PFAS IN SOIL SAMPLES FROM SEVERAL LOCATIONS IN AUSTRIA TESTED BY CHEMICAL ANALYSIS AND BIOASSAY USING TTR TR REPORTERGENE ASSAY

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Introduction

Over the last decade, per- and polyfluoroalkyl substances (PFASs) have become one of the most heavily investigated persistent organohalogen compound class of environmental concern. Due to regulatory action via the Stockholm Convention, global reduction, and phase-out activities of some long chain PFASs like PFOS and PFOA, are ongoing. This is urgently needed considering the widespread contamination of e.g., soil. The long chain PFASs are often substituted by other shorter chain PFASs with lower bioaccumulation potential, but with increased mobility. Due to limited information on their toxicity, these PFASs might be regrettable substitutes which need to be controlled. Therefore, tools are needed to assess toxic effects of PFAS mixtures for relevant toxicity end points such as endocrine disrupting properties.

Thyroid hormones play a critical role in the regulation of metabolism, and thyroid function is related to cardiovascular disease, fertility, and fetal neurodevelopment [Kim et al. 2018; Fenton et al. 2021]. In animal experiments, treatment of PFAS induced hypertrophy or hyperplasia of thyroid follicular cells in rat and lowered total and free T4 concentrations. It has been suggested that some PFASs may disrupt the thyroid hormone system in humans. Because PFOS, PFOA, and PFHxS are the most widely present PFASs, their association with thyroid dysfunction has been studied most as compared to other PFASs. Several studies showed that blood PFAS concentrations are negatively correlated with thyroid hormone concentrations.

Earlier publications indicated that many PFASs, like perfluorooctanoic acid (PFOA) were shown to potently bind to the thyroid transport protein transthyretin (TTR) thereby competing with the natural hormone thyroxine (T4) (Weiss et al. 2009; Young et al. 2021), which can lead to reduced plasma thyroid hormone levels. A fast and reproducible human cell-based reportergene bioassay which enabled comprehensive analysis of thyroid disruption potential of compounds i.e., the TTR-TRβ CALUX bioassay has been established (Collet et al. 2020; Behnisch et al. 2021). While earlier TTR-based bioassays, such as FITC-T4 assay only measures binding competition of a compound with T4 on the plasma protein TTR, the TTR-TRβ CALUX measures the integrated response of interference of compounds with several additional types of interaction points in the thyroid activity pathway (Behnisch et al. 2021). The strong dose-dependent TTR-T4 binding competition potency of PFOA could be confirmed in this bioassay already earlier (Sprengel et al. 2020; Young et al. 2021).

Here we applied TTR-TRβ CALUX bioassay based PFAS potency factors for thyroid hormone disruption potential on chemical analysis results and compared them with TTR-TRβ CALUX bioanalysis results of soil samples from several locations in Austria.

Materials and methods

The TTR-TRβ reporter gene bioassay was carried out by BioDetection Systems B.V. (BDS, Amsterdam, the Netherlands) under conditions described in detail previously (Behnisch et al. 2021). Soil samples were weighed (~5g dry weight) into 50 mL polypropylene tubes. For soil extraction, 20 mL of MeOH was added to each polypropylene tube and shaken for 60 minutes. The tubes were then centrifuged at 830g for 10 minutes. The supernatant was decanted into a labeled new polypropylene tube. After this, another 20 mL of MeOH was added to the soil and shaken for 30 minutes. This extract was also centrifuged at 830g for 10 minutes, after which the supernatant was decanted and added to the first fraction. The last step was repeated. The pooled fractions with a total volume of 60 mL were evaporated to 25 mL. Next, the collected soil extracts were diluted in 500 mL MilliQ water up to a final MeOH concentration of 5%. Further clean-up using a weak anion exchange (WAX) solid phase extraction (SPE) was performed. The cartridge was conditioned using 4 ml of MeOH with 0.1% NH_4OH, 4 ml of MeOH and 4 ml HPLC water. The sample was transferred to the cartridge at a rate of about 1 drop per second. The cartridge was washed using 4 ml 25 mM NH_4Ac pH 4 and 8 ml THF/MeOH (75:25). After washing, the cartridge was dried for 30 minutes applying vacuum. A 15 ml tube was placed under the cartridge and the sample was eluted using 4 ml of MeOH with 0.1% NH_4OH. The extract was evaporated to dryness under a gentle stream of N_2 and re-dissolved in 100 µL of DMSO followed by preparation of serial dilutions in DMSO in log-scale increments of 0.5. Five (5) µL of diluted PFASs and of the soil sample extracts were mixed with 100 µL TTR and 50 µL T4 (final concentrations 0.058 µM and 0.052 µM, respectively), both dissolved in Tris-buffer (pH 8.0) and incubated in a final volume of 155 µL overnight at 4 ^{O}C. As standard reference substance, a dilution series of PFOA (Sigma-Aldrich, Zwijndrecht, The Netherlands) in DMSO in log-scale increments of 0.5 was prepared (1.0*10-6 – 3.0*10-3 mol/L) and 5 µl of each dilution was incubated with 50 µl T4 and 100 µl TTR. TTR-bound T4 was separated from unbound (free) T4 by loading the total incubate on a pre-cooled Bio-Gel P-6DG (Bio-Rad Laboratories B.V., The Netherlands) column followed by 1 minute centrifugation (210 g). One hundred and forty (140) µL of the collected eluate containing the TTR-bound T4 was mixed with 500 µL serum-free assay medium consisting of DMEM/F12 supplemented with penicillin and 10 µg/mL streptomycin (P/S) and non-essential amino acids (NEAA). The eluate/assay medium mix was added to seeded and pre-incubated TRβ reporter gene cells (200 µL/well; in triplicate wells). After 24 h of exposure of the TRβ CALUX cells in a conditioned environment (37 °C, 5% CO_2, 100% humidity), the medium was removed, and the cells were lysed with 30 µL of a triton-lysis buffer. The induction of luciferase production was quantified after addition of 100 µL illuminate solution containing 15 µg of the substrate D-luciferin and subsequent measurement of luminescence on a luminometer (Mithras LB949, Berthold Technologies, Bad Wildbach, Germany). For quality control purposes, the cells were visually inspected daily under a microscope for signs of cytotoxicity, infection, and decrease in cell viability. Any dilution of a soil sample that affected cell viability was further subjected to exclusion. Each sample was analyzed in triplicate. Moreover, the respective reference compound was included on every microtiter plate to control for potential variations between plates. The vehicle controls were used as negative controls/procedure blanks and no displacement of T4 from TTR were observed.

Results and discussion

In vitro toxicity potency factors were established for thyroid hormone disruption potential using the TTR-TRβ reporter gene bioassay for major PFASs (Behnisch et al. 2021) listed in the German drinking water guideline (German Environment Agency, 2017). All PFASs listed here affected the T4 binding to TTR, an important plasma thyroid hormone transport protein. For all tested PFASs, potency factors based on PC80 values relative to PFOA (1.0) could be obtained and ranged between 0.0018 (PFBA) and 2.0 (PFOS). PFOA

was used here as reference compound because it is the most active bioaccumulating (blood and mother milk) PFASs so far tested.

Applying these in vitro potency factors from the TTR-TRβ reporter gene assay to the chemically determinate concentrations of PFAS congeners in these soil samples led to calculated PFOA-equivalent concentrations as indicated in Table 1. All soil samples showed thyroid hormone disruptive potential in the TTR-TRβ reporter gene bioassay and the PFOA-EQ measured correlated well (R^2 0.992) with these above calculated PFOA EQ from the chemical analysis for the major PFASs present in these mixtures (see Table 1 and Figure 1):

Table 1. Correlation between total PFOA-equivalents (BEQs) measured directly in the TTR-TRβ reporter gene assay and LC-MS-based PFOA equivalents (TEQs) calculated using PC80-based relative potency factors from TTR-TRβ reporter gene assay for several soil samples.

PFAS		Soil-1		Soil-2		Soil-3		Soil-4		Soil-5	
	Potency factor	PFAS (a)	PFOA-EQ (b)	PFAS (a)	PFOA-EQ (b)	PFAS (a)	PFOA-EQ (b)	PFAS (a)	PFOA-EQ (b)	PFAS (a)	PFOA-EQ (b)
PFBA	0.0018	0	0	0	0	1.6	0.0029	1.1	0.002	0	0
PFPeA	0.080	0	0	0	0	1.8	0.14	4.5	0.36	0	0
PFHxA	0.19	2	0.37	3.3	0.61	1.8	0.33	1.4	0.26	0.7	0.13
PFHpA	1.4	1.9	2.7	2.8	3.9	2.8	3.9	1.3	1.8	0	0
PFOA	1.0	10	10	5.7	5.7	15	15	5.7	5.7	2.8	2.8
PFNA	0.32	3.2	1	1.9	0.62	5.3	1.7	3.4	1.1	0.51	0.17
PFDcA	0.12	3.6	0.44	3.4	0.42	8.7	1.1	6.1	0.75	0.25	0.031
PFBS	0.052	0	0	0	0	0	0	0	0	0	0
PFHxS	1.6	0.91	1.4	1.5	2.3	1.1	1.7	1.3	2	0	0
PFHpS	1.0	0.25	0.24	0.69	0.67	0.25	0.24	0	0	0	0
PFOS	2.0	70	140	40	80	57	110	53	106	37	74
PFOSA	0.72	0	0	0	0	0.5	0.36	0	0	0	0
SUM TEQ (PFOA-TEQ)			**156**		**94**		**134**		**118**		**77**
Measured TTR-TRβ-CALUX (PFOA-BEQ)			**474**		**229**		**360**		**504**		**145**

(a) PFAS congener analysis results expressed as µg/kg d.w.

(b) PFAS congener analysis expressed as µg PFOA equivalents/kg d.w.

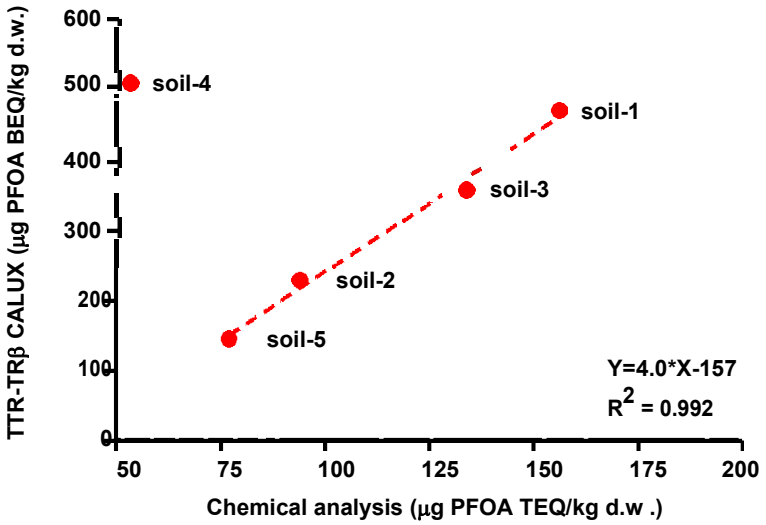

Figure 1. Correlation between PFOA-equivalents derived by TTR-TRβ reporter gene analysis and LC-MS analysis (using PC80-based potency factors from TTR-TRβ reporter gene) of soil samples.

Conclusion

In all soil samples the group- and effect-based TTR-TRβ reporter gene assay did show significantly higher PFOA-EQ as compared to the calculated-PFOA-EQ using chemical-based analysis. This indicates that other factors, e.g., specific but currently not tested PFAS and other unknown structural compounds, could also play a role in this sample. Overall, limited information on the composition of such industrial PFAS mixtures prevented us from determining the factors that were responsible for the different activities of the thyroid hormone disruption here studied. A good correlation between the results from chemical and biological analysis was observed.

Irrespectively, reduced thyroid function as indicated in our tests can be harmful for the human developmental effects in newborn babies and in wildlife species. The observation of thyroid disruptive activity of PFASs and their industrial mixtures in the TTR-TRβ reporter gene assay may indicate a potential for thyroid hormone disruption and associated transfer from the mother to the infant, making use of the selective plasma thyroid hormone delivery system (TTR) involved in placental transfer and delivery over the blood-brain barrier in the body (Collet et al. 2020). Further testing of other different, but ideally, well-characterized PFAS compounds, as well as more soil samples are needed to clarify the factors that influence the different activities determined in the TTR-TRβ reporter gene bioassay.

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