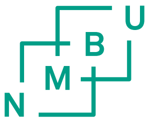

**Norwegian University of Life Sciences**
Faculty of Veterinary Medicine
Department of Basic Sciences and Aquatic Medicine

Philosophiae Doctor (PhD)
Thesis 2017:30

# Cocktails of persistent organic pollutants lead to behavioural and reproductive toxicity

## Blandinger av persistente organiske miljøgifter gir effekter på atferd og reproduksjon

Abdolrahman Khezri

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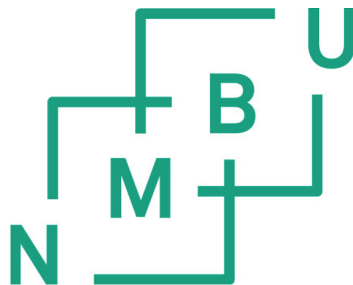
Philosophiae Doctor (PhD) Thesis

Abdolrahman Khezri

Faculty of veterinary medicine
Norwegian University of Life Sciences

Adamstuen (2017)

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# Summary in english

Persistent organic pollutants (POPs) are chemicals that widely distributed in the environment and biota that could cause neurobehavioural and reproductive toxicity. As humans and animals are exposed to a complex mixture of POPs, not to a single compound, and chemicals are known to have additive, synergic and antagonistic effects in a mixture setting, it would be beneficial to evaluate the mixture effect. The aim of this thesis was to evaluate the different parameters that could affect zebrafish behavioural activity, and evaluate the neurobehavioural and reproductive toxicity caused by a POP mixture in zebrafish larvae and maternally exposed mice, respectively.

During the last years, zebrafish larval behaviour has become a sensitive endpoint to evaluate the neurobehavioural toxicity that might be caused by different compounds. However, in some points behavioural results following exposure to identical chemicals of concern are inconsistent across the literature. In order to further understand this assay and how to interpret the results, zebrafish larvae were exposed to tetrabromobisphenol A (TBBPA) and bisphenol A (BPA) and the effect of rearing condition, larval age, and arena size were tested. Furthermore, a mechanistic approach was employed in order to test the behavioural response following BPA exposure. Results indicated that the prior photo-regime, larval age, and/or arena size can alter both the dose response and the direction of change following identical chemical exposures, Furthermore; previously identified mechanistic pathways may not explain the contrasting behavioural outcomes when using different methodology (**Paper I**).

Upon identifying the factors that can affect the locomotor activity in zebrafish larvae, we assessed the neurobehavioural toxicity caused by a POP mixture, and relevant sub mixes, in zebrafish larvae. The POP mixture was based on the levels found in human blood taken from the Scandinavian population. The experiment was also designed to investigate the time window of exposure and analyse the expression of genes involved in neurobehavioural development. Results revealed that the POP mixture increases the swimming speed of larval zebrafish following exposure between 48 to 96 hours post fertilization (hpf). This behavioural effect was associated with the perfluorinated compounds within the POP mixture, and more specifically with Perfluorooctane Sulfonate (PFOS). These findings indicated that the PFOS effect was equal to that of the mixture, which suggests no mixture effect on the endpoint tested. However, the expression of genes related to the dopaminergic,

histaminergic, cholinergic, GABAergic, serotonergic as well as those related to the stress response and neuronal maintenance were altered differently following exposure to the POP mixture and PFOS (**Paper II**).

In order to evaluate reproductive toxicity, mice were exposed to a POP mixture based on the levels found in Scandinavian food. Exposure began at the weaning of dams and continued through pregnancy and the lactation period. Pups that were maternally exposed had their testis and sperm collected at the age of 9 weeks. We evaluated testis morphometric and cauda sperm concentration as well as Deoxyribonucleic Acid (DNA) integrity in vas deferens and cauda sperm cells. Results demonstrated that the POP exposure impaired the seminiferous tubules, decreased sperm concentration and affected sperm DNA integrity in the vas deferens and cauda (**Paper III**).

This thesis demonstrates how methodology can have significant effects on behavioural outcomes in larval zebrafish and exemplify a crucial need for a greater understanding of how this test could reflect the toxicity of compounds and its application in safety assessment. Nevertheless, this thesis indicates that a human blood based POP mixture and PFOS can affect zebrafish behaviour, yet this effect could not be associated with any common differences in neuronal gene expression. Finally, the current findings highlight the reproductive toxicity of a food basket based POP mixture in male offspring of a mammalian model.

# Summary in Norwegian (Sammendrag)

Tungt nedbrytbare organiske miljøgifter (persistent organic pollutants, POPs) er kjemikalier som er vidt utbredt i miljøet og biota, og som blant annet kan gi toksiske effecter hos mennekser og dyr. Mennesker og dyr blir eksponert for en kompleks blanding av miljøgifter, ikke bare enkeltstoffer. Disse kjemikaliene kan, når de forekommer i blanding, gi additive, synergiske eller antagonistiske effekter. Det er derfor viktig å undersøke effekter av blandinger i tillegg til enkeltstoffer. Hensikten med denne avhandlingen var å undersøke ulike faktorer som kan påvirke atferd hos sebrafisk, samt å beskrive adferds- og reproduksjonstoksiske effekter etter eksponering med en blanding av POPs hos sebrafisklarver og maternalt eksponerte mus.

Atferdsendringer hos sebrafisklarver har de siste årene fått økt oppmerksomhet somet følsomt endepunkt for neurologiske effekter av forskjellige kjemikalier. I litteraturen beskrives imidlertid til dels motstridende resultater, selv etter eksponering med identiske kjemikalier. For å få mer kunnskap om atferdsstudier og om mulig bidra til å forklare årsaker til inkonsistente resultater, ble sebrafisklarver eksponert for Tetrabromobisphenol A(TBBPA) / Bisphenol A(BPA). Effekter av oppstallingsforhold og larvenes alder ble undersøkt. I tillegg til å teste atferdsendringer etter BPA eksponering, ble også mekanismer som kunne være forbundet med disse endringene undersøkt. Resultatene viste at lysregime, larvenes alder og/eller arenastørrelse kan påvirke atferd hos sebrafisklarver etter identiske kjemiske eksponeringer. I tillegg ble det funnet at tidligere studerte mekanismer ikke lenger kunne forklare de motstridende effektene på atferd når det ble brukt annen metodikk. (**Artikkel I**).

I artikkel 2 undersøkte vi atferdsendringer og neurologiske effekter hos sebrafisklarver etter eksponering med POP-blandinger. POP-blandingene var basert på publiserte blodnivåer fra den skandinaviske befolkningen. I forsøket inngikk også undersøkelse av sensitivitetsvindu for eksponering og undersøkelse av genuttrykk for gener involvert i neurologisk utvikling og atferd. Resultatene viste at POP-blandingen økte svømmehastigheten til larvene etter eksponering mellom 48-96 timer etter befruktning. Denne atferdseffekten var forbundet med de perfluorerte stoffene i POP blandingen, og mer spesifikt med Perfluorooctane Sulfonate (PFOS). Resultatet indikerte at PFOS var ansvarlig for de observerte effektene uten å bli påvirket av andre kjemikalier i blandingen. Selv om PFOS alene og POP-blandingen førte til

de samme atferdsendringene, var genuttrykket knyttet til eksponeringene forskjellig (**Artikkel II**).

For å undersøke reproduksjonstoksisitet, ble hunnmus eksponert for en POP-blanding basert på nivåer i maten til den skandinaviske befolkningen. Eksponeringen begynte ved avvenning og fortsatte gjennom svangerskapet og ammeperioden. Testikler og sædceller fra avkommene ble tatt etter avlivning ved 9 ukers alder. Vi registrerte morfometriske endepunkter i testiklene, spermiekonsentrasjon i cauda epidydimis og DNA-integritet i sædceller samlet fra både cauda, epidydimis og vas deferens. Resultatene viste at POP-eksponering førte til færre og mindre sædkanaler, nedsatt spermiekonsentrasjon og økt DNA-skade i spermiene (**Artikkel III**).

Avhandlingen viser at undersøkelsesmetoden kan ha betydelig innvirkning på utfallet i atferdsstudier av sebrafisklarver. Det er derfor et behov for bedre kunnskap om nytteverdien av slike tester i toksisitetsstudier. Vi fant at en POP-blanding basert på nivåer målt i humane blodprøver, samt PFOS alene, påvirket atferd hos sebrafisklarver på samme måte, men med ulikt genuttrykk. Avhandlingen viste også reproduskjonstoksiske effekter hos hannmus som var avkom etter mødre eksponert med en POP-blanding gjennom maten.

بێچووەماسییەکان. لێکۆڵینەوەکانمان نیشانیان دا کە تێکەڵاوی POPس کان بوو نە هۆی خێراتر مەلەکردنی ئەو بێچووەماسییانەی دە تەمەنی ٤٨ تا ٩٦ کاژێر دوای گەرادانان. ئێمە بۆمان دەرکەوت کە ئەو کاردانەوەیە لە سەر خێرایی مەلە کردن، بە هۆی بوونی مادەیەک بە ناوی PFOS دە ناو تێکەڵاوی POPس کان دا بوو. ئەوە دەریدەخا کە ئاستی ژاراویی بوونی PFOS بە تێکەڵاوبوون دەگەڵ ماددەکانی دیکە دانابەزێ. هاوکات بۆمان دەرکەوت کە هەرچەند ئاستی ژاراوییبوون و کارتێکەری PFOSو تێکەڵاوی POPس کان لەسەرجمووجۆڵی بێچووەماسییەکان وەک یەک وایە، بەڵام جێنەکانی کاریگەر لە سەر بەشەکانی جۆراوجۆری مێشک بە دوو چەشنی جیاواز گۆڕانکارییان بەسەر هاتبوو (مەقالەی دووەم).

بە مەبەستی لێکۆڵینەوە لە سەر ئەگەری کارتێکەری ژاراویی پ ۆ پ یەکان لەسەر زاووزێ، ئێمە ئەو پ ۆ پ ئەمان بەڕادەیەکی بەرامبەر دەگەڵ ئەو ڕادەیەی کە دە خواردنی خەڵکانی سکاندیناڤیا دا هەیە دەرخواردی مشکان دا. بۆ ئەنجامدانی ئەو تاقیکارییە پ ۆ پ ەکانمان دوای ئەوەی مشکەکانمان لە شیر بوونەوە تێکەڵ بە خۆراکی ئەوان کرد. ئەم چەشنە دەرخوارددانە تا ئاوس بوون و زان و شیردانی مشکەکان درێژەی هەبوو. بێچووە مشکەکان دە تەمەنی ٩ حەوتوویی دا بە شێوازێکی بێئازار کوژران و باتوو و سپێرمەکانیان کۆکرانەوە. شکڵی باتتوەکان، خەستیی سپێرمەکان، و ئاستی ساغبوونی ترشەی ناوەکی ناو سپێرمەکان خسترانە بەر لێکۆڵینەوە. ئاکامەکان نیشانیان دا کە پێگەیشتنی POPس کان لە دایکەوە شکڵی باتووی بێچووە مشکەکانی نێری تێکدابوو، ژمارەی سپێرمەکانی کەم کردبۆوە، و کاردانەوەی نەرێنی لە سەر ترشەی ناوەکی سپێرمەکان هەبوو. (مەقالەی سێیەم).

دەم تێزە دا ئێمە نیشانمان دا کە چۆنیەتی لێکۆڵینەوە لە سەر مەلەکردنی بێچووەماسییەکان دەتوانێ لە سەر هەڵسەنگاندنی جمووجۆڵی بێچووەماسییەکان کاریگەری هەبێت. ئێمە ئەو ڕاستیەمان بە هێنانەوەی نموونەیەک نیشان داوە، و باسی ئەوەمان کردووە کە پێویستە ئەم چەشنە تاقیکردنەوانە بە شێوازێکی ستاندارد ئەنجام بدرێن و ئاستی زانیاری لەسەر ئەم بابەتە پێویستە بەرز بێتەوە. بە تایبەت لە بەر ئەوە کە ئەم چەشنە تاقیکردنەوانە بە بەرفراوانی لەبارەی کارتێکەرییە نەرێنییەکانی مادە کیمیاییەکان ئەنجام دەدرێن. ئەم تێزە هەروەها دەریخست کە لە ناو کۆی POPس کان دا تەنیا PFOS لەسەر چۆنیەتی جمووجۆڵی بێچووەماسیەکان کاریگەری هەیە، هەر چەند کاریگەرییەکان لە سەر جینەکانی کاروباری مێشک و سیستێمی عەسەبی جیاواز بوون. دە کۆتایی دا ئەم تێزە جەختی کردەوە لە سەر ئەو ڕاستییە کە تێکەڵاوێک لە POPس کان دەتوانن کارتێکەری نەرێنییان لەسەر نێرەکانی گیانلەبەرێکی مەمکداردا هەبێ.

# Summary in Kurdish (کورته)


تاقمێک مادەی ئۆرگانیک بە ناوی (پەرسیستەنتە ۆرگانیچ پۆلۆتانتس، POPس، پ ۆ پ) کە دەتوانن کارتێکەری ژاراوییان لە سەر مێشک و سیستێمی عەسەبی هەبێت، بەبەرفراوانی دە ژینگە دا بڵاو بوونەتەوەو نافەوتێن . هەم مرۆڤ و هەم ئاژەڵ هاوکات بەرامبەری چەندین جۆر لەم مادە مەترسیدارانە دەبنەوە. ئەو ڕاستیە سەلمێندراوە کە تێکەڵاوبوونی ئەو مادانە پێکەوە دەتوانێ ببێتە هۆی گۆڕان دە ڕادەی ژاراویبوونی ئەوان بە بەراورد دەگەڵ ئەو کات کە ئەمانە تەنیان، واتە ئەم ڕادەیە دەتوانێ زۆر زیادتر یا خود زۆر کەمتر بێتەوە. کەوابێ لێکۆڵینەوە لەسەر کارتێکەرییە ژاراوییەکانی ئەو مادانە بە تێکەڵاوی دەتوانێ زۆر پڕبایەخ بێ .ئامانجی ئەم تێزە بریتییە لە باشتر ناساندنی خاڵە سەرەکی و گرینگەکانی بواری تاقیکردنەوەی جۆری مەلەکردن و جمووجۆڵی ماسی زێبرا. ئامانجەکانی دیکە بریتیی بوون لە ڕوونکردنەوەی چۆنیەتی شوێندانانی تێکەڵاوێک لە POPس کان لە سە ر سیستێمی عەسەبی، جۆری مەلەکردن و جمووجۆڵی ماسی زیبرا، و هەروەها لە سەر زاووزێی مشک.

دە ماوەی چەند ساڵی ڕابردوو دا جۆری مەلەکردن و جموجۆڵی ماسی زێبرا (جۆرە ماسیە کی بچووک) وەکوو نیشانەیەکی هەستیار بەرامبەر بە کارتێکەرییە ژاراویەکانی مادە جۆراوجورەکان لە سەر مێشک ناسراوە. تا ئەمرۆ، دەرەنجامی تاقیکردنەوەکان لە سەر جۆری مەلەکردن و جموجۆڵی ماسی زیبرا، پاش دەرخوارددانی مادە کیمیاییەکان یەکتر ناگرنەوە. بە مەبەستی باشتر تێگەیشتن لەم تاقیکردنەوەیە وچۆنیەتی لێکدانەوەی دەرەنجامە جۆراوجۆرەکان و چۆنیەتی کارتێکەری فاکتۆرە جیاوازەکان، دوو مادەی کیمیاوی بە ناوەکانی TBBPA و BPA مان لە سەر بێچووە ماسییەکان تاقی کردەوە. هاوکات ئێمە لێکولێنەوەمان لە سەر چەند فاکتۆڕی وەک ڕادەی نوور، ڕادەی گەورەیی ژووری تێداگەورەبوون، و تەمەنی بێچووەماسییەکان کرد. ئەنجامی ئەو پشکنینانە دەریخست کە هەمووی ئەو فاکتۆڕانە دە توانن کاریگەریی زۆریان لە سەر ئاستی ژاراوییبوونی مادە کیمیاییەکان هەبێت. جگە لەوانەش بۆمان دەرکەوت کە خێراییی مەلەکردنی بێچووە ماسییەکان بە دوای تێکەڵ کردنی BPA دەگەڵ ئاوی بێچووە ماسییەکان، کە پێشترگومان دە کرا بە هوی ئاندروژنیکە، دە توانێت هۆکاری دیکەی هەبیت (مەقالەی یەکەم).

پاش ئەوەی ئاکامی لێکۆڵینەوەکەی مەقالەی یەکەممان دەری خست کە فاکتۆڕە سەرەکییەکان چۆن کاریگەرییان لە سەر جمووجۆڵی بێچووەماسییەکان هەیە، دەستمان دا تاقیکردنەوە لە سەر چۆنیەتی کارتێکەری تێکەڵاوێک لە POPس کان لە سەر جمووجۆڵی بێچووەماسییەکان. ئاستی POPس کان وا داندرا کە بەرامبەر بێ دەگەڵ ئاستی ئەوان دە خوێنی خەڵکانی سکانیناڤیا دا. هەروەها ڕوونمان کردەوە کە بێچووەماسییەکان دە چ تەمەنێک دوای گەرادانان دا بەرامبەر بە کارتێکەریە ژاراوییەکانی POPس کان هەستیارترن. لێکۆڵینەوەشمان لەسەر ئەو بابەتە کرد کە چۆن POPس کان دەبنە هۆی گۆڕانکاری دە جینەکانی پێوەندیدار بە پێگەیشتنی مێشک و سیستێمی عەسەبی، و جینەکانی پێوەندیدار بە جمووجۆڵی

# Abbreviations

| | |
|---|---|
| **AchE** | **Acetylcholine Esterase** |
| **ADHD** | **Attention Deficit Hyperactivity Disorder** |
| **ANOVA** | **Analysis of Variance** |
| **AOT** | **Acridine Orange Test** |
| **AR** | **Androgen Receptor** |
| **BBB** | **Brain Blood Barrier** |
| **BDNF** | **Brain-Derived Neurotrophic Factor** |
| **BPA** | **Bisphenol A** |
| **BRCs** | **Brominated Compounds** |
| **CLC** | **Chlorinated Compounds** |
| **CNS** | **Central Nervous System** |
| **CRHF** | **Corticotropin-Releasing Hormone Factor** |
| **DDD** | **Dichlorodiphenyl Dichloro Ethane** |
| **DDE** | **Dichlorodiphenyl Dichloro Ethylene** |
| **DDT** | **Dichlorodiphenyl Trichloro Ethane** |
| **DFI** | **DNA Fragmentation Index** |
| **DHT** | **Dihydrotesterone** |
| **DNA** | **Deoxyribonucleic Acid** |
| **DPF** | **Day Post Fertilization** |
| **E2** | **Estradiol** |
| **FSH** | **Follicle-Stimulating Hormone** |
| **FTOH** | **Fluorotelomeralcohol** |
| **GABA** | **Gamma-Aminobutyric Acid** |
| **GnRH** | **Gonadotropin-Releasing Hormone** |
| **$H_2O2$** | **Hydrogen Peroxide** |
| **HBCD** | **Hexabromocyclododecane** |
| **HCB** | **Hexachlorobenzene** |
| **HCH** | **Hexachlorocyclohexane** |
| **HDS** | **High DNA Stainability** |
| **HPF** | **Hour Post Fertilization** |
| **HRH1** | **Histamine Receptor H1** |
| **IQ** | **Intelligence Quotient** |

| | |
|---|---|
| **LDL** | **Low Density Lipoprotein** |
| **LH** | **Luteinizing Hormone** |
| **LME** | **Linear Mix Model** |
| **MANF** | **Mesencephalic, Astrocyte-derived Neurotrophic Factor** |
| **MeHg** | **Methyl Mercury** |
| **NT** | **Neurotransmitter** |
| **PAH** | **Polycyclic Aromatic Hydrocarbon** |
| **PBDE** | **Polybrominated Diphenyl Ethers** |
| **PCB** | **Polychlorinated Biphenyl** |
| **PFC** | **Perfluorinated Compound** |
| **PFDA** | **Perfluorodecanoic Acid** |
| **PFHxS** | **Perfluorohexane Sulfonic Acid** |
| **PFNA** | **Perfluorononanoic Acid** |
| **PFOA** | **Perfluorooctanoic Acid** |
| **PFOS** | **Perfluorooctane Sulfonate** |
| **POP** | **Persistent Organic Pollutant** |
| **ROS** | **Reactive Oxygen Species** |
| **SCDT** | **Sperm Chromatin Dispersion Test** |
| **SCGE** | **Single Cell Gel Electrophoresis Assay** |
| **SCSA** | **Sperm Chromatin Structure Assay** |
| **SSRI** | **Selective Serotonin Receptor Inhibitor** |
| **T** | **Testosterone** |
| **T3** | **Triiodothyronine** |
| **T4** | **Thyroxine** |
| **TBBPA** | **Tetrabromobisphenol A** |
| **TBG** | **Thyroxin-Binding Globulin** |
| **THs** | **Thyroid Hormones** |
| **TRH** | **Thyrotropin-Releasing Hormone** |
| **TSH** | **Thyroid-Stimulating Hormone** |
| **TTR** | **Transthyretin** |
| **TUNEL** | **Terminal deoxynucleotidyl transferase dUTP Nick End Labeling** |

# List of Papers

Paper I – Toxicology letters (under revision).

**Toxin induced behavioural aberrations in larval zebrafish are dependent on minor methodological alterations.**

Thomas W. K. Fraser, Abdolrahman Khezri, Juan G. H. Jusdado, Anna M. Lewandowska-Sabat, Theodore Henry, Erik Ropstad

Paper II – *International journal of molecular sciences* **2017,** 18, (2), 291.

**A Mixture of Persistent Organic Pollutants and Perfluorooctanesulfonic Acid Induces Similar Behavioural Responses, but Different Gene Expression Profiles in Zebrafish Larvae.**

Abdolrahman Khezri, Thomas W. K. Fraser, Rasoul Nourizadeh-Lillabadi, Jorke H. Kamstra, Vidar Berg, Karin E. Zimmer, Erik Ropstad

Paper III – Toxicology and applied pharmacology (under review).

**Maternal exposure to a mixture of persistent organic pollutants (POPs) affects testis morphology, epidydimal sperm count and induces sperm DNA fragmentation.**

Abdolrahman Khezri, Birgitte Lindeman, Anette K. Krogenæs, Hanne F. Berntsen, Karin E. Zimmer, Erik Ropstad

# 1. Introduction

## 1.1 Persistent organic pollutants (POPs)

Nowadays numerous amounts of different chemicals are produced and applied in industrial products. Not all of these chemicals are safe and some of them have become a major concern for animal and human safety. Some of these chemicals are POPs for which their physiochemical properties give them the ability to 1) stay resistant to environmental degradation, 2) contaminate and distribute widely via different environmental compartments such as, soil, water and air, 3) accumulate in fatty tissue, and 4) induce a wide range of toxicity in humans and animals [1]. Due to some of these properties, the concentrations of POPs have biomagnified in living organisms and bioaccumulated from lower organisms to top predators and humans [2].

Although the production of POPs has been reduced over time, POPs have been released into the environment over the last several decades and some POPs have been detected in regions far away from their sources [3]. Due to their resistance to degradation, POPs have been distributed to remote locations via air and ocean currents and by the migration of contaminated fish and birds. Besides that, it has also been shown that climate change has had a great impact on POP distribution [4]. For instance, global warming increases the rate of POP degradation [5], enhances POP volatilization, has increased the level of air pollutants, and altered the balance of POPs between soil, water and air [6].

For the first time in 2001, the Stockholm convention classified a series of chlorinated POPs like hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs) as being compounds for which the manufacturing and emissions must be ceased [1]. The list was updated to include brominated and perfluorinated compounds in 2009 [7].

### 1.1.1 Brominated compounds (BRCs)

Different brominated compounds such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA) and polybrominated biphenyls (PBBs) (Figure 1) are used in a wide range of industrial products like plastics, textiles, electronic circuitry, furniture and building materials to reduce the chances of a fire hazard [8].

PBDEs consist of 209 different chemicals, have two halogenated aromatic rings and according to the number of added bromine groups, are categorized into penta, octa, and deca BDEs [8]. The amount of applied BDEs in electronic products in Nordic countries was about 5750 tonnes up to 1995 [9]. In 2004 the European Union (EU) banned the trading of products containing more than 0.1% of PentaBDE/OctaBDE by weight and waste containing > 0.25% PentaBDE was categorized as hazardous products [10]. Today, TBBPA is the most widely used brominated flame retardant. The European food safety authority, by evaluating 652 food samples from four different countries, listed TBBPA as a safe chemical and not as a POP [11]. Although the concentration of TBBPA in the human food basket is very low, its concentration is very high in aquatic biota [12].

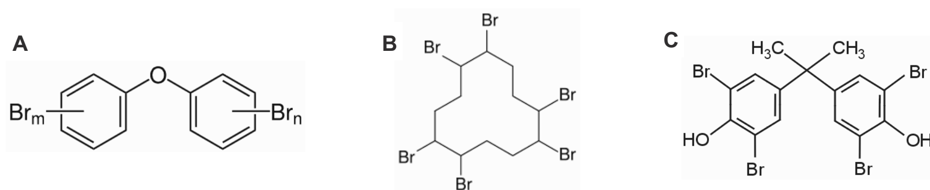



*Figure 1. An overview of different classes of BRCs. A) PBDE, B) HBCD and C) TBBPA. Figure was adapted from [13].*

### 1.1.2 Chlorinated compounds (CLCs) – PCBs

Polychlorinated biphenyls (PCBs) are a group of 209 colourless synthetic chemicals with a mutual biphenyl structure and different numbers (2-10) of chlorine atoms [14]. Based on the chlorine binding position to the biphenyl molecule, PCBs are divided into coplanar and non-coplanar isoforms [15] (Figure 2), which are highly lipophilic and mainly accumulate in adipose tissue in humans/animals [16]. PCBs are widely applied in different industrial products like lubricants, isolators, electrical equipment products, inks and paint as well as plastic products [17]. Human and animals are mainly exposed to PCBs via food [18] and dust [19]. Food born PCBs have a longer half-life compared to air born PCBs [17].

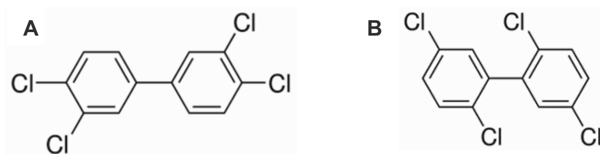



*Figure 2. Representative of different classes of CLCs. A) PCB 77 a coplanar and B) PCB 52 a non- coplanar product. Figure was adapted from [20].*

### 1.1.3 Other chlorinated compounds

Although the production of PCBs has been banned since 1979, Dichloro Diphenyl Trichloroethane (DDT) is a chlorinated insecticide that because of its application against malaria, is still in use [21]. Different isoforms of DDT are produced, with *p,p* -DDT and *o,p* –DDT known as the most persistent forms of DDT with half-lives between 2 – 15 years [22]. DDT mainly metabolizes to Dichlorodiphenyl Dichloro Ethylene (DDE) and Dichlorodiphenyl Dichloro Ethane (DDD), which are more persistent compared to the parent compound [23]. Dieldrin is another chlorinated pesticide, which was used between 1950 and 1970 against soil insects and yet significant levels of dieldrin in the environment has been detected [24]. Hexachlorobenzene (HCB) was introduced into the industry as an anti-fungal agent in 1933, later categorized as a POP by the Stockholm convention and banned from production, but is still produced as a by-product and released into the environment [25]. Hexachlorocyclohexane (HCH) has been known as a potent pesticide since the 1940s, is available in different isoforms including α, β, γ and δ, and today γ-HCH or lindane is the only isomer that exhibits strong insecticidal properties [26]. Chlordane is another chlorinated pesticide, introduced as termiticide in 1940, currently forbidden in the USA and Europe but still produced in China [27].

### 1.1.4 Perfluorinated compounds (PFCs)

PFCs are a large group of industrial compounds, have been produced since the 1950s and are widely used in water resistance products, breathable cloths, Teflon cookware, building materials, electrical products and other packaging materials [28]. PFCs have been widely detected in the environment, human's tissue [29] as well as wild life samples [30] and oral exposure to PFCs is the main route of exposure [31]. In contrast to BRCs and CLCs, which

accumulate in adipose tissue, PFCs mainly bind to proteins [32]. Because of the high energy atomic bounds in PFC structures, they are extremely persistent to degradation [33]. PFCs consist of a common partially or fully fluorinated alkyl chain (4–14 carbons), which can bind to different functional groups including carboxylic acids (PFOA, PFDA, PFNA), sulfonic acids (PFOS, PFHxS) and fluorotelomers groups (FTOH) [32] (Figure 3).

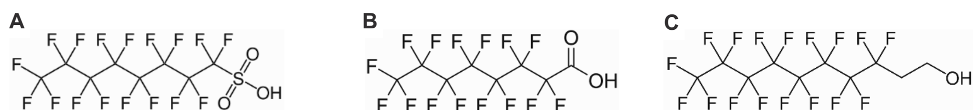



*Figure 3. An overview of different classes of PFCs. Carbon chain in PFCs can bind to A) sulfonic acids (PFOS), B) carboxylic acids (PFOA) and C) fluorotelomeralcohol (FTOH). Figure was adapted from [32].*

### 1.1.5 Perfluorooctanesulfonate (PFOS)

PFOS is the most predominant PFC in biota [34]. Previous publications have indicated that PFOS is the most concentrated PFC in human and wildlife brain tissue [32, 35] and has a half-life of up to 5.4 years in human blood serum [36]. Moreover, PFOS is known to be the most concentrated compound in Arctic wildlife, specifically in the liver of polar bears [37]. It has been shown that PFOS can be detected as linear or branched isoforms with different distributions. For instance, a study in polar bears showed that branched PFOS was predominant in the liver and blood, but not in the brain, muscle, or fat tissue, suggesting isoforms differ in their affinity to bind with locally specific proteins [38].

## 1.2 Toxicological effects of POPs

Although POP production has declined recently, different levels of them have been widely detected in human serum and breast milk from all over the world [39]. POPs have relatively high elimination half times in blood serum of both human and wild life and can cause a variety of toxic effects via different pathways including, enzyme induction, alteration in intracellular signalling, gene expression and disruption of the endocrine system [21].

The main targets of BRCs are the liver and thyroid hormone homeostasis [40]. For instance compounds including, BDE-47, 99, 209, TBBPA and BPA all induced thyroid toxicity at the

gene expression level and affect transcripts of phase II hepatic metabolizing enzymes in zebrafish [41]. In addition to thyroid and hepato toxicity, some studies have linked behavioural and reproductive toxicity to BRCs [42, 43].

It has been shown that planar and non-co-planar isoforms of PCBs induce toxicity via different pathways [20] and could directly or indirectly, play an important role in cancer development [44]. In addition, some of the PCBs can interfere with thyroid hormone function [45], can have anti-androgenic effects and result in a feminizing phenotype in males [46], increases the risk of cardiovascular disease as well as type 2 diabetes [47, 48], suppress the immune system [49], and are associated with an increased risk of asthma [50].

A wide range of toxicity effects have been associated with the perfluorinated compounds. It has been shown that the toxicity of PFCs is dependent on the length of the carbon chain and type of functional group. For instance, compounds with longer carbon chains or attached to sulfonic acid groups are more toxic than compounds with shorter carbon chains or attached to a carboxylic group [51, 52]. PFCs with fluorotelomer groups mainly metabolize to compounds with a carboxylic group [53, 54]. Exposure to PFCs in animal models has been linked to hepatotoxicity [55] and immunotoxicity [56] and changes in testosterone [57], oestrogen [58] and thyroid hormones [59]. Human epidemiological studies have revealed that PFC levels are correlated with thyroid function [60, 61], increased low-density lipoprotein (LDL) [62] and prostate cancer [63]. PFOS as a member of the perfluorinated compounds is known to be an endocrine disruptive compound [64], which can cause reproductive toxicity [65, 66], immunotoxicity [56, 67] and hepatotoxicity [55, 68]. Previous studies have reported that PFOS exposure could cause neurotoxicity and behavioural alterations in mice, rats and zebrafish [69-74].

## 1.3 Mixture effects of POPs

For many years, researchers have investigated the toxicity of single or a small group of different contaminants using different *in vitro* and *in vivo* models. However, humans are exposed to a complex mixture of different chemicals including POPs simultaneously, as different chemicals of concern have been detected in human blood and breast milk samples from all over the world [39]. Although different classes of compounds could compete with each other and show antagonistic effects, the greatest concerns regarding mixture exposures is low dose effect [75]. Chemicals in low dose effect have potential additive and synergic

effects, which refer to effects that are equal to or greater than the sum of the individual effects, respectively [75, 76].

In a study investigating the effect of maternal exposure to PCB (118, 138, 153, 180), *p,p'*-DDE and methyl mercury (MeHg) on neurophysiological response in 7 years children, a weak correlation was observed between PCBs, *p,p'*-DDE and endpoints. This borderline correlation was disappeared after adjusting the statistical model for MeHg [77]. In another study, a binary mixture of pesticides including, permethrin and cypermethrin, caused higher morphological defects, greater gene effects and more oxidative stress in zebrafish larvae, compared to the added effects of single chemical exposures [78]. Another study showed that bisphenol AF is more potent and caused more endocrine disrupting effect in zebrafish larvae when co-exposed with the antibiotic sulfamethoxazole [79]. Furthermore, androgen receptor (AR) antagonists at a very low individual concentration, showed additive effects when applied in a mixture scenario [80]. A study on adult rats exposed to a mixture of PBDE-47 and PCB153 revealed that, these compounds could have additive effects on developmental neurotoxicity [81]. Therefore, investigating the toxicological effects of POP mixtures is more environmentally relevant than studying the toxicity caused by a single POP.

## 1.4 Maternal exposure to POPs

POPs have been detected in human serum and breast milk in different locations, which can be considered as a major concern for fetus [82-86]. Although the placenta acts as a protective biological barrier that minimizes the transfer of POPs to the fetus, evidence suggests that even POPs with high molecular weights can penetrate through the placenta, a process that leads to the accumulation of POPs in the fetus. For example, a study conducted by the Environmental Working Group (EWG) revealed a total of 287 different chemicals including BRCs, CLCs and PFCs in umbilical cord blood, which among them 208, 217 and 180 compounds are well known to cause developmental toxicity, neurotoxicity and cancer, respectively [87]. In another study, the concentration of PFCs in cord blood was highly correlated with the levels in mother's blood at the time of delivery [88]. The same findings were reported for PFOA, PFOS, and PCBs [89]. Results from mice studies have indicated that following maternal exposure, PFOS accumulates in the brains of fetuses and pups, at concentrations higher than in the dams brain [90].

Neonates are quite sensitive to the different chemicals mainly because of detoxification mechanisms, which are not well developed during the pregnancy and even at the time of delivery and POPs, by crossing the placental barrier and excretion to breast milk, can target fetuses and neonates and cause various toxicological effects [91]. For instance, maternal plasma levels of PCBs, PBDE and DDT were significantly correlated with lower birth weight in both males and females in humans [92] and children prenatally exposed to PCBs showed higher degrees of $CD3^+$ T cells and activated B cells [93].

## 1.5 Neural development in humans and the role of the endocrine system

Human central nervous system (CNS) development is a complicated process that requires extension of the neurons to their exact location and establishing connections between different cell types and other neurons. CNS development starts with developing the ectoderm into the neural plate at 2 weeks post gestation (WPG). At gestational day (GD) 18, the neural plate further differentiates into the neural groove, which at the end of 3 WPG forms the neural tube. From approximately 26 ± 1 GDs, the neural tube starts to develop further into distinct parts including the telencephalon (forebrain), mesencephalon (midbrain), rhombencephalon (hindbrain) and spinal cord. At GD 48 – 51, different brain ventricles are developed and the CNS further matures via different processes including, proliferation, migration, differentiation, synaptogenesis, gliogenesis, myelination, and apoptosis [94].

Thyrotropin-releasing hormone (TRH) is released by the hypothalamus and stimulates thyroid-stimulating hormone (TSH) secretion from the anterior lobe of the pituitary, which triggers thyroid hormones (THs) production including, thyroxine (T4) and triiodothyronine (T3), by the thyroid gland in a high and lesser extent, respectively. THs bind with specific proteins such as thyroxin-binding globulin (TBG), transthyretin (TTR) and albumin. They reach different tissues and iodothyronine deiodinases enzyme converts T4 to T3, which is known as the functional form of TH [95]. At the end, both T3 and T4 exert a negative feedback on both the hypothalamus and the pituitary and regulate TSH release [96].

A large number of studies have highlighted the role of the maternal thyroid system in early brain development of the fetus. For instance, THs regulate different genes involved in neural proliferation and cell cycles in brain tissue [97]. It has also been shown that T3 regulates neuronal migration in the neocortex and cerebellum via regulating the expression of Reelin

protein [98, 99]. THs by activating neurotrophin brain-derived neurotrophic factor (BDNF), enhance the differentiation of neurons including, oligodendrocytes, astrocytes and microglia [95]. Moreover, THs are essential for the myelination of proteins by glial cells [100] and the normal maternal level of T3/T4 is important for regulating the levels of gamma-aminobutyric acid (GABA), monoamines (norepinephrine, epinephrine, dopamine and serotonin) and acetylcholinesterase (AchE) activity in offspring [101].

Neural cells communicate with each other as well as with other types of cells via synapses and the release and uptake of neurotransmitters (NT) via presynaptic cells. NT systems are developed from the mid of pregnancy in humans and continues later [94]. Some of the NTs are important in brain development and behaviour. For instance, acetylcholine (Ach) activates muscles, however, in the brain it has an inhibitory effect on the likelihood of action potentials being created by neurons. GABA and glutamate are known as major inhibitory and excitatory (increasing the likelihood of an action potential by neurons) neurotransmitters, respectively. Furthermore, dopamine is involved in motivated behaviour such as food and drug seeking and regulates insulin and nor epinephrine (important in the response to stress) release. Histamine is another NT, which regulates gut motility and its secretion decreases Ach and serotonin [102]. Serotonin is important in motor function and mood. Many of the antidepressant drugs known as selective serotonin reuptake inhibitors (SSRIs), which act via blocking the serotonin reuptake protein and increasing the level of serotonin molecules in synapses, improve mood, increase motor activity and induce anxiety like behaviour [103].

## 1.6 POPs and neurobehavioural toxicity

Although CNS development continues after birth, different populations of neural cells grow in a tight time window of pregnancy, when detoxification mechanisms are not well developed. As discussed previously, the placenta is not able to protect the fetus completely from unwanted chemicals and the blood brain barrier is not fully developed until 6 months after birth [104]. Moreover, neural cells have low repair capability [94] that makes them sensitive to a variety of environmental contaminants; hence, any damages could have notable long-term consequences.

Exposure to different chemicals during early life has been found to increase the susceptibility to diseases in later life stages. Although it is challenging to discuss the time of

exposure (early life stages) and observed behavioural effects in later life stages. However, a higher risk of attention deficit hyper-activity disorder (ADHD) in school-aged children has been found to occur with higher levels of PFCs in their plasma [105, 106] and parental PCBs [107]. In addition, children with higher levels of maternal PBDE had poorer mental development and a lower intelligence quotient (IQ) at school age [108]. As reviewed here [108], maternal exposure to BPA could be associated with neurobehavioural disorders such as depression and aggressive behaviour in children, however the effect is both age and sex dependent.

Neurobehavioural toxicity following POPs exposure has been reported in several animal models including, the chicken embryo [109], mice [110], rat [111, 112] and zebrafish [43, 113]. To date, the underlying cellular and molecular mechanisms in neurotoxicity following POPs exposure is not well understood. However, researchers investigating the effect of POPs in animal models have suggested that POPs could induce neurotoxicity via impairing neurotransmitter levels, intracellular signalling, or hormonal haemostasis [114].

Regarding to the neurotransmitter function, it has been reported that POPs could induce neurotoxicity and result in behavioural change via impairing the cholinergic [115], dopaminergic [69, 116, 117] and serotonergic [65] systems. In addition, POP exposure could interfere with intracellular signalling. For instance, BDE 47 and PFOS disrupted $Ca^{2+}$ haemostasis [118, 119] and exposure to Aroclor 1254 (a chlorinated mixture) as well as PFOS interfered with $Ca^{2+}$ mediated signalling [120, 121]. Another study revealed that PFOA and PCBs changed protein kinase C activity in brain tissue [109, 122] and exposure to PCBs altered membrane potential [123]. Moreover, the function and development of the brain are regulated by the endocrine system and specifically THs [124, 125]. It has been documented that PCB exposure induced neurotoxicity via a reduction in maternal and offspring serum T4 levels [126] and reductions in circulating thyroid hormones via occupying the TTR [127]. PBDE 47 depressed thyroid development and at the same time negatively affected the long-term learning and memory in rats [81].

## 1.7 Male reproduction and the role of the endocrine system

As depicted in Figure 4, gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus in a pulsatile mode and leads to luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from the anterior lobe of the pituitary, which binds to

the corresponding receptors in Leydig and Sertoli cells, respectively. In response to LH, Leydig cells secrete testosterone (T), which promotes spermatogenesis and is further metabolised to dihydrotesterone (DHT) and estradiol (E2) by Sertoli cells. Furthermore, Sertoli cells supply the nourishment for spermatogenesis and in response to FSH, they secret inhibin, which acts as a negative feedback on the anterior lobe of the pituitary and suppresses FSH secretion. In addition to inhibin, T, DHT, and E2 exert a negative feedback on the hypothalamus and suppress GnRH secretion, in turn LH/FSH are decreased [128].

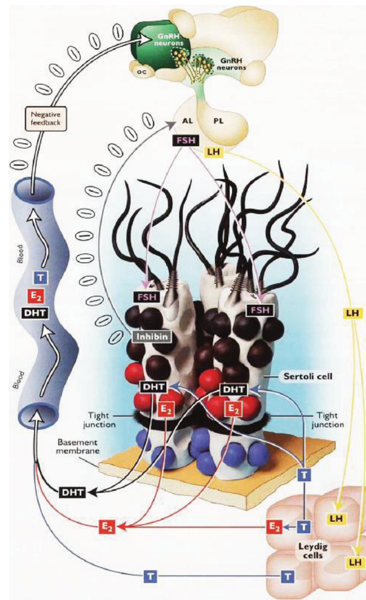



*Figure 4. An overview of the regulation of spermatogenesis via hormones secreted from the hypothalamus and pituitary. Image taken from* [128].

Spermatogenesis, taking place in the seminiferous tubules, consists of three distinct stages including, proliferation, meiosis, and differentiation and leads to the production and release of sperm cells into the lumen of seminiferous tubules [128]. Within spermatogenesis, spermatogonia undertake a series of mitotic divisions producing intermediate spermatogonia, which further develop to B spermatogonia via mitotic division. Primary and secondary spermatocytes raise via mitotic division from B spermatogonia and meiosis I division from primary spermatocytes, respectively. Secondary spermatocytes undertake meiosis II division and further develop to spermatids by taking a differentiation step [128].

One of the most important phenomena that takes place in the differentiation process is sperm DNA condensation, which is necessary for normal morphology of the sperm head, protecting the genomic material from damage and playing an important role in epigenetic regulation [129]. During DNA condensation, sperm DNA histones are replaced by protamines [130]. The histone/protamine ratio is critical for sperm cell function and it has been shown that sperm cells with less protamines and DNA condensation are more susceptible to chemical damage [131]. Sperm DNA integrity, which can be evaluated using the sperm chromatin structure assay (SCSA), is essential for successful fertilization as well as normal development of the fetus [132]. In SCSA, sperm cells are stained with acridine orange (AO), which emits green and red fluoresces after binding with double and single strand DNA, respectively [133]. Different useful parameters can be obtained via SCSA including, the DNA fragmentation index (DFI), which is calculated based on the ratio of red fluorescence sperm cells to total green and red fluorescence sperm cells, % DFI which is sperm cells with a moderate or high DFI and percentage of high DNA stainability (% HDS) that indicates sperm cells with less condensed DNA [133]. Today, SCSA is one of the most common procedures for determining the sperm DNA integrity and its relationship to fertility. For instance, it has been shown that human sperm cells with a DFI of $> 27\%$ and an HDS of $\geq 15\%$ are significantly correlated with a lower fertility rate [134-136].

## 1.8 POPs and reproductive toxicity

Along with industrialization, the incidence of testicular and breast cancer has increased consistently and the human sperm count in both North America and Europe has decreased persistently over the last 60 years [137]. It has been suggested that the endocrine disruptive properties of POPs make them able to cause reproductive toxicity [138]. An increasing body of evidence suggests that POPs, via activating on the apoptosis pathway, interfere with spermatogenesis and steroidogenesis could cause reproductive toxicity [139].

It has been shown that environmental contaminants and POPs can change the balance between pro-oxidant and antioxidant defence systems in the testis, increase reactive oxygen species (ROS) production, promote apoptosis in germ cells and decrease the number of sperm cells [140]. In addition to increasing the apoptosis rate in germ cells, POPs could trigger the apoptosis pathway in Sertoli cells, which nourish the germ cells, and result in a lower sperm count [141]. In addition to apoptosis in germ cells and Sertoli cells, POPs could

induce apoptosis in Leydig cells as well. As an example it has been shown that PFOS exposure triggered apoptosis in Leydig cells in rat offspring [66] and mice [142].

Environmental pollutants can also affect steroidogenesis in males. For instance, it has been reported that both BPA and PFOS decreased the testosterone level in rats [66, 143], whereas exposure to Aroclor 1254, a technical mixture of PCBs, stimulated testosterone production in rats [144].

Furthermore, it has been suggested that POPs can alter the protein structure in testis compartments. For instance, BPA affected the gap junction in the blood-testis barrier [145] and impaired the communication between Sertoli cells by altering the distribution of gap junction protein like connexin 43 [146].

In addition, reproductive toxicity could happen via targeting sperm DNA condensation and integrity. The main mechanism behind sperm DNA fragmentation is oxidative stress and ROS production [147]. It has been reported that some POPs such as PCBs [148] and BDE-209 [149] are able to induce sperm DNA fragments via enhancing oxidative stress and ROS production. However, sperm DNA damage and breaks in strand(s) may be caused by different internal/external factors as well [150].

## 1.9 Zebrafish as a model organism in toxicology

Zebrafish (*Danio rerio*) are originally from India and belong to the family Cyprinidae [151]. The last decade has seen a growing trend towards using zebrafish as an ideal laboratory model in different fields such as toxicology and biomedicine [152, 153]. This model organism shows great advantages over other laboratory animals. For instance, zebrafish have a small body size and can be raised in a relatively small animal facility. The developmental process of zebrafish is quick, most organs being fully developed by 96 hpf, and adults are sexually mature in 3 months. Spawning of the eggs is triggered by light; therefore, hundreds of synchronized embryos can be obtained at one time. These embryos are transparent and this allows researchers to observe the developmental process [152]. Larvae fully depend on the yolk until 7 day post fertilisation (dpf), therefore chemical exposure can be done without any unwanted food effect [154]. Another advantage is that the genome has been fully sequenced and different transgenic lines exist that can be used in order to validate different physiological pathways [155]. In addition, highly conserved signalling pathways are found

between zebrafish and humans with a high level of genomic homology which facilitates the translation of results from zebrafish to humans [156].

### 1.9.1 Neurodevelopment in zebrafish

As reviewed by [157], zebrafish neurodevelopmental stages can be divided into three distinct process including neurogenesis, axonogenesis and development of neural subtypes.

Development of the CNS starts with neuroectoderm differentiation at 6 hpf [158] and by 10 hpf the neural plate develops from the neuroectoderm [157]. During the primary and secondary neurulation process, the neural plate forms the neural tube by 12 hpf [159]. By 16 hpf, the neural tube will further differentiate and develop into the telencephalon, diencephalon, mesencephalon, and rhombencephalon [160]. During axonogenesis, a network of early axon "pioneers" will develop by 24 hpf [159]. The zebrafish brain further develops into the forebrain, diencephalon and telencephalon [161] and by 48 hpf the brain ventricles will develop [162]. From 48 hpf onward, neural subtype populations will develop. Serotonergic cell populations can be tracked from 48 hpf onwards [157]. Glutamic acid decarboxylase, an enzyme responsible for GABA formation, is expressed 2-3 dpf [163]. Cholinergic neurons can be identified at 3 dpf [164]. By 85 hpf, the first histaminergic neurons can be identified in the ventral hypothalamus [165] and the dopaminergic neuron population develops by 4 dpf [166]. Glutamatergic neurons can be identified by 4–5 dpf [167]. It has been shown that blood–brain barrier (BBB) development is undertaken between 3 to 10 dpf in zebrafish larvae and the BBB is similar to that in mammals, both structurally and functionally [168].

### 1.9.2 Locomotor activity in zebrafish larvae

Zebrafish locomotor activity first starts with coiling and a series of full body contractions at 18 hpf [169]. At 24 and 48 hpf, zebrafish larvae are able to twitch and perform a tail-flip behaviour in response to mechanical stimulation [170]. It has been reported that robust locomotor activity can be observed at the beginning of 5 dpf [161] and at this age a high number of larvae can be simultaneously studied in a high-throughput system, which produces a huge volume of data and increases statistical power [171].

Zebrafish larvae as a model organism in behavioural work have several advantages. For instance, it has been documented that the larval zebrafish nervous system displays a high

degree of structural and pharmacological conservation to mammals [172, 173] and zebrafish larvae are able to display a range of useful and quantifiable behaviour responses [174]. In addition, this model has a number of similarities with humans that make it ideal to study neurotoxicity. For instance, zebrafish have a great similarity to humans in terms of brain anatomy (for, mid and hindbrain), the peripheral nervous system with the associated components, enteric and autonomous nervous system, sensory organs (eye, ear, olfactory system) and exhibit different behavioural responses (social, memory and conditional) [175]. Moreover, zebrafish brain shares significant similarity in neurochemistry and pathological pathways to humans [176]. During the last years, several types of behavioural assays have been developed to evaluate the larval behavioural response in depth. For example, the startle C-bend turn [177] or the dark flash induced O-bend turning behaviour [178] are well established and used by different laboratories. For these reasons, the larval zebrafish behavioural assay has been introduced as a sensitive method to investigate the sub lethal effects of different environmental contaminants [179].

### 1.9.3 Locomotor activity following light/dark transition

This test has been developed to assess the locomotor activity in 4 to 7 dpf zebrafish larvae and known as a good indicator of neurotoxicity that might cause by different chemicals [180]. Zebrafish larvae are usually distributed in multi-well plates and subjected to alternating light and dark periods (10–20 min) in an automatic tracking system (Figure 5), which tracks larval activity based on a given threshold of movement during alternative light and dark phases [180]. Zebrafish larvae typically show freeze behaviour during the lighted period of the test, but high activity in response to the transition into darkness before a gradual reduction in activity over time [181]. Different locomotor activities can be recorded following the light/dark transition such as the distance moved, the number of times the larvae were active, and the swimming speed [51].

Although the number of papers using light/dark behavioural assay in zebrafish increases daily, as demonstrated in Table 1, different laboratories have applied different protocols in terms of larval age, arena/well plate size and alternating light/dark periods. However, in some cases, the protocol used is not well described and it has been shown that some of these differences are known to have consequences on the levels of basal activity [182]. A question that needs to be addressed is how these different protocols could affect the results in risk

assessment studies? In addition, how these contrasting results can be interpreted and translated to the realistic scenario?

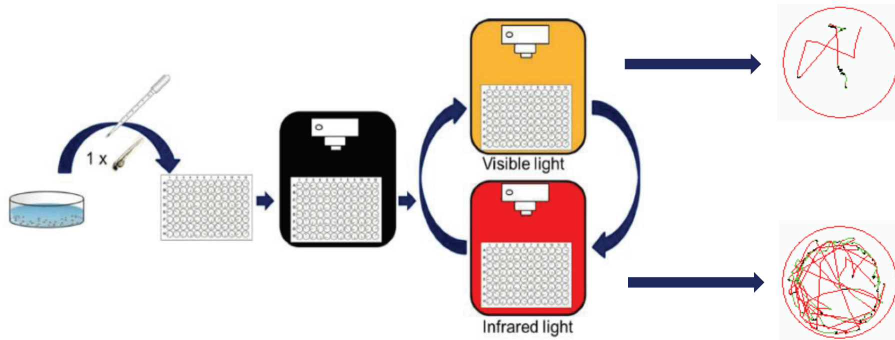



*Figure 5. An illustration regarding the experimental procedure and the dark/light behavioural test in zebrafish larvae. During alternative visible and infrared light (dark) periods, zebrafish larvae movement can be tracked. Figure adapted from [180].*

*Table 1. An overview of different protocols in light/dark transition assay in order to assess the zebrafish larval locomotor activity following exposure to different chemicals. D: dark, L: light, N.R: not reported.*

| **Larval age (dpf)** | **Plate** | **Volume of media (µl)** | **Alternating light/dark periods setup Acclimation → test (min each)** | **Reference** |
|---|---|---|---|---|
| 5 | 48 | 500 | L (180-240) → D / L / D / L / D / L / D (10) | [183] |
| 5 and 7 | 24 | 500 | L (10) → L (40) / [L (10) / D (5)] ×3 | [184] |
| 5 | 96 | N.R. | N.R. → D / L / D / L (10) | [185] |
| 6 | 96 | 500 | D (10) → L / D / L / D (10) | [186] |
| 5 | N.R. | N.R. | L (2) → L / D (4) | [187] |
| 5 | 24 | N.R. | L (30) → D / L / D (10) | [188] |
| 5 | 96 | N.R. | D (N.R.) / L / D / L (10) | [189] |
| 5 | 96 | 100 | L (20) → L / D / L / D / L / D (10) | [190] |
| 5 and 6 | 24 | N.R. | L (10) → L / D / L / D / L / D (20) | [74] |
| 6 | 48 | 750 | L (10) → D / L / D / L (10) | [51] |

## 1.10 Mice as a model organism in toxicology

Mice are the most used animal model in research and have several distinct advantages over other rodents. For instance, mice are small, easy to handle, have a short generation period of around 10 weeks, and an extensive amount of literature exists on their physiology and biochemical properties [191]. Mice have a set of unique criteria including, placentation, intrauterine development and lactation, which make them a useful model in toxicological studies and more specifically in reproductive toxicology [192]. It must be noted that the clearance rates for many toxicants have been shown to be higher in mice than in humans [193]. Therefore, in order to have good results for translation in to the human scenario, exposures need to be set at relatively higher levels compared to humans. However, a large amount of literature exists on using the mouse model to evaluate the reproductive toxicity of POPs. For instance, it has been shown that mice maternally exposed to PCBs had lower testis weight and reduced seminiferous diameter and sperm viability [194]. Another study revealed that *in utero* exposure to BDE209 resulted in an increase in offspring sperm hydrogen peroxide ($H_2O_2$) and DFI [149].

### 1.10.1 Spermatogenesis in mice

In mice, spermatogenesis consists of 16 steps, of which 12 take place in the seminiferous epithelium and steps 1-8 cover the round spermatid developmental stage, whereas steps 9-16 cover the elongation and differentiation process [195]. It has been shown that spermatogenesis is one of the targets for environmental toxicants. For instance, di-(2-ethylhexyl) phthalate exposure in mice significantly affected the number of germ cells in the F3 generation [196] and exposure to Aroclor 1254, significantly decreased the germ cell and sperm count [197].

# 2. Knowledge gaps and aim of the thesis

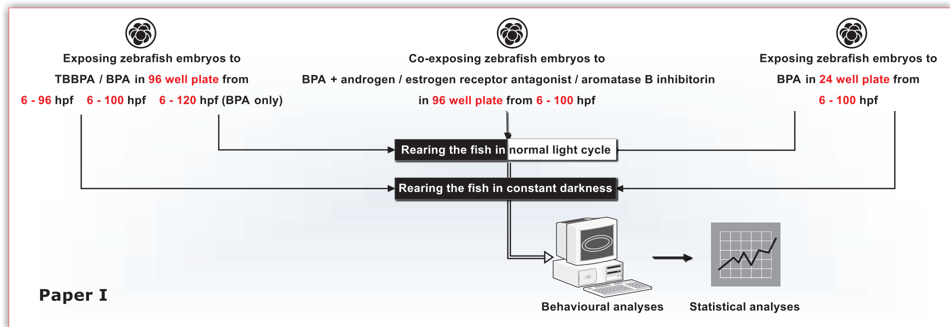
In recent years, there has been an increasing interest to assess the locomotor activity in zebrafish larvae following a light-dark transition as a useful endpoint to assess the sub lethal effects of different chemicals. However, despite the popularity of the zebrafish larval behavioural assay, no standard protocol has been developed and different publications reported different protocols [180]. The first aim of this thesis was to:

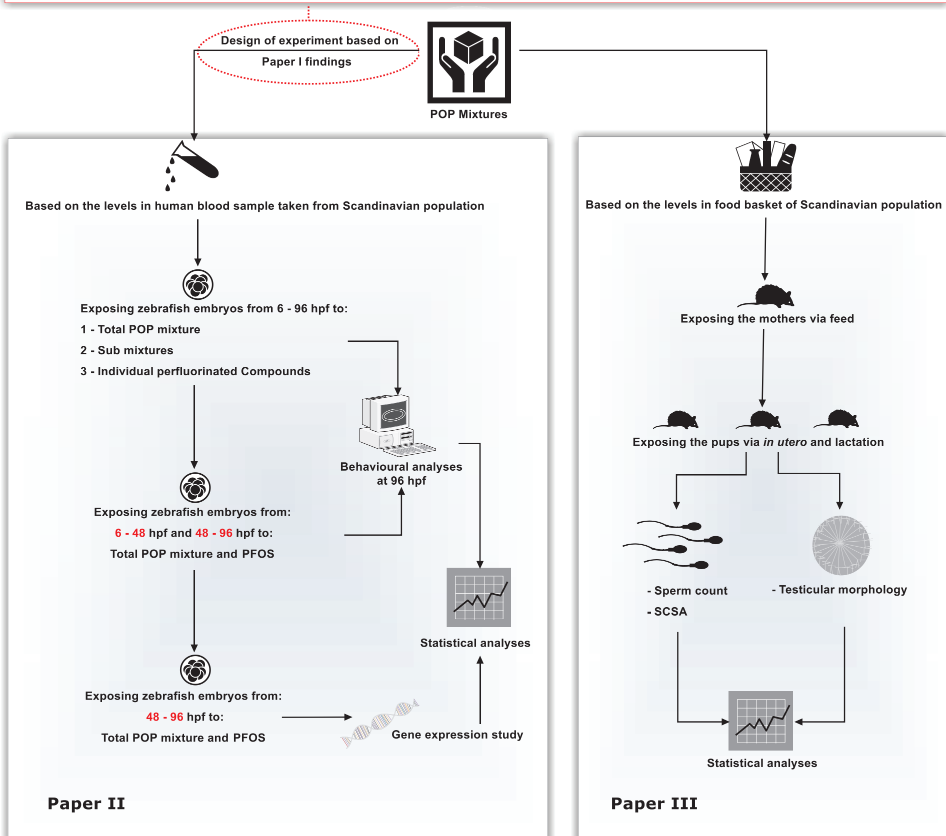
- Assess whether protocol manipulation can influence toxin responses in the zebrafish behavioural assay (**Paper I**).

Persistent organic pollutants (POPs) are widespread throughout the environment. Some of these POPs are reported to cause reproductive toxicity [198, 199] and neurobehavioural toxicity [117, 200]. In real daily life, humans are exposed to a complex mixture of POPs simultaneously. However, to date, studies conducted on the role of POPs in reproductive and neurobehavioural toxicity, have generally only considered single chemicals and not mixtures of them. How different chemicals in mixture scenarios exert toxicological effects is not clear, but it has been shown that chemicals in mixture scenarios can have additive [201, 202] or antagonistic effects [203]. Therefore, it is worth investigating how a mixture of POPs based on the actual level in a food basket and human blood samples could induce reproductive and neurobehavioural toxicity. This thesis aimed specifically to investigate:

- How a POP mixture, and its sub mixes, based on the individual levels in human blood samples in a Scandinavian population could induce neurobehavioural toxicity in zebrafish larvae (**Paper II**).
- How a POP mixture based on the individual levels in the Scandinavian food basket could induce reproductive toxicity in offspring mice (**Paper III**).

# 3. An overview of the thesis



# 4. Materials and methods

Within this thesis, in order to investigate different toxicological endpoints, a series of high-throughput and well established methods have been used. Details about protocols were given in the corresponding papers. Here only the composition of the POP mixtures is provided.

## 4.1 *In vitro* POP mixture

The *in vitro* mixture was developed at NMBU based on the mean of reported values of the different congener/substances in human blood taken from the Scandinavian population, reported in publications before 2012 [204]. In this thesis, the *in vitro* mixture was used in **paper II** and the concentration of the different compounds is presented at Table 2.

*Table 2. The composition and concentrations of chemicals used for in vitro POP mixture. Values reflecting the concentrations equal to 1× human plasma levels. (Pf) Perfluorinated mixture; (Br) Brominated mixture; (Cl) Chlorinated mixture; (Pf + Br) binary mixture of perfluorinated and brominated compounds; (Pf + Cl) binary mixture of perfluorinated and chlorinated compounds; (Br + Cl) binary mixture of brominated and chlorinated compounds. Table was adapted from [204].*

| Compounds | Concentration (nM) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Total** | **Pf** | **Br** | **Cl** | **Pf + Br** | **Pf + Cl** | **Br + Cl** |
| **Perfluorinated compounds (PFCs)** | | | | | | | |
| PFOA | 10.923 | 10.923 | | | 10.923 | 10.923 | |
| PFOS | 54.801 | 54.801 | | | 54.801 | 54.801 | |
| PFDA | 0.962 | 0.962 | | | 0.962 | 0.962 | |
| PFNA | 1.723 | 1.723 | | | 1.723 | 1.723 | |
| PFHxS | 7.873 | 7.873 | | | 7.873 | 7.873 | |
| PFUnDA | 0.990 | 0.990 | | | 0.990 | 0.990 | |
| **Polybrominated diphenyl ethers (PBDEs)** | | | | | | | |
| BDE-47 | 0.018 | | 0.018 | | 0.018 | | 0.018 |
| BDE-99 | 0.007 | | 0.007 | | 0.007 | | 0.007 |
| BDE-100 | 0.003 | | 0.003 | | 0.003 | | 0.003 |
| BDE-153 | 0.001 | | 0.001 | | 0.001 | | 0.001 |
| BDE-154 | 0.003 | | 0.003 | | 0.003 | | 0.003 |
| BDE-209 | 0.011 | | 0.011 | | 0.011 | | 0.011 |
| HBCD | 0.038 | | 0.038 | | 0.038 | | 0.038 |
| **Chlorinated compounds (CLCs) including:** | | | | | | | |
| **Polychlorinated biphenyls (PCBs)** | | | | | | | |
| PCB 28 | 0.050 | | | 0.050 | | 0.050 | 0.050 |
| PCB 52 | 0.034 | | | 0.034 | | 0.034 | 0.034 |
| PCB 101 | 0.024 | | | 0.024 | | 0.024 | 0.024 |
| PCB 118 | 0.196 | | | 0.196 | | 0.196 | 0.196 |
| PCB 138 | 0.615 | | | 0.615 | | 0.615 | 0.615 |
| PCB 153 | 1.003 | | | 1.003 | | 1.003 | 1.003 |
| PCB 180 | 0.490 | | | 0.490 | | 0.490 | 0.490 |
| **Other organochlorines** | | | | | | | |
| p,p`-DDE | 1.578 | | | 1.578 | | 1.578 | 1.578 |
| HCB | 0.410 | | | 0.410 | | 0.410 | 0.410 |
| α-chlordane | 0.026 | | | 0.026 | | 0.026 | 0.026 |
| Oxy-chlordane | 0.051 | | | 0.051 | | 0.051 | 0.051 |
| Trans-nonachlor | 0.092 | | | 0.092 | | 0.092 | 0.092 |
| α-HCH | 0.020 | | | 0.020 | | 0.020 | 0.020 |
| β-HCH | 0.182 | | | 0.182 | | 0.182 | 0.182 |
| γ-HCH (Lindane) | 0.020 | | | 0.020 | | 0.020 | 0.020 |
| Dieldrin | 0.063 | | | 0.063 | | 0.063 | 0.063 |

## 4.2 *In vivo* POP mixture

The *in vivo* mixture was previously developed at the Norwegian University of Life Science (NMBU). This POP mixture was designed based on the estimated daily intake (mg/kg/day) for humans in a Scandinavian population and constructed according to the reported mean values for a 70 kg person in publications prior to 2012 [204]. In this thesis, the *in vivo* mixture was used in **paper III** and the concentration of the different compounds is presented in Table 3. The *in vivo* POP mixture was given as a part of the feed to the mothers from weaning, during pregnancy and lactation. Therefore, pups were exposed *in utero* and via lactation for a total of 6 weeks. After weaning, feeding was continued using a reference diet that contained no POPs.

*Table 3. The composition and predicted concentrations of the in vivo mixture added to the feed. Values reflect the concentrations equal to 5000× human daily intake (low dose) and 100 000× human daily intake (high dose). Concentrations (ng) indicate the nominal concentration of compounds per (g) feed. Table was adapted from [204].*

| Compounds | ng/g feed | |
|---|---|---|
| | Low Dose | High Dose |
| **Perfluorinated compounds (PFCs)** | | |
| PFOA | 18.3 | 366.7 |
| PFOS | 10.8 | 216.7 |
| PFDA | 7.9 | 158.3 |
| PFNA | 5.8 | 116.7 |
| PFHxS | 4.9 | 97.5 |
| PFUnDA | 4 | 80 |
| PFDoDA | - | - |
| PFTrDA | - | - |
| **Polybrominated diphenyl ethers (PBDEs)** | | |
| BDE-47 | 40.4 | 808.3 |
| BDE-99 | 7.9 | 158.3 |
| BDE-100 | 6.3 | 125 |
| BDE-153 | 1.3 | 25 |
| BDE-154 | 2.5 | 50 |
| BDE-209 | 62.5 | 1250 |
| HBCD | 12.5 | 250 |
| **Chlorinated compounds (CLCs) including:** | | |
| **Polychlorinated biphenyls (PCBs)** | | |
| PCB 28 | 5.8 | 116.7 |
| PCB 52 | 13.8 | 275 |
| PCB 101 | 23.3 | 466.7 |
| PCB 118 | 40.4 | 808.3 |
| PCB 138 | 57.5 | 1150 |
| PCB 153 | 57.5 | 1150 |
| PCB 180 | 15.4 | 308.3 |
| **Other organochlorines** | | |
| p,p`-DDE | 119.6 | 2391.7 |
| HCB | 50 | 1000 |
| α-chlordane | 37.5 | 750 |
| Oxy-chlordane | 12.5 | 250 |
| Trans-nonachlor | 12.5 | 250 |
| α-HCH | 21.7 | 433.3 |
| β-HCH | 17.5 | 350 |
| γ -HCH (Lindane) | 23.8 | 475 |
| Dieldrin | 75 | 1500 |

# 5. Results and summary of the papers

## 5.1 Paper I:

**Toxin induced behavioural aberrations in larval zebrafish are dependent on minor methodological alterations**

Alterations in zebrafish motility are used to identify neurotoxic compounds, but few have reported how methodology may affect results. To investigate this, we exposed embryos to bisphenol A (BPA) or tetrabromobisphenol A (TBBPA) before assessing larval motility. Embryos were maintained on a day/night cycle (DN) or in constant darkness, were reared in 96 or 24 well plates (BPA only), and behavioural tests were carried out at 96, 100, or 118 (BPA only) hours post fertilisation (hpf). We found that the prior photo-regime, larval age, and/or arena size influence behavioural outcomes in response to toxin exposure. For example, methodology determined whether 10 µM BPA induced hyperactivity, hypoactivity, or had no behavioural effect. Furthermore, the minimum effect concentration was not consistent between different methodologies. Finally, we observed a mechanism previously used to explain hyperactivity following BPA exposure does not appear to explain the hypoactivity observed following minor alterations in methodology. Therefore, we demonstrate how methodology can have notable implications on dose responses and behavioural outcomes in larval zebrafish motility following identical chemical exposures. As such, our results have significant consequences for human and environmental risk assessment.

## 5.2 Paper II:

**A mixture of persistent organic pollutants and perfluorooctanesulfonic acid induces similar behavioural responses, but different gene expression profiles in zebrafish larvae**

Persistent organic pollutants (POPs) are widespread in the environment and some may be neurotoxic. As we are exposed to complex mixtures of POPs, we aimed to investigate how a POP mixture based on Scandinavian human blood data affects behaviour and neurodevelopment during early life in zebrafish. Embryos/larvae were exposed to a series of sub-lethal doses and behaviour was examined at 96 hours post fertilization (hpf). In order to determine the sensitivity window to the POP mixture, exposure models of 6 to 48 and 48 to 96 hpf were used. The expression of genes related to neurological development was also assessed. Results indicate that the POP mixture increases the swimming speed of larval zebrafish following exposure between 48 to 96 hpf. This behavioural effect was associated with the perfluorinated compounds, and more specifically with perfluorooctanesulfonic acid (PFOS). The expression of genes related to the stress response, GABAergic, dopaminergic, histaminergic, serotoninergic, cholinergic systems and neuronal maintenance, were altered. However, there was little overlap in those genes that were significantly altered by the POP mixture and PFOS. Our findings show that the POP mixture and PFOS can have a similar effect on behaviour, yet alter the expression of genes relevant to neurological development differently.

## 5.3 Paper III:

**Maternal exposure to a mixture of persistent organic pollutants (POPs) affects testis morphology, reduces sperm cells and induces sperm DNA fragmentation in mice**

Persistent organic pollutants (POPs) are widespread throughout the environment and some are suspected to induce reproductive toxicity. As animals and humans are exposed to complex mixtures of POPs, it is reasonable to assess how such mixtures could interact with the reproductive system. Our aim is to investigate how maternal exposure to a mixture of 29 different persistent organic pollutants, formulated to mimic the relative POP levels in the food basket of the Scandinavian population, could alter reproductive endpoints. Female mice were exposed via feed from weaning, during pregnancy and lactation in 3 exposure groups (control (C), low (L) and high (H)). Testicular morphometric endpoints, epididymal sperm concentration and sperm DNA integrity (in sperm from both vas deferens and cauda epididymis) were assessed in adult male offspring. We found that the number of tubules, proportion of tubule compartments and epididymal sperm concentration significantly decreased in both POP exposed groups. Cauda and vas deferens sperm showed different trends in the sperm chromatin structure assay. Nevertheless, epididymal sperm from both POP exposed groups showed increased DNA fragmentation. It is concluded that maternal exposure to a defined POP mixture relevant to human exposure can affect testicular development, sperm production and sperm chromatin integrity.

# 6. Discussion

## 6.1 Methodological considerations

### 6.1.1 Choice of TBBPA and BPA

The intention in **paper I** was to investigate whether we could replicate the results of other labs using the same compounds, and to what extent the methodology may influence repeatability. Therefore, as the POP mixture is unique, we used two compounds for which more than one study on larval zebrafish behaviour existed with contrasting outcomes, TBBPA and BPA. We then tested the reproducibility of data and how different parameters could affect behaviour in zebrafish larvae.

### 6.1.2 Zebrafish larvae behavioural assay

Zebrafish larval behaviour upon exposure to similar chemical(s) is not always consistent across the literature and the reasons behind these inconsistencies are not clear. In order to translate larval behaviour into realistic scenario one needs to understand how the assay works. Therefore, we decided to investigate the factors that might explain the inconsistent results; hence, different parameters in the behavioural assay were investigated in **paper I**. Most of the studies reported larval age as day post fertilization, which is not precise as the zebrafish develops rapidly. Therefore, different time points during 5 dpf were tested including 96, 100 and 118 hpf. Furthermore, different labs employ different photo-regimes during larval rearing, with larvae typically kept either in complete darkness or on a day/night cycle. As the prior light regime could affect the circadian rhythm [205] and development of the circadian rhythm is a key element in regulating behaviour [206], two photo-periods during rearing were compared, including a normal day/night cycle and total darkness. It has been suggested that the level of activity following light/dark transition could be related to anxiety level and anxious larvae display thigmotaxis (proximity to the arena wall) [207]. Therefore, in order to test whether contrasting toxicity results could be explained by the level of anxiety, zebrafish larvae were also tested for the level of thigmotaxis following rearing on a day/night cycle or in constant darkness.

Based on the findings in paper I, the larval zebrafish behavioural assay in **paper II** was undertaken according to the following criteria. Zebrafish larvae were kept in 96 well plates

instead of 24 well plates because the 96 well plates gave a better opportunity to include more concentrations at the same time on one plate. It has been shown that zebrafish larvae baseline locomotion during the dark phase of the test is not stable over the time of day and larvae show more activity in the morning compared to the evening [181]. Using 96 well plates also meant we were able to perform the test at a specific time point, which led to a reduction in variability and reduced any unwanted experimental effect. Furthermore, in order to reduce any possible general toxicity due to longer exposures, behavioural assays were conducted at 96 hpf. Lastly, in order to mimic a more realistic environmental scenario and provide the optimal conditions for larval development, larvae were reared on a day/night cycle.

### 6.1.3 *In vivo* and *in vitro* POP mixtures

Humans and animals are continually exposed to a variety of chemicals at the same time. It has been shown that different chemicals even at the non-observable adverse effect level can have additive and synergistic effects in mixture scenarios and the results from single compounds are not fully extensible to mixtures and realistic scenarios [208]. It is important to keep in mind that human and animals are exposed to mixtures of compounds both externally and internally. External dose is the dose that they are exposed to via oral, inhalation and skin routes whereas the internal dose is a dose that after absorption circulates via blood and distributes into the different tissues. Therefore, two different POP mixtures, one based on the level of compounds in a food basket of Scandinavian population (*in vivo* mixture) and the other one based on the levels of compounds in blood samples also taken from Scandinavian population (*in vitro* mixture), were previously made in the Reproductive and Environmental Toxicology group at NMBU.

The aim was to mimic the realistic scenario in human embryos and since zebrafish embryos are considered as a developmental model, and human embryos are developmentally exposed to chemicals via cord blood, we exposed the zebrafish embryo/larvae to the *in vitro* POP mixture to investigate the neurobehavioral effects (**Paper II**). On the other hand, in order to mimic the reproductive toxicity in human offspring, where mothers are exposed orally, mice were exposed through feed to the *in vivo* POP mixture and reproductive toxicity in their offspring was studied (**Paper III**).

### 6.1.4 Zebrafish and mice as experimental models

In the current thesis, the *in vivo* mixture was tested using a mouse model (**Paper III**). Reproductive toxicity in this thesis was a part of another bigger project, in which the effect of the *in vivo* mixture was tested on the behaviour, stress response and different organs including different parts of the brain, liver, adrenals and gut microflora. Since mice were initially used for the behavioural assay, they were treated in a way to minimize stress during pregnancy, lactation and rearing, therefore it was not possible to assess other reproductive toxicity endpoints. If the experiment had been planned specifically for reproductive toxicity, gonad sampling at the age of 3, 6 and 9 weeks in parallel with relevant hormone analyses would have been an ideal set-up, pups and testes would have been weighed and fresh sperm cells would have been used for motility assessment.

Our unpublished data indicate that the *in vivo* mixture alters the behavioural response in mice. The next aim was to screen the sub mixtures and individual compounds using zebrafish to identify which compounds may explain the neurotoxicity observed from the mouse work. As discussed before, the zebrafish behaviour assay is considered a high through put screening assay and good indicator of neurobehavioral toxicity for different compounds and chemicals. Therefore, in addition to the total *in vitro* POP mixture, different sub mixtures were tested using the zebrafish model (**Paper II**), which was not practical with the mouse model.

### 6.1.5 RT-qPCR

Quantitative real-time PCR (qPCR) is a sensitive method to assess gene expression in toxicological studies [209, 210]. Within this thesis, gene expression was analysed using qPCR and the number of used housekeeping genes was different in paper I and II. In **paper I**, all primers were designed based on previous and similar publications and gene expression data was normalized against *βactin1* and *ef1α* as the housekeeping genes. Although *βactin1* is the most used housekeeping gene in zebrafish studies [211], in order to get a reliable results in relative qPCR assay, it is necessary to have a stable reference gene under different experimental conditions. Since expression of genes may not remain stable even in untreated tissue [211], it is recommended to use more than one reference gene [212]. Therefore, in **paper II,** five different reference genes were evaluated using an online tool (Reffinder) for determining the best-suited reference genes. The Reffinder platform is a popular tool,

working on raw Cq values only, and allows testing the genes for stability based on three well-known algorithms including Bestkeeper, Normfinder and Genorm. Reference genes in paper II were selected based on Genorm algorithm because it has been shown that this algorithm is able to identify the appropriate number of reference genes while other algorithms are less sensitive and examines the stability of each single reference gene independently [213]. Genorm algorithm identified two stable genes (*ef1α* and *rps18*) instead of one single gene, which was suggested by Bestkeeper and Normfinder algorithms.

The q-PCR method used in this thesis has some drawbacks as well. For instance, it is difficult to discuss the causality relationship between gene expression and protein level for several reasons. Firstly, the steps involved in translating the mRNA into the proteins including the post modification process, are complicated and not well understood. Secondly, protein half-life is dependent on a series of conditions and in some cases proteins degrade quickly and lastly, despite growing technology, techniques identifying gene and protein expression are not precise and results contain a degree of error and noise [214]. Cell function is controlled by protein concentration not necessarily by gene expression and therefore, in future similar studies, it would be beneficial to include protein analyses in parallel with gene expression.

### 6.1.6 Sperm chromatin structure assay

Many publications have indicated that DNA fragmentation could be associated with exposure to different POP mixtures [215-217]. Different techniques have been developed in order to analyse the sperm DNA integrity including the sperm chromatin structure assay, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, Acridine Orange Test (AOT), Sperm Chromatin Dispersion Test (SCDT) and The Single Cell Gel Electrophoresis assay (SCGE, also known as comet assay) [133]. Mice offspring sperm cells in **paper III** were analyzed for DNA integrity using the sperm chromatin structure assay for some distinct reasons. Firstly, preparation of the samples for SCSA is fast compared to other methods. Secondly, SCSA is performed using flow cytometry, hence thousands of sperm cells can be evaluated in a few minutes resulting in a high $N$ for statistical analysis. Thirdly, SCSA is known as a precise method and variation is very small between different laboratories, allowing for repeatability of SCSA data. Fourthly, SCSA can evaluate sperm DNA fragmentation using multiple endpoints, and lastly SCSA data has been shown to have reasonable association with field fertility data [218].

Sperm motility is also known as a useful marker to assess male reproduction. It has been shown that environmental pollutants such as PCBs and BDEs alter sperm motility in humans [219, 220]. However, assessing sperm motility using methods like computer aided sperm analysis (CASA), demands fresh semen or semen diluted in protective extenders. Because of the load of the samples during necropsy, lack of appropriate extenders and the lack of a CASA expert, none of the mentioned endpoints was assessed in this thesis. Similarly, it has been shown that spermatogenesis is a target for environmental toxicants [221]. Assessing spermatogenesis process would be a good way to address the reduction in seminiferous tubules diameter/area. It is unfortunate that the study did not include any assessment of spermatogenesis process and ploidy analyses, mainly because of practical reasons. Considering the reported effects of POPs on sperm motility and spermatogenesis, including mentioned endpoints in future similar research would be beneficial.

### 6.1.7 Sperm count and testicular morphology

It has been shown that environmental toxicants in humans and different animal models could cause testicular toxicity and affect the number of sperm cells [222]. Sperm count and testis histology assessment are considered the classical and inexpensive routine tests for semen analysis and testicular toxicity, respectively. Sperm count as a final product of spermatogenesis in combination with evaluating the spermatogenesis unit (seminiferous tubules) will give us an appropriate overview of the exposure effect on spermatogenesis output. Within **paper III**, testicular morphology and sperm count were reported in mice offspring. Two different approaches were applied for evaluating the seminiferous tubules, the diameter and area measurements. The majority of studies have reported the diameter of the seminiferous compartment as a marker of testicular toxicity. However, seminiferous tubules are not completely round and epithelium thickness varies across each tubule, therefore diameter measures could be an approximate measure. An alternative for diameter is area measurements, where area occupied by seminiferous tubules itself and different compartments of it, including lumen and epithelium, can be precisely calculated.

### 6.1.8 Linear mixed model instead of analyse of variance (ANOVA)

In this thesis, behavioural data in **paper I** and **II** as well as reproductive endpoints in **paper III** were compared with control groups using linear mixed effect (LME) model. However, it seems more common to apply one-way ANOVA and Kruskal–Wallis analyses to compare

the endpoints in exposed groups with control in case of normally and non-normally distributed data, respectively. It has been shown that LME model has several advantages over ANOVA tests. For instance, missing data and non-homogenized variance from unequal sample size as well as correlated response in experimental unites (animals) are not well fitted into the ANOVA analyses [223]. LME model is suggested as an ideal method in animal research where sample sizes are not equal, some data is missing, or responses could be correlated [224]. The mixed term in LME model covers both dependent and random variables [225] and the main advantages of LME over ANOVA is its ability to include random effects in the analysis. Random variable is a set of possible values (such as test replicates) from an experiment that might have input on results.

In this thesis, endpoints were considered as dependent variables. However, since the behaviour assay is a sensitive measurement and biological replicates may affect the final output [182], test replicates were considered as random effects. Moreover, in our behavioural assay, some of the fish with normal morphological appearance did not respond to the light/dark transition, which resulted in some zero values that led to unequal variance, therefore LME model was considered as an option over ANOVA. In **paper III**, some of the pups were born from the same mother; and these could have some degree of correlated response. Therefore, in order to minimize the genetic and maternal effects; mothers were included as random variable. Considering mothers as a random effect in LME analyses not only will correct the maternal correlation but also reduces the number of animals used (if we consider only one pup per mother), which is in line with the Reduction of 3R principle. Moreover, some of the samples appeared to have a low population of sperm cells, therefore some of SCSA and sperm count results considered as outliers and excluded from final analyses, which resulted in unequal sample size, therefore LME analyses was employed instead of ANOVA.

## 6.2 General findings

### 6.2.1 Zebrafish behavioral assay as an indicator for neurotoxicity

In **paper I**, the behavioural toxicity of TBBPA and BPA under different experimental conditions was investigated. The neurotoxic effect of TBBPA has previously been reported

in zebrafish [226, 227], rat [228] and mice [229]. Similarly, exposure to BPA led to behavioural alterations in zebrafish [230, 231] and medaka [232].

Within paper I, we demonstrated the suitability and sensitivity of a zebrafish larvae behavioural assay to assess environmental contaminants. This assay is ideal for high-through put screening and provided us with highly repeatable results. These features can be useful especially in risk assessment studies where the safety of thousands of different chemicals is not yet documented. However, a difference in the lowest effective dose for both BPA and TBBPA was observed following different rearing conditions or larval age. Therefore, a question that needs to be addressed is, if protocol manipulation could give different results with the same toxin, how can we differentiate the toxicity of different compounds from experimental effects?

At this stage, it is difficult to determine the reasons that are behind the different behavioral responses upon exposure to the same compound. For instance, very little is known about how different rearing conditions could affect neural development, change larval physiology and the capacity to metabolize the chemicals, or enhance the degradation level of the compounds, which all may affect the behavioural outcome. On the other hand, locomotor activity is a complicated phenomenon and many other physiological and biochemical processes in sensory or endocrine systems are incorporated into the behavioural response [233]. All these knowledge gaps indicate that zebrafish locomotion and the mechanisms behind it are poorly understood and with all these uncertainties, interpreting zebrafish larval behavioural data needs to be done carefully and all these parameters need to be taken into consideration. As reviewed here [234], incorrect experimental set up in animal research is one of the main reasons that make data interpretation challenging. Furthermore, in order to assess the neurotoxicity of different environmental contaminants using zebrafish larval behaviour and in order to facilitate the reproducibility of experiments between different laboratories, a realistic experimental design with minimum effect on developmental process and a well described protocol is recommended.

Within **paper II** in this thesis, the neurobehavioural toxicity of a POP mixture consisting of 29 different compounds was investigated. Results indicated that the POP mixture increased the zebrafish larvae swimming speed starting at a concentration equal to 20× the human serum level, but at the same time decreased the time active and distance moved by larvae. This behavioural outcome in larvae was associated with perfluorinated compounds and more

specifically with PFOS. Neither the POP mixture nor PFOS induced hyperactivity in larvae when the exposure was undertaken before 48 hpf. These results suggest that PFOS potency is not affected by other compounds in the mixture and no mixture effect can be concluded regarding the behavioural aspect.

Our investigations in **paper I** revealed that behavioural toxicity may be dependent on methodology. Our pilot study showed that arena size and larval age did not affect the total mixture results. Therefore, it appears that the total mixture result is relatively robust. However, it is not possible to conclude whether similar observed neurobehavioral effects following POP/PFOS exposure would be observed again following protocol manipulation including rearing the larvae in complete darkness. Our results indicated that PFOS 70× increased swimming speed, while it affected only one of the investigated genes involved in neurobehavioural development. On the other hand, POP 10× had no effect on behaviour but affected the transcription level of genes involved in neuronal maintenance (*manf*), the stress response and endocrine regulation (*crhf*) and the histaminergic system (*hrh1*). These findings highlight the fact that a compound/mixture could have minimum neurotoxicity effect at a molecular level, while having an effect on a behavioural response or vice versa. Therefore, based on the experiences presented in this thesis, the zebrafish larval behavioural assay is a quick and affordable assay for chemical screening with a complex model organism, but at the same time, the results may be dependent on experimental conditions. Therefore, in order to understand whether a compound is neurotoxic or not, it is recommended to have another assay in parallel with the zebrafish larval behavioural assay.

The number of studies that investigated the behavioural responses in zebrafish larvae after exposure to a mixture of compounds are limited and in most of the cases they applied different experimental protocols compared to ours and analysed the behavioural responses differently [235], that might explain the differences in the results and make the data comparison challenging. Unfortunately, there is no way to say which of these protocols is more realistic, therefore translating the results to humans and interpreting the results in risk assessment studies must be performed conservatively.

Gene expression data revealed that although the POP mixture and PFOS resulted in the same behavioural outcome, the POP mixture affected a greater number of genes involved in different neural pathways, whereas PFOS affected only one gene. This could be explained by the presence of brominated and chlorinated compounds in addition to the perfluorinated in

our total POP mixture, possibly indicating a synergic or additive effect of compounds in a mixture setting. Similar mixture effects have been described after exposing human hepatoma cells (HepG2) to a mixture of PAHs and individual compounds within that mixture [236]. Results indicated that individual compounds in a mixture scenario showed an additive effect on apoptosis and on cell cycle blockage while had both additive and antagonistic effects in a gene expression assay.

### 6.2.2 Reproductive toxicity following exposure to *in vivo* POP mixture

Reproductive toxicity caused by the *in vivo* POP mixture has been shown in **paper III**. Results indicated that the POP mixture at both low and high concentrations significantly changed testis morphology, reduced epididymal sperm count and induced sperm DNA fragmentation.

Importantly, we demonstrated that maternal exposure to a mixture of POPs based on the levels in human food basket can induce reproductive toxicity in offspring. Reproductive toxicity in offspring following POPs exposure previously has been showed in other mammalian models, such as swine [237], sheep [238], mice [194] and rats [239]. Comparison between mixture studies and our results also need to be done in a conservative way since we had different classes of compounds and those studies that applied mixture; either used different individual compounds within the mixture or investigated the effects on a different animal model. As described previously, different mechanisms of action have been linked between POPs exposure and reproductive toxicity. Due to the agonistic or antagonistic effects of different compounds in a mixture setting, it is difficult to discuss which compound(s) mediated the reproductive toxicity in our mixture scenario. Similarly at this point, we cannot conclude any mixture effect. However, as mentioned before, because of practical reasons, it was not possible to break down the mixtures and expose the mice to individual compounds.

## 6.3 Relevance and limitations

Most of the previous publications have highlighted the importance of the chemical toxicity topic-using single or a small group of chemicals and within specific cell lines or animal models. However, due to the complex physiochemical cascades in the body, results obtained via cell line studies are often not easy to translate to the realistic scenario. The current thesis

depicted a more realistic scenario to mimic the human exposure using two well-known animal models for neurotoxicity and reproductive toxicity.

Based on previous studies, the concentrations of TBBPA and BPA in **paper I** were environmentally relevant. The maximum plasma level of TBBPA and BPA were reported as 2.3 nM and 16.4 nM, in a Norwegian and Swedish population, respectively [240, 241]. Moreover, urinary BPA has been reported even up to 11µM in 6-10 years Danish children [242]. However, we have shown that the toxicity results can be different depending on methodology. In this case, result interpretation must be done carefully since an environmental relevant level could turn out as toxic or not, depending on methodology.

In **paper II**, it has been documented that the POP mixture at a nominal concentration equal to human plasma levels had no effect on zebrafish larvae behaviour. However, significant behavioural effects were observed at 20 times the human plasma level. Although the uptake assay indicated that only around 10% of the PFOS within 100× the total POP mixture accumulated in the larvae. These results might be explained by poor epithelial permeability or biotransformation mechanisms and efflux transporters that decreased internal concentration of compounds in zebrafish larvae [243]. However, we cannot estimate how much PFOS accumulated in brain tissue or how much was just stuck to the larvae on the outside, the same conclusion is expandable to the rest of the chemicals within the POP mixture.

The gene expression study revealed that the POP mixture even at a concentration equal to 10 times higher than the human scenario can significantly affect the transcription level in some important CNS and endocrine genes. Although expression of the majority of genes was affected at a concentration equal to 70 times higher than human plasma level, this concentration could be important because it provides us with new insight into the pathways involved in neurotoxicity.

In **paper III**, the POP mixture resulted in reproductive toxicity in mice offspring. Although the doses given to the mice were higher compared to the estimated human daily intake, the plasma level for the majority of POPs in the low exposed mice ranged at a comparable level (1 – 20 times higher) to the human plasma level. Furthermore, the concentration of perfluorinated compounds was close to the reported values in polar bears [30]. By comparing the given doses and those measured in the offspring, it can be hypothesized that a portion of

the given POPs metabolized in mothers, a portion may have been prevented by the placenta and a degree could have been metabolised by the offspring. In addition, it is important to keep in mind that mice were exposed *in utero* and during lactation period (3 weeks) and after weaning they were put on a diet with no added POPs and the measurement was performed 6 weeks after weaning, which may have affected the actual concentration at the time of experiment.

In papers II and III, we have shown that both *in vitro* and *in vivo* POP mixtures could significantly induce neurotoxicity and reproductive toxicity. Although both mixtures were unique in terms of their chemical composition, effects were observed in high concentrations of the POP mixtures. However, those concentrations (70× higher than human serum level in paper II and low dose or 5000 times than human estimated intake in paper III) still could be environmentally relevant for several reasons. Firstly, both mixtures were designed based on the average values of the normal population, many individuals or sub-groups could have higher POP levels compared to the normal population. Secondly, as mentioned before, all tested concentrations were nominal and the actual concentrations of these chemicals might have been affected by practical steps of preparing the solutions (stuck to pipette tips etc.). Lastly, the mixtures were designed based on the levels in a Scandinavian population with relatively good environmental protection policy. It has been reported that the levels of environmental pollutants such as DDT is higher in developing countries with less advanced environmental protection policy (600 and 1300 μg/kg human milk in western Europe and middle east, respectively)[244]. The concentration of brominated compounds has been found to be higher in American breast milk compared to European breast milk samples [245, 246].

Epidemiological studies have correlated behavioural endpoints such as ADHD in children to POP levels in mother's blood samples [247, 248]. However, these observations are not always significant [249, 250]. The same contrasting results have been reported for reproductive toxicity in children and POP levels in their mothers [251, 252]. However, there are important points that should be kept in mind when interpreting current findings and correlate those to realistic scenarios.

Human epidemiological research has its own limitations and it is not always easy to estimate the precise timing of exposure to environmental toxicants. For instance, studies that have reported the chemicals in cord blood, measured reproduction endpoints several years later in children [251]. On the other hand, maternal toxicant levels may not always be representative

of the amount of fetal exposure mainly because of protective barriers and the affinity of chemicals to distribute in different organs. Moreover, another fact that makes the data interpretation difficult is that only a small portion of chemicals have been identified as toxic substances to humans and for most of the other chemicals, there is no data available, which doesn't necessarily mean they are safe.

Another problem is the behavioural definition, which is not consistent across the studies. For example, In Paper II, we documented that the POP mixture starting at a concentration equal to 100× higher than human serum level increased swimming speed but at the same time decreased the distance moved and swimming time. Therefore, the POP mixture resulted in both hyperactivity and hypoactivity in larvae simultaneously, depending on what endpoints we examine. The same discussion is true for term "-like" which is widely applied in different behavioural studies such as "obsessive–compulsive disorder (OCD)-like" or "anxiety-like". As deeply reviewed here [234], this definition is simply not good enough to correlate such behavioural studies to the human scenario.

Moreover, human epidemiological research is based on correlational observations not causation and because of bioethical consequences; it is not possible to perform a causal study in humans. Therefore, researchers have to rely on animal data, which has its own limitations. For instance, human developmental does not run synchronously to that of animals, therefore sensitive time-windows would be different between them. Furthermore, there is a spices difference in physiology and ability in metabolizing the toxicants. For instance, zebrafish own a series of specific properties, which might be important in behaviour. For example, the cerebral hemispheres and cortex in the telencephalon is not well developed, zebrafish have their own specialized sensory organ named the lateral line and larvae are not able to display as complex behavioural responses as adult zebrafish/mammals [175, 253]. Zebrafish larvae display clear differences in terms of the toxicokinetics compared with mammals. For instance, although mammals are mostly exposed to POPs via an oral route, three routes of absorption have been suggested in zebrafish including, active oral absorption, passive absorption through gills and diffusion through the skin [254]. In zebrafish, oral exposure would not be an active route of exposure up until 72 hpf [255], gills become functional at about 21 dpf and before that, ions and oxygen uptake happen via skin until 7 and 14 dpf, respectively [256]. The same goes for the mice model, their 3D structure of seminiferous tubes is different to that of humans and the number of produced sperm cells per gram of testis is lower in the mouse model [257].

Taken all together, the current findings indicated that a human relevant mixture of POPs could induce toxicity in living animal models. Some aspects of neurobehavioral and reproductive toxicity in current thesis are previously described for other animal models, indicating that mechanisms that lead to behavioural aberration in zebrafish and reproductive toxicity in mice are comparable with other studies. Therefore, data presented in this thesis are worth to consider as valuable screening results that also revealed some potential physiological and molecular pathways associated with behaviour and reproductive toxicity following exposure to a realistic mixture of POPs.

# 7. Conclusion and future perspectives

This thesis has documented that firstly, basal locomotor activity in zebrafish larvae depends on rearing conditions, larval age, and/or arena size. Secondly, based on applied protocols, BPA caused hyper-, hypo-activity, or have no behavioural effect in zebrafish larvae. Finally, following methodological manipulation, a suggested mechanism did not appear to explain the hypoactivity following BPA exposure. One may consider behavioural activity as a screening method but as we showed, locomotor activity is highly dependent on methodology and behaviour and is a complicated phenomenon that is controlled not only by the CNS, but also by sensory and endocrine systems. Therefore, further studies need to be carried out in order to:

- Find out the interaction between experimental factors and chemical degradation or fish physiology and develop a standard protocol for screening the chemicals using zebrafish larval behaviour.
- Validate the zebrafish larval behavioural response using mechanistic approaches.

In addition, this thesis has shown that a complex POP mixture based on blood concentrations relevant to the human scenario and a single compound therein, PFOS, can induce a similar behavioural response in zebrafish larvae, but affect the expression of genes involved in neurodevelopment differently. Down regulation in genes involved in inhibitory mechanisms might explain the higher swimming speed. However, here we investigated the behavioural response in larvae and a limited number of the genes involved in neurobehavioural. Therefore, more research is required to determine how POP mixture/PFOS can cause behavioural abbreviation via:

- Assess the biological effects of affected genes via combined molecular and protein analyses.
- Validate the role of possible signaling pathways in behaviour via mechanistic approaches.
- Rearing the exposed larvae and observe the behavioural effect at the latter stages of life.

The third major finding in this thesis was that a complex POP mixture constructed based on the levels in the Scandinavian food basket, induced reproductive toxicity in mice offspring and resulted in a change in testis morphology, reduced sperm count and increased sperm DNA fragmentation. The POP mixture used in the current thesis consisted of brominated, chlorinated and perfluorinated compounds, which are all categorized as endocrine disruptive compounds (EDCs). EDCs are able to act as agonists or antagonists for a variety of nuclear and membrane receptors, hence interfering with hormone activity, gene/protein expression and induced reproductive toxicity [258]. Further research should focus on:

- Exploring other physiological and molecular endpoints that may affect by POP exposure including, androgenic hormones, spermatogenesis process, etc.
- Investigating sperm cells motility following exposure and determining the reproduction outcome after POP mixture exposure.
- Investigating the observed toxicological endpoints following exposure to the sub mixtures or individual compounds.

# 8. referencess

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# Appendix: Paper I, II and III

# Paper I

1 **Toxin induced behavioural aberrations in larval zebrafish are dependent on minor methodological**

2 **alterations**

3

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13 **Abstract**

14 Alterations in zebrafish motility are used to identify neurotoxic compounds, but few have
15 reported how methodology may affect results. To investigate this, we exposed embryos to bisphenol
16 A (BPA) or tetrabromobisphenol A (TBBPA) before assessing larval motility. Embryos were
17 maintained on a day/night cycle (DN) or in constant darkness, were reared in 96 or 24 well plates
18 (BPA only), and behavioural tests were carried out at 96, 100, or 118 (BPA only) hours post
19 fertilisation (hpf). We found that the prior photo-regime, larval age, and/or arena size influence
20 behavioural outcomes in response to toxin exposure. For example, methodology determined
21 whether 10 µM BPA induced hyperactivity, hypoactivity, or had no behavioural effect. Furthermore,
22 the minimum effect concentration was not consistent between different methodologies. Finally, we
23 observed a mechanism previously used to explain hyperactivity following BPA exposure does not
24 appear to explain the hypoactivity observed following minor alterations in methodology. Therefore,
25 we demonstrate how methodology can have notable implications on dose responses and
26 behavioural outcomes in larval zebrafish motility following identical chemical exposures. As such,
27 our results have significant consequences for human and environmental risk assessment.

28

29 **Keywords**: endocrine disruption; neurotoxicology; bisphenol A; tetrabromobisphenol A; brain;
30 locomotion

31 **1. Introduction**

32 The zebrafish (*Danio rerio*) is an established vertebrate model in developmental biology and
33 is becoming increasingly popular as a tool for identifying neurotoxic compounds. In particular, a
34 growing number of studies use larval zebrafish motility during light/dark cycles to test new and
35 existing compounds for neurotoxicity (i.e. Jarema et al., 2015; Noyes et al., in press). Importantly,
36 the results from such tests share a general agreement with those of other vertebrate models and
37 cell lines (Ali et al., 2012; Irons et al., 2010; Rubinstein et al., 2006; Ton et al., 2006), thereby
38 demonstrating the potential for zebrafish larvae in toxicity screening. However, compared to rodent
39 models, zebrafish behavioural research is still in its infancy and there is little standardisation
40 between studies on larval motility. This is a concern, as little is known about how alterations in
41 methodology may influence toxicity testing.

42 The most common test when using larval zebrafish is to assess motility during alternating
43 periods of light and dark at approximately 120 hours post fertilisation (hpf). Here, one expects
44 control larvae to show freeze behaviour during periods of bright light, but a sharp increase in
45 motility following the onset of darkness (Jarema et al., 2015). However, activity levels are known to
46 be influenced by the timing of the experiment. For example, larvae are typically reported to become
47 more active in the light with increasing age (i.e. Esch et al., 2012) and the level of activity during the
48 dark period is also known to change throughout the day (MacPhail et al., 2009). The reasons behind
49 these observations are unclear, but the larvae are growing rapidly and the brain is continuously
50 maturing during this life period (Wullimann & Knipps, 2000). To date, little information exists as to
51 the extent of these changes in basal activity on toxin responses.

52 There are several examples within the literature of inconsistent dose and/or behavioural
53 responses with the same compound. For example, the brominated flame retardant
54 tetrabromobisphenol A (TBBPA) was recently reported to reduce larval motility, from 64 nM (Noyes
55 et al., in press) up to 5 μM (Chen et al., 2016), whereas the plasticiser bisphenol A (BPA) has been

56 found to induce hyperactivity with peak activity at 100 nM (Kinch et al., 2015; Saili et al., 2012). In
57 contrast, Jarema et al. (2015) reported no behavioural effects of TBBPA following developmental
58 exposure at concentrations between 1.2-3.8 µM. Similarly, Wang et al. (2013) found BPA induced
59 hypoactivity between 1-15µM, whereas Saili et al. (2012) found no behavioural effects at 1 or 10
60 µM. These studies all used minor alterations in methodology in comparison to one another, which
61 included differences in larval age and the time of testing, as well as water temperature, photo-
62 periods before and during testing, strain, and the number of light cycles during the test, to name but
63 a few. Of particular note, Noyes et al. (in press) and Saili et al. (2012) reared their embryos/larvae in
64 constant darkness prior to behavioural testing (R. Tanguay, personal communication), whereas
65 Jarema et al. (2015) and Wang et al. (2013) used a day/night (DN) cycle during rearing. This is of
66 particular interest, as photoperiods play an important role in fish developmental biology and the
67 development of circadian rhythms (Hurd and Cahill, 2002), but the effect on toxicity testing remains
68 unknown.

69 Initially, our objective was to investigate whether larval age during day five (*i.e*. 96, 100, and
70 118 hpf) or the photo-regime during rearing, continuous darkness *vs.* a DN cycle, could influence the
71 behavioural effects on larval zebrafish motility exposed to different doses of either BPA or TBBPA.
72 Leading on from this, we explored whether a mechanism previously identified to explain
73 hyperactivity in larval zebrafish exposed to BPA, whereby BPA acted via androgen receptors to
74 induce aromatase B expression (Kinch et al., 2015), could explain the hypoactivity we observed when
75 using an alternative methodology. In addition, we also assessed whether rearing larvae in constant
76 darkness could influence the level of anxiety like behaviour compared to larvae reared on a DN cycle,
77 and whether the arena size could influence behavioural results in response to BPA exposure.

## 2. Methods

**2.1 Chemicals.** Stock solutions of TBBPA (97% purity, Sigma Aldrich), BPA (>99% purity, Sigma Aldrich), fluvestrant (ICI, >98% purity, Sigma Aldrich), flutamide (FLU, Sigma Aldrich), fadrozole hydrochloride (FAD, ≥98% purity, Sigma Aldrich), and 17α-ethinyl estradiol (EE2, Sigma Aldrich) were prepared in dimethyl sulfoxide (DMSO, high performance liquid chromatography grade, Sigma Aldrich). The final concentration of DMSO in all test concentrations of TBBPA, BPA, ICI, FLU, FAD, EE2, and the solvent control, was 0.01%.

**2.2 Fish husbandry.** The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences, Oslo, Norway. The unit is licensed by the Norwegian Animal Research Authority (NARA) (www.mattilsynet.no) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway.

AB wild-type zebrafish were maintained at 28°C under a 14:10 light/dark photoperiod at the Norwegian University of Life Sciences (NMBU), Oslo, Norway. Adult care and breeding was in accordance with the local protocols. To generate embryos, adults were placed in spawning tanks in the afternoon, the fish were spawned following the cessation of light (08:00) the next day, and the embryos collected (09:00) and maintained in embryo media (60 µg/ml Instant Ocean® sea salts) until the time of exposure.

**2.3 Chemical exposure.** Fertilised embryos were transferred into clear polystyrene 96-well plates (Nunc™ MicroWell™) and continuously exposed under static conditions to TBBPA, BPA, or the solvent control from 6-7 hpf until the time of testing (i.e. the larvae were tested with the chemical present in the media). For BPA, five nominal concentrations ranging from 1 nM to 10 µM and a solvent control were equally distributed across two 96 well plates (n=8/concentration/96 well plate).

103 For TBBPA, five nominal concentrations, ranging from 150 pM to 1.5 μM, and a solvent control were
104 equally distributed across one 96 well plate (n=16/concentration). For EE2, one nominal
105 concentration of 10 nM was used together with a solvent control, and equally distributed across a 96
106 well plate (n=12/concentration). We selected this dose of EE2 as it is reported to induce aromatase B
107 overexpression in larval zebrafish (Brion et al., 2012; Chung et al., 2011). The highest concentrations
108 of both TBBPA and BPA were below those found to be teratogenic. For co-exposures of BPA with ICI
109 (1 μM), FLU (6.18 μM), or FAD (1 μM), embryos were distributed over one 96 well plate
110 (n=8/treatment). To assess thigmotaxis, larvae were distributed in 24 well plates and embryos were
111 exposed from 6-7 hpf until the time of testing. Only one treatment (10 μM and 100 nM BPA for the
112 DN cycle and constant darkness, respectively), plus the solvent control were used per plate
113 (n=12/concentration/replicate). In addition, 1 ml of exposure media was added to each well of a 24
114 well plate, whereas only 200 μl of media was used in 96-well plates. Prior to and following the
115 exposure, embryos were reared in an incubator at 28°C. The light cycle within the incubator was
116 14:10 light/dark. Where complete darkness was required, the embryos were reared in the same
117 incubator as the embryos on a DN cycle, but the well plate was wrapped in aluminium foil. All
118 experiments were repeated six times.

119 **2.4 Larval behaviour.** Behavioural tests were conducted using a ViewPoint® Zebrabox and the
120 accompanying video tracking software (ViewPoint Life Sciences, Lyon, France). A difference of ≥ 5
121 pixels between each consecutive frame (25 frames per second) was set as the threshold for the
122 detection of movement. Behavioural testing was undertaken at three time points for BPA, 96, 100,
123 and 118 hpf, two time points for TBBPA, 96 and 100 hpf, and one time point for EE2, 96 hpf. These
124 corresponded to tests beginning 90 minutes (09:00), 330 minutes (13:00), and immediately after the
125 cessation of light in the incubator (07:30), for 96, 100, and 118 hpf, respectively. For each time point,
126 different larvae were used to assess behaviour (i.e. no individual larvae was tested more than once).
127 Larval behaviour, including the cumulative distance travelled and the time spent active per minute,
128 were simultaneously measured for all larvae on a plate during a light-dark-light cycle that lasted for a

129 total of 40 minutes and consisted of 20 minutes of light, 10 minutes of darkness, and a final 10
130 minutes of light. The light level was set to 100 % on the ViewPoint software during the lighted
131 periods, and 0% during the dark periods when infrared light is used to track larval activity. When
132 using 24 well plates, the arena was split into two zones, a centre zone and an outer zone, to assess
133 thigmotaxis as detailed in Schnorr et al. (2012). Previous work has demonstrated that 5 dpf larvae
134 treated with anxiolytic compounds are more active in the inner zone, whereas anxiogenic
135 compounds increase the amount of movement in the inner zone (Schnorr et al., 2012; Richendrfer et
136 al., 2012). After the behavioural test, the larvae were inspected with a stereo microscope to identify
137 dead or deformed larvae. Deformities included spinal aberrations, yolk sac or cardiac edema,
138 aberrations in pigmentation, and loss of equilibrium. As we only had one behavioural testing unit, it
139 was not possible to compare the motility of fish kept on the DN cycle vs complete darkness from the
140 same batch of embryos at the same age. Therefore, for experiments using 96 well plates different
141 batches of embryos were used for fish kept on a DN cycle as to constant darkness, but the same
142 breeding populations were used for both. In contrast, when using 24 well plates the same batches of
143 embryos were used for larvae reared on a DN cycle and those reared in constant darkness, but here
144 those reared on a DN cycle were tested at 99 hpf (12:15) and those reared in constant darkness at
145 100 hpf (13:00).

146 With BPA, a further set of tests were used to study the effect of dark acclimation on larvae
147 reared on a DN cycle. Here, embryos were exposed to a solvent control or 0.1, 1, or 10 µM BPA
148 across four plates ($N$ = 16 conc$^{-1}$ plate$^{-1}$) and maintained on a DN cycle at 28°C. Behavioural tests
149 were carried out between 100-102 hpf (13:00-15:00). At 2, 4, 6, and 24 hrs prior to behavioural
150 testing, one plate per time point was covered in aluminium foil to induce dark acclimation. Larval
151 motility was then assessed during a behavioural test consisting of 20 mins of light, and 5 mins of
152 darkness.

153 **2.5 RNA isolation and RT-qPCR (reverse transcription-quantitative PCR)**. Larvae that had been
154 exposed to a solvent control or 10 nM EE2 from 6-96 hpf, and undergone behavioural analysis at 96
155 hpf, were sampled for RT-qPCR. Total RNA was isolated from 11-12 larvae per biological replicate
156 using the QIAzol lysis reagent (Qiagen, Hilden, Germany) followed by on column purification by
157 NucleoSpin RNA kit (Macherey-Nagel) including a DNase treatment after RNA isolation according to
158 the manufacturer's instructions. Two biological replicates per each treatment and control were
159 used.

160 RNA concentration was measured using a NanoDrop 1000 (Thermo Fisher Scientific,
161 Wilmington, USA). All samples had an OD A260/A280 ratio of ≥ 2.0. A total of 200 ng of RNA was
162 used for cDNA synthesis reaction using a Tetro cDNA synthesis kit (Nordic BioSite, Norway), and 5 ng
163 was used in the qPCR reaction in triplicate per sample using Express SYBR GreenER SuperMix with
164 premixed ROX (Invitrogen) according to the manufacturer's recommendations. Transcript levels
165 were analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the standard
166 cycling program: 50 °C for 2 minutes, 95 °C for 2 minutes, 40 cycles of 95 °C for 15 seconds, and 60
167 °C for 1 minute, and the melting curve analyses were applied. The transcript levels of AroB and vtg
168 were analysed with beta actin 1 and elongation factor alpha included as housekeeping genes. Primer
169 sequences are presented in **Table S1**.

170 Negative controls with no added template were included for all primer pairs (no template
171 control), and no RT control reactions for each sample and each primer pair were run in qPCR in order
172 to check for genomic DNA contamination (no RT control). Initial analysis of the RT-qPCR data was
173 performed using RQ Manager 1.2 (Applied Biosystems). A standard deviation of ≤ 0.3 per triplicate
174 was accepted. The fold change was calculated by ΔΔCt method i.e. transcript levels relative to the
175 control and normalized to the two housekeeping genes.

176 **2.6 Statistical analysis.** Data were transferred to R version 2.15.0 (R Development Core Team,
177 http://www.r-project.org) for behavioural analyses. Individual larvae were considered as the

178 experimental unit. Prior to statistical analysis, visual exanimation of the data confirmed that the
179 dose responses to BPA and TBBPA followed similar trends between each independent experiment.
180 Significance was assigned at $P < 0.05$. All dead and deformed larvae were discounted for behavioural
181 analyses. For both compounds, only motility during the dark phased was analysed as movement was
182 minimal during the light periods. For BPA, only the initial 5 minutes of the dark period was analysed
183 for comparisons to previous work (Saili et al., 2012; Kinch et al., 2015) whereas for TBBPA we used
184 the entire 10 minutes of the dark period. For the same reason, we analysed the cumulative distance
185 moved for TBBPA, but the cumulative time spent active for BPA. For the thigmotaxis analysis, we
186 also analysed the percentage of the total distance moved in the outer zone as in Schnorr et al.
187 (2012).

188 Data was checked for normality following visual examination of plots (i.e. histograms and/or
189 q-q plots). Linear mixed effect models were used for all analyses. For parametric data, the
190 dependent variable was the cumulative time spent active for BPA or the cumulative distance
191 travelled for TBBPA. Where data was non-parametric, these data were ranked and the rank was set
192 as the dependent variable. Dose and larval age were included as categorical independent variables,
193 and replicate was included as a random effect to account for any variability between tests. In the
194 initial models, dose and age were allowed to interact. If there was no significant interaction, then
195 the model was simplified by removing the interaction. To assess individual doses to the controls, we
196 used the contrast results provided within R for linear models.

197 **3. Results**

198 3.1 Larval age and rearing photoperiod can give contrasting results

199 For larvae exposed to BPA and maintained in constant darkness, there was no dose effect or
200 interaction with larval age (**Fig. 1A**). When using a DN cycle, 10 µM BPA exposure resulted in
201 consistent hypoactivity, but there was no interaction between dose and larval age (**Fig. 1B**).

202 There was a significant interaction between dose and larval age for larvae exposed to TBBPA
203 and maintained in constant darkness. Specifically, the low doses of 1.5 and 15 nM tended to be
204 hyperactive at 96 hpf, but hypoactive at 100 hpf whereas the high dose of 1.5 µM had no real
205 tendency at 96 hpf, but was hypoactive at 100 hpf (**Fig. 1C**). When using a DN cycle, there was no
206 interaction between dose and larval age, but 150 nM and 1.5 µM resulted in hypoactivity (**Fig. 1D**).
207 Therefore, the lowest observed effect concentration was 150 nM when using a day/night cycle,
208 compared to 1.5 nM when using constant darkness.

209 3.2 Dark acclimation gives contrasting results from day/night cycles

210 As using constant darkness vs a DN cycle led to contrasting dose responses, we wanted to
211 understand whether a short period of dark acclimation could modify the results obtained following
212 DN rearing. Therefore, we reared larvae on a DN cycle, but used a period of dark acclimation of
213 between 2-24hrs prior to behavioural testing. We found that using this methodology we were
214 unable to detect any behavioural effects of BPA exposure on zebrafish larvae (**Fig. 1E**).

215 3.3 Bisphenol A: testing a proposed mechanism

216 Previously Kinch et al. (2015) found co-exposure with the androgen receptor antagonist FLU
217 could rescue a behavioural effect attributed to BPA exposure, but co-exposure with the estrogen
218 receptor antagonist ICI did not. In contrast, were unable to rescue BPA induced hypoactivity with
219 either FLU or ICI, nor the aromatase inhibitor FAD (**Fig. 1F**). Indeed, the addition of FLU led to a
220 significant interaction effect with a greater reduction in activity compared to BPA alone. As a positive

221 control for aromatase B induction, we also exposed larvae to the synthetic estrogen 17α-ethinyl
222 estradiol (EE2). However, there was no effect on behaviour in 96 hpf larvae following exposure to 10
223 nM EE2 even though the transcript levels of the molecular markers for estrogenic exposure
224 vitellogenin (vtg) and aromatase B (AroB) were upregulated compared to controls (**Figure S1**).

225 3.4 Methodology influences the behaviour of the controls

226 We had determined that the photo-regime experienced during larval rearing could influence
227 our behavioural outcomes in larvae treated with BPA and TBBPA, but it was unclear why. However, it
228 was clear that the photo-regime prior to behavioural testing had pronounced effects on the
229 behaviour. For example, the total time spent active was significantly greater in those larvae reared
230 on a DN cycle compared to those in constant darkness irrespective of larval age. In addition, larvae
231 reared on a DN cycle and given a 2-24 hr period of dark acclimation where characterised by an
232 intermediate level of activity (**Fig. 2A**). Larval age was also found to influence behaviour as we found
233 consistent increases in the distance moved with age in larvae reared in constant darkness, but no
234 such trend in larvae reared on a DN cycle (**Fig 2A**). Furthermore, a large percentage of those larvae
235 maintained in constant darkness remained quite static in that they failed to move more than one
236 body length (> 4 mm) during the dark period of the behavioural test, but this effect decreased with
237 age (25, 12, and 5% at 96, 100, and 118hpf, respectively). In contrast, in larvae reared on a DN cycle
238 < 1% of the population were static during the dark period at any given age. In addition, larvae reared
239 on a DN cycle showed peak activity at minute 21, which is immediately after the onset of darkness
240 during the behavioural test (**Fig. 2B**), whereas those larvae reared in constant darkness showed peak
241 activity at minutes 23-26, several minutes after the onset of darkness (**Fig. 2C**). Finally, when
242 considering the lighted period of the behavioural test, irrespective of the rearing photo-regime,
243 larvae at 118 hpf began to show low levels of movement compared to the freeze behaviour shown
244 at 96 and 100 hpf (**Fig. 2BC**).

245 3.5 Thigmotaxis and arena size

246 As general activity is typically associated with the level of anxiety (Kalueff et al., 2013), we
247 asked whether keeping larvae in constant darkness *vs.* DN cycle could influence any other endpoint
248 of anxiety. Therefore, we assessed the degree of thigmotaxis, or wall hugging, in five-day-old larvae.
249 Consistent with our previous findings, larvae reared in constant darkness spent significantly less time
250 moving than those larvae maintained on a DN cycle also when using 24 well plates (**Fig. 3A**).
251 However, there was no effect of rearing photo-regime on the level of thigmotaxis (**Fig. 3B**).
252 Unexpectedly, in DN reared larvae, 10 µM BPA exposure in a 24 well plate resulted in hyperactivity
253 (**Fig. 3A**) in contrast to our previous finding of hypoactivity when using 96 well plates (see **Fig. 1B**).
254 Furthermore, BPA treated larvae showed significantly greater levels of thigmotaxis (**Fig. 3B**). When
255 using constant darkness, 100 nM BPA had no effect on activity levels or thigmotaxis.

256 **4. Discussion**

257 We found methodology to have a significant influence on the outcome of a larval zebrafish
258 assay when assessing behavioural responses to two known endocrine disruptors. Of particular
259 importance to toxicity testing, we found both larval age and the photo-regime resulted in significant
260 changes in the minimum toxic effect concentration for both compounds tested. In addition, the
261 same concentration of a given compound could increase, decrease, or have no effect on motility in
262 larvae of the same age depending on methodology. These results demonstrate the importance of
263 accurately recording and standardising methodology when conducting behavioural tests. In addition,
264 these results highlight the challenges of translating behavioural data from larval zebrafish to other
265 vertebrate models and their potential role in the risk assessment of new and existing compounds.

266 A major objective for toxicity testing is to determine lowest effect concentrations. We found
267 this value was dependent on methodology. Taking TBBPA as an example, the lowest effect
268 concentrations ranged from 1.5 nM to 150 nM. With BPA, we found significant effects following
269 exposure to 10 µM when using a DN cycle, but not when using constant darkness or a period of dark
270 acclimation following DN rearing. Furthermore, both compounds had age- and photo-regime specific
271 effects. Such differences have serious implications on the translation of large-scale screen studies
272 that rely on only one time point and/or photo-regime (Jarema et al., 2015; Noyes et al., in press). For
273 example, we have demonstrated that minor alterations in methodology determined whether or not
274 BPA tested positive for behavioural effects.

275 We found marked behavioural differences between non-treated DN and constant darkness
276 reared larvae. Most notably, compared to larvae reared on a DN cycle, those larvae reared in
277 constant darkness did not show a startle response, were slower to reach their peak levels of activity,
278 and a sizeable percentage of the population displayed no activity at all following the onset of the
279 dark test period. We could not associate these behavioural effects with any gross differences in
280 morphological staging between the two photo-regimes. Previously, Kazimi and Cahill (1999) also

281 reported that dark rearing had no gross effect on morphological staging. However, DN cycles are
282 essential for establishing behavioural rhythmicity in zebrafish larvae from as early as 2 dpf (Hurd and
283 Cahill, 2002) and light exposure from 2 dpf is required in order to establish the rhythmic expression
284 of clock genes (Hurd and Cahill, 2002), cell cycles (Dekens, 2003) and melatonin levels (Kazimi and
285 Cahill, 1999) in zebrafish. Rearing in constant darkness is also known to influence visual behaviour
286 (Bilotta, 2000) and long-term survival. For example, Villamizar et al. (2014) found that rearing larvae
287 under constant darkness up until 5 dpf, when they were transferred to a DN cycle, resulted in a
288 significant increase in mortality compared to controls at 30 dpf. Finally, it is unclear what effect the
289 photo regime itself may have on the behaviour of the test compound within the media, for example
290 its rate of degradation. Therefore, further work is required in order to understand whether these
291 factors may be influencing toxicity testing.

292 Of particular interest is how our alterations in methodology led to significant differences in
293 behavioural outcomes. For example, BPA induced hypo- or hyper-activity, or had no effect at all
294 dependent on methodology. Of particular note, we are unable to explain why 10 µM BPA leads to
295 hyperactivity following testing in a 24 well plate, but hypoactivity when using a 96 well plate.
296 However, well size can influence the degree of basal activity with larvae maintained in the larger
297 wells of a 24 well plate moving more than when maintained in a 96 well plate (Padilla et al., 2011).
298 We also provide evidence that BPA induced motility effects may have several modes of action. That
299 is, whereas androgen receptors and aromatase B overexpression appear to be associated with the
300 mechanism behind BPA induced hyperactivity in larvae reared in constant darkness (Kinch et al.,
301 2015), we found no support for this pathway in BPA induced hypoactivity in DN reared larvae.
302 Similarly, we found the potent aromatase B inducer 17α-ethinyl estradiol had no effect on larval
303 zebrafish behaviour when using a DN cycle. However, in support of Kinch et al. (2015), we were also
304 unable to rescue the BPA induced behavioural response with an estrogen receptor antagonist.

305 Larval zebrafish are viewed as an alternative to animal testing, including within
306 developmental neurotoxicity assessment (Coecke et al., 2007). Numerous behavioural endpoints are
307 available with this model, but how does one interpret an "effect" seen within zebrafish larvae to
308 human neurotoxicity? Here, it is generally agreed that as long as the molecular basis between the
309 two biological systems are similar, as it is between zebrafish and humans, then if one sees an
310 "effect" in zebrafish it is likely a similar effect will occur in humans (Coecke et al., 2007). However,
311 we highlight that the behavioural outcomes in zebrafish larvae can be contrasting or non-existent
312 depending on methodology. A lack of standardisation and validation has been identified as a barrier
313 to the broader acceptance of small fish models within toxicology (Planchart et al., 2016). Therefore,
314 there is a need to determine how alterations in methodology alter basal behaviour in order to gain
315 more insight into the mechanistic pathways and relevance of potential neurotoxins.

316 In conclusion, we found a dramatic effect of methodological practices on behavioural
317 outcomes, not only following exposure to toxic compounds, but also in basal activity levels. Notably,
318 the same concentration of a compound could lead to hyperactivity, hypoactivity, or have no
319 behavioural effect, depending on methodology. As such, inconsistencies within the literature may
320 reflect a lack of standardisation in methodological practices. We believe our results exemplify a need
321 for greater transparency in all methodological practices. In addition, we recommend further
322 validation of this test system in order to understand how differences in methodology influence basal
323 activity as this may improve our ability to translate larval zebrafish behaviour into other animal
324 models and thereby refine risk assessment.

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396 **Figure legends**

397 **Figure 1**. **Locomotor activity in larval zebrafish exposed to bisphenol A (BPA) and**
398 **tetrabromobisphenol A (TBBPA).** Dose responses were influenced by larval age and/or rearing
399 photo-regime. In (E), all larvae were originally reared on a DN cycle, but were given a period of dark
400 acclimation from 2-24 hrs prior to behavioural testing at 100-102 hours post fertilisation (hpf). In (F),
401 larvae were reared on a DN cycle and co-exposed to 10 µM BPA and either 6.18 µM flutamide (FLU),
402 1 µM fadrozole (FAD), or 1 µM fulvestrant (ICI) before behavioural testing at 98 hpf. In (A-E), an
403 asterisk indicates an overall significant effect (LME, $P$ <0.05) of dose compared to the control,
404 whereas subscript letters indicate a significant interaction between dose and larval age. In (F), all
405 BPA larvae showed significantly less activity compared to controls (not indicated on the graph for
406 clarity), and the subscript letter indicates a significant interaction effect between the control (in this
407 case FLU) and co-exposure with BPA. Values are means ± SE. For (A-E), $n$ 90-96 for all groups, for (F),
408 $n$ = 46-48 for all groups.

409 **Figure 2**. **A comparison of the controls between different methodologies and larval ages**. (A) The
410 time spent active during the initial 5 minutes of the dark period of the behavioural test. Larvae were
411 reared on either a day/night cycle (DN) or), under constant darkness (CD), or reared on a DN cycle
412 with a period of dark acclimation of between 2-24 hrs (DA). Different lower case letters indicate a
413 significant group effect (LME, $P$ < 0.05). Larvae were tested at 96, 100, or 118 hours post fertilisation
414 (hpf). Values are means ± SE, $n$ = > 90 group. The distance moved in the controls during the
415 behavioural test in larvae previously reared on (B) a day/night cycle or (C) in constant darkness and
416 tested at different hpf. Values are means ± SE, $n$ = 92-190 minute$^{-1}$ age$^{-1}$.

417 **Figure 3. Locomotor activity and thigmotaxis in larval zebrafish reared in 24 well plates**. (A) Time
418 spent active and (B) thigmotaxis in larvae reared on a DN cycle (DN) and exposed to 10 µM bisphenol
419 A (BPA), or reared under constant darkness (CD) and exposed to 100 nM BPA. Asterisk represents a

420 significant effect of either the rearing photo-regime or BPA (LME, $P < 0.05$). Values are means ± SE, $n$

421 = 70-72 for all groups.

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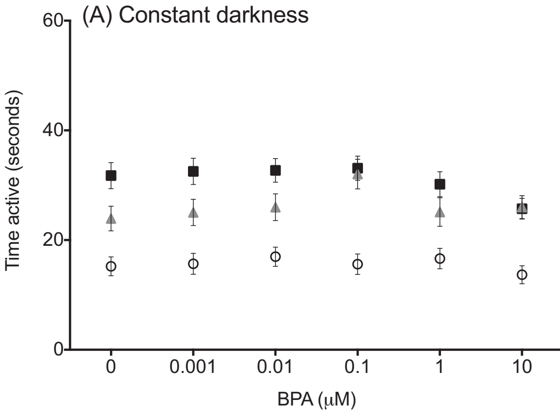
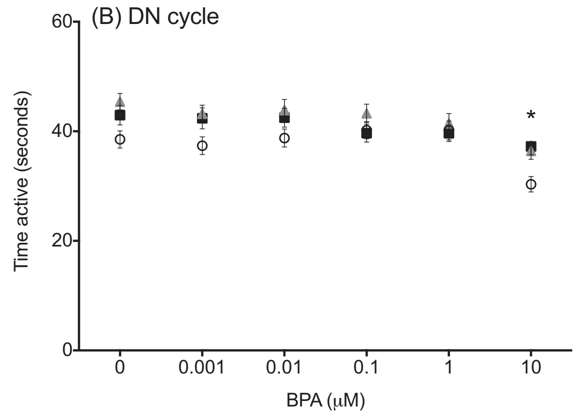
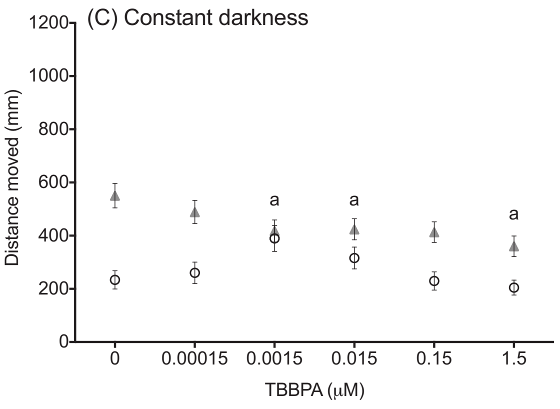
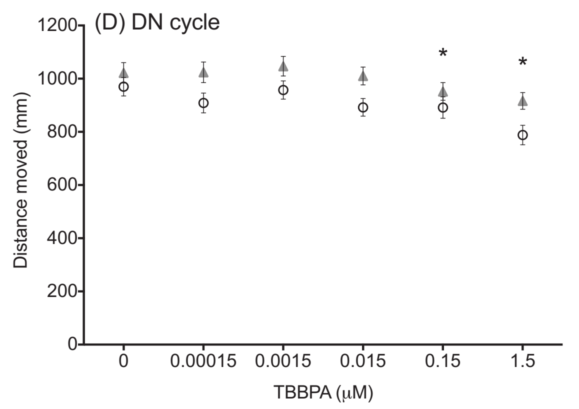
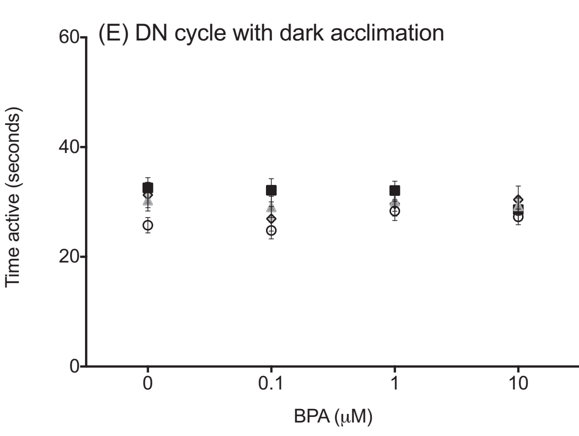
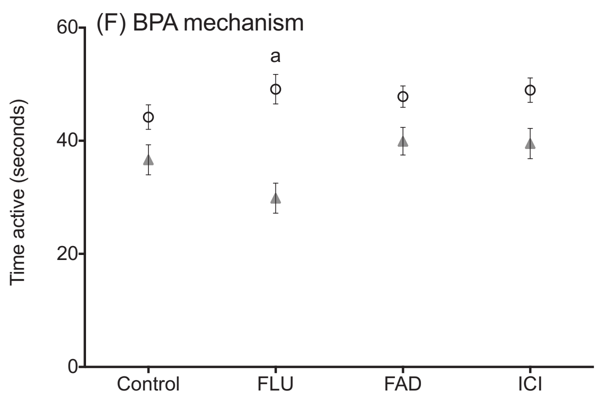

○ 96 hpf
▲ 100 hpf
■ 118 hpf
(A) Constant darkness
Time active (seconds)
BPA (μM)
0
0.001
0.01
0.1
1
10

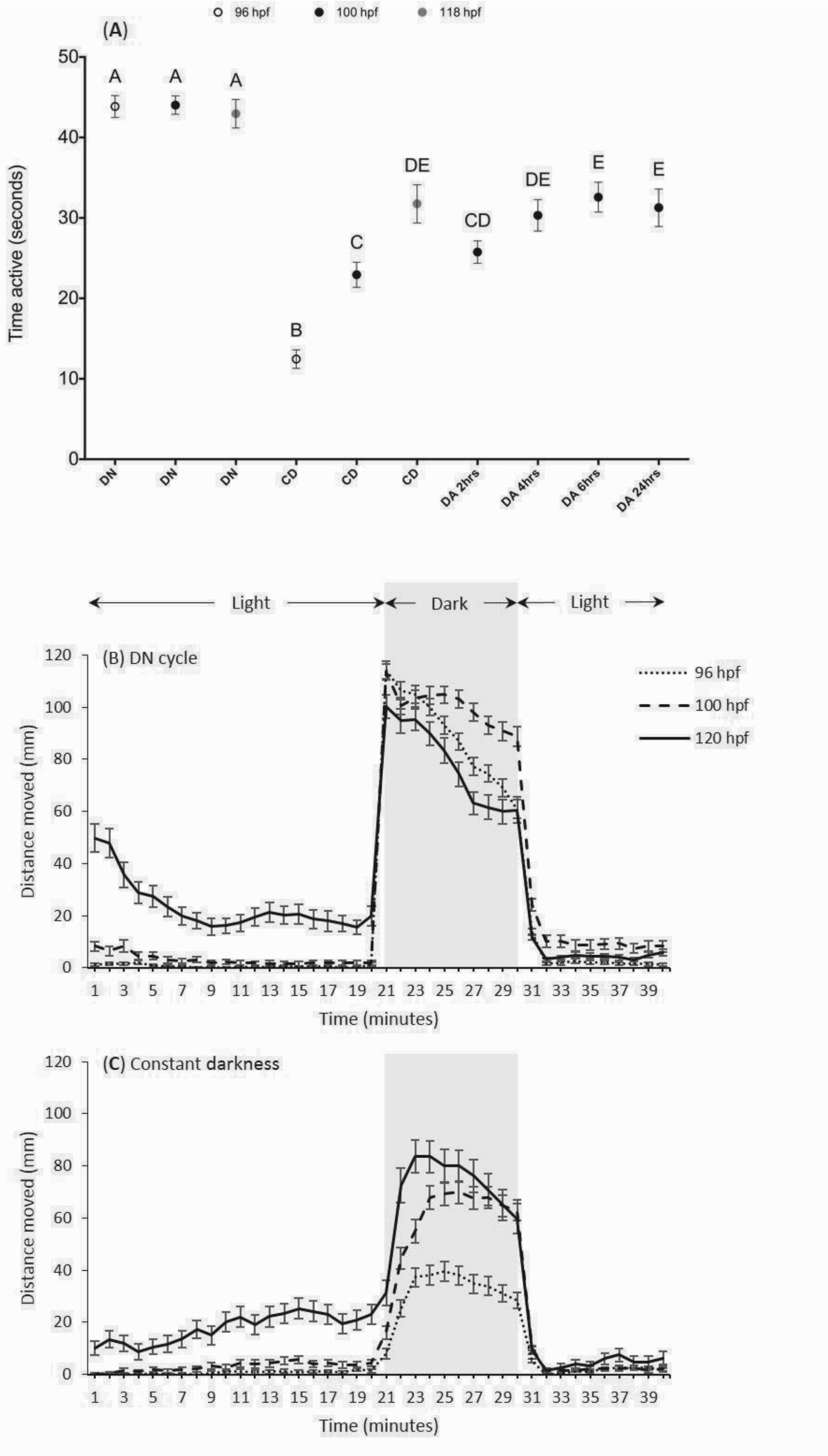

○ 96 hpf
▲ 100 hpf
■ 118 hpf
(B) DN cycle
Time active (seconds)
BPA (μM)
*


○ 96 hpf
▲ 100 hpf
(C) Constant darkness
Distance moved (mm)
TBBPA (μM)
0
0.00015
0.0015
0.015
0.15
1.5
a


○ 96 hpf
▲ 100 hpf
(D) DN cycle
Distance moved (mm)
TBBPA (μM)
*


○ 2 hrs
▲ 4 hrs
■ 6 hrs
◇ 24 hrs
(E) DN cycle with dark acclimation
Time active (seconds)
BPA (μM)
0
0.1
1
10


○ Control
▲ BPA
(F) BPA mechanism
Time active (seconds)
a
Control
FLU
FAD
ICI


96 hpf
100 hpf
118 hpf
(A)
Time active (seconds)
A
A
A
B
C
DE
CD
DE
E
E
DN
DN
DN
CD
CD
CD
DA 2hrs
DA 4hrs
DA 6hrs
DA 24hrs
Light
Dark
Light
(B) DN cycle
96 hpf
100 hpf
120 hpf
Distance moved (mm)
Time (minutes)
(C) Constant darkness
Distance moved (mm)
Time (minutes)

(A)

Time active (seconds)

100

75

50

25

0

*

*

DN Control

DN BPA

CD Control

CD BPA

(B)

% of the total distance moved in outer zone (thigmotaxis)

90

85

80

75

70

0

*

DN Control

DN BPA

CD Control

CD BPA

**Supplementary material**

**Title: Toxin induced behavioural aberrations in larval zebrafish are dependent on minor methodological alterations**

Authors: Thomas W. K. Fraser, Abdolrahman Khezri, Juan G. H. Jusdado, Anna Lewandowska-Sabat, Theodore Henry, Erik Ropstad

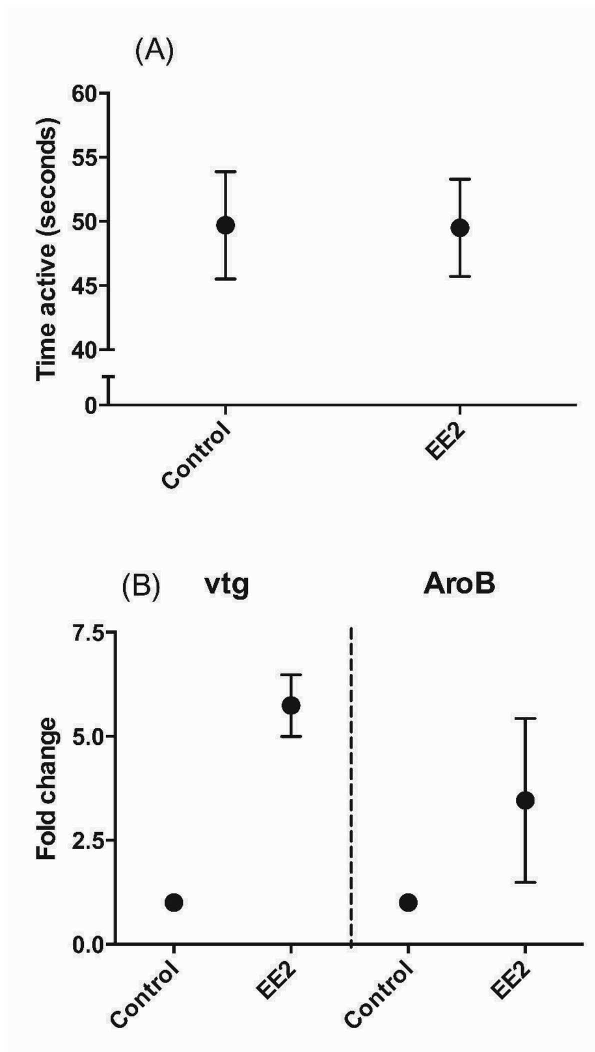



**Figure S1. The potent xenoestrogen 17α ethinyl estradiol (EE2) has no effect on behaviour.** Exposure to 10 nM EE2 had no effect on behaviour at 96 hpf (A) even though the transcript levels of the estrogenic markers vitellogenin (vtg) and aromatase B (AroB) were upregulated compared to the controls (B). Data are means ±SE, *n* = 69-70/group for the behavioural data and *n* = 2/group for the RT-qPCR data.

| Gene | Forward (5'-3') | Reverse (3'-5') |
|---|---|---|
| β actin 1[1] | CGAGCAGGAGATGGGAACC | CAACGGAAACGCTCATTGC |
| Elongation factor α[1] | CTTCTCAGGCTGACTGTGC | CCGCTAGCATTACCCTCC |
| Aromatase B[2] | AAAGAGTTACTAATAAAGATCCACCGGTAT | TCCACAAGCTTTCCCATTTCA |
| Vitellogenin 1A/B[3] | TGCGGAGTGCAAACAGTATGCAGT | GCAAGGCTGCAGTCAGTTCAATCTC |

**Table S1**. RT-qPCR primer sequences

[1]McCurley, A.T., Callard, G.V. 2008. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. BMC Mol Biol 9, 102. [2]Sawyer, S.J., Gerstner, K.A., Callard, G.V., 2006. Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: Gene specific tissue distribution, sex differences, developmental programming, and estrogen regulation. Gen Comp Endocrinol 147, 108-117. [3]Henry, T.B., McPherson, J.T., Rogers, E.D., Heah, T.P., Hawkins, S.A., Layton, A.C., Sayler, G.S., 2009. Changes in the relative expression pattern of multiple vitellogenin genes in adult male and larval zebrafish exposed to exogenous estrogens. Comp Biochem Physiol A 154, 119-126.

# Paper II

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# A Mixture of Persistent Organic Pollutants and Perfluorooctanesulfonic Acid Induces Similar Behavioural Responses, but Different Gene Expression Profiles in Zebrafish Larvae

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**Abstract:** Persistent organic pollutants (POPs) are widespread in the environment and some may be neurotoxic. As we are exposed to complex mixtures of POPs, we aimed to investigate how a POP mixture based on Scandinavian human blood data affects behaviour and neurodevelopment during early life in zebrafish. Embryos/larvae were exposed to a series of sub-lethal doses and behaviour was examined at 96 h post fertilization (hpf). In order to determine the sensitivity window to the POP mixture, exposure models of 6 to 48 and 48 to 96 hpf were used. The expression of genes related to neurological development was also assessed. Results indicate that the POP mixture increases the swimming speed of larval zebrafish following exposure between 48 to 96 hpf. This behavioural effect was associated with the perfluorinated compounds, and more specifically with perfluorooctanesulfonic acid (PFOS). The expression of genes related to the stress response, GABAergic, dopaminergic, histaminergic, serotoninergic, cholinergic systems and neuronal maintenance, were altered. However, there was little overlap in those genes that were significantly altered by the POP mixture and PFOS. Our findings show that the POP mixture and PFOS can have a similar effect on behaviour, yet alter the expression of genes relevant to neurological development differently.

**Keywords:** persistent organic pollutants; PFOS; zebrafish larvae; behavioural; neurotoxicity

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## 1. Introduction

Persistent organic pollutants (POPs) refers to groups of toxic environmental chemicals with a carbon-based structure, resistant to environmental degradation and widely distributed via soil, water and air [1]. Because of their lipophilic nature, POPs tend to bioaccumulate in top predators and humans [2]. Among different classes of POPs, chlorinated, brominated and perfluorinated compounds are the most persistent compound classes, widely detected in human adipose tissue, breast milk and serum samples from all over the world [3–7].

POPs are endocrine disruptors and have been shown to have a wide range of effects including impaired reproduction, carcinogenicity, and thyroid disruption, and can promote cardiovascular

disease and induce hepatic lesions [8,9]. Of particular concern is the lipophilic property of POPs that makes them capable of passing through biological barriers such as the placenta [10]. Indeed, several POPs are known to be neurotoxic [11–13] and have been associated with neurological diseases in children [14]. The complicated processes taking place during development make the brain and neural tissue sensitive to a variety of environmental contaminants [15,16]. Previous studies have demonstrated the ability of POPs such as perfluorooctanesulfonic acid (PFOS) to pass through the blood−brain barrier [17], causing neurotoxicity and behavioural alterations in mice, rats, and zebrafish [18–23]. As for the potential mechanisms, work in zebrafish has demonstrated that POPs such as PFOS can promote cell death in the brain following early life exposure which is then associated with altered behaviour [20]. Moreover, exposure can induce reactive oxidant species (ROS) [24] and estrogenic biomarkers [25], as well as influence the expression of genes related to metabolism and organogenesis [26]. Behavioural responses may also be related to dopaminergic deficits [27].

A large and growing body of literature has been published on the effectiveness of zebrafish as a model organism. These studies all indicate that zebrafish, due to their small size, high offspring rate, rapid development, short generation period, low cost, and transparent embryos, make a successful model organism for high-throughput screening studies [28–30]. In addition, recent work has highlighted the use of behaviour as a sensitive tool for assessing the sub-lethal effects of environmental pollutants [31–33] on both general toxicity [34] as well as neurotoxicity [31–33]. Furthermore, zebrafish have proven to be a useful model system for developmental neurotoxicity and investigating mechanistic pathways. For instance, previous studies have shown how the expression of central nervous system (CNS)-related genes in zebrafish can be impaired following exposure to different compounds [35–38].

The majority of toxicological studies have focused on the effects of single compounds only, whereas in reality we are exposed to complex mixtures of pollutants [39]. Indeed, environmentally relevant mixtures of POPs can induce biomarkers of estrogenic activity and induce cytochrome P4501A [40], impair reproductive function [40] and lead to behavioural aberrations [41,42]. However, less is known about which chemicals within these mixtures are influencing specific endpoints or how such mixtures interact on toxicological endpoints. This is a significant concern as several studies have demonstrated the potential of different compounds to have additive effects. For instance, it has been shown that a combination of 17α-ethinyl estradiol (EE2) and dibutyl phthalate (DBP) had greater effects on gonad, liver and gill development in zebrafish compared to EE2 and DBP exposures alone [43]. Similarly, co-exposing zebrafish larvae to PFOS and nano-ZnO led to more serious thyroid-disrupting effects than exposure to PFOS alone [44]. With this in mind, we recently developed a POP mixture based on Scandinavian blood data. Initial studies have shown that our POP mixture induces ROS production in a human hepatocarcinoma cell line [45]. Furthermore, individual compounds within the POP mixture and not the total POP mixture altered the transcriptional activity of the glucocorticoid receptor in the glucocorticoid receptor redistribution assay [46].

Animals and humans are exposed to POPs in a mixture scenario. Therefore, investigating the effects of environmentally relevant POP mixtures is more realistic than the effects of a single POP. Moreover, the research to date has tended to focus on observed behavioural responses following exposure to specific compounds or a group of them, rather than investigating the mechanistic pathways involved in the behavioural response. Therefore, the aim of this study was to investigate the possible neurobehavioural effects of an environmentally relevant POP mixture and sub-mixtures, derived from Scandinavian human blood data, on zebrafish larvae. The secondary aim was to investigate the impact of the POP mixture on the expression of genes relevant to brain development and behaviour during the early life stage of zebrafish.

## 2. Results

### 2.1. Total Persistent Organic Pollutant (POP) Mixture Increased Swimming Speed

The first part of the experiment was to screen the mixture for behavioural effects. We looked at three endpoints: the total distance moved, the total time spent active and the average swimming speed (Appendix A). From these, the average swimming speed was identified as the most robust behavioural response and used for further study. The total POP mixture at an equal concentration to that found in human plasma had no effect on larval swimming speed, while doses 20×, 100× and 200× higher than the human serum level resulted in significant increases in the average swimming speed (10%, 38% and 61% increase, respectively) compared with controls (Figure 1A). Based on the clear response at 100× higher than human serum level, and in order to minimize any possible general toxicity, this concentration was selected for further investigation.

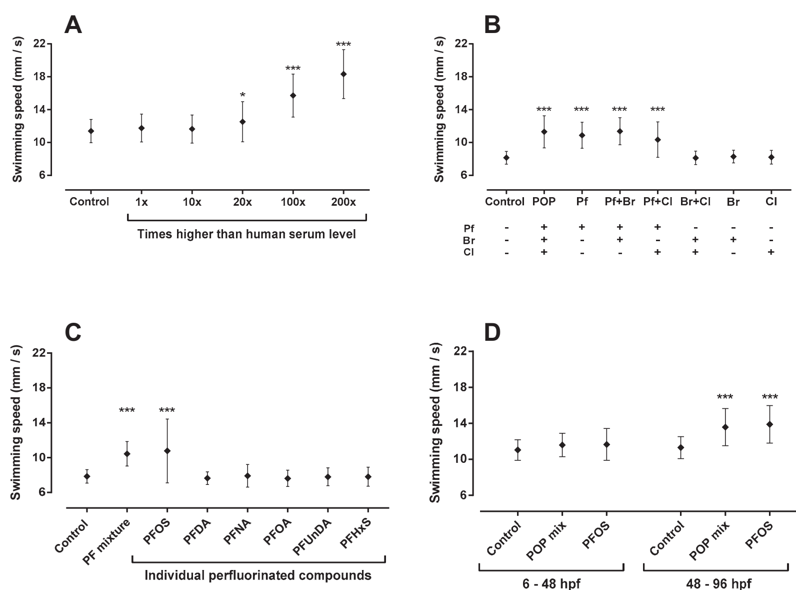


**Figure 1.** Swimming speed in zebrafish larvae exposed to a mixture of environmental pollutants, sub-mixtures and individual perfluorinated compounds. (**A**) swimming speed in zebrafish larvae upon exposure to five different concentrations of total persistent organic pollutant (POP) mixture; (**B**) swimming speed in zebrafish larvae upon exposure to sub-mixtures at the concentration equal to 100× higher than that found in human serum; (**C**) swimming speed after exposing the zebrafish to individual perfluorinated compounds (100× human serum level) compared to PF mixture; (**D**) PFOS and POPs sensitivity test (100× human serum level). (Pf) Perfluorinated mixture; (Br) Brominated mixture; (Cl) Chlorinated mixture; (Pf + Br) binary mixture of perfluorinated and brominated compounds; (Pf + Cl) binary mixture of perfluorinated and chlorinated compounds; (Br + Cl) binary mixture of brominated and chlorinated compounds. (+) contained; (−) not contained. Data are means ± SD. An asterisk identifies values that are significantly different from the solvent (0.05% DMSO) control (LME, * = $p < 0.017$, ** = $p < 0.0017$, *** = $p < 0.0001$).

### 2.2. Sub-Mixtures Containing Perfluorinated Compounds Increased Swimming Speed

The total POP mixture consisted of three main sub-mixtures, perfluorinated, brominated and chlorinated compounds. Therefore, the next step was to identify which groups contributed to the observed behavioural response following exposure to the total mixture. It can be seen from the data

in Figure 1B that neither the brominated or chlorinated compounds alone or in combination had any effect on swimming speed. However, exposure to mixtures containing perfluorinated compounds, at a concentration equal to 100× higher than human serum level, significantly increased swimming speed similar to what was observed following exposure to the total POP mixture.

### 2.3. Perfluorooctanesulfonic Acid (PFOS) Increased Swimming Speed

In order to identify the role of individual perfluorinated compounds in increasing the swimming speed, zebrafish embryos were exposed to the six different chemicals that made up the perfluorinated mixture. We found only PFOS significantly increased swimming speed in zebrafish larvae, similar to both the perfluorinated and total POP mixtures (Figure 1C).

### 2.4. PFOS Tissue Uptake in Larvae

We found the increase in swimming speed observed after exposure to the total POP mixture was mimicked by PFOS exposure. Based on this finding, we evaluated PFOS accumulation in 96 hpf zebrafish larvae after exposure to the total POP mixture at a concentration equal to 100× the human serum level. Our results showed that after 96 h exposure, 22% of the nominal PFOS concentration was detected. Of this, 49% accumulated in the larvae, 49% remained in the exposure medium and 2% was stuck to the wells.

### 2.5. 48–96 hpf as Developmental Window of Sensitivity

Based on our observed results, PFOS was the only compound that could explain the behavioural response in zebrafish larvae exposed to the total POP mixture. Next, we tested which phase of zebrafish neurodevelopmental is the most sensitive to PFOS and POP exposure. We observed that exposure from 48 to 96 hpf significantly increased swimming speed, whereas exposure from 6 to 48 hpf had no effect on swimming speed (Figure 1D). In addition, we observed that the insensitivity between 6 to 48 hpf was not related to the presence of the chorion as exposure between 24 to 48 hpf in dechorionated embryos did not increase swimming speed compared to the control (Appendix B).

### 2.6. POP Mixture and PFOS Altered Gene Expression Differently

We investigated the expression of a battery of genes involved in neurodevelopment and behaviour after exposure to the POP mixture and PFOS between 48 to 96 hpf. POP and PFOS exposure led to different gene expression profiles. Cluster analyses revealed that both PFOS 10× and PFOS 70× clustered together as did POP 10× and POP 70×. In addition, the distance between POP-exposed groups and control was greater than the distance between PFOS-exposed groups and the control (Figure 2).

Although differences in gene expression profiles were detected via cluster analysis, the expression of the majority of genes remained unchanged with only eight genes including *manf*, *crhb*, *hrh1*, *hdc*, *chrna7*, *sertb*, *bdnf* and *gabra1* being significantly affected. The POP exposure significantly affected the greatest number of genes, whereas PFOS exposure only affected one gene, *hrh1*. The genes *manf* and *hrh1* were significantly downregulated in both the POP 10× and 70×-exposed larvae. Transcription levels of *hdc*, *chrna7*, *sertb*, *bdnf* and *gabra1* were significantly decreased only in the POP 70× group, whereas *crhb* was significantly affected only in the POP 10× group. Finally, *hrh1* was the only gene that was significantly downregulated by both POP 70× and PFOS 70× exposures (Figure 3).

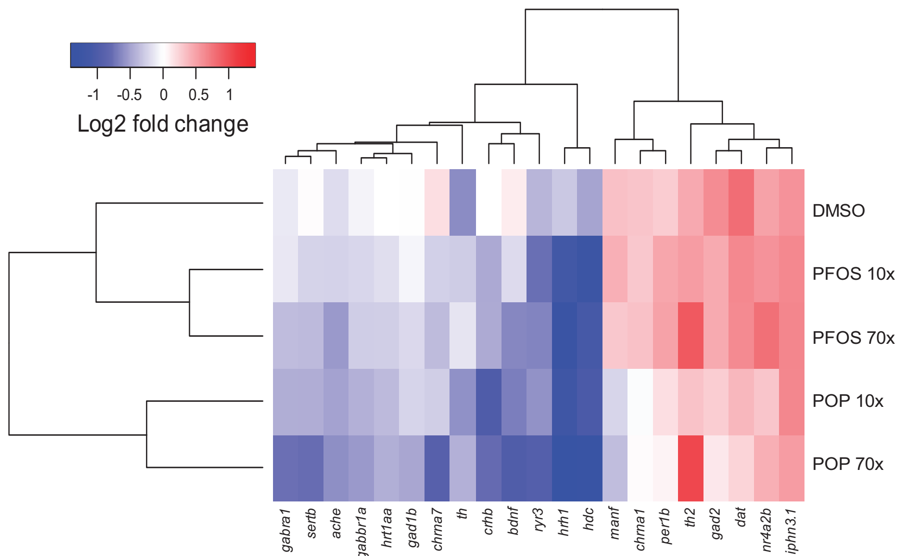


**Figure 2.** Euclidean distance and ward clustering on log2 normalized expression values. The heat map shows the differences in expression of 21 genes related to neurodevelopmental processes between the solvent control (0.05% DMSO) and exposed samples in 96 hpf zebrafish. Cluster analysis was performed on log2 expression values of five biological replicates.

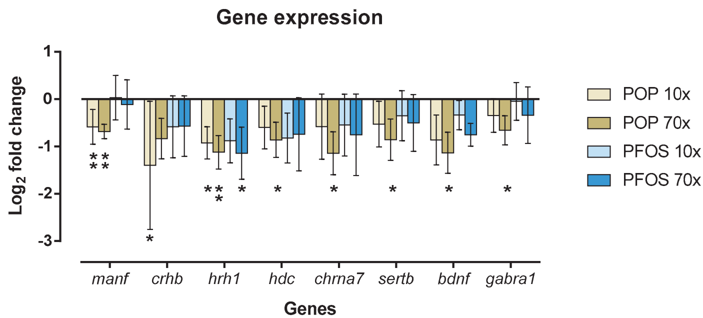


**Figure 3.** Transcription levels in genes relevant to behaviour following POP mixture and PFOS exposure. The line at zero indicates the gene expression in control groups (DMSO 0.05%). Data are presented as mean ± SD relative to control. An asterisk identifies genes expression levels that were significantly different from the solvent control (one-way ANOVA test, * = $p < 0.05$, ** = $p < 0.005$).

## 3. Discussion

Our aim was to determine whether a human POP mixture based on human blood levels from the Scandinavian population could induce behavioural effects following developmental exposure, using zebrafish as a model vertebrate system. Our results indicated that the total POP mixture significantly affected the swimming behaviour in zebrafish larvae starting at a concentration 20× higher than that found in human serum. Further investigations revealed that PFOS alone could mimic the behavioural response observed following exposure to the POP mixture. However, although the results from gene expression analysis revealed that both the POP mixture and PFOS altered the regulation of CNS-related genes, there was limited overlap in those genes significantly affected. Our work highlights the potential

developmental neurotoxicity of a POP mixture relevant to humans. To date, very little attention has been paid to the potency of mixtures of environmental pollutants on the induction of neurobehavioural toxicity. Previous work would suggest the results of single compounds are not fully translatable to mixture scenarios, mainly because of unknown interactions between different chemicals in complex mixtures [47]. We found the POP mixture increased the swimming speed in zebrafish larvae in a dose-dependent manner. This behavioural effect was associated with the perfluorinated compounds within the mixture, more specifically with PFOS. This result could be explained by the fact that PFOS was the compound with the highest concentration in the total and perfluorinated mixtures (5.46 μM in 100× mixtures), compared with PFOA (1 μM in 100× mixture) which was the second most concentrated compound. Of note, PFOS alone increased swimming speed to a similar extent as the total POP mixture, which suggests PFOS toxicity was not influenced by other compounds in the POP mixture. Previous studies have shown a hyperactive behaviour upon PFOS exposure in both zebrafish and rodents. For instance, zebrafish larvae exposed to 1.85 μM PFOS developed spontaneous activity and persistent hyperactivity [27]. Another study reported that PFOS in a wide range of concentrations (0.5 to 8 μM) increases the swimming speed in both 5 and 6 dpf zebrafish larvae [20]. It has also been shown that chronic prenatal exposure to PFOS (0.5 μM) for 120 days in zebrafish is able to increase the swimming speed in both parents and F1 larvae [18]. Similarly, mice exposed to 3 mg/kg/day PFOS displayed spontaneous activity [27] whereas other rodent studies have found that PFOS decreases locomotor activity [48,49].

Regarding the increase in swimming speed, we found that 48–96 hpf is the sensitive window for the total POP mixture and PFOS exposure, as exposures before 48 hpf had no effect on swimming speed. These results match those observed in earlier studies. For example, it has been shown that zebrafish larvae exposed to PFOS from 49 to 73 hpf had higher swimming speeds compared with groups exposed before 49 hpf and after 73 hpf [20]. Moreover, it has been reported that 16 μM PFOS exposure between 48 to 96 hpf in zebrafish larvae resulted in noticeable deformities (uninflated swim bladder, less developed gut, and curved spine), whereas larvae developmentally exposed to PFOS from 8 to 48 hpf did not develop any distinct deformities, even after exposure to 32 μM [26]. Another study reported that PFOS exposure before 48 hpf had no effect on the development of the swim bladder, while exposure after 48 hpf resulted in swim bladder deformities in 50% of the zebrafish larvae [50]. Different hypotheses have been suggested regarding the sensitivity of zebrafish larvae to PFOS exposure. For instance, this window of sensitivity might be related to the development of estrogenic receptors, which begin to be expressed after 48 hpf in zebrafish larvae, and could mediate PFOS toxicity [26]. However, although PFOS exposure does produce estrogenic effects in zebrafish [25], we have previously found exposure to 10 nM of the xenoestrogen 17α-ethinylestradiol (EE2) has no effect on behaviour at 96 hpf even though we detected an elevation in the expression of estrogenic response genes [51]. Furthermore, it seems that PFOS toxicity is not related to the presence of the chorion. Previous work has demonstrated that PFOS accumulates in 6 hpf-exposed embryos two hours after exposure, but absorption and accumulation of PFOS is accelerated in larvae after 48 hpf [20]. This increase in absorption at later life stages may explain why larvae were more sensitive to PFOS exposure at the later life stage.

We evaluated gene transcription after POP and PFOS exposure during the 48–96 hpf window. As previously reviewed [29,52,53], the different regions of the zebrafish brain are almost developed by 48 hpf and between 48–96 hpf the developmental processes for different neurotransmitter-expressing neurons is accelerating. Therefore, we hypothesized that those CNS processes that start to develop after 48 hpf in zebrafish larvae could mediate the POP and PFOS behavioural toxicity. Although both POP 70× and PFOS 70× exposure significantly increased the swimming speed, we found only one mutually affected gene (*hrh1*) between these exposure groups, whereas other genes involved in dopaminergic (*manf*), histaminergic (*hdc*), serotonergic (*sertb*), cholinergic (*chrna7*), GABAergic (*gabra1*), stress (*crhb*) and neural maintenance (*bdnf*) signalling were exclusively affected in POP-exposed groups. This could be explained by presence of brominated, chlorinated and perfluorinated compounds within

the mixture. Similarly, mixtures of polycyclic aromatic hydrocarbons (PAHs) had limited overlap on gene expression compared to individual compounds in rat liver [54]. The cluster analysis confirmed that the POP exposure altered the gene expression profile in a different manner compared with PFOS. This would suggest that the genes assessed here were either not involved in the observed behavioural responses or that PFOS has a different molecular pathway leading to the observed behavioural effects.

Previous studies have implemented the dopaminergic and serotonergic systems in the neurotoxicity of PFOS, but we found no clear evidence that these systems explained the increase in swimming speed in the current study. For example, PFOS increased the level of serotonin in different regions of the rat brain [55] and impaired the dopaminergic system in both mice and zebrafish [22,27]. Moreover, it has been shown that PFOS exposure upregulated *crhb*, which is a marker of the stress response [56]. However, the dopaminergic genes *sertb*, and *crhb* were not significantly affected by PFOS in this study. Additionally, although gene expression was more influenced by exposure to 70× compared to 10× of the POP mixture, including genes involved in inhibitory signaling pathways (*sertb*, *gabra1*) [57,58], it is unclear which systems may be behind the behavioural effects observed in the current study.

One of the main objectives of toxicity testing is to determine the lowest effect concentrations. Gene analyses data revealed that *manf*, *crhb* and *hrh1* genes were significantly downregulated upon POP exposure, even at a concentration only 10× higher than human serum level. *manf* is a dopaminergic neurotrophic factor that protects dopaminergic neurons from neurotoxic damage [59] and plays a supportive role in cell viability [60]. In addition to the stress response, *crhb* also plays an important role in thyroid-stimulating hormone (TSH) secretion [61]. There was also significant downregulation of *crhb* in this study, thereby suggesting a possible disruptive effect of the POP mixture on the hypothalamic-pituitary-interrenal (*HPI*)/hypothalamic-pituitary-adrenal (*HPA*), and hypothalamic-pituitary-thyroid (*HPT*) axis. *Hrh1* is a histamine receptor expressed widely in the CNS, and also regulates the immune response [62]. Therefore, although no behavioural effect was observed following the 10× exposure, changes in gene expression were observed at concentrations close to the human scenario. Further research is needed to explore the biological significance of these changes in gene expression and which compounds from the POP mixture are responsible for these changes.

Based on our results, exposure to a mixture of brominated and/or chlorinated compounds had no effect on swimming speed. Similarly, it has been reported that brominated compounds including BDE 47, 99, 100, 153, 154, 209 and HBCD, at concentrations within the range used in current study, had no significant effect on locomotor behaviour in 5 dpf zebrafish larvae [63]. Although some brominated and chlorinated compounds are known to influence larval zebrafish behaviour in contrast to our results, these compounds are either not in our mix or the effects were found at concentrations higher than those used in the current study [42,64].

We used larval zebrafish to assess a human-based POP mixture for behavioural effects. Differences in larval locomotor behaviour using the light/dark assay are generally associated with the level of anxiety [65], suggesting our mixture could lead to alterations in anxiety within humans. The concentrations tested were of relevance to humans, as we found effects levels only marginally higher (i.e., 10×) than those found in human blood serum. Here it is noted that the human-based POP mixture was based on the mean values within Scandinavians. Therefore, some individuals will have higher values than the mean, and the levels of environmental pollutants within humans varies between different countries and tends to be lower in more developed countries [66]. Furthermore, we could only recover 22% of the nominal value for PFOS at 96 hpf. It is unclear where the remaining 78% went, but the values attained per embryo (63 ng following exposure to 5.5 μM PFOS) were very similar to those values obtained by [20] following exposure between 0 and 5 days post fertilization (66 ng/embryo following exposure to 8 μM PFOS).

## 4. Materials and Methods

### 4.1. Mixtures and Chemicals

The POP mixtures were designed and made by the Norwegian University of Life Sciences, Oslo, and described in [67]. Relevant compounds and their levels in human plasma of a Scandinavian population were identified, and seven different mixtures were prepared and used in the current study, including; (1) total POP mixture containing perfluorinated, brominated and chlorinated compounds; (2) perfluorinated mixture (Pf); (3) brominated mixture (Br); (4) chlorinated mixture (Cl); (5) perfluorinated and brominated mixture (Pf + Br); (6) perfluorinated and chlorinated mixture (Pf + Cl); and (7) brominated and chlorinated mixture (Br + Cl). The compounds were mixed in concentration ratios relevant to the human serum level. The intention was that the dose of each mixture reflected the human plasma level of corresponding chemicals within that mixture. The chemicals included in the mixtures and their respective concentrations are shown in Table S1. All polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and other organochlorines were originally purchased from Chiron As (Trondheim, Norway). Hexabromocyclododecane (HBCD) and all perfluorinated compounds (PFCs) were obtained from Sigma-Aldrich (St. Louis, MO, USA), except PFHxS which was from Santa Cruz (Dallas, TX, USA). All stock solutions were formed in pure DMSO (Sigma-Aldrich).

### 4.2. Zebrafish Maintenance and Breeding

Adult AB strain zebrafish (*Danio rerio*) were housed with a 14:10 h light:dark cycle period in a carbon-filtered flow-through system. System water was kept at 28 ± 1 °C and prepared by adding 15.5 g of Instant Ocean® salt, 5.3 g of sodium bicarbonate and 1.5 g of calcium chloride per 100 L of tap water to attain a pH of 7.5–7.6 and conductivity of 500 µS/cm. Fish were fed daily, twice with Artemia and once with formulated feed (SDS 400, Essex, UK) and kept at a density equal to seven fish/L. For egg production, male and female adult zebrafish were held in breeding tanks equipped with a barrier and spawning net. The barriers were removed shortly after the onset of light in the morning and the fish paired for 30 min. Eggs collected from the breeding tanks were rinsed and kept in autoclaved system water at 28 °C until exposure.

### 4.3. Exposure Scenario

The study was performed at the Section for Experimental Biomedicine at the Norwegian University of Life Sciences in Oslo, Norway. The unit is licensed by the Norwegian Food Inspection Authority (NFIA) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (2014/225976). The study (2013/39783-2) was approved on 20/08/2013 by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) and NFIA.

Fertilized and healthy embryos at approximately 6 hpf were selected using a stereo microscope. Equal numbers of embryos for each treatment were distributed in a checker-box pattern across 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) (one embryo/well) and exposed statically in 200 µL of media. The final concentration of DMSO in all test concentrations and the solvent control was 0.05%. First, embryos were exposed separately to all seven mixtures over the concentration range 1× to 200× higher than human serum levels (three replicates). These concentrations were considered non-teratogenic based on a maximum mortality/deformity rate of 10% in any one group. These experiments were then followed by exposing the zebrafish embryos to individual chemicals from the PF mixture, including: perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid potassium salt (PFOS), perfluorodecanoic acid (PFDA), perfluorononanoic acid (PFNA), perfluorohexane sulfonate potassium salt (PFHxS), perfluoroundecanoic acid (PFUnDA) and the perfluorinated mixture itself at a concentration equal to 100× higher than human serum levels (three replicates). After each exposure, plates were placed into sealed transparent plastic bags and kept at 28 °C on a 14:10 h light: dark cycle until 96 hpf, when behavioural tests were undertaken.

*4.4. Locomotor Activity*

Behavioural assays were conducted on 96 hpf larvae during a light/dark/light cycle using a Viewpoint Zebrabox (Viewpoint Life Science, Lyon, France). This system consists of a 25-frame per second camera equipped with an infrared filter that is capable of tracking zebrafish movement through its supplied software (Video-Track software, ViewPoint Life Science, France). All tests were performed at 28 °C between 09:00 and 10:30. The test consisted of 10 min of acclimation when the light intensity was set to 100% (these data were excluded from final analyses), followed by a further 10 min of 100% light, 10 min of complete darkness, and a final 10 min of 100% light. Only locomotor activity during the dark period was analysed, as movement during the lighted periods was minimal as expected for this life stage [68]. Zebrafish larvae were distinguished from the background by introducing a 30-pixel threshold difference within the tracking software. In addition, short and large movements were defined as 5 and 8 mm per sec in the protocol, respectively. The total distance moved (mm) and the total time spent active (s) were recorded every 60 s. From this data, the mean swimming speed (mm/s) was calculated. Following locomotor assessment, larvae were evaluated using a microscope for any dead or malformed (spinal/tail aberrations, yolk sac or cardiac edema, aberrations in pigmentation, and loss of equilibrium) individuals to be excluded from behavioural analyses.

*4.5. PFOS Tissue Uptake in Larvae*

The analyses of embryos, medium and wells was done at the laboratory of Environmental Toxicology at the Norwegian University of Life Sciences. The laboratory is accredited by the Norwegian accreditation for testing PFOS in biological material according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137). The PFOS concentrations in the exposure media and whole-body tissues of zebrafish larvae were measured in embryos exposed from 6 to 96 hpf to the total POP mixture at a concentration equal to 100× higher than in human serum. Six zebrafish larvae from six individual wells were pooled as one sample and the exposure media was taken from the corresponding six wells (200 µL/well). Each well was then rinsed by methanol, which was then collected to measure any chemicals that may have resided on the wall of the well. PFOS was analysed according to [69] and references therein. Both linear and branched PFOS were included in analyses as recommended by [70]. The samples were extracted with methanol and clean up was accomplished using active carbon (EnviCarb, Supelco, Zwijndrecht, The Netherlands). Analysis was performed by the separation of compounds on a high-performance liquid chromatographer (HPLC) with a Discovery C18 column, connected to a C18 pre-column (Supelco, Sigma-Aldrich) and detection with liquid chromatography tandem mass spectrometry (MS-MS) (API 3000, LC/MS/MS System). The details of the analytical quality system have been described in [71]. Briefly, every analytical series included three procedural blanks (solvents), one blind (non-spiked clean), and two spiked clean samples for recoveries. The quality control parameters were within the accepted ranges for the method.

*4.6. Developmental Sensitivity Test*

In order to link the observed behavioural response with the neurodevelopmental stages, we exposed zebrafish embryos at two different time points. Zebrafish embryos were exposed to PFOS and then a total POP mixture at a concentration equal to 100× higher than human serum level from 6 to 48 hpf before being washed three times with autoclaved system water and exposed to 200 µL of the vehicle solution only (DMSO 0.05%) from 48 to 96 hpf. Simultaneously, on the same plate, another group of zebrafish embryos were exposed to 200 µL of vehicle solution during the first 48 h and then the exposure followed between 48–96 hpf by adding PFOS or the total POP mixture at 100× higher than the human serum level. This experiment was repeated in triplicate.

### 4.7. Gene Transcription Analysis

To determine the mRNA expression induced by the total POP mixture and PFOS, zebrafish larvae were exposed between 48–96 hpf. Concentrations were adjusted to 10× and 70× human serum level as the highest dose at which had no effect on swimming speed (HNSS) or the lowest dose at which there was a significant increase in swimming speed (LISS), respectively (Appendix C). Primers were designed to span exon-exon boundaries using Primer3-based algorithms available at (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and tested for dimers and efficiency using Vector NTI® advance software version 11 for windows and melting curve, respectively (Table S2). Total RNA was isolated from a pool of 10 embryos from each treatment using Trizol agent (Invitrogen, Carlsbad, CA, USA) and following the manufacturers' instruction. RNA concentration was measured by nanodrop, cDNA was prepared from 1 μg of DNase-treated total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers according to product specifications. Quantitative PCR (qPCR) was carried out on a LightCycler® 96 Real-Time PCR system (Roche, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master (Roche). Each cDNA sample was analysed in duplicate and composed of 5 μL mastermix, 2 μL primer mix (5 μM of each of forward and reverse), and 3 μL of each 10× diluted cDNA sample in a total volume of 10 μL. The cycling parameters were 10 min pre-incubation at 95 °C, followed by 45 cycles of amplification at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s, followed by a melting curve from 60 °C to 95 °C. qPCR assay was performed for five biological replicates. After the assessment of candidate reference genes (*hprt*, *rps18*, *ef1α*, *hmbs* and *bactin*) using the online RefFinder analysis available at (http://fulxie.0fees.us/), and based on the Genorm algorithm, *rps18* and *ef1α* were considered the most stable housekeeping genes for all exposure groups. The expression of each target gene transcript was normalized to the housekeeping genes and the fold change was calculated using the $\Delta\Delta C_t$ method, using the geometric averaging of the two reference genes [72].

### 4.8. Statistical Analyses

Locomotor activity data were transferred to R Studio (RStudio Team 2015, version 0.99.473 for windows, Boston, MA, USA, available at: http://www.rstudio.com/) for behavioural analyses. To test the effect of the total POP mixture, sub-mixtures and individual compounds on locomotor activity, a linear mixed effect model (LME) was employed with distance moved, time spent swimming or swimming speed as the dependent variable, mixture/compound concentration as a categorical independent variable, and test replicate as a random effect. Examination of the residual plots verified that no systematic patterns occurred in the errors (e.g., q-q plots). To assess individual doses to the controls, we used the contrast results provided within R. Due to multiple comparisons of the same data set (i.e., the same individuals were used to assess three behavioural endpoints, distance moved, time active, and swimming speed), the results were Bonferroni corrected to avoid Type I errors. Therefore, significance was assigned at $p < 0.017$ (i.e., 0.05/3). Gene expression data were analysed using one-way ANOVA test followed by Dunnett's post hoc test and the limit of significance was set at $p < 0.05$. Data were plotted using GraphPad Prism version 7.02 for Windows, (GraphPad Software, San Diego, CA, USA).

## 5. Conclusions

We aimed to assess the possible neurobehavioural toxicity of an environmentally relevant mixture of persistent organic pollutants (POPs), which was constructed based on Scandinavian human blood data. This study has shown that exposure to a complex mixture consisting of brominated, chlorinated and perfluorinated compounds, significantly affected the swimming speed of zebrafish larvae. The effect was related to the perfluorinated compounds, exclusively with perfluorooctanesulfonic acid (PFOS). These behavioural effects could not be associated with the difference in gene expression. Since behaviour is a complicated phenomenon, further work should investigate whether the POP mixture and PFOS affect additional molecular and physiological processes

related to behaviour such as the sensory system or endocrine hormone levels, and investigate the functional role of the genes affected by the POP mixture.

**Supplementary Materials:** Supplementary materials can be found at www.mdpi.com/1422-0067/18/2/291/s1.

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**Author Contributions:** Abdolrahman Khezri and Thomas W. K. Fraser conceived and designed the experiments; Abdolrahman Khezri performed the experiments; Abdolrahman Khezri, Thomas W. K. Fraser and Jorke H. Kamstra analysed the data; Rasoul Nourizadeh-Lillabadi, Jorke H. Kamstra and Vidar Berg contributed analysis tools; Abdolrahman Khezri wrote the paper. Karin E. Zimmer prepared the POP mixture. Erik Ropstad and Karin E. Zimmer have taken part in the planning and supervision and all authors contributed to drafting and reviewing the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

| | |
|---|---|
| BDE | Brominated diphenyl ethers |
| BDNF | Brain-derived neurotrophic factor |
| CHRNA7 | Cholinergic receptor nicotinic alpha 7 subunit |
| CNS | Central nervous system |
| CRHB | Corticotropin releasing hormone Beta |
| DMSO | Dimethyl sulfoxide |
| DPF | Day post fertilization |
| GABRA1 | Gamma-Aminobutyric Acid Type A Receptor Alpha1 Subunit |
| HDC | Histidine decarboxylase |
| HPF | Hour post fertilization |
| HRH1 | Histamine Receptor H1 |
| LME | Linear mixed effect |
| MANF | Mesencephalic astrocyte-derived neurotrophic factor |
| PBDE | Polybrominated diphenyl ethers |
| PCB | Polychlorinated biphenyl |
| PFDA | Perfluorodecanoic acid |
| PFHxS | Perfluorohexane sulfonate |
| PFNA | Perfluorononanoic acid |
| PFOA | Perfluorooctanoic acid |
| PFOS | Perfluorooctanesulfonic acid |
| PFUnDA | Perfluoroundecanoic acid |
| POPS | Persistent organic pollutants |
| ROS | Reactive oxygen species |
| SERTB | Serotonin transporter B |

## Appendix A

**Table A1.** Distance moved and swimming time in 96 hpf zebrafish larvae exposed to total POP mixture, sub mixtures and individual mixtures.

| Exposure Groups | Distance Moved (mm/10 min) | | | | | | Swimming Time (s/10 min) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 1× | 10× | 20× | 100× | 200× | Control | 1× | 10× | 20× | 100× | 200× |
| **Total POPs** | 742.1 ± 290.2 | 782.5 ± 373.4 | 735.9 ± 303.9 | 783.2 ± 271.7 | 722.1 ± 366.2 | 596.3 ± 400.8 * | 66.1 ± 27.1 | 67.2 ± 31.9 | 64.1 ± 25.3 | 64.6 ± 23.8 | 48.3 ± 26.8 ** | 34 ± 24.3 *** |
| **Pf** | 730.8 ± 345.3 | 730.5 ± 371.7 | 774.4 ± 385.3 | 722.8 ± 370 | 743.6 ± 306.7 | 552.4 ± 367.4 * | 61.7 ± 29.8 | 61 ± 32.3 | 66.2 ± 34.6 | 60.2 ± 31.7 | 47.6 ± 23.9 * | 32.6 ± 24.1 *** |
| **Pf + Br** | 784.8 ± 420.4 | 720.4 ± 326.2 | 672.8 ± 368.9 | 834.1 ± 331.1 | 657.2 ± 330.9 | 457.2 ± 321 *** | 66.9 ± 35.3 | 63.3 ± 29.3 | 55.7 ± 31.3 | 69.2 ± 28.4 | 42.9 ± 24 *** | 25.4 ± 18.5 *** |
| **Pf + Cl** | 667.4 ± 330.8 | 689.4 ± 305.7 | 675.9 ± 276.3 | 669.2 ± 313.3 | 632.4 ± 369.2 | 494.5 ± 313.8 * | 57.8 ± 27.3 | 59 ± 27 | 57.3 ± 24.7 | 54.9 ± 24.4 | 42 ± 28.7 * | 30.7 ± 23.9 *** |
| **Br** | 622.5 ± 309.6 | 562.6 ± 238.3 | 614.9 ± 283.4 | 622.8 ± 293.6 | 656.8 ± 238.3 | 679.6 ± 297.2 | 60.3 ± 30.1 | 52.4 ± 23.2 | 56.9 ± 27.3 | 56.7 ± 27.2 | 59.9 ± 23.4 | 62.6 ± 27.8 |
| **Cl** | 770.6 ± 341.4 | 684.1 ± 310.5 | 711.9 ± 269.8 | 738.1 ± 330.8 | 889.7 ± 374.6 | 838.8 ± 403.6 | 68.4 ± 30.1 | 58 ± 27.2 | 60.5 ± 24.4 | 64.1 ± 29.8 | 77 ± 32.6 | 71.3 ± 33.7 |
| **Br + Cl** | 677.7 ± 338.2 | 627.4 ± 319.9 | 641.7 ± 342.3 | 654.4 ± 329.8 | 640 ± 327.3 | 706.4 ± 277.5 | 62.6 ± 31.9 | 58.4 ± 31.4 | 58.1 ± 32.8 | 58.4 ± 29.4 | 57.7 ± 30.6 | 64.2 ± 26.9 |

The distance moved and swimming time by zebrafish larvae at 96 hpf after exposure to the total persistent organic pollutants (POP) mixture and different sub-mixtures at different concentrations (1 to 200× higher than human serum level). (Pf) perfluorinated mixture; (Pf + Br) co-mixture of perfluorinated and brominated compounds; (Pf + Cl) co-mixture of perfluorinated and chlorinated compounds; (Br) brominated mixture; (Cl) chlorinated mixture; (Br + Cl) co-mixture of brominated and chlorinated compounds. Data presented as means ± SE. Asterisk indicates a significant exposure effect compared to the control (LME, * = $p < 0.017$, ** = $p < 0.0017$, *** = $p < 0.0001$).

**Appendix B**

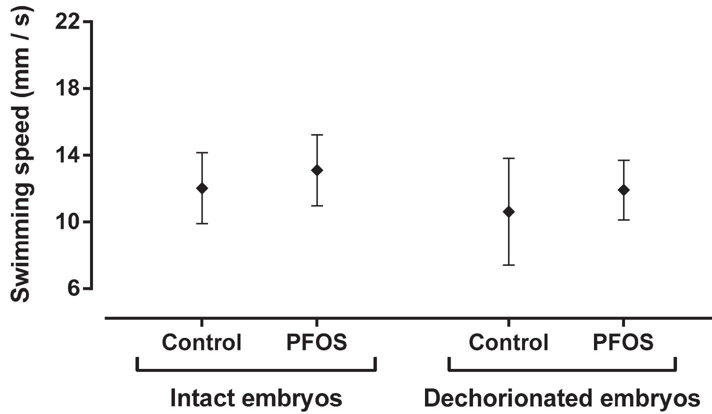


**Figure A1.** Swimming speed at 96 hpf in zebrafish larvae. Dechorionated and intact zebrafish embryos were exposed to perfluorooctanesulfonic acid (PFOS) (100× human serum level) between 24 to 48 hpf. Data presented as mean ± SD.

**Appendix C**

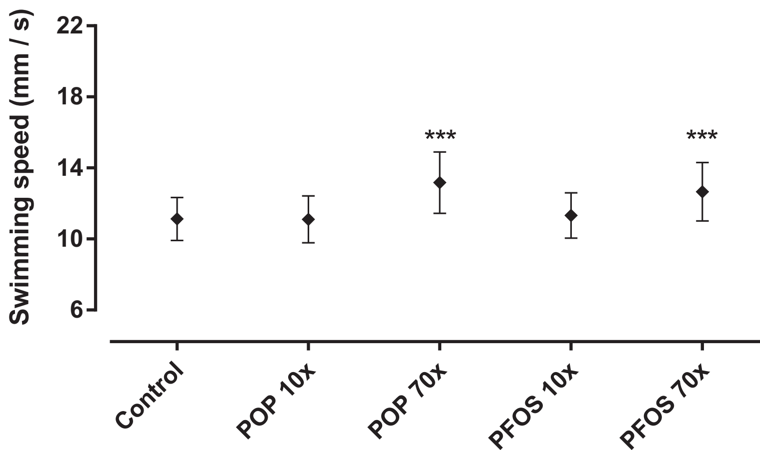


**Figure A2.** Swimming speed in 96 hpf zebrafish larvae after exposure to POP and PFOS at two different concentrations (10× and 70× higher than human serum level) between 48 to 96 hpf. In order to expose the zebrafish for gene expression analyses, concentrations were adjusted to 10× and 70× human serum level as the highest dose at which there was no significant effect on swimming speed (HNSS) or the lowest dose that consistently and significantly increased the swimming speed (LISS), respectively. Data presented as mean ± SD. An asterisk indicates a significant difference between the exposure group and the solvent control (LME, * = $p < 0.017$, ** = $p < 0.0017$, *** = $p < 0.0001$).

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# Supplementary Materials: A Mixture of Persistent Organic Pollutants and Perfluorooctanesulfonic Acid Induce Similar Behavioural Responses, but Different Gene Expression Profiles in Zebrafish Larvae

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**Table S1.** The composition and concentrations of chemicals in the persistent organic pollutant (POP) mixture. The value reflect the concentrations equal to 1× the mean human plasma level. (Pf) Perfluorinated mixture; (Br) Brominated mixture; (Cl) Chlorinated mixture; (Pf + Br) binary mixture of perfluorinated and brominated compounds; (Pf + Cl) binary mixture of perfluorinated and chlorinated compounds; (Br + Cl) binary mixture of brominated and chlorinated compounds. The table is adapted from [67].

| Compounds | Concentration (nM) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Total | Pf | Br | Cl | Pf + Br | Pf + Cl | Br + Cl |
| **Perfluorinated compounds (PFCs)** | | | | | | | |
| **PFOA** | 10.923 | 10.923 | | | 10.923 | 10.923 | |
| **PFOS** | 54.801 | 54.801 | | | 54.801 | 54.801 | |
| **PFDA** | 0.962 | 0.962 | | | 0.962 | 0.962 | |
| **PFNA** | 1.723 | 1.723 | | | 1.723 | 1.723 | |
| **PFHxS** | 7.873 | 7.873 | | | 7.873 | 7.873 | |
| **PFUnDA** | 0.990 | 0.990 | | | 0.990 | 0.990 | |
| **Polybrominated diphenyl ethers (PBDEs)** | | | | | | | |
| **BDE-47** | 0.018 | | 0.018 | | 0.018 | | 0.018 |
| **BDE-99** | 0.007 | | 0.007 | | 0.007 | | 0.007 |
| **BDE-100** | 0.003 | | 0.003 | | 0.003 | | 0.003 |
| **BDE-153** | 0.001 | | 0.001 | | 0.001 | | 0.001 |
| **BDE-154** | 0.003 | | 0.003 | | 0.003 | | 0.003 |
| **BDE-209** | 0.011 | | 0.011 | | 0.011 | | 0.011 |
| **HBCD** | 0.038 | | 0.038 | | 0.038 | | 0.038 |
| **Chlorinated compounds (CLCs) including** | | | | | | | |
| **Polychlorinated biphenyls (PCBs)** | | | | | | | |
| **PCB 28** | 0.050 | | | 0.050 | | 0.050 | 0.050 |
| **PCB 52** | 0.034 | | | 0.034 | | 0.034 | 0.034 |
| **PCB 101** | 0.024 | | | 0.024 | | 0.024 | 0.024 |
| **PCB 118** | 0.196 | | | 0.196 | | 0.196 | 0.196 |
| **PCB 138** | 0.615 | | | 0.615 | | 0.615 | 0.615 |
| **PCB 153** | 1.003 | | | 1.003 | | 1.003 | 1.003 |
| **PCB 180** | 0.490 | | | 0.490 | | 0.490 | 0.490 |
| **Other organochlorines** | | | | | | | |
| **p,p′-DDE** | 1.578 | | | 1.578 | | 1.578 | 1.578 |
| **HCB** | 0.410 | | | 0.410 | | 0.410 | 0.410 |
| **α-chlordane** | 0.026 | | | 0.026 | | 0.026 | 0.026 |
| **Oxy-chlordane** | 0.051 | | | 0.051 | | 0.051 | 0.051 |
| **Trans-nonachlor** | 0.092 | | | 0.092 | | 0.092 | 0.092 |
| **α-HCH** | 0.020 | | | 0.020 | | 0.020 | 0.020 |
| **β-HCH** | 0.182 | | | 0.182 | | 0.182 | 0.182 |
| **γ-HCH (Lindane)** | 0.020 | | | 0.020 | | 0.020 | 0.020 |
| **Dieldrin** | 0.063 | | | 0.063 | | 0.063 | 0.063 |

**Table S2.** Primer sequences for qPCR.

| Gene | Accession Number | Sequence | PCR Product Size |
| --- | --- | --- | --- |
| *Bactin* | FJ_915059.1 | F: CGAGCAGGAGATGGGAAC<br>R: CAACGGAAACGCTCATTGC | 101 |
| *Hprt* | NM_212986.1 | F: CAGCGATGAGGAGCAAGGTTATG<br>R: GTCCATGATGAGCCCGTGAGG | 102 |
| *Ef1α* | AM_422110.2 | F: TTGAGAAGAAAATCGGTGGTGCTG<br>R: GGAACGGTGTGATTGAGGGAAATTC | 90 |
| *Rps18* | NM_173234.1 | F: CATCCCAGAGAAGTTTCAGCACATC<br>R: CGCCTTCCAACACCCTTAATAGC | 104 |
| *Hmbs* | NM_201154.1 | F: GTGTGTGGAATTGGACAACAAAGTG<br>R: CGAGGGCTGATGATGAGATATTGC | 91 |
| *th1* | NM_131149.1 | F: TGGATCAGGATCACCCAGGA<br>R: GTAGACCTCCCGCCATGTTC | 149 |
| *th2* | NM_001001829.1 | F: CGTTCCGGGTTTTCCAGTGT<br>R: CGAGACGAGTCCAATCTGTGAA | 152 |
| *manf* | NM_001076629 | F: AGAGTGTGAAGTCTGTGTGGG<br>R: CGCTGTCAAACTTGACGTTGT | 77 |
| *hdc* | NM_001102593.1 | F: CTGGGCTCCACTGGTGTG<br>R: CTTGGACGGGTTGAAGACGA | 141 |
| *hrh1* | NM_001042731.1 | F: CGACCTCCACATGTTCACCA<br>R: CGTTGCAGAGCGGGTAAATG | 77 |
| *crhb* | NM_001007379.1 | F: CAATTACGCACAGATTCTCCTCG<br>R: GAAGTACTCCTCCCCCAAGC | 197 |
| *bdnf* | NM_001308649.1 | F: GGACACTTTCGAGCAGGTCA<br>R: CTCCAAAGGCACTTGGTTGC | 178 |
| *nr4a2b* | NM_001002406.1 | F: CGTACAGATCCAACCTGCCA<br>R: TATGGTGAGAGCGGCTATGC | 194 |
| *iphn3.1* | XM_005170940.2 | F: GAACAGCTCAGCGACTCTCA<br>R: TGTAGGAGGCTTGGGTGTTG | 161 |
| *per1b* | NM_212439.2 | F: AACGCTAAAGGTCCGTCTGT<br>R: CTTGTCCCCAACATGGACGA | 141 |
| *ache* | NM_131846.1 | F: CTCCAGGAACACTAGGCTGG<br>R: TACACAGCACCATGCGAGTT | 73 |
| *gabra1* | NM_001077326.1 | F: AGCCATCCTGATTTTTCGAGGG<br>R: AGCTTTTTCCAGCCAGAGCA | 121 |
| *chrna1* | NM_131445.1 | F: CTCGACCGACCTCTGGAAAC<br>R: GCAGGTCGAAGGGAAAGTGA | 176 |
| *chrna7* | NM_201219.2 | F: GAGTGGGACCTTGTGGAAGT<br>R: TCCGCATCACCACCGTAAAA | 100 |
| *gad2* | NM_001017708.2 | F: ATTGGCTAACCTCCACTGCC<br>R: CGAGCCAGTAGCATGGCATA | 184 |
| *gad1b* | NM_194419.1 | F: CTGTGACACCTGTGACTCCGTA<br>R: GTGTGCAACCCCGTACCAC | 181 |
| *gabbr1a* | XM_689405.6 | F: AACAACCGGAGCGACATTCT<br>R: CAGATTCCACATTCGCGCTG | 189 |
| *htr1aa* | NM_001123321.1 | F: CTACTCAACTTTCGGGGCGT<br>R: CACCGCCAAGCATTTATCCG | 145 |
| *sertb* | NM_001177459.1 | F: ACCCTGCCATATGTTGTGCT<br>R: AGCTGCATCTACCCATACGC | 135 |
| *ryr3* | XM_009294773.1 | F: GAGGCAACGTTCTTGTGCAG<br>R: CCGTCCTTTCACGCTGATTG | 191 |
| *dat* | NM_131755.1 | F: TCAAGTTCCTGCACAAACATCG<br>R: CACAAATTTCCAGCACAGTCTCC | 268 |

# Paper III

1 **Maternal exposure to a mixture of persistent organic pollutants (POPs) affects testis**
2 **morphology, epidydimal sperm count and induces sperm DNA fragmentation in mice**

3

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33

34 **Abstract**

35 Persistent organic pollutants (POPs) are widespread throughout the environment and some
36 are suspected to induce reproductive toxicity. As animals and humans are exposed to complex
37 mixtures of POPs, it is reasonable to assess how such mixtures could interact with the reproductive
38 system. Our aim is to investigate how maternal exposure to a mixture of 29 different persistent
39 organic pollutants, formulated to mimic the relative POP levels in the food basket of the
40 Scandinavian population, could alter reproductive endpoints. Female mice were exposed via feed
41 from weaning, during pregnancy and lactation in 3 exposure groups (control (C), low (L) and high
42 (H)). Testicular morphometric endpoints, epididymal sperm concentration and sperm DNA
43 integrity (in sperm from both vas deferens and cauda epididymis) were assessed in adult male
44 offspring. We found that the number of tubules, proportion of tubule compartments and epididymal
45 sperm concentration significantly decreased in both POP exposed groups. Cauda and vas deferens
46 sperm showed different trends in the sperm chromatin structure assay. Nevertheless, epididymal
47 sperm from both POP exposed groups showed increased DNA fragmentation. It is concluded that
48 maternal exposure to a defined POP mixture relevant to human exposure can affect testicular
49 development, sperm production and sperm chromatin integrity.

50 **Keywords:** Persistent organic pollutants (POPs), reproductive toxicity, testis morphology, sperm
51 count, Sperm Chromatin Structure Assay (SCSA)

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## Introduction

Persistent organic pollutants (POPs) refer to groups of toxic environmental chemicals with a carbon-based structure, which are resistant to environmental degradation and are widely distributed via soil, water and air (Hung *et al.*, 2016). Because of their lipophilic nature, POPs tend to biomagnify through the food chain and bioaccumulate from lower organisms to top predators and humans (Daley *et al.*, 2014). Among different classes of POPs, chlorinated, brominated and perfluorinated compounds are the most persistent compound classes, widely detected in human adipose tissue, breast milk and blood samples from all over the world (Knutsen *et al.*, 2008; Polder *et al.*, 2008; Linderholm *et al.*, 2010; Kim *et al.*, 2013; Pumarega *et al.*, 2016).

The lipophilic property of some POP families makes them capable of passing through biological barriers such as the blood-brain barrier (Rasinger *et al.*, 2014) as well as the placenta (Vizcaino *et al.*, 2014), a process which leads to accumulation of POPs in the fetus. Parental exposure to POPs has in epidemiological studies been associated with adverse effects in fetuses and neonates such as lower birth weight (Casas *et al.*, 2015; Robledo *et al.*, 2015), ADHD (Attention Deficit Hyperactivity Disorder) and depression (Strom *et al.*, 2014), decreased mental development (Gascon *et al.*, 2012), immune-related diseases and lung dysfunction (Cao *et al.*, 2016). In contrast, some classes of POPs like perfluorinated compounds do not accumulate in adipose tissue, but can bind to proteins and interfere with normal endocrine function (Jones *et al.*, 2003; Zhang *et al.*, 2013b).

Reproductive toxicity upon POP exposure has been reported in both females and males in different animal models including mice (Tseng *et al.*, 2013; Fiandanese *et al.*, 2016), rats (Zhou *et al.*, 2013) and zebrafish (Nourizadeh-Lillabadi *et al.*, 2009; Zhang *et al.*, 2016). It has been shown that some POPs induce reproductive toxicity via the disruption of steroidogenesis, interference

80 with normal hormonal balance by interaction with carriers/receptors, induction of oxidative stress
81 and/or interference with epigenetic mechanisms (Sharpe and Irvine, 2004; Jeng, 2014; Vested *et*
82 *al.*, 2014). Furthermore, some POP families are able to disrupt the blood-testis barrier, accumulate
83 in the testis and impact testicular function (Li *et al.*, 2009; Qiu *et al.*, 2013; Lu *et al.*, 2016).

84 Although several methods have been developed in order to study male fertility, testis
85 histology assessment is still considered a gold standard to assess testicular toxicity. The sperm
86 chromatin structure assay (SCSA) provides a measure of sperm DNA fragmentation and
87 compaction and is a valuable method to assess sperm quality (Evenson and Wixon, 2005). Sperm
88 DNA integrity is a key factor for the proper transmission of genetic material, and sperm chromatin
89 abnormalities or DNA damage may cause male infertility (Agarwal and Said, 2003). It has been
90 suggested that POP exposure could induce sperm DNA damage and decrease the DNA integrity
91 (Rozati *et al.*, 2002; Rignell-Hydbom *et al.*, 2005; de Jager *et al.*, 2009).

92 The majority of toxicology studies have focused on the effects of single compounds only,
93 whereas in reality we are exposed to complex mixtures of pollutants (Frederiksen *et al.*, 2009; Ni
94 *et al.*, 2013). How such mixtures interact on toxicological endpoints is relatively unknown, but is
95 a significant concern as several studies have demonstrated the potential of different compounds to
96 have additive effects. For instance, it has been shown that co-exposure to PCB 153 and methyl
97 mercury enhances developmental neurotoxic effects in mice (Fischer *et al.*, 2008). Further,
98 exposure to a mixture of lead, arsenic and organic mercury in pregnant mice enhanced maternal
99 toxicity compared to individual exposure (Bellés *et al.*, 2002). Recently we developed a complex
100 mixture based on the POPs found in Scandinavian food basket surveys, as well as human blood
101 and breast milk samples (Berntsen *et al.*, 2016a) and preliminary unpublished data indicates that
102 our POP mixture significantly affects behavior and stress responses in mice.

103 A key aspect in reproductive toxicity studies is that the exposure scenario must be
104 translatable to a human scenario. Therefore, investigating the effects of environmentally relevant
105 POPs in a mixture formula would be more realistic than investigating effects of single POPs. In
106 the current experiment, we investigated effects on testis morphology, sperm production and sperm
107 DNA integrity in male mice exposed *in utero* and through mother's milk to a complex POP mixture
108 reflecting POP ratios found in Scandinavian food.

109 **Material and methods**

110 *Animals, housing and husbandry*

111 The study was performed at the Section for Experimental Biomedicine at The Norwegian
112 University of Life Sciences in Oslo, Norway. The unit is licensed by the Norwegian Food
113 Inspection Authority (NFIA) and accredited by the Association for Assessment and Accreditation
114 of Laboratory Animal Care (www.aaalac.org). The study was approved by the unit's animal ethics
115 committee (Institutional Animal Care and Use Committee/IACUC) and NFIA.

116 All animals were group housed in open type III cages (Tecniplast, Buguggiate, Italy),
117 containing standard aspen bedding (Scanbur BK, Nittedal, Norway) and cellulose nesting material.
118 The animals had free access to their assigned feed. Tap water was available from standard drinking
119 bottles (Tecniplast, Buguggiate, Italy). The animal room was on a 12:12 light–dark cycle, with a
120 room temperature of 21 ± 2°C as well as 20 air changes per hour and 45 ± 5% relative humidity.
121 The cages, bedding, nesting material and water bottles were changed once a week. In-house bred
122 129:C57BL/6 females (which parents were obtained from Jackson Laboratory, Maine, USA), were
123 mated with non-brother males of the same hybrid and generation to produce the male specimens
124 used in the current study.

125 *Feed design, chemicals and exposure scenario*

126 The design and preparation of the POP mixture is described in (Berntsen *et al.*, 2016a). In
127 brief, relevant compounds and their estimated daily intake (EDI) levels for humans in Scandinavia
128 were identified and used as a basis for the mixture. Feed concentration ratios of the different
129 compounds were calculated to reflect these human EDIs. However, the clearance rates for many
130 toxicants have shown to be higher in mice than in humans (Walton *et al.*, 2001). When determining
131 doses to use, factors such as interspecies differences in compound metabolism and number of
132 distinct doses were taken into consideration. therefore, the doses were set higher. The allowed
133 maximum concentrations of different compounds were set to 5000 times and 100 000 times the
134 EDIs calculated for the basic mixture.

135 All polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyl (PCBs) and other
136 organochlorines were originally purchased from Chiron As (Trondheim, Norway).
137 Hexabromocyclododecane (HBCD) and all perfluorinated compounds (PFAAs), except
138 perfluorohexane sulfonic acid (PFHxS), which was from Santa Cruz (Dallas, USA), were obtained
139 from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were dissolved in an appropriate solvent
140 and added to corn oil (Asko, Norway), intended for human consumption. In all diets, Soybean oil
141 in the original feed recipe was exchanged with the same corn oil used for preparations of the
142 mixture. Four different diets were made, three exposure diets for pregnant mice, including control
143 (containing corn oil from which solvents had been evaporated), low dose (5000x EDI) and high
144 dose (100 000x EDI), and one reference diet for males and pups after weaning (containing
145 untreated corn oil only). The females used for breeding were randomly assigned to either the high
146 concentration exposure group (H) (16 females), low concentration exposure group (L) (16 females)
147 or control group (C) (15 females) and were exposed to the mixture of POPs through the feed. The

148 exposure started at weaning and continued through breeding and lactation until necropsy. The
149 offspring from the exposed mothers, were only exposed to POPs (6 weeks) via the placenta,
150 mother's milk and by nibbling of their mothers' feed before weaning. After weaning, pups were
151 given the reference diet with no added POPs.

152 *Sample collection*

153 In order to assess general toxicity, offspring mice were weighed at weaning and prior to
154 necropsy. Nine week old mice (15 pups per exposed group) were anesthetized by isoflurane
155 inhalation (4,5 % ISO at 700 mL airflow), euthanized by exsanguination and decapitation under
156 deep anesthesia. During exsanguination, in order to measure the actual concentration of chemicals
157 in plasma, a blood sample was collected using 1 mL EDTA coated syringes with a 23 G needle
158 (Terumo, Norway), transferred into a 1 mL EDTA coated microvette tube (Sarstedt, Norway) and
159 kept at 4 °C. Later on, samples were centrifuged at 5000 rpm / 4 °C for 10 minutes; plasma was
160 collected and stored at -80 °C until analyzes. In order to collect the testis and epididymis, the
161 abdominal cavity was opened. After collecting the internal organs, the testes were pushed forward
162 from the scrotal sac. The left testis was dissected and fixated in Davidson solution (30% *v/v*
163 formaldehyde, 15% *v/v* ethanol, 5% *v/v* glacial acetic acid and 50% *v/v* distilled water) for 24h at
164 4 °C for morphological studies. The right testis and the epididymis compartments (including cauda,
165 vas deferens and caput) from both sides were stored at -80 °C until further analyzes.

166 *POPs plasma concentration measurement*

167 The plasma concentrations of POPs were measured at the Norwegian University of Life
168 Sciences, Department of Food Safety and Infection Biology, Laboratory of Environmental
169 Toxicology as more thoroughly described by (Berntsen *et al.*, 2016b). One pooled sample from

170 each exposure group was measured. These pools also included samples of females used for other
171 studies in the project. For the lipophilic groups of chemicals, extraction of POPs from biologic
172 samples has been described by (Polder *et al.*, 2014). Separation and detection of the pesticides and
173 PCBs were performed on a high resolution gas chromatograph (HRGC) (Agilent 6890 Series,
174 Agilent Technologies, PA, USA). Detection of PBDEs (except from PBDE 209) and HBCD was
175 performed on a high-resolution gas chromatograph-low resolution mass spectrometer (HRGC-
176 LRMS) (Agilent 6890 Series, Agilent Technologies, PA, USA). Perfluorinated compounds were
177 analyzed according to (Bytingsvik *et al.*, 2012) and references therein. The samples were analyzed
178 by separation of compounds on a high-performance liquid chromatographer (HPLC) with a
179 Discovery C18 column, connected to a C18 pre-column (Supelco, Sigma-Aldrich) and detection
180 with liquid chromatography tandem mass spectrometry (MS-MS) (API 3000, LC/MS/MS
181 System). Since perfluorinated compounds do not accumulate in lipids, they were not measured
182 based on ng/g lipid weight in plasma.

183 *Absolute measures and proportions of seminiferous tubules*

184 Fixed testis were dehydrated, using 70 % ethanol, and embedded in paraffin. Subsequently,
185 5-µm sections were mounted on slides and stained with hematoxylin and eosin (H&E) according
186 to the local protocol. Images were taken using a digital camera (color view XC30, Olympus) and
187 software for image capture (CellSens Dimension v1.6, Olympus). Images were captured from
188 testis areas where round cross-sections of the seminiferous tubules were clear. In total, 15 samples
189 per exposure group and 20 round seminiferous tubules per sample were captured and measures of
190 the seminiferous tubules were taken using ImageJ v.1.51 software (National Institutes of Health,
191 Bethesda, MD, USA) for windows according to (Montoto *et al.*, 2012). Briefly, mean diameter of
192 the seminiferous tubule and lumen diameter were calculated across the minor and major axes. The

193 distance from the basal membrane to the luminal border was considered as epithelial thickness and
194 the final value was calculated as the mean value of measurements in each cross-section. The area
195 occupied by seminiferous tubules and lumen was measured and epithelium area was calculated by
196 subtracting the lumen area from the seminiferous area. The relative number of seminiferous
197 tubules was calculated as described in figure 1.

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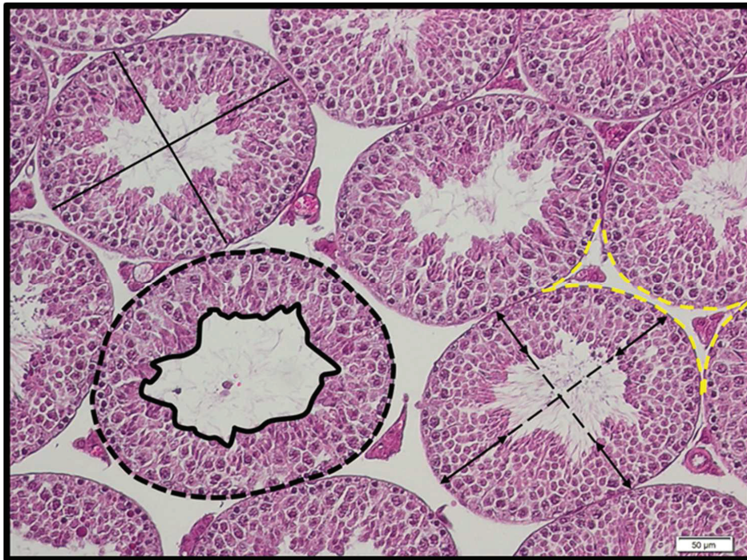


206 **Figure 1**. *Measurements of components of the seminiferous tubules. The thick dotted and*
207 *continuous black lines define the circumference of the seminiferous tubule and lumen, respectively.*
208 *The double-headed arrows, dotted and continuous black narrow lines show the epithelium*
209 *thickness, lumen diameter and seminiferous tubule diameter, respectively. The percentage of total*
210 *interstitial area in the testis was obtained by measuring the area occupied by the sum of all*
211 *interstitial space (dotted yellow line). The percentage of total area occupied by seminiferous*
212 *tubules was calculated by subtracting the obtained area occupied by interstitial area from the*
213 *image area (bold black borderline).*

214 *Epididymal sperm count*

215 One dissected cauda epididymis of each animal was weighed, and the sperm cells were
216 gently squeezed out into 800 µl of ice-cold TNE buffer (pH 7.4; 0.01 M Tris-HCl, 0.15 M NaCl,
217 1 mM EDTA). The cell suspension was carefully pipetted and 50 µl of cell suspension was fixed
218 by adding 50 µl of 0.2 % paraformaldehyde and kept at 4 °C for cell counting the next day whereas
219 the remaining suspension was used for the SCSA analyzis. A volume of 10 µl of each sperm
220 suspension was diluted 1:1 with trypan blue and counted in a Bürker chamber under light
221 microscopy.

222 *Sperm chromatin structure assay (SCSA)*

223 Cauda and vas deferens sperm were analyzed by SCSA following the standardized
224 procedures described by (Evenson and Jost, 2000) to determine sperm chromatin integrity. Briefly,
225 a few incisions were made in one cauda epididymis and the content carefully squeezed out into
226 800 µl of cold TNE buffer and passed through a 160-µm nylon filter. Sperm cells from both vas
227 deferenses were gently squeezed out using an L shape needle and handled as cauda sperm cells
228 and treated separately. Prior to analyzis, sperm samples were denatured for 30 seconds by adding
229 an acid solution (pH 1.2; 0.08 N HCl, 0.15M NaCl, 0.1 % Triton-X 100), and subsequently loaded
230 with acridine orange (AO) staining buffer (pH 6.0; 0.1 M citric acid, 0.2 M Na2PO4, 1 mM EDTA,
231 0.15 M NaCl and 0.6 µg/ml of AO). After 3-5 minutes incubation at 4 °C, ten thousand cells per
232 sample were analyzed by flow cytometry (LSRII; BD Bioscience, CA, USA). Three parameters
233 from the SCSA diagram were analyzed: DNA fragmentation index (DFI), which is the relationship
234 between sperm cells with red fluorescence and total (red and green) fluorescence. The % DFI or
235 percentage of cells with elevated DFI, was calculated from the DFI frequency histogram, and
236 represents cells with an increased level of single-stranded DNA and is defined as the percentage

237 of sperm that falls outside the main population in a sample. The main population was defined based
238 on the DFI distribution of a control sample. The highly DNA stainable (% HDS) represents sperm
239 cells with an elevated incorporation of AO into double-stranded DNA and is visualized as an
240 increased green fluorescence. HDS cells are considered sperm cells with an incomplete chromatin
241 condensation. For the flow cytometer set-up and calibration, a reference sample was established
242 consisting of batches of control cauda sperm cells stored at -80 °C. A positive control sample was
243 prepared by incubating the cauda sperm from control mice in DNase buffer (200 U/ml) with 1%
244 Triton X-100, at 37 °C for 1 hour. Reference and positive control samples were stored at -80 °C
245 until use. Flow data were obtained and SCSA parameters were calculated using The BD
246 FACSDiva software (v. 6.1.2) and FCS express (DeNovoSoftware, CA, USA; v.3), respectively.

247 *Statistical analyzes*

248 Data were analyzed using R Studio (RStudio Team 2015, version 0.99.473 for windows).
249 To test the effect of the POP mixture on endpoints, a linear mixed effect model (LME) was
250 employed. Morphometric measures, sperm count and SCSA data were introduced as dependent
251 variables, exposure group as a categorical independent variable, and mothers as a random effect.
252 Examination of the residual plots verified that no systematic patterns occurred in the errors (e.g.
253 q-q plots). To assess individual doses to the controls, we used the contrast results provided within
254 R. Prior to correlation analyzes, the normal distribution of data was tested using the Shapiro-Wilk
255 test. If necessary, the data were log-transformed to meet the requirements for a parametric test.
256 Correlations between endpoints were determined by Pearson correlation coefficient method in case
257 of normal distribution and by Spearman's rank correlation coefficient in case of non-normal
258 distribution. The limit of significance was set at $p < 0.05$. At the end, data were plotted using
259 GraphPad Prism version 7.02 for Windows, (GraphPad Software, San Diego, California, USA).

260 **Results**

261 *POP levels in mice plasma*

262 POP levels in dam and offspring plasma levels, adipose tissue and brain levels were
263 reported in (Berntsen *et al.*, 2016b). Here only offspring plasma levels are given. As demonstrated
264 in Table 1, plasma POP levels (ng/g ww) in high exposed mice were almost 5 to 32 times higher
265 than in low exposed mice except for β-HCH. The levels of all perfluorinated compounds and to a
266 lesser degree some PCBs turned out to be positive in the control offspring plasma.

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277 **Table 1**. *POP plasma levels in offspring mice. (*) break down products; (n.d) not detected; (n.m)*
278 *not measured. Table was adapted from (Berntsen et al., 2016b).*

| | Control | | Low | | High | |
|---|---|---|---|---|---|---|
| | ng/g wet weight | ng/g lipid weight | ng/g wet weight | ng/g lipid weight | ng/g wet weight | ng/g lipid weight |
| **Lipid Content (%)** | - | 0.4 | - | 0.3 | - | 0.4 |
| **Chlorinated compounds** | | | | | | |
| PCB 28 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| PCB 52 | n.d. | n.d. | 0.12 | 40.0 | 2.805 | 766.4 |
| PCB 101 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| PCB 118 | 0.282 | 72.0 | 0.369 | 123.3 | 11.829 | 3232.0 |
| PCB 138 | 1.158 | 296.2 | 3.984 | 1332.5 | 49.756 | 13594.6 |
| PCB 153 | 1.16 | 296.8 | 2.891 | 967.0 | 48.939 | 13371.3 |
| PCB 180 | 0.33 | 84.4 | 0.774 | 258.8 | 14 | 3825.1 |
| p,p'-DDE | n.d. | n.d. | 0.299 | 99.9 | 1.707 | 466.5 |
| HCB | 0.274 | 70.0 | 0.747 | 249.8 | 9.512 | 2599.0 |
| α - chlordane | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| oxy - chlordane | n.d. | n.d. | 1.285 | 429.7 | 13.659 | 3731.8 |
| trans-nonachlor | n.d. | n.d. | 0.359 | 119.9 | 6.22 | 1699.3 |
| α-HCH | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| β-HCH | n.d. | n.d. | 0.149 | 50.0 | 10 | 2732.2 |
| γ-HCH (Lindane) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Dieldrin | n.d. | n.d. | 1.355 | 453.0 | 16.829 | 4598.2 |
| **Brominated compounds** | | | | | | |
| BDE-47 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| BDE-99 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| BDE-100 | n.d. | n.d. | 0.09 | 30.1 | 0.61 | 166.6 |
| BDE-153 | n.d. | n.d. | n.d. | n.d. | 1.098 | 299.9 |
| BDE-154 | n.d. | n.d. | n.d. | n.d. | 1.951 | 533.1 |
| BDE-209 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| BDE-202* | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| BDE-196* | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| BDE-208* | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| BDE-207* | n.d. | n.d. | 0.159 | 53.3 | n.d. | n.d. |
| BDE-206* | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| HBCD | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Perfluorinated compounds** | | | | | | |
| PFHxS | 7.574 | n.m. | 16.385 | n.m. | 341.659 | n.m. |
| PFOS | 18.799 | n.m. | 30.443 | n.m. | 684.585 | n.m. |
| PFOA | 13.676 | n.m. | 27.558 | n.m. | 644.49 | n.m. |
| PFNA | 15.458 | n.m. | 24.799 | n.m. | 506.993 | n.m. |
| PFDA | 16.799 | n.m. | 29.038 | n.m. | 542.347 | n.m. |
| PFUnDA | 3.73 | n.m. | 6.571 | n.m. | 123.064 | n.m. |
| PFDoDA* | n.d. | n.m. | n.d. | n.m. | n.d. | n.m. |
| PFTrDA* | n.d. | n.m. | n.d. | n.m. | n.d. | n.m. |

279

280 *POP exposure affected testicular morphological endpoints*

281 Body mass was not affected by exposure neither at weaning nor at necropsy. However,
282 exposure to POPs altered the structure of seminiferous tubules. The POP mixture in both low and
283 high concentrations significantly reduced and increased the relative total number of seminiferous
284 tubules and the interstitial space, respectively (Figure 2A). Our findings showed that the
285 seminiferous tubule diameter decreased in both low and high exposed mice. However, this trend
286 was significant only in high exposed mice and lumen diameter as well as epithelial thickness were
287 not affected significantly by POP exposures (Figure 2B). Using area measures we found a
288 significant reduction in both lumen and epithelium area. As shown in Figure 2C, POP exposure in
289 both groups significantly decreased the area occupied by seminiferous tubules and lumen.
290 Epithelium area was also reduced significantly in low exposed mice. Our results showed that the
291 area measures correlated significantly with diameter measures (r = 0.911, 0.886 and 0.739, for
292 tubules, lumen and epithelium area, respectively).

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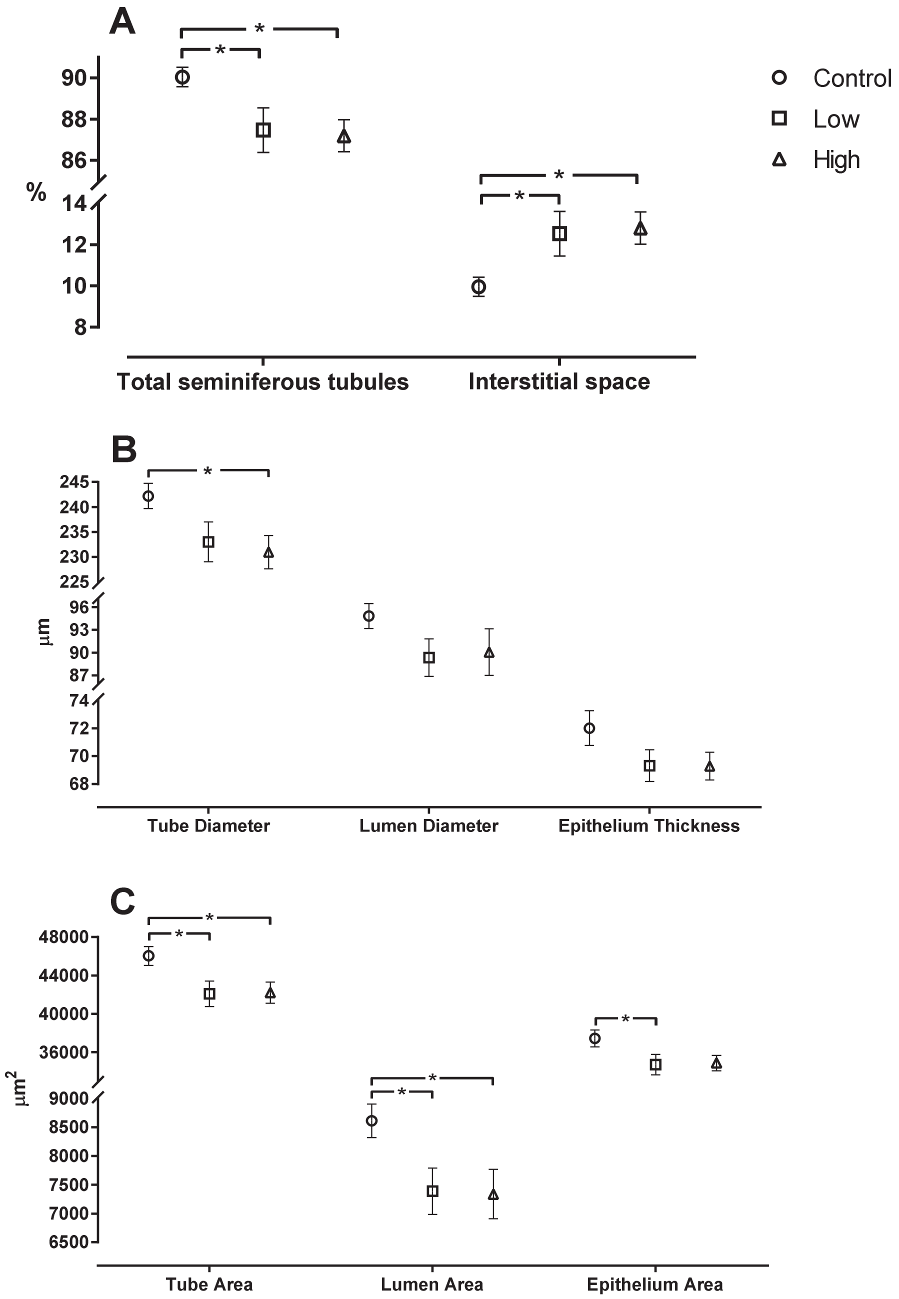


296

297 **Figure 2**. *Testis histomorphological measures in offspring mice after exposure to a mixture of*
298 *POPs. (A) Percentage of total seminiferous tubules and interstitium in the testes; (B) Diameter of*
299 *seminiferous compartments and (C) Circumference of tubules compartments. Data presented as*
300 *mean ± SE. Asterisk showing results significantly different from control by linear mixed effect*
301 *analyzes (* = $p < 0.05$).*

302

303 *The POP mixture affected the number of epididymal sperm cells*

304 POP exposure in both low and high groups resulted in a significant reduction in epididymal
305 sperm count (Table 2). However, there was no significant correlation between number of
306 epididymal sperm cells and total number of seminiferous tubules as well as epithelium
307 thickness/area.

308

309 **Table 2**. *Epididymal sperm count in offspring mice after exposure to low and high dose of POP*
310 *mixture. Data presented as mean ± SE. Asterisk showing significantly different from control by*
311 *linear mixed effect analyzes (* = $p < 0.05$, ** = $p < 0.005$).*

| | **Control** | **Low** | **High** |
|---|---|---|---|
| **Sperm count ($10^4$/mg cauda)** | 56.06 ± 4.95 | 42.58 ± 2.73* | 36.39 ± 3.78** |

312

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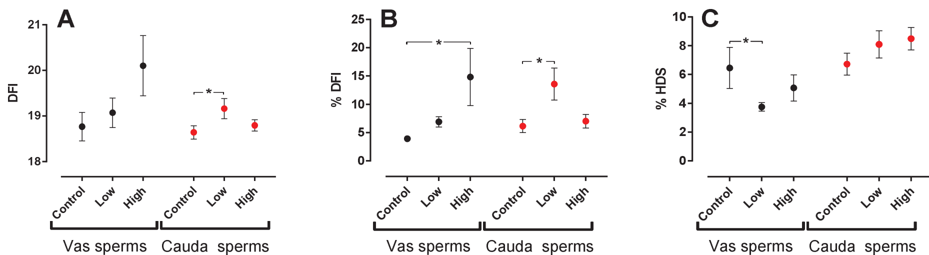
315

316 *The POP mixture-reduced sperm DNA integrity*

317 Sperm cells collected from POP exposed mice showed increased DNA fragmentation. As
318 demonstrated in Figure 3A and 3B, POP exposures increased both DFI and % DFI in vas sperm
319 cells, but this trend was statistically significant only for the % DFI in high exposed group. Vas
320 sperm cells in exposed mice showed a higher degree of DNA condensation, but this trend was
321 significant only in low exposed mice (Figure 3C). On the other hand, in caudal sperm cells, DFI
322 and the % DFI were significantly increased in low exposed mice (Figure 3A and 3B) and the %
323 HDS in exposed mice was not statistically different compared to control (Figure 3C). Overall
324 cauda sperm cells in exposed mice showed a higher % HDS trend.




325

326 **Figure 3**. *Sperm chromatin structure assay from collected sperm cells in vas deferens and cauda*
327 *epididymis in offspring mice after exposure to a mixture of POPs. (A) DNA fragmentation index;*
328 *(B) percentage of sperm cells with damaged DNA and (C) percentage of sperm cells with immature*
329 *DNA condensation. Data presented as mean ± SE. Asterisk showing results significantly different*
330 *from control by linear mixed effect analyzes (* = $p < 0.05$).*

331

332

333 **Discussion**

334 In this study, we demonstrate that our POP mixture, which made to mimic the realistic
335 human exposure scenario, significantly changed the area occupied by different parts of
336 seminiferous tubules, reduced the number of sperm cells and induced sperm DNA fragmentation
337 in the offspring of exposed mothers.

338 Although some brominated and chlorinated compounds, which are normally detected in
339 human blood, were not detected in exposed offspring mice, most of the POPs in the low exposed
340 group were found in the range of 1-20 times human blood levels. Oxy – chlordane, dieldrin, BDE-
341 100, perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) were found at
342 concentrations up to 70 times higher than in humans (Berntsen *et al.*, 2016b). In this study, the
343 plasma level (ng/g ww) of perfluorinated compounds in high exposed mice were close to what
344 has previously been reported in polar bears (Bytingsvik *et al.*, 2012). We noticed that some of the
345 chlorinated and all of the perfluorinated compounds were found in the control group. This can
346 probably be explained by air-borne contamination through inhalation of feed dust. However,
347 offspring were only exposed during fetal life and the suckling period, after which all animals were
348 given the same feed and placed in another room separated from the dams. The control dams had
349 lower levels of these same compounds than their offspring for unknown reasons (Berntsen *et al.*,
350 2016b).

351 To date, little attention has been paid to how mixtures of POPs can affect male
352 reproduction. Previous work suggested that the results of single compounds are not fully
353 translatable to mixture scenarios, mainly because of unknown interactions between different
354 chemicals in complex mixtures (Groten *et al.*, 2001). In this study, gestational and lactational
355 exposure to a mixture of POPs significantly decreased the seminiferous compartments area, while

356 no such significant trend was found when we applied diameter measurement; hence measuring the
357 area occupied by seminiferous compartments was more sensitive than measuring the
358 compartments diameter. These differences can be explained in part by the proximity of diameter
359 measures because not all of seminiferous tubules were completely round and we measured
360 diameter only in two axes. Although we found a close correlation between the values in both
361 approaches, most of the studies reported the tubular diameter as an endpoint and our results further
362 support the relationship between the effect of environmental pollutants and testicular structure. For
363 instance, it has been reported that mice exposed to Bis(2-ethylhexyl) phthalate (DEHP) or PCBs
364 (alone and in combination), showed smaller seminiferous tubule diameter, while the epithelial
365 thickness was not affected (Fiandanese *et al.*, 2016). Similar results were found in mice offspring
366 upon lactation exposure to a mixture of PCBs (101 + 118) (Pocar *et al.*, 2012). Another study
367 reported that *in utero* exposure to PCB (118+153) mixtures in a range equal to 3 times higher (ng/g
368 lipid wight) than the levels in this study, exerted subtle effects on developing fetal testis proteome
369 but did not significantly disturb testis morphology in sheep (Krogenaes *et al.*, 2014). There is little
370 published data on the effect of perfluorinated compounds on seminiferous tubule structure. It has
371 been documented that perfluorooctanoic acid (PFOA) disrupted the blood-testis barrier (Lu *et al.*,
372 2016), and accumulated in mice testis (Zhang *et al.*, 2014). Perfluorinated compounds like
373 perfluorooctane sulfonate (PFOS) could induce histopathological lesions such as vacuolations in
374 mice testis (Qu et al., 2016). Further studies need to be carried out in order to address the role of
375 perfluorinated compounds on testicular structure.

376 Our results indicated that exposure to a mixture of environmental pollutants significantly
377 affected the epididymal sperm count. Our finding is in agreement with previous studies where
378 PFOA was associated with a reduction in epididymal sperm cells in human (Vested et al., 2013)

379 and mice (Zhang et al., 2014). Current results further support the causal relationship between PFOS
380 and epididymal sperm count in mice as well (Wan et al., 2011). On the other hand, it has been
381 shown that sperm concentration in mice was not affected by maternal exposure to BDE-209 (Tseng
382 *et al.*, 2013) or a mixture of PCB (101+118) (Pocar *et al.*, 2012; Fiandanese *et al.*, 2016).
383 Furthermore, PCBs or *p,p'*-DDE levels in human plasma were not associated with the number of
384 produced sperm cells (Toft *et al.*, 2006; Jurewicz *et al.*, 2009; Haugen *et al.*, 2011). It has been
385 suggested that the number of seminiferous tubules are correlated with the number of produced
386 sperm cells (Montoto *et al.*, 2012). Our results revealed no significant relationship between
387 produced sperm cells and number of seminiferous tubules, which would indicate that, POPs could
388 interfere with spermatogenesis process via other pathways. It has been reported that PFOS
389 promotes apoptosis in germ cells and thereby decreases the number of caudal sperm cells (Qu *et*
390 *al.*, 2016). The fact that we in the present study detected a high level of different compounds even
391 in low exposed mice plasma, advocate for more research to determine the combined toxicities of
392 perfluorinated compounds and the classical POPs in spermatogenesis process.

393 Our SCSA analyzes suggest that the POP mixture could impair the cauda and vas deferens
394 sperm DNA integrity. The induction of DNA fragmentation in the high dose group was only
395 significant when analyzed in vas sperm. The reasons for this finding are not clear but could be
396 explained by the differences in cauda and vas deferens luminal fluid composition. It is reported
397 that sperm cells are more sensitive to DNA damage after incubation with vas deferens luminal
398 fluid suggesting that a program of abortive apoptosis occurs to a higher degree in vas than in cauda
399 sperm (Gawecka *et al.*, 2015). Effects of POPs on mice sperm DNA integrity are not consistent
400 across the literature. It has been reported that maternal exposure to BDE-209, increased the % DFI
401 (Tseng et al., 2013) and exposure to PCB 153 (75 times higher than our level in high exposed

402 group), did not affect the sperm DNA integrity (Oskam *et al.*, 2004). These results may be
403 explained by the complexity of the POP mixture. On the other hand, PCB 153 at a level equal
404 (ng/g w.w) to our high exposed mice, increased the % DFI in 40 week old goats (Oskam *et al.*,
405 2005). To our knowledge, the only study investigating the association between perfluorinated
406 compounds and sperm DNA integrity, revealed no associations in men from three geographical
407 regions (Specht et al., 2012). It is well documented that oxidative stress and reactive oxygen
408 species (ROS) are both involved in the induction of sperm DNA fragmentation (Lopes et al., 1998;
409 Wright et al., 2014). It has been shown that *p, p'*-DDE exposure, which was a part of our mixture,
410 could affect the sperm DNA fragmentation via ROS production and mitochondrial dysfunction
411 (Pant et al., 2014). Another study reported that exposure to PFOS and PCB 153 increased ROS
412 production in Sertoli cells (Zhang *et al.*, 2013a) and BDE 209 resulted in oxidative stress and
413 increased the level of $H_2O_2$ in testis tissue (Tseng et al., 2006). In the current study, cauda sperm
414 cells in exposed mice showed a lower degree of DNA condensation (% HDS) compared to vas
415 sperm cells. Sperm DNA condensation is a process that requires an exchange between histones
416 and protamines in sperm DNA (Chapman and Michael, 2003) and takes place while sperm cells
417 migrate through vas deferens, toward the cauda epididymis (Hingst *et al.*, 1995; Golan *et al.*,
418 1996). It has been shown that environmental toxicants can increase the % HDS via preventing the
419 cleavage of protamine 2, hence the chromatin would not be fully condensed (Evenson and Wixon,
420 2005). More research is needed to evaluate the role of POP mixtures in ROS induction in
421 reproductive organs as well as in the different phases of spermatogenesis.

422 The main goal of the current study was to determine whether a human POP mixture based
423 on the Scandinavian food basket could induce reproductive toxicity in male mice following
424 maternal exposure. Our results showed that the POP mixture had a significant effect on the

425 seminiferous tubule compartments, sperm production and sperm DNA fragmentation. The current
426 findings highlight the potential for reproductive toxicity following in utero and lactational
427 exposure to a human relevant POP mixture. Further experiments are needed to investigate possible
428 mechanisms of action and effects on male fertility.

429 **Conflict of interest**

430 The authors declare that there are no conflicts of interest.

431 **Acknowledgment**

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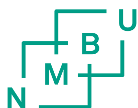


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