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In situ measurement of estrogenic activity in various aquatic systems using organic diffusive gradients in thin-film coupled with ERE-CALUX bioassay

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ABSTRACT

Organic-diffusive gradients in thin-film samplers (o-DGT), were developed and applied for accumulation of estrogen and estrogen-like compounds on a XAD18 resin and deployed *in situ* in the effluents of Beijing Gaobeidian Wastewater Treatment Plant (GWWTP) and Brussels North Wastewater Treatment Plant as well as in several aquatic systems in Belgium, including the Zenne River, the Belgian Oostende Harbor and the North Sea. Estrogenic compounds accumulate on the XAD18 resin and the estrogenic activity of the resin extract was measured with the Estrogen Responsive Elements-Chemically Activated LUciferase gene eXpression (ERE-CALUX) bioassay. With this result and by applying Fick's diffusion law, it is possible to calculate the estrogenic activity in the aquatic system, if the diffusion boundary layer (DBL) is known or negligible compared to the hydrogel diffusive layer thickness. The DBL thickness in our study varied from 0.010 to 0.023 cm and ignoring the DBL thickness would for instance, underestimate the estrogenic activity by 10–20%. Estrogenic activities in the secondary effluent of GWWTP were the highest (29 ± 4 ng E2-equivalents L^{-1}), while the lowest level was found at the Belgian Oostende Harbor (0.05 ± 0.01 ng E2-equivalents L^{-1}). Comparable estrogenic activities in water samples measured by o-DGT and grab sampling were obtained, confirming that o-DGT can be efficiently used in various aquatic systems. The advantage of our sampling and measuring method is that very low, time averaged estrogenic activities can be determined, with a minimum of sample treatment. The risk of sample contamination is very low as well as the cost of the whole analytical procedure.

1. Introduction

Endocrine disrupting chemicals (EDCs) represent a broad class of compounds that act on the hormonal system and consequently cause adverse health effects to humans and/or to other organisms (UNEP/WHO, 2013). They are widespread in the environment and they can pose severe health effects such as cancer, reproductive problems, body deformation (Roig et al., 2012). Most EDCs have been identified as having estrogenic activities (Lintelmann et al., 2003) and several studies have confirmed that low dose exposure (ng or ng L^{-1}) to these estrogenic EDCs can cause adverse effects on aquatic species for example reproductive impairment in seals, eggshell thinning in predatory birds, feminisation of fish and masculinisation in marine snails (Vethaakn and Legler, 2012; Ying et al., 2002; Duncan et al., 2015). Under the Water Framework Directive, the Environmental Quality Standards Directive was amended in 2013/39/EU and a watch list was established to require monitoring of other substances for which evidence suggest a possible risk to the environment. The first watch list, adopted in 2015 (Commission Decision 2015/495/EU), identified several substances with a clear estrogenic activity, including 2 natural hormones, 17β-estradiol (E2) and estrone (E1). Two pharmaceuticals, the anti-inflammatory diclofenac and the synthetic hormone 17α-ethinyl estradiol (EE2), were also put on the list. The Annual Average Environmental Quality Standards (AA-EQS) value proposed at the European level for E2 is set at 0.4 ng L^{-1} (Commission Decision 2015/495/EU). A trigger value of 3.8 ng E2-equivalents L^{-1} as hormonal activity in drinking water was suggested to monitor the water safety in the Netherlands (Brand et al., 2013). Hence, the determination of estrogenic activities in the aquatic environment becomes essential for the protection of aquatic ecosystems and ultimately for the protection of humans.

Two options exist to assess the risk from those substances for

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ecosystem and human health: (1) measuring the concentrations of estrogenic compounds that are listed by international regulations (US-EPA, EU Commission, WHO) by Liquid Chromatography (LC) or (Gas Chromatography – Mass Spectrometry/Mass Spectrometry (GC–MS/MS); (2) measuring directly the estrogenic activity using appropriate bioassays such as the Chemically Activated LUciferase gene eXpression (CALUX) estrogen cell line (Avbersek et al., 2011). Both approaches are complementary, each having its advantages and drawbacks. For example, identification and quantification of individual compounds can be obtained with LC or GC–MS/MS and sometimes even their source(s) can be traced back which is not possible with the bioassay. On the other hand, with the bioassay an integrated biological response for all compounds showing estrogenic activity in the sample extract can be obtained, taking even synergetic and antagonistic effects into account (Rajapakse et al., 2002; Leusch et al., 2010). Nevertheless, the greatest challenge to study estrogens in aquatic systems is that they are generally present at very low concentrations. This can be partially overcome by sampling large water volumes and pre-concentrating, but these handlings can lead to some artificial problems such as analyte contamination, post-sampling losses or changes, more time consumption, and increased costs in sample processing (Alvarez et al., 2005; Barreiros et al., 2016). In addition, the classic active sampling, in this study referred as spot/grab sampling, can only generate information on concentration levels of these estrogenic compounds on a small temporal and spatial scale. A solution for measuring low estrogenic levels in natural water systems and for obtaining a better temporal and spatial representation of the estrogenic activity in the aquatic system is the use of passive samplers *in situ* in the field. However, *in situ* measurement of the time-weighted average (TWA) estrogenic activity in natural aquatic systems remains challenging today.

In a recent paper, an analytical method combing a Diffusive Gradient in Thin-Films (DGT) passive sampler with the bioanalytical method of Estrogen Responsive Elements-Chemically Activated LUciferase gene eXpression (ERE-CALUX) bioassay to determine estrogenic activity in aquatic system was developed in our previous study (Guo et al., 2017). The DGT technique allows *in situ* pre-concentration of the estrogenic compounds during a period ranging from a few hours to several weeks depending on the estrogenic activity level in the aquatic system and the time and spatial scale one wants to study. The ERE-CALUX bioassay on the other hand allows the determination of estrogenic activities at low concentration levels, such as pg E2-equivalents L^{-1}. The combined method can effectively measure estrogenic activities at a detection limit of 0.026 ± 0.003 ng E2-equivalents L^{-1} for a 24 h *in situ* sampling period (Guo et al., 2017). From the amount of estrogenic compounds adsorbed on the XAD18-DGT resin, the estrogenic activity can be calculated using Fick's law. However, this equation can only be solved if the total diffusive domain of the DGT is known and in natural aquatic systems one must be aware that the thickness of the diffusion boundary layer (DBL), which is a part of the total diffusive domain of the DGT, is dependent on the turbulence or the flow conditions in that aquatic system (Garmo et al., 2006; Warnken et al., 2006).

Measurements of estrogenic activities in natural waters are rather scarce, hence our general objective was to determine these activities in a variety of aquatic environments: effluents from wastewater treatment plants (WWTPs), river, harbor and marine waters. Specific objectives are the following: 1) to investigate various exposure times of the o-DGT and to assess *in situ* the DBL thickness and its effect on estrogenic activity calculations; 2) to compare estrogenic activities obtained with o-DGT and grab sampling in all sampling locations; and, 3) to compare estrogenic activities obtained with our o-DGT in various water systems in Belgium and China with literature data. Moreover, suggestions for future application of o-DGT combined with CALUX bioassay are discussed.

2. Experimental section

2.1. Chemicals and reagents

17β-Estradiol (E2), methyl *tert*-butyl ether (MTBE), methanol (MeOH) and hydrochloric acid (HCl) were purchased from Sigma–Aldrich (Germany). Dimethyl sulphoxide (DMSO) was purchased from Merck (Germany). Hexane and acetone were purchased from Biosolve (The Netherlands; dioxin-grade). Amberlite™ XAD18™ was obtained from Rohm and Haas Company (USA) and agarose was obtained from Bio-Rad Laboratory (Spain). Dulbecco's Modified Eagle Medium (DMEM without phenol red (Gibco)), sodium pyruvate (100 mM, sterile-filtered), alpha-Minimal Essential Medium (α-MEM (Gibco)), penicillin–streptomycin, fetal bovine serum (FBS), L-glutamine (200 mM), trypsine (0.5% (Gibco)), phosphate buffered saline (PBS, 1 ×, pH 7.4), and trypsin without phenol red (10 ×, 0.5% (Gibco)) were purchased from Life Technologies (United Kingdom). Charcoal-stripped FBS was obtained from Biowest (France; through VWR). Luciferin reagent and lysis reagent were obtained from Promega (The Netherlands). Stabilizing buffer A, B and NucleoCounter cartridges were purchased from Chemometec (Denmark).

2.2. DGT theory and o-DGT preparation

The DGT technique (see Fig. 1) is based on Fick's first law of diffusion (Zhang and Davison, 1995). Analyte diffuses through the diffusion layer and is rapidly bound by the resin in the binding gel. For well mixed solutions, the concentration in bulk solution is constant outside the DGT unit and a constant concentration gradient is maintained in the diffusion layer during the deployment time. Thus, the measured concentration in solution by DGT can be calculated by Eq. (1):

$$C_{DGT} = \frac{M_{DGT}(\Delta g + \delta)}{DAt} \quad (1)$$

where C_{DGT} is the concentration of a target compound (organic or

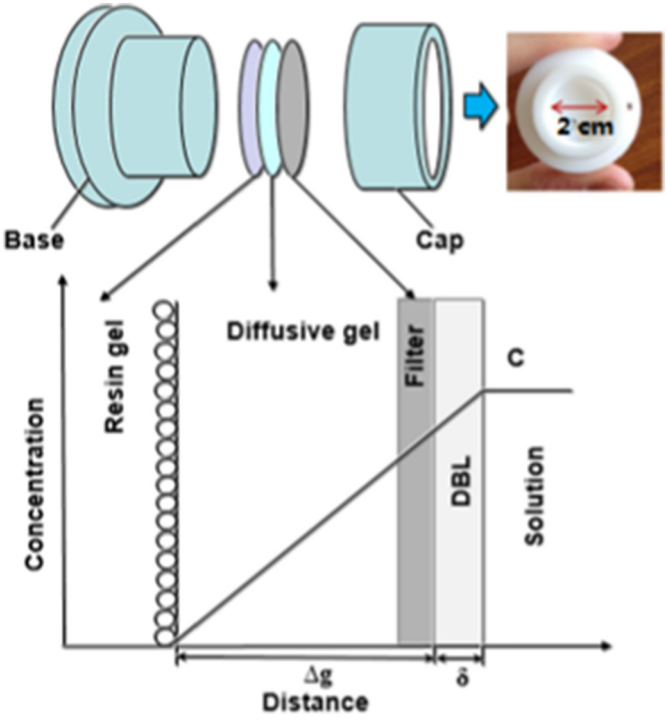

Fig. 1. The schematic diagram and principle of diffusive gradients in thin-film (DGT). DBL is the diffusive boundary layer and C is the concentration in solution.

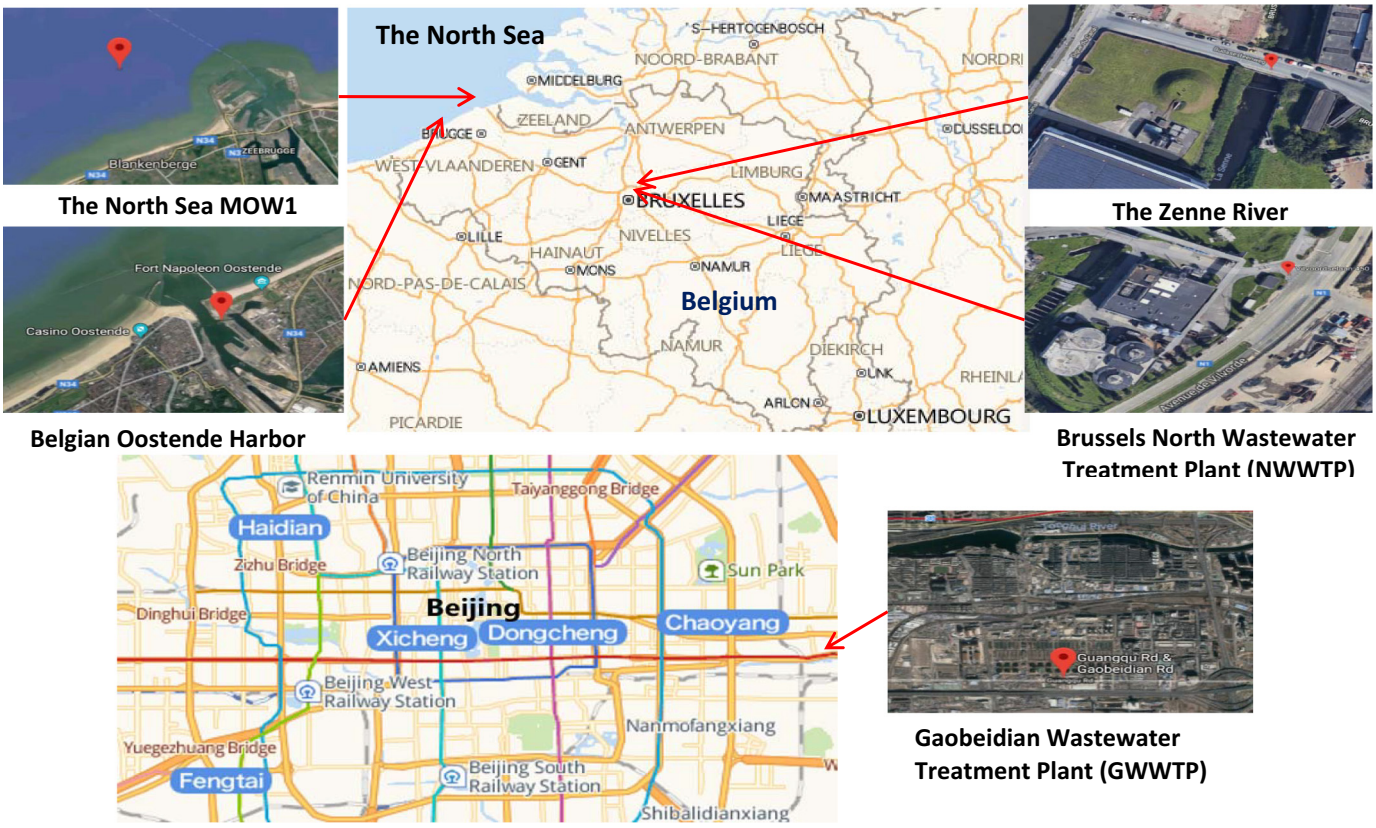

Fig. 2. Sampling locations in Belgium and in Beijing.

metallic in nature) in the water, M_{DGT} is the mass of a target compound accumulated on the binding gel, Δg is the thickness of the diffusion layer which includes the thickness of both diffusive gel and filter membrane, δ is the DBL thickness, D is the diffusion coefficient of a target compound in the diffusive gel, A is the exposure area and t is the exposure time.

Standard o-DGT samplers were made in Teflon to avoid the binding of organic pollutants to the classic ABS samplers (Guo et al., 2017) and they have an exposure area of 3.14 cm^2. The configuration contained the following items: a 0.050 cm thick XAD18 resin gel, a 0.075 cm agarose diffusive gel and a 0.017 cm filter membrane (HVLP Durapore from Millipore consisting of Hydrophilic PVDF: Polyvinylidene Fluoride, 0.45 μm). In brief, a 1.5% (w/v) agarose diffusive gel solution was prepared by dissolving 0.3 g agarose in 20 mL of Milli-Q water and 0.5 g (wet weight) of methanol conditioned XAD18 was mixed with 1.5% (w/v) warm agarose solution to form the resin gel. The XAD18 resins were purified by ultrasonic extraction with n-hexane and acetone, followed by vortex conditioning using methanol (5 min) prior to use. The detailed preparation and performance testing of o-DGT can be found in the previous study (Guo et al., 2017).

2.3. Diffusion boundary layer (DBL) measurement

The DBL at the water-membrane interface can affect the uptake of the target analyte, resulting in a bias during the field deployment (Challis et al., 2016). In the field, the DBL thickness can be determined by simultaneous deployment of multiple o-DGT samplers with different thicknesses of the diffusive gel layer. The key factor influencing DBL thickness is the turbulence intensity, which itself is dependent of the water flow characteristics in the aquatic system (Garmo et al., 2006; Warnken et al., 2006; Challis et al., 2016). In order to compare DBL results and potential differences based on experimental conditions, tests were carried out in the lab as well as in the field. Thus, triplicate o-DGT samplers with varying diffusive gel thickness (0.025, 0.050, 0.075 and 0.100 cm) were first deployed in a 9 L test volume containing a 16 ng L^{-1} E2 solution at a stirring rate of 300 rpm during a 4 h period. This test indicates of the DBL at laboratory condition, the δ-value in Eq. (1), can be neglected or not. In addition, the turbulence intensity in the field could differ from the one produced by the stirring system in the laboratory. Therefore, triplicate o-DGT samplers with 4 different thicknesses of diffusive gels were deployed for 6 h in the open effluent channel of the GWWTP (the wastewater flowrate is 1,000,000 m^3 d^{-1}) and in the open effluent channel of the NWWTP (the wastewater flowrate is 275,000 m^3 d^{-1}). Additionally, o-DGT samplers were deployed in the Belgian Oostende Harbor for 14 days where no biofouling effect was observed. The following equation presents the mass (M) of a given compound collected by the XAD18 resin gel as a function of the thickness of the diffusive layer.

$$\frac{1}{M_{DGT}} = \frac{\Delta g}{C_{DGT}AtD} + \frac{\delta}{C_{DGT}AtD} \quad (2)$$

Using this equation, the DBL thickness (δ) can be obtained from the slope and intercept of the plot of 1/M *versus* Δg (Warnken et al., 2006; Chen et al., 2013; Challis et al., 2016).

2.4. Estrogenic activity based on o-DGT and grab sampling during field deployment

The estrogenic activities were calculated from Eq. (1) using the experimentally determined DBL thickness (see Section 2.3). The pH of all water samples was adjusted to 3 using HCl to suppress microbial activity. Samples were stored in a cool box and transferred immediately to the laboratory, where they were extracted within 24 h.

To compare o-DGT and grab sampling techniques, both techniques were applied to the effluents of two WWTPs (Beijing and Brussels), in the Zenne River, at the Belgian Oostende Harbor and in the North Sea in

Belgium in Autumn (from September to October) (Fig. 2). A decreasing level of estrogenic activity was expected when moving from concentrated sites (WWTP) to the marine environment due to dilution of the freshwater with seawater (Baeyens et al., 1998), which prompted the need to adapt deployment times. In addition, the range of activity levels encountered in highly polluted systems, such as WWTPs, to less polluted systems, such as the marine environment, also provides a performance test for the o-DGT. For GWWTP, triplicate o-DGT samplers were deployed in the secondary and tertiary effluent for 6 h each. At the same location, 1 L water samples were collected by grab sampling at 0 h and 6 h and mixed together (1:1 v/v). Then 500 mL of the mixed water sample was extracted with Oasis HLB cartridges. At the Belgian Oostende Harbor and in the North Sea, triplicate o-DGT samplers were deployed for 2 weeks, and for each site 1 L water samples were collected by grab sampling at 0 h and 14 days to prepare the mixed water sample. For the NWWTP and the Zenne River, triplicate o-DGT samplers were deployed in the effluent of NWWTP and upstream Zenne River (2 km away from NWWTP) for 72 h, and at each site 1 L water samples were collected by grab sampling at 0 h and 72 h to prepare the mixed water sample. In the effluents of the NWWTP, o-DGT sampling was also compared with grab sampling at various time periods during the day to estimate short-term fluctuations. The o-DGT samplers were deployed in triplicate for each deployment time in the effluent of the NWWTP for 6, 24, 48 and 72 h. At the same location, grab samples (triplicates of 1 L each) were also taken at 0, 6, 24, 48 and 72 h.

The water temperature was 15.8–17.2 °C in the effluent of the GWWTP, 18.5–19.5 °C in the effluent of the NWWTP, 17.7–18.7°Cin the Zenne River, 12.6–15.9 °C at the Belgian Oostende Harbor, and 15.8–16.6 °C in the North Sea.

2.5. Sample extraction

From the mixed water sample, pre-filtrated 500 mL was extracted using Oasis HLB cartridges (200 mg, 5 cm^3, glass). The column was first conditioned with 3 mL MTBE, followed by 3 mL MeOH (methanol) and 3 mL Milli-Q water. Samples (500 mL) were loaded onto the cartridge, washed with 3 mL of 40% MeOH in Milli-Q water and then re-equilibrated with 3 mL Milli-Q water followed by 3 mL of 10% MeOH/ 2% NH_4OH in Milli-Q water. Estrogen-like compounds (either pure 17β-estradiol during method optimization, or the mix of unknown estrogenic compounds in a natural sample) were eluted using 40 mL of 10% MeOH in MTBE. Between the final washing step and the subsequent elution step, the system was placed under vacuum and the resin in the cartridge was dried for at least 30 min at room temperature (20 ± 2 °C). All solvents used were passed through the cartridge at a flow rate of approximately 5 mL per minute.

XAD18 resin gel samples were peeled off from the o-DGT samplers and transferred to the 33 mL extraction cell of the Dionex ASE200 extraction unit and subjected to the following treatment: acetone/*n*-hexane 1:1 (v/v) as extraction solvent, pressure of 1000 psi, extraction temperature of 50 °C, 2 extraction cycles with a 5 min static time (extraction period) and 6 min heating time, 60% flushing volume, and a final nitrogen purge of 60 s. Extracts were collected in 60 mL vials.

All samples were vacuum centrifuged (MiVac Quattro, Genevac) and resuspended in known volumes of n-hexane. Samples were light shielded and stored at room temperature until further analysis (typically within seven days).

From a practical point of view, the extraction method applied on the o-DGT is much more efficient than the extraction of grab water samples. The extraction of XAD18 resin gel (solid phase) with an automated extraction unit (ASE) is much less time consuming, less labor intensive and solvent reducing compared to the extraction of grab water samples (liquid phase) where a manual extraction setup including conditioning, washing and elution steps was applied.

2.6. Estrogenic activity analysis

VM7Luc4E2 cells (formerly known as BG1Luc4E2 cells, (NIEHS, 2016)) were used to carry out ERE-CALUX bioassay measurements, with tests performed according to the XDS LUMI-CELL agonist protocol (XDS, 2009) and the OECD TG 455 guidelines (OECD, 2012a, 2012b) but with certain modifications (Vandermarken et al., 2016). The detailed routine cell cultivation, seeding and dosing in 96 well plates, and analysis parameters were as described in the previous study (Guo et al., 2017). Results of the CALUX procedure, expressed as RLUs (Relative Light Units), were normalized to 100% of the RLUs maximally induced by E2 and the E2 standard and sample responses (*i*) were processed using a logistic function (with *i* representing the number of standard solutions or sample dilutions used to model the sigmoidal curves; typically 10 different standard concentrations and sample dilutions were used) (Elskens et al., 2011):

$$y_i = y_0 + \frac{(m\, x_i^h)}{(k^h + x_i^h)} + \varepsilon_i \quad (3)$$

where y_i represents the measured %RLU of standard or sample *i*; y_0 represents the background %RLU; *m* represents the maximum %RLU; x_i represents the amount of E2 standard solution or sample (pg in the former and liter or gram for the latter); *k* represents the amount of E2 standard solution or sample in the well at 50% of the maximum of the dose-response curve (= EC_{50}); *h* is the slope parameter (the slope at the inflection point corresponding to EC_{50}); and ε_i represents the residual term which results from the minimization of the Sum of Squared Residuals. Based on 15 independent full dose-response E2 standard curves, the mean EC_{50} value ± standard deviation from these analyses was 404 ± 54 fg/well and the limit of detection (LOD) was 24 ± 3 fg/well (Table 1). These values are comparable, both in absolute values and variance, to that of our previous study (Guo et al., 2017).

2.7. Statistical analysis

All o-DGT experiments were carried out in triplicate and the results were expressed as the mean value ± standard deviation. Statistical analysis was performed with GraphPad Prism 7.0 software. Analysis of variance (ANOVA) was performed to detect the difference of estrogenic effects obtained from o-DGT and grab sampling. Significant differences were set at the alpha significance level of 0.05.

3. Results and discussion

3.1. Effect of DBL

DGT samplers are different from other passive sampling techniques that transport inside the sampler (filter and hydrogel) is diffusive controlled. However, outside the DGT exists a zone where transport is also limited to molecular diffusion (the DBL) but the thickness of this zone, in contrast to those of filter and hydrogel, is not so well-defined. The DBL is a thin layer of viscous fluid close to a solid surface in contact with a moving stream in which the velocity varies from zero at the surface up to the boundary that corresponds to the free stream velocity.

Table 1
The parameters in logistic function and limit of detection (LOD) for dose-response E2 standard curves (n = 15).

Parameter	Mean value ± SD[a]
y_0	(4.0 ± 0.6) %
m	(95 ± 2) %
h	1.43 ± 0.08
k	(404 ± 54) fg/well
LOD	(24 ± 3) fg/well

[a] SD, standard deviations.

Its thickness is influenced by the turbulence intensity in the aquatic system; the higher the turbulence, the thinner the DBL (Garmo et al., 2006). Fortunately, the DBL thickness can be operationally determined without knowledge of the hydrodynamical characteristics of the aquatic system (see Section 2.3).

When using DGT in well-stirred, mixed solutions the effect of the DBL thickness can generally been ignored with the assumption that it is negligibly thin compared to the total thickness of Δg, *i.e.*, the sum of the thickness of the prefilter and the diffusive gel (Garmo et al., 2006). However, the effect of the DBL thickness on the target analyte measurement may be considerable (> 25%) for small values of Δg or for weakly mixed solutions (Challis et al., 2016; Huang et al., 2016a). In this case, the effect of the DBL thickness on the accuracy and uncertainty of o-DGT measurements should be considered. The DBL thickness is first studied in laboratory conditions. Triplicate o-DGT samplers with varying diffusive gel thickness (0.025, 0.050, 0.075 and 0.100 cm) were deployed in an estradiol solution mixed at high stirring rate (300 rpm). The DBL (δ), calculated with Eq. (2), amounted to 0.019 cm. In the field, mean DBL values of 0.022, 0.023, and 0.010 cm were observed in the effluent of the GWWTP, in the effluent of the NWWTP, and at the Belgian Oostende Harbor, respectively (Fig. 3). For the Zenne river and the Belgian coastal zone (station MOW1), DBL values were used based on values from the NWWTP and from the Oostend Harbor respectively because they are located close to them. The DBL values obtained in this study (0.010–0.023 cm) are by far smaller than those obtained from slow-flowing and static conditions for metals (0.044 cm, at 60 rpm), for sulfamethoxazole (0.076 cm, under quiescent conditions) and for thiamethoxam (0.091 cm, under static conditions) (Warnken et al., 2006; Chen et al., 2012; Challis et al., 2016), but comparable with 0.023 cm found for antibiotics in well-mixed wastewater (Chen et al., 2013). Under well mixed conditions, neither the DGT deployment time (6 h–14 d), nor the number of diffusive gel thicknesses (3–6) seemed to affect the linearity (R^2: 0.946–0.999) between 1/M and Δg (Table 2). As shown in Table 2, ignoring the DBL thickness would underestimate the estrogenic activities by ≈10–20% but there are examples in the literature, studying other compounds, that this may even increase to 40% (Huang et al., 2016b). Thus, field deployments of o-DGTs, that use Fick's law, should employ an experimental set-up with multiple diffusive layer thicknesses (Δg) to determine field DBL-values and to calculate bulk concentrations based on the total diffusive domain thickness.

3.2. Comparison of o-DGT with grab sampling methods

In general, results from the mixed water samples (grab sampling) and results from the o-DGT samples correspond well in all investigated aquatic systems (WWTP effluents, rivers, harbors and coastal seas) (Fig. 4) with no statistically significant difference (ANOVA, $p > 0.05$). The estrogenic activities obtained from o-DGTs ranged from (0.05 ± 0.01) ng E2-equivalents L^{-1} to (29 ± 4) ng E2-equivalents L^{-1} (Fig. 4), which are comparable to the range of (0.08 ± 0.01) ng E2-equivalents L^{-1} to (33 ± 1) ng E2-equivalents L^{-1} obtained from mixed water samples by grab sampling. Similar results or trends were found in literature data. For instance, there was an agreement between

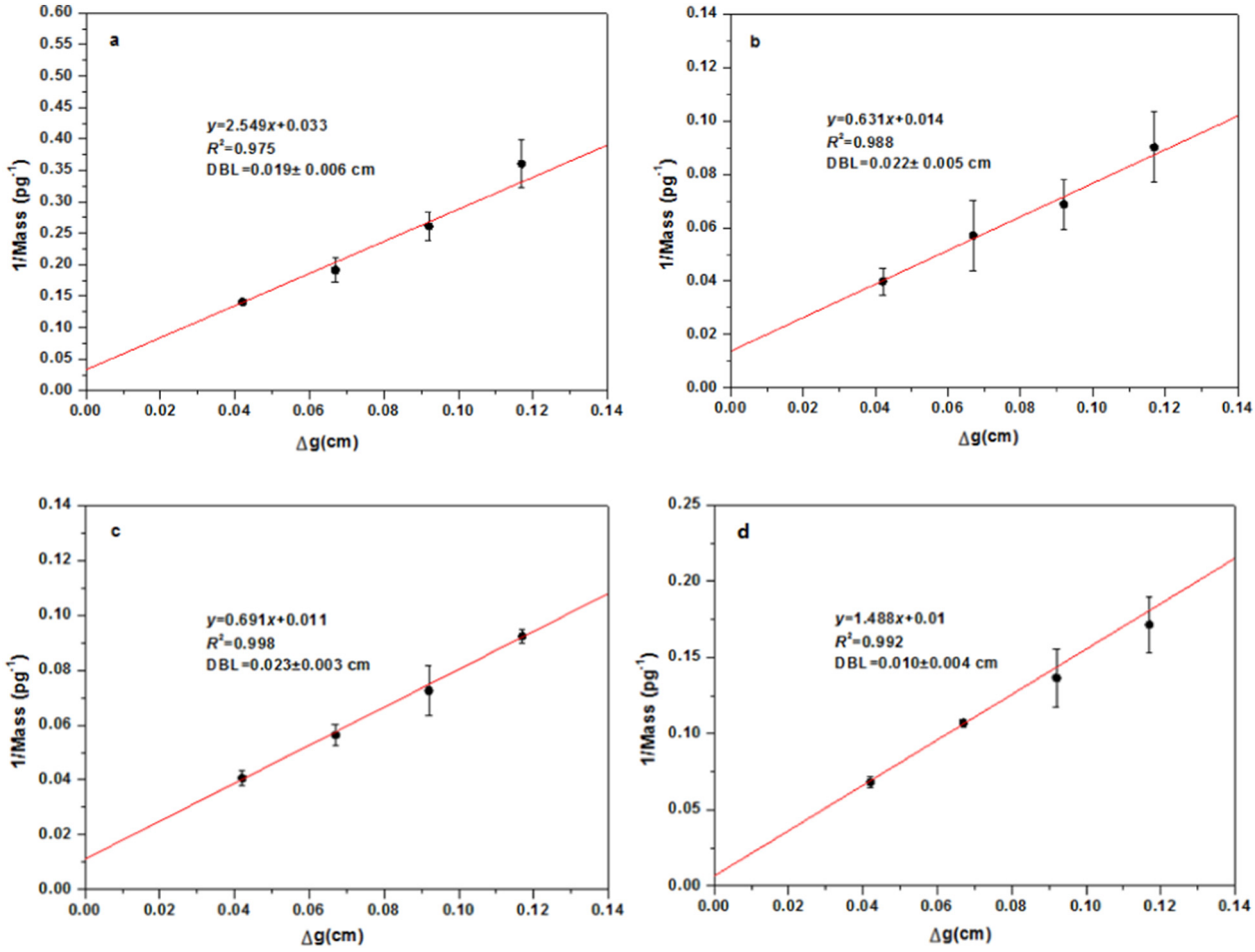

Fig. 3. DGT deployments in the laboratory (a), in the effluent of Beijing Gaobeidian Wastewater Treatment Plant (GWWTP) (b) in the effluent of Brussels Northern Wastewater Treatment Plant (NWWTP) (c) and at the Belgian Oostende Harbor (d). A plot is made of 1/mass of E2 on XAD 18 resin gel (in pg-1) *versus* the diffusive layer thickness (Δg, cm). The DBL is the thickness of the diffusive boundary layer obtained under laboratory conditions and during field sampling.

Table 2
Estimated the thickness of diffusive boundary layer (DBL) in different water in the world with DGT under well stirred conditions.

Chemicals	Applied sites	DBL thickness	Deployment time	R^2	Diffusive gel thickness	Reference
		mm	days		mm	
Zn, Cd and Pb	Wyre River, NW England	0.26 ± 0.017	3	0.996–0.999	0.16, 0.4, 1.2, 2.0	Warnken et al., 2006
Europium	Laboratory, Norway	0.27 ± 0.074	1	0.966–0.995	0.16, 0.4, 0.8, 1.2, 1.6, 2.0	Garmo et al., 2006
Thiamethoxam	Laboratory, Canada	0.22 ± 0.11	8	0.980	0.75, 1.0, 1.5	Challis et al., 2016
Orthophosphate	Nanyun River, China	0.16 ± 0.009	5	0.946	0.5, 1.0, 1.5, 2.0	Feng et al., 2016
Nitrate	Loders Creek, Australia	0.89 ± 0.39	1	0.965	0.5, 0.9, 1.3	Huang et al., 2016b
Estrogenic activity	The effluent of GWWTP, China	0.22 ± 0.05	0.25	0.988	0.25, 0.5, 0.75, 1.0	This study
	Belgian Oostende Harbor, Belgium	0.10 ± 0.04	14	0.992		
	Laboratory, Belgium	0.19 ± 0.06	0.17	0.975		
	The effluent of NWWTP, Belgium	0.23 ± 0.03	0.25	0.998		

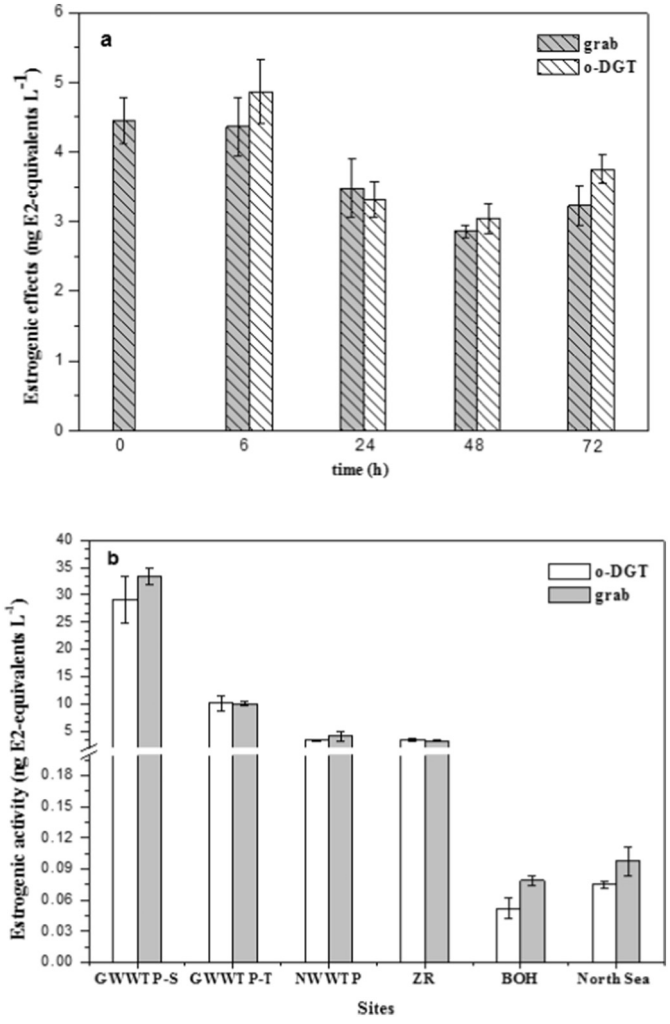

Fig. 4. Estrogenic activity (ng E2-equivalents L-1) measured by o-DGT and grab samplers, in the final effluents of the Brussels Northern Wastewater Treatment Plant (NWWTP) at different grab sampling times and different DGT retrieval times (a), and in the effluent of the GWWTP (secondary effluent: GWWTP-S and tertiary effluent: GWWTP-T) and the NWWTP, ZR (the upstream of Zenne River), BOH (Belgian Oostende Harbor) and in the North Sea (b). Bars represent the mean values ± standard deviation.

the concentrations of antibiotics, anionic pesticides and perfluoroalkyl substances detected by o-DGT and grab sampling in the aquatic environment (Chen et al., 2013; Challis et al., 2016; Guibal et al., 2017; Guan et al., 2018).

However, looking at grab sampling time series results, there are large variations in estrogenic activity, with the highest results at 0 h and 6 h and the lowest at 48 h at the NWWTP (Fig. 4a). This confirms the large variability in estrogenic activity in this type of WWTP effluent that is highly dependent on the quantity and the composition of the inflow. Hence, if grab sampling is applied, high frequency and short interval sampling is recommended. Furthermore, the peak events support the deployment of o-DGT which could be effectively used to monitor the TWA activity of estrogenic compounds instead of grab sampling, as evidenced by the ratio of estrogenic activity from o-DGT samples and mixed water samples (C_{DGT}/C_g ranged from 0.8 to 1.1 as indicated in Table 3). Taking the standard deviations associated with both methods into account, it is clear that longer deployment times allow for a better match between the two sampling methods, which leads to more representative results.

When comparing both methods from a practical point of view, grab sampling provides only “snapshots” of the estrogenic activity in the effluents of WWTPs, thereby possibly missing short-term peak events linked to variations in inflow conditions and operational parameters. Moreover, frequent sampling and sample treatment including transportation and storage of samples does not only increase the analysis cost but also introduces random variability in the results. Previous research (Chen et al., 2013) and the results presented above indicate that o-DGT may also encounter problems. For instance, organic matter, heavy metals, anions *etc.* could all affect the pollutants' behavior during uptake by o-DGT (Pan et al., 2012; Zheng et al., 2014; Xie et al., 2018). Thus, to reduce the impact of these factors, o-DGT should be deployed in several replicates, and the effect from interfering substances, including biofouling effects, should be documented and if possible corrected accordingly. Compared to grab sampling, o-DGT is a good sampling method to obtain TWA activities of estrogenic compounds, especially when dealing with classes of compounds that require intensive pre-concentration.

3.3. Estrogenic activity in different aquatic systems

The highest levels of estrogenic activity were encountered in WWTP effluents, whereas harbor and sea levels were an order of magnitude lower (Fig. 4b). The estrogenic activity in the effluent of the GWWTP

Table 3
(Grab) mixed water sample, and C_{DGT} estrogenic activities at the effluent of the Brussels Northern Wastewater Treatment Plant (NWWTP), Belgium. Data for estrogenic activities are presented as mean values (n = 3) ± 1 standard deviation.

Deployment time	(Grab) mixed water sample or C_g (ng E2-equivalents L^{-1})	C_{DGT} (ng E2-equivalents L^{-1})	C_{DGT}/C_g
6	4.4 ± 0.1	4.9 ± 0.5	1.1
24	4.1 ± 0.1	3.3 ± 0.3	0.8
48	3.0 ± 0.7	3.1 ± 0.2	1.0
72	3.7 ± 0.3	3.8 ± 0.2	1.0

(secondary effluent: 29 ± 4 ng E2-equivalents L^{-1} and tertiary effluent: 10 ± 1.4 ng E2-equivalents L^{-1}), was much higher than that from the effluent of the NWWTP, 3.3 ± 0.3 ng E2-equivalents L^{-1}. Literature states that traditional water treatment technology, such as coagulation, biological aerated filtration, and sand filter, showed poor performance in estrogenic activity reduction, but some advanced wastewater treatment technologies, such as ozonation and membrane filter, showed a better efficiency in estrogenic activity reduction (Chen et al., 2017; Guo et al., 2017). The NWWTP, with a 3 times lower effluent activity than the GWWTP, has used a modern tertiary treatment removing solids, organic matter, nitrogen and phosphorus since 2007 (Brion et al., 2015), which was effective in reducing the estrogenic activity in the effluent. Miège et al. (2009) reported that hormones (such as estrone, 17β-estradiol, 17α-ethinyl estradiol, *etc.*) are the most studied estrogenic-like pharmaceuticals and personal care products in WWTPs. These chemicals are closely followed by other highly expected substances such as the anti-inflammatory compounds (such as Ibuprofen, Diclofenac, Naproxen, *etc.*) and antibiotics (Miège et al., 2009).

Our values can be compared with values from sewage treatment plant effluents in Finland, 0.8–29.7 ng E2-equivalents L^{-1} (Välitalo et al., 2016) and in sewage treatment plant effluents in Slovenia, 2.1–48.2 ng E2-equivalents L^{-1} (Avbersek et al., 2011). However, these results are much higher than that in municipal effluent from the Netherlands, 0.4–1.0 ng E2-equivalents L^{-1} (van der Linden et al., 2008). The estrogenic activity observed in our upstream sampling station of the Zenne River (3.5 ± 0.2 ng E2-equivalents L^{-1}), was three times higher than previously reported values (0.94 ng E2-equivalents L^{-1}) in the downstream area of Zenne River (Vandermarken et al., 2018). This can be attributed to different factors such as: water discharge, occurrence of combined sewer overflow (CSO), interannual variability, sampling location (upstream or downstream Brussels). In the study by Vandermarken et al. (2018), the Zenne River was sampled under low water discharge conditions. The concentration of suspended matter was relatively low (50 to 100 mg L^{-1}) and the estrogenic activities peaked downstream of Brussels. In case of heavy rains, combined sewer systems are designed to overflow and evacuate excess wastewater directly to the Zenne river resulting in a drastic increase of the pollutant loads. In addition, surface river water, downstream of Brussels, is composed by > 50% of the NWWTP effluents (Brion et al., 2015). In our study, sampling was done close to the NWWTP discharge point in a CSO occurrence period (> 150 overflow events a year), which implies less retention, increased erosion, direct impact of urban surface runoff, and hence an increased load of active estrogenic substances.

The values found in this study were also higher than those in the surface waters of the Netherlands (0.2–0.5 ng E2-equivalents L^{-1}) (van der Linden et al., 2008), but significantly lower than those (6.63–84.5 ng E2-equivalents L^{-1}) in rivers receiving concentrated livestock effluent (Liu et al., 2018). Lower estrogenic activities were observed at the Belgian Oostende Harbor (0.05 ± 0.01 ng E2-equivalents L^{-1}) and at the North Sea (MOW1, 0.08 ± 0.003 ng E2-equivalents L^{-1}). This can be attributed to the distance from estrogenic input sources, dilution effects by non- or less-contaminated waters and attenuation by degradation. The estrogenic activities measured in Belgium waters, including effluents in the NWWTP and in surface water from the Zenne River, the Belgian Oostende Harbor and the North Sea were below the trigger value (3.8 ng E2-equivalents L^{-1}) of hormonal activity in drinking water (Brand et al., 2013). However, the environmental quality standard (EQS) for E2 of 0.4 ng L^{-1} (Commission Decision 2015/495/EU) is exceeded in all sites including the GWWTP, the NWWTP and the upstream of the Zenne River, but not at the Belgian Oostende Harbor and in the North Sea.

4. Conclusions

In this study, *in situ* estrogenic activity for different waters in Belgium and China was measured by a novel time-weighted average method combining o-DGT with the ERE-CALUX bioassay. Field sampling using o-DGTs implies an *in situ* measurement of the DBL thickness in order to correctly calculate the estrogenic activity. Although the flow rate in effluents of WWPTs in Beijing and Brussels and the Belgian Oostende Harbor were high enough to create well-stirred conditions, the effect of the DBL thickness on the accuracy of o-DGT results could not be neglected. The o-DGT provided comparable results to those obtained using mixed water samples by grab sampling in the investigated water bodies. Estrogenic activities in the WWTPs effluents were significantly higher than those measured in the sea. To monitor low trace levels of estrogenic compounds in natural aquatic systems and WWTPs, o-DGT combined with ERE-CALUX is a good option compared to grab sampling with LC or GC–MS/MS.

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