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Effects of Defined Mixtures of Persistent Organic Pollutants on DNA Damage in a Human Endocrine Cell Line (H295R) and in Mouse Lymphocytes Exposed in Vitro and in Vivo

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Abstract

A wide range of synthetic chemicals have been used extensively for decades. They are ubiquitous in the environment, and can therefore pose adverse effects on humans and wildlife. Mixtures of persistent organic pollutants (POPs) that are relevant to real –life exposure, are of emerging concern when it comes to their potentially adverse effects and the ability to interact with other substances.

The main objective of this study was to investigate exposure –relevant mixture of POPs, based on levels recently measured in blood and breast milk in Scandinavia, for the *in vitro* study. The *in vivo* study based the mixture on estimated daily intake measured in food. DNA damage was assessed by the method of choice; comet assay and used on the human adrenocortical carcinoma cell line –H295R and isolated lymphocytes from mice.

The effect of a mixture containing brominated, chlorinated and perfluorinated compounds (total mixture), and sub –mixtures containing respectively, brominated, chlorinated and perfluorinated compounds, were used on the cytotoxic potential, characterized by means of Alamar Blue® viability assay. The total mixture and the sub - mixtures were used to assess the percent of DNA damage in the adrenal cell line –H295R. Isolated lymphocytes from mice were used to assess the percent of DNA damage, both *in vitro* and *in vivo*, using the total mixture.

The *in vitro* exposure of the mice lymphocytes revealed a small, but significant increase in the percent of DNA damage lymphocytes for the exposure dose 10^{-8} when compared to the solvent control, and there were an indication that each mouse reacted differently to exposure and exposure dose. Furthermore, the *in vivo* exposure on mice lymphocytes did not show any significant change in DNA damage when compared to the unexposed control group, but the high group and the low group were significantly different from each other.

The *in vitro* exposure of the H295R cells gave a significant dose –response relationship for the perfluorinated sub –mixture and the total mix. Furthermore, exposure to the mixtures did not reveal any significant differences at the concentrations corresponding to the approximate levels of relevant POPs measured in human blood and breast milk. However, the highest dilution of the perfluorinated sub –mixture was cytotoxic to the H295R cells. The

perfluorinated sub –mixture caused an abrupt decline in viability, at the highest dilution (10^{-3}), while the other mixtures were stable at about >95 %.

Sammendrag

Et bredt spekter av syntetiske kjemikalier har vært brukt i flere tiår, de er allestedsnærværende i miljøet, og kan dermed utgjøre skadelige effekter på mennesker og dyreliv. Mikser av persistente organiske miljøgifter (POPs) som er relevante for virkelighetsnær eksponering, er av voksende bekymring når det gjelder deres potensielle negative effekter og mulighet for interaksjon seg i mellom.

Hovedmålet med denne studien var å undersøke eksponerings-relevante blandinger av POPs, basert på nivåer som er målt i blod og morsmelk i Skandinavia, for *in vitro* studiene. *In vivo* studiene baserte miksen på estimert daglig inntak fra mat. DNA-skade ble detektert ved metoden; Comet assay og anvendt på den humane binyrebark cellemodellen -H295R og isolerte lymfocytter fra mus, både *in vitro* og *in vivo.*

Effekter av miksen som inneholder bromerte, klorerte og perfluorerte forbindelser (total miksen), og sub-miksene som inneholder henholdsvis bromerte, klorerte og perfluorerte forbindelser, ble brukt for å studere cytotoksisitet, karakterisert ved Alamar Blue® levedyktighets assay. De samme miksene ble brukt til å studere prosent DNA-skade i binyrebark cellelinje-H295R. Isolerte lymfocytter fra mus ble brukt til å studere prosent DNA-skade, både *in vitro* og *in vivo*, med den totale miksen.

In vitro-eksponering av isolerte lymfocytter fra mus viste en liten, men signifikant økning i prosenten av DNA-skade, for eksponeringsdose 10^{-8} når den ble sammenlignet med løsningsmiddelkontrollen. Det var også en indikasjon på at hver mus reagerer forskjellig på eksponering og eksponerings doser. *In vivo* eksponeringen på lymphocytter isolert fra mus viste ingen signifikant endring i DNA-skade sammenlignet med den ikke-eksponerte kontroll gruppen, men den høye gruppen og den lave gruppen var signifikant forskjellige fra hverandre.

In vitro-eksponering av H295R cellene ga et signifikant dose –respon forhold for den perfluorerte sub –miksen og den totale miksen. Videre, eksponering til miksene viste ingen signifikant forskjell på de konsentrasjonene som tilsvarer de omtrentlige nivåene av relevante miljøgifter, målt i humant blod og brystmelk. Den høyeste fortynningen av den perfluorerte sub-miksen var cytotoksisk for H295R cellene. Den perfluorerte sub-miksen forårsaket en

drastisk reduksjon i levedyktighet, ved den høyeste fortynning (10^{-3}), mens de andre miksene var stabile over 95 %.

Abbreviations

°C:	Degrees Celsius
µl:	Microliter
µM:	Micromolar
AB Assay:	Alamar Blue Assay
BFR:	Brominated flame retardant
CO_2:	Carbon dioxide
DAPI:	4`.6-diamidino- 2- phenyllindole
DDT:	Dichlorodiphenyltrichloroethane
DMEM:	Dulbecco`s Modifies Eagle Medium
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
DNA:	Deoxyribonucleic acid
ECD:	Endocrine –Disrupting chemical
EDI:	Estimated daily intake
EDTA:	Ethylene Diamine – Tetra –acetic Acid
ELFO:	Electrophoresis solution
FBS:	Fetal bovine serum
H295R:	Human adrenocortical carcinoma cell line
H_2O_2:	Hydrogen peroxide
HBCD:	Hexabromocyclododecane
HCB:	Hexachlorobenzene

HCH:	Hexachlorocyclohexane
HepG2 cells:	Liver hepatocellular cells
LMP:	Low melting point
mg:	Milligram
ml:	Milliliter
mM:	millimolar
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
NMP:	Normal melting point
OCP:	Organochlorine pesticide
p,p` -DDE:	Dichlorodiphenyltrichloroethane
PBDE:	Polybrominated Diphenyl Ethers
PBS:	Phosphate buffered saline
PCB:	Polychlorinated biphenyls
PFC:	Perfluorinated compounds
PFDA:	Perfluorodecanoic acid
PFHxS:	Perfluorohexanesulfonic acid
PFNA:	Perfluorononaoic acid
PFOA:	Perfluorooctanic acid
PFOS:	Perfluorooctane sulfonate
PFUnDA:	Perfluoroundecanoic acid
POP:	Persistent organic pollutant
SCGE:	Single –cell gel electrophoresis

SD:	Standard error
SEM:	Standard error mean
Tris –Base:	Tris (2, 3-dibromopropyl) phosphate
Triton X-100:	Polyethylene glycol p-(1, 1, 3, 3-tetramethylbutyl)-phenyl ether
UNEP:	United Nations Environment Programme

Contents

1. Introduction

1.1 Persistent organic pollutants

The Second World War gave rise to an industry that led to production and use of synthetic chemical compounds. Scientists started to recognize their potential hazard on wildlife and humans (El-Shahawi et al., 2010). The book "Silent spring" by Rachel Carson (Carson 1962) was one of the first influential publications that raised awareness towards the persistent organic pollutants (POPs) (Wu et al., 2008).

Persistent organic pollutants (POPs) are according to the United Nations Environment Program (UNEP), "organic chemical substances that possess a particular combination of physical and chemical properties such that, once released into the environment, they; (i) remain intact for exceptionally long periods of time (many years), (ii)become widely distributed throughout the environment as a result of natural processes involving soil, water and most notably, air, (iii) accumulate in the fatty tissue of living organisms including humans, and are found at higher concentrations at higher levels in the food chain, (iv) are toxic to both humans and wildlife" (UNEP, 2013a).

The sources of emission are determined by where and how they are used, and the POPs may be released intentionally or unintentionally. Unintentionally released POPs are typically industrial chemicals or by –products that are released by volatilization or leakage. Pesticides are intentionally released POPs, and example of that is dichlorodiphenyltrichloroethane (DDT), which are released at their point of application (Vallack et al., 1998).

POPs are organic compounds which have the ability to migrate in air, soil and sediments. Furthermore, another major pathway for POPs is the atmospheric transport, which contributes to the global spread and distributions as well as the river and ocean current (Hardell et al., 2010b). They have the ability to accumulate in the food chains and can therefore be harmful for the health of humans and wildlife (El-Shahawi et al., 2010).

POPs can travel long distances due to their stability in the atmosphere, and they tend to migrate towards colder areas, where they descend because of the cold temperature (El-Shahawi et al., 2010). Even in arctic areas have POPs been detected, where no such substances are used or produced (Vallack et al., 1998). Most documented effects have been in birds and marine mammals (Jones and de Voogt, 1999). There are many concerns regarding

POPs, especially their ability to bioaccumulate in certain organisms and biomagnification can occur in top predators (Mackay and Fraser, 2000). Declines in marine population have been reported related to DDT and PCBs (Vasseur and Cossu-Leguille, 2006a).

Thousands of POP chemicals exist and they are typically hydrophobic and lipophilic and therefore stored in fatty tissue. POPs is a wide group of chemicals including organochlorine pesticides, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) among others (Jones and de Voogt, 1999) and several of them are listed in the Stockholm convention, to protect human health and the environment (Hardell et al., 2010a) and to reduce the production and release of POPs (Lammel and Lohmann, 2012). Dietary intake via especially products like fish and meat, are the main exposure source to POPs for humans (Hardell et al., 2010a).

Risk assessment of POPs has traditionally focused on the effect of single compounds, but in real life we are exposed to multiple compounds. Investigation of the effects of mixtures reflecting environmental contaminants is considered a key issue to modern toxicology (Carpenter et al., 2002) (Kortenkamp, 2007). This study focus on mixture effects of POPs derived from respectively levels measured in human blood and breast milk or food, in Scandinavia.

1.1.1. Perfluorinated compounds.

PFCs are man-made chemicals and do not occur naturally in the environment. They have been produced since the 1950s; because of their unique properties, such as anti-wetting or surfactant they are much used in industry and consumer products (Florentin et al., 2011). A fully fluorinated hydrophobic linear carbon chain attached to various hydrophilic heads, is a typical characterization for the perfluorinated compounds (PFCs) (Florentin et al., 2011). They are typically 4-14 atoms in length with a charged moiety, typically carboxylate or sulfonate (Eriksen et al., 2010). PFCs have an extreme resistance due to the carbon – fluorine (C –F) bond, this makes them resistant to degradation by heat, reactions with strong acids or bases and oxidizing agents or photolysis (Florentin et al., 2011).

Since PFCs have been used in the industry and as consumer products and because of their resistant to degradation, they are found several places in environment and wildlife (Haug et al., 2010). PFCs have a global occurrence and, they are found in water, sediment, fish, birds, marine mammals as well as blood and milk of humans (Hu and Hu, 2009), but they mainly distribute to liver and blood (Karrman et al., 2006). PFCs are widespread and their distribution and degradation in the environment is complex, the major exposure pathway for human is through food, but inhalation of dust may also be a potential source of exposure (D'Hollander et al., 2010).

PFOA and PFOS are the PFCs with the highest concentration found in human serum in Norway (Haug et al., 2010). They are also the most studied PFCs (Florentin et al., 2011), PFOS consists of 8 carbon atoms and a sulfonic acid group (Figure 1,A), while PFOA has 8 carbon atoms and a carboxylic acid group (Figure 1,B) (Olsen et al., 2007).

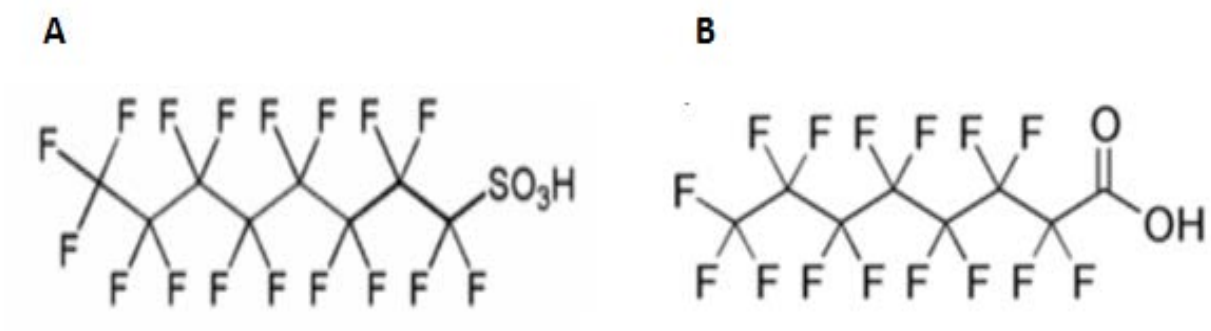


Figure 1. Chemical structure of PFOS (A) and PFOA (B)

1.1.2. Brominated Compounds

Brominated flame retardants (BFRs) contain a diversity of chemicals, polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) are some of them. During the last two decades they have drawn attention due to their environmental and human concerns. The main routs of release into the environment are via effluents from factories producing BFRs and plastic products. Hazardous waste, facilities that recycle plastic, metal from electronic equipment and accidental fires are other possible ways of release. The fate and behavior of the BFRs depend on their degree of bromination, higher brominated compounds are less mobile in the environment and often tend to end up in sediment near the emission source (Watanabe and Sakai, 2003).

1.1.2.1. Polybrominated diohenyl ethers (PBDEs)

Polybrominated diphenyl ethers (PBDEs) are organic chemicals; they all have a common structure and are widely used as flame – retardants in various industries. The compounds are halogenated and the structure is characterized with two benzene rings *(*Hites, *2004)* and different compositions of bromine atoms (Figure 2). There are 209 PBDEs and they are distinguished by the number of bromine atoms (1 -10) and their position (ortho –, meta –, para – position). PBDEs are found in water, soil, sediment and living organisms, reasons for this is that they are volatile and are insoluble in water (Yue and Li, 2013).

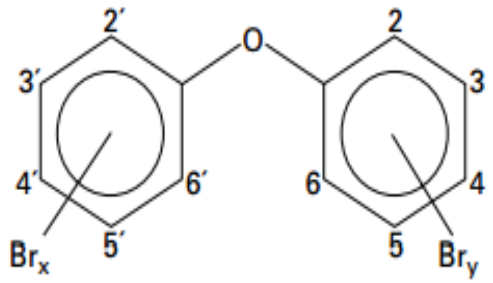


Figure 2. General chemical formula to PBDEs

Polybrominated diphenyl ethers (PBDEs) have been widely used since the 1970s. PBDEs are polymer additives and therefore not chemically bound to materials, they tend to leach into the surrounding environment. They are considered contaminants in the environment, due to their high production, lipophilicity and persistence. Over the past 30 years levels of PBDEs in humans and the environment have increased (He et al., 2008). PBDEs are used as flame retardants in a variety of construction materials, textiles and polymers for electronic equipment. PBDEs and polychlorinated biphenyls (PCBs) have a similar chemical structure (Song et al., 2009).

The variation in the roots of exposure is due to the variation of chemicals and their variation because of physiochemical properties and molecular weight. Diet appears as the main rout of exposure for the general human exposure, this is particularly from the lower brominated congeners. Fatty fish is the most important food group. Release from consumer products that

are treated with these compounds could also be a rout of exposure, due to inhalation of air. Work environment is also a place where people may be exposed (Watanabe and Sakai, 2003).

1.1.2.2. Hexabromocyclododecane (HCBD)

In addition to the PBDEs; Hexabromocyclododecane (HBCD) is also included in the brominated sub –mixture. HBCD is a brominated flame retardant with 16 possible stereoisomers, used for plastics and textiles. HBCD is highly lipophilic and accumulates in biota and is one of the most used BRFs (Heeb et al., 2005). The chemical structure of HBCD is shown in Figure 3 (Yamada-Okabe et al., 2005). HCBD is added as an additive or reactive component in a variety of polymers and over the past decades the demand of HBCD has increased (Wu et al., 2013).

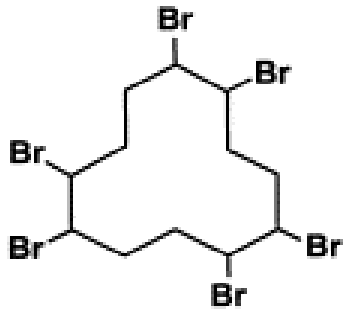


Figure 3. Chemical structure of HBCD

1.1.3. Organochlorinated Compounds

The organochlorines consist of a broad family of synthetic organic compounds, with chlorine substitutes. Most organochlorines are highly lipophilic (Mrema et al., 2013), and due to their persistence and bioaccumulation properties, some of them are grouped under Persistent Organic pollutants (POPs), The main exposure route for humans is via consumption of meat, fish and dairy products (Strom et al., 2014). Exposure can also occur via the placenta and primarily via breast milk (Klincic et al., 2014).

1.1.3.1. Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are synthetic organochlorine chemicals and they have been produced since the 1920s (Robertson and Ludewig, 2011). There are 209 different PCB compounds (congeners) and they all have different numbers and the composition of the chlorine substitutes in the molecule varies. Two six –carbon rings, benzene rings, are linked together by a carbon –carbon bond (Figure 4) (Carpenter, 2006). The origin comes from many different sources and PCB has been widely used in the industry and commercial application, ranging from capacitors, plasticizers in paint to rubber production, reasons for this is their stability and non –flammability properties (Robertson and Ludewig, 2011).

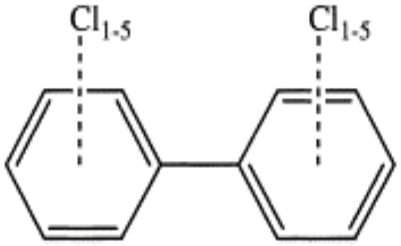


Figure 4. General chemical structure of PCBs

Obtained from; (Sabljic, 2001).

Since the late 1980s most industrialized countries banned the production and use of PCB, due to their bioaccumulating, toxic and persistent properties (La Rocca and Mantovani, 2006). PCBs are widespread in the environment both near and far from their source of origin. The most important contribution to the overall exposure in humans has shown to be the diet, dairy products, fish and other seafood in particularly (Domingo and Bocio, 2007). PCBs can be separatd into two categories; “dioxin – like” and “non – dioxin – like”. (Crinnion, 2011).

1.1.3.2. Organochlorine pesticides

Organochlorine pesticides (OCPs) are global pollutants due to their resistance and they were used extensively in agriculture decades ago (Shen et al., 2005), consisting essentially of carbon, hydrogen and several chlorine atoms (Mrema et al., 2013). OCPs are widespread in the environment and (Jaraczewska et al., 2006), and they have a tendency to occur in

mixtures with other once; they are unique and toxic chemicals, which are distributed to the environment to kill off pests. The pathways of exposure are multiple, ranging from drinking water to diet (Hernandez 2012).

Dichlorodiphenyltrichloroethane (p,p′-DDE) is the main metabolite found in the environment due to DDT decomposition, it is persistent and bioaccumulates. p,p` -DDE is frequently found in air, soil, water, organisms and humans and has neurotoxic properties (Wang et al., 2014). Although DDT were banned in many countries after 1970s, the pesticide is used to control vector borne diseases, like malaria, due to the lack of adequate alternatives (Mrema et al., 2013) (Dewan et al., 2013). Hexachlorobenzen (HCB), chlordanes, dieldrin and hexachlorocyclohexanes (HCHs) are also pesticides that were banned by many developing countries in the 1970s (Van Oostdam et al., 2004)

OCPs have the ability to cross the placenta and secrete into the breast milk, they are also rapidly absorbed in the small intestine. Levels are detected in blood and breast milk. Cancer, reproductive defects, endocrine disruptors, behavioral changes and DNA –methylation are health effects associated with these chemicals (Mrema et al., 2013).

1.2. Mixed exposure

POPs are widespread in the environment but risk assessments and study on chemicals have had most focus on effects of single compounds, even though humans typically are exposed to mixtures of chemical compounds in low doses (Carpenter et al., 2002) (Kortenkamp, 2007).

The effects of mixtures are less studied, due to their complexity, (Carpenter et al., 2002) and chemicals may act in an additive, antagonistic or synergistic way and thereby induce combined effects. Which furthermore can be different from what expected by summing the effects of single compounds and the variation in composition of chemicals may lead to changes in toxic effect (Kortenkamp, 2007). A great concern today is the synergistic interactions of chemical mixtures, and the fact that several chemicals can show a larger effect than what predicted, by increase the effect of other chemicals (Cedergreen, 2014).

The main focus on health risk assessment of chemical substances has been on effects from single compounds to determine the doses for toxical concern. However, the awareness

between environmental conditions and public health have increased (Sarigiannis and Hansen, 2012).

The exposure concentration to persistent organic pollutants varies between populations and the mixture effect is dependent on number of chemicals in the mixture and their respective concentration (Porta et al., 2012). In Sweden, market basket studies have been done on several estimations of POP mixtures; due to dietary intake have been done, since exposure to POPs mainly occurs via the diet (Tornkvist et al., 2011). Knowledge from such studies may be useful as a basis for the design of mixtures that resemble realistic exposure scenarios.

1.3. Endocrine disrupting compounds

An endocrine disruptor is defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al., 1996).

Endocrine –disrupting chemicals have been released into the environment since world war II (Colborn et al., 1993) and in the early 1990s the term endocrine disruptors (EDs) were introduced (Hotchkiss et al., 2008) and during the past decade a diverse range of chemicals have been shown to interfere with the endocrine system. Furthermore, Oestrogen mimics were the first endocrine disrupting chemicals (EDCs) to be described (Clotfelter et al., 2004) and endocrine –disrupting chemicals cover a wide range of chemicals, including pesticides, industrial by –products and manufactured products such as plastics (Welshons et al., 2003).

EDCs have some differences from the classical pollutants when it comes to toxicology; some EDCs are more toxic at a lower concentration and the effect is not always immediate (Clotfelter et al., 2004). Pesticides like DDT and its metabolites have been shown to induce eggshell thinning in fish –eating birds and PCBs have shown to interfere with embryogenesis and the development in juveniles (Vasseur and Cossu-Leguille, 2006b). Polybrominated diphenyl ethers (PBDEs) share some of the same structure as the thyroid hormones and therefore they have the ability to disturb the thyroid endocrine system (Chen et al., 2012).

The sources of exposure are in the air, water and from food. Characteristics of EDCs are that they are lipid – soluble and accumulate in the tissue. EDCs can have a negative effect on pituitary, thyroid glands and the reproduction (Clotfelter et al., 2004). The endocrine cells, H295R (Hecker et al., 2006) has been used a part of this thesis to investigate cell viability and DNA damage to mixed exposure.

1.4. Assessment of cytotoxicity

There are several cytotoxic assays in use for *in vitro* toxicology studies, like MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, Lactate dehydrogenase leakage assay (LDH), the neutral red assay (NR) and Alamar Blue Assay™ (AB Assay). MTT assay also involves a conversion, where the water soluble MTT (3-[4, 5 – dimethylthiazol – 2 –y1] -2, 5 –diphenyltetrazolium bromide) is converted to formazan, which is insoluble. Formazan accumulate in healthy cells, due to its imperable ability to the cell membrane. LDH leakage assay on the other hand is based on lactate dehydrogenase activity measurement and the conversion of lactate to pyruvate. The neutral red assay (NR) is also an assay to determine cell viability, the neutral red dye, determines the accumulation in lysosomes of viable healthy cells. (Fotakis and Timbrell, 2006).

The AB Assay was first established to monitor contamination in milk by bacteria and yeast, but was later applied to measure cell viability and cytotoxicity of mammalian cell cultures (O'Brien et al., 2000). A change in color from blue to red indicates that the cells are healthy and viable, since viable cells metabolically can reduce and convert resazurin. If changes in color do not appear, the cells are unhealthy or unable to convert the redox reagent, which indicates cytotoxicity. Due to this, AB Assay is a good method to assess cell viability and cytotoxicity (Rampersad, 2012).

AB Assay is a well-established method and was used in this thesis to measure cell viability on H295R cells exposed to the total mix, perfluorinated sub-mix, brominated sub-mix and the chlorinated sub-mix (Rampersad, 2012).

1.5. Genotoxicity

Every year an increasing number of pollutants with a genotoxic potential enters the environment, due to anthropogenic activities. Some of these genotoxicants may have the ability to induce DNA double –strand breaks (DSB) (Chankova et al., 2007). DNA damage is a marker for genotoxic effects (Anderson, 2006) and it is a consequence of endogenous sources and processes as well as exogenous sources (Li and Heyer, 2008). Assessment and evaluation of environmental agents that may have a genotoxic risk in humans is important to study and genotoxicity in rodents are a useful biological test model, to the investigation of toxicology (Recio et al., 2010).

Genotoxic potential is a risk factor for long –term effects, such as carcinogenic and reproductive toxicology (Bolognesi, 2003). The effect of pesticide mixtures seems to depend on the intensity and the length of the exposure, and the know genotoxic effect on OCPs are limited or the result contradict each other (Alvarado-Hernandez et al., 2013). Organochlorinated compounds like; HCB, HCH, DDT, DDE and PCB have been detected in fish and DNA damage in various degrees has been observed (Gonzalez-Mille et al., 2010). Perfluorinated compounds like PFOS and PFOA have been tested extensively, mainly in rodents and the widely used PFOA and PFOS have been tested for their genotoxic potential by using the human HepG2 cells, furthermore no increase in DNA damage was observed (Florentin et al., 2011). DDT and its metabolites showed in a study that they were able to induce DNA damage in human mononuclear cells, *in vitro* (Yanez et al., 2004).

Testing of genotoxicity varies, and thus depends on the type of cells that are used and micronucleus assay is a way to test for genotoxicity in mammalien cells like lymphocytes. Furthermore, bacterial reverse mutation assay (Ames Assay) is used as a test for revealing genotoxic effects in bacteria (Di Sotto et al., 2014). For the genotoxicity testing in this study, the alkaline comet assay was used on the adrenal cell line H295R cells and isolated mice lymphocytes.

The alkaline (pH>13) comet assay is simple, sensitive and versatile – combined with the fact that it is fast and economic makes it a good and reliable method. It is applied within areas of genotoxicity testing, human biomonitoring as well as molecular epidemiology and ecogenotoxicology (Collins, 2004). It is therefore a good method of choice for testing the genotoxicity of different chemicals in this thesis (Azqueta et al., 2011).

The comet assay, also called Single- cell gel electrophoresis (SCGE) is a well-established method for measuring DNA damage and repair in cells with nuclei. This method has been used since the 1970s when Peter Cook and colleagues studied and developed the model of nuclear structure which is based on the lysis of cells with nonionic detergent and high – molarities sodium chloride. But further modifications have been done, by Ostling and Johanson (1984) and Singh et al. (1988) (Collins, 2004).

Several variants of the comet assay have been developed during late decades, including alkaline – and neutral single–cell gel electrophoresis. Fluorescent *in situ* hybridization (FISH) comet is a less common variant. In this study the variant alkaline SCGE was used for all experiments. (Collins, 2004).

1.6. Cell models

As a part of this thesis the *in vitro* cell model H295R was used. The cell line is derived from a human adrenocortical carcinoma (Winther et al., 2013). The cell line was established by A.F. Gazdar et al in 1990 and the patient was a 48 years old black woman from Bahamas with a carcinoma of the adrenal cortex. The cells from the adrenal tumor was minced and established in a culture of HITES growth media where they grew as floating aggregated cells (Gazdar et al., 1990).

Isolated lymphocytes from mice, as both *in vitro* and *in vivo* models, were also used as a part of this thesis for genotoxicity testing. 129S1/SvImJ mice were used for the *in vitro* model and 129C57BL/6F1mice was used for the *in vivo* model.

1.7. Aim of study

POPs have been used extensively for decades. Humans and wildlife are steadily exposed to multiple POPs, which due to their persistency and chemical properties are associated with a wide range of health effects(Carpenter et al., 2002). However, consistent data on mixture effects of POPs are sparse, and the investigations of the combined effects reflecting relevant exposure are of increasing concern (Kortenkamp, 2007).

The main aim of this study was to assess the DNA damage to four mixtures of persistent organic pollutants, by using comet assay as the method of choice. The assay was conducted on an endocrine cell line, H295R.

The secondary aims were:

- Study lymphocytes from mice; both *in vitro* and *in vivo*, after expose to a mixture of persistent organic pollutants, to assess the DNA damage by the method comet assay.
- Assess of cytotoxicity of the mixtures by Alamar Blue® viability assay.

2. Materials and methods

2.1. Chemicals mixtures

Mixtures of the test compounds were designed and pre –made by Hanne Friis Berntsen and Karin Zimmer at the section of Experimental Biomedicine, Norwegian University of Life Sciences, Oslo. For *in vitro* experiments with the H295R cells, four mixes were used; a) total mixture (TM), containing all the listed test compounds, b) brominated mixture (Br), c) chlorinated mixture (Cl) and d) perfluorinated mixture (PFC). For the *in vitro and in vivo* study on mice lymphocytes, the TM was used for both exposures. The chemicals included in the different mixtures and their respective concentrations in the stock solution are shown in Table 1 and 2.

Dimethyl sulfoxide (DMSO) was used as a solvent control (Sigma –Aldrich ®, Saint Louis, MO, US).

Mixtures were stored in glass vials at -20ºC between experiments.

2.1.1. *In vitro* mixture

The *in vitro* POP mixtures used in this study were based on concentrations of relevant POPs measured in human blood and breast milk, according to recently studies of the Scandinavian population (Haug et al., 2010, Van Oostdam et al., 2004, Polder et al., 2009, Polder et al., 2008, Knutsen et al., 2008). The compounds were mixed in concentration ratios relevant to human exposure, the total mixture and the chlorinated sub –mixture is 10 times more diluted compared to the perfluorinated and the brominated sub -mixtures (Table 1).

2.1.2. *In vivo* mixture

The study also included *in vivo* exposures, where the TM was used. Composition of the TM mixture was the same as for the *in vitro* exposures, but the mixture was designed and made according to estimated daily intake (EDI) from food, in Scandinavian population (Knutsen et al., 2008, Haug et al., 2010, Kvalem et al., 2009, Tornkvist et al., 2011). Therefore the stock concentrations for the in vivo experiments are slightly different, the low group is 5000 times estimated daily intake while the high group is 100 000 times estimated daily intake via food (Table 2).

Table 1. The composition of mixtures used for *in vitro* exposure in the thesis, TM; total mix, PFC; perfluorinated sub – mixture, Br; brominated sub –mixture, Cl; chlorinated sub –mixture and their stock concentration.* Estimated concentrations of POPs in stock solution is 10000 times estimated concentration in human serum. ¤ Estimated concentration of POPs in stock solution is 1000 times estimated concentration in human serum. ** Purchased from Chiron AS (Trondheim, Norway). *** Purchased from Santa Cruz (Dallas, TE, US) and HBCD was purchased from Sigma –Aldrich (Saint Louis, MO, US).

	Stock concentration (mM)			
Chemicals	**TM¤**	**Br***	**Cl¤**	**PFC***
Brominated diphenyl ethers (BDEs)				
BDE-47**	0,018	0,177		
BDE-99**	0,006	0,062		
BDE-100**	0,004	0,038		
BDE-153**	0,015	0,153		
BDE-154**	0,003	0,027		
BDE-209**	0,011	0,113		
HBCD	0,038	0,383		
Polychlorinated biphenyls (PCBs)				
PCB-28**	0,05		0,041	
PCB-52**	0,033		0,033	
PCB-101**	0,024		0,024	
PCB-118**	0,196		0,196	
PCB-138**	0,615		0,615	
PCB-153**	1,003		1,003	
PCB-180**	0,491		0,491	
Other organochlorines				
p,p'-DDE**	1,579		1,578	
HCB**	0,411		0,411	
α – chlordane**	0,026		0,026	
oxy – chlordane**	0,052		0,052	
trans-nonachlor**	0,092		0,092	
α-HCH**	0,021		0,021	
β-HCH**	0,181		0,181	
γ-HCH (Lindane)**	0,021		0,021	
Dieldrin**	0,063		0,063	
Perfluorinated compounds				
PFOS***	54,671			546,701
PFDA***	0,963			9,629
PFOA***	10,922			109,223
PFNA***	1,724			17,239
PFUnDA***	0,993			9,927
PFHxS***	7,855			78,548

Table 2. The composition of the mixture used for the in vivo exposure in this thesis. The predicted feed concentrations are in ng/g and the two representative groups are illustrated in the table. * Purchased from Chiron AS (Trondheim, Norway), ** Purchased from Santa Cruz (Dallas, TE, US) and HBCD was purchased from Sigma –Aldrich (Saint Louis, MO, US).

	Predicted feed concentration in ng/g	
Chemicals in TM	**Low group 5000X**	**High group 100 000X**
Perflurinated compounds:		
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
Brominated compounds:		
BDE – 209*	62,5	1250
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
HBCD	12,5	250
Chlorinated compounds:		
PCBs		
PCB 138*	57,5	1150
PCB 153*	57,5	1150
PCB 101*	23,3	466,7
PCB 180*	15,4	308,3
PCB 52*	13,8	275
PCB 28*	5,8	116,7
PCB 118*	40,4	808,3
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

2.2. Human adrenocortical carcinoma cell line –H295R

The cell line used as a part of this thesis was H295R, Human adrenocortical carcinoma cell line (passage 5 to 13). These cells were obtained from the American Type Culture Collection (Igc Standards – ATCC CRL -2128, Igc Standards ATCC, Manassas, VA).

The H295R cells were maintained as a monolayer and were cultured in 75-cm² tissue culture Falcon™ plastic flasks (BD Biosciences, USA) with growth medium (13 ml in each flask), and stored at 37 degrees in a 5 % CO2, incubator. The complete medium was a composition of 500 ml of Dulbecco`s Modified Eagel Medium/Ham`s F12 medium (1:1) (GIBCO®, Invitrogen, Paisley, UK). Additionally 1 % ITS Premix (BD Biosciences, Bedford, MA, USA) and 2, 5 % Nu –Serum (BD Biosciences, USA) was added.

2.2.1. Expansion and plating of the H295R cells

The medium was changed three times a week, and the cells were sub-cultured/split once a week at approximately 80 % confluence. Growth medium was aspirated and 3 ml of trypsin-EDTA were added and removed quickly (washing step). 3 ml of trypsin –EDTA was added again and left for a few minutes; the cells were observed under the light microscope (Nikon TMS microscope, phase contrast, Melville, NY, USA) as they started to loosen from the bottom of the dish. Additionally, the bottom of the flask was washed with 3 ml of fresh medium, to make sure that the maximum of cells is obtained. After washing, all the content was transferred to a 15 ml falcon tube and the cells were centrifuged on 1250 rpm for 5 min in a Beckman GS -6R centrifuge (Beckman coulter, Ltd., Brea, CA, USA) and the supernatant was discarded. Cells were re – suspended in fresh medium, counted and transferred to new flasks containing 13 ml fresh medium. Seeding of the cells was done as described above, with exception in the last step, whereas for seeding; the cells were counted and diluted to $3x10^5$ cells/ ml and plated 1 ml pr. well in a 24 well culture dish. Cells were placed in an incubator at 37°C with 5 % CO_2.

2.2.2. *In vitro* exposure of the H295R cells

The four different mixtures used for the exposure studies were in the following range; 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. The mixtures were diluted 1/100 with a final DMSO concentration of 0.1% and a medium blank.

2.3. Isolation of lymphocytes from mice.

Histopaque®-1083 from Sigma Aldrich (Saint Louis, US), was used to isolate the lymphocytes for both the *in vitro* and the *in vivo* experiments. Histopague® -1083 is designed to separate different cells. Blood was added to a micro tube filled with 1 ml of RPMI -1640 medium (Gibco ®, life technologies), the medium was already supplemented with 10 % fetal bovine serum (Gibco ®, Invitrogen). Samples were left on ice for 30 minutes and after that underlayed with 100 µl Histopaque (Figure 5).

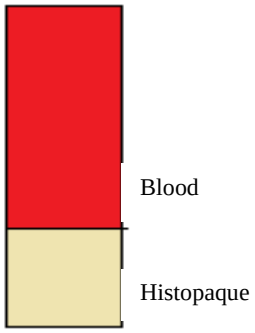


Figure 5. Illustration of the two layers after adding histopaque.

After adding the Histopaque carefully, the samples were spun in a Heraeus Fresco 21 Centrifuge (Thermo Scientific) on 500 xg for 3 minutes at 4ºC. During the centrifugation the Histopaque separates the lymphocytes from the erythrocytes and granulocytes, creating four layers in the micro tube (Figure 6). Since erythrocytes and granulocytes are heavier they sink to the bottom and the lymphocytes will remain floating in the plasma. A pipette was used to collect the lymphocytes, which appeared as a cloudy layer above the Histopaque.

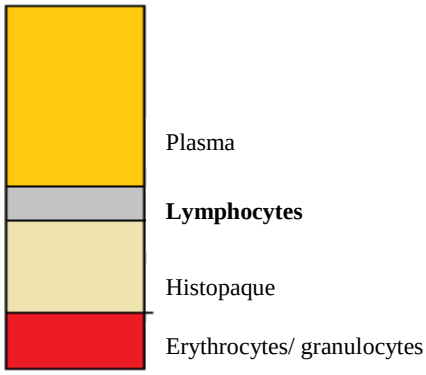


Figure 6. Illustration of the four layers after the samples has been spun.

2.4. Alamar Blue assay.

Alamar Blue™ (Invitrogen, Carlsbad, CA, USA) was used to test viability on exposed H295R cells. The ability to metabolically reduce and convert resazurin, a blue redox dye, to resorufin, a red dye, only occurs if the cells are viable (Rampersad, 2012). Resazurin is the active ingredient and a blue non - fluorescent dye (Figure 7A), which is reduced to the pink – colored, resorufin (Figure 7B), as a response to cellular metabolic reduction. If the color changes from blue to red, the cells are healthy and viable. If the color remains blue, it indicates cytotoxicity and the cells are unhealthy or unable to convert the redox reagent. The absorbance signal is proportional to the number of viable cells. Damage cells have a lower metabolic rate and thus a lower absorbance signal (Nakayama et al., 1997).

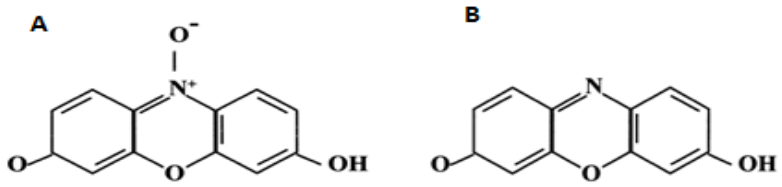


Figure 7. Chemical structure of Resazurin (A) and Resorufin (B)

Adapted from; (O'Brien et al., 2000).

2.4.1. Preparation of the H295R cells for AB assay.

The H295R cells were seeded at $3x10^5$ cells/well in a 24-well Falcon® PRIMARIA™ flat bottom plates (Becton Dickinson, Franklin Lakes, NJ, USA), with three replicates per sample. Plates were incubated at 37ºC for 24 hours and were then exposed for 48 hours to the TM, the perfluorinated mix, the brominated mix and the chlorinated mix in the same 24-well plates as they were seeded. The plate setup for each 24-well plate had three parallels with control, three parallels with solvent control (0, 1 % DMSO) and three parallels for each dilution, as shown in Figure 8.

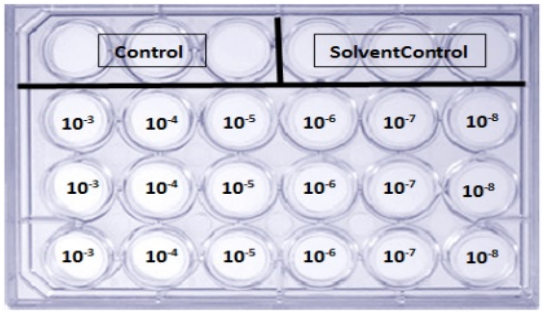


Figure 8. Illustration of the set -up for exposure experiments for the viability assay. Each plate was set up with a solvent control (DMSO) and control (medium blank) in triplicates. Each of the exposures was set up in triplicates for the six different dilutions and the final DMSO concentration in each well was 0, 1 %.

After 48 hours the medium was removed and 1 ml of fresh medium + 10 % alamar blue solution was added to each well. Then the cells were incubated at 37ºC in a 5 % CO_2, humidified atmosphere for three hours. From each well 100 µl subsample was added to triplicate wells in a transparent 96-well plate and absorbance was read at 570- and 600 nm using a VICTOR^{3TM} spectrophotometer (Perkin Elmer, Shelton, CT, USA).

The absorbance values were plotted in Excel to calculate cell viability. The template accounted for overlap in optical density spectra for both the oxidized and reduced form of AB, by subtracting the absorbance at the high wavelength (600 nm) from the low wavelength (570 nm). The template had a correction factor R_0, which represented the absorbance ratio (low wavelength/ high wavelength) of AB. The results were expressed as percent of living cells (viable) to solvent control (DMSO).

2.5. Comet assay (Single- cell gel electrophoresis)

The comet assay is a well-established method for measuring DNA damage and repair in cells with nuclei. This method has been used since the 1970s and with further modifications done, by Ostling and Johanson (1984) and Singh et al. (1988). The comet assay used in this thesis is the alkaline version of comet assay, which detects DNA strand breaks (single – or double stranded) and the procedure is done according to (Collins et al., 1996), with some modifications.

2.5.1. Preparation of solutions

The solutions for comet assay were prepared according to (Hudecova et al., 2010, Hudecová et al., 2012) . Microscope slides were pre –coated with a base layer of 100 µl of 1 % normal melting point agarose (Sigma Aldrich ®, Saint Louis, US) in distilled water and the slides were placed to dry for 24 hours. The cells were re –suspended in 1 % low melting point agarose (Sigma Aldrich ®, Saint Louis, US) in PBS buffer (Ca^{2+} and Mg^{2+} free) (Life technologies, Lonza).

Hydrogen peroxide (H_2O_2) was used as a positive control for all the experiments. H_2O_2 is a soluble gas and an oxidative biocide. Biocides can at high concentrations cause cellular damage at a molecular level. It removes electrons from susceptible chemical groups, oxidizes them, and is reduced in the process. Therefore it is commonly used as a positive control in the comet assay (Linley et al., 2012). Two dilutions of H_2O_2 were utilized; solution A and B. Solution A was made from 11 µl stock solution (30% w/v i.e 30 g / 100 ml; 9.82 M) in 1 ml of PBS = 100 mM. Solution B was made from 10 µl solution A in 1 ml of PBS = 1 mM. Several working solutions (30 µM, 50 µM, and 100 µM H_2O_2), were tested in a pilot study, and resulted in 30 µM as the chosen H_2O_2 concentration for a positive control.

The lysis solution was made by weighing out 146, 1 g NaCl, 37, 22 g EDTA (Sigma Aldrich ®, Saint Louis, US) and 1, 21 g Tris –Base. The chemicals were mixed with 900 ml of distilled water and placed on a stirrer, while the pH was adjusted to 10 by using a 10 M NaOH solution. When the solution had reached a pH of approximately 10, the distilled water was added to make a solution of 1 liter in total. On the day of experiment, the lysis solution was mixed with 1 ml of Triton –X/ 100 ml and stored in the fridge until used. The alkaline

solution was made by weighing out 12 g NaOH and 0, 37 g EDTA and mixing it on a stirrer with 1 liter of distilled water.

Samples were stained either with, 20 µl of SYBr Gold (Molecular probes, life technologies) (0,1 µl/ml in TE buffer (10 mM Tris –HCL, 1mM Na2EDTA, pH 7.5 – 8) or DAPI (Molecular probes, life technologies) (1 µg/ml DAPI solution in distilled water). The DAPI aliquots are stored at -20ºC until use.

2.5.2. Preparation of the H295R cells

H295R cells were exposed to the different mixtures, Br -, Cl -, PFC - sub mixture and the TM, which contained all the compounds from the three sub mixes for 48 hours. The cells were exposed in 24-well Falcon® PRIMARIA™ flat bottom.

On the day of experiment, the cells were washed with 1 ml of cool PBS, then 200 µl trypsin-EDTA was added to each well. The cells were watched carefully for a few minutes during the trypsinization and when the cells stated to “loosen” from the bottom, 1 ml of medium was added, and the then cells were transferred to the marked micro tubes. The tubes were centrifuged at 1250 rpm for 5 minutes in a Heraeus Fresco 21 Centrifuge (Thermo Scientific). The supernatant was discarded, and the cell pellets were re-suspended in 1 ml of medium. After centrifugation, the tubes with cells were kept on ice. The dilution of 10^{-6} was counted, in all the 24 well plates, by using a Burker counting chamber. A cell suspension of $1x10^{4}$cells/sample is optimal for the comet assay and was used in this experiment.

Tubes with the right concentration of suspension were mixed with 170 µl of 1% low melting point (LMP) agarose in PBS buffer (Ca^{2+} and Mg^{2+} free), vortexed for a few seconds and 70µl of the cell suspension was quickly placed onto the pre-coated microscopic slides (Thermo Scientific Frosted microscope slides). They were then covered with glass cover slips (Menzel –Glaser 18x18 mm) on the top to spread the gel, which was left to set at 4°C. Afterwards the cover slips were removed, the slides were placed in staining jars and left in lysis solution for 1 hour, at 4°C, to remove cellular membranes, cytoplasma and histones, leaving DNA as nucleoids.

After lysis, the microscopic slides were rinsed twice in ice-cold PBS, then they were transferred to an electrophoresis tank (BIO –RAD sub –cell® model 192) and covered with

the alkaline solution. They were then left to unwind for 20 minutes at 4°C, before the electric source was switched on (BIO –RAD, PowerPac™ Basic). The electrophoresis was run under the following conditions: 25 V, for 30 minutes, at 260-320 mA. Immediately after the electrophoresis, the microscopic slides were removed and placed into staining jars. They were covered with cool PBS, for 7 minutes at 4°C, followed by distilled water for 7 minutes to neutralize the microscopic slides. Finally, the microscopic slides were placed on paper and left to dry.

For the positive control, slides were submerged for five minutes in a 30 µM H_2O_2 at 4°C. This was repeated for all experiments.

2.5.3. Comet assay on mice lymphocytes

In this thesis comet assay experiments on mice lymphocytes were done to assess DNA damage both *in vitro* and *in vivo*. Figure 9 shows a scheme of the mice that were used. The seven mothers from the F0 –generation that were exposed *in vitro* were 129S1/SvImJ mice; they were mated with F0 –fathers (C57BL/6J) and were bought from Jackson Laboratory (Maine, USA). The F1 –generation that was exposed *in vivo* via feed are 129C57BL/6F1 hybrid mice, and a result of the mating between 129S1/SvImJ mice and C57BL/6J mice.

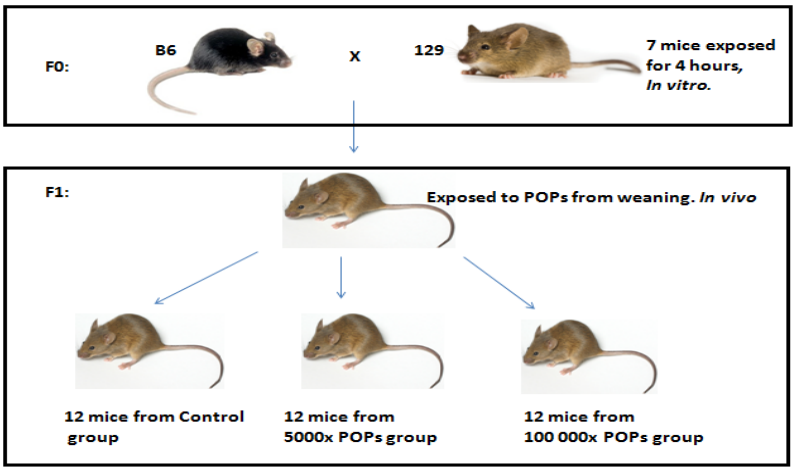


Figure 9. Scheme over the mice that were used for the experiments. F0, 129S1/SvImJ mice were used for the in vitro experiments and the F1, 129C57BL/6F1 hybrids were used for the in vivo experiments.

2.5.4. *In vitro* Comet assay

Blood was taken from seven female F0 129S1/SvImJ mice by terminal bleeding and then the lymphocytes were isolated and exposed for four hours to the total mixture of POPs at two different concentrations. The concentrations 10^{-5} and 10^{-8} were diluted 1/100 from the stock solution, in 0, 1 % DMSO.

After 30 minutes, the samples were mixed again and underlayed with 100 µl of Histopaque®-1083 (Sigma Aldrich). Samples were centrifuged at 500xG for three minutes at 4 °C in a Heraeus Fresco 21 centrifuge (Thermo Scientific), and the lymphocytes were transferred over to 1, 5 ml clear microtube (Genuine Axygen Quality, CA, USA), already filled with 1 ml RPMI medium. Samples were centrifuged again at 500xG for three minutes at 4 degrees. The supernatant was discarded by using a pipette. The mirotube containing the 10 µl of the total mix and 1 ml RPMI – medium were transferred to the microtube with the supernatant and left to incubate for 4 hours. After incubation, spin the sample again at 500xg for 3 minutes at 4°C, remove the supernatant and mix the sample with LMP. The comet assay procedure is described in section 2.5.2.

2.5.5. *In vivo* comet assay

Female hybrid 129C57BL/6F1 mice were used for the *in vivo* experiment. These mice had been eating feed containing POPs from when they were weaned (3 weeks old). The feed containing POPs was divided into groups; control feed, low concentration feed (5000 times) and high concentration feed (100 000 times), previously described in Table 2 and section 2.1.2. Blood was collected for the first repeat in week 13, which means that the mice had been eating the feed for 12 weeks. Blood for the two other repeats was collected after 15 weeks. For each repeat 12 mice were chosen from each group, including an extra mouse for positive control (Hydrogen peroxide). The preparation and procedure for the comet assay on *in vivo* exposure was done according to previous describes in section 2.5.2 and section 2.5.4.

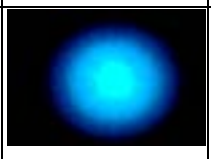
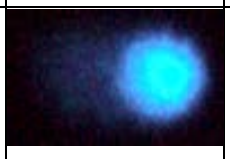
2.6. Scoring the comets

Scoring the comets can be done by image analysis using specialized software or visually. The first *in vitro* experiment in this thesis was scored visually and the two other experiments were scored by image analysis at Norwegian Institute for Air research (NILU) and at the University of Oslo (UIO).

2.6.1. Visual scoring

For the visual scoring, 50 comets per gel were scored from each slide, making it a total of 100 comets due to the set –up of two gels on each slide. The slides from the experiment on the H295R cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) and scored under the fluorescent microscope (Olympus IX81). The nuclei were divided into 5 categories, according to the amount of DNA in the tail. Category 0 has no DNA damage, category 1 up to 25 %, category 2, up to 50 %, category 3 up to 75 %, while category 4 is above 75% (Table 3). Staining with a DNA –binding dye makes DNA visible by using a fluorescent microscope (Collins, 2004).

Table 3. An overview over the different categories used for scoring the comets are illustrated with the number of the categories, DNA damage in % and a picture to illustrate.

Category	**0**	**1**	**2**	**3**	**4**
DNA damage	The nuclei without any damage, 0% DNA in the tail.	Up to 25% DNA in the tail	Up to 50% DNA in the tail	Up to 75 % DNA in the tail	75% or more DNA in the tail
Comets					

2.6.2. Software scoring

The *in vitro* and *in vivo* experiments on mice lymphocytes were scored at the Norwegian Institute for Air Research (NILU) and at the University of Oslo (UIO). The samples were stained with SYBR® Gold Nucleic Acid Gel Stain (SYBR® Gold) and scored using a fluorescent microscope (Leica DMI6000 B) at 20X objective (Figure 10) and a computerized

image analysis program; comet assay IV 4, 2 Perceptive Instruments Ltd . 50 comets per gel and 100 in total for each slide were also scored when using the software scoring and the tail intensity or % DNA in the tail for each comet was used further for analyzing the data. The DNA in the tail represents the frequency of DNA stand breaks.

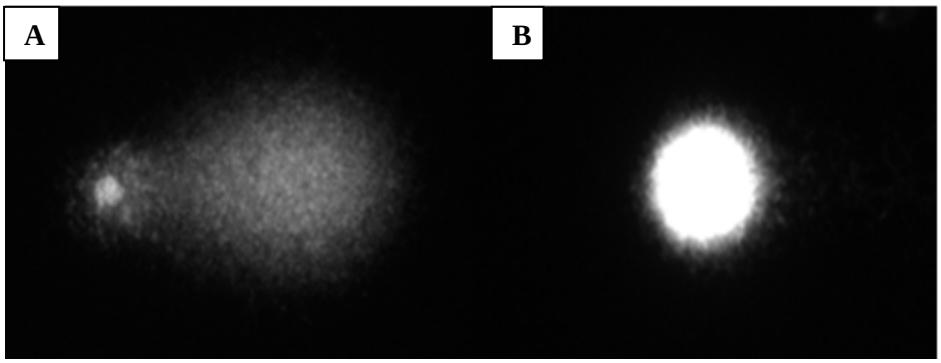


Figure 10. Images from the software scoring. A) Comet with damage, from the positive control (H_2O_2). B) Comet with barley any damage, from the solvent control (0, 1 % DMSO).

2.6. Ethics

The procedures involving live animals were performed according to Norwegian legislation and approved by the Norwegian Animal Research Authority (NARA).

2.7. Statistical analysis

The statistical analysis was performed using JMP 9 software (SAS Institute Inc, Cary, NC, USA). The distributions of dependent variables were tested for normality by the Shapiro – Wilk test. All studied endpoints, except for the *in vivo* exposed mouse lymphocytes, gave a satisfactory fit to the normal distribution. Hsu –Dunnett`s T –test was used to compare exposed groups to control. General linear models (GLM) were used. Percentage of DNA damaged cells were dependent variables. Independent variables were experiment (n=2 or 3) and dilution of mixtures entered as discrete variables. Differences between exposure groups and controls were assesses with the Hsu –Dunnet`s test. In experiments where live animals were used, effects of the individual and individual*exposure interactions were included as

additional explanatory together with dilution of the mixtures. Dose –response relationships were evaluated by fitting experiments as a discrete variable and dilution of the mixtures as a spline function. P – values < 0, 05 were considered statistically significant.

3. Results

3.1. Alamar Blue assay

The exposed H295R cells showed stable cell viability results > 95 % for all tested mixtures, except for the perfluorinated sub-mix, which caused an abrupt decline in viability at the lowest dilution of the mixture (Figure 11).

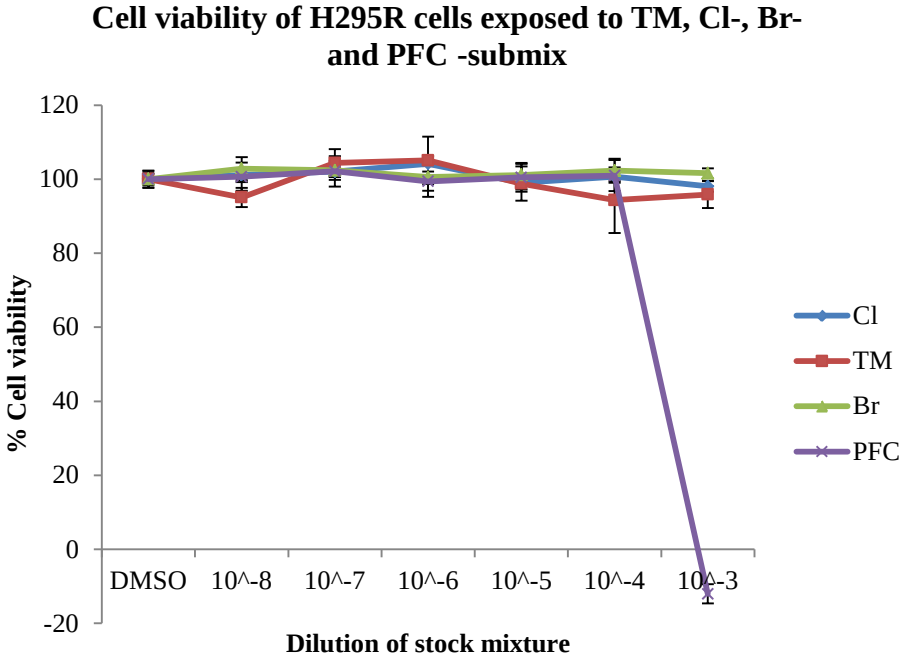


Figure 11. Cell viability in H295R cells after 48 hours of exposure to indicated dilutions of the TM, PFC -, Br -, and Cl –sub mixtures illustrated in different colors. Data represent percentage of cell viability, relative to control (DMSO) Obtained from three independent experiments.

3.2. H295R cells and DNA damage.

When accounting for difference between experiments and fitting the log transformed dose (e.g. dilution of stock solution) as a spline function, there was a significant dose –response relationship in the Comet assay with the perfluorinated sub –mixture and the total mixture. In the perfluorinated sub –mixture; DNA damage increased with increasing dose, whereas with the total mixture a curvelinear relationship was indicated (Figure 12c and d, respectively). For both mixtures the magnitude of increase in % DNA damage was moderate when compared with the solvent control (SC). With the chlorinated - and brominated – sub

mixtures there was no significant dose –response relationship in the comet assay (Figure 12a and b).

When entering dilution of mixture as a discrete variable, it was not many exposure groups that were different from the control. According to the Hsu –Dunnett the dilutions 10^{-3}, 10^{-4} and 10^{-5} were significantly different compared from solvent control (DMSO), for the chlorinated, perfluorinated, sub –mixtures and the total mix, respectively.

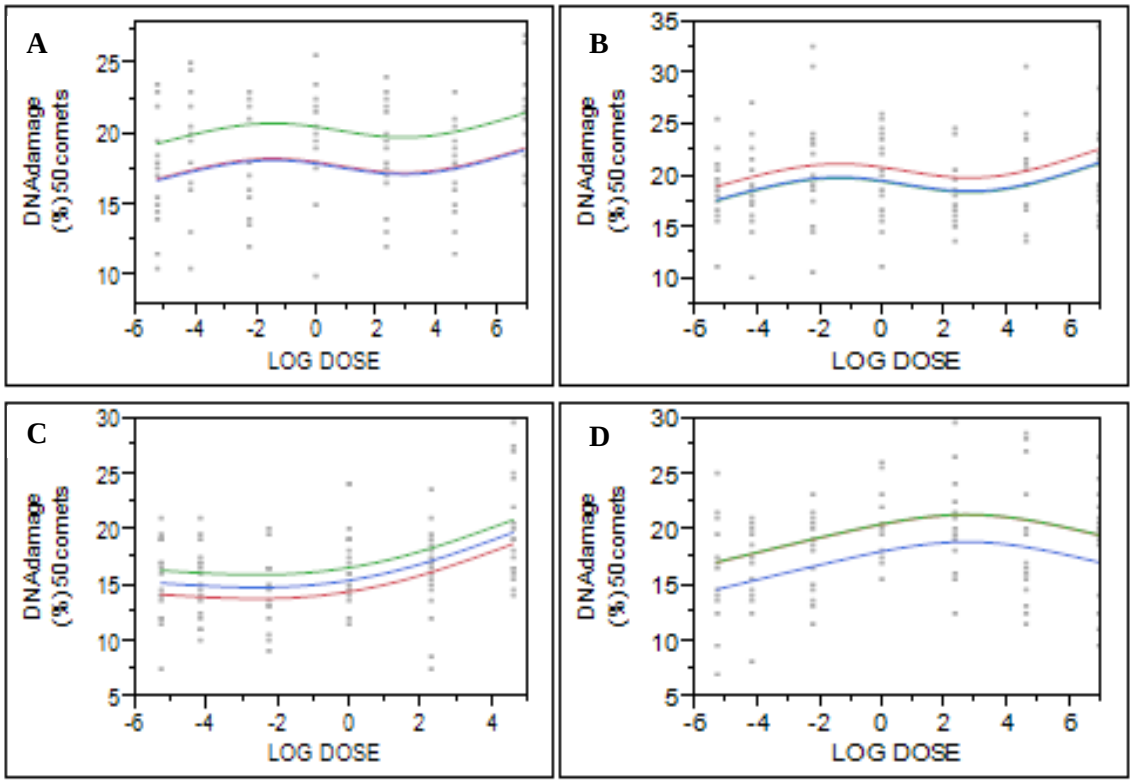


Figure 12. Percentage DNA damage in H295R cells exposed to the four different mixtures of POPs. A: mixture containing chlorinated POPs, B: mixture containing brominated POPs, C: mixture containing perfluorinated POPs and D: mixture containing total mixture of POPs. Data were fitted using dose/dilution as a spline function and experiment (N=2 or 3, respectively) as a discrete variable.

3.3. Lymphocytes and DNA damage

3.3.1. *In vitro* exposure

Exposure with the highest dilution of the total mixture, resulted in a small, but significant increase [mean (SEM) = 32, 7 % (2, 9) vs. 28, 1% (2, 3) for the solvent control; Figure 13] in the percentage of DNA damaged lymphocytes. Furthermore, there was a significant effect of mouse and the mouse* dilution interaction, indicating that individual mice reacted differently to exposure and exposure dose.

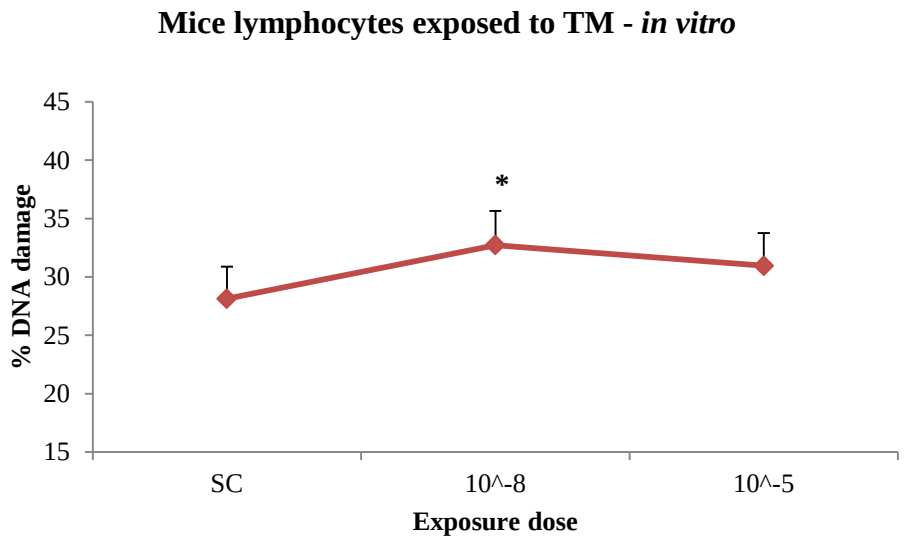


Figure 13. Mean (+SEM) percentage DNA damage in mice lymphocytes exposed for 4 hours with a mixture of POPs (TM) containing 29 different compounds. * Significantly different from solvent control (DMSO) (P < 0, 05; Hsu –Dunnett test).

3.3.2. ***In vivo* exposure**

Mouse lymphocytes exposed *in vivo* with either high or low concentration of a mixture of totally 29 different POPs did not show any significant change in DNA damage when compared with unexposed controls. However, the high –exposed group had a significantly higher DNA damage than the low exposed group [Mean (SEM) = 15, 2 % (0, 9) vs. 12, 4 % (1, 0) in percentage, P = 0,004]. The high- and low group differed significantly (Tukey HSD test; P < 0, 05) (Figure 14).

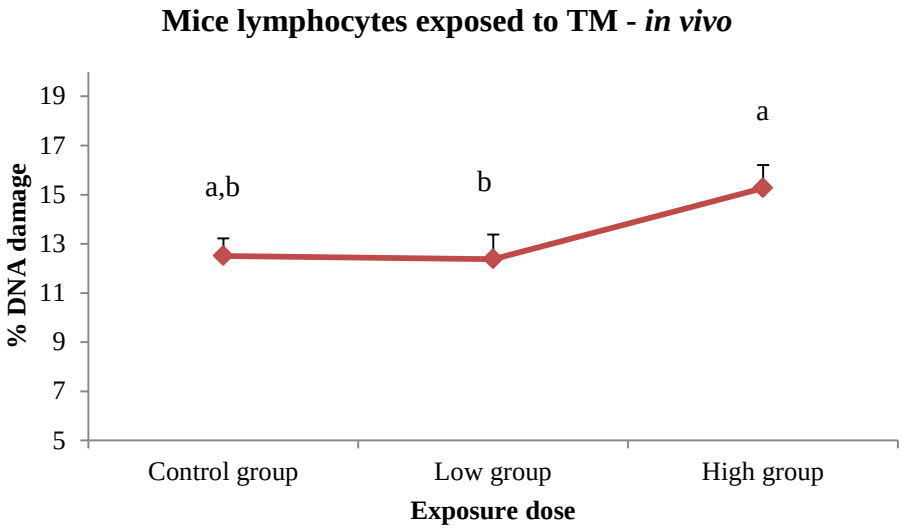


Figure 14. Mean (+SEM) percentage DNA damage in mouse lymphocytes exposed *in vivo* to a mixture of POPs (TM) containing 29 different compounds. The mice are group in unexposed control group, low group and a high group. [a,b)] Means with different superscript are significantly different (Tukey HSD test).

4. Discussion

The present study describes the exposure effects of POP mixtures that were designed based on levels of POPs normally found in human blood and breast milk or estimated levels in food. The mixtures used contained brominated, chlorinated and perfluorinated POPs (total mixture) and sub –mixtures of the three respective groups. Exposures were done on different cell systems, both *in vitro* and *in vivo*.

4.1. Cell viability

An aim in this study was to investigate the viability of H295R cell exposed to four different mixtures for POPs. The Alamar Blue® viability assay revealed approximately the same results, and the cells were viable at > 95 % for all the four mixtures, with one exception; the PFC mixture, which indicated a rapid decline at the highest dilution (10^{-3}), indicating this particular dilution of the PFC mixture to be cytotoxic. The TM however, did not indicate a rapid decline in the curve, even though it contains the three sub –mixtures, including the PFC mixture. The reason for this might be that the PFC mixture used in this study was 10000 times estimated concentration in human serum, whereas the TM was “merely” 1000 times estimated in human serum. This means that the concentrations of PFC in the TM are lower than in the PCF mixture.

As far one know, the combinations of compounds used in this study, has never been utilized before. However, a previous study on the viability of H295R cells exposed to single compounds of PFCs, indicated a dramatic decrease in viability for both PFOA and PFNA at the concentration 600 µM (Kraugerud et al., 2011). Thus, these single compounds have been shown to decrease viability at lower concentrations than was observed in this thesis on the PFC mixture. In the present study, the different concentrations in the PFC mixture was respectively 17,2 mM for PFNA and 109, 22 mM for PFOA from the stock solution, which is equal to the highest dilution used (10^{-3}). Interestingly, the total mixture which includes the PFC compounds, are ten times more diluted and the viability were stable > 95 %. The reason for this in unknown and it is difficult to compare single compound exposure studies to this

specific mixture used in the thesis. However, the concentration of compounds in the dilution 10^{-3} are slightly higher than real –life exposure.

A more recent study on cell viability of H295R cells has been done on three PCBs as single compound exposures at two different doses. The low dose exposures were equal for all the PCBs at 4 μM, whereas the high dose varied from 10 – 14 μM. The cell viability remained > 95 % for all the tested PCBs at both the respective concentrations (Tremoen et al., 2014). The PCB mixture used in this present study was also stable at > 95 % for all the dilutions, were the highest PCBs concentration for the chlorinated mixture was 1,003mM.

Summarized, the overall results show stable cell viability modeled by the *in vitro* H295R experiments, for all the different mixtures, even at high doses. With exception of the PFC mixture that among all tested mixtures, only the PFC mixture had acute cytotoxic effect, on the H295R cell line. It is however worth mentioning that the highest concentration range is not relevant to real –life exposure.

4.2. H295R cells and DNA damage

Another aim in this study was to assess the degree of DNA damage in H295R cells exposed to the four different mixtures of POPs. The results were based on cells exposed to the TM, Br, Cl and PFC mixtures. All results indicated approximately 15 -20 % DNA damage irrespective of the different mixtures used for exposure.

Most interestingly, is that the TM mixture (including all compounds) showed an increase in percent DNA damage up to the dilution 10^{-5} before a decrease occurred (Figure 13d), while the sub –mixtures alone do not indicate a decrease after the 10^{-5} dilution. It is reasonable to speculate that one or some of the other chemicals present in the total mixture may exert an antagonistic effect.

The PFC mixture however, caused an increase in DNA damage with increasing dose and the highest dilution of PFC mixture was cytotoxic for the H295R cells. A reason for this might be that the PFC mixture was estimated with a concentration containing higher amount of each compound compared to the TM, were PFCs and the other included compounds are estimated to be 10 times more diluted.

Both the chlorinated and the brominated mixture resulted in similar patterns of DNA damage (Figure 12), and neither showed a significant dose –response in the comet assay.

From what one know there are not much literature on the adrenal cell line -H295R and the evaluated effects on DNA damage from exposure of persistent organic pollutants (POPs). A study by Song et al. used the H295R cells to investigate DNA damage on two polybrominated diphenyl ethers, but no prominent tail formation was found and samples were no different from the control. However, the possibility that cells repaired any DNA damage formed during the incubation time (12 hours) could not be excluded (Song et al., 2009). In this study the cells were exposed for 48 hours, which is a four times longer exposure, so the fact that the cells may have repaired damaged DNA, should not be excluded in this study either.

Another study on PFOA exposure has shown to cause a significant increase in tail movement for concentrations from 50 – 400 µM for exposed HepG2 liver cells. Indicating that PFOA had genotoxic effects on HepG2 cells (Yao and Zhong, 2005).

One should be aware that, exposures in mechanistic cell models cannot be compared to real - life exposures, even though the concentrations are the same, since there are factors in the body that *in vitro* studies cannot imitate. However, the results from this present study exposed the H295R cells for 48 hours, and can give an indication of the genotoxic effect, due to POPs.

4.3. Lymphocytes and DNA damage

Assessing the percent of DNA damage from mice lymphocytes exposed *in vitro* and *in vivo* were also an aim for this study, and the Total mixture (TM) was used for both exposures. To the best of knowledge, assessing the effect on DNA damage on isolated mice lymphocytes has never been done before, with this designed mixture of POPs, containing 29 compounds.

4.3.1. *In vitro*

The results from the *in vitro* exposure indicate that each mouse reacts differently to the exposure of the TM. The exposure dose 10^{-8} is significantly different from the SC and the

percentage of DNA damage is also higher than for the exposure dose 10^{-5}. The results revealed a similar curve as the H295R cells exposed to the TM. As far as one knows there is not much literature on mixture effects on isolated lymphocytes.

However, a study done on different fish species, to determine the levels of POPs by using whole blood as an biological indicator, where evaluated using comet assay. Organochlorine pesticides (HCB, HCH, DDT and DDE) and PCB were detected and the results indicated that the DNA damage varied between the fish species (Gonzalez-Mille et al., 2010), which was similar to the results on the mice in this study, which also had variation between the individuals.

Another study assessed genotoxicity by utilizing comet assay on dolphin leukocytes exposed to a synthetic PCB mixture (Aroclor 1254). The doses used were consistent with blood concentration measured in wild specimens, and the results indicated a slight, but significant increase in DNA migration of treated cells as compared to controls (Taddei et al., 2001). The isolated lymphocytes in this study were also exposed to a mixture relevant to estimated levels in blood and breast milk, but in humans. Interestingly, the comet assay showed a significant increase in DNA damage at the dilution 10^{-8} which is lower that the estimated detected levels in blood and breast milk.

A study on isolated peripheral blood mononuclear cells (PBMC) incubated with p,p` -DDT, p,p`-DDE and p,p`-DDD at different concentrations (40,80 and 100 µg/ml) and different exposure time (24, 48 and 72 hours) all induced significant DNA damage (Yanez et al., 2004). Furthermore, the study we have done on isolated mice lymphocytes included an exposure to a complex mixture of 29 compounds, including the metabolite p,p` -DDE, and the exposure time were only 4 hours. A significant increase in DNA damage have also been detected in single PCB compounds (Sandal et al., 2008).

The results show that comet assay of exposed lymphocytes *in vitro* indicated individual differences and small significant increase in DNA damage. Furthermore, studies on larger population of individuals are needed and a larger dose –range should be explored to conclude.

4.3.2. *In vivo*

The results from the *in vivo* mixture revealed that the high dose exposure group had significantly higher DNA damage than the low dose exposure group. These results indicated a slight difference from both the *in vitro* exposure on the endocrine cell line (H295R) and the *in vitro* experiment on mice lymphocytes, exposed to the TM. The reason for this is difficult to speculate around, since the *in vivo* mixture is based on predicted levels detected in food. The two groups were therefore divided in a low-dose and a high-dose group, with a different estimation as illustrated in Table 2.

Previous research and available literature on isolated mice lymphocytes, exposed *in vivo* to mixtures of POPs has as far as we know never been done before. However, an *in vivo* study done on blood samples collected from 54 women from an area with a history of indoor pesticide spraying of DDT, showed that DDT and its metabolites were able to induce DNA damage, measured by comet assay (Yanez et al., 2004). From this present *in vivo* study the percentage of DNA damage lymphocytes were lower than for the *in vitro* exposure.

The results from this *in vivo* study on feed exposed mice indicated no significant changes in the DNA damage, when compared to the unexposed control group. However, the high group showed higher % of DNA damage, and was significantly different from low group. Additional *in vivo* study with this complex mixture is needed with a larger population. More *in vivo* studies with this complex mixture is needed to conclude further, with preferably a larger population and also in other research animals.

5. Conclusion

The findings obtained through this study showed, presumably for the first time the assessment of DNA damage due to the exposure of mixtures of POPs designed to mimic relative proportions and concentrations measured in human blood, breast milk and detected levels in food. The results were gained utilizing both *in vivo* and *in vitro* mechanistic studies on the adrenal cell line –H295R and isolated lymphocytes from mice.

The results from the *in vitro* exposure on mice lymphocytes revealed a small, but significant increase in the percentage of DNA damaged lymphocytes for the exposure dose 10^{-8}, which is an exposure dose lower than the levels detected in human blood and breast milk. Each individual mouse also reacted differently to the exposure and the exposure dose. The *in vivo* study resulted in neither of the two groups was significantly different from the unexposed control group, but the high group and the low group were significantly different from each other.

None of the doses from the dilution range used on the H295R cells revealed significant differences at the concentration corresponding to the approximate levels measured in human blood and breast milk.

On the other hand, the cell viability was stable > 95 % for all the tested mixture, with one exception for the PFC mixture, where the highest dilution was cytotoxic to the H295R cells.

Additional research on how exposure to mixtures of POPs can impact humans and wildlife is necessary to gain a better insight on their mechanisms and effects.

6. Future perspectives

The *in vitro* exposure on mice lymphocytes in this thesis, reveled a significant effect on a dilution below what is estimated in real –life and DNA damage was detected in various degrees for both the *in vitro* and the *in vivo* studies.

The literature on mixed exposure is sparse and effect studies on larger populations and concentration range are needed for both *in vitro* and *in vivo* studies. Also important for the study on mixed exposure is the ability of chemicals to act synergistic or antagonistic towards each other.

Safety assessments and risk assessments of DNA damaging agents or genotoxins are an important issue for the understanding of dose effects. The use of relevant dose –range in the exposure is important so the genotoxic effects are not overestimated. Understanding the mechanisms, that protect against genotoxins, at low doses, are of importance to get a better understanding on setting exposure limits.

To summarize, further study on designed mixtures, relevant to real –life exposure, their toxicity and the mechanisms behind are important areas of research.

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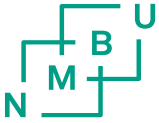

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