



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



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HIGHLIGHTS

- A high throughput T₄-TTR binding assay was developed in a 96-well microplate format.
- The dose response relationship of eight chemicals was determined in the assay.
- The thyroid hormone disrupting potency of 22 herring gull eggs was determined.

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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. For most of the 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 TH disrupting potency of 22 herring gull (*Larus argentatus*) egg extracts, collected from two different locations (Musvær and Reieren) in Norway, was evaluated to demonstrate the applicability of the assay for environmental samples.

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1. Introduction

Over the past decade, research in the field of endocrine disruption has greatly increased with regard to the occurrence of endocrine disrupting compounds in the environment and their effects in the ecosystem and humans (Crisp et al., 1998). Over the

years, various end points such as estrogenic, androgenic, progestogenic, glucocorticoid and thyroidogenic activities (Crisp et al., 1998) 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 (Boas et al., 2006). 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, T₄).

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Disruption of the binding of T₄ 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 T₄ binding to TTR (Gutleb et al., 2010; Weiss et al., 2009; Cao et al., 2010; Hamers et al., 2006). Interference with the plasma transport of THs may lead to disruption of the targeted transport and metabolism of T₄ 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 (Miller et al., 2009). Advanced bio-analytical methods have therefore been developed to evaluate disruption of T₄ transport. A classical method to assess such an effect uses ¹²⁵I labelled T₄ as the radioactive ligand in a competitive binding assay (Lans et al., 1993). The method was successfully applied in many studies and showed good sensitivity (Weiss et al., 2009; Meerts et al., 2000; Simon et al., 2013). However, due to the costly radioactive ligand, relatively complicated assay steps and safety issues related to the handling of ¹²⁵I 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 (Montaño et al., 2012; Huang et al., 2011). Similar to the ANSA-TTR assay, another bioassay, based on a previous study (Smith, 1977), 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 T₄ (Ren and Guo, 2012). 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-T₄ was applied, however in order to further enhance assay throughput, it was miniaturized in a 96 well microplate format. First, the new down-scaled protocol was optimized to achieve similar performance as the assay performed in cuvettes. Then, the new protocol was tested with eight compounds from seven different groups (OH-PCBs, OH-PBDEs, BFRs, PFASs, bromophenols, phthalates and antibacterial agents) known to interfere with the T₄-TTR binding. The observed activities were compared with those obtained in the classical radio-ligand binding assay. The T₄ displacing 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 (Muusse et al., 2015).

2. Materials and methods

2.1. Chemicals

Fluorescein isothiocyanate (FITC, >90%) and L-thyroxine (T₄, >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-T₄) has been synthesized and purified according to a previous study (Smith, 1977). 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 vol 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 vol of MilliQ water and centrifuged again at the same condition. The precipitate was then re-dissolved in 8 vol of 0.005 M of ammonium bicarbonate. A few drops of ammonia solution (10%, v/v) were added until the precipitate dissolved. 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-T₄) 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-T₄ 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 (Muusse et al., 2015). 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×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 Envirolgel 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 dimethyl sulfoxide (purity 99.8%, Sigma–Aldrich) for the bioassay.

2.4. Miniaturization of the TTR binding assay

The previous study (Ren and Guo, 2012) uses the TTR binding assay in cuvettes to measure the fluorescent labelled T₄ (FITC-T₄) 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 (Brack, 2003). 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-T₄, 8 μL of 3.6 μM TTR together with 80 μL of Tris-NaCl buffer was incubated for 5 min at room temperature. In each experiment, two sets of blanks (n = 3) were simultaneously tested in the same plate to estimate the percentage of replacement (T₄ displacing potency of the inhibitor) and to correct the original fluorescence caused by free FITC-T₄. The FITC-T₄-only blank (Blank 1) was prepared by 14.5 μL of 1 μM FITC-T₄ and 98 μL of Tris-NaCl buffer. The FITC-T₄-TTR blank (Blank 2) was prepared by 1 μL of 1 μM FITC-T₄, 8 μL of 3.6 μM TTR together with 90 μL Tris-NaCl buffer. 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).

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 T₄ and TTR, a saturation study was performed as described by Ren and Guo (2012). The protocol was conducted in the same way as the inhibition assay except that five different concentrations of substrate (FITC-T₄, from 0.025 μM to 1.0 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 T₄ displacing 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 IC₅₀ values were determined by the GraphPad Prism 6 software with 95% confidence. The IC₅₀ value represents the concentration of an inhibitor that is required for 50% inhibition. The concentration range chosen for most of the inhibitors in the dose response experiments was 1 pM–100 μM. The GraphPad Prism software was used to plot the curves and find appropriate fittings. The T₄EQ

values (g/g, lipid) of the tested herring gull egg samples were calculated based on their measured T₄ displacing potencies and their lipid weights measured in a previous study (Muusse et al., 2014).

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 (sample 15, 16, 19, 22, 23, 24) 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. (2000). 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) (Meijer et al., 2008).

3. Results and discussion

3.1. Saturation study

The curve obtained in the saturation study (Fig. 1) achieved a similar shape as reported by Ren and Guo (2012) that allowed the calculation of the FITC-T₄ and TTR dissociation constant (K_d). The K_d obtained in this study was equal to 261 μM (R² = 0.99).

3.2. TTR disrupting compounds tested using the miniaturized FITC-T₄/TTR binding assay

In total the T₄ displacing potency of eight compounds from seven selected groups of chemicals together with non-labelled T₄ was quantitatively determined by a concentration response experiments (n = 3) using the miniaturized FITC-T₄/TTR binding assay. A concentration-dependent decrease in T₄ binding to the TTR was observed for all the tested compounds (Fig. 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

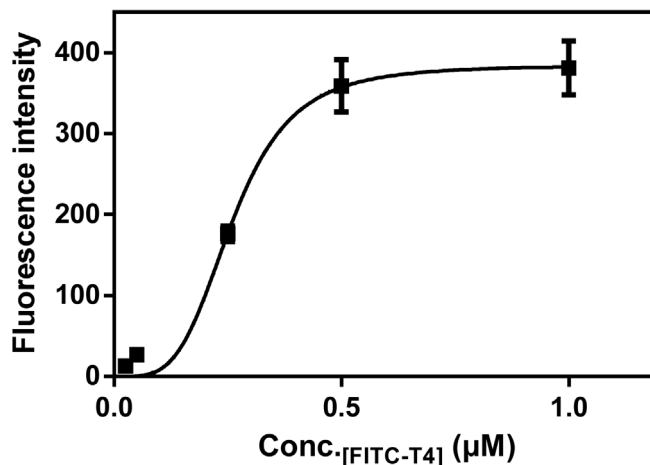


Fig. 1. Fluorescence measurements (n = 3) at different substrate (FITC-T₄) concentrations for the saturation study.

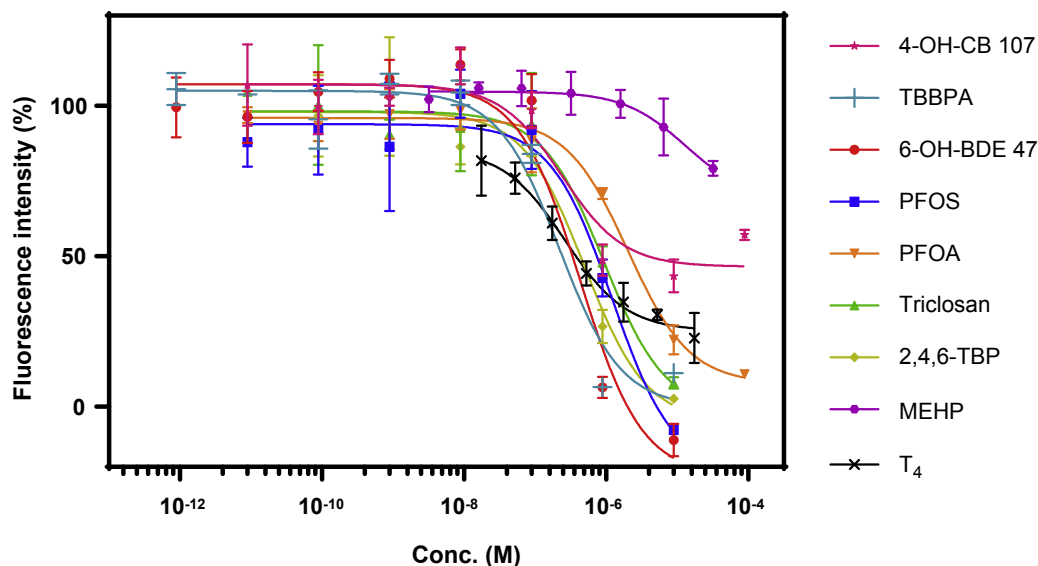


Fig. 2. Dose response curves of eight (potential) TH disruptors together with T_4 , tested by the miniaturized FITC- T_4 /TTR binding assay.

radio-ligand binding assay (Gutleb et al., 2010; Weiss et al., 2009; Cao et al., 2010; Hamers et al., 2006; Meerts et al., 2000).

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 μ M), 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 T_4 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 (Crofton et al., 2007); its T_4 displacing potency was determined by the radio-ligand binding assay in a recent study (Weiss et al., 2015). For MEHP, the concentration in human urine was found to be negatively correlated to the free T_4 and T_3 levels in serum (Meeker et al., 2007). 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.

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} 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 the FITC- T_4 /TTR assay performed in cuvette (260 ± 13 nM

and 323 ± 10 nM) (Ren and Guo, 2012), implying a successful miniaturization. Comparing with the values achieved by the radio-ligand binding assay, 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. 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. For the least potent compounds (triclosan and MEHP), the IC_{50} values were lower, indicating a possible higher sensitivity for these two compounds 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. The experiments were performed in triplicate. All individual extracts interfered with T_4 -TTR binding, although the T_4 displacing 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 T_4 displacing potency was

Table 1

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

Compounds	IC_{50} miniFlu-TTR (μ M)	IC_{50} RL-TTR (μ M)	RL-TTR Reference
4-OH-CB-107	0.24	0.024	Meerts et al. (2002)
TBBPA	0.22	0.031	Hamers et al. (2006)
6-OH-BDE-47	0.43	0.18	Hamers et al. (2006)
PFOS	1.21	0.94	Weiss et al. (2009)
PFOA	2.02	0.95	Weiss et al. (2009)
Triclosan	0.93	2.84	Weiss et al. (2015)
2,4,6-TBP	0.50	0.068	Meerts et al. (2000)
MEHP	13.08	31.55	DENAMIC ^a
T_4	0.26	0.08	Meerts et al. (2000)

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

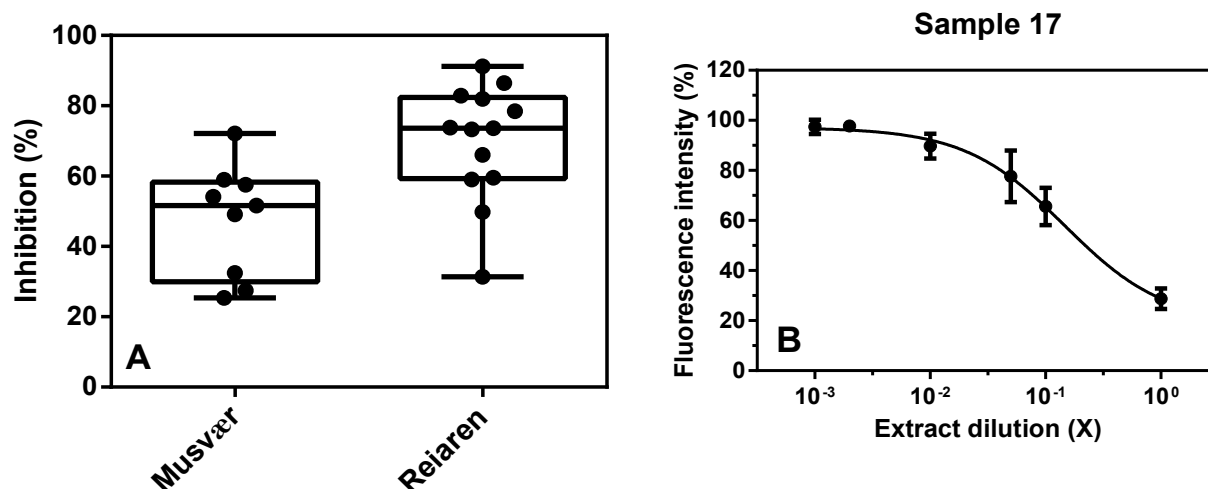


Fig. 3. Inhibition of the binding between T_4 and TTR measured in presence of 22 herring gull (*Larus argentatus*) egg extracts from two locations in Norway: The Musvær Island and the Reiareen Island (A) and a dilution study with a selected sample (#17) from the Reiareen Island (B).

observed (Fig. 3A), with lower TTR inhibition of egg extracts from Musvær Island than from Reiareen Island, albeit this difference was not statistically significant due to large intra-group variance.

Similarly, a lower aryl hydrocarbon receptor agonist potential was found in the eggs sampled from Musvær Island in a previous study (Muusse et al., 2015), 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, Fig. 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. (2007), reported that the TTR-binding potencies in indoor dusts collected from Japan were roughly 250–4100 ng/g (median value 820 ng/g).

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 (Berger et al., 2004; Verreault et al., 2005; Fångström et al., 2005). 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, per injection in GC. 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

Table 2
Weight, lipid content (Muusse et al., 2014) 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	Reiareen	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	1.68E-05	3.14E-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						1.12E-06

Table 3A comparison of the cost and time of the developed miniaturized fluorescence T₄-TTR (miniFlu-TTR) binding assay and the classical ¹²⁵I radio-ligand (RL-TTR) binding assay.

Assay	Cost of the ligands	Incubation	Format
miniFlu-TTR	€40 per 100 mg (FITC)	5 min	96 well microplate
RL-TTR	€780 per 100 μCi (0.3 mCi/mL, [¹²⁵ I]T ₄)	>8 h	vial

(Weiss et al., 2015). 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 (Gutleb et al., 2010; Cao et al., 2010; Lans et al., 1993; Chauhan et al., 2000).

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 (Boas et al., 2006). 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 T₄ 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 (Gutleb et al., 2010; Uacán-Marín et al., 2009).

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) (Ouyang et al., 2016; Booij et al., 2014), for which a fast and cost efficient bioassay is required. Comparing with the classical ¹²⁵I radio-ligand binding assay, the developed miniaturized fluorescence T₄-TTR binding assay is much more suitable for high throughput EDA with a thyroidogenic end point (Table 3), for its lower cost of ligands, shorter incubation time and 96 well microplate format that is compatible with high resolution fractionation.

4. Conclusion

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-T₄/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-T₄/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 sensitivities of T₄ and the eight tested compounds in the assay are slightly different from that of the radio-ligand binding assay. For the most potent TTR disrupting compounds (IC₅₀ < 100 nM in radio-ligand binding

assay), the sensitivity was roughly one order of magnitude lower. Possibly, this may be attributed to the relatively lower binding affinity of the probe caused by the steric hindrance of the FITC moiety. 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 (IC₅₀ ≥ 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.

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