92**	**1.87**	**2.21**	**3.61**	**6.61**	**7.65**	**10.30**	**9.97**
1,2,3,7,8-PeCDD	**1.19**	**1.26**	**1.30**	**3.58**	**5.24**	**5.65**	**2.32**	**2.94**	**4.61**	**6.46**	**9.42**
1,2,3,4,7,8-HxCDD	0.83	0.69	0.55	0.70	0.64	1.01	0.69	1.49	2.11	2.91	3.49
1,2,3,6,7,8-HxCDD	3.22	2.84	1.88	2.17	2.19	3.34	2.45	3.60	4.66	5.90	5.59
1,2,3,7,8,9-HxCDD	0.32	0.38	0.29	0.30	0.46	0.98	0.48	b)	b)	1.46	b)
1,2,3,4,6,7,8-HpCDD	3.88	3.07	2.36	4.26	3.13	5.00	2.26	3.30	4.06	4.26	5.41
OCDD	41.40	18.10	19.30	59.4	26.50	24.70	16.60	b)	33.5	20.60	44.30
non-ortho											
PCBs	10.2	20.7	37.0	36.0	45.3	74.6	56.5	53.7	65.2	93.6	96.6
PCB 77	20.5	12.3	7.4	9.6	6.3	10.1	7.1	8.4	7.8	10.6	8.3
PCB 81	**41.5**	**59.4**	**87.3**	**112.0**	**126.0**	**152.0**	**186.0**	**235.0**	**391.0**	**574.0**	**637.0**
PCB 126	11.5	14.3	16.7	21.5	23.6	28.0	36.1	45.7	80.8	128.0	131.0
PCB 169											
Mono-ortho PCBs											
PCB 105	**3.8**	**4.5**	**7.2**	**10.5**	**8.2**	**12.2**	**16.1**	26.0	50.0	88.5	87.7
PCB 114	0.3	0.3	0.4	0.6	0.4	0.6	0.8	1.1	2.4	4.1	4.7
PCB 118	**13.9**	**16.0**	**27.8**	**44.8**	**31.5**	**47.7**	**71.1**	**137.0**	**238.0**	**416.0**	**406.0**
PCB 123	a)	a)	a)	a)	a)	a)	a)	a)	a)	a)	a)
PCB 156	**1.9**	**2.1**	**3.6**	**5.7**	**4.1**	**6.2**	**9.1**	**18.6**	**35.0**	**68.2**	**68.4**
PCB 157	0.4	0.4	0.7	1.0	0.9	1.2	1.8	3.3	6.0	11.5	10.9
PCB 167	1.0	1.2	2.0	3.2	2.6	3.7	5.9	12.1	22.4	40.3	38.6
PCB 189	0.2	0.2	0.4	0.7	0.5	0.7	1.1	2.5	4.4	9.2	9.3
Indicator PCBs											
PCB 28	3.3	3.9	9.1	12.2	7.4	8.3	14.3	23.1	37.3	50.5	50.8
PCB 52	9.7	10.1	12.4	23.3	11.7	12.4	32.2	76.3	137.7	230.2	236.8
PCB 101	8.1	10.5	22.1	42.8	29.2	42.3	62.6	161.6	261.8	488.4	489.5
PCB 118	15.3	17.0	28.2	47.9	34.0	50.2	75.5	151.8	258.2	457.9	446.9
PCB 138	39.4	45.1	76.4	130.8	96.9	134.6	211.9	471.6	837.6	1619.5	1690.2
PCB 153	35.9	42.4	73.9	131.9	99.9	134.1	228.8	531.2	885.5	1698.7	1732.6
PCB 180	12.5	21.7	21.4	41.0	26.7	41.1	66.6	169.6	298.0	654.3	699.6

a) Interference.
b) Incorrect IR.
c) Compounds marked in bold contribute most to the total TEQ level within their class (>10%).

3.6 Indicator PCB

An alternative to the DR CALUX® assay could be the use of indicator PCB, although the succes of this test depends even more on a possible correlation between PCB and dioxin-like compounds, or dioxins only. Current limits for indicator PCB in eel in the Netherlands are 500, 200, 400, 400, 500, 500 and 600 ng/g eel for PCB 28, 53, 101, 118, 138, 153 and 180, respectively. Analysis of indicator PCB in the 153 samples showed 2 samples exceeding the limit for PCB 153.

Regarding the high contribution (85%) of dioxin-like PCB to the total TEQ level in highly contaminated samples, a good correlation is expected. This is confirmed by the data shown in Fig. 5A, demonstrating a good relation between the levels of the seven indicator PCB and the total TEQ levels in 39 of the eel samples, with a correlation coefficient

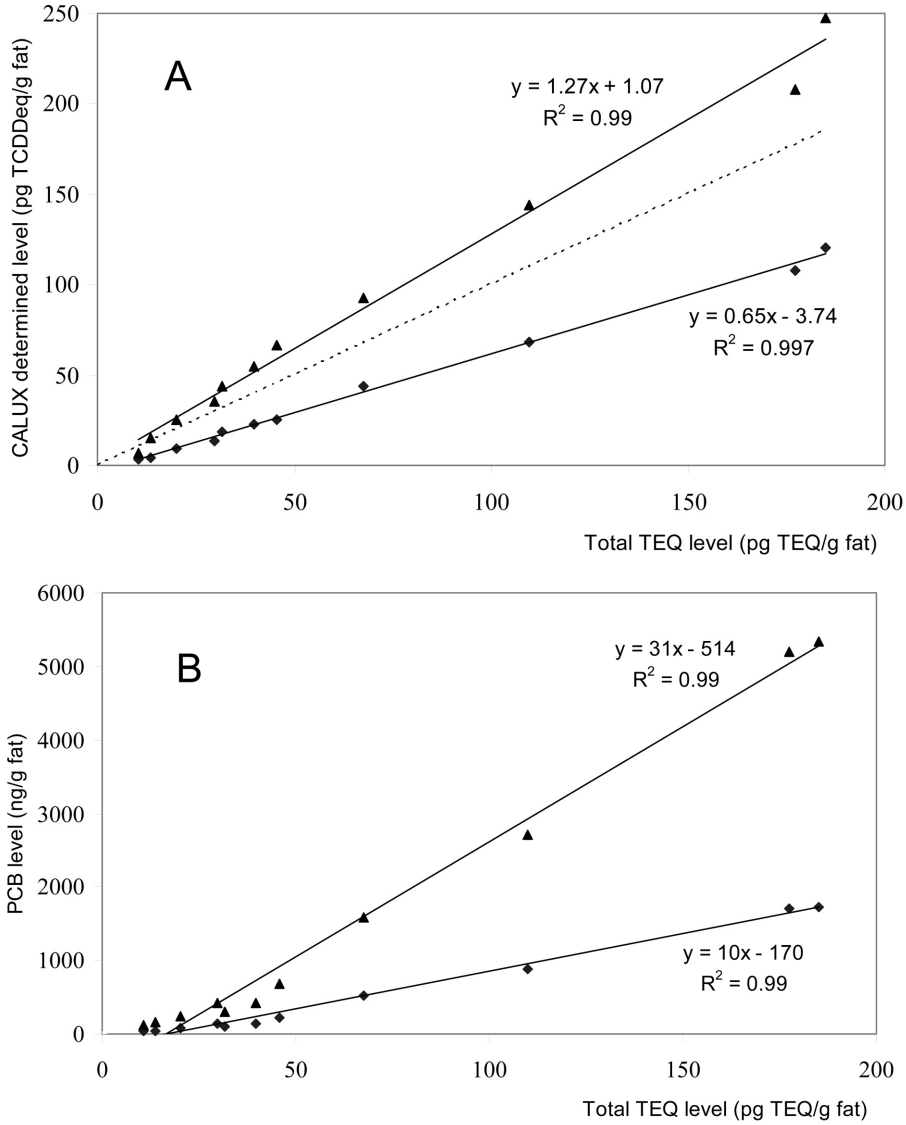

Figure 2. Comparison between total TEQ levels as estimated by DR CALUX® (A) or indicator PCB (B) with GC/MS determined total TEQ in 11 samples of pooled eel fat. Solid lines represent linear regression curves. Panel (A) includes DR CALUX® determined levels based on either the reference samples (triangles) or the TCDD calibration curve (diamonds). Panel (B) includes both the sum of indicator PCB (triangles) and PCB 153 (diamonds).

of 0.91. Based on this still limited dataset, a decision limit around 1000 ng/g for the indicator PCB appeared to be suitable for selection of samples exceeding the virtual limit of 44 pg TEQ/g eel. The future limit of 12 pg TEQ/g eel for the sum of dioxins and dioxin-like-PCB would of course require a much lower decision limit, being in the range of 100–300 ng/g eel. However, this requires the analysis of more samples around these much lower levels.

Although the results from the pool samples suggest a rather good correlation between dioxins and indicator PCB (Table 3), the results obtained with individual samples show the contrary (Fig. 5B; $r^2 = 0.18$). Of the five samples with dioxin levels higher than 6 pg TEQ/g eel, three had PCB levels lower than 500 ng/g eel. The correlation coefficient of the relation between indicator PCB and mono-ortho, or non-ortho PCB was much better, being, respectively, 0.95 and 0.72 (data not shown). It can be concluded that indicator PCB are suitable for screening for total TEQ levels in eel, but not for dioxins only.

3.7 Evaluation of the test strategy

Screening methods, like DR CALUX® assay are a prerequisite for testing larger sample numbers, both in monitoring

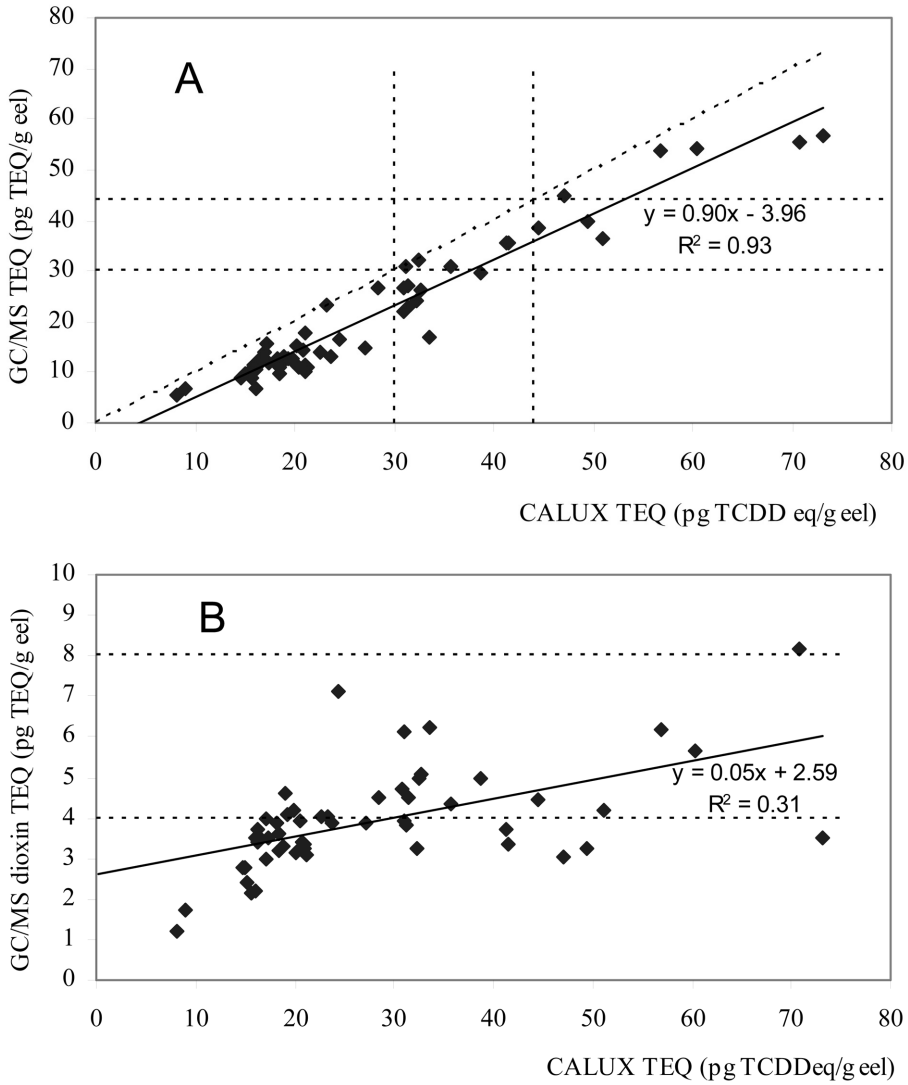

Figure 3. Comparison between total TEQ levels as estimated by DR CALUX® with GC/MS determined total TEQ (A) or dioxin levels (B) in 39 samples of eel including 21 samples exceeding the action limit of 30 pg TEQ/g. Solid lines represent linear regression curves, dashed lines the residue and virtual residue limits of 8 and 44 pg TEQ/g, respectively.

programs but especially during incidents. The DR CALUX® assay is a test that will respond to any Ah-receptor agonist present in the sample extract. The use of an acid silica clean up dramatically increases the specificity of the assay, but additional purification is required to split the dioxins and dioxin-like PCB. Alternatively, the decision limit can be set low enough to exclude false-negatives and limit the number of false-positive results. In the case of highly contaminated eel samples, the fact that dioxin-like-PCB were not yet included in the limit presented a major complication, since these compounds have been shown to contribute for more than 80% to the total TEQ level (Leonards, P. E. G., Lohman, M., de Wit, M. M., Booy, G. *et al*., 2000, RIVO-report C0034/00). This was confirmed in the present study showing a clear increase in the contribution of dioxin-like-PCB to the total TEQ-level in higher contaminated fish (Tables 3 and 4, Fig. 4). This is primarily due to an increased contribution of the mono-ortho PCB, which in the pooled fat samples increased from about 30 to 50%, as compared to a relatively stable contribution of the non-ortho PCB (35 – 40%) and a decreasing contribution of dioxins (30 – 15%). Regarding the poor response of the mono-ortho PCB, this could lead to a serious underestimation of the levels.

As demonstrated by the data, this problem was overcome by the use of a set of reference samples with a fixed contribution of dioxins, non-ortho and mono-ortho PCB. When the relative contribution of mono-ortho PCB to the total TEQ is significantly lower than the 45% in these reference samples,

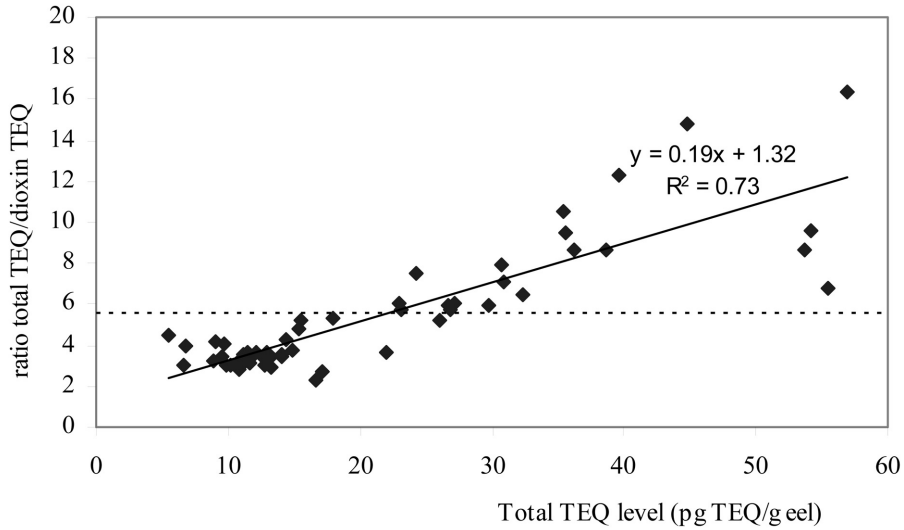

Figure 4. Relation between the total TEQ level and the ratio total TEQ/dioxins, as determined by GC/MS in 39 samples of eel. The dashed line indicates the ratio of 5.5 used for extrapolation of the official limit of 8 pg TEQ/g for dioxins to the virtual limit of 44 pg TEQ/g for total TEQ.

the levels are theoretically overestimated by the DR CALUX® assay and this is confirmed by the results obtained with lower contaminated pooled samples (Table 3, Fig. 2A). At these low levels, however, it is unlikely that this strategy will result in false-positive results. Similarly, the chances that the level in a sample where the total TEQ-level would primarily be due to mono-ortho PCB, would be seriously underestimated and as such be missed by the test (false-negative), appears to be very small. The alternative, the testing of much less samples due to the costs of the GC/MS method, is likely to result in many more samples with an unacceptably high TEQ level. The suitability of the test for screening for elevated total TEQ levels is further demonstrated by the comparison of the test data with the GC/MS data (Fig. 3A). Based on these data, the decision limit could actually have been increased to, *e. g.* 35 or 40 pg TEQ/g, thereby decreasing the fraction of false-positives (suspected but lower than 44 pg TEQ/g) from 10 to, respectively, 5 and 3%, without any false-negatives. Of course this should be re-evaluated for the much lower future EU-limit for dioxins and dioxin-like-PCB of 12 pg TEQ/g eel.

At present, the EU-limit for fish still includes only dioxins. There appears to be no direct relation between the dioxins and both the dioxin-like and indicator PCB in contaminated eel. This is shown by the fact that the relative contribution of PCB to the TEQ level in the pool samples increases in higher contaminated samples, whereas the relative contribution of PCDF decreases (Tables 3, 4). A similar trend was observed in the individual samples (data not shown). This indicates that the dioxins in the higher contaminated samples do not originate from PCB oil contaminated with these compounds (oxidation), as was, *e. g.* the case in the fat from the Belgian crisis in 1999. Therefore, the positive correlation between dioxins and dioxin-like-PCB levels in pooled eel (Table 3) appears to reflect a more general exposure to a low or high contaminated environment or feed. In the case of farmed eel, which in general contains much lower levels of these compounds, contamination is more likely to originate from the use of contaminated fish meal. It appears that the relative importance of mono-ortho PCB in other fish and also fish meal is rather low (< 15%) (Leonards, P. E. G., Lohman, M., de Wit, M. M., Booy, G. *et al*., 2000, RIVO-report C0034/00 and [18, 19]) and this could explain the relatively low levels of mono-ortho PCB and the relatively high contribution of PCB 126 in lower contaminated eel (Table 3). Regarding the lack of a direct relationship between dioxins and dioxin-like-PCB, and the important contribution of the non-ortho PCB 126 to the response, it can be expected that the test performs less well for selection of high levels of dioxins only. This is supported by the rather poor correlation between the DR CALUX® results and the dioxin levels (Fig. 3B). However, these data only represent the most contaminated samples and it is likely that samples with low DR CALUX® determined TEQ-levels will have much lower dioxin levels as well. This is supported by the data obtained with the pooled samples, actually showing a clear correlation between DR CALUX® determined TEQ levels and the dioxin levels (Table 3).

Furthermore, when plotting GC/MS determined dioxin levels of individual samples against the sum of dioxins and non-ortho PCB (Fig. 6) there appears to be a clear correlation for most samples. In general, the sum is at least twice that of dioxins only, with an extreme value of more than 5 for four samples. With a ratio of 2.5 and another ratio of

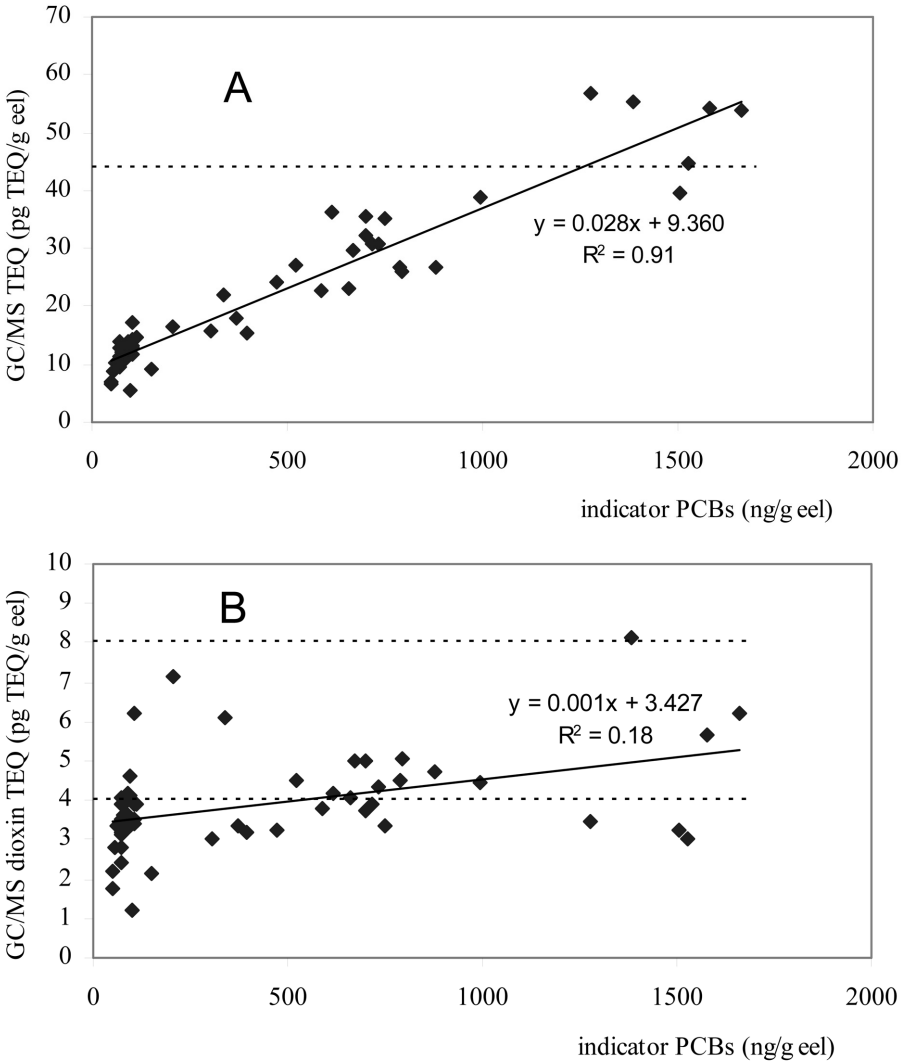

Figure 5. Comparison between levels of indicator PCB with GC/MS determined total TEQ (A) or dioxin levels (B) in 39 samples of eel including 21 samples exceeding the action limit of 30 pg TEQ/g. Solid lines represent linear regression curves, dashed lines the residue and virtual residue limits of 8 and 44 pg TEQ/g, respectively.

about 2 for mono-ortho PCB in higher contaminated samples (Table 3), a decision limit of 30 should detect samples with a dioxin level higher than 6. In the present study, only five samples exceeded this limit, but one showed a TEQ level below 30. Another 16 samples exceeding the decision limit of 30 (false-positives) had dioxin levels below 6 pg TEQ/g eel. When regarding the current limit for dioxins of 4 pg TEQ/g fish and an action limit for total TEQ of 20 pg TEQ/g, all 17 samples exceeding the limit would have been selected, together with another 16 (10% of total) samples (false-positive) not exceeding the limit of 4 pg TEQ/g fish. Although not ideal, this supports the use of the test for selecting out negative samples, even with a limit for dioxins only. A further improvement can be achieved by inclusion of an additional clean-up step for dioxins based on, *e.g.* activated carbon [4] or florisil (Hoogenboom, unpublished). On the other hand, dioxin-like PCB are included in the exposure limit (p-TWI) and will be included in the product limits this year.

3.8 Levels of dioxins and PCB in eel

The present dataset provides a lot of additional information on dioxin and PCB levels in eel, with a strong bias on wild eel, which represents only a minor fraction of the total eel production. The data confirm the results of previous studies that farmed eel in general contains low levels of these compounds, but that wild eel may in some cases contain rather

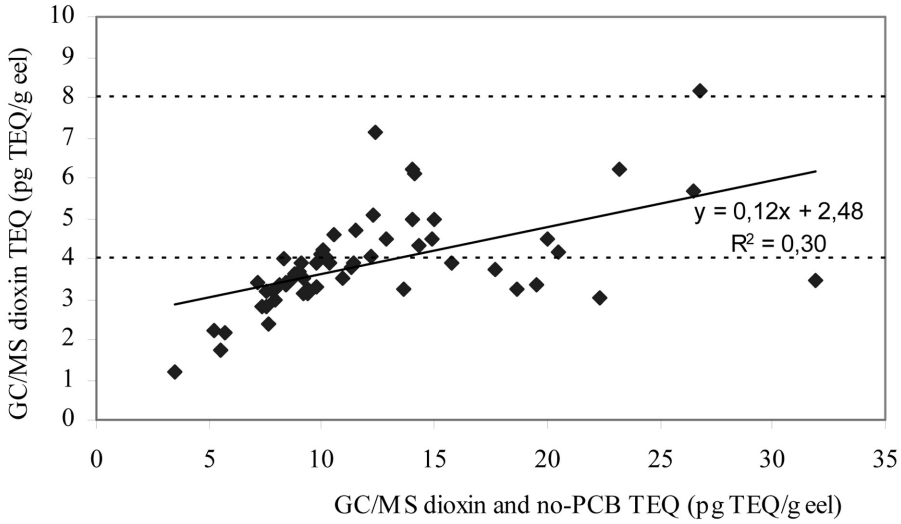

Figure 6. Relation between dioxin levels and the sum of dioxins and non-ortho PCB in 39 samples of eel, as determined by GC/MS. The correlation between the two parameters is shown by the regression line with a correlation coefficient of 0.30.

high levels. Furthermore, it should be stressed that levels in farmed eel are much easier to manipulate.

Another important issue is whether current limits for PCB in eel are still up to date. In the Netherlands, the current limits are 500, 200, 400, 400, 500, 500 and 600 ng/g eel for PCB 28, 53, 101, 118, 138, 153 and 180, respectively. In the present study, 4 out of 6 samples exceeding an indicator PCB level of 1000 ng/g also exceeded the virtual limit of 44 pg TEQ/g. Levels of PCB 153 in these 4 samples were 453, 602, 554 and 485 ng/g eel and thus in two cases below the limit for this congener. Furthermore, a limit of 400 ng/g for PCB 118 corresponds with a TEQ level of 40 pg TEQ/g, which would fill up most of the virtual limit of 44 pg TEQ/g, used in this study, and is far above the future EU limit of 12 pg TEQ/g eel.

4 Concluding remarks

We conclude that both the DR CALUX® assay and the indicator PCB may be used to select samples that may contain high levels of dioxins and dioxin-like PCB. However, the DR CALUX® assay performs better for detection of dioxins only, which is in agreement with the fact that this test is particularly suitable for detection of the most potent Ah-receptor agonists, whereas the indicator PCB focus more on dioxin-like PCB and in particular the mono-ortho PCB. It should be pointed out that in most other fish products and in food in general, these mono-ortho PCB are much less important in terms of their contribution to the total TEQ level (Leonards, P. E. G., Lohman, M., de Wit, M. M., Booy, G. *et al*., 2000, RIVO-report C0034/00 and Parsley, K., Wright, C., Thorpe, S., 1998, PCDDs, PCDFs and PCBs in marine fish, salmon and fish fingers, Report FD97/66 and [19]), and that the DR CALUX® bioassay is likely to perform much better as a screening assay than the indicator PCB. In addition, future reduction of TEF values for mono-ortho PCB may further reduce the importance of these compounds. In general, the use of a bioassay for screening for compounds with specific toxicological properties should obtain the preference above indirect chemical and immunological methods. The current study clearly demonstrates that a strategy based on relevant reference samples is superior to the use of a TCDD calibration curve.

The authors would like to thank Rob Coors, Richard van Helsdingen and Constant Onstenk (RIKILT) for their valuable contribution to the analysis of samples by DR CALUX® and GC/MS.

5 References

[1] Aarts, J. M. M. J. G., Denison, M. S., de Haan, L. H. J., Schalk, J. A. C., *et al*., *Organohal. Comp*. 1993, *13*, 361 – 364.

[2] Denison, M. S., Zhao, B., Baston, D. S., Clark, G. C., *et al*., *Talanta* 2004, *63*, 1123 – 1133.

[3] Gizzi, G., Hoogenboom, L. A. P., Holst, C. von, Rose, M., Anklam, E., *Food Addit. Contamin.* 2005, *22*, 472 – 481.

[4] Brown, D. J., Chu, M., van Overmeire, I., Chu, A., Clark, G. C., *Organohal. Comp*. *53*, 211 – 214.

[5] Bovee, T. F. H., Hoogenboom, L. A. P., Hamers, A. R. M., Aarts, J. M. M. J. G., *et al*., *Food Addit. Contamin.* 1998, *15*, 863 – 875.

[6] Hoogenboom, L. A. P., in: Watson, D. H. (Ed.), *Food chemical safety, volume 1 Contaminants*, Woodhead Publishing Ltd, Cambridge, UK 2001, pp. 91 – 106.

[7] Hoogenboom, L. A. P., Bovee, T. F. H., Portier, L., Bor, G., *et al*., *Talanta* 2004, *63*, 1249 – 1253.

[8] van Loco, J., van Leeuwen, S. P. J., Roos, P., Carbonnelle, S., *et al*., *Talanta* 2004, *63*, 1169 – 1182.

[9] EC, *Official Journal of the European Communities* 2002, *L209*, 5 – 14.

[10] Benisch, P. A., Allen, R., Anderson, J., Brouwer, A., *et al*., *Organohal. Comp.* 2001, *50*, 59 – 63.

[11] Hoogenboom, L. A. P., Goeyens, L., Carbonnelle, S., van Loco, J., *et al*., *Trends Anal. Chem.* 2006, *25*, 410 – 420.

[12] Bligh, E. G., Dyer, W. J., *Canad. J. Biochem. Physio.* 1959, *37*, 911 – 917.

13] Tuinstra, L. G. M. Th., Traag, W. A., van Rhijn, J. A., van de Spreng, P. F., *Chemosphere* 1994, *29*, 1859 – 1875.

[14] Van den Berg, M., Birnbaum, L., Bosveld, Brunström, B., Cook, P., *et al*., *Environ. Health Perspect.* 1998, *106*, 775 – 792.

[15] Tsutsumi, T., Amakura, Y., Nakamura, M., Brown, D. J., *et al*., *The Analyst* 2003, *128*, 486 – 492.

[16] De Boer, J., Stronck, C. N. J., Traag, W. A., Van de Meer, J., *Chemosphere* 1993, *26*, 1823 – 1843.

[17] Fernández, Gómara, B., Bordajandi, L. R., Herrero, L., *et al*., *Food Addit. Contam.* 2004, *21*, 983 – 991.

