Chapter 4

Miniaturization of a transthyretin binding assay using a fluorescent probe for high throughput screening of thyroid hormone disruption in environmental samples

Submitted to Science of the Total Environment

Xiyu Ouyang[a], Jean Froment[b,c], Pim E. G. Leonards[a], Guttorm Christensen[d], Knut-Erik Tollefsen[b], Jacob de Boer[a], Kevin V. Thomas[b], Marja H. Lamoree[a]

[a] Institute for Environmental Studies, VU University Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

[b] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway

[c] Department of Chemistry, University of Oslo (UiO), PO Box 1033, Blindern, N-0316 Oslo, Norway

[d] Akvaplan NIVA, FramCentre, N-9296 Tromsø, Norway

Abstract

Thyroid hormone (TH) disrupting compounds are potentially important environmental contaminants due to their possible adverse neurological and developmental effects on both humans and wildlife. Currently, the most successful bio-analytical method to detect and evaluate TH disruptors, which target the plasma transport of TH in environmental samples, is the radio-ligand thyroxine-transthyretin (T4-TTR) binding assay. Yet, costly materials and tedious handling procedures prevent the use of this assay in high-throughput analysis that is nowadays urgently demanded in environmental quality assessment. For the first time a miniaturized fluorescence T4-TTR binding assay was developed in a 96-well microplate and tested with eight TH disrupting compounds. The sensitivity of the newly developed assay was slightly lower than the radio-ligand binding assay, however, throughput was enhanced at least 100-fold, while using much cheaper materials. The thyroid hormone disrupting potency of 22 herring gull (*Larus argentatus*) egg extracts, collected from two different locations (Musvær and Reiaren) in Norway, was evaluated to demonstrate the applicability of the assay for environmental samples.

1. Introduction

Over the past decade, research in the field of endocrine disruption has greatly increase with regard to the occurrence of endocrine disrupting compounds in the environment and their effects in the ecosystem and humans.[1] Over the years, various end points such as estrogenic, androgenic, progestogenic, glucocorticoid and thyroidogenic activities[1] have been included in endocrine disruption studies. The thyroid system comprises the hypothalamus, the pituitary and the thyroid gland. It is vulnerable to endocrine-disrupting effects through different mechanisms, such as binding of xenobiotics to thyroid hormone (TH) transport proteins and interference with the plasma hormone transport of THs.[2] Together with thyroxine-binding globulin (TBG), transthyretin (TTR) is one of the most important plasma proteins associated with the transport of THs such as thyroxine (3, 3', 5, 5'-tetraiodo-L-thyronine, T_4). Disruption of the binding of T_4 with TTR has already been observed in the presence of various environmental contaminants, such as hydroxylated polychlorinated biphenyls (OH-PCBs), perfluoroalkyl and polyfluoroalkyl substances

(PFASs), hydroxylated polybrominated diphenyl ethers (OH-PBDEs) and other brominated flame retardants (BFRs); caused by their competition with the T_4 binding to TTR.[3–6] Interference with the plasma transport of THs may lead to disruption of the targeted transport and metabolism of T_4 that may ultimately lead to perturbations of natural functions of THs in adults as well as maturation and development in juvenile or fetal life stages of vertebrates.[7] Advanced bio-analytical methods have therefore been developed to evaluate disruption of T_4 transport. A classical method to assess such an effect uses ^{125}I labelled T_4 as the radioactive ligand in a competitive binding assay.[8] The method was successfully applied in many studies and shows good sensitivity.[4,9,10] However, due to the costly radioactive ligand, relatively complicated assay steps and safety issues related to the handling of ^{125}I as a radioactive tracer, the throughput of this approach is still rather limited. In addition to this classical radio-ligand binding assay, other approaches such as the TR-CALUX (thyroid hormone responsive chemically activated luciferase gene expression) assay and the ANSA (8-anilino-1-naphthalenesulfonic acid ammonium)-TTR competitive fluorescence displacement assay have been developed to assess the TTR binding capacity of TH disruptors in the environment.[11,12] Similar to the ANSA-TTR assay, another bioassay, based on a previous study[13], has also been developed to investigate the binding of OH-PBDEs to TH transport proteins (TTR and TBG) using the fluorescence probe fluorescein isothiocyanate (FITC) associated to T_4.[14] This assay was performed in cuvettes and the reported sensitivity for OH-PBDEs was comparable to the radio-ligand assay.

In the present study, the same principle of FITC-T_4 was applied, however in order to further enhance assay throughput, it was miniaturized in a 96 well microplate format. First, the new downscaled protocol was optimized to achieve similar performance as the assay performed in cuvettes. Then the new protocol was tested with compounds from seven different groups known to interfere with the T_4-TTR binding (OH-PCBs, OH-PBDEs, BFRs, PFASs, bromophenols, phthalates and antibacterial agents). The observed activities were compared with those obtained in the classical radio-ligand binding assay. The TH disrupting potency of herring gull extracts from two locations in Norway was evaluated using the new protocol to demonstrate the applicability of the assay for testing environmental samples. In addition, in order to further explain the activities determined using the bioassay, target analysis of OH-

PCBs based on gas chromatography (GC) using electron capture detection (ECD) was performed for the most potent sample, as high levels of PCBs have previously been detected in these samples.[15]

2. Materials and methods

2.1. Chemicals

Fluorescein isothiocyanate (FITC, >90%) and L-thyroxine (T_4, >98%) were supplied by Sigma-Fluka (Zwijndrecht, The Netherlands). Anhydrous pyridine (99.8%) and triethylamine (>99%) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Water was obtained from a Milli-Q Reference A+ purification system (Millipore, Bedford, MA, USA). Perfluorooctanoic acid (PFOA, 96%) and triclosan were purchased from Sigma-Fluka. Perfluorooctanesulfonic acid (PFOS, 98%) was purchased from RTI laboratories (Livonia, MI, USA). 2, 4, 6-tribromophenol (2, 4, 6-TBP, 99%) were purchased from Riedel-de Haen (Seelze, Germany). Mono (2-ethylhexyl) phthalate (MEHP, 100 µg/ml in methyl tert-butyl ether) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). 2,2',3,4',5,5'-hexachloro-4-biphenylol (4-OH-CB-146, 50 µg/mL in nonane), 2,2',4,4',5,5'-hexachloro-3-biphenylol (3-OH-CB-153, 50 µg/mL in nonane) and 2,2',3,3',4',5,5'-heptachloro-4-biphenylol (4-OH-CB-172, 50 µg/mL in nonane) were obtained from Wellington Laboratories (Guelph, ON, Canada). 2,3,3',4',5-pentachloro-4-biphenylol (4-OH-CB-107, 0.99 mg/g in 4-methyl-2-pentanol), 2,2',3,4',5,5',6-heptachloro-4-biphenylol (4-OH-CB-187, 2 mg/g in 4-methyl-2-pentanol), 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47, 1 mg/mL in dimethyl sulfoxide) and tetrabromobisphenol A (TBBPA, 1 mg/ml in dimethyl sulfoxide) were obtained from the group of Prof. Bergman, ACES, Stockholm University, Sweden.

2.2. Synthesis of the fluorescent probe

The fluorescent labelled thyroxine (FITC-T_4) has been synthesized and purified according to a previous study.[13] In short, 51.4 mM of FITC reacted with 25.7 mM of L-thyroxine in a pyridine/water/triethylamine medium (9:1.5:0.1, v/v/v) for one hour at 37 °C. The reaction products were precipitated by adding 20 volumes of 0.2 M

ammonium acetate buffer and collected after centrifugation (10 min, 1000 × g, Biofuge Stratos, Heraeus Instruments, Hanau, Germany). After removal of the supernatant, the precipitate was washed with 20 volumes of MilliQ water and centrifuged again at the same condition. The precipitate was then re-dissolved in 8 volumes of 0.005 M of ammonium bicarbonate. A few drops of ammonia solution (10%, v/v) were added to dissolve the precipitate. The obtained solution was applied to a Sephadex G-50 fine column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) which was previously equilibrated with a solution of 5 mM ammonium bicarbonate. The fluorescent impurities were thereafter removed by washing the column with 10 column volumes of 5 mM ammonium bicarbonate solution. The purified product (FITC-T_4) was eluted from the gel using MilliQ water and then freeze dried for 48 h under 0.7 mbar, -20 °C. Before use, the obtained FITC-T_4 was dissolved in Tris-NaCl buffer (50 mM Tris−HCl/100 mM NaCl, pH 7.4) and its concentration was measured by absorbance at 490 nm using a Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Herring gull egg collection and extraction

Sample collection and extraction was described in a previous study.[15] During spring 2012, 22 herring gull (*Larus argentatus*) eggs were collected from two locations in Norway: 1) the Musvær Island (69˚52'N, 18˚33'E), a remote island in the north of the country in the municipality of Tromsø and 2) the Reiaren Island (59˚8'N, 10˚27'E), a more populated area off the southeastern coast in the municipality of Tjøme. After collection all the eggs were frozen as fast as possible in order to prevent embryo development. Each egg was collected from a different nest without knowing the age. The egg yolk extraction was performed in an ultrasonic bath for 15 min with acetone and cyclohexane (3:2, v/v). After shaking for 1 h, the extracts were centrifuged for 10 min (1300 x g) and the lipids were removed by gel permeation chromatography (GPC; Waters 2695 separations module coupled to a Waters 486 absorbance detector at 254 nm) fitted with Envirogel columns (19 mm × 150 mm + 19 mm × 300 mm; Waters). The extracts were collected between 14.40 and 21.00 min and the dichloromethane used as mobile phase was evaporated under a gentle nitrogen stream in order to transfer the extracts into dimethylsulfoxide (DMSO, purity 99.8%,

Sigma–Aldrich) for the bioassay.

2.4. Miniaturization of the TTR binding assay

The previous study[14] uses the TTR binding assay in cuvettes to measure the fluorescent labelled T_4 (FITC-T_4) that binds to TTR by fluorescence enhancement. In this study, the protocol was downscaled to 96 well microplates (Greiner Bio-One, Frickenhausen, Germany), to support high-throughput screening in e.g. effect-directed analysis (EDA) of complex environmental samples.[16] Different materials (polypropylene, polystyrene) and well shapes (flat bottom, round bottom and V bottom) of microplates were tested. A white polypropylene plate with flat bottom wells was selected due to relatively lower T4 adsorption to the wall of the wells and good fluorescence measurement property. In the well, 14.5 µL of 1 µM FITC-T_4, 8 µl of 3.6 µM TTR together with 80 µL of Tris-NaCl buffer was incubated for 5 min at room temperature. Afterwards, 10 µL of the competitor to be tested (either a single compound, an egg extract or the buffer for the control wells, n=3) was added and the fluorescence intensity was measured at 490 nm for the excitation wavelength and 518 nm for the emission wavelength using a Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA). In each experiment, two set of blanks (n=3) were simultaneously prepared in the same plate to estimate the percentage of replacement (binding potency of the inhibitor) and to correct the original fluorescence caused by free FITC-T_4. The FITC-T_4 -only blank (Blank 1) was prepared by 14.5 µL of 1 µM FITC-T_4 and 98 µL of Tris-NaCl buffer. The FITC-T_4-TTR blank (Blank 2) was prepared by µl of 1 µM FITC-T_4, 8 µl of 3.6 µM TTR together with 90 µL Tris-NaCl buffer.

2.5. Data analysis of the assay

All the curve plotting and statistical treatment were carried out with the software GraphPad Prism 6 (GraphPad software, San Diego, CA, USA). In order to calculate the dissociation constant (K_d) between T_4 and TTR, a saturation study was performed as described by Ren and Guo.[14] The protocol was conducted in the same way as the inhibition assay except that different concentrations of substrate (FITC-T_4, from 0.025 µM to 1.5 µM) were tested at a fixed TTR concentration (1 µM). The

saturation study curve-fitting to the data was performed by the “One site – Specific binding with Hill slope” model.

The binding potency of the tested compounds or the extracts was estimated by the percentage of fluorescence intensity, after the inhibitors (or extracts) had been introduced. The percentage was calculated by equation 1:

$$\varepsilon = \frac{I_S - I_{B1}}{I_{B2} - I_{B1}} \times 100\% \quad (1)$$

In the equation, ε is the fluorescence intensity in percentage. I_S is the fluorescence detected in the wells containing single compound or extract, together with FITC-T4 and TTR. I_{B1} and I_{B2} are the fluorescence detected in the wells of Blank 1 and Blank 2, as defined in section 2.4.

The dose response curves were obtained using the “log (inhibitor) vs. response (three parameters)” model (n=3) and the IC_{50} values were determined by the GraphPad Prism 6 (GraphPad software, La Jolla California, USA) software with 95% confidence. The concentration range chosen for most of the inhibitors in the dose response experiments was 100 pM-100 μM. The GraphPad Prism software was used to plot the curves and find appropriate fittings.

2.6. Target analysis of five OH-PCBs

Target analysis of five OH-PCBs (4-OH-CB-107, 4-OH-CB-146, 3-OH-CB-153, 4-OH-CB-172 and 4-OH-CB-187) was performed for six egg extracts and an extraction blank. Prior to the GC analysis, partitioning and derivatization of the aqueous fractions were performed according to the method developed by Hovander et al.[17] The five OH-PCBs were subsequently analysed by an Agilent 6890 GC equipped with an electron capture detector (ECD, Agilent Technologies, Palo Alto, CA, USA) on a CP-Sil-8CB (25 m × 0.15 mm i.d., 0.12 μm film thickness, Agilent) column using hydrogen as carrier gas and nitrogen as makeup gas. The column-oven temperature program was: 60 °C (2 min), 50 °C/min up to 200 °C (0 min), 1 °C /min up to 230 °C (0 min), and 30 °C /min up to 300 °C (3min).[18]

3. Results and discussion

3.1. Saturation study

The curve obtained in the saturation study (Figure 1) achieved a similar shape as reported by Ren and Guo[14] that allowed the calculation of the FITC-T_4 and TTR dissociation constant (K_d). The K_d obtained in this study was equal to 261 µM (R^2 = 0.99).

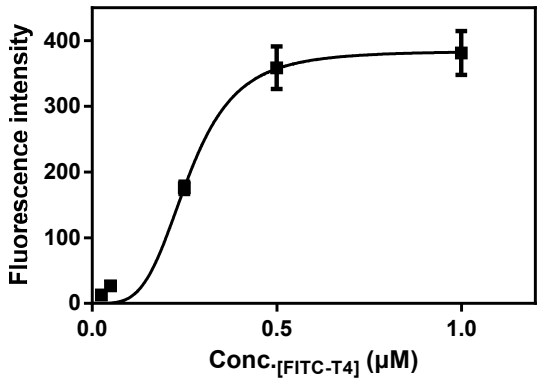

Figure 1: Fluorescence measurements at different substrate (FITC-T_4) concentrations for the saturation study.

3.2. TTR disrupting compounds tested using the miniaturized FITC-T_4/TTR binding assay

In total the binding potency of eight compounds from seven selected groups of chemicals together with non-labelled T_4 was quantitatively determined by a concentration response experiments using the miniaturized FITC-T_4/TTR binding assay. A concentration-dependent decrease in T_4 binding to the TTR was observed for all the tested compounds (Figure 2). Among the tested compounds, 4-OH-CB-107, TBBPA, 6-OH-BDE-47, PFOS, PFOA and 2,4,6-TBP are well-known TH disrupting compounds and their TTR binding potencies have previously been evaluated using the radio-ligand binding assay.[3–6,9]

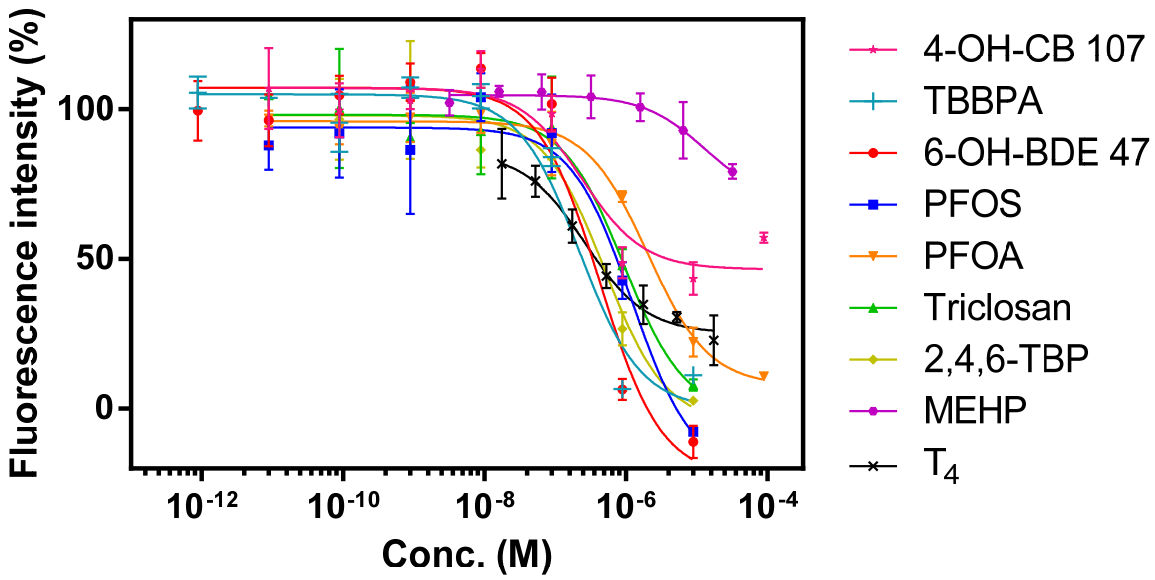

Figure 2: Dose response curves of eight (potential) TH disruptors together with T_4, tested by the miniaturized FITC-T_4/TTR binding assay.

In the miniaturized FITC-T_4/TTR assay, 4-OH-CB-107 showed a partial concentration-response curve, which was likely caused by the water solubility of the compound (0.2 µM), plus the initial solvent of 4-OH-CB-107 (4-methyl-2-pentanol) that was also poorly miscible with the aqueous working medium of the assay. Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) also displayed a concentration-dependent displacement of T4 from TTR, which was consistent with a dose-dependent decreases in total plasma T_4 in rats exposed to the same compound via oral exposure[19]; its binding potency was determined by the radio-ligand binding assay in a recent study.[20] For MEHP, the concentration in human urine was found to be negatively correlated to the free T_4 and T_3 levels in serum.[21] Therefore, MEHP was also expected to be a TH disrupting compound. In this assay, MEHP was proven to disrupt T_4/TTR binding according to the dose response curve, although the potency was relatively low compared to the other compounds in this study.

Table 1. IC_{50} values of the eight tested compounds obtained using non-labelled T_4 measured in the miniaturized FITC-T_4/TTR assay (FluTTR) and the radio-ligand binding assay (RLBA).

Compounds	IC_{50} FluTTR (µM)	IC_{50} RLBA (µM)	RLBA Reference
4-OH-CB-107	0.24	0.024	Meerts et al.[22]
TBBPA	0.22	0.031	Hamers et al.[6]
6-OH-BDE-47	0.43	0.18	Hamers et al.[6]
PFOS	1.21	0.94	Weiss et al.[4]
PFOA	2.02	0.95	Weiss et al.[4]
Triclosan	0.93	2.84	Weiss et al.[20]
2,4,6-TBP	0.50	0.068	Meerts et al.[9]
MEHP	13.08	31.55	DENAMIC*
T_4	0.26	0.08	Meerts et al.[9]

*Data from the EU FP7 project DENAMIC (Developmental Neurotoxicity Assessment of Mixtures in Children, http://www.denamic-project.eu/)

The binding potencies of the eight compounds were evaluated by their IC_{50} values, calculated from the corresponding dose response curves, and were compared with the IC_{50} values obtained from the radio-ligand binding assay (Table 1). The IC_{50} values of the most potent compounds (4-OH-CB-107, TBBPA and 2,4,6-TBP) measured by the miniaturized FITC-T_4/TTR assay were about 7-10 times higher than the values achieved by the radio-ligand binding assay. For the medium potent compounds (6-OH-BDE-47, PFOS, PFOA and non-labelled T_4), the IC_{50} values were also higher but only by a factor of 1-3. The IC_{50} value of non-labelled T_4 and 4-OH-BDE-47 obtained in the miniaturized FITC-T_4/TTR assay (262 nM and 430 nM) were very close to the original report of FITC-T_4/TTR assay performed in cuvette (260±13 nM and 323±10 nM).[14] For the least potent compounds (triclosan and MEHP), the IC_{50} values were even lower, indicating a possible higher sensitivity of a factor 2.5. For all nine compounds, a linear regression was performed for their IC_{50} values obtained by the two methods. A slope of (IC_{50} radio-ligand assay as X and miniaturized IC_{50} FITC-T_4/TTR assay as Y) 0.40 was calculated with a R^2 value of 0.9845.

3.3. Assessment of the TTR disrupting potency of herring gull egg extracts

Twenty-two herring gull egg extracts, collected from two different locations were

tested with the miniaturized FITC-T_4/TTR assay. The percentage of inhibition was determined by one minus percentage of fluorescence intensity. All individual extracts interfered with T_4-TTR binding, although the binding potency was considerably different between individual eggs (Fig. 3A). This observation is logical considering the long distance migration behavior of European herring gulls. An apparent trend in TTR binding potency was observed (Figure 3A), with lower TTR inhibition of egg extracts from Musvær Island than from Reiaren Island, albeit this difference was not statistically significant due to large intra-group variance.

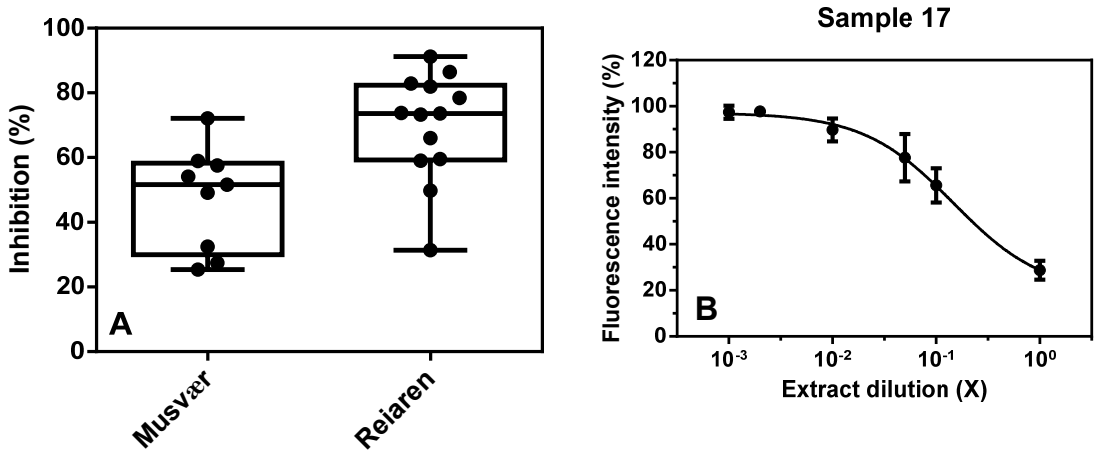

Figure 3: Inhibition of the binding between T_4 and TTR measured in presence of herring gull (*Larus argentatus*) egg extracts from two locations in Norway: The Musvær Island and the Reiaren Island (A) and a dilution study with a selected sample (#17) from the Reiaren Island (B).

Similarly, a lower aryl hydrocarbon receptor agonist potential was found in the eggs sampled from Musvær Island in a previous study[15], suggesting a possible generally lower pollutant load at Musvær Island. In addition, a full concentration response curve (CRC) was obtained for a representative extract (sample 17, Figure 3B). The TH disrupting activity (inhibition) of the samples was interpolated with the T_4 dose response curve in Fig. 2 to create the T_4 equivalent quotients (T_4EQ) for the sample (Table 2). As TH disruption is still a relatively less studied endpoint in environmental research, no T_4EQ value has been reported so far from similar matrices. Nevertheless, Suzuki et al.[23] reported that the TTR-binding potencies in indoor dusts collected from Japan were roughly 250-4100 ng/g (median value 820 ng/g).

Table 2: Weight, lipid content[24] and T_4EQ Values (mol/L in extract and g/g in lipid) of the 22 egg samples from two locations. The detected activities in sample 15, 16, 17, 21 and 23 were above the value range of the curve thus the maximum concentration of T_4 in the curve was used.

No.	Location	Weight (g)	%Lipid	Lipid (g/egg)	T_4EQ (mol/L, extract)	T_4EQ (g/g, lipid)
1	Musvær	34.78	24.70	8.59	1.02E-07	1.46E-08
2		30.76	27.09	8.33	3.63E-07	5.34E-08
4		28.05	24.35	6.83	5.30E-08	9.51E-09
5		29.37	29.08	8.54	6.58E-08	9.44E-09
6		25.13	30.07	7.86	8.18E-07	1.27E-07
7		25.73	35.72	9.19	5.38E-07	7.17E-08
8		24.26	32.04	7.77	4.39E-07	6.93E-08
9		23.59	31.07	7.33	5.42E-06	9.07E-07
10		30.02	26.27	7.89	7.21E-07	1.12E-07
11	Reiaren	28.01	28.79	8.06	3.83E-07	5.82E-08
12		28.58	23.72	6.78	1.79E-06	3.23E-07
13		28.00	22.20	6.22	9.35E-08	1.84E-08
14		30.56	19.07	5.83	8.64E-07	1.82E-07
15		30.97	22.06	6.83	>1.75E-05	>3.14E-06
16		22.74	25.21	5.73	>1.75E-05	>3.74E-06
17		34.96	18.78	6.57	>1.75E-05	>3.27E-06
18		31.14	21.29	6.63	8.72E-06	1.61E-06
19		51.91	11.60	6.02	8.24E-07	1.68E-07
20		29.84	19.79	5.91	7.86E-06	1.63E-06
21		32.94	17.54	5.78	>1.75E-05	>3.71E-06
22		48.01	10.79	5.18	9.42E-06	2.23E-06
23		47.05	13.43	6.32	>1.75E-05	>3.39E-06
Average						1.13E-06

3.4. OH-PCBs analysis of the selected samples

OH-PCBs are widely present TH disruptors in the environment. The five OH-PCBs for target analysis were chosen based on their relatively high concentrations detected in previous studies in seabird eggs.[25–27] With the analytical method used, the limits of detection (LODs) for 4-OH-CB-107, 4-OH-CB-146, 3-OH-CB-153, 4-OH-CB-172 and 4-OH-CB-187 were 0.49 ng, 0.48 ng, 0.55 ng, 0.29 ng, and 0.07 ng respectively. None of the OH-PCBs were detected above the LODs in the six extracts, suggesting the presence of other TH disruptors. Although TH disruption

has received increasing attention the recent years, comparing with other endpoints of endocrine disruption such as the well-studied and extensively documented estrogenic activity, only limited knowledge about potential TTR binders in the environment exist.[20] Nevertheless, it is becoming increasingly clear that the transport protein TTR is susceptible to interference from a large number of compounds including pollutant metabolites. Of these compounds, OH-PBDEs, BFRs or PFASs may be sufficiently persistent and bio-accumulative to enrich in bird eggs and warrant targeted analysis in future initiatives.[3,5,8,28]

3.5. Considerations

The thyroid system is a rather complex system. Disruption of TH transport is only one way a chemical can interfere with the thyroid hormonal system. Other pathways include interference with TH synthesis, the cellular uptake mechanisms, the TH receptor, the iodothyronine deiodinases, and the metabolism of THs in the liver [2]. Due to their structural similarities with THs, it is not surprising that (poly)hydroxylated metabolites of halogenated aromatic hydrocarbons would mainly interfere with TH transport. Measuring the disturbance of binding between T_4 and TTR is therefore relevant to understand the impact of metabolites coming of halogenated aromatic hydrocarbons. Compounds disrupting TH transport have been found in mature organisms, but their impact is greater in non-mature organisms.[3,29]

The intrinsic drawback of using *in vitro* bioassays to evaluate environmental samples is that no information on the identity of the compounds causing the observed effect is obtained. For an endocrine disruption endpoint like TH disruption, which has not yet been comprehensively studied, the target analysis of known disruptors alone is apparently not sufficient. The recent developments in high throughput effect-directed analysis (EDA) are expected to address this problem by applying finer fractionation powered by novel liquid chromatography techniques such as ultra-performance liquid chromatography (UPLC) and comprehensive two dimensional liquid chromatography (LC × LC)[30,31], for which a fast and cost efficient bioassay is required. The miniaturized FITC-T4/TTR assay is currently the only applicable assay for high throughput EDA with a thyroidogenic end point.

4. Conclusions

Although there are several assays available to measure TH disruption, none of the assays is easy to use, cheap and very suitable for the application to environmental samples. The miniaturization of the FITC-T_4/TTR assay into 96-well format developed in the present study offers the possibility of rapid screening of TH disrupting potencies in hundreds of environmental samples within a few hours. Compared to the classical radio-ligand binding assay, the FITC-T_4/TTR assay is especially suitable for high throughput EDA, due to the much lower costs of the ligand used, the shorter incubation time, and easier handling procedures. The sensitivity of the assay is slightly different from that of the radio-ligand binding assay, dependent of the compound. For the most potent TTR disrupting compounds (IC_{50}<100 nM in radio-ligand binding assay), the sensitivity was roughly one order of magnitude lower. It suggests that the assay is more suitable for screening samples that can be easily obtained in relatively large quantities, such as water and sediment samples. For the less potent compounds (IC_{50}≥100 nM in the radio-ligand binding assay), the sensitivities achieved by the two methods were similar.

The assay was successfully applied for the analysis of 22 extracts of herring gull eggs from two locations in Norway. All extracts showed activity in the assay. On average, the egg extracts collected from the southeastern coast of the Norway showed much higher inhibition than those collected from an island in the Arctic. Target analysis of five OH-PCBs was performed for a selection of six samples using GC-ECD but none of the OH-PCBs were detected above the LODs, suggesting the possible presence of other TH disrupting compounds.

Acknowledgements

Financial support from the EU Marie Curie EDA-EMERGE project (EU contract 290100), FORMAS MiSSE project (Swedish Research Council, 210-2012-131) and the Research Council of Norway are gratefully acknowledged. The authors thank Martine Muusse for preparing the egg extracts.

References

1. Crisp, T. M. *et al.* Environmental endocrine disruption: an effects assessment and analysis. *Environ. Health Perspect.* **106 Suppl,** 11–56 (1998).
2. Boas, M., Feldt-Rasmussen, U., Skakkebæk, N. E. & Main, K. M. Environmental chemicals and thyroid function. *Eur. J. Endocrinol.* **154,** 599–611 (2006).
3. Gutleb, A. C. *et al.* In vitro assay shows that PCB metabolites completely saturate thyroid hormone transport capacity in blood of wild polar bears (Ursus maritimus). *Environ. Sci. Technol.* **44,** 3149–3154 (2010).
4. Weiss, J. M. *et al.* Competitive binding of poly- and perfluorinated compounds to the thyroid hormone transport protein transthyretin. *Toxicol. Sci.* **109,** 206–16 (2009).
5. Cao, J. *et al.* Structure-based investigation on the binding interaction of hydroxylated polybrominated diphenyl ethers with thyroxine transport proteins. *Toxicology* **277,** 20–28 (2010).
6. Hamers, T. *et al.* In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. *Toxicol. Sci.* **92,** 157–173 (2006).
7. Miller, M. D., Crofton, K. M., Rice, D. C. & Zoeller, R. T. Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. *Environ. Health Perspect.* **117,** 1033–41 (2009).
8. Lans, M. C. *et al.* Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-p-dioxins and -dibenzofurans with human transthyretin. *Chem. Biol. Interact.* **88,** 7–21 (1993).
9. Meerts, I. A. *et al.* Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol. Sci.* **56,** 95–104 (2000).
10. Simon, E. *et al.* Effect-Directed Analysis To Explore the Polar Bear Exposome: Identification of Thyroid Hormone Disrupting Compounds in Plasma. *Environ. Sci. Technol.* **47,** 8902–8912 (2013).
11. Montaño, M. *et al.* New approaches to assess the transthyretin binding capacity of bioactivated thyroid hormone disruptors. *Toxicol. Sci.* **130,** 94–105 (2012).

12. Huang, R. *et al.* Chemical genomics profiling of environmental chemical modulation of human nuclear receptors. *Environ. Health Perspect.* **119,** 1142–1148 (2011).
13. Smith, D. S. Enhancement fluoroimmunoassay of thyroxine. *FEBS Lett.* **77,** 25–27 (1977).
14. Ren, X. M. & Guo, L.-H. Assessment of the binding of hydroxylated polybrominated diphenyl ethers to thyroid hormone transport proteins using a site-specific fluorescence probe. *Environ. Sci. Technol.* **46,** 4633–40 (2012).
15. Muusse, M. *et al.* Characterization of AhR agonists reveals antagonistic activity in European herring gull (Larus argentatus) eggs. *Sci. Total Environ.* **514,** 211–218 (2015).
16. Brack, W. Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? *Anal. Bioanal. Chem.* **377,** 397–407 (2003).
17. Hovander, L., Athanasiadou, M., Asplund, L., Jensen, S. & Wehler, E. K. Extraction and cleanup methods for analysis of phenolic and neutral organohalogens in plasma. *J. Anal. Toxicol.* **24,** 696–703 (2000).
18. Meijer, L. *et al.* Serum concentrations of neutral and phenolic organohalogens in pregnant women and some of their infants in the Netherlands. *Environ. Sci. Technol.* **42,** 3428–3433 (2008).
19. Crofton, K. M., Paul, K. B., DeVito, M. J. & Hedge, J. M. Short-term in vivo exposure to the water contaminant triclosan: Evidence for disruption of thyroxine. *Environ. Toxicol. Pharmacol.* **24,** 194–197 (2007).
20. Weiss, J. M. *et al.* Tracing thyroid hormone-disrupting compounds: database compilation and structure-activity evaluation for an effect-directed analysis of sediment. *Anal. Bioanal. Chem.* **407,** 5625–5634 (2015).
21. Meeker, J. D., Calafat, A. M. & Hauser, R. Di(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men. *Environ. Health Perspect.* **115,** 1029–34 (2007).
22. Meerts, I. a T. M. *et al.* Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat. *Toxicol. Sci.* **68,** 361–71 (2002).
23. Suzuki, G. *et al.* Dioxin-like and transthyretin-binding compounds in indoor

dusts collected from Japan: average daily dose and possible implications for children. *Environ. Sci. Technol.* **41,** 1487–93 (2007).

24. Muusse, M., Christensen, G., Langford, K., Tollefsen, K.-E. & Thomas, K. V. Aryl Hydrocarbon Receptor Agonists in European Herring Gull (Larus argentatus) Eggs From Norway. *J. Toxicol. Environ. Heal. Part A* **77,** 550–556 (2014).
25. Berger, U., Herzke, D. & Sandanger, T. M. Two trace analytical methods for determination of hydroxylated PCBs and other halogenated phenolic compounds in eggs from Norwegian birds of prey. *Anal. Chem.* **76,** 441–52 (2004).
26. Verreault, J. *et al.* New organochlorine contaminants and metabolites in plasma and eggs of glaucous gulls (Larus hyperboreus) from the Norwegian Arctic. *Environ. Toxicol. Chem.* **24,** 2486–2499 (2005).
27. Fängström, B., Athanasiadou, M., Athanassiadis, I., Weihe, P. & Bergman, A. Hydroxylated PCB metabolites in nonhatched fulmar eggs from the Faroe Islands. *Ambio* **34,** 184–7 (2005).
28. Chauhan, K. R., Kodavanti, P. R. & McKinney, J. D. Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. *Toxicol. Appl. Pharmacol.* **162,** 10–21 (2000).
29. Ucán-Marín, F. *et al.* Recombinant transthyretin purification and competitive binding with organohalogen compounds in two gull species (Larus argentatus and Larus hyperboreus). *Toxicol. Sci.* **107,** 440–50 (2009).
30. Ouyang, X. *et al.* Rapid screening of acetylcholinesterase inhibitors by effect-directed analysis using LC × LC fractionation, a high throughput in vitro assay, and parallel identification by time of flight mass spectrometry. *Anal. Chem.* **88,** 2353–60 (2016).
31. Booij, P. *et al.* Identification of photosynthesis inhibitors of pelagic marine algae using 96-well plate microfractionation for enhanced throughput in effect-directed analysis. *Environ. Sci. Technol.* **48,** 8003–11 (2014).