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Rapid Aryl Hydrocarbon Receptor Based Polymerase Chain Reaction Screening Assay for Polychlorinated Dibenzo-*p*-dioxins and Furans in Soil and Sediment

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Abstract

A method for the measurement of polychlorinated dibenzo-*p*-dioxin and furan (PCCD/F) toxicity equivalent quotient (TEQ) of soil and sediment matrices is described. The method includes extraction, isolation of the PCDD/Fs from interfering compounds, such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), and measurement of PCDD/F TEQ using the PROCEPT® aryl hydrocarbon (AhR) receptor based polymerase chain reaction (PCR) assay. The TEQ values obtained using the PROCEPT® assay correlate well with reference values generated from gas chromatography-high resolution mass spectrometry (GC-HRMS), with a linearity coefficient (R^2) of 0.94. Applied in a screening mode at 50 pg/g PCDD/F TEQ, the PROCEPT® assay yielded 5 false positive results (2.6%) and no false negative results for 196 analyses of spiked soils and environmental samples obtained from US EPA Superfund sites.

Keywords: Persistant Organic Pollutants, Dioxin, Ah-Receptor, PCR

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1. Introduction

Polychlorinated dibenzo-*p*-dioxins and furans (PCDD/F) are a class of persistent organic pollutants which are highly resistant to vertebrate metabolism and can accumulate in high concentrations in top predator species. Most of the toxic and biological effects of PCDD/Fs have been attributed to interaction with the aryl-hydrocarbon receptor (AhR), a cytosolic receptor protein found in most vertebrate tissues [1]. The world health organization (WHO) has assessed the relative toxicity of seventeen PCDD/F congeners (a PCDD/F compound containing a specific number and pattern of chlorine substituents) based on the ability of the congener to bind to the AhR, elicit AhR-mediated biochemical and toxic responses and be persistent and accumulate in the food chain [1]. These seventeen PCDD/F congeners have been assigned toxicity equivalent factors (TEFs) relative to the most toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Table 1), in order to provide consistent recommendations to national regulatory authorities for the establishment of monitoring levels. In 1998, the United States Agency for Toxic Substances and Disease Registry set a guideline screening concentration for PCDD/F in residential soils near contaminated sites of 50 pg/g (50 parts per trillion) [2].

The standard method for the determination of PCDD/Fs is to measure the concentration of each of the seventeen WHO PCDD/F congeners by gas chromatography-high resolution mass spectrometry (GC-HRMS) [3]. A toxicity equivalent quotient (TEQ) is then calculated from the sum of the concentration of each congener multiplied by its TEF value. The GC-HRMS method requires extensive sample preparation and a significant investment in capital equipment (>$500K). Therefore, a rapid screening method capable of measuring PCDD/F TEQs below 50 pg/g using less

expensive laboratory equipment could facilitate the more efficient and more thorough screening of residential soils.

This paper details a rapid *in vitro* AhR based PCR assay (PROCEPT®) for the measurement of PCDD/F TEQ and a chromatographic method for the isolation of PCDD/F from components of soil and sediment and other aryl hydrocarbon compounds. The assay utilizes the affinity of the aryl hydrocarbon receptor for PCDD/F compounds, forming complexes composed of PCDD/F, the AhR, an aryl hydrocarbon nuclear translocator protein (ARNT) and a specific DNA response element (DRE) [4-8]. This complex is then bound to a plastic strip coated with a polyclonal antibody specific for the c-terminus of the ARNT. Excess AhR, ARNT and DNA are washed away and the amount of DNA is amplified and measured using real-time polymerase chain reaction (PCR). The degree of interaction of individual PCDD/F congeners with the AhR is proportional to the TEF established by the WHO (Table 1), therefore, the amount of DNA measured by the PCR is proportional to the PCDD/F TEQ of the sample. However, the PROCEPT® assay does not provide information on the amount of each individual PCDD/F congener.

Several other classes of aryl hydrocarbon compounds also interact with the AhR [9], including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and brominated and mixed brominated/chlorinated dibenzo-p-dioxins and furans (Table 1). Therefore, in order to measure PCDD/Fs using the AhR-PCR assay, a sample preparation method capable of separating PCDD/Fs from other AhR active compounds is necessary. The method employed in this study is similar to the GC-HRMS sample

preparation method [3], but simplified by removing the graphitized carbon column and modified to provide additional decontamination from PAH and PCB compounds [9,10].

The PROCEPT® assay is based on a 96-well format, allowing for the measurement in duplicate of TEQ values for up to 38 unique samples including a 7 point calibration curve, assay blank, method blank and recovery standard. Measuring up to 38 samples simultaneously in 4-5 hours offers a significant time savings over GC-HRMS (>24 hours). However, further improvement in analysis time will require reduction in the time needed for extraction and sample clean-up.

Capital expenditures for the equipment needed to perform the assay (real-time PCR, 96-well plate shaker and 96-well plate washer) are approximately $40-50K, approximately one-tenth of the cost of a GC-HRMS instrument.

2. Experimental

2.1 Materials

Sodium sulfate and Florisil (Sigma-Aldrich) were washed with 1:1 hexane:methylene chloride using an accelerated solvent extraction apparatus (ASE100, Dionex) to remove organic impurities and then heated at 140°C for 24 hours and cooled in a dessicator. The 44% sulfuric acid silica, 10% silver nitrate silica, 2% potassium hydroxide silica, washed silica, toluene (99.9%, HPLC grade), methylene chloride (HPLC, >99.9%), hexanes (HPLC, >98.5%), acetone (histological, >99.5%) and heptane (HPLC, >99%) were used as received from Sigma-Aldrich. Brilliant QPCR mastermix (Stratagene) and the PROCEPT® AhR-PCR assay (Eichrom Technologies) were stored according to manufacturer's instructions until use. Standard solutions of 2,3,7,8-

tetrachlorodibenzo-*p*-dioxin in isooctane, and all other PCDD/F standards, were obtained from Cambridge Isotopes.

2.2 Preparation of Soil Samples

Spiked soils were prepared by drying playground sand and potting soil at 110°C for 48 hours and cooling in a dessicator. The potting soil was then pulverized using a mortar and pestle and sifted to remove large particles. An aliquot of the dried soil or sand was then weighed into a glass round bottom flask and hexane was added to a level ~3 cm above the soil level. A mixture of tetra-octa chlorinated dibenzo-p-dioxins was added to achieve the desired PCDD/F TEQ. The mixture was then equilibrated on a rotary evaporator at room temperature for 2 hours (100 rpm), and the hexane was slowly removed under vacuum. Additional "real world" samples were obtained through the United States Environmental Protection Agency Superfund Innovative Technology Evaluation (SITE) Program.

2.3 Soil Extraction

PCDD/Fs were extracted from the soils by accelerated solvent extraction (ASE) using a mixture of 70:30 toluene:acetone at 100°C and 1500 psi using a Dionex ASE100. Five grams of soil was added to a 33 mL stainless steel extraction cell (Dionex) equipped with a glass fiber filter (Dionex) and filled ~1/3 with diatomaceous earth dispersion agent (Dionex). Diatomaceous earth was then added on top of the soil to fill the remaining space in the cell and the cell was sealed by hand tightening. Each soil was then extracted for two static cycles of five minutes and flushed with 30% of the cell volume between

cycles. A sixty second nitrogen flush completed the extraction procedure and the extract was collected in a 200 mL glass bottle. Other extraction methods and solvent systems including Soxhlet extraction or ultrasonic extraction can be used provided sufficient recovery of PCDD/F can be demonstrated.

2.4 Extract Purification

Following extraction, 2 mL of dodecane was added to the soil extracts, and the extracts were concentrated to ~10 mL at 50°C using a stream of dry air. The extracts were then transferred to 40 mL glass vials with two 5 mL portions of hexane, and the removal of toluene and acetone was completed using a stream of dry air. Ten milliliters of hexane and 5 grams of 44% sulfuric acid silica gel were added to the concentrated extracts. The 40 mL vials were then sealed and gently agitated overnight to begin the removal of PAH compounds and other organics extracted from the soil matrix.

Immediately prior to use, multi-layer silica columns (from top to bottom in a 50 mL glass column, 17 mm i.d.: glass wool, 1 g washed silica, 2.5 grams 10% silver nitrate silica, 1 gram 2% potassium hydroxide silica, 1 gram washed silica, 10 grams 44% sulfuric acid silica, 2.5 grams dried sodium sulfate) and Florisil columns (from top to bottom in a 25 mL glass column, 14 mm i.d.: glass wool, 2 grams Florisil, 1.5 grams dried sodium sulfate) were prepared. The silica and Florisil columns were prewashed with 30 mL and 10 mL of hexane, respectively.

For each extract, a silica column was placed in series above a Florisil column in a fume hood. The extract/44% sulfuric acid silica mixture was then slurried and added to the silica column to complete the PAH compound removal. The transfer of the extract

from the vial to the column was completed using three 5 mL portions of hexane. Once the level of fluid reached the top of silica column bed, 45 mL of hexane was added to complete the elution of PCDD/F on the silica column. The entire PCDD/F eluate from the silica column was collected and passed through the Florisil column, which retains the PCDD/F compounds, but not the PCBs. Once the solvent level reached the top of the Florisil column bed, rinses of 10 mL 2% methylene chloride in hexane and 10 mL of 5% methylene chloride in hexane were performed to complete the separation of PCBs from the PCDD/F. The PCDD/F fraction was then eluted from the column using 15 mL of 50% methylene chloride in hexane and collected in a 20 mL glass test tube. The PCDD/F fraction was then concentrated at room temperature using a stream of dry air, transferred to a 1.5 mL glass vial with methylene chloride, evaporated and dissolved in 0.2 – 1.5 mL of heptane. A final batch contact with 50-100 mg of 10% silver nitrate silica was then performed in the 1.5 mL glass vial to remove any traces of PAH compounds remaining in the extract.

2.5 PROCEPT® AhR-PCR Assay

The PROCEPT® AhR-PCR was performed according to the manufacturer's instructions. All solution dispensing and measuring was performed using delivery pipets and plastic barrier pipet tips. Plastic wells were prepared by washing on a plate washer (BioTek ELx50) using the manufacturer supplied wash solution, adding 50 mL of the thawed antibody solution (stored at -20°C) to each well, equilibrating on a plate shaker (Heidolph Titramax 1000) for 60-90 minutes and washing on the plate washer to remove excess antibody. While the plastic wells were equilibrating, five microliters of each

purified soil extract, standard, assay blank, method blank or recovery standard were added to a 96-well plate of glass vials containing 25 microliters of the manufacturer supplied buffer solution. Samples, standards, assay blanks (heptane), method blanks (blank soil processed through the entire method) and recovery standards (known soil or PCDD/F standard mix processed through the entire method) were analyzed in duplicate (Figure 1). Twenty-five microliters of solution containing the AhR, ARNT and DRE (stored at -80°C and thawed immediately prior to use) were then added to each glass vial, and the plate of glass vials was equilibrated on the plate shaker for 60 minutes. Following equilibration, 30 microliters of solution from the glass vials was added to prepared plastic wells and equilibrated for 30 minutes on the plate shaker to bind the AhR/ARNT/DRE/PCDD/F complexes. Excess AhR, ARNT, and DRE were removed by washing on the plate washer.

2.6 Polymerase Chain Reaction

PCR reagents were prepared according to the manufacturer's instructions by adding 40% DNase free water, 50% 2x PCR mastermix ($MgCl_2$, Taq polymerase, DNA bases, buffer, ROX reference dye) and 10% primer/probe solution. Forty microliters of PCR reagent were added to each plastic well. The plastic wells were sealed using optically clear adhesive film (Applied Biosystems) and placed in the real-time PCR instrument (Stratagene Mx3000P). The PCR protocol included 2 minutes at 50°C, 10 minutes at 95°C and then forty cycles of 15 seconds at 95°C and 60 seconds at 60°C.

The output from the PCR instrument for each sample is a threshold cycle (Ct). The Ct is the number of real-time PCR temperature cycles at which the fluorescence

measurement exceeds a threshold level. By comparing the Ct value for an unknown sample to a calibration curve of known standards (Figure 1), the TEQ value for a sample extract can be calculated ($TEQ_{extract}$). The value of $TEQ_{extract}$ can then be used to determine the TEQ of the soil sample (TEQ_{sample}) using the following equation:

$$TEQ_{sample} = (TEQ_{extract} - MB)(V)(RF)/W$$

where $TEQ_{extract}$ is the value exported by the PCR instrument (pg/mL) by comparing the Ct for the unknown sample to a calibration curve of known standards (Figure 1). The MB is the method blank, V is the volume in mL of heptane in which the purified sample extract has been dissolved, RF is the recovery factor (ratio of the known TEQ of the recovery standard/measured value) and W is the dry weight of the soil sample in grams.

3. Results and discussion

3.1 Method Blank

Method blank results were generated by processing soils certified to be free of PCDD/F, PCB and PAH contamination. Method blank samples were analyzed after employing a final batch contact of the purified extract with 50-100 mg of 10% silver nitrate silica, with a final contact with 0.3 mL of concentrated sulfuric acid and with no further treatment following the elution of the PCDD/F fraction from the Florisil column.

The results for the method blanks (Table 2) show the importance of the final batch contact with silver nitrate silica or sulfuric acid. The final batch contact reduces the background response from impurities (likely PAH) in the solvents, column materials and glassware used to process the samples. Silver nitrate silica gel was chosen over sulfuric acid for subsequent analyses of soil samples due to observed faster kinetics, more efficient removal of PAHs found to have high response on the AhR-PCR assay and concerns of the long term stability of extracts stored over concentrated sulfuric acid.

PCDD/F compounds also have a small affinity for the 10% silver nitrate silica. However, no significant decrease in the response of PCDD/F standards was observed as long as the ratio of the volume of heptane used to dissolve the standard (microliters) to the mass of 10% silver nitrate silica (mg) was higher than 6.

The detection limit of the assay was estimated using three standard deviations of the assay blank. For a 5 gram sample, dissolved in 200 microliters of solvent, the calculated detection limit of the AhR-PCR assay is 1 pg/g (1 ppt). Actual achievable method detection limits will depend on the size of the sample, the volume of the final purified sample extract, and the amount of non-PCDD/F compounds in the sample which have a response on the AhR-PCR assay.

3.2 PAH/PCB Removal

Soil samples spiked with high levels of PAH mixtures and individual PCB standards were processed to verify the removal of these compounds from the PCDD/F fraction by the sample preparation method. The amount of PAH or PCB remaining in the PCDD/F fraction following the extract purification was determined by response on the

AhR-PCR assay (Table 3). The removal of PAH compounds was also verified using ultraviolet-visible spectrometry on the purified extracts. Greater than 99.999% of PAH compounds and greater than 99.8% of PCB-126 and 99.98% of PCB-169 were removed by the chromatographic sample preparation method.

Selected purified soil extracts were analyzed by GC-MS (Battelle Laboratories, Columbus, OH) to determine what types of compounds remain in the PCDD/F fraction following the extract purification. The GC-MS data revealed alkanes, alkenes, phthalates, silicones and benzophenone. At the levels observed in the GC-MS, none of these compounds was found to have significant response on the AhR-PCR assay or induce an antagonistic effect on the measurement of PCDD/F compounds.

3.3 Correlation with GC-HRMS

The AhR-PCR assay TEQ values were found to directly correlate with TEQ values obtained by GC-HRMS (Figure 2) with a correlation coefficient (R^2) of 0.94. This data includes 84 determinations of spiked soils and 112 determinations of environmental samples obtained from US EPA Superfund sites. The range TEQ of values obtained with the PROCEPT® AhR-PCR assay was 71-168% of the corresponding GC-HRMS result, with a mean value of 99% and a median value of 96%. The average relative standard deviation of the TEQ measured for 4-6 replicates of soil samples processed through extraction, clean-up and measurement on the PROCEPT® assay (196 total measurements) was 24%. The relative standard deviation of the PROCEPT® measurement itself for duplicate measurements of the same purified extract was 11%.

3.4 Screening at 50 pg/g

In Table 4, the same data plotted in Figure 2 is evaluated in a screening mode at an action level of 50 pg/g. The AhR-PCR assay correctly identified samples below 50 pg/g and samples above 50 pg/g, yielding no false negative values and five false positive values for the analysis of 112 environmental samples and 84 spiked samples. The five false positive results were observed for environmental samples obtained from US EPA Superfund sites. The false positive results are all well above the limit of detection and are likely due to compounds other than the 17 WHO PCDD/F congeners which have a response on the PROCEPT® AhR-PCR assay, but are not removed during the sample preparation method. Attempts to identify these compounds by GC-MS have been unsuccessful. However, compounds which could cause a high bias in the PROCEPT® measurement include compounds of similar structure to PCDD/F such as, polybrominated dibenzo-*p*-dioxins and furans, mixed chlorinated/brominated dibenzo-*p*-dioxins and furans, polychlorinated xanthenes (analogue of dibenzo-*p*-dioxin with one bridging oxygen replaced with a methylene group), polychlorinated thiophenes (sulfur analogue of furans) and PCDD/F congeners other than the 17 assigned TEF values by the WHO. A complete list of all cross-reactive compounds studied on the PROCEPT® assay can be found in reference [9].

4. Conclusions

The PROCEPT® rapid dioxin assay, an aryl-hydrocarbon based polymerase chain reaction assay, has been evaluated for the measurement of polychlorinated dibenzo-*p*-dioxin and furan toxicity equivalent quotients (TEQ) of soil and sediment samples. TEQ

values obtained using the PROCEPT® assay correlate well with TEQ values obtained using the standard gas chromatography-high resolution mass spectrometry method (R^2 = 0.94). Evaluation of the PROCEPT® assay in a screening mode at 50 pg/g (parts per trillion), yielded 0% false negative results and 2.6% false positive results for 196 TEQ determinations. False positive results are consistent for certain environmental samples and likely arise from the presence of dioxin-like compounds which are not removed during chromatographic purification of soil extracts.

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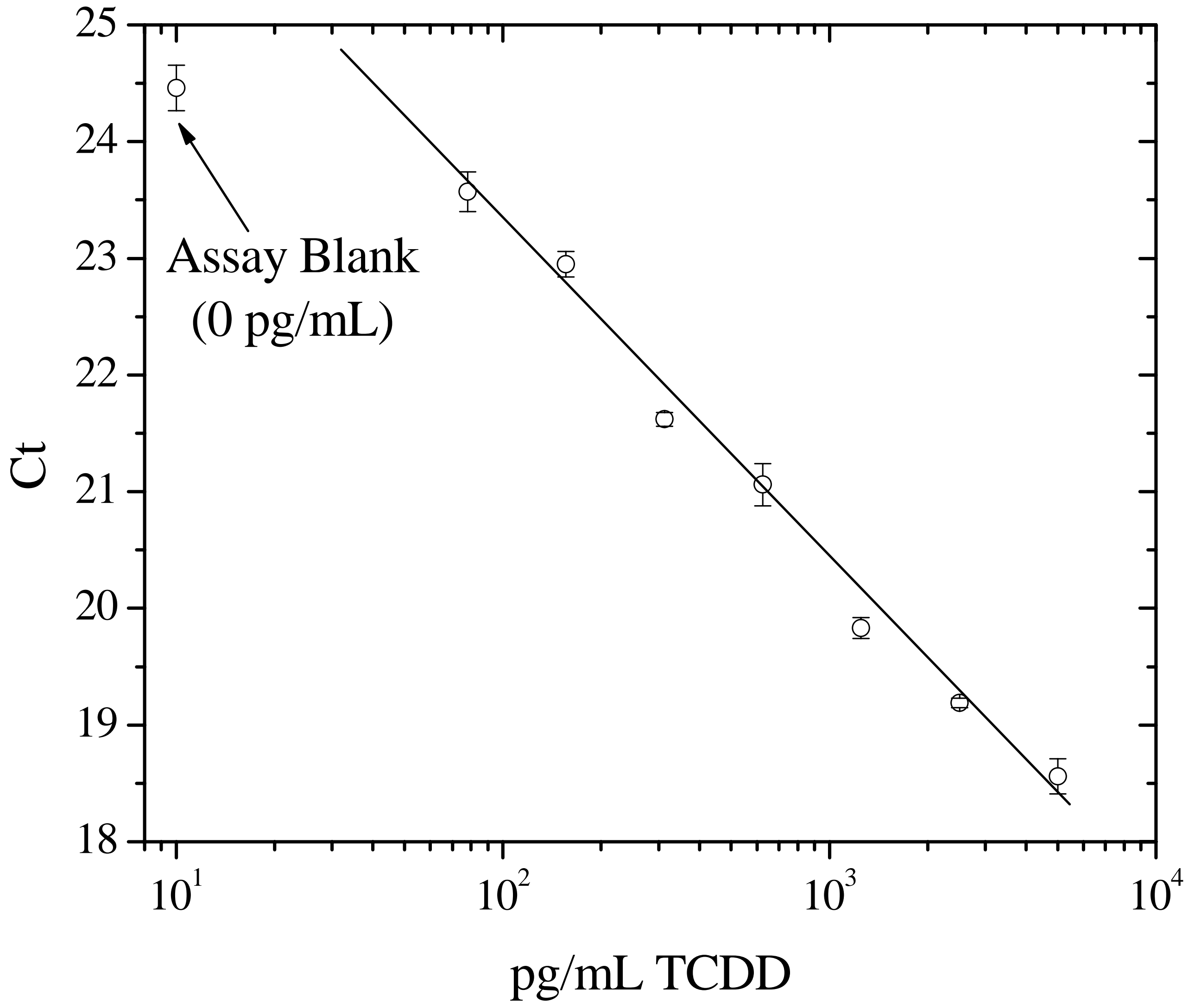

Figure 1. Calibration curve for AhR-PCR assay (Threshold cycle, Ct vs pg/mL 2,3,7,8-TCDD). Error bars determined from duplicate analyses.

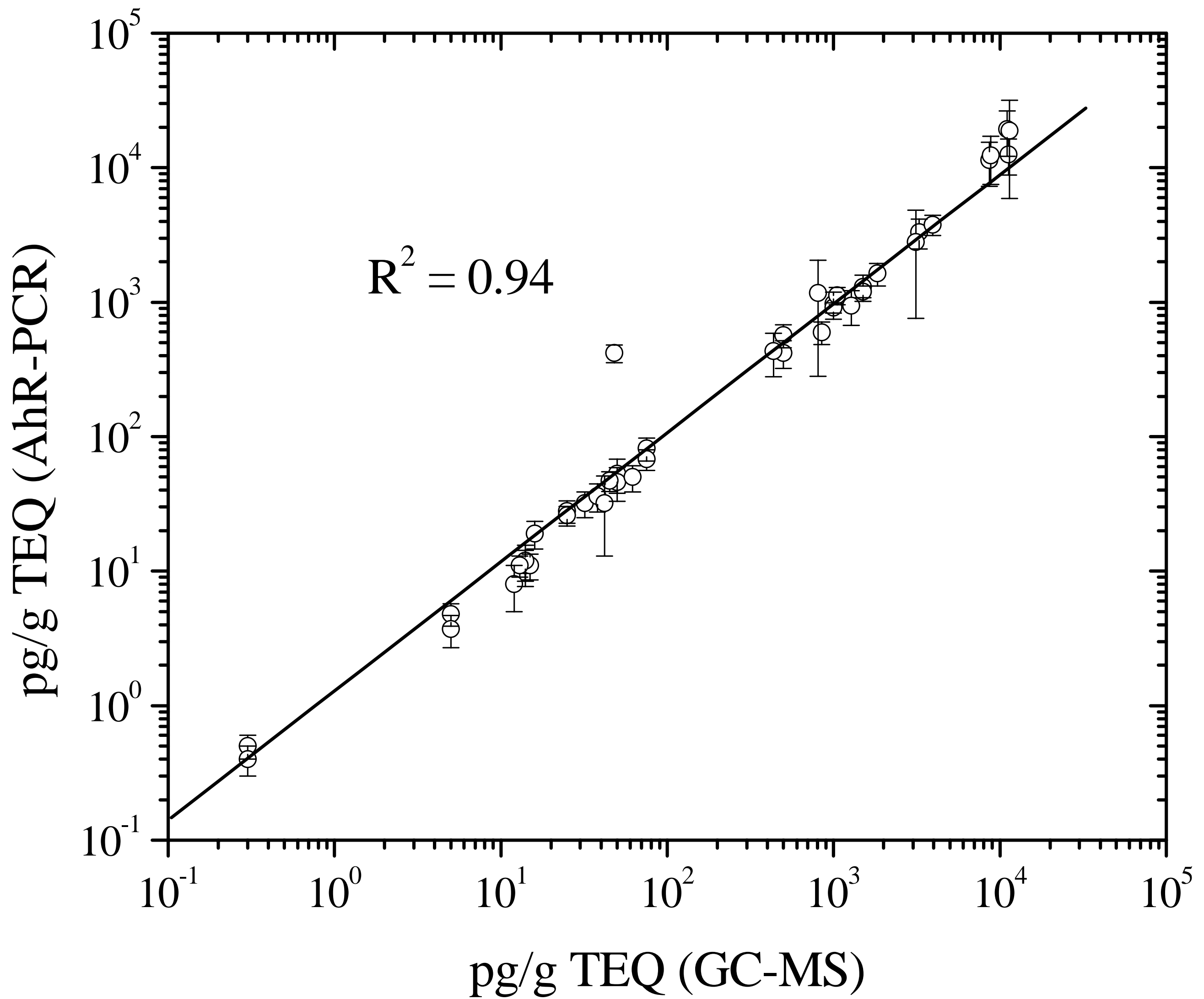

Figure 2. Correlation of AhR-PCR assay and GC-HRMS analysis of PCDD/F in soil and sediment. Error bars determined from 4-6 replicates processed through the entire method (extraction, purification and measurement).

Compound	WHO 2005 TEF	AhR-PCR Assay response factor
2,3,7,8 TCDD	1	1
1,2,3,7,8 PCDD	1	0.6
1,2,3,4,7,8 HxCDD	0.1	0.4
1,2,3,6,7,8 HxCDD	0.1	0.1
1,2,3,7,8,9 HxCDD	0.1	0.5
1,2,3,4,6,7,8 HpCDD	0.01	0.01
1,2,3,4,6,7,8,9 OCDD	0.0003	0.000003
2,3,7,8 TCDF	0.1	0.06
1,2,3,7,8 PCDF	0.03	0.1
2,3,4,7,8 PCDF	0.3	0.3
1,2,3,4,7,8 HxCDF	0.1	0.4
1,2,3,6,7,8 HxCDF	0.1	0.2
1,2,3,7,8,9 HxCDF	0.1	0.3
2,3,4,6,7,8 HxCDF	0.1	0.1
1,2,3,4,6,7,8 HpCDF	0.01	0.05
1,2,3,4,7,8,9 HpCDF	0.01	0.02
1,2,3,4,6,7,8,9 OCDF	0.0003	0.0005
PCB-77 (3,3',4,4')	0.0001	0.00003
PCB-81 (3,4,4',5)	0.0003	0.00005
PCB-105 (2,3,3',4,4')	0.00003	$<3 \times 10^{-7}$
PCB-114 (2,3,4,4',5)	0.00003	0.00001
PCB-118 (2,3',4,4',5)	0.00003	$<3 \times 10^{-7}$
PCB-123 (2',3,4,4',5)	0.00003	0.000009
PCB-126 (3,3',4,4',5)	0.1	0.01
PCB-156 (2,3,3',4,4',5)	0.00003	0.00003
PCB-157 (2,3,3',4,4',5')	0.00003	0.00004
PCB-167 (2,3',4,4',5,5')	0.00003	0.000001
PCB-169 (3,3',4,4',5,5')	0.03	0.001
PCB-189 (2,3,3',4,4',5,5')	0.00003	$<3 \times 10^{-7}$
Indeno(1,2,3-cd)pyrene	N/A	0.8
Benzo(k)fluoranthene	N/A	0.5
Benzo(b)fluoranthene	N/A	0.6
Dibenzo(a,h)anthracene	N/A	0.3
Benzo(a)pyrene	N/A	0.1
Benzo(a)anthracene	N/A	0.05
Chrysene	N/A	0.04
Benzo(g,h,i)perylene	N/A	0.004

Data reproduced from McAlister, et al., *Organohalogen Compnd* . **2006**, *68* , 940-943.

Table 1. AhR-PCR assay response factors vs. WHO TEF values for PCDD/F

Batch Contact	**Method Blank (pg/mL)[1]**	**Standard Deviation**
none	18	6
H_2SO_4	1.1	0.9
10% $AgNO_3$ Silica	0.4	0.3

[1]Six replicates, 5 gram blank soil, 200 uL final extract volume

Table 2. Method blank for AhR-PCR assay vs method of post-Florisil column treatment

Sample	number of replicates	Concentration (pg/g)	Average AhR-PCR Response Following Clean-up (pg/g)	Standard Deviation	% Contaminant Removed
PAH mix A[1]	5	1.E+09	2.9	0.9	>99.999%
PAH mix B[2]	5	2.E+09	3.2	0.9	>99.999%
PCB-126[3]	5	1.E+06	17	7	99.8%
PCB-169[3]	5	1.E+06	3.6	0.9	99.98%
Method Blank	5	N/A	3.4	0.9	N/A

[1]1000 ug/g of 3-methylcholanthrene, 7H-dibenzo(c,g)carbazole, Benzo[j]fluoranthene, dibenzo[a,e]pyrene, dibenzo[a,h]acridine, dibenzo[a,j]acridine, dibenzo[a,h]pyrene and dibenzo[a,i]pyrene

[2]2000 ug/g of acenaphthene, acenaphthylene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g,h,i]perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-c,d]pyrene, naphthalene, phenanthrene and pyrene

[3]% contaminant removed for PCB-126 and PCB-169 calculated using the response factor for these anlaytes on the AhR-PCR assay

Table 3. Decontamination of PAH and PCB from PCDD/F fraction

Result	Environmental Samples	Spiked Samples	total
False Positive	5 (4.5%)	0 (0.0%)	5 (2.6%)
False Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)
total samples	112	84	196

Table 4. Screening Results at 50 pg/g (50 parts per trillion)