FOOD CHEMICAL CONTAMINANTS

Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection: Interlaboratory Study

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An interlaboratory study was conducted for the determination of paralytic shellfish poisoning (PSP) toxins in shellfish. The method used liquid chromatography with fluorescence detection after prechromatographic oxidation of the toxins with hydrogen peroxide and periodate. The PSP toxins studied were saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins 2 and 3 (GTX2,3 together), gonyautoxins 1 and 4 (GTX1,4 together), decarbamoyl saxitoxin (dcSTX), B-1 (GTX5), C-1 and C-2 (C1,2 together), and C-3 and C-4 (C3,4 together). B-2 (GTX6) toxin was also included, but for qualitative identification only. Samples of mussels, both blank and naturally contaminated, were mixed and homogenized to provide a variety of PSP toxin mixtures and concentration levels. The same procedure was followed with samples of clams, oysters, and scallops. Twenty-one samples in total were sent to 21 collaborators who agreed to participate in the study. Results were obtained from 18 laboratories representing 14 different countries.

Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins produced by certain marine dinoflagellates (1). These toxins can accumulate to highly toxic levels in shellfish. As a result shellfish destined for human consumption must be monitored to ensure there is minimal risk from these toxins to the consumer.

The only collaboratively studied method available at present for the determination of PSP toxins in shellfish is the AOAC INTERNATIONAL mouse bioassay (2). This method has been used by regulatory agencies and industry in many countries for more than 40 years for routine monitoring of PSP toxins. However, a recent evaluation of the mouse bioassay has shown that it can significantly underestimate the true concentration of PSP toxins in shellfish (3). Because of this and the concern expressed in some countries towards animal assays, an alternative collaboratively studied method using chemical analysis is needed.

The purpose of the present work was to evaluate through an interlaboratory study a liquid chromatographic method using fluorescence detection after prechromatographic oxidation of the toxins to fluorescent derivatives (4). The results of the study are presented herein.

Interlaboratory Study

Twenty-one samples representing the 4 most commonly consumed shellfish species (mussels, oysters, clams, and scallops) were prepared for the study. The set included 1 practice sample, 3 blank samples, 5 artificially contaminated (spiked) samples, and 12 naturally contaminated samples. Collaborators were provided with the practice sample to assess their performance before beginning the study.

All samples were thoroughly homogenized in a blender. Spikes were added as appropriate, and the spiked samples were homogenized a second time. Subsamples of 6–12 g were packaged in polyethylene containers, sealed, coded, and held at or below –20°C until shipment.

Test samples (frozen) and standard solutions (refrigerated) were shipped to collaborators in Styrofoam boxes with ice-pack inserts. Collaborators were instructed to place the test samples in frozen storage and the standards in a refrigerator until the time of analysis.

Because of difficulties with obtaining sufficient quantities of PSP analytical standards for spiking, spiking levels and the

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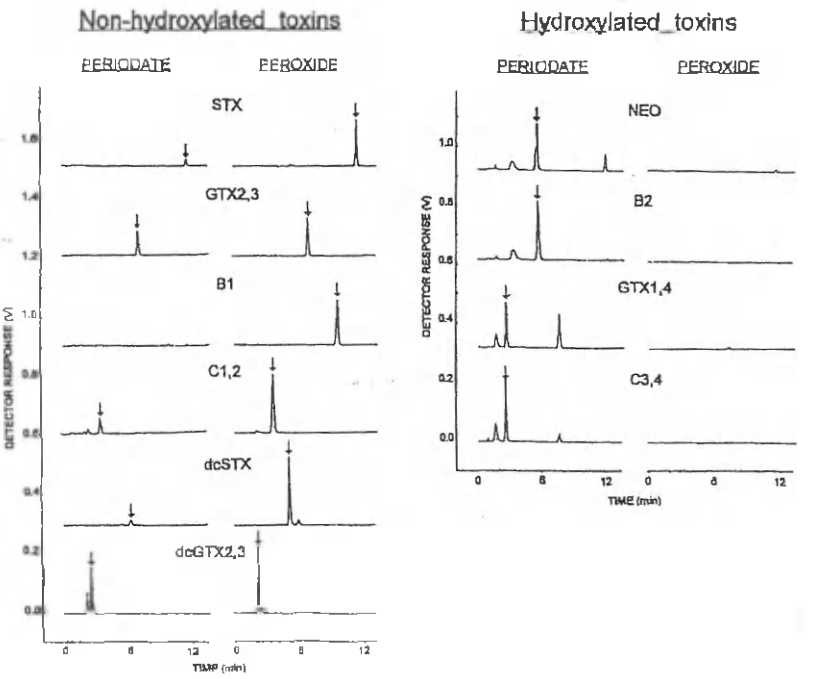

Figure 1. Chromatographic patterns showing oxidation products formed after periodate and peroxide oxidations of toxins included in this study. The same quantity of each toxin was used for each oxidation reaction. Arrows indicate peaks used for quantitation.

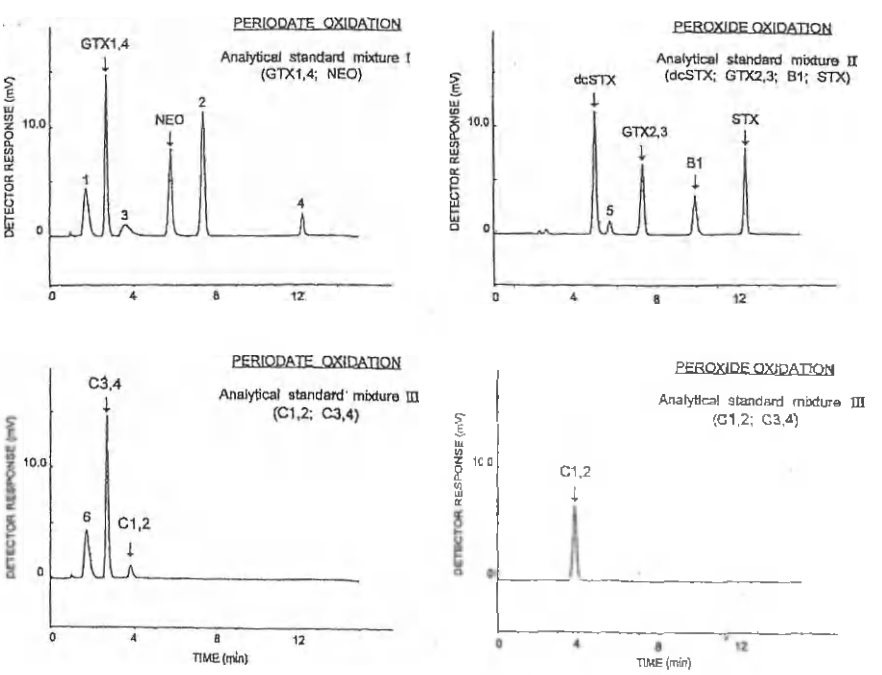

Figure 2. Typical chromatographic patterns obtained with 3 mixtures of analytical standards of PSP toxins. The hydroxylated toxins are oxidized with periodate and the nonhydroxylated toxins by peroxide. The arrows indicate peaks used for quantitation. Peaks 1 and 2 are secondary oxidation products of GTX1,4; peaks 3 and 4 are secondary oxidation products of NEO; peak 5 is a secondary oxidation product of dcSTX; and peak 6 is a secondary oxidation product of C3,4.

number of spiked samples were limited. A C-3 and C-4 (C3,4) analytical standard was not available from any known sources, and to include it in the study it was necessary to isolate it from the shellfish tissue. For that purpose we used New Zealand shellfish. C-1 and C-2 (C1,2) and C3,4 toxins were isolated and purified from the tissue. The toxins were quantified by hydrolyzing them to gonyantoxins 1 and 4 (GTX1,4) and gonyantoxins 2 and 3 (GTX2,3) and comparing to certified standards of GTX1,4 and GTX2,3. That mixture was used for calibration standard preparation and for spiking. For spiking 2 of the study samples a purified acetic acid extract of *Acanthocardia tuberculatum* from Spain containing decarbamoyl saxitoxin (dcSTX), B-1 (GTX5), and STX was used. A B-2 (GTX6) standard was not available for this study. However, laboratories were asked to qualitatively report the presence of B-2 toxin.

METHOD

Applicable to the determination of STX, neosaxitoxin (NEO), GTX2,3/GTX1,4, dcSTX, B-1, C-1 and C-2 (together), and C-3 and C-4 (together) in shellfish (mussels, clams, oysters, and scallops).

Principle

Samples are extracted by heating with acetic acid solution. The extracts are cleaned up using solid-phase extraction (SPE) C18 cartridges. After periodate and peroxide oxidation they are analyzed by LC with fluorescence detection. Most toxins (STX, C1,2, B-1, dcSTX, and GTX2,3) can be quantified after simple SPE-C18 cleanup. Extracts containing the toxins NEO, GTX1,4, C3,4, and B-2 have to be further purified by using SPE-COOH cleanup/separation. PSP toxin concentrations are calculated by comparison of peak areas or heights in the test samples with those of the standards.

Apparatus and Materials

(a) *LC system.*—The LC system must be capable of producing a binary graident. It must be equipped with an injection port capable of injecting up to 100 µL solution. A reversed-phase C18 column with dimensions of 15 cm × 4.6 mm id, 5 µm particle size (e.g., Supelcosil LC-18), should be used for separation of the toxin oxidation products. If a column of different dimensions is used, the gradient and flow-rate conditions must be altered to obtain the necessary separation of the PSP oxidation products within a ca 20 min chromatographic run. The suggested mobile phase gradient used to elute the PSP oxidation products consists of 2 mobile phases [*see Reagents*, (o)] under the following conditions: 0–5% mobile phase B in the first 5 min, 5–70% B for the next 4 min, and back to 0% B over the next 2 min; then at 0% B for another 3 min before the next injection. The flow rate is 2 mL/min. For monitoring the LC effluent, a dual monochromator fluorescence detector, with excitation set to 340 nm and emission to 395 nm, is required. It is a requirement that the detector be sensitive enough to produce a peak height response of at least 3 cm with a peak-to-peak baseline noise of 3 mm (10:1, signal/noise ratio) for an injection of 400 pg STX standard carried through the peroxide oxidation procedure under the chromatographic conditions described above. Figure 1 shows expected chromatographic responses for the toxins after periodate and peroxide oxidation. The chromatographic system must be able to separate with baseline resolution, the main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B-1, and STX, within a 20 min chromatographic run (Fig-

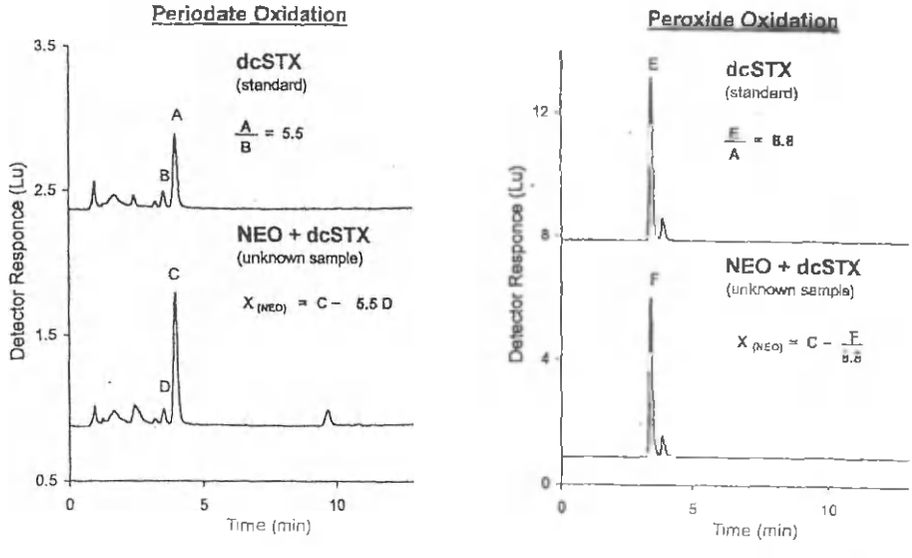

Figure 3. Chromatograms explaining calculation of NEO in the presence of dcSTX. A, B, and E are peak areas of dcSTX standard after periodate and peroxide oxidation; C, D, and F are peak areas of an unknown sample contaminated with dcSTX and NEO after periodate and peroxide oxidation.

Table 1. Interlaboratory study results for the determination of STX in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	$_{.}R$	HORRAT
Practice	Mussel	11(1)	471.00		41.16	8.74	115.25	0.49
1	Scallop	15(0)	1048.07		229.23	21.87	641.84	1.38
2	Clams	15(0)	338.27		90.88	26.87	254.46	1.43
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
5	Clams	15(0)	522.20		69.39	13.29	194.29	0.75
5D	Clams	15(0)	517.87		73.89	14.27	206.89	0.81
6	Clams	15	ND					
7	Mussel	14(0)	23.36		8.48	36.30	23.74	1.29
8	Mussel	15(0)	309.60		78.07	25.22	218.60	1.32
8D	Mussel	15(0)	315.53		70.28	22.27	196.78	1.17
9	Mussel	15(0)	132.47		33.38	25.20	9 3.46	1.16
10	Oyster	15(0)	136.13		46.14	33.89	129.19	1.57
10D	Oyster	15(0)	144.47		41.64	28.82	116.59	1.35
11	Oyster	15(0)	257.67		59.49	23.09	166.57	1.18
12	Mussel	15(0)	783.87		215.24	27.46	602.67	1.65
Spike 1	Mussel	15(0)	223.07	74.4	52.46	23.52	146.89	1.17
Spike 2	Mussel	15(0)	447.00	74.5	112.49	25.17	314.97	1.39
Spike 3	Mussel	15(0)	51.27	93.2	16.96	33.08	47.49	1.32
Spike 4	Mussel	15(0)	169.00	76.8	43.00	25.44	120.40	1.22
Spike 5	Mussel	11(0)	13.18		8.65	65.63	24.22	2.14

[a(b)]Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

ure 2). A chromatography data processing workstation is recommended to analyze chromatograms and generate reports.

(b) *Hot plate.*

(c) *Vortex mixer.*

(d) *pH meter.*

(e) *Benchtop centrifuge.*—Capable of 3600 × *g*.

(f) *Centrifuge tubes.*—Polypropylene, 50 mL.

(g) *Test tubes.*—Glass, graduated, 5 and 15 mL.

(h) *SPE C18 cartridges.*—Supelclean LC18 cartridges (500 mg/3 mL volume; Supelco; Bellefonte, PA).

(i) *SPE-COOH ion exchange cartridges.*—Bakerbond Carboxylic Acidsilane (COOH) bonded to silica gel (500 mg/3 mL volume; J.T. Baker, Phillipsburg, NJ). Phenomenex or Isolute SPE-COOH cartridges are also suitable with the procedure. However, other COOH-SPE cartridges were not evaluated and may require different eluting conditions. *Note*: The analyst must ensure that cartridges used in this method function correctly.

Reagents

(a) *Analytical standards of STX, NEO, GTX1,4, GTX2,3, C1,2, B-1, and dcSTX.*—As separate solutions in 0.1M acetic acid with concentrations ranging from 100 to 2000 µg/mL, obtained from National Research Council of Canada, Halifax, Canada. Prepare ca 2 mL of each working stock solution by 10- or 20-fold dilution of analytical standard solution with water to obtain working stock solutions with concentrations ranging from 8 to 100 µg/mL (take 200 or 100 µL analytical standard solution and diluting it to 2 mL). Do further dilutions as needed, making sure that the pH is about 4 (*see* note below).

Note: For convenience the standards can be combined into 3 mixtures by appropriate dilution of the stock solutions in water. Those solutions must be adjusted to about pH 4 with 0.1M acetic acid. All further dilutions should be made using 0.1mM acetic acid, so that the solutions are kept at about pH 4. The suggested mixtures are as follows: Mix I: *For periodate oxidation.*—GTX 1,4 and NEO; Mix II: *For peroxide oxidation.*—GTX 2,3, STX, B-1, and dcSTX; Mix III: *For periodate and peroxide oxidation.*—C1,2 and C3,4.

Figure 2 shows typical chromatograms of such mixtures.

It was observed that the sensitivity of the most positively charged toxins, STX, NEO, and dcSTX, decreased drastically over time when dilute aqueous solutions of standard mixtures were stored in glass vials. This appears to be due to adsorption of these toxins on the glass walls. To ensure stability, dilutions should be made with 0.1mM acetic acid as described above. It is recommended that dilute standard solutions be stored in plastic vials. However, if glass containers are used they must

Table 2. Interlaboratory study results for the determination of STX in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	15(0)	451.80		121.68	26.93	340.70	1.49
1	Scallop	15(0)	955.93		187.77	19.64	525.76	1.22
2	Clams	14(0)	294.57		82.80	28.11	231.84	1.46
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	15(0)	509.53		85.78	16.84	240.18	0.95
5D	Clams	15(0)	474.40		82.56	17.40	231.17	0.97
6	Clams	14	ND					
7	Mussel	12(0)	21.33		6.80	31.88	19.04	1.12
8	Mussel	15(0)	270.13		68.83	25.48	192.72	1.31
8D	Mussel	13(0)	269.08		49.81	18.51	139.47	0.95
9	Mussel	14(0)	119.64		38.16	31.90	106.85	1.45
10	Oyster	14(0)	140.00		45.24	32.31	126.67	1.50
10D	Oyster	15(0)	123.67		26.92	21.77	75.38	0.99
11	Oyster	15(0)	232.87		37.89	16.27	106.09	0.82
12	Mussel	14(0)	644.00		78.89	12.25	220.89	0.72
Spike 1	Mussel	13(0)	191.08	63.7	39.19	20.51	109.73	1.00
Spike 2	Mussel	15(0)	409.27	68.2	122.65	29.97	343.42	1.64
Spike 3	Mussel	11(0)	43.55	79.2	11.36	26.08	31.81	1.02
Spike 4	Mussel	15(0)	147.93	67.2	31.42	21.24	87.98	1.00
Spike 5	Mussel	9(0)	10.44		4.48	42.91	12.54	1.35

[a(b)]Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

be deactivated by soaking the vials overnight in diluted sodium hydroxide (1M). The vials are then rinsed with water, followed by methanol, and dried. The oxidation products are not affected by the type of vials; therefore, the oxidation reactions can be performed in glass autosampler vials without deactivation.

(b) *Matrix modifier.*—Used for periodate oxidation. A solution is prepared from an oyster extract and added as a reagent to the oxidation mixture. The presence of the matrix modifier increases the yield of oxidation products of the hydroxylated toxins (NEO, GTX1,4, and B-2), as well as recovery with spiked shellfish samples. The matrix modifier is prepared from a sample of blank (PSP-free) oysters, that is extracted and cleaned up on an SPE C18 cartridge exactly as described in the procedure (*see Sample Extraction* and *SPE C18 Cleanup*). The extract is permitted to sit at room temperature for 3 days to allow precipitation of coextracted material. The supernatant is then decanted or filtered through a 0.45 µm Acrodisc filter and stored in a refrigerator when not in use.

(c) *Distilled-deionized (Milli Q) water.*

(d) *Acetic acid.*—Glacial.

(e) *Methanol.*—Analytical grade.

(f) *Acetonitrile.*—Analytical grade.

(g) *Ammonium formate.*—0.3 and 0.1M aqueous solutions.

(h) *Ammonium acetate.*—0.01M aqueous solution.

(i) *Sodium chloride.*—0.3 and 0.05M aqueous solutions.

(j) *Sodium hydroxide.*—1M aqueous solution.

(k) H_2O_2.—10% aqueous solution (store in a refrigerator).

(l) *Sodium phosphate* (Na_2HPO_4).—0.3M aqueous solution.

(m) *Periodic acid solution.*—0.03M aqueous solution (store in a refrigerator).

(n) *Periodate oxidant.*—Prepare daily by mixing 5 mL each 0.03M periodic acid, 0.3M ammonium formate, and 0.3M Na_2HPO_4, and adjusting pH to 8.2 with 0.2M NaOH using a pH meter.

(o) *Mobile phase.*—(A) 0.1M ammonium formate; (B) 0.1M ammonium formate in 5% acetonitrile, both adjusted to pH 6 by adding 6 mL 0.1M acetic acid.

Shellfish Tissue Preparation

As with mouse bioassay (2), thoroughly clean outside of shellfish with fresh water; then open shellfish, remove tissue into homogenizer, and blend until homogeneous.

Sample Extraction

In a 50 mL polypropylene centrifuge tube mix 5.00 g (±0.10 g) homogenized shellfish sample with 3.0 mL 1% acetic acid solution on a Vortex mixer, cap it loosely (to avoid pressure build up during heating), and then place in boiling water bath (100°C) so that contents of tube are below the water line. Heat for 5 min. Do not place so many tubes in the bath at once that the water bath stops boiling or the temperature drops for more than 30 s. Remove sample from water bath, remix on Vortex mixer, and cool by placing in refrigerator or beaker of cold water for 5 min. Centrifuge for 10 min at 4500 rpm (3600 × *g*), and decant supernatant into 15 mL graduated conical test tube. Add 3 mL 1% acetic acid solution to centrifuge tube containing solid residue, mix well on Vortex mixer, and centrifuge again for 10 min at 4500 rpm (3600 × *g*). Collect supernatant into same graduated conical test tube that contains the first portion of extract and dilute to 10.0 mL with water.

SPE C18 Cleanup

Condition 3 mL SPE C18 cartridge (Supelco) with 6 mL methanol followed by 6 mL water. Add 1 mL (0.5 g shellfish equivalent) of above crude extract to cartridge. Keep flow rate between 2–3 mL/min for all elutions. Collect effluent into 5 mL graduated conical test tube. Do not let cartridge run dry. Wash cartridge with 2 mL water and combine washings with effluent.

Adjust extract to pH 6.5 with 1M NaOH using pH indicator paper (calibrated against a pH meter), and then adjust volume to exactly 4.0 mL with water. Use aliquots of this extract for oxidation with periodate and peroxide as described below. Also, analyze an aliquot of the extract without oxidation to verify that the PSP toxin peaks found in the chromatograms are indeed due to PSP and not to naturally fluorescent sample coextractives. To perform that, a volume of sample extract and matrix modifier is mixed with water instead of periodate oxidant before injecting it into the LC system. The resulting chromatogram will allow identification of peaks arising from naturally fluorescent sample coextractives. PSP toxins do not produce peaks under these conditions.

SPE-COOH Cleanup

SPE-COOH ion exchange cleanup is used only for extracts that contain N-1-hydroxylated PSP toxins after C18 cleanup. Condition a 3 mL SPE-COOH cartridge (Bakerbond, J.T. Baker) by passing 10 mL 0.01M ammonium acetate solution through it. Keep flow rate between 2–3 mL/min for all elutions. Do not let cartridge run dry. Discard effluent. Pass 2 mL aliquot (0.250 g shellfish tissue equivalent) of shellfish extract from SPE C18 cleanup through the cartridge and collect effluent in 15 mL graduated conical test tube marked as Fraction #1. Then pass 4.0 mL water through the cartridge and collect into the same tube. Adjust final volume to 6.0 mL in total. This fraction contains the C toxins. Then pass 4.0 mL 0.05M NaCl solution through the same cartridge, collect in 5 mL graduated conical test tube marked as Fraction #2. Ensure that final volume is 4.0 mL. This fraction contains the toxins GTX1,4, GTX2,3, B-1, B-2, and dcGTX2,3 (although

Table 3. Interlaboratory study results for the determination of NEO in shellfish after SPE-C18 cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	10(2)	565.50		113.31	20.04	317.27	1.15
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
5	Clams	7(1)	33.14		12.75	38.47	35.70	1.44
5D	Clams	9(1)	40.00		17.41	43.53	48.75	1.68
6	Clams	15	ND					
8	Mussel	14(0)	283.86		91.76	32.33	256.93	1.67
8D	Mussel	14(0)	278.29		70.58	25.36	197.62	1.31
9	Mussel	13(0)	140.08		45.88	32.75	128.46	1.52
12	Mussel	15(0)	871.07		227.63	26.13	637.36	1.60
Spike 1	Mussel	14(0)	222.64	55.7	88.03	39.54	246.48	1.97
Spike 2	Mussel	15(0)	455.40	56.9	116.94	25.68	327.43	1.43
Spike 3	Mussel	14(0)	158.29[d]		39.36	24.87	110.21	1.18
Spike 4	Mussel	12(0)	213.33[e]		85.77	40.21	240.16	1.99
Spike 5	Mussel	12(0)	344.08[e]		163.71	47.58	458.39	2.53

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

[d] NEO together with dcSTX but calculated as NEO.

[e] No NEO present in these samples. Peaks due to dcSTX but calculated as NEO.

Table 4. Interlaboratory study results for the determination of NEO in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	15(0)	642.80		147.95	23.02	414.26	1.35
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	10(0)	38.80		13.02	33.56	36.46	1.29
5D	Clams	10(0)	42.20		18.27	43.29	51.16	1.68
6	Clams	14	ND					
8	Mussel	13(1)	254.62		89.85	35.29	251.58	1.80
8D	Mussel	15(0)	266.20		92.93	34.91	260.20	1.79
9	Mussel	14(0)	97.64		46.37	47.49	129.84	2.09
12	Mussel	14(1)	842.93		199.36	23.65	558.21	1.44
Spike 1	Mussel	14(0)	212.29	53.1	68.05	32.06	190.54	1.59
Spike 2	Mussel	15(0)	492.43	61.6	159.58	32.41	446.82	1.82

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

not included in this study). Next pass 5.0 mL 0.3M NaCl solution through the cartridge, and collect in 5 mL graduated conical test tube marked as Fraction #3. Ensure that final volume is 5.0 mL. This fraction contains STX, NEO, and dcSTX. Proceed with oxidation and LC analyses.

If problems with detector sensitivity are encountered, each fraction can be concentrated. In that case, collect each fraction from ion exchange column into 50 mL round-bottom flasks instead of graduated conical test tubes and evaporate to ca 1 mL on rotary evaporator with water bath set at 45°C. Transfer solution into 5 mL graduated conical test tube using a Pasteur pipet. Rinse 50 mL round-bottom flask 3 times with ca 0.2–0.3 mL water each time, transferring rinse into graduated tube so that final volume of extract is 2.0 mL. *Note*: A graduated 2 mL volumetric flask can be also used. Analyze fractions #1, #2, and #3 by LC after periodate and peroxide oxidations as described below.

Oxidation Reactions

(a) *Periodate oxidation.*—All reagents and solutions used in the oxidation reactions are dispensed using autopipets (Eppendorf or equivalent) with disposable plastic tips. Add 100 µL sample extract after SPE C18 or ion exchange cleanup or standard solution to 100 µL matrix modifier solution in 1.5 mL microcentrifuge tube (or autosampler vial, if automated chromatographic analysis will be performed). Then add 500 µL periodate oxidant, and mix well on a Vortex mixer. Let solution react at room temperature for 1 min; then add 5 µL concentrated acetic acid, and mix. Let mixture stand for 10 min at room temperature before injecting 50–100 µL into LC system.

(b) *Peroxide oxidation.*—Add 25 µL 10% (w/v) aqueous H_2O_2 to 250 µL 1M NaOH in 1.5 mL plastic microcentrifuge tube (or autosampler vial, if automated chromatographic analysis will be performed), and mix. Then add 100 µL sample extract after SPE C18 or ion exchange cleanup or standard solution. Mix and let react for 2 min at room temperature. Add 20 µL concentrated acetic acid and mix solution. Inject 25–50 µL of this solution into LC system. (*Note*: Injecting more than 50 µL may cause peak broadening.)

Table 5. Interlaboratory study results for the determination of NEO in the presence of dcSTX in spiked shellfish after SPE-COOH cleanup—spike 3

Lab No.	NEO, µg/kg	Recovery, %
2	107	53.5
6[a]	137	68.5
12[a]	154	77.2
15	86	43.0
18	75	37.5
19[a]	92	46.0
22	112	56.0
23	153	76.5
Mean	114.48	57.28
S_R	30.49	
RSD_R	26.63	
R	85.37	
HORRAT	1.20	

[a] Calculation made in coordinator's laboratories using results provided by collaborators.

Table 6. Interlaboratory study results for the determination of dcSTX in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	9(0)	18.78		21.61	115.07	60.51	3.95
1	Scallop	13(0)	12.31		4.33	35.17	12.12	1.13
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
5	Clams	14(0)	8.14		2.71	33.29	7.59	1.01
5D	Clams	12(1)	7.42		1.83	24.66	5.12	0.74
6	Clams	15	ND					
7	Mussel	11(1)	10.55		2.02	19.15	5.66	0.60
11	Oyster	11(0)	3.45		1.63	47.25	4.56	1.26
12[d]	Mussel	—						
Spike 3	Mussel	15(0)	83.73	83.7	41.17	49.17	115.28	2.12
Spike 4	Mussel	15(0)	293.87	73.5	63.35	21.56	177.38	1.12
Spike 5	Mussel	14(1)	385.64	64.3	40.60	10.53	113.68	0.57

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

[d] Not calculated. Only 7 laboratories reported numerical data; the remainder reported as not detected.

Calculation

Each toxin is quantitatively determined in shellfish tissue by direct comparison to analytical standards at similar concentrations as anticipated in the sample [*see* below (**a**)]. For convenience, 3 analytical standard mixtures can be used for quantitating the toxins as described above in *Reagent* section.

STX, GTX2,3, B-1, and C1,2 produce single oxidation products with both oxidation reactions; dcSTX produces 2 oxidation products with both reactions. However, NEO, B-2, GTX1,4, and C3,4 each produce 3 peaks after periodate oxidation but only the second eluting peaks are used for quantitation (Figure 1). Because some PSP toxins (NEO and B-2; GTX1,4 and C3,4) give the same oxidation products, their quantitative determination can be done only after separation by COOH ion exchange chromatography, as described in *SPE-COOH Cleanup* section above. If present in sufficient concentration, dcSTX will interfere with NEO quantification after periodate oxidation. NEO can be quantified by using mathematical ratios of peaks after periodate and peroxide oxidation, as described below in (**b**).

(**a**) *Concentration of PSP (in µg/kg).*

$$\text{Toxin concentration, } \mu g/kg = \frac{Ax \times Cs \times Vx \times D \times 1000}{As \times M}$$

where Ax = peak area of the toxin in the extract analyzed; As = peak area of the nearest standard; Cs = concentration of standard (µg/mL); Vx = final volume of extract analyzed (mL); D = dilution factor. For extracts where PSP toxins are above the most concentrated standard mixture, dilute them with water, so that the toxin concentrations fall in the range of the standards, and reanalyze. Note the dilution factor. M = amount of matrix (g) carried through cleanup procedure (for example, 0.5 g for SPE C18 and 0.25 g for SPE-COOH cleanup); 1000 = factor to convert results from µg/g to µg/kg.

(**b**) *Calculating NEO in the presence of dcSTX.*—The peak area of NEO in a sample containing both NEO and dcSTX can be calculated in 2 ways. Figure 3 shows chromatograms after periodate and peroxide oxidation of a dcSTX standard and an unknown sample containing a mixture of NEO and dcSTX.

Mark the peak areas with letters as follows: dcSTX standard A = second (bigger) peak, periodate oxidation; B = first (smaller) peak, periodate oxidation; E = first (bigger) peak, peroxide oxidation; unknown sample C = second (bigger) peak, periodate oxidation; D = first (smaller) peak, periodate oxidation; F = first (bigger) peak, peroxide oxidation.

Method 1.—If concentration of dcSTX in sample containing mixture of NEO and dcSTX is significant to produce a sensitive, well-integrated first peak (peak D, Figure 3) with periodate oxidation, calculate the peak area corresponding to NEO (peak $X_{(NEO)}$) from the ratio of the 2 dcSTX peak areas produced with periodate oxidation of the standard.

For the dcSTX standard analyzed with periodate oxidation, the ratio of the 2 peak areas is calculated as:

$$\frac{A}{B} = \frac{X_{(dcSTX)}}{D} \Rightarrow X_{(dcSTX)} = \frac{D \times A}{B}$$

where $X_{(dcSTX)}$ is the peak area of dcSTX in peak C. The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - \frac{D \times A}{B}$$

Table 7. Interlaboratory study results for the determination of dcSTX in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	10(0)	12.60		12.38	98.25	34.66	3.18
1	Scallop	12(1)	9.88		3.89	39.37	10.89	1.23
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	11(0)	8.36		4.86	58.13	13.61	1.77
5D	Clams	10(0)	6.80		3.33	48.97	9.32	1.44
6	Clams	14	ND					
7	Mussel	13(0)	11.62	3.04	26.16	8.51	0.84	
11[d]	Oyster	—						
12	Mussel	10(0)	7.80	3.22	41.28	9.02	1.24	
Spike 3	Mussel	13(0)	69.08	69.1	17.09	24.74	47.85	1.03
Spike 4	Mussel	15(0)	276.00	69.0	52.83	19.14	147.92	0.99
Spike 5	Mussel	15(0)	396.27	66.0	93.72	23.65	262.42	1.29

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

[d] Not calculated. Only 6 laboratories reported numerical data; the remainder reported as not detected.

Table 8. Interlaboratoy study results for the determination of GTX1,4 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	11(0)	1437.55		649.76	45.20	1819.33	2.98
1	Scallop	10(0)	199.30		170.10	85.35	476.28	4.19
2	Clams	11(1)	285.27		60.08	21.06	168.22	1.09
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	11(0)	61.36		28.46	46.38	79.69	1.91
5D	Clams	11(0)	62.09		17.73	28.56	49.64	1.17
6	Clams	16	ND					
7	Mussel	15(0)	1489.13[d]		417.08	28.01	1167.82	1.86
8	Mussel	12(0)	572.67		156.87	27.39	439.24	1.57
8D	Mussel	13(0)	672.23		164.05	24.40	459.34	1.44
9	Mussel	13(0)	254.46		69.57	27.34	194.80	1.39
11	Oyster	12(0)	81.00		54.86	67.73	153.61	2.90
12	Mussel	10(0)	1815.70[d]		427.69	23.56	1197.53	1.61
Spike 1	Mussel	10(0)	240.10	60.0	115.87	48.26	324.44	2.43
Spike 2	Mussel	11(0)	1440.45[d]		274.83	19.08	769.52	1.26
Spike 5	Mussel	12(1)	2236.25[c]		571.98	25.58	1601.54	1.80

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

[d] GTX1,4 together with C3,4 but calculated as GTX1,4.

Table 9. Interlaboratory study results for the determination of GTX1,4 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	16(0)	1279.38		316.92	24.77	887.38	1.61
1[c]	Scallop							
2	Clams	13(1)	364.23		141.21	38.77	395.39	2.08
3	Oyster	15	ND[d]					
4	Mussel	15	ND					
5	Clams	13(0)	73.54		17.18	23.36	48.10	0.99
5D	Clams	13(0)	71.46		22.60	31.63	63.28	1.33
6	Clams	16	ND					
7	Mussel	14(1)	240.07		64.50	26.87	180.60	1.35
8	Mussel	15(1)	645.00		147.94	22.94	414.23	1.34
8D	Mussel	15(1)	674.73		120.99	17.93	338.77	1.06
9	Mussel	14(0)	235.57		44.94	19.08	125.83	0.96
11	Oyster	8(1)	48.88		13.03	26.66	36.48	1.06
12	Mussel	16(0)	2079.31		394.95	18.99	1105.86	1.33
Spike 1	Mussel	14(0)	267.50	66.9	86.80	32.45	243.04	1.66
Spike 2	Mussel	15(0)	628.07	78.5	167.26	26.63	468.33	1.55
Spike 5	Mussel	14(0)	265.21[e]		68.76	25.93	192.53	1.33

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] Not calculated. Half of the laboratories reported not detected.

[d] ND = Not determined.

[e] No GTX1,4 present in this sample. Peaks due to dcGTX2,3 (confirmed by peroxide oxidation) but calculated as GTX1,4.

Method 2.—If concentration of dcSTX in sample containing NEO and dcSTX is not significant enough to produce a well-integrated first peak (peak D, Figure 3) with periodate oxidation, then calculate the peak area corresponding to NEO [peak $X_{(NEO)}$] from the ratio of the dcSTX peak produced with periodate oxidation (peak A, Figure 3) and the dcSTX peak area produced with peroxide oxidation (peak E, Figure 3) in the standard.

For a dcSTX standard analyzed with periodate and peroxide oxidations, the ratio of the 2 peak areas is calculated as:

$$\frac{E}{A} = \frac{F}{X_{(dcSTX)}} \Rightarrow X_{(dcSTX)} = \frac{F \times A}{E}$$

where $X_{(dcSTX)}$ is the peak area of dcSTX in peak C. The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - \frac{F \times A}{E}$$

Application of the Method for Routine Analysis

To efficiently apply this method to routine analysis where large numbers of samples need to be analyzed, the following procedure is recommended.

(*1*) Analyze sample extracts after C18 SPE cleanup and periodate oxidation. If no peaks correspond to any of the PSP standards, the sample is negative and no further analyses are required.

(*2*) If any of the nonhydroxylated toxins, STX, dcSTX, GTX2,3, B1, or C1,2 are present but the N-hydroxylated toxins NEO, GTX1,4, and C3,4 are absent, then the nonhydroxylated toxins can be quantified by direct comparison to known standards carried through the periodate oxidation. However, the peroxide oxidation reaction is much more sensitive for B1, C1,2, and dcSTX and would be preferred if maximum sensitivity is required. The peroxide oxidation reaction may be used to confirm findings of all toxins obtained by the periodate oxidation.

(*3*) If both N-hydroxylated and nonhydroxylated toxins are present, the nonhydroxylated toxins are quantified after another portion of the sample extract is oxidized using hydrogen peroxide and the peaks compared to standards similarly oxidized. The reasons for this are that, with periodate oxidation, some of the hydroxylated toxins produce peaks that elute with their nonhydroxylated analogs, and that for some toxins (especially B-1, C1,2, and dcSTX) peroxide oxidation is much more sensitive.

(*4*) For quantification of any N-hydroxylated toxins, another portion of the sample extract is cleaned up using the SPE-COOH cartridge step and the appropriate fraction oxidized after periodate oxidation. This step separates C3,4 from GTX1,4, and B-2 from NEO, enabling all toxins to be quantified by direct comparison to standards carried through the same periodate oxidation procedure.

Table 10. Interlaboratory study results for the determination of GTX2,3 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	12(1)	3291.17		523.82	15.92	1466.70	1.19
1	Scallop	16(0)	2526.00		652.30	25.82	1826.44	1.86
2	Clams	15(1)	2537.73		479.37	18.89	1342.24	1.36
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	16(0)	119.75		21.78	18.19	60.98	0.83
5D	Clams	16(0)	113.75		24.44	21.49	68.43	0.97
6	Clams	16	ND					
7	Mussel	14(0)	348.64		92.95	26.66	260.26	1.42
8	Mussel	16(0)	813.13		252.98	31.11	708.34	1.89
8D	Mussel	16(0)	757.31		180.55	23.84	505.54	1.43
9	Mussel	16(0)	316.44		65.95	20.84	184.66	1.10
10	Oyster	16(0)	367.25		88.99	24.23	249.17	1.30
10D	Oyster	15(1)	336.20		78.60	23.38	220.08	1.24
11	Oyster	15(1)	673.00		182.03	27.05	509.68	1.59
12	Mussel	15(1)	1855.93		505.27	27.22	1414.76	1.87
Spike 1	Mussel	14(1)	227.36	75.8	42.71	18.79	119.59	0.94
Spike 2	Mussel	16(0)	529.31	88.2	167.47	31.64	468.92	1.80
Spike 5	Mussel	16(0)	954.31	79.5	293.49	30.75	821.77	1.91

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

Results and Discussion

Twenty-one laboratories from 16 different countries declared their interest in participating in the study, but 3 laboratories withdrew after receiving the standards and samples. One of them attempted to set up the LC system with our assistance but without success. Shipments of samples and standards were sent by courier to each participating laboratory. All shipments were received in good condition (still frozen or just thawed). Interlaboratory study results were received from 18 laboratories (14 different countries) in a period from 1 to 7 months after the samples were prepared. Data from 2 laboratories were not included in the report, because not enough information (missing data, calculations) was provided to verify results. Data of the remaining 16 laboratories were statistically analyzed and are summarized in Tables 1–16 for SPE C18 cleanup and SPE C18 and ion exchange (SPE-COOH) cleanups. Tables 17 and 18 show results obtained from blind duplicates for all toxins studied. All results are acceptable with HORRAT values <2.0.

The study samples included shellfish tissue from Spain, New Zealand, and the east and west coasts of Canada, and contained a variety of PSP toxin patterns. The reason for this selection was to test the method with geographically different matrixes/mixtures of PSP toxins. For example, sample 12 contained all 8 toxins studied. The practice sample and samples 8 and 9 contained most of the toxins, mainly GTX1,4, GTX2,3, NEO, STX, B1, and C1,2. Samples 10 and 11 contained mostly STX, GTX2,3, and C1,2, and samples 1 and 2 contained mostly GTX1,4, GTX2,3 and STX. Sample 5 contained mainly STX and traces of other toxins. Samples 7 and 12 and spikes 2 and 5 contained GTX1,4 together with C3,4 and the other toxins. Spike 3 was spiked with NEO and dcSTX together to determine whether participants could calculate NEO in the presence of dcSTX.

The participants used a variety of LC equipment: Agilent, Waters, Jasco, Hewlett Packard, Varian, Dionex, TSP (Thermo Separations Products), and a variety of brand name C18 analytical columns: Supelcosil, Hypersil, Chrompack, Chromsep, Nucleosil, and Luna. All systems met the criteria for sensitivity and toxin separation. Figure 4 shows some typical chromatographic results supplied by several participants. Autoinjection systems were used by most participants. Only 2 laboratories used manual injection. The suggested elution pattern was often modified to suit each individual LC system. The typical limits of detection/quantitation varied for different PSP toxins and depended very much on the sensitivity of the detectors used by the participating laboratories. The laboratories that did not achieve the required sensitivity had difficulties detecting low levels of PSP in the samples. This is one of the reasons that in the tables, some data from the 16 laboratories were not used for statistical analysis (e.g., Tables 3 and 4

Table 11. Interlaboratory study results for the determination of GTX2,3 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	15(1)	3314.33		594.49	17.94	1664.57	1.34
1	Scallop	16(0)	2347.94		522.01	22.23	1461.63	1.58
2	Clams	16(0)	2697.50		506.20	18.77	1417.36	1.36
3	Oyster	15	ND[c]					
4	Mussel	14	ND					
5	Clams	15(1)	132.00		25.33	19.19	70.92	0.88
5D	Clams	16(0)	115.94		28.43	24.52	79.60	1.11
6	Clams	15	ND					
7	Mussel	15(0)	342.80		80.23	23.40	224.64	1.25
8	Mussel	16(0)	804.63		164.42	20.43	460.38	1.24
8D	Mussel	16(0)	745.94		164.78	22.09	461.38	1.32
9	Mussel	16(0)	320.88		58.22	18.14	163.02	0.96
10	Oyster	15(1)	369.47		71.38	19.32	199.86	1.04
10D	Oyster	15(0)	341.60		86.11	25.21	241.11	1.34
11	Oyster	16(0)	701.31		203.93	29.08	571.00	1.72
12	Mussel	16(0)	1873.94		345.02	18.41	966.06	1.27
Spike 1	Mussel	15(0)	261.27	87.1	42.72	16.35	119.62	0.84
Spike 2	Mussel	16(0)	563.56	93.9	116.81	20.73	327.07	1.19
Spike 5	Mussel	16(0)	1024.88	85.4	174.72	17.05	489.22	1.07

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

with NEO results). Also, the laboratories experiencing too-low detector sensitivity had problems to correctly identify and integrate the GTX1,4 peaks analyzed after SPE C18 and the C1,2 peaks analyzed after SPE C18 or SPE-COOH because those peaks eluted at the front of the chromatogram together with background peaks. In that case, only some of the laboratories provided results that were used for statistical analyses (Tables 8, 14, and 15). Two laboratories experienced carry-over of an interfering peak at the retention time of B-1. Also, one laboratory did not report data for STX, dcSTX, and NEO (*see* the reason explained below), so those results are not included.

AOAC INTERNATIONAL provided guidance (computer spreadsheet program) on the statistical evaluation applicable to duplicates and/or Youden pairs but not for statistical processing of individual test samples. However, we applied the Dixon test to each of the individual test samples having a sufficient number of data points to obtain the between-laboratory statistical data for the test samples. HORRAT values (acceptable values are 0.5–2) indicate that variability of the results (expressed as relative standard deviation, RSD_R) for most of the toxins at various concentrations is in the acceptable range. The most variable results (high RSD_R%) were obtained for GTX1,4 after SPE C18 cleanup. Laboratories that failed to achieve good separation and sufficient sensitivity had difficulties in identifing and correctly quantifing the early eluting toxins: GTX1,4, C1,2 or C3,4, because the matrix/background peaks coeluted with those toxins. Overall results for GTX1,4 are better when the second cleanup/separation is applied where GTX1,4 elutes in Fraction #2. In this case, the sample coextractive peaks elute in Fraction #1 with the C toxins. For that reason, the results for C1,2 and C3,4 show a high variability at the concentrations of toxins that were present in the sample. The situation is better after ion exchange separation. The most common errors for GTX1,4, C1,2, and C3,4 included poor integration or misidentification of the peaks.

B-2 toxin was not included in the study for quantitative evaluation because of insufficient amount of the analytical standard available. However, after ion exchange cleanup most laboratories successfully detected and reported B-2 in certain samples at a concentration of about 30 µg/kg or greater. This indicates that once analytical standards are available for B-2, this method should be applicable to the quantitative detection of this toxin.

In spike 3, participants had to apply one of the methods (described in the procedure) for calculating NEO in presence of dcSTX. The exercise was not performed by all laboratories. Table 5 shows results for NEO in spike 3 by the laboratories that performed the calculation. For a number of results the calculation was performed by the study coordinator using data

provided by the participants. These results indicate that NEO can indeed be quantified in the presence of dcSTX with an acceptable RSD_R.

During the study, one laboratory reported a problem with sensitivity of the +2 charged toxins (STX, dcSTX, and NEO) in diluted standards. At that time, the Study Coordinator could not find an answer for the problem but the laboratory was requested to continue the analyses for the rest of the toxins. Meanwhile, our investigation led us to the discovery that significant adsorption of the most basic PSP toxins occurred on the surface of glass vials used for making and storing the standard solutions. However, performing the oxidation reactions in such vials does not cause any problem. As a result, the method has been modified with a note stating to use plastic or deactivated glass vials when handling the PSP standard solutions. No other laboratories reported such a problem, but in a few cases nonproportional peak areas for STX indicated a similar slight effect of adsorption/loss of the toxins. That problem might be a factor contributing to the variation of the results for these toxins especially for NEO.

None of the collaborators reported difficulty with any of the shellfish types analyzed. Also, statistically the method performed satisfactorily for all sample types studied.

In practice, to screen the samples for PSP presence only SPE C18 cleanup is needed. After periodate and peroxide oxidation of the extracts, most of the toxins can be quantified without error except NEO and GTX1,4. All PSP-positive samples suspected to contain NEO or GTX1,4 have to be confirmed by performing the SPE-COOH ion exchange separation because the GTX1,4 peak may contain C3,4 and the NEO peak may contain B-2.

As an additional part of this study, to help assess the accuracy of the method, a set of interlaboratory study samples was analyzed by the mouse bioassay (E. Buenaventura, Canadian Food Inspection Laboratory, Burnaby, BC, Canada) and by a lateral flow antibody-based strip test (Jellett Biotech , Halifax, NS, Canada) in the coordinator's laboratory. The results are presented in Table 19. Overall, the correlation among 3 methods is very good. To compare with the mouse bioassay total, PSP toxin values obtained by the collaboratively studied method were converted to STX equivalents using literature values (5). These results were in very good agreement over the full concentration range studied. The qualitative lateral strip test correlated very well with the total PSP values. The difference in results obtained by the mouse bioassay and the strip test for samples 7, 10, 10D, and 11 are due to the presence of C toxins in these extracts, which are well detected by the strip test but not the mouse bioassay. Spike 3 contained a substantial amount of B-1 toxin. This has a low STX equivalent value and thus was not detected by the mouse bioassay, although the

Table 12. Interlaboratory study results for the determination of B-1 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	11(0)	435.36		47.84	10.99	133.95	0.61
1	Scallop	13(0)	91.08		25.12	27.58	70.34	1.20
2	Clams	11(0)	31.00		7.36	23.74	20.61	0.88
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	13(1)	42.77		8.36	19.55	23.41	0.76
5D	Clams	13(1)	40.54		7.22	17.81	20.22	0.69
6	Clams	16	ND					
7	Mussel	15(0)	145.07		27.11	18.69	75.91	0.87
8	Mussel	15(0)	328.00		47.09	14.36	131.85	0.76
8D	Mussel	15(0)	334.13		51.52	15.42	144.26	0.82
9	Mussel	15(0)	119.80		25.34	21.15	70.95	0.96
10	Oyster	12(0)	37.67		9.26	24.58	25.93	0.94
10D	Oyster	14(0)	39.57		12.97	32.78	36.32	1.26
11	Oyster	14(0)	73.14		16.47	22.52	46.12	0.95
12	Mussel	15(0)	946.87		263.88	27.87	738.86	1.73
Spike 2	Mussel	14(0)	53.36		16.01	30.00	44.83	1.21
Spike 3	Mussel	15(0)	311.13	77.8	69.98	22.49	195.94	1.18
Spike 4	Mussel	15(0)	1212.53	75.8	239.77	19.77	671.36	1.27
Spike 5	Mussel	14(1)	691.00	86.4	223.40	32.33	625.52	1.91

[a/b] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

Table 13. Interlaboratory study results for the determination of B-1 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	14(0)	349.71		58.75	16.80	164.50	0.90
1	Scallop	12(1)	71.75		13.02	18.15	36.46	0.76
2	Clams	9(0)	27.11		4.43	16.34	12.40	0.59
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	12(0)	42.00		11.07	26.36	31.00	1.02
5D	Clams	12(0)	40.75		9.73	23.88	27.24	0.92
6	Clams	14	ND					
7	Mussel	13(0)	120.85		17.71	14.65	49.59	0.67
8	Mussel	14(0)	285.79		43.73	15.30	122.44	0.79
8D	Mussel	14(0)	274.14		52.47	19.14	146.92	0.98
9	Mussel	14(0)	123.71		29.11	23.53	81.51	1.07
10	Oyster	10(0)	30.60		5.99	19.58	16.77	0.72
10D	Oyster	12(0)	37.58		10.93	29.08	30.60	1.11
11	Oyster	12(0)	63.00		11.09	17.60	31.05	0.73
12	Mussel	14(0)	784.86		73.41	9.35	205.55	0.56
Spike 2	Mussel	13(0)	46.00		8.78	19.09	24.58	0.75
Spike 3	Mussel	13(0)	292.54	73.1	43.03	14.71	120.48	0.76
Spike 4	Mussel	14(0)	1064.71	66.5	147.33	13.84	412.52	0.87
Spike 5	Mussel	15(0)	619.67	77.5	85.39	13.78	239.09	0.80

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

Table 14. Interlaboratory study results for the determination of C1,2 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	12(0)	299.00		50.81	16.99	142.27	0.89
2	Clams	13(1)	253.62		56.83	22.41	159.12	1.14
3	Oyster	16	ND[c]					
4	Mussel	15	ND					
5	Clams	16(0)	256.50		74.66	29.11	209.05	1.48
5D	Clams	15(1)	239.13		50.81	21.25	142.27	1.07
6	Clams	16	ND					
7	Mussel	15(0)	912.40		301.90	33.09	845.32	2.04
8	Mussel	12(0)	118.08		63.81	54.04	178.67	2.45
8D	Mussel	10(1)	96.30		24.10	25.03	67.48	1.10
9	Mussel	9(0)	76.56		80.34	104.9	224.95	4.46
10	Oyster	11(0)	161.91		43.73	27.01	122.44	1.28
10D	Oyster	15(0)	189.73		58.51	30.84	163.83	1.50
11	Oyster	16(0)	357.69		58.20	16.27	162.96	0.87
12	Mussel	16(0)	256.50		67.38	26.27	188.66	1.34
Spike 2	Mussel	13(1)	734.85	73.5	108.97	14.83	305.12	0.88
Spike 5	Mussel	14(1)	1566.50	78.3	227.74	14.54	637.67	0.97

[a(b)] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

[c] ND = Not determined.

Table 15. Interlaboratory study results for the determination of C1,2 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
Practice	Mussel	14(0)	300.93		77.77	25.84	217.76	1.35
2	Clams	14(1)	261.07		66.24	25.37	185.47	1.30
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	14(1)	237.07		64.30	27.12	180.04	1.37
5D	Clams	15(0)	238.07		80.34	33.75	224.95	1.70
6	Clams	15	ND					
7	Mussel	15(0)	983.40		255.06	25.94	714.17	1.62
8	Mussel	9(0)	108.56		40.27	37.09	112.76	1.66
8D	Mussel	10(0)	93.40		35.75	38.28	100.10	1.67
9[d]	Mussel							
0	Oyster	14(0)	204.36		57.38	28.08	160.66	1.38
0D	Oyster	14(0)	200.71		48.51	24.17	135.83	1.19
1	Oyster	15(0)	349.07		81.27	23.28	227.56	1.24
2	Mussel	12(1)	257.75		39.23	15.22	109.84	0.78
Spike 2	Mussel	14(0)	859.64	86.0	204.53	23.79	572.68	1.45
Spike 5	Mussel	15(0)	1687.13	84.4	342.42	20.30	958.78	1.37

[a] Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier laboratories.

ND = Not determined.

Not calculated. Only 3 laboratories reported numerical data; the remainder reported as not detected.

ample was positive by the lateral strip test. From these comarisons it is clear that the collaboratively studied LC method ompares very well with the 2 other independent techniques in rms of accuracy. This is particularly positive considering iat the 3 methods detect the PSP toxins by entirely different iechanisms (chromatography/fluorescence, toxicity, and anbody recognition).

Collaborators' Comments

Most collaborators found the study long to perform, but most were able to complete it without difficulty. The Study Coordinator, B. Niedzwiadek, was able to respond to questions from the collaborators concerning many details of the method. As a result, a number of the steps in the final analytical procedure were clarified. Collaborators are thanked for their comments.

able 16. Interlaboratory study results for the determination of C3,4 in shellfish after SPE-COOH cleanup

ample No.	Matrix	Labs[a(b)]	Mean, µg/kg	Recovery, %	S_R	RSD_R, %	R	HORRAT
	Oyster	15	ND[c]					
	Mussel	15	ND					
	Clams	15	ND					
	Mussel	13(0)	837.15		227.24	27.14	636.27	1.65
	Mussel	10(1)	237.50		106.35	44.78	297.78	2.25
ike 2	Mussel	9(0)	724.78	80.5	180.88	24.96	506.46	1.49
ike 5	Mussel	15(0)	1425.07	79.2	301.97	21.19	845.52	1.40

Number of laboratories where a = number of laboratories retained after Dixon statistical outliers removed, and b = number of outlier aboratories.

ND = Not determined.

Table 17. Interlaboratory study results for the determination of PSP in shellfish after SPE C18 cleanup: Blind duplicates statistical treatment

PSP	Youden pairs	Matrix	Labs[a(b)]	Mean, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	r	R	HORRAT
STX	5 and 5D	Clams	15(0)	520.03	31.38	6.03	71.46	13.74	87.85	200.10	0.78
	8 and 8D	Mussel	15(0)	312.57	67.80	21.69	73.23	23.43	189.84	205.05	1.23
	10 and 10D	Oyster	15(0)	140.30	24.96	17.79	43.91	31.29	69.87	122.94	1.46
NEO	8 and 8D	Mussel	13(0)	280.00	43.14	15.41	83.82	29.94	120.79	234.70	1.55
dcSTX	5 and 5D	Clams	12(0)	7.46	0.68	9.08	2.02	27.04	1.90	5.65	0.81
GTX1,4	5 and 5D	Clams	9(0)	64.61	12.85	19.89	24.13	37.35	35.98	67.57	1.55
	8 and 8D	Mussel	11(0)	601.45	119.96	19.95	155.53	25.86	335.89	435.48	1.50
GTX2,3	5 and 5D	Clams	16(0)	116.75	15.77	13.51	23.15	19.83	44.15	64.82	0.90
	8 and 8D	Mussel	16(0)	785.22	135.94	17.31	220.26	28.05	380.64	616.72	1.69
	10 and 10D	Oyster	15(0)	347.13	66.64	19.20	81.10	23.36	186.59	227.09	1.25
B-1	5 and 5D	Clams	13(0)	41.65	5.26	12.62	7.82	18.78	14.72	21.90	0.73
	8 and 8D	Mussel	15(0)	331.07	27.86	8.42	49.17	14.87	78.01	137.68	0.79
	10 and 10D	Oyster	12(0)	38.58	8.35	21.63	11.69	30.30	23.37	32.73	1.16
C1,2	5 and 5D	Clams	15(0)	241.47	37.53	15.54	53.39	22.11	105.09	149.49	1.12
	8 and 8D	Mussel	8(0)	101.00	28.62	28.34	38.38	38.00	80.14	107.46	1.68
	10 and 10D	Oyster	11(0)	168.77	53.48	31.69	53.48	31.69	149.75	149.75	1.52

[a(b)] Number of laboratories where a = number of laboratories retained after Cochran statistical outliers removed, and b = number of outlier laboratories.

Table 18. Interlaboratory study results for the determination of PSP in shellfish after SPE-COOH cleanup: Blind duplicates statistical treatment

PSP	Youden pairs	Matrix	Labs[a(b)]	Mean, µg/g	S_r	RSD_r, %	S_R	RSD_R, %	r	R	HORRAT
STX	5 and 5D	Clams	15(0)	491.97	43.99	8.94	85.73	17.43	123.16	240.04	0.98
	8 and 8D	Mussel	13(0)	273.31	36.19	13.24	60.05	21.97	101.32	168.15	1.13
	10 and 10D	Oyster	14(0)	132.14	32.87	24.88	37.88	28.67	92.05	106.08	1.32
NEO	8 and 8D	Mussel	13(0)	262.65	67.73	25.79	89.95	34.24	189.65	251.79	1.75
	5 and 5D	Clams	10(0)	40.50	7.82	19.30	15.86	39.15	21.89	44.40	1.51
dcSTX	5 and 5D	Clams	9(0)	6.89	0.88	12.80	3.15	45.76	2.47	8.83	1.35
GTX1,4	5 and 5D	Clams	12(0)	73.92	11.10	15.02	19.36	26.19	31.09	54.20	1.11
	8 and 8D	Mussel	15(0)	659.87	85.84	13.01	135.04	20.47	240.36	378.12	1.20
GTX2,3	5 and 5D	Clams	15(0)	124.47	19.88	15.97	28.14	22.61	55.65	78.80	1.03
	8 and 8D	Mussel	16(0)	775.28	91.07	11.75	166.54	21.48	380.64	616.72	1.69
	10 and 10D	Oyster	14(0)	357.21	84.30	23.60	84.30	23.60	236.03	236.03	1.26
B-1	5 and 5D	Clams	11(0)	40.68	5.04	12.39	10.37	25.48	14.11	29.03	0.98
	8 and 8D	Mussel	14(0)	279.96	40.58	14.49	48.02	17.15	113.62	134.44	0.89
	10 and 10D	Oyster	10(0)	35.25	8.49	24.10	9.56	27.11	23.78	26.76	1.02
C1,2	5 and 5D	Clams	14(0)	230.43	46.06	19.99	62.14	26.97	128.97	174.01	1.35
	8 and 8D	Mussel	8(0)	100.94	19.99	19.80	40.52	40.14	55.96	113.45	1.78
	10 and 10D	Oyster	14(0)	202.54	37.12	18.33	52.66	26.00	103.93	147.46	1.28

[a(b)] Number of laboratories where a = number of laboratories retained after Cochran statistical outliers removed, and b = number of outlier laboratories.

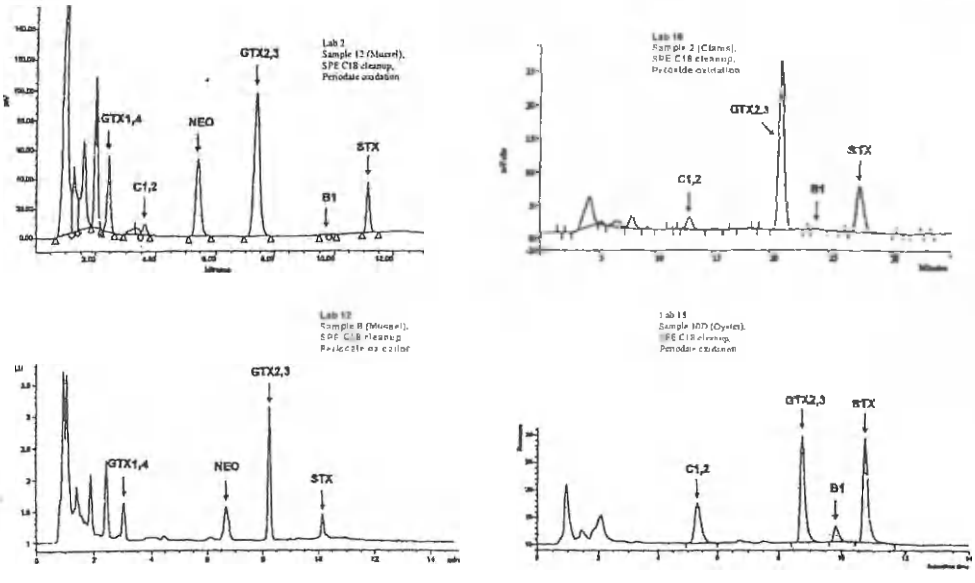

Figure 4. Typical examples of chromatograms from the collaborating laboratories.

Recommendations and Conclusions

The interlaboratory study was successfully completed by 16 laboratories representing 12 different countries from around the world. Collaborators were able to quantify STX, NEO, dcSTX, GTX2,3, GTX1,4, and B-1 at individual concentrations down to between one-tenth and one-twentieth of the common regulatory guideline level of 800 µg/kg (80 µg/100 g) STX equivalents. The C toxins were successfully quantified at levels down to about one-fiftieth to one-hundredth of the regulatory level in terms of STX equivalents, although in terms of µg/kg concentration units they were the least sensitive.

It is recommended that this method be accepted by AOAC INTERNATIONAL as an Official First Action method for the quantitative determination of the above-mentioned PSP toxins in shellfish.

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Table 19. Comparison of interlaboratory study results with mouse bioassay and Mist Alert tests results

		LC analysis			
Sample No.	Matrix	Total PSP, µg/100 g	Total PSP as STX equivalent[a], µg/100 g	Mouse bioassay, µg/100 g	Mist Alert tests, +/– (as T% of C)[b]
Practice	Mussel	634	310	380	+ (T = 0% of C)
1	Scallop	338	185	180	+ (T = 0% of C)
2	Clams	364	156	120	+ (T = 0% of C)
3	Oyster	ND[c]		<42	– (T = 100% of C)
4	Mussel	ND		<42	– (T = 100% of C)
5	Clams	104	65	55	+ (T = 0% of C)
5D	Clams	99	61	61	+ (T = 0% of C)
6	Clams	ND		<42	– (T = 100% of C)
7	Mussel	256	36	<42	+ (T = 0% of C)
8	Mussel	237	122	160	+ (T = 0% of C)
8D	Mussel	232	123	170	+ (T = 0% of C)
9	Mussel	90	48	58	+ (T = 0% of C)
10	Oyster	74	29	<42	+ (T = 25% of C)
10D	Oyster	70	26	<42	+ (T = 25% of C)
11	Oyster	140	54	<42	+ (T = 0% of C)
12	Mussel	673	346	470	+ (T = 0% of C)
Spike 1	Mussel	93	64	76	+ (T = 25% of C)
Spike 2	Mussel	372	149	190	+ (T = 0% of C)
Spike 3	Mussel	53	21	<42	+ (T = 25% of C)
Spike 4	Mussel	149	37	45	+ (T = 25% of C)
Spike 5	Mussel	592	91	73	+ (T = 0% of C)

[a] PSP relative toxicity values (ref. 5): STX–100%; NEO–88%; dcSTX–60%; GTX1,4–63%; GTX2,3–38%; B-1–5.1%; C1,2–3.2%; C3,4–2.2%.

[b] C and T line color interpretation: 50% of C< T≤100% of C; +, 0% of C ≤T≤50% of C.

[c] ND = Not determined.

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References

(1) Shimizu, Y. (1988) in *Handbook of Natural Toxins: Marine Toxins and Venoms*, A.T. Tu (Ed.), Marcel Dekker, New York, NY, p. 68

(2) *Official Methods of Analysis* (1990) 15th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, sec. **959.08**

(3) LeDoux, M., & Hall, S. (2000) *J. AOAC Int.* **83**, 305–310

(4) Lawrence, J.F., & Niedzwiadek, B. (2001) *J. AOAC Int.* **84**, 1099–1108

(5) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

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Topic 3.5

Brominated flame retardants and endocrine disruption*

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Abstract: From an environmental point of view, an increasing important group of organohalogen compounds are the brominated flame retardants (BFRs), which are widely used in polymers and textiles and applied in construction materials, furniture, and electronic equipment. BFRs with the highest production volume are the polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBP-A), and hexabromocyclododecane (HBCD). Because of their persistence and low biodegradation profile, several of the PBDE congeners accumulate in biota and are widely found in the aquatic food chain. Their levels in the environment and in humans have increased during the last decades, in contrast to compounds such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), for example. Humans may be exposed to PBDEs mainly through consumption of fatty food of animal origin (e.g., fish), but exposure through skin contact with textiles protected with flame retardants or through inhalation of BFRs volatilized from electronic and electric equipment may also occur. The levels of PBDEs in Swedish human milk showed a doubling in concentration every five years over the period 1972 to 1997 (2,2′,4,4′-tetraBDE being the predominant congener). The levels of penta- and hexa-BDEs increased at the same rate in ringed seals collected in the Canadian Arctic from 1981 to 2000. PBDEs exhibit a great variety of biological effects, depending on the bromine substitution pattern. PBDEs are potential endocrine disrupters, based on shared toxicity with the structurally related PCBs, polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-*p*-dioxins (PCDDs) (partial aryl hydrocarbon- [Ah-] receptor agonist and antagonist activity in vitro, thyroid toxicity, and immune effects), including developmental toxicity. The potency of TBBP-A to interact with thyroid hormone homeostasis is indicated from in vitro studies in which the compound competes with thyroxin (T4) for binding to transthyretin (TTR). So far, the toxicological profile of many BFRs is too incomplete and insufficient to perform an adequate risk assessment, and further information is required regarding the potential for endocrine disruption of these compounds that are of increasing environmental concern.

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* 75, 1617–2615 (2003).

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INTRODUCTION

There is growing concern about the possible harmful consequences of exposure to xenobiotic compounds that are capable of modulating or disrupting the endocrine system. This concern for endocrine-disrupting chemicals (EDCs) is directed at both humans and wildlife [4,38]. Several expert working groups [1,21,44,47,48] have concluded that there is increasing evidence of adverse effects in human and wildlife reproductive health, and have discussed the hypothesis that chemicals in the environment have caused these endocrine-mediated adverse effects. Endocrine disruption is a complex area to address and it is difficult to establish causal links between exposure to suspected EDCs and any measured effects. The most prominent and persistent organic pollutants that are associated or even causally linked with endocrine disruption in wildlife and in human individuals are the organohalogen compounds (OHCs), including dichlorodiphenyltrichloroethane (DDT) and metabolites, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). From an environmental point of view, an increasing important group of OHCs are the brominated flame retardants (BFRs)

Flame retardants constitute a diverse group of compounds used to prevent fires or minimize the extent of a fire. The worldwide annual production of flame retardants was estimated as 600 000 tons in 1992 [23]. There are three main categories of chemical flame retardants: halogenated hydrocarbons, organophosphorous compounds, and inorganic products often based on metallic hydroxides; these represent 45, 24, and 27 %, respectively, of the total market of flame retardants in 1999 (see ref. [41]). The BFRs are made up of structurally very different chemicals with a wide variety in physicochemical and reactivity characteristics [2]. Important BFRs are the polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBP-A), and hexabromocyclododecane (HBCD) (Fig. 1), high-production-volume chemicals that are widely used in polymers and textiles and applied in construction materials, furniture, and electric and electronic equipment. The annual market demand in 1999 has been estimated as 67 000 tons for PBDEs and 121 000 tons for TBBP-A [41]. The properties of PBDE congeners are variable as well [7,20]. There are three groups of industrial products of PBDEs with an average of five (pentaBDE), eight (octaBDE), or ten bromine atoms (decaBDE) in the molecule, while the theoretical number of possible congeners is 209. Further, photolytic degradation of decaBDE forms by debromination a large number of PBDE congeners not found in technical products of PBDEs [37]. PBDEs are highly lipophilic compounds and generally have a low biodegradation profile. In contrast to PCBs and DDT, the levels of some BFRs, such as PBDEs, show an increasing trend in wildlife and humans dur-

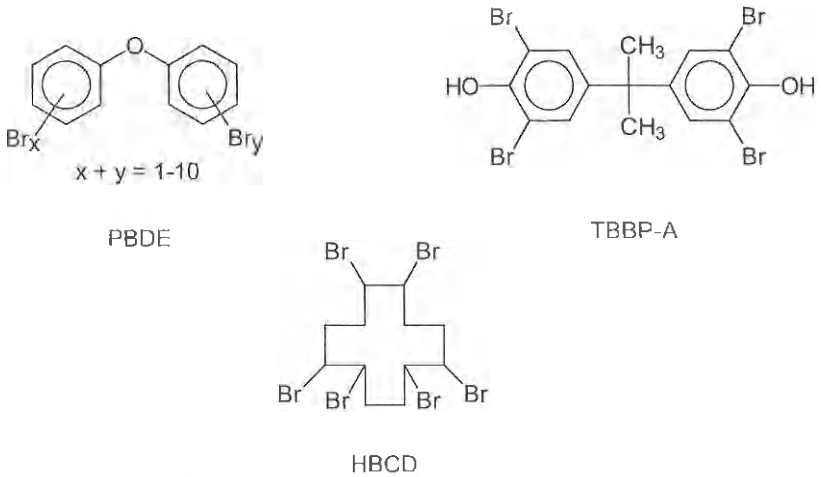

Fig. 1 Chemical structures of polybrominated diphenyl ethers (PBDE), tetrabromobisphenol A (TBBP-A), and hexabromocyclododecane (HBCD).

ing the last decades. Based on shared toxicity with the structurally related PCBs, PCDFs, and PCDDs, PBDEs are potential endocrine disruptors. Moreover, PBDEs may be contaminated with polybrominated dibenzo-*p*-dioxins and dibenzofurans (PBDDs/PBDFs), compounds that also can be formed during combustion of PBDEs. These substances are regarded as equally toxic as their chlorinated counterparts [26].

EXPOSURE DATA

PBDEs accumulate in environmental biota and are widely found in the aquatic food chain [24,36,49], including the Canadian Arctic [22]. Notably, they were found in top predators, such as the harbor seal and in the sperm whale; the latter finding indicates that PBDEs also occur in deep-sea food chains [8]. In the abiotic environment, a wide variety of PBDEs have been found, ranging from congeners with three to ten bromine atoms. From a quantitative point of view, decaBDE is the most dominant PBDE in sediments, up to milligram per kilogram (mg/kg) levels. Debromination of decaBDE may yield PBDE congeners with higher bioavailability and toxicity. Other important lower brominated PBDE congeners in sediment are 2,2′,4,4′-tetraBDE (BDE-47), 2,2′,4,4′,5-pentaBDE (BDE-99), 2,2′,4,4′,6-pentaBDE (BDE-100), and 2,2′,4,4′,5,5′-hexaBDE (BDE-153). These congeners can be found, for example, in sewage sludge and sediment up to several hundreds of micrograms per kilogram (μg/kg) dry weight [9,17], while, in addition, decabrominated BDE can be present into the mg/kg dry weight range [9].

Tissue analysis of wildlife and fish has shown that PBDEs can accumulate, with BDE-47, BDE-99, BDE-100, and BDE-153 being the dominant congeners present [7,11]. The first three are the predominant congeners present in ringed seal from the Canadian Arctic. In humans, these PBDEs have also been found in blood, adipose tissue, and milk with mean levels ranging between 4 and 16 ng/g lipid [10,32,39,42], and levels of approximately 200 ng/g lipid were reported recently in a pooled sample of human milk from the United States (levels of 132, 27, and 15 ng/g lipid of BDE-47, BDE-99, and BDE-153, respectively) [33]. Humans may be exposed to PBDEs mainly through the consumption of fatty food from animal origin (e.g., fish), but exposure through skin contact with textiles protected with flame retardants or through inhalation of BFRs volatilized from hot electrical equipment may also occur. For example, subjects working at an electronics dismantling plant had elevated plasma levels of higher brominated BDEs and TBBP-A [42].

An important observation is that, in contrast to PCBs and DDT, for example, the levels of PBDEs are increasing in human milk: a study in Sweden showed a doubling in concentration every five years over the period 1972 to 1997, with BDE-47 being the predominant congener [31] (Table 1). From 1998 to 2000, a decrease in PBDE levels was noticed that can be a consequence of the phase-out of commercial pentaBDE in Sweden [15]. The temporal trends and influence of age and gender on six BDE congeners was investigated on archived serum samples from Norway [43]. The sum of the BDEs increased from 0.44 ng/g lipids in 1977 to 3.3 ng/g in 1999, with BDE-47 being the most abundant congener. BFR levels in the different age groups were relatively similar, except for the age group of 0 to 4 years, which had 1.6 to 3.5 times higher serum concentrations, with breast milk considered the main source.

Table 1 Temporal trend of PBDEs in Swedish human milk[a].

	1972	1976	1980	84/85	1990	1994	1996	1997	1998	1999	2000
BDE-47	0.06	0.18	0.28	0.49	0.81	1.48	2.08	2.28	2.29	1.97	1.70
Sum of PBDE congeners	0.07	0.35	0.48	0.73	1.21	2.17	3.11	4.02	3.90	3.47	2.80

[a]Levels in ng/g lipid of pooled milk samples (after [15]).

PBDE concentrations showed an exponential increase in ringed seals collected from subsistence hunts in the Canadian Arctic in 1981, 1991, 1996, and 2000, with doubling of the penta- and hexa-BDE in less than five years; the current PBDE concentrations are 50 times lower than those of mono-ortho and non-ortho PCBs [22]. In fish and birds from the Baltic region, increasing levels of BDE-47, -99, and -100 have been reported since the 1970s but have begun to decline or level off in the 1990s [11], which can be a consequence of the phase-out of commercial pentaBDE in Sweden. Increasing levels of PBDEs in fish and birds are also reported for the U.S. Great Lakes, while PCBs levels have decreased during the same time frame. In different fish species collected in 2000 from the North Sea and the Celtic Sea, levels of BDE-47 were similar to levels of PCB 153 and *p,p*-DDE, whereas levels of hexachlorobenzene (HCB) and toxaphene (CHB-50) were lower (Fig. 2). The occurrence and fate of decaBDE is less well known, partly due to analytical difficulties and the fact that the compound has not been prioritized as an analyte, but also due to the different environmental fate of decaBDE as compared to other PBDEs. Further, little is known about other BFRs concerning environmental persistence, bioaccumulation, and toxicological effects, in spite of the fact that these compounds are also high-production-volume chemicals. TBBP-A has been detected in occupationally exposed persons, while HBCD has been found in wildlife and in environmental samples; no HBCD data on humans have so far been presented [2,3,42]. TBBP-A is rapidly excreted by mammals [16] while nothing is known about TBBP-A derivatives in this context. Thus, PBDEs have been steadily increasing over the last decades in biota (including humans), while much less has been published on other BFRs, such as TBBP-A and HBCD. Consequently, the question arises as to what extent these BFRs pose a risk to species higher in the food chain, in particular to top predators and humans. Human exposure probably occurs mainly via food in analogy to PCBs and related compounds, but occupational exposure (e.g., through handling electronic equipment) may also play a significant role. However, it should be noted that detailed information regarding the routes of human exposure to BFRs is presently lacking.

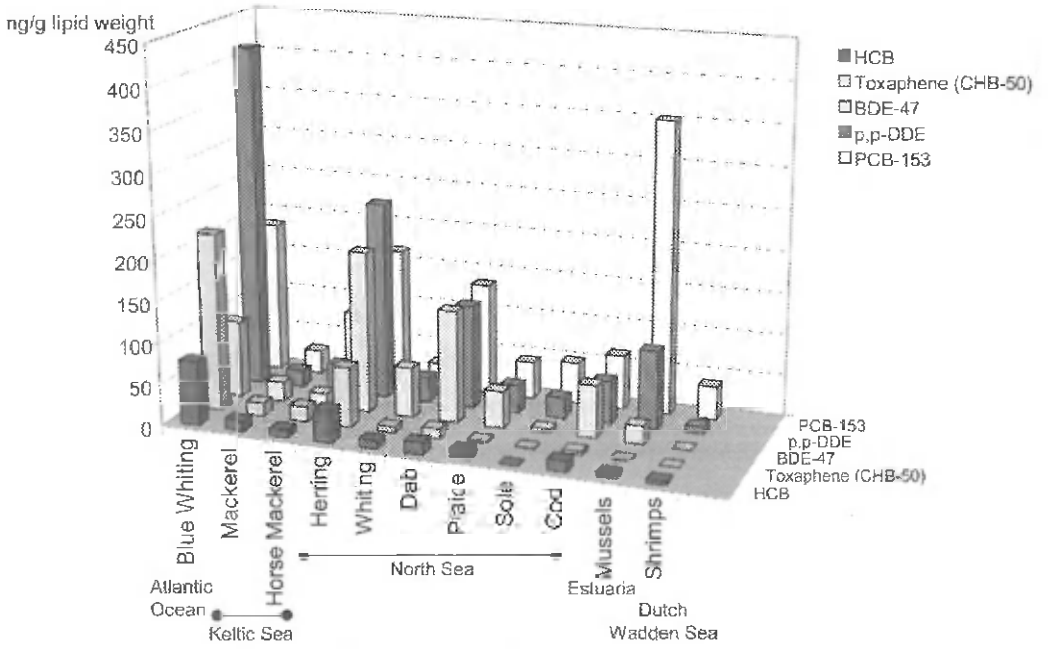

Fig. 2 Contaminant profile of fish, mussels and shrimp caught in 2000 in different waters (pooled samples of 25 animals each).

EFFECT DATA

For a review of the toxicity of BFRs, see refs. [7,19,24,25,27]. From these studies, it can be concluded that there is a lack of information regarding the potential for BFRs to cause endocrine disruption. Alteration of endocrine function by BFRs is realistic as there are striking resemblances in toxicological effects between the chlorinated aromatic hydrocarbons PCBs, PCDFs, and PCDDs and the PBDEs (i.e., partial aryl hydrocarbon- (Ah-) receptor agonist and antagonist activity in vitro, thyroid toxicity, and immune effects). Thus, PBDEs may produce toxic effects in a number of ways. The structural resemblance to thyroxin may explain interaction with the thyroid hormone system, and effects on thyroid function seem to be a sensitive endpoint. Decreased serum T4 levels were shown in mice exposed to a penta-BDE mixture [14] as well as to the 2,2′,4,4′-BDE congener (BDE-47) [18]. In addition, it was found that PBDEs after metabolic activation compete with the thyroid hormone (T4) for binding to transthyretin, the T4 transporting protein [29]. Following perinatal maternal exposure of rats to DE-71 (a commercial tetra- and penta-BDE mixture), reduced serum T4 was measured in the offspring showing its developmental toxicity [51]. Neonatal exposure to BDE-47 and BDE-99 has been found to induce neurotoxic effects in the adult animal [12]. In mice, the penta-BDE mixture also produced a decrease in the thymus/body weight ratio and in the antibody response to sheep red blood cells, and increased the activity of the hepatic cytochrome P450 mixed function oxidase system [14].

Using the in vitro CALUX-assay, pure PBDE-congeners appeared to act via the Ah-receptor signal transduction pathway as agonists, but mainly as antagonists [28]. A recent in vivo study with rats and commercial PBDE mixtures also indicated that hepatic induction of CYP1A1 and CYP2B activities could occur [50]. The former activity is of special relevance as this is an Ah-receptor-mediated process, which is common for dioxin-like compounds and PCBs, and most of the toxic responses (including the immunotoxicity) of dioxin-like compounds are mediated through binding to this receptor [34,35,46]. Persistent compounds with this type of mechanism are included in the toxic equivalency concept (TEF) and are now generally used in risk assessment procedures [45]. The CYP2B induction found in in vivo studies is also of toxicological interest, as this indicates the activation of multiple genes (e.g., glucocorticoid) associated with the phenobarbital responsive unit [50]. Finally, limited in vitro data indicate that some PBDEs and their hydroxylated metabolites can act as estrogenic compounds—data that suggest that in vivo metabolism of PBDEs might produce more potent pseudoestrogens [30]. The information about the toxicological effects of TBBP-A and HBCD is even more limited [25]. In vivo studies with TBBP-A and rats indicated that this compound can cause hepatotoxicity and disturbance of the haem synthesis [40]. In addition, it was shown that TBBP-A and lower brominated analogs can be potent competitors with T4 for binding to transthyretin (TTR) in vitro [30].

DISCUSSION

The toxicological profile of many BFRs is too incomplete and insufficient to perform an adequate human and ecological risk assessment. For a selected number of BFRs (including PBDEs and TBBPA), interactions with thyroid hormone homeostasis, estrogen, and Ah (dioxin) receptor have been reported. However, these studies are based only on in vitro or short-term experiments; therefore, significant gaps in knowledge exist for the situation of chronic and low-level exposure of humans and wildlife. Most notably, the PBDEs exhibit a high variety of biological effects, depending on the bromine substitution pattern. As these PBDEs occur in the environment in complex mixtures, the identification of biological and toxicological structure–activity relationships (SARs) is of great importance for understanding the mixture toxicity of this group of compounds. In addition, there is very little information available regarding useful biological or toxicological markers of low-level exposure to these compounds. Because of this lack of information and the apparently similar toxicity of structurally related PCBs and dioxins (partial Ah-receptor agonist and antagonist activity in vitro, thyroid toxicity, and immune effects) the European Union (EU) Scientific Committee for Toxicity, Ecotoxicity and the Environment (CSTEE)

concluded that further information is required regarding the potential of (PeBDE) for endocrine disruption and/or dioxin-like effects. This was concluded for both human and environmental risk assessment [5,6]. In response to the need for research on endocrine disruptors, the European Union allocated a budget of €20 million, and proposed four projects for funding, including a project on BFRs [13].

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REFERENCES

1. G. Ankley, E. Mihaich, R. Stahl, D. Tillitt, T. Colborn, S. McMaster, R. Miller, J. Bantle, P. Campbell, N. Denslow, R. Dickerson, L. Folmar, M. Fry, J. Giesy, L. E. Gray, P. Guiney, T. Hutchinson, S. Kennedy, V. Kramer, G. LeBlanc, M. Mayes, A. Nimrod, R. Patino, R. Peterson, R. Purdy, R. Ringer, P. Thomas, L. Touart, G. Van Der Kraak, T. Zacharewski. *Environ. Toxicol. Chem.* **17**, 68–8 (1998).
2. Å. Bergman. *Organohalogen Compd.* **47**, 36–40 (2000).
3. S. Bouma, D. Vethaak, P. Meininger, A. Holland. *De visdiefkolonie (Sterna hirundo) bij Terneuzen: blijven er problemen?* Rijksinstituut voor Kust en Zee/RIKZ, Middelburg, Rapport 2000.045 (2001).
4. T. Colborn, F. Vom Saal, A. Soto. *Environ. Health Perspect.* **101**, 378–384 (1993).
5. CSTEE. *Opinion of the EU Scientific Committee for Toxicity, Ecotoxicity and the Environment on the Results of the Human Risk Assessment of Pentabromodiphenyl Ether* [CAS N° 32534-81-9] carried out in the framework of Council Regulation (EEC) 793/93 on the evaluation and control of the risks of existing substances1 - Opinion expressed at the 16th CSTEE plenary meeting, Brussels, 19 June 2000.
6. CSTEE. *Opinion of the EU Scientific Committee for Toxicity, Ecotoxicity and the Environment on the Results of the Environmental Risk Assessment of Pentabromodiphenyl Ether* [CAS N° 32534-81-9], carried out in the framework of Council Regulation (EEC) 793/93 on the evaluation and control of the risks of existing substances - Opinion expressed at the 13th CSTEE plenary meeting, Brussels, 4 February 2000.
7. P. O. Darnerud, G. S. Eriksen, T. Johannesson, P. B. Larsen, M. Viluksela. *Environ. Health Perspect.* **109** (Suppl. 1), 49–68 (2001).
8. J. de Boer, P. G. Wester, H. J. Klamer, W. E. Lewis, J. P. Boon. *Nature* **394**, 28–29 (1998).
9. J. de Boer, K. de Boer, J. P. Boon. In *Handbook of Environmental Chemistry,* Vol. 3, Part K, *New Types of Persistent Halogenated Compounds*, J. Paasivirta (Ed.), pp. 61–95, Springer Verlag, Berlin (2000).
10. J. de Boer, A. van der Horst, P. G. Wester. *Organohalogen Compd.* **47**, 85–88 (2000).
11. C. A. de Wit. *Chemosphere* **46**, 583–624 (2002).
12. P. Eriksson, E. Jakobsson, A. Frederiksson. *Environ. Health Perspect.* **109**, 903–908 (2001).
13. European Union, 2002. Health and the Environment: European research on endocrine disrupters receives major boost (<http://europa.eu.int/comm/research/press/2002/pr1505en.html>).
14. J. R. Fowles, A. Fairbrother, L. Baecher-Steppan, N. I. Kerkvliet. *Toxicol.* **86**, 49–61 (1994).
15. D. Guvenius Meironyté. *Organohalogen contaminants in humans with emphasis on polybrominated diphenyl ethers*, Doctoral dissertation, University of Stockholm, Sweden (2002).
16. H. Hakk, G. Larsen, Å. Bergman. *Xenobiotica* **30**, 881–890 (2000).
17. R. C. Hale, M. J. La Guardia, E. P. Harvey, M. O. Gaylor, T. M. Mainor, W. H. Duff. *Nature* **412**, 140–141 (2001).
18. S. Hallgren and P. O. Darnerud. *Organohalogen Compd.* **35**, 391–394 (1998).
19. M. L. Hardy. *Chemosphere* **46**, 757–777 (2002).

20. M. Harju and M. Tysklind. *Organohalogen Compd.* **41**, 529–532 (1999).
21. P. T. C. Harrison, C. D. N. Humfrey, M. Litchfield, D. Peakall, L. K. Shuker. *IEH Assessment on Environmental Oestrogens: Consequences to Human Health and Wildlife*. Medical Research Council, Institute for Environment and Health, Page Bros., Norwich (1995).
22. M. G. Ikonomou, S. Rayne, R. F. Addison. *Environ. Sci. Technol.* **36**, 1886–1892 (2002).
23. IPCS, Environmental Health Criteria 192. *Flame Retardants, A General Introduction*, World Health Organization, International Programme on Chemical Safety, Geneva (1997).
24. IPCS, Environmental Health Criteria 162. *Brominated Diphenylethers*, World Health Organization, International Programme on Chemical Safety, Geneva (1994).
25. IPCS, Environmental Health Criteria 172. *Tetrabromobisphenol A and Derivatives*, World Health Organization, International Programme on Chemical Safety, Geneva (1995).
26. IPCS, Environmental Health Criteria 205. *Polybrominated Dibenzo-p-dioxins and Dibenzofurans*, World Health Organization, International Programme on Chemical Safety, Geneva (1998).
27. T. A. McDonald. *Chemosphere* **46**, 745–755 (2002).
28. I. A. T. M. Meerts, E. A. C. Luijks, G. Marsh, E. Jakobsson, Å. Bergman, A. Brouwer. *Organohalogen Compd.* **37**, 147–150 (1998).
29. I. A. Meerts, J. J. van Zanden, E. A. Luijks, I. van Leeuwen-Bol, G. Marsh, E. Jakobsson, Å. Bergman, A. Brouwer. *Toxicol. Sci.* **56**, 95–104 (2000).
30. I. A. T. Meerts, R. J. Letcher, S. Hoving, G. Marsh, Å. Bergman, H. G. Lemmen, B. van der Burg, A. Brouwer. *Environ. Health Perspect.* **109**, 399–407 (2001).
31. D. Meironyté, K. Norén, A. J. Bergman *Toxicol. Environ. Health Part A* **58**, 101–113 (1999).
32. M. Meneses, H. Wingfors, M. Schuhmacher, J. L. Domingo, G. Lindström, B. V. Bavel. *Chemosphere* **39**, 2271–2278 (1999).
33. O. Päpke, L. Bathe, Å. Bergman, P. Fürts, D. M. Guvenius, T. Herrmann, K. Norén. *Organohalogen Compd.* **52**, 197–200 (2001).
34. S. Safe. *CRC Crit. Rev. Toxicol.* **21**, 51–88 (1990).
35. S. Safe. *CRC Crit. Rev. Toxicol.* **24**, 87–149 (1994).
36. U Sellström. *Determination of some polybrominated flame retardants in biota, sediment and sewage sludge*. Doctoral dissertation, Stockholm University (1999).
37. U. Sellström, G. Soderstrom, C. de Wit, M. Tysklind. *Organohalogen Compd.* **35**, 447–450 (1998).
38. R. M. Sharpe and N. E. Skakkebaek. *Lancet* **341**, 1392–1395 (1993).
39. A. Sjödin, L. Hagmar, E. Klasson-Wehler, K. Kronholm-Diab, E. Jakobsson, A. Bergman. *Environ. Health Perspect.* **107**, 643–648 (1999).
40. J. A. Szymanska, J. K. Piotrowski, B. Frydych. *Toxicol.* **142**, 87–95 (2000).
41. C. Thomsen. *Brominated flame retardants-development and application of analytical methods for characterization of human exposure*, Doctoral dissertation, University of Oslo (2002).
42. C. Thomsen, E. Lundanes, G. Becher. *J. Environ. Monitor.* **3**, 366–370 (2001).
43. C. Thomsen, E. Lundanes, G. Becher. *Environ. Sci. Technol.* **36**, 1414–1418 (2002).
44. J. Toppari, J. C. Larsen, P. Christiansen, A. Giwercman, P. Grandjean, L. J. Guillette, B. Jegou, T. K. Jensen, P. Jouannet, N. Keiding, H. Leffers, J. A. McLachlan, O. Meyer, E. Müller, E. Rajpert-De Meyts, T. Scheike, R. Sharpe, J. Sumpter, N. Skakkebaek. *Male Reproductive Health and Environmental Chemicals with Estrogenic Effects*, Danish Environmental Protection Agency, Copenhagen (1995).
45. M. Van den Berg, L. S. Birnbaum, A. T. C. Bosveld, B. Brunström, Ph. Cook, M. Feeley, J. P. Giesy, H. Hanberg, R. Hasegawa, S. W. Kennedy, T. Kubiak, J. Ch. Larsen, F. X. R. van Leeuwen, A. K. D. Liem, C. Nolt, R. E. Peterson, L. Poellinger, S. Safe, D. Schrenk, D. Tillitt, M. Tysklind, M. Younes, F. Wærn, T. Zacharewski. *Environ. Health Perspect.* **106**, 775–792 (1998).
46. J. G. Vos, C. de Heer, H. Van Loveren. *Teratogenesis, Carcinogenesis, Mutagenesis* **17**, 275–284 (1997/1998).