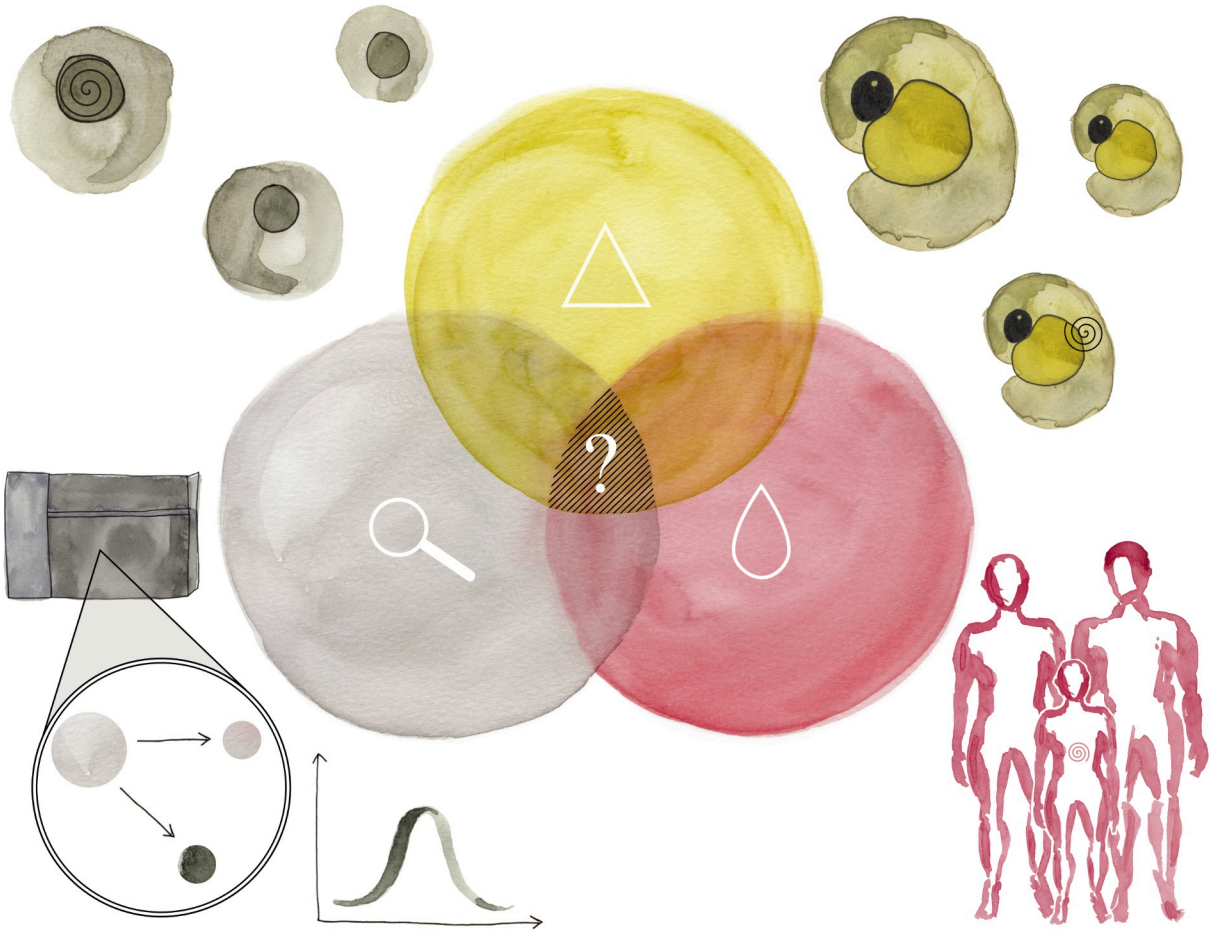
Stockholm University

# Exposure to Chemical Mixtures and Associated Health Risks

Focusing on Endocrine Disruption and the Swedish General Population

Josefin Engelhardt




Doctoral Thesis in Environmental Sciences at Stockholm University, Sweden 2025

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## Focusing on Endocrine Disruption and the Swedish General Population

**Josefin Engelhardt**

Academic dissertation for the Degree of Doctor of Philosophy in Environmental Sciences at Stockholm University to be publicly defended on Friday 28 March 2025 at 10.00 in De Geer-salen, Geovetenskapens hus, Svante Arrhenius väg 14 and online via Zoom, public link is available at the department website.

### Abstract

Humans are exposed to a wide range of environmental pollutants through multiple routes of exposure. Chemical exposure can result in endocrine-disrupting effects, manifesting at low concentrations, and are associated with several health problems, such as infertility, obesity, and autoimmune diseases. This thesis aims to evaluate the potential health effects of exposure to complex chemical mixtures. Two key objectives were to understand human chemical exposure and investigate whether the Swedish general population falls within the risk threshold for health effects related to this exposure. In **Paper I**, a literature review was conducted to investigate chemical exposure based on existing knowledge, compiling peer-reviewed literature on chemicals and their exposure levels in Swedish human blood. Additional chemicals were analyzed in Swedish human blood to examine exposure to chemicals currently used, such as per- and polyfluoroalkyl substances (PFAS, **Paper II**) and synthetic phenolic contaminants (**Paper III**). A 50-component chemical mixture was created based on human blood levels found in **Papers I-III** to better understand the potential endocrine-disrupting and developmental toxic effects. Persistent organic pollutants (POPs) were the most frequently studied and detected chemicals analyzed in human blood (**Paper I**), prompting the inclusion of four POP classes in the artificial mixture. New contaminants not previously found in Swedish samples were also identified, including hydrogen-substituted perfluoroalkyl carboxylic acids (**Paper II**) and synthetic phenolic antioxidants (SPAs) (**Paper III**). Some PFAS and SPAs were present in all individuals at high levels, motivating the addition of the SPAs into the chemical mixture. Mixture risk assessment strategies were employed to evaluate the potential adverse effects of exposure to POPs (**Paper I**) and PFAS (**Paper II**). The mixture comprises polychlorinated biphenyls (PCBs), brominated flame retardants, organochlorine pesticides, PFAS, SPAs, bisphenol A, and phthalates. The total mixture was tested as a whole and as six subgroup mixtures to identify the drivers of effect. The mixtures were evaluated using *in vitro* cell assays to screen for binding to estrogen receptor α, androgen receptor, and aryl hydrocarbon receptor (**Paper IV**). The developmental toxicity of the mixtures was examined using the zebrafish embryo toxicity (ZFET) test (**Paper V**). The effects associated with exposure to the total chemical mixture demonstrated estrogen and androgen activity at 10 and 15 times human blood concentration (xHBC), respectively, levels that fall within the human blood exposure range of the Swedish general population (**Paper IV**). Developmental effects were observed at 15 xHBC using the ZFET test (**Paper V**). This thesis identified that POPs, such as PFAS, chlorinated pesticides, and PCBs, continue to pose a health threat. Newly identified SPAs present a potential risk due to high exposure levels and need further investigation. The mixture risk assessment strategies employed in this thesis suggest that a risk of health impairments from known chemical exposure cannot be ruled out for the Swedish population. Although these risk assessments are only tentative, they add to the ever-growing body of evidence, showing that mixtures pose an unavoidable health concern to humans. Therefore, it is essential to manage and limit human exposure to the mixture of environmental pollutants to ensure the well-being of future generations.

**Keywords:** *Chemical mixtures, Human blood, Chemical exposure, Sweden, Human health, Synthetic phenolic antioxidants (SPAs), PFAS, Persistent organic pollutants, Mixture risk assessments, Endocrine disruption, in vitro, zebrafish embryo toxicity (ZFET) test.*

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*"I mean, anyone can let danger out but the really clever thing is finding somewhere for it to go afterwards"*

**- Tove Jansson, Sculptor's Daughter**

# Populärvetenskaplig Sammanfattning

Människor är idag omgivna av olika produkter som innehåller miljöföroreningar. En del av dessa kan släppas ut från produkterna och tas upp i våra kroppar. Om miljöföroreningen besitter liknande egenskaper som våra kroppsegna molekyler kan de störa naturliga processer och system i kroppen. Vanligtvis utvärderas en förorenings inneboende fara enskilt, även om exponeringen oftast sker som en blandning av många kemikalier. Den här avhandlingen har undersökt om den kemikalieblandning som finns i blodet hos den svenska befolkningen skulle kunna ge negativa konsekvenser för vår hälsa. Mer specifikt har blodhalter av miljöföroreningar som används idag bestämts och jämförts mot gränsvärden för att utvärdera risken för olika hälsoeffekter. Halterna av PFAS i blodet från svenska bloddonatorer var inte högre än resultat från andra studier. Ändå var halterna i många fall högre än gränsvärdena, vilket medför att risken inte gick att utesluta för effekter relaterade till fosterutveckling, sköldkörtelstörningar, ökat kolesterol i blodet och försvagat immunförsvar. Vidare undersöktes kemikalier som används i plast och skönhetsprodukter, där höga halter hittades av syntetiska antioxidanter. Syntetiska antioxidanter är ämnen som adderas i plast, mat, kläder och elektronik för att minska nedbrytningen av materialet. På grund av de höga halterna och att de hittades i majoriteten av de studerade individerna, bör flera undersökningar göras för att bestämma halterna i en större population och utvärdera farligheten av syntetiska antioxidanter.

För att förstå effekten av den kemikalieblandning vi har i oss så sattes en blandning av kemikalier ihop i detta projekt, som ska reflektera de ämnen och halter som finns i blod från den svenska befolkningen. Hormonstörande och fosterutvecklingsstörande effekter har sedan undersökts genom att exponera celler och zebrafiskembryon för kemikalieblandningen. Blandningen orsakade hormonstörande effekter vid tio gånger humannivån som vi har i blodet och fosterutvecklingsstörande effekter vid femton gånger humannivån. Eftersom dessa tester gjorts på celler och fiskembryon ska man vara försiktig med att dra för stora slutsatser med vilka effekter som det skulle kunna leda till i människor. Från de studier som gjorts i denna avhandling, kan man inte utesluta att en risk för hälsoeffekter föreligger i den svenska populationen på grund av exponeringen för miljöföroreningar.

# Popular Science Summary

Today, individuals are constantly exposed to numerous products that contain environmental pollutants. Some of these substances can be released from the products and enter into our bodies. When these pollutants share similar properties to those naturally occurring in our bodies, they can disrupt essential bodily processes and systems. Typically, the hazardous nature of a chemical is assessed individually. This thesis investigates whether the exposure to the combination of pollutants found in the blood of the Swedish general population could lead to adverse health effects. Specifically, the study measures and compares the current blood levels of environmental pollutants against health-based reference values to evaluate potential health risks. The detection of PFAS in Swedish blood donors matched levels observed in earlier studies, yet many were above the reference values. This suggests that health risks – particularly concerning fetal development, thyroid function, elevated cholesterol levels, and weakened immune responses – cannot be ruled out. Furthermore, high levels of environmental pollutants typically used in plastics and personal care products were identified in human blood. Of particular concern are synthetic antioxidants, which are added to various materials, including plastics, food, clothing, and electronics, to slow degradation. Given the significant concentration of these chemicals found in the blood and their presence in a large portion of the studied population, future research should aim to determine their levels among a broader population and evaluate the risks associated with synthetic antioxidants.

In this project, a chemical mixture was created to evaluate the effects related to the chemical mixture in our blood, reflecting the pollutants and levels in the blood of the Swedish general population. Hormone disruption and developmental effects were assessed by exposing cells and zebrafish embryos to the mixture. The mixture caused hormone-related effects at ten times the levels in human blood and developmental effects at fifteen times the levels. Since these effects have been assessed using cells and fish embryos, a translation to human consequences is limited. The conclusion from the studies included in this thesis is that a risk for health impairments in the Swedish general population from the chemical mixture in our blood cannot be ruled out.

# List of Papers

**Paper I**: Engelhardt AJ, Norström K, Weiss JM. 2022. Anthropogenic Organic contaminants analyzed in human blood and combined risk. *Expo Health*, https://doi.org/10.1007/s12403-022-00507-y

**Paper II**: Engelhardt AJ, Plassmann M, Weiss JM. 2025. An extended PFAS profiling of a Swedish subpopulation and mixture risk assessments using multiple approaches. *Environ. Int*., https://doi.org/10.1016/j.envint.2024.109214

**Paper III**: Engelhardt AJ, Athanassiadis I, Leonards P, Weiss JM. Multi-target analysis of synthetic phenolic compounds in human blood. S*ubmitted.*

**Paper IV**: Engelhardt AJ*, Struwe N*, Jansson A, Munic Kos V, Larsson M, Weiss J. Evaluating the endocrine-disrupting and oxidative stress potential of a 50-component human-relevant complex chemical mixture using in vitro tests. *Manuscript.*

**Paper V**: Zacari Fanali L*, Engelhardt AJ*, Weiss JM, Örn S. Toxicity assessment of a 50-component human-relevant chemical mixture using zebrafish embryos (*Danio rerio*). *Manuscript.*

*Joint first author, the authors contributed equally to the work

# Statement of Contribution

**Paper I**
Contributed to the conceptualization and methodology of the study. Conducted the data generation and was responsible for the data analysis, visualization, and interpretation of the results. Main author responsible for writing, reviewing, and editing the manuscript.

**Paper II**
Contributed to the conceptualization and methodology of the study. Conducted the experimental work and was responsible for data analysis, visualization, and interpretation of the data. Main author responsible for writing, reviewing, and editing the manuscript.

**Paper III**
Contributed to the conceptualization and methodology of the study. Assisted with the experimental work and was responsible for the data analysis, visualization, and interpretation of the data. Main author responsible for writing, reviewing, and editing the manuscript.

**Paper IV**
Contributed to the conceptualization, methodology, and funding of the study. Conducted some of the experimental work at research visits and was responsible for data analysis, visualization, and interpretation of the data together with the joint first author. Joint first author responsible for writing, reviewing, and editing the manuscript.

**Paper V**
Assisted with the experimental work and data analysis. Contributed to the visualization and interpretation of the data. Joint first author responsible for writing, reviewing, and editing the manuscript.

# Table of Contents

# Abbreviations and Definitions

| | |
|---|---|
| 2,4-DBP | 2,4-ditert butylphenol |
| 4-tOP | 4-tert octylphenol |
| ACN | Acetonitrile |
| AhR | Aryl hydrocarbon receptor |
| AO2246 | 2,2'-methylenebis(4-methyl-6-tert-butylphenol) |
| AO246 | 2,4,6-tris(tert-butyl)phenol |
| AO4426 | 4,4’-methylenebis(2,6-di-tert-butylphenol) |
| AR | Androgen receptor |
| BDE47 | 2,2',4,4'-tetrabromodiphenyl ether |
| BFR | Brominated flame retardant |
| BHA | Butylated hydroxyanisole |
| BHT | Bultylated hydroxytoluene |
| CA | Concentration addition |
| CB138 | 2,2',3,4,4',5'-Hexachlorobiphenyl |
| CB153 | 2,2',4,4',5,5'-Hexachlorobiphenyl |
| CB180 | 2,2',3,4,4',5,5'-Heptachlorobiphenyl |
| DC | Direct constant voltage |
| DDT | Dichlorodiphenyltrichloroethane |
| ECHA | European Chemicals Agency |
| EDC | Endocrine-disrupting chemical |
| EI | Electron ionization |
| ERα | Estrogen receptor α |
| ESI | Electrospray ionization |
| EU | European Union |
| GC | Gas chromatography |
| HBDB | Human blood database |
| HBM | Human biomonitoring |
| HI | Hazard index |
| HQ | Hazard quotient |
| LC | Liquid chromatography |
| m/z | Mass-to-charge ratio |
| MAF | Mixture allocation/assessment factor |
| MCR | Maximum cumulative ratio |

| | |
|---|---|
| MoA | Mode of action |
| MRA | Mixture risk assessment |
| MS | Mass spectrometer |
| NAMs | New approach methodologies |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| PBDE | Polybrominated diphenyl ethers |
| PBT | Persistent, bioaccumulative and toxic |
| PCB | Polychlorinated biphenyl |
| PFAS | Per- and polyfluoroalkyl substance |
| PFDA | Perfluorodecanoic acid |
| PFHxS | Perfluorohexane sulfonate |
| PFNA | Perfluorononanoic acid |
| PFOA | Perfluorooctanoic acid |
| PFOS | Perfluorooctane sulfonate |
| POP | Persistent organic pollutant |
| REACH | Registration, evaluation, authorization and restriction of chemicals |
| ROS | Reactive oxygen species |
| RF | Radio frequency |
| SEDB | Swedish exposure database |
| SPA | Synthetic phenolic antioxidant |
| SVHC | Substance of very high concern |
| xHBC | Times human blood concentration |
| ZFET | Zebrafish embryo toxicity |

Chemical, compound, substance, environmental contaminant, environmental-pollutant and contaminant are used interchangeably and are defined as anthropogenic (synthetic) organic chemicals

In this thesis, mixtures refer to unintentional chemical mixtures with different exposure sources at various time points. The mixtures discussed in this thesis will only consider known mixtures, *i.e.*, not cover the unknown chemical exposure. A mixture of more than 20 chemicals potentially acting through numerous modes of action is defined as a complex mixture within this thesis. Furthermore, the discussion will only cover mixtures of anthropogenic organic contaminants.

# 1 Introduction

In recent decades, we have seen an increase in a range of health impairments on a global scale [1-4]. The genome and lifestyle can only explain a part of this increase, and chemical exposure is a well-established risk factor for disease [5]. Examples of health implications linked to chemical exposure are fertility impairment, endocrine diseases such as thyroid disruption or diabetes, or immunological diseases such as allergies and autoimmune diseases [2-4, 6, 7]. A recent publication argues that we are outside the safe operating space of the planetary boundary for novel chemical entities [1]. Chemical pollution in 2015 was responsible for 16 % of all premature deaths globally – a staggering number that is three times higher than the percentage of malaria, tuberculosis, and AIDS combined [8]. The effects of chemical exposure have been estimated to cost society up to 6.2 % of the global economic output annually [8]. Humans have used synthetic organic substances during the last century, enabling the continuously evolving Technosphere [9]. Chemicals are used in many life-saving situations, such as medicine to cure diseases, sanitizers, and detergents to keep ourselves and our homes clean [10]. Other chemicals have significantly improved our quality of life and increased the efficacy of various products, such as pesticides in food production, water- and grease-repellant fabrics, preservatives, and plastics as food-contact materials [10]. The preservatives in our personal care products are important to keep the products safe for use [11], and the same goes for certain food-contact materials [12]. However, the convenience of some of these chemical solutions does not necessarily guarantee their overall benefit [10]. There are polycarbonate plastics made of bisphenol A and plastic additives such as phthalates, UV filters, and synthetic phenolic antioxidants (SPAs) [13]. Parabens are used in personal care products [11], and per- and polyfluoroalkyl substances (PFAS) are used in water-repellent fabrics [14]. These types of chemicals can exert endocrine properties, meaning that they can mimic hormones in our bodies [7]. Most people are exposed to a mixture of chemicals, and the realistic chemical exposure is chronic and complex [15-17]. At specific time points in our lives when the body develops and grows, the body is particularly sensitive to chemical exposure, *e.g.*, during fetal stages, childhood, and adolescence [7].

A mixture risk assessment is based on two key pillars: the defined chemical exposure and the inherent hazard associated with that exposure. Both pillars

must be known to assess the risk [18]. This thesis will focus on human exposure to anthropogenic chemicals (**Papers I-III**) and the impact this exposure might have (**Paper IV-V**), specifically on chemicals present in our blood.

## 1.1 Human Exposure to Chemicals

How a chemical is processed by a living organism is called ADME and stands for absorption, distribution, metabolism, and excretion. During absorption, a chemical can enter the body through different routes of exposure. When it comes to environmental contaminants, the routes are via food, water, air, or through the skin. The bioavailability of the chemical determines how well the chemical enters the body. Depending on the route of exposure and bioavailability, the chemical is then distributed in the body. A very polar chemical can be excreted in the urine almost immediately. Some compounds bind to proteins. A less polar or non-polar compound can bind to lipid-rich tissues and bioaccumulate. It needs to be metabolized before excretion through the process of biotransformation. Biotransformation includes Phase I, II, and III transformation [19]. In Phase I, the chemical is transformed into a more polar chemical by oxidation, hydroxylation, hydrolysis, or reduction. An important enzyme group for this reaction is the cytochrome P450 oxidases. Phase II metabolism includes the addition of a conjugate, such as a glucuronic acid or sulfone group. Phase III metabolism is the process of transportation outside of the cell using ATP-binding cassette transporters. When the chemical metabolite is polar enough, it can be excreted through urine or feces.

There are many things to consider when determining what chemicals are present in humans and the design of the experiment will determine which analytes can be measured. The quantifiable chemical space is limited by factors such as the choice of the sample matrix, standard availability, analytical method, and sample preparation method (Fig. 1). Additionally, chemicals can be divided into organic and inorganic chemicals. In this thesis, synthetic organic chemicals will be considered, further limiting the detectable chemical space.

Anthropogenic organic contaminants can be detected in human matrices such as blood, urine, breast milk, hair, and nails. Blood is a matrix that is relatively stable over time and is in contact with all organs in the body. Therefore, using blood to estimate internal human exposure is suitable when studying chronic chemical exposure in a general population. However, some polar compounds are metabolized fast and cannot be detected in blood, which limits the coverage of the detectable chemical space (Fig. 1). Other matrices frequently used are urine (which reflects the daily/weekly exposure and the excretion rate, and it is commonly used for polar compounds and contaminant metabolites) and human breast milk (which reflects the exposure to infants).

Analytical reference standards are essential for confidently identifying the analyte and determining its concentration in the sample. However, the availability of standards is low, and isotopically labeled standards are even more difficult to find, creating a critical bottleneck in method development [20]. The standard availability sets the limit between the quantifiable and detectable chemical space (Fig. 1). They are also necessary for conducting a recovery test to correctly understand how the analyte behaves throughout the method. A method is validated using a recovery test by fortifying a matrix with a known standard amount. Subsequently, the recovery is determined by comparing the known amount and the amount after being processed through the sample preparation.

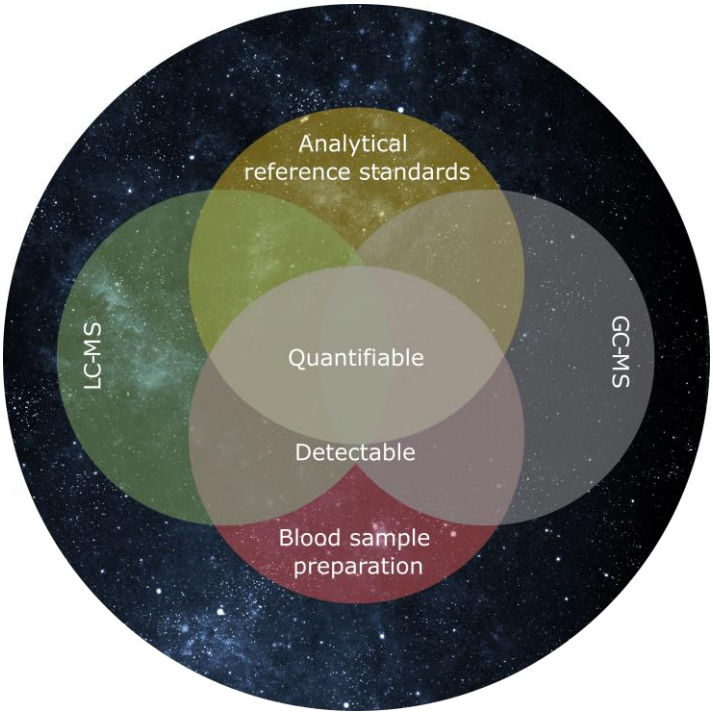


*Fig. 1. The detectable chemical space is limited to the analytical instrumental technique (liquid/gas chromatography mass spectrometry, LC-MS/GC-MS) and blood sample preparation method. The standard availability (analytical reference standards) further limits the quantifiable chemical space. Inspired by Black and coworkers [21].*

To analyze chemicals in human blood, the sample benefits from sample treatment to remove the biological matrix (including endogenous compounds such as lipids and proteins) and extract the analytes of interest. This is tedious work as the environmental chemicals are at 1000 times lower levels compared to

endogenous compounds [22]. The more steps included, the higher the risk of losing the analyte or contaminating the sample. Different solvents can be used for extraction depending on the chemical properties of the analytes. After the analytes are extracted, the sample needs to be separated from endogenous compounds that can interfere with the ionization and, thus, the signal intensity from the instrument. Proteins can be denatured during extraction, but lipids are usually extracted with the solvent and still present in the sample extract. The choice of clean-up method depends on the properties of the analytes of interest, such as stability, polarity, and chemical structure. Two common methods to detect the analytes are liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS). These methods are complementary, and utilizing both expands the covered chemical space (Fig. 1).

### 1.1.1 Persistent Organic Pollutants

To restrict and ban the most problematic groups of chemicals, the Stockholm Convention of Persistent Organic Pollutants (POPs) was implemented in 2004 [23]. The Stockholm Convention aims to protect human health and the environment from the effects of POPs. Twelve chemicals, the Dirty Dozen, were the first to be listed in the Stockholm Convention. These POPs were chlorinated chemicals with an aromatic ring structure. Since then, brominated, fluorinated, and non-halogenated compounds have been added to the list of POPs. POPs have persistent, bioaccumulative, toxic (PBT) properties and long-range transport potential. The Stockholm Convention currently covers 34 chemicals or groups of chemicals separated into three annexes: elimination (A), restriction (B), and minimization of unintentional production (C) [23]. Chemicals listed in the Stockholm Convention must be monitored in humans and environmental matrices, including human blood. Therefore, the availability of biomonitoring data is high for these POPs (discussed in **Paper I**). In Sweden, the Swedish Environmental Protection Agency is responsible for biomonitoring POPs. The most common exposure route of POPs is via contaminated food, especially from animal sources [24].

PCBs have been used commercially since the late 1920s as coolants and lubricants in electrical equipment. In 1966, the PCBs were detected in multiple compartments in the environment by Sören Jensen [25]. Due to the widespread pollution and their toxic properties, national bans were implemented shortly thereafter, and in 2004, PCBs were added to the Stockholm Convention of POPs. PCBs have exerted properties related to neurotoxicity, immune impairment, cardiovascular diseases, reprotoxicity, decreased fertility, and cancer [26]. Even if the production has ceased, PCBs are still present in the environment, but the levels are decreasing. PCBs are still found in humans, with 2,2',3,4,4',5'-Hexachlorobiphenyl (CB138), 2,2',4,4',5,5'-Hexachlorobiphenyl

(CB153), and 2,2',3,4,4',5,5'-Heptachlorobiphenyl (CB180) at the highest concentrations (Fig. 2, **Paper I**).

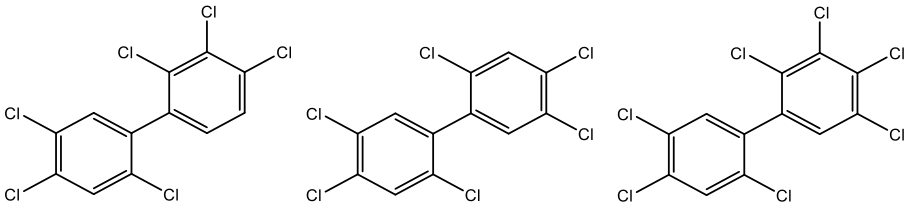


*Fig. 2. The three most commonly detected polychlorinated biphenyls (PCBs) in human blood. CB-138 – 2,2',3,4,4',5'-Hexachlorobiphenyl (left); CB153 – 2,2',4,4',5,5'-Hexachlorobiphenyl (middle) and; CB180 – 2,2',3,4,4',5,5'-Heptachlorobiphenyl (right).*

Organochlorine pesticides have been used since World War II as agricultural pest control. The environmental spread and toxicity of these chemicals, especially DDT (dichlorodiphenyltrichloroethane, Fig. 3), gained public awareness through Rachel Carson in her book "Silent Spring" [27]. Nine organochlorine pesticides were first added to the Stockholm Convention list of POPs. The toxicity of organochlorine pesticides includes neurological damage, endocrine disruption, hypertension, and oxidative stress [28].

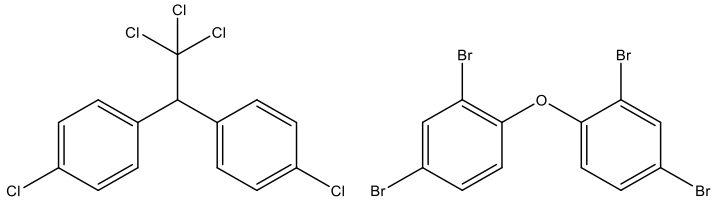


*Fig. 3. Two persistent organic pollutants frequently detected in human blood, p,p'-DDT – dichlorodiphenyltrichloroethane (left) and BDE47 – 2,2',4,4'-Tetrabromodiphenyl ether (right).*

Brominated flame retardants (BFRs), *e.g.*, polybrominated diphenyl ethers (PBDEs), have been used since the 1970s to reduce the flammability of everyday products, e.g., plastics, textiles, furniture, and electronic equipment. In the 1980s, findings of these chemicals in the arctic environment showed their persistence and long-range transporting properties [29]. Some BFRs are regulated regionally (in the European Union [EU]), and others are internationally banned in the Stockholm Convention. BFRs have toxic properties related to neurotoxicity, developmental toxicity, carcinogenicity, and endocrine disruption [30]. BDE47 (2,2',4,4'-tetrabromodiphenyl ether) is one of the PBDE congeners found at the highest concentrations in human blood (**Paper I**, Fig. 3)

PFAS have been used in consumer and industrial uses since the 1940s. PFAS is a chemical group comprising more than 10,000 chemicals [31]. PFAS are stable molecules used for their heat-resistant and water/grease-repellant properties. PFAS are spread in the environment and have high detection frequencies in humans (discussed in **Paper II**) [32-34]. Perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS, Fig. 4), perfluorooctanoic acid (PFOA, Fig. 4), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA), are the most frequently found PFAS in human blood. PFHxS (including its salts and PFHxS-related compounds); PFOS (including its salts and perfluorooctane sulfonyl fluoride); and PFOA (including its salts and PFOA-related compounds) are listed in the Stockholm Convention of POPs [23]. PFAS chemicals have toxic properties that can disrupt the immune system, hepatoxicity, lipid disruption, and reproductive and developmental toxicity (discussed in **Paper II**) [35]. To stop new PFAS exposure, Denmark, Germany, the Netherlands, Norway, and Sweden proposed restricting a broad range of PFAS uses within the EU, which was later suggested by the EU Chemicals Strategy for Sustainability [36, 37]. Sources of PFAS exposure have been identified as routes through food, water, indoor dust, air, and personal care products [38]. It is primarily food from animal sources which have the highest levels [39, 40].

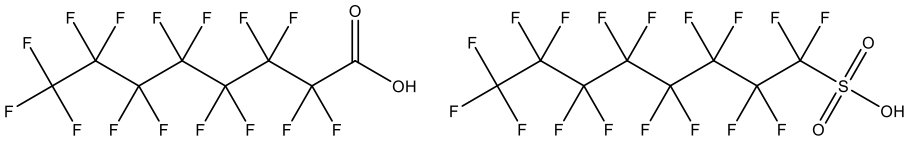


*Fig. 4. Two perfluoroalkyl substances (PFAS) frequently found at the highest concentration in human blood. PFOA – Perfluorooctanoic acid (left) and PFOS – Perfluorooctane sulfonate (right).*

## 1.1.2 Synthetic Organic Contaminants in Use Today

Synthetic organic contaminants addressed here are SPAs, parabens, bisphenols, UV filters, and phthalates. Many synthetic phenolic contaminants are unregulated, and human exposure to them is continuous from various sources, such as plastic materials used as food contact materials [13].

### 1.1.2.1 Synthetic Phenolic Antioxidants

SPAs are single or double-ringed phenolic structures with at least one tert-butyl group attached to the ring and some an alkyl chain attached to the ring. One tert-butyl group is positioned in the *ortho*-position relative to the hydroxyl group, which affects the polarity of the compound. The SPAs are a structurally diverse chemical class with a $\log K_{OW}$ (octanol-water partitioning

coefficient) between 2.8 and 9.5. The pKa value of the phenol group varies between 8.6 and 14, depending on the functional groups around it.

SPAs are used to slow down the oxidation, thereby preventing the degradation of products [41-43]. These chemicals are added to, *e.g.*, personal care products, food, clothing, and plastics [42-46]. A recent review concluded that external human exposure to these chemicals is high based on available data, but internal exposure levels are lacking, especially in Europe [13]. The available studies with human internal exposure in blood are from the United States and China and show high levels of some SPAs [47-51]. Both *in vitro* and *in vivo* studies have observed effects of SPAs related to endocrine disruption, neurotoxicity, developmental toxicity, and carcinogenicity [52, 53]. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Fig. 5) are used in a variety of products, including food and personal care products [54, 55]. Three SPAs are listed in the Candidate List of Substances of Very High Concern (SVHC) due to reproductive toxicity (2,4,6-Tris(tert-butyl)phenol [AO246] and 2,2'-methylenebis(4-methyl-6-tert-butylphenol) [AO2246], Fig. 5), endocrine disruption (4-tert octylphenol [4-tOP]) and PBT properties (AO246) [56-58]. Additionally, 4,4'-methylenebis(2,6-di-tert-butylphenol) (AO4426), 4-tOP, and AO246 are being assessed for persistent, bioaccumulative, and toxic properties [57-59]. AO4426 [59] and 2,4-di-tert-butylphenol (2,4-DBP) [60] are evaluated for endocrine disruptive properties, while AO2246 [56] and AO246 [58] are evaluated for reprotoxic properties.

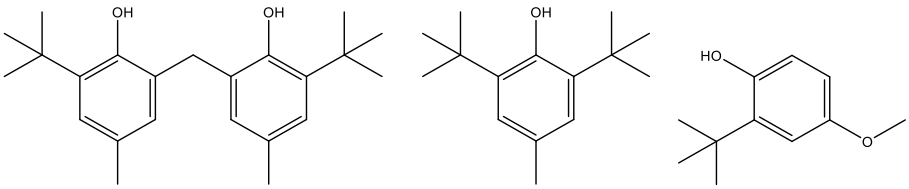


*Fig. 5. Three synthetic phenolic antioxidants. AO2246 - 2,2'-methylenebis(4-methyl-6-tert-butylphenol) (left), BHT - Butylated hydroxytoluene (middle), and BHA – butylated hydroxyanisole (right).*

### 1.1.2.2 Parabens

Parabens are one-ringed phenolic structures with an ester group placed at the *para*-position relative to the hydroxyl group (Fig. 6). The length of the ester alkyl chain determines the polarity, with $\log K_{OW}$ ranging between 2 and 3.6 and pKa of 8.5. Parabens are used in food [61-63], personal care products, and pharmaceuticals [64] but are also naturally occurring [65]. These chemicals are used as preservatives due to their anti-fungal and anti-microbial properties. Parabens, such as methylparaben, have been detected at relatively high levels

in human serum, urine, and seminal fluids [66-68]. The high molecular weight parabens have been primarily associated with endocrine disruption [69]. Methyl- and ethylparaben are considered safe to use in personal care products at up to a certain level [70]. However, five parabens (isopropylparaben, isobutylparaben, pentylparaben, phenylparaben, and benzylparaben) are banned in personal care products due to lack of hazard information [70].

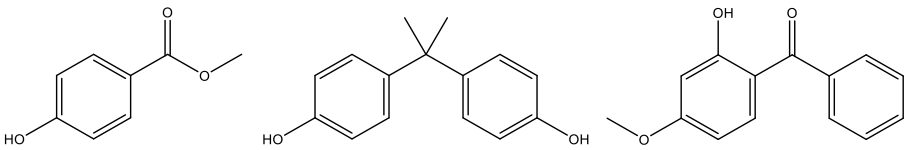


*Fig. 6. Three synthetic phenolic compounds. Methylparaben (left), bisphenol A (middle), and benzophenone-3 (right).*

### 1.1.2.3 Bisphenols

The general structure of bisphenols is two phenols linked by a bridge. The hydroxyl groups are in the para position of both rings. The bridge varies depending on the bisphenol, with bisphenol A having a methylene bridge (Fig. 6). The $LogK_{OW}$ of bisphenols varies between 3.2 and 6.9, and the pKa between 9.1 and 10.3. Bisphenols are monomers used in the production of polycarbonate plastic, food-contact materials, dental materials, and receipts [23-27]. Bisphenol A is restricted in food-contact materials and toys and banned in baby bottles due to its toxic properties related to fertility, skin allergy, and endocrine disruption[71]. Bisphenol A has been classified as endocrine-disrupting with effects related to metabolic derailment and obesity [72]. Other bisphenols are replacing bisphenol A in these products, raising concerns about their safety [73]. Bisphenols are found in human urine, with decreasing levels of bisphenol A and increasing levels of other bisphenols replacing the bisphenol A use [74].

### 1.1.2.4 UV filters

There are many types of UV filters, inorganic (*e.g.,* titanium dioxide), organic, (*e.g.,* UV-328), and phenolic organic UV filters (*e.g.,* benzophenones). The UV filters addressed within this thesis are phenolic structures that protect humans (via sunscreen) and products from harmful UV radiation. The $LogK_{OW}$ of the UV filters included here varies between 2.6 and 4.2, and the pKa between 6.6 and 9.7. UV filters are present in personal care products, plastics, textiles, tattoo- and printing ink [75-78]. The phenolic organic UV filters have been found in human serum, urine, amniotic fluid, fetal blood, cord blood, and seminal fluids [79, 80]. UV filters have been linked to endocrine effects [81].

For this reason, some benzophenones in sunscreens, such as benzophenone-3 (Fig. 6), are regulated [82].

#### 1.1.2.5 Phthalates

Another group of synthetic contaminants used in personal care products and as a plasticizer is phthalates, providing flexibility and durability to plastic materials (Fig. 7) [83]. Phthalates have been detected in humans and the environment since the 1970s [84]. These plastic additives, together with the SPAs and UV filters, are not chemically bound to the plastic polymer and have been seen to leach from plastic food contact materials [42, 85, 86]. Phthalates are usually analyzed in human urine, and similar trends are seen for bisphenols, with old phthalates decreasing and new replacement phthalates increasing [74]. Phthalates have been linked to endocrine disruption and reproductive and developmental toxicity [87].

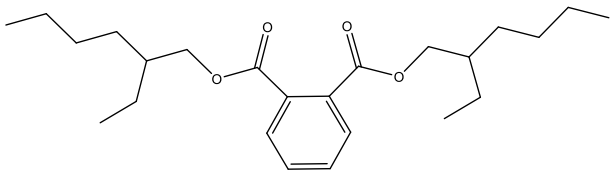

*Fig. 7. The phthalate Bis(2-ethylhexyl) phthalate, commonly referred to as DEHP.*

## 1.2 Chemical Toxicity

### 1.2.1 Endocrine Disruption

Hormones are the signaling molecules in the body and are used for communication between cells and organs. A hormone is defined as

> "Any substance released by a cell that acts on another cell near or far, regardless of the singularity or ubiquity of the source, and regardless of means of conveyance" [88]

There are eight endocrine glands in the human body: the hypothalamus, pituitary gland, pineal gland, thymus, pancreas, adrenal gland, thyroid gland, and ovary or testis (Fig. 8). These glands produce over 30 hormones, including estrogens, androgens, and thyroid hormones. Most studies focus on effects related to effects on the estrogen, androgen, thyroid, or steroidogenesis (EATS) pathways, but there are many other hormone modalities in the human body (non-EATS). This thesis studies the EATS (**Paper IV** and **Paper V**), except for the steroidogenesis pathway.

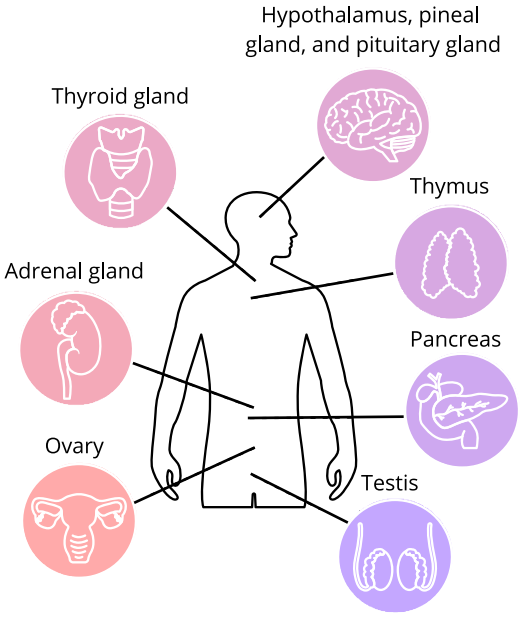


*Fig. 8.* ***Overview of the endocrine system****, with the eight glands: adrenal, thyroid, hypothalamus, pineal, pituitary glands, thymus, pancreas, and testis/ovary.*

When a hormone binds to its receptor, it causes a cascade of reactions leading to increased or decreased transcription (RNA) and translation (proteins). The hormone levels are relatively low compared to other endogenous chemicals. A hormone receptor can be placed on the cell membrane, in the cytosol, or in the nucleus.

A chemical with similar properties as a hormone can disrupt the endocrine system. A chemical affecting the endocrine system is called an endocrine-disrupting chemical (EDC), defined by the World Health Organization, International Program on Chemical Safety as

> "…exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations" [89].

At least five mechanisms can affect the endocrine system [90]. An EDC can cause endocrine disruption when the signaling itself is disrupted through the chemical binding of the EDC to the hormone receptor. EDCs can alter the hormone balance by binding to important transporting proteins and disrupting regulating pathways, which, in the end, also affects the hormone level. EDCs can cause an increased destruction of hormones through increased biotransformation and excretion. EDCs can disturb hormone synthesis or secretion.

Lastly, EDCs can trigger hormone stimulation, which could lead to hypertrophy (gross enlargement) or hyperplasia (excessive cellular development) and, eventually, tumorigenesis [90]. Due to the complex network of the various hormone signaling pathways, it is challenging to predict endocrine-disrupting properties. The effects caused by an endocrine disruptor can be diverse and affect multiple hormone systems and organs (Fig. 9).

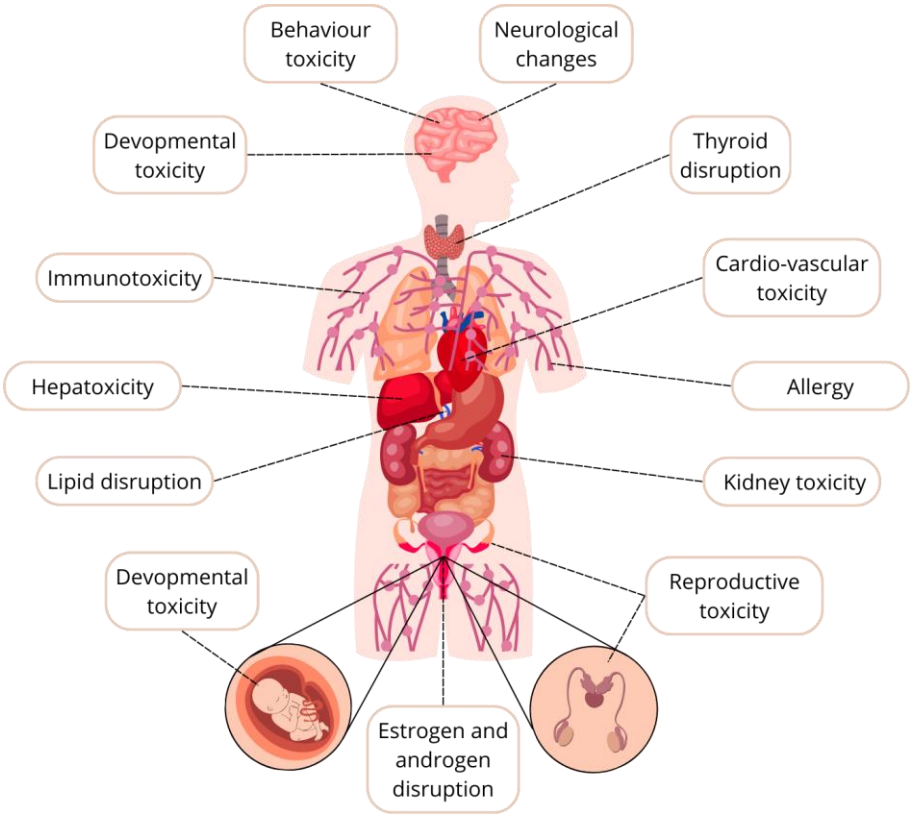


*Fig. 9.* ***Overview of toxicological effects*** *many of which can be related to the endocrine system, that could occur from chemical exposure.*

A functioning endocrine system is crucial to the development and function of the organs and tissues in the body. For example, an impaired hormone system can affect the reproductive system in females and males, fetal development, neurodevelopment, immune system, metabolism, and cardiovascular system [7]. Additionally, studies have found that early-life exposure to EDCs is linked to late-onset effects such as precocious puberty, cognitive and behavioral impairment, lowered IQ score, and autistic spectrum disorders [91-95]. There have also been studies suggesting links to obesity and type 1 and 2 diabetes mellitus [87, 96-99]. Many phenolic compounds have endocrine-disrupting

properties, such as bisphenols [100], parabens [101, 102], UV filters [103, 104], SPAs [105-107], and phthalates [108].

Structurally similar compounds can have similar effects even if the potency between the compounds can differ depending on the structure [35, 100]. Due to the structural similarities between synthetic phenolic compounds and several hormones, the relationship between phenolic structures and their endocrine activity has been studied previously [100]. The study defined structural requirements for a chemical to have endocrine-disrupting activity (Fig. 10). Predicting the binding to different endocrine receptors is a first-tier approach when toxicity data is lacking. However, human relevance is difficult to predict as many feedback loops and signaling pathways co-occur in a biological organism [109-112]. The hydroxyl group at the *para*-position of the A-ring (left in Fig. 10) seems essential for the binding to the endocrine receptors (estrogen, androgen, and thyroid). Other studies have shown estrogen receptor activities for structures with *meta*-hydroxyl and *ortho*-hydroxyl groups attached to a phenol ring. However, these show lower activity than *para*-hydroxyl structures [107, 113]. Substituents at the *meta*-positions in the A-ring seem to regulate the endocrine activity. For example, a bulky group is essential for binding the thyroid receptor, while the estrogen receptor is hindered by this, and the androgen receptor (AR) is unaffected [100]. The polarity of the substituents of the 2-position in the propane group (part of the methylene bridge) affected both the estrogenic and androgenic activity. A second ring (B-ring, right in Fig. 10) was not essential but enhanced the thyroid hormone and estrogenic activities [100].

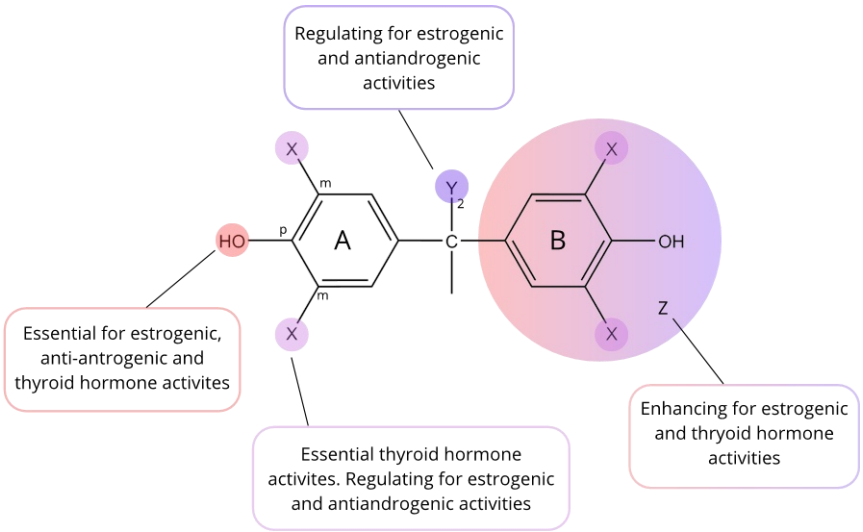


*Fig. 10.* ***Potential structural requirements*** *for endocrine-disrupting activity of phenolic compounds. Picture recreated from Kitamura and coworkers[100].*

### 1.2.2 Concentration-Response Association

Determining the toxicity of a chemical can be done *in silico [114]*, *in vitro* (**Paper III**), or *in vivo* (**Paper IV**). The studied effects can be confirmed in epidemiological data if the exposure is widespread (**Paper II**) [115]. A concentration-response curve can be made by studying an effect at numerous concentrations. These curves typically follow a sigmoidal curve shape, with a plateau when 100 % binding has occurred. The receptor disruption can be caused by an agonist (binding and stimulating the receptor) or an antagonist (binding and blocking the receptor) (Fig. 11). An *in vitro* assay can utilize the chemically induced translation of proteins through the nuclear receptor (Fig. 11) [116]. The cells in the assay have been genetically modified to produce the light-producing enzyme, *e.g.*, luciferase, instead of the intended enzymes. In the presence of luciferin and ATP, this enzyme produces light, which a spectrophotometer can detect (Fig. 11).

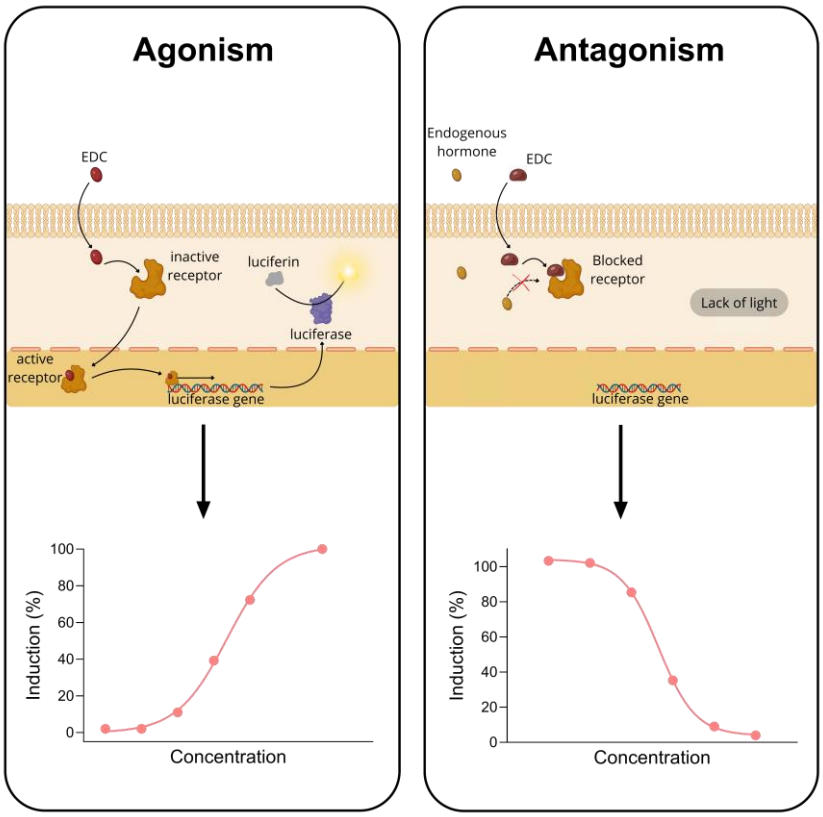


*Fig. 11.* ***Schematic overview*** *of the receptor-binding in vitro assay with genetically modified cells by adding the luciferase gene (top). Sigmoidal concentration-response curves generated from the observed intensities. Depending on which substrates are added, the agonistic binding (left) or the antagonistic binding (right) can be evaluated. Created with BioRender.com.*

The Replacement, Reduction, and Refinement (3R) principle should be considered before performing *in vivo* tests [117]. New Approach Methodologies (NAMs) use scientific techniques to predict the human-relevant toxicological effects to reduce the use of animal testing [118]. NAMs include modeling (*in silico*), abiotic determination of reactivity (*in chemico*), chemical analysis using omics techniques (genomics, transcriptomics, proteomics), and cell tests (*in vitro*).

Toxicokinetic modeling (*in silico*) can be used to screen thousands of chemicals for certain structural traits [119]. The chemicals with indications of toxicity can thereafter be tested *in vitro* for confirmation. *In vitro* assays can show the cellular response necessary for a toxic effect [120]. Cell-based tests (*in vitro*) are used to gain a mechanistic understanding of toxicity (**Paper IV**). Furthermore, using these tests can provide a quantitative estimate of the effect in the test system [120]. To further evaluate if the observed effect *in vitro* is also occurring in an organism, the next step would be to conduct *in vivo* experiments. In a biological system, *in vivo*, multiple pathways can be activated simultaneously. One interaction might lead to a signal causing an increased response, but simultaneously, another signal pathway activated inhibits the same effect. Due to the uncertainty and complexity of the biological organism, until the NAMs are further developed, animal studies are still needed to ensure human safety [121]. The zebrafish embryo toxicity (ZFET) test is one way to screen chemicals for toxic properties in a whole organism (used in **Paper V**). The test can be used to screen multiple general toxicity endpoints related to developmental effects, behavior effects, and endocrine disruption [122-125].

Extrapolating effects seen *in vivo* or *in vitro* to human toxicity is challenging[126]. *In vivo* experiments will have differences in toxicity based on animals, dose, and how the chemicals are administered to the animal. There is guidance on translating effects in animal studies (rat, mouse) to effects in humans using assessment factors ranging from size 10 to 10,000 [127]. These can be used for inter-/intraspecies variations or translating from acute to chronic dosing. Additionally, assessment factors compensate for uncertainties related to gaps and limitations between animals and humans [127].

When chemical exposure is well-established and widespread among human populations, and much scientific literature is available, epidemiological data can be complementary to evaluate the association between a chemical and a toxicological effect [115]. Using these associations, effect levels can be derived and compared with the exposure levels (used in **Paper II**)[128, 129]. Furthermore, in humans, it must always be considered that there can be more than one explanation for an effect, and therefore, epidemiological data alone cannot provide a toxicological link [115].

## 1.3 EU Chemicals Regulation

Different authorities and regulations regulate chemicals in the European Union (EU). The European Chemicals Agency (ECHA) oversees the regulation called Registration, Evaluation, Authorisation, and Restrictions of Chemicals (REACH) with the aim of removing hazardous chemicals from the market. This is done by placing the responsibility on the producers and importers of a chemical to register all chemicals above 1 ton/year/actor (Registration) [130]. Chemicals below this threshold are not risk-assessed within REACH. The company registering the chemical will then assess the chemical and provide data from suitable toxicity tests and propose risk management measures. A large body of evidence from multiple studies using various techniques called a weight-of-evidence approach, is often necessary to assess chemical safety [131]. The submitted data is then evaluated and chemicals of concern are selected for further assessment (Evaluation). ECHA will, based on the data provided, identify SVHC, such as chemicals with properties such as carcinogenic, mutagenic, or reprotoxic, PBT, very persistent, very bioaccumulative, or endocrine disruptive [132, 133]. For a chemical to be classified as an EDC within REACH and other frameworks within the EU, the chemical needs to meet three criteria. The chemical needs to show an endocrine activity (*in vitro*), cause an adverse effect (using *in vivo* multi-generational study), and that there is a biologically plausible link between the endocrine activity and adverse effect [134]. When these are met, the chemical is classified as an EDC and will be treated as an SVHC. Chemicals with SVHC properties will be placed on the Candidate list [135]. The chemicals on the Candidate list are prioritized based on volume and hazardous properties to be further evaluated. An obligation from using a chemical on the Candidate list is to notify ECHA if an article includes > 0.1 % w/w. Chemicals with cause for concern will be added to the Authorisation list. Chemicals on the Authorisation list can only be used for specific uses for a short time until a replacement is found. These chemicals cannot be used without authorization after a set deadline. Chemicals with an unacceptable risk can be restricted, entailing a complete ban or only permitted specific uses where the benefit outweighs the risk.

Other authorities in the EU have a specific focus, such as food and food-contact materials (European Food Safety Authority, EFSA). Several regulations focus on the intended use of a product, such as in the case of cosmetics (Cosmetic Products regulation). Depending on production volume and intended use, each substance can be regulated in separate frameworks entailing different legislative responsibilities [136].

## 1.4 Chemical Mixtures

In a real-life scenario, a chemical will be accompanied by hundreds of chemicals in the same organism that may or may not contribute to the same adverse effect. Assessing one chemical at a time can give information regarding that specific toxicity. However, it does not provide enough information for a real-life human exposure scenario. A mixture risk assessment (MRA) using human blood as an exposure matrix can be used to estimate the risk associated with cumulative chemical exposure.

The risk of observing an effect related to chemical exposure depends on the hazard presented by the chemical -meaning its potency-, the level of exposure, and the sensitivity of the individual affected. A MRA compares the exposure and hazard data from all known chemicals. The chemicals in a mixture can act through one or multiple pathways and affect endpoints in various compartments of the body. Whether to include chemicals acting through the same mode of action (MoA) or the same pathway in the risk assessment is yet to be understood [137]. The chemical exposure can be measured as external exposure, identifying and measuring all sources contributing to the exposure, or as internal exposure by measuring the blood or urine concentration in an individual.

### 1.4.1 Chemical Mixture Effects

There are several types of chemical mixtures. The mixture can be known, such as in a specific product, or undefined, such as in the case of the mixture of all chemicals in our body. Depending on the research question, the mixtures can be defined differently, such as all chemicals contributing to the same effect or all substances independent of the impact each chemical causes. The exposure of multiple chemicals through multiple routes and exposures is defined as cumulative exposure.

Mixture effects are complex to predict [138]. These mixture effects can be categorized into four mechanisms: concentration addition (CA), independent action (IA), synergistic effects, and antagonistic effects (Fig. 12). CA is a mixture concept applied to chemicals with the same MoA, claiming the additive effect from each component contributes to the observed effect [16], and it has proven to be a robust method working in most complex large mixture cases [16, 139, 140]. Several studies have shown that chemicals in a mixture additively can cause an impact both through the same MoA and different MoAs, even at very low levels [137, 141-149]. Each chemical will typically not cause an observable effect at these low levels. IA is a mixture model where each component acts strictly through a different MoA at different target sites [150]. Mixtures seem to follow IA when there are few mixture constituents, and each

chemical is at a detectible, commonly high level, causing different toxic effects [150]. It has been shown that CA and IA are indestinguishable at low effect levels [151].

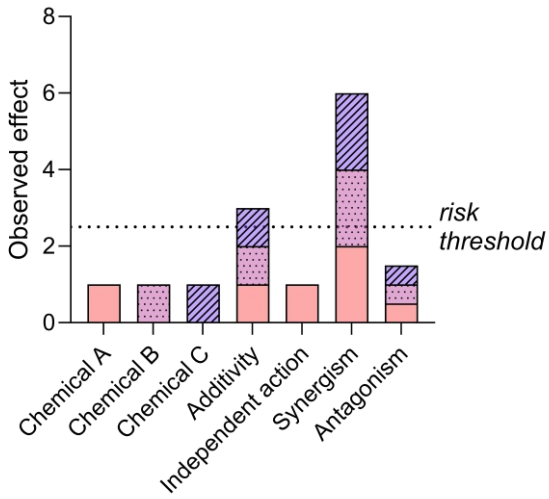


*Fig. 12.* ***Mixture effects.*** *Depending on their properties, the combined effect of chemicals A, B, and C can be additive, independent, synergistic, or antagonistic.*

When the observed effect from a mixture is greater than the predicted effects, synergistic effects are the cause (Fig. 12). Another term for this is the so-called "*cocktail effect*". One example could be if a chemical enhances the permeability of other substances through the skin or cell membrane. Without that specific chemical, the other chemicals would not reach the target cell and thus not cause an effect. PFAS have been identified to alter the membrane integrity, causing other chemicals to enter the cell [152-156]. This is an example of synergistic mixture effects. There has been a lot of discussion regarding how common synergistic effects are. Mixtures have been observed to only follow synergistic or antagonistic mixture effects in 5% of mixture cases [140, 157]. However, the results are dependent on which mixtures are studied. Most mixture studies focus on relatively few component mixtures with less than ten mixture components [140]. There is a lack of studies testing complex mixtures with full concentration-response curves and individual substance effect data [157, 158]. Therefore, the body of evidence for neglecting or confirming synergism is insufficient [157].

### 1.4.2 Mixture Risk Assessment

When using human internal exposure levels, the most frequently used MRA approach is to find effect levels from the literature for all the known chemicals within the exposure mixture contributing to the same endpoint and compare

the levels of the exposure and effect level. A critical bottleneck in the risk assessment is the availability of effect levels and exposure data for multiple compounds in the same individuals [18]. By dividing the exposure levels by the obtained effect levels, the quotient for each chemical is combined to summarize the total mixture risk (**Papers I** and **II**). When the exposure level exceeds the hazard level of a chemical, the risk of an effect occurring cannot be ruled out. This would correspond to the sum of these quotients exceeding 1 in a MRA. Human cumulative assessment studies are further limited to detectable chemicals with internal effect levels, restricting the number of chemicals that can be included in the MRA [18].

The level where an effect can be observed, an effect level can be derived using dose-response animal data or epidemiological data or described as relative potencies of a reference compound. Many human biomonitoring (HBM) guidance values are derived using animal studies. However, data from animal studies need to be extrapolated to human relevance using assessment factors [127]. When using these HBM guidance values, the Hazard Index (HI), a first-tier approach, is used (**Paper I**). Furthermore, HI requires that all included chemicals act on the same target organ [159]. However, HI can become problematic when applying a wide range of assessment factors to animal-derived effect levels [160]. In those cases, the point of departure index can be used instead, where a point of departure (PoD) value is used [160]. Epidemiological data can be used to derive PoD, but a prerequisite is that the exposure is widespread and that the association covers co-exposure to other contaminants and stressors (**Paper II**) [115]. The HBM guidance values for PFOS and PFOA used this approach [128]. Relative potency factors are used for chemicals with similar structures and normalize the potency of each chemical by a reference chemical, and the relative potencies are assumed to be the same between different species [159, 161]. These values have historically been used for dioxin and dioxin-like PCBs [159] but have recently been gaining more attention in other chemical classes, such as PFAS (used in **Paper II**)[161].

### 1.4.3 Mixture Allocation Factor

The process of weighing different risks related to their benefits can be defined as the risk tolerance within the government. Estimating the risk tolerance includes weighing the risk assessment perspectives, socio-economic interests, and political interests. The European Commission stated, in the EU Chemicals Stategy for Sustainability, a need for a mixture assessment/allocation factor (MAF) [37]. An MAF could be seen as defining the size of the "risk cup"; when full, a risk of adverse effects cannot be excluded [162]. Using an MAF would account for the unintentional mixture components when evaluating the toxicity of a single chemical in REACH by lowering the acceptable contribution from a single chemical. The size of the "risk cup" has been discussed in

human and environmental scenarios with suggestions of 5, 10, or 100 [163-167]. However, the suggested size is limited by the number of guidance values available, especially lacking for human effects. The appropriate size of the MAF is highly dependent on the mixture constituents and the effect studied, resulting in a general MAF with high uncertainty, which needs to be considered.

### 1.4.4 Uncertainty in Mixture Risk Assessment

Uncertainty will always be present when assessing the risk of adverse effects related to chemical exposure. For example, the certainty of an observed association when deriving an effect level will depend on the quality of the data provided, sample size, and body of knowledge available (**Paper II**). There are two types of uncertainties: inherent uncertainties, which are unpredictable, and knowledge uncertainties, which are associated with the lack of knowledge. Knowledge uncertainty can be reduced by further studying the exposure and hazards at an economical cost. Furthermore, uncertainty always depends on the available expertise an individual has at a given time.

To account for uncertainty is vital; otherwise, indications of risk are neglected. The Rio Declaration, an international treaty, states in Principle 15 that the precautionary principle should be used when there is uncertainty regarding the risk. It states that

> "In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation"[168].

There are numerous examples of when the precautionary principle could have made an impact but was not applied. Many of these cases could be solved by grouping similar chemicals in restrictions. For example, a grouping of PFAS could have hindered the fourteen-year gap between banning PFOS (2009) and PFHxS (2023), and many are still not regulated. PFOS was listed in the Stockholm Convention 2009, but it took ten more years before PFOA was listed and an additional three years to ban PFHxS [23].

# 2 Aims and Scope of Thesis

The overall aim of this thesis was to assess the potential health effects associated with exposure to complex chemical mixtures. To achieve this, the thesis focuses on two pillars: understanding human chemical exposure and investigating whether the Swedish general population is within the risk threshold for health effects related to chemical exposure.

The Swedish human chemical exposure was investigated using four objectives:

(a) To determine the already-known chemical exposure by employing literature searches and creating a database consisting of chemicals and their levels in human blood (**Paper I**)
(b) To identify knowledge gaps regarding chemical exposure in Sweden compared to analyzed worldwide (**Paper I**)
(c) To identify knowledge gaps regarding exposure to chemicals in use today (**Papers I** and **III**)
(d) To fill in the gap of chemicals lacking internal human blood concentrations by developing analytical methods to analyze SPAs, Parabens, UV filters, and bisphenols using complementary LC-MS and GC-MS (**Paper III**)

Four objectives were put forward to investigate whether the Swedish general population is within the risk threshold for health effects related to chemical exposure:

(e) To apply mixture risk assessment strategies to the known chemical exposure of POPs and PFAS (**Papers I** and **II**)
(f) To create a complex chemical mixture based on data from **Papers I** and **III** (**Paper IV**)
(g) To test the endocrine-disrupting potential, oxidative stress potential, and metabolic-disrupting potential of the complex chemical mixture using cell-based *in vitro* assays (**Paper IV**)
(h) To test the developmental toxicity in early-life exposure of the complex chemical mixture in a whole organism using the *in vivo* ZFET assay (**Paper V**)

This PhD project is part of the RiskMix project, which aims to understand the effects of chemical mixtures that humans are exposed to by using the ZFET test to evaluate toxicity.

# 3 Method

## 3.1 Human Exposure to Chemicals

**Paper I** reviewed the literature and compiled chemicals analyzed in human blood in the Swedish Exposure Database (SEDB) and worldwide in the Human Blood Database (HBDB). The two databases were compared to identify exposure knowledge gaps [32]. Chemicals identified as substances of concern in the mixture risk assessment conducted in **Paper I** were prioritized for analysis in **Paper II**.

To prioritize target analytes lacking human blood concentrations, three strategies were used (**Paper III**):

(1) Comparing the analytes in the two databases (SEDB and HBDB) revealed that Sweden covered the analysis of most halogenated compounds but missed the analysis of many compounds in production today, such as bisphenols, parabens, perfume additives, UV filters, halogenated phenols, solvents, and polycyclic aromatic hydrocarbons (Fig. 13). However, in Sweden, many of these analytes have been detected in urine [74, 169, 170].
(2) *In silico* models (QSAR) were applied to HBDB to assess which chemicals were thyroid hormone disruptive and, therefore, could be of concern to the general population [114]. Bisphenols, parabens, and organic UV filters were identified as potential thyroid disruptors and selected from this prioritization strategy (Fig. 13).
(3) To cover chemicals in use today, the chemical properties in HBDB were applied to chemicals in the Products Registry (managed by the Swedish Chemicals Agency) using OPERA QSAR modeling [171]. This prioritization method focused on the likelihood of exposure to the general population rather than the hazard. This method prioritized several SPAs and a novel bisphenol, diallyl-bisphenol A (Fig. 13). These chemical groups were included as target analytes when developing an analytical method in **Paper III**.

The strategies lead to the development of analytical methods focusing on SPAs, bisphenols, parabens, and UV filters.

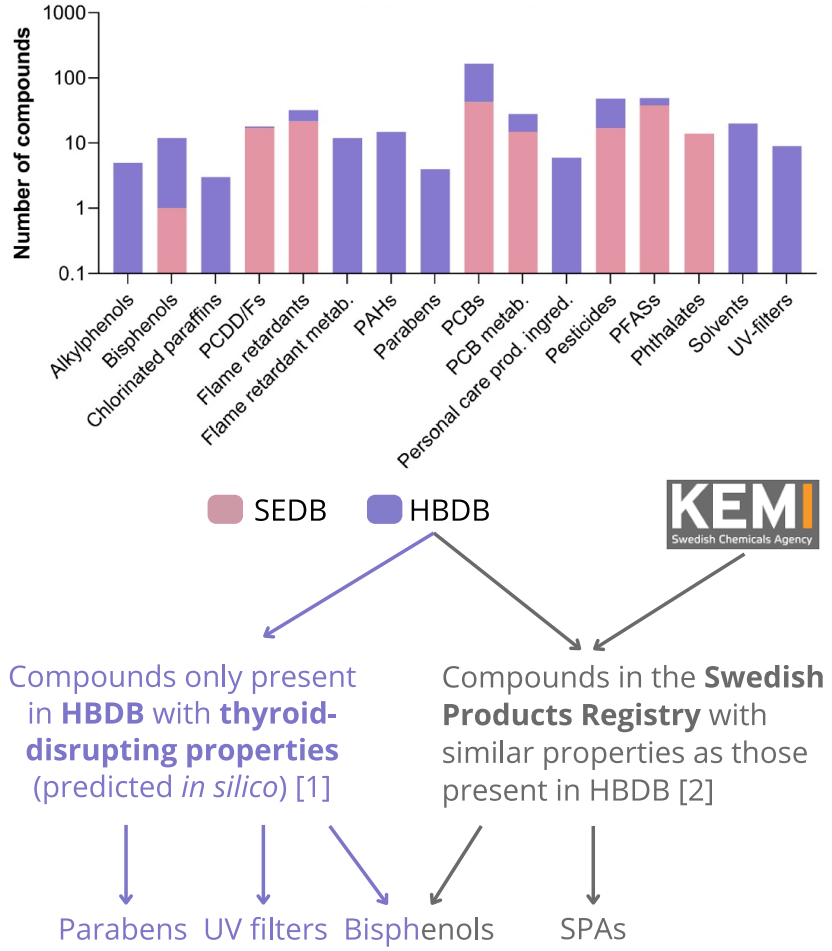


*Fig. 13* ***Prioritization strategy*** *for selecting contaminants for chemical analysis.*

### 3.1.1 Human Samples

Blood donors are individuals meeting specific health criteria to be able to donate blood. From a blood donation centre, 100 individual serum samples were taken from January to February 2020, to study individual variations. A pool constructed from all individuals ($Pool_{100}$) was also compiled to investigate the average exposure levels within the studied group. In **Paper II**, 60 individuals were analyzed for PFAS exposure, the 30 youngest and 30 oldest females and males. In **Paper III**, the 30 youngest individuals were analyzed for SPAs, bisphenols, parabens and UV-filters, 15 females and 15 males.

### 3.1.2 Target Analysis and Suspect Screening

**Target analysis** is the process where both the sample preparation and instrumental analysis are optimized to pinpoint a specific array of chemicals. Using

this method, the recovery of each analyte is determined, and the analytes in the samples are quantified. Commonly, the detection limit decreases using target analysis, *i.e.*, lower concentrations can be detected compared to other screening methods. This method was applied in **Paper II** and **Paper III**.

A **suspect screening** can be performed as a first-tiered approach to gain additional information on other chemicals in the sample. The exact mass of a list of suspected chemicals in e.g., human blood, without standards and method recovery, is added to the instrumental method to acquire identification data, such as $MS^2$ spectral and retention time information. Depending on the information gained, the data can be matched to a suspect feature with a 1-5 identification confidence level [172]. A high confidence level indicates that the exact mass and $MS^2$ spectra match with standards, while a lower confidence level indicates the same m/z or the sum chemical formula. This approach is particularly useful when a target analysis is done, and there are more chemicals with similar properties to the target analytes, but no standards are available. This method was used in **Paper II** and **Paper III**.

### 3.1.3 Sample Preparation and Clean-up

In **Paper II,** an established method was used to analyze PFAS in human serum. In **Paper III**, a new method was developed where multiple extraction solvents, clean-up methods, and analytical instruments were evaluated. Ultimately, both methods included acetonitrile (ACN) as an extraction solvent and for protein denaturation. Various clean-up methods exist, and the choice will depend on the structure of the analytes. In **Paper II**, a method using active carbon (EnviCarb™) was combined with formic acid (to protonate the PFAS molecules). In **Paper III**, the analytes performed better using an enhanced matrix remover (Bond-Elute EMR-Lipid™), compared to the EnviCarb™ and using a HybridSPE®-Phospholipid solid-phase extraction cartridge (unpublished data).

In **Paper III**, sampling and all sample preparation steps were checked for contamination before the sample analysis. Triplicate method blanks were included for each batch of eight samples. Furthermore, where blank contamination was observed, measures were taken to minimize the contamination, for example, by distilling solvents, washing the EMR powder, and avoiding plastic labware. To ensure that the quantified concentrations were correct, the samples were diluted and re-analyzed to ensure the concentrations were in the linear range of the calibration curve.

Many analytes, such as phenols, are metabolized in the human body by adding a conjugate group to increase polarity. These metabolites are also of interest when considering the total exposure of an analyte. By cleaving the conjugate

groups from the analytes, in a process called deconjugation, the total concentration of an analyte in blood can be determined. This can be done using an enzymatic deconjugation step (**Paper III**).

### 3.1.4 Analytical Instrumentation

Two methods are commonly used to analyze contaminants in human blood, LC-MS and GC-MS. LC is frequently used for more hydrophilic (polar) analytes where the sample is transported through a packed column using mobile phases. In reversed-phase LC, used in **Papers II** and **III**, the column is non-polar (C18 or similar), and the mobile phase gradient starts as very polar and gradually decreases the polarity in a pre-determined timed gradient. The analytes are transported through the column based on polarity. When the polarity of the mobile phase is more like the analyte than the column, the analyte follows the mobile phase out of the column and into the mass spectrometer. How much the analyte is retained on the column determines its retention time. In LC-MS, the most common way to ionize the analytes is electrospray ionization (ESI). ESI is a soft ionization technique that forms molecular ions, usually removing a single hydrogen atom from the analyte, when using negative ionization mode. The mobile phase (with the analytes) is sprayed through a high potential difference, causing the droplets to become charged. Thereafter, the droplets decrease in size until only the charged ion is left, caused by heat and nitrogen gas. These ions are then transported to the mass analyzer and separated based on mass.

GC is used for more hydrophobic (non-polar) analytes. The analytes are separated based on volatility using a temperature gradient. The ions are sent into a hydrophobically coated (stationary phase) narrow column and placed in an oven at a programmed temperature. An inert gas, the carrier gas, is pushed through the column (helium or argon) at constant pressure as the temperature increases over time. Depending on the properties of the column and the analytes, the analytes will stay put until they are volatile. After volatilization, the retention time, the time the chemical moves through the column, depends on the degree of interaction with the stationary phase. After the column, the analytes are pushed into the ion source. The ion source used in this thesis is electron ionization (EI). EI is a hard ionization technique, causing the ions to fragment. By reading the spectra, structural information can be gained. The ionization is caused by electrons from a heated filament bombarding the analytes with electrons. This causes the analyte to release an electron and form a positive radical. Depending on the energy and the stability of the ion, the radical can fragment further already in the ion source. After the ionization, the ions are further transported to the mass analyzer.

After the chromatographic separation of the analytes, the ions are separated on their mass-to-charge ratio (m/z) in the mass analyzer. Electrical and magnetic fields cause the separation in a vacuum. The use of multiple mass analyzers helps to detect not only the parent ion but also its fragment ions, often generating a compound-specific fingerprint of different m/z fragments. In target analysis, multiple reaction monitoring is used, where pre-determined ions are selected, usually the parent ion (used to confirm the correct identity) and at least one high-intensity fragment ion (used for quantification). In suspect screening, the MS spectrum is investigated to find compound-specific fingerprints. This thesis primarily focused on target analysis using standards, where the parent ion and fragments are known beforehand. There are two frequently used mass analyzer types: beam-type and trapping-type. Beam-type analytes are widely used in this research area, and the triple quadrupole is one example (used in **Paper III**, coupled to the LC and GC). The quadrupole consists of four parallel rods, switching between negative and positive currents in a pairwise manner. A radio frequency voltage is applied, and together, these build up the electrical field, permitting only a single m/z ratio ions to pass at a time. In the triple quadrupole, a collision cell is placed between two quadrupole sets. A selected m/z is fragmented in the collision cell through collision-induced dissociation with an inert gas (argon or nitrogen).

Besides triple quadrupole MS that focuses on specific target compounds, some mass spectrometers can scan for a wide range of compounds and be used for suspect. For instance Orbitrap is a high-resolution trapping-type mass analyzer using Fourier transformation to convert the secular frequencies of the ions into m/z. The mass accuracy of the Orbitrap is better than 1 ppm. Because of this, it is a useful tool for separating ions with a similar m/z ratio, as it has a higher identification confidence compared to low-resolution instruments (triple quadrupole). The Orbitrap used in this thesis (**Paper II** and **Paper III**) is a hybrid mass analyzer consisting of a triple quadrupole and an Orbitrap. Before fragmentation, the ions are collected in a C-trap, a wave-shaped trap that decreases the kinetic energy of the ions by collision with nitrogen gas. The ions are stored there until a set number of ions accumulate, then passed to the collision cell or the Orbitrap. The trap is emptied by applying direct constant voltage (DC) and alternating radio-frequency(RF) potentials. The collision occurs in a cell close to the C-trap, called a higher energy collisional dissociation collision cell. After fragmentation, the fragments are transported back to the C-trap and then to Orbitrap for m/z determination. When the ions enter the orbitrap, a DC voltage on the central electrode is applied, forcing the orbiting ion motion to contract, preventing the ions from colliding with the outer electrode, a process called electrodynamic squeezing. Each ion will oscillate back and forth around the inner electrode at a frequency determined by its m/z.

## 3.2 Chemical Toxicity

Two methods were used to determine the hazard profile of the chemical exposure. One method used effect levels derived from epidemiological and animal studies (**Paper I** and **Paper II**). The second method was to create a human-relevant artificial chemical mixture and assess the hazard using both *in vitro* (**Paper IV**) and *in vivo* methods (**Paper V**).

### 3.2.1 Effect Levels

HBM guidance values can be derived using animal studies (*in vivo*) and compensate for species and exposure differences using assessment factors [127]. In **Paper I**, available HBM guidance values from literature derived using animal and epidemiological studies were used to assess the risk of chemical exposure. The concentration where the effect differs from the background noise is seen as a point of departure value, where a higher concentration leads to a more severe effect in a concentration-response manner [128, 173]. The point of departure value, which can be derived using epidemiological studies, is defined as an effect level. Statistically significant effects between an observed effect and the concentration of an environmental contaminant can be used to determine a concentration above which a risk for adverse health effects cannot be excluded. Using epidemiological data means there is no need for assessment factors to compensate for interspecies differences. The downside is that confounding factors could trigger the effect, such as co-contaminants not determined in the studied population. Furthermore, many factors influence the associations observed in human epidemiological studies, including stress, chemicals, genetics, and lifestyle. Because of this, it is crucial to have a large enough population size and adjust for known potential cofactors. Furthermore, to better understand where along the concentration-response curve the population is, the association is carefully studied using interquartile associations to ensure statistical significance. A weight-of-evidence approach, where all available data is considered, is applied to ensure that the identified association is valid, where the same effect should also be observed *in vitro* and *in vivo*, along with a mechanistic understanding of why the chemical would cause this effect. The PFOS and PFOA HBM guidance values used in **Papers I** and **II** were derived using this approach [128, 129]. New PFAS effect levels for specific effects were derived and used in **Paper II**. Many of the endpoints included are well-characterized MoAs from PFAS exposure, for example, the thyroid hormone disruption in *in vitro* [174, 175] and *in vivo* experiments [175, 176]. Relative potency factors, used in **Paper II**, have also been derived for PFAS, with PFOA as the reference compound [161].

### 3.2.2 Human-relevant Chemical Mixtures

It is vital to consider mixtures with multiple compound classes to grasp the magnitude of mixture effects. Therefore, a 50-component chemical mixture was created (described in **Paper IV**, Fig. 14). This is one of the largest chemical mixtures used for toxicity testing, including POPs and other EDCs in human-relevant concentrations. The mixtures were tested in a concentration-response assessment *in vitro* (**Paper IV**) and *in vivo* (**Paper V**).

Each component concentration was taken from **Paper I**, using the average concentration from all data inputs (average, median, or geometric mean). Using the concentration from multiple studies, the maximum representation of the general population of Sweden could be considered. The chemical mixture created in this thesis reflects chronic exposure to the most abundant POPs and EDCs in a lifetime. Grouping chemicals into subgroup mixtures based on structures and function was used to minimize the number of tests. To identify the chemical group of concern, the compounds were divided into six subgroup mixtures which were tested separately (Fig. 14). The subgroup mixtures were the BFRs, PCBs (including the dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [2,3,78-TCDD), organochlorine pesticides, PFAS, phthalate, and phenol mixtures. The concentrations of the SPAs in the phenol mixture were taken from **Paper III**. The mixtures were tested at relative human blood concentrations (xHBC), see section 3.3 for further explanation of xHBC.

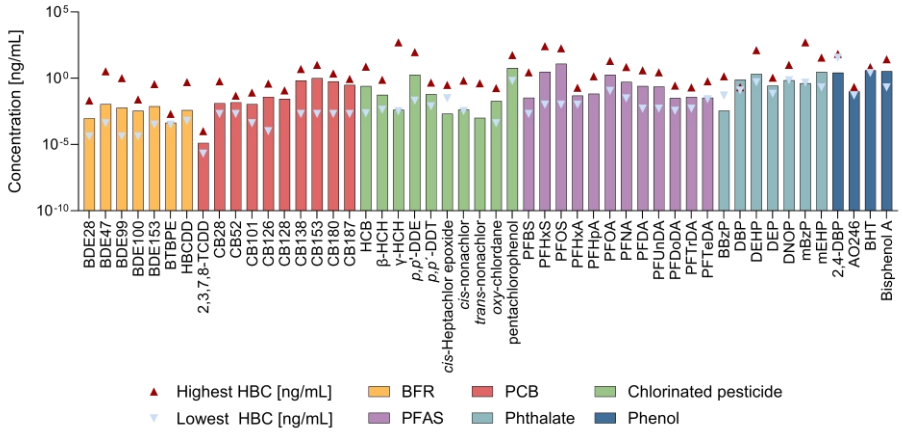


*Fig. 14.* ***Overview of the 50 components included in the mixture****. The concentrations (ng/mL) in human blood on the y-axis is in a logarithmic scale, details can be found in the supplemental materials of* ***Paper IV****. The triangles represent the lowest and highest human blood concentration (HBC) in the Swedish Exposure Database (SEDB, Paper I).*

#### 3.2.2.1 *In vitro* Assays

The chemical mixtures were tested in five cell-based *in vitro* assays in **Paper IV**. Four chemically activated luciferase expression (CALUX®) assays were utilized, namely dioxin responsive (DR-), estrogen receptor α (ERα-), AR-, and nuclear factor erythroid 2-related factor 2 (Nrf2)-CALUX, along with an adipocyte cell assay. *In vitro* assays with the ERα and AR receptors were used to evaluate the endocrine-disrupting properties of the mixtures. Binding to the aryl hydrocarbon receptor (AhR, used in DR-CALUX) is an example of a xenobiotic effect, primarily binding dioxins, polyaromatic hydrocarbons, and dioxin-like compounds [177]. Interactions lead to the upregulation of genes involved in xenobiotic metabolism, cell growth, and immune responses. Oxidative stress is when the levels of reactive oxygen species (ROS) are elevated in the cell [178]. ROS are reactive and can damage DNA, proteins, and lipids. However, ROS are essential for some mechanisms, especially in NADPH-dependent enzyme processes and the electron transport chain when ATP is formed. The oxidative balance in the cell is strictly regulated. The transcription factor Nrf2 is one mechanism against oxidative stress [179]. Since oxidative stress is an effect of unspecific toxicity, Nrf2 acts by activating detoxification pathways and antioxidant genes. An *in vitro* assay for oxidative stress using the Nrf2, Nrf2-CALUX, was tested in **Paper IV**. Some EDCs can also act as metabolism disruptors. Adipocytes are lipid-producing cells that can be affected by chemicals. These cells produce lipid droplets, which can be used to distinguish them from other cell types. This was measured microscopically to see if the adipocytes increased in numbers compared to other cell types (**Paper IV**).

#### 3.2.2.2 ZFET Test

The chemical mixtures were tested in *in vivo* to test the toxicity on an organism level using the ZFET test (**Paper V**). The data gained is more multidimensional using an organism than an *in vitro* test. Zebrafish (*Danio rerio*) is used as a model organism for its transparent embryo and quick reproductive phase. Of the human genes, 70% have orthologues in the zebrafish [180], making it a good human model, especially related to early lifetime exposure.

In the ZFET test, the embryo is exposed to a chemical or mixture of chemicals via the water in the plate, and effects are thereafter observed at specific time points (Fig. 15). After 24 hours post fertilization (hpf), early movement is measured by the number of tail coilings per minute. Tail coilings have been linked to neurotoxic effects [181]. At 48 hpf, the heart rate is monitored, and from 48-144 hpf, the hatching is checked hourly. The survival rate is checked throughout the experiment. Reduced heart rate, hatching time, and survival are considered a developmental toxicity endpoint [182]. After 6 days (144 hpf), the behavior of the surviving embryos will be checked by monitoring the

swimming distance in a shifting light and dark environment. Thyroid effects have been correlated to relative eye area [183-185], swim bladder inflation [186], and behavioral effects (reviewed by [187]). Furthermore, the embryos can be analyzed using other analytical methods to further determine more subtle effects, such as genomic, transcriptomic, proteomic, metabolomic, and lipidomic patterns.

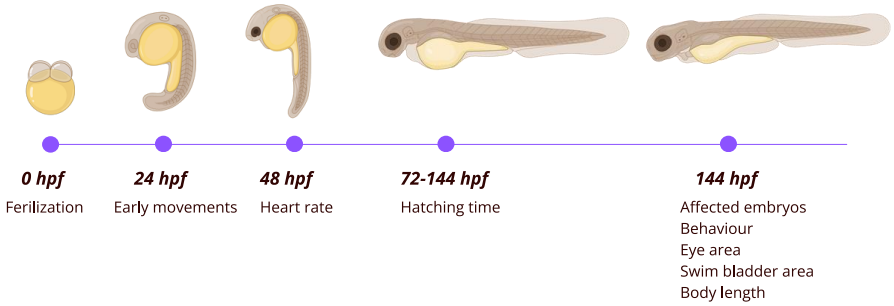


*Fig. 15.* ***An overview of the zebrafish embryo toxicity test (ZFET test)****, with the eight endpoints monitored at four time points. The exposure occurs directly after fertilization. Created with BioRender.com.*

## 3.3 Component-based MRA Methods

The risk of chemical exposure was assessed by comparing exposure levels to effect levels (**Papers I** and **II**). When comparing human chemical exposure to health-based guidance values, the HI is the most frequently used risk index. The HI is sometimes called the point of departure index when the effect level used is based on POD values. Within the scope of this thesis, both of these indices are called HI. Using HI for the MRA, the exposure and effect levels of each chemical must be available. Each chemical exposure level is then divided by the effect level (the same units are necessary). This quotient is called the hazard quotient (HQ). Thereafter, all the hazard quotients are added into a sum, HI, (Eq. 1). When the HI is 1 or higher, a risk of the effect occurring cannot be excluded [188]. HBM guidance values are usually used as the effect level for human risk assessments. These values are typically set with a certain margin of safety to account for uncertainties, either by applying assessment factors (when derived from animal studies) or using point of departure (when derived from epidemiological studies).

$$Hazard\ index\ (HI) = \sum HQ = \sum_{i=1}^{n} \frac{Blood\ level_i}{Effect\ level_i} \qquad \text{(Eq. 1)}$$

*where HQ is the hazard quotient for each component in the mixture, n is the number of components in the mixture, and i is the $i^{th}$ compound in the mixture.*

The maximum cumulative ratio (MCR) was calculated to identify the mixture contribution (**Paper II**). MCR is defined as the effect of the cumulative toxicity (the sum of the effect of all chemicals in the mixture) in relation to the most toxic chemical within the mixture ($HI_{max}$ the toxicological driver) (Eq. 2). If the MCR is 1, only the driver is causing an effect. If the MCR is close to the number of chemicals in the mixture, it would suggest that all chemicals contribute equally to the effect.

$$Maximum\ cumulative\ ratio\ (MCR) = \frac{HI}{HQ_{max}} \qquad \text{(Eq. 2)}$$

*where HI is the hazard index of the mixture, $HQ_{max}$ is the highest hazard quotient in the mixture.*

To test if a mixture follows the CA model, each mixture component is expressed as a fraction of the mixture. The component fraction is divided by the concentration of the component provoking a certain percent effect when tested individually (Eq. 3). By calculating the 5-100 % predicted effect, a predicted concentration-response curve can be plotted and compared to the observed concentration-response curve.

$$Predicted\ EC_{x,mix} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x,i}}\right)^{-1} \qquad \text{(Eq. 3)}$$

*where Predicted $EC_{x,mix}$ is the predicted concentration of the mixture causing x % effect, n is the number of mixture components, $p_i$ is the fraction of the $i^{th}$ mixture component in the mixture, $EC_{x,i}$ is the concentration of the mixture component causing x % effect when tested individually [189].*

# 4 Results and Discussion

## 4.1 Human Exposure to Chemicals

In **Paper I**, the chemical exposome was assessed by exploring the published literature for chemicals, resulting in 556 chemicals analyzed in human blood worldwide (HBDB). Of them, 440 chemicals were detected and quantified, and 166 were quantified in Swedish human blood (SEDB). The study revealed that 68 % of the chemicals detected in Swedish human blood were POPs. In **Paper I**, PFAS was identified as the chemical group of concern during the MRA. In **Papers II** and **III**, PFAS and synthetic phenolic compounds were analyzed in healthy blood donors. The number of chemicals detected in one individual in **Papers II** and **III** ranged between 17 to 30 chemicals, of the 73 targeted analytes. SPAs covered the highest molar contribution, adding to a total concentration in the µmolar range (Fig. 16).

Within the studied population, the prioritization strategy resulted in the findings of SPAs in the highest detection frequencies and high concentrations (Fig. 16). Detection frequencies and concentrations were also high for PFAS and parabens (Fig. 16). The levels of PFAS were in line with the latest reported concentrations in SEDB when comparing the results in **Papers I** and **II**. The chemical exposure pattern differed between females and males (Fig. 17). Males had significantly higher levels of ethylparaben (**Paper III**). In comparison, females had significantly higher levels of BHT and BHA, PFDA, and PFUnDA (**Papers II** and **III**). One reason for the lower levels of some substances in males could be the higher approved blood donation frequency of males (four times per year) compared to females (three times per year) [190]. Some differences in the concentration of the chemicals could also be due to user patterns, as many of these chemicals are used in personal care products.

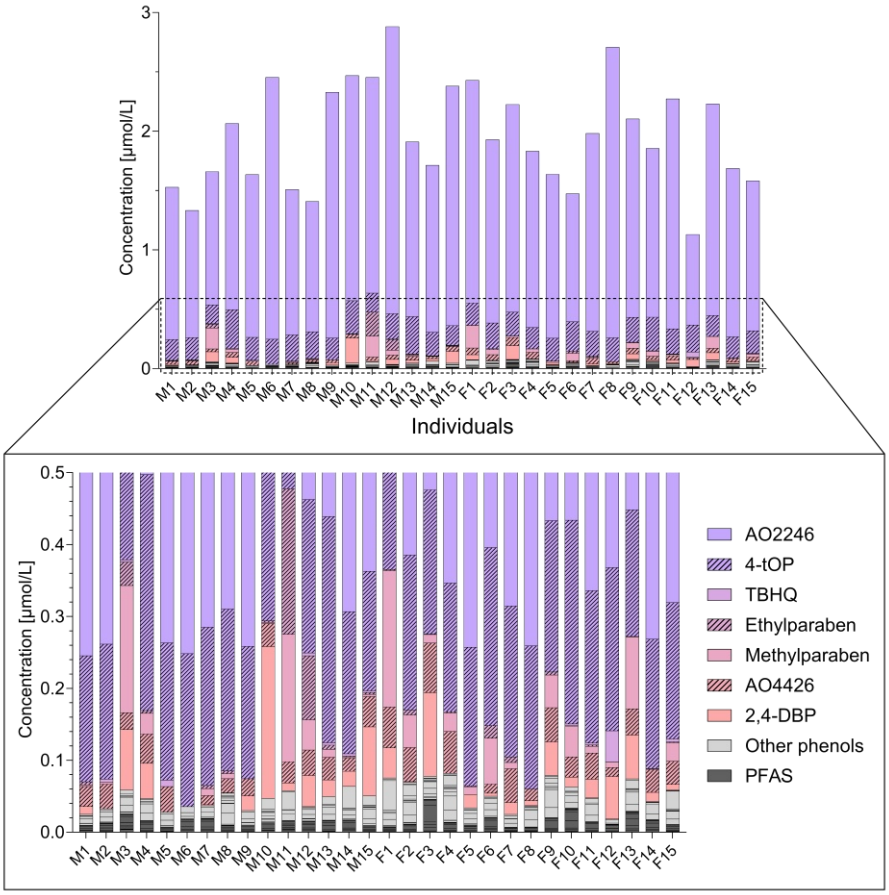


*Fig. 16. Composition of the mixture of chemicals found in human blood from Paper II and Paper III (n=30), males to the left and females to the right on the x-axis. Each compound is presented as the molar concentration, with the y-axis showing the contribution of each chemical. The lower graph shows the lower-concentration chemicals.*

As discussed in **Paper III**, AO2246, 4-tOP, and AO4426 were found in high concentrations in human blood. The analytes AO2246, 4-tOP, and 2,4-DBP were only semi-quantified as no recovery test could be done due to the unexpectedly high concentrations compared to the fortified native standard concentrations in the recovery blood samples. AO4426 could only be detected after the samples were diluted 10 times. Unknown contamination sources cannot be ruled out and should be further evaluated. Extensive measures were taken to verify that the high concentrations of SPAs were not due to background contamination (**Paper III**). The analyte 4-tOP was detected in the sampling blank, at levels 10 times below the levels in the samples. AO2246 was

not detected above the limit of quantification in any blanks or steps of the method, and the levels before and after dilution were not statistically different.

Even when using a targeted method, there is a small risk of quantifying a similar (endogenous) compound. For that reason, AO2246 and 4-tOP were identified using multiple analytical methods, such as GC-MS and LC-MS, or using two types of LC columns separating the analytes based on different properties. By doing this, the validity of the identification was strengthened (**Paper III**).

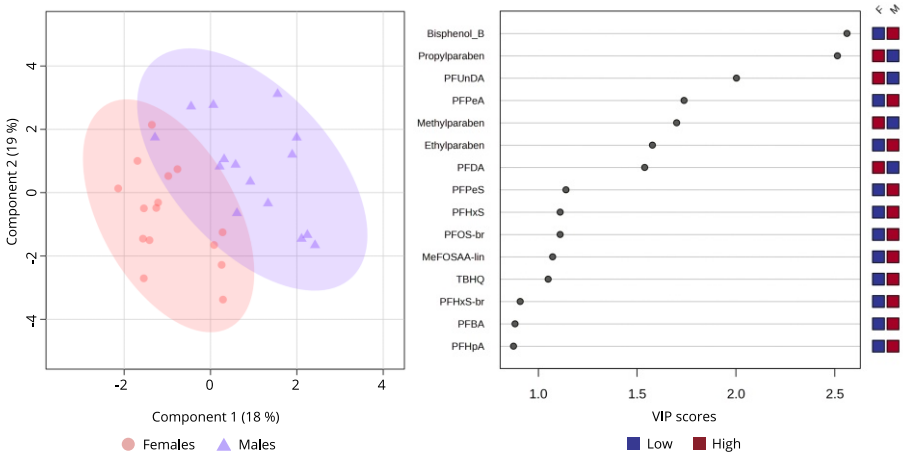


*Fig. 17. PLSDA plot of the chemical exposure patterns determined in Papers II and III, between males (purple, triangles) and females (pink, circles). The plot was created using Metaboanalyst.ca.*

In **Papers II and III**, a pooled sample of 100 individuals ($Pool_{100}$, including the individuals analyzed) was analyzed. The concentrations in the $Pool_{100}$ were similar to the mean level in the individual dataset, suggesting that the selected subset of individuals was representative of the total cohort (Fig. 18).

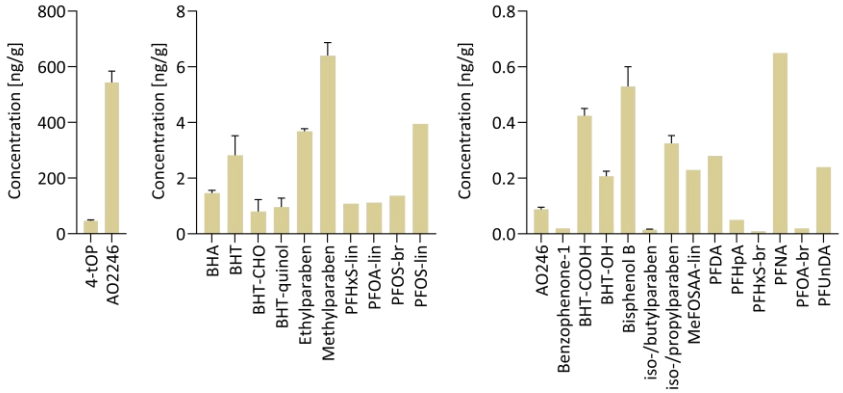


*Fig. 18.* ***Concentrations of PFAS and phenols in a pooled sample*** *of 100 individuals from Stockholm, Sweden, analyzed in Papers II and III.*

A few chemicals detected using target analysis and suspect screening have not been detected in human blood previously (Table 1). In **Paper II**, hydrogen-substituted perfluoroalkyl carboxylic acids (H-PFCAs) were identified in the studied population. In **Paper III**, three SPAs and bisphenol B were quantified.

*Table 1. Chemicals quantified for the first time in human blood within this thesis.*

| **Compound** | **Detection frequency** | **Estimated conc. range [ng/g]** | **Paper** |
|---|---|---|---|
| H-PFOA[a] | 28% | <LOD-0.037 | II |
| H-PFNA[a] | 12% | <LOD-0.031 | II |
| H-PFDA[b] | 62% | <LOD-0.078 | II |
| H-PFUnDA[a] | 48% | <LOD-0.035 | II |
| H-PFDoDA[b] | 12% | <LOD-0.025 | II |
| H-PFTrDA[b] | 10% | <LOD-0.029 | II |
| AO2246[a] | 93% | <LOD-29 | III |
| TBHQ[c] | 43% | <LOD-7.2 | III |
| Fenozan[b] | 73% | <LOD-4.7 | III |
| Bisphenol B[c] | 63% | <LOD-0.87 | III |

[a] – semi-quantified with standards
[b] – semi-quantified using a structurally similar standard
[c] – target analytes

## 4.2 Chemical Toxicity

Of the 166 chemicals found in Swedish blood, 17 % of the detected chemicals had HBM guidance values (**Paper I**). Examples of chemicals without HBM guidance values were PFAS (except for PFOS and PFOA), phthalates, bisphenols, and a few pesticides, PCBs, and flame retardants. Regarding the POPs in SEDB, it can be assumed that most of them have been evaluated for toxicity. In **Paper I**, although there was a limited number of HBM guidance values that could be used to assess the toxicological impact of the mixture, PFOS and PFOA were driving the toxicity and resulted in a HI above the risk threshold.

The PCBs and TCDD discussed in **Paper I** were below the risk threshold for harmful effects; however, the HQ was contingent on the number of PCBs evaluated. In **Paper IV**, the mixture showed an agonistic AhR activity (using DR-CALUX) at 0.1 xHBC, triggering the transcription of transformation enzymes (Table 2). By reviewing available data for the components in the PCB mixture, only TCDD and CB126 were expected to give an agonistic response in the DR-CALUX [191]. Interestingly, by comparing the relative potency factors from those studies with our values of the mixtures, the predicted activity was higher in that study compared to the observed activity for the PCB and total mixtures in **Paper IV**. It can be hypothesized that antagonistic activities of other compounds were affecting the observed activity. Non-dioxin-like PCBs, such as CB138, CB153, and CB180, found at relatively high concentrations in humans, are antagonists in the DR-CALUX assay [191]. The total mixture was statistically significantly less potent than the PCB mixture, which could be explained by the presence of BDE47, a known antagonist in the same assay [191]. No effects from the PCB mixtures were observed for the endpoints in the zebrafish embryos studied in **Paper V**.

The chlorinated pesticides in human blood were not a cause for concern using the HBM guidance values and levels reported in SEDB (**Paper I,** Table 2). The levels from SEDB were used to make the pesticide mixture, evaluated in **Papers IV** and **V**. The mixture showed antagonistic activity at 50 xHBC to the AR. However, in **Paper V**, the concentration-dependent effects of the mixture were decreased heart rate (10 xHBC) and mortality (100 xHBC) in zebrafish. As discussed in **Paper V**, pentachlorophenol, present at high concentrations in the pesticide mixture, has shown similar effects previously [192]. Decreased swim bladder area, linked to thyroid hormone disruption [193] (0.01 xHBC) and behavioral effects (1 xHBC), did not follow a concentration-dependent correlation (referred to as a non-monic concentration-response correlation). Non-monotonic concentration-response relationships have been observed previously, especially for endocrine-disrupting effects [7, 194, 195].

BFRs were not a cause for concern when assessed in **Papers I** and **IV** (Table 2). In **Paper V**, some non-monotonic correlations could be observed at 10 xHBC. The effects observed in **Paper V** (swim bladder area and body length) could potentially be linked to endocrine disruption, but further studies would be needed to determine the validity of this observation.

The exposure to PFAS was evaluated in four of the five papers in the thesis (Table 2). In **Paper I**, the concentrations of PFOS and PFOA caused the HI to exceed the risk threshold of 1. For this reason, PFAS exposure was investigated further in **Paper II**, where the levels indicated risk for adverse health effects related to developmental toxicity, immunosuppression, thyroid disruption, and increased serum cholesterol. At 10 xHBC, the mixture caused a reduced eye area of the zebrafish (**Paper V**), indicating thyroid effects. It was also seen as a potential effect of concern in **Paper II**, with almost all individuals above the risk threshold for increased free thyroid hormones. In **Papers IV** and **V**, the PFAS mixture caused oxidative stress, increased cell permeability of agonistic chemicals, and reduced swimming distance at concentrations above human relevance (>100 xHBC).

*Table 2.* ***Overview of the observed effects from Papers I, II, IV and V.*** *Average concentrations above the risk threshold (Papers I and II) and up to 15 times human blood concentrations (Papers IV and V) are indicated red. Other tested endpoints without an observed effect (marked green) or with an observed effect above 15 times human blood concentrations (marked orange).*

| Mixture | Assessment method | Reproductive | Development | Estrogen | Androgen | Thyroid | Immune system | Neurological | Xenobiotic | Liver | Lipid dysfunction | Oxidative stress | Assessed in paper |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PCBs | HBM-GV | | | | | | | | | | | | I |
| | *In vitro* | | | | | | | | | | | | IV |
| | *In vivo* | | | | | | | | | | | | V |
| Pesticides | HBM-GV | | | | | | | | | | | | I |
| | *In vitro* | | | | | | | | | | | | IV |
| | *In vivo* | | | | | | | | | | | | V |
| BFR | HBM-GV | | | | | | | | | | | | I |
| | *In vitro* | | | | | | | | | | | | IV |
| | *In vivo* | | | | | | | | | | | | V |
| PFAS | HBM-GV | | | | | | | | | | | | I |
| | Epi. data | | | | | | | | | | | | II |
| | *In vitro* | | | | | | | | | | | | IV |
| | *In vivo* | | | | | | | | | | | | V |
| Phthalates | *In vitro* | | | | | | | | | | | | IV |
| | *In vivo* | | | | | | | | | | | | V |
| Phenols | *In silico* | | | | | | | | | | | | [117] |
| | *In vitro* | | | | | | | | | | | | IV |
| | *In vivo* | | | | | | | | | | | | V |
| **Total tested mixture** | **HBM-GV** | | | | | | | | | | | | **I** |
| | ***In silico*** | | | | | | | | | | | | **[117]** |
| | ***In vitro*** | | | | | | | | | | | | **IV** |
| | ***In vivo*** | | | | | | | | | | | | **V** |

*HBM-GV: human biomonitoring guidance values*

In **Paper II** the exposure levels of PFAS were related to toxic endpoints in multiple MRAs. Within **Paper II**, five MRAs used newly derived guidance values based on epidemiological data (Fig. 19). Deriving effect levels from epidemiological data, the total chemical mixture and biological diversity are included in the association between an effect and the PFAS concentration. This is the only approach when human subjects are used to study concentration-response effects, as experiments with exposure to these substances in humans are, for many reasons, crossing ethical borders. PFAS are widespread in the environment and among humans [34]. Many PFAS are co-occurring, and identifying if more than one PFAS is causing the effect in humans can be difficult. Suppose PFOS is the toxicological driver for a specific effect; studying other lower-concentration PFAS might also reveal a significant concentration-response association influenced by the co-occurring PFOS. This, in turn, leads to a lower effect level compared to PFOS, regardless of the  toxicity of tha PFAS. This example underscores the necessity of a comprehensive weight-of-evidence approach to pinpoint the underlaying MoA. This effect was likely observed in **Paper II** but was not investigated further (Fig. 19). Most research has been focused on the most abundant PFAS congeners, PFOS and PFOA, but the body of evidence related to the toxicity of other PFAS is growing (**Paper II**) [35].

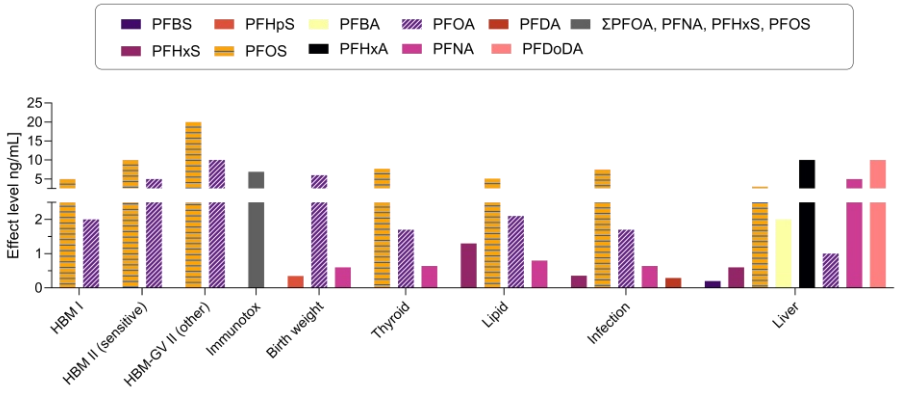


*Fig. 19* ***Overview of nine effect levels*** *used for the mixture risk assessments in Paper II. Further details on the derivation process is available within the paper.*

The phthalate mixture did not trigger any effects in human-relevant concentrations in the assays or tests applied in **Papers IV** and **V**. However, non-monotonic correlations were observed for a reduced eye area linked to thyroid disruption (**Paper V**) [196].

The phenol mixture resulted in effects from 10 xHBC depending on the endpoint studied (Table 2). Within the RiskMix project, QSAR modeling of the mixture components was done to predict thyroid disruption [114]. The models were also applied to the HBDB. The bisphenols, parabens, UV filters, and SPAs were identified as potential thyroid disruptors [114]. The four phenols in the mixture (BHT, AO246, and 2,4-DBP, and bisphenol A) were predicted to bind to 15 of the 19 receptors/proteins tested (Table 3). However, the mixture did not trigger thyroid-disrupting-related endpoints *in vivo* (**Paper V**). The heart rate decreased at 100 xHBC. In **Paper IV**, ERα induction was observed at 10 xHBC, AR antagonistic induction at 25 xHBC, and oxidative stress induction at 1000 xHBC.

*Table 3.* ***In silico thyroid-related protein binding of chemical in the phenol mixture****. The predictions were performed by Lena Golosovskaia, using in silico models in [114] with a confidence coefficient of 0.9. Blank cells indicate that the chemical binding could not be predicted at the set confidence level.*

| **Compound** | **BPA** | **2,4-DBP** | **BHT** | **AO 246** |
|---|---|---|---|---|
| TRHR antagonist | inactive | active | active | active |
| TSHR agonist | inactive | active | active | active |
| TSHR antagonist | inactive | inactive | | |
| DIO1 | inactive | active | | |
| DIO2 | inactive | active | | |
| DIO3 | inactive | active | active | active |
| NIS | active | | | |
| TPO | active | active | active | active |
| TTR | inactive | inactive | inactive | inactive |
| TRβ agonist | inactive | | | active |
| TRβ antagonist | active | | | active |
| AHR agonist | | inactive | inactive | inactive |
| CAR agonist | active | | active | active |
| CAR antagonist | active | active | | active |
| PPARδ agonist | inactive | | | |
| PPARδ antagonist | | | | |
| PPARγ agonist | | | | active |
| PPARγ antagonist | active | inactive | | |
| PXR agonist | active | | | active |

For the total chemical exposure assessed in this thesis, various effects were seen at human-relevant concentrations. In **Paper I**, the risk threshold was exceeded based on the total known POP exposure in SEDB and PFAS was identified as the driver of the toxicity. Most of the chemicals in the mixture were predicted to bind to thyroid-related receptors or proteins [114]. In **Paper IV**, the total mixture resulted in effects in human-relevant concentrations at 10

xHBC (Erα-CALUX with the phenol mixture as toxicological driver), 15 xHBC (antagonistic AR-CALUX with the phenol, PCB, and pesticide mixtures as toxicological drivers), 0.12 xHBC (DR-CALUX with the PCB mixture as toxicological driver). Adipogenic effects (cells differentiating to more adipocytes than other cells) were observed at levels above human relevance (100 xHBC). No oxidative stress was observed at the tested concentrations (up to 1000 xHBC). In **Paper V**, the total mixture caused a decrease in body length and hatching time at 15 xHBC, mortality and decreased heart rate at 22 xHBC, and reduced tail-coilings at 40 xHBC. The effects were seen earlier in the total mixture compared to the subgroup mixtures, which could indicate an additive effect from the subgroup mixtures through a phenomenon called "Something from nothing" [147].

Already in 1962 Rachel Carson wrote

> "We are accustomed to look for the gross and immediate effect and to ignore all else. Unless this appears promptly and in such obvious form that it cannot be ignored, we deny the existence of hazard." [27]

More than 60 years have passed, and legislation still fails to cover chemical mixtures. Although the conclusions that can be drawn from this thesis are tentative, the growing body of evidence that the mixture of environmental pollutants is disruptive to human development warrants action. For example, a correlation between EDCs and a lowering of IQ has been observed in 7-year-olds [95]. A shift in IQ by 2 points might not be detrimental to a normal functioning individual, but on a population level, it entails more people needing assistance and, thus, an increased societal cost. Chemical exposure is especially concerning for sensitive populations. Exposure to EDCs affects mothers, children, and families, placing a significant demand on healthcare providers and, thus, burdening society [197].

# 5 Concluding Remarks and Future Perspectives

In this thesis, the mixture assessment strategies to combine the same MoA (**Paper IV**), the same pathways (**Paper II**), and different pathways (**Papers I** and **V**) have been tested. In all methods used to assess the risk associated with the chemical mixture, at least one endpoint related to PFAS exposure was above the risk threshold in **Papers I** and **II**, and effects from the total mixture were observed at human-relevant concentrations in **Papers IV** and **V**.

It has been argued that the safety margins included in the HBM guidance values used in **Papers I** and **II** are insufficient to compensate for the effects of co-exposure to other chemicals [137]. When combining the exposure assessment from **Papers II** and **III**, the number of target PFAS and synthetic phenolic compounds determined in one individual reached up to 30. **Papers II-III** add 25 new blood contaminants in the Swedish population to the 166 contaminants listed in SEDB (**Paper I**). Consequently, 191 contaminants have been reported quantified in the blood of the general Swedish population. This aligns with a study detecting over 200 chemicals in one newborn [198].

Depending on the half-life of a chemical in the human body, some accumulate and are difficult to reduce after exposure. Chemicals with low persistence (such as phenols and phthalates) could be reduced by limiting the exposure [199]. Still, persistent chemicals (such as POPs) will take a long time to decrease in humans and will transfer to future generations during pregnancy and breastfeeding, even after being banned. Grouping structurally similar substances could avoid regrettable substitution, such as in the case of bisphenols and PFAS. To prevent exposure to hazardous chemicals in a population, regulating a chemical class within which there are one or more substances of concern could be done through grouping. Thus, until a chemical within that class has been proven safe, the chemical class should be regulated.

However, high exposure to a chemical without information about its hazards can still be damaging. In the case of SPAs, the high blood exposure warrants action. The precautionary principle should be applied to reduce exposure even if the hazard is low or unknown. This aligns with the environmental objectives system of the Swedish Government, which aims for a non-toxic environment,

with one cornerstone focused on reducing total chemical exposure regardless of hazardous properties [200].

To extend the chemical exposure assessment, the method used in **Paper III** could be applied to a different cohort and with an increased sample size. Ideally, the sampling should occur in a strictly controlled environment, avoiding the use of plastic equipment to prevent potential contamination. To further expand the knowledge of these chemicals, it would be interesting to include additional health biomarkers and records of diseases to understand potential health effects and user patterns to identify sources of exposure.

The results from the exposure to the artificial chemical mixture used in **Papers IV** and **V** showed adverse effects related to general developmental toxicity and endocrine disruption at human-relevant concentrations. Even if there are differences between a test system (*in vitro* and *in vivo*) and humans, *e.g.*, in regards to exposure pathway and ADME, observing an effect could still cause concern as the mechanistic effect was possible. By including 50 chemicals, almost twice as many as previously tested in other studies from various chemical classes, the test concentrations were closer to reflecting the "true" chemical exposure in human blood.Within the scope of this thesis, organic contaminants were evaluated for exposure and toxic effects. Nevertheless, many studies have shown that metals, such as cadmium and arsenic, are co-contaminants that could also cause toxic effects on the same endpoints [15]. Consequently, combinations of both organic and inorganic chemicals should be tested in complex mixtures for toxic effects. Hypothetically, combining 200 chemicals or more to test in *in vitro* and *in vivo* test systems might have resulted in observed effects at 1 xHBC. The observed effects from the mixture underscore the importance of testing realistic chemical mixtures to assess the effects in more accurate exposure scenarios.

In **Paper V**, a one-time exposure of the zebrafish embryo is used. In reality, we are exposed to chemicals throughout life. Furthermore, the human chemical exposome varies regarding the number of chemicals and their levels. Using the ZFET test, the total mixture showed effects earlier than observed from the subgroup mixtures, highlighting the importance of assessing complex mixtures in toxicological test systems. Because of the homology between the human and zebrafish genomes, effects can be studied to gain insight into potential human health effects. However, extrapolation is complex when no biochemical effect analysis is included in the assessment. Zebrafish embryos are especially useful for early lifetime exposure studies as exposure susceptibility is critical at developing life stages, such as in the womb, childhood, adolescence, and pregnancy. The uncertainties involved in MRAs, such as extrapolation between species, are something we have to accept, but where do we draw the line between risk and benefit? Do the effects need to be visible and

proven in humans before regulations are implemented, or should we trust the data from the collected evidence showing effects in a model organism? The toxicity of chemical exposure can be subtle and might only show toxicity after chronic exposure.

It would be valuable to further use of the zebrafish embryo as a model organism for human-related effects by shifting the focus towards evaluating more subtle health effects in zebrafish embryos at even lower concentrations of the chemical mixture. The study could be designed to reflect early-lifetime exposure and study late-onset toxicity in adult zebrafish. Including the newly detected chemicals discussed in this thesis is also motivated. The embryos could be analyzed using omics techniques, such as proteomics or lipidomics, to gain insights into biochemical health effects, making extrapolations between animals and humans easier. Furthermore, given the complexity of the immune system, similar to that of the endocrine system, it would be interesting to include biomarkers for immune-disrupting effects in the analysis to broaden the scope of the assessment.

When considering chemical exposure safety, the benefits of the use of chemicals must outweigh the risks. This is evaluated for prescription medicine on the market, but the risk-benefit analysis for human health is more complex to perform for environmental contaminants. The complexity arises from the difficulty of determining the benefits, and the potential adverse effects may not become apparent until 20 years after the exposure occurred [7].

Even if synthetic organic contaminants have been found at high concentrations in humans, most are not persistent, meaning decreasing the exposure leads to lower concentrations in blood [199]. Even though companies and regulators are responsible for preventing ongoing exposure to environmental contaminants, individuals can still reduce their exposure, at least in part, by making informed decisions. Avoiding these chemicals by decreasing the consumption of food contact plastic materials, canned food, fast food and fashion, bottled milk and pre-made purees for children, preventing dust from accumulating at home, and buying organic food when possible decreases the exposure to EDCs [199]. Notably, several sources of exposure to EDCs are beyond individual control [199, 201, 202]. However, making informed decisions to lower the exposure to these contaminants should not be the responsibility of an individual. Changing habits, making informed decisions, and choosing which products you buy is not a given for most individuals in most parts of the world and requires a deep understanding of toxicological studies. Instead, the responsibility to regulate and reduce human exposure to environmental pollutants lies with the governmental agencies and policymakers, an act vital to society.

# Additional Publications

Palm E, Engelhardt AJ, Tshepelevitsh S, Weiss JM, Kruve A. 2024. Gas Phase Reactivity of Isomeric Hydroxylated Polychlorinated Biphenyls. *JASMS*, https://doi.org/10.1021/jasms.4c00035

Dracheva E, Norinder U, Rydén P, Engelhardt J, Weiss JM, Andersson PL. 2022. In silico identification of potential thyroid hormone system disruptors among chemicals in human serum and chemicals with high exposure index. *Environ. Sci. Technol*. 56:12,8363-8372. https://doi.org/10.1021/acs.est.1c07762

Khabazbashi S, Engelhardt J, Möckel C, Weiss J, Kruve A. 2022. Estimation of the concentrations of hydroxylated polychlorinated biphenyls in human serum using ionization efficiency prediction for electrospray. *Analytical and Bioanalytical Chemistry*, 414, 7451-7460. https://doi.org/10.1007/s00216-022-04096-2

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