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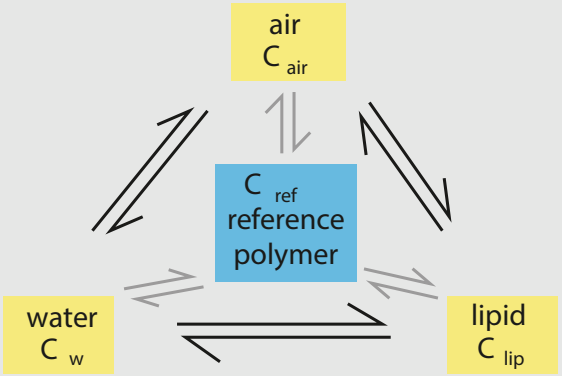
Using a reference partitioning phase to link exposure and effect assessment of hydrophobic organic chemicals

Novel equilibrium partitioning concepts and methods



Dorothea Gilbert
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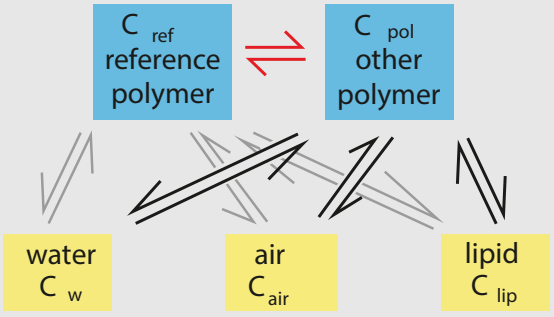

AARHUS UNIVERSITY

Technical University of Denmark

Using a reference partitioning phase to link exposure and effect assessment of hydrophobic organic chemicals

Novel equilibrium partitioning concepts and methods

PhD thesis

by

Dorothea Gilbert

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Summary

Hydrophobic organic chemicals (HOCs) constitute a large group of pollutants of concern. They are sparingly soluble in water and partition into organic phases, which makes it a challenge to determine the environmental exposure situation. For the same reason, it is not less challenging to assess the toxicity of these chemicals. The actual risk that these chemicals pose to environmental and human health can only be evaluated if exposure levels and toxic dose levels are linked on a scientifically sound basis. However, the scientific basis to establish such a link needs to be improved and further developed. This thesis advocates the use of concepts of chemical thermodynamics and further develops such concepts through the introduction of a reference partitioning phase. Equilibrium partitioning methods were applied and further developed in individual studies: (1) Polymers were considered a suitable reference partitioning phase for HOCs, and their sorptive capacities were compared to calibrate polymers and therewith polymer-based equilibrium partitioning methods against each other to increase the accuracy and precision of quantitative measurements of HOCs. This also facilitated derivation of multimedia partition coefficients, for example for HOC partitioning between lipid and water. (2) A silicone polymer served as reference phase to transfer a mixture of persistent organic pollutant (POPs) from human silicone implants into an *in vitro* assay that can detect the potential of chemicals to interfere with steroidogenesis. Measured concentrations in human silicone implants served to set test concentration levels of a POP mixture in silicone disks, which subsequently were used to control the exposure in the in-vitro assay by equilibrium partitioning. (3) Different exposure scenarios were studied in an *in vitro* biotransformation assay using molecular carriers as dosing vehicles for polycyclic aromatic hydrocarbons (PAHs). By the use of equilibrium partitioning methods, it was possible to estimate the PAH carrier-bound and enzyme-bound fractions. Further, some of the developed ideas and methods found their application in additional studies, in which equilibrium sampling using silicone devices was employed to thermodynamically assess the HOC exposure situation in the environment, and partitioning-controlled dosing methods were used for determining toxic effects of HOCs at better controlled and better defined exposure conditions. Finally, the application of equilibrium partitioning methods and the use of a reference partitioning phase allowed exposure to be expressed in terms of chemical activity, facilitating environmental exposure to be linked to exposure-effect relationships observed in laboratory studies. The study explores new paradigms, concepts and methods that contribute to an improved scientific base for chemical risk assessment.

Dansk Resumé

Mange miljøfremmede organiske stoffer er hydrofobe og har en lav opløselighed i vand. Grundet deres lave vandopløselighed vil hydrofobe organiske stoffer binde til og fordele sig ind i andre faser såsom jord, sediment og især lipider i levende organismer, hvilket gør det til en udfordring at vurdere deres skæbne i miljøet. Af samme grund er det ikke mindre udfordrende at vurdere toksiciteten af disse stoffer. Stoffernes reelle risiko for miljøet og menneskers sundhed kan kun vurderes, hvis eksponeringsniveauer og toksiske niveauer kan sammenlignes på et solidt videnskabeligt grundlag. I den forbindelse er det vigtigt, at det videnskabelige grundlag løbende forbedres og videreudvikles. Denne afhandling tager udgangspunkt i kemisk termodynamik og indfører brugen af en referencefase (*reference partitioning phase*) til at vurdere fordelingen af stoffer i miljøet og bestemme effektive koncentrationer for toksicitet. Ligevægtsfordelingsbaserede metoder blev anvendt og videreudviklet i de enkelte undersøgelser: (1) Polymerer blev vurderet til at være velegnede referencefaser for analytiske ligevægtsbaserede opsamlings- og doseringsteknikker. Fordelingen af hydrofobe organiske stoffer mellem forskellige polymerer blev bestemt for at kalibrere polymerer, og dermed ligevægtsmetoderne, mod hinanden og øge deres akkurathed og præcision. Derudover blev polymerer brugt som referencefase i bestemmelsen af multimedie fordelingskoefficienter, for eksempel fordeling mellem lipid og vand. (2) En silikonepolymer blev anvendt som referencefase til at overføre eksponering af persistente organiske stoffer (POPer) fra humane silikoneimplantater til et *in vitro*-assay med det formål at måle hormonforstyrrende effekter. Målte koncentrationer af POPer i silikoneimplantater dannede dermed basis for at indstille koncentration af en POP-blanding i en silikoneskive, som derefter blev brugt som reservoir til at styre eksponeringen i et *in vitro*-assay. (3) Forskellige eksponeringsscenarier blev undersøgt i et *in vitro*-biotransformations-assay ved hjælp af transportmolekyler som doseringsvehikler for polycykliske aromatiske hydrokarboner (PAHer). Ligevægtsfordeling blev i dette studie brugt til at måle bindingen af PAHer til transportmolekyler og enzymfraktioner. De udviklede koncepter og metoder blev derudover anvendt i en række yderligere studier. Ligevægtsfordelingsbaserede metoderne blev brugt til at bestemme toksiske virkninger af hydrofobe organiske stoffer under bedre definerede og kontrollerede eksponeringsforhold ved hjælp af silikonepolymer og lipid som reservoir/doseringsfase, og ligevægtsfordelingsbaserede metoder blev ligeledes brugt på forskellig vis til at måle POPer i miljøet ved hjælp af polymerer som opsamlingsfase. Flere af delstudierne viser tydeligt, at man kan bruge en polymer som referencefase til at bestemme eller kontrollere stoffernes kemiske aktivitet, hvilket muliggør sammenkoblingen af viden om eksponering i miljøet og eksponerings-effektsammenhænge bestemt i laboratorieforsøg. Det samlede studie fremsætter nye paradigmer, begreber og metoder, der bidrager til et forbedret videnskabeligt grundlag inden for kemisk risikovurdering.

Preface

This dissertation was submitted in partial fulfillment for the requirements of a PhD degree by the Technical University of Denmark. It deals with some fundamental aspects of environmental chemistry and toxicology of organic contaminants, and in particular with the partitioning behavior, bioavailability, endocrine activity and biotransformation of some hydrophobic organic chemicals. The theoretical concepts and methods presented may be of interest to the environmental chemist, the environmental toxicologist, and to scholars and students of environmental sciences.

The study was carried out between 2012 and 2015 at the Department of Environmental Science (Roskilde), Aarhus University, as primary place of employment, and at the Technical University of Denmark at the National Food Institute and the Department of Environmental Engineering. In addition, parts of this work were carried out at the US EPA laboratory in Duluth, Minnesota. The studies were funded both by the Department of Environmental Science, Aarhus University, and the National Food Institute (DTU).

Dorothea Gilbert
Roskilde, Denmark, 2015

List of publications

I Gilbert D, Witt G, Smedes F and Mayer P. 2015. Polymers as reference partitioning phase: polymer calibration for an analytically operational approach to quantify multimedia phase partitioning. To be submitted to *Analytical Chemistry*.

II Gilbert D, Mayer P, Pedersen M and Vinggaard AM. 2015. Endocrine activity of persistent organic pollutants accumulated in human silicone implants – dosing in vitro assays by partitioning from silicone. *Environment International* (in press). DOI: 10.1016/j.envint.2015.07.008.

III Gilbert D, Fitzsimmons P, Hoffmann A, Mayer P and Nichols J. Molecular carriers for substrate delivery: a cross-validation study on *in vitro* biotransformation of PAHs using fish liver S9 enzyme fractions (Manuscript in preparation)

Additional publications (minor contribution)

IV Jahnke A, Mayer P, McLachlan MS, Wickström H, **Gilbert D** and MacLeod M. 2014. Silicone passive equilibrium samplers as "chemometers" in eels and sediments of a Swedish lake. *Environmental Science Processes & Impacts* 16:464–472.

V Mäenpää K, Leppänen M, Figueiredo K, Mayer P, **Gilbert D**, Herve S, et al. 2015. Fate of polychlorinated biphenyls in a contaminated lake ecosystem: Combining equilibrium passive sampling of sediment and water with total concentration measurements of biota. *Environmental Toxicology and Chemistry* (in press). DOI: DOI: 10.1002/etc.3099.

VI van der Heijden SA, Hermens JLM, Sinnige TL, Mayer P, **Gilbert D**, Jonker MTO. 2015. Determining high-quality critical body residues for multiple species and chemicals by applying improved experimental design and data interpretation concepts. *Environmental Science & Technology* 49:1879–1887.

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Part I

Background

Chapter 1
Introduction

1.1. Motivation

Since the industrialization, man-made chemicals have emerged into the environment in increasing numbers and amounts. Currently, there are approximately 100.000 registered chemicals on the global market. Chemical pollution is named one of nine identified anthropogenic threats on planetary health that determine a "safe operating space" for humanity to develop [1, 2]. Rockström et al. introduced the concept of planetary boundaries, and defined those boundaries as thresholds for quantifiable control variables "set at a 'safe' distance from a dangerous level" on a global scale. Yet, as pointed out by the authors, the quality of such a safe distance depends on a societal judgment of acceptability of risk and uncertainty. It also depends on the uncertainty associated to the scientific knowledge about the natural limit [3]. Planetary boundaries for chemical pollution could not (yet) be defined, first of all because the natural limits for chemical burden are difficult to define due to the large number of chemicals and their mixtures, and the multitude of adverse effects they can cause in an equivalently large number of species; secondly because of the scarce scientific knowledge about the natural limits of the global assimilative capacity for chemical pollution [3].

Nevertheless, strategies have been developed and in varying degrees been implemented in form of policy actions (e.g. the European REACH regulation for the Registration, Evaluation, Authorisation and Restriction of Chemicals or the World Health Organization's International Programme on Chemical Safety, IPCS) to control chemical pollution at least on regional scales, namely chemical risk assessment procedures [4, 5]. Risk assessment of chemicals is a process that involves the steps of hazard identification, exposure assessment, effect assessment and risk characterization (Figure 1.1) [6], and is carried out by regulatory authorities while being strongly based on scientific knowledge and advances within environmental chemistry and toxicology.

While hazard is the inherent ability of an agent to cause harm, risk is the probability of such harm to occur as a result of exposure to such an agent [6]. That means for a toxic chemical posing a hazard to the health of living organisms, including humans, that it does not constitute a risk unless living organisms are exposed to it. Therefore, a crucial point in risk assessment is the linkage between exposure and effect assessment. This requires environmental chemistry and toxicology to be integrated on a common conceptual basis. For environmental organic chemicals, however, this is often done unsatisfactorily not least because scientific concepts for defining exposure [7] both within environmental chemical fate research and within the context of

toxicology need to be better understood by environmental chemists and toxicologists in the first place and must then be implemented by risk assessors. For example, sediment quality guidelines of major legislative bodies are in most cases still based on total concentrations rather than on freely dissolved concentrations, although there is scientific support that an exposure assessment that is based on total concentrations may overestimate the actual risk [8, 9]. Similarly, exposure to especially hydrophobic organic chemicals within (eco)toxicological effect assessment is often poorly defined, which not least is due to an insufficient exposure definition [10, 11, 12]. In this light, the importance of research on determining exposure within environmental chemistry and toxicological effect assessment becomes obvious.

The research undertaken within the here presented thesis (highlighted in Figure 1.1) touches mostly on elements of chemical exposure assessment but also on elements of chemical effect assessment, and builds on concepts of chemical thermodynamics [13, 14, 15] to understand the behavior of neutral hydrophobic organic chemicals (HOCs) in the environment and in interaction with biological targets.

1.2. Aims and research questions

The overall aim with this thesis is in attempt to improve the scientific conceptual basis for linking exposure and effect assessment. Therefore, more specific aims were to (i) introduce the concept of a reference partitioning phase for making exposure measurements comparable between and within different areas of their usage, i.e. toxicological research and chemical fate research, (ii) develop and apply methods that make use of thermodynamic working principles, and (iii) to explore the feasibility of a thermodynamic measure, i.e. chemical activity, for risk characterization. The thesis addresses the following research questions:

1. How can concepts from chemical thermodynamics be applied and expanded to increase the comparability, accuracy and precision of equilibrium partitioning methods to determine exposure?

2. How can environmental HOC exposure levels be transferred into laboratory tests for an effect assessment?

3. To which degree can partitioning projections be used to estimate levels of HOCs in organisms, and in particular at the site of toxic action?

1.3. Scientific base and scope of the thesis

Traditionally, chemical risk assessment relies on the evaluation of concentrations, both for assessment of a chemical's fate and effects, and consequently a risk quotient is defined as the ratio of the "predicted environmental concentration", PEC, and the "predicted no-effect concentration", PNEC [6] (see Figure 1.1). Several problems arise, however, by defining such risk quotient. First, concentrations, as by definition an

amount of chemical per unit volume or mass, are given in different units depending on the phase where measured and can thus not easily be compared. Conversion of units may seem a basic task; nevertheless it appears erroneous and unfunctional especially for ill-defined phases, e.g. sediment or fish, when phase density is not readily at hand. Second, concentrations cannot be used to assess the chemical equilibrium status of phases, as they do not reflect the capacity of phases to accommodate a chemical. These issues may disqualify a predicted environmental concentration as exposure parameter, at least for complex media such as soil, sediment or biota tissue [16, 14]. Third, the use of concentrations as dose parameter in toxicology seems only feasible if clearly defined [17, 18, 10]. It is crucial to explicitly state the phase to which a concentration unit refers to, and it is essential to also clearly define the "effective amount of chemical", which in the case of hydrophobic organic chemicals may be only a fraction of the total due to factors such as sorption, uptake and metabolism affecting bioavailability. Yet, toxicity data are commonly expressed in units of mol/L without the necessary care about bioavailability issues and differences in phase compositions, albeit these details being decisive in the case of tests with hydrophobic organic chemicals. Finally, there is a scientific debate on the definition of PNECs, especially since the use of so called no- (or low-) observed effect concentrations (NOEC or LOEC) that are sometimes used for their derivation has been critized [19, 20, 21].

In the past decades, significant advances have been made on chemical exposure assessment through the application of the thermodynamic concept of chemical activity and fugacity: It was in 1979 when Donald Mackay published "Finding fugacity feasible" in the journal Environmental Science and Technology [16] . In this contribution, he encouraged the application of the fugacity concept for predicting transport, distribution and bioaccumulation of toxic chemicals in the environment, recognizing that the prediction of phase equilibrium relationships by the help of the fugacity concept could be as powerful within environmental chemistry as it had proven to be in chemical engineering sciences. This has led to the development of sophisticated multimedia fugacity models to predict chemical fate and exposure [22, 23, 24, 25]. Besides, several measurement techniques have been developed that are based on thermodynamic working principles and have chemical activity or fugacity as direct measurement endpoint [26, 27, 28, 29, 30].

More recently, the chemical activity concept has also been explored by environmental toxicologists who rediscovered and continued the work of Ferguson [31] on the use of chemical potentials as indices of toxicity. Significant research has been done investigating chemical activity as exposure and toxicity parameter for hydrophobic organic chemicals and their mixtures [17, 32, 33, 34, 35, 36]. In particular, it appeared that organic chemicals exert baseline toxicity (narcosis) in the chemical activity range 0.01-0.1, which has been confirmed through experiments and modeling efforts [37, 38]. The power of chemical activity to be predictive for baseline toxicity and the utility of chemical activity to distinguish specific modes of toxic action is now increasingly recognized in the scientific community.

Mackay and co-workers [14] in a recent publication encouraged the use of chemical activity as an integrating concept in the assessment and management of environmental

contaminants, stating: "*It may at first seem unlikely that the thermodynamic concept of chemical activity can serve as a tool to link [...] very different aspects of environmental science and management. Closer examination reveals that chemical activity has the potential to provide this link if an effort is made to express and measure quantitative data on chemical presence not only in the traditional units of concentration but also as activities.*" Thus, one may alternatively define a risk quotient as the ratio of predicted environmental (chemical) activity (PEA) and predicted no-effect (chemical) activity (PNEA) (Figure 1.1).

In this light, this thesis is written with the ambition to overcome some of the inertia associated to the implementation of the chemical activity concept by giving an example how the concept can be put in practice. As the chemical activity concept is rather abstract, this thesis introduces a reference partitioning phase to realize the concept in practical terms. For example, by choosing a polymer as reference partitioning phase for partitioning-based measurement techniques, measurements can be directly compared on a polymer basis, possibly after calibration of these polymers against each other; and just like with chemical activities, the direction of transport processes can directly be deduced and exposure between different media be linked. In turn, a reference partitioning phase facilitates a straightforward determination of chemical activity, given knowledge about the activity coefficient. For the future, it is thus desirable that methods be developed that directly employ a reference partitioning phase, following the examples given in this thesis.

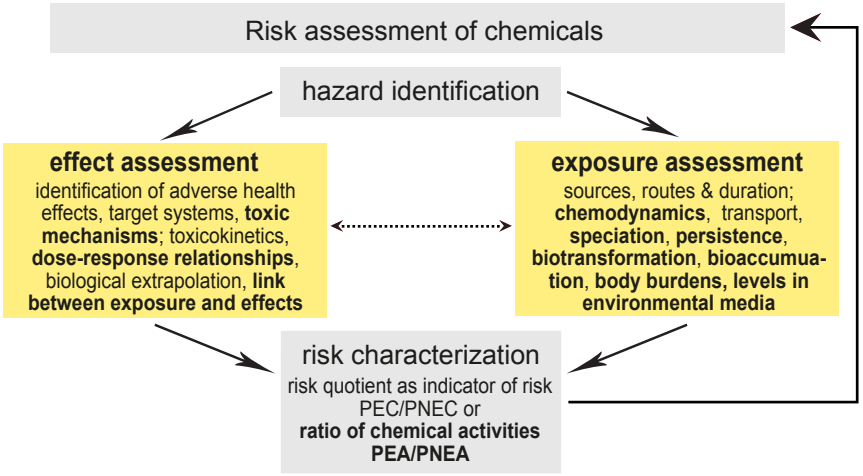

Figure 1.1. Steps in chemical risk assessment. Highlighted are the topics that this thesis deals with.

1.4. Structure of this thesis

The thesis is divided into three parts: The chapters in Part I provide an introduction and the background to the presented research themes and the thesis in particular. In Part II, three individual studies are presented that apply the concept of a reference partitioning phase (Manuscript I and II) and/or are concerned with the exposure of HOCs in *in vitro* assays (Manuscript II and III). Finally, in Part III, it is attempted to link environmental exposure levels of HOCs between exposure media and to toxicological effects on the basis of a reference partitioning phase and chemical activity. The major findings are discussed in perspective of chemical risk assessment and future research needs are highlighted.

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Chapter 2
Hydrophobic organic chemicals

Among all xenobiotic chemicals that contribute to chemical pollution, organic chemicals that are hydrophobic make up a major group of chemicals of concern. They comprise various chemical groups of environmental pollutants (e.g. petrochemicals, dioxins, flame retardants, preservatives, some pesticides and drugs etc.) and show wide structural diversity. Most concerning are perhaps the ones that enter(ed) the environment in large amounts and stay, i.e. persistent organic pollutants (POPs) and those that trigger severe toxic effects. Examples for organic chemicals of environmental concern are the aliphatic and aromatic hydrocarbons originating from the combustion of fossil fuels (e.g. polycyclic aromatic hydrocarbons, PAHs) or during the production of petroleum-based products (e.g. olefins and aromatics like benzene, toluene, xylene), industrial chemicals, e.g. polyhalogenated aromatic hydrocarbons like the polychlorinated biphenyls (PCBs) that have been widely used as coolant fluids and dielectrics until they were banned between 1970-1980, polybrominated diphenylethers (PBDEs) that are used as flame retardants, phthalates as plasticizers for synthetic plastic polymers, halogenated benzenes, which are important intermediates in the chemical industry, and organochlorine pesticides (e.g. chlorinated phenols or the prominent dichlorodiphenyltrichloroethane, DDT). Chemical structures for some of these organic chemicals are depicted in Figure 2.1.

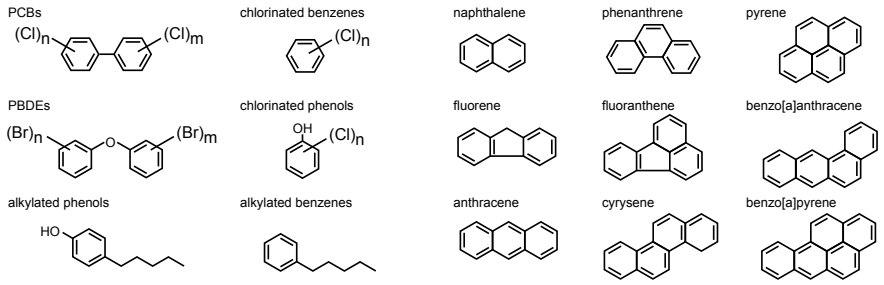

Figure 2.1. Chemical structures of some important hydrophobic organic chemicals.

These chemicals have in common that they are only sparingly soluble in water ($\ll$1 mmol/L), i.e. they are hydrophobic, meaning they dislike being surrounded by water

molecules. Generally, the water solubility of organic chemicals that only have a limited number of functional groups decreases with molecular size (more correctly, with the molecular surface area) and the degree of halogenation. This has important implications for their behavior in the environment, and to some extent also determines their interaction with biological structures. As another consequence, these chemicals tend to bind to solids, and partition to a larger extent into phases that offer them more favorable conditions for accommodation with regards to energetic costs for dissolution, i.e. organic phases in the broader sense of being carbon-based. The research presented in this thesis deals with such hydrophobic organic chemicals, as they are chemicals whose behavior and, in parts, toxicity can be described by thermodynamic considerations. Chemical thermodynamics provides a powerful conceptual basis for defining and understanding the term exposure, and allows predictions to be made based on measurable thermodynamic parameters.

Chapter 3
Chemical thermodynamics

3.1. Some fundamental thermodynamic concepts

The fate of HOCs in the environment and their bioavailability follow thermodynamic laws. Thermodynamics is concerned with the description of the transfer and transformation of energy. As energy is the capacity to do work, it is also what drives a chemical's transport and distribution as well as chemical reactions. Therefore, it is the energy status of a compound, or more precisely the change in the energy status, that is of interest when approaching a thermodynamically-based assessment of the behavior of organic chemicals. As a fundamental thermodynamic principle, any spontaneous process within a thermodynamic system and its surrounding is accompanied by a dispersal of energy, which follows from the Second Law of thermodynamics. In particular for chemicals, this implies that any compound strives to achieve a balanced energy status within a closed system.

The energy status of a chemical can be described by the chemical potential (μ, in units J/mol), whereby the molecule entities of the compound make up the total free energy, or the Gibbs free energy (G, in units of energy [J]) [1]. Following from the Gibbs-Duhem equation, any change in the chemical potential of a compound at constant temperature is related to a corresponding change in pressure that this chemical exerts onto its surrounding [2]. For an ideal gas, this is the gas pressure (P in Pa), for a real gas it is what Lewis [3] called *fugacity* (f,). Mathematically, this can be expressed in form of a differential equation, which upon integration provides equation 3.1 [4], with which the chemical potential of a chemical relative to a chosen standard state ($^\circ$) can be quantified:

$$\mu = \mu^\circ + RT \ln \frac{P}{P^\circ} \equiv \mu = \mu^\circ + RT \ln \frac{f}{f^\circ} \tag{3.1}$$

where R is the ideal gas constant [$\mathrm{J\,mol^{-1}\,K^{-1}}$], T the ambient temperature [K], and f° the reference fugacity [Pa]. For considerations of phase transfer processes of environmental organic chemicals, the pure liquid state of a chemical is commonly chosen as its reference state, and at given environmentally relevant conditions of ambient pressure and temperature, referred to as the standard state (marked $^\circ$). Hence, P° in this case is the saturated vapor pressure that the pure liquid substance at ambient temperature and pressure exerts into an ideal gas.

Equation 3.1 can also be rewritten in terms of *chemical activity* (a) as instead of gas pressure or fugacity [4, 5], because for ideal (gas) mixtures the partial pressure (P_i) of a compound can be expressed as

$$P_i = xP, \tag{3.2}$$

and fugacity can be expressed [4, 6] as

$$f = \gamma x f^{\circ} \tag{3.3}$$

where x is the mole fraction and γ the activity coefficient, both unitless. Thus, with chemical activity defined [1] as

$$a = \gamma x \tag{3.4}$$

equation eq 3.1 can be rewritten:

$$\mu = \mu^{\circ} + RT \ln a \equiv \mu = \mu^{\circ} + RT \ln x\gamma \tag{3.5}$$

Often, γ is given in inverse units of concentration when concentrations are used instead of mole fractions. The activity coefficient is an interesting parameter that quantifies the non-idealities of molecular interactions of different molecules (i.e. solute and solvent) in a mixture. In case of ideal mixtures according to Raoult's Law, $\gamma = 1$.

Chemical activity is a relative measure and quantifies the energetic state of a compound in a given state (e.g. dissolved) *relative* to the energetic state of the compound in its reference state (e.g. pure liquid) at the same temperature and pressure (equation 3.5). From equation 3.4, it follows that for ideal mixtures ($\gamma = 1$), the chemical activity can take a value of maximal 1 ($a = x$), as the mole fraction is defined between 0-1. For compounds that are solids at ambient temperature, the maximum chemical activity of 1, with the liquid state chosen as reference state, cannot be reached as additional energy is needed for the crystal structure of a solid to break and to transform the solid into a hypothetical liquid state. To estimate the maximum chemical activity (a_{max}) of solid organic chemicals, it may suffice to know the chemical's melting point temperature (T_m), and to ensure that the assumption of a constant energy of melting of $56\,\mathrm{J\,mol^{-1}\,K^{-1}}$ is justified [7]:

$$a_{max} = \frac{S_s}{S_L} = \exp\left[6.8\left(1 - \frac{T_m}{T}\right)\right] \tag{3.6}$$

S_L is the solubility of the chemical in its hypothetical subcooled liquid state and S_s the solubility of the solid state chemical.

3.2. Equilibrium partitioning theory

Equilibrium partitioning theory forms the basis for many environmental fate modeling approaches [8, 9]. Molecular phase transfer processes such as equilibrium partitioning can be described thermodynamically, i.e. in terms of the energetic changes a chemical undergoes upon phase transfer. Chemical equilibrium is a state of a system at which it has reached minimal Gibbs free energy. This is when the chemical potentials in any phase (i, j) of the system are balanced:

$$\mu_i = \mu_j \text{ or } a_i = a_j \tag{3.7}$$

In any multimedia system, the balancing of a compound's energy status between phases where it differs will be directed from higher to lower chemical potential. The underlying assumption thereby is that these phase transfer processes are rapid and reversible. The equilibrium criterion of equal chemical potentials (equation 3.7) of a chemical that is distributed in two phases i and j implies that the abundance of a chemical in these phases relative to each other (x_i/x_j) is due to a difference in the energy state of a compound in these phases, which can be deduced from the Gibbs-Duhem equation [2, 4]:

$$RT \ln \frac{x_i}{x_j} = -(RT \ln \gamma_i - RT \ln \gamma_j) \tag{3.8}$$

whereby $RT \ln \gamma$ is the partial molar excess free energy of a compound in a phase. The larger this molar excess free energy, the less favorable are the conditions for the chemical to be accommodated. Hence, a distribution of a compound between phases can be linked to differences in its energetic states in these phases, which is manifested in the Boltzmann equation (see, e.g. [4] or [1]). The mole fraction ratio, in principle, is a partition coefficient ($\hat{K}_{ij}$):

$$\hat{K}_{ij} = x_i/x_j = \gamma_j/\gamma_i \tag{3.9}$$

However, commonly used partition coefficients K_{ij} reflect concentration ratios:

$$K_{ij} = C_i/C_j = \frac{\gamma_j V_{mix}}{\gamma_i V_m \rho_i} \tag{3.10}$$

where V_{mix} stands for the molar volume of the mixture, V_m for the molar volume of the solute and ρ_i for the density of phase i.

Partition coefficients are important parameters to describe a chemical's phase transfer behavior. For example, the octanol-water partition coefficient K_{ow} is often used as a measure for a chemical's hydrophobicity and/or lipophilicity. Similarly, the bioconcentration factor (BCF), commonly used to estimate the accumulation of a chemical in biota from water, can be considered a biota-water partition coefficient. However, for such ill-defined phases like biota, certain assumptions have to be made, e.g. that compounds are mainly partitioning into the lipid fraction of an organism. This is not further problematic as long as it is kept in mind that such assignment of chemical mass to a dominating partitioning phases may lead to an overestimation of partition coefficients. Likewise, partition coefficients can be determined for sediment or soil and water, which often then are referred to as binding constant K_d [$\mathrm{L\,kg^{-1}}$].

Equilibrium partitioning theory provides a powerful tool for the exposure assessment of chemicals as with the knowledge of the concentration of a chemical in one phase and a partitioning coefficient, the concentration of that chemical can be projected into another phase. Moreover, if the concentration at the target site for toxicity within an organism is known, toxicity may be predicted based on thermodynamic phase partitioning considerations. The power of equilibrium partitioning theory has been demonstrated in various studies, especially within quality assessment of sediment and soil [10, 11] but the theory has also been criticized as an over-simplifying approach

[12, 13]. For example, not always are the phases considered homogeneous and is the transport of chemicals between environmental compartments or these phases passive and reversible; and neither can the mass of a chemical in an environmental thermodynamic system be considered constant due to metabolism and degradation processes. On the other hand however, equilibrium partitioning theory provides a solid thermodynamic concept to estimate the ultimate final stage of any thermodynamic phase transfer process and can also be used for modeling nonequilibrium situations [8].

3.3. On the use of a reference partitioning phase

Similarly to the *standard state*, introduced as a reference measure and point of departure to quantify changes in the energetic state of a chemical in relative rather than in absolute terms, it may appear useful to define a *reference phase* for phase partitioning considerations, a *reference partitioning phase*. While the standard state refers to a chemical's state (e.g. solid, liquid, gaseous, dissolved) under a chosen set of (standard) conditions, a reference phase may be considered the conceptual analog for a compound's "surrounding". Ideally, it may be a phase consisting of the same molecules as the solute itself such that $\gamma = 1$. In this case, and only then, standard state and reference phase may not be distinguishable. Hence, the pure liquid substance is both reference state of the chemical and reference phase for the chemical. To choose a reference partitioning phase for HOCs, the following is to consider: (i) Given rather high solute concentrations (mole fractions), one may choose a reference partitioning phase such that Raoult's Law applies, i.e. $\gamma \approx 1$. This is conceivable for mixtures of isomers and mixtures of congeners of organic chemicals, or organic phases, in which solute and solvent display the least intermolecular interactions, including non-aqueous phase liquids (NAPLs). In situations where solutes are in rather dilute solution, often encountered in the environment, one may choose a reference partitioning phase such that Henry's Law applies, i.e. $\gamma = const.$ over the lower end of the mole fraction scale, (ii) and such that γ is rather small (relative to activity coefficients in water). (iii) With regards to toxicological studies, a third criterion may be that γ be very similar across different organic chemicals. For example, with lipid or silicone as reference partitioning phase, HOCs exhibit rather low activity coefficients [14, 15] that are comparable to activity coefficients in an organic solvent phase (typically deviating within a factor of 3), but are much smaller than activity coefficients in water; also, activity coefficients of HOCs for silicone and lipid seem to be uniform between chemicals. While concentration-independent activity coefficients ensure that chemical activities are linearly related to the mole fraction or the concentration (equation 3.4), the second criterion is important in that it dictates intermolecular interactions between solute and solvent molecules not to become too dominating for the chemical to be accommodated in a phase. The third criterion finally determines whether toxic effects that are based on the same target site (e.g. narcosis) will fall within a rather narrow range of chemical activities. This is especially important to be able to predict certain forms of toxicity based on knowledge of a chemical's activity. However, such thermodynamically-based toxicity predictions are not yet widely established, and so

far received most attention within research on the narcotic mode of action of organic chemicals.

The choice of an appropriate standard state depends on the questions being asked, and so does the choice of an appropriate reference phase. For studying phase partitioning processes of organic chemicals within a toxicological context, a well-defined lipid phase such as triglycerides or phospholipids can serve as a reference partitioning phase. On the other hand, a nonpolar solvent, e.g. octanol, or a hydrophobic amorphous polymer (elastomer), e.g. silicone may constitute a suitable reference phase for quantitative aspects of the phase partitioning of organic chemicals. For nonpolar organic chemicals dissolved in a polar solvent like water at low solute fractions (dilute solutions), γ is typically by orders of magnitude above unity and varies considerably between solutes [4]. That is why from a thermodynamic perspective water does not constitute a suitable reference phase for hydrophobic organic chemicals.

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Part II

Individual Studies

Manuscript I

Polymers as reference partitioning phase: polymer calibration for an analytically operational approach to quantify multimedia phase partitioning

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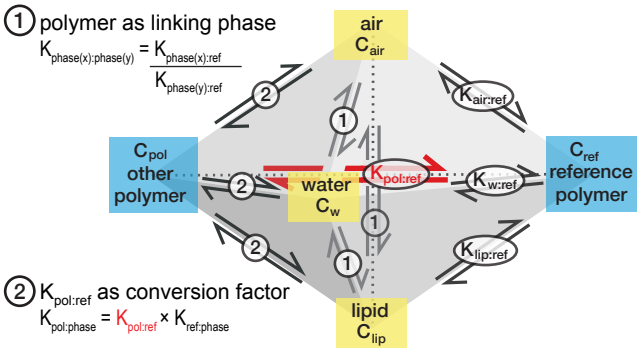

to be submitted to *Analytical Chemistry*

Chapter 4

Polymers as reference partitioning phase: polymer calibration for an analytically operational approach to quantify multimedia phase partitioning

Abstract

Polymers are applied for the enrichment of hydrophobic organic chemicals (HOCs) from various types of samples and media in many analytical partitioning-based measuring techniques. In the present study, we suggest polymers to be considered as a reference partitioning phase and introduce polymer-polymer partitioning as the necessary link for accurate polymer-based multimedia phase partitioning calculations. Polymer-polymer partition coefficients were determined for polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and a selection of organochlorine pesticides (OCPs) by equilibrating 13 silicones, including polydimethylsiloxane (PDMS), and one low-density polyethylene (LDPE) material in methanol-water solutions. The partition coefficients confirmed identical absorption capacities of PDMS materials for HOCs. However, they also revealed that within the group of silicones the absorptive capacity of polymers can differ as much as between silicones and LDPE, i.e. by up to a factor of typically six. Uncertainty in polymer volume due to imprecise coating thickness or the presence of fillers was identified as a source of constant deviation immanent to some polymer-based partition coefficients. New polymer-based (LDPE-lipid, PDMS-air) and multimedia partition coefficients (lipid-water, air-water) were calculated by applying the new concept of a polymer as reference partitioning phase and by using polymer-polymer partition coefficients as polymer-specific conversion factors. The present study encourages the use of polymer-polymer partition coefficients, recognizing that polymers can serve as a linking third phase for a quantitative understanding of equilibrium partitioning of HOCs between any two phases.

4.1. Introduction

A wide variety of polymers is applied in analytical chemistry for the enrichment and selective extraction of organic chemicals. Early on, polymers with a high sorptive capacity for organic chemicals have been used in both chromatographic separation approaches and sorbent extrac-

tion techniques [1]. Most of the latter rely on adsorption of analytes to high-affinity sites of the polymer, providing high enrichment from very dilute samples while facilitating selectivity of the extraction. In tradition of liquid-liquid extraction techniques, these sorbent extractions aim at complete analyte transfer from the sample matrix. Among them, solid-phase extraction (SPE) based on polymeric sorbent particles packed into cartridges or incorporated into membranes (Empore extraction disks) is the one most commonly used.

In the past decades, enrichment techniques that employ polymers as a partitioning phase have been developed. In 1990, Arthur and Pawliszyn [2] introduced solid-phase microextraction (SPME) as a technique that builds on partitioning as working principle. Thereupon, several other polymer-based partitioning methods such as stir-bar sorptive extraction [3], immobilized liquid extraction (ILE), and thin-film extraction evolved, reviewed by Baltussen [4] and Seethapathy and Górecki [5]. Not least, partitioning of chemicals into a polymer forms the basis for many passive samplers [6, 7, 8] and chemical sensors [9].

Later, partitioning-based methods have been further developed with the objective to measure other than mass-based parameters, i.e. to determine thermodynamic measures such as the freely dissolved concentration [10, 11], fugacity [12], or chemical activity [13, 14, 15] to better understand partitioning processes. Such measurement endpoints strictly require (i) thermodynamic equilibrium and (ii) the negligible depletion criterion to be fulfilled, i.e. that the polymer does not deplete the sampled phase [16]. Furthermore, partitioning-based methods have been developed to control freely dissolved concentrations in laboratory tests, e.g. for analytical passive dosing to determine binding and speciation of hydrophobic organic chemicals at controlled freely dissolved concentrations [17, 18].

Polymers for partitioning enrichment. Amorphous polymers, for example elastomers, are especially suited as partitioning phase because they allow the accommodation and mobility of solutes within the polymer network. They function thermodynamically as a liquid phase in which a chemical species can diffuse. Elastomers have a very low glass transition temperature and consist of flexible chainlike molecules that facilitate the accommodation of solutes. Indeed, partitioning of organic chemicals into silicone has been described as an absorption process [19, 20].

Elastomers possess a wide range of affinities for organic chemicals depending on their chemical structure [21]. Their sorptive capacity has been shown to be dependent on the spatial arrangement of the partitioning domains (amorphous parts) and the degree of cross-linking [22]. Silicone polymers are among the polymers most frequently used in partitioning enrichment methods because of their suitable physical properties, thermostability, chemical inertness, and compatibility with various matrices [23]. Furthermore, silicones were shown to be of a structure that facilitates diffusion of hydrophobic organic chemicals [24]. PDMS is one of the silicones most frequently applied in analytical chemistry [5]. Another important polymer is polyethylene, especially low-density polyethylene, which is mainly used for sampling of chemicals from environmental matrices [25].

Equilibrium partitioning. Parti-

tioning of a chemical species between phases is a process driven by differences in chemical potential between the phases until these differences are balanced, i.e. chemical equilibrium is reached. Hence, given infinite bath conditions (i.e. $V_{sample}/V_{polymer} >> K_{polymer:sample}$), maximum enrichment of a chemical in a polymer can be achieved at equilibrium (equilibrium passive sampling). Often, however, polymers are employed in the kinetic uptake regime, e.g. in most passive sampling approaches or as depletive sampling phase for extraction of analytes from complex matrices [26, 4].

For two phases i and j in equilibrium, measurement of a chemical's state variable in one phase, e.g. the concentration in a polymer, can be used to deduce the chemical's thermodynamic properties in another partitioning phase. This is because thermodynamic laws dictate that at equilibrium the chemical potential of a compound, which is a natural logarithmic function of the compound's chemical activity a (unitless), is identical in all phases:

$$\mu_i = \mu_j \leftrightarrow a_i = a_j \tag{4.1}$$

Thermodynamic measures related to chemical activity such as the activity coefficient (γ) or the fugacity (f) appear therefore useful to describe and understand phase partitioning phenomena. Chemical activity is proportional to the mole fraction of a solute x (mole solute per mole solvent) with the activity coefficient γ as proportionality factor:

$$a = \gamma x \tag{4.2}$$

The solute mole fraction can be converted to concentration C (mol/L) by normalizing it to the overall molar volume V_m (L/mol) of both solute and solvent.

Fugacity is the partial pressure (P in units of Pa) that a substance exerts into an ideal gas and is linked to concentration by the fugacity capacity Z (mol/m^3 Pa):

$$Z = \frac{a}{V_m \gamma f} \tag{4.3}$$

and

$$C = Zf \tag{4.4}$$

Both chemical activities and fugacities are useful parameters to determine chemical equilibrium and form the basis for thermodynamic environmental chemical fate models [27, 28, 29].

Polymer-based partition coefficients. Commonly used partition coefficients K_{ij} reflect the concentration ratio of a solute in two phases i and j that are in equilibrium:

$$K_{ij} = C_i/C_j \tag{4.5}$$

However, partition coefficients can also be understood thermodynamically as the inverse ratio of the activity coefficients or as the ratio of the fugacity capacities of two partitioning phases i and j:

$$\hat{K_{ij}} = x_i/x_j = \gamma_j/\gamma_i = Z_i/Z_j \tag{4.6}$$

Hence, for phases in equilibrium, it is possible to project concentrations, activity coefficients or fugacity capacities from one phase to another with knowledge of the partition coefficient – an essential tool to understand chemical fate in multimedia systems.

For a large number of organic chemicals, experimentally determined polymer-based partition coefficients can be found in the literature, mostly for environmental phases like water and air. However, for a single analyte and one given polymer such partition coefficients can range over several orders of magnitude [30].

This illustrates that determining accurate and precise partition coefficients for phases of very different capacity for the solute is challenging, especially for highly hydrophobic compounds. Di Filippo et al. [30] critically assessed the quality of silicone-water partition coefficients from the SPME literature and pointed out that variation of these partition coefficients largely resulted from methodological errors but may also be due to uncertainty in fiber coating thickness and polymers source. Indeed, detailed polymer specifications are often not available from the suppliers. Rusina et al. (2007) [31] compared PAH sorption of passive sampling polymers and their study revealed that polymer-water partition coefficients for LDPE, EXACT (ethylene/octane copolymer) and PDMS differed by up to 1.3 log units for selected PAHs, whereas within a group of silicone rubbers the partition coefficients differed by at most 0.4 log-units. These ranges show major sorption differences between polymer classes and indicate that also within a polymer class, e.g. within the class of silicone polymers, the sorptive capacity of polymers can vary.

Polymers as a reference partitioning phase. Ideally, one would always use the same polymer as sampling phase in all devices, which would allow using tabulated reference partition coefficients. Moreover, it would facilitate comparing and linking measured concentrations in passive samplers on a polymer basis. In this regard, polymers can be considered a reference partitioning phase for analytical measurements in general. In consideration of the multitude of passive sampler materials available, it is common practice to determine new material-specific partition coefficients through experiments, which for environmental phases like water or air is not trivial. Such partition coefficients are difficult to determine experimentally because the partitioning phases involved exhibit extremely different solvation properties for HOCs. In this case, a polymer can offer intermediate solubility. We suggest a complementary approach to deriving new partition coefficients by using polymer-polymer partition coefficients as conversion factors to existing polymer-media partition coefficients $K_{ref:med}$ (eq 4.7a). In this regard, a well-defined polymer can serve as a reference partitioning phase (subscript ref), also to for calculating multimedia partition coefficients (eq 4.7b).

$$K_{pol:med} = K_{ref:med} \times K_{pol:ref} \tag{4.7a}$$

$$K_{med_i:med_j} = K_{ref:med_i} / K_{ref:med_j} \tag{4.7b}$$

Polymer-polymer partition coefficients quantify differences in the sorptive capacity of polymers and can be experimentally determined with high accuracy and precision. Moreover, multimedia partition coefficients can be calculated based on individual polymer-based partition coefficients: the polymer as third partitioning phase then merely serves as a connective linking phase (Figure 4.1). Such calculated partition coefficients may be superior not only for quantifying environmental multimedia partitioning but also for determining partitioning behavior of HOCs in miscible phases. Multiplication of polymer-based partition coefficients may then be less erroneous than the direct experimental determination of a partitioning coefficient.

Aims. The general aim of the study is to establish a new strategy for deriving

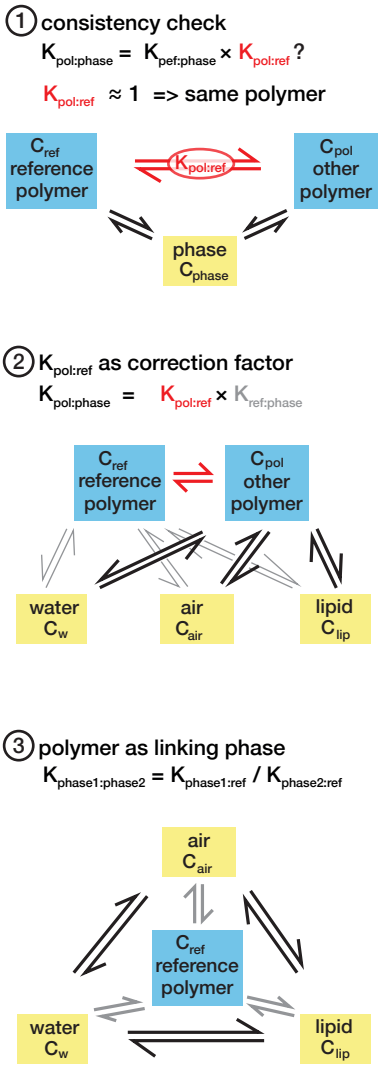

Figure 4.1. Concept maps on the application of polymers as reference partitioning phase.

partition coefficients in which a polymer serves as a reference partitioning phase in multimedia systems. More specific aims are to (1) systematically investigate partitioning of hydrophobic organic chemicals between various elastomers, i.e. silicone polymers and LDPE, that are applied in polymer-based analytical enrichment techniques, (2) apply polymer-polymer partition coefficients as conversion factors to derive new polymer-specific partition coefficients based on existing data, and (3) derive multimedia partition coefficients by using a polymer as a reference partitioning phase, facilitating a multimedia phase partitioning understanding.

4.2. Material & Methods

Chemicals. PAHs (naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene and benzo(a)pyrene), PCBs (IUPAC congeners 3, 28, 31, 40, 44, 49, 52, 99, 101, 105, 110, 118, 128, 138, 149, 151, 153, 156, 170, 180, 187, 188, 194, 198, 209), and OCPs (α-HCH, β-HCH, γ-HCH, HCB, o'p-DDT, o'p-DDE, p'p-DDT, p'p-DDE, p'p-DDD, trans-nonachlor) were obtained in purity $\geq$ 98% (detailed information, including abbreviations, can be found in the Supporting Information). The solvents methanol ($\geq$ 99.9 %), ethylacetate ($\geq$ 99.5 % for analysis), and iso-octane ($\geq$ 99.5 % for analysis) were all purchased from Merck (Darmstadt, Germany); acetone from Rathburn Chemicals Ltd. Ultrapure water (Millipore, MA, USA) was used.

Co-exposure of polymers in methanolic solution. Separate experiments were conducted for PAHs and organochlorine chemicals. The

polymeric materials were equilibrated in a methanol-water solution. A 60:40 % v/v methanol-water ratio was chosen to facilitate fast mass transfer of the chemicals and to avoid floating of the polymers. To introduce the chemicals, one spiked polymer material was added to each setup. Silicone polymers and LDPE only have minimal absorptive capacity for methanol [32, 31]: In a mixed solution of 60 % methanol and 40 % water, the fraction of methanol in the polymers was estimated to be not greater than 2 % in silicone polymers ($\leq$ 0.06 % in LDPE). Given that methanol-silicone partition coefficients of HOCs are rather low [33, 34], the mass fraction of excess analyte in the polymer due to methanol should be below 10 %. However, methanol may have a limited effect on the polymer itself as indicated from the data by Smedes et al. [34]. Swelling can increase the free volume in and the flexibility of silicone polymers [35], which in consequence may influence the sorptive capacity of the polymers. As this influence will likely be of similar magnitude for different elastomers, it will thus not have a large effect on polymer-polymer partition coefficients.

In total 14 polymeric materials were included in the study (Table 4.1), comprising silicone sheets, silicone coatings, silicone tubing, silicone-coated SPME fibers as well as lay-flat LDPE tubing. All these have been reported suitable or potentially suitable as passive sampling materials. Further, silicone materials that proofed applicable for passive dosing were included, i.e. medical-grade silicone elastomer [36] and silicone O-rings (Smith2010a), as well as silicone rods [18]. The pure PDMS membrane from Speciality Silicone Products Inc. (in the following referred to as 'SSP'), Altesil silicone sheets and/or silicone O-rings and the silicone elastomer MDX4-4210 were chosen as reference materials for cross-validation of determined partition coefficients between experiments (Table 4.2).

Polymer preparation. Whenever possible, materials were cut into pieces of ca. 20 to 100 mg (LDPE, Altesil, SSP, Biscasil, silicone tubing, silicone rods). Silicone O-rings had a highly standardized weight (223 $\pm$ 1.8 mg). Smaller O-rings (weight 46.5 $\pm$ 0.1 mg) were used in the PAH-experiment. Tubes were opened longitudinally. Nagasep silicone hollow fibers were knotted to bundles of ca. 10 mg. Silicone coated fibers were cut into pieces of 3 cm and 5 cm respectively and bundled together with a piece of copper wire. The fiber coating silicone volume was estimated to ca. 2.5 μL based on reported fiber coating thickness and total fiber length (Supporting Information, p. S4). MDX4-4210 silicone elastomer was prepared as described by Smith et al. [36] and 500.0 $\pm$ 2.5 mg were casted into 20-mL glass vials. For experiments with the DC1-2577 polymer, 10-mL glass vials were coated with the polymer: Appropriate amount of DC1-2577 polymer was diluted in pentane and a small volume of this solution was pipetted onto the side walls of pre-weighed 10-mL glass vials that laid horizontally on a roller (30 rpm; IKA ROLLER 10 basic). The vials were left rolling over night in a fume hood to allow the pentane to evaporate off and the silicone polymer to polymerize. Vials were weighed again and the exact weight of the silicone coating noted. For experiments with Silastic Type A silicone, coated glass jars (diameter = 6 cm, volume=120 mL) were used [14]. All polymers were cleaned three times

Table 4.1. Polymer materials for which partition coefficient were determined. Densities (at $23 \pm 2\,°C$) are given where available.

material name	application	polymer specification	density (g/cm^3)	supplier	polymer thickness
LDPE lay-flat tubing	air/water passive sampling	low-density polyethylene	0.930	Brentwood Plastics Inc. (USA)	70 μm
MDX4-4210 biomedical elastomer	analytical passive dosing	poly(dimethylsiloxane), dimethylvinyltermi-nated/trimethylated silica	1.10	Dow Corning (USA)	ca. 2 mm
SSP-M823 membrane	in-tissue passive sampling	poly(dimethyl)siloxane	1.12-1.16	Silicone Speciality Products Inc. (USA)	254 μm
O-ring (red)	passive dosing	silicone	1.20	Altec (UK)	1.78/2.40 mm
Polymicro-fiber	SPME	silicone	na	Polymicro Technologies Inc. (USA)	30 μm
Fiberguide-fiber	in-situ passive sampling in sediment	silicone	na	Fiberguide Industries (USA)	10 μm
Nagasep		poly(dimethyl)siloxane	na	Nagayanagi Co Ltd. (Japan)	40 μm
Biscasil	passive sampling in water	organopolysiloxane mixture	na	Biscor Ltd. (UK)	ca. 60 μm
Altesil silicone sheet (translucent)		silicone	1.20	Altec (UK)	1 mm
AM-tubing (biomedical-grade)	stir-bar sorptive extraction (SBSE)	silicone	na	A-M systems Inc. (USA)	0.80 mm
rod (white)	sorptive bioaccessibility extraction (SBE)	silicone	1.20	Altec (UK)	$\oslash = 2$ mm
rod (translucent)		silicone	na	Altec (UK)	$\oslash = 2$ mm
DC1-2577	ex-situ passive sampling of sediment	octamethyltrisiloxane/ poly(dimethyl/methyl phenyl)methoxysiloxane	0.88	Dow Corning (USA)	ca. 10 μm
Silastic Type A (Brand Medical Adhesive Silicone)		silicone	1.15	Dow Corning (USA)	ca. 8 μm

overnight in each ethylacetate, acetone and methanol. Residual solvent was allowed to evaporate off for 24 h. The exact weight or volume of each polymer was recorded.

Partitioning experiments. A summary of the partitioning experiments is provided in Table 4.2. Based on previous studies that demonstrated that the transfer of analytes from a methanolic solution into silicone can be accomplished within days [36, 37, 38] or weeks [34], equilibration times of 10 days for PAHs and 3 months for organochlorine chemicals in silicone polymers, and 6 months for organochlorine chemicals in LDPE were deemed long enough. Rather than the transport of HOCs through methanolic solution, it is likely diffusion within polymers which is the rate-limiting step for equilibration of HOCs between polymers. Rusina et al. [24] determined diffusivities of HOCs in silicone and LDPE, which suggest that diffusive equilibration within the polymers may require only a few days under the equilibration conditions chosen here, i.e. 20 °C and agitation.

In a first experiment, vials with casted MDX4-4210 PDMS silicone were spiked with PAHs. PAHs were dissolved in methanol at each 10 mg/L and 1 mL of this solution was added into each of triplicate vials to allow partitioning of the PAHs into the silicone [18]. This loading step was repeated after 72 h. After loading, the vials were repeatedly washed shortly with ultrapure water to remove any residual loading solution. Triplicates of each silicone material were placed into the spiked vials and allowed to equilibrate (Table 4.2).

In a second experiment, PCBs and OCPs were spiked into casted silicone (MDX4-4210). The chemicals were dissolved in iso-octane and then combined to a mixture with each analyte present at ca. 2 mg/L in our laboratory. The solution was up-concentrated 20-fold by reducing the solvent under a gentle stream of nitrogen. One hundred microliter of this stock solution (4 μg of each analyte) were added onto the casted silicone of seven replicate vials. The solvent was evaporated off under a gentle stream of nitrogen, which was confirmed gravimetrically. Silicone polymers and LDPE were equilibrated in such vials (Table 4.2). Finally, jars and vials with thin silicone coatings (Silastic Type A and DC1-2577 silicone) were equilibrated with selected reference polymers. The vessels were placed horizontally on a roller (IKA roller 10 basic, Germany) to ensure good contact of the methanol-water solution with the silicone coating. At the end of each experiment, the methanol-water solution was removed and polymers were collected, shortly rinsed with water to remove adhering methanolic solution, and blotted dry on lint-free tissue.

Chemical analysis. PAH concentrations were measured in both polymer extracts and methanol-water solutions. PAHs were extracted from MDX4-4210 silicone with twice 5 mL of methanol, and extracts were combined. All other polymers were extracted with excess of methanol (using ca. a 50-fold of the polymer volume). The extraction efficiency was confirmed with a second extraction with a smaller volume of methanol, which yielded concentrations generally less than 5 % of the first extract or below the limit of quantification. PAHs were analyzed by HPLC with a fluorescence detector (Agilent 1100 series, G1321A FLD). Excitation wavelength was set to 260 nm and PAHs were monitored at emission wave-

Table 4.2. Overview over polymer equilibration experiments. Highlighted are the polymers that had been spiked with the chemicals.

	polymers	analytes	equilibration conditions	setup
exp 1	**MDX4-4210**, SSP, Altesil, silicone rod, AM-tubing, O-ring, Biscasil, Polymicro-fiber	PAHs	10 days at $20 \pm 2\,^{\circ}$C, 250 rpm	3x20-mL vials, ca. 1 g total silicone mass, 10 mL methanolic solution
exp 2a	**MDX4-4210**, SSP, Altesil, silicone rod, AM-tubing, O-ring, Biscasil, Polymicro-fiber, Fiberguide-fiber, Nagasep hollow fiber	PCBs, OCPs	3 months at 20 °C, 1000 rpm	7x20-mL vials, ca. 1.25 g total silicone mass, 10 mL methanolic solution
exp 2b	LDPE, **MDX4-4210**, Altesil, O-ring	PCBs, OCPs	6 months at 20 °C, 250 rpm	3x20-mL vials (from experiment 2a), ca. 1 g total polymer mass, 10 mL methanolic solution
exp 3	DC1-2577, SSP, **Altesil**, translucent rod	PCBs, OCPs	1 month at $20 \pm 2\,^{\circ}$C, rolling	4x10-mL vials, ca. 320 mg total silicone mass, 2 mL methanolic solution
exp 4	SilasticA, SSP, **Altesil**	PCBs, OCPs	1 month at $20 \pm 2\,^{\circ}$C, rolling	3x20-mL vials, ca. 225 mg total silicone mass, 10 mL methanolic solution

lengths 350, 420, 440 and 500 nm. A CP-Ecospher 4-PAH column (Varian Inc., Palo Alto, CA) was used. Sample volumes of 30 μL were injected onto the column at 28°C and PAHs were separated by gradient elution at a constant flow rate of 0.5 mL min-1. Initially, 50:50 % v/v methanol/water was used as mobile phase (12 min). The methanol content was then increased (5 min) to reach 75 %, and thereafter further increased (28 min) to reach 100 % (15 min). Peaks were quantified using Agilent Chemstation software (version B.03.01) with a nine-point external calibration curve.

For measurement of PCB and OCP concentrations, polymers were extracted with iso-octane using a volume equaling at least a 20-fold of the polymer volume, and adding CB-55 as recovery standard. The extractions were repeated with the same volume as in the first extraction, and the extracts were finally combined. Recoveries confirmed extraction efficien-

cies between 96 % and 121 % (average 103 %). Aliquots of the combined extracts were transferred into GC-vials and CB-155 added as internal standard for quantification. Samples were analyzed by GC-μECD (Agilent 7890A) equipped with a pre-column (SGE Analytical Science) and dual columns (J&W DB-5: 60 m x 250 μm x 0.25 μm and DB-1701: 60 m x 250 μm x 0.25 μm) for separation. Hydrogen was used as mobile phase. Samples were injected at 270°C (1 μL on each column, splitless); equilibration time was set to 1 min. The temperature program was as follows: 90°C (1 min), 90 to 180°C ramped 25°C/min (hold time 2 min), 180 to 238°C ramped 1.5°C/min (hold time 2 min), 238 to 280 °C ramped 3°C/min (hold time 10 min). Chromatograms were quantified based on internal standard signals and nine-point external calibration curves in Agilent's Chemstation software.

QA/QC measures. The GC method for the analysis of PCBs and the OCPs is accredited (ISO 17025) and subject to regular in-house quality control measures and inter-laboratory comparisons. It is used for regular monitoring of chemicals in various environmental matrices. Limits of quantification using this method were determined to be in the range 0.22-0.35 ng/mL, except for CB-3 (8.8 ng/mL). The measured concentrations in the samples were typically 100-fold above the detection limit. For analysis of PAHs by HPLC-FLD, direct injection of polymer extracts or methanol-water samples, hence minimal sample manipulation, has earlier proved to provide data of very high precision [18]. External HPLC standards showed excellent linearity in the concentration range from 0.1 to 1000 ng/mL. PAH concentrations in the samples were in the range of approximately 20-200 ng/mL. Solvent blanks were included in both HPLC and GC analyzes, in which the analytes could not be detected or else were below the limit of quantification.

Data treatment. For the PAH dataset, concentrations in the measurement replicates of each vial (n=3) were first averaged (CV $<$ 3 %) and the true replicates (concentrations in polymers from separate vials) used to determine polymer-polymer partition coefficients (eq 4.8). Polymer-polymer partition coefficients were determined as the ratio of the measured analyte concentrations in polymers (C_{pol}) of each replicate vial and then averaged:

$$K_{pol.x:pol.y} = \frac{\sum_{i=1}^{n} (C_{pol.x_i}/C_{pol.y_i})}{n} \tag{4.8}$$

For the PCB dataset, an outlier test was performed on the analyte concentrations in replicate polymers (n = 4 to 7) prior to calculation of partition coefficients. Values outside the 95 % probability interval for a normal distribution with the given mean concentration and its standard deviation were excluded (177 out of 3570). Concentrations in replicate polymers had relative standard deviations typically between 2-5 %, except for the analytes β-HCH, o'p-DDT and p'p-DDT ($CV > 10\%$ for most polymers in all experiments), which thus were excluded from the entire data set. Relative standard deviations in analyte concentrations in polymers equilibrated in Silastic Type A-coated jars (experiment 4) were generally higher than 5% and are shown as tentative results.

The mass balance for each chemical in the individual experimental setups was calculated as the ratio of total recovered analyte mass and the initially spiked analyte mass. Concentrations

in the methanol/water phase were only measured in the PAH-experiment, and else were estimated using polymer-solvent partition coefficients from Smedes et al. [34]. These coefficients were not available for DDT isomers and their metabolites (DDX), HCH isomers, TNC and CB-3, CB-40, CB-188, CB-194, CB-198, CB-209), for which then only the mass fraction in the polymer phase was calculated. Recoveries were compared across all experiments for each analyte, and in case recoveries fell significantly below the overall median (deviation > 5x the median absolute deviation [MAD]), the mass balance was deemed incomplete and data were excluded (i.e. DDE-isomers, p'p-DDD, TNC, α-HCH, CB-3 and CB-40 in polymers equilibrated in Silastic-coated jars).

Polymer-polymer partition coefficients for the chosen reference polymers ($K_{Altesil:SSP}$, $K_{O-ring:MDX4-4210}$, $K_{Altesil:ring}$, $K_{Altesil:MDX4-4210}$) from separate experimental setups (excluding SSP-Altesil partition coefficient from Silastic Type A-coated jars) showed agreement within a 10 % margin of deviation and thus were averaged. The uncertainty of these pooled values was determined as the between-experiment variation by calculating the relative standard deviations (RSDs), as the within-experiment variability had proved to be very small.

For materials that were not equilibrated together, partition coefficients were calculated as the product of polymer-polymer partition coefficients for the reference polymers Altesil, SSP and/or MDX4-4210 and silicone O-rings (eq 4.7). Because polymers were equilibrated with at least two reference polymers, calculated polymer-polymer partition coefficients could be cross-validated through comparison of the estimates obtained via different reference polymers. These estimates were averaged. Uncertainties of calculated polymer-polymer partition coefficients were estimated by error propagation of the input data (Supporting Information, p. S4).

4.3. Results & Discussion

Mass balance. Recoveries of PAHs ranged from 87 % to 112 % (average 102 %, CV 7 %). For PCBs and HCB for which a complete mass balances could be calculated, recoveries ranged between 87 % and 119 % (average 99 %, CV 5 %). For the remaining analytes, the mass fraction in the polymers was between 79 % and 110 % (on average 92 %, CV 7 %), except for CB-3, p'p-DDD, and HCH isomers for which recoveries were between 60-76 %, 58-88 % or < 42 %, respectively. These values seem reasonable, considering that the methanol/water phase is neglected in the mass balance, and that the respective polymer-water partition coefficients are very low. For example, partition coefficients for HCH isomers [39, 38] suggest a distribution where up to 60 % of analyte will be in the methanol/water phase.

Polymer-polymer partition coefficients. Polymer-polymer partition coefficients were compiled for 13 silicone polymers and one LDPE material for 10 PAHs, 7 OCPs and 25 PCB congeners. The final data set includes 1988 experimentally determined polymer-polymer partition coefficients and 1074 calculated polymer-polymer partition coefficients (not considering the inverse $1/K_{pol:pol}$), which are tabulated in the Supplementary Material. Polymer-polymer partition coefficients were determined with high ac-

curacy, as data from independent experiments proved to be in very good agreement (Figure S1 and S2 in Supporting Information). Precision of the experimentally determined partition coefficients was very high with relative standard errors (RSE) < 5 % for the majority (96 %) of the data, and RSEs not larger than 11 %. Also calculated polymer-polymer partition coefficients exhibited excellent precision: RSEs were not larger than 8 %, which demonstrates that multiplication of polymer-polymer partition coefficients can result in precise estimates. Note that uncertainty was also reduced by cross-validating calculated values via two different reference polymers.

Polymer-polymer partition coefficients ranged within 0.834 log units from zero depending on the polymer combination (Figure 4.2, an enlarged version of subfigure A is available in the Supporting Information). Maximum values were reached for the silicone polymer DC1-2577 ($0.105 < |\log K_{DC:silicone}| < 0.834$) and LDPE ($0.0007 < |\log K_{LDPE:silicone}| < 0.724$) each in combination with all other silicone polymers. Without these two materials, polymer-polymer partition coefficients spanned within 0.247 log units from zero. Three groups of polymer-polymer partition coefficients can be distinguished (Figures 4.2B and 4.2C): (1) For identical polymers, $\log K_{pol:pol}$ are expected to lie within a very narrow range around zero. (2) For polymers of the same chemical structure whose volume however is poorly defined, e.g. due to the presence of fillers, $\log K_{pol:pol}$ are expected to show a constant offset from zero for all analytes. (3) For polymers differing in chemical structure, $\log K_{pol:pol}$ values are expected to be analyte-specific and hence to span over a wider range.

$\log K_{pol:pol}$ taking a value in the narrow range around zero (Figure 4.2B1) were identified as partition coefficients for polymer combinations of MDX4-4210, SSP and Nagasep hollow fibers, silicone rods (Altec), and biomedical silicone tubing – the first two materials being specified as pure PDMS polymers by the manufacturers. These results suggest that Altec silicone rods, both white and translucent, and the silicone tubing may consist of PDMS, which however will need to be confirmed by polymer analysis. Notably, Altesil as well as the Polymicro-fibers and Fiberguide-fibers in combination yielded partition coefficients in a narrow range from zero (Figure 4.2C-I) but not in combination with identified PDMS-like materials (Figure 4.2A), indicating that Altesil silicone polymer and the silicone fiber coatings may be similar in chemical structure, yet different from pure PDMS.

For silicone O-rings and Biscasil each in combination with PDMS or PDMS-like polymers, partition coefficients showed an offset of 0.1 to 0.2 log units from zero that was constant across all analytes for given polymer combinations (Figure 4.2B-II). Both silicone O-rings and Biscasil are composite materials. Silicone O-rings were analyzed by scanning electron microscopy and were shown to contain diatomeous earth as filler in the polymer matrix likely to improve the stability of the polymer. The rings also contain iron oxide, which gives the rings their red color. SEM analysis of Biscasil revealed the presence of $CaCO_3$ in the polymer matrix (Figure S4 in Supporting Information).

Biscasil is a material consisting of silicone coated onto glass fiber fabric; hence, the exact polymer volume is unknown. This may have caused a further off-

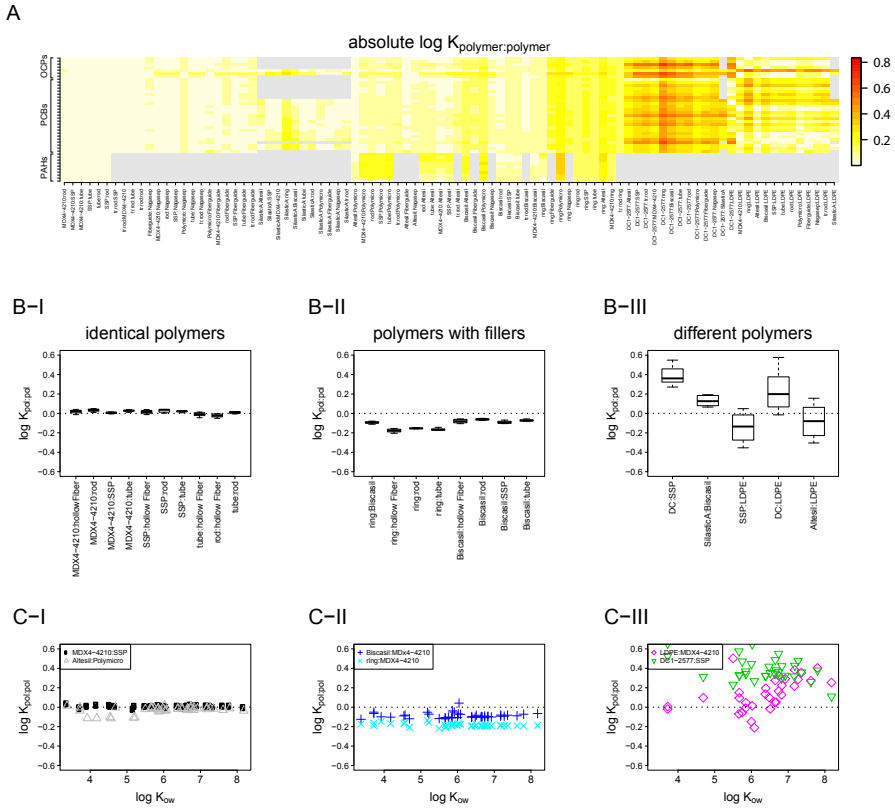

Figure 4.2. Polymer-polymer partition coefficients. (A) Visual representation of partition coefficients in a heatmap with light colors indicating similar sorptive capacity of polymers (spaces filled grey). (B) Data summarized across analytes allowed distinguishing three groups: identical polymers (I), polymers with fillers (II), and polymers of different chemical structure (III). Boundaries of boxes represent the 25th and 75th percentile with the median indicated; whiskers show the 10th and 90th percentiles. (C) Example data are plotted against log K_{ow} to facilitate an analyte-specific analysis of polymer-polymer partition coefficients.

set of polymer-polymer partition coefficients and added measurement uncertainty. In fact, such uncertainty is intrinsic for any volume-based measurements on thin polymer coatings. Also the coating thickness of silicone-coated fibers has been shown to vary [40], which is reflected in the present polymer-polymer partitioning dataset: (1) an offset of log $K_{pol:pol}$ from zero can be observed, e.g., for coated fibers, Biscasil and the coating polymers Silastic Type A in combination with polymers of defined mass, and (2) associated standard deviations for those partition coefficients are higher than standard deviations of polymer-polymer partition coefficients for polymers of known mass. For example, relative standard deviations (RSDs) of polymer-polymer partition coefficients for combinations of the two fibers and Biscasil (n=106) were for 56 % of the data > 5 %. In summary, partition coefficients involving polymers that contain fillers or polymers whose volume is uncertain may deviate by a constant factor from the true value. Such deviation can be quantified by equilibrating such polymers with identical polymeric material of defined volume. Determined polymer-polymer partition coefficients can then be used as universal conversion factors.

Third, there was a group of polymer-polymer partition coefficients that showed a non-constant offset from zero. Across analytes, log $K_{pol:pol}$ spanned more than 0.1 log-unit for given polymer combinations (Figure 4.2B-III), reflecting analyte-specific partitioning. Such behavior is typical when the involved partitioning phases interact differently with the solutes, i.e. when activity coefficients of the compounds differ between polymers. As expected, this was the case for combinations of silicone polymers and LDPE. The present data clearly show that also DC1-2577 and Silastic Type A in combination with PDMS materials exhibit such a wide range of log $K_{pol:pol}$. We therefore have to conclude that their polymer structure must differ from the PDMS structure. Indeed, the material specifications for DC1-2577 reveal that the polymer is not a pure PDMS silicone polymer. Possibly the presence of phenyl groups in the structure may be responsible for the higher affinity of more hydrophobic solutes to DC1-2577 compared to PDMS. Altesil in combinations with all other PDMS or PDMS-like materials also showed a rather wide span of log $K_{pol:pol}$ across all analytes with interquartile ranges larger than 0.05 log-units, which suggests that functional side groups other than methyl-groups may be attached to the siloxane backbone of this silicone polymer. Polymer-polymer partition coefficients when plotted against the octanol-water partition coefficient (log K_{ow}) can reveal a detailed picture of how polymers differ in their capacity to absorb HOCs (Figure 4.2C). For example, increasing $K_{pol:pol}$ with increasing log K_{ow} may indicate hydrophobic affinity sites in one polymer that are not present in the other polymer. This seems to be the case for LDPE compared to PDMS, which is in agreement with earlier findings [31]. Interestingly, also Altesil and the Polymicro-fibers showed a greater affinity than PDMS for more hydrophobic PAHs, but not for PCBs.

New partition coefficients. Applying polymer-polymer partition coefficients as conversion factors, new polymer-specific partition coefficients can now be derived from existing experimentally determined partition coefficients (eq 4.7). Accuracy of such derived coefficients will

Table 4.3. Calculated polymer-specific and multimedia partition coefficients (extract from tables in SI).

	$K^{a}_{lip:LDPE}$ g/g	$K^{b}_{lip:DC}$ g/g	log $K^{c}_{DC:w}$ L/kg	log $K^{d}_{PDMS:air}$ L/kg	log $K^{e}_{lip:w}$ L/kg	log $K^{f}_{air:w}$ L/L
HCB	4.52	8.98	5.25	6.37	6.22	-1.31
CB-28	12.95	8.64	5.79	7.11	6.74	-1.63
CB-31	14.53	8.65	5.74	–	6.70	–
CB-44	18.95	5.69	6.20	7.68	6.97	-1.92
CB-49	16.50	8.41	6.15	–	7.08	–
CB-52	18.96	7.92	6.11	7.56	7.01	-1.80
CB-99	16.22	12.08	6.63	–	7.71	–
CB-101	18.38	11.93	6.53	8.11	7.62	-1.88
CB-105	17.66	10.81	6.81	8.47	7.85	-2.13
CB-110	18.25	9.01	6.64	–	7.59	–
CB-118	16.00	14.65	6.70	8.40	7.88	-2.04
CB-128	18.94	7.80	7.21	8.70	8.11	-2.02
CB-138	19.50	12.36	7.11	8.69	8.20	-1.99
CB-149	22.33	10.58	6.95	–	7.98	–
CB-151	19.66	10.09	6.88	–	7.88	–
CB-153	18.39	17.08	6.98	8.56	8.22	-1.89
CB-156	18.74	16.87	7.08	8.74	8.31	-2.08
CB-170	20.78	13.14	7.50	9.07	8.62	-2.04
CB-180	19.73	19.66	7.29	9.08	8.58	-2.14
CB-187	18.29	14.55	7.14	9.03	8.31	-2.20

[a] $K_{lip:LDPE} = K_{lip:SSP} \times K_{SSP:LDPE}$ ($K_{lip:SSP}$ from Jahnke et al. [41])
[b] $K_{lip:DC} = K_{lip:SSP} \times K_{SSP:DC}$ ($K_{lip:SSP}$ from Jahnke et al. [41])
[c] $\log K_{DC:w} = \log K_{Altesil:w} + \log K_{DC:Altesil}$ ($K_{Altesil:w}$ from Smedes et al. [34])
[d] $\log K_{PDMS:air} = \log K_{LDPE:air} + \log K_{MDX:LDPE}$ ($K_{LDPE:air}$ from Khairy et al. [42]; note that MDX4-4210=PDMS)
[e] $\log K_{lip:w} = \log K_{lip:SSP} + \log K_{Altesil:w} + \log K_{SSP:Altesil}$ ($K_{lip:SSP}$ from Jahnke et al. [41] and $K_{Altesil:w}$ from Smedes et al. [34])
[f] $\log K_{air:w} = \log K_{Altesil:w} - \log K_{LDPE:air} + \log K_{PDMS:Altesil} - \log K_{PDMS:LDPE}$ ($K_{Altesil:w}$ from Smedes et al. [34] and $K_{LDPE:air}$ from Khairy et al. [42])

largely depend on the input data, whose quality should be cross-validated. New polymer-based partition coefficients are listed in Table 4.3 and include lipid-LDPE and PDMS-air partition coefficients for HOCs. Jahnke et al. [41] determined polymer-lipid partition coefficients for thin silicone sheets, yet for LDPE such data have never been reported. Conversely, polymer-air partition coefficients for a larger set of organic chemicals have so far only been determined for LDPE and polyurethane foam (PUF) but are lacking for silicone, although silicone has been suggested as promising sampling phase for diffusive passive air sampling of volatile and semi-volatile organic chemicals [43].

Also multimedia partition coefficients were computed based on polymer-based partition coefficients (Table 4.3), whereby the polymer phase then merely served as reference partitioning phase. Using such approach, it is especially important that any differences in the polymers' sorption capacity for HOCs be numerically consid-

ered, i.e. quantified through a polymer-polymer partition coefficient. As an example, we computed lipid-water partition coefficients ($K_{lip:w}$) and air-water partition coefficients ($K_{air:w}$) as product of lipid-polymer [41] and polymer-water [34] or polymer-air [42] partition coefficients, respectively, and corrected with the appropriate polymer-polymer partition coefficients for sorption differences between polymers (eq 4.7).

Comparison to literature data. The derived partition coefficients $K_{LDPE:w}$, and $K_{lip:w}$ when compared with partition coefficients from the literature generally show good agreement (Figure 4.3). This demonstrates that polymer-polymer partition coefficients can be applied to translate from one to another polymer-specific partition coefficient with high accuracy and supports the conceptual framework outlined in Figure 4.1. Calculated LDPE-water partition coefficients deviated not more than 0.5 log units from LDPE-water partition coefficients reported by Choi et al. [44], Smedes et al. [34] and Fernandez et al. [45] (Figure 4.3A). Notably, Fernandez et al. had obtained LDPE from a different source whereas all other LDPE partition coefficients were determined for LDPE from Brentwood Plastics. Also, the here calculated PDMS-air partition coefficients, which are based on experimentally determined LDPE-air and LDPE-PDMS partition coefficients, differ not more than 0.3 log units from experimentally determined values (CB-28, CB-52, CB-101 and HCB) given by Sprunger et al. [46].

Calculated lipid-water partition coefficients in contrast showed only partial agreement with data from the literature (Figure 4.3). Several reasons can be identified: (i) Partition coefficiencts are temperature-dependent. Hence, lipid-water partition coefficients determined at higher temperatures are expected to be smaller. For that reason, partition coefficients reported by Quinn et al. [47] were temperature-corrected (Supporting Information, p. S5) using the Van't Hoff relationship and partitioning enthalpies that were calculated as suggested by Geisler et al. [48]. (ii) Partitioning phases must be well-defined. In the case of lipid-water partition coefficients compared here, different lipid surrogates were used: our derived partition coefficients refer to a nonspecific storage lipid because storage lipids generally show similar capcacity for accommodating HOCs [41, 48]. Jabusch et al. [49] worked with triolein, a chemically well-defined triglyceride, whereas Geisler et al. calculated general partition coefficients for storage lipids based on polyparameter linear free energy relationships. (iii) Finally, the number of conversion steps determines accuracy and precision of calculated partition coefficients: Here, lipid-water partition coefficients were calculated as the product of three terms, namely a polymer-water partition coefficient, a polymer-lipid partition coefficient and a polymer-polymer partition coefficient as conversion factor.

Application. Polymer-polymer partition coefficients will aid calculating multimedia partitioning coefficients such as for lipid-water, sediment-water or water-air partitioning, which within environmental chemistry can help understanding chemical fate and thermodynamic phase relationships. Including a polymer as a third phase may also be practical when determining partitioning coefficients for HOCs between miscible phases, e.g. mixed solvents. The main advantage would

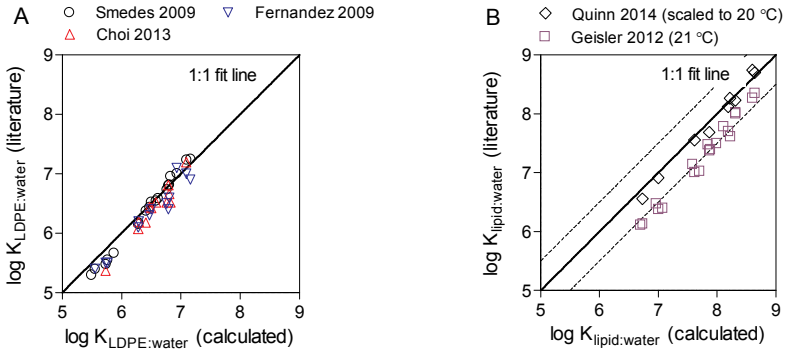

Figure 4.3. Comparison of partition coefficients from this study with partition coefficients from the literature. Partition coefficients were calculated as the product of reported polymer-based partition coefficients at 20 °C and specific polymer-polymer partition coefficients from this study.

thereby be that HOCs are easy to measure in a solid-state polymer phase, which would not mix with the other phases. Finally, polymer-polymer partition coefficients when applied as polymer-specific conversion factors to literature values allow for a consistency check of published partition coefficients. Differences between partition coefficients that exceed the magnitude of the specific polymer-polymer partition coefficient will reveal inconsistencies and systematic errors. Polymer-polymer partition coefficients may therefore serve as tool to scrutinize existing partition coefficients and improve quality assurance.

Implications. Availability, accuracy and precision of partition coefficients are essential components for progress in research within analytical chemistry. Deriving new partition coefficients that are accurate and precise via polymer-polymer partition coefficients will therefore have great value especially within passive sampling of organic chemicals. Just as new passive sampling materials widen the possibilities for the field application of passive samplers can the availability of partition coefficients also extend the extrapolation possibilities. For example, recently developed coated jars for passive sampling of whole sediment [14, 50] enabled to target measurements at freely dissolved concentrations, which however required the availability of partition coefficients specific for the silicone polymer used. Such polymer-specific partition coefficients can now be easily obtained by applying polymer-polymer partition coefficients as conversion factors to existing data. In the case of polymer-water partition coefficients, this will facilitate a more accurate determination of freely dissolved aqueous concentrations [50]. Beyond that, they will also facilitate other phase translations, e.g. the translation of

measured concentrations in passive samplers to equilibrium partitioning concentrations in lipids, which may be relevant in a regulatory context for assessing bioaccumulation potential of HOCs or be of interest for toxicologists. PDMS-air partition coefficients are presented within this study in prospect that they will advance the development and application of silicone-based passive air samplers.

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Manuscript II

Endocrine activity of persistent organic pollutants accumulated in human silicone implants – dosing *in vitro* assays by partitioning from silicone

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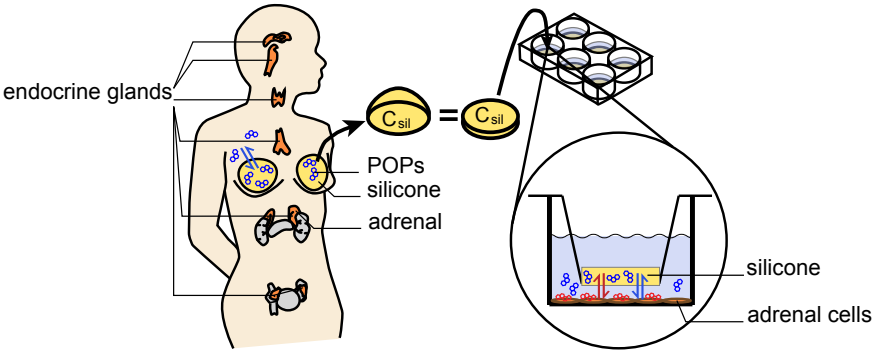

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Chapter 5

Endocrine activity of persistent organic pollutants accumulated in human silicone implants – dosing *in vitro* assays by partitioning from silicone

Abstract

Persistent organic pollutants (POPs) accumulated in human tissues may pose a risk for human health by interfering with the endocrine system. This study establishes a new link between actual human internal POP levels and the endocrine active dose *in vitro*, applying partitioning-controlled dosing from silicone to the H295R steroidogenesis assay: (1) Measured concentrations of POPs in silicone breast implants were taken from a recent study and silicone disks were loaded according to these measurements. (2) Silicone disks were transferred into H295R cell culture plates in order to control exposure of the adrenal cells by equilibrium partitioning. (3) Hormone production of the adrenal cells was measured as toxicity endpoint. 4-nonylphenol was used for method development, and the new dosing method was compared to conventional solvent-dosing. The two dosing modes yielded similar dose-dependent hormonal responses of H295R cells. However, with the partitioning-controlled freely dissolved concentrations (C_{free}) as dose metrics, dose-response curves were left-shifted by two orders of magnitude relative to spiked concentrations. Partitioning-controlled dosing of POPs resulted in up to 2-fold increases in progestagen and corticosteroid levels at C_{free} of individual POPs below the femtomolar range. Silicone acted not only as source of the POPs but also as a sorption sink for lipophilic hormones, stimulating the cellular hormone production. Methodologically, the study showed that silicone can be used as reference partitioning phase to transfer *in vivo* exposure in humans (silicone implants) to *in vitro* assays (partition-controlled dosing). The main finding was that POPs at the levels at which they are found in humans can interfere with steroidogenesis in a human adrenocortical cell line.

5.1. Introduction

Exposure and effects of POPs. Humans and wildlife are continuously exposed to chemicals among which persistent organic pollutants (POPs) are

of special concern because they resist metabolism and accumulate in animals and humans. POPs have for long been included in biomonitoring campaigns [1], which allow linking local contamination sources to increased human body burdens and enable monitoring of environmental POP levels over time [2]. However, the complex composition of human tissue and matrices challenges not only chemical analysis but also the translation of measurements to a well-defined and meaningful exposure parameter. For instance, lipid normalization assumes lipids to be the only sorption phase and disregards differences in lipid composition [3]. A recent study by Allan et al. (2013) explored the feasibility of measuring POPs in explanted human silicone prostheses. Silicone is a well-defined partitioning phase, hence minimizing interfering matrix effects and facilitating chemical analysis and data interpretation. Typically, silicone implants remain in the body for a time long enough for equilibration of POPs with the silicone. Allan et al. found good correlation between concentrations of measured POPs in silicone and lipid-normalized biomonitoring data, and therefore proposed to include silicone samples in regular human biomonitoring.

Concern has been raised that exposure to POPs may lead to adverse human health effects. POPs such as PCBs, PBDEs, and organochlorine pesticides were shown to cause multiple forms of toxicity including immunotoxicity, carcinogenicity, neurotoxicity, as well as reproductive and developmental toxicity through endocrine activity. Endocrine disruption may be the result of perturbation in the synthesis, metabolism, transport and/or receptor binding of hormones. POPs showing structural similarities to endogenous hormones can bind to steroid receptors and evoke hormone-mediated responses (e.g. [4]). They can also indirectly trigger endocrine responses through binding to the aryl hydrocarbon receptor (e.g. [5]). Also non-receptor mediated effects on the endocrine system have been observed, e.g., through disturbed hormone synthesis [6]. Evidence for a causal relationship between human tissue residues of POPs and endocrine dysfunction is however very limited, in part because linking *in vitro* and *in vivo* exposure is difficult, and because the complexity of the endocrine system cannot be captured in experimental models.

Mixture toxicity. Importantly, the toxicity of single chemicals can be modified when present in a mixture through toxicokinetic interactions with co-solutes, solubility addition [7] or a joint action at the target site, which can enhance or inhibit the effect of endogenous hormones [8, 9]. It has also been shown that minor changes in mixture composition can cause larger shifts of endocrine responses [10]. Several studies investigated the combined effect of POPs on the endocrine system but only few have approached mixture toxicity testing with the aim to link actual human exposure levels to endocrine effects. Yet, experimental approaches for testing POPs at environmentally relevant concentrations have been presented: Several authors followed the strategy of extracting POPs from environmental matrices like water [11], sediment [12], or animal tissue (e.g. [13, 14]) and subsequently testing the collected extract *in vitro*. Nonetheless, only the mixture that is present in human tissue reflects the actual internal human exposure. Therefore chemical extracts from human matrices have also been subjected to *in vitro*

testing (e.g. [5]). While all these approaches facilitate an effect-directed analysis and testing of non-target chemicals, their main shortcomings are that (i) the mixture composition will be operationally defined and (ii) final test levels in the *in vitro* test cannot readily be compared to levels in the original sample matrix without further phase partitioning considerations.

In vitro tests. The majority of *in vitro* assays for screening endocrine disrupting effects of chemicals aims at detecting hormone receptor activity. Complementary to that, the H295R assay has been developed specifically to investigate possible interferences of chemicals with steroidogenesis [15]. The assay uses the human adrenocortical carcinoma cell line H295R [16], in which the entire adrenal steroidogenesis pathways are conserved. H295R cells can produce all the hormones that are found in the adult adrenal cortex and the gonads, what makes them a unique *in vitro* model to study disturbances of steroidogenesis. Developments of the assay over the past decade have resulted in a standardized test protocol for testing endocrine disruptive effects of chemicals [17, 15]. However, very hydrophobic chemicals are difficult to introduce into *in vitro* tests. Metabolism, evaporation, degradation, and sorption to surfaces of the vessel and to medium constituents can cause substantial losses of test chemicals, and hence non-constant exposure [18, 19]. Often hydrophobic chemicals are introduced using a co-solvent, which can lead to unrealistically high test concentrations above the solubility limit. Moreover, a solvent may alter a chemical's interaction with the biological target [20] and can itself trigger responses of the biological test system, even at very low concentrations [21]. Besides, testing of hydrophobic chemicals requires careful definition of the dose beyond nominal concentrations because it is primarily the freely dissolved chemical that is thought to be toxicologically active rather than the bound form of a chemical.

Passive dosing. To overcome these dosing challenges, partitioning-based dosing methods have been developed, also known as "passive dosing", which apply silicone as a reservoir for hydrophobic test chemicals in *in vitro* test systems [22]. Exposure is then controlled by equilibrium partitioning from silicone, and maintained constant. Passive dosing represents a superior dosing technique particularly for testing mixtures of POPs. For example, Rojo-Nieto et al. [23] recreated the seawater mixture composition of polycyclic aromatic hydrocarbons (PAHs) in an aquatic toxicity test, and Schmidt et al. [24] applied passive dosing for controlling both level and composition of PAH mixtures. Most recently, Claessens and coworkers [25] applied passive dosing using silicone sheets that before had been employed as passive equilibrium samplers such that the chemical mixture from the marine environment could be directly transferred into an algal toxicity test.

Passive dosing has successfully been applied and tailored toward various standard *in vitro* assays [26, 27, 28, 29] and has shown to improve the sensitivity of the assays while facilitating a better understanding of the obtained toxicological data.

In the present study, passive dosing was applied to reconstitute *in vivo* human POP exposure levels in an *in vitro* system by spiking silicone with POPs to levels as found in explanted human breast

silicone prostheses [30]. Thereby silicone functions not only as dosing donor for the POPs but also as a reference partitioning phase, allowing linking in vivo and in vitro exposure.

Aim of the study. This study aimed to investigate possible effects of internal human POP levels on human steroidogenesis. More specific aims were to (1) establish passive dosing in the *in vitro* H295R assay using 4-nonylphenol as a model chemical, (2) explore the effect of silicone as a sorption sink for steroid hormones, and (3) directly link human *in vivo* levels of POPs to endocrine effective concentrations *in vitro* using silicone as a reference partitioning phase.

5.2. Methods

Chemicals and materials. A technical mixture of 4-nonylphenol isomers (CAS-no. 84852-15-3; purity *geq* 99 %) was obtained from Acros Organics (Germany). PCB 52, 101, 105, 110, 118, 138, 153, 180, p'p-dichlorodiphenyltrichloroethane (p'p-DDT), p'p-dichlorodiphenyldichloroethylene (p'p-DDE) and hexachlorobenzene (HCB) were in purity *geq* 99 % and purchased from Promochem (Germany) and Riedel-de-Haën (Germany). PBDE 47, 99, 100, 153, 154 came from Cambridge Isotope Laboratories (USA) in purity *geq* 98 %. Prochloraz (95 %) and forskolin (*geq* 98 %) were obtained from Ehrenstorfer (Germany) and Sigma-Aldrich (Denmark), respectively. All solvents were obtained at highest purity from Merck (Germany) or Rathburn Chemicals (Scotland). Dimethyl sulfoxide (DMSO) (*geq* 99.5 %) was from Sigma-Aldrich (Denmark). Ultrapure water from a Milli-Q Integral 5 water purification system (Merck Millipore, USA) was used. Silicone disks (ø = 10 mm) were cut from translucent silicone sheets (Altesil; Altec Products Ltd., UK) in thicknesses of 1 mm (84.1 ± 1.5 mg), and 2 mm (on average 192.8 mg, CV=2 %, n=75). The thicker disks were used in all final experiments, offering a higher sorption capacity for nonpolar hormones and better stability in the inserts. Disks were washed with mild detergent, rinsed with water and then solvent-washed by soaking three times over night in each ethylacetate, acetone and ethanol. Finally, they were washed three times in sterile ultrapure water.

Loading of silicone disks. The loading procedure is described in the Supplemental Material (p. 119). Briefly, disks were spiked with a methanolic solution of 4-NP or with an acetonic solution of POPs. The solvent was allowed to evaporate off, forcing the chemicals into the silicone [31]. This yielded concentrations of 4-NP between 193-6173 μmol/kg$_{silicone}$. POP levels in silicone disks covered the concentration range found in human silicone breast implants (Figure 5.1), ranging from 1.93-1931 Σnmol/kg$_{silicone}$ (0.638-638 Σng/g$_{silicone}$). Note that partitioning of POPs from the passive dosing silicone will be similar to that from breast implant silicone (likely a mixture of silicone polymers), as the sorption capacity of different silicone polymers typically differs by less than a factor of three (chapter 4 23; unpublished results). Control disks were loaded with pure solvent. Triplicate disks loaded to the same dosing level (including solvent controls and blanks) were placed together in one vial and pre-equilibrated with the cell culture medium (4 mL each) for 24 h at 1200 rpm at room temperature, protected from

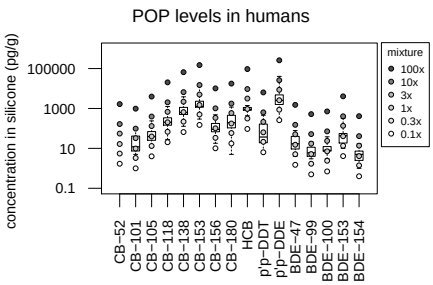

Figure 5.1. POP concentrations in silicone in the tested mixtures in this study (circles) and in human breast silicone prostheses (boxes) as published by Allan et al. 2013 [30]. Borders of boxes indicate the 25th and 75th percentiles; whiskers extend from the minimum to the maximum. Note: CB-52 was included in the test mixture but not measured by Allan et al.

light. Using silicone-water partition coefficients ($K_{sil:w}$), concentrations in silicone ($C_{silicone}$) were converted to equilibrium partitioning concentrations in pure water (C_{free}) using equation 5.1 (see Supplemental Material, Table S1).

$$C_{free} = C_{silicone}/K_{sil:w} \qquad (5.1)$$

H295R cell culture. H295R cells were cultured as described previously [32, 15]. Briefly, the cells were obtained from the American Type Culture Collection (ATCC CRL-2128 NCI-H295R, LGC Standards, Sweden) and cultured under sterile conditions at 37 °C in a humidified 5 % CO2 atmosphere in Dulbecco's Modified Eagle Medium/Ham's F-12 nutrient mixture with added HEPES buffer and L-glutamine, without phenol-red (InVitrogen, Denmark) to which 2.5 % NuserumTM (BD Bioscience, USA) and a mixture of insulin, transferrin and selenous acid (ITS-premix; VWR, Denmark) was added (1 %). Only cells between the fifth and the twelfth passage were used. For experiments, cells were seeded at a density of 3×10^5 cells per well in flat-bottom 24-well cell culture surface-treated plates (Corning®Costar®) and after 24 h the culture medium was renewed and exposure initiated.

Exposure. A new setup for *in vitro* passive dosing was developed based on the format described by Kramer et al. [27]: Instead of placing silicone disks in the bottom of cell culture plates, we seeded cells on the bottom of the wells following the standard OECD protocol, and employed CellCrown®well plate inserts (Scaffdex Oy, Finland) as a hanging silicone disk holder (see Supplemental Material, Figure S1). Initially, we investigated to which degree silicone can function as a sorption sink for steroid hormones, and whether the presence of silicone affects cell viability: Control wells were filled with cell culture medium (1.275 mL), whereas treatment wells in addition received a silicone disk (84 mg). Cells were incubated for 48 h as described above. This experiment (n=4 in each treatment group) was conducted three times: in the first two runs immunoassays were used to quantify solely estradiol and testosterone, while in the last run LC-MS/MS was used for quantification of nine steroid hormones (see below).

For passive dosing of 4-NP and the POP-mixture, the cell culture medium was replaced with the pre-equilibrated medium (1 mL) at the day of exposure start and prepared silicone disks (193 mg) were placed into the wells; also the inserts were filled with pre-equilibrated medium (250 μL). Both solvent-treated and blank

silicone disks were included as controls. Each treatment was tested in triplicates. In parallel, cells were exposed to chemicals by traditional DMSO-spiking following the OECD protocol with the modification that a total of 1.25 mL of culture medium was added to each well. Intermediate dosing solutions were prepared in the cell culture medium. The final DMSO concentration in the test was 0.1 % vol/vol. 4-NP was dosed via DMSO at nominally 0.3, 0.9, 2.8, 9.4 and 28.3 μmol/L$_{medium}$. The POP-mixture was tested by DMSO-dosing at six concentrations in the range 0.87-894 Σnmol/L$_{medium}$. For each cell batch tested, a quality control plate was processed using prochloraz and forskolin as positive controls [15]. Each treatment was tested in triplicate wells, and DMSO-controls in six replicate wells of each plate. The experiments with both 4-NP and the POPs were repeated twice on different days, resulting in three independent data sets.

All incubations were terminated after 48 h by collecting the exposure medium (and silicone disks) and replacing it with 0.5 mL of fresh medium for the cell viability MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously [32].

Hormone analysis. In the first preliminary experiments, only progesterone, 17β-estradiol and testosterone were quantified by dissociation-enhanced lanthanide fluorescent immunoassays (DELFIA®; PerkinElmer, USA), whereas in the final experiments steroid hormones were quantified by HPLC coupled to a tandem mass spectrometer (HPLC-MS/MS) as described earlier [33] and in the Supplemental Material. Changes in hormone concentration when quantified by the imuunoassays were previously shown to be in good agreement with those measured by HPLC-MS/MS [34]. Solid-phase extraction cartridges were used to extract hormones from the cell culture medium [32], while silicone disks were extracted directly with methanol. All extracts were reduced under nitrogen at 50 °C to dryness, and the residues re-dissolved in either acetonitrile/water (2:3) for HPLC analysis or in human serum surrogate for DELFIA analysis (for details see Supplemental Material).

Data analysis. Data points that deviated more than 5 times the median absolute deviation (MAD) from the median of a group were excluded. Data points for test concentrations that visibly or likely caused cytotoxicity are shown but were not considered for dose-response fitting. Hormone data from the individual experiments underwent first the non-parametric Kruskal-Wallis test (GraphPad Prism 5) to test whether treatment groups differed ($p < 0.05$). Given statistical significance, dose-response curves were then fitted (GraphPad Prism 5) using a 4-parameter log-logistic model with the Hill-slope allowed to be fitted or set to 1.0, the bottom of the curve fixed to the average response value from the controls, and the top fixed to the maximal response measured (see Supplementary Material). Similarly, dose-response curves were fitted to the mean of the normalized data from the three repeated experiments, given that two of the individual experiments had shown statistically significant changes in hormone levels. Normalization was done by dividing each absolute measured hormone concentration with the average hormone concentration from control-treatments on each plate.

Table 5.1. Distribution of hormones between silicone and cell culture medium. Hormones were quantified by HPLC-MS/MS, except 17β-estradiol. The mean with the standard deviation of 4 replicate measurements is given.

hormone	mass fraction in silicone[a]	$D_{silicone:medium}$ **[L/kg]**
androstenedione	0.70 ± 0.024	36.02 ± 3.92
dehydroepiandrosterone	0.64 ± 0.026	27.42 ± 3.19
testosterone	0.62 ± 0.019	25.16 ± 1.83
17β-estradiol[b]	0.70 ± 0.056 | 0.73 ± 0.031	37.27 ± 9.72 | 20.24 ± 2.88
progesterone	0.85 ± 0.023	86.30 ± 13.91
17α-hydroxyprogesterone	0.56 ± 0.022	19.03 ± 1.51
cortisol	0.0028 ± 0.00033	0.042 ± 0.0048
corticosterone	0.07 ± 0.003	1.20 ± 0.031

[a] based on $m_{silicone}$ = 84 mg and $Vmedium$ = 1.275 mL
[b] data from two quantifications by DELFIA

5.3. Results

Hormone partitioning into silicone. Cell viability was not compromised by hanging a silicone disk above the cells during exposure (see Supplemental Material, Figure S4). Steroid hormones of low polarity, i.e. progestagens, androgens and estrogens, accumulated in the nonpolar silicone as expected, and partitioning was in favor of the silicone phase for these hormones (Table 5.1). In contrast, the two corticosteroid hormones cortisol and corticosterone having two additional polar hydroxyl groups attached to the steroidal skeletal structure, remained in the aqueous phase. For the more nonpolar hormones, a small amount of silicone was thus sufficient for their quantitative enrichment from the medium, which facilitated the analytical quantification. Even more important, silicone therefore functioned as a sorption sink, which in turn stimulated the cellular hormone production, similarly as in an *in vivo* environment where released hormones are continuously removed by the blood stream and redelivered by hormone-producing tissue to maintain homeostasis. Yet, it has to be noted that silicone in dimensions for this *in vitro* setup could not act as an infinite sink. Nevertheless, H295R cells increased the production of those hormones that accumulated in silicone (Figure 5.2 and Figure S3 in the Supplemental Material), and the increase correlated to the silicone-medium distribution coefficient of the hormones (see Supplemental Material, Figure S2).

4-NP-induced effects on steroidogenesis. No pronounced cytotoxicity was detected by the MTT assay when 4-NP was dosed from silicone up to Cfree = 0.19 μM (Supplemental Material, Figure S5-C), but at concentrations above. It is thus likely that cytotoxicity was triggered also at this highest passive dosing level, although the MTT assay did not indicate it. 4-NP when dosed via DMSO up to concentrations of 28 μM did not appear to be cytotoxic (Supplemental Material, Figure S5-D). Note, however that Preuss et al. [35] observed cytotoxicity of 4-NP isomers above 18 μM. 4-NP sig-

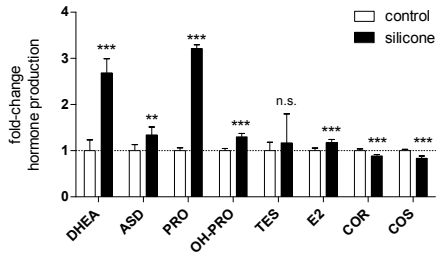

Figure 5.2. Hormone production in H295R cells in the presence and absence of silicone. Means of 4 replicate measurements are shown. All hormones were quantified by HPLC-MS/MS except 17β-estradiol (E2), which was quantified by DELFIA. Error bars indicate the 95 % confidence interval. Abbreviations: DHEA = dehydroepiandrosterone, PRO = progesterone, OH-PRO = 17α-hydroxyprogesterone, COR = cortisol, COS = corticosterone, ASD = androstenedione. Statistical significance between treatments (t-test) is indicated as follows: n.s. = not significant, $^{**}p < 0.01$, $^{***}p < 0.001$.

nificantly altered the hormone concentration profile of H295R cells in both dosing modes similarly. The hormone production was generally upregulated in a dose-dependent manner, which was more pronounced under DMSO-dosing than under passive dosing (see Supplemental Material, Figure S6). Cytotoxicity seemed to dominate the cellular responses at the highest test doses, likely causing the observed drop in hormone levels. 4-NP had the strongest effect on progestagen production, with the concentrations of progesterone and 17α-hydroxyprogesterone shown to be elevated by at most a factor 8.7 (DMSO-dosing) or 6.5 (passive dosing) relative to control treatments. In contrast, corticosteroid hormone production seemed unaffected, with the exception that under DMSO-dosing the levels of corticosterone increased by up to 70 %. Androstenedione and testosterone production increased with higher doses by up to a factor 2.3 until presumably cytotoxic events at the highest test dose triggered the hormone levels to fall to basal levels or below. Similarly, estrogen production (estrone and 17β-estradiol) increased. These results confirmed our preliminary experiment that had revealed marked effects of 4-NP on progesterone levels and moderate effects on testosterone and 17β-estradiol (data not shown).

By passive dosing, hormonal responses were observed at concentrations of freely dissolved 4-NP (C_{free}) more than two orders of magnitude below the nominally defined concentration by DMSO-spiking (Figure 5.3). For example, EC50 values (concentrations causing 50 % effect) for progesterone and 17α-hydroxyprogesterone, the hormones most affected, were reduced by factors of 201 and 168, respectively, by passive dosing (see Supplemental Material, Table S3). Several reasons can explain this apparent discrepancy: (1) Obviously, the two dose parameters are defined differently: while C_{free} refers to freely dissolved molecules in the exposure medium, the nominally defined spiked dose refers to the total amount of test substance added to a defined volume of exposure medium. Consequently, for chemicals that bind to medium constituents or surfaces, nominal concentrations can largely exceed the concentration of unbound molecules [36]. (2) Losses of test substance cannot be compensated when doses are spiked once-only. However, partitioning-controlled dosing from silicone compensates losses, and exposure concentrations are held constant [22]. (3) Finally, H295R cells indeed may be more sensitive in a passive dosing

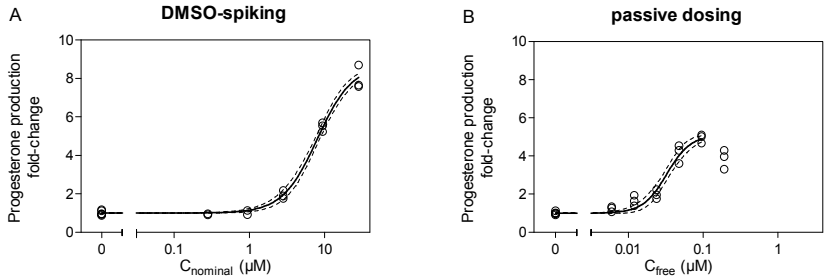

Figure 5.3. Progesterone response of H295R cells to 4-nonylphenol by DMSO-spiking (A) and passive dosing (B). With the freely dissolved concentration C_{free} as dose parameter, dose response-curves are left-shifted by more than two orders of magnitude relative to nominally defined concentrations by DMSO-spiking. Nonlinear regression lines are shown together with their 95 % confidence bands.

setup because their hormone production and metabolism are likely maintained at a higher rate due to silicone acting as a sink for steroid hormones.

POP-induced effects on steroidogenesis. The mixture of POPs introduced by equilibrium partitioning from silicone caused the H295R cells to increase the overall steroid hormone production (Figure 5.4). In contrast, no effect was observed when the POP mixture was added by DMSO-spiking (data not shown). The POP mixture did not cause measurable effects on cell viability in neither dosing mode (Supplemental Material, Figure S5-A and B). Arguably, the spiked test concentrations were very low (nanomolar range) with regard to common test concentrations often being in the micromolar range (e.g. [37, 38]). With passive dosing, hormonal responses were observed right within the range of POP concentrations found in human silicone breast implants. These translate to concentrations of individual freely dissolved POPs even below the femtomolar range (for derivation of C_{free}, see Supplemental Material, Table S1), supporting that hormonal responses can occur at very low exposure levels [39].

Increasing hormone concentrations with increasing POPs exposure was a general trend for all hormones synthesized by the cytochrome P450 enzyme CYP17 and the hydroxysteroid dehydrogenase 3β-HSD. In contrast, this trend was not conserved for hormones metabolized through 17β-HSD, i.e. testosterone. Pregnenolone was measured in one experiment, and levels increased up to 2-fold. Similarly, levels of progesterone and 17α-hydroxyprogesterone increased maximal 73 % and 70 %, respectively. The results from all three experiments showed elevated cortisol levels after exposure of the cells to POPs by upto 120 %, 98 % and 65 %, respectively, with statistical significance (based on a Kruskal-Wallis test comparing the overall treatment group means from the three independent experiments). For corticosterone levels, a non-significant

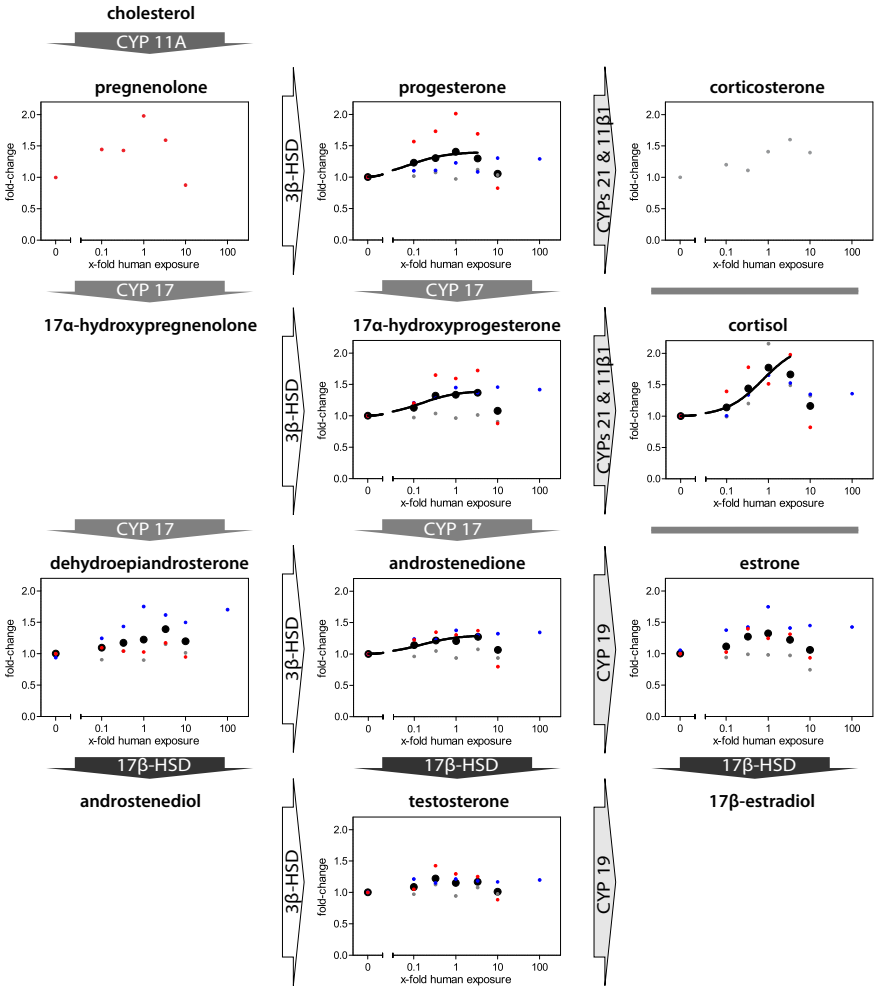

Figure 5.4. Hormonal responses of H295R cells to a mixture of POPs introduced by equilibrium partitioning from silicone. HPLC-MS/MS data from three independent experiments are shown in distinct colors. Nonlinear regression lines through the mean (black) of the data from the independent experiments are shown given statistically significant differences between control and treatments in at least two out of the three experiments. Pregnenolone and corticosterone were measured in only one experiment. Exposure levels on the x-axes are expressed relative to the median POP concentrations found in human silicone implants (median (1x) = 19.32 Σnmol/kg$_{silicone}$). The lowest and highest test doses were outside the human exposure range.

trend toward an increase was observed. Androstenedione concentrations were slightly elevated (up to 46 %). One experiment also gave indication for elevated concentrations of dehydroepiandrosterone (85 %) and estrone (75 %). No effect on testosterone was observed in any of the experiments. 17β-estradiol seemed not to be affected as indicated by the DELFIA hormone quantification in the preliminary experiment; concentrations were at or below the quantification limit for HPLC-MS/MS analysis and are not reported here.

Interestingly, maximal effects were reached at the dose corresponding to the median human exposure level (Figure 5.1). Hormone production did not significantly increase further at higher exposure levels, and in some cases hormone levels were observed to decline after reaching peak values. At present, we cannot explain this final drop in hormone concentrations since test doses were clearly below the range where cytotoxicity is expected.

In summary, the results suggest an activating effect of POPs on steroid hormone synthesis early in the steroidogenic pathway, being most pronounced for the progestagens and corticosteroid hormones, whereas the effect on hormones further downstream in the synthesis pathway seemed to be buffered.

5.4. Discussion

Impact of the dosing mode. The dosing mode plays a crucial role in *in vitro* toxicity testing: On the one hand it affected the actual biological responses measured while on the other hand it determined the dose metrics, and hence data interpretation and understanding of results. The results of our experiments illustrate both these aspects: (i) No effect was obvious when conventional DMSO-spiking of POPs was applied, while subtle effects were observed with partitioning-controlled dosing. (ii) With DMSO-spiking, exposure was expressed as the total spiked concentration, which however did not facilitate an understanding of the dose-response relationship because the actual effective concentration (C_{free}) remained unknown. With partitioning-controlled dosing, in contrast, exposure was defined by the concentration in silicone, allowing a linkage between *in vitro* exposure levels and actual human body burdens. Furthermore, passive dosing directly controlled C_{free}, which is believed to represent the toxicologically effective concentration [40, 41] and can be compared to concentrations of unconjugated chemicals in human matrices. Qualitative and quantitative changes in toxicological responses as a consequence of controlled and constant exposure by passive dosing have been observed before [28].

Comparison of results with earlier findings. In previous studies, PBDEs (e.g. [37]) and PCBs (e.g. [38]) were tested in the H295R assay as single compounds and at much higher concentrations than in the present study. Kraugerud et al. [38] measured an increased activity of 3β-HSD, as well as upregulation of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMGR) and the steroidogenic acute regulatory protein (StAR), which regulates the level of cholesterol, the precursor of all steroid hormones. The authors suggested that these altered enzyme activities may potentially be responsible for increased steroid hormone levels. A recent study by van den Dungen et al. [42] supports this hypothesis.

Canton et al. [37] found PBDE metabolites to have an inducing effect on aromatase (i.e. the enzyme converting androgens to estrogens), and in the study by He et al. [43] indeed higher 17β-estradiol levels were measured. All together, these studies suggest that the POPs that we included in our mixture have the potential to stimulate steroidogenesis. However, a complex mixture of these chemicals still may show varying toxicological effects depending on the mixture composition [8, 10]. Results from studies that tested POP mixtures extracted from environmental matrices in the H295R assay generally support our finding of elevated steroidogenesis: Zimmer et al. [14] tested POPs extracted from burbot and measured an increase of cortisol and/or 17β-estradiol levels, depending on the mixture composition, and in one case observed decreased testosterone levels. Similarly, Grund et al. [12] and Gracia et al. [11] reported increased 17β-estradiol levels, and in one case decreased testosterone levels, upon exposure of H295R cells to POPs extracted from sediment of the river Danube and from waste water, respectively. We could not quantify 17β-estradiol with our HPLC-MS/MS method, but we observed slightly increased estrone levels in one experiment. Interestingly, all these *in vitro* results point in the same direction as findings by Warner et al. [44] who found a positive association between blood serum PCB levels and an upregulation of enzymes involved in estrogen synthesis in daughters of the Michigan Fisheaters' Cohort.

Implications and perspectives. If POPs indeed interfere with steroidogenesis, effects would not only be expected in the adrenal but also in other steroidogenic organs. It remains uncertain whether POPs can modify the hormone secretion *in vivo* because complex hormonal feedback mechanisms exist to maintain homeostasis. However, such mechanisms are not fully developed during fetal life [45]. Hence, especially during early life, altered steroid hormone levels could have detrimental consequences. Obesity [46], diabetes [47] as well as timing of puberty [48] have been associated with exposure to certain POPs. Still, the question remains how such correlations link to POP-induced hormonal changes. Rennert et al. [49] reported a positive correlation between increased levels of dehydroepiandrosterone sulfate in children and POP levels in breast milk of their mothers, concluding that there could be a link between early exposure to POPs and acceleration of adrenal puberty. Our results indicated increased dehydroepiandrosterone levels in the adrenal cell line H295R upon exposure to POPs, supporting this finding.

We only tested a mixture of selected POPs, while the actual exposure comprises many more chemicals. One would therefore expect effects of real mixtures to be larger in magnitude or to occur at even lower exposure levels. It is also worth mentioning that the endocrine effects we observed would be underestimated if POPs did not yet reach chemical equilibrium between the body tissue and silicone breast implants or if the silicone in the *in vitro* assay was not fully equilibrated with the exposure medium (but note that the medium was pre-equilibrated for 24 h). "Real" mixtures could be studied by first equilibrating silicone in human tissue and then using this silicone as donor for passive dosing. However, this approach would also transfer endogenous hormones from the tissue

into the test, which potentially could obscure the effects of xenohormones. On the other hand, including hormones as part of chemical test mixtures can also be seen as the future challenge: Research within the field of endocrine disruption should be directed at investigating how foreign chemicals interact with the *in vivo* chemical environment, much as suggested in the human exposome concept [50].

5.5. Conclusions

POPs at concentrations as found in human silicone breast implants showed to increase the production of progestagens and corticosteroids in adrenocortical carcinoma cells *in vitro* when dosed by equilibrium partitioning from silicone. Evidence is thus given that POPs at human *in vivo* levels possess the potential to interfere with steroid hormone synthesis. This study also demonstrated that silicone can serve as a partitioning reference phase to translate exposure between *in vivo* and *in vitro* media.

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Manuscript III

Molecular carriers for substrate delivery: a cross-validation study on *in vitro* biotransformation of PAHs using fish liver S9 enzyme fractions

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Manuscript in preparation

Chapter 6

Molecular carriers for substrate delivery: a cross-validation study on *in vitro* biotransformation of PAHs using fish liver S9 enzyme fractions

Abstract

The intrinsic metabolism of chemicals is a crucial parameter in the evaluation of their toxicity and bioaccumulation potential. Recently, *in vitro* assays using fish liver S9 fractions have been developed to measure enzymatic biotransformation of chemicals as an alternative to whole animal tests. However, it is challenging to provide a well-defined substrate supply when working with hydrophobic chemicals because of sorptive losses and their low aqueous solubility which often requires the use of a co-solvent for dosing. To avoid the use of co-solvent and to ensure sufficiently fast delivery of hydrophobic organic chemicals, several molecular carriers were considered, and the non-ionic surfactant Tween 80 (polysorbate), β-hydroxypropyl cyclodextrin and bovine serum albumin (BSA) chosen for delivery of PAHs into an *in vitro* biotransformation assay with S9 fractions from rainbow trout (*Onchorhynchus mykiss*). Among the carriers, Tween as a co-sorbent was the only one showing additive binding behavior in the S9 test system. Apparent biotransformation rate constants in carrier treatments were similar to or within a factor of two lower than rates in treatments with solvent-spiking. In the case of Tween, these differences could be partly explained by the lower fraction of PAH bound to S9 due to the presence of Tween as secondary binding phase. Measured PAH biotransformation rate constants showed very good agreement if substrate delivery occurred by molecular carriers or by a co-solvent. In all cases, clearance rates increased with increasing log K_{ow} of the PAHs, displaying a log-log linear relationship.

6.1. Introduction

Biotransformation and chemical risk assessment. Bioaccumulation is one of the criteria used to identify chemicals of very high concern, i.e. chemicals belonging to the group of persistent, bioaccumulative and toxic (PBT) chemicals. For chemical risk assessment, the European regulation of chemicals REACH strictly requires bioaccumulation screening. Hydrophobic organic chemicals (HOCs) possess chemical

properties that favor their accumulation in biota and thus pose a hazard to environmental and human health through potential toxic effects. For a risk assessment of these chemicals, reliable tools to quantify bioaccumulation are needed and hence strategies for estimating metabolic transformation rates. Such estimation of metabolic transformation is in the best case based on measured bioconcentration factors or bioaccumulation factors [1]. Bioaccumulation screening involves increasingly predictive models, which often rely on quantitative-structure-activity relationships (QSARs) [2], or still on a simplistic correlation of bioaccumulation factors to octanol-water partitioning. Such models, however, can only give accurate estimates if chemical biotransformation is taken into account. The large number of chemicals that need to undergo risk assessment therefore urges the development of effective screening methods, possibly only relying on *in silico* and *in vitro* data and not involving expensive animal testing.

Biotransformation *in vitro* models. To this end, *in vitro* models for hepatic metabolism have been developed – first for mammals by the pharmaceutical industry [3, 4, 5], and more recently by ecotoxicologists for fish [6, 7, 8, 9]. Biotransformation of chemicals happens through evolutionary conserved enzymatic pathways that are similar in fish and humans. Mainly enzymes of the cytochrome P450 (CYP) family are responsible for chemical breakdown. These enzymes are located in the endoplasmatic reticulum within cells, mainly in the hepatic tissue. They can be isolated by homogenizing liver tissue and then separating the microsomal enzyme fraction by centrifugation ($>$ 9000g) from cell debris, which is why the collected supernatant is commonly referred to as S9.

Regardless of whether these *in vitro* models employ hepatocytes, microsomes or S9 fractions, they all are challenged when hydrophobic chemicals need to be tested. This is because (i) hydrophobic chemicals are difficult to introduce into aqueous solution. (ii) They require very sensitive analytical methods for quantifying product formation and especially substrate depletion given that first-order transformation kinetics can only be observed at low initial substrate concentrations. (iii) Substrate supply must not limit the biotransformation kinetics. Commonly a carrier solvent, typically acetone or dimethylsulfoxide (DMSO), is used for substrate administration. This then raises several issues: first, the solvent, although added in small proportion, for example 0.5 % or 1 %, may impact enzyme activity by modifying the S9 protein structure, i.e. it may appear to be toxic. Second, this dosing mode does not guarantee complete dissolution of the substrate [10, 11, 12]. Both these issues can lead to an underestimation of biotransformation rate constants. Third, exposure to initially high concentrations due to solvent-spiking is far from the environmental exposure reality where exposure is characterized by small concentrations over a longer period of time. This further complicates in-vitro-to-in vivo extrapolations.

New developments. In order to overcome these limitations, few alternative approaches to solvent-spiking of *in vitro* biotransformation assays have been investigated: Lee et al. [13] applied a passive dosing approach using a thin-film polymeric sorbent phase for substrate delivery by partitioning. Obvi-

ously, such solvent-free systems have the advantage of sustaining the activity of the biological system. Another advantage was that assays could be conducted at low initial substrate concentrations, controlled by chemical partitioning. Furthermore, quantification of the chemical concentration in the polymer instead of in an aqueous enzyme suspension facilitated instrumental analytical performance. However, the estimation of biotranformation rates as a relative change in chemical concentration in the polymer and/or the aqueous medium with reference to a control system without the biological matrix relied on the assumption that the polymer-medium partitioning rate constant and the medium-polymer back-partitioning rate constant would not be affected by the presence of biological matrix. Yet, mass transfer rates of HOCs through aqueous boundary layers can be enhanced by dissolved organic carbon species [14, 15], which is especially effective in rich media [16]. Another study by Smith et al. [17] also used a polymer-based passive dosing system to study microbial biodegradation of two PAHs. They employed loaded silicone O-rings as a partitioning source for PAHs and measured bacterial mineralization rates by quantification of carbon dioxide evolution.

Both these polymer-based dosing approaches suffer one fundamental limitation: the dosing kinetics may become rate-limiting for biotransformation at high turnover rates by the biological system. A mathematical kinetic model would still allow estimating biotransformation rate constants but the first challenge would be to find an accurate model, and second, such model likely is not applicable to rapidly metabolized chemicals. The only way to overcome this limitation is to substantially enhance the substrate delivery kinetics. For sorbent-phase dosing systems, one option could be to improve the area-to-volume ratio while minimizing polymer thickness to reduce the diffusive resistance within the polymer. This has been shown feasible by Reichenberg et al. [18] who developed sediment passive samplers using μm-thin silicone coatings on glass jars to achieve fast sampling kinetics. In a pilot study, we undertook similar effort and investigated silicone-to-medium partitioning kinetics of PAHs in an aqueous system in the presence and absence of S9 in μm-thin silicone-coated vials (see Supporting Information, SI).

Molecular carriers for dosing. As long as chemicals will have to enter the aqueous phase by diffusion through an aqueous boundary layer (ABL), biotransformation is likely to be limited by this transport process, i.e. limited by substrate supply. Sufficiently fast delivery from a third phase may thus only be possible when dosing occurs from molecular-scale carriers. In fact, it is molecular carriers that transport insoluble chemicals in multicellular organisms. Substantial research into this topic has been undertaken by the pharmaceutical industry for the development of delivery of hydrophobic drugs. Consequently, it is within the pharmaceutical research where carrier-based dosing is currently being investigated the most. For example, many chemotherapy drugs are hydrophobic and require the use of cosolvent, which however often is toxic itself and causes unwanted side-effects. In effort to develop solvent-free formulations of such drugs, various carriers were suggested, among them endogenous carrier proteins such as

serum albumins ([19, 20, 21], cyclodextrins [22, 23, 24], liposomes [25], and non-ionic surfactants such as polysorbates [26, 27] and alkyl polyglycosides. Within the environmental sciences, molecular carriers have mainly been studied in the context of contaminant transport or in the light of modifying the bioavailability of HOCs [28, 29, 30].

In the present study, we investigated molecular carriers for dosing of hydrophobic chemicals in an *in vitro* biotransformation assay. Understandably, a dosing approach without any potentially toxic solvent is thought advantageous. Yet, it has to be assured that the carrier of choice does not cause toxicity. Further, molecular-scale carriers can ensure fast delivery of HOCs in an aqueous enzyme suspension, and hence solve the issue of substrate supply kinetics as rate-limiting factor for biotransformation. In return, addition of another sorption phase will necessarily reduce the chemical activity of HOCs, which in turn will result in lowered biotransformation rate constants.

To choose carriers for *in vitro* dosing of HOCs, we set up a list of criteria: a good carrier should (i) not interact with the biological material, (ii) be water-soluble, (iii) possess a high affinity/capacity for the test chemical, (iv) exhibit sufficiently fast release of test chemical (dissociation kinetics), (iv) exhibit well-defined binding behavior with regards to sorbent-sorbate interactions and sorbent-sorbent interactions in systems with multiple binding phases, and (v) be stable under the conditions of the test (pH, temperature, etc.). To this end, we chose the non-ionic surfactant Tween 80 (a polyoxyethylene sorbitol ester, with oleic acid as the primary fatty acid), bovine serum albumin (BSA) and β-hydroxypropyl cyclodextrin (HPCD) (Figure 6.1). HPCD is a cyclic oligosaccharide consisting of seven linked glycopyranose units. While Tween, being a surfactant, enhances solubilization of HOCs by micelle formation, BSA and HPCD are carriers that bind HOCs through noncovalent binding or complex formation, respectively. Binding of HOCs to BSA is likely a partitioning process, and previous studies indicated a log-log linear dependency of BSA binding constants and K_{ow}, e.g. for PAHs [31, 30], although binding of HOCs to the active site in CYP enzymes has been described to be mainly driven by hydrogen bonding [32]. In contrast, cyclodextrins accommodate HOCs within their hydrophobic cavity, whereby the steric geometry of the cavity determines to a large extent the binding affinity to HOCs [33]. Surfactants on the other hand enclose HOCs by micelle formation, an aggregation process that happens above a certain threshold concentration of the surfactant, the critical micelle concentration (cmc). We investigated the solubility enhancement of PAHs by Tween 80 in a pilot experiment, which showed that in the concentration range 0.03-3 g/L, logarithmic PAH binding constants correlated perfectly with log K_{ow} ($y = 1.06x - 0.236$; $R^2 = 0.977$). This given, Tween seemed a promising carrier that would bind PAHs in a well-behaved manner. Note that the reported cmc is in the range 13-30 mg/L [34, 35].

Theory. *In vitro* biotransformation can be quantified by measuring either the rate of metabolite formation [36, 3, 4] or the depletion of substrate over time in the incubation medium [37, 38, 39, 40, 30, 41]. In this study, we determined depletion rate constants k_{dep} (1/min), assuming first-order kinetics of the enzymatic bio-

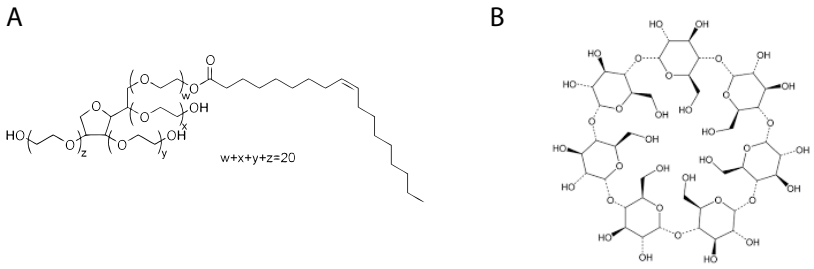

Figure 6.1. Structure of Tween 80 and β-hydroxypropylcyclodextrin (HPCD) that were used as molecular carriers for PAHs.

transformation reaction:

$$k_{dep} = \frac{\ln\left(C_0/C(t)\right)}{t} \qquad (6.1)$$

where C_0 (mol/L) is the initial substrate concentration in the incubation medium, and $C(t)$ is the substrate concentration as a function of time (t). Initial substrate concentrations below 1 μM are believed to be sufficiently small [42] to satisfy that saturation of the enzyme does not influence the measured biotransformation rate constant, i.e. it is assumed that the initial substrate concentration then be much below the Michaelis constant, $C_0 \ll K_M$. However, Lo et al. [43] observed a concentration dependence of biotransformation rate constants in an *in vitro* fish-liver S9 assay with PAHs at initial substrate concentration even below 1 μM, which was reflected in a up to 12-fold underestimation of the actual *in vitro* biotransformation rate constant.

In vitro intrinsic clearance rate are commonly subjected to an extrapolation procedure to estimate an in vivo whole-body metabolism rate constant [8], which in turn is used for the prediction of bioconcentration factors [6, 39]. The in-vitro-to-in-vivo extrapolation hinges on the assumption that only chemical in the freely dissolved form is available for enzymatic metabolism, and therefore a scaling factor is introduced into the extrapolation model to account for the relative difference in bioavailability of substrate to biotransforming enzymes in the *in vitro* system and in the intact tissue (i.e. through the estimation of unbound fractions in the *in vitro* system and in blood, respectively). However, it appeared that BCFs estimated through a mass-balance model, including unbound fractions a scaling factor, were by approximately 3 orders of magnitude larger than experimentally determined BCFs [44]. In contrast, BCF estimates, for which a scaling factor equaling one [6] had been used in the model, predicted BCFs better. Escher and co-authors therefore hypothesized that "chemicals bound to proteins can desorb rapidly and thus contribute to metabolic turnover of the chemicals"' which essentially questions the widely accepted view that only the freely dissolved chemical determines measured biotransformation rate constants.

Aim of the study. The first aim of this study was to explore the feasibility of molecular carriers for introducing HOCs into an aqueous *in vitro* test system. The second aim was to use such carrier-based dosing approach for cross-validating the performance of the current *in vitro* fish S9 biotransformation model that uses a co-solvent for dosing. Finally, by investigating the relationship between bound/unbound chemical fractions in an S9 system with an additional sorption phase and biotransformation rate constants, we aimed to scrutinize the bioavailability assumption behind in-vitro-in-vivo extrapolations.

6.2. Material & Methods

Chemicals and materials. The PAHs naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene and benzo[a]pyrene (99.5 %) were obtained from Sigma-Aldrich (MO, USA) or Sigma-Aldrich (Germany), Acros (Belgium), Fluka (Germany) and Cerilliant (TX, USA, all in purity > 98 %. Bovine serum albumin (Cohn fraction V, lyophilized powder, acid-free, purity ≥ 96 %, cat.-no. A5611) was purchased from Sigma-Aldrich (MO, USA). Tween 80 (art. no. 822187) was from Merck (Germany), and β-hydroxypropyl cyclodextrin (product name Cavasol W7 HP) from Wacker Chemie AG (Germany). All solvents used (acetone, methanol, acetonitrile) were of analysis-grade. Milli-Q water was used.

S9 fractions. S9 fractions from rainbow trout (*Oncorhynchus mykiss*) liver were collected as described by Johanning et al. [42]. Liver homogenates from five individual female or five male fish of approximately 470-540 g were pooled and stored in aliquots of 0.5 mL at -80°C. S9 from fish of either gender was used for the assays. The protein concentration was measured by a modified Lowry assay (Sigma, Technical bulletin TP0300) and enzyme activity characterized as described in Nichols et al. [30].

The glucose-6-phosphatase assay to test carrier tolerance of S9. To test whether the carriers themselves, i.e. Tween, BSA and HPCD, would affect the enzyme activity, the glucose 6-phosphatase activity was measured by quantifying the activity of the enzymes that are located in the endoplasmatic reticulum. The protocol is based on the methods described by Hübscher and West [45] and Ames [46] and was modified slightly (details in Supporting Information). Briefly, in this assay glucose 6-phosphate (G6P) is supplied as substrate to S9 for conversion by the enzyme glucose-6-phosphatase to glucose and inorganic phosphate. Liberated phosphate is then measured by addition of the complex-forming ammonium molybdate and ascorbing acid as reducing agent, leading to the development of a specific blue color in the reaction mix, which is quantified by measurements of absorbance at 820 nm on a spectrophotometer. Ethylene diamine tetraacetic acid (EDTA) and potassium fluoride (KF) are incorporated into the incubation to inhibit alkaline and acid phosphatases which also catalyze the metabolism of glucose 6-phosphate. We assumed that the carriers would not reduce substrate (G6P) availability, and also confirmed this for BSA. Reaction mixtures were prepared in a 100 mM Tris buffer (pH 7.8), adding the carriers Tween, BSA and HPCD to final concentrations of 0.035-31 g/L (Tween), 0.1-10 g/L (BSA), or 1-

100 g/L (β-HPCD), glucose 6-phosphate (28.5 mM final concentration), and the S9 suspension (1 g/L final concentration). Incubations were stopped after 20 min by adding aqueous solution of trichloroacetic acid (10 % w/v). Finally, glucose-6-phosphatase activity was expressed by the rate constant for phosphate production by means of total amount of phosphorous produced (nmol) per unit time (min) and mass S9 (mg).

Carrier loading. Based on reported PAH binding constants for BSA [44] and HPCD [47], and the results from the pilot binding experiments with Tween, the capacities of the aqueous carrier solutions at the chosen test concentrations were estimated to ensure that PAHs would solubilize. Stock solutions of individual PAHs were prepared in acetone at concentrations between 4.6 - 30 mg/L. One hundred microliter of these solutions were added into 4-mL brown amber glass vials To create a thin film of PAH microcrystals on the side walls of the vials, the vials were placed horizontally on a roller, rotating at 25 rpm, and the solvent was allowed to evaporate off. Thereafter, aqueous carrier solution was added to dissolve the PAH crystals: BSA and HPCD were each at a concentration of 100 g/L, Tween at a concentration of 1 g/L in a 100 mM potassium phosphate buffer (pH 7.8 at 11°C). The vials were then left rotating on the roller overnight. Naphthalene was directly dissolved in the BSA- and HPCD aqueous solutions (each at 100 g/L) and then diluted to the desired concentration. To dissolve naphthalene in aqueous Tween solution, a concentrated Tween solution (20 g/L) was prepared in potassium phosphate buffer and then diluted to the desired concentration (Tween at 1 g/L, naphthalene at 20 μM). Dissolution of PAHs in the aqueous carrier solutions was further facilitated by sonicating the vials in a warm water bath for approximately 2 h. Finally, subsamples of the carrier solutions were taken for analysis by high-pressure liquid chromatography (HPLC). Recovery was calculated as follows, with m_0 as the mass of chemical initially added:

$$recovery = \frac{C_{measured} \times V_{solution}}{m_0} \quad (6.2)$$

In vitro biotransformation assays. PAH metabolization by the S9 enzyme fractions was quantified by measuring substrate depletion. The *in vitro* assay was conducted as described before [42, 30] with some modifications: In order to setup a test system entirely free of organic solvent, we omitted, after careful evaluation, the addition of alamethicin, a pore-forming agent, which normally is dissolved in methanol and then added for supporting Phase II glucuronidation. However, for metabolization of PAHs, phase I hydroxylation is likely the rate-limiting step. Indeed, our test results indicated that alamethicin is not required for PAH clearance by rainbow trout liver S9 enzyme fractions. Further, in experiments run by conventional solvent spiking, the final acetone concentration was reduced to 0.5 %. Reaction mixtures of a total volume of 1 mL (1.25 mL for fluoranthene; 1.5 mL for naphthalene) were prepared in 100 mM potassium phosphate buffer (pH 7.8 at 11°C), and contained 2 mM β-NADPH (nicotinamide adenine dinucleotide phosphate), 2 mM UDPGA (uridine 5'-diphospho-glucuronic acid), 5 mM GSH (glutathion) and 0.1 mM PAPS (adenosine 3'-phosphate 5-phosphosulfate) as cofactors. S9 was added to a final con-

centration of 1 g/L and the mix preincubated for 10 min. PAHs were then added either with acetone as carrier (0.5 % vol/vol final concentration) or with a molecular carrier in aqueous phosphate buffer (added in a volume ratio for 10x dilution in the incubation mixture). Three treatments were processed in parallel: S9 to which PAH substrate was delivered by solvent dosing ("active solvent control"), S9 to which substrate was delivered by a molecular carrier ("carrier"), and heat-denatured S9 to which substrate was added by solvent dosing ("inactive control"). This latter treatment was included to account for possible abiotic losses (e.g. volatilization) or non-enzymatic degradation. Each treatment consisted of three replicates, i.e. in total nine vials were processed in parallel (sometimes requiring two persons working simultaneously). Initial PAH concentrations ranged between 0.12 μM and approximately 2.0 μM and were matched for the two dosing modes. Reaction mixtures were incubated in 4-mL glass vials (for the more volatile PAHs naphthalene and fluorene, vials were capped) in a water bath at 11°C. Subsamples of 100 μL from these incubation mixtures were transferred at predetermined time intervals to vials containing 300 μL ice-cold acetonitrile, vortexed for 20 s, and stored on ice until centrifuged. Sampling times varied between PAHs and were adjusted appropriately to reliably quantify depletion over time. Active and denatured samples were collected at all sampling times. Biotransformation was terminated by adding 300 μL of ice-cold acetonitrile to each incubation mixture. Finally, all samples were centrifuged at 3000g for 6 min at 4 °C, and the supernatant transferred to LC-vials for analysis by HPLC (see below). Samples were stored at 4°C and analyzed within 24 h.

Determining PAH sorption to S9 in the presence of carriers. An equilibrium partitioning method that builds on passive dosing of hydrophobic chemicals from silicone [48, 47] was applied to study the sorption of PAHs and solubility enhancement in S9 suspensions augmented with molecular carriers. The method has been applied previously to determine binding and speciation of PAHs without phase separation of solutions or suspensions [48, 47, 49]. Freely dissolved concentration in the sample are controlled by equilibrium partitioning of PAHs from silicone, while the total concentration in the incubation solutions are then measured as dependent variable. Five hundred milligram of silicone polymer MDX4-4210 (Dow Corning, USA) was casted into 10-mL glass vials and the polymer allowed to cure; silicone was loaded with a PAH mixture (naphthalene, phenanthrene, anthracene, fluoranthene, pyrene) from methanol as described previously [47]. We followed a sequential equilibration strategy as suggested by Gouliarmou et al. [47] and equilibrated alternating the potassium phosphate buffer and S9 suspension with the carriers (S9+acetone, S9+BSA, S9+Tween or S9+HPCD). Vials were thoroughly rinsed with small aliquots of water between each run to avoid carry-over of solutions. Two sets of 12 vials were used in parallel (one for Tween and β-HPCD, the other for acetone and BSA) to equilibrate triplicates at four S9 concentration levels (0.25, 0.5, 1.0 and 2.0 g/L), the carrier concentration kept constant (BSA 10 g/L; β-HPCD 10 g/L; Tween 0.1 g/L; acetone 0.5 % vol/vol as in the final tests). Incubation mixtures were prepared in potassium phosphate buffer as in the biotransforma-

tion assays, but did not contain the cofactors. Vials containing 2 mL volumes of the S9 suspensions or the phosphate buffer (100 mM) were incubated at 11°C on an orbital shaker (1000 rpm; IKA) for two hours (buffer solution) or for 24 h (S9+carrier suspensions). Three additional vials for each S9+carrier mixture at the highest S9 concentration of 2 g/L were incubated for 48 h or 72 h to confirm equilibration. In a separate experiment, equilibration kinetics were determined for S9+HPCD by taking subsamples of three replicate vials at fixed time points. After equilibration, subsamples were collected and mixed with acetonitrile/water (75/25 % vol/vol) in ratios of 1:10 to 1: 50 depending on the S9 and carrier concentration. Protein was precipitated by centrifugation as described above, and the supernatant collected for analysis. Water samples were diluted in 75/25 % vol/vol acetonitrile/water.

Enhanced capacities of S9 suspensions, free and bound fractions as well as S9-binding constants were determined as measurement endpoints. The enhanced capacity E is a measure that quantifies the relative increase in solute capacity of aqueous solutions or suspensions due to the presence of additional binding phases. Typically, pure water is chosen as reference:

$$E = C_{tot}/C_w \tag{6.3}$$

whereby C_{tot} is the total solute concentration and C_w is the solute concentration in pure water. The inverse of the enhanced capacity equals the fraction of freely dissolved species (f_{fd}), as molecules can be considered "freely dissolved" in pure water:

$$f_{fd} = 1/E \tag{6.4}$$

Finally, enhanced capacities are related to the binding constants (K_S) of a sorbent S in solution or suspension:

$$E = K_S[S] + 1 \tag{6.5}$$

where $[S]$ is the concentration of the sorbent in the solution or suspension. In case of multiple sorption phases, the enhanced capacity of solutions or suspensions due to additional sorbents must be taken into account:

$$E = K_{s_1}[S_1] + E_{S_2} + E_{S_3} + ... + E_{S_i} \tag{6.6}$$

where S_1 is the primary sorbent, S_2 the secondary sorbent, and S_3 the tertiary sorbent, etc. and E_{S_i} is the enhanced capacity of the solution or suspension at a given concentration of S_2, S_3 or S_i. In the present study, our primary sorbent phase of interest was S9, since it is the active site where biotransformation actually takes place, and the molecular carriers for dosing were considered a secondary binding phase, such that eq 6.6 can be rewritten as

$$E = K_{S9}[S9] + E_{carrier} \tag{6.7}$$

Because C_{free} is the controlled variable in the experiments, S9-bound fractions in the presence of a carrier ($f_{S9}^{carrier}$) can be calculated as

$$f_{S9}^{carrier} = \frac{C_{S9+w} - C_w}{C_{S9+carrier+w}} \tag{6.8}$$

by assuming additive binding in the S9-carrier-water systems. C_{S9+w} is the total solute concentration in aqueous S9 suspensions, C_w is the solute concentration in pure water (C_{free}), and $C_{S9+carrier+w}$ is the total solute concentration in aqueous S9 suspensions with carrier molecules. Similarly, the S9-bound fraction in absence of a carrier ($f_{S9}^{\emptyset}$) can be calculated as

$$f_{S9}^{\emptyset} = \frac{C_{S9+w} - C_w}{C_{S9+w}} \tag{6.9}$$

Thus, it follows that

$$\frac{f_{S9}^{carrier}}{f_{S9}^{\emptyset}} = \frac{E_{S9}}{E_{S9+carrier}} = \frac{f_{fd}^{carrier}}{f_{fd}^{\emptyset}} \tag{6.10}$$

whereby E_{S9} is the enhanced capacity of an aqueous S9 suspension, $E_{S9+carrier}$ is the enhanced capacity of an aqueous S9 suspension with carrier molecules, $f_{fd}^{carrier}$ is the fraction of freely dissolved solute in S9 suspensions with carrier molecules, and $f_{fd}^{\emptyset}$ is the fraction of freely dissolved solute in S9 suspensions without carrier molecules.

PAH analysis by HPLC. Prior to analyzing samples from the biotransformation and binding experiments, samples containing HPCD or Tween at various concentrations were spiked with PAHs. These measurements confirmed that Tween and HPCD did not interfere with the HPLC analysis of PAHs. Precipitation of S9 by centrifugation had previously proven to remove protein sufficiently from samples without retaining analytes [9]. PAHs were analyzed by an Agilent HPLC system (1260 series) coupled to a fluorescence detector (FLD G1321B) and equipped with a Hypersil Green PAH 5 μm column (100 x 2.1 mm; part-no. 31105-102130 Thermo Scientific, Pennsylvania, USA). The FLD was operated in multi-emission mode, excitation set to 260 nm and emission wavelength set to 350 nm and changed to 440 nm at 2.5 min run time (channel A), while simultaneously monitoring at 420 nm (channel B). Water and acetonitrile were used as mobile phase (90/10 % vol/vol H2O/acetonitrile and 5/95 % vol/vol H2O/acetonitrile), at a ratio 35:65. Samples of 20 μL were set on the column; solvent flow rate was set to 0.6 mL/min, and PAH eluted isocratically. Chromatograms were analyzed using Agilent's software Chemstation based on 10-point external calibration curves (linear, forced through the origin).

6.3. Results & Discussion

Carrier tolerance of S9. Tween 80, BSA and β-HPCD did not appear to have a pronounced effect on G6P activity (Figure D.1 in Supporting Information), which is an enzyme thought to reflect the enzymatic activity of S9. In the presence of Tween 80, G6P activity was reduced by less than 10 % under test conditions ([Tween] = 0.1 g/L). β-HPCD at the actual test concentration ([HPCD]=10 g/L) did not affect the G6P activity with statistical significance (t-test, $p > 0.05$) although at higher concentrations G6P activity was reduced by up to 22 %. In contrast, BSA at the highest and tested concentration (10 g/L) increased the G6P activity by 11%. Based on these results, toxicity of the molecular carriers to S9 should thus not affect PAH metabolism kinetics within an uncertain margin of about 10 %. **Carrier loading.** Direct dissolution of naphthalene in aqueous solutions of BSA and β-HPCD resulted in the desired concentration; in aqueous Tween solution recovery was only 83 % (Table D.1 in Supporting Information). For the remaining PAHs, re-dissolution of microcrystals gave recoveries ranging between 50-97 % in Tween solutions, 79-107 % in BSA solutions, and 54-84 % in β-HPCD solutions (Table D.1 in Supporting Information), with the lowest recoveries observed for fluorene. It is obvious from these results that the carrier loading approach through the formation of microcrystals by solvent evaporation has a limited applicability domain toward rather

non-volatile substrates. In aqueous β-HPCD solutions, the larger PAHs pyrene and benzo[a]pyrene were only poorly recovered. β-HPCD forms inclusion complexes with PAHs depending on molecular size and steric arrangement of the molecule [33], which may have limited the recovery. With few exceptions (phenanthrene, pyrene), the PAH concentration in the aqueous carrier solutions could be controlled with good precision (CV < 10 %). Overall, the carrier loading protocol seemed sufficient for the purpose of producing spiking solutions for an *in vitro* assay.

***In vitro* biotransformation of PAHs.** Regardless of the dosing mode, i.e. solvent-dosing or molecular carrier-based dosing, logarithm-transformed depletion rates (k_{dep}) increased linearly with *log* K_{ow}, and generally agreed with each other (Figure 6.2). A positive correlation between substrate hydrophobicity and biotransformation rate constants has been described earlier [50, 32, 51]. Biotransformation kinetics were lowest for naphthalene, and only in acetone- and Tween-dosed treatments could depletion rate constants for naphthalene be reliably quantified (for BSA, a linear regression 6.1 on the time-concentration data series did not yield slopes significantly different from zero while the β-HPCD data were not reproducible). Generally, precision of the determined depletion rates was very good with relative standard deviations between replicates below 5 %, and a between-experiment variation < 10 %. Depletion rates in carrier-dosed setups were on average by a factor 2.4 lower than those in acetone treatments, and this factor was most uniform across PAHs for Tween (mean=2.3; CV 16 %) while it varied more in the β-HPCD data set ($0.9 < k_{dep}^{carrier}/k_{dep}^{acetone} < 3.5$) and in the BSA data set ($1.9 < k_{dep}^{carrier}/k_{dep}^{acetone} < 4.9$). In the latter two cases, the largest difference between depletion rates (carrier- vs. acetone substrate delivery) was observed for fluorene. On the other hand, the smallest differences between depletion rates, determined by the different forms of substrate supply, were observed for benzo[a]pyrene. Interestingly, when fluoranthene was introduced by β-HPCD to the S9 test system, the depletion rate constant was not significantly different (t-test $\alpha = 0.053$) from the depletion rate constant determined by acetone spiking. This indicates that more than just the freely dissolved fraction at chemical equilibrium between S9, β-HPCD and water must have reached the active sites for enzymatic biotransformation within the S9 fraction, as the addition of a secondary binding phase (the carrier) in the S9 system inevitably leads to a reduction in the freely dissolved fraction.

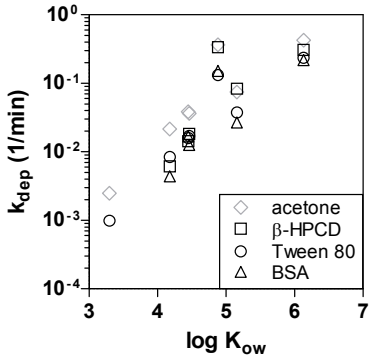

Figure 6.2. Depletion rate constants (k_{dep}) for the *in vitro* biotransformation of PAHs by fish liver S9 enzymes under different dosing regimes.

Binding of PAHs in S9-carrier suspensions. An equilibrium partitioning method that controls the chemical activity in a sample [48, 47] was employed to determine the speciation of PAHs in S9 suspensions with and without added molecular carriers. The time series measurements in the S9+β-HPCD system with S9 present in a concentration of 2 g/L indicated that S9+carrier suspensions equilibrated with the silicone PAH reservoir within 24 h (Figure D.2 in Supporting Information).

Using eq 6.3, enhanced capacities were calculated and plotted against the concentration of S9 (Figure 6.3). In S9 suspensions with BSA as secondary binding phase, no capacity enhancement was observed for naphthalene (Figure D.3 in Supporting Information). Enhanced capacities of S9+BSA suspensions when extrapolated to a zero-concentration of S9 showed an enhanced capacity due to BSA alone (at a concentration 10 g/L) of approximately a factor 20 whereas the maximum capacity enhancement for naphthalene by S9 alone was approximately only 4-fold (Figure SD.3 in Supporting Information), which explains why increasing S9 concentrations did not further enhance the capacity of the solution. Tween caused only a small capacity enhancement of diluted S9 suspensions for naphthalene whereas β-HPCD clearly contributed to a higher capacity of the S9 suspensions.

An estimation of binding constants from the enhancement curves using the original linear model (eq 6.7) was only possible for the Tween data set. Intercepts of the linear regressions in this case predicted enhanced capacities of an aqueous Tween solution of concentration 0.1 g/L fairly well (Figure D.4 in Supporting Information): The in-

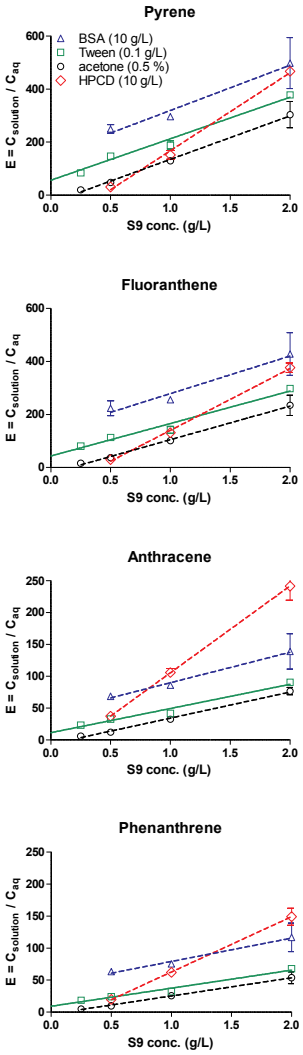

Figure 6.3. Enhanced capacity of S9 suspensions augmented with Tween, BSA or β-HPCD. Data points show the mean and SEM of three replicates. Only the Tween data could be fitted to the original linear model $E = K_{S9}[S9] + E_{carrier}$. Parallel slopes indicate similar S9 binding constants.

Table 6.1. S9 binding constants K_{S9} and p-values from the ANCOVA analysis (significance threshold $\alpha = 0.05$) comparing K_{S9} in carrier-dosed treatments against K_{S9} in the acetone-dosed treatment.

	acetone		Tween 80		BSA		β-HPCD	
	$K_{S9} \pm$ SE L/kg		$K_{S9} \pm$ SE L/kg	p-value	$K_{S9} \pm$ SE L/kg	p-value	$K_{S9} \pm$ SE L/kg	p-value
naphthalene	1386	153.9	na	na	na	na	9655	629.1	<0.0001
phenanthrene	28334	2345	28228	2147	0.973	36545	11492	0.452	86420	6757	<0.0001
anthracene	40934	1948	38047	2820	0.430	47818	14052	0.598	136382	11408	<0.0001
fluoranthene	126632	9660	122552	8908	0.761	141621	43430	0.716	233112	11440	<0.0001
pyrene	163772	12456	161427	10689	0.888	170639	49498	0.885	293986	11261	<0.0001

tercepts were by a constant factor of ca. 4 larger than enhanced capacities of aqueous Tween solutions predicted from Tween binding constants for PAHs that had been determined in another experiment through $E_{Tween}([Tween] = 0.1g/L) = K_{Tween:water} * 0.1g/L + 1$, however at room temperature ($20 \pm 2\ ^{\circ}C$). As protein partition coefficients of HOCs generally increase with decreasing temperature, the intercepts seem good estimates for the actual Tween enhancement factors in aqueous solution at 11 $^{\circ}C$. For all other dosing vehicles, the model could not predict the theoretical y-intercept $E_{carrier}$, and only in the case of acetone were the actually observed enhancement factors at [S9]=0.25 g/L estimated correctly. Therefore, the domain of the linear model was constrained to the S9 concentration range 0.5-2.0 g/L for BSA and β-HPCD, and to the concentration range 0.25-2.0 g/L for the acetone treatment. Thus, we conclude that Tween as secondary sorbent contributed additively to PAH binding in S9 suspensions. In contrast, the molecular carriers BSA and β-HPCD appeared to display non-additive PAH binding behavior at low S9 concentrations, and it could be hypothesized that this may be due to an interaction of S9 with these carriers (e.g. [49]). Though this phenomenon was not obvious in the S9+acetone systems, the intercepts of the capacity enhancement curves, which were below unity (except for naphthalene), give indication that the S9+acetone system neither followed the additive model (eq 6.7) over the entire S9 concentration range.

Enhancement curves for acetone, Tween and BSA were almost perfectly parallel (Figure 6.3). The slopes of the linear regressions yielded S9 binding

constants K_{S9} and were compared by an analysis of covariance (ANCOVA) using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla California, USA, www.graphpad.com). This proved that S9 binding constants were not statistically different between acetone, Tween and BSA treatments ($p \gg 0.05$; Table 6.1), which in turn suggests that the carriers did not modify the S9 binding behavior. S9 binding constants K_{S9} also agreed very well with binding constants that had been previously determined by a thin-film passive dosing method (Figure D.5). In contrast, apparent S9 binding constants in presence of β-HPCD were increased by a factor 2-3 (for naphthalene by a factor of 7), indicating a possible interaction of β-HPCD with S9. This may in part explain why the measured depletion rate constant k_{dep} in S9 systems with β-HPCD was equal to the one in an S9 system without molecular carriers in the particular case of fluoranthene (Figure 6.5).

Based on eq 6.10, ratios of S9-bound fractions $f_{S9}^{carrier}/f_{S9}^{\emptyset}$ were calculated as the inverse of capacity ratios $E_{S9}/E_{S9+carrier}$ to see whether they would correlate to the relative change in depletion rate constants when introducing an additional sorption phase into S9 test systems. Since eq 6.10 is based on the assumption of additive binding, only the Tween data can be interpreted with confidence. As introducing a secondary binding phase reduces the freely dissolved concentration (i.e. chemical activity) of a substance in a system, S9-bound PAH fractions were as expected lower in S9 systems with the carriers Tween, BSA or β-HPCD than in S9 systems that were solvent-spiked. However, the higher the S9 concentration in the system, the lower becomes the importance of a carrier, and eventually ratios of S9-bound fraction approximate unity (Figure 6.4). The S9

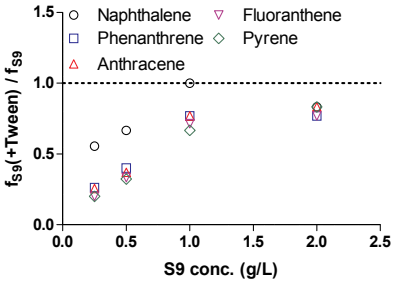

Figure 6.4. Ratios of S9-bound PAH fractions in S9 suspensions with and without Tween as a function of the S9 concentration in the *in vitro* assays.

concentration in all biotransformation assays was 1 g/L and therefore S9-bound PAH fractions in acetone, BSA and β-HPCD treatments were determined from the actually measured enhanced capacities in the carrier-S9 suspensions at [S9] = 1 g/L. In the case of S9+Tween suspensions, for which the binding model had appeared to be valid, enhanced capacities were deduced from the fitted slope and intercept of the linear model (eq 6.7; slopes are given in Table 6.1; intercepts $E_{Tween}([S9] = 1g/L)$ were 9.04, 11.34, 43.63 and 55.87 for phenanthrene, anthracene, fluoranthene, and pyrene respectively).

Generally, ratios of depletion rates in S9 suspensions with and without carriers corresponded well to ratios of S9-bound fractions (Figure 6.5), showing that differences in S9-bound fractions were reflected in differences in the enzymatic biotransformation of PAHs. For Tween in par-

ticular, differences in the biotransformation activity of S9 enzymes likewise correspond to changes in free fractions (eq 6.10), as PAH binding proved to be additive in mixed S9-Tween systems. The somewhat lower depletion rate ratios for Tween than the corresponding ratios of S9-bound fractions (Figure 6.5) may in part be due to an enzyme-inhibiting effect of Tween on S9 that we had already observed in the G6P assay. Naphthalene data were excluded, as depletion rates were not measurable with all carriers and also because linear regression of measured enhanced capacities of S9 suspensions did not yield binding constants for all dosing treatments.

Carriers for substrate supply. The results of this study showed that organic molecules that consist of both hydrophilic and hydrophobic domains such as the surfactant Tween, the biological macromolecule BSA, and the cyclic oligosaccharide β-HPCD can serve as carrier molecules and substrate delivery vehicles for PAHs in an *in vitro* assay. However, β-HPCD appeared unsuited in the particular fish liver S9 biotransformation assay as it altered PAH binding to S9, which in turn precluded the use of a simple additive binding model to predict PAH speciation in the S9-carrier system. With BSA, S9 PAH binding constants seemed to be conserved, but binding interactions seemed to occur at low concentrations of S9. On the one hand, BSA has the advantage of being a naturally occurring protein with hydrophobic binding domains, which even seemed to support CYP enzyme activity. On the other hand, it constitutes a binding phase of rather variable properties; PAH binding in particular has been shown to depend on protein treatment [44] and is likely also de-

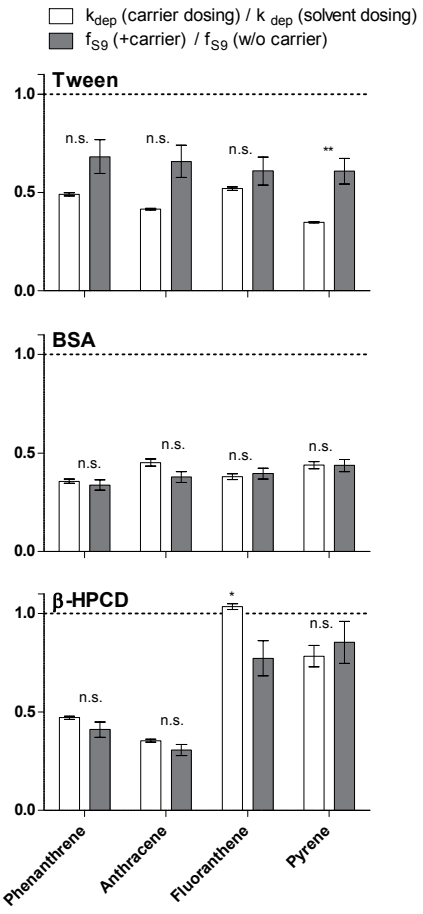

Figure 6.5. Correlation of ratios of depletion rate constants with ratios of S9-bound PAH fractions to investigate whether a change in the S9-bound PAH fraction would be reflected in a similar change in depletion rate constants.

pendent on the protein's properties that result from the applied method of manufacturing. It is also important to consider that adding BSA into an assay that already uses S9 protein suspensions further increases the risk of protein precipitation. Tween proved to be the only carrier displaying predictable additive PAH binding that was independent of the concentration of the primary sorbent S9. On the downside, it also slightly affected the activity of CYP enzymes. To reduce these adverse effects, one could possibly further lower the Tween concentration, but the concentration window is very narrow, as Tween is only effective as carrier above its critical micelle concentration. Rather than proposing that molecular carriers should be considered an alternative for introducing HOCs into biotransformation assays, we conclude that such alternative dosing approach proved to be appropriate as confirmatory measure to validate the accuracy of carefully conducted *in vitro* biotransformation assays, in which the amount of solvent for dosing of HOCs is minimized to as low as 0.5 % vol/vol.

Effect of third-phase binding of HOCs on biotransformation and implications. Earlier, researchers who investigated biotransformation of chemicals have expressed their concern on the effect of chemical binding on intrinsic hepatic clearance rates [44, 51]. It is commonly assumed that the unbound fraction of chemical determines enzymatic biotransformation rate constants [13, 30, 51, 43]. While this is true to the extent that only freely dissolved chemicals can partition from the intracellular medium (or aqueous medium in an *in vitro* assay) to the actual site of biotransformation, which supposedly lies within a macromolecular protein assembly [32], it has also been described in the analytical chemistry literature that under certain circumstances the readily desorbing bound chemical fraction can contribute to mass flux toward a sink [14]. Specifically, it was noted that this may be the case when the rate limiting step for an uptake process is diffusion through the an aqueous boundary layer, the concentration of the sorbed analyte is high, and when desorption from the matrix is fast [14]. In the case of BSA and β-HPCD, it is thus possible that also the carrier-bound PAH fraction may have contributed to a flux of PAH toward active site for biotransformation within the S9 matrix, which in turn may have increased the apparent biotransformation rate constant. Consequently, the assumption that only the freely dissolved fraction of a chemical is metabolized may not hold in all instances, which then would lead to an underestimation of biotransformation in in-vitro-to-in-vivo extrapolations, and hence to an overestimation of bioaccumulation factors.

6.4. Conclusions

This study showed that *in vitro* biotransformation rate constants can be reliably determined if a carefully validated dosing protocol is followed. Carrier-based dosing confirmed that a carrier solvent such as acetone in a very low concentration (0.5 % vol/vol) did not impair the enzymatic activity of S9 fractions to a degree that it would bias the determined depletion rate constants, as depletion rates agreed within a factor of ca. 2.5 between the different dosing regimes. The underestimation of biotransformation rate constants that may result from initial substrate concentrations that are too high is, by comparison, on the order of one magnitude

[43]. Rather than experimental issues in the determination of intrinsic clearance rates, the assumption of C_{free} controlling biotransformation rate constants is a much larger issue for the uncertainty of BCF estimates. Our study suggests that it may suffice to assume that S9-bound chemical fractions control biotransformation rate constants, as relative differences in *in vitro* biotransformation rate constants due to the presence of secondary sorption phase scaled equally to the ratio of S9-bound chemical fractions as to the ratio of free fractions.

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Part III

Closure

Chapter 7
Linking exposure and effect levels on the basis of a reference partitioning phase and chemical activity

7.1. Introduction

The ultimate task of chemical risk assessment is to weigh chemical exposure against exposure levels causing adverse effects, and thereupon to characterize the risk resulting from chemical pollution. Traditionally, such risk characterization relies on a comparison of predicted environmental concentrations (PEC) and predicted no-effect concentrations (PNEC), whereby variability both in exposure and effect measurements, including differences in susceptibility, is accounted for by the use of uncertainty factors. The severity and the nature of adverse effects are then evaluated during a risk characterization through comparison of toxic concentration levels and by the use of toxic equivalency factors (TEFs). However, two major issues hamper such risk characterization: (1) PEC/PNEC ratios are problematic because concentrations do not necessarily allow for multimedia comparisons. (2) The severity of adverse effects or the toxic potency of a chemical is defined as a relative measure – a closer look reveals that the choice of the reference for such relative values is not always justifiable.

The concepts of a reference partitioning phase and chemical activity can help to overcome some of the challenges in risk characterisation: A reference partitioning phase can offer a common basis for concentration-based measurements, and chemical activity is a dimensionless measure that only depends on the chosen reference state for a chemical and thus can be compared across media. The chemical activity concept has not yet been fully realized within chemical risk assessment practices, although the theoretical basis exists and has been scrutinized by the scientific community [1, 2, 3, 4]. Webster et al. [5] put forward the use of equilibrium lipid concentrations, complementary to fugacity as exposure parameter, as a measure to compare exposure levels across ecosystems and between different media. However, the conversions of conventional concentration measures into equilibrium lipid concentrations relied on the assumption of chemical equilibrium between phases, and required knowledge of the fugacity capacities of the phases or knowledge of the respective partition coefficients. Advantageously, exposure data expressed on the basis of a common reference partitioning phase, such as lipid as suggested by Webster et al., are much more intuitively understood.

Lipid appears to be a suitable reference partitioning phase for hydrophobic organic chemicals (HOCs) for the obvious reason of it being an environmental phase of interest to estimate bioaccumulation and (lipid) membrane-induced toxicity (narcosis) [6, 7].

Thermodynamically, it constitutes a suitable reference phase because lipid activity coefficients of HOCs are small (e.g. compared to water) and, importantly, similar between HOCs [8, 9], which allows an instant comparison of chemical activity between chemicals.

With regards to the latest developments made in techniques such as equilibrium passive sampling [10] and partitioning-controlled dosing (passive dosing) [11, 12, 13, 14], that employ a polymer as partitioning phase, I suggest that also polymers, and in particular silicones, be considered a reference partitioning phase – with the main advantages being that (1) a polymer as partitioning reference phase links directly to chemical measurement techniques and (2) concentrations expressed on a polymer-basis can either be compared directly or be linked to chemical activity in a straight-forward way.

In the following, this will be demonstrated with data from the studies by Jahnke et al. [15] and Mäenpää et al. [16], in which polymer-based equilibrium partitioning methods were used to determine PCB exposure in a pristine lake in Sweden and in a contaminated lake in southern Finland. In both studies, the aim had been not only to measure freely dissolved concentrations in multiple phases of the lake ecosystems by the use of silicone passive samplers, but rather to assess the chemical equilibrium status of the environmental compartments in these ecosystems. While Mäenpää et al. used chemical activity as exposure parameter and measure for the equilibrium status of the ecosystem, Jahnke et al. based the assessment of the ecosystem's equilibration status on ratios of equilibrium partitioning concentrations measured in silicone, which in fact represented chemical activity ratios. Thus, both studies provide data that can be used for a true comparison of the PCB exposure situation in two distinct ecosystems. With the aim to illustrate the use of a polymer as a reference partitioning phase for a thermodynamically-based risk assessment of some organohalogen compounds, the results of both these studies will be put in relation to endocrine toxicity data that were determined in chapter 5 under equilibrium-partitioning controlled exposure conditions in an *in vitro* assay. In this effort, silicone-based concentrations from the individual studies will be converted to chemical activities. In addition, critical body residues of some narcotic organic chemicals in three different species that were determined by van der Heijden et al. [18] will be expressed on the scale of chemical activities to further validate the as hitherto established chemical activity range of 0.01-0.1 for baseline toxicity (narcosis) [19, 9, 20, 21, 22, 23].

7.2. Methods

Chemical activities (a) were estimated from silicone-based concentrations (C_{sil}) given in Jahnke et al. [15] and Gilbert et al. [17] as follows:

$$a = \frac{C_{sil}/K_{sil:w}}{S_L} \equiv \frac{C_{free}}{S_L} \tag{7.1}$$

where S_L is the subcooled liquid aqueous solubility. S_L values for PCBs were estimated according to van Noort et al. [24] as $\log S_L(mol/L) = -0.575([N_{Cl}] -$

$0.33[N_{ortho-Cl}]) - 1.38$ where N_{Cl} is the number of chlorine atoms in the molecule and $N_{ortho-CL}$ the number of chlorine atomos in *ortho* position. For PBDEs, subcooled liquid solubilities were calculated as the ratio of the subcooled liquid vapor pressure (P_L°; Pa) and the Henry's law constant (H; Pa m^{-3} mol^{-1}), taking parameter values from Tittlemier et al. [25]. For hexachlorobenzene (HCB), p'p-DDT and p'p-DDE, S_L values were taken from Suntio et al. [26].

Also concentrations of the endogenous hormones testosterone, progesterone, 17β-estradiol and cortisol were converted into chemical activities. Reference values for freely circulating hormone concentrations in humans ([27] and references therein) were used to estimate chemical activities of hormones according to eq (7.1), and subcooled liquid solubilities were estimated from the Yalkowsky equation [28]:

$$a_{max} = \exp\left[6.8\left(1 - \frac{T_m}{T}\right)\right]. \tag{7.2}$$

with T_m being the melting point temperature (K) and T the ambient temperature (K).

The CBR data set published by van der Heijden et al [18], i.e. total lipid-based lethal concentrations (C_{lipid}; mmol/kg total lipid), were converted into chemical activities by assuming a constant lipid activity coefficient $\gamma_{lip} = 0.3$ L/mol for all chemicals [8] and calculating chemical activity as

$$a = \gamma_{lip} C_{lip} \tag{7.3}$$

Such calculated chemical activities were cross-validated by also estimating freely dissolved concentrations via a lipid-water partition coefficient $K_{lip:w}$ (determined by van der Heijden et al. [18]) , and then calculating chemical activity as the fraction of saturation using the subcooled liquid solubility (for substances that are solids at ambient temperature) or simply the aqueous solubility (for substances that are liquids at ambient temperature, i.e. pentylbenzene and 1,2,4-trichlorobenzene):

$$a = \frac{C_{lip}/K_{lip:w}}{S_L} \tag{7.4}$$

Subcooled liquid solubilities were estimated as $S_L = S_w/a_{max}$, where the maximum chemical activity a_{max} was calculated from the Yalkowsky equation as above.

7.3. Results & Discussion

Linking critical body residues of narcotic chemicals to chemical activity. Total lipid-based critical body residues in *Lumbriculus variegatus, Hyalella azteca* and *Poecilia reticulata* (guppies) that van der Heijden et al. [18] determined for a a set of simple organic chemicals acting as baseline toxicants were all within the expected chemical activity range 0.01 to 0.1 (Figure 7.1), regardless of whether the conversion was based on a lipid activity coefficient that was assumed to be the same for all chemicals, i.e. $\gamma = 0.3$ L/mol, or on the fraction of aqueous solubility , i.e. C_{free}/S_L

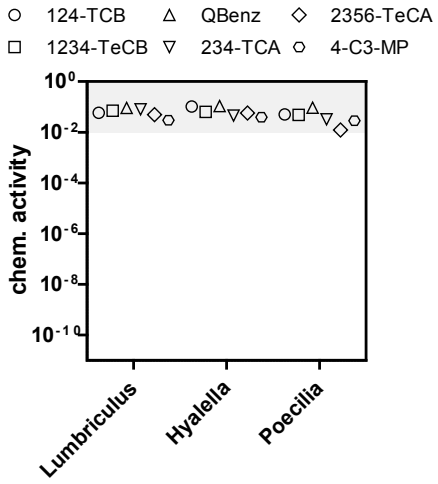

Figure 7.1. Chemical activity of some narcotic compounds tested by van der Heijden et al. (2015) [18]. Abbreviations: TCB=trichlorobenzene, TeCB=tetrachlorobenzene, QBenz=pentylpenzene, TCA=trichloroaniline, TeCA=tetrachloroaniline, 4-C3-MP=4-chloro-3-methylphenol.

(Figure 7.2). On the one hand, these results confirm the established chemical activity range for baseline toxicity and show once more that this range is independent of the structural features of organic chemicals and independent of tested species, in line with earlier findings [19, 29, 20, 21, 23]. On the other hand, this conversion shows that the critical body residue concept [30, 31], and in particular the target lipid model for narcosis [6] is in agreement with the Ferguson principle [19, 21]. The good agreement of chemical activities estimated through both eq 7.3 and eq 7.4 indicates that indeed it seems to be fair to assume that $gamma_{lipid} = 0.3$ L/mol – a value reflecting the approximate activity coefficient of PAHs in storage lipids [8]. Nevertheless, further research should be directed at determining lipid activity coefficients for a structurally diverse set of organic chemicals to validate this estimate.

Chemical activity of endocrine active substances. Effective aqueous concentrations of hormones, i.e. C_{free}, ranged within 6 orders of magnitude while effective freely dissolved concentrations of the organohalogen compounds tested by Gilbert et al. fell within 3 orders of magnitude below the range observed for hormones (Figure 7.3A) and were in the femto- to picogram per liter concentration range. At a first glance, it may seems counterintuitive to find effective (aqueous) concentrations of xenoestrogens below effective concentrations of endogenous hormones. However, as both the organohalogen chemicals and the steroid hormones (except cortisol) are rather hydrophobic, a comparison of the chemicals' "activity" is complicated by the fact that the wide variability in interactions of these solutes with water is in fact what

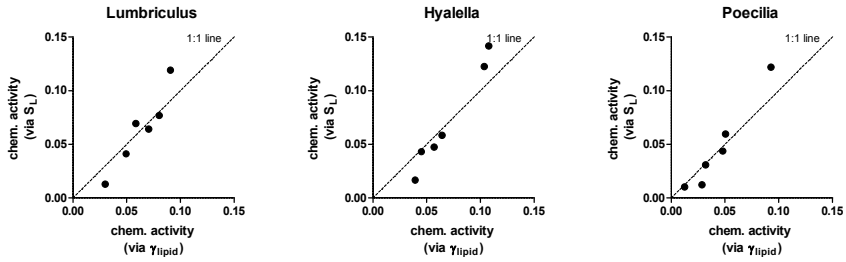

Figure 7.2. Comparison of chemical activities of some narcotic compounds [18] that were estimated from equilibrium partitioning freely dissolved concentrations and subcooled liquid solubilities S_L or by assuming a constant lipid activity coefficient γ_{lipid} of 0.3 L/mol.

is reflected in the aqueous effective concentrations of these chemicals. When effective concentrations are compared not on the basis of aqueous medium but rather on the basis of a more appropriate reference phase for hydrophobic chemicals such as silicone (Figure 7.3B), it becomes clear that effective concentrations of both endogenous hormones and xenohormones are in fact within the same and a much narrower range that only spans approximately 2 orders of magnitude.

Such change of point of view also changes our perception of toxic potency. Toxic potency is typically defined as a ratio of an effective concentration of a test chemical and that of a reference chemical. Several concepts in toxicology such as that of the toxic equivalency factors (TEFs) [32, 33], the related concept of relative effective potencies (REPs), e.g. [34], and the approach of calculating17β-estradiol-equivalent factors or -concentrations for comparing toxic potencies of endocrine active chemicals all rely on a comparison of effective concentrations *between* chemicals. Thus, the definition of "potency" is dependent on the metrices used to describe exposure and consequently must be judged with great care.

If chemical activity is chosen as exposure parameter instead of the concentration in a reference phase such as silicone, it appears that the overall picture of relative potencies of the organohalogen compounds and endogenous hormones is conserved (Figure 7.3C). In addition, chemical activity as exposure parameter provides means to quantify the "excess toxicity" of these endocrine active compounds, i.e. to estimate an activity ratio as the ratio of an effective chemical activity to baseline toxic chemical activity, as has been suggested by Mackay et al. [21]. Note, that this is then not a comparison of activity between chemicals but rather an equivalent to a "toxic ratio" [35, 36], which essentially distinguishes a specific form of toxicity of a chemical from its baseline toxicity.

Apparently, for endocrine active compounds such an activity ratio (or toxicity ratio), i.e. the ratio of chemical activity required for baseline toxicity and the chemical activity required for endocrine activity, seems to equal or greater than 10^5. This is

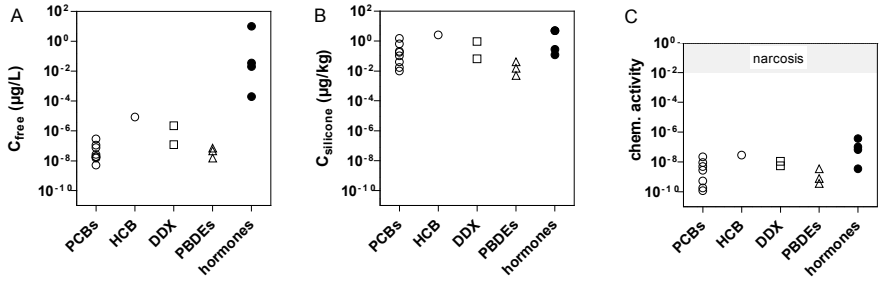

Figure 7.3. Three exposure parameters for endocrine toxicity data from Gilbert et al. (2015) in comparison: (A) freely dissolved aqueous concentrations (C_{free}), (B) Concentrations in silicone as reference partitioning phase, and (C) chemical activity. While freely dissolved aqueous concentrations span over five orders of magnitude, the data collapse into a much narrower range when expressed on the basis of a reference partitioning phase or as chemical activity.

an even larger factor than has been observed for chemicals exerting toxicity through a specific mode of action [37, 14]. While narcosis is a non-specific and reversible form of toxicity thought to be triggered through interactions of chemicals with the lipid cell membrane, and thus the minimum toxicity any compound can theoretically exert, toxicity through a specific mode of action is believed to involve a chemical reaction or chemical interactions through formation of covalent bonds of the toxicant with a specific molecular target, which does not require a rather high fraction of saturation like narcosis [35, 21]. Endocrine disruption as a toxicity endpoint on the other hand seems to happen at even lower fractions of saturation, and it may be hypothesized that this is due to the fact that not only a specific molecular interaction occurs but rather an entire regulatory circuit is targeted. It is interesting that effective chemical activities of the endocrine active compounds considered here (i.e. some endogenous steroid hormones, polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs), hexachlorobenzene (HCB) as well as DDT and its metabolite DDE) all fall within the approximate chemical activity range 10^{-7} to 10^{-9}. This leads to the hypothesis that endocrine target(s) may be of a nature, or embedded in a phase, such that hydrophobic chemicals exhibit similar phase activity coefficients. Only dedicated studies that are directed at a broader range of endocrine-related toxicity outcomes can challenge this hypothesis.

Chemical activity of PCBs in the environment. The studies by Jahnke et al. and Mäenpää et al. both allow an assessment and comparison of the environmental exposure situation to PCBs (Figure 7.4). PCBs occur at rather low fractions of saturation, i.e. at chemical activities below 10^{-7} in an ecosystem without direct contamination sources. By contrast, chemical activities of PCBs in a contaminated ecosystem are elevated by up to three orders of magnitude. While these exposure levels are far from the range where baseline toxicity is expected, it is worrisome that

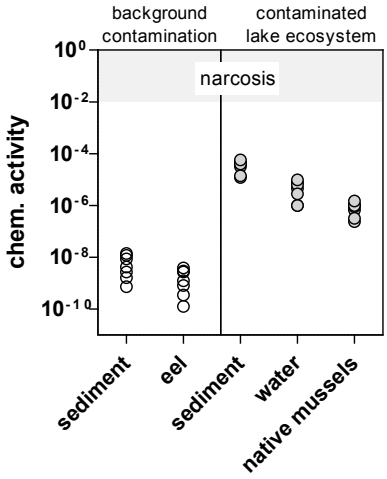

Figure 7.4. Environmental exposure levels of PCBs: Multimedia comparison of an uncontaminated with a contaminated ecosystem on the basis of chemical activity. Data from Jahnke et al. (2014) were converted into chemical activity, and data from Mäenpää et al. (2015) were directly plotted. The same PCB congeners are displayed as in the study by Gilbert et al. (2015).

these actual environmental PCB levels coincide with the chemical activity range, in which PCBs may possibly exert endocrine activity (Figure 7.3C). In terms of a risk characterization, this actually means that the risk quotient, here re-defined as the ratio of the predicted effective activity (PEA) and the predicted environmental exposure activities (PNEA), in the particular case of PCBs raises concern.

7.4. Conclusions

Exposure levels of hydrophobic organic chemicals when determined as equilibrium partitioning concentrations in a suitable reference partitioning phase allow a direct readout and comparison of the "activity" of chemicals. In other words, patterns in exposure data on the basis of, e.g., silicone or lipid can directly mirror the patterns that are revealed on a chemical activity basis. A partitioning reference phase for hydrophobic organic chemicals is therefore thought useful for the ultimate translation of concentrations into chemical activities, and thus a thermodynamic understanding of exposure. Finally, chemical activity proved to be an inevitable measure to understand how different forms of toxicity, in particular baseline toxicity and endocrine disruption, scale to different levels of exposure.

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Chapter 8
Conclusions

8.1. Synthesis and discussion of the main findings

The objective of this thesis was to introduce and apply a thermodynamic reference phase for linking exposure and effect assessment of hydrophobic organic chemicals within chemical risk assessment. The study's aim was thus to introduce a thermodynamic reference phase as a new concept, to further develop partitioning-based methods that apply a reference partitioning phase, and to finally show that the new conceptual and methodological advances can support a thermodynamically-based chemical risk characterization. While thermodynamic concepts and models have been introduced and are to some degree applied for current exposure assessment, and to a very limited degree also for the effect assessment, a firm link between exposure and effect assessment based on chemical thermodynamics has not yet been realized. This study therefore aimed to answer (1) how concepts from chemical thermodynamics can be applied and expanded to increase the comparability, accuracy and precision of methods to determine exposure, (2) how environmental HOC exposure levels can be transferred into laboratory tests for an effect assessment, and (3) to estimate levels of HOCs in organisms, and in particular at the site of toxic action, based on partitioning projections.

First, it was found that clear concepts and definitions of exposure parameters do exist (from chemical thermodynamics) on which comparisons of exposure levels between media can and should be based on, but that these ought to be brought from the conceptual level into practice by the development and application of dosing methods and measurement techniques that are built on a working principle that allows to measure and control thermodynamic exposure parameters. It appeared that such methods need to be linked to each other to move the basis for exposure comparison from the abstract level of a unifying thermodynamic exposure parameter, such as chemical activity, to a more operational domain. This was achieved by introducing a reference partitioning phase, and it was shown (chapter 4) that in the particular case of a polymer as reference partitioning phase, method calibration and thus an improvement of accuracy and precision of quantitative chemical measurements is possible through polymer calibration. Further, it was demonstrated that through the use of a reference partitioning phase exposure levels can be transferred across media, e.g. from the environment and into laboratory toxicity tests (chapter 5). Finally, this allowed a straightforward linkage of environmental exposure and exposure-effect relationships observed in controlled laboratory studies. Moreover, the use of a reference

partitioning phase facilitated a straightforward translation of chemical concentrations into chemical activities.

Second, it was found that the chemical activity scale provides new insights for an evaluation of the actual risk associated to environmental chemical exposure, as certain mechanisms of toxicity seem to be linked to rather narrow ranges of chemical activity. In particular, it became apparent that chemical activities of PCBs in a study area without direct PCB input sources are in the same range as chemical activities of PCBs in humans, and that these are at a level at which compounds may exert endocrine action.

Third, the individual studies all demonstrated that by applying equilibrium partitioning theory, projections of chemical concentrations (or chemical activities) can be made toward a biologically relevant phase for bioaccumulation, i.e. lipids, or toward a toxicologically relevant site of interest, e.g. specific enzymes: All studies encompassed within this thesis (chapters 4, 5, 6), and also the ones to which I contributed besides [1, 2, 3], deal to some degree with the aspect of determining internal levels of chemicals. Lipids play a dominant role in bioaccumulation of chemicals, and in particular membrane lipids are the toxic target for narcosis. In the studies by Jahnke et al. [1] and Mäenpää et al. [2], concentrations of hydrophobic organic chemicals in biota lipids were estimated based on equilibrium partitioning projections using partition coefficients involving a lipid phase. In the studies by Jahnke et al. [1] and Mäenpää et al. [2] , this was done with the aim to estimate the thermodynamic potential of PCBs to bioaccumulate. In contrast, in the study by van der Heijden et al. [3], these estimations served to set the upper limit for investigating membrane toxicity mediated narcotic effects of some organic chemicals, while in the manuscripts II and III (chapter 5 and 6) were specifically concerned with determining the concentration of HOCs at a specific toxicological target site, i.e. in adrenal cells or at the active sites for biotransformation in S9 fractions (P450 enzymes).

8.2. Implications

This study questions some of the current fundamental scientific paradigms in chemical risk assessment and highlights their shortcomings, while affirming that the progress made in recent years through the use of thermodynamic concepts proves beneficial to better link exposure and effect assessment. With the introduction of a reference partitioning phase, the here presented work elaborates thermodynamic equilibrium concepts, realizing them by means of new equilibrium partitioning methods for determining and controlling exposure. This not only facilitates the implementation of established thermodynamic concepts for chemical risk assessment, but also provides measurements and empirical observations of a new quality. Specifically, the concept of a reference partitioning phase may help to better understand the scales of partitioning processes, which is an essential prerequisite to understand multimedia phase partitioning, to design new partitioning-based methods, and to interpret exposure and effect data. Calibrating methods for determining exposure and methods for detecting toxic effects of HOCs based on a polymer as reference partitioning phase has the po-

tential to further reduce the uncertainty in chemical risk assessment, and therewith to reduce the uncertainty associated to risk management decisions. In this regard, the thesis also contributes to defining "a 'safe' distance from a dangerous level" of chemical pollution [4]. Within this thesis also the chemical activity concept was explored and an example provided how chemical activity can be applied for risk characterization based on ratios of effective and environmental chemical activities.

8.3. Future research needs

For measurements of HOCs in a reference partitioning phase to be directly linked to chemical activity, future research should be directed at determining activity coefficients in possible reference phases such as silicone or lipid. Direct methods for their determination have been suggested, e.g. by Reichenberg et al. [5] and Mayer et al. [6], and should be complemented with modeling efforts to evaluate to which degree activity coefficients agree between chemicals and within reference phases. Future research should also be directed at the development of dosing methods that in their working principle make use of lipid as reference partitioning phase. This should improve the link between external exposure concentrations and internal contaminant concentrations. Further, dosing from lipids can provide means to directly set test concentrations directly within or below the range where narcosis is expected (40-160 mmol/kg lipid), without the need for further phase partitioning or chemical activity calculations. Similarly, a concentration range on a polymer-basis has been linked to narcosis. For chemical activity, or more precisely ratios of exposure and effect chemical activity ratios, to become a unifying risk indicator, an important question to tackle is how different forms of toxicity, beyond narcosis, link to chemical activity. If such link can be established, the chemical activity scale may become a useful tool to predict toxicity, to quantify potency on a chemical activity basis and thereupon to rank toxic endpoints, and to hypothesize about the nature of a toxic target site.

8.4. Concluding remarks

This thesis integrated aspects of exposure and effect assessment on the basis of chemical thermodynamics. It expanded the thermodynamic conceptual framework by introducing a reference partitioning phase, which aided to put a powerful concept from chemical thermodynamics into practice. It was shown that a polymer as reference partitioning phase can provide a unifying basis to determine and control the chemical activity of HOCs. With that, the thesis gives an example how a link between environmental exposure and exposure-effect relationships can be established, much as envisioned by Mackay et al. [7]. This is essential to better characterize and manage risks resulting from chemical pollution.

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Appendices

Appendix A
Additional publications

Silicone passive equilibrium samplers as "chemometers" in eels and sediments of a Swedish lake

Jahnke A, Mayer P, McLachlan MS, Wickström H, **Gilbert D** and MacLeod M. 2014. *Environmental Science Processes & Impacts* 16:464–472. DOI: 10.1039/c3em00589e.

Abstract: Passive equilibrium samplers deployed in two or more media of a system and allowed to come to equilibrium can be viewed as 'chemometers' that reflect the difference in chemical activities of contaminants between the media. We applied silicone-based equilibrium samplers to measure relative chemical activities of seven 'indicator' polychlorinated biphenyls (PCBs) and hexachlorobenzene in eels and sediments from a Swedish lake. Chemical concentrations in eels and sediments were also measured using exhaustive extraction methods. Lipid-normalized concentrations in eels were higher than organic carbon-normalized concentrations in sediments, with biota-sediment accumulation factors (BSAFs) of five PCBs ranging from 2.7 to 12.7. In contrast, chemical activities of the same pollutants inferred by passive sampling were 3.5 to 31.3 times lower in eels than in sediments. The apparent contradiction between BSAFs and activity ratios is consistent with the sorptive capacity of lipids exceeding that of sediment organic carbon from this ecosystem by up to 50-fold. Factors that may contribute to the elevated activity in sediments are discussed, including slower response of sediments than water to reduced emissions, sediment diagenesis and sorption to phytoplankton. The 'chemometer' approach has the potential to become a powerful tool to study the thermodynamic controls on persistent organic chemicals in the environment and should be extended to other environmental compartments.

Fate of polychlorinated biphenyls in a contaminated lake ecosystem: Combining equilibrium passive sampling of sediment and water with total concentration measurements of biota.

Määenpää K, Leppänen M, Figueiredo K, Mayer P, **Gilbert D**, Herve S, et al. 2015. *Environmental Toxicology & Chemistry* DOI: 10.1002/etc.3099.

Abstract: Passive equilibrium sampling devices (ESDs) can be applied to study and monitor the exposure and fate of hydrophobic organic chemicals (HOCs) on a thermodynamic basis. ESDs can for instance be used to determine (1) freely dissolved concentrations, (2) chemical activity ratios and (3) HOC concentrations in lipids at thermodynamic equilibrium with the sediment or water. Our aim was to assess the exposure status for polychlorinated biphenyls (PCBs) in a contaminated lake ecosystem along its discharge course using ESDs for measurements in sediment and water and combine them with analyses of biota. The ESDs (silicone rubber and polyethylene) were applied to determine freely dissolved concentrations and chemical activities of PCBs in the water column and sediment pore water, and to estimate chemical activities and PCB concentrations in model lipids at thermodynamic equilibrium with water and sediment. Chemical activities of PCBs were higher in sediment than in water, which implies that sediment functions as a source of PCBs and that there is net diffusion from sediment to the water column. Measured lipid-normalized PCB concentrations in biota were generally below the calculated concentrations in lipids at thermodynamic equilibrium with the sediment, indicating that the organisms were below the equilibrium partitioning level relative to the sediment. The versatility of ESDs and their usefulness for a thermodynamic understanding of chemical exposure and fate was successfully demonstrated.

Determining high-quality critical body residues for multiple species and chemicals by applying improved experimental design and data interpretation concepts

van der Heijden S, Hermens JLM, Sinnige TL, Mayer P, **Gilbert D**, Jonker MTO. 2015. Determining high-quality critical body residues for multiple species and chemicals by applying improved experimental design and data interpretation concepts. *Environmental Science & Technology* 49:1879–1887. DOI: 10.1021/es505078r

Abstract: Ecotoxicological effect data are generally expressed as effective concentrations in the external exposure medium and do thus not account for differences in chemical uptake, bioavailability, and metabolism, which can introduce substantial data variation. The Critical Body Residue (CBR) concept provides clear advantages, because it links effects directly to the internal exposure. Using CBRs instead of external concentrations should therefore reduce variability. For compounds that act via narcosis even a constant CBR has been proposed. Despite the expected uniformity, CBR values for these compounds still show large variability, possibly due to biased and inconsistent experimental testing. In the present study we tested whether variation in CBR data can be substantially reduced when using an improved experimental design and avoiding confounding factors. The aim was to develop and apply a well-defined test protocol for accurately and precisely measuring CBR data, involving improved (passive) dosing, sampling, and processing of organisms. The chemicals 1,2,4-trichlorobenzene, 1,2,3,4-tetrachlorobenzene, 2,3,4-trichloroaniline, 2,3,5,6-tetrachloroaniline, 4-chloro-3-methylphenol, pentylbenzene, pyrene, and bromophos-methyl were tested on Lumbriculus variegatus (California blackworm), Hyalella azteca (scud), and Poecilia reticulata (guppy), which yielded a high-quality database of 348 individual CBR values. Medians of CBR values ranged from 2.1 to 16.1 mmol/kg wet weight (ww) within all combinations of chemicals and species, except for the insecticide bromophos-methyl, for which the median was 1.3 mmol/kg ww. The new database thus covers about one log unit, which is considerably less than in existing databases. Medians differed maximally by a factor of 8.4 between the 7 chemicals but within one species, and by a factor of 2.6 between the three species but for individual chemicals. Accounting for the chemicals' internal distribution to different partitioning domains and relating effects to estimated concentrations in the target compartment (i.e., membrane lipids) was expected to but did not decrease the overall variability, likely because the surrogate partition coefficients for membrane lipid, storage lipid, protein, and carbohydrate that were used as input parameters did not sufficiently represent the actual partitioning processes. The results of this study demonstrate that a well-designed test setup can produce CBR data that are highly uniform beyond chemical and biological diversity.

Appendix B
Supporting Information to Manuscript I

Supporting information for: Polymers as reference partitioning phase: polymer calibration for an analytically operational approach to quantify multimedia phase partitioning

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Table S1: List over chemicals, their purity and supplier.

chemical	short name[a]	$log\ K_{ow}$[b]	purity (%)	supplier
Polychlorinated biphenyls				
4-Chlorobiphenyl	CB-3	4.69	99	Ultra Scientific
2,4,4'-Trichlorobiphenyl	CB-28	5.67	99.78	Promochem
2,4',5-Trichlorobiphenyl	CB-31	5.67	99.8	Promochem
2,2',3,3'-Tetrachlorobiphenyl	CB-40	5.66	99.1	Dr. Ehrenstorfer
2,2',3,5'-Tetrachlorobiphenyl	CB-44	5.75	99	Cambridge Isotope Laboratories
2,2',4,5'-Tetrachlorobiphenyl	CB-49	5.85	98	Cambridge Isotope Laboratories
2,2',5,5'-Tetrachlorobiphenyl	CB-52	5.84	99.59	Promochem
2,2',4,4',5-Pentachlorobiphenyl	CB-99	6.39	99.6	Dr. Ehrenstorfer
2,2',4,5,5'-Pentachlorobiphenyl	CB-101	6.38	99.7	Promochem
2,3,3',4,4'-Pentachlorobiphenyl	CB-105	6.65	>98	Cambridge Isotope Laboratories
2,3,3',4',6-Pentachlorobiphenyl	CB-110	6.48	98	Cambridge Isotope Laboratories
2,3',4,4',5-Pentachlorobiphenyl	CB-118	6.74	99.8	Promochem
2,2',3,3',4,4'-Hexachlorobiphenyl	CB-128	6.74	99.5	Dr. Ehrenstorfer
2,2',3,4,4',5'-Hexachlorobiphenyl	CB-138	6.83	99.91	Promochem
2,2',3,4',5',6-Hexachlorobiphenyl	CB-149	6.67	99	Promochem
2,2',3,5,5',6-Hexachlorobiphenyl	CB-151	6.64	98	Cambridge Isotope Laboratories
2,2',4,4',5,5'-Hexachlorobiphenyl	CB-153	6.92	99.9	Promochem
2,3,3',4,4',5-Hexachlorobiphenyl	CB-156	7.18	99.2	Promochem
2,2',3,3',4,4',5-Heptachlorobiphenyl	CB-170	7.27	99.0	Dr. Ehrenstorfer
2,2',3,4,4',5,5'-Heptachlorobiphenyl	CB-180	7.36	99.5	Promochem
2,2',3,4',5,5',6-Heptachlorobiphenyl	CB-187	7.17	98.0	Dr. Ehrenstorfer
2,2',3,4',5,6,6'-Heptachlorobiphenyl	CB-188	6.82	98	Cambridge Isotope Laboratories
2,2',3,3',4,4',5,5'-Octachlorobiphenyl	CB-194	7.80	98	Cambridge Isotope Laboratories
2,2',3,3',4,5,5',6-Octachlorobiphenyl	CB-198	7.62	98	Cambridge Isotope Laboratories
Decachlorobiphenyl	CB-209	8.18	99	Promochem

Table S1: List over chemicals, their purity and supplier [continued].

chemical	short name[a]	$log\ K_{ow}$[b]	purity (%)	supplier
Organochlorine pesticides				
α-Hexachlorocyclohexane	alpha-HCH	3.72	98.3	Dr. Ehrenstorfer
β-Hexachlorocyclohexane	beta-HCH	3.72	98.4	Dr. Ehrenstorfer
γ-Hexachlorocyclohexane	gamma-HCH	3.72	98.5	Dr. Ehrenstorfer
Hexachlorobenzene	HCB	5.50	99.5	Dr. Ehrenstorfer
o'p-Dichlorodiphenyldichloroethylene	o'p-DDE	6.00	99	Dr. Ehrenstorfer
o'p-Dichlorodiphenyltrichloroethane	o'p-DDT	6.79	99	Dr. Ehrenstorfer
p'p-Dichlorodiphenyldichloroethane	p'p-DDD	6.02	99.8	Promochem
p'p-Dichlorodiphenyldichloroethylene	p'p-DDE	6.51	99.7	Promochem
p'p-Dichlorodiphenyltrichloroethane	p'p-DDT	6.91	98.5	Dr. Ehrenstorfer
trans-nonachlor	TNC	6.08	99	Dr. Ehrenstorfer
Polycyclic aromatic hydrocarbons				
Naphthalene	NAPH	3.37	>99	Sigma
Acenaphtene	ACE	3.92	99	Aldrich
Fluorene	FLE	4.18	99	Fluka
Phenanthrene	PHEN	4.57	98	Acros
Anthracene	ANT	4.54	99	Acros
Fluoranthene	FLU	5.22	99	Aldrich
Pyrene	PYR	5.18	>99	Fluka
Benz(a)anthracene	BAA	5.91	99	Aldrich
Chrysene	CHR	5.86	99	Cerilliant
Benzo(a)pyrene	BAP	6.04	98	Aldrich

[a] PCBs are numbered according to IUPAC

[b] for PCBs and HCB from Hawker and Connell;[S1] for HCHs and TNC experimental data from the EPI-Suite database were taken;[S2] for DDX from Howard and Meylan 1997;[S3] for PAHs from Mackay[S4]

Estimation of fiber coating volume

In a recent study, Witt et al.[S5] determined coating thicknesses of PDMS-coated glass fibers from Fiberguide Industries and fibers from Polymicro Technologies Inc. : the average coating thickness was 12.67 $\pm$ 1.68 μm and 26.51 $\pm$ 4.02 μm, respectively. The glass core diameter was determined to be 207.30 $\pm$ 1.36 μm and 107.25 $\pm$ 0.77 μm, respectively. Fiberguide fibers were cut to a total length of 30 cm, Polymicro fibers to a length of 20 cm. The silicone coating volume was estimated to 2.63 μL and 2.23 μL respectively using eq S1:

$$V = \pi b(d+b)h \tag{S1}$$

where b is the coating thickness, d the glass core diameter and h fiber length.

Error propagation

For derived partition coefficients ($\hat{K}$) that were obtained as a product of partition coefficients, the uncertainties $\hat{e}$ were calculated by adding the relative (fractional) errors of each component (either the standard deviation or the standard error of the mean) in quadrature:

$$\frac{\hat{e}}{\hat{K}} = \sqrt{\sum_{i=1}^{k} (e_i/K_i)^2} \tag{S2}$$

where K_i are the individual input partition coefficients with each their uncertainty e_i. Pooled errors (e_p) for averaged polymer-polymer partition coefficients were obtained by taking the weighted mean of the individual errors of the input measures (e_i):

$$e_p = \frac{\sqrt{\sum_{i=1}^{k} e_i^2}}{} \tag{S3}$$

Temperature correction of lipid-water partition coefficients

Triolein-water partition coefficients (triolein as surrogate for a storage lipid) that Quinn et al.[S6] determined in an experiment at 37 °C ($K_{triolein:water}$) were scaled to 20 °C using the van't Hoff equation:

$$log\,K_{lipid:water[T_1]} = log\,K_{lipid:water[T_2]} - \frac{\Delta H}{2.303R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad \text{(S4)}$$

where T_1 and T_2 are the respective temperatures (293.15 K and 310.15 K, respectively). The partitioning enthalpies ΔH were estimated from the model suggested by Geisler et al.:[S7]

$$\Delta H_i = 10.51L_i - 49.29S_i - 16.36A_i + 70.39B_i - 66.19V_i + 38.95 \quad \text{(S5)}$$

which is based on a polyparameter linear free energy relationships (pp-LFERs) established by Abraham et al.[S8] and modified by Goss et al.[S9] Therein, the variables L_i, S_i, A_i, B_i and V_i are solute descriptors that quantify molecular interactions of a solute in condensed phases. The coefficients in front of these variables are phase descriptors describing capacity differences of the partitioning phases, here lipid and water, for interactions with a solute. They were estimated by Geisler et al.[S7] through calibration of a pp-LFER model against experimental lipid-water partition coefficients and found to be suitable also for estimating ΔH. In order to estimate ΔH, we used the solute descriptors reported for PCBs (Table S2) by Geisler et al.:[S7]

Table S2: Solute descriptors for estimating partitioning enthalpies ΔH, and temperature-corrected triolein-water partition coefficients from Geisler et al.[S7]

	L	S	A	B	V	dH (kJ/mol)	$K_{triolein:water}$[20°C]
CB-28	7.9	1.33	0	0.15	1.69	-44.88	6.56
CB-52	8.14	1.48	0	0.15	1.81	-57.69	6.91
CB-101	8.87	1.61	0	0.13	1.94	-66.44	7.56
CB-118	9.4	1.59	0	0.11	1.94	-61.29	7.69
CB-138	9.77	1.74	0	0.11	2.06	-72.74	8.12
CB-153	9.59	1.74	0	0.11	2.06	-74.63	8.27
CB-156	10.2	1.72	0	0.09	2.06	-68.64	8.23
CB-170	10.58	1.87	0	0.09	2.18	-79.99	8.70
CB-180	10.42	1.87	0	0.09	2.18	-81.67	8.75
CB-187	9.86	1.87	0	0.09	2.18	-87.55	8.82

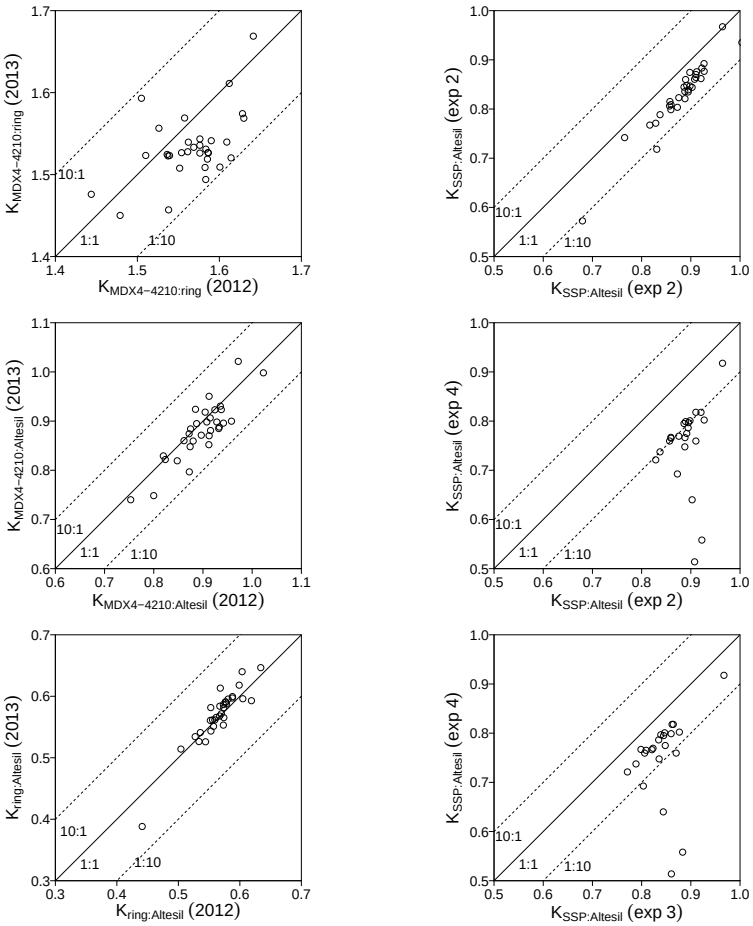

Figure S1: Polymer-polymer partition coefficients for reference materials. 'Experiment 2' refers to equilibrations in MDX4-4210 silicone coated vials. 'Experiment 3' refers to equilibrations in vials coated with DC1-2577. 'Experiment 4' refers to equilibrations in jars coated with Silastic Type A. Altesil-SSP partition coefficients from the experiment with Silastic-coated jars were not considered for data pooling. Silastic-polymer partition coefficients for the analytes visibly deviating in the reference plots were excluded.

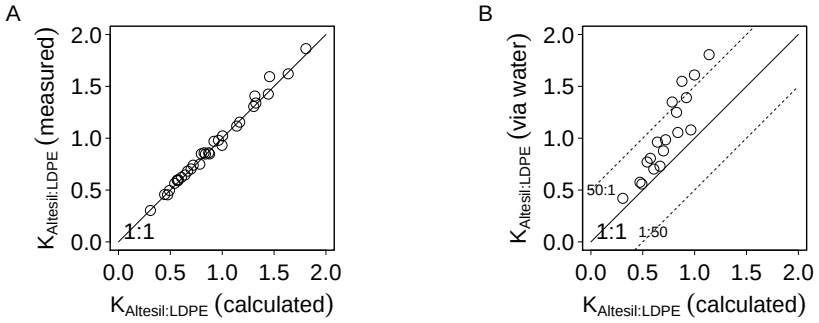

Figure S2: Altesil-LDPE partition coefficients. (A) Calculated partition coefficients were in very good agreement with experimentally determined values (n=1). (B) Polymer-polymer partition coefficients agreed less when compared to the ratio of published polymer-water partition coefficients.[S10]

Figure S3: Visual representation of polymer-polymer partition coefficients in a heatmap. Light colors indicate similar sorptive capacity of polymers.

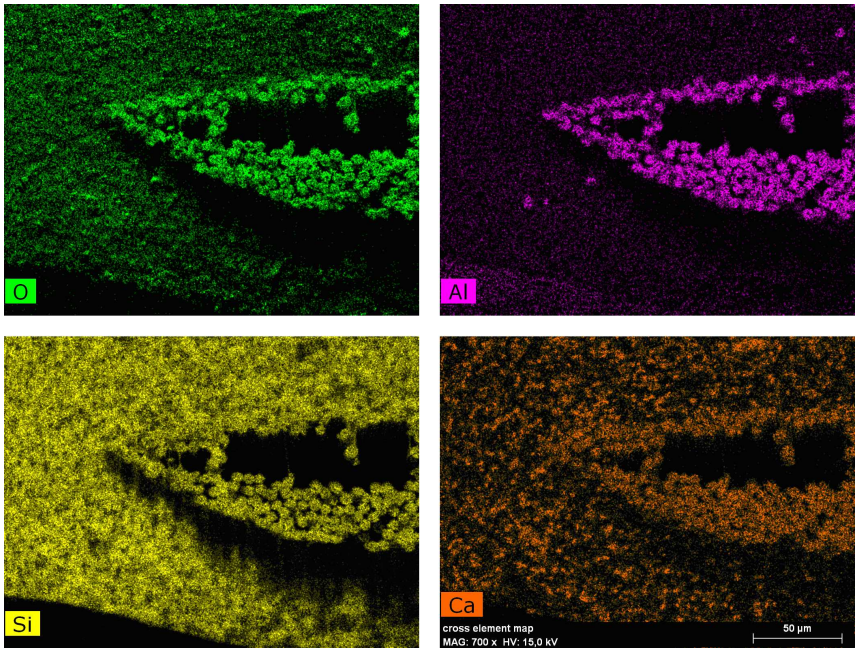

Figure S4: SEM image (element map) of Biscasil. $CaCO_3$ was found as filler.

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Appendix C
Supporting Information to Manuscript II

Supplemental Material

Endocrine activity of persistent organic pollutants accumulated in human silicone implants - dosing in vitro assays by partitioning from silicone

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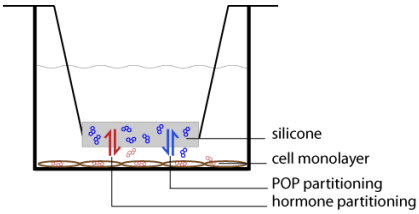

Figure S1: Passive dosing setup for the H295R *in vitro* assay.

Loading of silicone disks

Disks were placed in individual vials and autoclaved to avoid microbial contamination during the loading procedure and pre-equilibration.

4-nonylphenol: Stock solution was prepared in methanol (2.67 mg/mL), and diluted two-fold in a 5-step geometric series. 100 µL of loading solution were spiked onto individual disks of ca. 193 mg, the solvent then allowed to evaporate off for 4h.

POP mixture: Individual chemicals were dissolved in isooctane and combined to a mixture that reflected the concentration ratios of POPs in silicone implants. CB-52 was included as it is frequently measured in human blood. Reported lipid-normalized concentrations for CB-52 (Polder et al. 2008) were converted to equilibrium silicone concentrations via the lipid-silicone partition coefficient (Jahnke et al. 2008). The isooctane stock solution was diluted 120-fold in acetone serving then as loading solution for the two highest dosing levels, and to prepare further dilutions. 50 µL (500 µL for the 100x dosing level) of loading solution were added onto individual disks. Solvent was allowed to evaporate off in a hood, and its complete removal was confirmed gravimetrically.

Table S1: Conversion of concentrations in silicone to C_{free}.

	M	$C_{silicone}$ [a]	log $K_{sil:w}$ [b]	C_{free} [c]	C_{free}
	g/mol	ng/kg	L/kg	ng/L	fmol/L
PCB-52	291.99	17	5.81	2.6E-05	0.09
PCB-101	326.43	10	6.28	5.1E-06	0.02
PCB-105	326.43	40	6.42	1.5E-05	0.05
PCB-118	326.43	204	6.42	7.7E-05	0.24
PCB-138	360.88	658	6.76	1.1E-04	0.32
PCB-153	360.88	1498	6.72	2.9E-04	0.79
PCB-156	360.88	101	6.72	1.9E-05	0.05
PCB-180	395.32	176	6.99	1.8E-05	0.05
HCB	284.80	942	5.05	8.4E-03	29.48
p'p-DDT	354.49	64	5.71	1.2E-04	0.35
p'p-DDE	318.03	2599	6.07	2.2E-03	7.01
BDE-47	485.79	15			
BDE-99	564.69	5			
BDE-100	564.69	7			
BDE-153	643.58	41			
BDE-154	643.58	4			
4-nonylphenol	220.35	2.6E+08	4.51 [d]	7.8E+3	3.6E+4

[a] median level
[b] from Smedes et al. 2009
[c] $C_{free} = C_{silicone}/K_{sil:w}$
[d] from Escher et al. 2011

Hormone extraction from silicone

Two milliliter of methanol were added to each disk (and 20 µL of internal standard mix in case of HPLC-MS/MS analysis) and vials left to stand overnight at room temperature. The extraction was repeated with 2 mL of fresh methanol, and extracts were combined.

All methanolic hormone extracts were evaporated to dryness under a stream of nitrogen at 50 °C (SpeedVac, Thermo Fisher Scientific, USA). Samples for subsequent HPLC analysis were reconstituted in 80 µL acetonitrile, thereafter adding 120 µL of water. Samples for DELFIA analysis were reconstituted in human serum surrogate (Diluent I, PerkinElmer) and were further processed as instructed in the assay kits.

Hormone analysis by HPLC-MS/MS

Progesterone, 17α-hydroxyprogesterone, androstenedione, dehydroepiandrosterone, 17β-estradiol, estrone, cortisol, corticosterone, testosterone and pregnenolone were analyzed on an Agilent 1100 HPLC system coupled to a Quattro Ultima Triple Quadrupole mass spectrometer (Waters Corp., USA) as described before (Mortensen et al. 2007). Details are given in Table S2. The deuterated hormones 17β-estradiol-d3, progesterone-c2, testosterone-d2, and methyltestosterone-d3 (RIKILT, The Netherlands) served as internal standards. Hormones were quantified using a seven-point external calibration curve. The limit of quantification (LOQ) was 0.05 ng/mL for testosterone, progesterone and estrone; 0.10 ng/mL for the remaining hormones, except for dehydroepiandrosterone (LOQ = 1.0 ng/mL). Recoveries in all cases were > 70%. 17β-estradiol was detected in most samples below the LOQ, corticosterone was in few samples below LOQ.

Table S2: HPLC-MS/MS method details.

	estrone, 17β-estradiol and 17β-estradiol-d3	**remaining hormones**
column	Atlantis C18 (Waters, USA)	Ascentis Express C8 (Supelco)
injection volume	50 µL	10 µL
mobile phase	65/35% vol/vol methanol/water mixture with 0.01% ammonia (0.15mL/min, isocratic)	acetonitrile/water with 0.2% formic acid (flow rate 0.25 mL/min, gradient elution)
detection	ESI-	ESI+

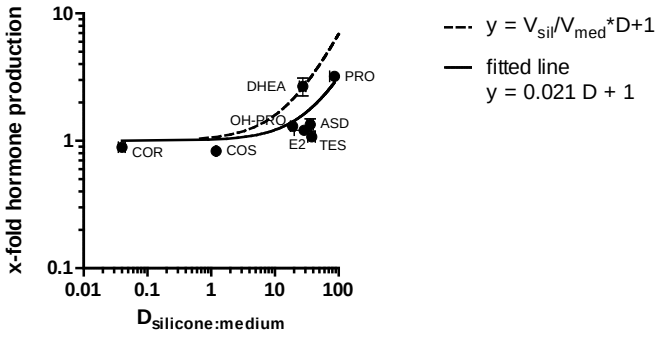

Figure S2: Increase in hormone production in the presence of silicone as a function of the silicone-medium distribution coefficient ($D_{silicone:medium}$) of the hormones. The dotted line indicates the predicted increase in hormone production with D as the only controlling variable at the given silicone/medium volume ratio (V_{sil}/V_{medium} = 0.059).

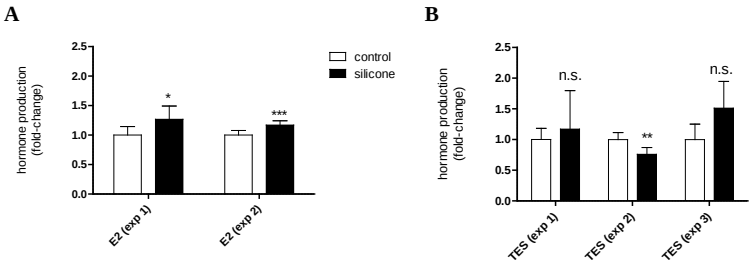

Figure S3: 17β-Estradiol (E2) and testosterone (TES) production in the presence and absence of silicone. Data from the first two experiments (exp 1, exp2) were obtained by immunoassay (DELFIA) analysis of hormones; in the third experiment HPLC-MS/MS analysis was performed. Bars show the mean of technical replicates, error bars show the standard error of the mean. Statistical significance between control and silicone treatments (t-test) is indicated as follows: n.s. = not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

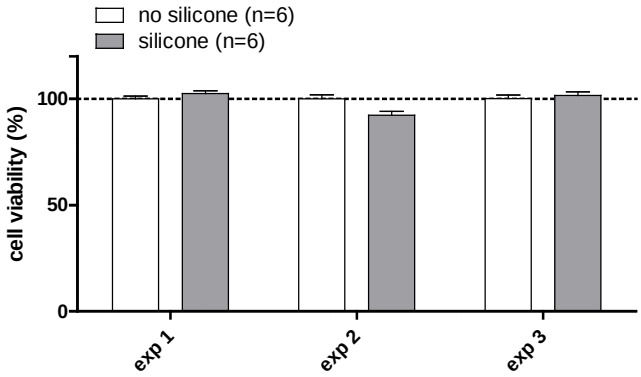

Figure S4: Cell viability as measured by the MTT assay for H295R cells cultured in parallel in the presence and absence of silicone (mean + SEM from three independent experiments). The average viability in blank treatments without silicone was defined 100 %.

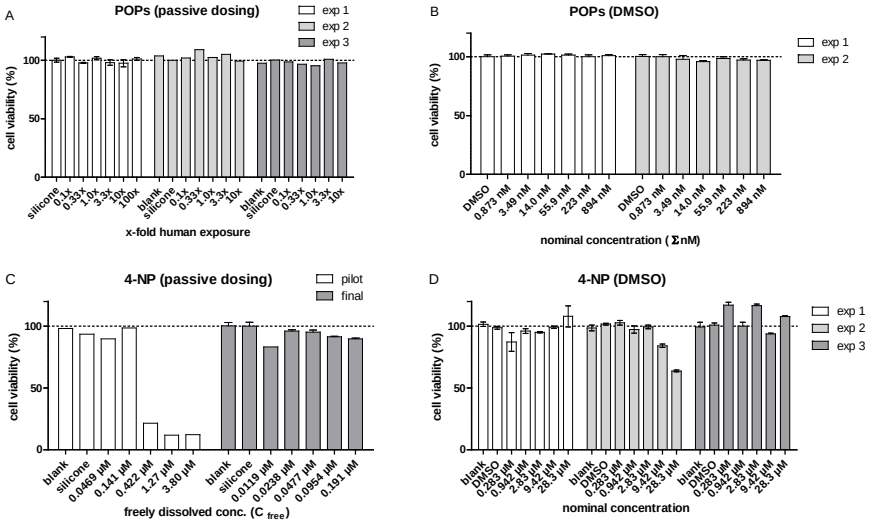

Figure S5: Cell viability in passive dosing and solvent-spiking experiments with POPs (A and B) and 4-NP (C and D) as determined by the MTT assay. Note that cell viability in the pilot passive dosing experiment with 4-NP (C, left) included test doses above the highest final dose, which were clearly cytotoxic. Bars show the mean, error bars show the standard error of the mean and are sometimes smaller than the bar borders. The average viability of experimental controls (blanks and solvent blanks) was set to 100%.

Dose-response curve modelling

A four-parameter log-logistic model was used for dose-response curve modelling (Graph Pad Prism 5):

$$y = y_{min} + \frac{(y_{max} - y_{min})}{1+10^{(logEC50-x)slope}} \quad \text{(eq S1)}$$

where *y* is the response predicted by the model (dependent variable), *x* the dose in log units of concentration (independent variable), y_{min} the response (average) in the control treatment (fixed), y_{max} the maximum response observed (fixed), *logEC50* the dose causing 50% effect (fitted), and *slope* the Hill slope (in inverse concentration units), which was preferably fitted, or fixed depending on model performance (R^2).

Table S3: Estimated EC50s for 4-nonylphenol with the 95% confidence interval and nonlinear regression parameters using the 4-parameter log-logistic model (eq S1).

	DMSO-dosing		passive dosing	
	EC50 (µM)	LL [a]; UL [b]; slope [c]	EC50 (µM)	LL [a]; UL [b]; slope [c]
progesterone	6.63 (5.93, 7.41)	0.32; 2.77; 2.36±0.239	0.033 (0.029, 0.037)	10.61; 54.13; 2.72±0.335
OH-progesterone	6.39 (5.89, 6.94)	1.47; 12.35; 2.14±0.143	0.038 (0.035, 0.041)	19.8; 129; 2.68±0.216
corticosterone	2.80 (1.41, 5.55)	5.64; 9.56; 1.0 [d]	--	--
estrone	5.44 (3.83, 7.73)	0.0960; 0.224; 1.85±0.478	--	--
testosterone	2.90 (2.35, 3.59)	0.410; 0.933; 2.18±0.513	0.038 (0.0063, 0.23)	2.47; 3.84; 0.365±0.182

[a] lower limit (LL) constrain (ymin) in nonlinear regression model in ng/mL (DMSO-dosing) or ng/g silicone (passive dosing)
[b] upper limit (UL) constrain (ymax) in nonlinear regression model in ng/mL (DMSO-dosing) or ng/g silicone (passive dosing)
[c] fitted slope ± standard error
[d] fixed slope

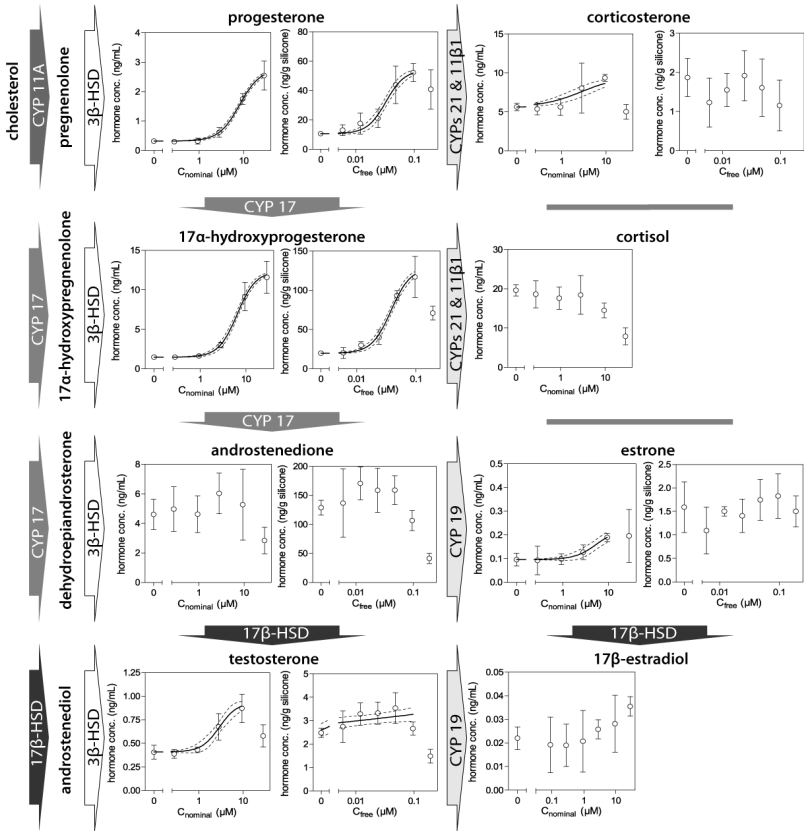

Figure S6: Hormonal responses of H295R cells to 4-nonylphenol introduced by DMSO-spiking ($C_{nominal}$; left) or by equilibrium partitioning from silicone (C_{free}; right). Symbols show the mean and the 95 % confidence interval of 3 (for controls n=6) replicate measurements. Nonlinear regression lines with their 95 % confidence belts are fitted given statistically significant differences between control and treatments (Kruskal-Wallis $p < 0.05$). Cytotoxic test doses were excluded from curve fitting. 17β-estradiol was quantified by DELFIA, the remaining hormones by HPLC-MS/MS. Note that comparison of the y-axis ranges between DMSO and passive dosing treatments allows deducing the enrichment factor for hormone accumulation in silicone.

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Appendix D
Supporting Information to Manuscript III

Glucose-6-phosphate assay

Binding of glucose-6-phosphate (G6P) to BSA was evaluated at BSA concentrations ranging from 0.1 to 2 g/L. The incubation mixtures also contained denatured S9 at 1 mg/ml. Solutions were incubated for 1 h at room temperature and vortexed every 15 min before they were loaded onto the ultracentrifuge cartridges (MW-cutoff 10000 g/mol). Recovery of G6P ranged between 90 % to 100 %.

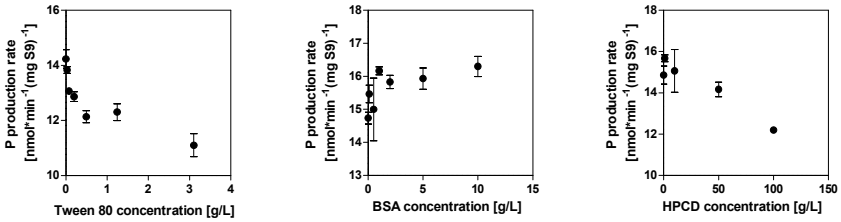

Figure D.1. Effects of Tween 80, BSA and β-hydroxypropyl cyclodextrin (HPCD) on the enzymatic activity of S9 as quantified by measurements of the glucose-6-phosphatase activity (i.e. phosphorous production).

Table D.1. Recoveries (%) of PAHs in aqueous solutions of Tween 80, BSA and β-HPCD. If not otherwise indicated, values in parentheses indicate the relative standard deviation of three replicates.

	Tween 80	BSA	β-HPCD
Naphthalene	83.0 (n=1)	102.1 (n=1)	100.4 (n=1)
Fluorene	49.1 (8.1%)	78.7 (4.1%)	54.5 (5.2%, n=2)
Phenanthrene	83.6 (6.2%)	98.5 (32%)	64.8 (21%)
Anthracene	77.8 (3.7%)	106.5 (0.8%)	84.3 (2.4%)
Fluoranthene	89.1 (2.0%)	89.1 (2.0%)	79.8 (0.2%)
Pyrene	94.1 (13%)	94.1 (13%)	59.7 (24%)
Benzo[a]pyrene	97.3 (10%)	97.3 (10%)	53.8 (13%)

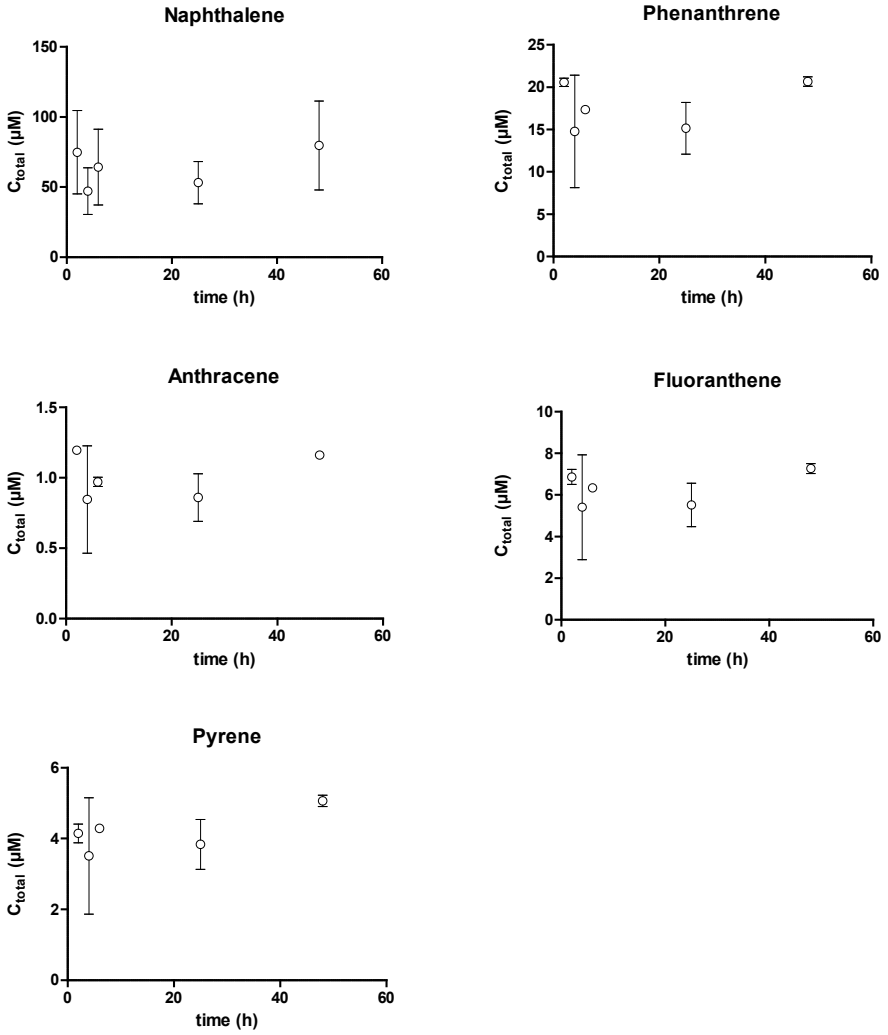

Figure D.2. Sorption kinetics for PAHs in aqueous solution of S9 (2 g/L) + HPCD (10 g/L) in passive dosing vials at 11 °C at 1000 rpm (incubation volume = 2 mL).

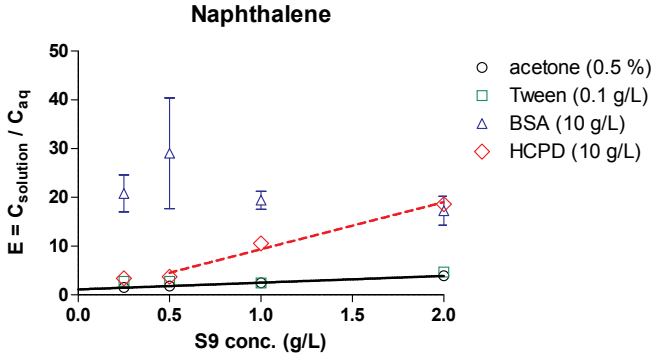

Figure D.3. Enhanced capacities of S9 suspensions for napthalene with and without added molecular carriers.

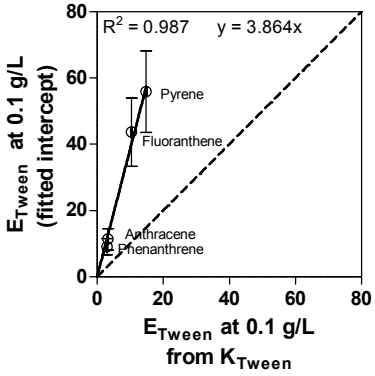

Figure D.4. Enhanced capacities of an aqueous Tween solution (0.1 g/L) for PAHs as predicted from Tween binding constants (K_{Tween} and the fitted intercepts from experimentally determined enhancement factors in solutions with Tween as secondary binding phase. Note that the x-values represent enhancement factors at 21 °C whereas the binding experiments with S9-Tween suspensions were conducted at 11 °C, which may explain the deviation of the points from the 1:1 line by a approximately a factor of four.

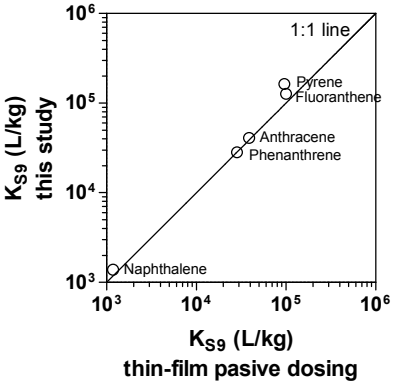

Figure D.5. Comparison of PAH S9 binding constants K_{S9} from the present study (data from the acetone-dosing treatment are shown) with S9 binding constants that were determined through a thin-film passive dosing method.

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It has been a long journey through the past 3.5 years on this PhD study, both scientifically and personally, on which there were many people to help me who shall be acknowledged here. In the first place, I want to thank Philipp Mayer as advisor and teacher, as someone who listened and responded to my ideas, and who I could always ask my questions. Often, Philipp answered those with another question that made me come back to him with a new thought a few days later, which again he would question, etc. – a long ping-pong-like game that I enjoyed so much, which sometimes then brought us to a discussion of rather philosophical matter. So indeed this has been a way toward a degree of *doctor philosophiae* with him. I am grateful he considered me as a colleague on an equal footing, and for all the trust he put in me.

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The whole second year of my PhD study, I worked in the Molecular Toxicology group, at the Division of Toxicology & Risk Assessment at DTU Food in Søborg, Copenhagen, headed by Anne Marie Vinggaard. Anne Marie never hesitated to provide me with the materials or the contacts I needed to make the next step. The integration in her wonderful team had made it easy for me to get started, and gave me a good framework for learning, exchange, getting help and last but not least a good portion of laughter. All the team be acknowledged here. The project at DTU-Food also involved Mikael Pedersen and his group at the Division of Food Chemistry who I all thank for their professional collaboration.

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Curriculum vitae

Dorothea Gilbert studied the interdisciplinary bachelor's program Biogeosciences at the Friedrich Schiller University Jena, Germany, giving her a foundation in environmental sciences with strong emphasis on geosciences, environmental microbiology and environmental chemistry. She completed these studies with an exchange year at the University of Joensuu, Finland, where she worked in the group of Matti Leppänen on her final bachelor's thesis project. Dorothea then continued her education with a master's program in Environmental Sciences, choosing ecotoxicology as major, at the University of Gothenburg, Sweden. Finally, she came in 2010 to Denmark to work in the group of Philipp Mayer at the National Environmental Research Institute in Roskilde (now Aarhus University) to complete her master studies with a project on microorganism-facilitated contaminant transfer. Ultimately, the fruitful collaboration let her continue further with the here presented PhD study.

This PhD thesis deals with some fundamental aspects of the exposure and toxicity assessment of hydrophobic organic chemicals (HOCs), and advocates the use of a reference partitioning phase to better link exposure levels and toxic dose levels on the basis of chemical thermodynamics. Equilibrium partitioning methods were applied and further developed in individual studies, which investigated the partitioning of HOCs between environmental media and polymer phases, the endocrine activity of some persistent organic pollutants and the enzymatic transformation of PAHs in fish liver subcellular fractions. The theoretical concepts and methods presented may be of interest to the environmental chemist, the environmental toxicologist, and to scholars and students of environmental sciences.

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