Direct effect based approaches applied to the screening of emerging substances

Deliverable Report

AD 16.3

WP 16 - Emerging Substances

Deadline: December 2017

Upload by Coordinator: 19 December 2017

Entity	Name of person responsible	Short name of institution	Received [Date]
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2 Introduction

To expand the knowledge on the identity of chemicals to which humans are exposed, the exploration of emerging chemicals is pursued within WP16 through complementary chemical and biological approaches. Suspect and non-target screening strategies using high resolution mass spectrometry (HRMS) will be implemented within the application of Effect-Directed Analysis (EDA) that uses a combination of (*in vitro*) bioassays, preparative fractionation and chemical analysis. In the context of tracking emerging substances, EDA may be used in a bottom-up approach entailed to first characterise the biological activity associated to particular fractions of a HBM sample then to guide the chemical identification work on this particular fraction to identify possible marker(s) of exposure responsible for this activity. These approaches aim to address the complex question of emerging substances using complementary angles respectively based on exposure assessment and hazard characterisation. Finally, their combination is expected to contribute to risk assessment and support to policy by providing data related both to real-world human exposure and to the potential toxicity of the considered substances. In addition, results from the work done in this WP16 task may serve as input for other WPs by providing candidates for further evaluation and eventual inclusion in future human biomonitoring campaigns.

This deliverable focuses on effect-based approaches used in EDA, after providing background information related to the general definition, principle, and possible application/use of EDA. In addition, an inventory has been made to provide an overview of the main existing approaches in this field, further used as a support the selection of more particular toxicological endpoints of *in vitro* bioassays that will be implemented for hazard characterisation and prioritisation of human samples in the specific context of our HBM4EU project and its related WP16.

3 Background information

3.1 Definition and principle

Effect-based approaches to direct the chemical analysis and identification of Chemicals of Emerging Concern (CECs) have already been applied in various research fields including environmental sciences, pharmaceutical sciences and food safety (Jonker et al., 2015a; Groh and Muncke, 2017). At the present time, these approaches remain clearly less applied in the human biomonitoring area.

In addition to suspect and non-target screening, EDA is one of the pillars in the identification of CECs in a wide variety of matrices. In the field of environmental sciences, the EDA concept has evolved in the course of the last two decades into a powerful tool to explore what bioactive chemicals are present in a sample. The application of *in vitro* bioassays inherently facilitates a prioritisation of samples, extracts or fractions to be studied for the presence of toxic CECs.

In EDA, both chemistry based and biology based techniques are used in an iterative mode in order to identify the substances of interest, as shown in Figure 1.
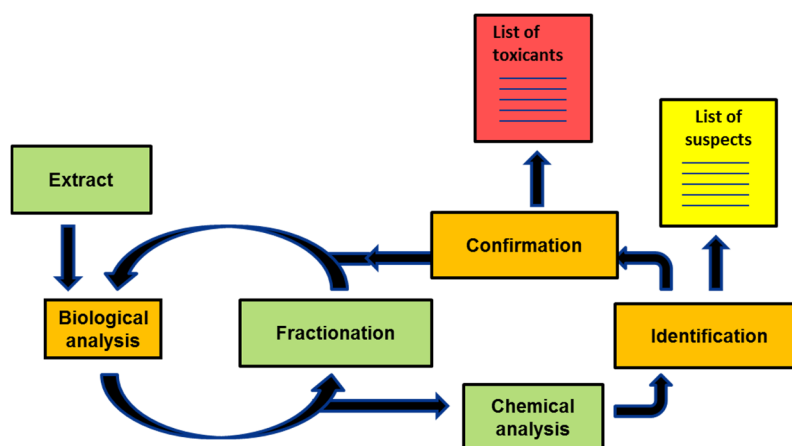

Figure 1. Schematic representation of EDA

Basically, EDA comprises i) biological/toxicological testing to focus on active compounds, ii) fractionation to reduce the complexity of the sample to be tested and iii) identification and confirmation strategies using HRMS techniques and chemical/mass spectral databases for structural elucidation of the concerned substances. Recently, these building blocks of EDA were discussed in a comprehensive review focusing on the use of EDA as support for environmental quality monitoring of aquatic environments (Brack et al., 2016) as well as a demonstration program on the effect-based and chemical identification of toxic compounds in surface waters (Tousova et al., 2017).

In any EDA study, the choice of endpoint and effect-based testing system intrinsically drives what type of chemicals will be identified. This choice then appears as a crucial issue especially in the context of looking for an extended range of emerging substances with various physico-chemical properties and subsequent various potential biological effects. A typical approach in EDA is to measure the biological activity is through, e.g. nuclear receptor binding (e.g. ER, AR, AhR)or the interaction with transport proteins (e.g. TTR-binding for thyroid hormone disruption).

In this general context, this background section will first describe the main analytical and technical aspects related to EDA and will secondly summarise its current application in environmental, food and human matrices respectively.

3.2 Advancements in analytical chemistry

To allow the identification of a broad range of bioactive compounds with diverse physico-chemical characteristics it is first necessary to use non-discriminating, non-destructive sample extraction and clean-up procedures. However, this non selective ambition is facing a practical limitation considering the non-compatibility of the detection systems with too complex biological samples. To address this complexity issue, high resolution fractionation approaches have been reported both based on liquid chromatography (LC) (Booij et al., 2014; Jonker et al., 2015b) and gas chromatography (GC) (Pieke et al., 2013; Jonker et al., 2017). To further increase the chromatographic separation resolution, comprehensive two-dimensional LCxLC can be used for specific applications to very complex extracts [Ouyang et al., 2016]. To obtain less complex, small volume fractions multi-well plates (96, 384, 1536) may be used in combination with miniaturised *in vitro* bioassays. Identification is facilitated by the correlation between bioassay response of fractions that contain only a few chemical peaks/signals and the accurate masses obtained by HRMS.

The suitability of EDA for the identification of CECs has significantly improved by the advancements in the field of mass spectrometry and the availability of HRMS equipment. In addition, chemical (e.g., ChemSpider or PubChem) and mass spectral database development (e.g., Exposome Explorer, Neveu et al., 2017) in combination with freely available software tools and those from MS instrument manufacturers have aided the development of strategies for suspect screening and identification of unknowns. For the identification of environmental toxicants (Krauss et al., 2010; Weiss et al., 2011), scientists relied on the progress that was made towards identification of unknowns in the field of metabolomics (Kind and Fiehn, 2007; Draper et al., 2009). Although the identification success rate has increased as a result of the developments in the field of HRMS, identification is still a difficult task. Indeed, this significant challenge of identification imposes to mobilise multidisciplinary competences including advanced mass spectrometry and bioinformatics/data processing related expertise and resources. These necessary competences are present and will be used within WP16 (through dedicated actions defined in the 2nd AWP) with regard to (1) structural elucidation of not yet known chemical structures from MS and MS/MS data and (2) implementation of consolidated MS reference libraries for suspect screening of already known markers of exposure.

3.3 Application of EDA on environmental samples

So far, EDA studies were mainly applied to abiotic compartments of the environment such as water and sediment. In biological matrices, however, only a few applications of EDA have been described as reviewed by Simon et al., 2015.

In the aquatic environment, the development and implementation of EDA strategies for investigative monitoring has been explored in the last decade, partly fuelled by European legislation (e.g. the Water Framework Directive). Within NORMAN, the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances, joint activities to promote the development and implementation of EDA, bioassays and nontarget screening in the indoor and outdoor environment have resulted in the advancement of the EDA strategies (Houtman et al., 2006; Weiss et al, 2011; Schmitt et al., 2012; Booij et al., 2014; Muz et al., 2017). The bioassays used have primarily been reporter gene assays for the AhR, ER, or AR, binding assays for transthyrethin (TTR), or for genotoxicity. While in these abiotic environmental compartments contaminants are often predominant in the detectable compound inventory, the presence of the "endogenous metabolome" hampers detection of contaminants.

Other specific challenges related to samples of biological origin (including food and human samples) are the presence of endogenous hormones that may influence the readout of bioassays aiming at endocrine activity, the presence of highly abundant matrix constituents affecting bioassay performance (e.g. lipids) and the high sensitivity necessary for the chemical analysis due to the usually low levels of bioactive chemicals. Regarding the identification, the possibility of the presence of not only parent compounds, but certainly also metabolites, also appears as a major issue to take into account. A successful EDA study focusing on thyroid hormone disruptive compounds in polar bear plasma has been reported by Simon et al. (2013), who identified linear and branched nonylphenol and mono- and dihydroxylated octachlorinated biphenyls.

3.4 Application of EDA on food and food packaging materials

Employing classical approaches such as targeted analysis to characterise the chemical composition of food contact materials (FCMs) and successively testing single compounds for biological activities is inadequate, as it will neither provide any information on compounds that are not explicitly known to be present in the material, nor account for the total, integrated biological activities of all the compounds present in the product (i.e. 'the cocktail effect'). To address these shortcomings, an EDA strategy can be applied, as has been exemplified in previous studies based on *in vitro* tests for genotoxicity (such as Ames test, p53 activation), cytotoxicity, and/or endocrine activity (such as ER, AR, AhR, PPARα/γ activity) in combination with advanced analytical chemistry to identify CEC in FCMs (Groh and Muncke, 2017; Rosenmai et al., 2017)

3.5 Application of EDA on human samples

EDA as a combination of bioassays, fractionation and chemical analysis has to our knowledge very rarely been used for identifying human samples. It has been applied for identifying androgens in human urine samples using an androgen receptor (AR) reporter gene assay with the purpose of using the assay in doping control (see study 14 and 15 in Table 1).

However, bioassays have been applied on human samples to identify correlations between bioassay activities and exposure to persistent organic pollutants (POPs) or human health endpoints without identifying the chemicals that gave rise to the activity. In this way, the bioassay readout has served as a biomarker for effect and/or exposure.

The application of EDA to human samples such as blood, urine, amniotic fluid, milk, or meconium, has great potential, as it may provide insight into which chemicals are found in the human body (i.e. the internal exposome) that we have not paid attention to so far. This knowledge is expected to support the mechanistic understanding of adverse effects through the selection of appropriate endpoints and appropriate *in vitro* bioassays to measure them (Escher et al., 2017).

4 Inventory of existing bioassay analyses of human samples

In this section, an inventory of suitable and existing *in vitro* bioassays is first described, from which a selection will be made for further EDA work in human samples in the specific framework of the HBM4EU project and its related WP16. This inventory is given in Table 1 and includes 23 studies, where a single or a combination of a few bioassays has been used to study associations with either POP exposure or with human health endpoints such as male reproductive diseases or breast cancer.

The majority of the studies have been performed by a Danish (n = 12) and a Spanish (n = 12) research group from Aarhus University (no. 6-8,11-12, 27, 29,31-35) and Granada University (no. 13-23 and 28), respectively. Four studies (no. 1-3,15) have been performed by people from the Bio Detection Systems company, the Netherlands, whereas the remaining studies originate from different research groups.

4.1 Bioassays used so far

Most focus so far has been on detection of arylhydrocarbon receptor (AhR) activity using reporter gene assays in human samples such as blood, follicular fluid, or milk from populations in Europe or Greenland. Indeed, a large set of lipophilic persistent organic pollutants (POPs) present the ability to bind AhR through their partially common mode of action at molecular level. Consequently, bioassays based on this particular receptor are relevant in screening for this large group of compounds. In many cases the bioassay activity has been compared with the total TCDD-equivalents (TEQs) of PCBs and dioxins based on quantification by chemical analysis. The advantage of using the AhR assay is that any interference by endogenous hormones is not occurring with this assay.

In other studies, estrogenic activity as measured by activation of the estrogen receptor (ER) using reporter gene assays or MCF7 proliferation has been determined, but in all cases human breast cancer cells have been the fundamental part of the assay. This estrogenic potency measurement is particularly employed for instance for screening endocrine disrupting chemicals.

Androgenic activity has been also measured using reporter gene assays based on either CHO cells, U2OS cells or yeast cells with a read out reflecting either agonism or antagonism of the receptor.

5 Selected approaches

5.1 Arylhydrocarbon Receptor bioassay

In front of the very wide range of possible "emerging chemicals" to be addressed in the frame of the HBM4EU project and its related WP16, a priority action was focused on halogenated chemicals in their global screening for CEC in human samples. Indeed, this large category of compounds may encompass a large range of exposure markers (e.g. not yet known/addressed novel BFRs, phthalates, bromo-/chlorophenols, chlorinated paraffins…, including parent compounds and their transformation products). Moreover, these halogenated compounds exhibit typical isotopic pattern possibly used as a filter for extracting potential signals of interest from untargeted mass spectrometric profiles.

As many halogenated chemicals are known to activate the AhR and as this bioassay is one of the most thoroughly tested bioassay on human samples, we will logically select this assay for a first round of measuring bioactivity. Methodologies for extraction of POPs and for running the bioassay are well-established. The intention is to identify one or more CECs with AhR activity. AhR activity is of increasing importance as this receptor is not only involved in liver metabolism and reproductive toxicity but also in the function of the immune system as well as in stem cell differentiation in the bone marrow.

5.2 Transthyretin binding assay

Thyroid hormone disruption can be effected through a variety of pathways, e.g. interference with the metabolism of thyroid hormones, their excretion or their transport through the body. Thyroid hormones are only sparingly soluble in blood plasma, through which thyroid hormones reach the location in the body where they have their effect. For this transport, several proteins are present in vertebrates, e.g. albumin, thyroxine-binding globulin (TBG) and transthyretin (TTR). Although not the most dominant transport protein, TTR is of importance because of its capacity to transport T4, but also xenobiotics, across the placenta and the blood-brain-barrier (Meerts et al., 2002). A recent overview of compounds having the potential to disrupt the thyroid hormone system was given by Weiss et al., 2015, in which also the various assays that employ binding to TTR are listed. Historically, the interference of environmental pollutants with the thyroid hormone system is assessed by the competition with the radiolabelled endogenous hormone T4 in the binding to TTR.

Using this assay, thyroid hormone disruption has been studied in-depth in polar bears (e.g. Bytingsvik et al., 2013) including an EDA study in polar bear plasma, revealing several highly metabolised (by hydroxylation) hepta- and octachlorinated biphenyls (Simon et al., 2011 and 2013). Although very reliable, this laborious and rather costly T4*-TTR radioligand binding assay was not capable of sufficiently high throughput for effective application in EDA. Recently, a modification of the assay into an assay that utilises fluorescence was reported (Ren and Guo, 2012), further adapted for high throughput EDA in 96 well plate format by Ouyang et al., 2017.

Table 1: Overview of existing Effect-Directed Analysis (EDA) approaches

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
1	*AhR activity*	DR-CALUX from BDS (rat H4IIE hepatoma cell line)	Total fat from 1 mL plasma extracted in 97% hexane/ 3% diethyl ether. Passed through two acid silica columns topped first 20% and then 30% H2SO4 to remove matrix components. Evaporated under nitrogen and re-dissolved in 8µL of DMSO	Maternal blood samples ("Rhea" mother–child cohort study in Crete and the Hospital del Mar cohort in Barcelona)	Plasma dioxin-like activity was negatively associated with anogenital distance (AGD) in male newborns. The estimated change in AGD per 10 pg CALUX®–toxic equivalent/g lipid increase was –0.44 mm (95% CI: –0.80, –0.08) after adjusting for confounders	Vafeiadi et al. 2013
2		DR-CALUX from BDS (rat H4IIE hepatoma cell line)	-do-	-do-	Dioxin-like activity in maternal blood was higher in the children born by mothers in the upper tertile of the "high-fat diet" score during pregnancy, compared to the lower and middle tertiles and were positively correlated (Spearman's rho = 0.49, P b 0.001)	Papadopoulou et al.2013
3		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	n-hexane extraction of blood serum or follicular fluid aliquots (1-1.5 mL) and removal of acid labile matrix components	106 serum and 9 follicular fluid samples collected from infertile women	TEQ values in human extracts correlated well with the sum of four major PCB congeners (153, 138, 180, 118) chemically determined in both serum and follicular fluid	Pauwels et al. 2000
4		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	The fat in the milk samples was extracted by addition of sodium oxalate and ethanol to the milk and subsequent extractions with diethylether and n-pentane, respectively. Clean-up on a silica column.	16 Danish human milk samples	TEQ levels in the total extract of the 16 human milk samples were correlated to the TEQ levels determined by GC/MS (R= 0.73). Most samples gave higher TEQs with the CALUX assay.	Laier et al. 2003
5		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	2 ml blood was extracted by n-hexane and passaged through a silica column. DMSO (7.5 µL) was added, and the extract was diluted to a total volume of 750 µL with minimal essential medium.	101 men 20–40 years of age were evaluated on sperm parameters, sex hormone levels and lifestyle factors	Age and the frequency of fish and egg consumption were independent positive determinants of serum AhR activity. Two-fold increase in CALUX-TEQ was associated with a decreased testosterone, a pronounced drop in semen volume but increased sperm concentration.	Dhooge et al, 2006

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
6		AhR assay from Denison (mouse Hepa1.12cr hepatoma cell line)	POPs were extracted at Le Centre de Toxicologie, Sainte Foy, Quebec, CA, using ethanol and hexane, followed by cleaning on FlorisilCNa2SO4 column using 2 ml serum	338 males from Greenland (Inuit's), Sweden, Poland and Ukraine	The variation of AhR serum activity may reflect different pattern of POP exposure, genetics and/or life style factors. No consistent correlations between AhR activities and two POP markers were found.	Long et al. 2006
7		AhR assay from Denison (mouse Hepa1.12cr hepatoma cell line)	POPs were extracted at Le Centre de Toxicologie, Sainte Foy, Quebec, CA, using ethanol and hexane, followed by cleaning on FlorisilCNa2SO4 column using 2 ml serum	357 serum samples from Greenland (South West Coast, North Coast and East Coast)	85% of the Inuit samples elicited agonistic AhR transactivity in a district dependent pattern. The AhR transactivity was inversely correlated to the levels of sum POPs, age and/or intake of marine food	Long et al. 2007
8		AhR assay from Denison (mouse Hepa1.12cr hepatoma cell line)	POPs were extracted at Le Centre de Toxicologie, Sainte Foy, Quebec, CA, using ethanol and hexane, followed by cleaning on FlorisilCNa2SO4 column using 2 ml serum	70 inhabitants from 6 different Greenlandic districts and young Danish volunteers	The AhR-TEQs of the Inuits were significantly higher than that of the Danes. AhR-TEQ of Inuit were positively associated with plasma POPs after adjustment for age and/or the ratio of n-3 to n-6 fatty acids, whereas no correlations were found for the Danish samples	Bonefeld-Jørgensen and Long, 2010
9		AhR CALUX from XDS (mouse H1L6.1c3 hepatoma cell line stably transfected with the pGudLuc6.1 reporter)	0.5 ml whole blood was extracted with 0.5 ml tert-butyl methyl ether and resuspended in 200 ml hexane and this stock extract was used for creating dilutions to be analysed in the bioassay.	10 human blood samples from volunteers under different dietary regimens	Close to 1,000-fold higher TEQ values were found by using the bioassay compared to analytical chemistry, suggesting that human blood contains a relatively high level of AHR agonists apart from PCBs/dioxins /furans. A vegetarian diet increased AhR activity.	Connor et al. 2008
10		AhR CALUX from XDS (mouse H1L6.1c3 hepatoma cell line stably transfected with the pGudLuc6.1 reporter)	10 g human plasma was mixed with 30 mL of acetone followed by extraction with 10 mL n-hexane. The concentrated extract (5 ml) was processed through an acid silica column in series with an activated carbon column. Dioxins were eluted and resuspended in 1.2 mL of n-hexane.	496 blood samples were collected for GC-HRMS and CALUX analyses	A significant correlation was established between the bioassay and chemical method for 17 PCDD/F congeners (R = 0.64)	Van Wouwe et al. 2004

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
11	*ER activity*	MVLN assay (stably transfected human breast cancer cells expressing ERα and ERβ)	3.6 ml serum was extracted by SPE on an OASIS HLB column followed by HPLC fractionation on a Spherisorb Si60 normal phase column. The first fraction included most POPs while leaving out endogenous hormones	358 men: Greenlandic Inuit's, Swedish fishermen, and inhabitants from Poland and Ukraine.	No ER agonistic activity was found in Inuits, while 12 – 24% of the European samples had detectable ER activity. On the contrary, 71% of Inuit serum samples antagonised ER compared to 7 – 30 % in the other regions.	Jørgensen et al. 2006
12		MVLN assay (stably transfected human breast cancer cells expressing ERα and ERβ)	3 ml serum was extracted by SPE on an OASIS HLB column followed by HPLC fractionation on a Spherisorb Si60 normal phase column. The F3 fraction included the PFAS. Estriol and Estetrol hormones were removed by weak anion exchange.	397 pregnant women from Aarhus Birth Cohort	52% of the PFAS serum extracts activated ER, and 46% enhanced the E2-induced ER transactivation. Positive linear concentration-response associations were found between the PFAS serum levels and the ER transactivation.	Bjerregaard-Olesen et al. 2016
13		MCF7 proliferation (human breast cancer cells)	200 mg adipose tissue was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	400 women with various diseases undergoing surgical treatment at Granada University Hospitals and Almeria Hospital, Spain	65% of the α-fractions and 76% of the β-fractions showed measurable estrogenicity. The mean estradiol equivalent (EEQ) unit was 750 pM EEQ/g lipid in the α-fraction and 903 pM EEQ/g lipid in the β-fraction.	Rivas et al. 2001
14		MCF7 proliferation (human breast cancer cells)	200-500 mg adipose tissue was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	458 women with various diseases undergoing surgical treatment at Granada University Hospitals and Almeria Hospital, Spain	75% of the α-fractions and 82% of the β-fractions showed measurable estrogenicity. The mean estradiol equivalent (EEQ) unit was 515 pM EEQ/g lipid in the α-fraction and 697 pM EEQ/g lipid in the β-fraction.	Fernandez et al. 2004
15		MCF7 proliferation (human breast cancer cells)	200 mg adipose tissue (breast tissue from cases; abdominal tissue from controls) was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no.	198 breast cancer cases and 260 age and hospital matched controls undergoing non-cancer-related surgery. Cases and	Overall, the cancer risk was not associated with the total effective xenoestrogen burden (TEXB) in the α-fraction. However, among cases with a body mass index (BMI) below the	Ibarluzea et al. 2004

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
			1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	controls were recruited at the three largest public hospitals serving Granada and Almeria provinces in Spain.	median (28.6 kg/m^2), the odds ratio (OR) was 2.44 (95% CI 1.22–9.58) for women in the highest quartile of TEXB in the α-fraction versus those in the lowest. After including the TEXB of the β-fraction in the logistic regression model, the OR increased to 3.42 (95% CI 1.22–9.58) for those in the highest tertile versus those in the lowest.	
16		MCF7 proliferation (human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	50 male newborn cases with congenital malformations and 114 male newborn controls matched by gestational age, date of birth, and parity. Cases and controls were registered at the San Cecilio University Hospital, Granada, Spain	The total effective xenoestrogen burden (TEXB) from the α-fraction was detectable in 72% and 54% of case and control placentas, respectively. Cases had an OR for detectable versus non-detectable TEXB of 2.82 (95% CI, 1.10–7.24) compared with controls.	Fernandez et al. 2007
17		MCF7 proliferation (human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	489 newborns from four cohorts in the Spanish Children's Health and Environment (INMA) study.	For the tertile with the highest total effective xenoestrogen burden in the α-fraction (TEXB-α), higher TEXB-α was associated with increased birthweight for boys (β=148.2 g, 95% CI: 14.01, 282.53) but not for girls.	Vilahur et al. 2013
18		MCF7 proliferation (human breast cancer cells)	200 mg adipose tissue was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. No HPLC fractionation was performed in this study.	386 study participants were recruited among patients undergoing non-cancer-related surgery. 34 of the included participants suffered from type 2 diabetes.	The total effective xenoestrogen burden (TEXB) was not associated with type-2-diabetes.	Arrebola et al. 2013

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
19		MCF7 proliferation (human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	489 children from four cohorts in the Spanish Children's Health and Environment (INMA) study.	Overall, no association was found between the total effective xenoestrogen burden in the α-fraction (TEXB-α) and the mental scores at 1-2 years of age. However, in tests of motor development at 1-2 years of age, boys in the third tertile of exposure scored on average 5.2 points less compared to those in the first tertile (p=0.052). The association disappeared at 4-5 years of age.	Vilahur et al. 2014a
20		MCF7 proliferation (human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	97 male and 95 female newborns from four cohorts in the Spanish Children's Health and Environment (INMA) study.	Boys in the highest tertile of total effective xenoestrogen burden in the α-fraction (TEXB- α) presented on average a decrease of 0.84% in AluYb8 DNA-methylation compared to those in the first tertile (p < 0.001), while no significant effects were found in girls (p = 0.134).	Vilahur et al. 2014b
21		MCF7 proliferation (human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	Discovery study: 181 mother-child dyads. Replication study: 126 mother-boy dyads. The dyads were included from four cohorts in the Spanish Children's Health and Environment (INMA) study.	No genome-wide significant associations were found between the total effective xenoestrogen burden in the α-fraction (TEXB-α) and DNA methylation in either study group.	Vilahur et al. 2016
22		MCF7 proliferation (human breast cancer cells)	3 mL serum was extracted with hexane:ethyl ether (1:1). The organic phases were passed through a Bond Elut PCB cartridge. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the	186 breast cancer cases and 196 frequency-matched controls were included from MCC-Spain, which is a population-based multicase-control study.	Cases had higher geometric mean total effective xenoestrogen burden in the α-fraction (TEXB-α) and the β-fraction (TEXB-β) than controls. The fully adjusted odds ratios (OR) for breast cancer comparing the second and third tertiles of TEXB-α with the first tertile	Pastor-Barriuso et al. 2016

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
			endogenous hormones were included in fraction β.		were 1.77 (95%CI: 0.76, 4.10) and 3.45 (95%CI: 1.50, 7.97), respectively. For TEXB-β the ORs were 2.35 (1.10, 5.03) and 4.01 (1.88, 8.56), respectively.	
23		MCF7 proliferation (human breast cancer cells)	200 mg adipose tissue was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh) no. 1097. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β.	55 women newly diagnosed with breast cancer and scheduled for surgery at San Cecilio University Hospital, Granada, Spain Breast adipose tissue was collected at the time of surgery and abdominal adipose tissue was collected at four time points after surgery (<6 months; 12 months, 12-18 months, and >18 months).	The total effective xenoestrogen burden in both the α-fraction (TEXB-α) and the β-fraction (TEXB-β) increased during the first 6-12 months after surgery, and then decreased lightly over time.	Fernandez et al. 2017
24		MCF7 prolifera-tion assay (human breast cancer cells)	4 mL serum was extracted with an OASIS HLB 6 cc 500 mg extraction cartridge. Slow elution by 3 mL of methanol followed by 2 mL ethyl acetate. The aqueous phase was extracted 3 x by 1 mL n-heptane/ethyl acetate (1:1). Supernatants were reconstituted in 125 µL n-heptane /ethyl acetate (9:1).	30 pregnant and 60 non-pregnant Danish women plus 211 serum samples from pregnant Faroese women	The estrogenicity of the serum from Danish controls exceeded the background in 22.7 % of the cases, while the same was true for 68.1 % of the Faroese samples.	Rasmussen et al. 2003
25	*AR activity (agonism)*	Yeast androgen assay expressing human androgen receptor and β-galactosidase	Reverse phase LC dual 96-well fraction collection set-up followed by offline LC/QTOFMS/MS and bioactivity screening	Human male and female urine samples by healthy volunteers from the laboratory	These androgens were identified: Testosterone, 5α-dihydro-testosterone (DHT), androsterone, etiocholanolone, gestagen, a new undocumented designer steroid	Nielen et al. 2006
26		AR-CALUX from BDS (stably	Enzymatic deconjugation followed by LLE with methyl-tert-butylether	Five males and five females provided urine samples	These androgens were identified: Testosterone, DHT, epi-testosterone,	Houtman et al. 2009

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
		transfected human U2OS cell line)			epi-DHT, androsterone, etiocholanolone	
27	*AR activity (agonism & antagonism)*	AR assay, transient transfected CHO cells with hAR and MMTV--LUC	POPs were extracted from 3.6 ml serum samples by SPE using Oasis HLB cartridges followed by HPLC fractionation in order to obtain the fraction (F1) containing POP mixtures but free of endogenous hormones	Adult male serum (n = 261) from Greenland, Sweden, Poland, and Ukraine	The study groups differed significantly with respect to AR antagonistic activity, which was increased in the Inuits and decreased in the European study groups; No difference in AR agonistic activity was found.	Krüger et al. 2007
28	*AR activity (antagonism)*	PALM reporter gene assay	1.0 g of placenta homogenates were extracted with acetonitrile. The supernatant was filtered and fractionated by preparative HPLC on a Spherisorb Si60 normal phase column. 27 fraction were collected.	29 male newborn cases with congenital malformations and 60 healthy male controls were included from San Cecilio University Hospital, Granada, Spain	The multivariable statistical analyses indicated a statistically significant positive association between the total effective xenobiotic burden of anti-androgens (TEXB-AA) of the HPLC fraction collected during minutes 1–2 (F2) and the risk of malformations (odds ratio: 2.33, 95% CI: 1.04–5.23).	Arrebola et al. 2015
29	*ER and AR*	MVLN assay (ER), AR assay, transient tranfections	*ER and AR assays*: POPs were extracted from 3.6 mL serum samples by SPE using Oasis HLB cartridges followed by HPLC fractionation in order to obtain the fraction (F1) containing POP mixtures but free of endogenous hormones	121 men and 119 women from Greenland	The xenohormone transactivities differed between districts as well as between genders. Associations between transactivities and age, n-3/n-6 and smoker years were observed. The xenoestrogenic and xenoandrogenic transactivities correlated negatively to the POPs for the combined female and male data, respectively.	Krüger et al. 2008a
30	*ER & AhR*	DR-CALUX & ER-CALUX (human breast carcinoma T47D.Luc cell line stably transfected with pEREtataLuc construct)	5 mL male serum were treated with 2 mL methanol and extracted 3 times with n-hexane:diethyl ether (1:1); the extracts were evaporated and dissolved in 1 mL dichloro-methane. For determination of ER activity, the solvent was replaced with DMSO in one-half of the crude extract; the second half of the sample was placed on a sulfuric acid-activated silica column and eluted with	150 individual male blood samples from residents of two areas of eastern Slovakia, which are differently contaminated with PCBs	In human male serum samples, high levels of PCBs were associated with a decreased ER-mediated activity and an increased dioxin-like activity	Pliskova et al. 2005

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
			n-hexane: diethylether mixture, evaporated, and redissolved in DMSO			
31	*ER, AR & AhR*	MVLN assay (ER), AR assay (transiently transfected) & AhR assay (Denison)	*AhR assay*: POPs were extracted at Le Centre de Toxicologie, CA as described under [6]. *ER and AR assays*: POPs were extracted from 3.6 mL serum as described under [17].	262 adult males (54 Inuits from Greenland, 69 from Poland, 81 from Sweden, and 58 from Ukraine	Negative correlations between xenobiotic-induced receptor activities and sperm DNA damage were found for Inuits having relatively lower xenoestrogenic, lower dioxin-like activity, and lower sperm DNA damage, but higher xenoandrogenic activity. In the European groups, xenobiotic-induced receptor activities were found to be positively correlated with the DNA damage.	Long et al. 2007
32		MVLN assay (ER), AR assay (transiently transfected) & AhR assay (Denison)	*AhR assay:* POPs were extracted at Le Centre de Toxicologie, CA as described under [6]. *ER and AR assays:* POPs were extracted from 3.6 ml serum as described under [17].	319 men from Warsaw (Poland), Greenland, Kharkiv (Ukraine), and Sweden	No strong and consistent associations between xenobiotic activity and semen quality measures was observed in the four populations. However, when combining data across populations, some associations between ER activity and sperm parameters were found.	Toft et al. 2007
33		MVLN assay (ER), AR assay (transiently transfected) & AhR assay (Denison)	*AhR assay:* POPs were extracted at Le Centre de Toxicologie, CA as described under [6]. *ER and AR assays:* POPs were extracted from 3.6 mL serum as described under [17].	The study included 53 Greenlandic Inuits and 247 Europeans (Sweden, Warsaw (Poland) and Kharkiv (Ukraine)).	For Inuits, ER and AhR activities and DNA fragmentation were inversely correlated. For Europeans, a positive correlation between AR activity and DNA fragmentation was found. For Europeans correlations between ER and AR activities and DNA stainability was observed.	Krüger et al. 2008
34		MVLN assay (ER), AR assay (transiently transfected) & AhR assay (Denison)	*AhR–CALUX assay*: Dioxin-like POPs were extracted by SPE-Supelco using 2 ml serum. *ER and AR assays*: POPs were extracted from 3.6 ml serum as described under [17].	31 Inuit breast cancer cases and 115 controls from Greenland	Associations between breast cancer risk and AR agonistic activity were found. Cases elicited a higher frequency of ER agonistic activity, but a lower AhR activity.	Bonefeld-Jørgensen et al. 2011
35		MVLN assay (ER),	*AhR–CALUX assay*: Dioxin-like POPs were extracted by SPE-Supelco using 2 ml serum.	232 Inuit samples from Greenland	The transactivities correlated negatively to the POP levels and were associated to the lifestyle characteristics.	Krüger et al. 2012

No	Activity	Bioassay used	Sample preparation in brief	Human population or cohorte	Observed associations	Reference
		AR assay (transiently transfected) & AhR assay (Denison)	*ER and AR assays*: POPs were extracted from 3.6 ml serum as described under [17].			

6 References

Arrebola, J. P., Pumarega, J., Gasull, M., Fernandez, M. F., Martin-Olmedo, P., Molina-Molina, J. M., Fernandez-Rodriguez, M., Porta, M., and Olea, N., Adipose tissue concentrations of persistent organic pollutants and prevalence of type 2 diabetes in adults from Southern Spain. Environ. Res., 2013. 122: p. 31-37.

Arrebola, J. P., Molina-Molina, J. M., Fernandez, M. F., Saenz, J. M., Amaya, E., Indiveri, P., Hill, E. M., Scholze, M., Orton, F., Kortenkamp, A., and Olea, N., A novel biomarker for anti-androgenic activity in placenta reveals risks of urogenital malformations. Reproduction, 2015. 149(6): p. 605-613

Bjerregaard-Olesen C, Ghisari M, Bonefeld-Jørgensen EC 2016. Activation of the estrogen receptor by human serum extracts containing mixtures of perfluorinated alkyl acids from pregnant women. Environ Res. 151:71-79.

Bonefeld-Jorgensen EC, Hjelmborg PS, Reinert TS, Andersen BS, Lesovoy V, Lindh CH, et al. 2006. Xenoestrogenic activity in blood of european and inuit populations. Environ Health 5:12.

Bonefeld-Jorgensen EC, Long M. 2010. Dioxin-like activity in the blood of Greenlandic Inuit and Danish women: a pilot study. Int J Circumpolar Health. 69(2):181-194.

Bonefeld-Jorgensen EC, Long M, Bossi R, Ayotte P, Asmund G, Krüger T, Ghisari M, Mulvad G, Kern P, Nzulumiki P, Dewailly E. 2011. Perfluorinated compounds are related to breast cancer risk in Greenlandic Inuit: a case control study. Environ Health 10:88.

Booij, P., Vethaak, A.D., Leonards, P.E.G., Sjollema, S.B., Kool, J. Voogt, P. de, Lamoree, M.H. 2014. Identification of photosynthesis inhibitors of pelagic marine algae using 96-well plate microfractionation for enhanced throughput in Effect-Directed Analysis. Environmental Science & Technology 48, 8003-8011.

Brack, W., Ait-Aissa, S., Burgess, R.M., Creusot, N., Di Paolo, C., Escher, B.I., Hewitt, L.M., Hilscherova, K., Hollender, J., Hollert, H., Jonker, W., Kool, J., Lamoree, M., Muschket, M., Neumann, S., Rostkowski, P., Ruttkies, C., Schollee, J., Schymanski, E.L, Schulze, T., Seiler, T., Tindalll, A.J., De Aragão Umbuzeiro, G., Vrana, B., Krauss, M. 2016. Effect-directed analysis supporting monitoring of aquatic environments — An in-depth overview. Science of the Total Environment 544, 1073-1118.

Bytingsvik, J., Simon, E., Leonards, P.E.G., Lamoree, M., Lie, E., Aars, J., Derocher, A.E., Wiig, O., Jenssen, B.M., Hamers, T. 2013. Transthyretin-binding activity of contaminants in blood from polar bear (Ursus maritimus) cubs. Environmental Science & Technology 47, 4778-4786.

Connor KT, Harris MA, Edwards MR, Budinsky RA, Clark GC, Chu AC, et al. 2008. Ah receptor agonist activity in human blood measured with a cell-based bioassay: Evidence for naturally occurring ah receptor ligands in vivo. J Expo Sci Environ Epidemiol 18:369-380.

Dhooge W, van Larebeke N, Koppen G, Nelen V, Schoeters G, Vlietinck R, et al. 2006. Serum dioxin-like activity is associated with reproductive parameters in young men from the general flemish population. Environ Health Perspect 114:1670-1676.

Draper, J., Enot, D.P., Parker, D., Beckmann, M., Snowdon, S., Lin, W., Zubair H. 2009. Metabolite signal identification in accurate mass metabolomics data with MZedDB, an interactive m/z annotation tool utilising predicted ionisation behaviour 'rules'. BMC Bioinformatics 10, 1–16

Escher, B.I., Hackermüller, J., Polte, T., Scholz, S., Aigner, A., Altenburger, R., Böhme, A., Bopp, S.K., Brack, W., Busch, W., Chadeau-Hyam, M., Covaci, A., Eisenträger, A., Galligan, J.J.,

Garcia-Reyero, N., Hartung, T., Hein, M., Herberth, G., Jahnke, A., Kleinjans, J., Klüver, N., Krauss, M., Lamoree, M., Lehmann, I., Luckenbach, T., Miller, G.W., Müller, A., Phillips, D.H., Reemtsma, T., Rolle-Kampczyk, U., Schüürmann, G., Schwikowski, B., Tan, Y-M., Trump, S., Walter-Rohde, S., Wambaugh, J.F. 2017. From the exposome to mechanistic understanding of chemical-induced adverse effects. Environment International 99, 97-106.

Fernandez, M. F., Rivas, A., Olea-Serrano, F., Cerrillo, I., Molina-Molina, J. M., Araque, P., Martinez-Vidal, J. L., and Olea, N., Assessment of total effective xenoestrogen burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect. Anal. Bioanal. Chem., 2004. 379(1): p. 163-170.

Fernandez, M. F., Olmos, B., Granada, A., Lopez-Espinosa, M. J., Molina-Molina, J. M., Fernandez, J. M., Cruz, M., Olea-Serrano, F., and Olea, N., Human exposure to endocrine-disrupting chemicals and prenatal risk factors for cryptorchidism and hypospadias: a nested case-control study. Environ. Health Perspect., 2007. 115 Suppl 1: p. 8-14.

Fernandez, M. F., Pumarega, J., Porta, M., Molina-Molina, J. M., Arrebola, J. P., and Olea, N., Changes in the total effective xenoestrogen burden (TEXB) of breast cancer patients during an 18-month post-surgical follow-up. Reprod. Toxicol., 2017. 69: p. 212-220.

Groh, K.J. and Muncke, J. 2017. In Vitro Toxicity Testing of Food Contact Materials: State-of-the-Art and Future Challenges. Comprehensive Reviews in Food Science and food Safety, 16, 1123-1150.

Houtman, C.J., Booij, P., Jover, E., Pascual del Rio, D., Swart, K., Velzen, M. van, Vreuls, R., Legler, J., Brouwer, A. & Lamoree, M.H. 2006. Estrogenic and dioxin-like compounds in sediment from Zierikzee harbour identified with CALUX assay-directed fractionation combined with one and two dimensional chromatography analyses. Chemosphere 65, 2244-2252.

Houtman CJ, Sterk SS, van de Heijning MP, Brouwer A, Stephany RW, van der Burg B, et al. 2009. Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays. Anal Chim Acta 637:247-258.

Ibarluzea, J. M., Fernandez, M. F., Santa-Marina, L., Olea-Serrano, M. F., Rivas, A. M., Aurrekoetxea, J. J., Exposito, J., Lorenzo, M., Torne, P., Villalobos, M., Pedraza, V., Sasco, A. J., and Olea, N., Breast cancer risk and the combined effect of environmental estrogens. Cancer Cause Control, 2004. 15(6): p. 591-600.

Jonker, L.W., Lamoree, M.H., Houtman, C.J., Kool, J. 2015a. Methodologies for Effect-Directed Analysis: environmental applications, food analysis and drug discovery. Chapter in: J. Kool and W. Niessen (eds.), Analyzing biomolecular interactions by mass spectrometry. Chapter 4: 111-163. ISBN: 978-0-470-77040-5. Wiley-VCH 2015.

Jonker, L.W., Lamoree, M.H., Houtman, C.J., Hamers, T., Somsen, G.W., Kool, J. 2015b. Rapid activity-directed screening of estrogens by parallel coupling of liquid chromatography with a functional gene reporter assay and mass spectrometry. Journal of Chromatography A 1406, 165-174.

Jonker, L.W., Zwart, N., Stöckl, J.B., Koning, S. de, Schaap, J., Lamoree, M.H., Somsen, G.W., Hamers, T., Kool, J. 2017. Continuous fraction collection of gas chromatographic separations with parallel mass spectrometric detection applied to cell-based bioactivity analysis. Talanta 168, 162-167.

Kind, T., Fiehn, O. 2007. Seven golden rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. BMC Bioinformatics 8, 1–20

Krauss, M., Singer, H., Hollender, J. (2010). LC–high resolution MS in environmental analysis: from target screening to the identification of unknowns. Analytical Bioanalytical Chemistry 39, 943–951.

Krüger T, Ghisari M, Hjelmborg PS, Deutch B, Bonefeld-Jorgensen EC. 2008a. Xenohormone transactivities are inversely associated to serum POPs in Inuit. Environ Health. 7:38.

Krüger T, Hjelmborg PS, Jonsson BA, Hagmar L, Giwercman A, Manicardi GC, Bizzaro D, Spano M, Rignell-Hydbom A, Pedersen HS, Toft G, Bonde JP, Bonefeld-Jorgensen EC. 2007. Xenoandrogenic activity in serum differs across European and Inuit populations. Environ Health Perspect. 115(1):21-27

Krüger T, Long M, Ghisari M, Bonefeld-Jørgensen EC. 2012. Combined Effect of Persistent Organic Pollutants in the Serum of Greenlandic Inuit: Xenoestrogenic, xenoandrogenic and dioxin-like activities. Biomarkers 17(8):692-705.

Krüger T, Spano M, Long M, Eleuteri P, Rescia M, Hjelmborg PS, Manicardi GC, Bizzaro D, Giwercman A, Toft G, Bonde JP, Bonefeld-Jorgensen EC. 2008. Xenobiotic activity in serum and sperm chromatin integrity in European and inuit populations. Mol Reprod Dev.75(4):669-680.

Laier P, Cederberg T, Larsen JC, Vinggaard AM. 2003. Applicability of the calux bioassay for screening of dioxin levels in human milk samples. Food Additives & Contaminants 20:583-595.

Long M, Andersen BS, Lindh CH, Hagmar L, Giwercman A, Manicardi G-C, et al. 2006. Dioxin-like activities in serum across european and inuit populations. Environmental Health 5:14-14.

Long, M, Deutch, B, Bonefeld-Jorgensen, E 2007. AhR transcriptional activity in serum of Inuits across Greenlandic districts. Environmental Health 6 (1): 32.

Long M, Stronati A, Bizzaro D, Kruger T, Manicardi GC, Hjelmborg PS, et al. 2007. Relation between serum xenobiotic-induced receptor activities and sperm DNA damage and sperm apoptotic markers in european and inuit populations. Reproduction 133:517-530.

Meerts, I.A.T.M., Assink, Y., Cenijn, P.H., Van den Berg, J.H.J., Weijers, B.M., Bergman, A., Koeman, J.H., Brouwer, A. 2002. Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat. Toxicological Sciences 68: 361-371.

Muz, M., Krauss, M., Kutsarova, S., Schulze, T., Brack, W. 2017 Mutagenicity in surface waters: synergistic effects of carboline alkaloids and aromatic amines. Environmental Science & Technology, 51: 1830-1839

Neveu, N., Moussy, A., Rouaix, H., Wedekind, R., Pon, A., Knox, C., Wishart, D.S., Scalbert, A. 2017. Exposome-Explorer: a manually-curated database on biomarkers of exposure to dietary and environmental factors. Nucleic Acids Research 45, D979-D984.

Nielen MW, Bovee TF, van Engelen MC, Rutgers P, Hamers AR, van Rhijn JH, et al. 2006. Urine testing for designer steroids by liquid chromatography with androgen bioassay detection and electrospray quadrupole time-of-flight mass spectrometry identification. Anal Chem 78:424-431.

Ouyang X, Froment J, Leonards PE, Christensen G, Tollefsen KE, de Boer J, et al. 2017. Miniaturization of a transthyretin binding assay using a fluorescent probe for high throughput screening of thyroid hormone disruption in environmental samples. Chemosphere 171:722-728.

Ouyang, X., Tousova, Z., Slobodnik, J., De Boer, J., Leonards, P.E.G., Lamoree, M.H. 2016. 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. Analytical Chemistry 67, 179-191.

Papadopoulou E, Vafeiadi M, Agramunt S, Mathianaki K, Karakosta P, Spanaki A, et al. 2013. Maternal diet, prenatal exposure to dioxins and other persistent organic pollutants and anogenital distance in children. Sci Total Environ 461-462:222-229.

Pastor-Barriuso, R., Fernandez, M. F., Castano-Vinyals, G., Whelan, D., Perez-Gomez, B., Llorca, J., Villanueva, C. M., Guevara, M., Molina-Molina, J. M., Artacho-Cordon, F., Barriuso-Lapresa, L., Tusquets, I., Dierssen-Sotos, T., Aragones, N., Olea, N., Kogevinas, M., and Pollan, M., Total Effective Xenoestrogen Burden in Serum Samples and Risk for Breast Cancer in a Population-Based Multicase-Control Study in Spain. Environ. Health Perspect., 2016. 124(10): p. 1575-1582.

Pauwels A, Cenijn PH, Schepens PJ, Brouwer A. 2000. Comparison of chemical-activated luciferase gene expression bioassay and gas chromatography for pcb determination in human serum and follicular fluid. Environ Health Perspect 108:553-557.

Pieke, E., Heus, F., Kamstra, J., Mladic, M., Velzen, M. van, Kamminga, D., Lamoree, M., Hamers, T., Leonards, P., Niessen, W., Kool, J. 2013. High-resolution fractionation after gas chromatography for Effect Directed Analysis. Analytical Chemistry 85, 8204-8211.

Plíšková M, Vondráček J, Canton RF, Nera J, Kočan A, Petrík J, et al. 2005. Impact of polychlorinated biphenyls contamination on estrogenic activity in human male serum. Environmental Health Perspectives 113:1277-1284.

Rasmussen TH, Nielsen F, Andersen HR, Nielsen JB, Weihe P, Grandjean P. 2003. Assessment of xenoestrogenic exposure by a biomarker approach: Application of the e-screen bioassay to determine estrogenic response of serum extracts. Environ Health 2:12.

Ren, X.M., Guo, L.-H. 2012. Assessment of the binding of hydroxylated polybrominated diphenyl ethers to thyroid hormone transport proteins using a site-specific fluorescence probe. Environmental Science and Technology 46: 4633-4640.

Rivas, A., Fernandez, M. F., Cerrillo, I., Ibarluzea, J., Olea-Serrano, M. F., Pedraza, V., and Olea, N., Human exposure to endocrine disrupters: standardisation of a marker of estrogenic exposure in adipose tissue. APMIS, 2001. 109(3): p. 185-197.

Rosenmai, A.K., Bengtström, L., Taxvig, C., Trier, X., Petersen, J.H., Svingen, T., Binderup, M.-L., Van Vugt-Lussenburg, B.M.A., Dybdahl, M., Granby, K., Vinggaard A.M. 2017. An effect-directed strategy for identification of hazardous chemicals in food contact materials of paper and board. Food Chem. Toxicol., 106, 250-259.

Schmitt, S., Reifferscheid, G., Claus, El., Schlusener, M., Buchinger, S. 2012. Effect directed analysis and mixture effects of estrogenic compounds in a sediment of the river Elbe. Environmental Science and Pollution Research 19: 3350-3361

Simon, E., Bytingsvik, J., Jonker, W., Leonards, P.E.G., De Boer, J., Jenssen, B.M., Lie, E., Aars, J., Hamers, T., Lamoree M.H. 2011. Blood plasma sample preparation method for the assessment of thyroid hormone-disrupting potency in Effect-Directed Analysis. Environmental Science & Technology 45, 7936-7944.

Simon, E., Velzen, M. van, Brandsma, B., Lie, E., Løken, K., Boer, J. de, Bytingsvik, J, Jenssen, B.M., Aars, J., Hamers, T., Lamoree, M.H. 2013. Effect-directed analysis to explore the polar bear exposome: the identification of thyroid hormone disrupting compounds in plasma. Environmental Science & Technology 47, 8902-8912.

Simon, E., Lamoree, M.H., Hamers, H., Boer, J. de. 2015. Challenges in effect-directed analysis with a focus on biological samples. Trends in Analytical Chemistry 67, 179-191.

Toft G, Long M, Kruger T, Hjelmborg PS, Bonde JP, Rignell-Hydbom A, Tyrkiel E, Hagmar L, Giwercman A, Spano M, Bizzaro D, Pedersen HS, Lesovoy V, Ludwicki JK, Bonefeld-Jorgensen EC. 2007. Semen quality in relation to xenohormone and dioxin-like serum activity among Inuits and three European populations. Environ Health Perspect. 115(1):15-20.

Tousova, Z., Oswald, P., Slobodnik, J., Blaha, L., Muz, M., Hu, M., Brack, W., Krauss, M., Di Paolo, C., Tarcai, Z., Seiler, T., Hollert, H., Koprivica, S., Ahel, M., Schollée, J.E., Hollender, J., Suter, M.J-F., Hidasi, A.O., Schirmer, K., Sonavane, M., Ait-Aissa, S., Creusot, N., Brion, F., Froment, J., Almeida, A.C., Thomas, K., Tollefsen, K.E., Tufi, S., Ouyang, X., Leonards, P., Lamoree, M., Osorio Torrens, V., Kolkman, A., Schriks, M., Spirhanzlova, P., Tindall, A., Schulze, T. 2017. European demonstration program on the effect-based and chemical identification and monitoring of organic pollutants in European surface waters. Science of the Total Environment 601-602, 1849-1868.

Vafeiadi M, Agramunt S, Papadopoulou E, Besselink H, Mathianaki K, Karakosta P, et al. 2013. In utero exposure to dioxins and dioxin-like compounds and anogenital distance in newborns and infants. Environ Health Perspect 121:125-130.

Van Wouwe N, Windal I, Vanderperren H, Eppe G, Xhrouet C, Massart AC, et al. 2004. Validation of the calux bioassay for pcdd/f analyses in human blood plasma and comparison with gc-hrms. Talanta 63:1157-1167.

Vilahur, N., Molina-Molina, J. M., Bustamante, M., Murcia, M., Arrebola, J. P., Ballester, F., Mendez, M. A., Garcia-Esteban, R., Guxens, M., Santa Marina, L., Tardon, A., Sunyer, J., Olea, N., and Fernandez, M. F., Male specific association between xenoestrogen levels in placenta and birthweight. Environ. Int., 2013. 51: p. 174-181.

Vilahur, N., Fernandez, M. F., Bustamante, M., Ramos, R., Forns, J., Ballester, F., Murcia, M., Riano, I., Ibarluzea, J., Olea, N., and Sunyer, J., In utero exposure to mixtures of xenoestrogens and child neuropsychological development. Environ. Res., 2014a. 134: p. 98-104.

Vilahur, N., Bustamante, M., Byun, H. M., Fernandez, M. F., Santa Marina, L., Basterrechea, M., Ballester, F., Murcia, M., Tardon, A., Fernandez-Somoano, A., Estivill, X., Olea, N., Sunyer, J., and Baccarelli, A. A., Prenatal exposure to mixtures of xenoestrogens and repetitive element DNA methylation changes in human placenta. Environ. Int., 2014b. 71: p. 81-87.

Vilahur, N., Bustamante, M., Morales, E., Motta, V., Fernandez, M. F., Salas, L. A., Escaramis, G., Ballester, F., Murcia, M., Tardon, A., Riano, I., Santa-Marina, L., Ibarluzea, J., Arrebola, J. P., Estivill, X., Bollati, V., Sunyer, J., and Olea, N., Prenatal exposure to mixtures of xenoestrogens and genome-wide DNA methylation in human placenta. Epigenomics, 2016. 8(1): p. 43-54.

Weiss, J.M., Simon, E., Stroomberg, G., De Boer, R., De Boer, J., Linden, S. van der, Leonards, P.E.G., Lamoree, M.H. 2011. Identification strategy for unknown pollutants using high resolution mass spectrometry: androgen disrupting compounds identified through effect-directed analysis. Analytical Bioanalytical Chemistry 400, 3141-3149.

Weiss, J.M., Andersson, P.L., Zhang, J., Simon, E., Leonards, P.E.G., Hamers, H., Lamoree, M.H. 2015. Tracing thyroid hormone disrupting compounds: database compilation and structure activity evaluation for an effect-directed analysis of sediment. Analytical Bioanalytical Chemistry 407, 5625-5634.