Effects of mixtures of endocrine disrupting chemicals

Thyroid disruption and behavioural effects in fish models

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EFFECTS OF MIXTURES OF ENDOCRINE DISRUPTING CHEMICALS – THYROID DISRUPTION AND BEHAVIOURAL EFFECTS IN FISH MODELS

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Dissertation abstract

Wildlife and humans are continuously exposed to thousands of man-made compounds, including chemicals that are able to act as endocrine disruptors (EDCs). These pollutants are able to affect vital processes including brain development, reproduction, metabolism and growth. An organism can be especially sensitive to these chemicals if the exposure occurs during early developmental stages, at so-called "windows of exposure" when organs that rely on endocrine regulation are still being developed. EDCs and other pollutants are present in the environment as complex mixtures, which can be difficult to handle from a risk assessment point of view. It is therefore important to evaluate effects of EDC mixtures after exposure during early development. The majority of the work in the current thesis was done by exposure studies using zebrafish. These studies take place within the EDC-MixRisk project, where an interdisciplinary whole mixture approach is used to assess effects of human-relevant EDC mixtures. This thesis is focused on three of the mixtures designed and produced within the EDC-MixRisk project, based on chemicals measured in serum of pregnant women and associated with adverse effects on neurodevelopment (MIX N0) or negatively associated with birth weight (MIX G0 and MIX G1) in their children.

Effects on behaviour and expression of genes related to the thyroid system were assessed in larval zebrafish after acute (48h) exposures using automatic locomotion tracking and qPCR. MIX N0, MIX G0 and MIX G1 were all found to significantly affect the locomotion of larval zebrafish at concentrations 100 times higher than the mean serum concentration measured in pregnant women (100X). Effects on thyroid receptor expression (*thra* and *thrb*) and deiodinases (*dio1* and *dio2*) were also observed for MIX N0 and MIX G1 at this concentration. For MIX G0, effects on gene expression (*thra, thrb* and *dio2*) were found already at 0.01X–1X concentrations (i.e., up to 100 times lower than the mean concentrations measured in women).

Next, we compared the two mixtures (MIX G0 and MIX G1) linked to adverse effect on growth by measuring locomotion over a longer period of time and found that the more complex MIX G1, which had the same total concentration as MIX G0 but consisted of more compounds, had an attenuated effect compared to MIX G0 immediately after exposure. However, when locomotion was measured one month later fish were still affected and moving less than compared to controls (hypoactive distance travelled) after MIX G1 exposure while MIX G0 no longer had an effect.

We also assessed the impact of environmental enrichment (EE) on exposure to the EDC mixtures. Our results showed that the rearing environment can affect the outcome of behavioural assays later in life for zebrafish acutely exposed to EDC mixtures. Additionally, adult fish reared in a barren or enriched environment and thereafter exposed to EDC mixtures can respond differently in a behaviour assay.

The final study included in this thesis was a field study of wild perch from sites in Sweden contaminated with known EDCs. We found that lifelong exposure to PFASs (one of the chemical classes present in the EDC mixtures described above) in a contaminated lake can affect both the thyroid system and immune defence in wild perch.

Keywords: Endocrine disrupting chemicals, ecotoxicology, zebrafish, perch, locomotion, behaviour, thyroid disruption, EDC mixtures

Svensk sammanfattning

Djur och människor exponeras kontinuerligt för tusentals syntetiska ämnen, inklusive kemikalier som kan störa kroppens endokrina system s.k. endokrinstörande ämnen (EDCs). Dessa föroreningar kan påverka vitala processer inklusive hjärnans utveckling, reproduktion, metabolism och tillväxt. Organismer är särskilt känsliga för dessa kemikalier om exponeringen sker under tidiga utvecklingsstadier, vid kritiska "exponeringsfönster" när organ som är beroende av endokrin reglering fortfarande utvecklas. EDCs och andra föroreningar finns i miljön som komplexa blandningar, vilket kan vara svårt att hantera ur riskbedömnings-synpunkt. Det är därför viktigt att utvärdera effekterna av EDC-blandningar efter exponering under tidig utveckling. Huvuddelen av arbetet i denna avhandling gjordes genom exponeringsstudier med zebrafisk. Dessa studier genomfördes inom ramen för EDC-MixRisk-projektet, där ett tvärvetenskapligt tillvägagångssätt och en “whole mixture approach” användes för att bedöma effekter av human-relevanta EDC-blandningar. Arbetet fokuserar på tre blandningar designade och producerade inom EDC-MixRisk. Blandningarna bestod av kemikalier som uppmätts i serum från gravida kvinnor och som associerats med negativa effekter på neuroutveckling (MIX N0) eller födelsevikt (MIX G0 och MIX G1) i kvinnornas barn.

Effekter på beteende och genuttryck relaterat till sköldkörtelsystemet analyserades hos zebrafiskyngel efter akuta exponeringar med hjälp av automatisk spårning av lokomotion samt qPCR. MIX N0, MIX G0 och MIX G1 visade sig alla ha signifikanta effekter på lokomotion i zebrafiskar vid koncentrationer 100 gånger högre än den genomsnittliga serumkoncentrationen uppmätt hos gravida kvinnor (100X). Genuttrycket av sköldkörtelhormonreceptorer (*thra* och *thrb*) och deiodinaser (*dio1* och *dio2*) påverkades också vid exponering för MIX N0 och MIX G1 vid denna koncentration. För MIX G0 noterades effekter på genuttryck (*thra, thrb* och *dio2*) redan vid 0,01X–1X (dvs upp till 100 gånger lägre än medelkoncentrationerna uppmätta i gravida kvinnor).

Därefter jämförde vi de två blandningarna (MIX G0 och MIX G1) kopplade till tillväxt genom att mäta lokomotion över en längre tidsperiod och fann att den mer komplexa MIX G1, som hade samma totala koncentration som MIX G0 men bestod av fler kemikalier, hade en lägre effekt jämfört med MIX G0 omedelbart efter exponering. När lokomotion mättes en månad senare var de MIX G1-exponerade fiskarna däremot fortfarande påverkade och rörde sig mindre än kontrollgruppen medan MIX G0 inte längre hade någon effekt.

Vi undersökte också effekterna av miljöberikning på exponering av EDC-blandningar. Våra resultat visade att miljöberikning kan påverka resultatet av beteendeanalyser senare i livet för zebrafiskar som exponeras akut för EDC-blandningar. Dessutom kan vuxen zebrafisk reagera annorlunda i en beteendeanalys efter exponering för EDC-blandningar beroende på om deras miljö under uppväxten är berikad eller inte.

Den sista studien som inkluderades i denna avhandling var en fältstudie gjord på vild abborre från platser i Sverige kontaminerade med kända EDCs. Vi fann att livslång exponering för PFASs (en av de kemikaliegrupper som finns i EDC-blandningarna som beskrivs ovan) i en förorenad sjö kan påverka både sköldkörtelsystemet och immunförsvaret hos vild abborre.

List of abbreviations

2-OH-PH:	2-hydroxyphenanthrene
3-PBA:	3-phenoxybenzoi acid
actβ:	Beta-Actin
AGD:	Anogenital distance
BPA:	Bisphenol A
DDT:	Dichlorodiphenyltrichloroethane
DEHP:	Di-(2-Ethylhexyl) Phthalate
dio1:	Iodothyronine deiodinase type 1
dio2:	Iodothyronine deiodinase type 2
dio3:	Iodothyronine deiodinase type 3
DMSO:	Dimethyl sulfoxide
DPP:	Dipentyl phthalate
EATS:	estrogen, androgen, thyroid, and steroidogenesis' modalities
EDC:	Endocrine Disrupting Chemical
gapdh:	Glyceraldehyde-3-phosphate dehydrogenase
HCB:	Hexachlorobenzene
MBP:	Mono-butyl Phthalate
MBzP:	Mono-benzyl phthalate
MEP:	Mono-ethyl phthalate
MEHP:	Mono-(2-Ethylhexyl) Phthalate
MINCH:	1,2-Cyclohexane dicarboxylic acid diisononyl ester
MINP:	Mono-isononyl phthalate
PCBs:	polychlorinated biphenyls
PFASs:	Polyfluorinated alkyl substances
PFHxS:	Perfluorohexane sulfonate
PFNA:	Perfluorononanoic acid
PFOA:	Perfluorooctanoic Acid
PFOS:	Perfluorooctane Sulfonate
p,p'DDE:	p,p'-dichlorodiphenyldichloroethylene
qPCR	Quantitative real-time polymerase chain reaction
RIA:	Radioimmunoassay
RNA:	Ribonucleic acid
rplp0:	Ribosomal protein, large, P0
SELMA:	Swedish Environmental Longitudinal, Mother and child, Asthma and allergy
SMACH:	Similar mixture approach
SMRI:	Similar mixture risk indicator
T3:	Triiodothyronine
T4:	Thyroxine
TCS:	Triclosan
TH:	Thyroid Hormone
thra:	Thyroid Hormone receptor alpha
thrb:	Thyroid Hormone receptor beta
TSH:	Thyroid-Stimulating hormone
VTG:	Vitellogenin
WQS:	Weighted Quantile Sum
XETA:	*Xenopus* Embryonic Thyroid Assay
x hsc:	Times human serum concentration

List of papers

This doctoral thesis is based on the following publications/manuscripts, which are referred to in the text by their Roman numerals:

Paper I Caporale, N[§]., Leemans, M[§]., **Birgersson, L**[§]., Germain, PL[§]., Cheroni, C[§]., Borbély, G., Engdahl, E., Lindh, C., Bardini Bressan, R., Cavallo, F., Chorev, N.E., D'Agostino, G.A., Pollard, S.M., Rigoli, M.T., Tenderini, E., Lopez Tobon, A., Trattaro, S., Troglio, F., Zanella, M., Bergman, Å., Damdimopoulou, P., Jönsson, M., Kiess, W., Kitraki, E., Kiviranta, H., Nånberg, E., Öberg, M., Rantakkoko, P., Rudén, C., Söder, O., Bornehag, CG[#]., Demeneix, B[#]., Fini, JB[#]., Gennings, C[#]., Rüegg, J[#]., Sturve, J[#]., and Testa, G[#]. (2022). *Accepted for publication in Science.* From cohorts to molecules: adverse impacts of endocrine disrupting mixtures.

[§] co-first authors
[#] senior authors

Paper II **Birgersson, L**. & Sturve, J. *Manuscript*. Early exposure to human-relevant mixtures of endocrine disrupting chemicals affects thyroid related mRNA expression and behaviour in zebrafish larvae.

Paper III **Birgersson, L**., Odenlund, S., & Sturve, J. *Manuscript*. Effects of environmental enrichment on exposure to human-relevant mixtures of endocrine disrupting chemicals in zebrafish.

Paper IV **Birgersson, L**., Jouve, J., Jönsson, E., Asker, N., Andreasson, F., Golovko, O., Ahrens, L., & Sturve, J. (2021). Thyroid function and immune status in perch (*Perca fluviatilis*) from lakes contaminated with PFASs or PCBs. *Ecotoxicology and Environmental Safety, 222*, 112495. https://doi.org/10.1016/j.ecoenv.2021.112495

Additional papers

The following studies were performed during the course of the doctoral studies but are not included in the thesis:

- Asnicar, D., Ašmonaitė, G., **Birgersson, L**., Kvarnemo, C., Svensson, O., & Sturve, J. (2018). Sand Goby—An Ecologically Relevant Species for Behavioural Ecotoxicology. *Fishes, 3*(1), 13.

- Goodrich, H. R., Bayley, M., **Birgersson, L**., Davison, W. G., Johannsson, O. E., Kim, A. B., Le My, P., Tinh, T.H., Thanh, P.N., Do Thi Thanh, H., & Wood, C. M. (2020). Understanding the gastrointestinal physiology and responses to feeding in air-breathing Anabantiform fishes. *Journal of fish biology, 96*(4), 986-1003.

- Carney Almroth, B., Cartine, J., Jönander, C., Karlsson, M., Langlois, J., Lindström, M., Lundin, J., Melander, N., Pesqueda, A., Rahmqvist, I., Renaux, J., Roos, J., Spilsbury, F., Svalin, J., Vestlund, H., Zhao, L., Asker, N., Ašmonaitė, G., **Birgersson, L**., Boloori, T., Book, F., Lammel, F., & Sturve, J. (2021). Assessing the effects of textile leachates in fish using multiple testing methods: From gene expression to behavior. *Ecotoxicology and Environmental Safety, 207*, 111523.

- Carney Almroth, B., Asker, N., Ašmonaitė, G., **Birgersson, L**., Book, F., Lammel, T., & Sturve, J. (2021). Teaching practices in science education related to chemical usage, their hazards and risks. *Integrated Environmental Assessment and Management, 17*(2), 482-483.

Table of contents

1. Introduction

1.1. Environmental contaminants

Humans and wildlife are continuously being exposed to large numbers of anthropogenic (man-made) chemicals present in the environment. These compounds can be found in water, air, food and consumer products worldwide and belong to diverse chemical classes with widespread use and different exposure routes. The chemicals have been designed to serve a variety of different functions, and they originate from a range of different sources. It is difficult to estimate exactly how many chemicals are currently present on the market as data is not accessible in all places but there is an estimated number between 75 000 to 140 000 on the market in Europe and the US. Toxicity data, as well as information about persistence and bioaccumulation is only known for a fraction of these chemicals (Johnson *et al*., 2020). Furthermore, laboratory studies are normally performed using single chemical exposures while chemicals are normally present as complex mixtures of varying composition and concentration in the environment.

1.2. Endocrine disruption - background and definition

A subset of the environmental contaminants are known or suspected to be endocrine disrupting chemicals (EDCs), and can affect the health and development of humans and wildlife (Zoeller *et al*., 2012; Bergman *et al*., 2013; Gore *et al*., 2015). An EDC is defined as "*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub) populations*" (IPCS, 2002). The endocrine system is a complex regulatory system where hormones are secreted by glands and organs and act as chemical messengers. Functioning endocrine systems are required for regulation of vital processes such as development, growth, metabolism, and reproduction. Disruption can occur through several different modes of action, either *directly* by interacting with the hormone receptors by blocking or mimicking hormones, or *indirectly* by e.g., affecting the synthesis or rate of metabolism of a hormone (Tabb & Blumberg, 2006). Disruption can have effects on for instance growth, reproduction, neurodevelopment, and immune function and has been linked to the increasing occurrence of endocrine related diseases in humans (Bergman *et al.,* 2013).

Nearly 1000 chemicals are recognised as known or potential EDCs. However, considering the large numbers of chemicals on the market and the

fact that many of these have not been tested for endocrine disruptive properties, it is likely that there are more potential EDCs than what has been shown experimentally thus far (Bergman *et al*., 2013). The concentrations of EDCs are higher in urban areas, but these compounds are found worldwide and can be detected even at remote locations (Ahrens *et al*., 2010; Weber and Goerke, 2003). EDCs belong to many different chemical classes and include pesticides, perfluorinated compounds, flame retardants, metals, dioxins and plasticizers, food packaging materials, and personal care products (Schug *et al*., 2016; Godfray *et al*., 2019; Gore *et al*., 2015).

Some EDCs are persistent compounds with the ability to bioaccumulate, causing them to remain a problem for decades after being banned from production (Boas *et al*., 2012). Other EDCs do not persist long-term *in vivo* (including Bisphenol A (BPA) and phthalates) but the continuous exposure to low levels of these chemicals leads to internal steady-state concentrations of them in humans and animals (Diamanti-Kandarakis *et al*., 2009).

Adverse effects of EDCs on reproduction in wildlife exposed to EDCs have been in focus since the publication of *Silent Spring* (Carson, 1962). Reproductive endpoints are generally sensitive and relatively easy to quantify with established methods. As a result, a large number of papers focusing on reproductive effects of EDCs in wildlife have been published over the last decades (Gore *et al.*, 2018). However, the endocrine system consists of many other parts besides the gonads and sex hormones. Disruption of other endocrine modalities have also been explored in the recent decade but the estrogens, androgens, thyroid and steroidogenesis related pathways (commonly referred to as the EATS-modalities) are still the focus of most published literature (Kucheryavenko *et al*., 2020; Martyniuk *et al*., 2022).

1.3. Endocrine disruption - key concerns

Currently the field of EDC research is well explored for some compounds, particularly in mammals, but there are still a number of uncertainties and an urgent need for further studies. Three key issues regarding EDC exposures include the knowledge gaps concerning low dose effects, mixture effects and exposures during sensitive periods during development (Bornehag *et al.,* 2012; Bornehag, & Gennings, 2018).

1.3.1. Low dose effects and non-monotonic dose response curves

EDCs differ from “traditional” toxicants as they do not always follow the typical dose-response curves and can have more subtle effects. Traditional toxicology assumes that the dose makes the poison (based on Paracelsus’s declaration), with greater response at a higher concentration of a chemical. In contrast, EDCs can instead have non-monotonic dose response curves

(reviewed by Vandenberg *et al*., 2012) including biphasic curves, or "U" or inverted "U"-shapes with different effects at low and high concentrations. Effects of a chemical exposure at low doses may not be predictable based on previous studies and tests performed with higher concentrations (Vandenberg *et al*., 2012; Zoeller *et al*., 2012; Lagarde *et al*., 2015). In addition, effects might not be detected with established test methods as these may not be sensitive enough to capture all detrimental effects.

1.3.2. Developmental exposure and late effects

As mentioned, wildlife and humans are continuously exposed to chemicals. This occurs throughout their entire life spans, although an individual's exposure is likely to vary in concentration and chemical composition over time depending on factors such as location, season and temperature. Exposures to EDCs can be particularly detrimental if they occur during early developmental stages, at so-called "windows of exposure". As hormones are involved in regulation of foetal growth and development, disruption at this stage can permanently affect the development of tissues and organs and cause irreversible adverse effects and increase the risk of disease in adults (Yoon *et al*., 2014; Bergman *et al*., 2013, Braun, 2017, Barouki *et al*., 2012). These diseases include metabolic disorders, impaired reproduction and disrupted neurodevelopment (Skakkebaek, 2016; Ghassabian & Trasande, 2018; Alonso-Magdalena *et al*., 2010; Engdahl & Rüegg, 2020).

1.3.3. Chemical mixtures

Although toxicological studies and risk assessment are traditionally focused on single compounds, compounds in the environment are normally present as mixtures. Thus, risk assessment and legislation are not based on the actual, "real life" exposure scenarios. Mixtures may, for instance, have additive or synergistic effects and could cause adverse impact at concentrations which are lower than the thresholds where single compounds have an effect (e.g., Kortenkamp, 2007; Kortenkamp, 2014; Celander, 2011). While single substance exposures are useful for determination of mechanism of action etc. in laboratory studies, mixture exposures are important in order to understand "real life" exposure situations and the potential effects on human and environmental health. Failing to take combined exposures into account could lead to underestimation of the risks posed by these exposures (Kortenkamp & Faust, 2018).

Mixtures can be assessed with a components-based approach by testing the individual components separately and comparing these to effects of a mixture of said components, or by a "whole-mixture approach" (Bopp *et al*., 2018). The whole mixture can be an intentionally produced product (such as a

formulated mixture of pesticides) or an unintentionally formed mixture of contaminants in the environment. Mixtures with environmentally relevant components and concentrations can for example be designed either based on testing samples of water, extracts from water, sediment or effluents from sewage treatment plants or based on chemical analysis of water, serum, tissues of humans or wildlife (Berntsen *et al.,* 2017; Berg *et al* 2016; Fini *et al*., 2017). Complex environmental mixtures can also be fractionated and tested further to find single chemicals or chemical groups with biological activity that act as drivers of an effect (Brack *et al*., 2016).

A whole-mixture approach based on epidemiology is used within the EU-project EDC-MixRisk which this thesis is a part of. EDC mixtures with environmental relevance were designed based on the pregnancy cohort study SELMA (Bornehag *et al.,* 2012) where chemicals in serum and urine of pregnant women were associated with adverse effects in their children (expanded in Section 1.6).

1.4. The thyroid system and thyroid disruption

Thyroid disruptors are of particular concern as thyroid hormones (THs) are essential for development, metabolism, and growth in vertebrates including fish (Mullur *et al*., 2014, Williams, 2008; Blanton and Specker, 2007; Power *et al*., 2001; Walpita *et al*., 2009). TH-regulation is also important for smoltification in salmonids (Björnsson *et al*., 2011) and metamorphosis in vertebrates with metamorphic stages, including flatfish and amphibians (e.g., Power *et al*., 2008).

The activity and synthesis of thyroid hormones triiodothyronine (T3) and thyroxine (T4) can be disrupted by chemicals interfering with hormone production, transport (e.g., *mct8* and *oatp1c1*), thyroid receptors (Thra and Thrb), and regulatory enzymes, such as the iodothyronine deiodinases (Dio1, Dio2, Dio3). The latter are involved in regulation of the availability and disposal of THs, including the conversion of T4 to T3 (Brown *et al*., 2004; Zoeller, 2007). Deiodinases 1 and 2 are involved in synthesis of T3, the active form of thyroid hormone, via deiodination and biosynthesis of T4, while Deiodinase 3 deactivates the THs by deiodination of the inner ring of T4 and T3 (Bianco and Kim, 2006, Freitas *et al*., 2016). Known thyroid disruptors include phthalates, phenols (e.g., Bisphenol A and triclosan), pesticides (like DDT and Chlorpyrifos), polybrominated flame retardants, Perfluorinated compounds and polychlorinated biphenyls (Boas *et al*., 2009; Zoeller 2010; Mughal *et al*., 2018).

1.5. EDCs in the aquatic environment

The aquatic environment can act as a sink for the anthropogenic chemicals and contains mixtures of chemicals that fish and other biota are continuously being

exposed to (van der Oost *et al*., 2003). These mixtures include EDCs that enter via effluents from wastewater treatment plants, industrial effluents and land runoff (Söffker and Tyler, 2012). Fish are in direct contact with the contaminants in water and sediment and can take up chemicals through their digestive system, gills and skin (Kwong *et al*., 2008; Weber and Goerke, 2003). Endocrine disruption near sewage treatment plants and pulp and paper mills are well-studied in fish (e.g., Purdom *et al*., 1994, Lange *et al*., 2011; Larsson and Förlin, 2002; Hewitt *et al.,* 2008). Examples of effects include feminization through presence of eggs in testes or presence of the egg-yolk protein vitellogenin (VTG) in male fish (Larsson *et al*., 1999, Purdom *et al*., 1994, Jobling *et al.,* 2005, Lange *et al*., 2011), effects on sex ratios (Lange *et al*. 2008), courtship (Gore *et al*., 2018), nest building and mating behaviours (Söffker and Tyler, 2012). Whole population effects of EDCs on fish are more difficult to assess for practical reasons. One such example is the study by Kidd *et al.,* (2007) where a whole-lake exposure to a low concentration of 17-ethynylestradiol (EE2) caused feminization of male fish and impacted gonadal development in fathead minnow, eventually causing the species to nearly become extinct in the exposed lake.

One approach for assessing the impact of pollutants on an aquatic organism is to measure the biological effects in said organism using biomarkers. Biomarkers measure biological changes in response to chemical exposure (Van der Oost *et al*., 2003). Examples of well-established biomarkers for chemical exposure in fish include measurements of vitellogenin levels in serum and ethoxyresorufin-O-deethlyase (EROD) activity which measures cytochrome P4501A induction. VTG levels can be induced in the serum of male and/or juvenile fish after exposure to compounds with estrogenic activity while EROD activity gives an indication of exposure to planar hydrocarbons (Allen *et al*., 1999; Stegeman & Hahn, 1994). Additionally, measurements of the activity of glutathione reductase, glutathione S-transferase, catalase and acetylcholinesterase (AChE) or the expression of related genes are examples of markers used for environmental monitoring to evaluate the health status in sentinel fish species (e.g., Asker *et al*., 2016; Hylland *et al*., 2017; Hanson *et al*., 2020). Exposure to emerging contaminants including non-estrogenic EDCs may require new markers to assess effect.

1.6. The EDC-MixRisk project

The PhD project described in this thesis was focused on the effects of exposure to endocrine disrupting compounds in fish. The main part of the project took place within the framework of the EDC-MixRisk project, an interdisciplinary, EU-financed Horizon 2020 project which started in 2015. This project aimed to promote use of safer chemicals for the next generations (https://edcmixrisk.ki.se/).

The research was interdisciplinary and combined epidemiology and biostatistics, experimental studies. The workflow of the EDC-MixRisk project is illustrated in Figure 1.

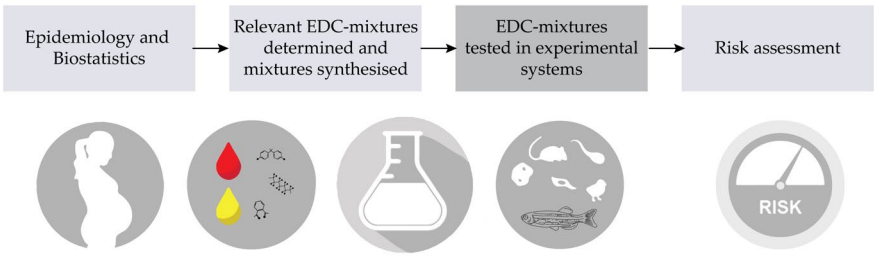


Figure 1. Overview of the EDC-MixRisk project workflow. The work presented in **Paper I–III** of this thesis is part of the third stage (testing mixtures in experimental systems), and our results from zebrafish are being used as a basis for risk assessment in the fourth stage.

1.6.1. EDC mixtures

Production of the mixtures used within the EDC-MixRisk project is described in more detail in **Paper I** and in (Bornehag *et al*., 2019). In summary, EDC mixtures related to three different health domains (neurodevelopment, growth and metabolism, and sexual development) were determined based on the association of the chemicals with adverse health outcomes in the Swedish pregnancy cohort study SELMA (Bornehag *et al*., 2012). 2,325 pregnant women from Värmland, Sweden, were included in this cohort. During week 10 of the pregnancy, blood and urine was sampled and 20 chemicals were analysed in the samples. These compounds included 10 phthalate metabolites (phthalate monoester metabolites from phthalate diesters), phenols (triclosan and BPA) and 8 perfluorinated compounds (PFASs). Weighted quantile sum (WQS) regression was used to identify subsets of the twenty chemicals that were associated with selected health outcomes for each health domain. The following three individual mixtures were designed:

- MIX N, for *Neurodevelopment*: compounds negatively associated with language delay (fewer words spoken) at 30 months were included in the mixture designated MIX N0 (for neurodevelopment) for 594 of the children from 1,874 mother-child pairs. A delayed language development in early childhood is linked to a lower cognitive function later in life (Peyre *et al*., 2017).
- MIX G, for *Growth*: compounds negatively associated with birth weight in both sexes for 1874 mother-child pairs. Lower birth weight

is linked to a higher risk for metabolic disease in humans later in life (Barker, 2012).

- MIX S, for *Sexual development*: compounds negatively associated with anogenital distance (AGD, the distance between the anus and genitals) in boys at 21-22 months of age for 184 mother-child pairs. AGD can be used as a marker for androgen exposure and a shorter AGD in infant boys is linked to genital anomalies (Schwartz *et al*., 2019).

Mixing proportions in each mixture were determined based on the measured or estimated serum concentrations to reflect the mean exposure levels in the maternal samples from the SELMA cohort. Next, the mixtures were synthesised by the EDC-MixRisk partner at the Division of Occupational and Environmental Medicine, Lund University and stock solutions with the relevant ratios of compounds for each health domain were sent out to the partners responsible for experimental testing. Mixtures were then tested using a whole-mixture testing approach in a number of experimental systems (*in vitro* and *in vivo*). The concentrations we used were coordinated across the models in the projects and are described as factors of the mean human serum concentrations (hsc) (See also section 2.1.2.). Finally, the estimates of adverse outcomes in the experimental models were linked to the human exposures using a "Similar Mixture Approach "(**Paper I**).

The EDC-MixRisk project was split into two rounds, where the initial mixtures (designated "MIX N0", "MIX G0" and "MIX S0", where 0 refers to the first round of identified mixtures) were based on chemical analysis of 20 different compounds measured in the samples from SELMA women. The second round consisted of new versions of the mixtures (mixture 1, designated "MIX N1", "MIX G1" and "MIX S1") were prepared using the same procedure as for the previous ones. A total of 54 chemicals were analysed in this round (compared to the 20 for mixture 0) and the association with adverse health outcomes was repeated to determine new mixtures. The chemicals included phenols, phthalates, PFASs, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs).

Table 1. Composition and concentration of EDC mixtures that were identified and tested in experimental systems within the EDC-MixRisk project.

Chemical class	Mixture component (parent compound)	Name	Concentration in mixtures (mol/L)		
			MIX N	MIX G0	MIX G1
Phthalates	MEP (DEP)	Mono-ethyl phthalate	2.70E-07	2.34E-08	3.204E-08
	MBP (DBP)	Mono-butyl phthalate	2.26E-08	2.00E-08	2.855E-08
	MBzP (BBzP)	Mono-benzyl phthalate	1.05E-08	9.1E-09	5.68E-09
	MINP (DINP)	Mono-isononyl phthalate	2.06E-08	1.79E-08	-
	MEHP (DEHP)	Mono-ethyl hexyl phthalate	-	1.24E-08	2.051E-08
	DPP	Dipentyl phthalate	-	-	4.9E-10
Phenols	BPA	Bisphenol A	4.2E-09	-	-
	TCS	Triclosan	-	2.6E-09	3,00E-10
PFASs	PFOS	Perfluorooctane sulfonate	1.03E-08	8.9E-09	1.048E-08
	PFOA	Perfluorooctanoic acid	-	2.9E-09	3.89E-09
	PFHxS	Perfluorohexane sulfonate	3.2E-09	2.8E-09	3.28E-09
	PFNA	Perfluorononanoic acid	1.1E-09	-	-
Others	3-PBA	3-Phenoxybenzoic acid	-	-	1.1E-10
	p,p'DDE	p,p'-dichlorodiphenyldichloroethylene	-	-	5.9E-10
	HCB	Hexachlorobenzene	-	-	1.6E-10
	MINCH (DINCH)	1,2-Cyclohexane dicarboxylic acid diisononyl ester	-	-	5.2E-10
	2-OH-PH	2-hydroxyphenanthrene	-	-	1.36E-09

The persistent compounds (PCBs, OCs, PFASs, PBDEs) were measured in serum samples while the other compound classes were quantified in urine samples. This PhD project focused on two of the three health domains (neurodevelopment and growth) and tested these mixtures using zebrafish as a model organism. Compositions and concentration of EDCs in the three mixtures presented in this thesis (MIX N0, G0 and G1) are included in Table 1.

1.7. Examples of EDCs of relevance for the current project

As mentioned above, **Papers I** – **III** in this thesis are focused on laboratory exposures of zebrafish to EDC mixtures defined within the EDC-MixRisk project. Among the main chemical classes in the mixtures are phthalates, PFASs and phenols (BPA and triclosan) (see Table 1 and Figure 2). In **Paper IV**, perch from Swedish lakes contaminated with PFASs or PCBs were sampled. In the following sections, I will give a brief overview of the main groups of chemicals which have relevance for this thesis.

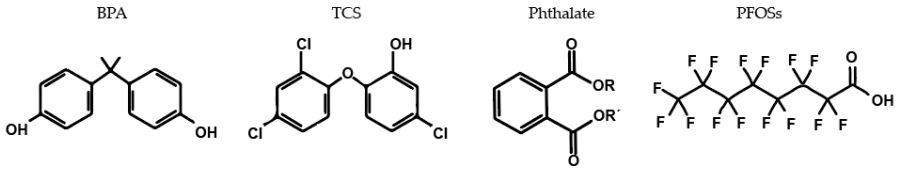


Figure 2. Examples of chemical structures of a selection of EDCs with relevance for the current thesis. The phenols (BPA and TCS), phthalates and PFASs (including PFOS) are all present in the EDC mixtures which were developed in the EDC-MixRisk project and tested in zebrafish (**Paper I–III**). For the field study with perch, PFASs together with PCBs were present in the contaminated lakes where wild perch were caught (**Paper IV**).

1.7.1. Phthalates

Phthalates are esters of phthalic acid, which are commonly used as plasticizers to make plastics softer, more flexible and durable. Phthalates are found in various products including polyvinyl chloride products, medical devices, personal care products, food containers, toys, adhesives and cleaning materials (Bergh *et al*., 2011). The high usage of phthalates and their tendency to leak from the end products or during production has resulted in high levels of phthalates found in water, air and dust (Gani *et al.,* 2017; Kashyap *et al*., 2018). In fish, behavioural effects have been found after exposure to various phthalates (Asnicar *et al.,* 2018; Tran *et al*., 2021).

1.7.2. Triclosan

Triclosan (TCS) is a biocide with antibacterial and antifungal properties used in consumer products such as soap and toothpaste (Dann *et al*., 2011; Rodricks *et al*., 2010). Effects of TCS have for example been reported on the androgen and estrogen receptors, and the thyroid axis (Ishibashi *et al*., 2004; Pinto *et al*., 2013; Gee *et al*., 2008). In zebrafish, effects have, for instance, also been shown on development (Oliveira *et al*., 2009), lipid metabolism (Ho *et al*., 2016), acetylcholinesterase activity, and behaviour (Pullaguri *et al*., 2020).

1.7.3. Bisphenol A

BPA is a plastic monomer which is used for production of several polycarbonate plastics and is found in many consumer products (e.g., food packaging, toys, construction materials and polycarbonate bottles) with high production volumes. The usage is now restricted in products such as infant feeding bottles. BPA can leak from products such as can linings and food containers affecting both humans and wildlife (Rochester, 2013, Vandenberg *et al*., 2007). It is currently one of the most well-studied EDCs and it has long been known to have estrogenic properties by being a weak agonist for the estrogen receptors (alpha and beta) (e.g., Dodds and Lawson 1936, Dodds and Lawson 1938; Alonso-Magdalena *et al*., 2012). BPA can also have effects on other systems including thyroid and androgen function (Boas *et al.,* 2012; Romano *et al.,* 2015).

1.7.4. Per- and polyfluoroalkyl substances

PFASs are persistent, surface-active anthropogenic compounds that can bioaccumulate through food webs. PFASs have a fluorinated carbon chain structure, are highly stable and have surface tension lowering properties which makes them suitable to use as surfactants (Buck *et al*., 2011). PFASs are used in a range of industrial products as well as numerous consumer products; as surfactants in paper and textile products, in food containers, in pesticides and in aqueous film forming foams (AFFFs) that are used for fire suppression (Kissa, 2001). Their persistent properties and extensive usage have caused them to be ubiquitous all over the world (e.g., Ahrens *et al*., 2010). PFASs can severely affect the health of aquatic organisms (Houde *et al*., 2011) and have been shown to have thyroid disruptive properties (Shi *et al.,* 2009; Chen *et al*., 2018; Wang *et al*., 2020) as well as behaviour disrupting effects in fish (Ulhaq *et al*., 2013; Huang *et al*., 2010; Spulber *et al*., 2014).

1.7.5. Polychlorinated biphenyls

PCBs are lipophilic and highly stable chemicals that consist of paired phenyl rings with various degrees of chlorination (Zoeller, 2007). PCBs were used as flame retardants, coolants, lubricants and plasticizers until their production was banned during the 70s-80s. They are still present in the environment globally and exposure continues due to their persistent properties (Boas *et al*., 2012). PCBs have well documented effects on thyroid hormone levels (Katarzyńska *et al*., 2015, Ahmed, 2013), are structurally similar to T4 which enables them to act as an analogue for this hormone by interacting with the receptor (Zoeller, 2007, Boas *et al*., 2012). In addition, PCBs are able to bioaccumulate or bioconcentrate in food webs and have been shown to occur at higher concentrations in piscivorous predators as well as fish (Houde *et al*., 2008; Burreau *et al*., 2006; Ruus *et al*., 2012).

1.8. Environmental enrichment

While some recommendations and guidelines for zebrafish care have been proposed (e.g., Aleström *et al*., 2020), there are still few standards that are generally accepted when it comes to environmental enrichment (EE) in the holding tanks for fish, including zebrafish. EE includes social enrichment, nutritional enrichment, occupational enrichment, sensory enrichment and structural enrichment (Näslund & Johnsson, 2016 and Stevens *et al*., 2021). Structural EE, i.e., adding physical structures or objects to the environment that animals are kept in, is one factor that has been linked to improved welfare and reduced stress in captive animals (Singhal *et al*., 2014; Young, 2003). Beneficial effects of structural EE have been extensively demonstrated in rodents but are less explored for fish (Stevens et al., 2021). Furthermore, studies that consider EE for ecotoxicological studies with fish models (e.g., Wilkes *et al*., 2012; Weber, & Ghorai, 2013) are even more rare and zebrafish used for laboratory experiments are normally kept in barren tanks (Kistler *et al*., 2011). The reluctance to introduce enrichment in fish tanks can for example be motivated by concerns regarding negative effects on standardisation and reproducibility, increased variability in data, cost and difficulty keeping tanks clean (Lidster *et al*., 2017).

Beneficial examples of EE effects demonstrated in fish models include fertility, reduced anxiety, improved learning, increased forebrain cell proliferation, brain size and reduced aggression, and stress levels (plasma cortisol concentrations) (Collymore *et al*., 2015; DePasquale *et al*., 2016; Carfagnini *et al*., 2009; von Krogh *et al*., 2010; Pounder *et al*., 2016; Näslund & Johnsson, 2016).

1.9. Behavioural ecotoxicology

Behavioural ecotoxicology is a growing discipline which aims to find novel and sensitive behavioural endpoints that could be used for sublethal toxicity screening of environmental contaminants (Hellou, 2011). Automatic tracking of fish larval locomotion during alternating dark and light periods can be a sensitive endpoint for sub-lethal toxicity of xenobiotics (Rihel *et al.,* 2010; MacPhail *et al.,* 2009). Once the swim bladder has developed, larval zebrafish show a distinct swimming pattern in response to alternating dark/light cycles recorded with high-throughput tracking (reviewed in (Basnet *et al*., 2019)). Locomotion tests in zebrafish can be used as a complement to developmental toxicity tests and used to screen for effects of neuroactive drugs and environmental pollutants. Different classes of chemicals with a suspected neurotoxic effect have previously been screened by locomotion tracking in fish larvae (Ulhaq *et al.,* 2013; Asnicar *et al.,* 2018; Ašmonaitė *et al.,* 2016). Experimental studies assessing locomotion after EDC exposures have shown both hyperactivity (induction of locomotion) and/or hypoactivity (reduction of locomotion) compared to the control treatment.

1.10. Research aims

The overall aims of this thesis were to (i) expand the knowledge on effects of mixtures of endocrine disrupting chemicals using fish models (ii) to evaluate the influence of environmental enrichment on the effects of EDC mixture exposure and (iii) to study endocrine disruption in wild fish and examine potential biomarkers for thyroid disruption in fish from areas contaminated with known thyroid disrupting chemicals.

This was achieved through the following specific objectives:

- To identify and characterise the effect of acute exposure to human-relevant mixtures of EDCs associated with growth or neurodevelopment in the EDC-MixRisk project, using gene expression analysis and locomotion as endpoints in zebrafish embryos (**Paper I and II**).
- To evaluate and compare the effects of two human relevant EDC mixtures with different complexity on zebrafish locomotion immediately after exposure and up to one month after exposure (**Paper II**).
- To examine interacting effects of environmental enrichment and exposure to EDC mixtures, on locomotion, swimming activity, social preference, and gene expression in juvenile and adult zebrafish (**Paper III**).
- To study whether thyroid- and immune-related parameters were affected in wild perch from Swedish lakes contaminated by known endocrine disruptors, specifically PFASs or PCBs (**Paper IV**).

2. Methodological considerations

This section consists of an overview of the experimental design and endpoints analysed in the papers and manuscripts on which this thesis is based. More detailed descriptions of the experimental and analytical procedures are presented in the individual research papers/manuscripts. The thesis focuses on the effects of endocrine disrupting chemicals in two fish models; the zebrafish, which is a commonly used species for laboratory testing and the European perch, which have ecological relevance for contamination in Swedish lakes.

2.1. Laboratory studies

The thesis is based on four different studies, three of which were performed with EDC mixtures from the EDC-MixRisk project in the laboratory and one field study with wild fish.

2.1.1. Experimental model

Zebrafish **(**ZF**)** (*Danio rerio*) are used as a vertebrate model species for several research areas including biomedicine, genetics, environmental toxicology, and pharmacological research and have become one of the most commonly used laboratory animal models (Stevens *et al*., 2021). They have several technical and practical advantages as a research model as they have a relatively short generation time, a fully sequenced genome, are relatively inexpensive to maintain and have a high fecundity (Basnet *et al.,* 2019; Ankley and Johnson, 2004). According to European legislation (Directive 2010/63/EU) zebrafish larvae are considered to be *in vitro* systems until initiation of exogenous feeding (Strähle *et al*., 2012), around 72h after hatching.

In **Paper I**, wild type (AB) zebrafish larvae were exposed to an EDC mixture (MIX N0) for 48h to examine effects on gene expression and mobility. In **Paper II** zebrafish larvae were exposed to MIX G0 and G1 for 48h, again to examine effects on gene expression and mobility. In addition, effects on locomotion after exposure to selected concentrations of MIX G0 and G1 were followed for 30 days after exposure. In **Paper III,** zebrafish larvae and adults were used to study the effects of environmental enrichment on EDC exposure using two approaches: i) larvae were acutely exposed to MIX G1 (based on long term effects in **Paper II**) and raised with/without enrichment. Effects on behaviour were assessed after 30 days and 3 months ii) adult ZF raised with or without enrichment were exposed to MIX G0 and effects on gene expression and behaviour were assessed. The exposure designs are expanded further in (Section 2.1.3.).

2.1.2. Substances tested

The exposure studies in larval and adult zebrafish (**Paper I-III**) used EDC mixtures that were designed and produced within the EDC-MixRisk project. The general process is described further in Section 1.6.1., as well as in **Paper I** and by (Bornehag *et al*., 2019). The tested EDC mixtures were associated with adverse effects related to neurodevelopment or growth in the offspring of the SELMA study. Composition and relative concentrations of the EDCs included in MIX N0, G0, N1 and G1 are illustrated in Figure 3. All mixtures were obtained from the EDC-MixRisk partner at the Division of Occupational and Environmental Medicine, Lund University. Additionally, BPA (**Paper I**, supplementary material) was used for single compound testing and was also obtained from the chemists in Lund. MIX N1 and single exposure to triclosan were also performed but results are not presented in the papers/manuscripts included in this thesis. The two mixtures associated with shorter anogenital distance in boys (S0 and S1) were tested by others within the consortium (Bornehag *et al.,* 2019; Repouskou *et al*., 2019; and Mentor *et al*., 2020) but not by us.

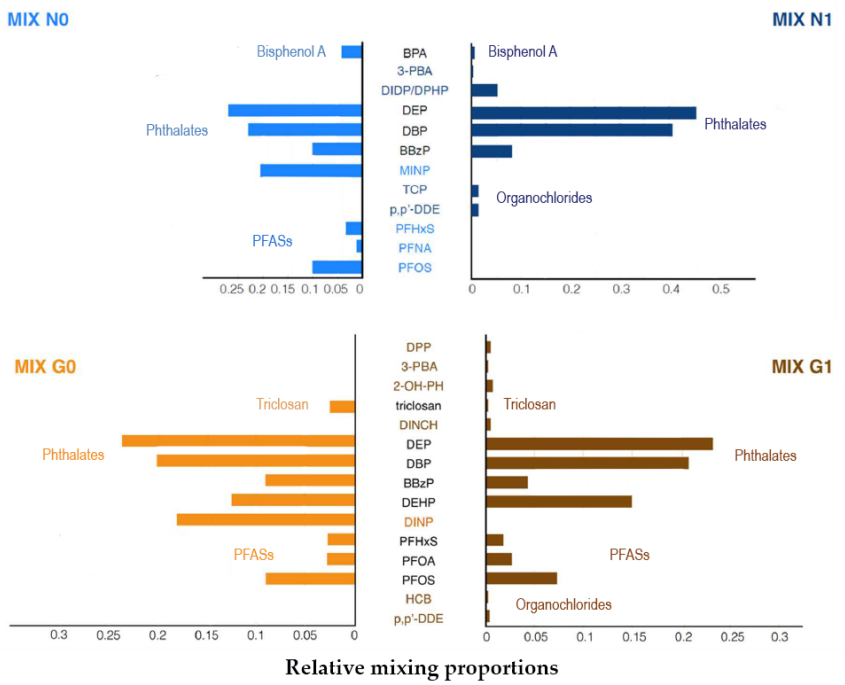


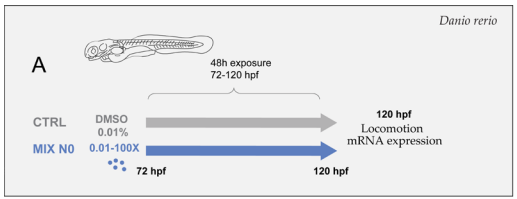
Figure 3. (previous page). Chemical composition of the two distinct mixtures associated with neurodevelopmental delay (N0 and N1) and the two mixtures associated with adverse effects on growth (G0 and G1) with relative concentrations of each compound (ratio of total mixture concentration). Note that MIX N1 is included for comparison but results for this mixture are not included in the manuscripts in this thesis.

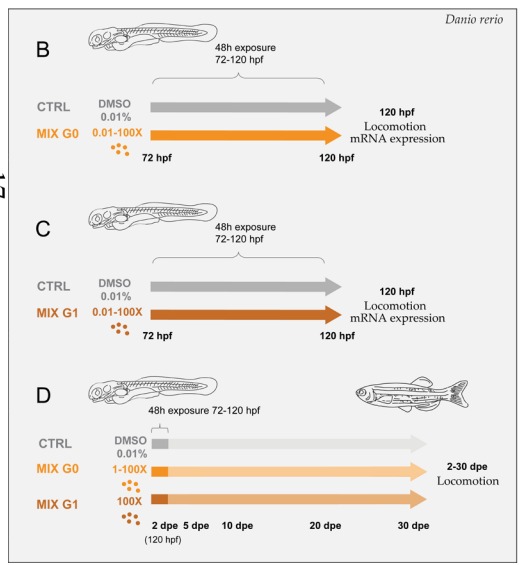
2.1.3. Exposure design

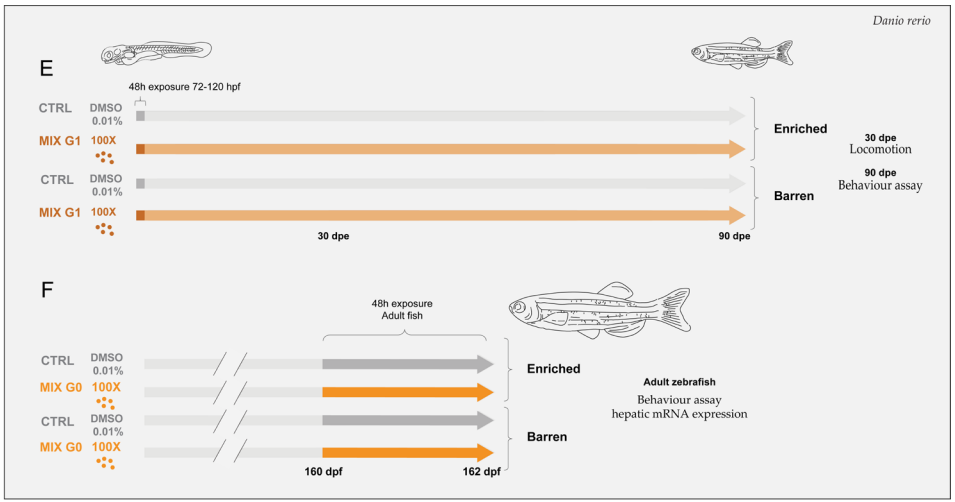
An overview of the experimental design used for each paper/manuscript included in the current thesis is presented in Figure 4. As mentioned in section 2.1.2**,** zebrafish were exposed to three of the EDC mixtures from the EDC-MixRisk project (MIX N0, G0 and G1) for **Papers I–III**. The tested concentrations of the mixtures (0.01-100X) were selected in collaboration with colleagues in the EDC-MixRisk project and are presented as they correspond to the exposure levels in pregnant women from the SELMA study, where 1X human serum concentration represents the geometric mean concentrations determined in the pregnant women's serum. Each laboratory exposure was done with AB zebrafish and was static and done through embryo medium. 48h exposure durations were used in all experiments. DMSO was used as a solvent and was included at the same concentration in all treatments including Controls (0.01% v/v).

In **Paper I and II** hatched larvae were exposed from 72 hpf-120 hpf when they were used for automatic locomotion tracking and sampled for mRNA expression (3 days - 5 days old) at concentrations N0 (designated MIX N in **Paper I**), MIX G0 and MIX G1 were tested between 72-120 hpf at 0.01-100X concentrations. In parallel with experiments performed with one of the other *in vivo* models in EDC-MixRisk, *Xenopus laevis*, gene expression and mobility of ZF larvae was assessed after an early, acute exposure to MIX N0 or G0. Single concentrations of BPA used in Paper I were selected based on the full mixture concentration as well as concentration of BPA in 1-100X mixture concentrations.

Paper II was split into two parts, experiment 1 examined acute effects of MIX G0 and G1 (0.01-100X) on gene expression and locomotion. Experiment 2 determined later effects on locomotion after early acute exposure. Tested concentrations of MIX G0 (1-100X) and G1 (100X) were selected based on the results from acute exposures and acute exposure was followed by continuous measurements of locomotion for five time points between 2 and 30 days after start of exposure. The concentrations were selected based on the acute exposures, which showed more effects of G0 than G1.


Danio rerio
A
48h exposure
72-120 hpf
CTRL
DMSO
0.01%
MIX N0
0.01-100X
120 hpf
Locomotion
mRNA expression
72 hpf
120 hpf


Danio rerio
B
48h exposure
72-120 hpf
CTRL
DMSO
0.01%
MIX G0
0.01-100X
120 hpf
Locomotion
mRNA expression
72 hpf
120 hpf
C
48h exposure
72-120 hpf
CTRL
DMSO
0.01%
MIX G1
0.01-100X
120 hpf
Locomotion
mRNA expression
72 hpf
120 hpf
D
48h exposure 72-120 hpf
CTRL
DMSO
0.01%
MIX G0
1-100X
MIX G1
100X
2-30 dpe
Locomotion
2 dpe
(120 hpf)
5 dpe
10 dpe
20 dpe
30 dpe


Danio rerio
E
48h exposure 72-120 hpf
CTRL
DMSO
0.01%
MIX G1
100X
Enriched
30 dpe
Locomotion
CTRL
DMSO
0.01%
MIX G1
100X
Barren
90 dpe
Behaviour assay
30 dpe
90 dpe
F
48h exposure
Adult fish
CTRL
DMSO
0.01%
MIX G0
100X
Enriched
Adult zebrafish
Behaviour assay
hepatic mRNA expression
CTRL
DMSO
0.01%
MIX G0
100X
Barren
160 dpf
162 dpf

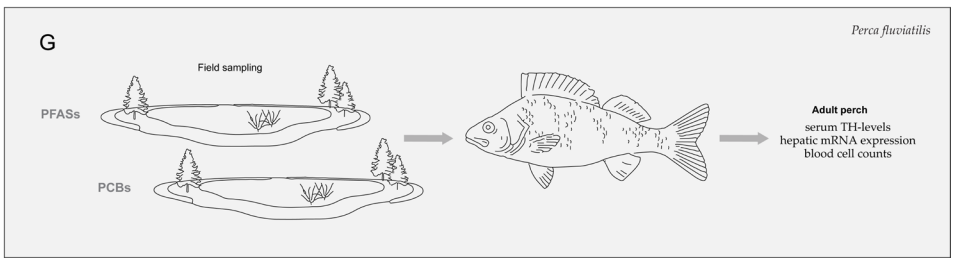

Perca fluviatilis
G
Field sampling
PFASs
PCBs
Adult perch
serum TH-levels
hepatic mRNA expression
blood cell counts

Figure 4. (previous page). Overview of the experimental setup in the experimental work included in the current thesis. In **Paper I** (**A**) hatched wild type zebrafish embryos were exposed via water to MIX N0 (N for neurodevelopment) for 48h (72-120 hpf). Larval locomotion was assessed through automatic locomotion tracking with a protocol of alternating dark/light cycles and the expression of selected endocrine related genes was tested using qPCR. In **Paper II** (**B-D**) larvae were first acutely exposed to MIX G0 and G1 (G for growth, both associated with low birth weight in children from the pregnancy cohort) (**B-C**). Exposure was done in the same manner as for MIX N in **Paper I**. Gene expression and larval locomotion was assessed at 120 hpf after 48h exposure. Additionally, **Paper II** includes a study of the longer-term effects of selected concentrations of the MIX G0 and G1 (1-100X resp. 100X) on locomotion (**D**). Acute exposure (72-120 hpf) was followed by automatic locomotion tracking at 2, 5, 10, 20 and 30 days post exposure (dpe). In **Paper III**, the interaction effect between environmental enrichment and EDC mixture exposure was assessed by exposing larvae acutely (CTRL or MIX G1, 100X) and raising the fish in enriched or barren tanks until 3 months of age (**E**) and by exposing adult ZF raised in enriched or barren environment to MIX G0 (**F**). **Paper IV** included a field study of wild perch from sites contaminated with known EDCs, PFASs and PCBs in two areas of Sweden (**G**). Endpoints included measurement of thyroid hormone levels, TH-related mRNA expression and blood cell counts.

Paper III was also separated into two experimental parts. Experiment 1 included long term effects of early acute exposure to MIX G1 (100X) subsequently raised in barren tanks or tanks with structural environmental enrichment. In Experiment 2, behavioural tests were performed in adult ZF after acute exposures. These exposures were done with MIX G0 as neither mixture had been tested in adult ZF previously and as acute exposures with MIX G0 gave a more pronounced effect on locomotion and gene expression.

In addition, BPA and TCS were used for single compound testing as these are two of the main differences between MIX N0 and G0. Locomotion and gene expression was assessed in order to determine if the effects observed with MIX N0 and MIX G0 are driven by either of the single compounds. Results for TCS are not included in the manuscripts while BPA is presented in **Paper I**.

2.2. Field study

In order to assess thyroid disruptive and immunotoxic effects of lifelong exposure to PCBs and PFASs (which are also included in the EDC-mixtures described above), in wild fish, we sampled wild perch from lakes in two different regions of Sweden. One site contaminated with PFASs (Lake Sänksjön) originating from a nearby airfield and two sites contaminated with PCBs (lakes Oxundasjön and Rosersbergsviken), with one reference site included for each area (**Paper IV**).

2.2.1. Model species

The European perch (*Perca fluviatilis*) is a common species in both fresh- and brackish aquatic environments throughout Europe and northern Asia (http://www.fishbase.org). Perch have been used for biomonitoring in Swedish lakes and the Baltic Sea for decades (e.g., Förlin *et al*., 1995; Hanson *et al*., 2009; Hanson *et al*., 2020) as well as in studies of PFASs distribution and accumulation (e.g., Ahrens *et al.,* 2010, Åkerblom *et al*., 2017). The liver transcriptome of European perch was recently sequenced (Förlin *et al*., 2019) which enabled us to design primers for gene expression analysis in **Paper IV**.

2.2.2. Study design

In **Paper IV**, thyroid disruption and immunotoxic effects were evaluated in wild female perch collected from contaminated areas in Sweden; one site contaminated with PFASs close to Kallinge, and two sites polluted with PCBs, north of Stockholm. Each site has been polluted for decades. Lake Sänksjön is located in Kallinge, in south-eastern Sweden. A fire training facility at a nearby military airfield was identified as a point source for PFASs in groundwater, streams, and lakes in this area and has been associated with elevated serum levels of PFASs in the local human population (Andersson *et al*., 2019; Li *et al*., 2018; Li *et al*., 2020). Lake Oxundasjön, north of the town of Upplands Väsby in Sweden has been shown to contain high levels of PCBs originating from local industries during the 1960s–80s. Levels in the sediment are estimated to be as high as five tonnes (Hållén *et al*., 2017; Karlsson and Viktor, 2014). The bay of Rosersbergsviken is located in Mälaren, downstream of Oxundasjön and also contains elevated PCB-levels (Karlsson, 2014; Karlsson *et al*., 2014).

Effects on the thyroid system in perch were evaluated by measuring thyroid hormone (T3) levels in plasma using a radioimmunoassay. Levels of hepatic mRNA coding for thyroid-related genes (selected based on the TH-disruptive results in ZF in **Paper I–III**) were quantified using real-time PCR. Further, the levels of immune-relevant blood cells (lymphocytes, granulocytes, and thrombocytes) were analysed in order to determine whether the immune system had been affected by long-term exposure to the contaminants.

2.3. Endpoints

The following section provides additional information or comments on the endpoints used in the manuscripts and papers included in this thesis.

2.3.1 qPCR

mRNA expression was analysed using Quantitative real time RT-PCR. RNA extractions were done with the Qiagen RNeasy® Plus Mini Kit while cDNA synthesis, and real time qPCR were performed with kits from BioRad according to the manufacturer's instructions. Pooled whole larval zebrafish (**Paper I-II**), adult zebrafish liver samples (**Paper III**) and adult perch liver samples (**Paper IV**) were used for the studies. The genes tested through qPCR were selected based on initial results within the EDC-MixRisk project showing TH disruptive properties in *Xenopus laevis* using the XETA assay (**Paper I**). Additionally, receptors from other endocrine modalities (estrogenic, androgenic and glucocorticoid systems) and a number of targets based on homologs of differentially expressed genes affected by the mixture in the human neurodevelopmental systems were tested. Primers were selected from literature or designed by us using NCBIs Primer BLAST or Primer3. Sequences are presented in Supplementary tables in each paper or manuscript.

2.3.2. Radioimmunoassay

A radioimmunoassay (RIA) protocol was utilised in **Paper IV** to determine the levels of total thyroid hormone (T3) in the blood plasma of perch. The procedure was based on the protocols used in (Einarsdóttir *et al*., 2006; Rotllant *et al*., 2003). In addition, we measured T3 levels with a Perkin Elmer ELISA kit and obtained the same results. However, total T4 levels were not possible to measure using either of our in-house methods, although these have successfully been used to measure T4 of other fish species before.

2.3.3. Blood cell count

Perch blood cells (number of lymphocytes, granulocytes, and thrombocytes) were counted microscopically in stained blood smears on glass slides (**Paper IV**). The white blood cell percentage (WBC) was calculated as the sum of the lymphocytes, granulocytes, and thrombocytes.

2.3.4. Zebrafish locomotion/mobility

In the current thesis, automatic behaviour tracking was used to determine locomotion endpoints in larval/juvenile zebrafish. The ViewPoint® automatic behaviour tracking system (ViewPoint Life Science, Montreal, CN) and an infrared camera were used to track locomotion. The locomotion parameters Distance travelled (Distance), Duration of movement (Duration) and Number of movements (Activity) were recorded and analysed with the ViewPoint® Zebralab software. All three parameters are presented in **Paper II** and **III**,

while only distance travelled is included in **Paper I**. The word *mobility* (referring to distance travelled) is used in **Paper I** while *locomotion* is used in **Paper II-III.** All locomotion testing was performed during the same time of day and with constant temperature as these factors can impact swimming activity. All concentrations were included on each tracked plate and placement of fish in the well plates was spread over the plate to account for possible plate effects. For **Paper I-II** a protocol with four alternating dark/light periods (5 min each, total protocol time 40 min) was used. This protocol was adapted from earlier studies in our laboratory (Ašmonaitė *et al*., 2016; Asnicar *et al*., 2018) where it was used to assess behavioural changes in zebrafish and sand goby at sublethal concentrations after metal ion or EDC-exposure. For **Paper III** a longer protocol with ten dark/light periods (100 min total). A longer protocol with more cycles can allow for evaluation of habituation effects if the locomotion response decreases over time as the stimulus is repeated (MacPhail *et al*., 2009; Ašmonaitė *et al*., 2016).

2.3.5. Juvenile and adult zebrafish behaviour

For **Paper III**, behaviour in older zebrafish (at 90 days and ca 160 days of age) was assessed through digital recording of individual fish in customised behaviour tanks for 5 min after a 10 min acclimatisation period. Videos were scored manually to determine swimming activity (number of grid lines crossed while swimming), social preference (time spent in the zone next to a group of conspecifics) and latency to approach (time until fish approached the group). Tank IDs were coded as to allow filming and scoring to be "blind", and filming was performed in a randomised order.

3. Results and discussion

An overview of the main results for exposure to MIX N0 (**Paper I**), G0 and G1 (**Paper II**) and G0 or G1 combined with enrichment (**Paper III**) as well as the results of perch field sampling (**Paper IV**) are presented in Figure 5.

3.1. Paper I - Acute effects of MIX N0 in zebrafish larvae

In **Paper I**, we measured effects of a human-relevant mixture associated with delay in language development in the SELMA study. Effects of the mixture associated with neurodevelopment (MIX N0) on expression of thyroid related genes in zebrafish larvae exposed for 48h (72-120 hpf) were tested after initial results of the XETA assay in *Xenopus* tadpoles, which showed that MIX N0 had the potential to disrupt the thyroid system. Experiments in zebrafish larvae were then performed in parallel with exposures in *Xenopus* tadpoles. Our results show that MIX N0 altered the gene expression for the thyroid receptors *thra* and *thrb* in zebrafish larvae while expression of the deiodinases *dio1, dio2* and *dio3* was not affected. Taken together with the XETA results and gene expression results in *Xenopus*, the mixture was determined to have TH-disruptive properties *in vivo*.

Other endocrine modalities were also considered by measuring common targets of EDC exposures on gene expression level including the estrogen receptor (*er*), androgen receptor (*ar*) and glucocorticoid receptor (*gr*), phosphoenolpyruvate carboxykinase (*pepck*), and peroxisome proliferator-activated receptor gamma (*ppar-γ*) expression in zebrafish larvae. Neither of these additional targets were found to be significantly altered by exposure to MIX N0 or MIX G0 in zebrafish for 48h.

Furthermore, differentially expressed targets identified after exposure in the *in vitro* systems were assessed in zebrafish but not found to be altered after MIX N0 exposure at the tested concentrations. See Supplementary Table S6 in **Paper I** for a list of all measured genes.

The locomotion assay with automatic tracking was included to assess the impact of MIX N0 on zebrafish larvae at an organismal level. There was a significant induction of locomotion (normalised distance travelled) at the 100X concentration. Locomotion was also slightly increased at the 10X concentration, but this difference was not significant compared to control.

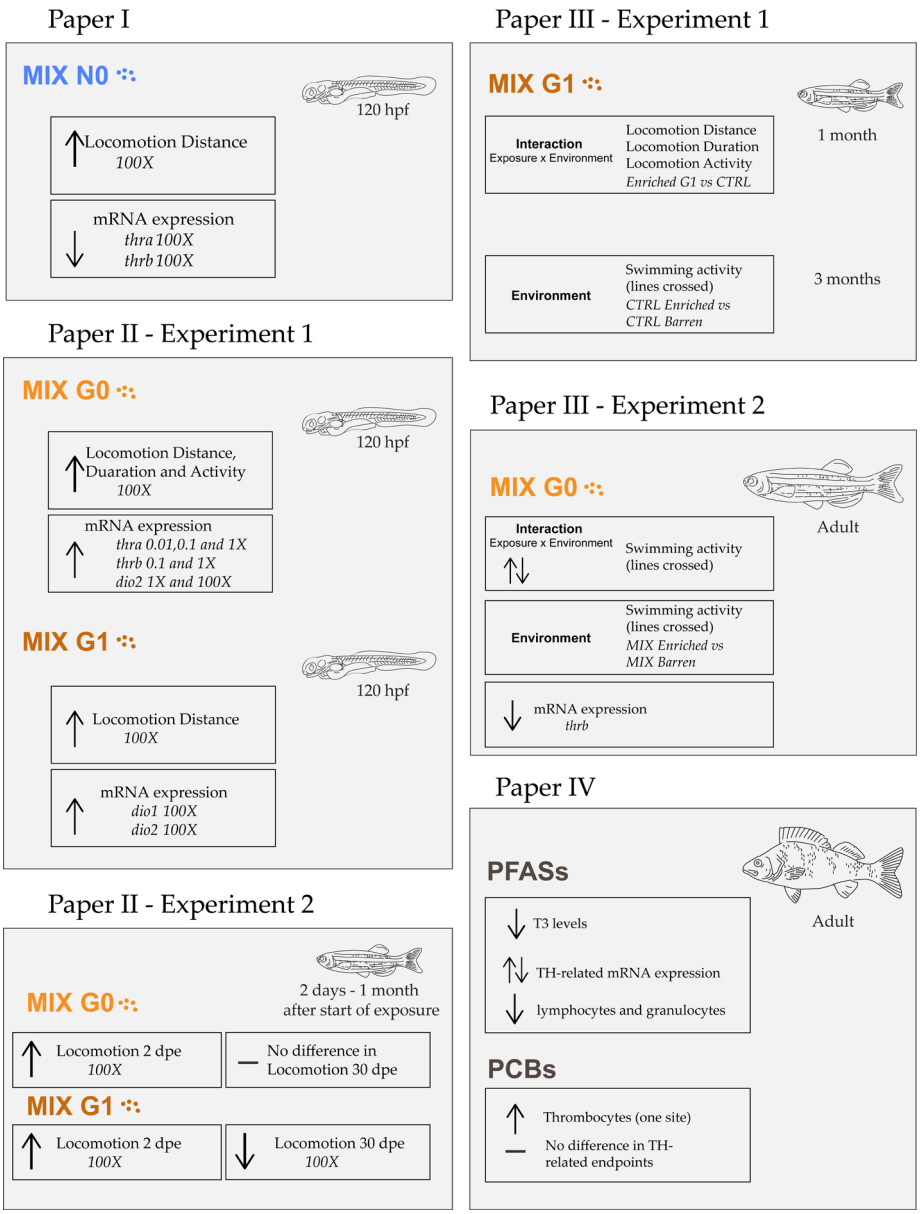


Figure 5. Overview of main results observed in the work included in the current thesis.

Given the importance of thyroid hormone for maturation of the central and peripheral nervous system (Williams, 2008), it is possible that the locomotor effects seen on both zebrafish larvae and *Xenopus* tadpoles in **Paper I** were an outcome of thyroid disruption. However, as locomotion is a complex, integrated response, other modes of action are also possible. Changes in locomotion after EDC-exposures have for instance been associated with neurotoxicity, altered muscle development, muscle malformations, motor neuron malfunction and impaired eye development in zebrafish (Zhang et al., 2011; Baumann et al., 2016; Christou et al., 2020). The exact mechanism behind the changes in zebrafish locomotion observed in this thesis would require further studies to define. In general, the mechanistic pathways leading to behavioural changes in fish after EDC exposures are often unclear and should be explored further.

Paper I demonstrates the innovative approach of the EDC-MixRisk project, combining scientific expertise, methodology and analyses from epidemiology, biostatistics, environmental chemistry and experimental models, where the results produced during this PhD project make up one part of the experimental studies. Our locomotion results were linked to the epidemiological data through a 'similar mixture approach' (SMACH), described in more detail in **Paper I**. Briefly, this strategy aims to i) assess what percentage of the study population had a mixture exposure that was sufficiently similar to a the reference mixture (MIX N0); and ii) to calculate a similar mixture risk indicator (SMRI, see Marshall *et al*., 2013) for cohort study participants which were determined to have sufficiently similar mixtures to MIX N0. Based on results from zebrafish locomotion data (normalised distance travelled), 91% of the pregnant women were determined to have sufficiently similar exposures to MIX N0. Children who had maternal prenatal concentrations sufficiently similar to MIX N0 with the highest decile values of SMRI were 3.3 times more likely (odds ratio) to have language delay at 30 months of age than the children with SMRI values in the lowest decile (P value = 0.043 based on zebrafish).

3.2. Paper II - Effects of MIX G0 and G1 in zebrafish larvae and juveniles

As a continuation of the exposures performed with MIX N0, we assessed acute effects of MIX G0 and G1 in larval zebrafish during experiment 1 in **Paper II**. Both mixtures were based on a negative association with birthweight in the SELMA cohort (Bornehag *et al*., 2012) but MIX G0 was designed and produced at an earlier stage of the project while G1 was designed during the second "round" of the project and is based on a larger number of analytes. It

can be noted that while MIX G0 is less complex than MIX G1, both mixtures have the same total concentration. Meaning that some individual components are present in MIX G1 at lower concentrations compared to the components in MIX G0.

As shown in Figure 4, the exposure protocol and endpoints for these experiments were the same as the one used for **Paper I**. The mRNA expression of thyroid-related genes was significantly altered by exposure to both mixtures. MIX G0 had an effect at lower concentrations (0.01X, 0.1X and 1X) compared to MIX G1 (100X).

The locomotion of the zebrafish at 120 hpf was significantly altered by both MIX G0 and MIX G1 at the highest tested concentration (100X), with hyperactivity for both mixtures compared to controls. Compared to results for MIX N0 in **Paper I**, the induction was considerably lower after acute MIX G0 exposure and even lower after MIX G1 exposure (Figure 6). As mentioned above, the observed changes in locomotion could be explained by several different modes of action, disruption of the thyroid axis being one of them.

For experiment 2 in **Paper II,** we followed the effects on locomotion over a longer period of time by continually tracking locomotion of larvae/juveniles at five timepoints until one month after EDC-exposure. At the last tested timepoint (30 days post exposure), the effects of MIX G0 were no longer significant while fish that had been exposed to MIX G1 had a significantly reduced locomotion (hypoactivity) compared to the control.

In summary, the effects of MIX G0 and MIX G1 on zebrafish locomotion differed depending on the time point locomotion was measured at. The less complex mixture (MIX G0) had a greater effect on mRNA expression at lower concentrations and a more pronounced acute effect on locomotion in experiment 1. In contrast, the longer term (1 month) effects in experiment 2 were only significant for the more complex mixture (MIX G1).

3.3 Paper III - Interaction between environmental enrichment and effects of MIX G0 or G1 exposure in zebrafish

In **Paper III,** we performed additional studies with acute exposures to the growth-related mixtures (MIX G0 and MIX G1) in zebrafish. Beneficial effects of structural enrichment have been documented in zebrafish and other fish species (e.g., reviewed by Stevens *et al*., 2021). However, despite the fact that zebrafish are now among the most common animal models used for laboratory studies, there is still no consensus for their housing conditions. There is also a large knowledge gap when it comes to the impact of the physical

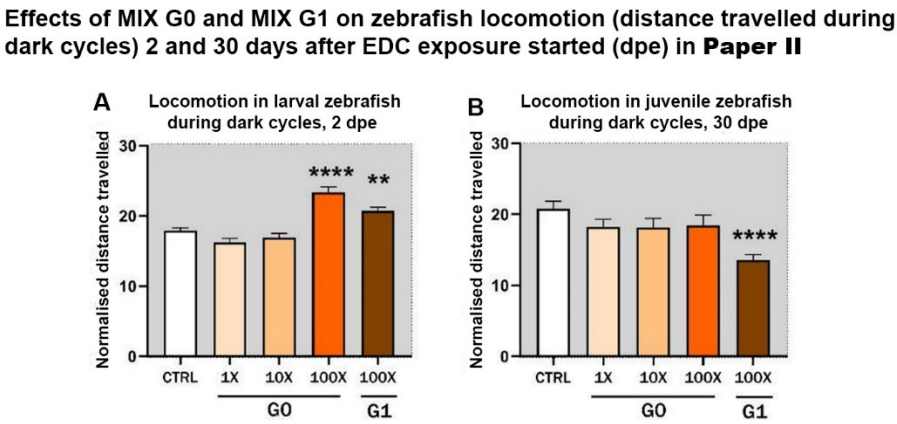


Figure 6. Comparison of zebrafish locomotion measured 2 and 30 days after the start of exposure to MIX G0 or the more complex MIX G1 in experiment 2 of **Paper II**. Normalised locomotion (measured as distance travelled) of zebrafish larvae exposed to MIX G0 (1X, 10X, 100X) or G1 (100X) was measured at five timepoints between 2 dpe and 30 dpe (days post start of exposure). Shown here are the results for the first and last time points measured, i.e., at 2 dpe (**A**) 30 dpe (**B**) for distance travelled during dark cycles. Note that the 2 dpe time point corresponds to the locomotion assay used for acute results in experiment 1 of **Paper II** and for MIX N0 exposure in **Paper I** (120 hpf). Data is presented as mean ± SEM and statistically significant differences between control and treatments are indicated with asterisks (*).

surroundings on the effects of chemical exposures in (eco)toxicological studies. To our knowledge, the study in **Paper III** is the first to explore the combined effect of enrichment and EDC mixture exposure in fish.

We used zebrafish to test the combined effects of environmental enrichment and exposure to the two human-relevant EDC mixtures MIX G0 and MIX G1. Behavioural effects were analysed using different endpoints depending on the age of the tested fish. The study in **Paper III** was also separated into two experiments. During experiment 1, an acute exposure to MIX G1 at 100X or CTRL was performed between 72 hpf-120 hpf (see Figure 4) (the 100X concentration was selected based on the results of acute exposures in experiment 1 of **Paper II**). Exposed fish were separated into two groups each (i.e., a total of four treatments) and raised in an enriched or barren environment. After one month, locomotion tracking was performed. After three months behaviour was assessed in custom made behavioural tanks, and three parameters were analysed (swimming activity, social preference, and latency to approach a group of conspecifics). In experiment 2, unexposed adult zebrafish raised in enriched or barren tanks were acutely exposed to MIX G0 (100X) or CTRL for 48h and behaviour was assessed in the same custom-made tanks. We selected MIX G0 for this experiment as we had previously found that MIX G0 had more of an effect than MIX G1 on larval zebrafish (for both locomotion and gene expression).

After one month, we found that enrichment had a significant interaction effect with the MIX G1-exposure (shown for distance travelled during light cycles in Figure 7.A). After three months, the EE fish were more active than control fish. In acutely exposed adult fish, there was a significant interaction effect between exposure and enrichment (see example in Figure 7.B). Additionally, the latency to approach conspecifics was longer in EE fish compared to barren fish, suggesting that EE fish might be less anxious.

Enrichment did not have an impact on the hepatic gene expression of TH-related genes measured in our study. An evaluation of the impact of EE on commonly used molecular biomarkers and expression of other genes in fish (including genes related to neurodevelopment in brain samples) after chemical exposure may be of interest for future studies.

It can be noted that EE in the form of sheets with images of gravel underneath the containers was also included in experiment 2 of **Paper II**. Since this external modification is preferred by zebrafish (Schroeder *et al*., 2014) while also inexpensive and does not impact water quality, hinder cleaning of the tanks or interfere with chemical exposures, it is likely to be suitable also for use in toxicological studies. Structural enrichment in the form of plastic plants or plastic structures (which are commonly used for aquaria) are less suitable for EDC-exposures.

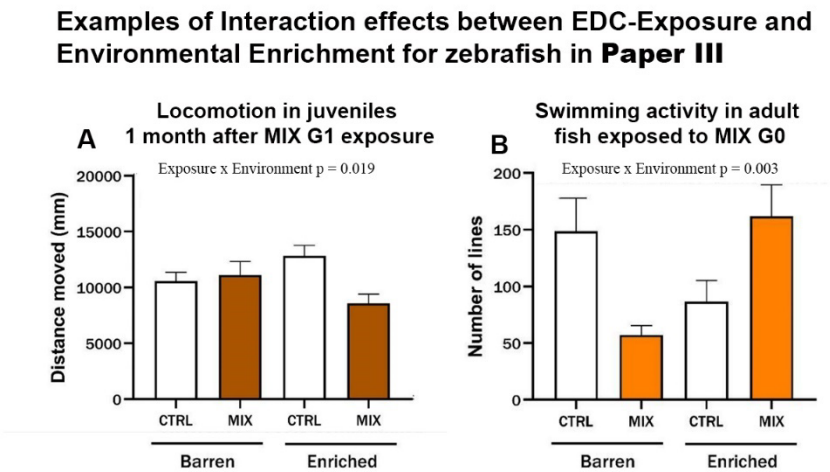


Figure 7. Examples of significant interaction effects between EDC-exposure and Environmental enrichment on behaviour at different time points in zebrafish used for **Paper III. (A)** Locomotion of zebrafish larvae exposed to MIX G1 at the 100X concentration (MIX) or control (CTRL) for 48h and thereafter raised in an enriched or barren environment for one month. Presented are the results for distance travelled during Light cycles. **(B)** Swimming activity (number of gridlines crossed during swimming) in adult zebrafish raised in an enriched or barren environment and thereafter exposed to MIX G0 at the 100X concentration (MIX) or control (CTRL) for 48h. Data is presented as mean ± SEM and significant interaction effects (Exposure x Environment) are shown by exact p-values.

3.4. Comments regarding Paper I-III

As previously noted, the word *mobility* in **Paper I** is used instead of *locomotion* (referring to distance travelled in the locomotion assay). The data presented in **Paper I** is normalised to the first minute during the assay and presented as photo motor curves with locomotion plotted for each minute while locomotion in **Paper II** and **Paper III** is presented as bar graphs summarising all locomotion performed during dark resp. light cycles for each concentration. MIX N0 had the most potent effect on locomotion of the three mixtures tested (Figure 8). As MIX N1 contains mostly phthalates and did not significantly affect locomotion (results not published), we chose to focus on MIX G1 for the second "round" of mixtures (see section 1.6.1.).

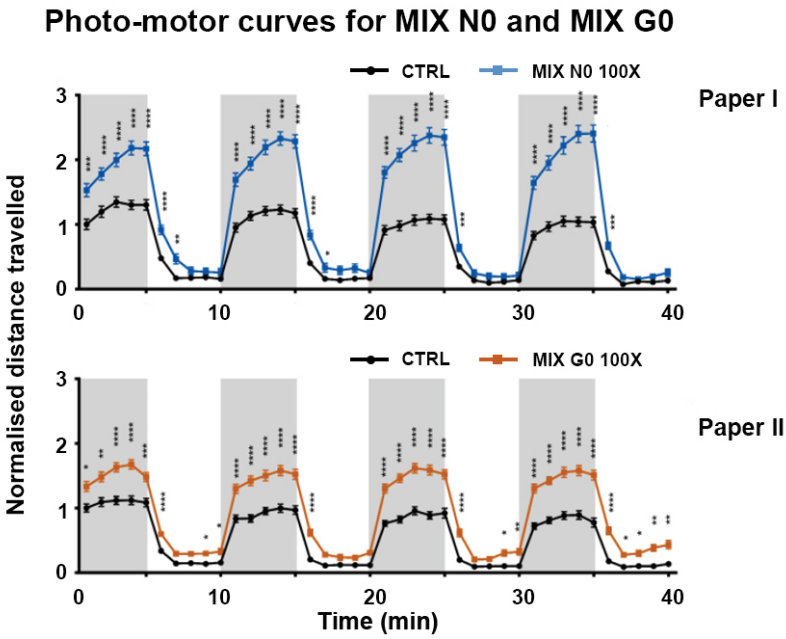


Figure 8. Comparison of locomotion results (normalised distance travelled) for MIX N0 (**Paper I**) and MIX G0 (**Paper II**) in zebrafish larvae after 48h exposure at the 100X concentration (for each mixture). Differences between CTRL and mixture concentrations were analysed using multiple t-tests with an FDR approach, FDR (Q)= 5%.

3.4.1. Single chemical exposures

Considering the whole mixture approach used for this thesis, each single compound present in the mixtures was not individually tested. Both MIX N0 and G0 consist of three main groups of chemicals: phthalates, phenols and perfluorinated compounds (Table 1). However, single exposures to TCS and

BPA were performed as these are two of the main differences between MIX N0 and G0. BPA results are presented in (**Paper I**) and TCS results are Unpublished. Neither of these single compounds were found to be responsible for the main effect of their respective mixture.

3.4.2. Considerations regarding concentrations and exposure times

The concentrations used in **Papers I - III** were selected in cooperation with the EDC-MixRisk consortium. Acute exposures were used in all experimental systems with some adjustments depending on physiological and developmental differences between the models. During the selected time frame for exposure, zebrafish were at the eleutheroembryo-stage, meaning that they are not surrounded by the chorion but were not yet free feeding embryos (Hanisch *et al*., 2010). At this stage, embryos are free swimming, have developed organs and have increased activities of detoxification processes which have been suggested to be similar to the processes in the adult zebrafish (Wiegand *et al*., 2000; Hanisch *et al*., 2010).

Furthermore, we do not expect concentrations (and also possibly composition/ratios) of mixture components in the zebrafish exposure medium to exactly correspond to the exposure situation of human foetuses in the SELMA-study. Differences are expected due to dissimilarities in e.g., uptake, metabolism and clearance (Smirnova *et al*., 2021). Uptake of the chemical exposure in zebrafish embryos at the stage of exposure used here (72-120 hpf) occurs mainly through diffusion over the skin. In an adult fish it would also include uptake over the gills which are not yet functional in the embryos until ca 14 dpf (Kimmel *et al*., 1995) and through eating, which starts ca 72h after hatching in zebrafish. Embryos/larvae used in **Paper I** and **Paper II** (experiment 1) are considered *in vitro* systems according to European legislation (2010/63/EU) as experiments were terminated before the onset of feeding.

Future studies could also include long term exposures to low concentrations of EDC mixtures which are a closer representation to real life-scenarios and similar to the situation for perch in **Paper IV.**

Together, the results of **Paper I-II** showed that MIX N0, MIX G0 and MIX G1 have disruptive effects on TH-related gene expression at concentrations from 0.01 to 100 times the geometric mean measured in pregnant women in the SELMA study (Bornehag *et al*., 2012). It can be noted that several women in the cohort had actual serum levels much higher than the 1X concentration and of a similar magnitude to the nominal concentrations tested in this thesis (the 95th percentile for compounds in MIX G0 varied depending on the mixture component but were generally between 2 to 10-fold the geometric mean of the human serum concentration (Smirnova *et al*., 2021).

3.4.3. Impacts of EDCs on fish behaviour

Our results on zebrafish locomotion and behaviour (**Paper I** –**III**) are in line with several previous studies of single chemical effect on fish behaviour. A range of EDCs have previously been shown to affect behaviour in adult and larval fish. For example, disruption of sexual behaviours, prey capture ability, schooling, feeding behaviour, predator avoidance responses, and the ability to perceive chemical alarm substances have been documented (Söffker and Tyler 2012; Zhou *et al.,* 2000; Scott and Sloman, 2004; Wibe *et al*., 2004, Breckels and Neff, 2010).

Locomotion-related endpoints in behaviour studies with fish models can be used as indicators of sub-lethal toxicity and can be a tool for detection of anxiety-like behaviours as well as visual-motor dysfunction in fish (Ali *et al*., 2012, Egan *et al*., 2009). As zebrafish larvae have a well-defined repertoire of locomotor responses, they are suitable models for assessment of environmental contaminants (Basnet *et al*., 2019; Ali *et al*., 2012).

EDC exposures can lead to non-monotonic responses in larval locomotion, where low concentrations have a stimulatory effect on swimming while high concentrations have a suppressing (toxic) effect on fish larval locomotion (as seen for di-butyl phthalate in Asnicar *et al.,* 2018). Hyperactivity as a result of EDC exposure has been observed in several studies (Spulber *et al.,* 2014; Ellis *et al.,* 2012; Saili *et al.,* 2012) and may be a response that enables larvae to avoid a polluted area but may be costly and not beneficial in the context of macro-environmental pollutants, (Asnicar *et al.,* 2018). Furthermore, it can be noted that these locomotion- based endpoints do not fully cover the complexity of animal behaviours, and that other important behavioural disruptions may be overlooked.

3.5. Paper IV - Field study with wild perch from EDC-contaminated lakes

In **Paper IV**, results are presented for an evaluation of thyroid disruption and immunotoxic effects in European perch caught from areas with long-term chemical pollution to two types of known thyroid disruptors (PCBs resp. PFASs). These exposure situations can be considered as "natural experiments" and they gave us the unique opportunity to evaluate the effects of thyroid disruptive chemicals on wild fish. Several previous studies demonstrate thyroid disruptive effects of PCBs and PFASs on fish in laboratory studies (e.g. Brown *et al.,* 2004), but studies showing conclusive effects in wild fish are still limited. Our results show that perch collected from the site chronically polluted by PFASs had significantly higher hepatic mRNA expression of *dio3*, and lower levels of thyroid hormone T3 in plasma (Figure 9.A-B). They also had lower mRNA expression levels of *thra* and *dio2* than the reference site

from the same area (Figure 9.C). Perch from the PFAS-contaminated site also had significantly lower levels of lymphocytes and granulocytes compared to reference fish. These findings suggest that chronic exposure to PFASs can disrupt both the thyroid system and immune system of wild perch, which may be of concern to the health of the aquatic environment.

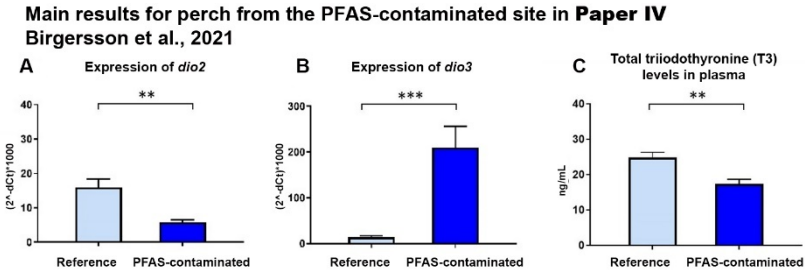


Figure 9: Main results for thyroid disruption in wild perch in the study presented in **Paper IV**. Significantly different hepatic mRNA expression of *dio2* and *dio3* **(A** and **B)** and of levels of total triiodothyronine (T3) in plasma **(C)** was demonstrated in perch caught at the PFAS-contaminated lake Sänksjön compared to the reference lake Skärsjön. Data are presented as mean ± S.E.M. and asterisks (*) indicate statistical significance between the groups ($p < 0.05$).

No clear effects were observed for the measured parameters in perch caught at the PCB-contaminated site. However, the PCB-area provided us with an additional reference site which we could compare the PFAS-area to. This was beneficial as it gave us more insight into the baseline levels of thyroid hormone and mRNA expression levels in perch, which are still largely unexplored in previous studies.

To our knowledge, our work in **Paper IV** is one of the first studies showing that life-long exposure to PFASs in the ng/L range may exert these adverse effects in wild perch. In addition, the results in **Paper IV** suggest that the expression of *dio2* and *dio3* could be suitable as biomarkers for thyroid disruptive chemicals, including PFASs.

Further method development for evaluation of perch samples is needed in order to include data for T4 and TSH measurements, which would be valuable for further assessment of the perch thyroid status. Neither could be measured by RIA or ELISA in our study but T4 levels in perch have recently been measured in perch plasma using mass spectrometry (Kupprat *et al*., 2021) which could be an alternative.

4. Conclusions and future perspectives

The overall aim of this thesis was to advance our knowledge of the effects that EDC mixtures have on fish models. During the main part of the work, I examined the effects of human-relevant mixtures that were studied with a whole mixture approach (**Paper I-III**). Three EDC mixtures associated with adverse effects in two different health domains of the EDC-MixRisk project were tested. Besides being of human-relevance, these mixtures are also relevant for environmental exposure situations as the components in the mixtures are commonly found in the environment. These experimental studies are complemented by a field study with wild perch caught from areas with long-term chemical pollution to known EDCs (**Paper IV**) which, in the case of the PFASs compounds, are also found in the EDC mixtures tested in zebrafish.

In **Paper I**, our results show that a human-relevant mixture of EDCs at concentrations of a similar magnitude of those found in human serum concentrations had an effect on the zebrafish model. Collectively, the results from the EDC-MixRisk consortium in **Paper I** demonstrate a novel approach for assessing exposure to mixtures of EDCs which could hopefully act as an inspiration for new, improved environmental toxicology research. It is a complement, rather than a replacement, of the currently used strategies for assessment of chemical mixtures. It is, for instance, not suitable for the testing of newly produced compounds, which are not already measurable in epidemiological cohort studies. Nevertheless, this whole mixture approach is a promising new tool for working with human- or environmentally relevant mixtures of pollutants.

When comparing the effects of MIX G0 and G1, two mixtures of different complexity related to the same adverse outcome, we found that MIX G0 had a more pronounced effect on locomotion and gene expression immediately after exposure (**Paper II**). Different effects between mixture 0 and mixture 1 (for MIX N0 vs N1 and G0 vs G1) were also observed in other model systems within the EDC-MixRisk consortium (e.g., Leemans, 2019). However, after a longer period of time, the complex mixture still had a significant effect on the locomotion while MIX G0 did not. Interestingly, the effect was the opposite of that observed immediately after exposure, as larvae were now hypoactive compared to controls instead of hyperactive. In conclusion, our results suggest that effects of complex mixtures may be more difficult to detect immediately after an exposure but could have a more pronounced and possibly more detrimental effect later in life. Endpoints measured at later life-stages are also important to consider in such cases.

In **Paper III**, we show that the rearing environment influenced the behavioural outcome measured after an EDC-exposure in zebrafish. Even a

simple enrichment in the form of a sheet with an image of gravel under the tanks led to a significant interaction effect on locomotion. Similarly, an interaction effect was observed in acutely exposed adults. While we did not find an impact of enrichment on the expression of the tested genes in adult fish, further studies should assess other common biomarkers for suitability of enrichment in ecotoxicological studies. Our study in **Paper III** is the first to evaluate the impact of enrichment on EDC mixture exposure. It is important to consider using EE when studying exposure of chemicals in fish models.

Lastly, in **Paper IV**, we demonstrated that wild fish exposed to PFASs in a real-life exposure scenario had lower levels of thyroid hormone T3 in plasma, higher mRNA expression of *dio3* and lower levels of *thra* and *dio2* than the reference site (non-polluted) from the same area. The levels of immune-relevant blood cells were also significantly different from the reference site. Our study also suggests that the levels of thyroid hormone (T3) and expression of *dio2* and *dio3* may be suitable as markers for thyroid disruptive chemicals, including PFASs, in fish.

Overall, the general conclusion of this thesis is that mixtures of EDCs including mixtures of PFASs have biological effects in fish models. It is therefore important to continue to investigate the effects of these classes of chemicals, especially as mixtures, and to develop new tools (e.g., biomarkers) for assessing their effects.

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