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Identification of Tetrabromobisphenol A Diallyl Ether as an Emerging Neurotoxicant in Environmental Samples by Bioassay-Directed Fractionation and HPLC-APCI-MS/MS

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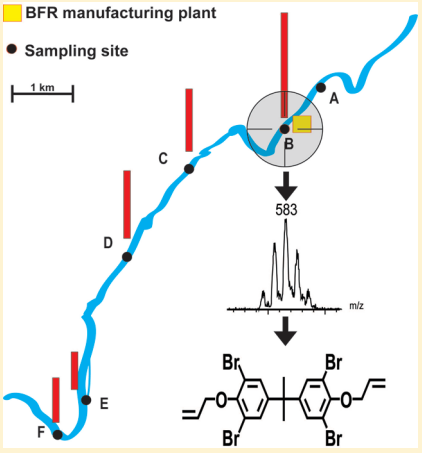
Supporting Information

ABSTRACT: Brominated flame retardants (BFRs) have been widely used as additives in products to reduce their flammability. Recent findings suggested that some BFRs exhibit neurotoxicity and thus might pose a threat to human health. In this work, a neurotoxicity assay-directed analysis was developed, combining sample cleanup, fractionation, chemical identification, and bioassay. Viability of primary cultured cerebellar granule neurons (CGNs) was used to evaluate the neurotoxicity of extracts or separated fractions from environmental samples. Tetrabromobisphenol A diallyl ether (TBBPA DAE) was identified as the causative toxicant in sediment samples collected from a river near a brominated flame retardant (BFR) manufacturing plant in South China. Liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) was optimized to determine TBBPA DAE levels in the potent fractions and to confirm TBBPA DAE as the key neurotoxicant. On the basis of comparison with the structure of other TBBPA derivatives, the 1-propenyl group in TBBPA DAE appears to be the cause for the neurotoxic potency. The levels of TBBPA DAE in samples along the river were found at up to 49 ng/L for river water, 10 183 ng/g dry weight (dw) in surface sediments, and 42 ng/g dw in soils. According to the distribution of TBBPA DAE in the environmental samples, the manufacturing plant was identified as the release source of TBBPA DAE. To our knowledge, this study is the first to demonstrate potential neurotoxicity induced by TBBPA DAE in real environmental samples.



INTRODUCTION

Neurotoxicity induced by environmental pollutants has been of major public and scientific concern due to the serious health threats to humans, especially infants and children.[1] Brominated flame retardants (BFRs) have been beneficially used in many consumer products such as polymers, furniture, computers, and television sets. However, large production volumes, modes of application, and intrinsic chemical-physical properties of some BFRs have also rendered these to be found at increasing levels in the environment, wildlife, and humans. Some of the BFRs have recently attracted much attention due to their persistence in the environment and widespread global distribution.[2,3] BFRs have also been studied in regard to their potential as developmental neurotoxicants.[4] It was reported that polybrominated diphenyl ethers (PBDEs) possess developmental neurotoxicity on primary fetal human neural progenitor cells (hNPCs).[5] Hexabromocyclododecanes (HBCDs) and tetrabromobiphenol A (TBBPA) are toxic to primary cultured cerebellar granule neurons (CGNs).[6,7] Sites contaminated with BFRs often contain complex mixtures of different chemicals including byproduct, transformation products of BFRs, and other unknowns. It might thus be difficult to determine which pollutants are the major causative neurotoxicants in such environmental sample. Bioassay-directed chemical analysis, which integrates sample extraction, cleanup procedures, multiple fractionation, concentration, and bioassay steps, has emerged as a practical method in identifying causative toxicants in environmental samples.[8] Previous studies have applied bioassay-directed analysis to evaluate the toxicity and identify key toxicants in environmental samples collected from contaminated sites, indoor environments, and biosamples.[9−11] Therefore, bioassay-directed chemical analysis could be considered a potentially useful tool for identification of organic neurotoxicants in environmental samples.

In this study, an extensive protocol for identification of neurotoxic BFRs in environmental samples was developed. We (1) isolated potent fractions using neurotoxicity assay-directed fractionation from sediments collected near a BFR manufacturing plant, (2) identified a TBBPA derivative, tetrabromobisphenol A diallyl ether (TBBPA DAE) as a key developmental

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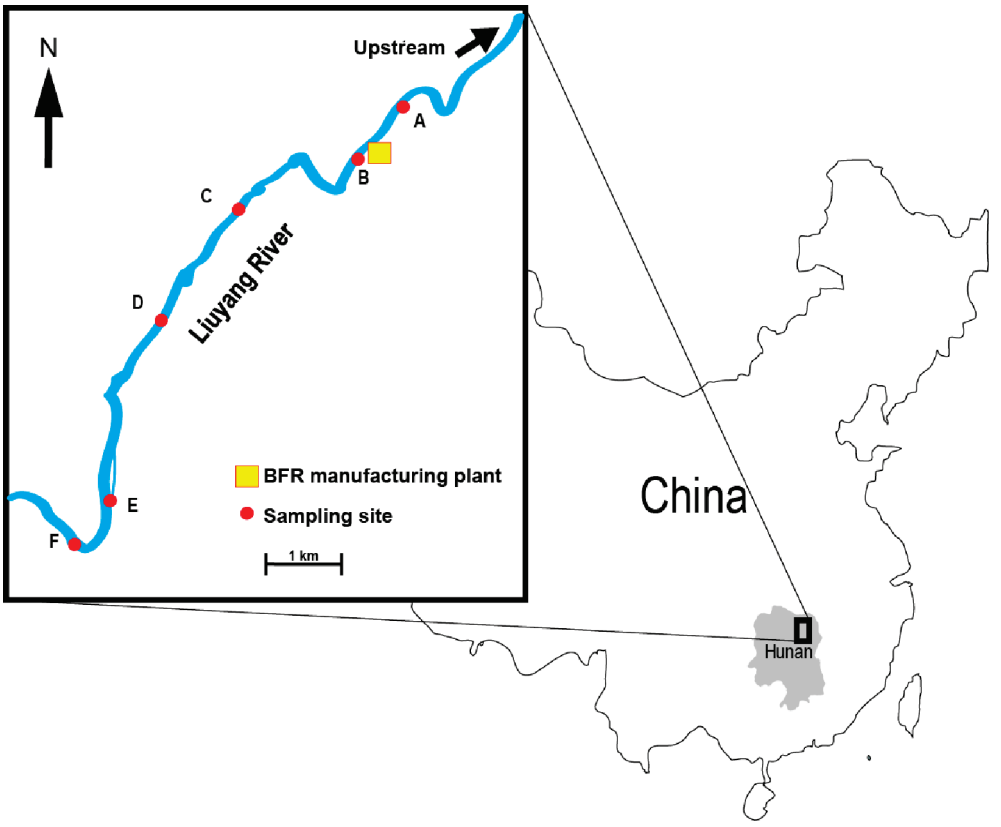

Figure 1. Sampling map. Site A, 0.8 km upstream of the outlet. Site B, 0.5 km downstream of the outlet. Site C, 3.1 km. Site D, 5 km. Site E, 6.5 km. Site F, 7.7 km.

neurotoxicant in the potent fraction based on liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) and gas chromatography coupled electron capture negative ionization mass spectrometry (GC-ECNI-MS), and (3) developed a liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (HPLC-APCI-MS/MS) method to determine the levels of TBBPA DAE in environmental samples.

CHEMICALS

TBBPA 2,3-dibromopropyl ether (TBBPA DBPE) (CAS 21850-44-2, purity unknown), TBBPA DAE (CAS 25327-89-3, 99%), TBBPA 2-hydroxyethyl ether (TBBPA OHEE) (CAS 4162-45-2, 98%), TBBPA diglycidyl ether (TBBPA GE) (CAS 3072-84-2, purity unknown), and TBBPA (CAS 79-94-7, 97%) were purchased from Sigma-Aldrich and purified with HPLC before use. Information on other chemicals used in this study is provided in the Supporting Information.

SAMPLE COLLECTION

Soil, sediment, and water samples were collected upstream and downstream of the outlet of a BFR factory at Liuyang River in Liuyang City, Hunan Province, in southern China in October 2009. The sampling sites are shown in Figure 1. A total of 18 samples of surface sediment, agricultural soil, and river water were collected in this study. Site A is located 0.8 km upstream of the outlet. Sites B, C, D, E, and F are, respectively, located 0.5, 3.1, 5, 6.5, and 7.7 km downstream of the outlet (Figure 1). Solid samples were packed in aluminum foil followed by polyethylene bags and stored in an ice box after collection. River water samples were collected using 500 mL glass bottles and stored in an ice box. After transport to the laboratory, the samples were stored at −20 °C until use.

BIOASSAY

The neurotoxicity of fractions or compounds was evaluated by the cytoxicity of primary CGNs. Primary CGNs were prepared from 7 day old Sprague−Dawley pups according to the method of Reistad et al. with the following minor modifications.[7] Cerebellum was collected in sterile Hanks balanced salt solution (HBSS) buffer supplemented with 35 mM glucose and then digested with HBSS containing trypsin (2 mg/mL) and DNase (0.1 mg/mL) for 15 min at 37 °C. After washing with HBSS buffer three times, the tissues were gently dissociated several times in 5 mL of basal medium eagle (BME) containing DNaseI (0.1 mg/mL) with a Pasteur pipet. After a 5 min incubation, the suspension was collected and centrifuged at 200*g* for 5 min at 4 °C. The supernatant was discarded, and the precipitate was resuspended with 5 mL of BME medium supplemented with 25 mM KCl. The suspension was gravity filtered through a cell strainer (BD, Falcon) and then plated in BME (containing 10% FBS, 25 mM KCl, 100 μg/mL streptomycin, 100 unit/mL penicillin, and 2 mM L-glutamine) in 96-well Costar plates precoated with 10 μg/mL of poly-L-lysine in sterile Milli-Q water (300 000 cells/cm^2, 100 μL medium/well). The plates were incubated in a 37 °C humidified incubator with 5% CO_2. Sixteen hours after plating, cytrarabine (Ara-C) was added to the cultures (final concentration was 2.5 μg/mL, which has no effect on CGNs viability) to prevent the growth of glial cells.

CGNs were exposed to technically pure TBBPA DBPE, TBBPA DAE, TBBPA OHEE, and TBBPA GE at different concentration levels to investigate their potential neurotoxicity and determine the dose that induces 50% loss of cell viability (EC50). Paraquat was used as a positive control because of its high neurotoxicity on developing CGNs, and its EC50 ($EC50_{paraquat}$) was also determined.[12] For exposure experiments, stock solutions of the above chemicals were diluted 200 times with BME to treat CGNs. Final dimethyl sulfoxide (DMSO) concentration was less than 0.5% v/v, which has no effect on the viability of CGNs. After 7 days of incubation, the cell viability was tested using Yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[13] MTT dissolved in HBSS was added to the cells at a final concentration of 0.05 mg/mL and incubated for 4 h at 37 °C to allow the reduction of MTT to produce the formazan product. The medium was removed, and cells were dissolved in DMSO. Thereafter, the formazan blue was extracted with 100 μL of DMSO, and the absorbance of the solution was measured at a wavelength of 595 nm with 650 nm as a reference wavelength. Viability results were expressed as percentages. The absorbance measured from solvent control-treated cells (DMSO in BME, 0.5% v/v) was taken to be 100%.

Sample Preparation for Neurotoxicity Assay-Directed Analysis. For the neurotoxicity-directed fractionation procedure, six sediment samples collected along the river (Figure 1) were used to evaluate the potential neurotoxic hazard. In brief, 6 g of dry sediment mixed with 15 g of anhydrous Na_2SO_4 was extracted with dichloromethane (DCM) at 100 °C, 1500 psi, and 2 cycles using an accelerated solvent extractor (ASE 300, Dionex). The extract was evaporated to 5 mL and then fractionated using gel permeation chromatography (GPC) (AccuPrep, J2-Scientific; Bio-Beads S-X3) with DCM as the mobile phase for clean up.

Primary Fractionation. The eluate from GPC was concentrated and solvent exchanged with 1 mL of hexane. Solid-phase extraction (SPE) cartridge (Bakerbond, 6 mL, 500 mg silica (SiOH) absorbents, Mallinckrodt Baker Inc., Germany) were selected for further cleanup and normal phase (NP) based primary fractionation because of its previous use in sample pretreatments of BFRs and other nonpolar chemicals.[14] After preconditioning the SPE cartridge with 3 mL of DCM and 6 mL of hexane, the concentrated extract from GPC was loaded into the SPE. In order to provide guidelines for this fractionation step, pilot experiments were conducted using HBCDs and PBDEs as marker compounds due to their differences in polarity and potential neurotoxicity.[5,6] SPE elution recovery results (Table S1, Supporting Information) showed that PBDEs could be eluted by 12 mL of DCM/hexane (1:9, v/v), and this fraction was designated as primary fraction 1 (F1). Further addition with 12 mL of DCM/hexane (2:8, v/v) resulted in elution of HBCDs, which was designated as primary fraction 2 (F2). Further details on primary fractionation design are given in the Supporting Information.

Each fraction was then concentrated to 600 μL (equal to 0.1 mL/g dried sediment) in which 200 μL was solvent exchanged with 10 μL of DMSO for neurotoxicity assay. The remaining 400 μL fraction was stored at 4 °C until further fractionation or chemical analysis.

Secondary Fractionation. If the primary fractions tested positive in the biotest, the fraction with highest potency was then processed by a secondary fractionation step with reverse-phase (RP) chromatography to further screen potential neurotoxic

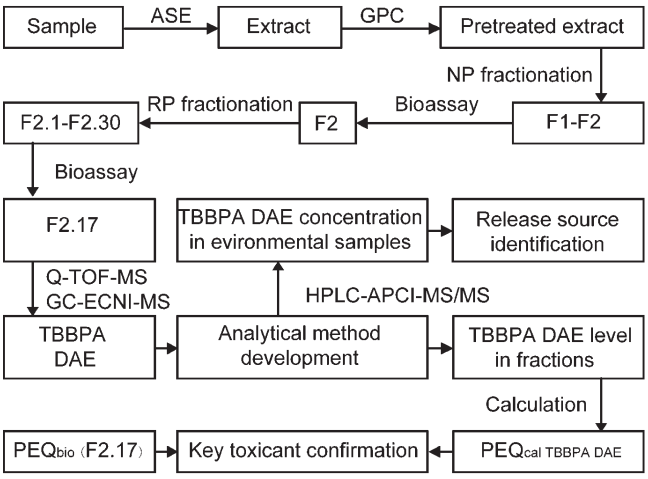

Figure 2. Bioassay-directed protocol. Sediment sample with the highest neurotoxicity was fractionated, and TBBPA DAE was identified as the potent neurotoxicant in the fractions.

fractions (Figure 2). The potent primary fraction(s) (400 μL of stock solution) was solvent exchanged with 1 mL of methanol for the secondary fraction using a Waters Auto Purification System (Waters, USA) coupled with 2767 Sample Manager (Waters, USA), 2525 Binary Gradient Module (Waters, USA), and Column/Fluidic Organizer (Waters, USA) equipped with a reverse-phase RP C18 semipreparation HPLC column (ZORBAX Eclipse XDB columns, 9.4 mm ×250 mm, 5 μm, USA). The mobile phase consisted of methanol (A) and water (B). The gradient program started at an initial composition of 80:20 A/B and ramped to 100:0 A/B in 10 min and held for 20 min. The flow rate was set at 4 mL/min. Fractions were automatically collected every 1 min to yield 4 mL portions. These secondary fractions were named F*X*.1, F*X*.2, F*X*.30 (*X* is 1 or 2 according to the primary fractions). Every fraction was then solvent exchanged with 20 μL of DMSO for neurotoxicity assay. The DMSO fractions from both primary and secondary fractionation steps were diluted 200 times with BME and then exposed to CGNs 24 h after cell plating for neurotoxicity assay (triplicate per fraction). After exposure, each well contained 100 μL of BME dissolving the amount of chemicals equal to 0.1 g of the sample (equal to 1 g of sample per microliter test medium). The exposed cells were treated for 7 days followed by cell viability assay. In order to assess potential background toxicity, procedural blanks using 15 g of anhydrous Na_2SO_4 were also processed in accordance to the above-described method.

Identification of Neurotoxicity-Inducing BFRs. The potent secondary fractions were used for chemical identification. LC-Q-TOF-MS (Waters micromass Q-ToF micro, USA) and GC-ECNI-MS (7000A triple quadrupole GC/MS, Agilent, USA) with a capillary column DB-5HT (15 m × 0.25 mm i.d., 0.10 μm film thickness, J&W Scientific, USA) were used to screen and identify the chemicals in the potent fractions (details on instrumental parameters are provided in the Supporting Information). In order to identify the chemicals in the most potent secondary fraction, 10 μL of that fraction was freeze dried and dissolved in 100 μL of methanol. Then 20 μL was injected and delivered by the mobile phase (methanol, 1 mL/min) into Q-TOF-MS at full scan from 50 to 1000 *m*/*z*. The parameters for the mass spectrometer are summarized as follows: corona current, 10 μA; source

temperature, 120 °C; probe temperature, 630 °C; cone gas flow, 40 L/h; desolvation gas flow, 100 L/h.

To further confirm the identity of the potent chemicals, 2 μL of the most potent fraction was freeze dried and dissolved in 200 μL of hexane, and 1 μL was injected into GC-ECNI-MS operated in full scan at 50−1000 m/z. The oven temperature was initially at 110 °C (held for 5 min), increased to 200 °C at a rate of 20 °C/min (held for 4.5 min), and finally increased to 220 at 10 °C/min (held for 15 min). Methane was used as moderating gas at an ion source pressure of 2.4×10^{-3} Pa. Helium was used as the carrier gas at a flow rate of 10 mL/min. The interface and ion source temperatures were 200 and 230 °C, respectively.

Development of LC-APCI-MS/MS Analysis Method. In order to further confirm whether neurotoxicity in the samples can be explained by the presence of TBBPA DAE, the levels of TBBPA DAE in the potent fractions were measured in order to calculate the neurotoxicity contribution. A qualitative and quantitative method for analyzing TBBPA DAE in potent fractions and environmental samples was developed using LC-APCI-MS/MS (Quattro Ultima triple quadrupole mass spectrometer (Waters, USA)). Details on method, instrument parameters, recovery evaluation, and real sample determination are described in the Supporting Information. Levels of TBBPA DAE in primary and secondary fractions from sediment extracts were diluted and directly determined using HPLC-APCI-MS/MS. All concentrations were reported on a dry weight basis.

Key Neurotoxicant Confirmation. The contributions of TBBPA DAE to the bioassay-measured total neurotoxicity in the fractions were quantified.[15,16] The neurotoxicity of TBBPA DAE was expressed relative to that of paraquat by calculating the paraquat equivalence factor (PEF value) for TBBPA DAE using the formula $PEF_{TBBPA\ DAE} = EC50_{paraquat}/EC50_{TBBPA\ DAE}$. The paraquat equivalent ($PEQ_{cal\ TBBPA\ DAE}$, μmol/g sediment) chemically derived by TBBPA DAE was used to express the neurotoxicity induced by TBBPA DAE in potent fractions equal to 1 g of sediment. $PEQ_{cal\ TBBPA\ DAE}$ was calculated by multiplying $PEF_{TBBPA\ DAE}$ and the concentration of TBBPA DAE in the correspondent extracts or fractions. The measured neurotoxicity in the extracts or fractions was expressed as paraquat equivalents (PEQ_{bio}, μmol/g sediment).

RESULTS AND DISCUSSION

Neurotoxicity of Fractions. In vitro neurotoxicity assay based on the CGN cell model has previously been developed, well characterized, and used to study the neurotoxicant effect on the differentiation and maturation of CGNs and also the neurotoxicity of pollutants, including some BFRs.[12,17−19] Therefore, cell viability of the developing CGNs was selected as the biological end point to evaluate the neurotoxicant of the active fractions and chemicals.

None of the F1s from sediments had an effect on cell viability (Figure 3). F2s from sediments collected downstream of the plant (sites B−F) showed high neurotoxicity, while site A located upstream of the plant showed no significant neurotoxicity. The selected PBDEs could be ruled out as the main cause to induction of neurotoxicity as they were eluted in F1 according to the pilot study (Table S1, Supporting Information). The F2 of site B produced the highest neurotoxicity (72% cell viability loss). F2 from sediment B was then further fractionated using an RP C18 column to isolate the potent neurotoxicant(s) and avoid potential interferences from other compounds during identification.

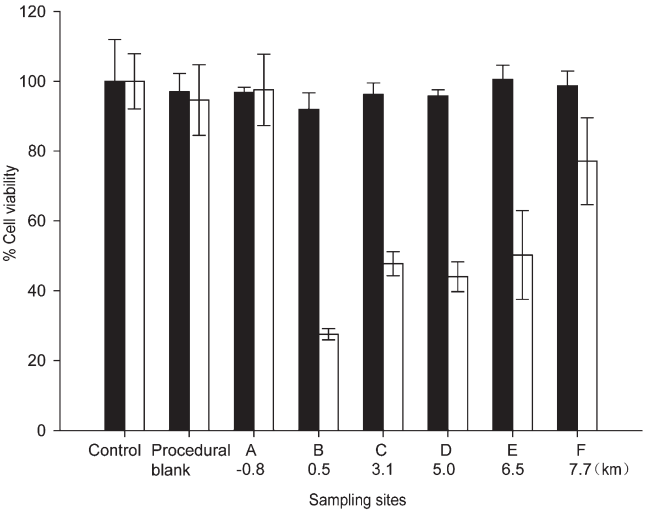

Figure 3. Cell viability of CGNs after treatment with primary fractions 1 (black bar) and 2 (white bar) of sediments in the six sampling site.

The results showed that F2.17 exhibited the highest neurotoxicity (41% cell viability reduction) among the secondary fractions and was therefore considered as the major toxic fraction (Figure S1, Supporting Information).

Chemical Identification of TBBPA DAE. *Qualitative Identification of TBBPA DAE.* The full scan mass spectrum (m/z = 50−1000) of F2.17 using HPLC-Q-TOF-MS is shown in Figure 4. High relative abundance of the 78.692/80.703 ions suggests the presence of bromine. Given the sampling location, this suggested that the detected chemical was a BFR or byproduct or degradation product from the BFR manufacturing plant. Both the ions at m/z = 582.816 and 541.774 (Figure 4A) probably contain 4 bromine atoms because the proportion of the isotope ions is 1:4:6:4:1, which is similar to the theoretical value. The ions in our study between about 291 and 293 (m/z = 290.967 in Figure 4A, m/z = 292.740 in Figure 4B, m/z = 291.8 in Figure S2, Supporting Information) provided useful information for identification of the unknown BFR. It was previously found that under negative ionization conditions, ions at 290.9 of TBBPA occur because of the presence of 4-isopropylene-2,6-dibromophenol.[20,21] Furthermore, the mass spectrum of TBBPA derivative (TBBPA DBPE) ions at 292.9 had previously been identified as 4-isopropylene-2,6-dibromophenol-oxonium.[22] The 4-isoproylene-phenol-based ions were also identified as possible fragments of BPA or its halogenated derivatives such as TBBPA.[23] The precursor fragments at 582.816 (Figure 4A) were similar to that of TBBPA DAE, which was previously reported by another research group using electron spray ionization mass spectrometry (ESI-MS) in a short symposium paper.[24] Therefore, it was suggested that the mass spectrum could be assigned to TBBPA or its derivatives. To further confirm the identification of the chemical(s), TBBPA and four commercially available TBBPA derivatives (TBBPA DBPE, TBBPA DAE, TBBPA OHEE, and TBBPA GE) were analyzed (Figure S3, Supporting Information). Mass spectra of F2.17 and TBBPA DAE were found to be the similar under negative APCI full scan mode (Figure S3, Supporting Information). Ions at m/z = 582.816 and 541.774 should be formed from breakage of the ether bond in TBBPA DAE. Positive APCI full scan ions also demonstrated the presence of TBBPA DAE in F2.17 (Figure S5, Supporting Information).

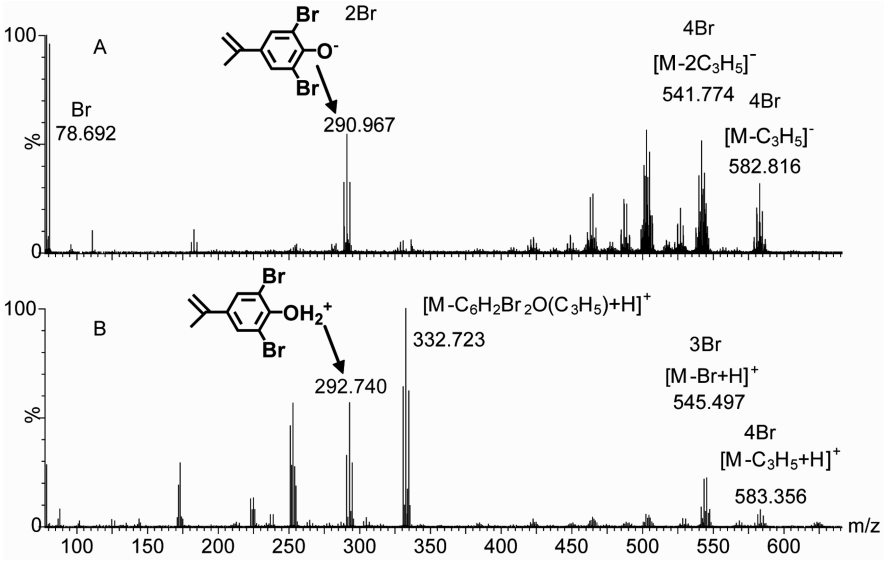

Figure 4. Mass spectra of F2.17 under negative (A) and positive (B) full scan mode using APCI-Q-TOF-MS.

Because of the thermodegradation of TBBPA DAE, GC-ECNI-MS yielded a broadened peak (data not shown). However, the same ions between F2.17 and TBBPA DAE standard also demonstrated that TBBPA DAE was collected in F2.17 (Figure S2, Supporting Information). In addition, HPLC-PDA (photodiode array) (Waters 2695, USA) provided the same UV adsorption spectrum of TBBPA DAE and F2.17 (Figure S6, Supporting Information). Therefore, TBBPA DAE was considered to be qualitatively detected in F2.17, and the possibility of the other three TBBPA derivatives in this fraction can be ruled out due to their different specific mass spectra (data not shown).

Method Development for Quantitation of TBBPA DAE. To quantitatively determine TBBPA DAE in the samples, HPLC-APCI-MS/MS was used in negative-ionization mode under optimized conditions. The Q1 scan for TBBPA DAE did not produce molecular ions but exhibited three major precursor ions. The fragment $[M - C_3H_5]^-$ is formed due to cleavage of the phenyl ether bond, whereas the other two ions $[M - C_3H_5 - CH_3 - Br]^-$ and $[M - Br]^-$ were caused by the corona current producing radical ions (O^-, OH^-, or H_2O^-) at the C−Br bond (Figure S7A, Supporting Information).[25] This is different from the displacement reactions induced by O^- attack of the carbon atom at the C−Br bond that usually play a dominant role in the APCI ionization of most halogenated flame retardants (HFRs).[25,26] The APCI ionization behavior of TBBPA DAE is also different from that of atmospheric pressure photoionization (APPI) reported recently. Under APPI mode, TBBPA DAE tend to form $[M + O_2]^-$ and $[M + O_2 - HBr]^-$ ions.[27]

The fragment at $m/z = 583$ ($[M - C_3H_5]^-$) was chosen as the precursor ion (Figure S7A, Supporting Information). The collision energy was optimized at 40 eV, which offered the most intensive signal under multiple reaction monitoring mode (MRM). The $[M - 2C_3H_5 - CH_3]^-$ ion (m/z = 527) was found to be the most intense fragment and sufficiently stable to be selected as the product ions for MRM quantification (Figure S7B, Supporting Information) (ions of m/z = 543 > 487 were used as confirmation transition ions). A high methanol proportion (90%) in the mobile phase was selected as the initial condition, and TBBPA DAE was eluted at 8.51 min (Figure S8A, Supporting Information). Only one single UV peak present at 8.51 min demonstrated the high purity of TBBPA DAE standard (Figure S8B, Supporting Information). The signal intensity repeatability (RSD) on column was 6.4%. Linear regressions showed R equal to 0.9996 (Figure S9, Supporting Information).

Table 1. TBBPA DAE Levels in Environmental Matrices[a]

	sample		
site	water (ng/L)	sediment (ng/g, dw)	soil (ng/g, dw)
A	1.5	143.4	24.7
B	49.0	10183.4	41.7
C	24.9	5933.3	10.7
D	nd	7368.6	11.8
E	nd	4639.3	2.3
F	nd	4414.6	nd

[a] nd: below limit of detection.

TBBPA DAE Levels in Potent Extract and Fractions. Using HPLC-APCI-MS/MS, 10.05 μg/g TBBPA DAE was detected in sediment B. After secondary fractionation, 5.98 μg/g TBBPA DAE was collected in F2.17. The loss of TBBPA DAE during the secondary fractionation might be caused by solvent exchange and also the fact that not all TBBPA DAE was collected into F2.17 during the automatic fractionation step. High levels of TBBPA DAE can also be detected at sites C, D, E, and F (Table 1). Therefore, TBBPA DAE was speculated to be the causative neurotoxic chemical in the sediments.

Toxicity of TBBPA DAE. Neurotoxicity evaluation of the four pure standards of TBBPA derivatives revealed that only TBBPA DAE was found to induce high neurotoxicity (Figures S10 and S11, Supporting Information). Only the two $[CHCH_2]$ functional groups in the TBBPA DAE molecule are different from the other TBBPA derivatives (Figure S3, Supporting Information). It has been reported that the double bonds in fatty acid are associated with apoptosis of cultured neuronal cell, and the unsaturated bond in piperine might be the cause of lipid peroxides in membranes of granule neurons.[28,29] Therefore, the $[CHCH_2]$ group

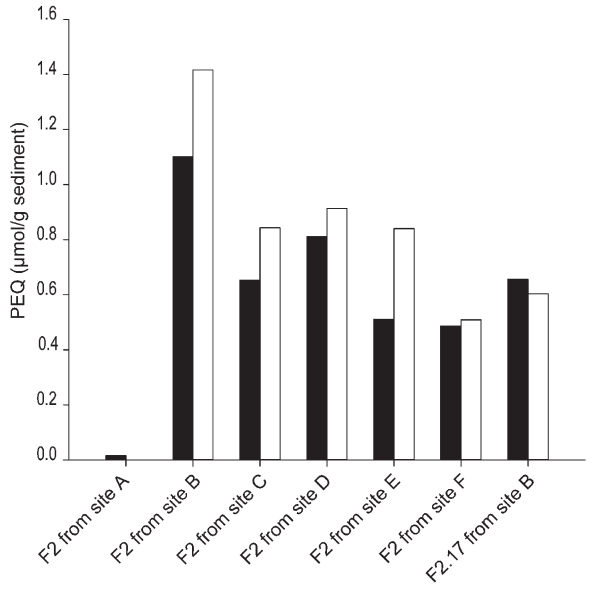

Figure 5. Comparison of TBBPA DAE derived $PEQ_{cal\ TBBPA\ DAE}$ (black bar) with neurotoxicity assay measured PEQ_{bio} (white bar) in sediment extracts.

together with the TBBPA structure seems to play a critical role in the neurotoxicity. Therefore, it is suggested that the potential neurotoxicity contributed by functional groups should be seriously considered when designing novel TBBPA derivatives.

Neurotoxicant Confirmation. The relative contribution of TBBPA DAE to the measured PEQ_{bio} can now be evaluated by combining the results of quantitative analysis and biological assay. PEQ_{bio} was calculated according to the single-point estimation using nonlinear regression according to the dose−response curve of paraquat (Figure S12, Supporting Information).[15,16] The $PEQ_{cal\ TBBPA\ DAE}$ accounted for 61−93% of PEQ_{bio} in F2 from all of the sediment samples (Figure 5). After further fractionation of the extract from site B, $PEQ_{cal\ TBBPA\ DAE}$ accounted for 109% of total PEQ_{bio} in F2.17. Therefore, it is demonstrated that TBBPA DAE was most likely the major causative neurotoxicant in the collected sediment.

Furthermore, the human liver carcinoma Hep G2, human breast cancer MCF-7, and mouse leukemic monocyte macrophage RAW 264.7 cell lines were also used to investigate the non-neurotoxic potencies (see method section in the Supporting Information). Results showed that none of the sediment fractions or pure TBBPA DAE standard significantly affected the activity of Hep G2, MCF-7, and RAW 264.7 cell lines (data not shown). This suggested that the sediment and TBBPA DAE standard do not induce cytotoxicity on non-neuronal cells.

Previous studies suggested that TBBPA possesses endocrine disruption potency and neural and reproductive toxicity.[7,30,31] These end points could also be extended to TBBPA DAE due to its similar structure to TBBPA, and these potential risks should be evaluated for TBBPA DAE. It is also suggested that whole organism testing and/or exposure to environmental samples, rather than sample extracts, need to be performed in order to more accurately predict bioavailability and risk of this compound.

In the past three decades, bioassay-directed analysis has been used for screening key toxicants and evaluating the toxicity of pollutants in environmental samples. Specific end points such as endocrine-disrupting potency, genetic toxicity, aquatic toxicity, and aryl hydrocarbon receptor activity have often been applied for bioassay-directed analysis.[8−10,32−35] In this study, the well-characterized primary developing CGNs was for the first time used to guide the neurotoxicant fractionation. Positive identification of TBBPA DAE as an emerging neurotoxic BFR demonstrates the importance of integrating neurotoxicity assay with chemical analysis to identify unknown or nonpriority chemicals that might pose a threat to wildlife and human health. The neurotoxicity-directed analysis can also be applied to identify other groups of nonpolar organic pollutants in environmental samples. Although cell viability of neurons may be less sensitive than other end points, such as intracellular signal,[36] cell migration,[5] differentiation, or gene expression,[12] our results indicated that this end point is sufficient in guiding the fractionation and subsequent isolation of neurotoxic pollutants from sample extracts. Other neurotoxicity end points are still expected to be integrated into bioassay-directed analysis in the future to improve the sensitivity and specificity of the methods.

Identification of Release Source of TBBPA DAE. An ESI method for quantitative determination of TBBPA DAE with an IDL of 50 pg was previously reported in a symposium short paper.[24] However, no details on experimental parameters, mass spectra, and recovery were available. During parameter optimization for an ESI method in our laboratory we could not find any precursor ion around $m/z = 583$ (see the Supporting Information for parameters). Recently, a highly sensitive method to determine TBBPA DAE with APPI-MS/MS was reported with an instrument detection limit (IDL) of 12 pg.[27] The developed APCI-based method in our lab provided an IDL of 40 pg. Although APPI showed a slightly higher sensitivity, this mass spectrometer ionization source is not commonly available in most analytical laboratories. Using the optimized pretreatment method, the recovery was 77% (RSD = 15%) for sediment, 81% (RSD = 14%) for soil, and 85% (RSD = 15%) for water samples ($n = 5$). TBBPA DAE was positively detected in nearly all water, surface sediment, and soil samples, with comparatively high levels of TBBPA DAE found in sediments (Table 1). The level of TBBPA DAE detected in the upstream sediment was 143.4 ng/g and significantly increased to 10 183.4 ng/g after the BFR manufacturing plant effluent (site B) and decreased to 4414.6 ng/g at 7.7 km downstream, which indicated that the release source of TBBPA DAE is from the plant. The levels of TBBPA DAE in river water was 1.5 ng/L upstream, 49.1 ng/L at the effluent outlet, and 24.9 ng/L at 3.1 km downstream. High levels of TBBPA DAE in the sediment and positive detection in water at 3 km downstream indicates that TBBPA DAE might be persistent in the aqueous environment once it is released. Levels of TBBPA DAE found in the soil also decreased with the increase of distance from the outlet but were significantly lower than those found in sediment. The ratios of average levels of TBBPA DAE in sediment and soil were larger than 100, which suggested that TBBPA DAE was mainly released through the river effluent from the manufacturing plant and not through the air.

TBBPA is one of the most widely used BFRs with an increasing production volume and about 60% of the total BFR market.[37] In addition to its direct application as BFR, approximately 20% is used to produce TBBPA derivatives and oligomers, such as TBBPA DAE. This compound is used as a reactive BFR in polystyrene foams and as precursor compound for a series of other TBBPA derivatives and polymers. Previously, Covaci et al. noted that nearly no information on analytical methodology and environmental distribution could be found for TBBPA DAE.[38]

Recently, in another review on novel BFRs, it was speculated that TBBPA DAE might become a widespread pollutant due to its possible resistance to environmental degradation and long-range atmospheric transport potential.[39] To our knowledge, the only published environmental study on TBBPA DAE analyzed its presence in herring gull eggs and lake trout, and the results suggested that TBBPA DAE is potentially bioaccumulative and persistent in the environment.[27,24] Our study provided additional details on TBBPA DAE including environmental hazard, analytical methodology, and environmental distribution. The presence of TBBPA DAE in these environmental samples suggested that further assessments on the environmental hazard around TBBPA DAE point sources are needed.

■ ASSOCIATED CONTENT

Supporting Information. Additional experimental details, tables, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Goldman, L. R.; Koduru, S. Chemicals in the environmental and developmental toxicity to children: A public health and policy perspective. *Environ. Health Perspect.* **2000**, *108*, 443–448.

(2) Schecter, A.; Pavuk, M.; Papke, O.; Ryan, J. J.; Birnbaum, L.; Rosen, R. Polybrominated diphenyl ethers (PBDEs) in US mothers' milk. *Environ. Health Perspect.* **2003**, *111*, 1723–1729.

(3) Hites, R. A. Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environ. Sci. Technol.* **2004**, *38*, 945–956.

(4) Eriksson, P.; Jakobsson, E.; Fredriksson, A. Brominated flame retardants: A novel class of developmental neurotoxicants in our environment? *Environ. Health Perspect.* **2001**, *109*, 903–908.

(5) Schreiber, T.; Gassmann, K.; Gotz, C.; Hubenthal, U.; Moors, M.; Krause, G.; Merk, H. F.; Nguyen, N. H.; Scanlan, T. S.; Abel, J.; Rose, C. R.; Fritsche, E. Polybrominated Diphenyl Ethers Induce Developmental Neurotoxicity in a Human in Vitro Model: Evidence for Endocrine Disruption. *Environ. Health Perspect.* **2010**, *118*, 572–578.

(6) Reistad, T.; Fonnum, F.; Mariussen, E. Neurotoxicity of the pentabrominated diphenyl ether mixture, DE-71, and hexabromocyclododecane (HBCD) in rat cerebellar granule cells in vitro. *Arch. Toxicol.* **2006**, *80*, 785–796.

(7) Reistad, T.; Mariussen, E.; Ring, A.; Fonnum, F. In vitro toxicity of tetrabromobisphenol-A on cerebellar granule cells: Cell death, free radical formation, calcium influx and extracellular glutamate. *Toxicol. Sci.* **2007**, *96*, 268–278.

(8) Brack, W. Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? *Anal. Bioanal. Chem.* **2003**, *377*, 397–407.

(9) Brack, W.; Klamer, H. J.; Lopez de Alda, M.; Barcelo, D. Effect-directed analysis of key toxicants in European river basins a review. *Environ. Sci. Pollut. Res.* **2007**, *14*, 30–38.

(10) Suzuki, G.; Takigami, H.; Watanabe, M.; Takahashi, S.; Nose, K.; Asari, M.; Sakai, S. I. Identification of brominated and chlorinated phenols as potential thyroid-disrupting compounds in indoor dusts. *Environ. Sci. Technol.* **2008**, *42*, 1794–1800.

(11) Nielen, M. W. F.; Bovee, T. F. H.; van Engelen, M. C.; Rutgers, P.; Hamers, A. R. M.; van Rhijn, I. H. A.; Hoogenboom, L. R. A. P. Urine testing for designer steroids by liquid chromatography with androgen bioassay detection and electrospray quadrupole time-of-flight mass spectrometry identification. *Anal. Chem.* **2006**, *78*, 424–431.

(12) Hogberg, H. T.; Kinsner-Ovaskainen, A.; Hartung, T.; Coecke, S.; Bal-Price, A. K. Gene expression as a sensitive endpoint to evaluate cell differentiation and maturation of the developing central nervous system in primary cultures of rat cerebellar granule cells (CGCs) exposed to pesticides. *Toxicol. Appl. Pharmacol.* **2009**, *235*, 268–286.

(13) Folch, J.; Yeste-Velasco, M.; Alvira, D.; de la Torre, A. V.; Bordas, M.; Lopez, M.; Sureda, F. X.; Rimbau, V.; Camins, A.; Pallas, M. Evaluation of pathways involved in pentachlorophenol-induced apoptosis in rat neurons. *Neurotoxicology* **2009**, *30*, 451–458.

(14) Gauthier, L. T.; Potter, D.; Hebert, C. E.; Letcher, R. J. Temporal Trends and Spatial Distribution of Non-polybrominated Diphenyl Ether Flame Retardants in the Eggs of Colonial Populations of Great Lakes Herring Gulls. *Environ. Sci. Technol.* **2009**, *43*, 312–317.

(15) Villeneuve, D. L.; Blankenship, A. L.; Giesy, J. P. Derivation and application of relative potency estimates based on in vitro bioassay results. *Environ. Toxicol. Chem.* **2000**, *19*, 2835–2843.

(16) Brack, W.; Schmitt-Jansen, M.; Machala, M.; Brix, R.; Barcelo, D.; Schymanski, E.; Streck, G.; Schulze, T. How to confirm identified toxicants in effect-directed analysis. *Anal. Bioanal. Chem.* **2008**, *390*, 1959–1973.

(17) Kodavanti, P. R. S.; Derr-Yellin, E. C. Differential effects of polybrominated diphenyl ethers and polychlorinated biphenyls on [H-3] arachidonic acid release in rat cerebellar granule neurons. *Toxicol. Sci.* **2002**, *68*, 451–457.

(18) Reistad, T.; Mariussen, E.; Ring, A.; Fonnum, F. In vitro toxicity of tetrabromobisphenol-A on cerebellar granule cells: cell death, free radical formation, calcium influx and extracellular glutamate. *Toxicol. Sci.* **2007**, *96*, 268–278.

(19) Chen, S.; Charness, M. E. Ethanol inhibits neuronal differentiation by disrupting activity-dependent neuroprotective protein signaling. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 19962–19967.

(20) Saint-Louis, R.; Pelletier, E. LC-ESI-MS-MS method for the analysis of tetrabromobisphenol A in sediment and sewage sludge. *Analyst* **2004**, *129*, 724–730.

(21) Tollback, J.; Crescenzi, C.; Dyremark, E. Determination of the flame retardant tetrabromobisphenol A in air samples by liquid chromatography-mass spectrometry. *J. Chromatogr. A* **2006**, *1104*, 106–112.

(22) Koppen, R.; Becker, R.; Jung, C.; Piechotta, C.; Nehls, I. Investigation of extraction procedures and HPLC-DAD/MS for the determination of the brominated flame retardant tetrabromobisphenol A bis(2,3-dibromopropylether) in environmental samples. *Anal. Bioanal. Chem.* **2006**, *384*, 1485–1492.

(23) Gallart-Ayala, H.; Moyano, E.; Galceran, M. T. Liquid chromatography/multi-stage mass spectrometry of bisphenol A and its halogenated derivatives. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 4039–4048.

(24) Ismail, N.; Pleskach, K.; Marvin, C. H.; Whittle, D. M.; Keir, M.; Helm, P. A.; Tomy, G. T. Temporal trends of flame retardants in Lake Ontario lake trout (1979−2004). *Organohalogen Compd.* **2006**, *68*, 1808–1811.

(25) Zhou, S. N.; Reiner, E. J.; Marvin, C.; Helm, P.; Riddell, N.; Dorman, F.; Misselwitz, M.; Shen, L.; Crozier, P.; MacPherson, K.; Brindle, I. D. Development of liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry for analysis of halogenated flame retardants in wastewater. *Anal. Bioanal. Chem.* **2010**, *396*, 1311–1320.

(26) Kato, Y.; Okada, S.; Atobe, K.; Endo, T.; Matsubara, F.; Oguma, T.; Haraguchi, K. Simultaneous Determination by APCI-LC/MS/MS of Hydroxylated and Methoxylated Polybrominated Diphenyl Ethers Found in Marine Biota. *Anal. Chem.* **2009**, *81*, 5942–5948.

(27) Letcher, R. J.; Chu, S. G. High-Sensitivity Method for Determination of Tetrabromobisphenol-S and Tetrabromobisphenol-A Derivative Flame Retardants in Great Lakes Herring Gull Eggs by Liquid Chromatography-Atmospheric Pressure Photoionization-Tandem Mass Spectrometry. *Environ. Sci. Technol.* **2010**, *44*, 8615–8621.

(28) Klumpp, S.; Selke, D.; Ahlemeyer, B.; Schaper, C.; Krieglstein, J. Relationship between protein phosphatase type-2C activity and induction of apoptosis in cultured neuronal cells. *Neurochem. Int.* **2002**, *41*, 251–259.

(29) Unchern, S.; Saito, H.; Nishiyama, N. Death of cerebellar granule neurons induced by piperine is distinct from that induced by low potassium medium. *Neurochem. Res.* **1998**, *23*, 97–102.

(30) Meerts, I. A. T. M.; van Zanden, J. J.; Luijks, E. A. C.; van Leeuwen-Bol, I.; Marsh, G.; Jakobsson, E.; Bergman, A.; Brouwer, A. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol. Sci.* **2000**, *56*, 95–104.

(31) Kuiper, R. V.; van den Brandhof, E. J.; Leonards, P. E. G.; van der Ven, L. T. M.; Wester, P. W.; Vos, J. G. Toxicity of tetrabromobisphenol A (TBBPA) in zebrafish (Danio rerio) in a partial life-cycle test. *Arch. Toxicol.* **2007**, *81*, 1–9.

(32) Houtman, C. J.; Van Oostveen, A. M.; Brouwer, A.; Lamoree, M. H.; Legler, J. Identification of estrogenic compounds in fish bile using bioassay-directed fractionation. *Environ. Sci. Technol.* **2004**, *38*, 6415–6423.

(33) Kawanishi, M.; Takamura-Enya, T.; Ermawati, R.; Shimohara, C.; Sakamoto, M.; Matsukawa, K.; Matsuda, T.; Murahashi, T.; Matsui, S.; Wakabayashi, K.; Watanabe, T.; Tashiro, Y.; Yagi, T. Detection of genistein as an estrogenic contaminant of river water in Osaka. *Environ. Sci. Technol.* **2004**, *38*, 6424–6429.

(34) Chou, P. H.; Matsui, S.; Misaki, K.; Matsuda, T. Isolation and identification of xenobiotic aryl hydrocarbon receptor ligands in dyeing wastewater. *Environ. Sci. Technol.* **2007**, *41*, 652–657.

(35) Zhen, H. J.; Wu, X. Q.; Hu, J. Y.; Xiao, Y.; Yang, M.; Hirotsuji, J.; Nishikawa, J.; Nakanishi, T.; Ike, M. Identification of Retinoic Acid Receptor Agonists in Sewage Treatment Plants. *Environ. Sci. Technol.* **2009**, *43*, 6611–6616.

(36) Liao, C. Y.; Li, X. Y.; Wu, B.; Duan, S. M.; Jiang, G. B. Acute enhancement of synaptic transmission and chronic inhibition of synaptogenesis induced by perfluorooctane sulfonate through mediation of voltage-dependent calcium channel. *Environ. Sci. Technol.* **2008**, *42*, 5335–5341.

(37) Law, R. J.; Allchin, C. R.; de Boer, J.; Covaci, A.; Herzke, D.; Lepom, P.; Morris, S.; Tronczynski, J.; de Wit, C. A. Levels and trends of brominated flame retardants in the European environment. *Chemosphere* **2006**, *64*, 187–208.

(38) Covaci, A.; Voorspoels, S.; Abdallah, M. A. E.; Geens, T.; Harrad, S.; Law, R. J. Analytical and environmental aspects of the flame retardant tetrabromobisphenol-A and its derivatives. *J. Chromatogr. A* **2009**, *1216*, 346–363.

(39) Covaci, A.; Harrad, S.; Abdallah, M. A. E.; Ali, N.; Law, R. J.; Herzke, D.; de Wit, C. A. Novel brominated flame retardants: A review of their analysis, environmental fate and behaviour. *Environ. Int.* **2011**, *37*, 532–556.