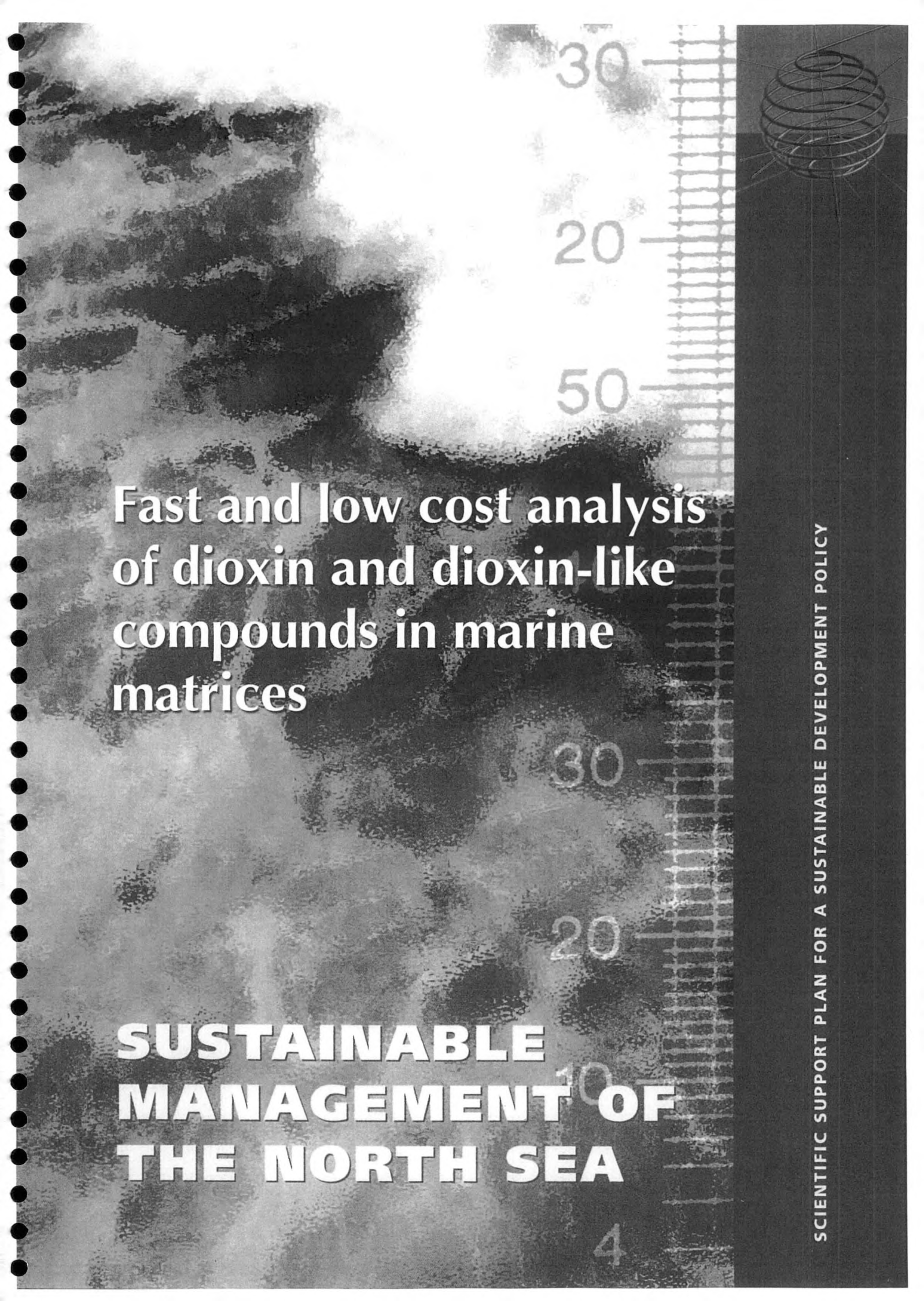

Fast and low cost analysis of dioxin and dioxin-like compounds in marine matrices

SUSTAINABLE MANAGEMENT OF THE NORTH SEA

SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY

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Programme "Sustainable Management of the North Sea"

Fast and low cost analysis of dioxin and dioxin-like compounds in marine matrices.

University of Liège
Laboratory of Mass Spectrometry
Centre of Analysis of Traces Residues (CART)

Final Report

Debacker V., Research assistant (Ulg)
Windal I., Research assistant (ISP)
Danis B., Research assistant (ULB)
Dubois Ph., Research associate (ULB)
De Pauw E., Professor (Ulg)

Involved scientific teams :

De Pauw Edwin, Coordinator
Debacker Virginie
Laboratory of Mass Spectrometry
Centre of Analysis of Traces Residues (CART)
University of Liège

Bouquegneau Jean-Marie, Partner
Oceanology
University of Liège

Dubois Philippe, Partner
Danis Bruno
Marine Biology Laboratory
Université Libre de Bruxelles

Joiris Claude, Partner
Laboratory of Ecotoxicology
Vrije Universiteit Brussels

Goeyens Léo and Baeyens Willy
Windal Isabelle
Laboratory of Analytical and Environmental Chemistry
Institue of Public Health

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Contents

1. Introduction, scope of the project.

The polychlorinated lipophilic compounds polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) do not occur naturally but as by-products of chemical synthesis, industrial processes and ocasionally of interaction between other organic contaminants in the environment. These compounds, widely distributed through atmospheric deposition, have raised much concern as they combine marked biological persistence with high toxicity even at low doses. In particular, 2,3,7,8-TCDD is regarded as the most toxic congener and serve as a reference to assign 'Toxic Equivalent Factors' (TEFs) to other congeners.

These pollutants exert a very specific toxic action mediated by the cellular aryl hydrocarbon receptor (Ah receptor) for which they display a high binding affinity. Once bound to the Ah receptor, they induce the expression of the genes CYP450. The CYP1A1 is one of the first protein to be expressed in an organim exposed to dioxins and dioxin-like compounds.

A wide range of toxic effects has been observed in animals including symptoms like dermal toxicity, immunotoxicity, hepatotoxicity, severe weight loss, reproductive effects, teratogenicity and endocrine toxicity.

The Chemically Activated LUciferase gene eXpression (CALUX) in vitro cell bioassay is an emerging analytical tool that gains ground in the assessment of dioxins and dioxin-like compounds in blood (Murk *et al.*, 1997, Koppen *et al.,* 2000, Zicardi *et al.,* 2000, Van Wouve *et al.*, 2003), sediments (Murk *et al.*, 1996, Vondracek *et al.*, 2001, Behnisch *et al.*, 2002, Sronkhorst *et al.*, 2002), food matrices, (Tsutsumi *et al.*, 2003, Cederberg *et al.*, 2002) and milk (Bovee *et al.*, 1998, Van Overmeire *et al.*, 2002) (reviews : Behnisch *et al.*, 2001a, b). CALUX is a reporter gene assay that detects dioxin-like compounds based on their ability to activate the aryl hydrocarbon receptor (AhR). The genetically modified hepatoma cells used for CALUX assays respond to dioxin-like compounds (AhR ligands) exposure with the induction of luciferase. Following cell lysis and addition of luciferin, the luciferase present in the cells produces a luminescent signal proportional to the cells' receptor activity. The measured luminescence is converted into a toxic equivalency (TEQ) value by the direct comparison of the response for a given sample to a dose-response curve obtained with 2,3,7,8 tetrachlorinated dibenzo-*p*-dioxin (TCDD). For a detailed description of the mechanism the authors refer to Van Overmeire *et al.* (2001).

Since CALUX analyses provide a biological and overall response for all dioxin-like compounds, the interpretation of the results is more complex than with chemical analyses.

The Mass Spectrometry Laboratory has developed and validated fast methods of analysis for dioxins, furans and coplanar PCBs in marine matrices. They require high-resolution mass spectrometry for indentification and quantification of target compounds.

In order to reduce costs and delays, a three levels strategy of analysis is investigated by gathering the reference method (high resolution mass spectrometry), a low resolution mass spectrometric technique and bio-assays.
The aim of this project was to provide the authorities with efficient, validated monitoring tools for dioxin-like compounds based on the application of this three-level analysis strategy, by performing the various techniques in parallel on selected samples.

2. Summary of the first activity report.

Sampling and analysis were certainly the main tasks accomplished during the first year of activities. Different techniques were used in parallel in the different laboratories and extracted samples were shared between the teams to pursue analysis. Validated and known techniques such as the high resolution mass spectrometry (GC-HRMS) and the analysis of CYP1A1 were immediately applied to field samples, whereas the emerging Calux technique still needed improvments and validation. This was accomplished during the first year of the programme (Van Overmeire *et al.*, 2002, Van Wouve *et al.*, 2003, Windal *et al.*, 2003). Not all specified matriced could be analysed during this first year and analysis continued during the second year.

3. Objective set for the second year of activity.

- To finish the analysis in all matrices.
- To modify and adapt extraction and clean-up before HRMS analysis for sediment samples.
- To exchange results obtained using the different techniques (CYP1A1, Calux and GC-HRMS) to compare the methods, their usefulness and efficiency.
- To determine the organochlorine levels in the different chosen biological samples and interpret them both in terms of toxicity and in terms of comparison with what is described for similar samples elsewhere.

4. Materials and methods.

4.1 Sampling.

Sampling was taken in charge by the Marine Biology Laboratory (Ph. Dubois) of the Université Libre de Bruxelles in 2002 for benthic fishes, starfishes, mussels and sediments, as follow:

4.1.1 Subtidal samples.

Fishes (*Pleuronectes platessa, Limanda limanda, Platichthys flesus, Solea solea, Merlangius merlangus, Gadus morhua*), starfishes (*Asterias rubens*), and sediment cores were collected during RV Belgica campaign 0209b on the Belgian and Dutch continental shelves between April 8th and 12th; 2002 (chief scientist: Ph. Dubois) (Table 1).

Station	Position (°N, °E)		Sediment cores (Reineck)	*A.rubens* cyt P450	*A.rubens* DLC analysis	*P.platessa*	*L. limanda*	*P.flesus*	*S.solea*	*M.merlangus*	*G.morhua*
710	51°26.45	3°08.32	3	5	6	6	6			28	-
250	51°31.00	3°19.00	3	5	5	1	17	2		28	7
S01	51° 25.00	3° 34.20	3	5	30			1	10	6	1
545	51°43.51	3°02.84	3	5	-		3			19	2
435	51°34.92	2°47.25	3	6	-	3	2		2	20	6
ZD2	51°37.20	3°11.30	3	5	30		3			10	1

Table 1. Samples collected during Belgica campaign 0209b

Three Reineck cores (1 sample/core) were taken at every station, for determination of total organic matter, PCBs, furanes, and dioxins. Samples (5 upper cm of each core) were immediately deep-frozen (-20°C). Epifauna was collected by trawling (3m beam trawl, 6mm mesh, 2kt, counter-current, 15min).

Five starfishes were dissected to collect the pyloric caeca (3ml) for analysis of cytochrome P450. These samples were immediately stored in liquid nitrogen. Remaining pyloric caeca, if present were pooled and deep-frozen. Twenty other starfishes (when available) were then dissected to collect the pyloric caeca (for PCBs, furans and dioxins analysis). These samples were kept frozen at -20°C.

Livers of flatfishes were dissected and stored (3ml) in liquid nitrogen. Remaining parts of the livers were pooled and deep-frozen. Head and inner organs of flatfishes were then removed and the body stored at –20°C (for PCBs, furans and dioxins analysis). Round fishes were deep-frozen as a whole.

4.1.2 Intertidal samples.

Starfishes (*Asterias rubens*), mussels (*Mytilus edulis*), and sediments were sampled in the intertidal zone in Ambleteuse (France), Nieuwport, Ostende, Wenduine and Knokke, referred

to hereafter as respectively: AMB, NP, OS, WD and KN. Starfishes and sediments were processed as described above. Mussels were deep-frozen as a whole.

4.1.3 Marine birds and mammals.

Additional samples of a seabird species, the common guillemot (*Uria aalge*) and a marine mammal (*Phocoena phocoena*) collected at the Belgian coast by the MARIN group were also included in the present study.

4.2 Determination of the organic content in sediment samples.

Sediments were dried at 70°C until a constant weight is recorded, then carbonised at 450°C during four hours and weighed. The difference between the two weighings corresponds to the organic content oxidised during carbonisation and eliminated as aqueous vapour and CO_2.

4.3 Analytical procedure using GC-HRMS to detect dioxins and dioxin-like compounds.

4.3.1 Extraction and clean-up.

All analyses were carried out in the Laboratory of Mass Spectrometry, Centre for the Analysis of Trace Residues (CART), University of Liège. Seventeen 2,3,7,8-substituted ^{13}C-labeled PCDD/Fs congeners, and 4 dioxin-like coplanar PCBs (IUPAC n° 77, 81, 126, 169) were quantified in the samples. All glassware were thoroughly cleaned using different solvents respectively methanol, acetone, toluene and a solution of dichloromethane hexane (50 :50, V :V). Samples were weighed prior lyophilisation (24 hours). After being finely crushed the dry tissues were inserted in a steel extraction cell which was placed in the Accelerated Solvent Extractor (ASE 200, Dionex). This machine using organic solvents operates under high pressure and temperature conditions (hexane, 10 minutes at 125°C and 1500psi) and allows the extraction of the different organic compounds present in the biological matrice. The extracts are placed in a tared balloon, filtered on Na_2SO_4 anhydre to eliminate the water. The remaining solvents are then totally evaporated. The tared balloon is weighed to precisely know the amount of extracted fat. A solution of hexane/dichloromethane is added to the extracted fat and the sample stored in a vial prior to purification. The fat extracts are then spiked with a mixture containing seventeen ^{13}C-labeled 2,3,7,8-substituted dioxins isomers, 4 c-PCBs isomers (EDF-4144, LGC Promochem) and 8 mono-ortho-PCB isomers (Campro Scientific WP-LCS). The purification is managed in a semi-automatic system made of four different columns. The first column is a HCDS column (High Capacity Disposable Silica) which is a silica-modified column (28 gr. aid silica, 6 gr. neutral silica, 16 gr. basic silica); the second one is a Na_2SO_4 column made of neutral and acid silica; the third one is a basic

aluminium column; and the fourth one is an active coal column (Power-Prep, Fluid Management System, U.S.A).

The sample migrates through the first two column where the fat and the proteins are eliminated by the acid action, and then through the basic aluminium column which retains the PCBs, PCDDs and PCDFs. These are removed by the hexane/dichloromethane (50:50, V:V) and the hexane/dichloromethane (98:2, V:V) and pass through the coal column where the PCDDs, PCDFs and coplanar PCBs are recuperated while the other PCBs carry on and are removed. The ethyl/benzene (50:50, V:V) and the toluene take down the PCDDs and the PCDFs present in the aluminium column. As the PCDDs and PCDFs are trapped in the top of the column, the toluene is sent in the opposite way to remove them. The purified extracts in the toluene are concentrated using a turbovap and are later transferred into 4 µl of nonane. The rest of the toluene is evaporated under atmospheric pressure conditions.

4.3.2 Instrumental analysis to analyze PCDD/Fs.

The different congeners present in the sample are then analysed using a **g**as **c**hromatography equipped of a capillary column of 40 m coupled to a **h**igh **r**esolution **m**ass **s**pectrometer (GC-HRMS, Autospec Ultima)). They can be quantified and their concentration calculated when compared to the added internal ^{13}C standard (Eppe, 1996 ; Windal, 2001). Congeners which produce a peak less than three times the signal to noise ratio in ^{12}C of the spectrometer are considered as 'non detected' (ND). Results are expressed either as pg/g of lipid weight (dry weight for sediments) or in terms of toxicity, using WHO TEF (Van den Berg, 1998) as pg TEQ/g of lipids weight (Table 2). The TEQs values were calculated using the 'upper bound' limit which means that for a non detected congener the minimum value considered in the TEQ calculation is its limit of quantification.

Molecules of PCDD/Fs	**Human TEF (WHO)**	**Bird TEF (WHO)**	**Fish TEF (WHO)**
2,3,7,8 TCDD	1	1	1
1,2,3,7,8 PeCDD	1	1	1
1,2,3,4,7,8 Hx1CDD	0.1	0.05	0.5
1,2,3,6,7,8 Hx2CDD	0.1	0.01	0.01
1,2,3,7,8,9 Hx3CDD	0.1	0.1	0.01
1,2,3,4,6,7,8 HpCDD	0.01	0.001	0.001
OCDD	0.0001	0.0001	<0.0001
2,3,7,8 TCDF	0.1	1	0.05
1,2,3,7,8 Pe1CDF	0.05	0.1	0.05
2,3,4,7,8 Pe2CDF	0.5	1	0.5
1,2,3,4,7,8 Hx1CDF	0.1	0.1	0.1
1,2,3,6,7,8 Hx2CDF	0.1	0.1	0.1
1,2,3,7,8,9 Hx3CDF	0.1	0.1	0.1

2,3,4,6,7,8 Hx4CDF	0.1	0.1	0.1
1,2,3,4,6,7,8 Hp1CDF	0.01	0.01	0.01
1,2,3,4,7,8,9 Hp2CDF	0.01	0.01	0.01
OCDF	0.0001	0.0001	<0.0001
PCB 77 (3,3',4,4' Tétrachlorobiphényle)	0.0001	0.05	0.0001
PCB 81 (3,4,4',5 Tétrachlorobiphényle)	0.0001	0.1	0.0005
PCB 126 (3,3'4,4',5 Pentachlorobiphényle)	0.1	0.1	0.005
PCB 169 (3,3',4,4',5,5' Hexachlorobiphényle)	0.01	0.001	0.00005
PCB 80 (2,2',4,5,5' Tetrachloro-)			
PCB 105 (2,3,3',4,4' Pentachloro-)	0.0001	0.0001	< 0.000005
PCB 114 (2,3,4,4',5 Pentachloro-)	0.0005	0.0001	< 0.000005
PCB 118 (2,3',4,4',5 Pentachloro-)	0.0001	0.00001	< 0.000005
PCB 123 (2',3,4,4',5 Pentachloro-)	0.0001	0.00001	< 0.000005
PCB 156 (2,3,3',4,4',5 Hexachloro-)	0.0005	0.0001	< 0.000005
PCB 157 (2,3,3',4,4',5 Hexachloro-)	0.0005	0.0001	< 0.000005
PCB 167 (2',3',4,4',5,5' Hexachloro-)	0.00001	0.00001	< 0.000005
PCB 189 (2,3,3',4,4',5,5' Heptachloro-)	0.0001	0.00001	< 0.000005

Table 2: Molecules analysed and their corresponding TEF considering either human, bird or fish values (Van den Berg *et al.*, 1998).

4.3.3 Identification and quantification of mono-ortho PCBs using MS/MS analysis.

Although less sensitive compared to the HRMS, tandem mass spectrometry (MS/MS) was used to analyse the mono-*ortho* PCBs as these compounds are present in much higher proportions –more than a 1000 times - than the dioxins. PCB congeners (IUPAC n°. 105, 114, 118, 123, 156, 157, 167, 189) are quantified.

All analyses were performed by tandem in time mass spectrometry (GC/MS/MS) using a ThermoQuest Trace GC PolarisQ ion trap mass spectrometer (Austin, Tx, USA) and a Hewlett-Packard (Palo Alto, CA, USA) 6890 Series gas chromatograph, the latter equipped with a Rtx 5-MS (40m x 0.18 mm x 0.20 µm) capillary column (Restek, Evry, France). The ion trap was set at 250°C, with the transfer line at 300°C. Electron impact was used as ionisation mode, with an energy of 70 eV. GC conditions were optimised to separate the 12 non- and mono-*ortho* PCBs and the 7 marker PCBs as follows: splitless injection of 1 µl at

140°C, initial oven temperature of 140°C for 1 min, then increased at 25°C/min to 180°C held for 1 min, then increased at 2°C/min to 210°C held for 8 min, finally increased at 3°C/min to 280°C and held for 2 min. He (N60, Air Liquide, France) was used as the carrier gas.

4.3.4 Adaptation of the procedure to analyze sediment samples.

Sediment samples were analyze as a whole without size grain fractionation, however large shell fragments were avoided. Samples were weighed (± 30g) and dried overnight in an oven at 75°C. Samples were extracted using the ASE system as described above but using toluene instead of hexane as the extracting solvent. No addition of acid silica was necessary. Samples were spiked prior to extraction with 10µl of a mixture containing seventeen ^{13}C-labeled 2,3,7,8-substituted dioxins isomers (EDF-4144, LGC Promochem), 4 c-PCBs isomers and 8 mono-ortho-PCB isomers (Campro Scientific WP-LCS). The purification step is also managed in a semi-automatic system made of three different columns: the first one is a Na_2SO_4 column made of neutral and acid silica; the second one is a basic aluminium column; and the third one is an active coal column (Power-Prep, Fluid Management System, U.S.A). The purified extracts in the toluene are concentrated using a turbovap and are later transferred into 4 µl of nonane. The rest of the toluene is evaporated under atmospheric pressure conditions.

Samples of a reference material used as a quality control (Campro Scientific, WMS-01) were inserted in each set of analysis. Results obtained and their comparison with the certified values are presented in Table 3. The comparison indicate that results obtained for 6 out of 17 PCDD/Fs congeners are systematically higher (over estimation) than the certified reference values. For c-PCB congeners, on the contrary, results are below that of the certified reference values (under estimation).

Congeners pg/g	Analysed concentration n = 4	Certified concentration x ± 2σ	Comparaison
2378-TCDD	18.9 ± 1.9	17.7 ± 5.6	
12378-PnCDD	10.7 ± 2.5	7.96 ± 2.8	
123478-Hx_1CDD	10.5 ± 1.7	8.66 ± 2.7	
123678-Hx_2CDD	20.1 ± 7.2	20.8 ± 4.8	
123789-Hx_3CDD	27.0 ± 11.5	17.3 ± 8.0	> 2 σ
1234678-HpCDD	328.0 ± 72.4	293 ± 63	
OCDD	2199.5 ± 500.7	1899 ± 456	
2378-TCDF	92.6 ± 18.8	52.5 ± 16	> 2 σ
12378-Pn_1CDF	18.1 ± 1.7	12.6 ± 5.0	> 2 σ

23478-Pn_2CDF	23.3 ± 7.4	18.5 ± 6.1	
123478-Hx_1CDF	80.8 ± 2.7	67.3 ± 24	
123678-Hx_2CDF	49.3 ± 16.5	20.3 ± 8.7	> 2 σ
123789-Hx_3CDF	1.64	*2.68 ± 4.0	
234678-Hx_4CDF	20.6 ± 9.1	16.0 ± 8.0	
1234678-Hp_1CDF	433.8 ± 18.0	299 ± 73	> 2 σ
1234789-Hp_2CDF	14.6 ± 5.8	15.1 ± 4.6	
OCDF	749.2	509 ± 157	> 2 σ
PCB-77	152.7 ± 124.5	1717 ± 520	< 2 σ
PCB-81	143.2 ± 153.6	*75 ± 79	
PCB-126	15.5 ± 1.6	84.9 ± 35	< 2 σ
PCB-169	1.3 ± 0.03	7.97 ± 5.3	< 2 σ

Table 3: Comparison of the results obtained after analysis with those of a certified sediment sample.

4.3.5 Quality assurance.

A recovery standard (RS, EDF-4145, LGC Promochem) is also added to the purified extracts to calculate the percentage of recovery for each considered congener. Recoveries of internal PCDD/Fs standards ranged typically between 60 to 120%.
Limits of quantification for PCDD/Fs ranged between 0,0006 to 0,3 pg TEQ/g lipid weight depending of the considered congeners. For all combined PCDD/Fs congeners the limit of quantification is of 0,79 pg TEQ/g lipids weight.

4.4 CALUX bioassay: analytical procedure.

4.4.1 Material and reagents.

The pGudLuc 6.1 cell line was supplied by Xenobiotic Detection Systems Inc (USA). The genetically modified cell line responds to dioxin-like chemicals with the induction of firefly luciferase in a time-, dose-, and AhR-dependant manner (Garrison *et al.*, 1996). The hexane (pestanal, for residue analysis grade), the sulfuric acid (95-98%, ACS reagent), the toluene (for pesticide residue analysis) and silica gel 60 for column chromatography were purchased from Fluka Sigma-Aldrich (Germany). Acetone and ethyl acetate were for gas chromatography, suprasolv grade and were purchased from Merck (Germany). Anhydrous sodium sulfate was ultra-resi analyzed grade and was obtained from Baker (The Netherlands).

The standard solution of 2,3,7,8 TCDD (50pg/μL) was purchased from AccuStandard Inc (New Haven, USA).

4.4.2 Extraction.

Biotic samples were lyophilized during 24-48h depending on the sample size. Dry samples were extracted with hexane by Pressurized Liquid Extraction (PLE) using a Dionex (Sunnyvale, CA, USA) ASE 200 extractor. 33mL cells were filled with freezed-dried samples and sodium sulfate. The extraction conditions were 125°C, 1500 PSI, 2 static cycles of 5 minutes. The extracts were then dried on sodium sulfate before concentration and gravimetric determination of fat. A part of the fat was analyzed by CALUX and the other part was analyzed by GC-HRMS.

Sediment samples were dried in an oven at 60°C, sieved at 1mm and subsequently extracted with toluene by Pressurized Liquid Extraction (PLE) using a Dionex (Sunnyvale, CA, USA) ASE 200 extractor. The extraction conditions were 125°C, 1500 PSI, 2 static cycles of 5 minutes. The extracts were concentrated to dryness and re-suspended in a known volume of hexane.

4.4.3 Clean-up for biotic samples.

A 25 ml Pyrex disposable column (Sigma Aldrich, Germany) was filled, from bottom to top, by 1.9 g of sodium sulfate, 2.8 g (for samples up to 0.25 g fat) or 5.6 g (for samples up to 0.6 g fat) or 8.4 g (for samples up to 1. 5 g fat) of 33% (w/w) sulfuric acid silica gel and 1.9 g of sodium sulfate, and rinsed with 30mL, 45 mL hexane and 60 mL of hexane, according to the amount of sulfuric acid silica gel. A 10 ml Pyrex disposable column (Sigma Aldrich, Germany) was filled from bottom to top by 0.7 g of sodium sulfate, 0.34 g of X-CARB (Xenobiotic Detection Systems Inc., USA) and 0.7 g of sodium sulfate, and rinsed with 5 mL of acetone, 20 mL of toluene and 10 mL of hexane. The acidic silica column was then placed on top of the carbon column. The fat was weighted and dissolved in 5 mL hexane. The extract was then loaded on the sulfuric acid silica gel column, the recipient was rinsed 2 times with 5 mL of hexane which were added on the column too. The column was then eluted with 15, 30 or 45 mL of hexane, according to the size of the acidic silica column. When the elution was completed, the acidic silica column was removed and the carbon column was eluted with 8 mL of a hexane-acetone (90/10) mixture. This fraction was discarded since it is toxic for the cells. The fraction containing the coplanar PCB was subsequently eluted with 15 mL of hexane/ethyl acetate/toluene (80/10/10), and the fraction containing the PCDD/Fs was then eluted with 20 mL of toluene. Extracts were concentrated to dryness in a centrifuge under vacuum and re-suspended in a known volume of hexane. Prior to dosing the plate, the extracts in hexane (1mL) were transferred in 4μL of DMSO using a centrifuge under vacuum and, finally, 400μL of medium were added to each extract in DMSO.

4.4.4 Clean-up for sediment samples.

A 25 ml Pyrex disposable column (Sigma Aldrich, Germany) was filled, from bottom to top, by 1.9 g of sodium sulfate, 2.0 g of deactivated neutral alumina, 3 g of 33 % (w/w) sulfuric acid silica gel, 1 g of silver nitrate 5 wt % on silicagel 60 (Aldrich, Germany), 2.8 g of 33 % (w/w) sulfuric acid silica gel and 1.9 g of sodium sulfate, and rinsed with 60 mL of hexane. A 10 ml Pyrex disposable column (Sigma Aldrich, Germany) was filled from bottom to top by 0.7 g of sodium sulfate, 0.34 g of X-CARB (Xenobiotic Detection Systems Inc., USA) and 0.7 g of sodium sulfate, and rinsed with 5 mL of acetone, 20 mL of toluene and 10 mL of hexane. The acidic silica column was then placed above the carbon column. The extract of sediment in hexane was then loaded on the sulfuric acid silica gel column, the recipient was rinsed 2 times with 5 mL of hexane which were added onto the column too. The column was then eluted with 45 mL of hexane. The rest of the procedure is the same than for biotic samples.

4.4.5 Determination of the percentage recovery.

One extra sample of fat or sediment is spiked with ^{14}C 2,3,7,8 TCDD and the sample is purified following the same procedure than the samples. The extracts collected after the carbon column are then analyzed for radioactivity. The comparison to the reference allows for the calculation of the percentage recovery.

4.4.6 Preparation of the plate.

Cells were cultured in phosphate buffered saline medium with L-Glutamine (Gibco, UK), supplemented by 8% of fetal bovine serum (Hyclone Laboratories, Utah, USA), 45.5 unit.mL^{-1} of penicillin and 45.5 unit.mL^{-1} streptomycin (Gibco, UK), at 37°C and 5% CO_2 in an atmosphere saturated with water. For the CALUX bioassay, 96-well culture plates were seeded with 200µL of cell suspension at a density of 8.10^4 cells.mL^{-1}.

4.4.7 Dosing the plate.

After 20-24h of incubation, the medium was removed from the plate and replaced by the extract in DMSO + medium, prepared as described above (200µL per well, each analysis being performed in duplicate). On each plate, 10 solutions of TCDD in DMSO (25000, 12500, 6250, 3125, 1562, 781, 391, 195, 98 and 49 fg TCDD.µL^{-1} DMSO) were analyzed to draw the calibration curve. The same solution of 750 fg TCDD.µL^{-1} DMSO (prepared independently from calibration solutions) was analyzed in 6 wells, distributed over the plate for quality control (see below) and DMSO alone was analyzed in 4 wells. No analysis was performed in the external wells.

4.4.8 Reading the plate.

After 20-24h of incubation, cells were examined microscopically for obvious toxicity. Each well was rinsed with 75 µL of PBS-Buffer (Gibco) and 30 µL of cell culture lysis reagent (5x) (Promega, Madison, USA) were added to each well to lyse the cells. The plate was shaken for 20 minutes at room temperature before being placed in the luminometer (Lucy 1, Anthos, Austria) for another 10 minutes. After the addition of 50 µL of luciferase assay reagent (Promega, Madison, USA), the light output was integrated over 15 s after a delay time of 5 s, and results were expressed in Relative Light Unit (RLU).

4.4.9 Analysis of data.

The average RLU value measured for the DMSO alone was subtracted from all RLU values, and the average RLU measured for the 2 wells containing the same extract or solution was calculated. The best equation fitting the calibration curve was calculated using a four-variable Hill equation. This equation was used to convert the measured RLU value into data expressed in pg TEQ/sample.

4.4.10 Setting quality controls.

Before starting the validation, strict quality control criteria were set up at different levels. These original quality criteria were designed for this CALUX-bioassay and some of them are quite different of what can be used in chemical analysis or other kind of bioassays.

1. The first quality control was set up to check the quality of the plates. In that purpose, a standard solution of 2,3,7,8 TCDD (750 $fg.\mu L^{-1}$ DMSO) is prepared independently from the calibration solutions and is analyzed in 6 wells, distributed over the plate. Its concentration is chosen to perform the measurements in the lower linear part of the calibration curve, where measurements of real samples are performed. The mean of the measurements as well as the standard deviation are plotted on a control chart for which classical criteria are applied (Figure 1). Results must vary within the limits of the actual concentration (1.5 pgTEQ/well) ± 20%. A second criterion imposes that the RSD associated with the 6 measurements has to be lower than 15%. Higher RSD indicates that the cell culture, and consequently the response of the cells are not homogeneous. Both percentages of variation were decided based on previous data. Figure 1 shows the results obtained in our lab during 7 months, using different standard solutions or cell culture reagents, different cells and obtained for different operators, including students in formation. 85% of the plates were accepted, based on those criteria. The mean of all data (outliers not included) is 1,50 pg TEQ/well with an RSD of 10 %.

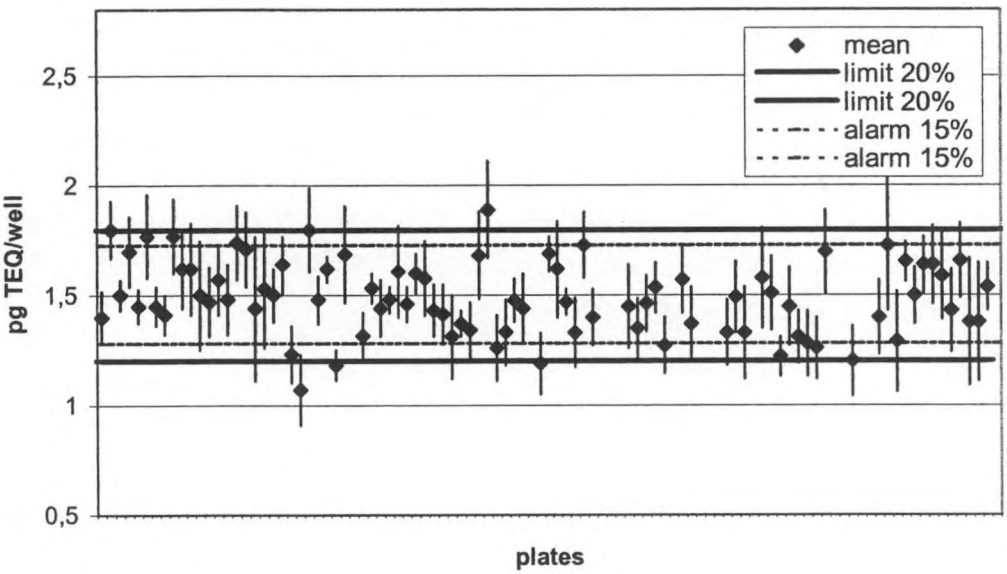


Figure 1: Control chart set up with the average and standard deviation associated with 6 measurements of the same standard solution of 2,3,7,8 TCDD on each plate.

2. A second quality control was set up to check both the contamination and toxicity of the extract that may be introduced by the procedure. In that purpose, the final extract of the procedural blank is spiked with the standard solution of 2,3,7,8 TCDD used for the quality control of the plates. The CALUX response of the latter mixture (standard solution + procedural blank) can not exceed the range average ± SD calculated for the standard solution. Responses in excess indicate contamination of the procedural blank with Ah ligands, whereas lower responses indicate the presence of some toxic compounds in the procedural blank poisoning the cells. With the procedure here described both toxicity and contamination of the extracts can be detected.

3. A quality control sample (cod liver oil for biotic samples or a contaminated river sediment for sediment samples) was analyzed within each serie of samples, as it is usually done within the frame of analytical method's accreditation. A control chart is set up for which classical control criteria are applied. Results are discussed in detail in the next paragraphs.

4. Quantification limits: when setting quantification limits, 3 factors were taken into account: 1) the dose-response curves of some dioxin-like compounds do not reach the same maximum as the 2,3,7,8 TCDD dose-response curve and 2) the antagonistic effect of some compounds is lower when working at lower concentration (data not shown) 3) quantification at concentration below our 7^{th} point of calibration (781 fg/well) is less precise. Consequently, it was decided to quantify only the samples giving results in the lower half of the calibration curve (concentration<3125 fg/well) and above the concentration of 781 fg/well.

Real samples are analyzed by series of 13, plus a procedural blank, a quality control sample (QCS) and an additional sample designed for the determination of the percentage recovery (see material and method). The percentage recovery is used to correct the results.

4.4.11 Application of CALUX to marine matrices and comparison with chemical analysis.

The trueness of CALUX measurements is much more difficult to evaluate. Chemical analyses of all dioxin-like compounds would be impossible, and TEF values are not available for every Ah ligands. Furthermore, non-additive interactions between compounds would not be taken into account. The chemical analysis of PCDD/Fs and c-PCBs will be used as the reference method. The 2 methods are compared for the analysis of dioxin and dioxin-like compounds in flatfishes (*Limanda limanda* and *Solea solea*), marine mammals (Harbour porpoises, *Phocoena phocoena*), seabirds (common guillemot *Uria aalge*), starfishes (*Asteria rubens*) and mussels (*Mytilus edulis*) collected at the Belgian coast.

4.5 Data analysis .

Statistical analysis of the results was performed using Statistica 5.1 for Windows. Results were considered to be significant at the 5 % critical level ($p < 0.05$) and highly significant at the 1 % critical level ($p < 0.01$).

4.6 Cytochrome P450 immunopositive protein (CYP1A IPP) quantification.

CYP1A IPP content was quantified using a competitive-ELISA. The method is fully described elsewhere (Danis *et al*, submitted). Briefly, the ELISA was carried out using competition between the CYP1A IPP contained in the starfishes pyloric caeca and a biotinylated CYP1A from ß-naphtoflavone (BNF)-injected trout (*Oncorhyncus mykiss*). Multiwell plates (96 wells) were coated with Anti-CYP1A (rabbit anti-fish CYP1A peptide, polyclonal antibody; Biosense, Norway). Wells were washed with phosphate-buffered saline (PBS), and nonspecific binding sites were blocked with PBS-Bovine serum albumin (BSA). Wells were washed again and biotinylated microsomes of BNF-injected trout were added (except for the blank wells). Starfishes samples or standards (with normalised protein concentration of 100 $\mu g\ ml^{-1}$) were then added to the wells. Competition was allowed to take place for 2 hrs, and after five washing steps extravidin-HRP was added to all the wells. The plate was then incubated for 45 min and the wells were washed again using PBS. Chromogen TMB (Biosource, UK) was added to all the wells and the plate was incubated in the dark for 10 min. Sulfuric acid was then added to stop the reaction and absorbance was measured at 450 nm using a micro plate reader (Packard, Spectracount). Final results were expressed as induction factors, *viz.*, the ratio of CYP1A IPP levels between samples and starfishes from the station displaying the lowest CYP1A IPP response.

5. Results and discussion.

5.1 Analyses of sediment samples .

5.1.1 Levels of PCDD/Fs, c-PCBs using GC-HRMS and Mo-PCBs using GC/MS-MS.

5.1.1.1 Subtidal stations.

The characteristics of the surface sediments sampled ranged from relatively fine (stations 250, ZD2, 545 and 435) to intermediate (station 710) and very fine composition at station SO1 enriched with silt and clay situated at the mouth of the Scheldt estuary.
Concentrations of PCDD/Fs and c-PCBs and TEQ values detected are reported in Table 4. As a whole for PCDD/Fs, our results can be divided in 2 categories : a first category gathering all stations but SO1 with very low to low concentrations ; the second category being represented by station SO1 alone with higher concentrations. This situation is clearly reflected in Map 1.
In general, spatial heterogeneity can be observed both between and within the stations. This was also observed when determining the organic content of these sediments samples as shown in Table 6.

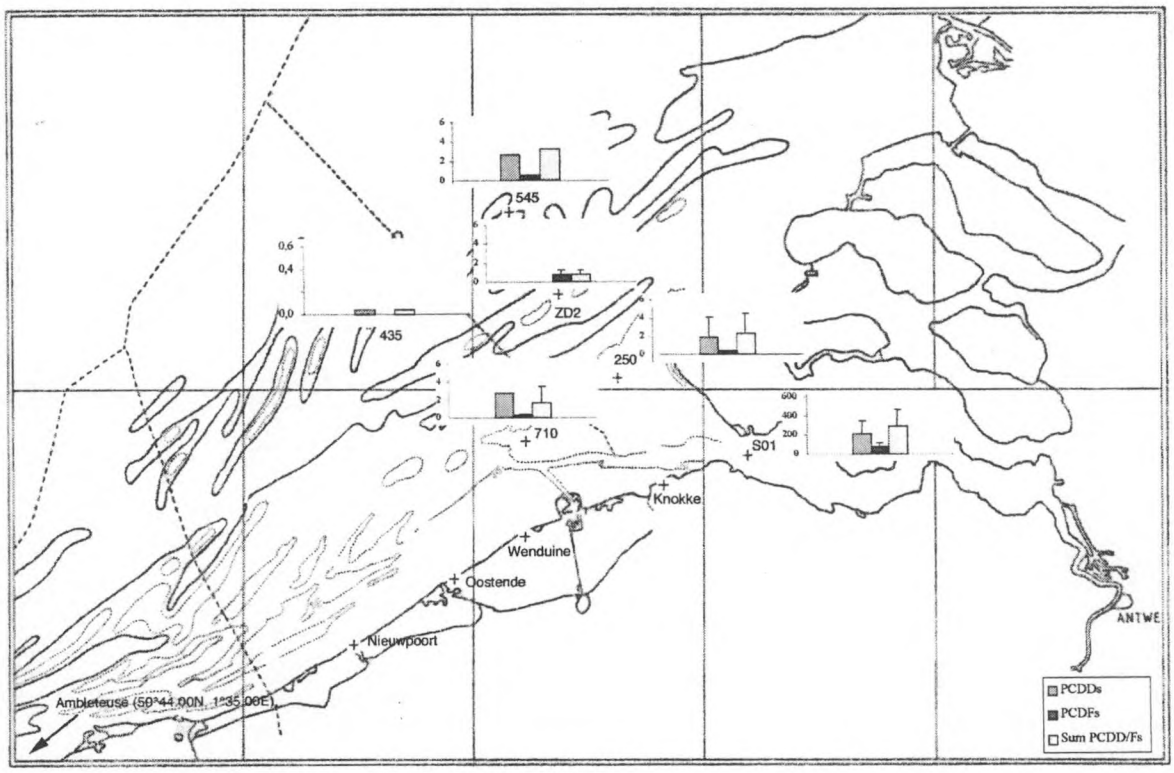
Low levels: most of the 17 PCDD/Fs congeners remain undetected or below the limit of quantification. Generally, concentrations of PCDDs were higher than those of PCDFs except for station ZD2. PCDDs profile is dominated by OCDD followed by HxCDDs as reported in other studies (Eljarrat *et al.*, 2001 ; Frignani *et al.*, 2001). On the contrary, for PCDFs, less chlorinated congener like the TCDF is present and almost detected in all samples except at station 435.

High levels: on the contrary to the low levels stations, all PCDDs congeners are detected at SO1 with also a clear dominance of OCDD. PCDFs congeners are dominated by hepta- and hexachlorinated furans. Mean concentration and TEQ of respectively 289.3 pg/g dw and 8.4 pg TEQ/g dw are within the range of what is described elsewhere for other coastal areas (Eljarrat *et al.*, 2001) and remains below the European Sediment Quality Objective set at 20 pg TEQ/g dw (Evers *et al.*, 1996).
For c-PCBs, high concentrations are detected both at stations SO1 and 710, the mix being dominated by congener 77. It is also worth noting that results obtained at these particular stations greatly vary within replicates, from a factor 10 (SO1) to a factor 100 (710).

Analysis of Mo-PCBs in subtidal sediment samples was unsuccessfull and results are thus unavailable for this matrice.

Congener pg/g dw	Subtidal stations SO1		545		710		435		250		ZD2	
TCDD	1.1	2.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PeCDD	0.4	0.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HxCDD 1	0.6	1.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HxCDD 2	1.4	3.3	ND	ND	ND	ND	0.04	ND	0.04	ND	ND	0.04
HxCDD 3	1.0	2.7	ND	ND	ND	ND	<Loq	ND	0.2	ND	ND	ND
HpCDD	14.9	38.6	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	ND	<Loq
OCDD	95.1	260.7	<Loq	2.7	ND	2.8	<Loq	<Loq	<Loq	3.5	<Loq	<Loq
[Sum PCDDs]	**114.3**	**309.9**		**2.7**		**2.8**	**0.04**		**0.27**	**3.5**		**0.04**
TCDF	5.0	10.0	<Loq	0.1	0.2	0.2	<Loq	<Loq	0.1	0.1	0.1	0.1
PeCDF 1	2.0	4.1	<Loq	<Loq	<Loq	ND	<Loq	ND	<Loq	ND	<Loq	<Loq
PeCDF 2	1.8	5.1	ND	ND	0.09	ND	<Loq	ND	0.05	ND	0.06	0.05
HxCDF 1	6.0	15.3	ND	ND	ND	ND	<Loq	ND	0.1	0.3	0.1	0.1
HxCDF 2	4.0	9.7	ND	ND	ND	ND	<Loq	ND	0.2	ND	0.1	0.1
HxCDF 3	ND	ND	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND
HxCDF 4	4.5	5.7	ND	ND	ND	ND	<Loq	ND	ND	ND	0.1	ND
HpCDF 1	26.0	50.8	ND	0.5	<Loq	<Loq	<Loq	ND	<Loq	ND	0.7	<Loq
HpCDF 2	ND	4.3	ND	ND	ND	ND	<Loq	ND	ND	ND	ND	ND
OCDF	ND	ND	ND	ND	ND	ND	<Loq	ND	ND	ND	ND	ND
[Sum PCDFs]	**49.2**	**105.1**		**0.6**	**0.4**	**0.2**			**0.4**	**0.4**	**1.2**	**0.4**
[Sum PCDD/Fs]	**163.5**	**415.0**		**3.2**	**0.4**	**3.0**	**0.04**		**0.7**	**3.9**	**1.2**	**0.4**
TEQ PCDD/Fs	**5.1**	**11.7**	**0.11**	**0.12**	**0.16**	**0.12**	**0.11**	**0.11**	**0.17**	**0.14**	**0.18**	**0.14**
77	260.6	0.5	<Loq	<Loq	251.4	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq
81	9.6	22.2	1.1	2.3	45.3	2.7	2.3	1.8	1.9	1.6	2.0	1.3
126	<Loq	1.8	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq
169	0.1	0.3	ND	ND	<Loq	ND	<Loq	ND	<Loq	0.3	<Loq	<Loq
[Sum c-PCBs]	**270.4**	**24.8**	**1.1**	**2.3**	**296.7**	**2.7**	**2.3**	**1.8**	**1.9**	**1.9**	**2.0**	**1.3**
TEQ c-PCBs	**0.13**	**0.18**	**0.10**	**0.10**	**0.13**	**0.10**	**0.10**	**0.10**	**0.11**	**0.10**	**0.10**	**0.10**

Table 4: Concentrations (pg/g dry weight) and TEQs (pg TEQ/g dry weight) of PCDD/Fs, c-PCBs detected in sediment collected in six distinct subtidal stations. TEF values used are human TEFs, < Loq : smaller than the limit of quantification, ND : not determined.



Map 1: Concentrations of PCDD/Fs congeners (pg/g dry weight) in sediments sampled at different subtidal stations.

5.1.1.2 Intertidal stations.

Results of PCDD/Fs and c-PCBs congeners are described in Table 5. In these intertidal sediments, those sampled at Nieuwport displayed the higher levels both in concentrations and TEQs. At this station, most of the 17 PCDD/Fs congeners were detected and quantified compared to the other stations for which many congeners remained undetected or below the limit of quantification. These results suggest that a ponctual and very localised source of contamination is present at the Nieuwport station.

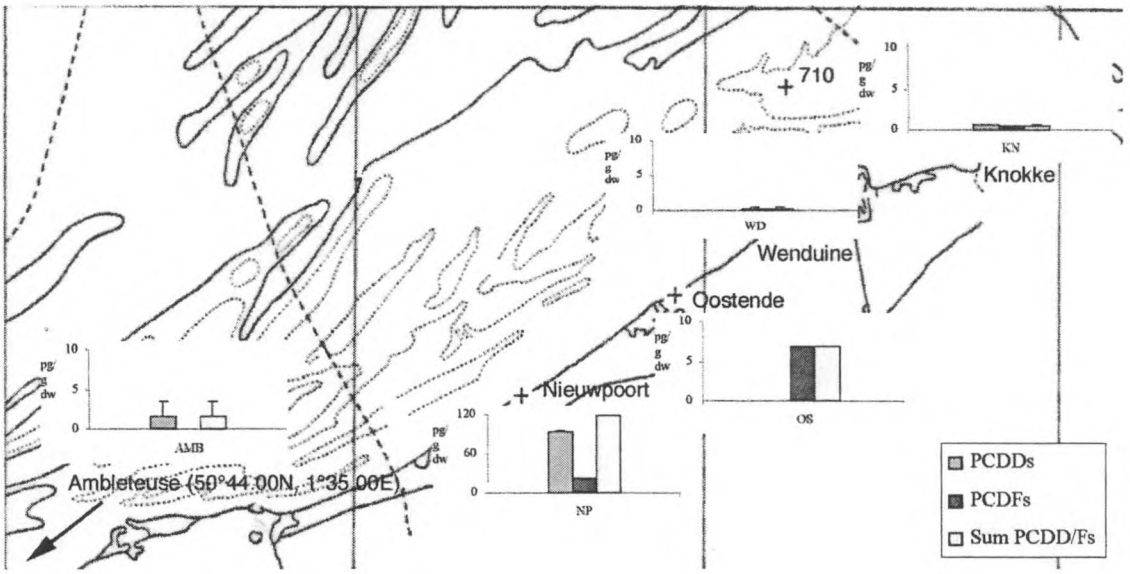
Congeners distribution of PCDD/Fs are similar to what is described above for subtidal sediments.

The highest TEQs values, also detected at Nieuwport, are well below the European Sediment Quality Objective set at 20 pg TEQ/g dw (Evers *et al.*, 1996).

Analysis of Mo-PCBs in subtidal sediment samples was unsuccessfull and results are thus unavailable for this matrice.

Congener pg/g dw	Intertidal stations AMB		NP		OS		WD		KN	
TCDD	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.6
PeCDD	1.8	ND	0.5	0.4	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq
HxCDD 1	ND	ND	0.6	0.3	ND	ND	ND	ND	ND	ND
HxCDD 2	ND	ND	1.0	1.0	ND	ND	ND	ND	ND	ND
HxCDD 3	1.1	0.2	1.0	0.9	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq
HpCDD	ND	<Loq	15.6	20.1	<Loq	<Loq	<Loq	<Loq	ND	<Loq
OCDD	ND	ND	75.9	70.8	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq
[Sum PCDDs]	**2.9**	**0.2**	**94.6**	**93.6**						**0.6**
TCDF	ND	<Loq	5.5	5.4	0.1	<Loq	<Loq	<Loq	ND	ND
PeCDF 1	ND	ND	0.4	0.5	6.8	ND	0.3	ND	0.41	ND
PeCDF 2	ND	ND	2.1	2.2	ND	ND	ND	ND	ND	ND
HxCDF 1	ND	ND	1.8	1.7	<Loq	ND	<Loq	<Loq	ND	ND
HxCDF 2	ND	ND	1.9	1.7	0.1	ND	0.05	0.08	ND	ND
HxCDF 3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HxCDF 4	ND	ND	3.4	3.2	ND	ND	ND	ND	ND	ND
HpCDF 1	ND	ND	6.6	8.8	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq
HpCDF 2	ND	ND	0.6	0.9	ND	ND	ND	ND	ND	ND
OCDF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
[Sum PCDFs]			**22.2**	**24.3**	**7.0**		**0.4**	**0.1**	**0.4**	
[Sum PCDD/Fs]	**2.9**	**0.16**	**116.8**	**117.9**	**7.0**		**0.4**	**0.1**	**0.4**	**0.6**
TEQ PCDD/FS	**2.0**	**0.1**	**3.4**	**3.3**	**0.5**	**0.1**	**0.1**	**0.1**	**0.1**	**0.7**
77	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq	17.9
81	ND	ND	<Loq	<Loq	<Loq	1.2	<Loq	<Loq	<Loq	2.9
126	ND	ND	<Loq	1.4	<Loq	<Loq	<Loq	<Loq	<Loq	<Loq
169	ND	ND	0.1	0.1	ND	ND	ND	ND	ND	ND
[Sum c-PCBs]			**0.1**	**1.5**		**1.2**				**20.7**
TEQ c-PCBs	**0.10**	**0.10**	**0.10**	**0.14**	**0.10**	**0.10**	**0.10**	**0.10**	**0.10**	**0.10**

Table 5: Concentrations (pg/g dry weight) and TEQs (pg TEQ/g dry weight) of PCDD/Fs, c-PCBs detected in sediment collected in five distinct intertidal stations. TEF values used are human TEFs < Loq : smaller than the limit of quantification, ND : not determined.



Map 2 : Concentrations of PCDD/Fs congeners (pg/g dry weight) in sediments sampled at different intertidal stations.

5.1.2 Organic contents of coastal and subtidal sediments.

Sediments sampled at station SO1 displayed the highest organic content of all subtidal stations as shown by Table 6. Sediments sampled at SO1 were characterized by a finer composition clearly enriched with clay and silt from the Scheldt estuary.

Subtidal stations	Percentage of organic matter Mean ± sd
710	0.34 ± 0.02
SO1	3.14 ± 0.20
435	0.36 ± 0.02
545	0.31 ± 0.02
ZD2	0.29 ± 0.01
250	0.34 ± 0.05
Intertidal stations	**Percentage of organic matter Mean ± sd**
AMB	0.22 ± 0.01
OS	0.38 ± 0.04
NP	4.71 ± 0.13
KN	0.32 ± 0.01
WD	0.53 ± 0.35

Table 6: Percentage of organic matter (%) expressed as mean ± standard deviation of sediment samples collected at 5 intertidal and 6 subtidal stations.

Sediments sampled at station NP displayed the highest organic content of all interdinal stations.

For both intertidal and subtidal sediments, results indicate that the highest concentrations of PCDD/Fs are detected in sediments displaying the highest total organic content (tables 4, 5 and 6).

5.1.3 CALUX bioassays.

The CALUX procedure set up for fat was slightly modified for the analysis of sediments. Indeed, PAHs can be present at high concentration in sediment, whereas the concentration of PAHs in fat is usually very low since they are quickly metabolized. The PAHs can give an important response in CALUX, even after 24h incubation (Behnisch *et al.*, 2003).
Most of the PAHs, but not all, are degraded on the acidic silica gel. To insure that all PAHs are degraded, silver nitrate is used besides the acidic silica gel during the clean-up (the complete degradation of PAHs was confirmed by chemical analysis).
Validation was performed with a river sediment sample highly contaminated with PAHs.

5.1.3.1 Dioxins fraction.

There is slightly more variation of the results according to the dilution, when compared to the results obtained for fat. Nine samples of the same sediment have been analyzed at 2 or 3 different dilutions (Figure 2) during 2 months. The RSD of all data, at the different dilution is 18%.
More data are needed for a complete evaluation of the validation of CALUX.

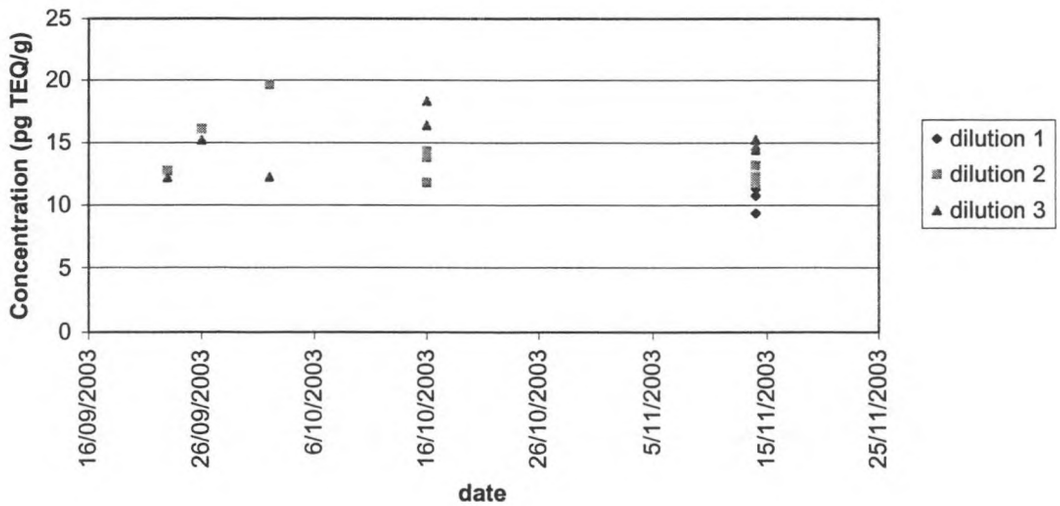


Figure 2: Control chart set up with CALUX results of the dioxin fraction of a quality control sample of sediment.

5.1.3.2 PCBs fraction.

The analysis of the PCBs fraction could not be performed with the procedure applied here since some compounds introduced by the procedure, decrease or suppress the CALUX response of the PCBs fraction. In order to analyze the PCBs fraction, the procedure should be modified.

5.1.3.3 Application of CALUX to marine sediments and comparison with chemical analysis.

On the 22 sediment samples analyzed by CALUX and GC-HRMS, only 5 samples were above the concentration of 0.5 pg TEQ/g for PCDD/F (see Tables 4 and 5: GC-HRMS results). The other samples were at low to extremely low concentrations, for which only few (and sometimes no) congeners can be detected. Results are then presented in 2 sets: high and low concentration.

High concentrations.

Results are more complex for sediment than for fat: the concentrations measured in CALUX increases when the extract is diluted (Figure 3) since the dose-response curves of TCDD and the extract are not parallel.

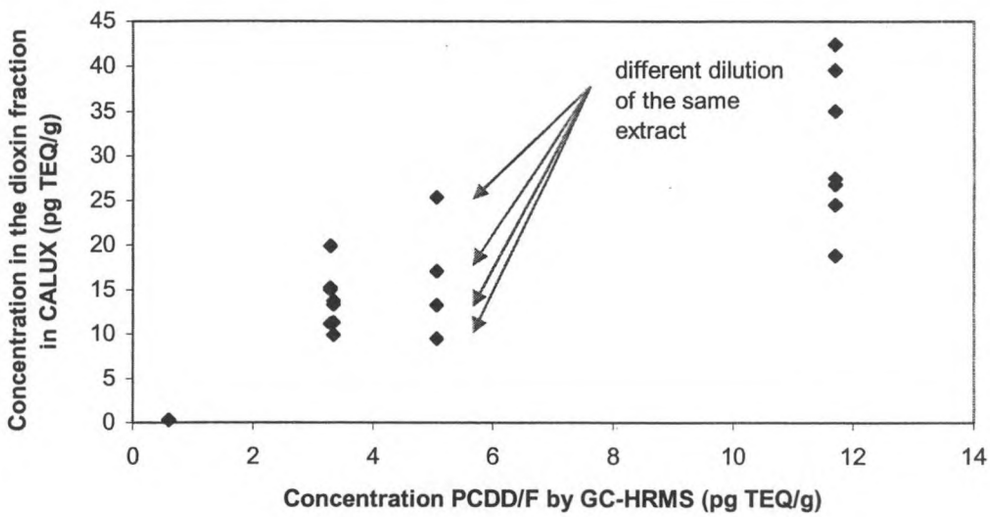


Figure 3: Comparison of CALUX results for the dioxins fraction and concentrations of PCDD/Fs by GC-HRMS for sediment samples (different dilution, high concentration).

Since antagonistic and toxic effects are at the minimum when the sample is diluted, the Figure 4 was drawn with only the dilution giving a result close to the LOQ (0.78 pg TEQ/well).
More data would be needed for a better comparison, but these first data indicates that CALUX and GC-HRMS are correlated, and that CALUX measure approximately 4 times more than

GC-HRMS. Different parameters can explain this difference: 1) the difference between TEF and REP, 2) antagonistic or synergistic effects, 3) the quantification limits and the presence of interferences in GC-HRMS analysis and 4) the presence of other AhR ligands.

In order to investigate the relative importance of the different parameters, the concentrations measured by GC-HRMS were multiplied by the REP instead of the TEF. These values are represented by open circles in Figure 4. The values calculated in this way are very close to the values calculated using the TEF and represented by the dotted line with a slope of 1 in the Figures. The difference between TEF and REP is thus not responsible of the observed discrepancy between the two methods for the sediments.

Synergistic effects are not common in CALUX. One example is corticosteroids: corticosteroids alone induce a very weak response in CALUX, but dramatically enhance the response of TCDD. Corticosteroids are degraded during the clean-up as probably all compounds of this class which would be susceptible to enhance the CALUX response. Antagonistic effects are much more common and are described for PCBs (review : Safe, 1997), α-naphtoflavone (Wilhelmsson *et al.*, 1994), PCN, hexachlorobenzene (Windal *et al.*, 2003). During the clean-up, most of the PAH are degraded, PCBs and hexachlorobenzene are discarded from the dioxin fraction, but some other antagonistic compounds may still be present. The result of the dioxin fraction can thus be underestimated compared to results for which no interaction occurs between compounds.

When some congeners that represent an important part of the TEQ value are not detected in GC-HRMS, or when there is an interference for one of these congeners, the concentrations measured by GC-HRMS can be largely underestimated. In CALUX analysis, this bias of measurement is not observed since one global response, expressed in pg TEQ/g, is measured for all AhR ligands. In this case, the discrepancy between the two methods can be due, at least in part, to the limit of quantification or the lack of selectivity of the GC-HRMS analysis.

The higher values measured by CALUX for the dioxin fractions, compared to the PCDD/F concentrations measured by GC-HRMS could also be due to the presence of other AhR ligands.

The list of the already identified AhR ligands is long. A review on classical AhR ligands was published by van Birgelen, 1998. The authors list RElative Potencies (REP) of some AhR agonists for different bioanalyses and provide a diagnosis of the pollutants' sources, fates and levels in the environment. Additional information is available in reviews from Denison and Heat-Pagliuso (1998), Denison *et al.* (1999, 2002, 2003), Hoogenboom *et al.* (1999) and Seidel *et al.* (2000).

GC-HRMS analyzes only the PCDD/F. CALUX analyzes PCDD/F+ other AhR ligands. Consequently, a good correlation between CALUX and GC-HRMS imply that the ratio of PCDD-Fs/other AhR ligands is constant. For the sediments, the difference observed for the two methods is probably due to the presence of other AhR ligands.

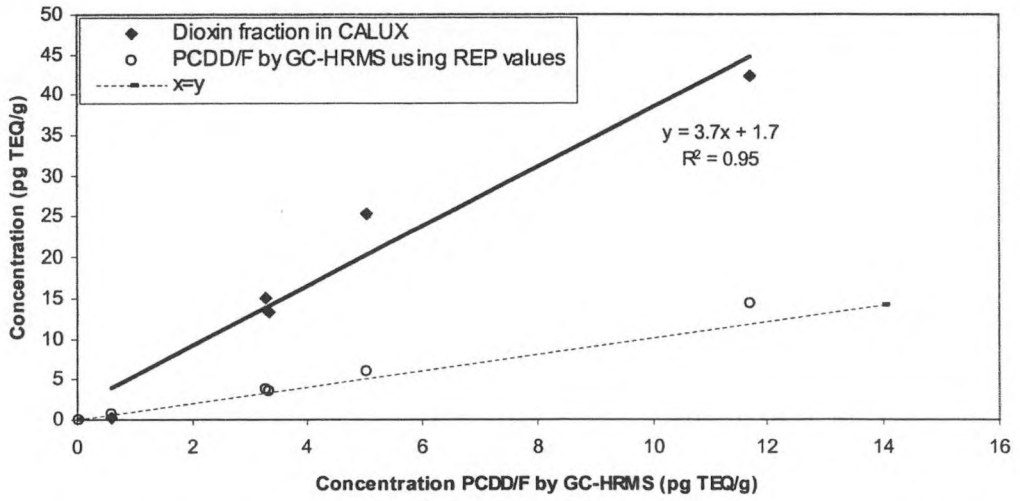


Figure 4: Comparison of CALUX results for the dioxin fraction and concentrations of PCDD/Fs by GC-HRMS for sediment samples, when the dilution, in CALUX, is chosen so that the measurement is closed to the limit of quantification.

Low concentrations.

All samples were above the quantification limits in CALUX, but only few congeners were detected in GC-HRMS. Therefore, there is no correlation at these extremely low levels (Figure 5).

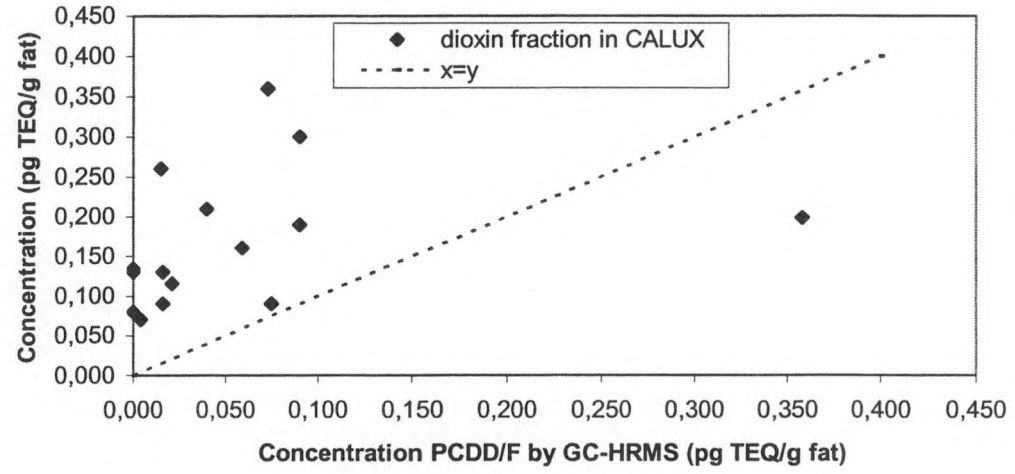


Figure 5: Comparison of CALUX results for the dioxin fraction and concentrations of PCDD/Fs by GC-HRMS for sediment samples (very low concentrations).

5.2 Analyses of starfishes (*Asteria rubens*).

5.2.1 Levels of PCDD/Fs, c-PCBs using GC-HRMS and Mo-PCBs using GC/MS-MS.

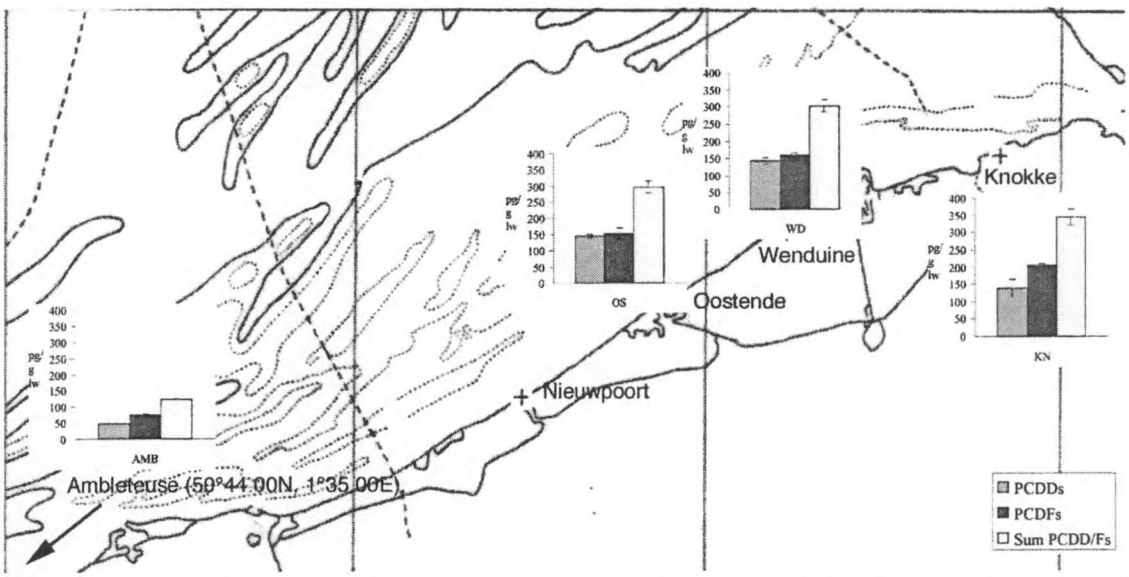
Levels of dioxin and dioxin-like compounds detected in pyloric caecas of starfishes sampled along the Belgian coast are presented in Table 7. Replicates were analysed for each of the stations except for that of Nieuwport for which the sample was lost.

Asteria rubens	**AMB** n = 3	**NP** n = 1	**OS** n = 3	**WD** n = 2	**KN** n = 2
Congeners					
TCDD	0.82 ± 0.04	-	7.7 ± 0.5	7.9 ± 0.7	11.8 ± 1.5
PeCDD	2.06 ± 0.26	-	7.8 ± 0.2	8.0 ± 0.1	7.7 ± 0.6
HxCDD 1	1.03	-	2.7 ± 0.6	3.4 ± 0.2	3.0 ± 0.3
HxCDD 2	3.2 ± 0.7	-	11.7 ± 0.8	12.3 ± 1.4	10.9 ± 1.3
HxCDD 3	1.51 ± 0.12	-	4.6 ± 0.5	5.2 ± 0.8	4.3 ± 1.0
HpCDD	10.8 ± 1.2	-	32.9 ± 2.6	31.9 ± 0.8	30.6 ± 7.0
OCDD	30.3 ± 1.0	-	78.2 ± 5.0	75.1 ± 8.6	69.9 ± 15.4
Total PCDDs	**47.3 ± 4.2**	-	**145.6 ± 5.3**	**143.7 ± 9.8**	**138.2 ± 26.5**
TCDF	70.25 ± 4.65	-	124.0 ± 14.9	134.9 ± 8.3	156 ± 2.7
PeCDF 1	< Loq	-	0.9 ± 0.3	< Loq	1.5 ± 1.2
PeCDF 2	6.8 ± 0.4	-	22.0 ± 3.8	21.4 ± 0.4	44.4 ± 9.2
HxCDF 1	0.58 ± 0.44	-	0.4 ± 0.2	0.74	0.66
HxCDF 2	< Loq	-	< Loq	< Loq	0.47
HxCDF 3	< Loq	-	< Loq	< Loq	< Loq
HxCDF 4	0.6 ± 0.06	-	3.5 ± 0.3	2.83 ± 0.01	4.3 ±1.0
HpCDF1	< Loq	-	3.61	< Loq	< Loq
HpCDF2	< Loq	-	< Loq	< Loq	< Loq
OCDF	< Loq	-	< Loq	< Loq	< Loq
Total PCDFs	**75.5 ± 1.4**	-	**151.9 ± 17.6**	**159.5 ± 7.3**	**206.7 ± 3.4**
Total PCDD/Fs	**122.8 ± 3.4**	-	**297.5 ± 18.9**	**303.1 ± 17.1**	**345 ± 23**
TEQ Fish PCDD/Fs	**8.6 ± 2.8**	-	**34.8 ± 2.6**	**35.6 ± 1.0**	**51.8 ± 2.3**
TEQ Human PCDD/Fs	**14.1 ± 2.2**	-	**41.7 ± 3.0**	**42.7 ± 1.3**	**60.1 ± 1.8**
c-PCBs					
PCB 77	*	-	7288 ± 141	6359 ± 1176	-
PCB 81	*	-	96.1 ± 2.4	229 ± 47	-
PCB 126	*	-	1029.93	988 ± 85	-
PCB 169	*	-	92.7 ± 2.4	95.4 ± 4.4	-

Total c-PCBs	*	-	**7621± 483**	**7671 ± 1312**	-
TEQ Fish c-PCBs	*	-	**5.5 ± 0.3**	**5.7 ± 0.6**	**7.1 ± 3.0**
TEQ Human c-PCBs	*	-	**97.1 ±7.8**	**100.4 ± 8.7**	**137.2 ± 58.6**
Mo-PCBs		-			-
PCB 123	19.4 ± 32.8	-	181.7 ± 56.1	**2.2** ± 0.1	-
PCB 118	45.8 ± 39.7	-	1.7 ± 1.5	225.1 ± 30.0	-
PCB 114	0.2 ± 0.2	-	0.7 ± 0.7	-	-
PCB 105	13.0 ± 0.6	-	37.8 ± 12.8	42.5 ± 2.3	-
PCB 167	2.4 ± 2.1	-	11.6 ± 12.8	14.0 ± 0.6	-
PCB 156	4.3 ± 1.5	-	13.3 ± 3.6	15.2 ± 0.7	-
PCB 157	1.3± 0.1	-	3.7 ± 0.7	4.6 ± 0.03	-
PCB 189	0.5 ± 0.2	-	26.2	2.0 ± 0.7	-
Total Mo-PCBs	**86.9 ± 8.3**	-	**259.3 ± 62.6**		-
TEQ Fish Mo-PCBs	**0.4 ± 0.04**	-	**12.1 ± 18.8**	**1.5 ± 0.2**	-
TEQ Human Mo-PCBs	**10.8 ± 0.2**	-	**36.1 ± 7.5**	**37.2 ± 3.6**	-

Table 7: Concentrations (pg/g lipids weight) and TEQs (pg TEQ/g lipids weight) of PCDD/Fs, c-PCBs and Mo-PCBs (ng/g lw) expressed as mean ± standard deviation detected in starfishes, *Asteria rubens*. < Loq : smaller than the limit of quantification. * : very low recoveries, unvalid result. - : not available.

The lowest levels of PCDD/Fs were recorded at the Ambleteuse station and appear to increase towards the Knokke station (Map 3). Although results are not available for Ambleteuse and Nieuwport, it can be noted that the same trend is observed for c-PCBs.



Map 3: Concentrations of PCDD/Fs congeners in starfishes (*Asteria rubens*) at different intertidal stations.

Although a direct effect of the Scheldt estuary on organisms collected at the Knokke station is most probable (OSPAR, 2000), our results suggest the influence of ponctual and very localised source of contamination at this station.

5.2.2 Comparison with Cytochrome P450 immunopositive protein (CYP1A IPP) induction.

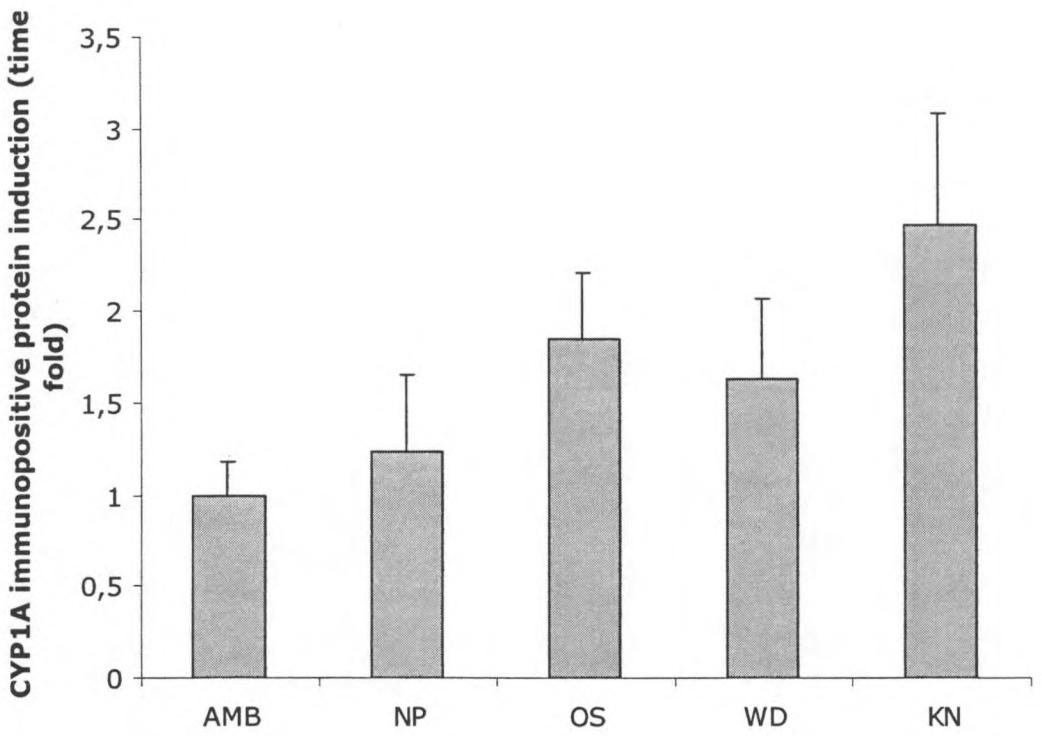


Figure 6: CYP1A1 induction in starfishes (*Asteria rubens*) collected at different intertidal stations along the Belgian coast.

The induction of a CYP1A immunopositive protein (CYP1A IPP) was measured using competitive ELISA in starfishes collected in the different stations (Figure 6). Significant differences (pANOVA=0.005) were detected between the different stations: individuals from Knokke displayed higher CYP1A IPP levels. The lowest induction value was recorded at both stations of Ambleteuse and Nieuwport. Values measured in organisms from Ostende and Wenduine are quite similar and do not differ significantly. Correlations were calculated between measured contaminant levels and CYP1A IPP induction. When considering concentrations, significant correlations were found between CYP1A induction and PCDD levels in sediments, and levels of various congeners in mussels (OCDD, PCDDs, PCB 77 and c-PCBs). Some of these correlations were negative (with PCDDs in sediments, PCB 77 and PCBs in mussels). The r value as well as the level of significance of these correlations was

very similar (r ranging between 0.88 and 0.91; p ranging between 0.03 and 0.05). Regarding TEQ measurements, significant correlations were found between CYP1A IPP induction and levels of PCDFs in mussels and various contaminant levels in starfishes (TCDD, PeCDF, HxCDF4, PCDFs and PCB 126). All these correlations were positive with determination coefficients ranging from 0.88 to 0.99 and their associate probability ranging from 0.02 to 0.05.

These results suggest that dioxin and dioxin-like contaminants contained in the sediment compartment represent a potential and non neglectable route of exposure to benthic organisms such as proposed by Danis *et al.* (2003) studying PCB uptake pathway in starfishes.

5.3 Analyses of mussels (*Mytilus edulis*).

5.3.1 Levels of PCDD/Fs, c-PCBs using GC-HRMS and Mo-PCBs using GC/MS-MS.

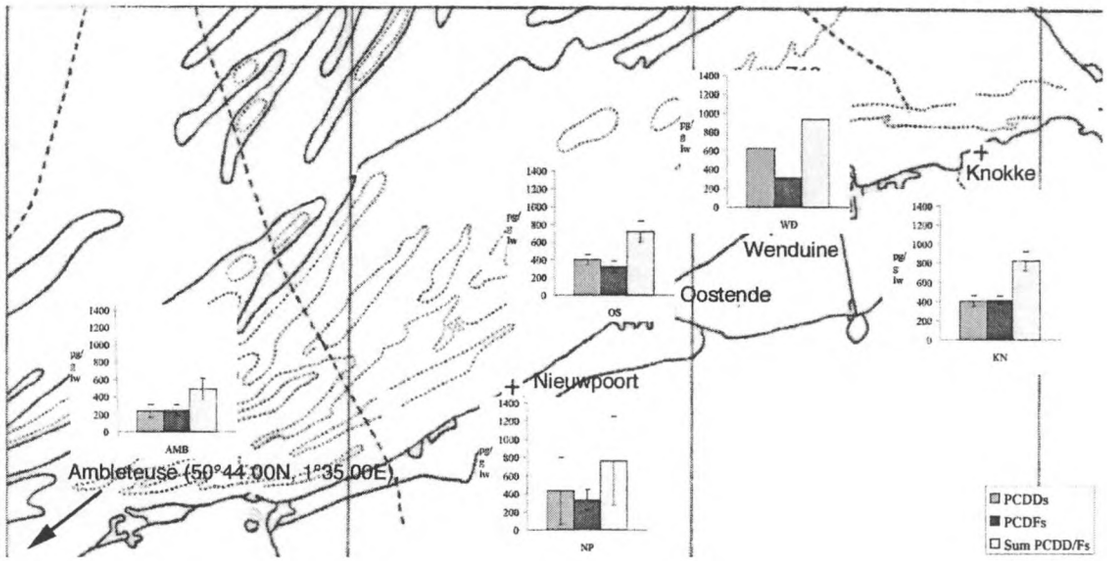
Mean levels of PCDD/Fs, c- and Mo-PCBs detected in whole soft tissues of mussels collected at different intertidal stations along the Belgian coast are presented in Table 8. As these animals are commonly consummed by humans, toxic equivalents using human TEFs values have been added.

Mytilus edulis	**AMB** n = 3	**NP** n = 3	**OS** n = 3	**WD** n = 1	**KN** n = 2
Congeners					
TCDD	< LOQ	4.6	5.7 ± 0.4	7.1	13.1 ± 1.2
PeCDD	3.3 ± 0.3	6.3 ± 1.0	5.8 ± 0.8	6.4	7.5 ± 0.7
HxCDD 1	4.7 ± 1.6	9.0 ± 5.9	3.9 ± 0.1	4.5	5.2 ± 0.02
HxCDD 2	7.4 ± 3.2	9.9 ± 4.7	10.1 ± 1.8	13.3	12.5 ± 1.5
HxCDD 3	6.1 ± 1.7	6.5 ± 4.0	5.6 ± 1.2	6.0	6.2 ± 1.4
HpCDD	57.3 ± 15.3	87.3 ± 68.8	75.1 ± 4.7	97.3	86.0 ± 0.1
OCDD	165.0 ± 62.0	314.4 ± 293.9	293.0 ± 52.7	489.3	277.6 ± 55.0
Total PCDDs	**240.0 ± 73.9**	**430.6 ± 369.6**	**399.1 ± 60.0**	**623.8**	**408.1 ± 59.5**
TCDF	162.2 ± 43.9	176.5 ± 25.8	168.5 ± 30.9	130.5	178.3 ± 28.7
PeCDF 1	13.3	12.2	13.6 ± 1.2	15.3	21.0 ± 1.1
PeCDF 2	36.9 ± 8.1	46.7 ± 14.0	42.9 ± 3.1	39.5	60.8 ± 4.6
HxCDF 1	6.3 ± 3.3	19.1 ± 15.6	10.1 ± 3.9	11.7	19.1 ± 2.5
HxCDF 2	4.9 ± 2.1	7.1 ± 4.6	4.5 ± 0.9	4.8	8.2 ± 0.4
HxCDF 3	0.34	< Loq	0.24	< Loq	< Loq
HxCDF 4	6.9 ± 4.4	11.9 ± 8.9	7.7 ± 0.1	8.0	12.5 ± 1.0
HpCDF1	13.8 ± 4.4	32.6 ± 25.9	29.9 ± 9.7	34.4	46.6 ± 4.6
HpCDF2	0.8 ± 0.5	4.8 ± 5.7	1.9 ± 0.4	1.8	3.2 ± 0.2

OCDF	12.5 ± 4.3	33.4 ± 22.7	42.3 ± 22.1	62.6	65.6 ± 5.4
Total PCDFs	**248.4 ± 63.0**	**332.2 ± 114.6**	**321.5 ± 62.1**	**308.7**	**415.2 ± 38.8**
Total PCDD/Fs	**488.4 ± 122.6**	**762.8 ± 484.0**	**720.6 ± 120.1**	**932.4**	**823.3 ± 98.3**
TEQ Fish PCDD/Fs	**33.7 ± 5.6**	**47.0 ± 14.5**	**46.8 ± 2.2**	**46.0**	**68.4 ± 1.5**
TEQ Human PCDD/Fs	**41.5 ± 8.13**	**54.3 ± 14.2**	**55.8 ± 4.0**	**53.3**	**77.7 ± 3.2**
co-planar PCBs					
PCB 77	4121 ± 611	3762 ± 1805	3292 ± 2533	3149	3269 ± 1757
PCB 81	534 ± 460	902.4 ± 719.4	195.1 ± 16.6	179.1	120.6 ± 15.5
PCB 126	833 ± 77	705.4 ± 63.8	861.0 ± 113.0	843.0	927.1 ± 39.9
PCB 169	85 ± 8	82.6 ± 5.9	86.3 ± 13.4	97.0	116.6 ± 5.6
Total c-PCBs	**5574 ± 970**	**5452 ± 2428**	**4435 ± 2672**	**4268**	**4433 ± 1818**
TEQ Fish c-PCBs	**4.8 ± 0.4**	**4.2 ± 0.1**	**4.7 ± 0.8**	**4.6**	**5.1 ± 0.4**
TEQ Human c-PCBs	**84.7 ± 7.8**	**71.8 ± 6.1**	**87.3 ± 11.7**	**85.6**	**94.2 ± 4.2**
Mo-PCBs					
PCB 123	-	-	90 ± 153	291 ± 99	3.6 ± **2 .2**
PCB 118	-	-	29.4 ± 45.6	4.2 ± 1.3	306 ± **139**
PCB 114	-	-	0.1 ± 0.2	2.4 ± 1.1	35.6 ± **60.6**
PCB 105	-	-	33.3 ± 29.6	46.6 ± 65.8	71.3 ± **38.7**
PCB 167	-	-	3515 ± 6064	15.0 ± 1.3	24.2 ± **9.2**
PCB 156	-	-	16.4 ± 9.0	0.8 ± 1.2	26.4 ± **10.9**
PCB 157	-	-	78.1 ± 127.8	6.5 ± 2.9	11.3 ± **7.7**
PCB 189	-	-	22.3 ± 36.5	120 ± 163	61.2
Total Mo-PCBs	-	-	**3773 ± 6060**	**487 ± 335**	**361 ± 80.6**
TEQ Fish Mo-PCBs	-	-	**18.9 ± 33.0**	**2.4 ± 1.7**	**2.5 ± 1.2**
TEQ Human Mo-PCBs	-	-	**121 ± 152**	**51.2 ± 35.5**	**75.1 ± 48.4**

Table 8: Concentrations (pg/g lipids weight) and TEQs (pg TEQ/g lipids weight) of PCDD/Fs, c-PCBs and Mo-PCBs (ng/g lw) expressed as mean ± standard deviation detected in mussels, *Mytilus edulis*. < Loq : smaller than the limit of quantification. - : not available.

Similarly to what has been observed for starfishes, lowest PCDD/Fs concentration are found at Ambleteuse and progressively increase towards the Knokke station (Map 4). For PCBs, however, no clear increasing tendency is observed. A large variation in between the results obtained at the same station is also observed for Mo-PCBs (large standard deviations).



Map 4: Concentrations of PCDD/Fs congeners in mussels (*Mytilus edulis*) at different subtidal stations.

5.3.2 CALUX bioassay.

5.3.2.1 Validation.

Separation of PCBs and PCDD/Fs.

During the clean-up, the extract is split into a "dioxins fraction" and a "PCBs fraction" on the carbon column placed after the acidic silica column. The specifications given by the carbon producer are as follow:

1) all c-PCBs are in the PCBs fraction except the PCB 169 for which maximum 30% can be present in the dioxin fraction.

2) as OCDD tends to bind strongly to the carbon, a minimum of 50% OCDD is collected in the dioxins fraction.

The quality of the separation was confirmed in our Laboratory using a standard solution of c-PCBs and analysis by GC-MSMS: only 18 % of PCB 169 was found in the dioxin fraction, and the remaining part was in the PCB fraction with a mean percentage recovery of 80%. When performing experiments with ^{14}C 2,3,7,8 TCDD for the determination of the percentage recovery, a mean of 7% of ^{14}C 2,3,7,8 TCDD was recovered in the PCBs fraction (n=12, RSD=38%) and 78% was recovered in the dioxins fraction (n=15, RSD=8%).

Dioxin fraction.

The repeatability, the intermediate precision and the impact of the column size on the results has been evaluated using 30 results of the cod liver oil used as quality control sample. The control chart set up with these data in one year is illustrated in Figure 2 .

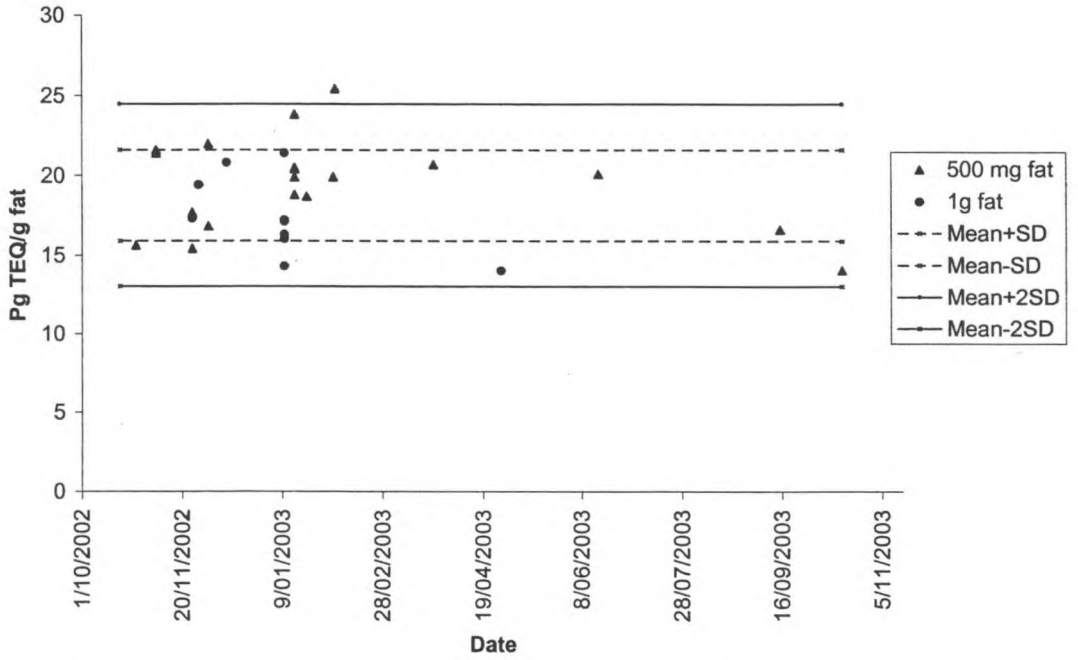


Figure 7: Control chart set up with CALUX results of the dioxin fraction of a quality control sample of cod liver oil.

As described above, the amount of acidic silica gel used for clean-up is adjusted to the amount of fat to be analyzed. Nevertheless, the size of the column has no statistically significant effect on the results ($p=0.78$), and all data are then considered as one set in the following discussion.

The relative standard deviation (RSD) for repeatability is 9% and the RSD for intermediate precision (within-lab reproducibility) is 15%. As the RSD associated with the measure of the standard solution used to check the quality of the plate is 10%, the sample preparation is very reproducible.

The quantification limits of the method depend of the quantity of fat used for the measurement: the procedure applied here has been used with 0.5 or 1 g of fat (different acidic silica column size); the procedure applied for 1g of fat can be used for amounts up to 1.5 g fat. In this case (1.5 g fat), the quantification limit of the method is 1.25 pg TEQ/g fat. If required, bigger acidic silica columns can be used to analyze higher amounts of fat and lower the detection limits.

PCBs fraction.

The RSD for intermediate precision of the PCBs fraction, calculated for 20 samples of cod liver oil (Figure 8), is higher than for the dioxin fraction: 22% compared to 15%. In the same way, the RSD associated with repeatability is higher for the PCB fraction (19%) than for the dioxin fraction (9%). The difference may be due to some losses by evaporation of the PCBs, and to the presence of toxic compounds in this fraction. Nevertheless, the RSD associated with the measurements remains below the 30% proposed by the European Commission (commission directive 2002/69/EC and 2002/70/EC) for bioassays used as screening for the determination of dioxins in food and feedstuff.

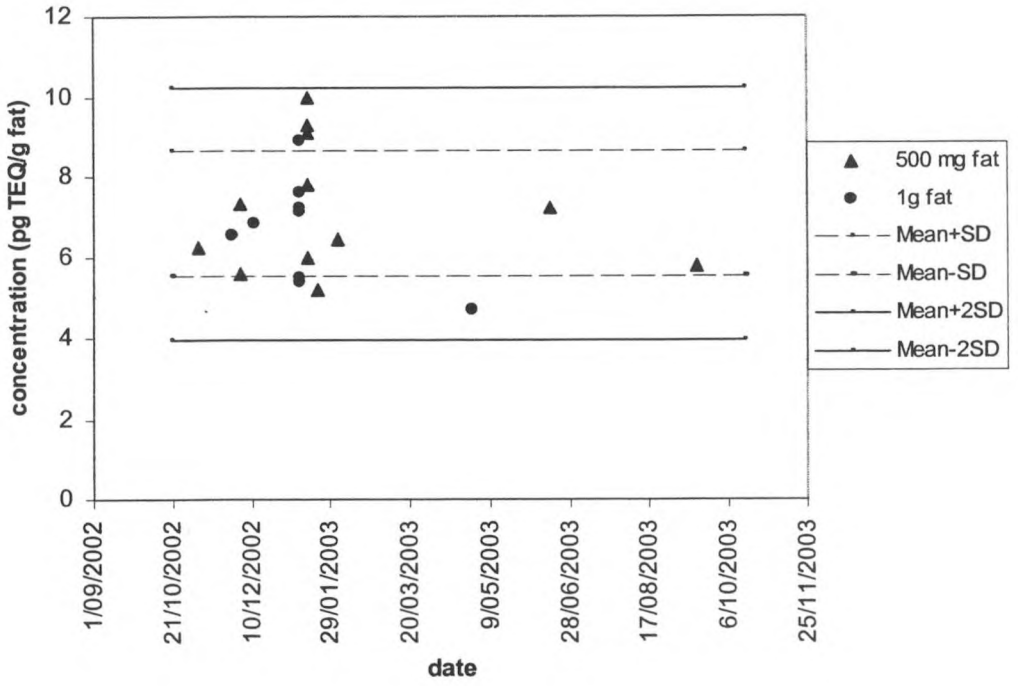


Figure 8: Control chart set up with CALUX results of the PCBs fraction of a quality control sample of cod liver oil.

5.3.2.2 Dioxin fraction

Results obtained for the dioxins fraction in CALUX and chemical analysis of PCDD/Fs are highly correlated for mussels and starfishes with R^2 values of respectively 0.73 and 0.77 and $p<<0.001$ (Figure 9). However, the ratio of CALUX /chemical results varies between 4 and 12 with an average of 8 (SD=1.9) that contrasts to the average ratio for seabird, marine mammals and fishes of 2.8 (SD=1.1, range 1.3-5.4) (see details in Table 10).This difference between vertebrates and invertebrates may be linked to the difference in Ah receptor: an AhR homologue has been identified in several invertebrates, but is unable to bind typical AhR

ligands (Hahn, 2002). Since the biotransformation systems in invertebrate are different from those of vertebrates, it would not be surprising that the ratio between PCDD/Fs and other classical AhR ligands is different in vertebrates or invertebrates. For example, PAH are known to bioaccumulate in mussels (Narbonne *et al.*, 1992) and to be quickly metabolized in vertebrates (Postlind *et al.*, 1993, Denison *et al.*, 1998, Hamer *et al.*, 2000, Vondracek *et al.*, 2001).

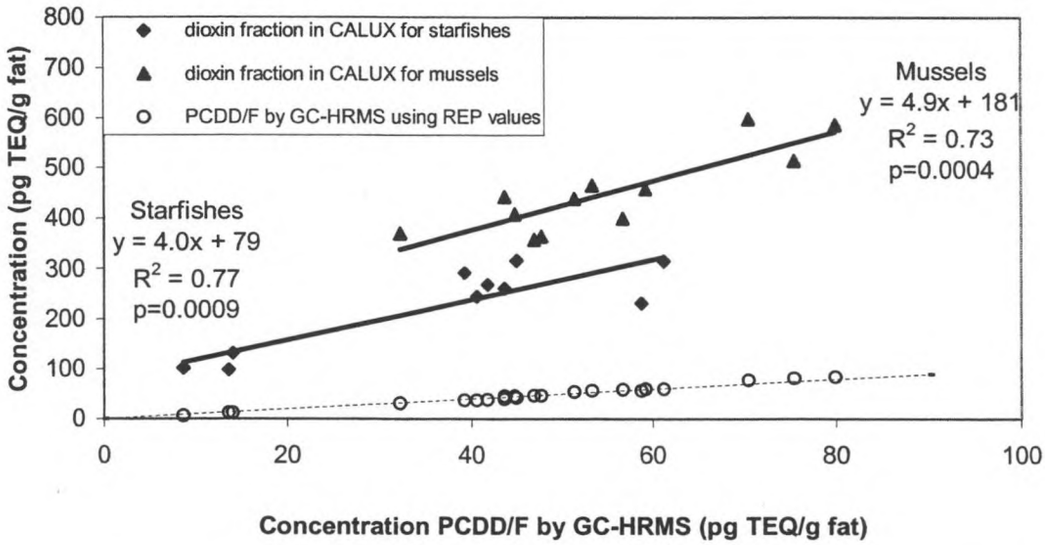


Figure 9: Comparison of CALUX results for the dioxins fraction and concentrations of PCDD/Fs by GC-HRMS for mussels and starfishes samples.

5.3.2.3 PCBs fraction.

Mussels.

Since the range of PCBs concentrations measured in mussels is narrow, and considering the quite high relative standard deviation associated with the measurements in CALUX (22%), no correlation is found between concentrations of c-PCBs measured by GC-HRMS and CALUX for this matrice (Figure 10). Nevertheless, the concentrations measured in CALUX are roughly one third of the concentrations measured by GC-HRMS.

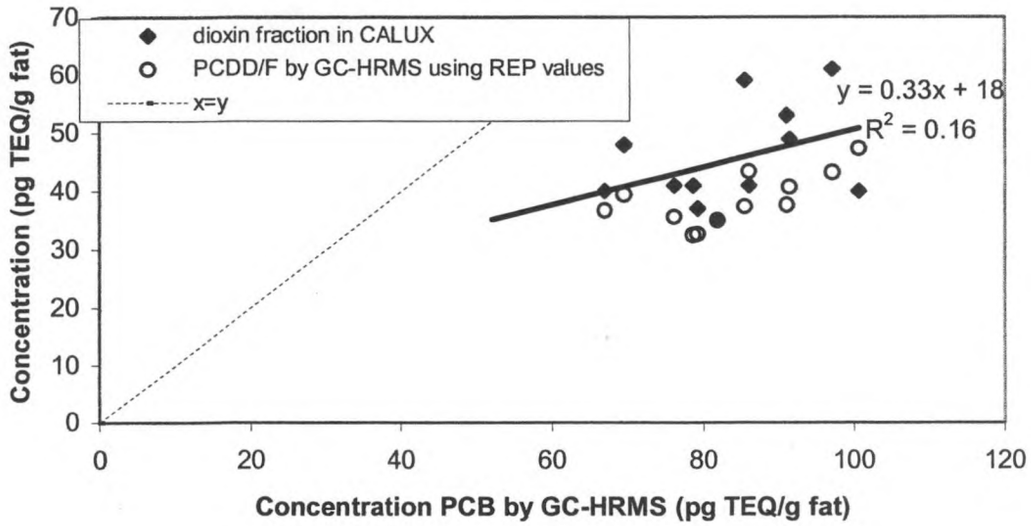


Figure 10: Comparison of CALUX results for the PCB fraction and concentrations of cPCB by GC-HRMS for mussels samples.

Starfishes.

PCBs results are not available for starfishes in CALUX and/or in GC-HRMS.

5.4 Analyses of benthic fishes : dab (*Limanda limanda*) and Dover sole (*Solea solea*).

5.4.1 Levels of PCDD/Fs, c-PCBs using GC-HRMS and Mo-PCBs using GC/MS-MS.

Mean levels of PCDD/Fs, c- and Mo-PCBs detected in muscles of dab (*Limanda limanda*) and Dover sole (*Solea solea*) collected at different subtidal stations on the Belgian continental shelf are presented in Table 10. Individuals of similar length were selected at each station in order to perform analysis in fishes of the same age class. The chosen individuals are adult fishes between 2.5 and 4 years old. As these fishes are commonly consummed by humans, toxic equivalents using human TEFs values have been added.

	710	**ZD2**	**250**	**435**	**S01**	**545**
Subtidal stations	n = 2	n = 3	n = 2	n = 2	n = 3	n = 3
Weight (g)	158.5 ± 7.8	173.3 ± 32.1	180.0 ± 28.3	120.0 ± 14.1	126.7 ± 5.8	101.7 ± 7.6
Length (cm)	24.0 ± 0.0	26.0 ± 2.6	25.0 ± 0.0	25.0 ± 0.0	24.0 ± 0.0	22.0 ± 1.0
% extracted lipids	0.8 ± 0.4	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.9 ± 0.2	1.0 ± 0.1
Congeners pg/g lw						
TCDD	6.5 ± 0.2	10.5 ± 4.5	17.5	8.0 ± 1.9	9.1 ± 3.5	19.3 ± 13.5
PeCDD	6.5	11.4 ± 2.2	17.1	12.4 ± 4.0	4.7	14.1 ± 1.6
HxCDD 1	< LOQ	3.0	1.9	2.6 ± 0.4	2.0	3.8

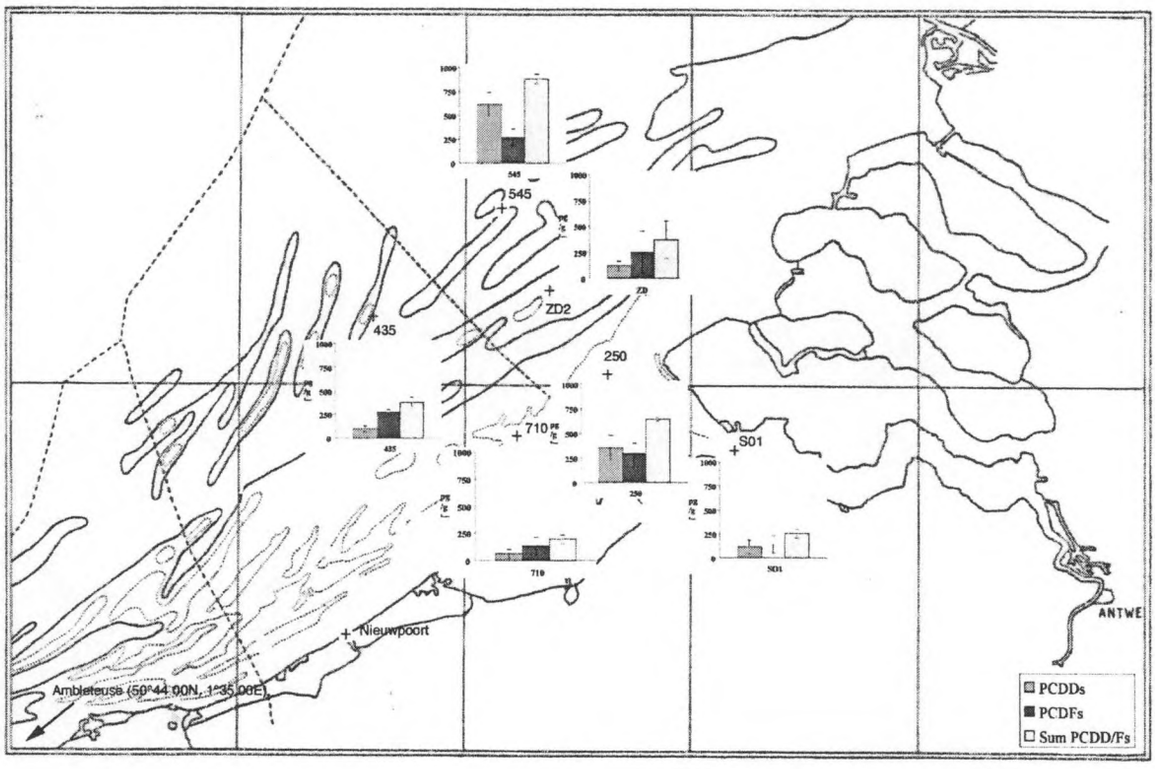
HxCDD 2	5.8 ± 1.5	12.7 ± 0.2	22.0 ± 8.9	9.6 ± 2.5	8.8 ± 3.4	16.0 ± 2.6
HxCDD 3	< Loq	2.6	< Loq	2.8 ± 1.0	1.9	3.3
HpCDD	14.9 ± 7.4	28.1 ± 15.3	55.5 ± 25.0	17.5 ± 12.3	19.5 ± 4.7	131.1 ± 35.6
OCDD	63.8	90.4 ± 15.9	257.7 ± 119.5	47.5 ± 13.9	109.7 ± 19.7	428.6 ± 88.7
Total PCDDs	**62.4 ± 41.0**	**116.8 ± 47.1**	**353.4 ± 127.6**	**100.3 ± 32.3**	**113.3 ± 68.2**	**611.6 ± 128.6**
TCDF	54.5 ± 63.2	66.0 ± 54.8	138.5	163.8 ± 24.1	42.5 ± 25.6	111.1 ± 21.9
PeCDF 1	10.5 ± 9.3	14.0 ± 3.9	17.3 ± 5.1	16.7 ± 0.8	11.5 ± 6.6	12.6 ± 3.0
PeCDF 2	40.1 ± 12.2	52.6 ± 8.4	90.4 ± 3.6	57.3 ± 1.1	< Loq	63.0 ± 5.0
HxCDF 1	4.1 ± 2.2	7.7 ± 2.0	9.4	5.7 ± 1.2	3.8	8.8 ± 1.5
HxCDF 2	3.4 ± 1.9	4.6 ± 0.8	4.1	4.4 ± 0.6	2.8	4.7 ± 0.9
HxCDF 3	< Loq	< Loq	< Loq	< Loq	< Loq	< Loq
HxCDF 4	2.9 ± 0.9	5.2 ± 0.2	5.5	4.5 ± 1.1	< Loq	6.5 ± 0.6
HpCDF 1	14.4 ± 3.4	32.6 ± 7.4	51.6 ± 1.2	11.8 ± 2.0	16.8 ± 4.4	62.0 ± 42.8
HpCDF 2	< Loq	0.8	< Loq	0.4	< Loq	< Loq
OCDF	7.9	100.7 ± 0.6	54.0 ± 7.4	11.5 ± 3.0	71.4 ± 90.8	23.3 ± 3.6
Total PCDFs	**133.6 ± 81.1**	**249.4 ± 202.4**	**291.9 ± 109.0**	**275.9 ± 22.3**	**138.8 ± 88.8**	**268.9 ± 87.1**
Total PCDD/Fs	**195.9 ± 40.1**	**366.2 ± 180.5**	**645.3 ± 18.6**	**376.2 ± 54.6**	**252.1 ± 45.4**	**880.4 ± 50.0**
Fish TEQ PCDD/Fs	**34.5 ± 5.7**	**41.9 ± 27.6**	**69.4 ± 28.8**	**61.1 ± 3.9**	**14.5 ± 4.3**	**64.1 ± 8.4**
Human TEQ PCDD/Fs	**37.9 ± 9.1**	**45.9 ± 0.2**	**74.9 ± 32.2**	**69.5 ± 5.4**	**17.4 ± 5.8**	**71.9 ± 8.4**
c-PCBs						
PCB 77	2304 ± 2108	3793 ± 1579	8088 ± 3384	5031 ± 163	1500 ± 309	516 ± 56
PCB 81	249 ± 264	525 ± 590	158 ± 29	193 ± 39	93 ± 39	20 ± 4
PCB 126	688 ± 116	846 ± 159	1054 ± 386	1167 ± 280	258 ± 65	148 ± 23
PCB 169	77 ± 10	127 ± 69	120 ± 51	169 ± 70	65 ± 39	86 ± 23
c-PCBs (pg/g)	**3318 ± 2498**	**5291 ± 2533**	**9421 ± 2977**	**6561 ± 552**	**1916 ± 269**	**707 ± 54**
Fish TEQ c-PCBs	**3.8 ± 0.9**	**4.9 ± 2.6**	**6.2 ± 1.6**	**6.4 ± 1.4**	**1.5 ± 0.3**	**0.8 ± 0.1**
Human TEQ c-PCBs	**69.8 ± 12.0**	**86.3 ± 46.7**	**107.5 ± 38.7**	**118.9 ± 28.7**	**26.6 ± 6.7**	**15.1 ± 2.4**
Mo-PCBs						
PCB 123	1.9	3.9	0.01	2.4 ± 0.4	3.1 ± 2.7	2.9 ± 0.5
PCB 118	98.7	358 ± 342	0.5	133 ± 9.5	138.4 ± 83.1	72.1 ± 25.3
PCB 114	0.7	5.7 ± 7.6	0.01	1.3 ± 0.2	0.9 ± 0.2	1.1
PCB 105	19.2	67 ± 42	0.2	37.4 ± 1.7	34.8 ± 22.2	17.8 ± 17.9
PCB 167	9.0	18.7 ± 8.7	0.1	14.3 ± 2.3	24.0 ± 19.9	7.2 ± 0.6
PCB 156	7.9	15.0 ± 7.3	0.03	10.2 ± 0.4	9.2 ± 4.9	5.5 ± 2.7
PCB 157	2.6	1.8 ± 1.0	0.01	3.7 ± 1.0	2.8 ± 0.5	3.7 ± 0.9

PCB 189	< Loq	6.7 ± 8.5	0.001	< Loq	1.1 ± 1.2	1.7 ± 0.3
Total Mo (ng/g)	**140.0**	**473 ± 414**	**0.8**	**202 ± 4**	**214.3 ± 135.3**	**102.9 ± 47.9**
Fish TEQ Mo PCBs	**0.7**	**2.4**	**0.004**	**1.0 ± 0.02**	**1.1 ± 0.7**	**0.5 ± 0.2**
Human TEQ Mo PCBs	**17.7**	**54.7 ± 46.5**	**0.09**	**12.9 ± 16.9**	**24.4 ± 14.0**	**10.5 ± 10.3**

Table 9: Concentrations (pg/g lipids weight) and TEQs (pg TEQ/g lipids weight) of PCDD/Fs, c-PCBs and Mo-PCBs (ng/g lw) expressed as mean ± standard deviation detected in the muscle of common dab, *Limanda limanda* and Dover sole, *Solea solea.* < Loq : smaller than the limit of quantification.

As shown in Map 5, fishes collected at station 545 display the higher mean PCDD/F concentrations of respectively 611.6 and 268.9 pg/g lw. Station 250 rank second with mean PCDD/F concentrations of respectively 353.4 and 291.9 whereas intermediate levels are found in both stations ZD2 and 435. The lowest levels were detected at stations 710 and SO1. Several reasons might be considered to explain these low levels encountered at station 250 : 1) in the mouth of the Scheldt estuary, currents and heavy traffic might prevent the deposition of contaminated particles ; 2) currents from the estuary slowly slide south along the Belgian coast before going up north and mixing towards station 250, where deposition could occur ; 3) the Dover soles sampled at station SO1 are summer residents (from April to June) and could thus only reflect a short time exposure to organochlorines (Amara, 1999).

In general, mean PCDF concentrations are higher than mean PCDDs except at stations 250 and 545. For PCDDs, the octachlorinated congener (OCDD) is prevalent in terms of concentrations. However, as its TEF value is very low (0.0001), its contribution to the TEQ will remain extremely small. Interestingly, this congener is also abundant in the subtidal sediment samples analysed in this study. This congener is recognized as the most prevalent dioxin in sediment in relation to atmospheric particles from combustion processes (Loganathan *et al.*, 1995, Bonn, 1998). At station 435, a different PCDD profile is observed, with a marked reduction of the OCCD (roughly 4 time fold less) whereas TCDD and PeCDD both increase. These two compounds are amongst the most toxic (TEFs = 1) and will largely contribute to the TEQ value.



Map 5: Concentrations of PCDD/Fs congeners in Dover sole (*Solea solea*) at subtidal station SO1 and in dab (*Limanda limanda*) at subtidal stations 710, 545, ZD2, 435 and 250.

In terms of TEQs, the highest to lowest values of pg TEQ/g lw at the different stations are found as follows : 250 > 545 > 435 > ZD2 > 710 > SO1. Dioxins and furans together contribute for more than 88% of the total TEQs, leaving only a small contribution to c- and Mo-PCBs as shown in Figure 11.

These TEQ levels, ranging from 14.5 and 69.4 pg TEQ/g lw are within the range of those described for other fishes species of similar lipidic content, collected in the North Sea (Karl *et al.*, 2002).

The maximum level for fish and fish products (Tolerable Weekly Intake) set by the European Council and into force from 1st of July 2002, is of 4 PCDD/Fs pg WHO-TEQ/ kg fresh weight (European Council Regulation, 2001). From our results, it was estimated that a person weigthing an average of average of 70 kg would ingest 0,8 pg TEQ/ kg of body weight per meal containing approximatively 100 g. of fish.

Comparing both species analysed in this study, our results also show that whereas the Dover sole displays the lowest TEQ value – 17.4 pg TEQ/g lw -, levels detected in the dabs are clearly much higher – 59.8 pg TEQ/g lw.

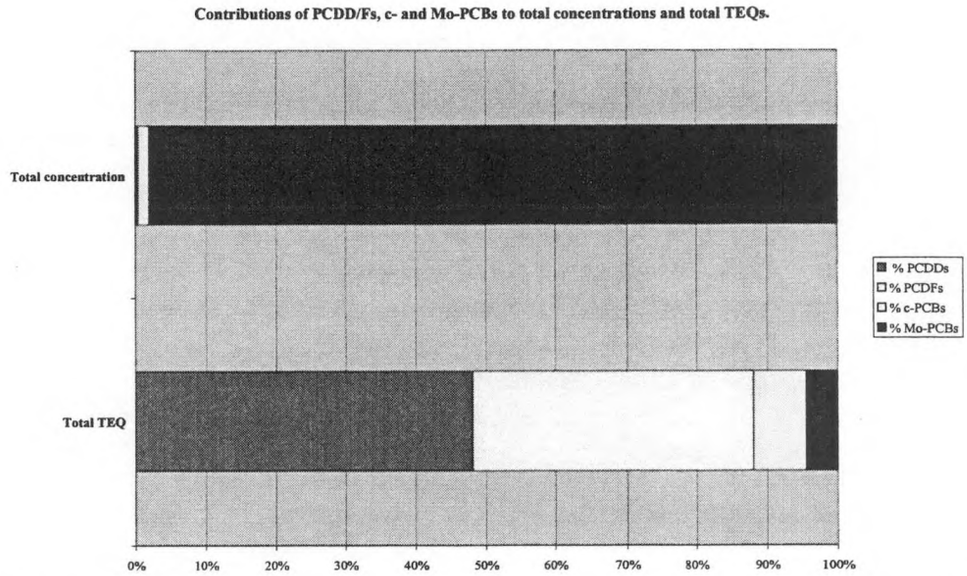


Figure 11: Contributions % of the PCDD/Fs congeners to total concentrations and TEQs.

5.4.2 Comparison with level detected in fresh water species.

In general, mean PCDD/F concentrations detected in marine fishes are largely higher – at least ten fold – to those reported for fresh water species (de Boer *et al.*, 1993). This observation is still valid when comparing our results with those obtained by Thomé *et al.*(2003), analysing 2 common species present in the Meuse river hydrographic basin.
On the contrary, c-PCBs levels are significantly much lower than those reported for fresh water species.

5.4.3 CALUX bioassay.

5.4.3.1 Dioxin fraction

Eight samples of flatfishes (6 samples of *Limanda limanda* and 2 samples of *Solea solea*) were analyzed by CALUX. The correlation between the dioxin fraction in CALUX and PCDD/F analysis by GC-HRMS is illustrated (Figure 12). The parameters show that results are highly correlated with a R^2 value of 0.91 and $p<<0.001$.

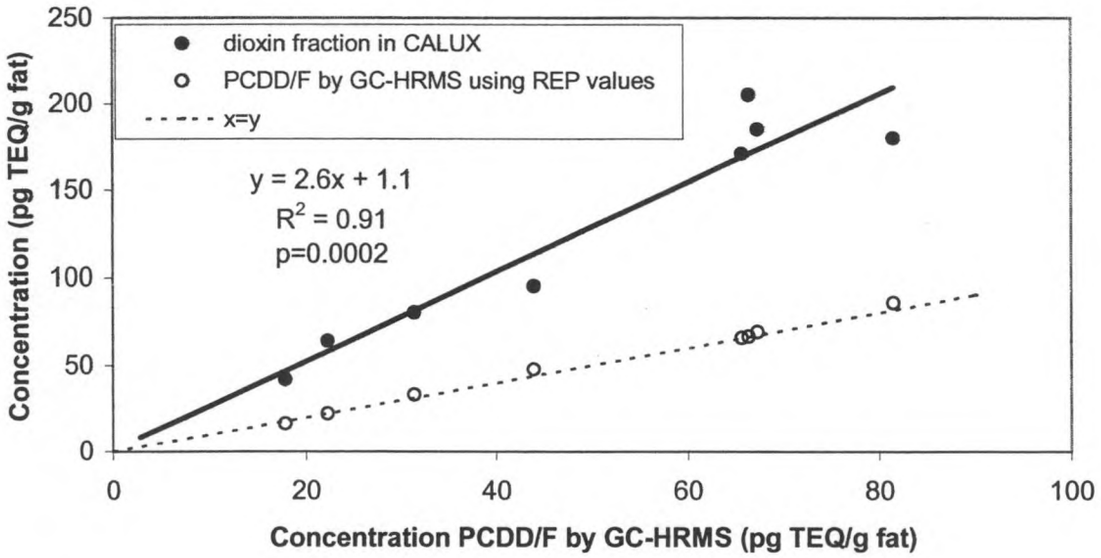


Figure 12: Comparison of CALUX results for the dioxins fraction and concentrations of PCDD/Fs by GC-HRMS for flatfishes samples.

The average of the ratio CALUX/GC-HRMS is 2.6 with a RSD of 13% (Table 10) which means that CALUX results are approximately 2.6 times higher than GC-HRMS results.

For fishes, most of the congeners were detected by GC-HRMS. The higher values measured by CALUX for the dioxin fractions, compared to the PCDD/F concentrations measured by GC-HRMS are then probably due to the presence of other AhR ligands.

GC-HRMS analyzes only the PCDD/F. CALUX analyzes PCDD/F+ other AhR ligands. Consequently, a good correlation between CALUX and GC-HRMS imply that the ratio of PCDD-Fs/other AhR ligands is constant.

	Dioxin fraction in CALUX/PCDD-F*			c-PCB/PCDD-F (chemical analysis)*		
	average	RSD (%)	n	average	RSD (%)	n
Fishes	2.6	13	8	0.22	26	14
Marine mammals**	3.2	21	10	2.1	68	10
Seabirds	2.1	42	12	1.4	65	10
Starfishes°	6.5	24	11	2.5	15	5
Mussels	8.4	18	12	1.6	24	12

Table 10: Ratio between dioxin-like compounds according to the matrices.

* concentrations expressed in pg TEQ/g fat

** upper bound concentrations for GC-HRMS results

° one sample discarded since the PeCDD and 2,3,4,7,8 PCDF are not detected

5.4.3.2 PCBs fraction.

PCBs results are not available for flat fishes in CALUX and/or in GC-HRMS.

5.5 Analyses of a pelagic seabird, the common guillemot *Uria aalge.*

5.5.1 Levels of PCDD/Fs, c-PCBs using GC-HRMS and Mo-PCBs using GC/MS-MS.

Mean levels of PCDD/Fs, c- and Mo-PCBs detected in livers of males common guillemots collected during the wintering seasons 1993 to 2002, are presented in Table 11. Compared to the other matrices analysed during this study, guillemots presented by far, the highest concentrations and that is true for all considered congeners. Such high levels are not unusual for birds which tend to have lower monooxygenase activities than mammals, so are likely to be less efficient in detoxifying organochlorine compounds (Walker *et al.*, 1987, Senthil Kumar *et al.*, 2002a). Comparable levels of PCDD/Fs have been described for other fish-eating species, such as the white-tailed sea eagle (*Haliaetus albicilla*) in Germany and Poland (Kannan *et al.*, 2003). This comparison is particularly interesting as some of the sea eagles analysed were severely emaciated, a common characteristic with more than half of the birds sampled along the Belgian coast. In these particular emaciated sea eagles, the authors report very high organochlorine concentrations in the tissues as is the case for Belgian guillemots. In addition, concentrations of PCDD/Fs detected in 4 severely emaciated guillemots collected at the Belgian coast largely exceed a proposed NOAEL (No Adverse Effect Observed Level) of approximatively 26500 pg/g lw in livers, proposed by Kannan *et al.*, 2003. It is suggested that these 4 individuals might be adversely affected by such high organochlorines levels.

	Belgian coast n = 21	
	pg/g lipid weight	pg TEQ/g lipid weight
% extracted lipids	2.7 ± 1.4	
Congeners		
TCDD	491 ± 434	491 ± 434
PeCDD	1529 ± 1441	1529 ± 1441
HxCDD 1	532.1 ± 486.3	27 ± 24
HxCDD 2	3032 ± 3164	30 ± 32
HxCDD 3	307 ± 302	31 ± 30
HpCDD	1231 ± 1159	1.2 ± 1.2
OCDD	715 ± 505	0.07 ± 0.05
Total PCDDs	**7778 ± 6879**	**2109 ± 1941**
TCDF (pg/g)	322 ± 537	326 ± 540
PeCDF 1	286 ± 325	29 ± 33

PeCDF 2	6170 ± 5586	6170 ± 5586
HxCDF 1	1356 ± 1412	274 ± 693
HxCDF 2	1126 ± 1197	113 ± 120
HxCDF 3	23 ± 20	2.3 ± 2
HxCDF 4	1239 ± 1282	124 ± 128
HpCDF 1	507 ± 501	5 ± 5
HpCDF 2	38 ± 33	0.4 ± 0.3
OCDF	64 ± 23	0.006 ± 0.002
Total PCDFs	**11084 ± 9954**	**6899 ± 5966**
Total PCDD/Fs	**18862 ± 16659**	**9008 ± 7847**
c-PCBs		
PCB 77	24305 ± 22509	1156 ± 1131
PCB 81	2508 ± 1877	244 ± 186
PCB 126	62430 ± 44197	5605 ± 4062
PCB 169	53727 ± 67153	53.7 ± 67.2
c-PCBs (pg/g)	**141693 ± 106581**	**7693 ± 4746**
Mo-PCBs		
ng/ lw		
PCB 123	36.7 ± 28.6	0.4 ± 0.3
PCB 118	6343 ± 4682	63.4 ± 46.8
PCB 114	143 ± 170	14.3 ± 17.0
PCB 105	2326 ± 2175	232.6 ± 217.4
PCB 167	985 ± 1095	9.9 ± 11.0
PCB 156	1560 ± 1470	156.0 ± 147.0
PCB 157	480 ± 437	48.0 ± 43.0
PCB 189	512 ± 422	5.1 ± 4.2
Total Mo-PCBs	**12270 ± 9869**	**518.2 ± 459.6**

Table 11: Concentrations (pg/g lipids weight) and TEQs (pg TEQ/g lipids weight) of PCDD/Fs, c-PCBs and Mo-PCBs (ng/g lw) expressed as mean ± standard deviation detected in the livers of common guillemots, *Uria aalge*.

The mean concentrations of PCDFs is approximatively 1.4 fold greater than the concentrations of PCDDs as described in other studies (Kannan *et al.*, 2003, Senthil Kumar *et al.*, 2002b). Among PCDFs congeners, 2,3,4,7,8-Pe_2CDF is the more abundant, representing ± 45% of the total PCDD/Fs concentrations. This congener has been shown to preferentially accumulate in liver tissues of several fish-eating water birds (Choi *et al.*, 2001, Senthil Kumar *et al.*, 2002c, Kannan *et al.*, 2003). High 2,3,4,7,8-Pe_2CDF concentrations in bird tissues have been attributed to exposure to technical PCBs mixtures, which contain this congener as a major impurity (Wakimoto *et al.*, 1988). Among PCDDs congeners, hexachlorinated congeners are more abundant and in particular 1,2,3,6,7,8-Hx_2CDD.

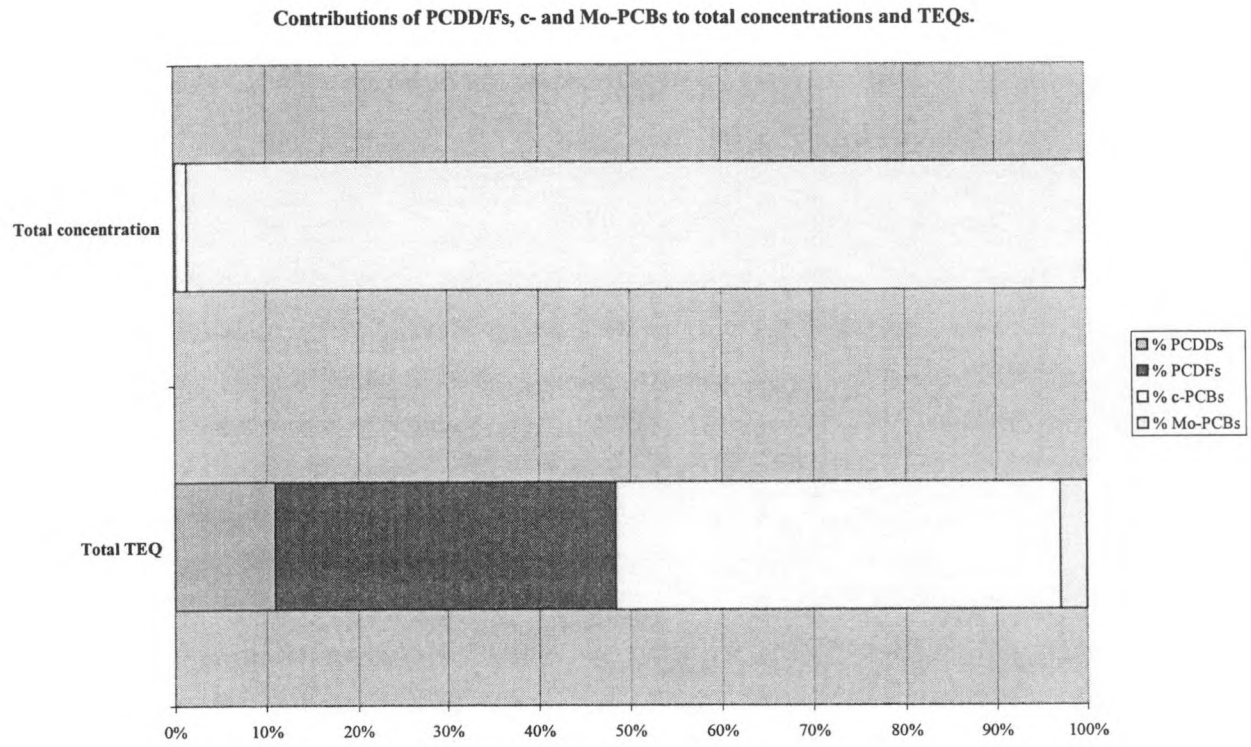


Figure 13: Contributions % of PCDD/Fs, c- and Mo-PCBs to total concentrations and TEQs.

In terms of total concentrations, congeners of Mo-PCBs contribute for more than 98%, with a mean value up to 12270 ng/g lw (Figure 13). However, in term of TEQs, these congeners contribute for less than 3%. This is partly due to their low TEF values compared to c-PCBs but it has also been shown that seabirds have a greater capacity to metabolize Mo-PCBs congeners compared to other marine predators such as mammals (Kannan *et al.*, 1993, Minh *et al.*, 2000). On the contrary, c-PCBs TEFs values for birds are higher than for other matrices (such as human or fishes) and consequently these congeners dominates the mix representing almost 50% of the total TEQs as shown in Figure 13.

Among c-PCBs congeners, PCB 126 makes most of the concentration immediately followed by PCB 77 as shown in Figure 14. This pattern has been described for other predatory birds and suggest a better metabolisation of PCB 77 compared to PCB 126 (Senthil Kumar *et al.*, 2002a,c, Kannan *et al.*, 2001, 2003).

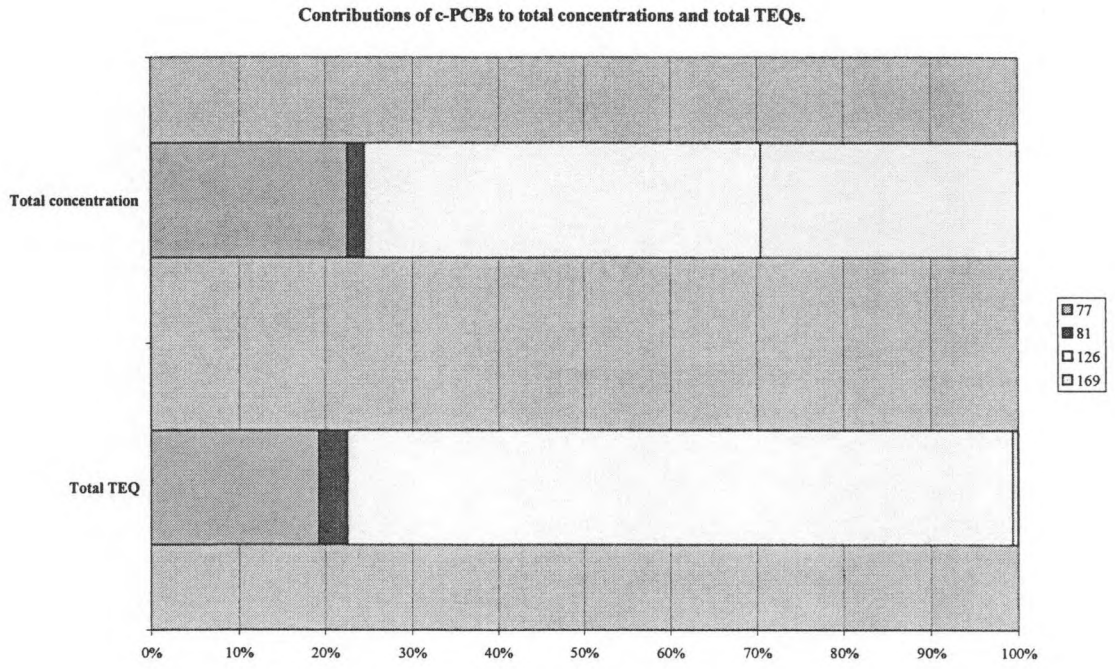


Figure 14: Contributions of c-PCBs congeners to total concentrations and TEQs.

5.5.2 Comparison with results obtained in livers of guillemots collected in Brittany (France).

Results obtained on common guillemots sampled along the Belgian coast were compared with those obtained on individuals collected in Brittany (France) after the Erika oil spill as shown in Figure 15.

Concentrations of PCDD, PCDF are respectively 3.0 and 5.3 fold greater in guillemots collected at the Belgian coast. The pattern of distribution is also slightly different with approximatively equal concentrations of PCDD and PCDFs whereas at the Belgian coast furans clearly are predominant. It is also worth noting that the range of concentrations detected in Brittany is much narrower (smaller standard deviation) than at the Belgian coast, meaning that for some individuals the concentrations reached are extremely high. These differences could be due to : 1) a far better body condition has been observed for the guillemots collected in Britttany which means that organochlorines stored in the fat deposits have not yet been concentrated as it is the case during an emaciation process ; 2) a cleaner open Atlantic environment compared to the Southern North Sea.

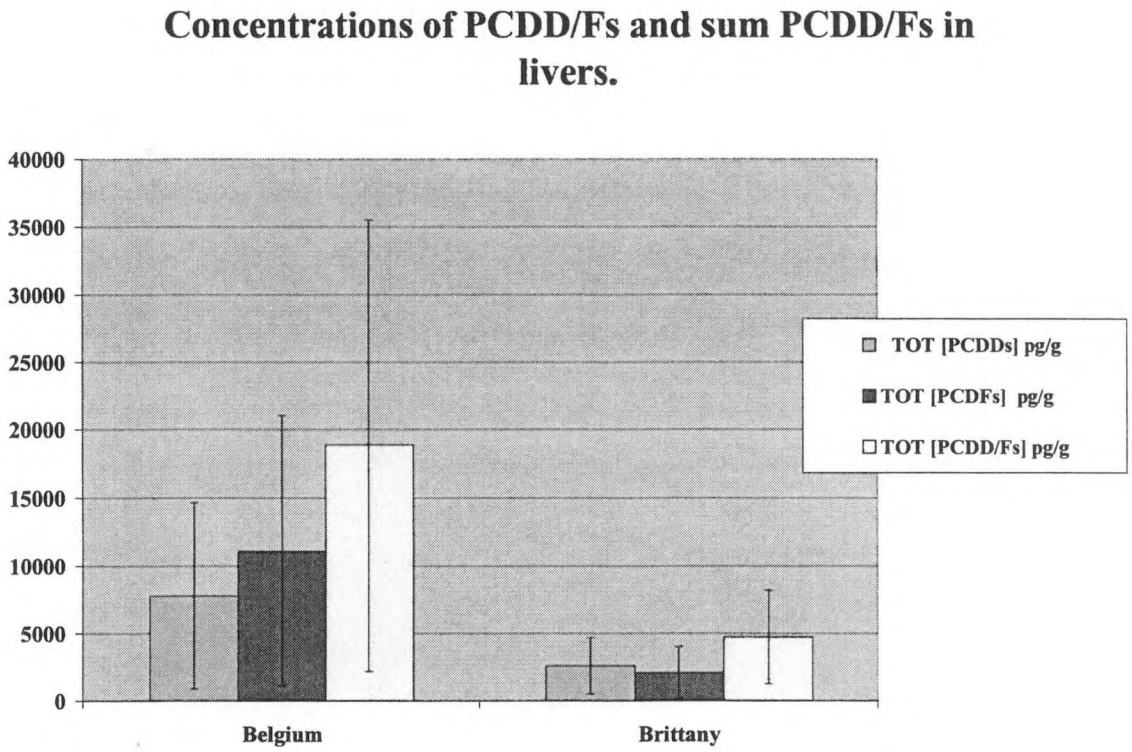


Figure 15: Concentrations of PCDD/Fs and sum of both in livers of common guillemots from different geographical origins.

5.5.3 CALUX bioassay.

5.5.3.1 Dioxin fraction

On the 11 samples of seabirds analyzed by the 2 methods, one sample is considered as an outlier (in CALUX and in GC-HRMS, determination by box plots) and is not included in the statistic calculation. CALUX and chemical measurements results are still correlated, but less than for the other matrices. This observation implies that the ratio between PCDD/Fs and other AHR ligands varies more for seabirds than for the other matrices. As the ratio between chemical analysis of c-PCB and PCDD/Fs varies also much more for seabirds than for the other matrices (Table 10), it is believed that this variation is linked to environmental consideration: different origin or feeding of the birds, different health status and metabolism,...

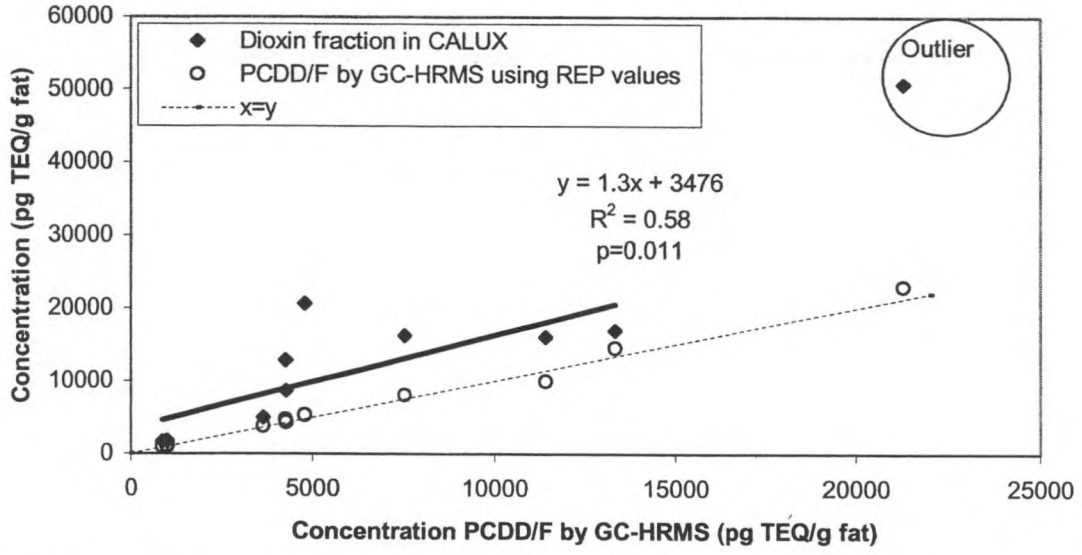


Figure 16: Comparison of CALUX results for the dioxin fraction and concentrations of PCDD/Fs by GC-HRMS for seabirds samples.

5.5.3.2 PCBs fraction.

For seabirds, the concentrations measured in the PCBs fraction in CALUX are highly correlated to the c-PCB concentrations measured by GC-HRMS (Figure 16). However, and in contrast with results obtained for dioxins, CALUX results are always lower than GC-HRMS results. When the c-PCB concentrations measured by GC-HRMS are multiplied by the REP instead of the TEF (values represented by open circles in the Figure) the values calculated are usually close to the results obtained in CALUX. This means that the difference between TEF and REP explains most of the discrepancy between CALUX and chemical analysis.

In chemical analysis, the coplanar PCB 126 is usually responsible of more than 90% of the PCBs TEQ. As the REP for the PCB 126 is roughly one third of the TEF, the concentrations measured in CALUX is roughly one third of the concentration measured by GC-HRMS (Figure 17).

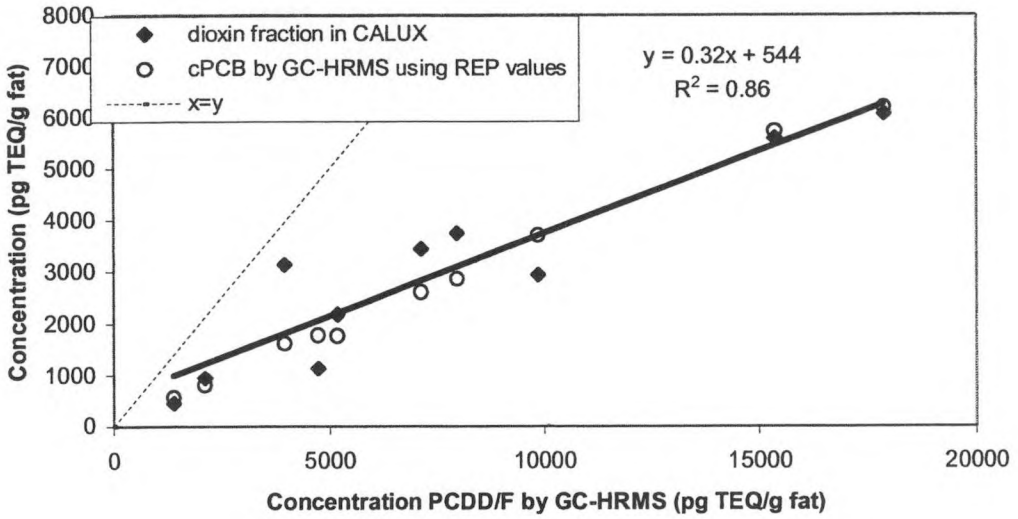


Figure 17: Comparison of CALUX results for the PCBs fraction and concentrations of c-PCB by GC-HRMS for seabirds samples.

5.6 Analyses of the harbour porpoise *Phocoena phocoena.*

5.6.1 Levels of PCDD/Fs, c-PCBs using GC-HRMS and Mo-PCBs using GC/MS-MS.

Levels of PCDD/Fs, c- and Mo-PCBs were analysed in the blubber of 20 harbour porpoises collected stranded on the Belgian and French coasts, between 1995 and 2001. One individual, a juvenile cachectic female displayed much higher levels of all combined dioxin-like contaminants compared to the other porpoises and is considered as an outlier. This individual was removed from the set presented in Table 12.

	Blubber	
n = 19	pg/g lipid weight	pg TEQ/g lipid weight
% extracted lipids	72.8 ± 16.6	
Congeners pg/g lw		
TCDD	0.4 ± 0.2	0.3 ± 0.2
PeCDD	0.7 ± 0.3	0.5 ± 0.3
HxCDD 1	< Loq	0.02 ± 0.0
HxCDD 2	0.8 ± 0.5	0.03 ± 0.03
HxCDD 3	< Loq	0.02 ± 0.0
HpCDD	< Loq	0.03 ± 0.0
OCDD	< Loq	0.001 ± 0.0
Total PCDDs	**1.4 ± 1.2**	**1.6 ± 0.7**
TCDF	2.1 ± 1.1	0.2 ± 0.1

PeCDF 1	1.0 ± 0.3	0.03 ± 0.02
PeCDF 2	2.1 ± 1.3	1.1 ± 0.6
HxCDF 1	0.8 ± 0.5	0.05 ± 0.05
HxCDF 2	0.8 ± 0.4	0.04 ± 0.04
HxCDF 3	< Loq	0.02 ± 0.0
HxCDF 4	0.4 ± 0.1	0.03 ± 0.01
HpCDF 1	< Loq	0.03 ± 0.0
HpCDF 2	< Loq	0.002 ± 0.0
OCDF	< Loq	0.002 ± 0.0
Total PCDFs	**11.3 ± 6.9**	**2.7 ± 1.5**
Total PCDD/Fs	**12.1 ± 7.9**	**4.4 ± 2.1**
c-PCBs		
PCB 77	1232.2 ± 2261.6	0.05 ± 0.1
PCB 81	130.6 ± 172.8	0.005 ± 0.009
PCB 126	67.1 ± 48.9	4.7 ± 4.9
PCB 169	28.2 ± 13.8	0.3 ± 0.1
c-PCBs (pg/g)	**511.1 ± 1440.1**	**5.0 ± 5.0**
Mo-PCBs ng/g lw		
PCB 123	7.6 ± 8.4	0.6 ± 0.8
PCB 118	528.5 ± 423.3	44.1 ± 39.0
PCB 114	1.9 ± 1.6	0.8 ± 0.8
PCB 105	104.4 ± 98.7	8.3 ± 8.2
PCB 167	94.8 ± 76.7	0.8 ± 0.7
PCB 156	17.7 ± 19.4	7.4 ± 9.3
PCB 157	30.8 ± 41.6	14.0 ± 21.1
PCB 189	13.1 ± 12.6	1.1 ± 1.2
Total Mo-PCBs	**798.7 ± 648.3**	**77.1 ± 70.9**

Table 12: Concentrations (pg/g lipids weight) and TEQs (pg TEQ/g lipids weight) of PCDD/Fs, c-PCBs and Mo-PCBs (ng/g lw) expressed as mean ± standard deviation detected in the blubber of harbour porpoises *Phocoena phocoena*. < Loq : smaller than the limit of quantification.

Although harbour porpoises are also marine top predators, they display a totally different pattern of concentrations and congeners profile distribution compared to common guillemots. A first striking observation is that the mean PCDD/F concentrations are the lowest of all the studied matrices of this program. Of the seventeen PCDD/F congeners, 8 remain undetected in the blubber samples. Those undetected congeners are some hexa-, hepta- and octachlorinated dioxins and furans as shown in Figure 18.

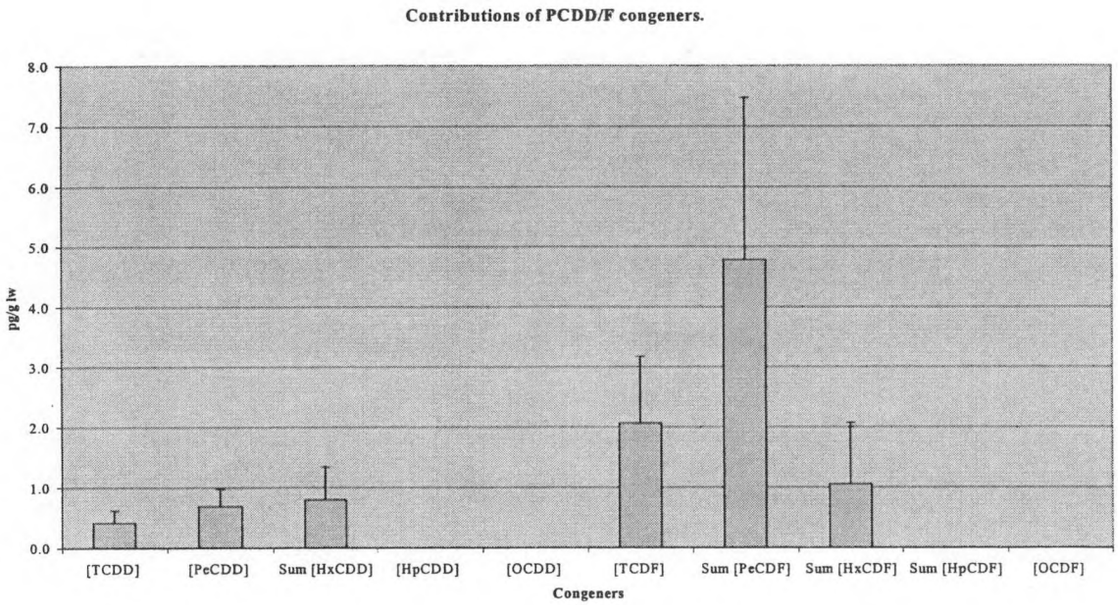


Figure 18: Contributions of PCDD/F congeners to concentrations.

Compared to PCDD/Fs, levels of both PCBs and more specifically Mo-PCBs are particularly important. These congeners represent almost 90% of the total TEQ value (Figure 19). In particular, congeners 118 and 105 make most of the concentration and TEQ (Figure 20).

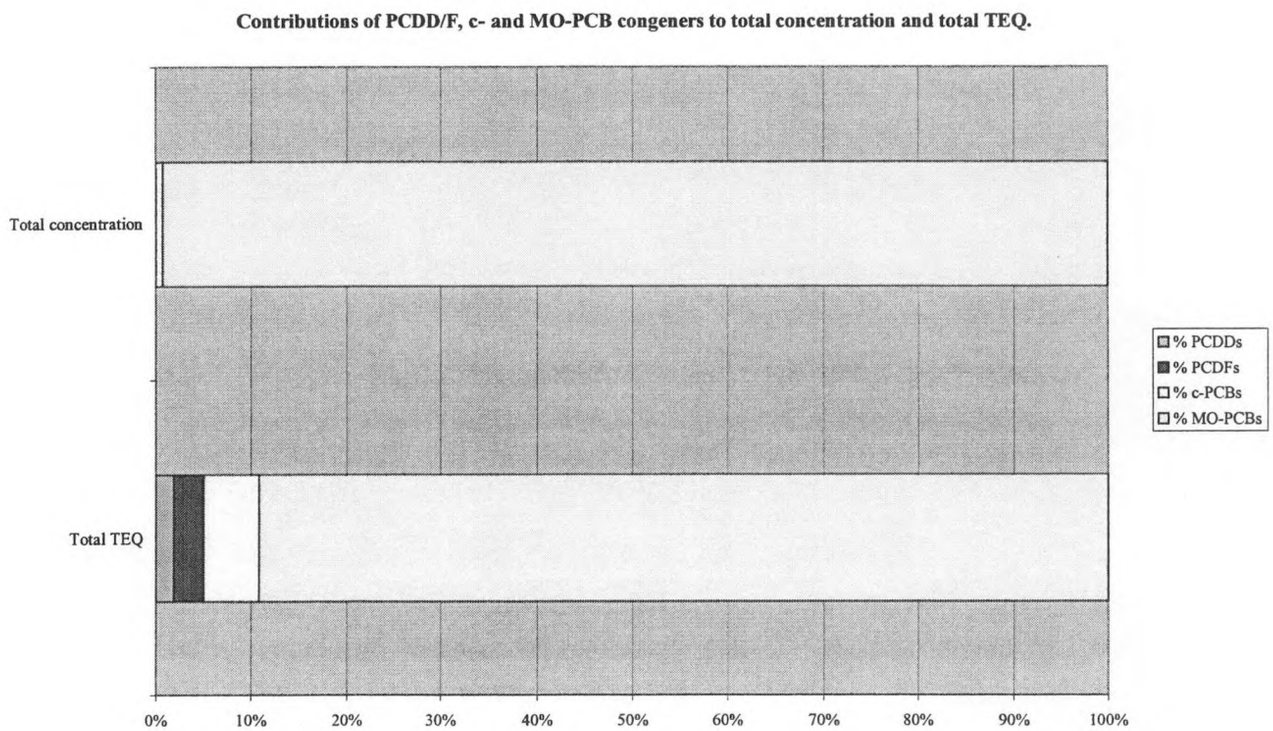


Figure 19: Contributions of PCDD/F, c- and Mo-PCBs to total concentrations and total TEQs.

The mean TEQs of all combined dioxin-like contaminants detected in this study are relatively low, 86.2 ± 73.6 pg TEQ/g lw, and yet they are slightly higher (but still in the same order of magnitude) than those reported for harbour porpoises collected in the Wadden Sea (Bruhn *et al.*, 1999). In general, low levels of dioxins and furans are also reported in other studies, suggesting a more efficient capacity to metabolise and eliminate these pollutants (Green *et al.*, 1996 ; Muir *et al.*, 1996).

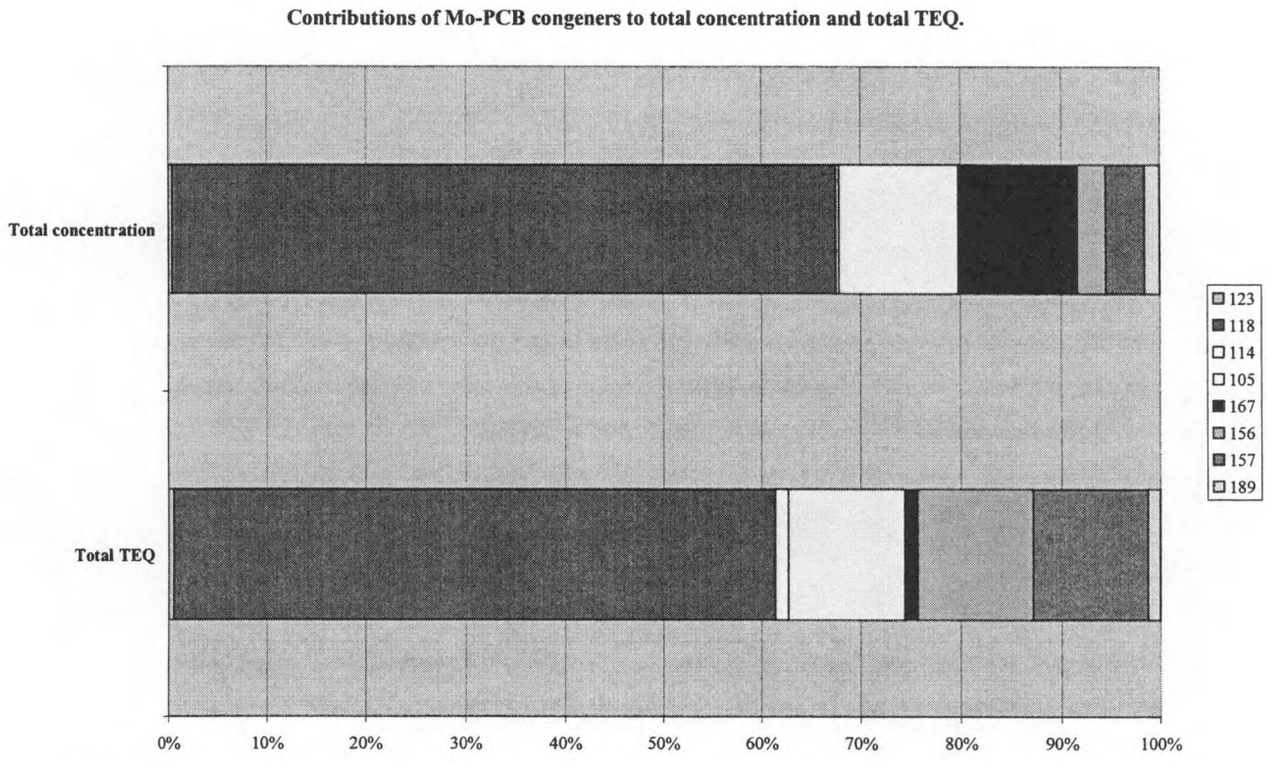


Figure 20: Contribution of Mo-PCB congeners to total concentration and total TEQ.

A first comparison showed that although females tend to concentrate higher levels of dioxin-like contaminants than males, their toxicity is lower whereupon they show a selection of less toxic congeners. However, these results were not statistically significant. Apart from sex, it appears that the individuals' body condition, as already mentionned for seabirds, influenced the pollutants' distribution in the tissues. Emaciated animals show much higher contaminant levels than non emaciated ones both in terms of concentrations and TEQ values (Beans *et al.*, 2003).

5.6.2 CALUX bioassay.

5.6.2.1 Dioxin fraction

The correlation between the results of the dioxins fraction in CALUX and PCDD/Fs analysis by GC-HRMS is illustrated in Figure 21 (n=18 samples of common porpoises blubber).

At these low or very low levels, some and sometimes most of the congeners of PCDD/Fs are not detected in GC-HRMS. The concentrations measured by GC-HRMS are then underestimated when the lower bound values are used. In CALUX analysis, this bias of measurement is not observed. As a consequence, we observed that the ratio CALUX/ PCDD-Fs increases to 10 when the concentrations measured by GC-HRMS decrease (and that the number of non-detected congeners increases) (Figure 22).

When the upper bound values are used instead of the lower bound values for GC-HRMS results, the correlation between CALUX and GC-HRMS is better (Figure 21) and the ratio between CALUX and GC-HRMS results is more constant and varies between 2 and 4 (Figure 22).

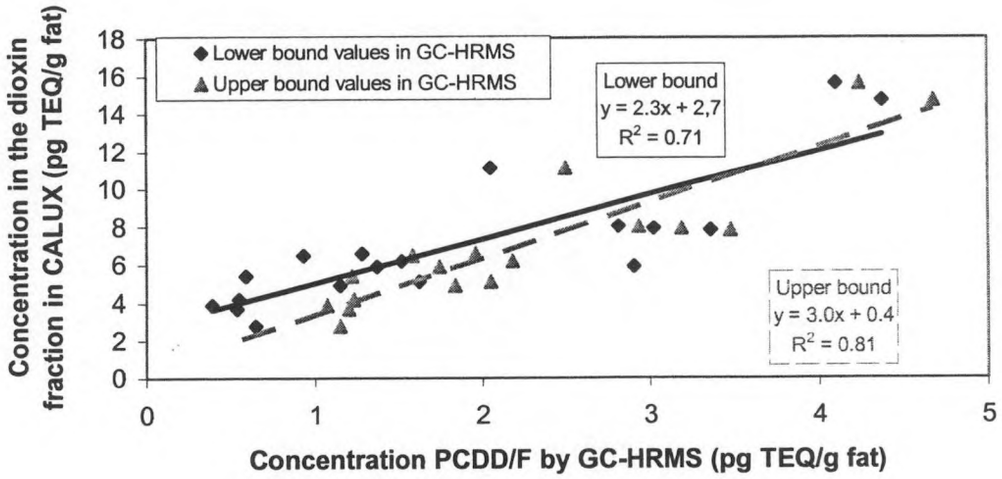


Figure 21: Comparison of CALUX results for the dioxins fraction and concentrations of PCDD/Fs by GC-HRMS for marine mammals samples.

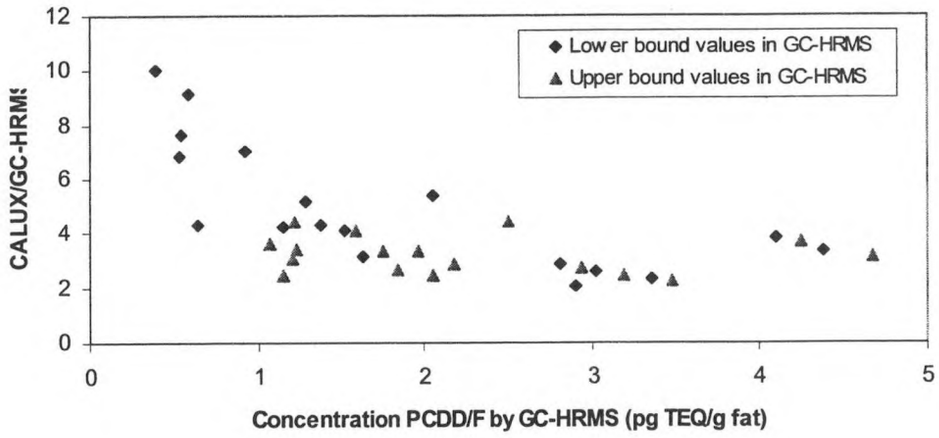


Figure 22: Variation of the ratio CALUX/GC-HRMS for marine mammals samples when lower or upper bound values are used in GC-HRMS.

5.6.2.2 PCB fraction.

PCB results are not available for marine mammals in CALUX and/or in GC-HRMS.

5.7 Comparison of the levels detected in the different marine matrices.

Our results indicate that :

- The highest range of concentrations for all dioxin-like compounds were encountered in the guillemots' tissues , followed in a decreasing order by mussels, starfishes, benthic fishes marine mammals and sediments ;
- In terms of TEQs, using Human TEFs for all considered matrices, the same decreasing order is observed ;
- PCDD/F levels in marine mammals' blubber are within the same range of those detected in sediment samples ;
- substantial concentrations of PCBs are found in all considered samples but the sediments. For this last matrice, results of Mo-PCBs were unfortunately not available;

As shown in Figure 23, the contributions (%) of the different congeners to the total TEQs values considerably differ depending of the considered matrices. This is partly explained by the fact that TEF values varies according to the matrices (see Table 2). However, accumulation of individual congener in the different considered species greatly depends upon their capacity to metabolise and excrete the compounds. This capacity is species specific as it has been discussed for birds and mammals in this study.

Unfortunately, contributions of the congeners in sediments samples could not be plotted as Mo-PCBs results were not available.

Interestingly, Mo-PCBs represent more than 97% in terms of concentrations for the five matrices, but however, only contributes predominantly to the total TEQs (almost 90%) in marine mammals. The opposite situation is observed in benthic fishes for which PCDD/Fs are the prevalent congeners.

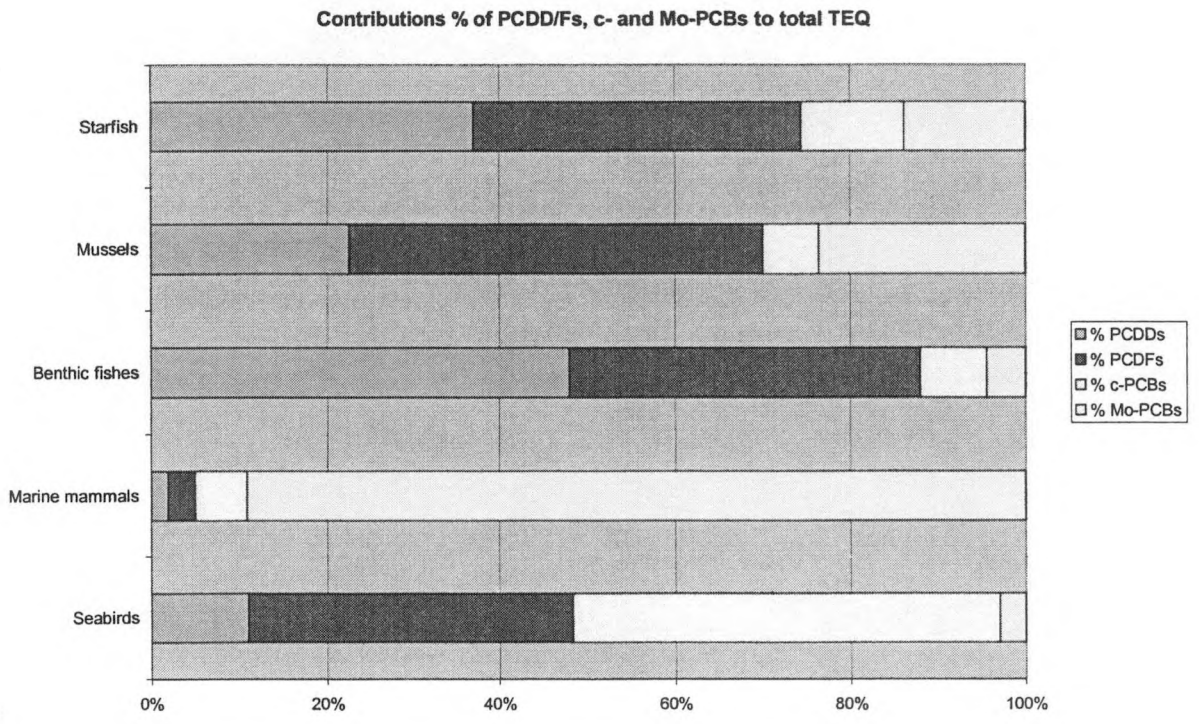


Figure 23: Contributions % of the PCDD/Fs, c- and Mo-PCBs to the total TEQs values in the different marine matrices analysed during this study. TEFs were used as follow : fish TEF were applied to starfishes, mussels and benthic fishes ; bird TEF were used for seabirds and human TEFs were used for marine mammals.

6. General conclusions and perspectives.

This two year programme aimed at assessing different techniques of analysis for dioxin and dioxin-like compounds on chosen marine samples. These different techniques have clearly shown their complementarity, each providing specific information.

6.1 CALUX bioassays versus GC-HRMS.

Good correlations are observed between the dioxins fraction measured in CALUX and GC-HRMS analysis of PCDD/Fs for all matrices investigated and that, from very low (pg TEQ/g fat) to very high concentrations (10 ng TEQ/g fat). CALUX measures approximately 2.8 times more than GC-HRMS for flatfishes, seabirds and marine mammals. The difference is probably due to the presence of other AhR ligands. In the case of mussels and starfishes (invertebrates), CALUX measures approximately 8 times more than GC-HRMS. The good correlations observed imply that the ratio PCDD-Fs/other AhR ligands is quite constant.

Good correlation is also observed between the PCBs fraction measured by both methods of c-PCB for seabirds. However, for those samples CALUX measures approximately 3 times less than chemical analysis. The discrepancy between the 2 methods is mainly due to the

difference between TEF and REP, especially for the PCB 126 which contributes for more than 90% of the TEQ PCB.

More information is available when PCBs and dioxins are analyzed separately in CALUX: the interpretation may be different if only the sum of PCBs and dioxins is considered.

At low concentration, when some congeners that represent an important part of the TEQ value are not detected in GC-HRMS, or when there is an interference for one of these congeners, the concentrations measured by GC-HRMS can be largely underestimated. In CALUX analysis, this bias of measurement is not observed since one global response, expressed in pg TEQ/g, is measured for all AhR ligands. A better correlation between the 2 methods is observed when the upper bound values (when available) are used for GC-HRMS results, instead of the lower bound values.

CALUX and GC-HRMS are complementary. On the one hand, CALUX is relatively cheap, rapid, quantitative, reproducible and sensitive. Moreover, CALUX is able to detect contamination with AhR ligands other than PCDD/Fs and dioxin-like PCBs. Its application to the numerous samples needed for environmental monitoring or environmental research would be easier than GC-HRMS determination of PCDD/Fs and dioxin-like PCBs. On the other hand, chemical analyses provide additional information on the contamination pattern.

6.2 Cytochrome P450 immunopositive protein (CYP1A IPP) induction versus GC-HRMS.

Good correlations were found between the induction of the CYP1A1 activity and measured concentrations of dioxin and dioxin-like contents in starfish samples, indicating an increased enzyme production in exposed organisms. It was also highly correlated to the most toxic TCDD congener. Furthemore, other correlations between CYP1A1 induction in starfishes and concentrations of some congeners in the sediment samples were also noted.
These results indicate that detection of the induction of this biomarker can successfully be used as a screening tool in exposed organisms.

6.3 Levels of dioxins and dioxin-like compounds in various marine matrices.

This two year programme also clearly aimed at determining the levels of dioxins and dioxin-like compounds in various marine matrices. This step is of prime importance in the actual context as few data are available in the literature regarding marine samples and particularly lack when speaking of edible species.

Several important findings in terms of conservation and sustainable management of the marine environment could be summarised as follow:

- Levels of all combined dioxins and dioxin-like compounds in mussels and benthic fishes are below the Tolerable Daily Intake set by the European Commission. Although human consumption of the analyzed species seems not a risk, surveillance and controls should be maintained especially due to persistence and high toxicity of these compounds;
- Of the 2 edible fish species considered, the Dover sole (*Solea solea*) presents the lowest concentration and TEQ values. However, this is a ponctual result and a more thorough investigation should confirm this observation;
- Our results suggest that localised and ponctual source of contamination could influence the organochlorine levels detected in organisms at some stations (Nieuwport and Knokke) ;
- Acumulation pattern clearly are species dependent, depending of the metabolisation capacity of the considered species. However, despite the lower detoxifying ability noted for seabirds compared to other species, the particularly high levels encountered in the livers of guillemots collected at the Belgian coast, are to be considered in relation to their declining body condition;

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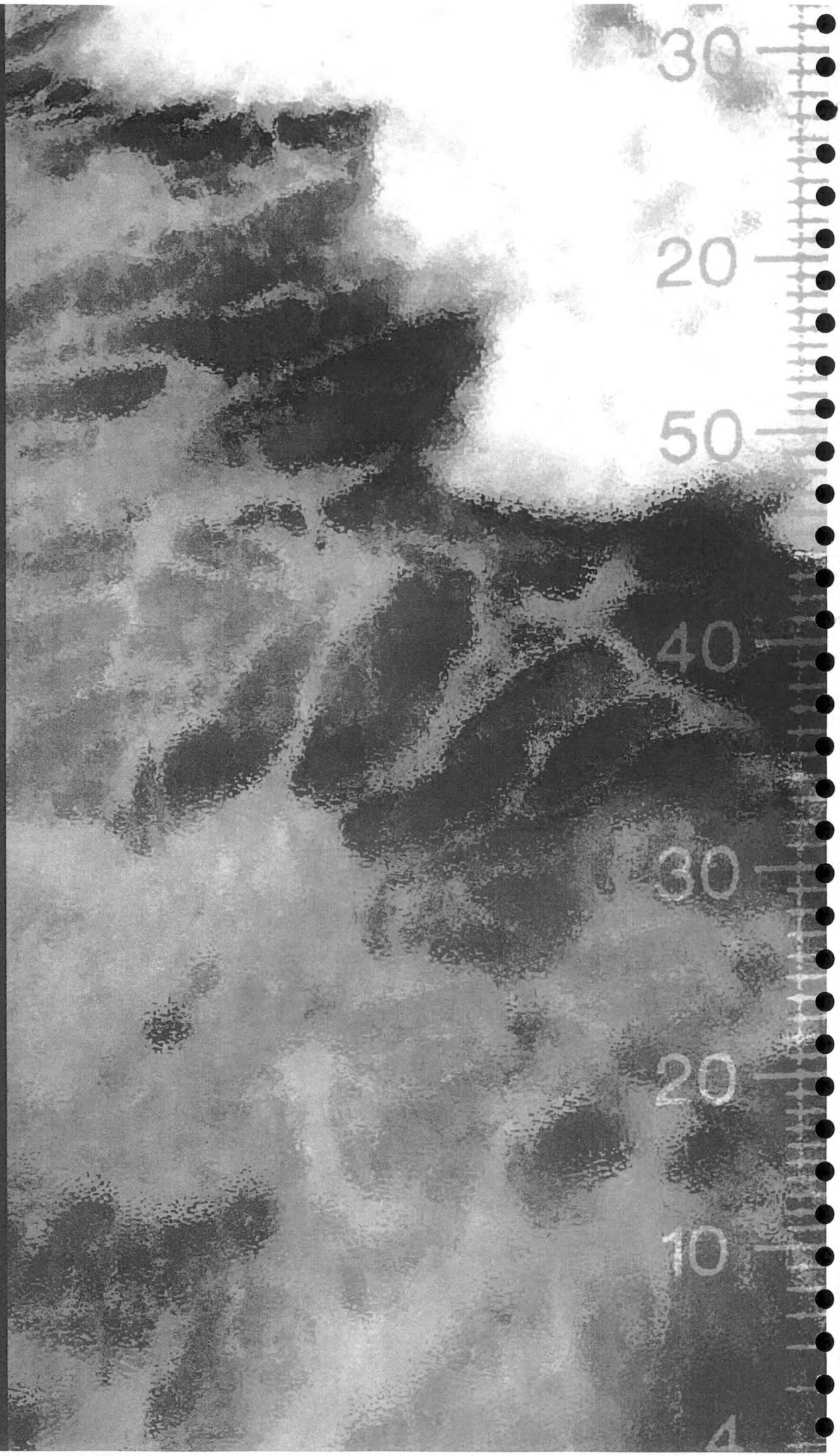

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