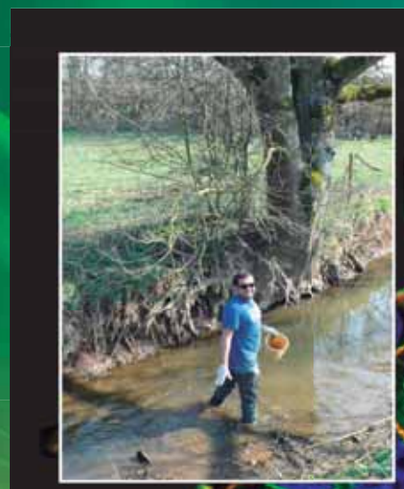

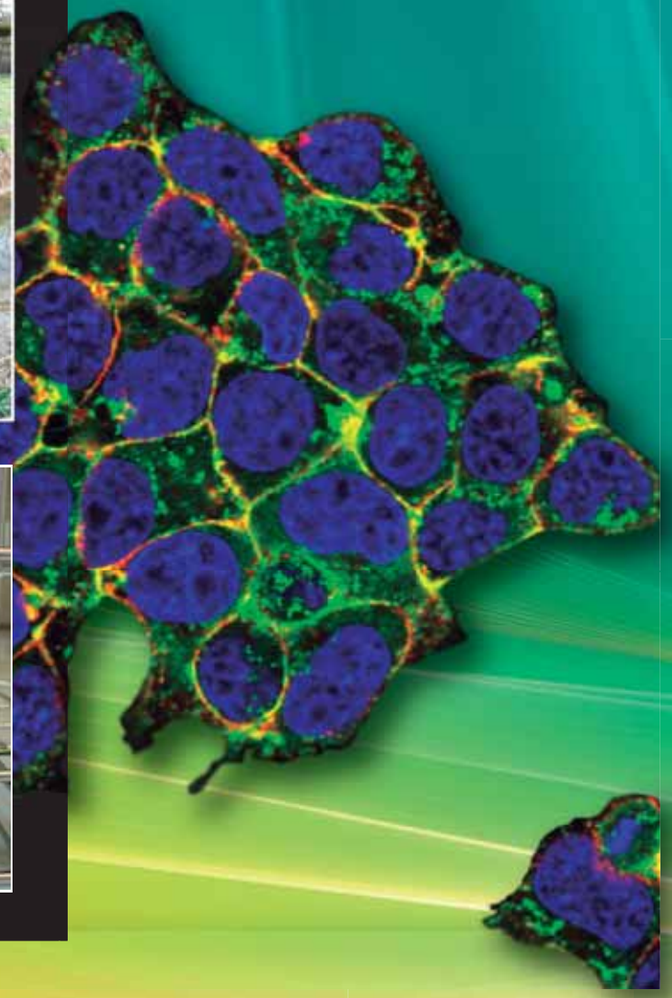

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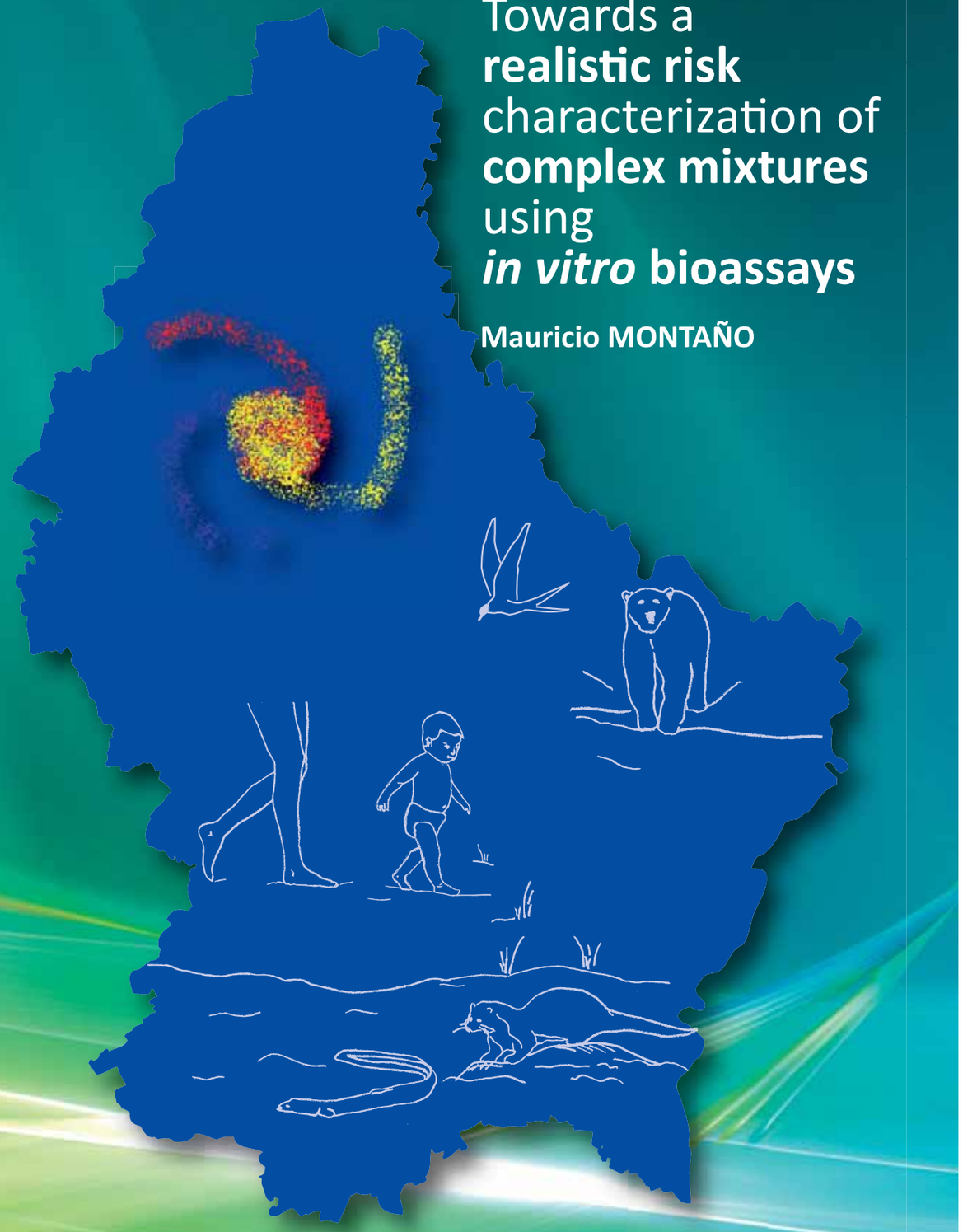

Towards a **realistic risk** characterization of **complex mixtures** using ***in vitro*** **bioassays**

Mauricio MONTAÑO

Towards a realistic risk characterization of complex mixtures using in vitro bioassays

Mauricio Montaño

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Towards a realistic risk characterization of complex mixtures using in vitro bioassays

Mauricio Montaño

Thesis

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Mauricio Montaño Garcés

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“It is important to strive for excellence but not to be subdued by it”

Prof. David Furlow

“What is science if not…

a handful of facts written in white parchment,

a mouthful of fruitful speculation,

and a slippery brain thought;

mixed together with the sparkling feeling

of an inquisitive hearth in an instance of lucidity.”

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CHAPTER 1

General introduction and thesis outline

Bioassay-based risk characterization of complex mixtures

"A daunting enterprise is to consider a very realistic exposure scenario that involve chemical mixtures"
(Letcher *et al.* 2010)
"A vast number of chemicals pervade our environment.
Exposures, weather simultaneous or sequential, are to chemical mixtures"
(Mumtaz 2010)

The air we breathe, the food we eat, the medicines we take, the clothes we wear, the water we swim and even the surfaces on which we crawl, walk, rest and sleep are all composed of a mixture of chemicals. Whether naturally or human produced, many are unknown to us, of a fair amount we are aware off, but only from a fraction we know their potential effects and interactions on human, wildlife and the environment.

Evaluation of potential human and environmental health hazards from exposure to chemical mixtures via the food chain and in the environment presents one of the most difficult challenges for risk assessment. While every living organism is exposed to mixtures of compounds, these mixtures are never the same. Governmental agencies, scientists and ultimately all individuals of our modern society are faced with the need of a proper assessment of the risk of complex mixtures to humans or the environment. For instance, food to be placed in the market, drinking water to be provided to the population and dredged spoils to be dumped; are all matrices for which certain risk assessment procedures exist. However, current legislation and regulation on risk assessment remain based on applied and basic sciences obtained with single substances (Mumtaz 2010). To cover this deficiency, research programs on mixture toxicology have been developed in recent years as well as the establishment of toxicological evaluation frameworks for chemical mixtures (Feron and Groten 2002; Groten *et al.* 2001; WHO 2009). Developing a suitable testing strategy to evaluate the potential risk of mixtures to humans and the environment is of very high scientific and societal interest.

Testing strategies involve chemical and biological methods to characterize the toxic potency of mixtures in several steps within the risk assessment cycle including hazard assessment, exposure assessment and monitoring. Along with chemical methods, *in vitro* and *in vivo* bioassays have been developing during the last decades to achieve the required quality and assurance standards for complex mixture testing (OECD 2012; OSPAR 1997; OSPAR 2009; WHO 2009).

Sediments are one example of a complex matrix of interest for environmental risk characterization. Sediments are fundamental to the wellbeing of aquatic ecosystems furnishing support, provision and regulation services (Gerbersdorf *et al.* 2011). However, sediments act as a storage compartment, serving as "secondary sources" of persistent organic pollutants (POPs) for other environmental compartments and organisms (Nizzetto *et al.* 2010). Not only can this impair the

health of the benthic, and thus aquatic, ecosystems, it encompasses an important route of exposure for aquatic food chains including fish, birds, mammals and eventually humans (Bosveld *et al.* 2000; de Boer *et al.* 2001; De Mul *et al.* 2008; Leonards *et al.* 1997; Moermond *et al.* 2004; Murk *et al.* 1998; Nyman *et al.* 2003; Schipper *et al.* 2009; van Leeuwen *et al.* 2007; Verslycke *et al.* 2005).

In response to the need of sediment risk characterization a series of integrated-effect based frameworks has been proposed. These include toxicity profiling (Hamers *et al.* 2010), effect directed analysis (EDA) (Brack *et al.* 2007), and tiered alternatives based on a series of tool-boxes combining *in vivo* and *in vitro* bioassays (Schipper *et al.* 2010).

In vitro bioassays have been developed to suit the complex mixture characterization needs covering endpoints such as survival, growth, reproduction, sensitization, genotoxicity, mutagenicity, neurotoxicity and particularly endocrine disruption (ED) (EC 2006; OECD 1992; OECD 2012; OSPAR 1997). In addition to general toxicity, assays for more mechanism-specific endpoints such as aryl-hydrocarbon receptor (AhR) activation, thyroid hormone disruption (THD), estrogenicity and steroidogenesis, among others, has been applied for risk characterization of mixtures of compounds in several complex matrices including sediments.

Despite the extensive and intensive improvements achieved in the development of *in vitro* methods, still some challenges remain to make them fully applicable for risk characterization of complex mixtures (Besselink *et al.* 2004; Hoogenboom *et al.* 2013; Hoogenboom *et al.* 2006b; Houtman *et al.* 2006b; Schipper *et al.* 2010; Van der Burg *et al.* 2010). These issues concern either the relevance or the reliability of the bioassay (OECD 2005). This thesis aims to better understand and further improve the relevance and reliability of *in vitro* bioassays for a biobased risk characterisation of complex mixtures, with special focus on POPs in sediments.

Mixture complexity

Compounds in mixture could greatly influence the behaviour of a potentially toxic substance increasing or decreasing its potential to produce an effect. Mixture components could influence a compounds fugacity from its exposure matrix, its susceptibility to degradation and even its bioavailability (Spurgeon *et al.* 2011). Once within the organism compounds could affect each other their absorption, distribution, metabolism, excretion and ultimately their capacity to exert effects at target sites. Interesting examples are the reduced absorption of tetracycline by several cationic ions due to reduced solubility and chelation (Reviewed by Spurgeon *et al.* 2011), and the increased metabolism of polyaromatic hydrocarbons

(PAHs) due to the presence of cytochrome P450 (CYP)-inducing dioxins (Shimada and Fujii-Kuriyama 2004).

Two recent publications compile the current knowledge and perspectives of mixture toxicology (Mumtaz 2010; van Gestel *et al.* 211). Both agree on how much we have already advanced in the understanding of chemical mixtures but how large is the extent of what we still do not know.

Complex mixture risk characterization

Since humans and their environments are exposed to a wide variety of substances, there is increasing concern in the general public about the potential adverse effects of the interactions between those substances when present simultaneously in a mixture. Although interactions between toxic compounds (including antagonism, potentiation, and synergism) have been described, they are not likely to occur at low exposure levels in a way that they are toxicologically significant (SCHER *et al.* 2012). For chemicals with common modes of action it is to be expected that they will act jointly to produce combination effects that can be described by dose/concentration addition. For current risk assessment of chemical mixtures, especially the lack of exposure information is limiting as well as the limited number of chemicals for which sufficient information on their mode of action is available (SCHER *et al.* 2012).

Whenever risk characterization of chemical contaminants is required, regulatory standards for environmental mixtures has been and still remains being heavily based on targeted chemical analysis. Examples are the water framework directive (WFD) (EC 2000), the Air Quality Directive (EC 2008a), Waste Framework Directive (EC 2008b), and dredged spoils licensing system (OSPAR 2008). Chemical analysis of contaminants has evolved to be a highly selective, exact, and sensitive technique for mixture characterization. However, chemical analysis of complex matrices and trace contaminants usually requires extensive temporal, technical and human resources. In addition, the steady flow of newly identified pollutants, including metabolites; adds up to the difficulties because it increases the demand for synthesis of pure standards and the need of continuous development of purification methods. Particularly in relation to complex mixture characterization another disadvantage of chemical targeted analysis is the inability to detect not yet known toxic compounds, including transformation products, or compounds present in concentrations below the limit of chemical detection (Schipper *et al.* 2010). Non-targeted analysis of emerging contaminants has been proposed as a solution to this shortcoming. However, up to date only very few compounds have been identified in environmental samples, and there is a need for the development of more

databases and libraries, as well as more efficient data mining methods (Zedda and Zwiener 2012).

Moreover, toxicological information about those newly discovered compounds often is lacking and current assessment methods for chemical mixtures based on chemical characterization, do not take proper account of joint actions and combined effect of chemicals in the mixture (SCHER *et al.* 2012).

Application of bioassays for complex mixture characterization

In addition to chemical methods, another possible approach to risk characterisation or safety evaluation of complex mixtures is testing toxic effects of the entire mixture (Groten *et al.* 2001). This approach is suitable for partially characterized but also for uncharacterized mixtures (Ragas *et al.* 2011). *In vivo* and *in vitro* bioassays, biosensors, and bioassay directed identification methods, have quickly evolved as the most important tools for whole mixture testing (Brack 2003; Chobtang *et al.* 2011; Hamers *et al.* 2010; Hoogenboom *et al.* 2013; Murk *et al.* 2013; Thain *et al.* 2008).

Initially, *in vivo* methods have been developed mainly for chemical risk characterisation (OECD 2012). They include assays with a variety of species such as annelids, crustaceans, amphibians, insects, fish and birds; and a variety of end points such as acute toxicity, growth, development, reproduction and metamorphosis (OECD 2012). Several of these assays have been further developed for monitoring of aquatic ecosystems (OSPAR 1997; OSPAR 2009), and adapted to be used for complex environmental mixtures (Schipper *et al.* 2009; Thain *et al.* 2008; Vethaak *et al.* 2005).

In vivo bioassay can provide a stronger relation of causality between contaminants and ecological responses and are expected to take bioavailability and toxicokinetics into account (Legler *et al.* 2002b; Maas and Vand den Heuvel-Greve 2005; Schipper *et al.* 2009). However, as stated by John E. Thain, Dick Vethaak and Ketil Hylland "As the level of biological complexity increases, so too does the ecological relevance of any contaminant effect; however, this is in turn mirrored by decreasing responsiveness, detectability, and mechanistic understanding" (Thain *et al.* 2008). Additional challenges of *in vivo* assays are the occurrence of false positives induced by matrix factors that can impair the health of the test animals (e.g. sulphur, ammonium or low oxygen levels) and to reduce natural variability. Solutions to these problems has been achieved through standardization (Thain *et al.* 2008), the use of cultured species (Schipper *et al.* 2008) and artificial synchronization methods (Gutleb *et al.* 2007b).

Newer developments include *in vitro* bioassays developed for a suit of toxic mechanisms including dioxin-like toxicity and endocrine disruption (ED) (EC 2006; OECD 1992; OECD 2012; OSPAR 1997). Among *in vitro* bioassays for complex mixtures, mechanism-based assays have been favoured and applied to several fields of complex mixture risk characterization such as EU regulations for food (EC 1883/2006) and feed (EC 152/2009) (Hoogenboom *et al.* 2013), sediments (OSPAR 1997), and dredged materials (OSPAR 2008). In addition, mechanism-based reporter-gene assays to test ED endpoints have been successfully applied to human fluids (Murk *et al.* 1997; Van Wouwe *et al.* 2004b), food and feed (Hoogenboom *et al.* 2006a; Hoogenboom 2002; Hoogenboom *et al.* 2006b); and to environmental matrices including air pollution (Hamers *et al.* 2000), pore water (Koh *et al.* 2002; Murk *et al.* 1996), soil (Nording *et al.* 2007), and sediments (Legler *et al.* 2002a; Stronkhorst *et al.* 2003).

Compared with *in vivo* tests, *in vitro* bioassays can handle a broader range of doses with usually much lower sample quantity. They require less initial investment, infrastructure, human resources and time, which make them adaptable to high throughput (Blaauboer 2008; Bolt and Hengstler 2008; Murk *et al.* 2013). A very important advantage in developing *in vitro* bioassays is to accomplish with the reduction and ultimately the replacement of animal use for toxicological testing (Jacobs *et al.* 2008). Compared with chemical analysis, *in vitro* bioassays are capable of detecting any potent substance in the mixture, including not yet known agonist or substances below chemical detection limits (Schipper *et al.* 2010). Therefore, *in vitro* bioassays indeed take into account join action and combined effect of active compounds in the mixture. In addition, in vitro methods are usually performed with extracts which reduces the presence of unwanted matrix factors (Schipper *et al.* 2010). Depending on the compound classes of interest, destructive cleaning methods can be applied to further remove matrix components.

The development of bioassay test methods has focused on standardization, transferability and reproducibility (OECD 2005). For instance, an important prerequisite for the application of *in vitro* bioassays in a licensing system is a high standard of quality assurance and quality control to guarantee the data on which the assessment is to be based (Schipper *et al.* 2010; Stronkhorst *et al.* 2003; US EPA 1995). Hence a great deal of effort has been place to develop standardized protocols for *in vitro* bioassays (Besselink *et al.* 2004; Hoogenboom *et al.* 2013; Hoogenboom *et al.* 2006b; Houtman *et al.* 2006b; Van der Burg *et al.* 2010). Furthermore, emphasise has been placed on standardization of sample preparation methods (Houtman *et al.* 2007; Schwirzer *et al.* 1998; Seiler *et al.* 2008; Van Wouwe *et al.* 2004a) as well as an understanding of the assays most critical parameters to achieve reliable results (Hoogenboom *et al.* 2011; Windal *et al.*

2005). However, *in vitro* bioassay still poses some challenges which can influence their relevance and reliability for their use in complex mixture risk characterization.

Relevance and reliability of *in vitro* bioassays

"The committee foresees that *in vitro* assays will make up the bulk of the toxicity test in its vision"
(Committee on toxicity testing and assessment of environmental agents.
"Toxicity testing in the 21st century." (NRC 2007)

The international harmonized criteria for scientific validation and regulatory acceptance of hazard test methods defined validation as "the process by which the method's reliability and relevance are established" (OECD 2005).

Bioassay relevance and the issue of metabolic activation

The relevance of a test method is defined as "the extent to which the test method correctly measures or predicts the (biological) effect of interest, as appropriate." (OECD 2005). It is ultimately an indication of how meaningful and useful are its results for the specified purpose.

Complex mixture frameworks, particularly for environmental applications, have addressed the importance of using relevant methods for risk characterization (Benedetti *et al.* 2012; Hamers *et al.* 2010; Schipper *et al.* 2010). Particularly Schipper and co-workers (2010) have considered the most important elements for a rational application of *in vitro* and *in vivo* bioassays to dredged sediments licensing.

One of the validation principles in the harmonized criteria (called the "Solna Principles") is the relationship between the test method's end point and the biological phenomenon of interest (OECD 2005). An important aspect of this principle is whether metabolic capability is considered within the relevance evaluation.

The importance of biotransformation within the assessment of potential risks of substances has been increasingly raised attention and concern (Coecke *et al.* 2006; Murk *et al.* 2013). In this direction the USA National Research Council has emphasized the need to include metabolite function and effects within testing strategies (NRC 2007). Currently biotransformation is not generally considered within *in vitro* toxicity testing frameworks; with the exception of genotoxicity and hepatotoxicity assays (Coecke *et al.* 2006). Nevertheless, there are plenty of examples of toxic effects depending on biotransformation of parent compounds including allergens, polyaromatic hydrocarbons (PAHs), methoxychlor, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) (Coecke *et al.* 2006; Legler *et al.* 2002a; Marsh *et al.* 2006; Qiu *et al.* 2007).

An example of particular interest for their thyroid hormone disruptive (THD) potency, is the metabolic activation of PCBs and PBDEs into OH-metabolites (Brouwer *et al.* 1998; Meerts *et al.* 2000). Interestingly, these hormone-like hydroxyl (OH-) metabolites are retained in plasma due to their capacity for binding to thyroid hormone binding proteins (THBPs) like transthyretin (TTR), thyroxin-binding globulin (TGB) and albumin (Brouwer and van den Berg 1986). This binding protects them from further degradation and excretion, whereas they are transported via these proteins to various tissues and over selective barriers to the brain and the placenta (Meerts *et al.* 2002; Morse *et al.* 1993).

POP bioactivation and THD effects

Monitoring programmes of exposed population have shown the accumulation of OH-PCBs and OH-PBDEs in humans and wildlife (Bergman *et al.* 1994; Letcher *et al.* 2010; Sandau *et al.* 2000). Also THD *in vitro* analysis of pure standards from POP metabolites has shown a greater toxicological relevance of OH-metabolites compared to the parent compounds (Freitas *et al.* 2011; Hamers *et al.* 2008; Lans *et al.* 1993; Lans *et al.* 1994; Meerts *et al.* 2001; Song *et al.* 2008).

However, the *in vitro* analysis of metabolites present in complex, and especially biological matrices, has not been straightforward (Hamers *et al.* 2008; Meerts *et al.* 2000; Schriks *et al.* 2006; Simon *et al.* 2010; Simon *et al.* 2011). Several attempts has been made to analyse the potency of POP metabolites, either produced with exogenous metabolizing systems (Hamers *et al.* 2008; Meerts *et al.* 2000; Schriks *et al.* 2006), or from biological matrices such as plasma (Simon *et al.* 2010; Simon *et al.* 2011). These attempts have been hampered by low biotransformation efficiencies, presence and toxicity of parent compounds and interfering matrix components; or it has required extensive clean-up procedures which could still not prevent the presence of interfering co-extractants in the analysis.

The limitations of chemical analysis to measure the total burden of POP metabolites along with the difficulties experienced in the application of bioassays to their total THD potency has opened a debate on the toxicological and health relevance of POP metabolites for wildlife and humans (Liu *et al.* 2012; Wan *et al.* 2009; Wiseman *et al.* 2011).

Bioassay reliability: achievements and challenges

The reliability of a test method is defined as “the extent of reproducibility of results from a test within and among laboratories over time, when performed using the same standardised protocol.” (OECD 2005).

The tendency of higher quantified potency with in vitro bioassays compared to chemical analysis has been labelled as overestimation of the toxicity, and has been

used to criticize *in vitro* bioassays. There are, however, established reasons to explain those differences between reporter-gene assays such as the dioxin-receptor activation assay DR.Luc and the standard chemical analysis with capillary gas chromatography coupled to high resolution mass spectrometry (GR-HRMS) (Hoogenboom *et al.* 2011; Windal *et al.* 2005). Bioassays include the toxicity of active compounds even present in levels below the limit of chemical detection, as well as unknown compounds or compounds that cannot be analysed due to practical reasons. Also potential interactions between compounds cannot be excluded, and differences may exist between relative toxic potencies used to calculate the total toxic potency of the chemically analysed compounds and the relative toxic potencies of those compounds in the in vitro bioassays (Van den Berg, 1988). In addition to these fundamental issues, a series of technical recommendations has been offered to improve reporter-gene assay reliability (Hoogenboom *et al.* 2011; Schipper *et al.* 2010; Windal *et al.* 2005), including:

- The use of cleaned and fractionated extracts to isolate desired active compounds
- The use of appropriate solvents, absorbents and materials to avoid contamination, analysis of procedural blanks for background signal must be included.
- The relevance of the cellular model for the intended toxicological end-point
- The variation of response with the analysed dose and the use of multiple sample dilutions for reliable potency quantification

As stated by Goeyens and co-workers " observed discrepancies should not be understood as absence of success in the initial efforts but rather as encouragements with positive effects on the pace of further development (Goeyens *et al.* 2010).
Two aspects that could influence the outcome of *in vitro* bioassay based quantification of toxic potencies are: 1) the occurrence sometimes of responses higher than the theoretical maximum based on the positive control for the assay (a phenomenon referred to as supramaximal (SPMX) effect) , and 2) the possible consequences of overconcentration of sample extracts during solvent change.

The SPMX effect in in vitro bioassays

The phenomenon that a response for a particular ligand is significantly higher than the maximal response of the endogenous substance, has been observed for *in vitro* estrogenic assays with several pure substances (Freyberger and Schmuck 2005; Kitamura *et al.* 2005; Legler *et al.* 1999; van Lipzig *et al.* 2005) and also for reporter-gene dioxin-like assays in pore water (Jonker *et al.* 2006), crude and refined petroleum products (Vrabie *et al.* 2009), and sediments (Murk *et al.* 1996) even after thorough clean-up (Baston and Denison 2011).

Weather an SPMX effect is an assay-based artefact or a real-life phenomenon with toxicological relevance is still unknow for certain. It has been demonstrated that activation of protein kinase C and inhibition of protein synthesis can synergistically enhance aryl-hydrocarbon (AhR)-dependent gene expression using purified compounds and under experimentally controlled conditions. But the importance for the potency quantification for environmental samples remains to be confirmed (Baston and Denison 2011). Estrogenic SPMX effect produced by genistein in breast cancer cells stably transfected with a luciferase reporter-gene (T47D.Luc) was ascribed to post-transcriptional stabilization of the firefly luciferase reporter enzyme, increasing the bioluminescence signal which would imply that an SPMX effect is not a biologically relevant phenomenon (Sotoca *et al.* 2010).

As the quantification of the estrogenic and/or dioxin-like activity of environmental contaminants, both individually as well as in mixtures, is based on the response relative to that of estradiol or TCDD respectively; an SPMX effect could lead to overestimation of the quantified potency. Although a normalization method has been offered for potency quantification of SPMX inducer sediments (Baston and Denison, 2010), the drivers and underlying causes of SPMX effects in in vitro bioassays remain an intriguing and relevant phenomenon for toxicology and particularly for toxicological risk characterization. Unfortunately the occurrence of SPMX effects has not been consistently reported in scientific literature, making it difficult to draw conclusions from the sometimes seemingly contradictory results.

Influence of stock concentration on the quantification outcome

It has been observed that original extract stock concentrations can significantly influence the observed estrogenic potency of waste treatment plant effluents (Murk *et al.* 2002). The authors suggested a loss of compounds during solvent exchange when the maximum solubility is approached. For dioxin-like compounds this has not been studied before. Nevertheless, it is a common practice to concentrate the sample as much as possible to be able to quantify the toxicity of less polluted samples. In practise a range of 1-200 g sediment equivalents/ml of DMSO are being used for testing in in vitro bioassays (e.g. Nording *et al.* 2007; Ocampo-Duque *et al.* 2008) without knowing the consequences for the ultimate dioxin-toxicity quantification. In addition, overloading clean-up columns needed for preparing samples to be tested in the dioxin-like DR.Luc results in residues of unwanted compounds such as PAHs to be present in the final extract (Schwirzer *et al.* 1998), including alkylated- and nitro-PAHs (Dindal *et al.* 2011). These compounds can induce AhR-dependent activity whereas their mechanism of toxicity is totally different from that of dioxins (Vondrácek *et al.* 2001). Most PAHs can be metabolised by cytochrome P450 enzymes that are present in H4IIE rat

hepatoma cells of the DR-Luc, hence increasing the time of exposure to 24 hours or more facilitates more PAHs in the sample to be metabolized reducing their influence on the bioassay response (Hamers *et al.* 2000; Vondrácek *et al.* 2001).

It is evident that for reliable in vitro bioassay-based risk characterisation the influence of sample preparation, initial sample extract stock concentration, and interpretation of SPMX effects need to be properly addressed.

In vitro bioassay characterization of sediments

"Secondary sources (of POPs) already represent a significant fraction of the total source inventory…" (Nizzetto *et al.* 2010)

Sediments probably are one of the most complex matrices for environmental risk characterization as they even can accumulate very lipophilic compounds bound to organic material including historical contaminations. In addition biotransformation products are being formed by local microbial communities. For proper functioning of aquatic ecosystems, healthy sediments provide fundamental furnishing support, provision and regulation services (Gerbersdorf *et al.* 2011). Contaminated sediments are an important route of exposure for aquatic food chains including fish, birds, mammals and eventually humans (Bosveld *et al.* 2000; de Boer *et al.* 2001; De Mul *et al.* 2008; Leonards *et al.* 1997; Moermond *et al.* 2004; Murk *et al.* 1998; Nyman *et al.* 2003; Schipper *et al.* 2009; van Leeuwen *et al.* 2007; Verslycke *et al.* 2005).

Sediment quality guidelines (SQG) have been developed by several governmental agencies (Iannuzzi *et al.* 1995) in order to safeguard a good environmental quality and provision of safe food. With few exceptions, including The Netherlands (Schipper *et al.* 2010)), the assessment of sediment compliance remains based on a chemical analytical approach (OSPAR 2004; OSPAR 2008). Despite the establishment of bioassay response level for assays such as dioxin-like and estrogenic activity (Hamers *et al.* 2010; Stronkhorst *et al.* 2003).

In response to the need for demonstration of *in vitro* bioassay suitability and reliability for sediment risk characterization, a series of integrated-effect based frameworks has been proposed. These include: 1) toxicity profiling of sediments with a battery of specific bioassays (Hamers *et al.* 2010); 2) effect directed analysis (EDA) which integrates toxicity testing, fractionation and non-target chemical analysis (Brack *et al.* 2007); and 3) a tiered alternative based on a series of tool-boxes combining *in vivo* and *in vitro* bioassays (Schipper *et al.* 2010).

Sediment hazard assessment, and particularly in relation to endocrine disruption (ED) effects (e.g. estrogenic, androgenic, thyroid hormone disturbance), has revealed that mainly the compounds in polar fractions of sediment extracts have

ED potencies (Brack *et al.* 1999; Houtman *et al.* 2006a; Legler *et al.* 2002a; Legler *et al.* 2003; MODELKEY 2013), particularly after the application of EDA (Brack *et al.* 2002; Brack *et al.* 2005; Higley *et al.* 2012; Lübcke-von Varel *et al.* 2011).

Nonpolar fractions of sediment extracts have been reported to exhibit very little or no *in vitro* ED effects (Higley *et al.* 2012; Lübcke-von Varel *et al.* 2011). Nevertheless, lipophilic POPs such as PCBs and PBDEs, present within the nonpolar fractions of sediments, are known to induce developmental, steroidogenic and THD effects *in vivo* (Chiu *et al.* 2000; Legler 2008). Even more striking, severe perturbations of thyroid hormone-dependent metamorphosis of amphibians (Gutleb *et al.* 2007a; Gutleb *et al.* 2007b) has been observed after exposure to nonpolar sediment extracts, not to the polar ones. This putative contradiction between in vitro and in vivo toxic effects of compounds present in the lipophilic sediment fraction requires further understanding of the mechanisms underlying the false negative *in vitro* responses and solutions to be able to better apply *in vitro* hazard characterization of sediments.

Thesis outline

This PhD aims at increasing the relevance and reliability of *in vitro* bioassay for complex mixtures by bringing further the understanding of above-mentioned challenges and developing suitable solutions to them. The focus of the current PhD research is on the use of *in vitro* bioassays for testing POPs for dioxin-like potency and for thyroid hormone disruption (THD) in the risk characterization of persistent organic pollutants (POPs) in sediment samples. This includes the issues of SPMX for which lessons can be learned from the better studied estrogenic compounds; of *in vitro* metabolism for THD compounds; and of sample preparation. The lessons learned will also be applicable to POPs in other, often less complex, matrices such as sea food. Figure 1 depicts the outline of this PhD study.

In **Chapter 2** a meta-analysis is performed to study the relevance hydroxylated compounds in humans and wildlife. . It reviews reported body burdens of halogenated phenolic contaminants (HPCs), including OH-POP in different tissues from humans and wildlife species. Their concentrations are analysed in relation to those of their putative parent compounds and the probable sources of exposure. The blood plasma levels are compared with known in vivo and in vitro toxicological threshold concentrations. This enables interpretation of the internal exposure and the toxicological implications thereof for humans and wildlife.

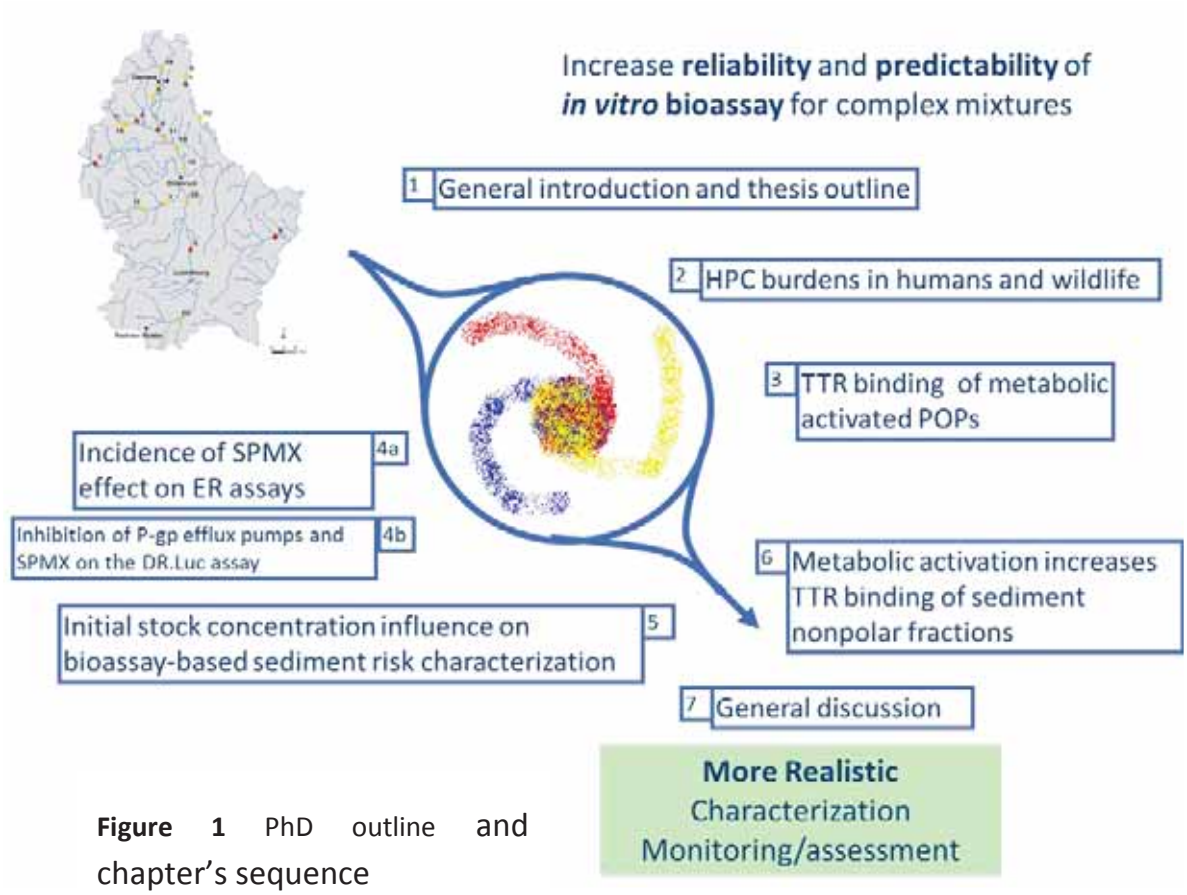

Figure 1 PhD outline and chapter's sequence

Given the toxicological relevance of the OH-POPs ,**Chapter 3** aims at providing solutions to the long standing problem of the in vitro production and analysis of OH-POP metabolite potency to bind plasma thyroid hormone binding proteins (THBPs) when present in biological matrices. In this chapter : 1) co-extractants are identified and quantified in the microsomal extracts after in vitro metabolisation; 2) the potency of these co-extractants to inhibit the thyroid hormone- transthyretin (T_4-TTR) plasma carrier protein competitive binding assay are quantified; 3) a method is developed to selectively extract metabolites and eliminate disturbing co-extractants; and 4) the newly developed method is applied to analyze the potency of bio-activated extracts from the model compounds CB 77 and BDE 47 . To support these objectives, 5) a selective chromatographic method was developed to analyze silylated derivatives from CB 77 and BDE 47 OH-metabolites and co-extractants, and 6) a non-radioactive T_4-TTR competitive fluorescence displacement method was set up in a 96-well plate .

The **chapters 4 and 5** are committed to tackle the issues of SPMX and sample extract concentration which are crucial to assess the reliability of *in vitro* bioassays for quantification of the toxic potencies of complex mixtures.

As the SPMX effect has been more elaborately reported for *in vitro* estrogenicity assays, a detailed meta-analysis was performed of the assays, compounds and conditions in which the effect is observed (**Chapter 4a)**. It includes a detailed analysis of assay characteristics for the most common SPMX inducers diethylstilbestrol (DES), genistein and bisphenol A, and the likelihood of producing a SPMX effect.

Several SPMX inducers also block cellular efflux pumps in vivo and in vitro (Anselmo *et al.* 2012; Georgantzopoulou *et al.* 2013). Therefore the hypothesis was tested that efflux pump blockers present in environmental matrices would increase the internal concentration of bioassay agonists and thus cause the SPMX. In **Chapter 4b** a 96-well plate cellular efflux pump inhibition assay (CEPIA) was adapted to the DR.Luc cell line and subsequently the influence evaluated of various environmentally relevant efflux pump inhibitors on the 2,3,7,8-tetrachlorodibenzo-p-dioxine (TCDD) response.

Considering the influence that the contaminant load can have on the performance of in vitro bioassays, we hypothesize that the use of overconcentrated stocks of sediment extracts can potentially cause wrong quantifications of toxic potencies. **Chapter 5 reveals** the effects of initial stock concentrations, on the quantified dioxin-like potency of cleaned nonpolar sediment extracts in an in vitro reporter gene assay. In addition the role of including sonication assisted dissolution and exposure time are studied. The consequences of sample preparation-related false positive or negative quantified toxic potencies of the sediment is related to safe levels set in sediment quality guidelines (SQG) and the impact on the management decision process. Suggestions are made to improve and standardize sample preparation practise and bioassay-based sediment potency quantification minimizing their modulating impact on its risk characterization.

Finally, in **Chapter 6** of this thesis, all methods and concepts developed in previous chapters are applied to non-polar extracts from highly or less contaminated sediments collected in Luxembourg, with the focus on the relevance of metabolic activation in *in vitro* analysis of the THD potency of the sediment extracts in the TTR-competitive binding bioassay. The extracts are either roughly split into a lipophilic and polar fraction using solvent partition fractionation, or fractionated into eight sub fractions with increasing polarity by normal phase HPLC. The model compounds CB77, BDE47 and the sediment extract fractions were tested for TTR binding potency before and after metabolic activation. The TTR competitive binding potency of the metabolic extracts is tested in the recently developed non-radioactive 96-well plate ANSA-TTR assay, including removal of lipids from the extracts to avoid interferences in the assay.

Chapter 7 discusses the implications of our results to improve the relevance and reliability of *in vitro* bioassay applied for risk characterisation of complex mixtures from sediments and other matrices. It considers various aspects of the newly developed methods and knowledge acquired within this PhD project on *in vitro* bioassay risk characterization of sediments and other complex mixtures. It includes future perspectives for the application of in vitro bioassays in this field and discusses remaining issues of concern and the knowledge gaps to tackle with further research.

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CHAPTER 2

Persistent toxic burdens of halogenated phenolic compounds in humans and wildlife

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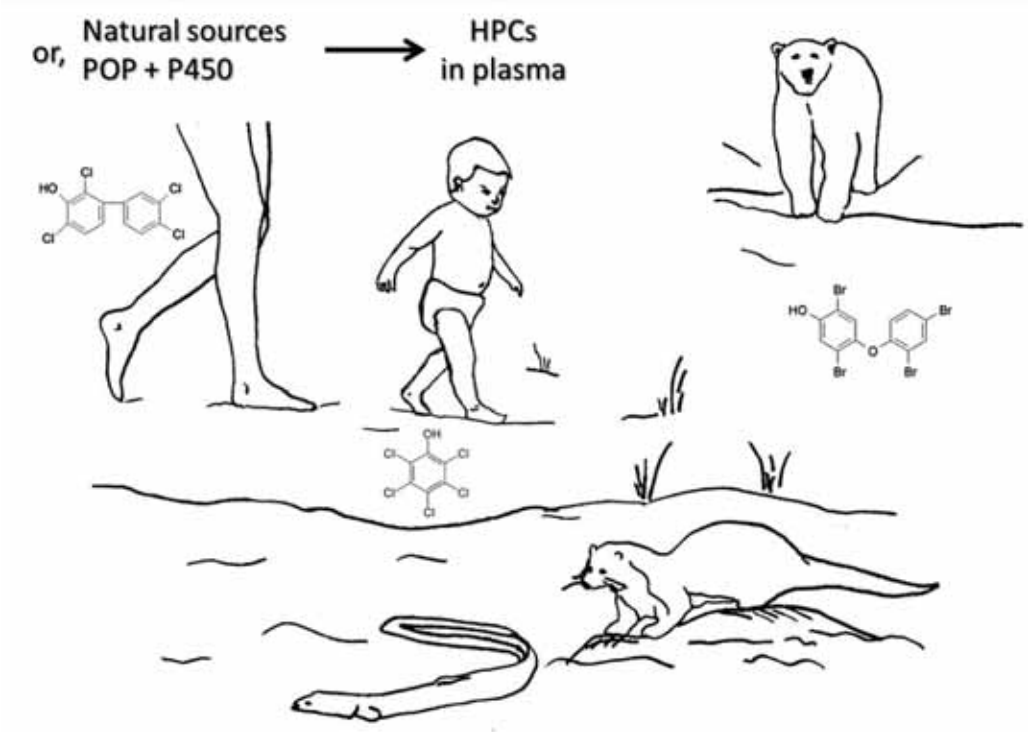
Abstract

Halogenated phenolic compounds (HPCs) including hydroxylated polychlorobiphenyls (OH-PCBs) and hydroxylated polybromodiphenyl-ethers (OH-PBDEs) can be persistent organic pollutant (POP) metabolites or natural marine compounds. Structurally similar to thyroid hormones (THs), they are retained in blood, transported through selective barriers, and the cause of endocrine and neuronal POP effects. This study presents a meta-analysis of HPC burdens in human and wildlife tissues, including OH-PCBs, OH-PBDEs, Pentachlorophenol, and polybromophenols. HPC blood plasma levels were also compared to known *in vitro* and *in vivo* toxicological effect concentrations. Blood, highly perfused, and fetal tissues contained highest levels of HPCs. Plasma concentrations of analyzed OH-PCBs/PBDEs ranged from 0.1 to 100 nM in humans and up to 240, 454, 800, and 7650 nM for birds, fish, cetaceans, and other mammals, respectively. These concentrations fully fall within the i*n vitro* effect concentrations reported in literature for HPCs of 0.05–10000 nM. We strongly advise to further study HPC blood levels in the general population, children, and fetal tissue to establish background levels and the risk at sensitive development stages. As not all HPCs are, or can be, chemically analyzed, the application of additional bioanalysis might reveal an even greater toxicological relevance of HPCs. In addition, metabolic activation should always be included within *in vitro* hazard assessment of POPs.



Introduction

Persistent organic pollutants (POPs) such as polychlorobiphenyls (PCBs) and polybromodiphenyl-ethers (PBDEs) are widely distributed in biotic and abiotic compartments, but a reduction or a leveling in biota has been observed during recent years (Letcher *et al.* 2010). However, contaminated environmental compartments such as soils and sediments serve as storage and act as "secondary sources" (Nizzetto *et al.* 2010). These POPs are accumulated by the organisms, magnified through the food chain (Kelly *et al.* 2008), and can be transformed into hydroxylated- (OH- containing) metabolites (Letcher *et al.* 2000; Malmberg *et al.* 2005; Murk *et al.* 1994). Monitoring and effects analysis of POPs is mostly focused on the parent compounds, for which standards and analytical techniques are widely available, extraction procedures are relatively straightforward to apply, and environmental concentrations are higher compared to OH-PCBs/PBDEs and other halogenated phenolic compounds (HPCs). However, effects of PCBs and PBDEs in the endocrine and neuronal systems have been attributed to the action of their OH-metabolites (Brouwer *et al.* 1998; Dingemans *et al.* 2011; Fonnum *et al.* 2006).

Chlorinated and brominated HPCs are usually reported and analyzed in separate publications. Among HPCs, OH-PCBs and 4-hydroxy-heptachlorostyrene (4OH-HpCs) are believed to be metabolites from anthropogenic PCBs and octachlorostyrene, respectively (Bergman *et al.* 1994; Sandau *et al.* 2000). OH-PBDEs and bromophenols (BPhs) are naturally present in the marine environment (Guitart *et al.* 2011; Malmvärn *et al.* 2005; Teuten *et al.* 2005) or they could be metabolites of PBDEs (Marsh *et al.* 2006; Qiu *et al.* 2007). A number of tri-BPhs are commercially produced as flame retardants and wood preservatives (Howe *et al.* 2005). Pentachlorophenol (PCP) was used as wood preservative but is also a metabolite from hexachlorobenzene (HCB) (Renner 1988). These HPCs have been detected and quantified in blood from humans, other mammals, birds, and fish (Athanasiadou *et al.* 2008; e.g.Sandau *et al.* 2000; Valters *et al.* 2005; Verreault *et al.* 2005a).

The basis for the retention of HPCs is their structural resemblance with the thyroid hormones (THs) 3,3',5,5'-tetraiodo-L-thyroxin (thyroxine, T_4) and 3,3',5-triiodo-L-thyronine (T_3) (Lans 1995). Therefore HPCs with the hydroxyl group in the para or meta position, with one, but preferably two, halogens in the adjacent position (Lans *et al.* 1993) bind with high affinity to thyroid-hormone-binding proteins (THBP) such as transthyretin (TTR), thyroxin-binding globulin (TGB), and albumin (ALB) (Marchesini *et al.* 2008; Ucan-Marin *et al.* 2010). This protects them from phase II metabolism and excretion, by retaining them in blood and facilitating their transport over selective barriers to the brain or the fetus (Meerts *et al.* 2002; Morse *et al.* 1993).

The competition of HPCs with thyroid hormones (THs) for the available transport proteins may cause direct effects on thyroid hormone homeostasis (Gutleb *et al.* 2010) but more importantly it allows delivery of HPCs instead of hormones to tissues (Zoeller *et al.* 2007), including endocrine targets (He *et al.* 2008; Meerts *et al.* 2001) and the neuronal system (Dingemans *et al.* 2011). In fact, endocrine, developmental, and behavioral effects were observed upon direct exposure to 4-OH-CB107 (e.g.Meerts *et al.* 2004b)

Three recent publications from 2000, 2005, and 2010 have thoroughly reviewed the concentrations of various HPCs in wildlife (Kawano *et al.* 2005; Letcher *et al.* 2010; Letcher *et al.* 2000). To our knowledge, there is no comparable analysis available for human populations. In addition, observed concentrations of HPCs either in humans or in wildlife have not been compared with relevant toxicological information. Considering the increasing evidence of HPC toxicity, it is of great relevance to review the reported concentrations in relation to their observed effects. The aim of this meta-analysis is to review reported tissue-specific HPC body burdens of humans and wildlife species, to analyze their concentration in relation to their putative parent compounds, and to relate them to relevant toxicological threshold concentrations. This will allow a more realistic assessment of the potential hazard of this class of compounds and the relevance of metabolic activation of POPs.

Methods

Literature search. Key words from the references cited by a recent POP exposure review (Letcher et al. 2010) were used in a scientific bibliographic search through the databases Scopus (Elsevier B.V.) and PubMed (NCBI). The keywords were classified into four categories: parent compounds (e.g., PCB or "polychlorinated biphenyls"), chemical group (e.g., metabolites or hydroxylated), biological compartment (e.g., tissue or blood), and endpoint (e.g., effect or level). From the reference and citation list from each retrieved publication, relevant publications were also collected.

Selection criteria. Papers with reported OH-PCB and/or OH-PBDE concentrations in tissues, published in peer-reviewed journals in English up to 2012 were included. Reports for PCP, tri-BPhs OH-HpCs, and TBBPA were only included in the analysis if OH-PCBs and/or OH-PBDEs were also reported. For separate publications reporting the same population and same sampling campaign, the first publication from the group or that one in which the data had been described with the highest level of detail was chosen for the database.

Collected data. The following information was tabulated from each publication when reported: bibliographic data, subject or population data (species, gender,

age, year, region, exposure level, and number of individuals), % lipid content, individual concentrations of most abundant congeners, as well as lowest, average (arithmetic or geometric mean, or median), and highest reported sums of congeners from PCBs, PBDEs, OH-PCBs, OH-PBDEs, PCP, tri-BPhs and OH-HpCs (database accessible through the WUR library E-depot: http://edepot.wur.nl/257263, excel file available upon request to the corresponding author). From each publication more than one entry could result depending of the number of species, tissues or conditions reported.

Analysis and calculations. Concentrations were harmonized to ng/g wet weight (ng/g ww) for all tissues. When the % of lipid was not reported, an average value from similar publications was used. Plasma, serum, and whole blood were all considered as blood and its density was set at 1 g/mL. Blood concentrations of HPCs were transformed into nanomol/liter (nM) units to allow comparison with *in vitro* toxicity data. The average molecular weights from the collected congeners used to calculate the nM values were 378.8 for OH-PCBs and 500.9 for OH-PBDEs. Exact molecular weights were used for PCP (266.4), 4-OH-HpCs (361.5), and TBPs (330.7). The uncertainty of these calculations could lead to up to 80% variation, which is however below the data dispersion within reports. To reduce uncertainty from differences in sample composition and detection capabilities, results from the lowest, average, and highest reported values were combined in boxplots to show the tissue concentrations and variability in humans and wildlife. Graphs and regressions were made in SigmaPlot (SPSS).

Results and discussion

In total, 75 publications met the inclusion criteria to be in the database. Four studies with experimentally exposed animals were included into the database because they give insight in the mechanism of retaining and effects of metabolites, but not into the quantitative analysis of naturally exposed animals and humans. As a result, 211 entries were included in the quantitative analysis (Table 1). Although a reduction in OH-PCBs has been observed through the past decade (data not shown), reports before 2004 surveyed especially contaminated populations, whereas recent studies reported mainly on general populations, confounding a potential temporal analysis and perhaps influencing the overall results toward higher burdens.

In addition to the HPCs included in the analysis, data for hydroxyl-polybromobiphenyls (OH-PBBs) have been reported in polar bear adipose, brain and liver, and ringed seal blubber at similar or even higher concentrations compared with OH-PBDEs (Gebbink *et al.* 2008; Letcher *et al.* 2009). However, OH-

PBBs seem to have a preference for adipose tissue retention (Gebbink *et al.* 2008) and their potency to bind THBPs is yet to be investigated.

Species and tissue concentrations of HPCs.

Reported human and wildlife tissue concentrations were, in general, 10 to 100 fold higher for OH-PCBs (Figure 1A) compared to OH-PBDEs (Figure 1B). Blood and highly perfused tissues such as liver and brain contained higher OH-PCBs/PBDEs compared with muscle, blubber, and adipose tissue (Figure 1). Levels in tissues and body compartments with few entries such as human milk and umbilical cord; fish eggs, cetacean cerebrospinal fluid, milk and liver; and polar bear brain and liver are listed in the Supporting Information but were not used in the analysis.

Studied human populations were mainly from North America, Japan and Europe with a few exceptions from Nicaragua and India. Human OH-PCB/PBDE concentrations in tissues were the lowest among species. Values of OH-PCBs in plasma from human males were considerably higher compared to those for mixed population and females (Supporting Information, Figure SF1). Higher OH-PCBs also have been observed in male dolphin plasma (Houde *et al.* 2006). Although the authors attribute this to higher CYP activity induced by elevated PCB burdens, lower levels in females could also be the result of maternal transfer to offspring via lactation and deposition in eggs (Jörundsdóttir *et al.* 2009; Jörundsdóttir *et al.* 2010).

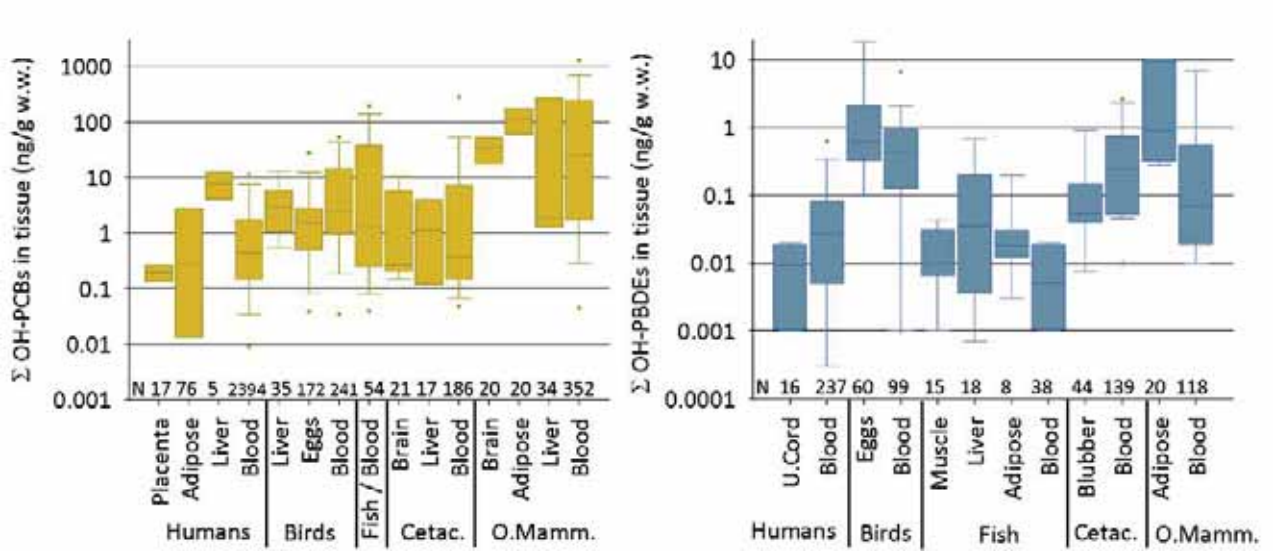


Figure 1 Distribution of lowest, average and highest reported concentrations of OH-PCB (A) and OH-PBDE (B) human and wildlife tissues including blood. Boxplots include data from the highest, average, and lowest sums of congeners reported in every entry for a range of tissues from humans and animals discriminated by species groups. Cetac. (cetaceans) and O.Mam. (other mammals). Values below boxplots indicate the number of all individuals included. Note the different scales.

Table 1.
References of HPCs in human and wildlife tissues discriminated by species group and tissue

Species group	Tissue	No entries [a]	Sum of individuals [b]	References
Humans	Placenta/Umbilical Cord	1 / 2	17 / 39	Gómara *et al.* 2012; Kawashiro *et al.* 2008; Otake *et al.* 2007
	Liver / Milk / Adipose	1 / 2 / 3	5 / 23 / 76	Fernandez *et al.* 2008; Guvenius *et al.* 2003; 2002; Kawashiro *et al.* 2008; Nomiyama *et al.* 2010b
	Blood	62	2553	Athanasiadou *et al.* 2008; Bergman *et al.* 1994; Cuadra *et al.* 2006; 2009a; Dallaire *et al.* 2009b; Dirtu *et al.* 2010; Eguchi *et al.* 2012; 2002; Fängström *et al.* 2005b; Glynn *et al.* 2011; Guvenius *et al.* 2003; Hagmar *et al.* 2001; Hovander *et al.* 2006; Kawashiro *et al.* 2008; Kunisue and Tanabe 2009; Meijer *et al.* 2008; Nomiyama *et al.* 2010b; 2007; Park *et al.* 2009c; Qiu *et al.* 2009; Rylander *et al.* 2012; Sandanger *et al.* 2004; Sandau *et al.* 2000; 2002; Sjödin *et al.* 2000; Soechitram *et al.* 2004; Weiss *et al.* 2006; Zota *et al.* 2011
Birds	Eggs	15	172	Fängström *et al.* 2005a; Jörundsdóttir *et al.* 2009; 2010; Verreault *et al.* 2005b
	Liver	6	45	Jaspers *et al.* 2008; Klasson-Wehler *et al.* 1998
	Blood	23	262	Fernie and Letcher 2010; Helgason *et al.* 2010; Klasson-Wehler *et al.* 1998; Kunisue and Tanabe 2009; Liu *et al.* 2010; McKinney *et al.* 2006a; Olsson *et al.* 2000; Park *et al.* 2009a; Verreault *et al.* 2005a
Fish	Fat [c] / Eggs	1 / 1	8 / 15	Zhang *et al.* 2010
	Liver / Muscle	2 / 2	18 / 15	Strid *et al.* 2010; Wan *et al.* 2010; Zhang *et al.* 2010
	Blood	22	70	Campbell *et al.* 2003; Li *et al.* 2003; Nomiyama *et al.* 2011b; Valters *et al.* 2005
Cetaceans	Blubber / Liver / Milk	4 / 3 / 1	64 / 34 / 8	Hoekstra *et al.* 2003; Kelly *et al.* 2008; McKinney *et al.* 2006b
	Brain	6	21	Kunisue *et al.* 2007; Montie *et al.* 2009
	Blood	24	253	Bennett *et al.* 2009; Hoekstra *et al.* 2003; Houde *et al.* 2006; Houde *et al.* 2009; Kelly *et al.* 2008; Nomiyama *et al.* 2010a; Nomiyama *et al.* 2011a; Weijs *et al.* 2009
Other mammals	Fat [c] / Brain / blubber	2 / 2 / 1	50 / 22 / 1	Gebbink *et al.* 2008; Letcher *et al.* 2009; Sandala *et al.* 2004
	Liver	4	34	Gebbink *et al.* 2008; Park *et al.* 2009b
	Blood	20	283	Bergman *et al.* 1994; Bytingsvik *et al.* 2012; Gebbink *et al.* 2008; Gutleb *et al.* 2010; Kunisue and Tanabe 2009; Routti *et al.* 2008; Routti *et al.* 2009; Sandala *et al.* 2004; Sandau *et al.* 2000; Verreault *et al.* 2005a; Weijs *et al.* 2009
Laboratory exposed animals				
Rat, sheep, dogs and mouse	Various tissues, mainly blood	2 / 2 / 3	4 / 16 / 14	Berg *et al.* 2010; Bergman *et al.* 1994; Qiu *et al.* 2007; Verreault *et al.* 2008

[a] An entry is the report of HPCs (OH-PCBs and OH-PBDEs, and PCP, OH-HpCs and tri-BrPhs when included) in a given species and a give tissue. [b] Total number of individuals for the given entries (sum of n)
[b] Adipose tissue

Only six publications reported HPCs in fish and included mainly trouts, carps, and amberjacks whereas sharks and tuna seem to be the only marine species so far surveyed (Nomiyama *et al.* 2011b; Strid *et al.* 2010; Wan *et al.* 2009; Wan *et al.* 2010). Given their allegedly lower metabolic activity, reported OH-PCBs in fish plasma range up to 100 ng/g ww. These might also demonstrate a lower phase II metabolic activity. However, these values are highly influenced by two reports from Detroit River fish (Li *et al.* 2003; Valters *et al.* 2005) while others reported concentrations up to 0.4 ng/g ww in fish blood from the Great Lakes and Japan (Campbell *et al.* 2003; Nomiyama *et al.* 2011b).

Reported data in birds included, among others, gulls, falcons, eagles, and albatrosses. The concentrations of OH-PBDEs in bird eggs were remarkably high, averaging around 1 ng/g ww which was double compared to reports of levels in blood (Figure 1B). Similar patterns of OH-PCBs and OH-PBDEs in bird blood and eggs (Fängström *et al.* 2005a) and their presence before embryonic development (Verreault *et al.* 2005b) strongly indicate their maternal transfer, but interspecies differences remain to be studied (Jörundsdóttir *et al.* 2010). Furthermore, opposite to OH-PCBs, OH-PBDEs concentrations in birds are higher compared to fish, similar to cetaceans and to other mammals.

In the category of other mammals, mainly seals and polar bears plus a few reports on cats and dogs were found. They were the highest among the species, while these results are strongly influenced by very high polar bear levels. Even after removing one outlier entry, (Gebbink *et al.* 2008) polar bear blood was still up to 267 ng OH-PCBs/g ww.

Developments on congener analysis

Organic synthesis development and resources available to researchers influenced the number and type of congeners reported on publications. A number of 31–56 OH-PCB congeners, mainly tetra to nona chlorinated, were synthesized and identified in human and wildlife plasma between 1994 and 2002 (Bergman *et al.* 1994; Hovander *et al.* 2002). From the available standards, approximately 11–18 congeners were reported in literature until 2009. The most abundant reported congeners in humans and wildlife were: 4-OH CB 107, 3-OH CB 153, 3'-OH CB 138, 4-OH CB 146, and 4-OH CB 187, although the congener profile varies within species and locations. Analysis in birds and fish revealed also the importance of 4-OH CB 120, 4-OH CB 199, and 4-OH CB 202 (Li *et al.* 2003; McKinney *et al.* 2006a). In 2006, 80% of the OH-PCBs in bottlenose dolphins (*Trupsiops truncatus*) from the Atlantic were still unidentified congeners (Houde *et al.* 2006), whereas in 2009, 40% of measurable OH-PCBs identified in sharks, cetaceans and mammals were mono to tetra congeners (Kunisue and Tanabe 2009; Nomiyama *et al.* 2010a; 2011b). Some

of these contributing congeners have been identified as: 4'-OH CB 25/26/31, 2'-OH CB 61, 4-OH CB 61, 3-OH CB 66, 4-OH CB69, and 4-OH CB 79. Lower halogenated congeners structurally resemble closer T_3 than T_4 which may result in lower binding affinity to plasma THBPs proteins, but reports of this group of congeners in humans is still lacking and the collected data in wildlife populations is insufficient to analyze the potential hazard implications.

Lower (Lacorte and Ikonomou 2009; Marsh *et al.* 2003) and higher (Rydén *et al.* 2012; Yu *et al.* 2010) brominated OH-PBDEs have meanwhile been synthesized and identified in humans and wildlife. The most abundant congeners, 4'-OH BDE 17, 2-OH BDE 68, 4-OH BDE 42, 3-OH BDE 47, 6-OH BDE 47, 4'-OH BDE 49, and 4-OH BDE 92, have remained the same up to date and therefore big uncertainties are not to be expected.

There is still a large proportion of unidentified congeners, particularly OH-PCBs. From the potential 209 PCB and 209 PBDE congeners, only 62 PCB and 12 BDE congeners have been consistently reported to be present in human and wildlife tissues. Several OH-metabolites may be produced from each of these parent compunds (Marsh *et al.* 2006; Montaño *et al.* 2012; Murk *et al.* 1994; Qiu *et al.* 2007). Considering the production of 4–6 metabolites per parent compound, the total number of metabolites could rise up to 400 congeners. Not all of these metabolites will be selectively retained in the blood and transported over placental and blood–brain barriers; however, this demonstrates the potential extent and complexity of an issue which clearly deserves further studies.

Relationship to parent compounds.

Although a relative large number of OH-PCB congeners have not been analyzed, their levels in human blood significantly correlated with that of parent PCBs ($P < 0.0001$, Figure 2A) in agreement with previous results (e.g.Park *et al.* 2007). This correlation is less evident for blood concentrations in other species and for other tissues such as eggs or liver. The ratio of ∑OH-PCBs to ∑PCBs was highest in human blood at low PCB burdens (Figure 2B). At concentrations above 10 ng ∑PCBs/g w.w. the ratio was stable at 0.2 (approximately 5 nM ∑OH-PCBs). This stabilization could be the result of equilibrium between phase I and phase II metabolism and the THBP carrying capacity. However, several human and wildlife studies comparing control and contaminated populations found considerably higher metabolite to parent ratios on individuals with higher POP burdens (Athanasiadou *et al.* 2008; Cuadra *et al.* 2006; Hovander *et al.* 2006; Routti *et al.* 2009). Furthermore, rats and sheep exposed to PCBs had up to 17 times more OH-PCB than PCB in blood at the end of the exposure period (Berg *et al.* 2010; Bergman *et al.* 1994).Therefore, it is plausible to suggest that at higher PCB burdens, phase II metabolism cannot

completely counterbalance the production of metabolites, and also the THBPs can transport much higher quantities of metabolites when required.

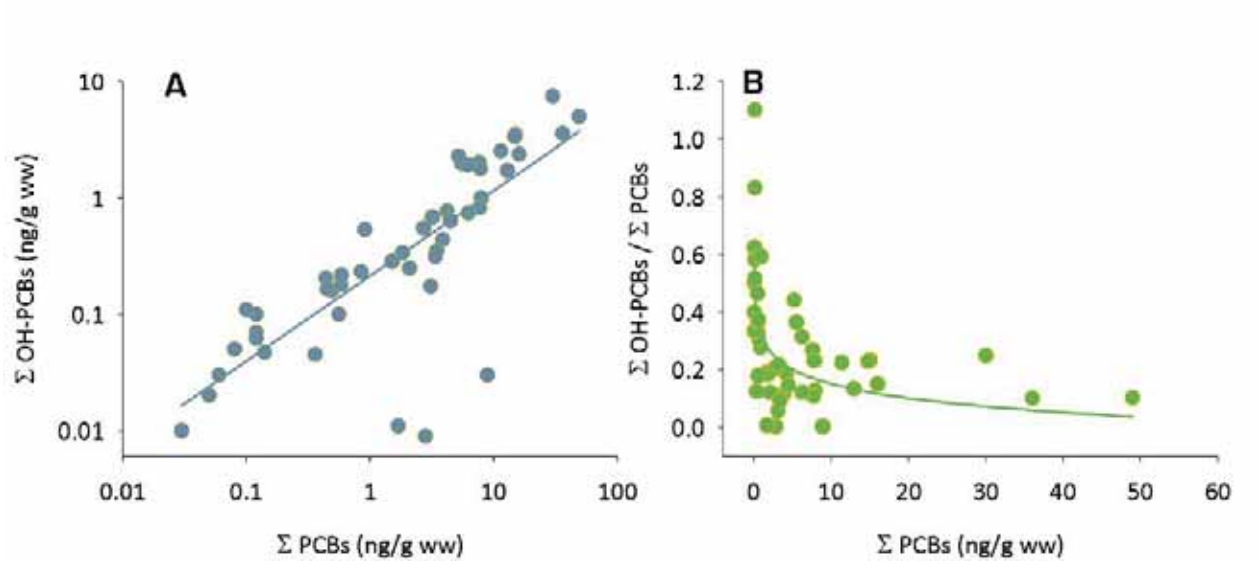


Figure 2 Correlation between absolute or relative metabolite concentrations and parent compounds for PCBs in human blood. Circles represent the average sum of congeners reported from each human entry. The (A) linear regression between ∑PCBs and ∑OH-PCBs (y=0.21 + 0.14*x**)** and the (B) logarithmic regression between ∑PCBs and the parent to metabolite ratios (y=0.31 - 0.072*$\log_{10}[x]$) were both highly significant, $P < 0.0001$.

The metabolite/parent ratios are important indicators of the balance between production, retention and excretion of metabolites, as are important to establish potential source relationships. The ∑OH-PCBs/∑PCBs were high in blood, brain, umbilical cord and eggs (Figure 3A), which is in agreement with a higher burden of OH-PCBs in highly perfused tissues and active transport through selective barriers (Meerts *et al.* 2002). Children have more than twice as high ∑OH-PCB/∑PCB ratios than adults (Figure 3C). This result may be influenced by studies of particularly exposed populations (Cuadra *et al.* 2006; Fängström *et al.* 2005b). The ratios reported in literature and discussed here probably represent an underestimation, particularly on OH-PCBs reports before 2010.

The tissues with the highest ∑OH-PBDE/∑PBDE are blood, brain and especially fetal tissue like eggs and umbilical cord (Figure 3B and D). These results therefore confirm an important transfer of OH-PBDEs into fetal tissues and suggest higher transfer efficiencies compared to OH-PCBs. For coastal and marine populations the main source of OH-PBDEs might not be metabolism of PBDEs but direct uptake of mainly ortho OH-PBDEs (Figure 4) directly from marine sources (Malmvärn *et al.* 2008). This can explain the absence of correlation between ∑OH-PBDEs and the ∑PBDEs. In blood from inland populations, mainly meta and para OH-BDE congeners were present (Figure 4), supporting existing evidence toward metabolism from parent BDEs as their source (Marsh *et al.* 2006; Montaño *et al.* 2012; Qiu *et al.* 2007).

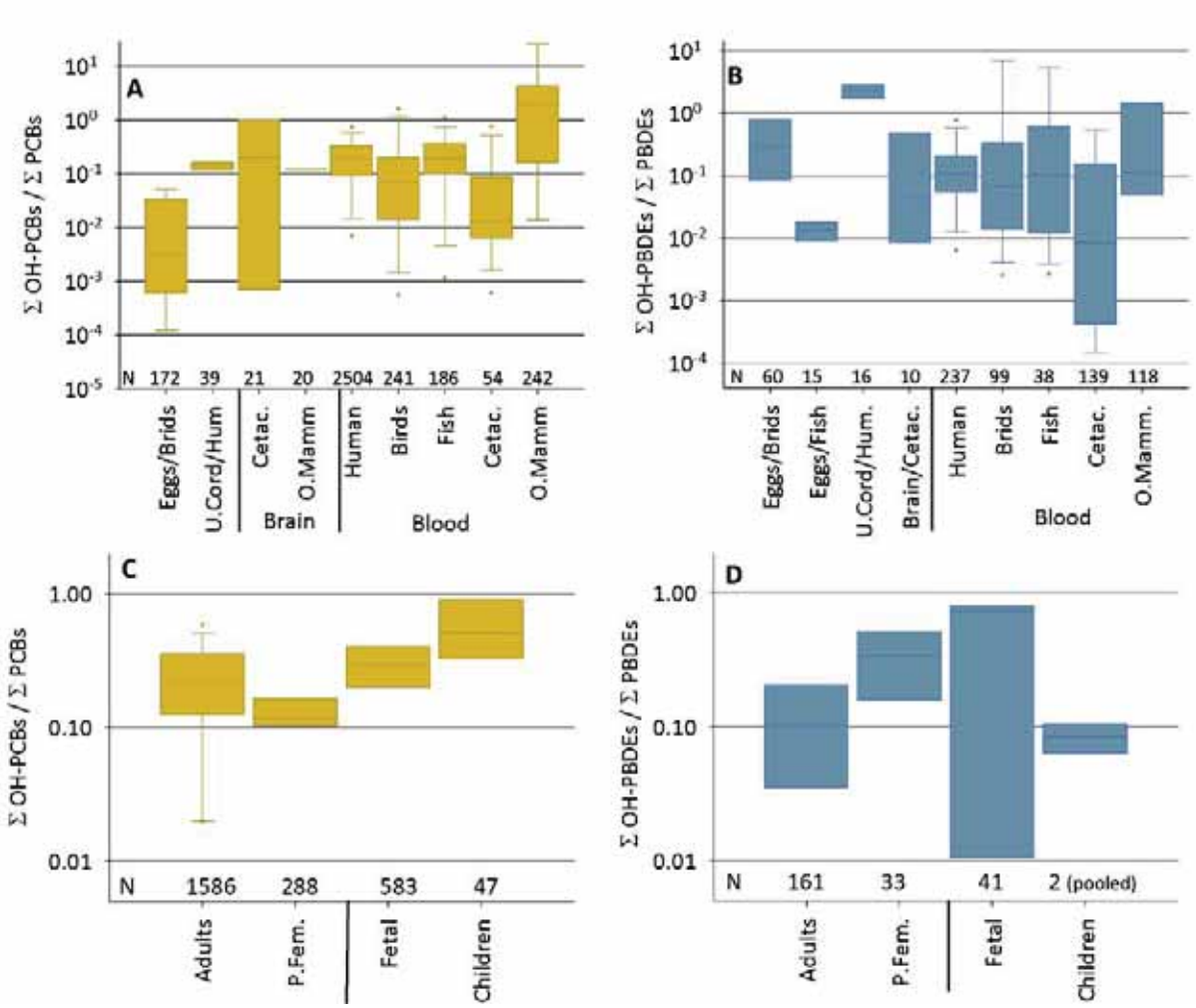


Figure 3 Ratio of ∑OH-PCBs/-PBDEs to ∑PCBs/PBDEs in tissues of various species (A and B) and in human blood (C and D). Boxplots represent the distribution of the ratio from the average sums of (A and C) OH-PCB and (B and D) OH-PBDE congeners to the sum of their respective parent compounds. U.Cord. (umbilical cord tissue), Hum. (Humans), Cetac. (cetaceans), O.Mam. (other mammals) and P.Fem. (pregnant females). Values below boxplots indicate the number of all individuals included.

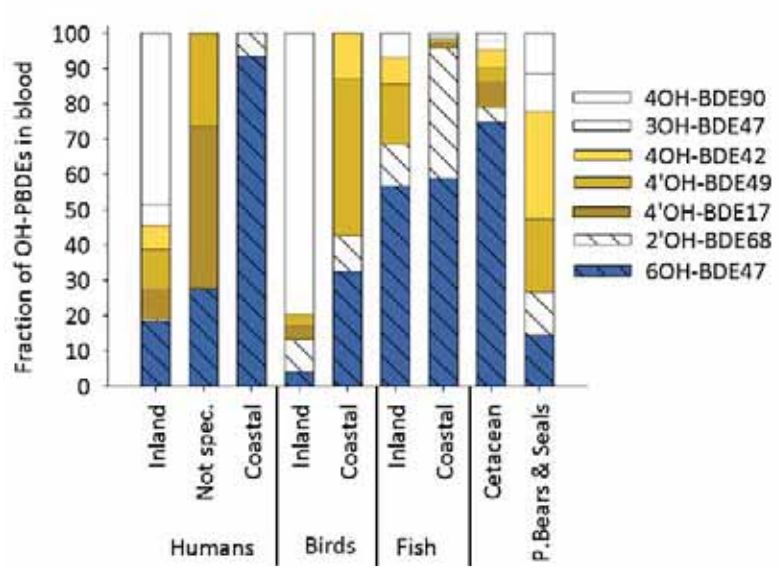


Figure 4 Relative presence of OH-PBDE congeners in blood calculated based on the reported ng/g ww average levels per species group distinguishing inland and coastal populations.

HPC plasma retention

The increased concentration of HPCs in relation to their putative parent compounds mainly in plasma but also in other highly perfused tissues (Figure 2) has been explained on the basis of their structural resemblance to thyroid hormones and hence their binding to the THBPs (Brouwer *et al.* 1998; Lans 1995). The main purpose of the THBPs is to provide a multicomponent buffer system to assess the even distribution of THs through the body (Schreiber 2002) which otherwise will permeate into cell membranes and disappear from the bloodstream (Mendel *et al.* 1987).

In general, HPCs competitively bind TTR with similar potency compared to T_4 (Supporting Information, Table ST1) whereas most of them have lower affinity than T_4 for TBG. However, as the affinity of TBG for T_4 is almost 3 orders of magnitude higher than that for TTR, similar concentrations of, for example, some OH-PBDEs will equally compete with T_4 for TTR as they will do for TBG. This indicates that TBG contributes as well to the HPCs retention in plasma. ALB demonstrated similar affinity for HPCs compared to THs (Ucan-Marin *et al.* 2010); however, the importance of ALB on HPC retention and transport is yet to be asserted.

The concentrations of thyroid binding proteins vary among species and within stages of development (Supporting Information, Table ST2). In combination with their binding affinity, it is estimated that in humans TBG, TTR, and ALB carry 65%, 15%, and 20% of the bound T_4, and that one in three TBG molecules, one in 300 TTR molecules, and one in 3000 ALB molecules carry one T_4 molecule bound to it under physiological conditions. Therefore, about 100 nM of proteins have bound-T_4, and at least 200 nM of TGB and 4000 nM of TTR are available so a large amount of HPCs can be bound without direct competition with THs. This might explain the absence of consistent correlations between plasma HPC and TH concentrations (Rolland 2000). Instead, the THBPs deliver the HPCs to the tissues where they can exert direct effects and/or interfere with TH sensitive processes altering feedback balances and hence TH homeostasis (Gabrielsen *et al.* 2011; Murk *et al.* 2013). It is therefore important to consider the HPC burdens in perspective of their various mechanisms of toxicity.

HPC burdens in relation to effect levels

Besides OH-PCBs and OH-PBDEss, 4OH-HpCs and halogenated phenols such as PCP, TBBPA and triBPhs bind to the THBP (Table 2). PCP was the major HPC found in human blood, whereas for all other species OH-PCBs were the most abundant HPCs (Figure 5). Birds had relatively higher brominated HPC levels, probably due to the fact that most publications reported on piscivorous coastal bird populations

characterized by a major exposure to tri-BPhs and OH-PBDEs from marine sources (Figure 5).

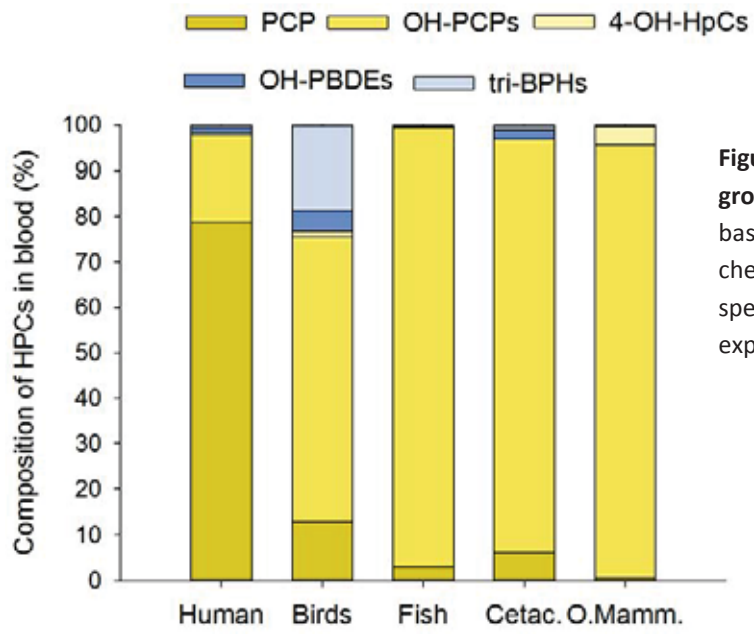


Figure 5 Composition of HPC chemical groups in blood. The composition is based on the average ∑HPCs per chemical group for all entries per species group, expressed in nM as explained in the Methods section.

This study aims at comparing the HPC body burdens of humans and wildlife to known effect concentrations. To this end, all reported concentrations were expressed in molar units, allowing sensible combination of relatively heavy brominated with also much lighter HPCs. The range of HPC concentrations in human blood was 0.1–100 nM including PCP and 0.2–20 nM without PCP (Figure 6). The total HPC range in human blood is one fold higher when PCP concentrations are included. Highest reported PCP values were 187 nM in blood from Swedish men (Sjödin *et al.* 2000). But it seems that PCP concentrations have decreased in recent years to levels below 40 nM (approximately 10 ng/mL) (Zheng *et al.* 2011). The results reveal that children and fetal cord blood HPC concentrations are similar to those in adults (Figure 6). HPC concentrations in wildlife plasma were in the range of 1–240 nM in birds, 0.2–454 in fish, 0.1–800 nM in cetaceans, and were highest (0.05–7650 nM) in other mammals (Figure 6); as mentioned before, there is apparently still a portion of OH-PCBs not yet quantified.

Among the relevant *in vitro* end-points (references in Supporting Information, Table ST3), thyroid hormone competitive binding has been most widely studied. THBP competition is observed from 5 to 1000 nM, whereas a higher quantity (200–50000 nM) is generally required to trigger T_3 dependent proliferative effects in the T-screen (Figure 6). Effects of HPCs on nuclear receptors are structurally dependent and required concentrations above 5 nM to act as agonist or above 1000 nM as antagonist. However, activation of thyroid hormone receptor (THR) by HPCs has

been observed already from 0.03 nM. HPCs inhibit aromatase and other steroidogenic enzyme activities from 500 nM, whereas gene expression in the same pathway is altered above 1000 nM. Neuronal effects on Ca^{2+} homeostasis and neurotransmitters have been observed above 200 nM; however, abnormal dendritic development is shown after exposure to OH-PCB concentrations as low as 0.005 nM.

Thyroid hormone homeostasis, estrogenic, behavioral, and neuronal effects were observed in offspring from pregnant rats exposed to 4-OH CB107 (Meerts *et al.* 2002; Meerts *et al.* 2004a; Meerts *et al.* 2004b). Acute toxicity in adults, and various developmental effects in embryos were observed on zebrafish exposed to 6-OH BDE47, while liver concentration were 170–940 nM and 300 nM, respectively (Boxtel *et al.* 2008). Similar developmental arrest was obtained after zebra fish exposure to three OH-BDE congeners (Usenko *et al.* 2012). Finally, exposure to a combination of PCB118 and PCB153 during sheep gestation influenced fetal growth, adrenal development, cortisol production, (Zimmer *et al.* 2011) and offspring sexual dimorphic behavior (Gutleb *et al.* 2011). In this case, 3-85 nM 4-OH CB107 and 4-OH CB146 metabolites were found in plasma from the same lactating ewes 50 days after birth (Berg *et al.* 2010) (Figure 6). Few *in vivo* exposure studies with either OH-PCBs/PBDEs or parent compounds report plasma effect concentrations allowing comparison with OH-PCB/PBDE levels in humans and wildlife.

Conclusions, knowledge gaps and recommendations

Plasma HPC (OH-PCB, OH-PBDE, PCP, OH-HpCs, and tri-BhPhs) concentrations reported ranged from 0.1 to 100 nM in humans and up to 240, 454, 800, and 7650 nM for birds, fish, cetaceans, and other mammals. Although still only a selection of HPCs can be and were analyzed, these concentrations are well within the range of observed *in vivo* and *in vitro* developmental, endocrine, and neuronal effects. Metabolic activation and the retention of OH-metabolites in plasma should be more explicitly considered within a more realistic hazard assessment of POPs, especially when this is based on *in vitro* analyses.

General population studies and the surveillance of a single population are required to determine background levels and temporal trends. These studies should especially pay attention to levels in fetuses (umbilical cord blood) and children as the scarce information present indicate relatively high concentrations of OH-PCBs and OH-PBDEs that are of concern. The results of fetal tissues, including human cord blood and bird eggs, suggest a greater maternal transfer efficiency of OH-PBDEs compared to that of OH-PCBs, an important aspect of exposure that must be further investigated.

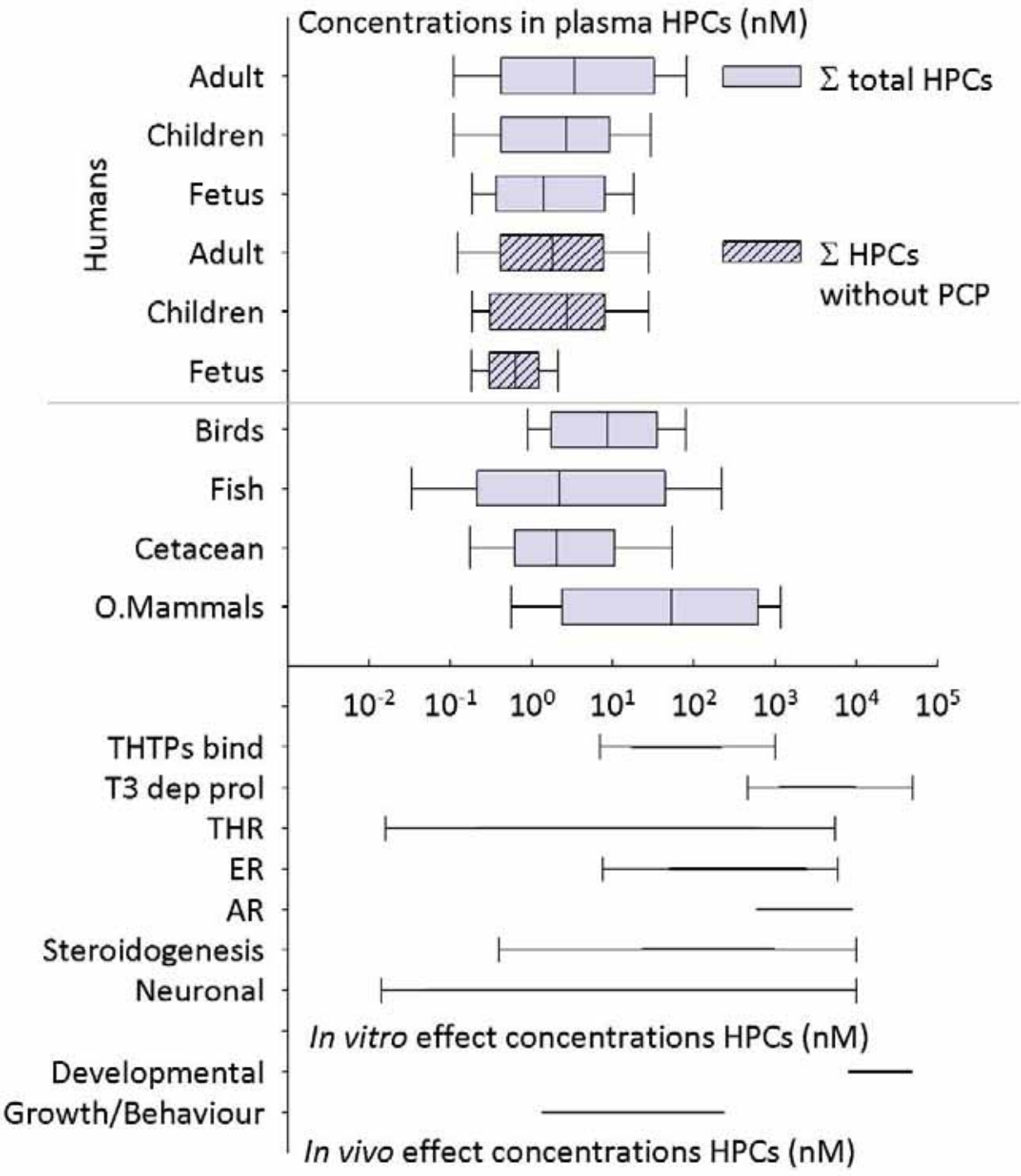


Figure 6 HPC concentrations in plasma from humans and wildlife compared to *in vitro* and *in vivo* effect concentrations. HPCs included reports from OH-PCBs and OH-PBDEs and also PCP, OH-HpCs, tri-BrPhs and TBBPA when included. Boxplots represent the distribution of highest, average and lowest sums of congeners in blood from humans and animals (nM) calculated from values reported in the literature included in this study. Cross-hatched boxes indicate human HPC concentrations without PCP. Lines indicate *in vitro* effect concentrations (nM) for THBP binding, T-screen, thyroid hormone (TH)R, estrogen (E)R and androgen (A)R receptor activation, steroidogenesis, neuronal outgrowth, and in vivo effect concentrations for mammalian growth and development. Details and references can be found in Supporting information, Table ST3.

Arctic top predators hold HPCs concentrations comparable to total TH transport protein capacity. Studies on nonarctic top predators and other important piscivores and carnivores are warranted. Two studies reported OH-PCB or OH-PBDE plasma concentrations in pets. These animals live in close association with house dust and lick their fur; they could serve as sentinel species to study indoor exposure of especially small children.

Biological methods should be developed to be able to include the toxic potencies of HPCs that cannot be chemically detected because standards do not (yet) exist or they occur in levels below the limit of detection while they do contribute to the combined HPC risk. Retrospective studies applying those more comprehensive methods to stored samples (including blood plasma from *in vivo* experiments and umbilical cord) may reveal an even greater toxicological relevance of OH-PCBs/PBDEs than already indicated by the results of our current meta-analysis.

Suporting information

Available supporting information includes: the list of individual congeners included in the database; the concentrations of HPCs not included in Figure 1; Figure S1 depicting the distribution of reported concentrations of OH-PCBs and OH-PBDEs in human plasma; Table ST1 with the summary of competitive binding parameters of hormones and HPCs to transport proteins; Table ST2 with the levels of hormone carrying proteins and thyroid hormones in various species and Table ST3 with the sources of information for the *in vitro* effect concentrations displayed in the Figure 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table ST1.
Summary of competitive binding parameters of hormones and HPCs to transport proteins [a]

Compound or group	TTR		TBG		Albumin		Species	References [c]
	IC_{50} [b] (nM)	RP [d]	IC_{50} (nM)	RP [d]	IC_{50} (nM)	RP		
T_4	13-260		1-85		7.9		Human	1, 2, 3, 4, 5, 6, 7, 9, 10, 11
	10				5.11		Birds	10, 11
T_3	216-1600		14-30		23.88		Human	4,5,6,8, 10, 11, 12
	5.99				1.12		Birds	10, 11
OH-PCBs	7-95	0.4-8	1380 - >10000	> 0.02			Humans	2,4, 13
	12.5 / 1.1 [e]	0.8 / 3.1 [e]			4.19 / 0.89 [e]	1.2 / 1.2 [e]	Birds	10, 11
PCP	33	0.6					Humans	5
4-OH-HPCs	71.74	1.1					Humans	14
OH-PBDEs	7 - 900	0.2 – 4	60 - >7500	0.01-1.5			Humans	5, 6, 7, 14, 15
	7.7-12 / 5-69 [e]	1.3-2 / 0.1-1 [e]			4-5 / 0.8-1 [e]	1-1.2 / 1-1.4 [e]	Birds	9, 10
Tri-BPhs	4 -67	0.9 -10					Humans	3, 5
PCP [*]	33.2	0.6	n.r. [*]	- -			Humans	5
TBBPA [*]	18.8	1	n.r. [*]	- -			Humans	5

[a] For a complete table with data from each reference and each compound see database at Wageningen Library e-depot (www.library.wur.nl)

[b] In some publications the IC50 is reported as the Ki (affinity constant) where Ki= IC50/(1+([ligand]/Kd), and Kd is the dissociation constant of the competitor-protein reaction.

[c] References: 1) Nilsson *et al.* 1975, 2) Lans *et al.* 1993, 3) Meerts *et al.* 2000, 4) Marchesini *et al.* 2006, 5) Marchesini *et al.* 2008, 6) Cao *et al.* 2010, 7) Montaño *et al.* 2012, 9) Nilsson and Peterson 1975 , 10) Ucan-Marin *et al.* 2009 , 11) Ucan-Marin *et al.* 2010, 12) Ren and Guo 2012a , 13) Cheek *et al.* 1999, 14) Sandau *et al.* 2000, 15) Hamers *et al.* 2008, 16) Weiss *et al.* 2009.

[d] RP values are reported mainly in competition with radioactive T4 (references: 1,2,3,6,9,10,11), with T4 linked to a chip (references: 4 and 5) and with 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANSA) (references: 6 and 7)

[e] Ki in competition with T4 / and Ki in competition with T3

Table ST2.
Levels of hormone carrying proteins and thyroid hormones in various species

Species	Tissue	TTR (µM)	TBG (µM)	Albumin (µM)	TT_4 (nM)	fT_4 (pM)	TT_3 (nM)	fT_3 (pM)	References [a]
Humans [b]	Plasma	5	0.24-0.5	600	23-200	9-25	1-4	1-9	1, 2, 3, 4, 5
	Cord plasma		0.6–1.3				0.4-0.8		6
	Cerebrospinal fluid (CSF)	0.42	0.0028	2.3	2.4	70			1
Larger mammals[c]	Plasma	+ [d]	+	+	9-100	9-30	0.5-3.0	2-8	7,8
Smaller mammals[c] including rodents	Plasma	+	+ dev [d]	+	20-80	14-36	0.8-1.7	1-7	7,8
Seals (Mothers / Pups)	Plasma	+	+	+	25 / 60 [e]		0.8 / 2 [e]		9
Birds [f]	Plasma	+		+	3-95	1-60	2-8	3-12	8, 10
Fish	Plasma	+	+ dev [d]	+	4-23	2-600	0.3-14	21-419	10, 11
Amphibians	Plasma		+ dev [d]	+	0.5-6		0.1-1.5		12

[a] References: 1) Schreiber 2002, 2) Dallaire *et al.* 2009, 3) Hagmar *et al.* 2001, 4) Nomiyama *et al.* 2010, 5) Zota *et al.* 2011, 6) Sandau *et al.* 2002, 7) Joasoo *et al.* 1975, 8) Hulbert 2000, 9) Gabrielsen *et al.* 2011, 10) Nøst *et al.* 2012, 11) Blanton and Specker 2007, 12) Fort *et al.* 2007

[b] The euthyroid human range is: 60-140 nM total T4, 10-25 pM free T4, 1-3 nM total T3 and 3-8 pM free T3 (Hulbert 2000);

[c] Larger mamals include (but not exclude): monkey, cattle, sheep, goat, buffalo, horse, swine and dog. Smaller mammals include (but not exclude): cat, rabbit, and rat (Joasoo *et al.* 1975)

[d] The protein presence has been confirmed (+) and only at a particular developmental stage (+ dev)

[e] mothers / pups;

[f] THs values reported by Nost and co-workers (2012) in Northern Fulmar are considerably higher compared to values summarized by Hulbert (2000).

Table ST3.

Sources of information for the *in vitro* effect concentrations and OH-POP concnetrations found after *in vivo* exposure displayed in the Figure 6.

End-point	References
In vitro	
THBP binding	Cao *et al.* 2010; Cheek *et al.* 1999; Hamers *et al.* 2006; Lans *et al.* 1993; Marchesini *et al.* 2008; Meerts *et al.* 2000; Montaño *et al.* 2012; Ren and Guo 2012b; Ucan-Marin *et al.* 2009; Ucan-Marin *et al.* 2010
T3 dependent proliferation	Ghisari and Bonefeld-Jorgensen 2005; Schriks *et al.* 2006
Nuclear receptor interactions	
Thyroid hormone receptor THR	Freitas *et al.* 2011; Kimura-Kuroda *et al.* 2008; Li *et al.* 2010
Estrogen receptor	Hamers *et al.* 2006; Kitamura *et al.* 2005; Kojima *et al.* 2009; Kramer *et al.* 1997; Meerts *et al.* 2001; Mercado-Feliciano and Bigsby 2008; Moore *et al.* 1997
Androgen receptor	Hamers *et al.* 2006; Kojima *et al.* 2009
Steroidogenetic effects	Cantón *et al.* 2006; He *et al.* 2008; Song *et al.* 2008
Nauronal effects	Dingemans *et al.* 2010; Hendriks *et al.* 2010; Kimura-Kuroda *et al.* 2005; Kimura-Kuroda *et al.* 2007
In vivo	
Pregnant rats exposed to 4-OH CB 107	Meerts *et al.* 2002; Meerts *et al.* 2004b; Meerts *et al.* 2004a
Zebrafish exposed to 6-OH BDE 47	Boxtel *et al.* 2008; Usenko *et al.* 2012
Pregnant sheep exposed to a mixture of PCB 118 and 153	Berg *et al.* 2010; Gutleb *et al.* 2011; Zimmer *et al.* 2011

CHAPTER 3

New approaches to assess the transthyretin binding capacity of bioactivated thyroid hormone disruptors

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Abstract

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl-ethers (PBDEs) are metabolized into hydroxylated metabolites (OH-PCBs/PBDEs), which can disrupt the thyroid hormone homeostasis. Binding of these metabolites to transport proteins such as transthyretin (TTR) is an important mechanism of their toxicity. Several methods to quantify the competitive thyroxine (T_4) displacement potency of pure metabolites exist. However, quantification of the potency of *in vitro* metabolized PCBs and PBDEs has drawbacks related to the coextraction of compounds disturbing the T_4-TTR competitive binding assay. This study identifies and quantifies the major coextractants namely cholesterol, saturated andnonsaturated fatty acids (SFA and NSFA) at levels above 20 nmolper mg equivalent protein following various extraction methods.Their TTR binding potency was analyzed in a downscaled, nonradioactivefluorescence displacement assay. At concentration factorsneeded for TTR competitive binding, at least 10 µM of thesecoextracts is present, whereas individual SFA and NSFA disturbthe assay from 0.3µM. The effectiveness of the *in vitro* metabolismand extraction of the model compounds CB 77 and BDE 47 waschemically quantified with a newly developed chromatographicmethod analyzing silylated derivatives of the OH-metabolites andcoextractants. A new method to selectively extract metabolites andlimit coextraction of disturbing compounds to less than 5 nmol permg equivalent protein is presented. It is now possible to make adose-response curve up to 50% inhibition with bioactivated CB 77and BDE 47. The toxic potencies of bioactivated persistent organicpollutants (POPs) should be taken into account to prevent seriousunderestimation of their hazard and risk.

Introduction

Metabolic activation of polychlorinated biphenyls (PCBs)and polybrominated diphenyl-ethers (PBDEs) has attracted scientific and societal interest and concern. These persistent organic pollutants (POPs) are accumulated by organisms and magnified through the food chain (Kelly *et al.*, 2008). Upon uptake, PCBs and PBDEs can be transformed by phase I metabolic enzymes into hydroxylated compounds (Marsh *et al.*,2006; Morse *et al.*, 1995). These metabolites can cause direct effects on the thyroid hormone homeostasis (Freitas *et al.*,2011; Gutleb *et al.*, 2010; Meerts *et al.*, 2002), interact with the estrogenic receptor (Hamers *et al.*, 2006; Meerts *et al.*, 2001),increase steroidogenic gene expression (Song *et al.*, 2008), and might be the underlying cause for observed neurological effects of PBDEs (Dingemans *et al.*, 2011).

Several of the effects of hydroxylated PCB and PBDE metabolites (OH-PCB/PBDEs) are driven by their competition with 3,3',5,5'-tetraiodo-L-thyronine (thyroxine, T_4) to bind the plasma transport proteins, their subsequent accumulation in tissues, and their specific transport over selective barriers (Meerts *et al.*, 2002; Morse *et al.*, 1993). OH-PCBs and OH-PBDEs have a structural similarity with the thyroid hormones T_4 and 3,3',5-triiodo-L-thyroxine (T_3). Therefore, they bind with high affinity to specific transport proteins such as transthyretin (TTR) and thyroxin-binding globulin (TBG) (Lans *et al.*, 1994; Marchesini *et al.*, 2006; Meerts *et al.*, 2000). As a result of this binding, OH-POP levels have been shown to be similar or up to 30 times higher in plasma than the respective parent compounds in mammals (up to 2.8 µg/g wet weight) (Gebbink *et al.*, 2008) and as high as the parent compounds in humans (up to 22 ng/g wet weight) (Sjodin *et al.*, 2000). This mechanism is then the basis for the selective retention of OH-PCB/PBDEs in various tissues and their transport through selective barriers to the brain or the fetus (Meerts *et al.*, 2002; Morse *et al.*, 1993). Given the high plasma levels and the toxicological relevance of the metabolite effects, it is very important to quantify the toxic potencies of these OH-POPs in order to take them into account for toxicological hazard and risk assessment of PCBs and PBDEs.

Mainly three *in vitro* methods to measure the potency of competitors to displace T_4 from TTR and TBG have been reported. The radio-ligand binding assay (RBA) uses radioactive T_4 (T_4*) in equilibrium with T_4, TTR, and the competitor at 4°C. The amount of unbound T_4* is a measure of the competitor's potency (Lans *et al.*, 1993). The plasmon resonance– based biosensor assay (Biacore) uses immobilized T_4, which interacts with a mobile phase containing TTR or TBG and the competitor. The availability of free TTR for binding the immobilized T_4 is a measure of the competitor's potency (Marchesini *et al.*, 2006). Finally, the fluorescence

displacement method is based on the measurement of the fluorescence emitted by a fluorophore when bound to proteins such as TTR, TGB, or albumin. A competitor will displace the fluorophore from its position in the protein, thereby reducing the fluorescence as a measure of the compound's potency to displace T_4 (Nilsson and Peterson, 1975).

These methods have all been successfully used to measure the competitive binding of pure substances, but efforts to measure the metabolite potency of biologically metabolized PCBs and PBDEs have encountered several problems. The analysis of bioactivated extracts using *in vitro* biotransformation techniques with microsomal fractions has been hampered by the low biotransformation efficiencies, presence of toxic parent compound concentrations in the extracts, and the presence of interfering matrix components coextracted from the metabolizing system (Hamers *et al.*, 2008; Meerts *et al.*, 2000; Schriks *et al.*, 2006). In contrast to the parent compounds, the OH-metabolites do not endure destructive clean-up steps, and their physical/chemical qualities make it difficult to separate them from potentially interfering substances in the extract. Recent attempts to remove the interfering matrix components from extracted tissues by exhaustive clean-up procedures and 30 to 100 times sample dilution have been reported, but these could still not prevent interference with the analysis by matrix compounds coextracted from plasma and muscle samples (Simon *et al.*, 2010, 2011).

Well-known compounds that interfere with T_4 binding to TTR are the free fatty acids. The fact that human plasma free fatty acids present in micromolar concentrations can directly inhibit T_4 binding to TTR was the reason for the decision not to use T_4-TTR binding assays to determine the plasma T_4 levels in humans in the clinical setting (Shaw *et al.*, 1976). In addition, the free T_4 * used in the RBA is prevented from binding to TTR at higher fatty acid concentrations, and it is transported through the column causing an overestimation of the competitive potency (Simon *et al.*, 2010; Weiss *et al.*, 2009). However, the identity concentration and T_4-TTR binding potency of the interfering coextractants from bioactivated extracts are not yet known. This information is required in order to solve the problem of disturbing coextractants and thus enable to study the toxic potencies of the ubiquitous OH-metabolite from POPs, such as PCBs and PBDEs.

In addition, a method to be able to quantify the TTR binding potency of the OH-metabolites without the use of radioactivity and expensive equipment and/or in relatively great quantities such in cuvettes is desired.

Therefore, this study aims to (1) identify and quantify the concentration of coextractants in microsomal extracts, (2) quantify the potency of coextractants to inhibit the T4-TTR competitive binding assay, (3) develop a method to selectively

extract metabolites and eliminate disturbing coextractants, and (4) apply the newly developed method to analyze the potency of bioactivated extracts from CB 77 and BDE 47, which are used as representative model compounds. To support these objectives, we developed (5) a selective chromatographic method to analyze silylated derivatives from CB 77 and BDE 47 OH-metabolites and coextractants and (6) a T_4-TTR competitive fluorescence displacement method in a 96-well plate setup according to a published method for cuvettes (Nilsson and Peterson, 1975).

Materials and methods

Standards and reagents. The model compounds 3,3',4,4'-tetrachlorobiphenyl (CB 77, 99.0%) and 2,2',4,4'-tetrabromodiphenylether (BDE 47, 98.8%) were purchased from Sigma-Aldrich Co. (Steinheim, Germany) and Chiron AS (Trondheim, Norway), respectively. CB 77 metabolites including 2-OH-3,3',4,4'-CB (2-OH CB 77) and 5-OH-3,3',4,4'-CB (5-OH CB 77) were previously synthesized in our laboratory (Lans *et al.*, 1993), whereas 4'-OH-3,3',4,5'-CB 79 (4'-OH CB 79) was purchased from LGC Promochem (Middlesex, U.K.). BDE 47 metabolites including 2'-OH-2,3',4,5'-BDE (2'- OH BDE 66) and 6-OH-2,2',4,4'-BDE (6-OH BDE 47) were provided by Ake Berkgman and were synthesized as previously described (Marsh *et al.*, 2003). 6'-OH-2,2',4,5'(6'-OH BDE 49), 5-OH-2,2',4,4'-BDE (5-OH BDE 47), 4-OH-2,2',3,4'-BDE (4-OH BDE 42), 4'-OH-2,2',4,5'-BDE (4'-OH BDE 49), and 3-OH-2,2',4,4'-BDE (3-OH BDE 47) were purchased from Accustandar (New Haven, CT). The internal standard for chromatographic analysis 2,2',3,3',4,4',5,5',6,6' decaCB (CB 209) was obtained from Riedel-de Haen (Seelze, Germany). Fatty acids including dodecanoic acid, myristic acid, and palmitic acid were purchased from Supelco (Bellefonte, PA), whereas linoleic acid, oleic acid, stearic acid, and arachidonic acid were purchased from Sigma-Aldrich Co. (Steinheim, Germany). Cholesterol was purchased from Vel (Leuven, Belgium). N,O-bis(trimethylsilyl)-trifluoroacetamidetrimethylchlorosilane (BSTFA–TCMS) (9:1) solution and lipid removal agent (LRA) were purchased from Supelco (Bellefonte, PA). Hexane (dioxin analysis grade) and all other solvents (chromatographic grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands) or from Sigma-Aldrich Co. (Steinheim, Germany). Stock solutions were prepared in dimethyl sulfoxide (DMSO) for bioassays, in iso-octane for storage, and in hexane (dioxin analysis grade) for chromatographic analysis. β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), 3,3',5,5'-tetraiodo- L-thyroxin (T_4), prealbumin from human plasma (TTR), and the fluorescent probe 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANSA) were purchased from Sigma-Aldrich Co. Concentrations of T4 and ANSA were routinely determined by UV-visible spectrometry with a Beckman Coulter DU 800 spectrophotometer. Extinction coefficients were 6180 $M^{-1}cm^{-1}$ at 325 nm in buffer phosphate and NaOH at pH 11 for T_4 (Nilsson and Peterson, 1975) and 6300 $M^{-1}cm^{-1}$ at 350 nm in buffer phosphate at pH 7.5 for ANSA (Cao *et al.*, 2010). All

extraction and storage materials were exclusively made of borosilicate glass prewashed with hexane, and polytetrafluoroethylene screw cap vials were used when required.

Microsomal incubations. In vitro bioactivation of PCBs and PBDEs has been previously described (Murk *et al.*, 1994) and was applied with few modifications given below. Compounds were metabolized using liver S9 fraction from β-naphthoflavone and phenobarbital-treated Sprague Dawley rats obtained from Xenotech (Lenexa, Kansas). Incubations were performed in a borosilicate glass tube in 1 ml phosphate buffer (PB, 100 mM, pH 7.5), S9 fraction (1 mg protein/ml), and parent compound (10 µM in 1% DMSO). After 5 min preincubation in a shaking water bath at 37°C, the reaction was initiated with the addition of 100 µl of 10 mM NADPH in PB. Additional 100 µl of 10mM NADPH was added every 20 min during the incubation period. Metabolism was stopped after 90 min (Harju *et al.*, 2007) by denaturation of microsomal proteins with 700 µl of ice-cold methanol (negative controls at t = 0). Some incubations were spiked with 4-OH CB 79, 2-OH BDE 66, and 6-OH BDE 47 (2 µM) to quantify extraction recovery and to study the potential production of dihydroxy metabolites. Coextractants and metabolites were analyzed as silylated derivatives by Gas chromatography/mass spectrometry (GC/MS) as described further below.

Metabolite extraction methods. To qualify and quantify the amount of coextractants from the incubation mixture, S9 control incubations were spiked with 2 nmol 4-OH CB 79/mg-eq protein in DMSO and extracted using four methods published in literature for POP OH-metabolites. The first method (M1) was a liquid-liquid extraction from the incubation mixture with diisopropyl ether (DIPE), and acidification was performed only during the third extraction step (Murk *et al.*, 1994). The second method (M2) was a modification of the previous one with a centrifugation step before extraction (Hamers *et al.*, 2008). Except for the use of methyl tert-butylether (MTBE) instead of DIPE, M1 and M2 were performed as published. The third and the fourth extraction methods (M3 and M4) correspond to the crude and the phenolic organohalogen extraction methods published by Hovander *et al.* (2000). When required, the protocols were downsized to fit the incubation volume. All extracts were evaporated under gentle N_2 flow and redissolved in 1 ml of hexane for GC analysis or transferred into 50 µl of MeOH for bioanalysis.

Chromatographic analysis. We developed a selective ion chromatographic method to analyze coextractants and metabolites in one run. GC/MS was performed on a HP 6890 coupled to an Agilent 5973 mass spectrometer all from Agilent Technologies. Helium 99.99% was used as a carrier gas at a constant flow of 1.2 ml/min; the inlet temperature was set at 250°C, whereas the transfer line was kept at 280°C. Injection volume was 1 µl. Separation was achieved through an Rxi-5ms capillary column, 30 m . 0.25 m, 0.25 µm film thickness (Restek). The temperature

program was set to 80°C for 2 min, raised at 30°C min−1 to 220°C, which was held for 15 min, and then raised at 15°C min−1 to 300°C and held for 3 min, for a total of 30 min. Electron ionization (EI) was used at an ion source temperature of 230°C and an electron energy of 70 eV. Chromatographic data were recorded and analyzed with Chemstation (Agilent Technologies). High-sensitivity electron impact (EI) mass spectra from silylated metabolites were obtained with GC/MS-MS (Supplementary data 1).

Extracts, POP OH-metabolites, selected fatty acids, and cholesterol were silylated by mixing 20 µl of compound standard solution or extract in hexane with 40 µl of BSTFA–TCMS (9:1), which contained 1 µg/ml of CB 209 as internal standard (IS), in GC vials. They were heated on a plate at 70°C for 30 min and then transferred into inserts for GC analysis. Identification of coextractants and produced metabolites was achieved through analysis of fragmentation patterns and retention time from their trimethylsilyl derivatives (Table 1) and their comparison with standards. Quantification was done using a multicompound external calibration, which was run with every analysis (Fig. 1A). The limit of detection of the OH-metabolites was calculated from the blank microsomal extract base line and subsequently confirmed by the analysis of three extracts of blank microsomal incubations spiked with OH-metabolites at the limit of detection level (Table 1).

ANSA displacement assay. The basic principle of the fluorescence displacement assay is the shift in fluorescence peak of the ANSA fluorescence probe, after binding to transport proteins such as human serum albumin, TTR, and TBG. The shift in the fluorescence peak is from 515 to 465 nm with a large increase in quantum yield. Fluorescent displacement of ANSA bound to TTR by competitors has been published using spectrometric detection with cuvettes (Cao *et al.*, 2010, 2011; Nilsson and Peterson, 1975; Nilsson *et al.*, 1975). We developed a method in a 96-well plate setup for TTR binding according to the same principle, which was optimized for a variety of method variables including plate type, measurement angle, emission filter, and sensitivity settings. The equilibrium between bound and unbound ligand is very sensitive for incubation conditions, and therefore, incubation volume, time, temperature, reactants, and solvent concentrations were optimized and standardized (Supplementary data 2). Subsequently, the ANSA-TTR fluorescence coefficient was obtained after titration of increasing ANSA concentrations [ANSA] into a 0.5µM TTR solution in PB. From the linear part of the curve between [ANSA] and the fluorescence intensity (Fλ), the fluorescence coefficient (B) was obtained (Moller and Denicola, 2002). The amount of bound ANSA was then calculated for any given [ANSA] where [ANSA]bound = Fλ/B. From the curve between [ANSA]bound and [ANSA], the ANSA dissociation constant (Kd) and the number of binding sites (Bmax) can be calculated after fitting the data to a dose-response model. The binding or association constant (Kb) will be the inverse

of Kd. The effects of the carrier solvents DMSO and MeOH on the ANSA fluorescence and the ANSA-TTR displacement were also evaluated.

TTR binding experiments. Dose-related binding of T_4, coextractants, and metabolite standards to TTR was assessed in the ANSA-TTR displacement assay. Standard solutions in MeOH (2.5% final concentration) were mixed in a round-bottom 96-well plate (Greiner Bio-One) with 0.6 µM ANSA and 0.5 µM TTR in 100 µl PB at pH 7.5. After 2 h of incubation at 4°C, the plate was gently shaken for 10 s and measured in a Biotech Synergy 2 plate reader (Winooski, VT). ANSA fluorescence was excited using a 380 ± 20 nm filter, and the emission was measured with a 475 ± 20 nm filter. Negative controls without TTR, ANSA-TTR positive controls, and T_4 0, 100, and 600nM displacement controls were included on every plate. Variation coefficients for repeatability and reproducibility were determined using replicates on separate plates and separate experiments, respectively. The detection limit was set at thrice the SD of the control without TTR.

Data were expressed as fluorescence relative to the ANSA-TTR maximal fluorescence (positive control) unless otherwise stated. The 20 or 50% inhibition potency (IC20 or 50) for the competitors was obtained after fitting the dose-related decrease in relative fluorescence units (RFU) into a dose-response model using SigmaPlot 2001 (SPSS Inc.). Subsequently, the IC50 was used to calculate the competitors' dissociation constant (KdI) (Yung and Prusoff, 1973) and then its inverse to render their binding (association) constant KbI. These inhibition parameters were compared with the potency of T_4 tested under similar conditions.

New method for low-fat metabolite extraction. A new method for low-fat metabolite extraction (M5) was developed based on M3 and M4 (Hovander *et al.*, 2000), with modifications based on various experiments performed during this study. Preliminary T_4-TTR competition experiments with microsomal extracts performed with the Biacore showed that selected fatty acids and cholesterol bind to the dextran-coated chip surface (data not shown). To selectively retain fatty acids, the incubation mixture (1 ml) was mixed with 50 µl of a 10% dextran solution from Leuconostoc spp. in phosphate buffer in addition to 50 µl of 6M HCl for acidification. This mixture makes the coextractants precipitate, and the subsequent centrifugation (3 min, 1500 g, 4°C) further compacted the flocks. The incubation mixture including the flocs was extracted twice with 1 ml of hexane:MTBE (1:1, vol/vol). After vigorous vortexing and centrifugation (1 min, 1500 g), the organic phases were transferred into a second test tube containing 1 ml of an aqueous KCl solution (1%, wt/wt). After gentle mixing and centrifugation (30 s, 1000 g), the organic phase was transferred to a clean test tube, and the KCl solution was reextracted with 1 ml of hexane:MTBE. The combined organic phases were reduced to a 50 µl extract (20 mg-eq protein/ ml) under gentle N_2 flow.

Fatty acids and other higher molecular weight lipids were separated from the metabolites with a LRA minicolumn made with 0.8 g of LRA and 0.3 g of Na_2SO_4

packed on top of a glass wool plug in a Pasteur pipette. The LRA minicolumn was washed with 2 ml of hexane:MTBE (1:1) before the 50 µl extract was transferred to the minicolumn. The extract tube was rinsed twice with 150 µl of hexane:MTBE (1:1), which was transferred into the LRA column as well. This eluate from the LRA column (350 µl from the 50 µl extract and 2 . 150 µl rinsing) was discarded. Then the column was further eluted with 1600 µl of the same solvent, which was collected into a prewashed test tube where it was dried ml) for chemical analysis or 50 µl MeOH (20 mg-eq protein/ml) for bioanalysis.

TABLE 1

Relative retention time (RRT), limit of detection and GC/MS (EI) fragmentation data for CB-77, BDE-47 and the trimethylsilylated derivatives from selected fatty acids, cholesterol, and their metabolites.

Original compound		RRT [a]	Limit of detection (nmol/mg protein) [b]	Quantif. m/z	Qualif. ions MS m/z (rel.int. %)
	CB 77 (3,3',4,4') [d]	0.454	0.30	292	220 (40), 185 (5)
	BDE 47 (2,2',4,4')	0.759	0.10	486	326 (90), 484 (70), 324 (60)
Silylated derivatives					
Fatty Acids [e]	Dodecanoic acid (12:0)	0.266	0.30	257	272 (2), 117 (70), 73 (70)
	Myristic acid (14:0)	0.304	0.30	285	300 (5), 117 (70), 73 (70)
	Palmitic acid (16:0)	0.363	0.30	313	328 (5), 117 (70), 73 (70)
	Linoleic acid (18:2)	0.447	0.01	337	352 (8), 117 (70), 73 (70)
	Oleic acid (18:1)	0.450	0.01	339	354 (7), 117 (70), 73 (70)
	Stearic acid (18:0)	0.466	0.30	341	313 (20), 117 /(50), 73 (70)
	Archidonic acid (20:4)	0.574	0.01	79	91 (90), 117 (90), 238 (40)
Cholesterol		1.088	0.003	329	458; 368
CB-77	2-OH CB 77 (3,3',4,4') [d]	0.590	0.03	365	380 (70), 329 (50), 93 (30)
metabolites	5-OH CB 79 (3,3',4,5')	0.620	0.03	365	380 (70), 329 (50), 93 (30)
	4'-OH CB 79 (3,3',4,5')	0.733	0.01	365	380 (70), 329 (50), 93 (30)
BDE-47	6'-OH BDE 49 (2,2',4,5')	0.872	0.10	559	574 (60), 324 (60)
metabolites	6-OH BDE 47 (2,2',4,4')	0.877	0.03	559	574 (60), 324 (60)
	5-OH BDE 47 (2,2',4,4')	0.931	0.03	574	559 (100), 320 (50)
	2'-OH BDE 66 (2,3',4,4')	0.955	0.03	559	574 (60), 324 (60)
	4-OH BDE 42 (2,2',3,4')	0.956	0.10	574	559 (90), 320 (40)
	4'-OH BDE 49 (2,2',4,5')	0.956	0.10	574	559 (90), 320 (40)
	3-OH BDE 47 (2,2',4,4')	0.981	0.03	559	574 (50), 320 (50)
Internal Standard (IS)	CB 209 (2,2',3,3',4,4',5,5',6,6')	1.00	- -	498	428; 214

[a] Retention times relative to CB 209 on a Rxi®-5ms capillary column, 30m x 0.25m x 25 µm. RRT had a variation coefficient within experiments no larger than 1%. [b] Limit of detection calculated from a blank microsomal extract base line and confirmed by extracts from spiked incubations at the limit of detection level. As the total protein was 1 mg/mL the units nmol/mg protein are equivalent to µM. [d] PCBs and BDEs systematic numbers according to Ballschmiter and co-workers (1980) reviewed by Mills and co-workers (2007) followed by the list of numerical chlorine or bromine substituent localizers. [e] Fatty acid common names followed by their structure (number of carbons : number of double bonds) according to R.O. Adolf and F.D.Gunstone (http://www.aocs.org/member/division/analytic/fanames.cfm).

TTR binding of CB 77 and BDE 47 metabolic extracts. The microsomal blank, OH-POP spiked incubations, and incubations with CB 77 and BDE 47 were extracted with the optimized M5 method. Metabolites were identified and quantified after silylation using GC/MS as described above. Extracts in MeOH were serially diluted up to 64 times and used to measure their ANSA-TTR fluorescence displacement potency relative to that of T_4 based on the linear part of the curve. The bioassay T_4-Eq was compared with the T4 equivalents calculated from the chemically analyzed metabolite concentrations multiplied by the T_4 equivalent factors from our own study or from literature (Hamers *et al.*, 2008). In the absence of complete dose-response curves, significant differences with extraction blanks and negative incubations were assessed with ANOVA followed by a LSD post hoc comparison (SPSS Inc.).

Results

Identification and quantification of coextractants

After extraction of blank microsomal S9 fraction incubations with four published methods, the chromatographic profiles of the extracts included various SFA, NSFA, and cholesterol (Fig. 1). For identification, the relative retention times and mass spectrometric characteristics were compared with standards when available (Table 1).

TABLE 2

Mean concentration expressed in nmol/mg-eq[a] protein of fatty acids, cholesterol, and % of recovery for 4-OH CB 79 in the extracts obtained with published methods and with the developed method

Compound	M1 Murk *et al.* (1994)	M2 Hammers *et al.* (2008)	M3 Hoovander *et al.* (2000) Crude	M4 Phenolic	M5 New method
Dodecanoic (12:0)	<LQ[b]	<LQ	<LQ	0.62 ± 0.57	0.07 ± 0.02 [c]
Myristic (14:0)	0.68 ± 0.50	0.40 ± 0.062	0.38 ± 0.25	1.5 ± 0.70	0.19 ± 0.15
Palmitic (16:0)	12 ± 1.5	7.6 ± 0.62	6.9 ± 0.44	15 ± 4.5	2.47 ± 0.94
Stearic (18:0)	12 ± 1.3	6.5 ± 0.78	5.2 ± 0.69	11 ± 2.3	1.23 ± 0.67
Total SFA [d]	24 ± 2.0	15 ± 1.00	12 ± 0.86	19 ± 5.1	4.0 ± 1.2
Arachidonic (20:4)	4.9 ± 1.60	2.9 ± 0.58	3.1 ± 0.047	8.0 ± 4.8	0.12 ± 0.2
Linoleic (18:2)	1.0 ± 0.12	0.4 ± 0.56	0.85 ± 0.20	1.7 ± 1.2	0.17 ± 0.03
Oleic (18:1)	0.55 ± 0.16	0.37 ± 0.04	0.50 ± 0.13	1.0 ± 0.5	0.17 ± 0.04
Total NSFA	6.5 ± 1.6	3.6 ± 0.81	4.5 ± 0.24	8.2 ± 1.2	0.53 ± 0.33
Cholesterol	26 ± 2.6	4.2 ± 0.32	18 ± 1.4	0.07 ± 0.05	5.3 ± 2.7
4-OH CB 79 (%) [e]	89 ± 12	76 ± 8.0	89 ± 9.6	106 ± 21	84 ± 13

[a] All extracts were made from 1 mg protein and dissolved in 1mL of hexane for GC/MS analysis therefore concentration was equivalent to µM. [b] LQ: limit of quantification. [c] Standard deviation from two separate experiments, each with duplicate incubation injected twice in GC/MS. [d] Palmitoleic acid (16:1) was identified in M1 and M2 but not quantified due to a lack of standard. [e] 4-OH PCB 79 original concentration in the incubation mixture was 2 nmol/mg protein

Per milligram original microsomal protein (mg-eq protein), the four methods extracted 12–24 nmol SFA, 3.6–8.2 nmol NSFA, and 0.1–26 nmol cholesterol. The M1 produced the highest quantity of coextractants, whereas the M2 limited the amount of SFA and NSFA extracted to almost 50% compared with M1 (Table 2). M3 had slightly lower quantities of SFA, NSFA, and cholesterol compared with the extract obtained with M1.

Furthermore, the phenolic extract obtained with M4 contained only detectable quantities of cholesterol, but the extraction roughly doubled the amount of SFAs and NSFAs, particularly that of arachidonic acid. Now that the coextractants have been identified and quantified, their relevance in disruption of the competitive TTR binding assay will be analyzed.

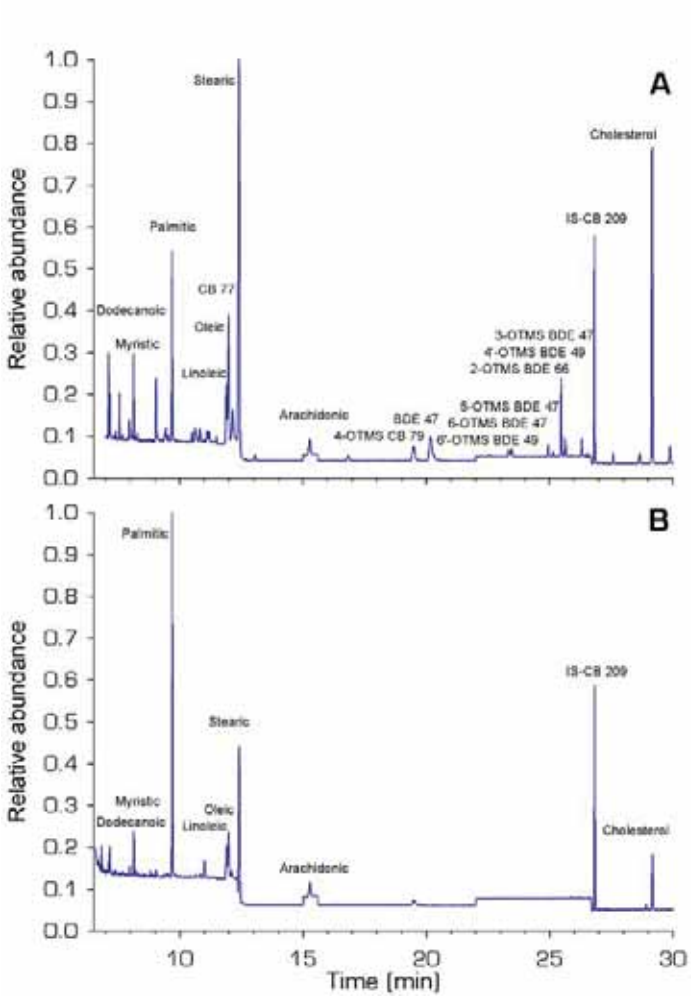


Figure 1. Chromatogram from silylated derivatives of a standard mixture (A) and of a S9 microsomal blank extracted with M1(B). Silylation internal standard (IS) was CB 209; surrogate standards were 4-OH CB 79, 2-OH BDE 66, and 6-OH BDE 47. Fatty acids were named with their common name.

ANSA fluorescence displacement assay

The ANSA fluorescence displacement assay was optimized for ANSA-TTR binding. The intensity of ANSA bound to TTR was optimal when performed in a round-bottom 96-well plate and the fluorescence measured from the bottom, with a 380±20 nm excitation filter and a 475±20 nm emission filter. The optimal ANSA concentration was 0.6 μM and TTR 0.5 μM. Equilibrium conditions were achieved after 2 h of incubation at 4°C with gentle shaking before measurement. For more detailed results, see Supplementary data 2.

ANSA in the absence of TTR gives background fluorescence, and neither MeOH nor DMSO (<20%) interfered with this background fluorescence. However, the fluorescence of ANSA in the presence of TTR was significantly reduced by DMSO and by MeOH above 2.5% (Fig. 2).

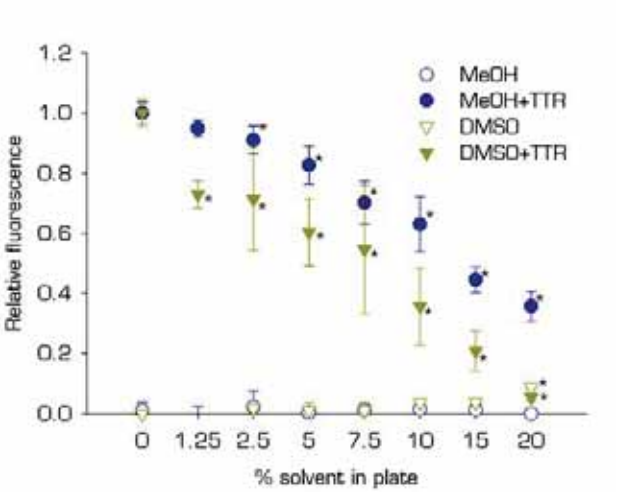


Figure 2. MeOH (circles) and DMSO (triangles) effect on the fluorescence intensity of 0.6μ ANSA in buffer (white) or with to 0.5μM TTR (black). Data represent the average fluorescence relative to an ANSA-TTR solution ± SD of three independent experiments in triplicate. The zero relative fluorescence represents the ANSA blank without TTR. * indicates significant differences (p < 0.05) after an ANOVA with Dunnett's *post hoc* comparison.

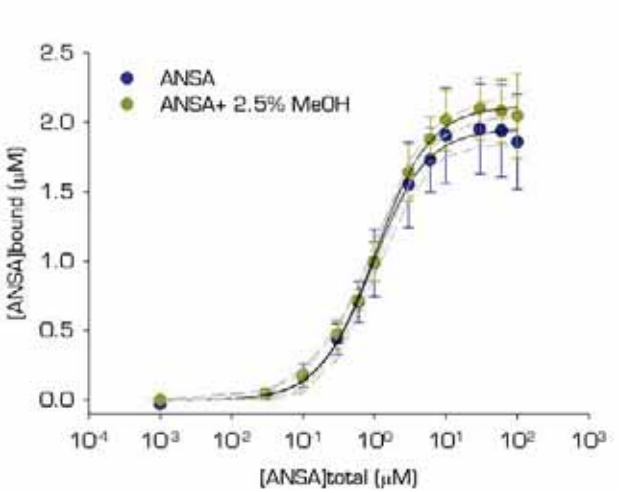


Figure 3. Association binding of ANSA to 0.5 μM TTR in phosphate buffer (black) and in phosphate buffer with 2.5% MeOH (gray). Data represent the calculated [ANSA] bound concentration ± SD of two independent experiments in triplicate. Lines represent the predicted values (solid) ± 95% confidence intervals (dashed) after the data were adjusted to a Hill model.

From the titration of ANSA into 0.5µM TTR, the ANSA Kb was calculated to be 1.06±0.13x10^6 M^{-1} (Fig. 3). There was indeed a significant interaction between the effect of 2.5% MeOH and the effect of ANSA concentration (ANOVA, P<0.05); MeOH increased fluorescence at high ANSA concentrations (> 6µM). However, the overall effect of 2.5% MeOH on the ANSA titration into TTR was not significant (p < 0.01) and particularly not at lower ANSA concentrations (Fig. 3). ANSA Kb in the presence of 2.5% MeOH was 0.97±0.08x106 M^{-1} which was, in consequence, not significantly different from the one without MeOH (p < 0.01). Therefore, 2.5% was chosen as maximal solvent concentration for further ANSA-TTR experiments.

Using a dose-response design, T_4 reduced the fluorescence signal from 0.6 µM ANSA plus 0.5 µM TTR in a dose-dependent manner with an IC20 of 80±16 nM, an IC50 of 265±8.2 nM (Fig. 4A) and a limit of detection of 60±12nM T4. The T_4 binding constant was 6.0±0.2x10^6 M^{-1} (Table 3). The repeatability was typically 5%, and the reproducibility was 9% when the same lot of TTR was used and was up to 15% when experiments were performed with different TTR lots. Figure 4B shows the full ANSA-TTR displacement curves for the four OH-CB 77 and OH-BDE 47 metabolite standards. Their relative potencies compared with that of T_4 are given in Table 3.

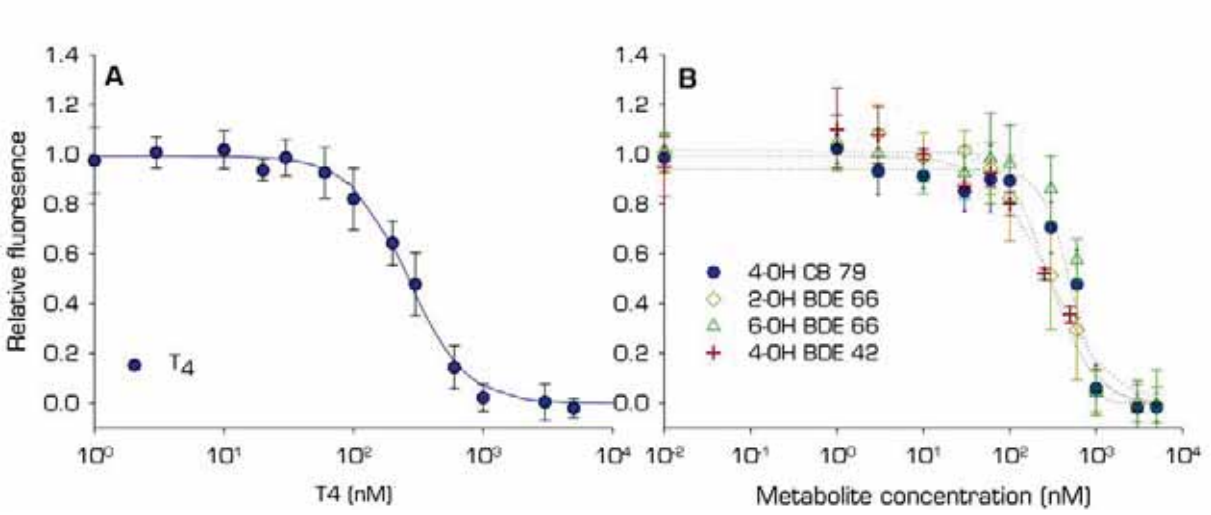


Figure 4. Fluorescence displacement curve of T_4 (A), PCB 77, and BDE 47 OH-metabolites (B) titrated into a solution of 0.6µM ANSA and 0.5µM TTR. Data represent the average of fluorescence intensity relative to an ANSA-TTR solution ± SD of at least three different experiments in triplicate. Lines represent the predicted values after the data were adjusted to a Hill model.

TTR competitive binding of coextractants

The S9 coextractants identified in the extracts of the four extraction methods all displaced ANSA from TTR with the exception of cholesterol and stearic acid (Figs. 5A and B). The SFAs significantly displaced ANSA fluorescence from 1 µM on, with dodecanoic acid and myristic acid fully displacing ANSA at 30 µM, whereas palmitic acid only partially displaced it (Fig. 5A). NSFAs had a significant effect already from 0.3 µM and completely displaced ANSA fluorescence at 30 µM (Fig. 5B). Inhibition

potencies of fatty acids were 5 to 30 times lower than that of T_4 and 2–10 times lower than the OH-metabolites. The fatty acid TTR binding constants are one order of magnitude lower compared with T_4 (Table 3). Given the TTR finding potency of the SFAs and NSFAs and their concentrations in the extract, it is important to further reduce the coextracted levels in the metabolic extract.

TABLE 3
ANSA-TTR displacement parameters for T_4, co-extractants and selected metabolites from CB 77 and BDE 47.

Group	Compound	Range (μM)	IC 20 (μM) [a]	IC 50 (μM) [a]	Kb (10^6 M^{-1})	T_4Eq
Hormone	T_4	0.001-5	0.12	0.26 ± 0.1 [b]	6.0	1
SFA	Dodecanoic (12:0)	0.01-300	3.0	11 ± 0.94	0.2	0.02
	Myristic (14:0)	0.01-300	1.5	6.5 ± 0.75	0.24	0.04
	Palmitic (16:0)	0.01-300	1.0	n.d. [c]	3.1 [d]	0.12 [d]
	Stearic (18:0)	0.01-300	>300	>300	-	<0.001
NSFA	Arachidonic (20:4)	0.01-300	0.40	2.3 ± 0.25	0.76	0.12
	Linoleic (18:2)	0.01-300	1.8	6.5 ± 0.83	0.24	0.04
	Oleic (18:1)	0.01-300	0.72	3.3 ± 0.38	0.48	0.08
	Cholesterol	0.01-30	>30	>30		<0.01
PCB metabolites	4'-OH CB 79	0.001-5	0.26	0.51 ± 0.02	3.10	0.52
BDE metabolites	2'-OH BDE 66	0.001-5	0.13	0.23 ± 0.02	5.51	0.93
	6-OH BDE 47	0.001-0.5	0.48	0.63 ± 0.02	2.50	0.42
	4-OH BDE 42	0.001-5	0.97	0.29 ± 0.02	5.36	0.93

[a] Concentration in the ANSA displacement assay plate which produced the 20 or 50% inhibition compared with the maximal inhibition of T_4

[b] T_4 IC_{20} was 80±16 nM, and it was used to calculate the extracts bioassay T_4 equivalents (T_4-Eq)

[c] n.d.: not determined

[d] Kb and T_4Eq calculated at the IC_{20} level

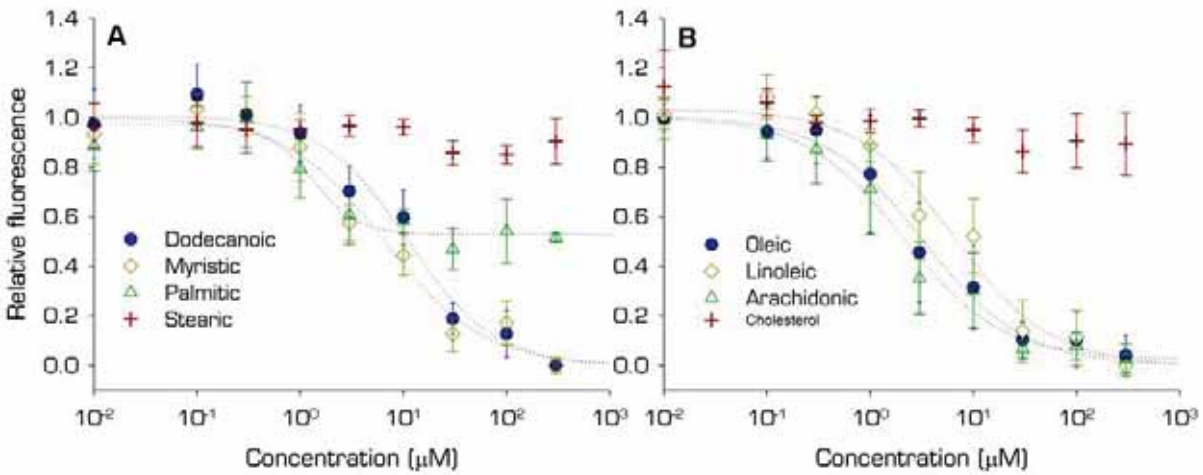


Figure 5. Competitive TTR binding by SFAs (A), NSFAs, and cholesterol (B) titrated into a solution of 0.6 μ M ANSA and 0.5 μ M TTR. Data represent the average of fluorescence intensity relative to an ANSA-TTR solution ± SD of at least three different experiments in triplicate. Lines represent the predicted valuesafter the data were adjusted to a Hill model.

Reduction of coextractants in the metabolic extract

A new extraction method M5 was developed to limit the coextractant concentrations and their effects in the TTR competition assay. The new method included protein denaturation and lipid flocculation with the addition of 10% dextran at pH < 2, liquid-liquid extraction, and a LRA minicolumn.

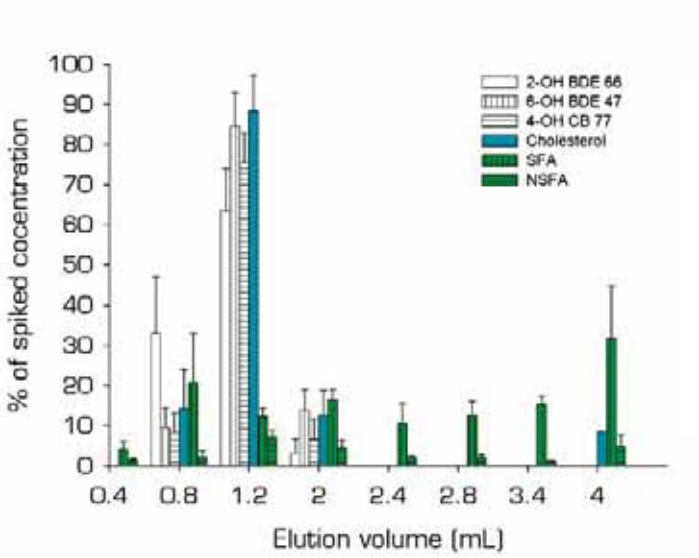


Figure 6. Elution profile of a standard mixture through an 80-mg LRA minicolumn. Concentrations of OH-metabolites, cholesterol, SFA, and NSFA were obtained after GC/MS analysis of eluates. In the M5, the first 0.4 ml is discarded as is everything after 2 ml. Data represents average ± SD from two independent experiments each with four separate columns.

The addition of 10% dextran at pH < 2 already reduced SFAs 50% compared with M3 to 6 nmol/mg eq protein and NSFAs to 2 nmol/mg eq protein. The LRA minicolumn improved this removal further. Figure 6 shows the elution profile of coextractants and OH-POP metabolites through the LRA minicolumn. The metabolites eluted from 0.4 to 2 ml (Fig. 6). SFA eluted constantly from the column, whereas NSFA and particularly oleic and linoleic acids eluted mainly between 1.2 and 2.4 ml. Arachidonic acid did not elute before 4 ml (Fig. 6) and therefore could be reduced to 0.12 nmol/mg-eq protein, which corresponds to a reduction of more than 21 times compared with M1 (Table 2). With M5, 5 to 10 times less SFAs and 7 to 20 less NSFAs were extracted than with any of the other methods tested (Table 2). Cholesterol concentration was five times lower than with M1, three times lower than with M3, but considerably higher compared with M4 (Table 2).

A 0.5 mg-eq protein/ml microsomal blank extract prepared with M5 and spiked with 3 nmol T_4/mg-eq protein produced a displacement curve that was not significantly different from that obtained spiking MeOH with a similar amount of T4 (Fig. 7). However, the undiluted microsomal extract significantly competed with ANSA-TTR binding above 0.25 mg-eq protein/ml with a competitive potency of 0.3 nmol T4-Eq/mg-eq protein (Fig. 8).

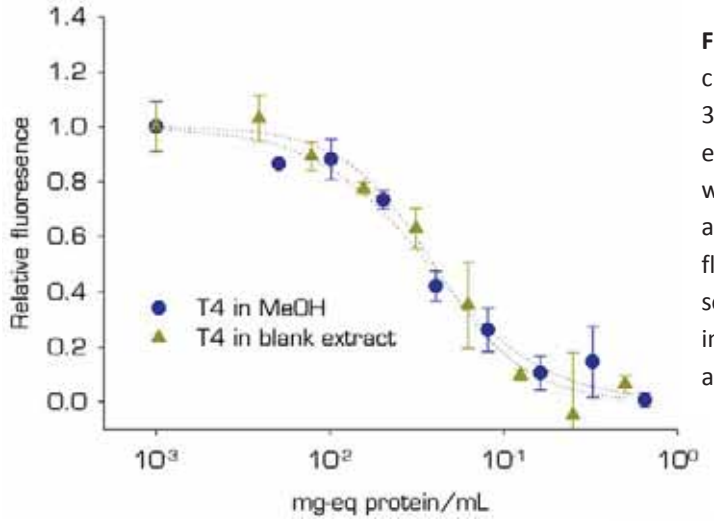
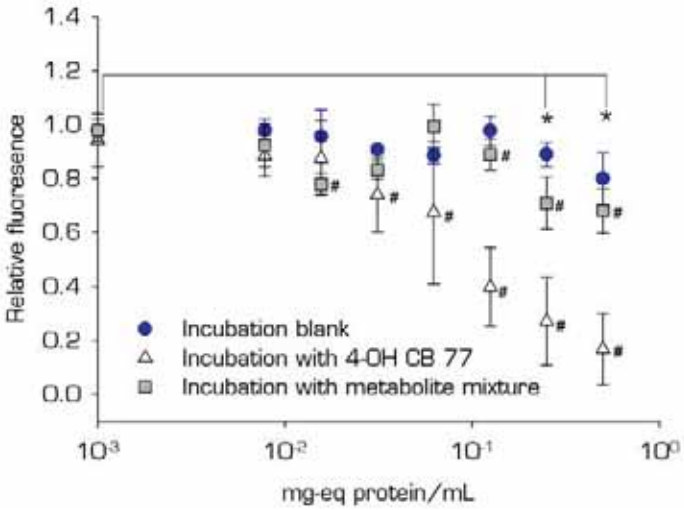


Figure 7. M5 extract effect on the T4 displacement curve. A solution of 4 µM T4 in MeOH (circles) or 3 µM T4 in 0.5 mg-eq protein/ml microsomal blank extract obtained with the M5 method (triangles) was serially diluted into a solution of 0.6 µM ANSA and 0.5 µM TTR. Data represent the average of fluorescence intensity relative to an ANSA-TTR solution ± SD of at least three different experiments in triplicate. Lines represent the predicted values after the data were adjusted to a Hill model.

Figure 8. Competitive TTR binding of serially diluted M5 extract (0.5 mg-eq protein/ml) of S9 incubation blank (circles), S9 incubation spiked with 2 nmol T_4-Eq of 4-OH CB 77/mg-eq protein (triangles) or with 0.3 nmol T_4-Eq/mg-eq protein of an OH-metabolite mixture (containing 4-OH CB 77, 2-OH BDE 66, and 6-OH BDE 47) and extracted with the M5 method. Data represent the average fluorescence relative to 0.6 µM ANSA and 0.5 µM TTR solution ± SD of at least two separate extracts in triplicate. * indicates significant differences of incubation blank compared with the ANSA-TTR control, and # indicates differences of spiked incubations compared with the incubation blank ($P < 0.05$) after an ANOVA with LSD *post hoc* comparison.



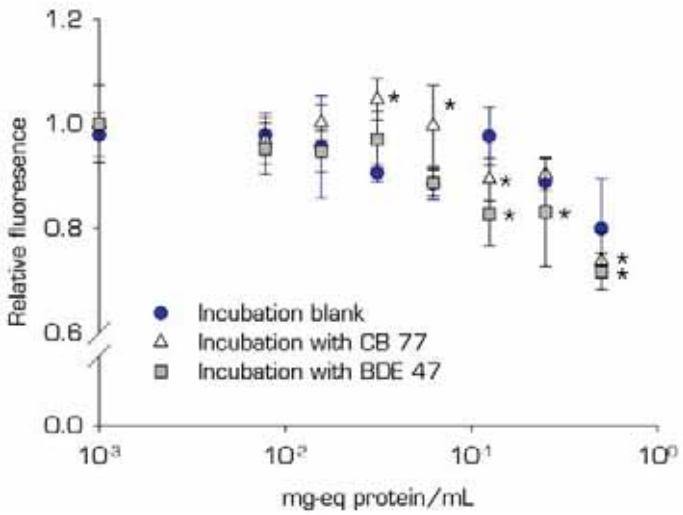


Figure 9. Effect of CB 77 and SBDE 47 metabolic incubation extracts. Microsomal incubation blanks with DMSO (circles), with CB 77 (triangles), and with BDE 47 (squares) were extracted according to the M5 method; the 0.5 mg-eq protein/ml extracts were diluted up to 68 times and titrated into a solution of 0.6 µM ANSA and 0.5 μM TTR. Data represent the average of fluorescence intensity relative to the maximum of an ANSA-TTR solution ± SD of at least two separate extracts in triplicate. * indicates significant differences when comparing with incubation blank in DMSO ($p < 0.05$) after an ANOVA with LSD *pos hoc* comparison.

TTR competitive binding by extracts with bioactivated CB 77 and BDE 47

Finally S9 microsomes were incubated during 90 min with 10 µM CB 77 or BDE 47 and extracted with the newly developed M5 method. The extracts were chemically analyzed and tested in the ANSA TTR displacement assay (Table 4). MS details of silylated metabolites are presented and discussed in Supplementary data 1. In vitro metabolism of CB 77 produced mainly 4-OH CB 79, which accounted for 68% of the total metabolite fraction, followed by 20% of 5-OH CB 77 and 16% of 2-OH CB 77. The most abundant metabolite from BDE 47 was 4'-OH BDE 49 with 48% of the total followed by 3-OH BDE 47 and 5-OH BDE 47, which accounted for 25% each, and trace amounts of 2'-OH BDE 66 and 4-OH BDE 47.

Considering the final concentration of metabolites, the calculated metabolic rates were 33 and 6 pmol mg protein^{-1}min^{-1} for CB 77 and BDE 47, respectively. Metabolic extracts from both CB 77 and BDE 47 reduced ANSA fluorescence in a dosedependent manner, but the differences with the dilution range of the incubation blank were small and not always statistically significant (Fig. 9). Incubations stopped at t = 0 were not significantly different from incubation blanks (solvent controls; data not shown). At the T_4 IC20 level, the bioassay T_4-Eq was 0.3 nmol T_4-Eq/mg-eq protein for the CB 77 metabolic extract and 0.6 nmol T_4-Eq/mg-eq protein for the BDE 47 bioactivated extract.

TABLE 4

Mean concentration (nmol/mg eq protein) of CB 77 and BDE 47 OH-metabolites in the extract after microsomal incubation and their respective concentrations in the MeOH extract and final concentration in the ANSA displacement assay.

	Extract		Assay plate
Solvent	Hexane	MeOH	Buffer + 2.5% MeOH
Volume	1 mL	50 µL	100 µL
Conc. factor	1	20	1/40
Protein-eq conc.	1 mg/mL	20 mg/mL	0.5 mg/mL
2-OH CB 77	0.10 ± 0.02	2.0	0.1
4-OH CB 79	0.41 ± 0.08	8.2	0.2
5-OH CB 77	0.12 ± 0.03	2.4	0.1
Sum OH-PCBs	**0.60 ± 0.10**	**13**	**0.3 (0.14 T_4Eq)**[a]
6-OH BDE 47	<L.D		
5-OH BDE 47	0.05 ± 0.02	1.0	0.03
2'-OH BDE 66	<L.D		
4-OH BDE 47	<L.D		
4'-OH BDE 49	0.10 ± 0.01	2.0	0.05
3-OH BDE 47	0.06 ± 0.01	1.2	0.03
Sum OH-BDEs	**0.21 ± 0.02**	**4.2**	**0.11 (0.38 T_4Eq)** [a]

[a] Calculated T_4 equivalents from the chemically analyzed metabolite concentrations multiplied by the T4 equivalent factors from our own study or from literature (Hamers *et al.* 2008).

Despite the interference at higher extract concentrations, spiked S9 microsomal incubations were extracted and successfully quantified. An extract spiked with 2 nmol T_4-Eq of 4-OH CB 77/mg-eq protein produced 2.3 T_4-Eq/mg-eq protein, and the extract spiked with 0.3 T_4-Eq OH-metabolite mixture/mg-eq protein.

Discussion

A method was developed to reduce the concentrations of the coextractants from bioactivated mixtures of PCB and BDE that disturb the TTR binding assays. With an optimized method for silylation, the identities and concentrations of extracted fatty acids and OH-metabolites were obtained, and their TTR binding potencies were quantified in a downscaled, nonradioactive TTR binding ANSA assay in a 96-well plate. The greatly improved removal of especially fatty acids from the microsomal extracts allows quantification of the TTR binding potency of the *in vitro* produced metabolites at the IC20 level.

Optimization of the S9 Extraction Method

The published metabolite extraction methods also are efficient in extracting lipids including free fatty acids, cholesterol, and acidic phospholipids, which are neutral at low pH. The observed fatty acid composition was very similar to that reported for human liver microsomes and trout S9 liver fractions, whereas the sum of fatty acids and cholesterol was only 4–10% of the total reported lipid content (Belina *et al.*, 1975; Escher and Fenner, 2011; Waskell *et al.*, 1982). Therefore, M1, M2, and M3 are already efficient at limiting the coextracted fatty acid content. Nevertheless, fatty acids can also be produced by hydrolysis of coextracted phospholipids. This was observed in the increase of NSFAs and particularly the arachidonic acid content in the M4 extract (Table 2). Neutral phospholipids such as phosphatidylserine have a higher arachidonic acid concentration (Waskell *et al.*, 1982); it was likely hydrolyzed under the highly basic conditions during the final extraction step in M4 increasing the coextracted free fatty acid. The addition of dextran in M5 partially reversed the transfer equilibrium of free fatty acids to the organic phase by their retention in the flock; the LRA minicolumn selectively retained heavier NSFA. And finally, lipolytic conditions were avoided in the newly developed method.

Interaction of coextractants with TTR binding

The inhibitory effect of fatty acids and particularly NSFA on the competitive protein-binding assay has long been known by clinicians who tried to develop a method to determine the free T_4 in plasma by testing its binding potency to transport proteins. The most potent inhibitors of T_4 binding to TBG and TTR are

short-chain SFA and long-chain NSFA (Shaw *et al.*, 1976). We confirmed this with our downscaled ANSA displacement assay (Table 3). The fatty acids' binding constants obtained here were similar to reported values (Tabachnick and Korcek, 1986), but their IC50 were lower than previously reported values (Shaw *et al.*, 1976). These differences are commonly due to variation in protein source and purity (Nilsson *et al.*, 1975). Although triglycerides have been found not to interfere with the assay (Shaw *et al.*, 1976; Simon *et al.*, 2010), the influence from the small coextracted amount of phospholipids is uncertain. Under M5 conditions, however, the LRA minicolumn is expected to completely retain both triglycerides and phospholipids.

It was not known whether the fatty acid disruption of the T_4-TTR binding is the result of their direct interaction with T_4 (Shaw *et al.*, 1976; Simon *et al.*, 2010) or due to their interaction with the TTR binding sites (Tabachnick and Korcek, 1986). As the ANSA-TTR fluorescence displacement assay presented here does not require the presence of T_4 and FA did not interfere with ANSA fluorescence, it is suggested that the observed inhibitory effect is caused by interaction of the FA with the TTR. Considering reported results with the T_4-TTR RBA and results presented here, it is reasonable to suggest that observed assay disturbances are a combined effect of two mechanisms: a direct interaction of FA with T_4 at high fatty acid concentrations (observed in the RBA assay) and TTR competitive inhibition by FA at lower concentrations (observed here). This would explain observed U shapes with muscle extracts in the RBA competitive binding assay (Simon *et al.*, 2010). In summary, a method that does not include T_4 for the competitive binding measurements, like the ANSA-TTR fluorescence displacement assay, is one step further reducing the disturbing action of coextractants.

The obtained ANSA-TTR binding constant (1.06×10^6 M^{-1}) in the 96-well ANSA assay was in agreement with previously published values between 1 and 36×10^6 M^{-1} (Cao *et al.*, 2010; Cheng *et al.*, 1977; Nilsson *et al.*, 1975). The calculated T_4 binding constant (6.0 . 106 M−1) was one order of magnitude lower compared with published data (10×10^7 M^{-1}) for the ANSATTR assay with cuvettes (Cao *et al.*, 2010; Nilsson *et al.*, 1975). Lans *et al.* (1993) reported a considerably lower T_4 binding constant (2×10^7 M^{-1}) in the RBA. This difference in relative binding affinity was reflected in the obtained T_4 IC50, which was 3 to 10 times higher in the ANSA assay than previously reported values (Cao *et al.*, 2010; Lans *et al.*, 1993; Marchesini *et al.*, 2008; Meerts *et al.*, 2000). These differences could also be attributed to variations in protein sources. However, despite the lower sensitivity obtained in the 96-well plate ANSA displacement assay, the T_4-relative potencies for OH-POP metabolites were in agreement with literature (Cao *et al.*, 2010; Lans *et al.*, 1993; Marchesini *et al.*, 2008; Meerts *et al.*, 2000). In addition to the advantage of

excluding potential interaction of FA with T_4 from TTR competitive binding incubations, the ANSA displacement assay has several other advantages compared with previously used methods (Lans *et al.*, 1993; Marchesini *et al.*, 2006): (1) It does not require radioactive materials, (2) the cumbersome and sometimes not reproducible immobilization of hormone to the biosensor chip is not needed, and (3) expensive biosensor equipment is not required. The downscaling of the ANSA fluorescence displacement method into a 96-well plate format brings the additional advantages: (1) reduction of amounts of chemicals needed, (2) considerable reduction in analysis time, and (3) possibility for high throughput.

Presence of Interfering Coextractants

A typical microsomal incubation mixture (1 mg protein in 1 ml) is mostly concentrated in 50 µl of MeOH or DMSO (so the coextractants are 20 times concentrated) followed by 40-times dilution in the TTR competitive assay solution. Thus, the final dilution factor usually is 2. Under these conditions, an undiluted extract obtained with M1 would have a concentration in plate of 12 µM SFA and 3.3 µM NSFA (Table 2), which is well above their IC50s in the ANSA displacement assay (Table 3). The newly developed method reduced the amount of coextractants by 10 times compared with M1. An extract obtained with the modified method (M5) and finally diluted twice as mentioned above will contain less than 2.0 µM SFA and 0.26 µM NSFA, which are concentrations below the IC50 for these compounds in the ANSA displacement method (Tables 2 and 3). When the extract is tested "undiluted," some interference with the analysis is to be expected. As can be seen from Figure 8, the blank microsomal extract will reduce ANSA binding at concentrations above 0.25 mg-eq protein/ml. This means that an extract diluted four times will have a FA below their competitive concentration, thus enabling an undisturbed analysis. This was corroborated by the results of Figure 7 where the ANSA displacement by T_4 in microsomal extract, prepared according to M5, was equal to the one in MeOH. This performance in a microsomal extract in a TTR competition assay is a considerable improvement compared with previously published results with fish and plasma, in which larger dilution regimes and cumbersome clean-up procedures were used (Simon *et al.*, 2010, 2011).

The metabolic profile and metabolic rates of CB 77 were in accordance with published results (Murk *et al.*, 1996), except that 6-OH-CB 77 was not detectable (Table 4). Similarly, the obtained results agreed with proportions and metabolic rates published for BDE 47 (Hamers *et al.*, 2008; Harju *et al.*, 2007). Metabolic extracts from CB 77 and BDE 47 reduced ANSA fluorescence significantly above 0.12 mg-eq protein/ml. However, due to the effects of the coextractants at concentrations above 0.25 mg-eq pr/ml it is not possible to prepare a full dose-

response curve with the amount of metabolites produced under the chosen conditions. Therefore, quantification has to be performed at a concentration around or below the IC50 for PCB and PBDE OH-metabolites (Table 4). The calculated bioassay T_4-Eq from the bioactivated CB 77 and BDE 47 was higher in comparison with the chemically obtained values (Table 4). An effect often seen with bioassays as also OH-metabolites present in levels below the limit of chemical detection will be contributing to the response in the bioanalysis.

Conclusions

SFA and, particularly, NSFA were identified along with cholesterol as the major coextractants upon OH-PCB and OH-BDE extraction from a PCB 77 and BDE 47 metabolic incubation with S9 liver fraction. The observed effect from the metabolic extracts in the ANSA fluorescence displacement assay is mainly due to the coextracted free fatty acids. A method to strongly reduce the coextraction of matrix components by including fat flocculation, liquid-liquid extraction, and a LRA minicolumn was developed. The extract obtained was used in the downscaled ANSA-TTR competitive fluorescence displacement assay to quantify the T_4 equivalents from samples spiked with OH-metabolites. GC-MS analysis of silylated derivatives was suitable to quantify the production of T_4-like OH-metabolites from the model POP compounds. With bioactivated CB 77 and BDE 47 it is now possible to make a dose-response curve up to 50% ANSA-TTR inhibition. To make a full dose-response curve, the efficiency of the biotransformation should be further enhanced. The developed new extraction and clean-up method can also be applied to other biological matrices such as plasma or tissue extracts.

Supplementary data

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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CHAPTER 4a

Meta-analysis of supramaximal effects in *in vitro* estrogenicity assays

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Abstract

In scientific literature, several estrogenic compounds are reported to induce responses in vitro that are significantly higher than that of estradiol (E2). These supramaximal (SPMX) estrogenic effects do not occur consistently and seem to differ depending on the cellular models applied. This study analyzes the possible underlying causes, mechanisms, and drivers for SPMX estrogenic effects in in vitro functional assays reported in the peer-reviewed literature. For the 21 natural and industrial chemicals identified as SPMX inducers, the culture and exposure conditions varied greatly among and between the assays. Detailed information on assay characteristics, however, sometimes lacked. Diethylstilbestrol, genistein, and bisphenol A were selected to build a database. The meta-analysis revealed that the occurrence of SPMX effects could be related to a number of specific assay characteristics: (1) the type of serum used to supplement the exposure medium, (2) the end point used to quantify the estrogenic potency (endogenous or transfected), (3) the number of estrogen response elements, and (4) and the promoter's nature. An SPMX response was not reported for expression of endogenous genes, assays that used African green monkey kidney (COS-1) cell line or with chloramphenicol transferase as the reporter gene. There were no indications that solvent concentration in culture, exposure period, or cell model influenced the occurrence of an SPMX effect. It is important to understand the mechanism behind this phenomenon because in vitro assays for estrogenicity are used extensively to characterize and quantify the estrogenic potency of compounds, mixtures and environmental extracts.

Introduction

There has been considerable effort over the last two decades to develop *in vitro* assays capable to screen and assess the estrogenic activity of environmental pollutants, industrial chemicals, natural chemicals, and estrogenic pharmaceuticals (reviewed by Charles, 2004; Mueller, 2002, 2004; Sato *et al.*, 2004; Scrimshaw and Lester, 2004; Zacharewski, 1997). These assays include competitive ligand-binding assays (Berthois *et al.*, 1986; Korach *et al.*, 1978; Migliaccio *et al.*, 1992), cell proliferation assays (Maggiolini *et al.*, 2001; Parez *et al.*, 1998; Soto *et al.*, 1995), induction of protein expression (Gehm *et al.*, 1997; Parez *et al.*, 1998), and recombinant receptor/reporter gene assays using various mammalian cell lines (Jausons-Loffreda *et al.*, 1994; Legler *et al.*, 1999; Miller *et al.*, 2000; Pons *et al.*, 1990; Ranhotra and Teng, 2005; Wilson *et al.*, 2004) and yeast strains (Arnold *et al.*, 1996; Routledge and Sumpter, 1996).

One interesting and often ignored feature of many *in vitro* assays is the ''supramaximal effect,'' occurring when the response for a particular ligand is significantly higher than the maximal response obtained for estradiol, the natural occurring ligand. Figure 1 displays three types of agonistic responses that can be distinguished: agonists that induce a supramaximal (SPMX) effect, compounds that induce ''submaximal'' responses, significantly lower than that of E2, and those with responses in between supra- and submaximal responses, named ''maximal.'' The presence of SPMX effects is not consistently reported in scientific literature, and sometimes the reports seem to be contradictory. Figure 1 also shows a ''putative supramaximal'' curve, where the response is reported as high as E2 maximum but without actually showing its maximum while the shape of the curve suggests that an SPMX response could have been reached at higher concentrations. For interpretation of the assay results, it is important to know whether this effect is related to a real suprastimulation of the gene expression that might also occur in vivo or whether it is an artefact from the assay applied.

An SPMX estrogenic response can be traced back at least to 1994 when Makela *et al.* (1994) discuss ''the exceeded 17β-estradiol–induced activation'' of both zeranol and zeralenone using an MCF-7 cell proliferation assay. Different names have been coined for the same effect by different authors; for instance, ''superagonism'' (Gehm *et al.*, 1997, 2004; Legler *et al.*, 1999; Ter Veld *et al.*, 2006), ''superagonist'' (Basly *et al.*, 2000; Harris *et al.*, 2005; Levenson *et al.*, 2003; Mueller *et al.*, 2004), ''superinduction'' (Freyberger and Schmuck, 2005), ''supramaximal'' (Van Lipzig *et al.*, 2005a, 2005b; Wilson *et al.*, 2004), and recently ''overactivation'' (Soto *et al.*, 2006).

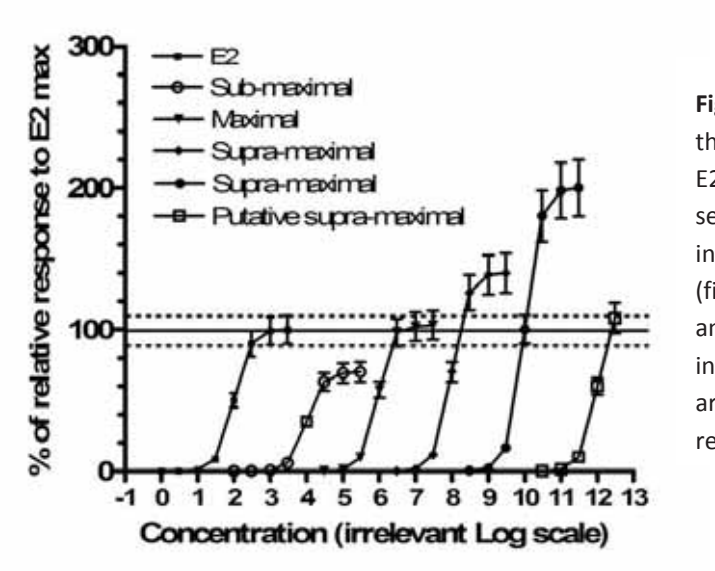

Figure 1. Simulated dose-response shapes as they appear in in vitro literature compared to E2 (filled squares) for which the maximum is set at 100%. Data represent submaximal induction (filled diamonds), maximal induction (filled triangles), SPMX induction (open circles and open squares), and putative SPMX induction (open diamonds). All simulated SEs are ± 10% of the percentage of relative response.

Many authors only mention the presence of a maximal response greater than that induced by estradiol (Buterin *et al.*, 2006; Han *et al.*, 2002; Kitamura *et al.*, 2005; Kuiper *et al.*, 1998; Kuruto-Niwa *et al.*, 2002, 2005, 2007; Makela *et al.*, 1994; Van Der Woude *et al.*, 2005; Wilson *et al.*, 2004), while others do not describe it in their results or mention it in their discussion even when evidently an SPMX response was obtained. Interestingly, reports of the SPMX estrogenic effect are not consistent among publications even for compounds analyzed with the same system. SPMX effects also have been observed in other reporter gene assays particularly for dioxinlike compounds as the Dioxin Receptor mediated Luciferase reporter gene assay (Jonker *et al.*, 2006; Murk *et al.*, 1996; Vrabie *et al.*, 2009).

The mechanism for an SPMX effect response remains obscure, only a few authors have discussed possible explanations and no one its implications. Stimulation of the estrogen receptor (ER) and/or coactivators expression or effects on luciferase stability have been suggested as potential underlying mechanisms in T47D cells stably transfected with a luciferase reporter gene (Legler *et al.*, 1999). In addition, some authors have used this later statement to support their observations without adding further insights on the mechanistic explanation (Harris *et al.*, 2005; Kuruto-Niwa *et al.*, 2002; Wilson *et al.*, 2004).

It has been questioned whether the ER is actually involved in the SPMX effect. This is based on the low affinity for the ER of compounds like bisphenol A (BPA) or resveratrol, while at the same time, a SPMX effect is observed on functional *in vitro* assays (Ashby *et al.*, 1999; Freyberger and Schmuck, 2005; Gehm *et al.*, 1997). However, several authors have demonstrated the ability of fluvestrant (ICI 182,780), an ER antagonist, to completely block the response supporting the idea that an SPMX response is ER mediated (Gehm *et al.*, 1997; Mueller *et al.*, 2004; Van Lipzig *et al.*, 2005b).

To our knowledge, only two papers have been published that have considered in a systematic manner the SPMX effect problem. Gehm *et al.* (1997) reported the SPMX effect of resveratrol in MCF-7 cells on the transiently transfected luciferase reporter gene induction and on cell proliferation but not on the induction of progesterone receptor mRNA measured by reverse transcription-PCR. They suggested that the resveratrol SPMX effect might depend on the target gene and the cell type. In addition, using the MDA-MB-231 ER-negative cell line, they demonstrated the requirement of both an ER and an estrogen response element (ERE) in the same reporter gene construct to obtain a resveratrol SPMX effect (Gehm *et al.*, 1997). Further on, they studied resveratrol SPMX activity with a number of MDAMB- 231 subclones stably transfected with a variety of intact, mutated, or none ERa expression vectors and one or two consensus EREs-tk81-Luc reporter gene plasmids (Gehm*et al.*, 2004). Among theirmost important conclusionswere the independence of resveratrol SPMX effect on the transfection procedure and its dependence on the cell type, the target gene, the number ofEREs in the clone construct, and the integrity of both activation function domains. The last finding led to the suggestion that the greater activity of ‘‘resveratrol-liganded ER’’ compared to ‘‘E2-liganded- ER’’ may result from enhanced interaction of these two domains. Increased cofactor binding to genistein (GEN) and/or quercetinboundERwas recently suggested as a cause for anSPMXresponse in ER-U2OS-Luc reporter gene assay (Sotoca *et al.*, 2008).

Chrysene metabolites and specifically 2-hydroxy-crysene (2-OHCHN) SPMX behavior was investigated using the ER-CALUX assay (Van Lipzig *et al.*, 2005b). Besides the blockage effect by the antiestrogenic compound ICI discussed previously, the authors studied the interaction with the aryl hydrocarbon receptor (AhR) and the ER nuclear concentrations under different patterns of stimulation. They found that coincubation with the AhR antagonist 3´,4´-dimethoxyflavone (DMF) could not block the 2-OHCHN SPMX effect, which suggest that a ‘‘putative role for the AhR in the increased estrogenic response of 2-OHCHN can be excluded.’’ However, DMF at low concentrations induced an increase in E2 and 2-OHCHN luciferase activity. Besides, higher ER nuclear concentrations were observed with DMF alone or in combination with 2-OHCHN compared to controls. The suppressive effect of the AhR on the ER nuclear concentration and the ER-dependent transcriptional activity is well known (Safe *et al.*, 1991). Therefore, the authors suggest that SPMX estrogenic effect could be partially due to a decreased ability of the AhR to inhibit ER expression, resulting in higher ER concentrations or enhanced ER transcriptional activity. Freyberger and Schmuck (2005) supported this suggestion based on SPMX effects of resveratrol and other flavonoids in MCF-7 cells. Since these compounds have been characterized as AhR antagonists, they

claimed the same AhR inhibition mechanism and thus explain the increased flavonoids estrogenic signaling. Nonetheless, based on the SPMX effect of BPA obtained with a U2-OS cell line supposedly devoid of endogenous steroid receptors, it has been proposed that cross-reactivity of different receptors can be excluded. Instead of this, a possible modulation of the several kinases involved in the ER activation has been suggested as a potential mechanism (Ter Veld *et al.*, 2006).

In summary, there is neither clear explanation given in literature for the mechanism of action causing the SPMX estrogenic effect nor has the discussion over the regularly observed SPMX effects in *in vitro* assays been consistently addressed. As the quantification of the estrogenic activity of environmental contaminants, both individually as well as in mixtures (Murk *et al.*, 2002), is based on the response relative to that of estradiol, an SPMX effect could lead to overestimation of the estrogenic hazard of the evaluated substance and/or mixture. Besides, reported SPMX effects are not consistent either across studies or among compounds. Therefore, SPMX is an intriguing and relevant phenomenon to toxicology and toxicological risk assessment, and a systematic approach is needed to understand the conditions for the SPMX effect to occur. Especially whether it is an assay-based artifact or a real-life toxicological effect. A better understanding of the SPMX estrogenic effect will help to unravel its possible causes, determine future research, and suggest proper precautions when applying *in vitro* assays for estrogenicity risk characterization procedures. For this purpose, a literature meta-analysis of publications with functional estrogenic *in vitro* assays was performed. Compounds with responses above estradiol maximal response were identified. Three of the most frequent SPMX effect inducers—diethylstilbestrol (DES), GEN, and BPA were selected to study further. A database was built with 134 entries compiling the nature, characteristics, and responses of these three compounds on functional *in vitro* assays. A relative response meta-analysis was performed using a response variable. Correlations of SPMX effect with assays nature and characteristics were established and discussed.

Materials and Methods

Compound selection. Scientific reviews from 1997 to 2006 related to in vitro assays for estrogenic substances were collected and evaluated for compounds with responses above the maximal response observed for estradiol (Charles, 2004; Gray *et al.*, 1997; Mueller, 2002, 2004; Scrimshaw and Lester, 2004; Soto *et al.*, 2006; Zacharewski, 1997). As a result, a total of 42 scientific papers were reviewed to identify compounds that exerted the SPMX effect. Synthetic hormones, plasticizers, flame retardants, pesticides, and natural substances were found to show responses above the estradiol maximal response in one or more studies. Out of this group of

SPMX inducers, an industrial, a natural, and a synthetic compound were chosen to build the database. Based on the availability of sufficient reports to allow analysis, DES, GEN, and BPA were selected. Nonylphenol was not included in the scope of this research although it has been broadly studied during the last decades (Bandiera, 2006) and has shown SPMX effect under given circumstances. The reason to exclude it was the inconsistencies found in scientific literature regarding exact identity of the nonylphenol used. For instance, some papers analyzed the technical mixture (CAS no 84852-15-3), while others analyzed the pure compound with linear or side alkyl chain. The main problem was that many papers did not state clearly what kind of nonylphenol was analyzed or did not provide a CAS number.

Database development for the three selected compounds. A scientific literature search was performed using the bibliographic databases: PubMed, CAB Abstracts, Web of Science, and Scopus. The search strategy used several combinations of the following keywords: compound names or abbreviations, cell model, and end point–specific names or abbreviations. Papers which reported *in vitro* analysis of a selected compound were collected whether or not an SPMX effect was observed. In addition, relevant articles concerning the selected compounds were collected from the reference and citation list of each publication. All publications in English peer-reviewed journals until March 2007, complying with the following inclusion criteria, were taken into consideration for the database development:

- Estrogenicity studies of the selected compounds in functional *in vitro* cell models, so competitive ligand binding assays were excluded.
- Studies in which 17β-estradiol was used as a standard.
- Studies in which exposures were performed without phenol red as this gives estrogenic interferences (Berthois *et al.*); effectively, this means studies since 1986.
- Studies which provide sufficient data or graphics to establish the quantitative relationship between the compound and estradiol maximal responses.

The compound, assay, and response characteristics that were recorded and classified are reported in Table 1. Whenever the assay characteristics were referenced, the original publication was collected to obtain the appropriate information. Each database entry is the assay's characteristics of a specific compound with a given cell model and a given end point reported in a study. Therefore, a paper which analyzes a variety of compounds or uses a variety of cell models will appear with more than one entry.

Response variable normalization. In order to perform an analysis of the relationship between compound maximal responses and assay characteristics, the compound's maximal response was expressed as a percentage relative to the estradiol maximal response for the same entry arbitrarily set to 100%. The response of the solvent control was set to 0%. The SDs at the maximal response of

both estradiol and the compound studied were expressed as percentage relative to the estradiol maximum as well. To be able to decide whether a compound response is significantly higher than the maximum response of estradiol, the compound's relative response was expressed as a t value (t_c) calculated according to Equation 1, assuming two independent sample means with equal sample sizes. The obtained t_c was compared with the one-tailed tabulated t value (critical value) which depends of the number of experiments reported (degrees of freedom) in the original publication. The response was classified as submaximal for a negative t_c lower than the critical value, as SPMX for a positive t_c higher than the critical value, and as maximal when the null hypothesis could not be rejected ($p < 0.05$). Several publications reported dose-response curves without visible maximum (Fig. 1, last curve). These putative SPMX cases were considered as maximal responses during the analysis.

Table 1

Characteristics recorded from each publication.

Compound	Name, CAS No, purity, and provider
Assay	Cell line, original (wild type) or transfected, solvent, solvent percentage, serum type, percentage serum, endpoint, exposure period, and measurement method. *For transfected cell lines:* Transfection (stably or transiently), ER type (endogenous, ERα or ERβ), ER source (human, mouse, rat, etc), number of ERE's in reporter construct, ERE characteristics, type of reporter gene, and reporter/gene characteristics.
Estradiol response	Response units, response of solvent control and estradiol maximal response [a], standard deviation of E_2 maximal response and the number of independent measurement were the standard deviation was calculated from.
Compound response	Lowest tested concentration, highest tested concentration, maximal response concentration, maximal response compared to E_2 maximal response[a] and compound standard deviation at maximal response[a].

Note. E2, 17b-estradiol.[a] Minimum and maximum responses and SDs were estimated from the figures when they were not given by the authors.

$$t_c = \frac{\left[\%C\max - \%E2\max\right]}{\sqrt{\frac{S_C^2}{n_C} + \frac{S_{E2}^2}{n_{E2}}}}$$

Equation 1

Where t_c= t-value calculated for the comparison of compound and estradiol relative response means %Cmax = maximal response of the compound relative to maximal estradiol response %E2max = maximal response of estradiol minus solvent control arbitrarily set to 100 S_{E2} = standard deviation of estradiol at maximal response relative to estradiol response S_C = standard deviation of compound response at maximal response relative to estradiol response n_C = number of independent assays from which S_C was calculated, usually n_C=3 n_{E2} = number of independent assays from which S_{E2} was calculated, usually n_{E2}=3.

Database analysis. The database was split into three data sets: mammalian cell entries, yeast entries, and rainbow trout entries to account for species differences. A descriptive analysis was performed comparing the proportion of submaximal (t_c significantly <0), maximal (t_c not significantly different from 0), and SPMX (t_c

significantly >0) entries within assay characteristic. The proportion of entries with SPMX effect is given within brackets. Because of the limitation of its application to the data set, the Kruskal-Wallis nonparametric test using t_c as continuous variable only was applied to substantiate the qualitative results comparing average t_c values between groups (p values given within brackets). When considered appropriate, subsets of the database were evaluated to underline trends on particular cell models or end points.

Results

Twenty-one chemicals including DES, dihydrotestosterone, 4-nonylphenol, 4-tert-octhylphenol, BPA, and some of its metabolites, methoxyclor, 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane genistein, quercetin, resveratrol, zeralenone, zeranol, coumestrol as well as some benzo[a]pyrene, and crysene metabolites were identified as SPMX inducers. The complete list is included as Supplementary data 1.

Database development and data quality

Based on the availability of sufficient reports to allow analysis, DES, GEN, and BPA were selected. A total of 127 publications on *in vitro* estrogenic responses for DES, BPA, and GEN could be collected, of which 79 complied with the selection criteria. The references of the publications that were not included are listed as Supplementary data 2. The 79 studies provided 133 entries: 33 for DES, 64 for GEN, and 36 for BPA, summarized in Table 2. Among the entries, 80% were for mammalian systems while 16% were results obtained in yeast and 4% in fish hepatocyte cultures.

Analysis of the data quality revealed that only 2% of the publications mentioned the Chemical Abstract Service (CAS) Registry Number of the analyzed substance while only 14% mentioned its purity. However, 96% included the compound's origin, and through the provider, it was possible to trace the CAS numbers and purity in 86 and 88% of the cases, respectively. Important information about the assay characteristics was frequently lacking. For instance, 15% did not clearly state the solvent used in the analysis whereas 24% omitted its concentration. Serum type supplemented into the medium was not provided in 4% of the papers and 13% of them lacked its concentration. Information regarding maximal responses and SDs were not frequently reported by the authors. Those values were estimated from the publication graphs in 91 and 81% of the entries for E2 and compound maximal responses, respectively. The complete database is included as Supplementary data 3. The highest SPMX responses found were 143% for DES, 255% for GEN, and 233% for BPA (Beck *et al.*, 2005; Kitamura *et al.*, 2005; Wilson *et al.*, 2004).

TABLE 2

Number of database entries and references of maximal induction by the compounds DES, GEN, and BPA compared to E2 per cell model and end point.

Cell model	End point	DES		BPA		GEN		Total
		No[a]	References	No[a]	References	No[a]	References	no.[a]
MCF-7	Luc	3	Sanoh *et al.* (2003), Demirpence *et al.* (1993), and Freyberger and Schmuck (2005)	6	Freyberger and Schmuck (2005), Fujimoto and Honda (2003), Kitamura *et al.* (2005), Matthews *et al.* (2001), and Yoshihara *et al.* (2004)	10	Freyberger and Schmuck (2005), Fujimoto and Honda (2003), Harris *et al.* (2005), Ki *et al.* (2003), Maggiolini *et al.* 2001), Le Bail *et al.* (1998), and Zierau *et al.* (2005)	19
	Cell prol	6	Han *et al.* (2002), Jones *et al.* (1998), Kanai *et al.* (2001), Morito *et al.* (2001), Okubo *et al.* (2001), and Olsen *et al.*(2005)	8	Han *et al.* (2002), Kanai *et al.* (2001), Olsen *et al.* (2005), Brotons *et al.* (1995), Nakagawa and Suzuki (2001), Olea *et al.* (1996), Parez *et al.* (1998), and Samuelsen *et al.* (2001)	14	Maggiolini *et al.* (2001), Le Bail *et al.* (1998), Seo *et al.* (2006), Han *et al.* (2002), Morito *et al.* (2001), Kuruto-Niwa *et al.* (2007), Makela *et al.* (1994), Yap *et al.* (2005), Hsieh *et al.* (1998), Hwang *et al.* (2006), Kinjo *et al.* (2004), Matsumura *et al.* (2005), Schmidt *et al.* (2005), and Murata *et al.* (2004)	28
	GFP			2	Kuruto-Niwa *et al.* (2002) and (2005)	3	Kuruto-Niwa *et al.* (2002) and (2007) and Miller *et al.* (2000)	5
	CAT					1	Matsumura *et al.* (2005)	1
	PR prod			3	Olea *et al.* (1996), Parez *et al.* (1998), and Krishnan *et al.* (1993)			3
HeLa	Luc	2	Ranhotra and Teng (2005)	3	Ranhotra and Teng (2005) and Sato *et al.* (2004)	4	Yap *et al.* (2005), Ranhotra and Teng (2005), and Sato *et al.* (2004)	9
	CAT	1	Shelby *et al.* (1996)			1	Makela *et al.* (1994)	2
	Cell prol					1	Maggiolini *et al.* (2001)	1
T47D	Luc	1	Wilson *et al.* (2004)	3	Buterin *et al.* (2006), Legler *et al.* (1999), and Meerts *et al.* (2001)	3	Wilson *et al.* (2004), Buterin *et al.* (2006), Legler *et al.* (1999), and Meerts *et al.* (2001)	7
	Cell prol					2	Seo *et al.* (2006) and Zava *et al.* (1997)	2

TABLE 2 - Continued

Cell model	End point	DES		BPA		GEN		Total
		No[a]	References	No[a]	References	No[a]	References	no.[a]
Ishikawa	Luc	2	Mueller *et al.* (2004)	2	Mueller *et al.* (2003) and (2004)	4	Mueller *et al.* (2003) and (2004)	8
	APhos	2	(Le Guevel and Pakdel (2001) and Wagner and Lehmann (2006)			3	Wagner and Lehmann (2006), Pisha and Pezzuto (1997), and Wober *et al.* (2002)	5
U2-OS	Luc			2	Ter Veld *et al.* (2006)	2	Van Der Woude *et al.* (2005)	4
H293	Luc	2	Kuiper *et al.* (1998)			4	Kuiper *et al.* (1998) and Wang *et al.* (2001)	6
COS-1	Luc	4	Ashby *et al.* (1999) and Pennie *et al.* (1998)	2	Pennie *et al.* (1998)			6
Rainbow trout hep	Vit prod	2	Olsen *et al.* (2005) and Pelissero *et al.* (1993)	2	Olsen *et al.* (2005) and Shilling and Williams (2000)	1	Pelissero *et al.* (1993)	5
Yeast	B-Gal	6	Morito *et al.* (2001), Ashby *et al.* (1999), Arnold *et al.* (1996), Beck *et al.* (2005), and Gaido *et al.* (1997)	3	Yoshihara *et al.* (2004), Routledge and Sumpter (1996), and Rutishauser *et al.* (2004)	8	Zierau *et al.*, (2005), (Morito *et al.* (2001), Kinjo *et al.* (2004), Beck *et al.* (2005), Routledge and Sumpter (1996), and Collins *et al.* (1997)	17
	GFP	2	Beck *et al.* (2005) and Bovee *et al.* (2004)			3	Beck *et al.* (2005) and Bovee *et al.* (2004)	5
TOTAL		**33**		**36**		**64**		**133**

Cell models: human epithelial breast carcinoma (MF-7), human cervical cancer (HeLa), human ductal breast carcinoma (T47D), human endometrial adenocarcinoma (Ishikawa), human osteosarcoma (U2-OS), human embryonal kidney (H293), African green monkey kidney (COS-1), rainbow trout hepatocytes (Rainbow t hep) and yeast. End points: luciferase induction (Luc), GFP, CAT, b-galactosidase (B-Gal), cell proliferation (cell prol), alkyl phosphatase activity (APhos), vitellogenin production (Vit prod), and progesterone receptor production (PR prod).

[a]An entry is the report of a specific compound with a given cell model and a given end point. Therefore, a paper which analyzes a variety of compounds or uses a variety of cell models will appear with more than one entry.

Response variable

In the mammalian database, 33% of the compound responses fell above the tabulated t value indicating SPMX, while from the yeast entries 18% were considered SPMX. When discriminating by compound, 13, 32, and 48% of all DES, BPA, and GEN entries, respectively, fell above the critical value and, thus, were considered SPMX. Table 3 shows the percentage of entries considered SPMX, discriminated by cell model and end point.

Cell model

The cell model appeared to be an important factor in the occurrence of the SPMX effect (Fig. 2A). T47D (78%) and U2-OS (75%) cell lines had more frequent occurrences of SPMX compared with, for example, HeLa (42%) or H293 (33%). Indeed, significant differences were found between cell model t_c averages ($p = 0.033$). When only cell models with luciferase induction as the end point were selected, again cell model t_c averages showed significant differences between cell models ($p = 0.018$), with SPMX proportions of T47D-Luc (86%) and U2-OS-Luc (75%) higher than the other cell models.

Interestingly, estrogenic responses with COS-1 cells never were SPMX; while MCF-7 cells had entries with values well distributed from submaximal to SPMX relative responses (Table 3 and Fig. 2A). Despite the differences, the cell model only accounted for a small fraction of the data variation. There was no apparent effect of the cell model when each compound was evaluated independently. The relative responses for BPA, GEN, and DES in Rainbow trout hepatocytes were always below the estradiol maximum response (Table 3).

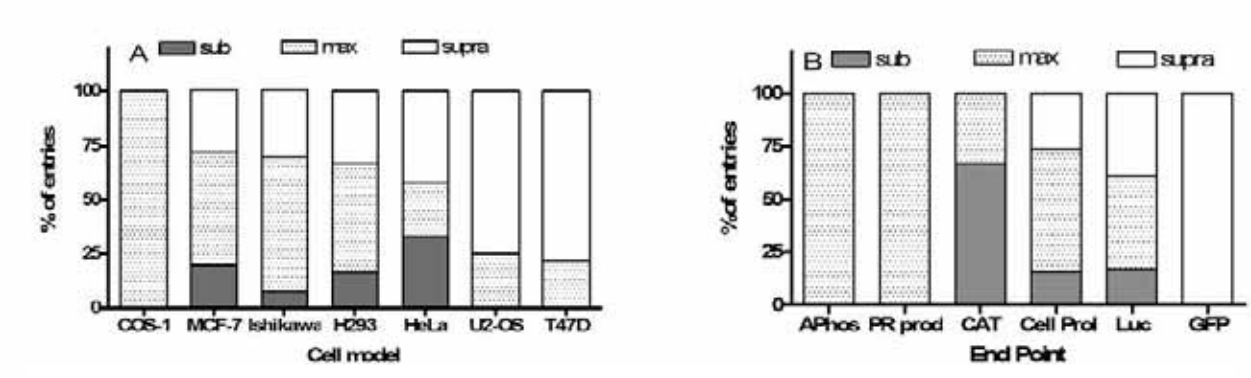

Figure 2. Percentage of submaximal (sub), maximal (max), and supramaximal (supra) entries of mammalian cell lines discriminated by cell model (A): MCF-7 (n = 56), HeLa (n = 12), T47D (n = 9), Ishikawa (n = 13), U2-OS (n = 4), H293 (n = 6), and COS-1 (n = 6); and by end point (B): Luc (n = 59), GFP (n = 5), CAT (n = 3), cell proliferation (n = 31), alkyl phosphatase activity (APhos, n = 5), and progesterone receptor production (PR prod, n = 3).

Table 3

Percentage of entries above the supra-maximal (SPMX) critical value[a] discriminated by cell model and end point.

Cell model	End point	%[b]	n[c]	Cell model	End point	%[b]	n[c]
MCF-7	Luc	16%	19	Ishikawa	Luc	50%	8
	GFP	100%	5		APhos	0%	5
	CAT	0%	1	U2-OS	Luc	75%	4
	Cell prol	25%	28	H293	Luc	33%	6
	PR prod	33%	3	COS-1	Luc	0%	6
HeLa	Luc	56%	9	Rainbow trout hep	Vit prod	0%	5
	CAT	0%	2	Yeast	GFP	40%	5
	Cell prol	0%	1		B-GAL	12%	17
T47D	Luc	86%	7				
	Cell prol	50%	2				

Cell models: human epithelial breast carcinoma (MF-7), human cervical cancer (HeLa), human ductal breast carcinoma (T47D), human endometrial adenocarcinoma (Ishikawa), human osteosarcoma (U2-OS), human embryonal kidney (H293), African green monkey kidney (COS-1), rainbow trout hepatocytes and yeast. End points: luciferase (Luc), green fluorescence protein (GFP), chloramphenicol transferase (CAT), β-galactosidase (B-GAL), cell proliferation, alkyl phosphatase activity (APhos), vitellogenin production (Vit prod), and progesterone receptor production (PRprod).

[a] entries above the SPMX critical value are those with *t* values above the one-tailed t, tabulated for degrees, of freedom reported in the publication and $p<0.05$,

[b] percentage of entries above the SPMX critical value,

[c] number of entries for the given cell model and end point.

End point

Despite apparent differences in the proportions of submaximal, maximal, and SPMX entries within end points in Figure 2B, no significant differences were found between the end points t_c averages. However, transfected chloramphenicol transferase (CAT) reporter gene cells did not produce SPMX responses on either MCF-7 or HeLa cell lines (Table 3). On the other hand, mammalian cells transfected with green fluorescence protein (GFP) reporter gene consistently gave SPMX responses (Fig. 2B), while the same reporter gene transfected into yeast gave SPMX response on three cases out of five (Table 3). There were in addition no differences on the SPMX effect occurrence among cell proliferation measurement methods.

Cell culture characteristics

Neither solvent nor its concentrations in culture seemed to have a significant effect on the three compounds relative maximum responses in mammalian cell lines. All mammalian entries together did not show any correlation between relative responses and serum concentration. However, within cell proliferation entries, a weak trend toward higher t_c values was observed with increasing serum concentrations in the exposure medium (data not shown). Interestingly, cell proliferation assays consistently never gave SPMX responses when human serum was used in culture (data not shown but available within Supplementary data 3).

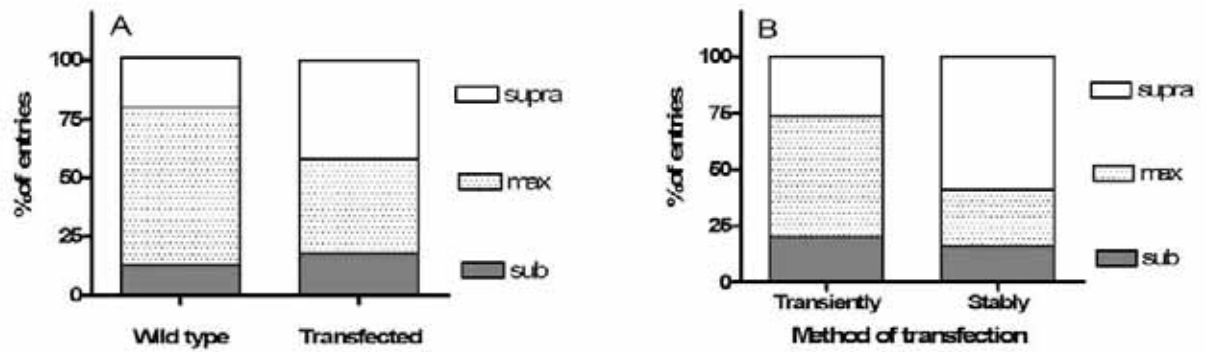

Figure 3. Percentage of submaximal (sub), maximal (max), and supramaximal (supra) entries of mammalian cell lines discriminated by genetical modification (A): wild type (n = 39) and transfected (n = 67); and for transfected mammalian cell lines discriminated by the type of transfection (B): transiently transfected (n = 35) and stably transfected (n = 32).

Transfected cell lines

Illustrated by Figure 3A, the proportion of SPMX entries for transfected cells was higher (42%) compared with wild-type cells (21%) ($p = 0.026$). Transfected T47D and Ishikawa cell lines exhibited higher SPMX proportions (86 and 50%, respectively) compared to the values in proliferation assays with its wild-type cells (50 and 0%, respectively). It is interesting to note that the likeliness of obtaining an SPMX effect apparently was not influenced by the origin of the ER, whether it was endogenous (T47D) or introduced (U2-OS). Among transfected entries, the stability of transfection had a significant influence on the occurrence of an SPMX effect (Fig. 3B). Considering mammalian cell lines, stably transfected cells had a higher SPMX proportion (59%) compared to transiently transfected cells (26%) ($p = 0.029$). Particularly for BPA, the SPMX incidence was higher in stably transfected cells (70%) compared to transiently transfected cells (10%) ($p = 0.028$). Also yeast cell lines stably transfected gave a higher SPMX proportion (27%) compared with transiently transfected ones which do not have a single SPMX entry ($p = 0.022$).

There were no significant differences between the proportions of SPMX for the three compounds together between cells expressing endogenous receptors (27%) compared to cells with transfected receptors, either ERα (48%) or ERβ (35%), despite a visible trend. Only for luciferase induction as the end point was the number of entries (59) sufficient to compare SPMX in models with endogenous receptors (24%) with those with transfected ERα (52%) or ERβ (35%). In addition, the source of the transfected receptors (i.e., human, rat or mouse) did not significantly influence the relative occurrence of SPMX responses.

A clear increase in the SPMX proportion was observed with increasing number of EREs (Fig. 4A). The t_c average values of mammalian cell entries grouped by the number of consensus EREs, transfected upstream of the reporter plasmid, were significantly different ($p = 0.003$). Furthermore, in yeast transfected with one consensus ERE, the SPMX occurrence was lower than in cells transfected with two EREs ($p = 0.041$) (Fig. 4B).

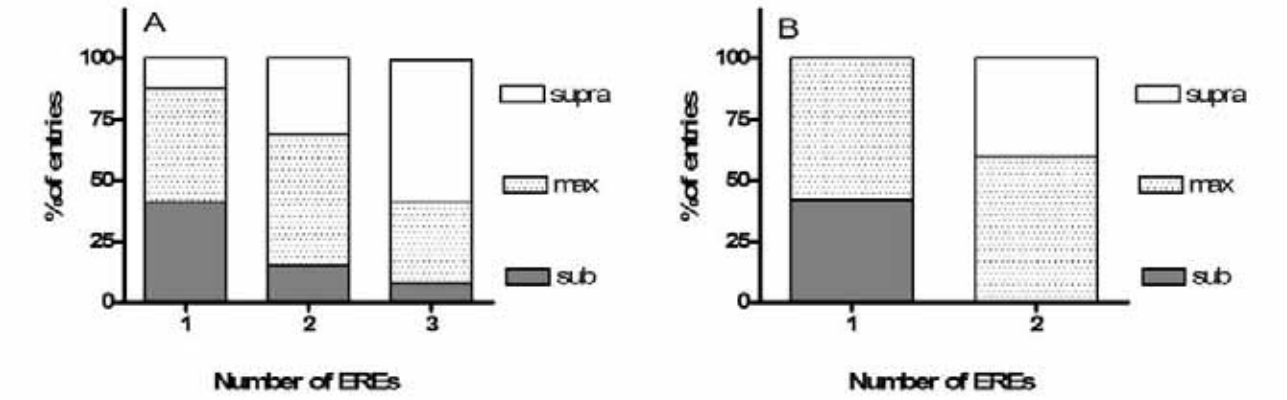

Figure 4. Percentage of submaximal (sub), maximal (max), and supramaximal (supra) entries discriminated by the number of EREs in the reporter plasmid used in (A) mammalian cell lines (n1 = 17, n2 = 13, n3 = 36) and (B) yeast cell lines (n1 = 12, n2 = 10).

Also, the context of the promoter within the clone construct was related to the incidence of SPMX response of the compounds. When thymidine kinase (tk) was present as a promoter, the proportion of SPMX entries were lower compared to TATA box and C3 promoters (Fig. 5).

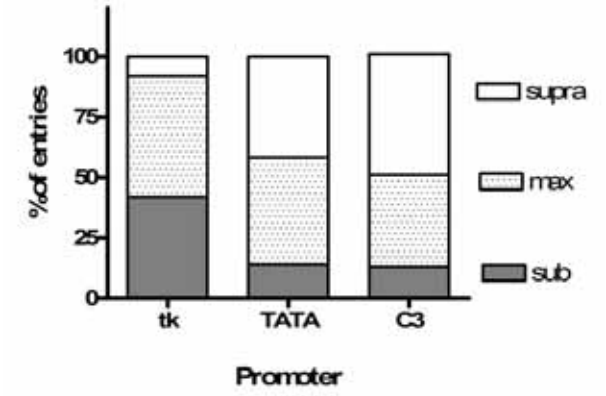

Figure 5. Percentage of submaximal (sub), maximal (max), and supramaximal (supra) entries of mammalian reporter cell lines discriminated by the promoter type: tk promoter (n = 12), TATA box (n = 36), and C3 promoter (n = 8), included with the ERE into the reporter plasmid construct.

Discussion

The existence of SPMX responses and the putative inconsistency of this SPMX occurrence in *in vitro* assays for estrogenicity present a challenge for the quantification and interpretation of the estrogenic potency of compounds and environmental extracts. When the SPMX character of a response is ignored, it could lead to overestimation of the relative potency compared to the current standard E2 dose-response. And when the existence of an SPMX dose-response is recognized, it is difficult to interpret its meaning for estrogenicity assessment. Several ubiquitous compounds have been reported to produce an SPMX effect in *in vitro* assays for estrogenicity under diverse (seemingly unrelated) circumstances. As results frequently are not consistent across studies, this has led to scientific debates about the nature of its cause and relevance for the *in vivo* situation. This study is the first to present a systematic analysis of data published on the estrogenicity of compounds that have been reported to induce SPMX effects. The correlation between the occurrences of SPMX with assay characteristics has been examined, with special attention for cell type, target gene, and clone characteristics.

Upon entering the published information into a database, the lack of detailed information became apparent. For example, unclear compound identification including its purity and lack of information on the identity and concentrations of reagents in the assays made it difficult to interpret the consequences of the absence or occurrence of SPMX effects in published studies. It was impossible to include detailed clone construct characteristics like ERE spacing, promoter spacing, ERE sequence or source, and reporter sequence or source in the analysis; the information provided was not sufficient even when referenced to previous published data.

Another possible source of confusion is the transformation of the response data for graphical presentation. For example, when using induction factors, the maximum value depends greatly on the control value (commonly the value with relatively the greatest variability). In several cases, the absolute data were not available, while in other cases the maximum values were not shown and the curve stopped at 100% with the shape suggesting a putative SPMX response. To avoid inexplicable inconsistencies in future in vitro publications that could perhaps have been related to differences in method details, good reporting practices are crucial.

Three estrogenic compounds were selected—DES, BPA, and GEN—that are frequently tested in addition to E2 to validate new *in vitro* methods for estrogenicity and to serve as a reference for other potentially estrogenic compounds and samples. Therefore, the total number of publications was sufficient for building the database and for investigating the main trends. Many

publications did not aim at comparing the full dose-response relationship between E2 and other agonists and therefore lacked its maximal response. This was the main reason for exclusion of some publications from the database (see Supplementary data 2). Heterogeneity of results between publications was expected, but in addition, ample data dispersion was found for the variables within single categories. For instance, MCF-7 or luciferase entries separately have large variations in the occurrence of SPMX and thus in their t_c ranges. This confirmed the inconsistency in the SPMX effect occurrence and its apparent independence of the cell model or end point. None of the variables alone, although significant in some cases, could explain the substantial variation in the occurrence of SPMX. This actually was to be expected given the limitations in the input data mentioned above. Only very dominant explaining variables could have been identified in the current meta-analysis of the available literature data. The best explaining variable as indicated from this analysis should ideally be further studied under experimentally controlled conditions.

There is a general trend within the database toward a higher frequency of SPMX effects with reporter gene assays. The influence of cell culture variables like serum type and concentration, exposure period, and solvent concentrations was not apparent. The effects of ethanol on the occurrence of SPMX in cell proliferation assays and ERa transcriptional activity in human breast cancer cells has previously been reported (Fan *et al.*, 2000; Singletary *et al.*, 2001). Bhat *et al.* (2001) suggested that the SPMX effect induced by resveratrol (Gehm *et al.*, 1997) could actually be due to the use of ethanol as solvent. The data in our study, however, do not support this suggested effect of ethanol. Also the concentration of serum has been suggested to influence the occurrence of SPMX (Soto and Sonnenschein, 1984; Strouken *et al.*, 1994). Again, this indication is not in accordance with the results from our study. Our results do nevertheless suggest that no SPMX effect occurs when human serum is used, although a limited number of assays with cell proliferation as end point used human serum. The suggested influence, however, is supported by several reports of different effects on cell proliferation between human and fetal bovine serum (McAlinden and Wilson, 2000; Soto and Sonnenschein, 1984; Soto *et al.*, 1986). Further experimental analysis of the putative influence of serum type on the occurrence of an SPMX cell proliferation effect is required to evaluate this issue.

The frequency of SPMX is higher in transfected cell lines compared to wild-type cells with endogenous ERs and end points such as alkyl phosphatase and progesterone receptor production in mammalian cell lines and vitellogenin production in rainbow trout cells (Table 3). This is in agreement with the previously reported suggestion that the occurrence of SPMX depends on the targeted gene

(Gehm *et al.*, 1997). This may even explain the visible trend of a cell model significant difference, as all entries for U2OS, and seven out of nine for T47D were stably transfected reporter gene end points (Table 3). These results indirectly suggest an influence of clone characteristics on the SPMX effect occurrence.

Of the transfected mammalian cell lines, the stably transfected cells showed a higher SPMX incidence than the transiently transfected ones. The same difference was observed for transfected yeast cell lines. For instance, two seemingly comparable assays with 3xERE-TATA-LUC transiently transfected H293 cells were performed by different laboratories. While Kuiper *et al.* (1997) reported GEN SPMX, Hwang *et al.* (2006), however, did not. The differences might be accounted for by major differences between the two methods related to transfection method, ER source, and plasmid construction.

We found a significant dependence of the SPMX effect on the number of EREs in the plasmid construct of transfected cell lines. No SPMX was described for models with only one ERE upstream of the CAT reporter gene. The plasmids of the Luc and GFP reporter genes all have two or more ERE's. Several authors reported increased E2 induced response with multiple EREs (Catherino and Jordan, 1995; Ponglikitmongkol *et al.*, 1990; Sathya *et al.*, 1997; Tyulmenkov *et al.*, 2000). In addition, both the number of EREs and their spacing, proximity to the promoter and the promoter nature, are suggested to influence the occurrence of synergistic estrogenic responses (Sathya *et al.*, 1997). Synergism could be related to SPMX responses, and exposure media contain many compounds of which their possible role in inducing estrogenic responses is not known. To our knowledge, there are currently no publications addressing the multiple ERE synergistic effect with ligands other than E2. Nonetheless, the dependence of SPMX effect on the number of EREs and on the nature of the promoter revealed in our meta-analysis may indicate that other compounds like GEN and BPA can induce this ERE transcriptional synergism as well. In that case, the observed SPMX effects in specific transfected cell lines indicate a stronger transcriptional synergism induced by GEN and BPA compared to that induced by E2. It is not clear why the compounds did not induce SPMX in COS-1 cell line. It is important to note that for these cells, only two sources were available, both from the same laboratory, so the variation in conditions is limited. In both papers, the use of two consensus EREs with the tk promoter is reported. Both characteristics are associated here with a lower likeliness for SPMX responses.

This meta-analysis confirms the inconsistent occurrence of SPMX effects, which could be related to a number of specific assay characteristics: (1) the type of serum used to supplement the exposure medium, (2) the end point used to quantify the estrogenic potency (endogenous or transfected), (3) the number of EREs, and (4) the nature of the promoter. It is important to understand the mechanism behind

this phenomenon because *in vitro* assays for estrogenicity are used extensively to characterize and quantify the estrogenic potency of compounds, mixtures, and environmental extracts. To our knowledge, the influence of significantly different maximum responses of compounds in a mixture to the mixture response has not been addressed. For the quantification of the estrogenic potency of mixtures, concentration addition is assumed (Jonker *et al.*, 2005), but when some compounds induce SPMX, this assumption is not valid. In addition, the estrogenic equivalency factors are derived from the EC_{50} value of the compounds of interest compared to that of E2. A significantly higher maximum will invalidate the equipotent assumption and thus derive a miscalculation of the compounds potency. The estrogenic potency of complex mixtures such as environmental extracts is determined by diluting the extract to a response, which falls into the linear part of the standard dose-response curve. The consequences of the SPMX phenomenon could result in overestimation of the estrogenic potency if the SPMX is the consequence of a molecular artefact of the cell model. But perhaps the SPMX effect only occurs at high concentrations and not at concentrations that could occur in blood. To be able to decide how to interpret and deal with the occurrence of SPMX in certain cell models, it is important to understand its underlying mechanism. Is it an artefact or a mechanism that is relevant for the in vivo situation as well? This cannot be resolved by further analysis of already reported results but requires dedicated additional research to unravel this intriguing phenomenon.

Supplementary data

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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CHAPTER 4b

Inhibition of Pg-p efflux pumps and supramaximal (SPMX) effect on *in vitro* dioxin bioassay

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Organohalogen compounds, **74**, 197-200 (2012)

Introduction

In vitro bioassays are increasingly applied to complex extracts to relatively quantify estrogenic, androgenic, progestagenic and dioxin-like activity. A casual feature of these bioassays is the "supramaximal effect" (SPMX), occurring when the response induced by the tested compound is significantly higher compared to the natural ligand (Montaño *et al.* 2010). This effect has been observed after incubation with some pure substances on estrogenic receptor (ER) mediated assays (Montaño *et al.* 2010), but also after incubation with complex mixtures like oils (Vrabie *et al.* 2009), oil contaminated pore water (Jonker *et al.* 2006), and sediments (Baston and Denison 2011; Murk *et al.* 1996) on dioxin receptor (DR) mediated luciferase reporter gene assays. The causes of this effect are still uncertain; a recent literature study of estrogenic SPMX effect inducers pointed towards the characteristics of the transfected cell lines (Montaño *et al.* 2010), while a mechanistic study with genistein on an ER-mediated luciferase assay suggested a post-transcriptional stabilization of the firefly luciferase reporter enzyme (Sotoca *et al.* 2010).

The multi-xenobiotic resistance (MXR) is a phenomenon in which over-expression of cellular efflux proteins results in enhancement of protection of organism against environmental contaminants (Bard 2000). Cellular efflux pumps like P-glycoprotein (P-gp also known as MDR1) are expressed in peripheral and barrier tissues of several marine organisms (Kurelec *et al.* 2000). Diverse environmental contaminants inhibit the MXR mechanism *in vitro* (Georgantzopoulou *et al.* 2012), and *in vivo* (Anselmo *et al.* 2012), and some of these contaminants potentiate the toxic effect of an efflux pumps substrate (Anselmo *et al.* 2012).

P-gp is present in significant amounts in the rat liver hepatoma cell line H4IIE (Mehta *et al.* 1994), which is a common cell line used in the DR-mediated luciferase reporter gene assay (DR.Luc) (Murk *et al.* 1996). We therefore hypothesized that P-gp efflux pump blockers will potentiate the effect of a dioxin-like agonist and perhaps induce a SPMX effect on the H4IIE based DR.Luc assay. To test this hypothesis, we adapted the 96-well plate cellular efflux pump inhibition assay (CEPIA) (Georgantzopoulou *et al.* 2012) to the H4IIE.Luc cell line, and measured the inhibition potency of verapamil as model P-gp inhibitor, pentachlorophenol (PCP) and 2,3,7,8-tetrachlorodibenzo-p-dioxine (TCDD). Then we tested verapamil and various environmentally relevant efflux pump inhibitors, alone and in co-exposure with TCDD on the DR.Luc assay.

Materials and methods

Compounds were >98% pure and purchased from Sigma-Aldrich, except for PCP and 4'-OH-3,3',4,5'-CB 79 (4'-OH CB 79) which were purchased from Accustandard and LGC Promochem, respectively. All compounds were dissolved in DMSO and then diluted in phosphate buffer saline (PBS) for the CEPIA assay or cellular media for the DR.Luc assay, with a final DMSO concentration

of 0.2% (v/v) for single exposures and 0.4% (v/v) for co-exposures. H4IIE.Luc cells were a kind gift from Prof. Michael Denison and were cultured and plated for assays as previously published (Vrabie *et al.* 2009).

Adaptations of the CEPIA assay on H4IIE.Luc cells were based on the previously standardized method by Georgantzopoulou and co-workers (Georgantzopoulou *et al.* 2012). Calcein-AM is a nonfluorescent P-gp substrate which can be metabolized by cytosolic esterases into the fluorophore calcein. Calcein is not a P-gp substrate, thus upon P-gp inhibition calcein will accumulate inside the cell and increase the fluorescence response (Georgantzopoulou *et al.* 2012). Cells were incubated for 30 min with 100 µL of PBS alone or with test compounds. Then they were supplemented with calcein-AM as a model substrate at final concentrations between 0.05 and 5 µM in 100 µL of PBS, and incubated for 15 to 180 min. Fluorescence was measured in a Biotech Synergy 2 plate reader at 485 nm excitation and 530 nm emission wavelengths. Standard curves were finally obtained with 3 µM calcein-AM exposure for 45 min.

For the DR.Luc assay, H4IIE.Luc cells were exposed to TCDD standards (0.2% DMSO v/v), P-gp pump inhibitors alone and in combination with TCDD at the EC_{50} and maximum levels (final conc. 10 and 100 pM respectively, 0.4% DMSO v/v) in 100 µL of fresh media for 24 hours. Inhibitor concentrations were chosen according to their *in vitro* CEPIA inhibitory concentration (Georgantzopoulou *et al.* 2012). Viability was determined by the measurement of the cells metabolic activity with 4 µM resazurin in 200 µL cell media for 3 hours (Montaño *et al.* 2011), immediately followed by the luciferase analysis on the same plate as previously published (Vrabie *et al.* 2009). Both measurements were performed in a Biotech Synergy 2 plate reader.

All experiments were performed in triplicate and repeated twice. Data points represent mean ± SD of relative fluorescence units (RFU) or luminescence units (RLU), relative to TCDD maximum response at 100 nM minus DMSO control. The dose response data were fitted to a Hill equation with SigmaPlot 2001 (SPSS Inc.), and 50% inhibitory concentrations (EC_{50}) were calculated from the modeled data. Statistical analysis was performed with ANOVA followed by Duncan (Figures 1A and 1B) or Tukey post-hoc comparison ($P<0.01$) (SPSS, Inc).

Results and discussion

H4IIE.Luc CEPIA assay

First, the optimal conditions for the CEPIA assay with H4IIE.Luc cells were determined. After incubation of H4IIE.Luc cells with calcein-AM, a significant increase in fluorescence was observed after 180 min with 0.5-1 µM, after 60 min with 2 µM or after 15 min above 3 µM (Figure 1A). The background fluorescence observed without inhibitor is likely due to calcein-AM hydrolysis before it is withdrawn from the cell. Incubation with the P-gp inhibitor verapamil (100 µM) induced a significant increase in fluorescence after 180 min with 0.5 µM calcein-AM, and after 15 min above 1 µM (Figure 1B). A concentration of 3 µM calcein-AM for 45 min was chosen to obtain a signal sufficiently high to properly observe inhibition.

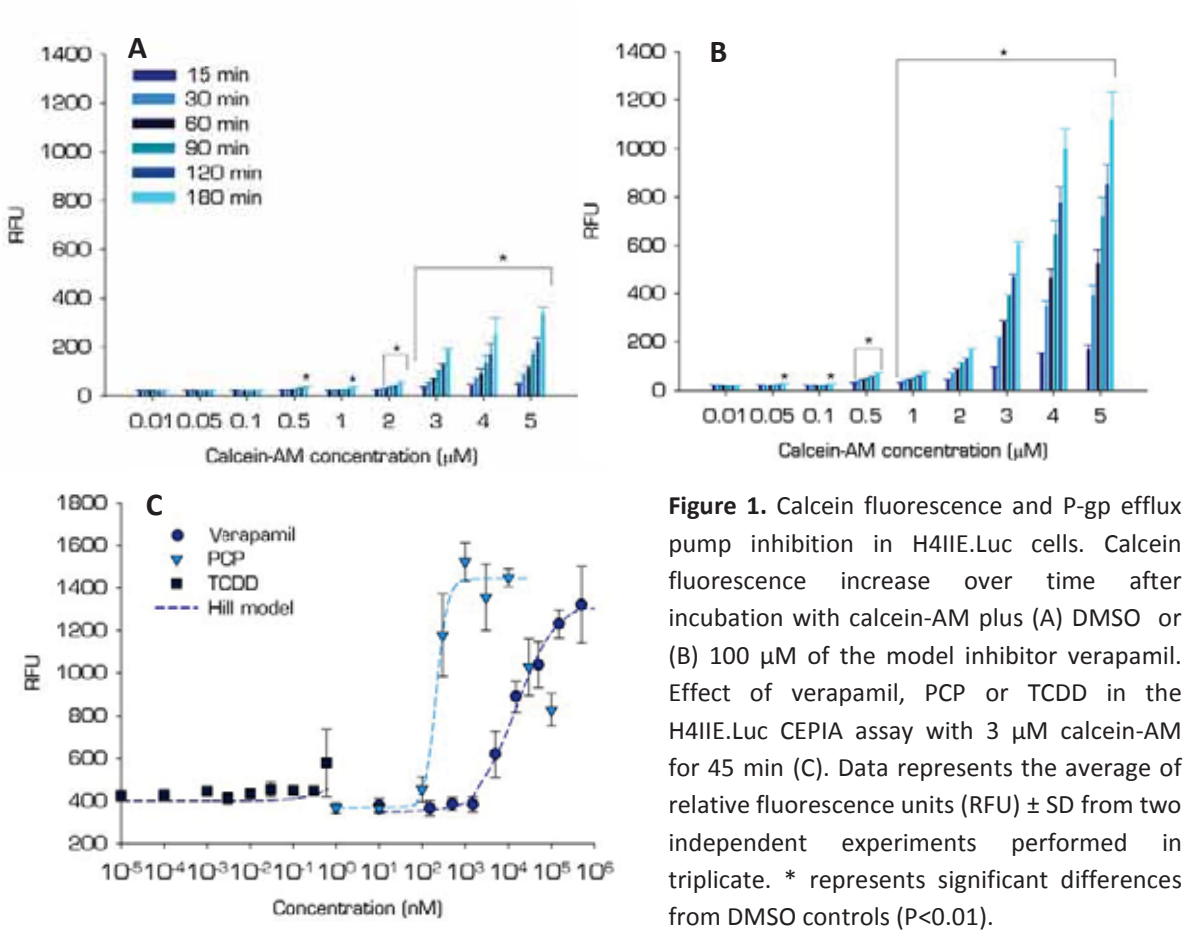

Figure 1. Calcein fluorescence and P-gp efflux pump inhibition in H4IIE.Luc cells. Calcein fluorescence increase over time after incubation with calcein-AM plus (A) DMSO or (B) 100 µM of the model inhibitor verapamil. Effect of verapamil, PCP or TCDD in the H4IIE.Luc CEPIA assay with 3 µM calcein-AM for 45 min (C). Data represents the average of relative fluorescence units (RFU) ± SD from two independent experiments performed in triplicate. * represents significant differences from DMSO controls (P<0.01).

The P-gp inhibition of verapamil, PCP and TCDD was then evaluated with the CEPIA assay optimized for H4IIE.Luc cells. Verapamil and PCP produced a dose-related increase in calcein fluorescence, while TCDD did not significantly increase the fluorescence compared to the DMSO controls (Figure 1C). The significant reduction in fluorescence observed above 30 µM PCP was due to cytotoxicity which was visually confirmed under the light microscope and by the cell viability assay (data not shown). The EC_{50} of verapamil and PCP were 16±2.5 µM and 0.21±0.02 µM, respectively, which was 3-6 times lower compared to inhibitory concentrations reported for P-gp over-expressing Madin-Darby canine kidney MDCKII-MDR1 cells (Georgantzopoulou *et al.* 2012). TCDD is known to induce P-gp mRNA expression (Maher *et al.* 2005) but to the best of our knowledge it is not known whether TCDD is a P-gp substrate. Up to 600 nM TCDD did not inhibit Ca-AM efflux, a concentration which is far above the EC50 used for mixture toxicity studies causing SPMX effects (Vrabie *et al.* 2009).

DR.Luc assay

First, the effect of PCP on the TCDD AhR-mediated response was evaluated in the DR.Luc assay. TCDD produced a dose-related response in the AhR-mediated DR.Luc assay with a limit of detection of 1.3±2.8 pM, an EC_{50} of 14±2.0 pM and an ECmax

at 100 nM (Figure 1A). These parameters are comparable to previously reported standardized results (Besselink *et al.* 2004). DMSO and the PCP concentration that gave maximal P-gP inhibition (1 µM PCP), were added to a full TCDD dose-response curve (0.4% DMSO) and compared with TCDD alone (0.2% DMSO). If considered isolated, 1 µM PCP induced a significant increase of TCDD luciferase production at concentrations above 100 pM (Figure 1A). However, the overall effect of DMSO or PCP compared to TCDD alone was not significant, as the effect of TCDD plus 1 µM PCP compared to TCDD plus DMSO was also not significantly different (ANOVA, P>0.01).

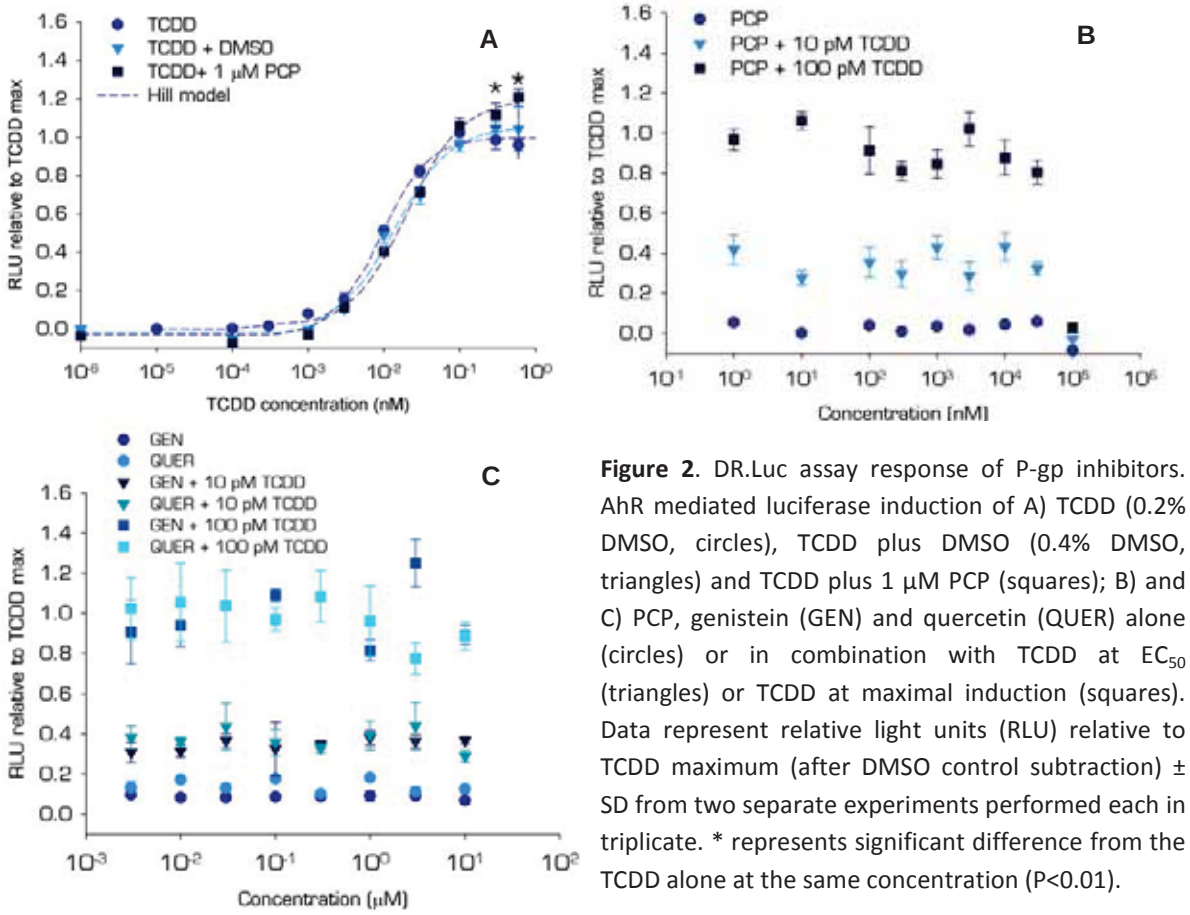

Figure 2. DR.Luc assay response of P-gp inhibitors. AhR mediated luciferase induction of A) TCDD (0.2% DMSO, circles), TCDD plus DMSO (0.4% DMSO, triangles) and TCDD plus 1 µM PCP (squares); B) and C) PCP, genistein (GEN) and quercetin (QUER) alone (circles) or in combination with TCDD at EC_{50} (triangles) or TCDD at maximal induction (squares). Data represent relative light units (RLU) relative to TCDD maximum (after DMSO control subtraction) ± SD from two separate experiments performed each in triplicate. * represents significant difference from the TCDD alone at the same concentration (P<0.01).

PCP did not induce a dioxin-like response alone, nor increased the response of 10 or 100 pM TCDD (Figure 2B). Verapamil, bisphenol-A, genistein, quercetin, indol-3 butiric acid, indol-3-acetic acid, and 4-OH CB 79 were tested alone and in co-exposure in the DR.Luc assay with similar negative results (data not shown). There was therefore no evidence that inhibitors of P-gp efflux pumps, at the concentrations tested, could induce luciferase production or potentiate that of TCDD on the DR.Luc assay.

Genistein and quercetin are known for inducing the SPMX effect in the ER-mediated reporter gene assays with more than 2 ERE's in the reporter construct (Montaño *et al.* 2010). Quercetin has been reported to inhibit P-gp activity (Georgantzopoulou *et al.* 2012). However both failed to induce AhR-mediated activity or to increase the response induced by 10 or 100 pM TCDD (Figure 2C). Therefore the proposed mechanism of post-transcriptional luciferase stabilization (Sotoca *et al.* 2010) does not apply to luciferase produced in the H4IIE.Luc cells and might not be the most important mechanism behind the SPMX effect.

Conclusions

The CEPIA assay was successfully adapted to the H4IIE.Luc cell line. The model P-gp inhibitor verapamil and PCP were more potent in this cell line compared to results obtained with the P-gp over-expressing MDCKII-MDR1 cell line. Under the DR.Luc assay conditions there was no evidence that P-gp efflux pump inhibitors modified or potentiated the activity of TCDD. Neither genistein nor quercetin, two potent SPMX inducers on ER-mediated assays, induced any signal on the DR.Luc assay, nor influenced the luciferase induction by TCDD. Future work should be focused on testing the consequences of efflux pump inhibition with an AhR-agonist which is a P-gp substrate, as this could result in intracellular accumulation of this AhR-agonist.

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CHAPTER 5

Initial sample extract stock concentration affects *in vitro* bioassay-based toxicological risk characterization

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Based on:

Journal of soil and sediments (submitted)

Abstract

Bioassays have become an alternative for sediment risk profiling, including potential compliance with sediment quality criteria (SQC). Despite their initial limited specificity and matrix sensitivity; *in vitro* functional bioassays have evolved through standardization and validation to provide a confident toxicological hazard estimate of sediments. Sample preparation proves to be a key aspect for bioassay improvement. It is standard practice to use a high single stock concentration of extracts to further dilute test concentrations from and perform the analysis. However, a high contaminant load in an extract (more than 20 g sediment equivalents (SEQ)/mL DMSO) oversaturates solubility in carrier solvents and overloads the clean-up columns which may result in residual PAH levels. These problems may cause respectively under- or over-estimation of the quantified dioxin-like toxic potency. Effects of initial stock concentrations and preparation, including sonication assisted dissolution, and exposure period influenced the outcome of quantified dioxin-like potency as observed for cleaned nonpolar sediment extracts from various locations in Luxembourg. Sonication assisted dissolution of the stock before serial dilution strongly reduced the standard variation of the outcomes. Taking into account the aspects revealed in this study in addition to already described important issues for quality control, the *in vitro* bioassays based on Bio-TEQs can be applied in a comprehensive monitoring program to determine whether sediments comply with health and safety standards for humans and the environment. For the generally applied sediment quality criteria, advices are given for maximum initial stock concentrations to achieve reliable bioassay outcomes.

Introduction

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBDEs) are widely distributed in the environment (Eljarrat and Barceló 2009), and constitute a concern for human and wildlife health (Carpenter 2006; Legler 2008; Letcher *et al.* 2010). Due to the lipophilic and persistent nature of such POPs they are mainly associated with sediments and lipids. Sediments act as a storage compartment serving as "secondary sources" of POPs for other environmental compartments and organisms (Heugens *et al.* 2001; Jonker *et al.* 2006; Moermond *et al.* 2004; Nizzetto *et al.* 2010; Vethaak *et al.* 2005).

In response to this problem sediment quality criteria (SQC) have been developed to: a) evaluate the need for sediment remediation on impacted sites, b) assess potential impacts of dredged waste, and c) regulate discharges to protect human health or the environment (Iannuzzi *et al.* 1995). Assessment of sediment compliance with SQC has been and continues to be based on a chemical analytical approach (OSPAR 2004; 2008). However, this definition of maximum acceptable concentration (action levels, ALs), focuses mainly on acute effects of legacy contaminants, ignoring effects from recently identified pollutants and neglecting their potential mixture effects (Schipper *et al.* 2010). In recent years, bioassays have been increasingly applied for sediment quality assessment and have become a standardized alternative for sediment risk characterization (Besselink *et al.* 2004; Brack *et al.* 2009; Hamers *et al.* 2010; Schipper *et al.* 2010; Stronkhorst *et al.* 2002). However, *in vivo* bioassays have been criticised for their lack of specificity, their sensitivity to matrix components and their poor reproducibility (Schipper *et al.* 2010). Therefore, *in vitro* assays such as the reporter gene assays for dioxin-like (DR-Luc) and estrogenic substances (ER-Luc) have been intensively fostered for sediment toxicity profiling (Hamers *et al.* 2010; Schipper *et al.* 2010). *In vitro* bioassay response-based safe levels are being developed either relating it to chemical safety standards or by translating the *in vitro* toxic potency into potential ecotoxicological risk. Two examples are the comparison to guidelines for the DR-Luc (also referred to as DR-CALUX) activity in marine harbour sediments (Besselink *et al.* 2004; Stronkhorst *et al.* 2002), and the translation to safe levels for estrogenic effects in vivo of ER-Luc responses of estuary sediments and suspended particles (Hamers *et al.* 2010).

An important prerequisite for the application of *in vitro* bioassays in a licensing system is a high standard of quality assurance and quality control to guarantee the quality of the data on which the assessment is to be based (Klamer *et al.* 2005; Schipper *et al.* 2009; Stronkhorst *et al.* 2003; US EPA 1995). False positives (disqualification) can imply unnecessary high costs, false negatives can imply

environmental or human health risks. Therefore, bioassay protocols, sample preparation methods and data interpretation must include GLP specifications in terms of selectivity, repeatability and reproducibility. Several of these issues already have been tackled through improvement on the bioassay performance and validation (Besselink *et al.* 2004; Haedrich *et al.* 2012; Hoogenboom *et al.* 2013; Houtman *et al.* 2006b; Van der Burg *et al.* 2010) and standardization of sample preparation (Houtman *et al.* 2007; Schwirzer *et al.* 1998; Seiler *et al.* 2008; Van Wouwe *et al.* 2004). Nevertheless, there are still several issues remaining, which could considerably influence the quantification, resulting in an over- or under-estimation of the toxic potency of samples and invalidate the risk judgement.

For example, the occurrence of more than maximal responses (supramaximal effects, SPMX) has been reviewed for reporter-gene assays for estrogenic substances (Montaño *et al.* 2010), as well as dioxin-like responses in pore water (Jonker *et al.* 2006), crude and refined petroleum products (Vrabie *et al.* 2009), and sediments (Murk *et al.* 1996), even after thorough clean-up (Baston and Denison 2011). This effect could lead to an over-estimation of the quantified potency of chemicals and extracts (Baston and Denison 2011; Montaño *et al.* 2010). Despite efforts to unveil the underlying mechanisms for SPMX effects (Montaño *et al.* 2012; Sotoca *et al.* 2010), it is not yet clear whether this is an assay-based artefact or represents a real-life toxicological effect. By quantification of the toxic potency at lower test concentrations this effect probably can be avoided, especially in cases of biphasic dose-response curves as has been shown for the ER-Luc (Montaño *et al.* 2010). For FR-Luc responses, however, this needs to be studied further.

Also sample preparation can alter the quantification of the toxic potency because it will inevitably influence the composition of the mixture tested (Seiler *et al.* 2008). Examples are loss of compounds by evaporation, introduction of compounds from filters or soxhlet sockets and solvent change implying a different solubility of compounds (Windal *et al.* 2005). An issue related to sample preparation that, to our knowledge, has not been properly addressed is the influence of the initial sample extract stock concentration on the final quantified toxic potency. It is a common practice to concentrate the sample as much as possible to be able to quantify the toxicity also in less polluted samples. When it is clearly stated within the publications, which regularly is not the case, commonly sediment extracts are concentrated to stocks of 10 to 50 g sediment equivalents (SEQ)/mL before analysis, which are then serially diluted in carrier solvent and media for exposure (Céspedes *et al.* 2004; David *et al.* 2010; Grote *et al.* 2005; Houtman *et al.* 2006a; Schmitt *et al.* 2011; Schwab *et al.* 2009; Vondrácek *et al.* 2001; Wölz *et al.* 2008; 2010a; 2010b; 2011). In a few cases the extracts have been concentrated to stock solutions below 10 g SEQ/mL (Galluba and Oehlmann 2012; Kinani *et al.* 2010;

Klamer *et al.* 2005; Louiz *et al.* 2008; Ocampo-Duque *et al.* 2008; Sanctorum *et al.* 2007; Thomas *et al.* 2004). In some other cases the extracts have been concentrated to stock solutions above 100 g SEQ/mL and as high as 200 g SEQ/mL (Besselink *et al.* 2004; Brack *et al.* 2008; Chao *et al.* 2011; Nording *et al.* 2007; Sanctorum *et al.* 2007).

Indeed, it has been observed that original extract stock concentrations can significantly influence the observed estrogenic potency of waste treatment plant effluents (Murk *et al.* 2002). The authors suggested a loss of compounds during solvent exchange when the maximum solubility is approached. When the initial stock was more diluted the final estrogenic potency was higher. Also storage temperature of the stocks and sonication of stock extracts before further dilution in carrier solvent and before exposure are not stated explicitly within the experimental section of most publications. Sonication usually greatly enhances the dissolution of compounds and therefore modifies the actual exposure concentrations in the bioassay. However, so far this effect has not been studied.

When a sample is cleaned more thoroughly the initial stock concentrations can be more concentrated. With an acid silica column the labile compounds are degraded and most PAHs are separated from the dioxins and PCBs. The use of an acid silica column (Besselink *et al.* 2004) is a standard clean-up procedure in the analysis of dioxin-like potency of sediments. Mostly a standard amount of sample is extracted and loaded. However, an overloaded column would allow other compounds to be present in the final extract (Schwirzer *et al.* 1998), including alkylated- and nitro-PAHs (Dindal *et al.* 2011, own unpuglished data) which can induce arylhydrocarbon (AhR) activity whereas their mechanism of toxicity is totally different from that of dioxins (Vondrácek *et al.* 2001). Most PAHs can be metabolised by cytochrome P450 enzymes that are present in H4IIE rat hepatoma cells of the DR-Luc. Increasing the time of exposure to 24 hours or more allow more PAHs in the sample to be metabolized reducing their influence on the bioassay response (Hamers *et al.* 2000; Vondrácek *et al.* 2001). Previously, differences in Bio-TEQs following 24h and 48h exposures were observed for sediment extracts with potencies between 0.04 and 0.10 ng Bio-TEQ/g SEQ obtained with 30 g SEQ/mL initial stocks (Vondrácek *et al.* 2001).

Considering the influence that actual contaminant load could have on the performance of a dioxin-receptor bioassay, we hypothesize that the use of concentrated stocks of sediment extracts could potentially cause wrong estimations of potency. Hence, this study focuses on the effects of initial stock concentrations, including sonication assisted dissolution and exposure time, on the quantified dioxin-like potency of cleaned nonpolar sediment extracts. The consequences of the ultimate effect on the quantified toxic potency of the

sediment are related to safe levels set in sediment quality criteria (SQC) and the impact on the management decision process. Suggestions are made to improve and standardize sample preparation practise and bioassay-based sediment potency quantification minimizing their modulating impact on its risk characterization.

Materials and Methods

Chemicals. Hexane (Hx, dioxin analysisgrade) and all other solvents (chromatographic grade) were purchased fromBiosolve BV (Valkenswaard, The Netherlands) or from Sigma-Aldrich Co(Steinheim, Germany). All other chemicals (incl. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) were purchased from Sigma-Aldrich Co (Steinheim, Germany) unless otherwise stated. All extraction and storage materials were exclusively made of borosilicate glass, and polytetrafluoroethylene screw cap vials were used when required. All materials were prewashed with hexane. Clean sand from Fontainebleu (Paris, FR) was dried overnight at 105 ^{o}C and extracted twice with the ASE method described below to obtain control sand. A portion of 30 g of cleaned sand was spiked with a solution containing TCDD in Hex and rotatory mixed for 72 hours for a final concentration of 10 pmol TCDD/g (3.2 ng TCDD/g).

Sediment sampling and preparation. Sediment samples were collected in 2010 and 2011 from various locations at the rivers Alzette, Our, Clerve and Woltz in the Grand Duchy of Luxembourg. To test the effects of stock concentration, sonication and exposure time, a subset of locations was selected to exemplify sediments with a range of contamination profiles from industrial/urban (Alz 1,Alz 10 and Alz 19), urban (Alz 17, Our 4), agricultural (Our 3) and woodland (Woltz 1) locations (Figure 1). Location labels correspond to the sampling position along the river and the overall codes from the full sampling campaigns were unchanged to allow comparison with other reports and publications (Montaño *et al.* 2013, submitted).

Composite samples of 4 cm deep surface sediments were collected manually from natural sedimentation zones covering at least 50 m along the river course. At least 8 sub-samples were mixed (1 kg), transported to the laboratory and stored in glass flasks at 4°C. The samples were transferred onto hexane washed aluminium plates, frozen at –20°C and then dried using an Alpha 2-4 LSC freeze-dryer (Christ, Germany) for at least 48 hours. Dried >63 µm sediment fraction was obtained using a sieving machine (Retsch, Germany), and stored at 4 °C in glass bottles in darkness until extraction.

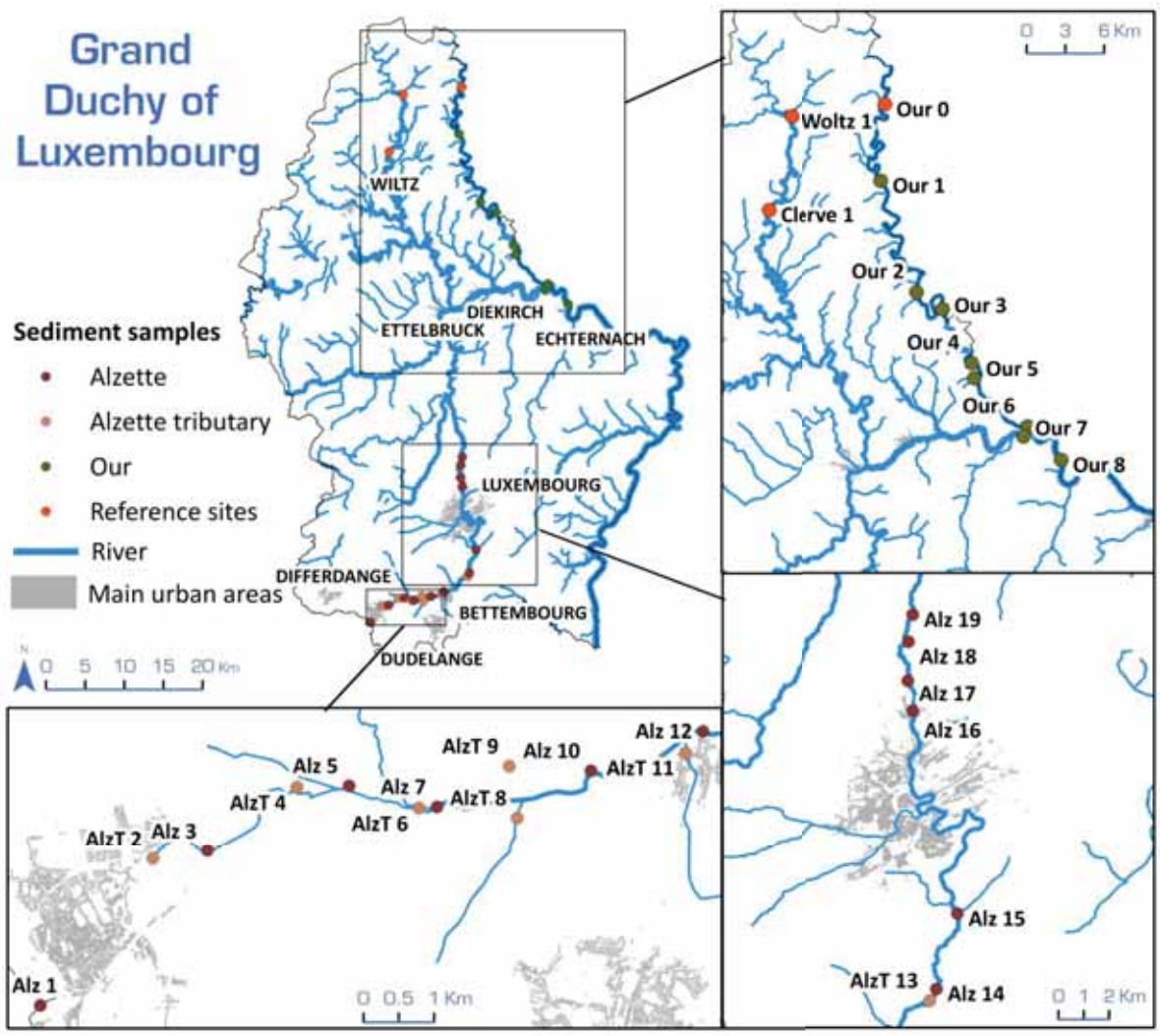

Figure 1 Sediment sampling locations within the Grand Duchy of Luxembourg

Preparation of crude extracts. A total of 5g of <63 µm dried sediment from each location, control sand and TCDD spiked sand were extracted with a mixture of Hex:acetone (Ac, 3:1, v/v) in an accelerated solvent extraction equipment (ASE200; Dionex, Sunnyvale, CA,USA) with three extraction cycles at 100 °C and 2,000 psi (Houtman *et al.* 2007). A mixture of 2 g dried Na_2SO_4 to eliminate remaining humidity and 1 g of Cu powder (Sigma-Aldrich Co., Steinheim, Germany) for sulphur removal were added into the ASE eluate collection bottles. The bottles were rotated for 30 min, then the extract transferred over a dried Na_2SO_4 filled funnel to remove residual humidity, flushed with additional 10mL Hx:Ac (3:1 v/v) and evaporated until near dryness under vacuum.

Fractionation. Polar and nonpolar fractions were obtained as previously published (Legler *et al.* 2002; Montaño *et al.* 2013, submitted) with slight modifications. Briefly, the dried ASE-extract was washed twice with 1 mL Hx. The hexane portions were combined, then filtered through a 1 g Na_2SO_4 filled Pasteur pipette to retain hexane insoluble particles, and evaporated to 500 µL under a gentle N_2 flow

(nonpolar fraction). This nonpolar fraction was further cleaned with a multilayer sulphuric acid silica column (Houtman *et al.* 2004), which consisted of 1 g Na_2SO_4 on top of 3 g 33% sulphuric acid silica on top of a silanized glass wool plug. The column was pre-washed with 8 mL of Hx:diethyleter (DEE, 97:3). The extract was transferred onto the column including two rinses with 50 µL of Hx:DEE (97:3) before elution with 8 mL of solvent. The eluate of both the sample and the rinses were collected into a cleaned tube and evaporated at room temperature under a gentle N_2 flow to near dryness.

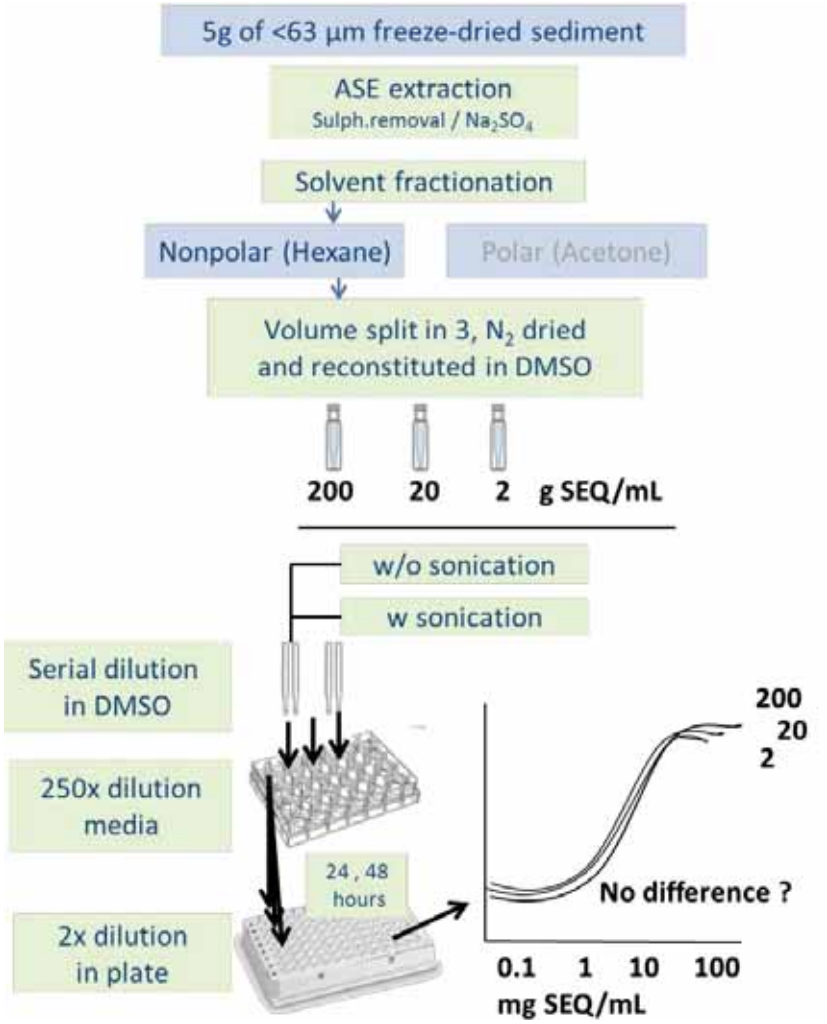

Figure 2 Graphical representation of the experimental design. Nonpolar sediment extracts were prepared to obtain three initial stocks with 2, 20 and 200 g SEQ/mL. From these initial stocks dilutions were made resulting in comparable sediment equivalent (SEQ) concentrations on the assay plate, and were tested in the DR.Luc assay.

Preparation of stocks. Cleaned nonpolar fractions were re-dissolved in 3 mL of hexane. The extract was split into three portions with a different volume; each was dried under a gentle N_2 flow, and transferred into DMSO with 90 s sonication to prepare three stocks with concentrations of 200, 20 and 2 g SEQ/mL (Figure 2). Extract stocks were stored at 4 °C until analysis in the bioassay. After 90 s

sonication, unless otherwise stated, an aliquot of each stock was serially diluted up to 10,000 fold in DMSO to obtain the exposure dilutions. Final SEQ/mL medium exposure dilutions from the different initial stock concentrations were as similar as possible. The effect of sonication assisted dissolution was tested on extracts with initial stock concentrations of 20 g SEQ/mL and 2 g SEQ/mL and not with the stocks at 200 g SEQ/mL based on pilot experiments revealing problems with such highly concentrated extracts. The effect of exposure period was tested for stock extracts with 2 g SEQ/mL.

DR.Luc bioassays. DR.Luc bioassays were performed with rat hepatoma H4IIE.Luc cells (kind gift from Prof Michael Denison) following previously developed assay protocols (Legler *et al.* 1999; Murk *et al.* 1998). Cell culture conditions, bioassay conditions and luciferase assay have been published elsewhere (Besselink *et al.* 2004; Vrabie *et al.* 2009). In short: cells were exposed in triplicate with the addition of 100 µL of growth medium containing TCDD, or the samples in a final concentration of 0.25% DMSO. Every experiment included a complete TCDD calibration curve while in addition each plate contained solvent and standard quality controls. Recovery was controlled for each extraction batch with 3 g of spiked sand which contained 3.2 ng TCDD/g of sand.

Cell viability was determined by the measurement of the total cells' metabolic activitybefore the end of the exposure period with 100 nM resazurin in 200 µL cell media for 2 hours (Montaño *et al.* 2011). Fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Then the plates were prepared for the luciferase assay and measured in a Biotech Synergy 2 plate reader as described before (Vrabie *et al.* 2009).

Parameter calculation and estimation of uncertainty. Experimental data were normalized to the maximal response given by 100 pM TCDD in the bioassay or to their own maximal induction when above that of TCDD as suggested by Baston and Denison (2011). The data were fitted to a three-parameter Hill function (Sigma Plot (Systat)) which assumes a logistic distribution of the data and provides an estimation of the maximum, slope and the effect concentration responsible for 50% of the luminescence effect (EC50). Based on these parameters the effect concentrations responsible for 20% of the effect (EC20) were also calculated for each experiment. Bioassay derived TCDD equivalents (Bio-TEQs) were calculated by relating the luminescence induced by the extract with that of the positive control 2,3,7,8-TCDD for the concentrations that gave a TCDD EC20 as well as EC50 equivalent response. All reported SEQ values are expressed in dry weight. The influences of initial stock sediment-equivalent concentration, sonication and exposure time on the final Bio-TEQ concentrations were analyzed.

Results

Effect of initial stock concentration

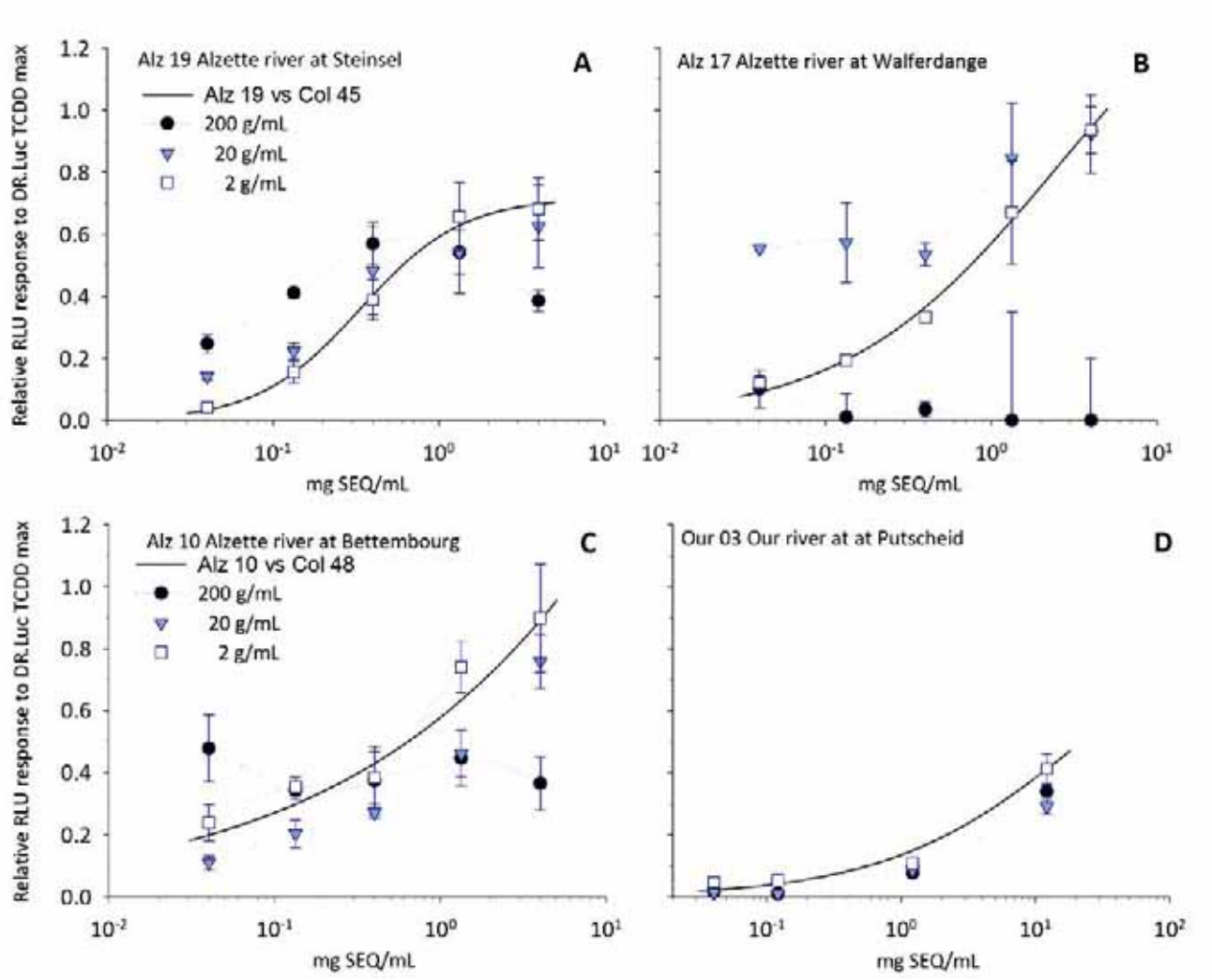

Figure 3 Effects of the initial stock concentration of cleaned nonpolar sediment extracts on their DR.Luc response. Cleaned nonpolar sediment extracts were prepared at initial stock concentrations of 200, 20 and 2 g SEQ/mL. These stocks were further serially diluted in DMSO and tested in the DR.Luc assay. Data represent the mean ± SD relative response to the maximal obtained by 100 pM TCDD in the DR.Luc assay from two separate experiments tested in triplicate. The line represents the predicted values after the data from the stock of 2 g SEQ/mL was adjusted to a Hill model. Cells exposed to the highest concentrations from the all the stocks of 200 g SEQ/mL and from the stock of 20 g SEQ/mL from Alz 19 and Alz 10, showed reduced cell viability to 71% compared to controls.

Figure 3 shows the DR-Luc bioassay responses induced by dilutions of cleaned nonpolar sediment extracts that were prepared from 3 different initial stock concentrations with 10x concentration difference. The best sigmoidal dose-response curves were obtained with the most diluted initial stocks whereas with the most concentrated (200 g/ml) stocks no good dose-response curve could be prepared except for the least polluted Our3 sediment (Figure 3D).

Calculation of dose-response parameters from 200 g SEQ/mL of cleaned nonpolar extracts from Alz 10, Alz 17 and Our 4 was not possible (Figure 3B and C). The effect of initial stock concentration becomes negligible for extracts from less polluted sediments with dioxin-like activity lower than 0.4 ng Bio-TEQ/ g SEQ (Table 1) as it was the case for Our 3 (Figure 3D).

Although all experiments from each location had the same extract concentrations in the plate, the 200 g SEQ/mL stocks from Alz1 and Alz 19 resulted in a lower quantification of the dioxin-like toxic potency compared to the potency from stocks with initial concentrations of 20 and 2 g SEQ/mL. Except for samples Alz 1, Alz 19 and Our 4 the differences between the result obtained with the initial stock of 20 g SEQ/mL and those obtained with the initial stock of 2 g SEQ/mL were not significant (Table 1). Identical results were obtained when all the Bio-TEQs were calculated with the EC20 although with considerably higher standard deviations.

Table 1

Dioxin-like activity expressed as Bio-TEQ (ng/g SEQ dry wt)[a] of the nonpolar fraction of sediments obtained with three different initial stock concentrations[b]

Label	Location	Initial stock concentration in DMSO (g SEQ/mL)								
		200			20			2		
Alz 1	Barbuerg	6.9	±	2.1[e]	16	±	3.2[e]	40	±	17
Alz 10	Bettembourg	NC[c]	±		2.8	±	0.32	2.1	±	0.45
Alz 17	Walferdange	NC[c]	±		1.2	±	0.23	0.70	±	0.18
Alz 19	Steinsel	0.76	±	0.05[e]	1.5	±	0.14[e]	2.1	±	0.19
Our 3[d]	Putsheid	0.26	±	0.06	0.28	±	0.04	0.21	±	0.04
Our 4	Bettel	NC[c]	±		0.64	±	0.12[e]	1.2	±	0.16
Woltz 1[d]	Maulusmillen	0.16	±	0.08	0.24	±	0.07	0.34	±	0.06

[a] Bio-TEQ (ng/g SEQ) ± SD obtained from at least two separate experiments, assayed with at least four concentrations twice in triplicate.

[b] In all cases the extracts were sonicated prior to the preparation of serial dilutions.

[c] NC: not possible to calculate dose-response parameters

[d] calculated with the EC20

[e] Significantly different from the results obtained with 2 g SEQ/mL

Effect of sonication

Cleaned nonpolar extracts with 20 and 2 g SEQ/mL in DMSO were tested before and after sonication. The main effect of sonification was the reduction in standard deviation, especially at the higher stock concentration (20 g/mL). The differences between the quantified potency of 2 g SEQ/mL extracts with and without sonication were negligible except for higher standard deviations without sonication. For 20 g SEQ/mL extracts, this effect of lower standard deviation in the sonicated extracts was even higher, especially those from the more polluted sediments with Bio-TEQ values >1 ng/g SEQ (e.g. Alz 1, Alz 10 and Alz 19). With the more polluted sediments the dose-response curves of extracts without sonication suggested a higher potency (Figure 4), although the standard deviation then is so high that the values with and without sonication overlap (Table 2).

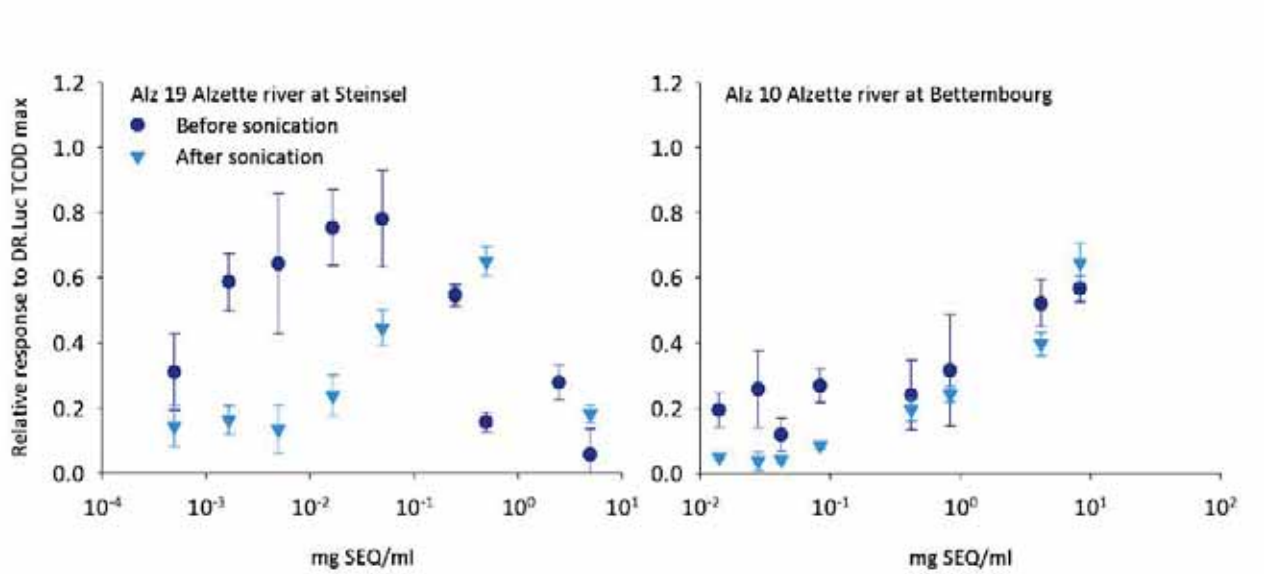

Figure 4 Effect of sonication on the dose-response of cleaned nonpolar sediment extracts. Cleaned nonpolar sediment extracts obtained with 20 gSEQ/mL stocks, were tested before and after 2 min sonication. Data represent the mean ± SD relative response to the maximum response obtained by 100 pM TCDD in the DR.Luc assay from two separate experiments tested in triplicate. Decrease of activity above 0.1-1 mg SEQ/mL for Alz 19 correlated with a reduction in cell viability (Data not shown).

Table 2

Dioxin-like activity expressed as Bio-TEQ (ng/g SEQ dry wt)[a] of the 20 g SEQ/mL nonpolar fraction of sediments with and without sonication[b]

Label	Location	Sonication					
		Without			With		
Alz 1	Barbuerg	272	±	266	26	±	5.5
Alz 10	Bettembourg	0.59	±	0.22	0.41	±	0.15
Alz 17	Walferdange	1.3	±	0.87	1.1	±	0.14
Alz 19	Steinsel	196	±	324	4.1	±	1.5
Our 3	Putsheid	0.36	±	0.16	0.34	±	0.08
Our 4	Bettel	0.78	±	0.23	0.98	±	0.14
Woltz 1	Maulusmillen	0.12	±	0.11	0.16	±	0.05

[a] Bio-TEQ (ng/g SEQ) ± SD obtained from at least two separate experiments, assayed with at least four concentrations twice in triplicate.
[b] Initial stock concentration was 20 g SEQ/mL.

Effect of exposure period for highly contaminated sediments

The luciferase induction by extracts from cleaned nonpolar sediment extracts was higher for 6 hours exposure than 24 or 48 hrs (Figure 5). This effect was greater for the more polluted locations independent of the initial stock concentration (2 g SEQ/mL). The dioxin-like potency values obtained at 48 hours for these three locations were closest to chemically measured values (Data not shown). The differences between responses at 24- and 48-hours were smaller or even negligible for extracts from locations with lower contaminant concentrations (Figure 5b).

Dioxin-like potency of nonpolar sediment extracts from Luxembourgish basins

Samples from various Luxembourgish basins (Figure 1) with a variety of environmental pressures were tested after sonication initially with a stock concentration of 20 g SEQ/mL and the potency was quantified after 24 h (or 48 hours) of exposure (Table 3). Based on indications obtained during sampling, sample organoleptic characteristics and especially the extract colour and behaviour during clean-up, potentially highly contaminated samples were also tested with a stock concentration of 2 g SEQ/mL at 24 and 48 hours (Table 3).

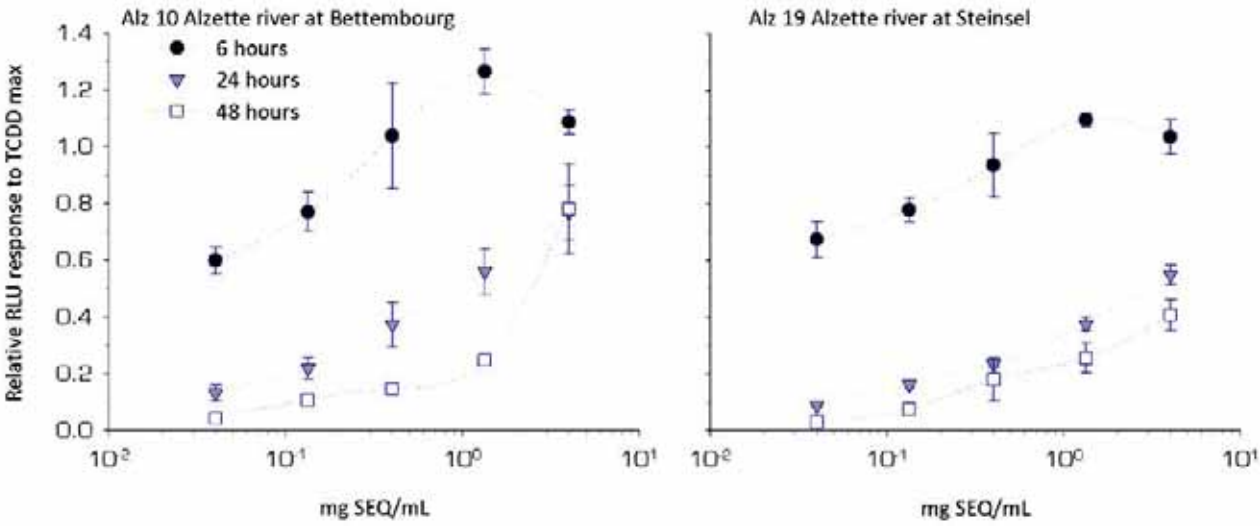

Figure 5. Effects of the exposure period on the DR.Luc dose response of cleaned nonpolar sediment extracts. Cleaned nonpolar extract of sediments were dried under N_2 and re-dissolved in DMSO at initial stock concentrations of 2 g SEQ/mL. These stocks were further serially diluted in DMSO for exposure and measurement in the DR.Luc assay at 6 hours (with conditioned medium), 24 hours and 48 hours. Data represent the mean ± SD relative response to the maximal response induced by TCDD after the same exposure period obtained from two separate experiments tested in triplicate.

Toxic potencies at control locations such as Clerve 1 and Our 0 were below 0.03 ng Bio-TEQ/g SEQ, but Woltz 1 showed an activity of 0.24 ng Bio-TEQ/g SEQ, which is considerably higher in comparison. Dioxin-like activity from locations at the Alzette river was higher from the point of entrance into the country all the way through Esch-sur-Alzette (40 ng Bio-TEQ/g SEQ) and decreased to 1-2 ng Bio-TEQ/g SEQ along its further course towards the north of Luxembourg (Table 3). Alzette tributaries had activities between 0.2 and 3.2 ng Bio-TEQ/g SEQ. Samples from the Our river showed a variable dioxin-like potency along its course but considerably lower compared to samples from the Alzette river (Table 3).

Table 3

Dioxin like toxic potency of nonpolar fraction of sediments from various Luxembourgish basins[a]

Label	Location / tributary	Bio-TEQ[b] 24 (48) hours (ng/g SEQ)	Label	Location	Bio-TEQ[b] 24 (48) hours (ng/g SEQ)
Alz 1	Barbuerg	40 (18) [c]	Clerve 1	Kaaspelt	0.027
AlzT 2	Lallange / Dipbech	0.86	Woltz 1	Maulusmillen	0.24
Alz 3	Amont Schiflange	8.9 (0.30)[c]	Our 0	Moulin de Kalborn	0.013
AlzT 4	Kiemelbaach	1.6 (0.85)	Our 1	Dasburg-bridge	0.13
Alz 5	Aval Kiemelbaach	2.2 (1.2)	Our 2	Stolzemborg	0.12
AlzT 6	Lameschermillen / Mess	0.65	Our 3	Putsheid	0.28
Alz 7	Lameschermillen	5.4[c]	Our 4[c]	Bettel	1.1 (0.12)[c]
AlzT 8	Op der Klaus / Kälbaach	3.2[c]	Our 5	Bettelaval STEP	0.066
AlzT 9	Op Bich / Mierbech	1.7	Our 6	Wallendorf-bridge	0.097
Alz 10	Bettembourg	2.1 (0.48)[c]	Our 7	Reisdorf / Sûre	0.13
AlzT 11	Bettembourg / Diddeleng.	0.23	Our 8	Dillingen / Sûre	0.12
Alz 12	AvalBettembourg	2.7			
AlzT 13	Roeser/Crauthemergruef	0.16			
Alz 14	Avalroeser	0.85			
Alz 15	Hesperange	1.3			
Alz 16	Beggen	1.7			
Alz 17	Walferdange	1.2 (0.16)			
Alz 18	Laag	0.25			
Alz 19	Steinsel	2.1[c]			

[a] Alz: Alzett river, AlzT: Alzett tributary, Our: Our river, Clerve: Clerve river and Woltz: Woltz river. T: tributary. Label numbers correspond to the river flow.

[b] Bio-TEQ (ng/g SEQ) obtained from at least two separate experiments, assayed with at least four concentrations twice in triplicate after 24 and 48 hours of exposure. 48 hours results were included only when they were different from the values at 24 hours. Standard deviation within experiments was less than 28%.

[c] Potency calculated with initial stock concentration of 2 g SEQ/mL

Discussion

For a confident bio-assay based toxicological hazard and risk characterisation of sediments, it is important that the outcomes of the bio-analysis are reliable, and relevant. This study examined the consequences for the quantified toxic potency of non-polar sediment extracts depending on the initial stock concentration, sample preparation and exposure duration in an *in vitro* bioassay for dioxin-like potency in cleaned nonpolar sediment extracts from various locations in Luxembourg. The initial stock concentrations prepared were 2, 20 and 200 g sediment equivalents (SEQ)/mL DMSO; from each stock, dilutions were prepared in DMSO and tested in the DR-Luc assay. Especially when using the highest (200 g SEQ/mL) initial stock concentrations, the quantified Bio-TEQs/g sediment were significantly lower compared to those based on the less concentrated initial stocks. The underestimation was 2-5 times for the least and most polluted sediments respectively compared to the Bio-TEQs based on the 2 g SEQ/mL stock. For the most polluted sediments even using the 20 g/mL initial stock resulted in an underestimation of the Bio-TEQs. The tendency towards higher quantified potency

at lower initial stock concentrations, the extent of the differences and the difficulties to obtain a proper dose response at higher initial stock concentrations (Table 1) support the hypothesis that part of the active compounds is lost due to oversaturation of the DMSO solution and precipitation of a fraction of the original amount present in the extraction solvent. Sonication assisted dissolution of the stock before serial dilution strongly reduced the standard deviation, but was not enough to dissolve all the relevant compounds in over-concentrated initial stocks. Sonication was necessary to produce reproducible results. Differences in quantified toxic potency due to over-concentration has been previously reported for extracts of waste treatment plant effluent (Murk *et al.* 2002), but to our knowledge this is the first report of this effect for sediment in a dioxin-like activity bioassay.

Due to differences in water solubility between PAHs and POPs of different lipophilicity depending on the number of halogens and the substituted positions (Huang and Hong 2002; Shiu and Mackay 1986), the ratios between dissolved and precipitated compound will differ depending on the degree of overconcentration. When the extracts are diluted further, especially without sonication, the precipitate is not transferred to the dilution vials. The initial stock concentration of 2 g SEQ/mL DMSO was low enough to prevent underestimation of the sediment Bio-TEQs. This results in a limit of quantification (LOQ) of 80 pg Bio-TEQ/g SEQ. Depending on the required LOQ, the initial stock concentration could be increased to 20 g SEQ/mL (LOQ 8 pg Bio-TEQ/g SEQ), and for relatively clean sediments this could even be 200 g SEQ/mL (LOQ 0.8 pg Bio-TEQ/g SEQ). Observed effects of sonication assisted dissolution of stocks before pipetting and exposure strongly suggests its inclusion in future testing protocols.

Not only overconcentration of initial stocks, but also overload of clean-up columns can impair quantification of the toxic potencies of sediments. Recently, it was demonstrated that extracts obtained by ASE and acid silica clean-up, also used by us, contained similar amounts of dioxins compared to more exhaustive analytical methods used for high resolution mass spectrometry (Dindal *et al.* 2011). Acid silica clean-up is needed to prevent false-positive responses due to PAHs that activate the AhR (Hamers et al., 2000; Puglisi et al., 2007), but do not induce dioxin-like toxicity because they are easier metabolised and give rise to other mechanisms of toxicity. However, overload of the acid silica column cause AhR active PAHs to run through the column and reach the final extract (Dindal *et al.* 2011; Schwirzer *et al.* 1998). The presence of PAHs in the extract of highly contaminated sediments is revealed when testing during 6, 24 and 48 hours of exposure as the PAHs can be metabolised by the DR-Luc cells that are H4IIE rat hepatoma cells capable of P450 activity (Figure 5). Quantification of dioxin-like potency at 24 hours or later has been suggested as an alternative to exhaustive clean-up as easily biodegradable

PAHs would be already metabolized (Hamers *et al.* 2000). When the extracts originate from less PAH-polluted sediments (Bio-TEQ below 0.01 ng/g SEQ at 24 hours) or are more thoroughly cleaned (in our case with 1 g SEQ/ g 33% acid silica column) small or no differences can be observed between the dioxin like response at 24 and 48 hours (Stronkhorst *et al.* 2002; Vondrácek *et al.* 2001). Sediment samples with potencies above the intervention value of 1 ng TEQ/g (Iannuzzi *et al.* 1995; OSPAR 2007; Stronkhorst *et al.* 2002) should be retested at longer exposure times to verify their dioxin-like activity. Testing of bio-available concentrations can also be applied to better assess the risk characterization of highly contaminated sediments (Puglisi *et al.* 2007).

The sediments in the Alzette basin showed higher DR.Luc activity due to dioxin-like compounds compared to other European water bodies (OSPAR 2007), most likely due to industrial activities in the south of Luxembourg as the level of pollution in this area is well known (Kurtenbach and Gallé 2008). To our knowledge this is the first report of dioxin-like activity of sediments from the Alzette River. The Bio-TEQ levels up to 48 ng/g are similar to what has been found in small Mediterranean rivers near to waste water discharges (David *et al.* 2010; Kinani *et al.* 2010), and up to 7 ng Bio-TEQ/g during flood events in the Rhine (Wölz *et al.* 2010a; 2010b). Towards the north of Luxembourg the BIO-TEQs are gradually lower, probably due to mixing with cleaner sediments from tributaries. The Our basin sediments are considerably less polluted with dioxins compared to the Alzette basin. The Bio-TEQs are low in the upper Our sediments, but these increase towards the town of Vianden and then slightly decrease until the junction with the river Sûre. These sediment Bio-TEQ levels correlate very well with the reported contamination profile of fish at similar locations (Boscher *et al.* 2010; Dauberschmidt and Hoffmann 2001; Hugla *et al.* 1998).

Sediment risk characterization using bio-analysis

Several sediment quality criteria (SQC) have been proposed depending on the protection goals (Table 4). For example a SQC intervention value of 1 ng TEQ/g triggers remedial action (Stronkhorst *et al.* 2002) and also is a limit for disposal of dredged spoils (Iannuzzi *et al.* 1995). Also governmental agencies and local governments have established screening levels as well as thresholds for the protection of human and ecological receptors from 0.01-0.61 ng TEQ/g (Reviewed by Iannuzzi *et al.* 1995; Stronkhorst *et al.* 2002). For *in vitro* bioassays SQC safe levels have been established for the dioxin-like reporter-gene assay from a target level of 0.002 ng TEQ/g to a threshold value of 0.025-0.050 ng TEQ/g (OSPAR 2007; Stronkhorst *et al.* 2002). Finally, safe levels for top predators have been estimated

to be between 1.75×10^{-4} and 0.012 ng TEQ/g (Murk *et al.* 1998; Schipper *et al.* 2010) (Table 4).

To be able to apply the DR.Luc assay to decide whether sediments comply with these SQC for TEQs, the initial stock concentration has to be high enough to produce a signal equivalent to 3 pM TCDD (approximately EC20) at 0.2% DMSO (but in no case more than 0.4).

Table 4 presents the SEQ/mL exposure medium and consequently the SEQ/DMSO that would be needed to significantly quantify the potency of sediment contaminated at the level of the SQC. If the risk characterization goal is to know whether the sediment potency is above the intervention SQC an initial stock concentration of 0.5 g SEQ/mL DMSO will provide suitable results without overconcentration problems. Only when the TEQ values also below the SQC need to be quantified a higher initial stock concentration is required.

Table 4

Initial stock concentrations derived from different sediment quality criteria (SQC)

Risk characterization goal	SQC ng TEQ/g sediment [a]	mg SEQ /ml exposure medium [b]	Initial stock g SEQ/ml DMSO [c]
Sediment intervention value [d]	1	0.96	0.48
DR-CALUX threshold value [e]	0.025-0.05	39-19	19-9.6
Protection of human and ecological receptors [f]	0.01-0.61	96-1.6	48-0.79
Safe sediment levels for wildlife [g]	1.75×10^{4} -0.01	5.5×10^{3}-96	2.7×10^{3}-48

[a] Data usually reported as ng TEQ/kg but expressed as ng/g for comparison with Tables 1, 2 and 3.
[b] Amount of sediment required in plate to produce a signal equivalent to 3 pM TCDD (0.96 pg/mL)
[c] Concentration of initial stock needed for 0.2% DMSO in exposure medium.
[d] (Iannuzzi *et al.* 1995), [e] (Stronkhorst *et al.* 2002) [f] (Iannuzzi *et al.* 1995) [g] (Schipper *et al.* 2010)

To monitor whether at a certain location there is a risk for human and wildlife; then the initial stock concentration should at least be 10 g SEQ/mL up to 50 mg SEQ/mL. The latter would result in over-concentration problems revealed in this study. Although the limit of detection with the DR-Luc is much lower than with chemical analytical techniques, it is not sufficient to quantify the really low levels required to establish conformity with some safe levels for wildlife without additional and rigorous clean-up. For instance a stock concentration of above 2 kg/mL would be necessary to produce a response equivalent to the safe sediment level of 3 pg TEQ/g organic C (equivalent to 0.18 pg TEQ/g sediment assuming sediment with 6% organic carbon) in Eurasian otters for hepatic vitamin A reduction (Murk *et al.*

1998). Values presented in Table 4 should be of guidance to decide the amount of sediment to be used and initial sample stock concentration in DMSO.

It will be, however, no problem to analyse sediments at the intervention value as already 0.5 SEQ/mL DMSO would be enough (Table 4). Even for highly polluted locations such as Alz 19 and Our 4, this concentration would not give problems with over-concentration-related underestimation of the TEQs/g sediment. . However, when Alz 19 and Our 4 potency would be tested at initial stock concentrations of 20 or 200 g SEQ/ml their toxicity would have been underestimated (Table 1) in a way that these locations might not be considered for intervention or banning which would be the decision when they are tested at 2 g SEQ/mL (Table 1). The TEQs/g sediment of Alz 1 and Alz 10 would be exceeding the intervention level at any concentration tested, although testing over-concentrated extracts of relatively polluted sediments without sonication might overestimate the toxic potency of the sediment. The same will be the case with too high residual PAH levels in highly contaminated extracts after 6-24 hours of exposure. Underestimation of the toxic potencies (false negative response) is unwanted because of safety risks but unnecessary classification of sediments above the SQC may result in unrequired expenses for dredging, storage and remediation.

Most samples from the Alzette river had dioxin-like toxic potencies close to or above the intervention SQC (Tables 3 and 4), and some samples closest to the France border (Alz 1, Alz 4 and Alz 5) and Our 4 after the city of Vianden greatly exceeded the intervention value of 1 ng TEQ/g sediment, even after 48h of exposure to reduce the false positive contributions of PAHs. These locations should require immediate attention and further studies in the area to determine the need for remediation

This study reveals that a higher contaminant load in the initial extracts from sediments can affect the reliability of the quantification of the toxic potency obtained with the *in vitro* bioassay. As a result there could be either underestimation because of solvent oversaturation and loss of active compounds through precipitation, or overestimation because of overload of clean-up columns and relative dissolution of the more potent compounds after sonication of the over-concentrated extract. In addition to the advice to standard apply sonication within the testing protocols, it also is advised to adapt initial stock concentrations both to the risk characterization goal as well as to the contamination level of the sample. In case of a relatively high dioxin-like toxicity the nature of the dioxin-like activity should be confirmed at longer exposure times to reduce the impact of possible residual PAHs in the extract.

Taking into account these aspects in addition to already described important issues such as using a validated dioxin-stock and positive samples for statistical process control charts; the *in vitro* bioassays based Bio-TEQs can be applied in a comprehensive monitoring program to determine whether sediments comply with health and safety standards for humans and the environments.

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CHAPTER 6

Metabolic activation of nonpolar sediment extracts results in enhanced thyroid hormone disrupting potency

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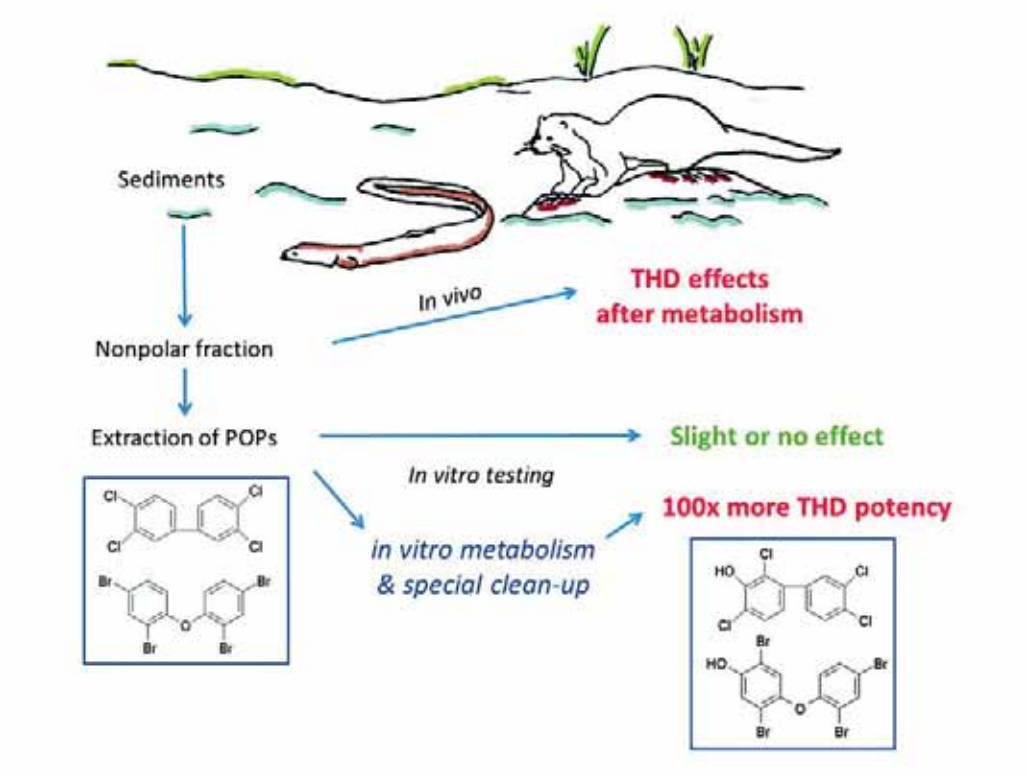
Abstract

Traditional sediment risk assessment predominantly considers the hazard derived from legacy contaminants that are present in nonpolar sediment extracts, such as polychlorinated biphenyls (PCBs), dioxins, furans (PCDD/Fs) and polyaromatic hydrocarbons (PAHs). Although *in vivo* experiments with these compounds have shown to be thyroid hormone disrupting (THD), *in vitro* their THD potency is not observed in nonpolar sediment extracts. This is hypothesized to be due to the absence of *in vitro* biotransformation which will result in bioactivation of the lipophilic compounds into THD hydroxyl metabolites. This study reveals that indeed metabolically activated nonpolar contaminants in sediments can competitively bind to thyroid hormone transport proteins. Sediment fractions were incubated with S9 rat microsomes, and the metabolites were extracted with a newly developed method that excludes most of the lipids to avoid interference in the applied non-radioactive 96-well plate TTR competitive binding assay. Metabolic activation increased the TTR binding potency of nonpolar fractions of POP-polluted sediments up to 100 times, resulting in potencies up to 240 nmol T_4 equivalents/g sediment equivalent (nmol T_4-Eq/g SEQ). Without bioactivation, medium polar and polar fractions also contained potent TTR-binding compounds with potencies from 1.6 to 17 nmol T_4-Eq/g SEQ. This demonstrates that a more realistic *in vitro* sediment THD risk characterization should also include testing of both polar and medium polar sediment extracts for THD, as well as bioactivated nonpolar sediment fractions.



Introduction

Sediments furnish support, provision and regulation services which are fundamental to the well-being of aquatic ecosystems (Gerbersdorf *et al.* 2011). However, they also serve as storage and secondary sources of persistent contaminants (Nizzetto *et al.* 2010), entailing an important route of exposure for aquatic food chains starting with benthic organisms including mollusks (Verslycke *et al.* 2005), fish (de Boer *et al.* 2001; Moermond *et al.* 2004; Montaño *et al.* 2011; Schipper *et al.* 2009; Zimmer *et al.* 2011), birds (Bosveld *et al.* 2000), and mammals (Leonards *et al.* 1997; Murk *et al.* 1998; Nyman *et al.* 2003), which ultimately will lead to human exposure (De Mul *et al.* 2008; van Leeuwen *et al.* 2007). Especially sediments in industrial and harbor areas have shown to possess high levels of persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), dioxins and furans (PCDD/Fs), polybrominated diphenyl ethers (PBDEs) and polyaromatic hydrocarbons (PAHs) (Houtman *et al.* 2006; Sanctorum *et al.* 2007).

The relevance of bioassays in hazard, impact and risk assessment of sediments has been increasingly addressed (Schipper *et al.* 2010). *In vitro* assays for general toxicity, genotoxicity, mutagenicity and endocrine disruption, have been proposed within integrated effect-based frameworks for site-specific sediment quality assessment (Hamers *et al.* 2006; Schipper *et al.* 2010) and effect-directed analysis (EDA) (Brack *et al.* 2007).

Risk assessment of organic contaminants has focused traditionally on nonpolar compounds such as PCBs, PCDD/Fs and PAHs, but polar compounds have been increasingly reported to be important in the overall sediment toxicological potency (Brack *et al.* 1999; Di Giorgio *et al.* 2011; Houtman *et al.* 2006; Legler *et al.* 2002; Legler *et al.* 2003), particularly after the application of EDA (Brack *et al.* 2002; Brack *et al.* 2005; Higley *et al.* 2012; Lübcke-von Varel *et al.* 2011). They rightly argue, that polar fractions comprise a significant amount of hazardous compounds and should be considered for sediment risk assessment (Lübcke-von Varel *et al.* 2011). Although nonpolar (lipophilic) POPs such as PCBs and PBDEs have been shown to induce steroidogenicity and thyroid hormone disruption *in vivo*, the nonpolar fractions of sediments in which they are present hardly exert any *in vitro* toxic effects (Higley *et al.* 2012). Indeed, alterations of thyroid hormone-dependent cell proliferation on the T-screen assay (Gutleb *et al.* 2005) and severe perturbations of thyroid hormone-dependent metamorphosis of *in vivo* exposed amphibians (Gutleb *et al.* 2007a; Gutleb *et al.* 2007b) have been observed after exposure to nonpolar sediment extracts from various contaminated locations in The Netherlands; despite the seemingly low potency of nonpolar sediment extracts (Lübcke-von Varel *et al.* 2011).

We therefore hypothesize that an important overlooked aspect of *in vivo* sediment hazard assessment is the importance of metabolic activation of lipophilic contaminants. Several toxicological studies have demonstrated that endocrine disruption from nonpolar compounds occurs via metabolic activation to more hormone-like compounds capable of exerting a range of toxic effects (Boxtel *et al.* 2008; Gutleb *et al.* 2010; Meerts *et al.* 2004; Usenko *et al.* 2012). Well-known examples are the increased estrogenic effect of methoxychlor (Legler *et al.* 2002) and the thyroid hormone disruptive capacity of hydroxylated (OH-) metabolites from PCBs and PBDEs (OH-PCBs and OH-PBDEs) (Freitas *et al.* 2011; Lans *et al.* 1993; Marchesini *et al.* 2008; Meerts *et al.* 2000; Schriks *et al.* 2007).

Lipophilic pollutants that are characteristically present in the nonpolar sediment fractions are taken up, accumulated, and biomagnified through the food chain (Kelly *et al.* 2008). In the liver they are transformed via phase I metabolism into OH-compounds which are structural analogues of the thyroid hormones (Marsh *et al.* 2006; Morse *et al.* 1995). Interestingly, these hormone-like OH-metabolites are retained in plasma due to their capacity for binding to thyroid transport proteins like transthyretin (TTR), thyroxin-binding globulin (TGB) and albumin (Hamers *et al.* 2008; Lans *et al.* 1994; Marchesini *et al.* 2008). This binding protects them from further degradation and excretion, whereas they are transported via these proteins to various tissues and over selective barriers to the brain and the placenta (Meerts *et al.* 2002; Morse *et al.* 1993). OH-PCBs and OH-PBDEs accumulate in plasma of birds, cetaceans and other mammals including man, at levels often higher than their respective parent compounds (Montaño *et al.* 2013).

The need for inclusion of metabolic activation has been acknowledged already for decades (Meerts *et al.* 2000). Indeed, it has been a priority within the development of *in vitro* testing alternatives (Coecke *et al.* 2006; Murk *et al.* 2013). Exogenous biotransfomation systems, however, only transform a relatively low percentage of the parent compounds into OH-metabolites (Morse *et al.* 1995; Murk *et al.* 1994). When the parent compounds are not separated from the metabolites they may interfere with the assay, for example by being cytotoxic to cells before the amount of metabolite is high enough to induce a measurable effect (Schriks *et al.* 2006). In addition, co-extractants from the enzymatic preparations can interfere with the bioassay (Hamers *et al.* 2008; Montaño *et al.* 2012; Simon *et al.* 2010). Recently, a method was developed that efficiently extracts OH-PCB/PBDE metabolites while limiting the co-extraction of lipids and almost eliminating the interferences with the TTR binding assay (Montaño *et al.* 2012). This extraction method coupled to an improved fluorescence based TTR competitive assay (ANSA-TTR) was successfully developed and validated with standards of POPs (Montaño *et al.* 2012). This

development has opened the possibility to test TTR binding potency of metabolically activated extracts.

The current research investigates the role of metabolic activation on TTR competitive binding potency of polar and nonpolar sediment extracts from highly or less contaminated sediments in Luxembourg. The extracts were either roughly split into a lipophilic and polar fraction using solvent partition fractionation, or fractionated into eight sub fractions with increasing polarity by normal phase HPLC. The model compounds CB77 and BDE47 and the sediment fractions were tested for TTR binding potency before and after metabolic activation with rat S9 microsomes as positive controls. The TTR competitive binding potency of the metabolic extracts was tested in a recently developed non-radioactive 96-well plate ANSA-TTR assay, including removal of lipids from the extracts to avoid interferences in the assay. The responses were expressed as nmol thyroid hormone (thyroxine, T_4) equivalents/g sediment equivalent (nmol T_4-Eq/g SEQ).

Materials and Methods

Chemicals. The model compounds 3,3´,4,4´-tetrachlorobiphenyl (CB77, 99.0%) and 2,2´,4,4´-tetrabromodiphenylether (BDE47, 98.8%) were purchased from Sigma-Aldrich Co. (Steinheim, Germany) and Chiron AS (Trondheim, Norway), respectively. The CB77 metabolite 4´-OH-3,3´,4,5´-CB79 (4´-OH CB79) was purchased from LGC Promochem (Middlesex, UK). Hexane (Hx, dioxin analysis grade) and all other solvents (chromatographic grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands) or from Sigma-Aldrich Co (Steinheim, Germany). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and methanol (MeOH). β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), 3,3´,5,5´-tetraiodo-L-thyroxin (T_4), human transthyretin (TTR), lipid removal agent (LRA) and the fluorescent probe 8-anilino-1-naphthalenesulphonic acid ammonium salt (ANSA) were purchased from Sigma-Aldrich Co (Steinheim, Germany).

Concentrations of T_4 and ANSA were routinely determined in cuvettes by UV-visible spectrometry with a Beckman Coulter DU 800 spectrophotometer. Extinction coefficients were 6180 $M^{-1}cm^{-1}$ at 325 nm in phosphate buffer (PB) and sodium hydroxide (NaOH) at pH 11 for T_4 (Nilsson *et al.* 1975) and 6300 $M^{-1}cm^{-1}$ at 350 nm in PB at pH 7.5 for ANSA (Cao *et al.* 2010). All extraction and storage materials were exclusively made of borosilicate glass and polytetrafluoroethylene screw cap vials were used when required. All materials were prewashed with hexane. Sand from Fontainebleu (Paris, FR) was dried overnight at 105°C and extracted twice with the ASE method described below to obtain clean sand.

Sediment sampling and preparation. Sediment samples were collected in 2010 and 2011 from 30 locations at the rivers Alzette, Our, Clerve and Woltz in The Grand

Duchy of Luxembourg. A subset of locations was selected to exemplify sediments with a range of contamination profiles and levels from industrial/urban (Alz 1, Alz 10 and Alz 15), urban (Alz 17), agricultural (Our 3) and woodland (Woltz 1) locations (Figure 1). Location labels correspond to the sampling position along the river and the overall codes from the full sampling campaigns were unchanged to allow comparison with other reports and publications.

Composite samples of 4 cm deep surface sediments were collected manually from natural sedimentation zones covering at least 50 m along the river course. At least 8 sub-samples were mixed (1 kg), transported and stored in glass flasks at 4°C. The samples were transferred into aluminium plates, frozen at –20°C and then dried using an Alpha 2-4 LSC freeze-dryer (Christ, Germany) for at least 48 hours. The sediment was sieved using a sieving machine (Retsch, Germany) and the >63 µm fraction was stored in darkness at 4°C into glass bottles until extraction.

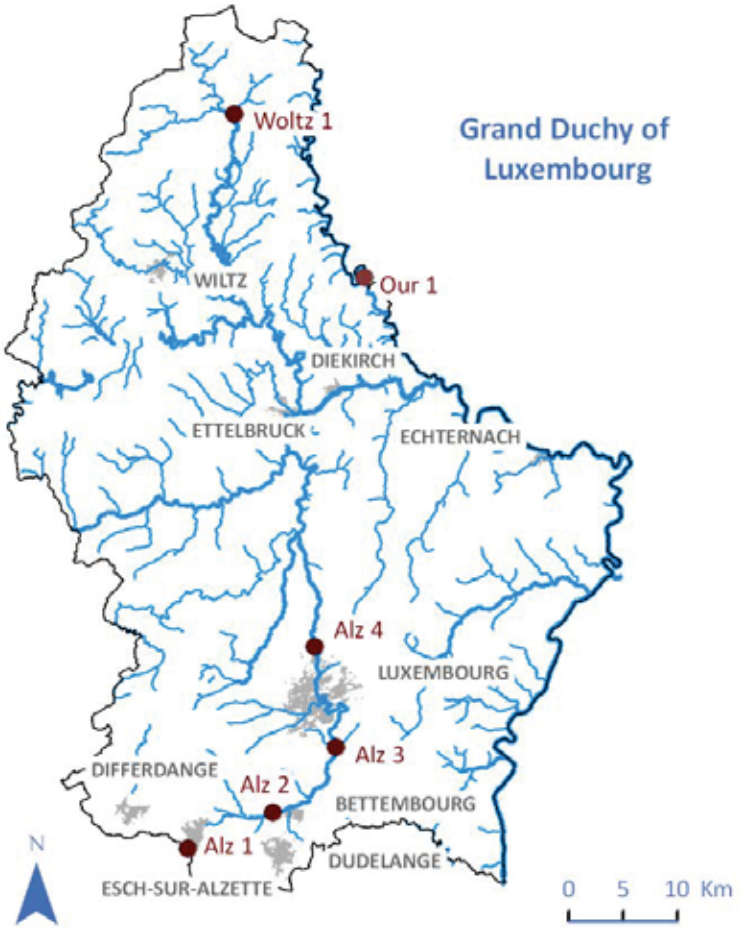


Figure 1. Sediment sampling sites within the Grand Duchy of Luxembourg. Samples were taken from the rivers Alzette (Alz, industrial/urban), Our (agricultural) and Woltz (woodland).

Extraction. A total of 5 g of <63 µm dried sediment from each location and cleaned sand were extracted with Hx and acetone (Ac, 3:1, v/v) with accelerated solvent extraction (ASE200; Dionex, Sunnyvale, CA, USA) with three extraction cycles at 100°C at 2,000 psi (Houtman *et al.* 2007). A mixture of 2 g dried sodium sulphate

(Na_2SO_4) to eliminate remaining moisture and 1 g of Cu powder (Sigma-Aldrich Co., Steinheim, Germany) for sulphur removal were added into the ASE collection bottles. The bottles were rotated for 30 min, then transferred over a dried Na_2SO_4 filled funnel, flushed with additional 10 mL Hx:Ac (3:1 v/v) and evaporated until near dryness under vacuum.

Solvent partition fractionation. Polar and nonpolar fractions were obtained as previously described (Legler *et al.* 2002). Briefly, the dried ASE-extract was washed twice with 1 mL Hx. The hexane portions were combined, then filtered through a 1 g Na_2SO_4 filled Pasteur pipette to retain hexane insoluble particles, and evaporated to 500 µL under a gentle N_2 flow (nonpolar fraction). The remaining acetone soluble precipitate was dissolved in acetone, filtered through the same 1 g Na_2SO_4 filled Pasteur pipette to re-dissolve the particles carried with the hexane, and evaporated under a gentle N_2 flow to near dryness (polar fraction). The nonpolar fraction was further cleaned with a multilayer sulphuric acid silica column (Houtman *et al.* 2004), which consisted of 1 g Na_2SO_4 on top of 3 g 33% sulphuric acid silica on top of a silanized glass wool plug. The column was activated with 8 mL of Hx:diethyleter (DDE, 97:3). The extract was transferred into the column including two rinses with 50 µL of Hx:DEE (97:3) before elution with 8 mL of solvent. Both the rinse and the eluate were collected into a cleaned tube and evaporated under a gentle N_2 flow to near dryness. The cleaned nonpolar fraction and the polar fraction were transferred into 250 µL of DMSO to obtain extracts at 20 g sediment equivalents (SEQ)/mL. Extract stocks were stored at 4°C until bioassays were performed.

GPC-NPC fractionation. ASE-extracts were cleaned up using Gel Permeation Chromatography (GPC) equipped with two polystyrene-divinylbenzene columns in series (10 µm, 50 Å, 600 x 25 mm, Polymer Laboratories) and with 10 mL/min dichloromethane (DCM) as eluent (Houtman *et al.* 2002; Weiss *et al.* 2009). The major part of the matrix elutes in the first fraction up to ca 18 min following injection. Based on the results of the elution profile of test compounds (Houtman *et al.* 2002) the fraction 16:30 – 24:00 min was collected.

Each GPC fraction was fractionated into eight sub-fractions with increasing polarity by Normal Phase (NP) High Pressure Liquid Chromatography (HPLC) as previously described (Fernandez and Bayona 1992). A semi-preparative column (Waters µPorasil, 7.8 x 300 mm, particle size 10 µm) with the following HPLC program (Weiss *et al.* 2009); mobile phase initially 100% Hx (5 min), 1%/min of DCM for 5 min, 4%/min of DCM for 25 min to 100% DCM (10 min) followed by 10%/min of acetonitrile (ACN) for 5 min, and then back to 100% DCM (5 min) and finally 6%/min to 100% of Hx to re-equilibrate (10 min). The fractions collected were at time 0-4 min (F 1), 5-17 min (F 2), 18-22 (F3), 23-26 (F4), 27-31 (F5), 32-40 (F6), 41-54 (F7) and 55-70 min (F8). Fractions were evaporated under a gentle N_2 flow to

near dryness and then transferred into 250 µL of DMSO to obtain fractions at 20 g SEQ/mL. Extract stocks were stored at 4°C until bioassay.

Metabolic activation and metabolite extraction. In vitro bioactivation of PCBs and PBDEs has been described (Murk *et al.* 1994) and was applied with few modifications as published (Montaño *et al.* 2012). Briefly, incubations were made in a borosilicate glass tube with 1 mL PB (100 mM, pH 7.5), S9 fraction (1 mg protein/mL, β-napthoflavone and phenobarbital treated rats, Xenotech, KA), and 10 µL extract or compound solution to obtain 1% DMSO. The tubes were pre-incubated at 37°C for 5 min and incubated for 60 min with the addition of 100 µL of 10 mM NADPH in PB every 20 min. Metabolism was stopped by denaturation of microsomal proteins with 700 µL of ice-cold MeOH and the metabolites extracted according to a recently published low-fat method for metabolite extraction (Montaño *et al.* 2012). Briefly, the fat was flocculated with 50 µL of 10% dextran (*Leuconostoc* spp., Sigma-Aldrich, Steinheim, Germany) and the protein denaturated with 50 µL of 6M HCl. Metabolites where extracted with 2x1 mL Hx:MTBE (1:1, v/v). The extract was transferred into a test tube with 1 mL of potassium chloride (KCl) in water (1%, w/w) and after centrifugation transferred again into a clean tube. The KCl was re-extracted with 1 mL of solvent and after combination of the organic phases reduced to about 50 µL extract (20 mg equivalent (mg-eq) protein/mL) under a gentle N_2 flow. The incubation extract was further cleaned-up with a lipid removal agent mini-column (Montaño *et al.* 2012) made of 0.8 g of LRA and 0.3 g of Na_2SO_4 packed on top of a glass wool plug in a Pasteur pipette. The mini-column was pre-washed with 2 mL of Hx:MTBE (1:1), then the extract was quantitatively transferred into the column and the tube was washed with 2x150 µL of Hx:MTBE. The column was further eluted with 1600 µL of the same solvent, which were collected into a prewashed test tube, dried under gentle N_2 flow, and re-dissolved in 50 µL MeOH, which corresponds to 20 mg-eq protein/mL and 4 g SEQ/mL.

Every metabolic activation experiment included a blank incubation with DMSO (S9 blank), recovery controls with various concentrations of spiked 4-OH CB 79, metabolic activation controls with spiked CB77 or BDE47 (2 µM), and negative controls with either POP or sediment fractions where denaturation was done at time zero.

The enzyme transformation rates (6-30 pmol metabolites per mg protein^{-1} mL^{-1}) are the limiting factor in the metabolic incubation. Therefore, above a certain threshold, increasing amounts of metabolically labile POPs in the sample will yield the same amount of metabolites and therefore the extracts will produce equal TTR binding activity. Serial dilutions of the incubation extract are hence not useful to calculate the TTR competitive potency in relation to sediment concentration. Instead, serial dilutions of the fractions were prepared in DMSO followed by duplicate incubations of each dilution incubated separately to find the

concentration for which the TTR binding activity was not different from the S9 blank.

Sediment fluorescence interference. It was checked whether the sediment fractions could possibly give a fluorescence signal that would disturb the ANSA-TTR assay measurement. Sediment fractions were serially diluted up to 4000 times in MeOH. These dilutions were mixed with 100 µL PB at pH 7.5 in a round-bottom 96-well plate (Greiner Bio-One) and had a MeOH final solvent concentration of 2.5%. The fluorescence was measured with a 380 ± 20 nm filter, and the emission was measured with a 475 ± 20 nm filter. Sediment fluorescence interfered with the ANSA-TTR assay even after the metabolic incubation extraction. Therefore, the highest sediment concentration which produced a signal below the MeOH control values plus three standard deviations was chosen as the highest concentration to be tested in the ANSA-TTR assay.

ANSA displacement assay. A recently published non-radioactive 96-well plate method was used for the measurement of TTR competitive binding (Montaño *et al.* 2012). The ANSA fluorophore increases its fluorescent yield when bound to transport proteins like TTR whereas the signal is reduced when the ANSA is displaced by competition with thyroid hormones and some halogenated phenolic contaminants. Sediment fractions, metabolically activated extracts or controls diluted in MeOH were mixed with 0.6 µM ANSA and 0.5 µM TTR in 100 µL PB at pH 7.5 in a round-bottom 96-well plate (Greiner Bio-One). All tests were performed in triplicate and at 2.5% final MeOH concentration. After 2 h of incubation at 4°C, the plate was gently shaken for 10 s and measured in a Biotech Synergy 2 plate reader (Winooski, VT). ANSA fluorescence was excited using a 380 ± 20 nm filter, and the emission was measured with a 475 ± 20 nm filter. Negative controls without TTR, ANSA-TTR positive controls, and 0, 100, and 600 nM T_4 displacement controls were included on every plate to calibrate the different exposure plates.

Data analysis and statistics. Competitive binding was expressed as T_4 equivalents (T_4-Eq), which was calculated with responses between 20% and 70% competitive binding by interpolation into a T_4 standard curve. The highest stock concentration that is tested for all sediment extract fractions is the concentration that can be tested without interference from auto-fluorescence in the ANSA-TTR assay. In combination with the limit of detection of compounds in the ANSA-TTR assay this determines the limit of detection per g of sediment equivalent (SEQ). Therefore the limit of detection differs between the samples. The potency of metabolically activated fractions was expressed based on the lowest fraction concentration capable to produce a significant competitive binding compared with the S9 blank. This sediment concentration was equated to the T_4 concentration which produced a signal at the limit of detection to provide a conservative estimate of the fraction potency expressed as nmol T_4-Eq/ g SEQ. Comparisons with ANSA-TTR (for S9 blank, recovery and positive controls) and comparison with S9 blank (for metabolically

activated fractions) were assessed with ANOVA followed by Dunet's *post hoc* comparison (SPSS Inc.). Graphs were made in SigmaPlot® (SPSS).

Results and Discussion

Background fluorescence of sediment extracts

The highest concentrations of sediment extracts that did not interfere with the ANSA-TTR fluorescence measurement at 380 nm varied greatly between samples (Figure 2), from: 0.5 (Alz 1) to 500 (Our 3 and Woltz 1) mg SEQ/mL DMSO stock (Table 1). For every location the crude polar extracts showed higher background fluorescence than the nonpolar extracts (Table 1, solvent fractionation), probably due to residual humic substances in the extract and/or total hydrocarbon content, particularly substituted PAHs (Law 1981; Patra 2003).

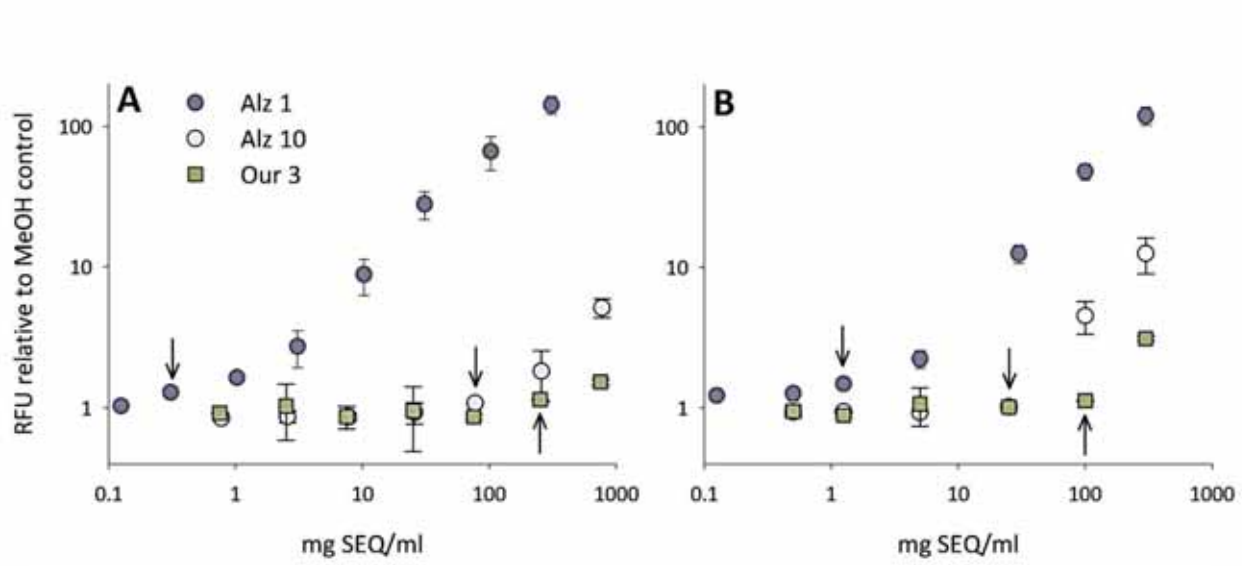


Figure 2 Background fluorescence in cleaned nonpolar (A) and polar (B) sediment extracts prepared with solvent partition. Data represent the relative fluorescence units (RFU) (380nm excitation and 475nm emission) relative to the control solvent (MeOH) (set to one) ± standard deviation from two independent experiments performed in triplicate. The arrow indicates the maximum concentration tested in the ANSA-TTR-assay (also see Table 1).

The GPC-cleaned HPLC-NP fractions with the highest fluorescent signal were F2-3 and F6-7 which corresponded to fractions containing PAHs (F2), nitro substituted PAHs (NO_2-PAHs, F3), quinones (F6) and nitrogen heterocycles (N-PAHs, F7) (Fernandez and Bayona 1992). Fractions with the lowest fluorescence signal contain long chain alkyl benzenes and PCBs (F1), Keto-PAHs and quinones (F4-F5), OH-PAHs and tertiary N-PAHs (F8) (Fernandez and Bayona 1992).

The fluorescence of nonpolar sediment fractions is suggested to indicate the degree of contamination with PAHs (Patra 2003). Our results are in accordance with this hypothesis, as a decreasing pollution gradient has been reported from upstream to downstream Alzette samples (from Alz 1 to Alz 17, Table 1). Our 3 and Woltz 1 sediments are from non-industrial areas and have accordingly low PAH-levels (Zheng *et al.* 2011). The extracts from solvent and HPLC-NP fractionations showed similar profile of responses (Table 1).

Table 1

Highest concentration of sediment extract equivalent per mL of DMSO stock (mg SEQ/mL) with a fluorescence signal below the solvent control in the ANSA-TTR assay

	Alz 1	Alz 10	Alz 15	Alz 17	Our 3	Woltz 1
Solvent fractionation						
Clean nonpolar	0.5	100	100	100	250	250
Polar	1.2	25	25	25	100	100
GPC-NPC fractionation						
F1	25	100	100	100	100	500
F2	0.5	0.5	5.0	5.0	5.0	25
F3	1.2	100	5.0	100	500	1.2
F4	1.2	100	100	100	500	500
F5	1.2	100	100	100	500	100
F6	0.5	5.0	5.0	100	100	25
F7	0.5	5.0	25	100	100	100
F8	5.0	100	100	100	500	5.0

TTR binding by sediment extract fractions.

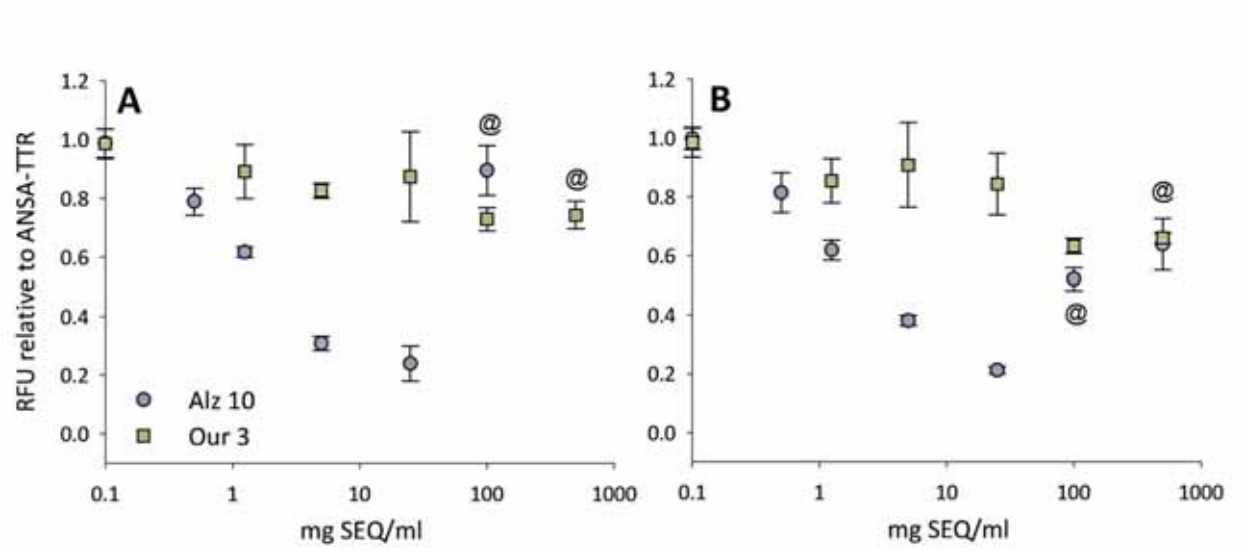


Figure 3 TTR competitive dose responses from sediment solvent fractions. Non auto-fluorescent dilutions of nonpolar (A) and polar (B) solvent fractions in the ANSA-TTR assay as relative fluorescence units (RFU) relative to the solvent control with ANSA and TTR set at one. Responses for with background fluorescence (above given in Table 1) are indicated with a @. The data represent the avg ± std from two independent experiments, measured in triplicate. The zero value corresponds to maximum replacement by 600 nM T_4 in the MeOH control.

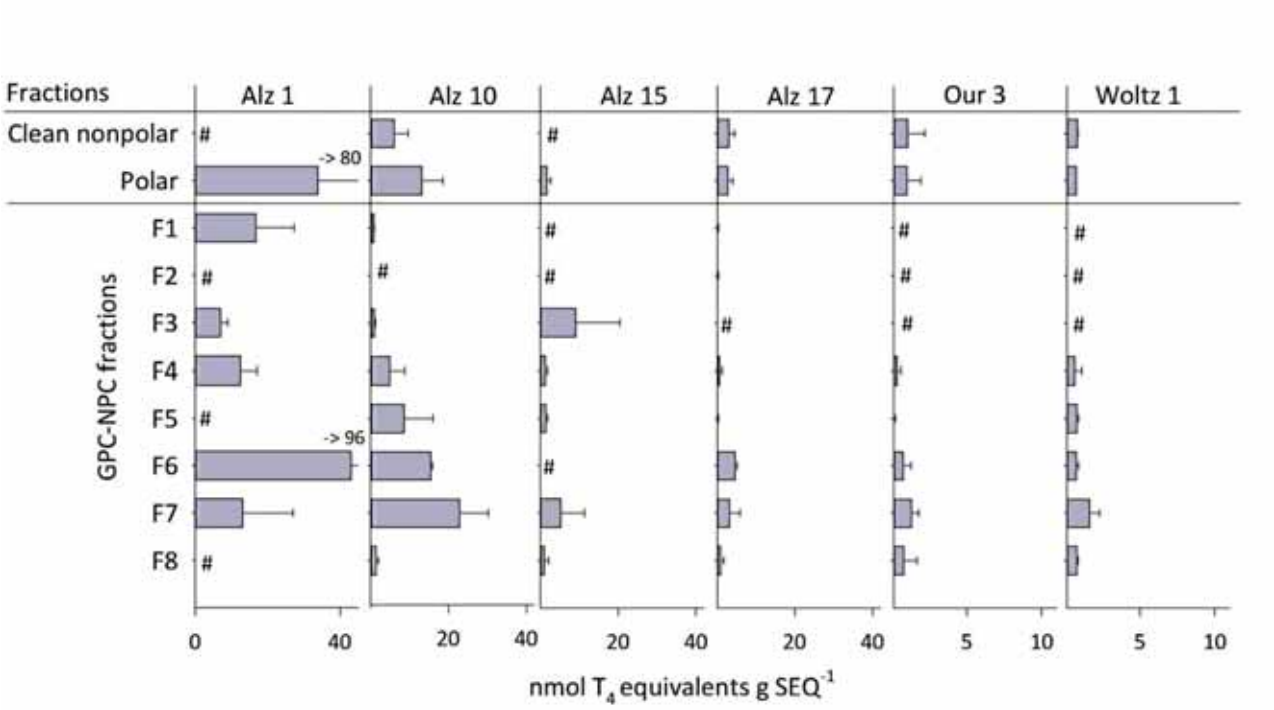


Figure 4 TTR competitive binding of sediment fractions. Fractions from solvent fractionation (nonpolar and polar) and from GPC-NPC fractionation (F1 to F8) were measured in the ANSA-TTR displacement assay at non-autofluorescent concentrations. Data represent the TTR binding potency expressed as nmol T_4 per gram of sediment equivalent (SEQ) ± std from two independent experiments measured in triplicate. # represent responses below limit of detection (<LOD). → response beyond figure-axis value. Note the differences in scale between the Alz- and the non-industrial locations.

To determine the TTR-binding potency of compounds present in the sediment extract fractions before metabolic activation, at least six non-fluorescent dilutions of each sediment fraction were tested in the ANSA-TTR assay. Sand control extracts did not displace ANSA-TTR. The limit of detection (LOD) for T_4 in the ANSA-TTR was 60 nM and the maximal SEQ-concentration that could be tested for each sample differed based on the back-ground fluorescence (Table 1), hence the LOD for the sediment fractions ranged from 0.12 to 120 nmol T_4-Eq/g SEQ (Table 2).

Extract fractions that did displace ANSA-TTR florescence, did so in a dose dependent manner, and the extracts that were tested in a concentration above the maximum concentration given in Table 1 clearly produced an auto-fluorescence increase (Figure 3). Figure 4 presents the TTR-binding potency of the sediment extract fractions, which is up to 80 nmol T_4-Eq/g SEQ without bioactivation. The gradient for decreasing pollution from Alz 1 to Alz 17 clearly is reflected, as are the low toxic potencies for the non-industrial areas Our 3 and Woltz 1 that barely gave a response above the LOD. For the more polluted Alz 1 and Alz 10 sediments, the nonpolar extracts clearly gave a much lower response than the polar extracts (Figure 4).

Although more detailed, the same results were visible for the GPC-cleaned HPLC-NP fractions, which were also from <LOD up to 96 nmol T_4-Eq/g SEQ (Figure 4). TTR competitive binding was primarily observed for F4 to F7 whereas less polar fractions (F1 to F3) had none or low activities (Figure 4). It is important to note that fractions F2 and F3 were tested at lower SEQ concentrations due to their auto fluorescence (Table 1) and this might be the cause of observed levels below LOD compared to other fractions. The observed TTR binding potency profile of GPC-NP fractions was in agreement with published results from testing the TTR-binding potency in a radio-active method with fractions prepared according to an identical separation method (Weiss *et al.* 2009) and with a similar but multi-separation procedure (Lübcke-von Varel *et al.* 2011). In both cases the majority of TTR binding activity was recovered in the polar fractions up to 4 nmol T_4-Eq/g SEQ (Lübcke-von Varel *et al.* 2011). The active compounds present in the polar sediment fractions should be further studied to determine whether they are hazardous. They might be natural compounds of plant origin that will be easily metabolized and excreted and thus never pose a risk to sediment-associated aquatic organisms and their consumers including humans. EDA is currently being applied to identify the compounds causing TTR competitive binding in polar fractions (Weiss *et al.* Unpublished).

TTR binding by metabolically activated sediment fractions

The S9 blank incubation produced a small but not significant competitive binding to TTR. Figure 5 shows that the recovery and response of the spiked 4-OH CB 79 was good, as was the ANSA-TTR displacement response of the bioactivated extracts from the S9-mix spiked with the positive controls CB77 and BDE47 in agreement with recently published results (Montaño *et al.* 2012).

TTR binding potency of solvent partitioned polar fractions and all GPC-NP fractions except F1 were in general not modified or lower after metabolic activation. Residual fluorescence was observed in fractions F2, F3, F6 and F7 even after metabolic incubation and low fat extraction; therefore, dilutions below 1 g SEQ/mL were used for ANSA-TTR analysis. Metabolic activation of PAHs present in these fractions, if any, might have not been detected.

Table 2 shows the LODs and the potency of the solvent partitioned nonpolar and the HPLC-NP F1 fractions before and after biotransformation. The assay LODs after biotransformation were higher mainly because of required additional dilution of the S9-extracts (Montaño *et al.* 2012) and because of residual fluorescence (Table 2).

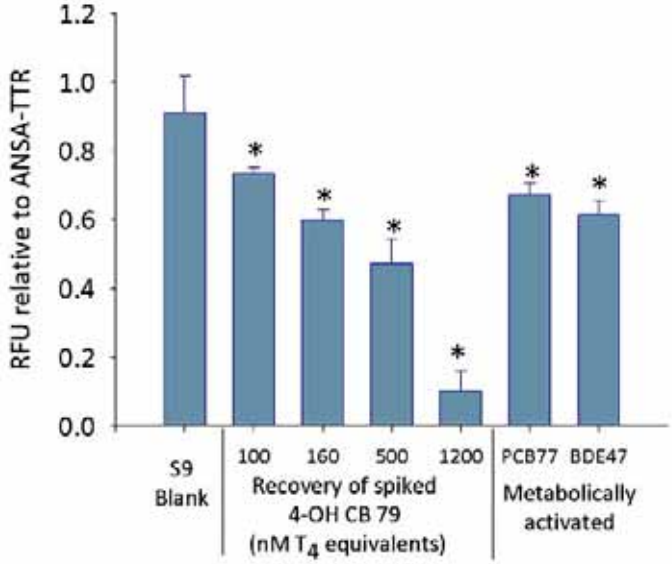


Figure 5 ANSA displacement from TTR by metabolic incubation controls. DMSO-spiked S9 blank,, spiked 4-OH CB 79, and metabolically activated CB 77 and BDE 47 were incubated with rat S9 liver fraction from which the metabolites were extracted, cleaned and transferred into MeOH for measurement in the ANSA displacement assay at a final concentration of 0.5 mg-Eq protein/mL. Data represents the relative fluorescence units (RFU) std from at least three independent experiments with duplicate S9-incubations, measured in triplicate. Maximal fluorescence without inhibitor is set at 1, maximal displacement by 600 nM T_4 is set at 0. * is significantly different from control (P<0.05).

Table 2

Limit of detection (LOD) and TTR binding potency before and after metabolic activation of solvent partitioned nonpolar and HPLC-NP F1 fractions of sediments expressed in nmol T_4-Eq/g SEQ.

	Solvent partitioned nonpolar				HPLC-NP F1			
	LOD [a]		Potency [b]		LOD [a]		Potency [b]	
	Before	After	Before	After	Before	After	Before	After
Alz 1	120	60	<LOD	60	2.4	12	17	120
Alz 10	0.60	60	6.1	<LOD	0.60	12	0.89	60
Alz 15	0.60	6.0	<LOD	60	0.60	12	<LOD	12
Alz 17	0.60	1.8	3.1	6.0	0.60	12	0.60	240
Our 3	0.24	1.8	1.0	5.9	0.60	12	<LOD	240
Woltz 1	0.24	1.8	0.84	5.9	0.12	12	<LOD	12

[a] LODs were calculated with the ANSA-TTR limit of detection (see materials and methods) and the maximal sediment concentration in Table 1.
[b] The potencies are expressed as nmol T_4 Eq/mg SEQ.

Figure 6 provides two examples of the dose related response of incubation extracts from separate fraction dilutions. Increased displacement of ANSA-TTR fluorescent binding was observed for all cleaned nonpolar and F1 fractions of sediments after metabolic activation (Table 2). An exception was the metabolically activated nonpolar fraction from Alz 10 for which the tested bio activated sample had a response below the LOD. Most likely due to the use of a 25 times diluted extract after metabolic activation compared to the original nonpolar solvent partitioned extract to avoid background fluorescence. Higher fluorescence of nonpolar extracts after metabolic activation might also indicate the formation of more fluorescent

metabolites. This will not be one of the classical POPs such as PCBs and PBDEs as the increase in fluorescence did not occur in the HPLC-NP fractionated F1 fraction. It would be interesting to apply EDA to identify which fluorescent compounds are produced after biotransformation.

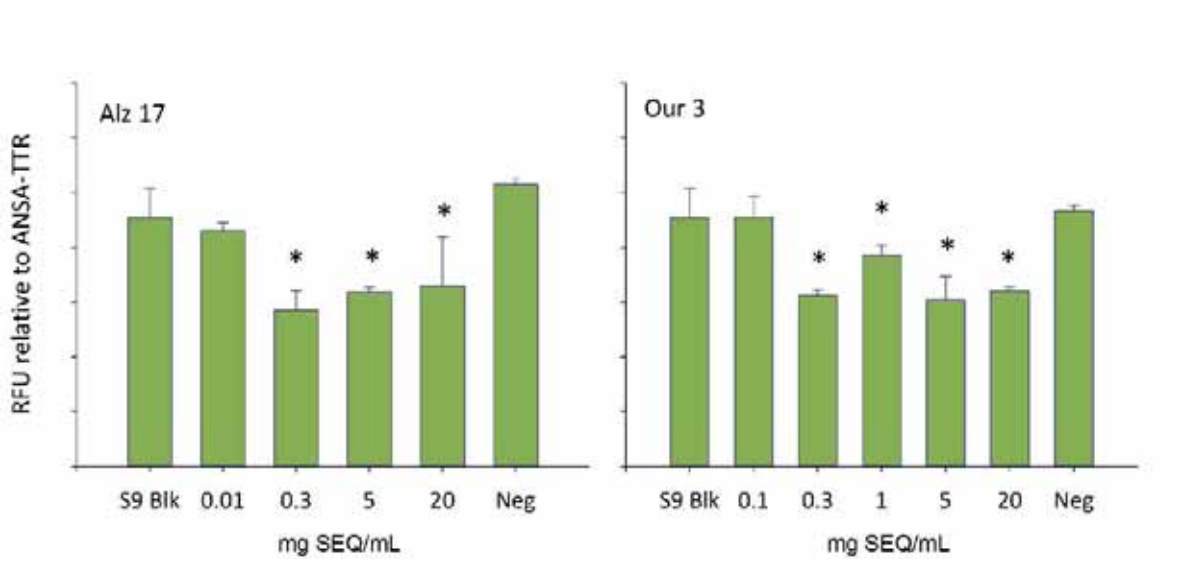


Figure 6 Examples of displacement of ANSA-TTR fluorescence by metabolically activated HPLC-NP F1 sediment extracts. Various dilutions of cleaned nonpolar and HPLC-NP F1 fractions were incubated with S9 microsomes. Blank corresponds to the S9 blank whereas Neg corresponds to the incubation of the highest concentrations of the sediment fraction where incubation was stopped at time = 0. The metabolite extracts were diluted in MeOH and measured in the ANSA-TTR displacement assay. Data represents an example of the relative fluorescence units (RFU) relative to the positive control with ANSA and TTR (equal to one) ± standard deviation from two separate experiments, with duplicated incubation measured in triplicate from the HPLC-NP F1 fractions of Alz 17 and Our 3. * is significantly different from S9 blank (P<0.05)

Applicability of biotransformation and in vitro testing of sediment extracts

The TTR competitive binding potencies of solvent partitioned nonpolar extracts and HPLC-NP F1 fractions from all locations were 10 to 100 times higher after metabolic activation than the potency of the same fractions without activation. This thyroid hormone disrupting potency of POPs accumulated in the sediment would have been unnoticed without metabolic activation, resulting in an underestimation of the toxic hazard for animals exposed to these compounds. For several species of aquatic organisms including fish and mammals direct or indirect (via the food chain) accumulation of sediment-associated POPs has been shown. They are able to metabolize them into OH- POPs right after absorption, after POP mobilization from lipid depots during migration (van Ginneken *et al.* 2009), or during larval development before becoming free feeding (Foekema *et al.* 2012). Once produced

OH- metabolites tend to be retained in the body bound to TTR where they are relatively protected from further metabolism (Montaño *et al.* 2013) and even can cross the placenta and blood brain barrier (Meerts *et al.* 2002; Morse *et al.* 1993). Because of this, and the many studies relating OH-metabolites to several of the observed POP effects (Dingemans *et al.* 2011; Fonnum *et al.* 2006), it is vital to include the bio activation of POPs in sediment hazard identification and quantification.

The approaches presented in our study allow a semi-quantitative detection of potential hormone disrupting POPs that until now were unnoticed. Further developments could make the approach quantitative. One of the limitations of the currently applied *in vitro* metabolic activation based on S9 microsomes (Montaño *et al.* 2012) or recombinant CYP enzymes (Erratico *et al.* 2011) is the low metabolic activation efficiencies of about 5% (Murk *et al.* 1994). Although the presently applied low-fat extraction method allows further concentration of the extract than before (Montaño *et al.* 2012) and allowing good classification of the THD potency of sediments, it is not yet possible to produce a full ANSA-TTR dose-response curve due to the low *in vitro* OH-metabolite yield. Technical improvements could include other methods for biotransformation possibly in combination with specific extraction of TTR-binding compounds as happens *in vivo*.

We currently applied 2 methods for separation of the nonpolar POPs, solvent based partitioning and HPLC-NP based fractionation. The first method is fast, cheap and easy but yields more crude nonpolar extracts than the second method that yields a more specific nonpolar fraction and also is a good basis for further identification of the active compounds. A new fractionation method combining the advantages of both methods could reduce the problem of background fluorescence, therefore increasing the method sensitivity and dynamic range. However, the currently presented methods allow a fast screening of sediments for the risk of potential THDCs that are not yet detected with the *in vitro* and chemical tests currently applied.

Our results demonstrate that metabolic activation of nonpolar POPs present in sediments should be considered in a more realistic risk assessment framework of sediments.

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CHAPTER 7

General discussion

Exposure to chemical mixtures occurs simultaneously, sequentially and chronically (Mumtaz 2010). Examples where there is a need of complex mixture assessment are food and drinking water compliance with regulations and dredged soils safety. Policy makers and governmental agencies are therefore faced with the need to assess the risk of these mixed exposure and the potential effects on human and wildlife wellbeing and health. As policy makers and risk assessors take decisions upon risk characterization results of complex mixtures, the methods applied shall be therefore **relevant** and **reliable**.

This thesis aimed to better understand and further improve the relevance and reliability of *in vitro* bioassays for a bioassay-based risk characterisation of complex mixtures, with special focus on POPs in sediments. In a meta-analysis of the scientific literature on the presence of bio-activated POPs and other hydroxylated phenolic compounds (HPCs) it was shown that these HPCs are present in blood of humans and wildlife at concentrations equal to or higher than their parent concentrations and these concentrations are similar to those inducing *in vivo* and *in vitro* toxicological effects (Chapter 2). The importance of metabolic activation on the relevance of *in vitro* bioassays for complex mixture testing was demonstrated through the strong increase of the thyroid hormone disrupting (THD) potency of persistent organic pollutants (POPs) and lipophilic sediment extracts after bio-activation (Chapter 3). To be able to solve the persistent problem of co-extractants from microsomal incubations interfering binding of thyroxine (T4) with the plasma carrier protein transthyretin (TTR) in the competitive T4-TTR binding assays, first, saturated and non-saturated free fatty acids were identified as being the interfering factors described and discussed in the literature. Secondly a method was developed to remove them with a dedicated sample-clean-up. Finally, it was possible to quantify the potency of metabolized POPs to competitively bind TTR in a newly developed non-radioactive microtiter ANSA-TTR bioassay (Chapter 3).

Two aspects to improve the reliability of *in vitro* bioassays for complex mixtures of toxic compounds were studied: 1) the occurrence of supramaximal (SPMX) effects that could result in overestimation of the toxic potency (Chapter 4a); and 2) the possible consequences of overconcentration of sample extracts during solvent change that could result in underestimation of the toxic potency (Chapter 5). The result of a meta-analysis of SPMX effects in functional *in vitro* estrogenicity assays revealed that the likelihood of the occurrence of a SPMX effect was strongly correlated to the use of specific reporter-gene assays (Chapter 4a). The relevance of this finding was discussed as well as an approach to reliably use these assays to quantify estrogenic potencies. To study a possible mechanism behind the SPMX mechanism, a 96-well plate cellular efflux pump inhibition assay (CEPIA) was adapted for the dioxin-like DR.Luc cells. Although the cells were sensitive for p-glycoprotein (P-gp) efflux pump inhibitors, no relationship was found with SPMX effects in this cell line (Chapter 4b). The study of the influence of the initial sediment extract stock concentration revealed that the use of concentrated stocks, above 20 g sediment equivalents (SEQ)/mL DMSO resulted in an underestimation of the obtained bioassay TCDD equivalents (Bio-TEQs). Overload of clean-up columns impaired proper quantitation of the toxic potency of sediments due to residual contaminants and sonication assisted dissolution reduced the results variability in the analyses (Chapter 5).

The approaches, methods and concepts developed in this PhD contribute to a more realistic and reliable in vitro bioassay-based risk characterisation of nonpolar sediment extracts (Chapter 6).

The importance of being relevant

When important or costly decisions have to be based on certain testing methods, the method relevance is one of the two validation components that are relevant according to the international harmonized criteria (OECD 2005). In this thesis the focus for the method's relevance was on the often not considered bio-activation of the lipophilic parent compounds which has been recognised to be crucial for the potential toxicological effect of pollutants (Coecke *et al.* 2006; Murk *et al.* 2013; NRC 2007).

Relevance of POP bio-activation: evidence from human and wild life exposure

A lot of evidence is available that halogenated phenolic compounds (HPCs), including naturally, commercially and metabolically produced OH-PCBs, OH-PBDEs, pentachlorophenol (PCP), and tribromo-phenols (tri-BPhs), are highly present in human and wildlife plasma (Chapter 2, Bergman *et al.* 1994; Letcher *et al.* 2010; Montaño *et al.* 2013a; Sandau *et al.* 2000) and that they are responsible for at least an important part of the endocrine disrupting potential of POPs (Dingemans *et al.* 2011; Fonnum *et al.* 2006). However, metabolites are rarely reported or considered in risk assessment together with the parent compounds whereas they are remarkably similar to thyroid hormones (TH) and therefore capable to interact and interfere at different levels with the TH system (Murk *et al.* 2013).The cumulative concentration of HPCs in plasma ranged from 0.1 to 100 nM in humans, up to 240 nM in birds, 454 nM in fish, 800 nM in cetacean and 7650 nM in cetaceans and other mammals. These HPC levels are at effect level in in vitro or in vivo bioassays, but in addition a quality of toxicological concern is the fact that these compounds can be actively transported to the fetus and brain because they competitively bind to T4 plasma transport proteins (Meerts *et al.* 2002; Morse *et al.* 1995). When bound in these proteins the HPCs are relatively protected against further metabolism. The explains why the ratios of metabolites to putative parent compounds were higher for fetal tissues such as eggs and umbilical cord, which is of great concern given the sensitivity for developmental toxicity of these early life stages. Binding to the plasma transport proteins TTR and thyroid hormone binding globulin (TBG) is not the only way OH-POP and other HPCs can interfere with the thyroid hormone system. The HPCs can also interfere with enzymes in charge of TH deiodination or conjugations and with the thyroid hormone receptor (TR) activity (Murk *et al.* 2013), and with other endocrine targets such as steroid glands and induce alteration of neuronal development (Dingemans *et al.* 2010; Freitas *et al.* 2011; Song *et al.* 2008).

Coastal populations, including birds, typically had higher concentrations of brominated HPCs especially of naturally produced *ortho* OH-PBDEs (Chapter 2, Montaño *et al.* 2013a). This observation confirmed the suggestion that that OH-BDEs found in marine organisms and humans originate also from naturally occurring OH-PBDEs and biotransformation of naturally occurring MeO-PBDEs (Liu *et al.* 2012; Wan *et al.* 2009; Wiseman *et al.* 2011). Inland populations were characterized by higher proportions of *meta*- and *para*- OH-PBDE

congeners which confirms the existing evidence of metabolism of PBDEs as their source (Marsh *et al.* 2006; Qiu *et al.* 2007).

The HPC blood burdens were expressed in molar units to allow comparison with relevant *in vitro* and *in vivo* effect concentrations which are within the nM range or even below the reported HPC burdens in blood (Chapter 2, Montaño *et al.* 2013a). Given the revealed toxicological relevance of HPCs, it becomes evident that POP bio-activation is important for bioassay-based THD risk characterization of POPs.

Relevance of POP bio-activation: evidence from in vitro bioassay testing

In order to be able to *in vitro* test the potential hazard of POPs after in vivo metabolisation, an approach had to be developed to get rid of co-extractants from the microsomal incubation that interfere with in vitro testing of disruption of thyroid hormone binding to plasma carrier proteins. First a thorough chemical analysis was performed to identify and quantify the saturated and not saturated fatty acids and with cholesterol which were suggested to be the most problematic co-extractants (Chapter 3, Montaño *et al.* 2012a). Next, it was confirmed that the nM concentrations of these co-extractants in the microsomal extract were indeed the major cause of interference in the TTR competitive binding assay was. To solve this problem, a new clean-up method was developed which strongly reduced the residual concentration of free fatty acids by including fat flocculation, liquid-liquid extraction, and a lipid removal mini-column. With this method a reduction the co-extractant concentrations were reduced up to 20 times compared with traditional OH-POP extraction methods (Hamers *et al.* 2008; Hovander *et al.* 2000; Murk *et al.* 1994). At the same time the complexity of the clean-up procedure was reduced compared to published alternatives (Simon *et al.* 2010; Simon *et al.* 2011).

The extract thus obtained was used in a newly adapted and downscaled non-radioactive ANSA-TTR competitive fluorescence displacement assay to quantify the thyroxin (T_4) equivalents of POP OH-metabolites present in microsomal extracts. This method avoids the direct use of T_4 when testing extracts, as T4 has been observed to interact with the fatty acids as well (Simon *et al.* 2010). Apart from the benefits of using a non-radioactive method; the downscaled ANSA-TTR method is fast (ca. 3 hr instead of overnight) and suitable for high throughput screening (Chapter 3, Montaño *et al.* 2012a).

Under these conditions, extracts from metabolically activated POPs displaced ANSA-TTR fluorescence up to 50%. This allows quantification of the capacity of POPs in lipophilic extracts of sediments and other POP mixtures to produce OH-metabolites. This means an important step forward in the realistic hazard characterisation of such extracts, as testing these extracts without biotransformation would result in false negatives. Negatives because hormone-like hydroxylated compounds will not be present in nonpolar fractions, false because it is known that several POPs in these extracts such as PCBs and PBDEs are in vivo metabolised into hormone-like hydroxylated compounds. . To be able to obtain full TTR competitive dose-response curves, the efficiency of the biotransformation should be increased from the current 2-5% to ideally up to 10%. (see discussion below).

Including bio-activation in *in vitro* bioassays for risk characterization

Any *in vitro* bioassay intended to determine the potential toxic potency of metabolically liable compounds or matrices suspected of containing them, shall be evaluated in regard to its metabolic capability when assessing its relevance. The failure to involve metabolic activation will severely affect the method's false negative rate. (OECD 2005)

The importance of POP biotransformation into THD OH-POP metabolites has been confirmed (Montaño *et al.* 2012a; Montaño *et al.* b, subm.). But POPs are not the only ones and the list is non-exhaustive: pharmaceuticals such as acetaminophen, carbamazepine and lidocaine; allergens, pesticides such as methoxychlor and vinclozolin and environmental contaminants such as PAHs are bioactivated into potent metabolites (Coecke *et al.* 2006; Jacobs *et al.* 2008). In addition POP are present in a variety of complex matrices which would require metabolic activation for a proper risk characterization; including: food, feed, cosmetics, biological fluids and tissues, air deposition, soils etc.

The most important methods for the application metabolizing systems in *in vitro* bioassays are: *in silico*, *in vitro* with subcellular systems, *in vitro* with self-contained systems, and *ex vivo* with perfused tissues (Jacobs *et al.* 2008). However, it is not an easy task as the paucity of published studies involving the use of exogenous metabolising systems for ED is problematic, considering its relevance, and reports up to date are still very scarce.

Two extensive documents prepared for the ECVAM and the OECD reviewed the knowledge and the developments of metabolizing systems for *in vitro* bioassays (Coecke *et al.* 2006; Jacobs *et al.* 2008). Therefore, here only important aspects of the inclusion of metabolizing systems resulting from this thesis will be discussed.

How to increase the metabolic yield

The difficulties experienced to measure OH-POP metabolites from biological matrices have been attributed primarily to the low metabolic rates obtained *in vivo* and *in vitro* after exposure to POPs, something also observed in this thesis (Chapters 2 and 3, Montaño *et al.* 2012a; Montaño *et al.* b, submitted). Reported *in vitro* metabolic yield of POPs using mixed type induced microsomes has been 4-6% for CB77 (Montaño *et al.* 2012a; Murk *et al.* 1994), 0.3-2% for BDE47, and ranged from 0.2-1.2% for BDE99 (Erratico *et al.* 2011; 2012; Hamers *et al.* 2008; Montaño *et al.* 2012a). This metabolic yield is barely enough to achieve effect concentrations after extraction, concentration in DMSO and re-dilution of metabolites in media (Montaño *et al.* 2012a). Increasing the time of incubation is no solution as the maximum is normally achieved during the first 30 minutes (Erratico *et al.* 2011; 2012; Harju *et al.* 2007). An increase in the protein content would also increase considerably the potentially disturbing co-extractants (Montaño *et al.* 2012a), while the use of higher parent compound concentrations may cause substrate inhibition and increase the risk of toxicity in the subsequent *in vitro* assay. Also methods have been published in which the NADPH is being recycled during the metabolic reactions. This could perhaps increase the amount of metabolites formed without an increase in co-extractant residues.

An alternative method is the use of readily available specific recombinant CYP450 enzymes. However, the metabolic yields when using enzyme concentrations from 10-30 pmol/mL (aprox. 0.25 mg protein/mL) for BDE47 and BDE99 were 0.06-0.1%, which is considerably lower when compared with microsomes (Erratico *et al.* 2011; 2012, unpublished results). As the preparations have low protein content, an increase in enzyme concentration could render a higher amount of metabolites in the incubation extracts. In addition, recombinant CZP450, especially obtained in *E.coli*, contains a very low amount of saturated fatty acids, mainly arachidonic acid, in the enzymatic preparation (unpublished results). This extractant derive from neutral phospholipids such as phosphatidylserine is one of the most potent among the free fatty acids to bind TTR (Montaño *et al.* 2012a).

An important aspect to consider when applying activated microsomal and/or cystosolic fraction as a metabolizing system is the potential metabolism of the co-extractants via their mixed-function oxidases (MFOs). One of the major co-extractants from microsomal incubations is cholesterol (Montaño *et al.* 2012a) which could be metabolized into pregnolenone, which can further be converted into steroid hormones. It is very important to consider this potential interference when including this metabolizing system into ED *in vitro* bioassays. Once again, recombinants enzymes can become useful as it is possible to exclude the steroidogenic activity from the incubation.

In summary recombinant CYP450 enzymes could provide solutions to some of the problems related to co-extractants and be useful for specific applications, but their lower metabolic yield will remain a drawback on their application as metabolizing systems for *in vitro* bioassays. In addition, for high throughput purposes this alternative might be impractical as several different enzymes will be required to simulate the full potential biological activity.

When testing the activity of microsomal extracts in cellular functional assays, still about 95% of the parent compounds are present, which could induce cytotoxicity before the metabolites are dosed at effect concentrations (Schriks *et al.* 2006). To be able to reduce the presence of parent compounds, the phenolic fraction from the microsomal incubation could be isolated (Hovander *et al.* 2000). The neutral compounds are extracted first under acidic conditions, and then the pH is elevated to neutralize the phenolic substances for extraction. Besides the considerable reduction of parent compound concentration this technique also reduces cholesterol from the final extracts as a big proportion is removed in the neutral extraction step (Montaño *et al.* 2012a). However, the extreme basic conditions of the phenolic extraction caused the hydrolysis of phospholipids and triglycerides present in the microsomal incubation, and thereby doubled the amounts of free non-saturated and saturated fatty acids in the extract (Montaño *et al.* 2012a). The possible impact of these concentrations of fatty acids on the membrane stability and physiological functions of the *in vitro* bioassay cell model need to be studied before drawing conclusions based on the bioassay results.

Reliable methods for reliable results

The method reliability is the other component of method validation according to the international harmonized criteria (OECD 2005). As with chemical analyses, it is very important to be aware of the specificities of any in vitro assay is the first line of action to avoid wrong estimation of the quantified potency of complex mixtures and therefore to apply the method in a reliable way for the intended application. Anyone applying *in vitro* bioassays should be aware of recommendations provided by validation protocols and the studies analysing the assays most critical parameters, aware of phenomena such as the SPMX effect and their potential implications, and also be aware of the alternatives to cope with these issues to maintain or even improve the reliability of *in vitro* assays.

Reliability improvement of in vitro bioassays has learned lessons from comparable developments of chemical analyses that started decades ago. Great advances have been achieved with standardization, transferability and reproducibility of *in vitro* bioassays (Besselink *et al.* 2004; Hoogenboom *et al.* 2013; Hoogenboom *et al.* 2006; Houtman *et al.* 2006; Van der Burg *et al.* 2010) up to their inclusion in regulatory testing frameworks (EC 2006; OECD 1992; OECD 2012; OSPAR 1997). Two aspects that could influence the outcome of *in vitro* bioassay based quantification of toxic potencies, but that are not yet tackled in current protocols are: 1) the occurrence sometimes of responses higher than the theoretical maximum based on the positive control for the assay (a phenomenon referred to as supramaximal (SPMX) effect), and 2) the possible consequences of overconcentration of sample extracts during solvent change.

The potential causes of SPMX effect in in vitro bioassays

An analysis of the peer-reviewed literature resulted in twenty one compounds which were reported at least once to produce SPMX effect in functional *in vitro* estrogenicity assays. This group of SPMX inducers included a variety of chemical structures such as synthetic hormones, plasticizers, flavonoids and PAH metabolites (Chapter 4a, Montaño *et al.* 2010). Nevertheless, the end-points, culture conditions and assay characteristics varied greatly between assays.

The correlation of assay variables and the likelihood of producing a SPMX of all reports from diethylstilbestrol (DES), genistein (GEN) and bisphenol A (BPA) revealed that its occurrence could be related to a number of specific assay characteristics: 1) the type of serum used to supplement the exposure medium, 2) the end point used to quantify the estrogenic potency (endogenous or transfected). Indeed, a higher incidence of SPMX effect on reporter-gene assays was observed, and it was related to the plasmid construction characteristics, including 3) the type of transfection, 4) the number of response elements and 5) the promoter nature (Montaño *et al.* 2010). There were, however, no indications that solvent concentration in culture, exposure period, or cell model influenced the occurrence of an SPMX effect.

Weather SPMX effect is an assay based artefact or a real-life phenomenon with toxicological relevance, still is unknown. Interestingly the dose-response curve was biphasic, where the maximum of the first part is the same as the maximum of the positive control (estradiol). For the Dr-Luc assay it has been demonstrated that activation of

protein kinase C and inhibition of protein synthesis can synergistically enhance AhR-dependent gene expression with standards. The SPMX effect produced by genistein on an estrogen receptor (ER) reporter-gene assay (T47D.Luc, Legler *et al.* 1999) was ascribed to post-transcriptional stabilization of the firefly luciferase reporter enzyme, increasing the bioluminesence signal. Therefore, it was concluded that the SPMX effect is not a biologically relevant phenomenon (Sotoca *et al.* 2010).

Sediment extracts have been shown to induce SPMX effects in the DR-Luc assay before (Murk *et al.* 1996). However, none of the potent SPMX inducers genistein or quercetin in the T47D.Luc assay (Sotoca *et al.* 2010) induced or increased the signal of TCDD in the DR.Luc assay with hepatoma cells (H4IIE.Luc). This reporter gene cell line has a similar gene construction and identical reporter enzyme (Chapter 4b, Montaño *et al.* 2012b). These results contrast with the suggestion that the SPMX effect produced by genistein is due to post-transcriptional stabilization of the firefly luciferase reporter enzyme increasing bioluminisence signal (Sotoca *et al.* 2010), although it cannot be excluded that post-transcriptional regulation of firefly luciferase differs between the hepatoma cells and the breast cancer cells.

Another putative explanation for the occurrence of SPMX was tested, namely inhibition of the cellular efflux pumps as has been demonstrated for several compounds that also can be found in sediment extracts (Anselmo *et al.* 2012). Cellular efflux pump inhibition has been shown to increase the toxicity of substrates for those pumps by increasing their cellular concentrations. Under the DR.Luc assay conditions, however, there was no evidence that P-gp efflux pump inhibitors modified or potentiated the activity of TCDD. Also genistein nor quercetin, the two potent SPMX inducers on ER-mediated assays, influenced the luciferase induction by TCDD (Chapter 4b, Montaño *et al.* 2012b). It is important to know whether the SPMX mechanisms are relevant for the in vivo situation as well. However, for the potency quantification for environmental samples it seems to be most suitable to quantify the toxic potency in the lower part of the dose-response (Sotoca *et al.* 2008). This approach is supported by the fact that the SPMX dose-response curves often are biphasic with a maximum of the first part of the curve at the maximum induction level of the positive control.

Unless a full dose response curve is made from complex matrix extracts, it cannot be known whether SPMX effects occur. Single dose analysis is common practice in monitoring studies, and it is assumed that the dose-response curves of the sample and the standard are parallel and most important that the maximal responses are identical (Windal *et al.* 2005). When this is not true, which is common for complex matrices, the quantified potency is a function of the dose. To avoid this problem multiple dilutions of a sample have to be used to calculate a reliable Bio-TEQ concentration as has been shown for the DR.Luc assay (Windal *et al.* 2005).

In addition a normalization procedure for DR.Luc assays has been proposed for cleaned sediment extracts which gave SPMX effects (Baston and Denison 2011). The described method for potency quantification of samples inducing a SPMX signal result in a Bio-TEQ quantification closer to chemically obtained results (Baston and Denison 2011).

Influence of stock concentration in the potency quantification outcome

Sediment extracts from the Alzette and the Our rivers in Luxembourg, prepared with various initial stock concentrations were analysed in the DR.Luc assay. The concentration of the initial stock influenced the quantified potency and the use of concentrated stocks, above 20 g sediment (SEQ)/mL caused an underestimation of the obtained Bio-TEQs (Chapter 5, Montaño *et al.*, subm.a). These results, along with higher variability when excluding dissolution assisted sonication from the protocol supported the hypothesis that at higher stock concentrations, part of the active compounds are lost due to oversaturation of the DMSO solution. This is expected to occur via precipitation of a fraction of the original amount present in the extraction solvent. A high initial extract concentration can also overload clean-up columns and impair proper quantitation of the toxic potency of sediments. Analysis of samples at longer exposure periods was necessary for highly contaminated samples to reduce the impact of metabolic liable compounds such as PAHs, even when using the lowest initial stock concentration.

When testing contaminated samples at initial stock concentrations of 20 or 200 g SEQ/mL the extract toxicity would have been underestimated in a way that these locations might not be considered for intervention or banning, which would be the decision when they were tested at 2 g SEQ/mL. On the other hand, testing without sonication assisted dissolution or highly contaminated samples at shorter exposure times would cause an overestimation of the quantified potency. Underestimation of the toxic potencies (false negative response) is unwanted because of safety risks but unnecessary classification of sediments above the SQC (false positive result) may result in unrequired expenses for dredging, storage and remediation.

Suitable sample amounts for reliable results

Because the initial stock concentration has an important influence on the quantification of sediment extracts (Chapter 5, Montaño *et al.* subm.a), method validation should include analysis of the suitable range for the initial sample amount, instead of a single value.

The first step to limit the influence of sample overload is to choose an initial sample weight according to the final risk characterization goal. For example when the goal is to determine whether sediment toxicity exceeds the intervention value of 1 ng TEQ/g sediment, the initial stock concentration should ideally have 0.5 g sediment equivalent (SEQ)/ml of DMSO (Chapter 5, Montaño *et al.* subm.a). The minimal amount of sample to be reliably quantified for a sample with potency at or above the intended goal should be calculated.

Furthermore, already available knowledge about the pollution load of a site, sample organoleptic characteristics and the extraction behaviour can provide indications of the need of a lower initial stock concentration. The ability to compare results based on different sample loads depends on the provision of a validated range of initial sample amounts for which the quantified result will remain statistically the same. Otherwise, the results might not be comparable.

Application of the developed approach for risk characterization of sediments

The concepts developed in this thesis in order to better understand the challenges of *in vitro* bioassay application to complex mixtures, and the tools developed to solve these issues, were applied to sediment samples from more or less contaminated sites in Luxembourg (Chapter 6, Montaño *et al.* subm.b). Nonpolar sediment fractions were incubated with S9 rat microsomes, and the metabolites were extracted with the newly developed method that excludes most of the lipids to avoid interference in the applied non-radioactive 96-well plate TTR competitive binding assay. Metabolic activation increased the TTR binding potency of nonpolar fractions of POP-polluted sediments up to 100 times, resulting in potencies up to 240 nmol T_4 equivalents/g sediment equivalent (nmol T4-Eq/g SEQ).

The information about contamination levels in Luxembourgish Rivers is in general scarce. There have been various publications on metals and PCBs contamination in fish in the North of Luxembourg (Boscher *et al.* 2010; Dauberschmidt and Hoffmann 2001; Hugla *et al.* 1998). There are also data on metals, organic matter, estrogenic substances and antibiotics on specific basins available (Kurtenbach and Gallé 2008; Pailler *et al.* 2008; 2009). In addition, the Centre de Recherche Public – Gabriel Lippmann has surveyed for several years the populations of macrobenthos, phytobenthos, macrophytes and fish for the Luxembourgish government (data not published) with the purpose to report within the Water Framework Directive (EC 2000).

The profile of dioxin-like activity observed for the Our River was in close agreement with PCB analysis in fish (Montaño *et al.* subm.a). In addition, the Bio-TEQs from both the Alzette and the Our rivers were in agreement with the ecological status as determined by the global standardized biological index (IBGN), the pollution sensitivity index (IPS) and the river fish index (CRP-GL, unpublished results). Interesting examples of this correlation are the two tributaries from the Alzette River joining at the industrial area of Bettembourg, the Kälbaach and the Mierbech. Most Alzette tributaries showed considerably lower dioxin-like activity in comparison to the main stream, with the exception of these two; in agreement with a classification of “bad” ecological status.

The TTR binding potency from nonpolar sediment samples before and after bioactivation were also in agreement with the general contamination profile (Montaño *et al.* subm.b). An exception is the particularly higher TTR binding potency after metabolic activation of the Our River sample in comparison with the more dioxin-like active Alzette samples. However, this location at the Our River has shown particularly high PCB contamination in fish (Dauberschmidt and Hoffmann 2001; Hugla *et al.* 1998).

Concluding remarks

Finally, we are confronted with the question whether the lessons learned and the methods developed have really made the use of *in vitro* bioassays more relevant and reliable towards a realistic risk characterization of complex mixtures.

Complex mixtures are diverse in nature and composition; however, the concepts developed here have a wider application to other anthropogenic and environmental matrices as well as to other chemical products and contaminants. These concepts include the toxicological importance of bioactivation for contaminant risk characterization (Montaño *et al.* 2013a; Chapter 2), the relevance of including metabolising system in *in vitro* bioassay testing (Montaño *et al.* 2012a; Chapter 3), and the influence of sample preparation, bioassay method parameters and responses on the risk characterization outcome (Montaño *et al.* 2010; Montaño *et al.* subm. a; Chapters 4 and 5).

Although the presently applied low-fat extraction method allows higher concentration of the extracts than any method applied before (Montaño *et al.* 2012a; Chapter 3), and results in a good classification of the THD potency of sediments, the amount of metabolites produced without getting problems with co-extractants is still not enough to produce a full ANSA-TTR dose-response curve. The approach served to prove that contradictory *in vivo* and *in vitro* bioassay results of the POP contaminants present in the nonpolar fraction of sediments were derived from the absence of metabolic activation. This demonstrates that a more realistic *in vitro* sediment THD risk characterization should also include testing of bio-activated nonpolar sediment fractions.

Phenomenon such as the SPMX effect and the influence of initial extract concentration of samples will influence the results precision and accuracy (Montaño *et al.* subm.a; Chapter 5). These aspects in addition to already described important issues to control the bioassay reliability enable the application *in vitro* bioassays based Bio-TEQs to determine whether sediments comply with health and safety standards for humans and the environment.

An important aspect of this thesis has been the convergence of every topic on increasing our understanding of toxicological effects and especially of the POPs stored in sediments (Montaño *et al.* 2012a; Montaño *et al.* subm.a and b; Chapters 2, 5 and 6). As a result of these studies it is evident that the toxic potency of bioactivated POPs cannot be neglected when considering their toxicological effects and should be an integral part of future POP risk assessment initiatives to prevent serious underestimation of their hazard.

The research presented in this thesis contributes to a better understanding of the challenges that *in vitro* bioassay applications face for the risk characterization of complex mixtures. New tools to reduce the burden of these challenges have been developed. This increases the relevance and reliability of the *in vitro* methods when applied to complex mixtures and therefore renders a more realistic assessment.

Future perspectives

As has been discussed above the application of *in vitro* bioassays for hazard and risk characterisation of complex mixtures have good perspectives when a number of important aspects for further improvement of relevance and reliability of the assays and the relevance of OH-POPs toxicity will be taken care of.

The most important issues for further research and development are:

1. Get better insight in the HPC body burdens of the general human population and specific groups at risk including fetuses (umbilical cord blood) and young children. The scarce information at present indicates relatively high concentrations of OH-PCBs and OH-PBDEs that are of concern.
2. The relevance of and differences in transfer over the placenta and blood-brain barrier of OH-PCBs, OH-PBDEs and other HPCs should be further investigated.
3. Studies on HPC levels in non-arctic top predators and other important piscivorous and carnivorous species are warranted, considering that arctic top predators even hold HPC body burdens high enough to saturate their total TH transport protein capacity.
4. Considering the limitations of chemical analysis of HPCs, bioassays such as the ANSA assay developed in this thesis should be applied to blood samples as it might reveal the real exposure to and possibly greater toxicological relevance of OH-POPs.
5. For increased efficiency of analysis the nonradioactive ANSA-TTR 96-well plate method to quantify thyroid hormone disrupting potency could be coupled to on-line clean-up and fractionation columns.
6. The low fat metabolite extraction method developed could be adapted for application to other biological matrices such as blood. This would facilitate body burden monitoring mentioned as priority above.
7. To be able to judge the relevance of the SPMX responses for the *in vivo* situation and *in vitro* bioassay responses, further research should be devoted to unravel its mechanism including the relationship with the potency to block efflux pumps of several SPMX inducing compounds.
8. The influence of initial sample stock concentration should be evaluated for all currently used *in vitro* bioassays and matrices tested.
9. The initial sample quantity should be included in the standard operation protocols of *in vitro* assay methods that are applied for decision making.
10. Alternative approaches to increase metabolic yield should be developed if exogenous metabolizing systems are to be coupled to *in vitro* bioassays for risk characterization of complex mixtures.

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CHAPTER 8

Summary

This thesis aims to better understand and further improve the relevance and reliabilityof *in vitro* bioassaysfor a biobased risk characterisation of complex mixtures, with special focus on persistent organic pollutants (POPs) in sediments.

In **Chapter 1** the importance of complex mixture characterization in modern society is introduced. The methods available, their current advantages and their disadvantages for complex mixture testing are described. With the shift from policy oriented chemical testing towards the inclusion of *in vitro* bioanalysis, important challenges have to be overcome to ensure a relevant and reliable quantification of the toxic potency of complex mixtures. These challenges are explained in the introduction, including the status of development and validation of those aspects for reliable testing. One of the main advantages that in vitro bioanalysis has to offer is the possibility to quantify the toxic potency of compounds for which chemical analytical methods have not or hardly been developed, for example because standards do not yet exist. Hydroxylated metabolites of POPs are an example of a toxicologically relevant group of compounds that can exert endocrine disrupting effects, but they cannot yet be routinely analysed. A selection of yet unsolved issues are further studied and discussed in this thesis, as outlined in the “approach and structure of the thesis”.

In **Chapter 2** a meta-analysis is performed to study the occurrence and relevance of hydroxylated (OH) compounds in humans and wildlife. Reported body burdens of halogenated phenolic contaminants (HPCs), including OH-POP in different tissues from humans and wildlife species, are reviewed in relation to the concentration of their putative parent compounds to be able to reveal relevant exposure routes and sub-populations at risk. Highest OH-POP levels were found in blood plasma, and highly perfused and fetal tissues. Plasma concentrations of analysed known HPCs ranged from 0.1-100 nM in humans and up to 240, 454, 800 and 7650 nM for birds, fish, cetaceans and other mammals, respectively. Reported metabolite blood plasma levels also are compared with relevant toxicological threshold concentrations from toxicological studies, and appeared to fully fall within the i*n vitro* (0.05–10000 nM) and in vivo (3-940 nM) effect concentrations reported for OH-POPs. Given the sensitivity of early developmental stages, and information lacking about the general population, it is advisable to determine HPC background blood levels in children and fetal tissue .

Given the toxicological relevance of the OH-POPs, **Chapter 3** aims at providing solutions to the long standing problem of the *in vitro* production and analysis of OH-POP metabolite thyroid hormone disrupting (THD) potency via binding to plasma thyroid hormone binding proteins (THBPs). In sediments and for example seafood, the POPs occur as parent compounds that would only become THD after metabolisation (hydroxylation). Several methods have shown the competitive

thyroxine (T_4) T_4 displacement potency of pure metabolites. However, *in vitro* metabolization of, among others, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers(PBDEs) followed by in vitro quantification of their potency has encountered drawbacks related to the co-extraction of compounds disturbing the T_4-TTR competitive binding assay. The present study identifies and quantifies the major co-extractants, cholesterol and saturated and non-saturated fatty acids (SFA and NSFA), at levels above 20 µM (20 nmol per mg protein in the incubation mixture) following various extraction methods. A new method is presented to *in vitro* metabolise parent compounds into OH-metabolites followed by selective extraction of metabolites while four-fold reducing co-extraction of the disturbing compounds. In addition a microplate-format non-radioactive fluorescence displacement assay was developed to quantify the TTR binding potency of the metabolites formed. The effectiveness of the *in vitro* metabolism and extraction of the OH-metabolites of the model compounds CB 77 and BDE 47 was chemically quantified with a newly developed chromatographic method analyzing silylated derivatives of the OH-metabolites and co-extractants. Due to the mentioned improvements, it is now possible to make a dose-response curve up to 50% inhibition with OH-metabolites extracted from bioactivated CB 77 and BDE 47. Without taking the toxic potencies of bio-activated POPs into account with bioanalysis, the hazard and risk posed by POPs will be seriously underestimated.

The **chapters 4 and 5** are committed to tackle the issues of supramaximal (SPMX) responses and sample extract concentration which are crucial to reliably quantify of the toxic potencies of complex mixtures with *in vitro* bioassays.

A SPMX effect is the phenomenon that compounds induce a maximum response in an assay that is significantly higher than that of the positive control. As the positive control is used to quantify the toxic potency of a sample, this could result in over-estimation of its toxic potency. As this has been most elaborately reported for *in vitro* estrogenicity assays, a meta-analysis was performed of such assays, compounds and conditions in which the effect is observed (**Chapter 4a)**.For the 21 natural and industrial chemicals that could be identified as SPMX inducers, the culture and exposure conditions varied greatly among and between the assays. Relevant information on assay characteristics, however, sometimes lacked. Diethylstilbestrol (DES), genistein (GEN) and bisphenol A (BPA) were selected to build a database. The meta-analysis revealed that the occurrence of SPMX effects, could be related to a number of specific assay characteristics: 1) the type and concentration of the serum used to supplement the exposure medium; 2) the endpoint used to quantify the estrogenic potency (endogenous or transfected reporter gene), 3) the number of EREs (estrogen responsive elements) used before the reporter gene, and 4) the nature of the promoter's. There were no indications

that solvent concentration in culture, exposure period or cell model influenced the occurrence of SPMX. It is important to understand the mechanism behind this phenomenon because *in vitro* assays for estrogenicity are used extensively to characterize and quantify the estrogenic potency of compounds, mixtures and environmental extracts.

Several SPMX inducers also have been reported to block cellular efflux pumps *in vivo* and *in vitro* (Anselmo et al. 2012; Georgantzopoulou et al. 2013). Therefore it was hypothesized that efflux pump blockers present in environmental matrices could increase the internal concentration of bioassay agonists and thus cause the SPMX. In **Chapter 4b** this hypothesis was tested by adapting a 96-well plate cellular efflux pump inhibition assay (CEPIA) to the H4IIE rat hepatoma cell line used for the DR.Luc reporter gene assay for dioxin-like compounds. The influence of various environmentally relevant efflux pump inhibitors on the 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD) response was tested. Under the DR.Luc assay conditions there was no evidence that P-gp efflux pump inhibitors modified or potentiated the activity of TCDD. Neither genistein nor quercetin, two potent SPMX inducers on ER-mediated assays, induced any signal on the DR.Luc assay, nor influenced the luciferase induction by TCDD. Future work should be focused on testing the consequences of efflux pump inhibition with an AhR-agonist which is a P-gp substrate, as this could result in intracellular accumulation of this AhR-agonist.

It is standard practice to use a high single stock concentration of extracts to further dilute test concentrations from and perform the analysis. However, a high contaminant load in an extract may oversaturate the solubility of the extracted compounds in carrier solvents and overload the clean-up columns which may reduce the efficiency of polyaromatic hydrocarbons (PAHs) elimination from the extract. These problems may cause respectively under- or over-estimation of the quantified dioxin-like toxic potency. Therefore **Chapter 5** focuses on the effects of initial stock concentrations, including sonication assisted dissolution and exposure time, on the quantified dioxin-like potency of cleaned nonpolar sediment extracts. Indeed, more than 20 g sediment equivalents (SEQ)/mL DMSO) as initial stock concentrations resulted in underestimation of bio-TEQ levels in the sediments as observed for cleaned nonpolar sediment extracts from various locations in Luxembourg. An overload of extract on clean-up columns caused an over-estimation of the dioxin-like potency at 24 hours of exposure, probably due to limited removal of PAHs that can induce false positive responses in the in vitro assays. Sonication assisted dissolution of the stock before serial dilution strongly reduced the standard variation of the outcomes. Taking into account the aspects revealed in this study, in addition to already described important issues for quality control, the in vitro bioassays based bio-TEQs can be applied in a comprehensive

monitoring program to determine whether sediments comply with health and safety standards for humans and the environment. For the generally applied sediment quality criteria, advices are given about maximum initial stock concentrations to achieve reliable bioassay outcomes.

The methods and concepts developed for metabolic activation of compounds in non-polar sediment extracts and in *in vitro* analysis of the TTR-competitive binding are applied in **Chapter 6** to extracts from highly or less contaminated sediments collected in Luxembourg. Nonpolar fractions of sediment extracts were incubated with S9 rat microsomes, and the metabolites were extracted with a newly developed method that excludes most of the lipids to avoid interference in the non-radioactive 96-well plate transthyretin (TTR) competitive binding assay. Metabolic activation increased the TTR binding potency of nonpolar fractions of POP-polluted sediments up to 100 times, resulting in potencies up to 240 nmol T_4 equivalents/g sediment equivalent (nmol T_4-Eq/g SEQ). Without bioactivation, medium polar and polar fractions also contained potent TTR-binding compounds with potencies from 1.6 to 17 nmol T_4-Eq/g SEQ. This demonstrates that a more realistic *in vitro* sediment THD risk characterization should also include testing ofboth polar and medium polar sediment extracts for THD, as well as bioactivated nonpolar sediment fractions. Without bioactivation THD potency is not observed in nonpolar sediment extracts, although in *in vivo* experiments PCBs and PBDEs, and not with dioxins or PAHs, have shown to be thyroid hormone disrupting (THD), demonstrating this bio-activation is toxicologically relevant and therefore required for sediment hazard characterisation.

Chapter 7 discusses the implications of our results to improve the relevance and reliability of *in vitro* bioassay applied for risk characterisation of complex mixtures from sediments and other matrices. The evidence obtained to support the relevance of POP bio-activation is considered both from the exposure perspective as well as the toxicity perspective. Various features of the newly developed methods and knowledge acquired within this PhD project are discussed in relation to *in vitro* bioassay risk characterization of sediments towards a realistic *in* vitro bioassay-based risk characterization of complex mixtures. Some important aspects for the inclusion of metabolizing systems within *in vitro* bioassay are discussed. In addition, alternatives to deal with the SPMX effect and the definition of suitable sample amounts to improve *in* vitro bioassay reliability are offered. The suitability of the developed approach application is considered for the risk characterization of sediments. Furthermore, an analysis is made to decide whether this thesis have made *in vitro* bioassays more reliable and relevant for risk characterization of complex mixtures. Finally, it provides some concluding remarks and aspects for further applications and research.

CHAPTER 9

Netherlandse sammevatting

Het doel van het voorliggende proefschrift was het beter begrijpen en betrouwbaarder toepassen van *in vitro* bio-assays voor karakterisering en kwantificering van het potentiële risico van complexe mengsels van contaminanten, met speciale aandacht voor persistente organische verontreinigingen (POPs) in sedimenten.

In **Hoofdstuk 1** wordt het belang van karakterisering van het risico van complexe mengsels van stoffen in de moderne samenleving geïntroduceerd. De beschikbare methoden voor het testen van complexe mengsels van stoffen worden beschreven en de voor- en nadelen daarvan besproken. Er is in toenemende mate aandacht binnen het beleidsgericht testen van chemicaliën voor het bij de beoordeling betrekken van *in vitro* bioanalyse. Om een relevante en betrouwbare kwantificatie van de toxische potentie van complexe stoffenmengsels zeker te stellen moeten echter nog wel belangrijke knelpunten worden opgelost. Hiervoor is gedegen kennis nodig van het proces van monsterbewerking tot en met de interpretatie van de uitkomsten. Deze aspecten die belangrijk zijn voor betrouwbare bioanalyse worden toegelicht in de introductie, evenals de status van ontwikkeling en validatie ervan. Als een van de belangrijkste voordelen van *in vitro* bioanalyse is de mogelijkheid om de toxische potentie te kwantificeren van stoffen, waarvoor nauwelijks of geen chemische analytische methoden zijn ontwikkeld, bijvoorbeeld omdat standaarden nog niet bestaan. Bij voorbeeld gehydroxyleerde metabolieten van POPs zijn een toxicologisch relevante groep stoffen die hormoonverstorende effecten kunnen uitoefenen, maar deze stoffen kunnen nog niet routinematig worden geanalyseerd. In hoofdstuk 1 is een selectie van tot nog toe onopgeloste onderwerpen geselecteerd die in dit proefschrift nader worden bestudeerd en besproken, zoals aangegeven in de “benadering en structuur van het proefschrift”.

In **Hoofdstuk 2** is een meta-analyse uitgevoerd van beschikbare gegevens over het voorkomen en de relevantie van gehalogeneerde contaminanten met een phenol groep (HPCs) in mensen en dieren in het wild. Gerapporteerde gehaltes in het lichaam van HPCs waaronder veel gehydroxyleerde POP-metabolieten (OH-POPs) in verschillende weefsels van mensen en wilde dieren, zijn beoordeeld in relatie tot de concentratie van hun vermoedelijke moederstoffen. Op basis hiervan kunnen de meest relevante blootstellingsroutes en subpopulaties met de hoogste interne gehaltes worden opgespoord. De hoogste OH-POP niveaus zijn geconstateerd in bloedplasma en in goed doorbloed en foetaal weefsel. Plasmaconcentraties van geanalyseerde bekende HPCs lagen tussen respectievelijk 01.-100 nM in mensen en tot 240, 454, 600 en 7650 nM voor vogels, vissen, walvissen en andere zoogdieren.

De gerapporteerde bloedplasma-niveaus van de HPCs zijn eveneens vergeleken met de drempelwaarden voor toxicologische effecten. Het bleek dat de in bloed aangetroffen gehaltes volledig binnen de *in vitro* (0.05 – 10000 nM) en *in vivo* (3-940 nM) effectconcentraties voor deze stoffen te vallen. Omdat daar nog niet veel over bekend is, gezien de gevoeligheid van vroege ontwikkelingsstadia wordt geadviseerd is om beter uit te zoeken wat de gehaltes aan HPCs zijn in bloed van kinderen en foetaal weefsel in ook de algemene bevolking.

Gezien de toxicologische relevantie van de OH-POPs, wil **Hoofdstuk 3** oplossingen geven voor het knelpunt van de *in vitro*-productie van OH-POP metabolieten en de schildklierhormoomverstorende (THD) potentie hiervan via binding aan schildklierhormoon transport eiwitten in plasma (THBPs). In sedimenten en in bijvoorbeeld vis, schaal- en schelpdieren komen POPs voor als moederstoffen die pas THD worden na metabolisatie (hydroxylering). Dit is aangetoond met zuivere metabolieten, maar blijkt erg moeilijk aan te tonen voor de ouderstoffen zoals PolyChloor biphenylen (PCBs) en PolyBroom diphenyl ethers (PBDEs) in combinatie met i*n vitro* metabolisatie. Wanneer de OH-metabolieten moeten worden geëxtraheerd om vervolgens *in vitro* hun THD potentie te bepalen, bleek co-extractie van stoffen de TH-TTR competitieve binding assay te verstoren. In de huidige studie worden eerst de belangrijkste probleemstoffen geïdentificeerd- en gekwantificeerd. De grootste problemen werden veroorzaakt door co-extractie van cholesterol en verzadigde en onverzadigde vetzuren (SFA en NFSA). Verschillende extractiemethoden en zuiveringsmethoden zijn vervolgens ontwikkeld tot . een nieuwe methode waarbij de opbrengst van *in vitro* gemetaboliseerde OH-metabolieten wordt verhoogd en de co-extractie van de verstorende stoffen met een factor vier wordt verminderd. Daarnaast werd een nieuwe assay op microschaal ontwikkeld op basis van niet-radioactief fluorescent substraat om de TTR bindings potentie van de gevormde metabolieten te kwantificeren. De effectiviteit van het *in vitro* metabolisme en de extractie van de OH-metabolieten van de modelstoffen CB 77 en BDE 47 werden chemisch gekwantificeerd met een eveneens nieuw ontwikkelde chromatografische methode om gesilyleerde derivaten van de OH-metabolieten en co-extracten te analyseren. Op basis van de bereikte verbeteringen kan nu een dosis-response curve gemaakt met 50% remming van de TTR binding door in vitro bio-geactiveerde OH-metabolieten van CB 77 en BD 47. Het is erg belangrijk om rekening te houden met de toxische potenties van bio-geactiveerde POPs in de bioanalyse, om te voorkomen dat het risico van POPs ernstig wordt onderschat.

In de **hoofdstukken 4 en 5** worden de kwesties van supramaximale (SPMX) responsen en overconcentratie van POP-extracten verder onderzocht omdat deze de betrouwbaarheid van het kwantificeren van de toxische potenties van milieuextracten met complexe mengsels in in vitro bioassays ernstig kunnen beïnvloeden. Een SPMX-effect is het verschijnsel dat stoffen in een assay een maximale response veroorzaken die beduidend hoger is dan die van de positieve controle. Omdat de positieve controle wordt gebruikt om de toxische potentie van een monster aan te relateren, kan SPMX resulteren in overschatting van zijn toxische potentie. Omdat SPMX effecten het meest is gerapporteerd voor *in vitro* estrogeniteits assays, werd hiermee een meta-analyse uitgevoerd om te analyseren welke eigenschappen van assays, stoffen en condities samenhangen met het optreden vandit effect (**Hoofdstuk 4a.**). Van de 21 natuurlijke en industriële chemicaliën waarvoor SPMX werd beschreven, werden diethylstilbestrol (DES), genestein (GEN) en bisphenol A (BPA) geselecteerd om een database mee te bouwen Soms ontbrak echter essentiële informatie over relevante assay karakteristieken. De meta-analyse toonde aan dat het voorkomen van SPMX-effecten kon worden gerelateerd aan een aantal specifieke assay-eigenschappen: 1) het type en de concentratie van het serum dat gebruikt werd voor het blootstellingsmedium; 2) het eindpunt dat waarmee de estrogene potentie werd gekwantificeerd (endogeen of getransfecteerd reporter gen); 3) het aantal EREs (estrogen responsieve elementen) dat is gebruikt voor het reporter gen, en 4) de aard van de promotoren. Er waren geen aanwijzingen dat de concentratie oplosmiddel in kweek, blootstellingsduur of cel model het optreden van SMX zouden beïnvloeden. Het is van belang het mechanisme achter dit verschijnsel te begrijpen om er om op de juiste wijze met deze *in vitro* assays de estrogene potentie van stoffen, stoffenmengsels en milieu-extracties te typeren en te kwantificeren.

Over meerdere SPMX-inducers is tevens gerapporteerd dat zij het cellulaire efflux pompjes *in vivo* en *in vitro* blokkeren (Anselmo et al. 2012; Georgantzopolou et al. 2013). Daarom werd de hypothese getoetst of het blokkeren van de cellulaire efflux pompjes door stoffen die aanwezig zijn in milieumatrixen, de interne concentratie van de andere agonisten in het mengsel zouden kunnen doen toenemen en zo de SPMX veroorzaken. In **Hoofdstuk 4b** werd deze hypothese getest door een 96 well plaat cellulaire efflux pomp remmer assay aan te passen aan de H4IIE rat hepatome cellijn die gebruikt wordt voor de DR-Luc reporter gen assay voor dioxine-achtige stoffen. Verschillende milieu-relevante stoffen remden inderdaad de P-gp efflux pompjes, maar veroozaakten geen SPMX effect op de

2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD) respons. Ook de P-gP remmersgenesteine en quercetine beïnvloedden evenmin maximale de luciferase-inductie door TCDD. Mogelijk dat voor het testen van de gevolgen van efflux pomp remming een AhR-agonist die een P-gp substraat is moet worden gebruikt, omdat dit resulteert in intracellulaire accumulatie van deze AhR-agonist.

Het is standaard praktijk om een hoge stock-concentratie van non-polaire extracten te gebruiken om lagere testconcentraties voor de analyse verder uit te verdunnen. Bij sterk vervuilde monsters, bijvoorbeeld van sediment, kan mogelijk de oplosbaarheid van de geëxtraheerde stoffen overschreden worden waardoor een deel van de stoffen neerslaat en dus niet mee verdund wordt. Ook kunnen de clean-up kolommen overbelast raken, wat de efficiency van het verwijderen van polyaromatische koolwaterstoffen (PAKs) vermindert. Deze problemen kunnen onder- dan wel overschatting van de uiteindelijke gekwantificeerde dioxine-toxiciteit veroorzaken. Deze effecten worden in **Hoofdstuk 5** bestudeert waarna oplossingen worden uitgewerkt. Aspecten van de monsteropwerking die moeten worden gestandaardiseerd om tot een betrouwbare inzet van in vitro bioassays te komen zijn de initiële stock concentraties afhankelijk van het soort monster, de inzet van sonificatie voor beter in oplossing brengen van stoffen uit het mengsel en het verlengen van de blootstellingstijd in de DR-Luc assay zodat de invloed van de PAKs op de uitkomst te verminderen . Aanbevolen wordt om nooit met meer dan 20 g sediment equivalenten per ml oplosmiddel (SEQ/ml DMSO) als initiële stock concentratie te gebruiken om onderschatting van met de bioassay gekwantificeerde TEQ (bio-TEQ) te voorkomen. Dit is uitgetest op gezuiverde non-polaire sedimentextracten van verschillende locaties in Luxemburg. Een overmaat van extract op clean-up kolommen veroorzaakte een overschatting van de dioxine-achtige potentie bij 24 uur blootstelling, vermoedelijk door te geringen verwijdering van de PAKs die vals-positieve responses veroorzaken. Sonificeren van de stockoplossing wordt altijd aanbevolen voordat hieruit verdeer wordt doorverdund. Dit reduceerde de standaardvariatie van de uitkomsten sterk. Rekening houdend met de in deze studie bekend gemaakte aspecten en in aanvulling op de reeds beschreven belangrijke onderwerpen voor kwaliteitscontrole, kunnen de op *in vitro* bioassays gebaseerde bio-TEQs worden gebruikt om vast te stellen of sedimenten gezondheids- of milieu risico's vormen of de getelde milieustandaarden overschrijden

De methoden en benaderingswijzen die in dit proefschrift zijn ontwikkeld zijn in **Hoofdstuk 6** toegepast op extracten van meer of minder vervuilde sedimenten uit Luxemburg. Non-polaire fracties van sedimentextracten zijn geincubeerd met S9 rat microsomen voor bioactivatie van de mogelijk aanwezige POP en de zo ontstane metabolieten werden geëxtraheerd met een nieuw ontwikkelde methode die de gehaltes van de meest storende matrix componenten (vooral vetten) voldoende verlaagd voor verdere bio-analyse. Vervolgens is *in vitro* analyse uitgevoerd met de nieuwe niet-radioactieve 96-wells plaat transthyretine (TTR) competitieve binding assay. Door de metabole activatie nam de TTR bindings potentie van non-polaire fracties tot 100 keer toe. De meer polaire fracties van de sediment extracten waren wel zelf in staat tot TTR-binding, dus zonder bio-activatie. Naar verwachting bevatten deze stoffen zelf veelal al OH-groepen die nodig zijn voor een hormoon achtige werking. Ook *in vivo* experimenten met PCBs en PBDEs hebben aangetoond dat deze stoffen slechts schildklierhormoonverstorend (THD) zijn na bioactivatie. De resultaten van dit werk tonen aan dat voor een meer realistische *in vitro* risico karakterisering van sedimenten zowel het testen van medium-polaire als ook van non-polaire sedimentextracten met bio-activatie moet omvatten om onderschatting van de THD-potentie te voorkomen. **Hoofdstuk 7** bediscussieert de implicaties van de resultaten van het gepresenteerde onderzoek om de relevantie en de betrouwbaarheid van *in vitro* bioassay te verbeteren voor risicoschatting van complexe mengsels van stoffen uit milieumatrixen.. Verschillende onderwerpen van de nieuw ontwikkelde methoden en verkregen kennis binnen dit AIO-project, worden besproken in relatie tot *in vitro* bio-analyse van sedimenten voor een realistische *in vitro* bioassay-gebaseerde beoordeling van de toxische potentie van complexe mengsels van contaminanten in de beleidspraktijk. Ten slotte bevat de discussie beschouwingen over de perspectieven voor de ontwikkeling van verdere toepassing en verder onderzoek.

APPENDIX

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About the author

Curriculum vitae

Mauricio Montaño Garcés was born in Bogotá D.C, the capital city of Colombia in August 1976. He finished high school at the Instituto Diversificado Albert Einstein in 1994 and soon after, he enrolled at the Chemistry Department from the Universidad Nacional de Colombia. For his bachelor thesis, he validated a multi residue method for pesticide analysis in strawberry (*Fragraria* spp.) by GC-ECD/NPD at the pesticide residue laboratory (LARP), under the supervision of Prof. dr. Jairo Arturo Gurrero Dallos. He obtained the degree of Chemist in 2000.

He was then granted a fellowship from the International Atomic Energy Agency (IAEA) to make an internship on pesticide residue methods for environmental samples at the Environmental Analysis Laboratory of Universidad de Costa Rica, under the supervision of Prof. dr. Elizabeth Carazo.

He obtained his qualification as Lead Auditor of ISO 9000, 14000 and 17025 standards between 2002 and 2006, in which he worked at Bureau Veritas Colombia as lead auditor and auditor trainer.

In 2006, he was granted a Netherlands Fellowship Programme (NFP) fellowship from the Netherlands Organization for International Cooperation (NUFFIC) to study a master in Environmental Sciences at Wageningen University (The Netherlands). During this period, he worked as an intern at the Norwegian School of Veterinary Science, Department of Production Animal Sciences, where he worked on the steroidogenic effects of POP mixtures from Cod liver oil under the supervision of Prof. dr. Erik Ropstad and Prof. dr. Tinka Murk. His master thesis focussed on supra-maximal (SPMX) effects in *in vitro* bioassays for estrogenicity, at the Division of Toxicology of Wageningen University under the supervision of Prof. dr. Tinka Murk. He obtained the degree of MSc in Environmental Sciences with a “cum laude” distinction in 2008.

In 2009 he obtained an “aide de formation recherché” AFR PhD grant from the Fond National de la Recherche Luxembourg (FNR) to develop the project resulting in the present thesis at the Centre de Recherche Public – Gabriel Lippmann under the supervision of Prof. dr. Tinka Murk and Dr. Arno Gutleb.

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The SENSE Research School declares that **Mr. Mauricio Montaño Garces** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 64 ECTS, including the following activities:

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- Market in Luxembourg" round table, Luxembourg, 27 June 2012
- Ecotoxicology
- Advance statistics

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- Topics in Environmental Mixture Toxicity
- Exposure assessment I: chemical exposure assessment analysis and modelling
- Integrated Ecosystem Assessment in Regional Management
- Environmental Policy Analysis and Evaluation
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Management and Didactic Skills Training

- Board member, Luxembourg Life Sciences Association ASBL, 2011-2012
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Oral Presentations

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