Effects of four environmental pollutants on Syrian hamster embryo cells

Effekter av fire miljøgifter på syriske hamsterembryoceller

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Summary

The main objective of this thesis was to investigate the carcinogenic potential of the three toxicants Perfluorooctane sulfonate (PFOS), Perfluorononanoic acid (PFNA) and Tetra-bromobispehenol-A (TBBPA), using the Syrian Hamster Embryo (SHE) Cell Transformation assay (CTA). The SHE CTA is an *in vitro* assay based on the morphological change of cell colonies when exposed to carcinogenic compounds. The cells used were primary cells isolated from Syrian hamster embryos. The SHE CTA has been used for several decades (Rivedal 1982), and the Organization for Economic Co-operation (OECD) has recently performed a prevalidation study and made a recommended protocol with a photo catalogue to help the scoring of the transformed colonies (Maire et al. 2012a; Maire et al. 2012b). Concentrations between 0.1 nM and 550 µM were tested in the current study, based on a dose range-finding experiment for each of the chemicals.

In addition to the SHE CTA, the DCF assay was used to investigate if exposure to PFOS, Perfluorooctanic acid (PFOA), PFNA and TBBPA would increase the production of reactive oxygen species (ROS) in SHE cells. This was tested to investigate if increased ROS production could be one of the mechanisms behind potential carcinogenicity of the compounds. In the experiments with the perfluorinated compounds concentrations between 1 µM and 1 mM were tested. In the experiment with TBBPA concentrations between 10 nM and 1 mM were tested.

The SHE CTA of PFOS did not give a valid answer, because of too low transformation frequency in the positive control Benzo-a-Pyrene (B[a]P). PFNA gave an inconclusive result, with only one concentration significantly different from the negative control Dimethyl sulfoxide (DMSO) in the experiments. TBBPA on the other hand gave a positive response in the SHE CTA with two positive concentrations, and it is therefore possible to say that TBBPA causes transformation in SHE cells. On the basis of the DCF assay experiments, it is unlikely that increase in ROS production is a part of the mechanisms behind these perfluorinated compounds' cytotoxic and carcinogenic potential. It was not possible to detect if TBBPA caused an increase in ROS production based on the assay used in this thesis.

Sammendrag

Hensikten med denne studien var å undersøke de tre kjemikaliene Perfluorooctane sulfonate (PFOS), Perfluorononanoic acid (PFNA) og Tetrabromobispehenol-A (TBBPA) sitt karsinogene potensiale. Dette ble gjort ved å utføre Syriskhamsterembryo (SHE) celletransformasjonsanalyse (CTA). Dette er en *in vitro* analyse basert på endring i cellekoloniers morfologi etter eksponering av karsinogene stoffer. Cellene benyttet er primærceller, isolert fra 13 dager gamle Syriske hamsterembryoer. Denne analysen har blitt benyttet i flere tiår og Organization for Economic Co-operation (OECD) har gjort en pre-valideringsstudie og lagd en anbefalt protokoll, samt en fotoguide for å hjelpe med scoringen av transformerte kolonier. Konsentrasjoner mellom 0,1 nM og 550 µM ble testet av de ulike kjemikaliene, basert på doseområde tester utført for hvert enkelt kjemikalie.

I tillegg til analysen av SHE celle transformasjon ble det utført en DCF analyse. Dette ble gjort for å se om eksponering av SHE celler av PFOS, Perfluorooctanic acid (PFOA), PFNA og TBBPA ville øke produksjonen av reaktive oksidanter (ROS) i cellene. Dette ble gjort for å undersøke om økt ROS produksjon kunne være en grunn til eventuelt karsinogent potensiale. I eksperimentene med de perfluorerte stoffene ble konsentrasjoner mellom 1 µM og 1 mM undersøkt. I eksperimentet med TBBPA ble konsentrasjoner mellom 10 nM og 1 mM testet.

Resultatene fra analysen etter eksponering av SHE celler for PFOS kunne ikke gi et gyldig resultat, fordi det var for lav transformasjonsrate på den positive kontrollen Benzo-a-Pyrene (B[a]P). PFNA eksponeringen ga et usikkert resultat med bare en signifikant konsentrasjon i tillegg til den positive kontrollen. TBBPA ga derimot et positivt resultat i SHE CTA med to konsentrasjoner signifikant forskjellig fra den negative kontrollen Dimethyl sulfoxide (DMSO). På bakgrunn av DCF forsøkene ser det ikke ut til at de perfluorerte stoffene testet her gir økning i ROS produksjon i forhold til DMSO kontrollen. Det var ikke mulig å undersøke om TBBPA ga økning i ROS produksjon basert på testen benyttet i denne studien.

Abbreviations:

°C:	Degrees Celsius
µg:	Microgram
µl:	Microliter
µM:	Micromolar
AUC:	Area under the curve
BFR:	Brominated flame retardant
B[a]P:	Benzo-a-Pyrene
BP-A:	Bisphenol-A
CMF-PBS:	Calcium-and magnesium-free phosphate buffered saline
CO_2:	Carbon dioxide
CTA:	Cell transformation assay
DDT:	Dichlorodiphenyltrichloroethane
DCF:	2',7'-dichlorofluorescin
DCFH:	2',7'-dichlorodihydrofluorescein
DCFH-DA:	2',7'-dichlorodihydrofluorescein diacetate
DRF:	Dose range finding
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
EURL ECVAM:	European Union Reference Laboratory for alternatives to animal testing

FBS:	Fetal bovine serum
H_2O_2:	Hydrogen Peroxide
H295R cells:	Human adrenocortical carcinoma cells
HBSS:	Hank's Balanced Salt solution
HepG2 cells:	Liver hepatocellular cells
Klif:	Klima- og forurensningsdirektoratet
MC:	Medium change
ml:	Milliliter
mM:	Millimolar
MTF:	Morphological transformation frequency
$NaHCO_3$:	Sodium Bicarbonate
ng:	Nanogram
nM:	Nanomolar
$O_2^{\cdot-}$:	Superoxide anion
OECD:	Organization for Economic Co-operation
·OH:	Hydroxyl radical
PBDE:	Polybrominated diphenyl ether
PCB:	Polychlorinated biphenyl
PE:	Plating efficiency
PFC:	Perfluorinated chemical
PFNA:	Perfluorononanoic acid

PFOA:	Perfluorooctanic acid
PFOS:	Perfluorooctane sulfonate
POPs:	Persistent organic pollutants
ROS:	Reactive oxygen species
RPE:	Relative plating efficiency
SHE:	Syrian hamster embryo
TBBPA:	Tetrabromobispehenol-A
TPA:	12-O-tetra-decanoyl-phorbol-13-acetat

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1. Introduction

1.1 Persistent organic pollutants

Synthetic chemical compounds have been found in the environment and in humans. Many of the compounds are persistent organic pollutants (POPs). POPs are generally defined as persistent, organic substances, which bioaccumulate, possess toxic characteristics and cause significant adverse effects to human health or to the environment, both near to and distant from their sources (UN-ECE). POPs are prone to long-range transboundary atmospheric transport and deposition, and are therefore globally distributed. There have even been found POPs in the Arctic, where no such substances are produced (El-Shahawi et al. 2010).

POPs may be released both intentionally and unintentionally. Unintentionally release of POPs may happen by volatilization or leakage, both during a products lifetime and after ultimate disposal, while pesticides, such as dichlorodiphenyltrichloroethane (DDT), are intentionally released at their point of application (Vallack et al. 1998).

There is a global concern related to the effects of POPs. As a consequence, the Stockholm Convention on persistent organic pollutants was adopted in 2001. This requires countries to take necessary measures to reduce and in some cases eliminate the release of POPs into the environment. The aim is to protect human health and wildlife (UNEP). Because of the Stockholm convention several POPs, like Perfluorooctane sulfonate (PFOS) and DDT, are banned in most countries. However, some countries still use them, and because of their persistency and lipophilic character they will stay in the environment, and reach high concentrations in top predators in many decades to come (El-Shahawi et al. 2010).

Animals on the top of the ecosystem, like polar bears and humans, are most prone to exposure to POPs, as these tends to bioaccumulate, and biomagnify up the food chain (Wania & Mackay 1999). POPs have shown to give adverse health problems such as birth defects, immunological, behavioral, neurological and reproductive discrepancies and cancer in humans (El-Shahawi et al. 2010). The main exposure source for humans is dietary intake of dairy products, meat and fish (Hardell et al. 2010).

There are several groups of chemicals within the definition of POPs. These include among others polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), various

organochlorine pesticides such as DDT and perfluorinated compounds (PFCs) (Hardell et al. 2010). In this thesis the focus will be on the PFCs.

1.1.1 Perfluorinated compounds

Perfluorinated compounds are synthetically produced fluorinated compounds composed of a carbon backbone. They are usually 4-14 atoms in length with a charged moiety, typically carboxylate or sulfonate. The carbon fluorine bond is extremely strong, and this makes the PFCs very resistant to degradation by heat, reactions with strong acids or bases, and oxidizing agents or photolysis (Florentin et al. 2011; Melvin E. Andersen et al. 2007).

PFCs are used in a wide range of industrial products because of their anti-wetting and surfactant properties. Perfluorinated compounds have been extensively used in industry in products as pesticides, herbicides, refrigerants, anesthetics, lubricants and insecticides and in consumer products as surfactants, oil and water repellant coating for cookware, carpets or textiles (Eriksen et al. 2010; Florentin et al. 2011; Xiao-Zhong & De-Cong 2009). The distribution and degradation of PFCs in the environment is complex, and gives a complicated exposure pattern. Most scientists conclude that food is the major exposure pathway for humans, but the ingestion of dust can also be a potential source of PFC exposure (D'Hollander et al. 2010).

Since the PFCs are very resistant to degradation they are found several places in the environment and wildlife. In this thesis I will focus on three PFCs; PFOS, PFOA and PFNA, which are the three PFCs with highest concentration found in human serum in Norway (Haug et al. 2010).

1.1.2 Perfluorooctanesulfonic acid

PFOS is the PFC found in highest concentration in human serum in the Norwegian population (Haug et al. 2010). PFOS has 8 carbon atoms and a sulfonic acid group at the end (*Figure 1).* Its half-life is of 5.4 years in humans (Olsen et al. 2007). PFOS has been widely used as a surfactant since the 1950s, but has recently been added to Annex B of the Stockholm Convention, because of its adverse health effects (Buck et al. 2011; Jacquet et al. 2012a). This has led to a reduction in PFOS levels in the environment (Calafat et al. 2007).

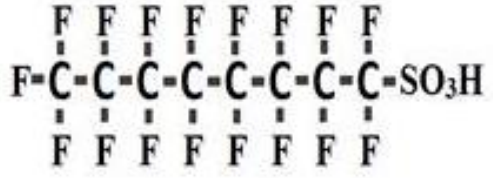

Figure 1: *Chemical structure of PFOS ($C_8HF_{17}O_3S$).*

PFOS is found to induce adverse health effects like cancer, hormone disruption, immunotoxicity and neonatal mortality (Yong et al. 2010). Exposure to 600 µM PFOS resulted in a dose responsive increase in oestradiol and a smaller increase in progesterone and testosterone secretion in the *in vitro* human adrenocortical carcinoma (H295R) cells (Kraugerud et al. 2011).

PFOS is not mutagenic, but has shown to have carcinogenic potential in the Syrian hamster embryo (SHE) cell transformation assay (CTA). It has also shown to be carcinogenic in rodents. Chronic exposures to PFOS in rats have been associated with tumor development in the liver, pancreas and testis (Eriksen et al. 2010). Production of reactive oxygen species (ROS) has also been investigated after exposure to PFOS, but the results point in different directions (Florentin et al. 2011; Jacquet et al. 2012a). Florentin (2011) did not detect an increase in ROS production in liver hepatocellular (HepG2) cells after PFOS exposure, while Eriksen et al. (2010) found an increase in ROS production in HepG2 cells.

1.1.3 Perfluorooctanoic acid

Perfluoroooctanoic acid (PFOA) is a PFC which consists of an 8 carbon-chain with a carboxylic acid group (*Figure 2)*. It has a half-life of 3.8 years in humans (Olsen et al. 2007), and the concentration of PFOA in human serum is in Norway found to be 4.1 ng/ml (Haug et al. 2010). PFOA is used as a surfactant and has been used for many decades as an essential processing aid in the manufacture of fluoropolymers such as polytetrafluoroethylene (Buck et al. 2011).

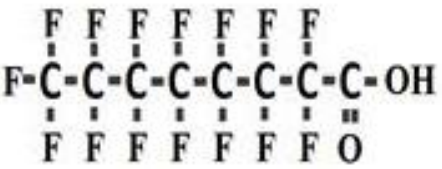

Figure 2: *Chemical structure of PFOA ($C_8HF_{15}O_2$).*

Effects have been found on the liver but not on the kidneys in mice treated with PFOA in the drinking water. Altered transcription of genes related to cell communication, growth, apoptosis and fatty acid metabolisms in rats have also been detected after exposure to PFOA (Xiao-Zhong & De-Cong 2009). But only small changes in hormone secretion have been detected in H295R cells (Kraugerud et al. 2011).

PFOA acts as a tumor promoter in Syrian hamster embryo cells and has been shown to be carcinogenic in rodents (Florentin et al. 2011; Jacquet et al. 2012b). Chronic exposures to PFOA have in rats been associated with tumor development in the liver, pancreas and testis (Eriksen et al. 2010). Eriksen et al. (2010) found that PFOA increased ROS production in HepG2 cells by 1.52-fold compared to unexposed cells. PFOA generated the most ROS compared with the other PFCs. However, Florentin et al. (2011) did not find an increase in ROS production after exposure to PFOA in HepG2 cells (Florentin et al. 2011).

1.1.4 Perfluorononanoic acid

Perfluorononanoic acid (PFNA) is also found in human serum in Norway (Haug et al. 2010). PFNA is a PFC with 9 carbon atoms and a carboxyl group at the end (*Figure 3)*. It has been manufactured since 1975, principally for producing fluoropolymer dispersions, especially polyvinylidene fluoride (Buck et al. 2011).The half-life for PFNA in human serum has not been estimated yet (Nelson et al. 2010). In rats the half-life for PFNA is longer than for PFOA (Ohmori et al. 2003). Since most countries have stopped producing PFOS, its concentration has declined in the environment, and the values found in humans and wildlife are decreasing. The amount of PFNA produced is on the other hand increasing (Lau et al. 2007).

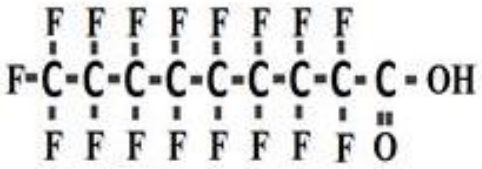

Figure 3: *Chemical structure of PFNA ($C_9HF_{17}O_2$).*

In the H295R *in vitro* cell assay only small changes in hormone secretion were detected after exposure to PFNA (Kraugerud et al. 2011). Exposure to PFNA in human HepG2 cells also caused only a small increase in DNA damage at cytotoxic concentration levels (Eriksen et al.

2010). In addition it has been discovered that PFNA can have endocrine disruptive effects in rodent models and in *in vitro* systems (Kraugerud et al. 2011).

Eriksen et al. found that the production of ROS after exposure to PFNA had an insignificant increase in the human HepG2 cells, except at 1 and 2 mM where the production was decreased (Eriksen et al. 2010). To the best of my knowledge there have not yet been done any experiments to test if PFNA is carcinogenic without being mutagenic.

1.2 Brominated flame retardants

Brominated flame retardants (BFRs) are brominated compounds incorporated into plastic products and polymers to reduce flammability (Alaee et al. 2003). They can be used in products such as electronic equipment, textiles and isolation material. BFRs can be released into the environment with effluents from factories producing BFRs and plastic products. Other ways BFRs may be released to the environment are from hazardous waste, facilities recycling plastics, metals from electronic devices and accidental fires (Watanabe & Sakai 2003).

BFRs, such as PBDEs and TBBPA have been found in human milk and the blood of the general population (Watanabe & Sakai 2003). Some BFRs have serious health effects, such as thyrodigenic, estrogenic, and dioxin-like activities (Watanabe & Sakai 2003).

Research has also shown that some BFRs have effects on the thyroid gland, the liver and the kidneys. Effects on behavioral development have also been detected (Darnerud 2003). One of the main routes of exposure of the general human population to BFRs such as for example PBDEs, particularly the lower brominated congeners, is through the diet, as is the case with PCBs and PCDDs/DFs. But for less persistent BFRs, inhalation of air polluted by these compounds is the main exposure pathway. People may also be exposed in the work environment where dismantling of electronic components, shredding and smelting of electronic components take place (Watanabe & Sakai 2003).

1.2.1 Tetrabromobisphenol-A

Tetrabromobisphenol-A (TBBPA) is a brominated flame retardant produced via bromination of bisphenol-A (BP-A) (*Figure 4)* (Alaee et al. 2003). It is one of the most frequently used BFRs (Reistad et al. 2005). TPPBA is a phenolic, weakly acidic and hydrophobic compound, and is as BFRs usually are, reactive, which means that it binds covalently to the material it is supposed to protect. Some of the reactive BFRs may not polymerize and can be released to the environment (de Wit 2002). TBBPA has a half-life of two days and can thus not go under the characterization persistent organic pollutant. Non persistent BFRs such as TBBPA do not biomagnify, hence leading to direct exposures via inhalation being the most likely route of exposure (Sjodin et al. 2003). TBBPA has been found in river sediments in Sweden and Japan and in workers occupationally exposed to BFRs (Sjodin et al. 2003).

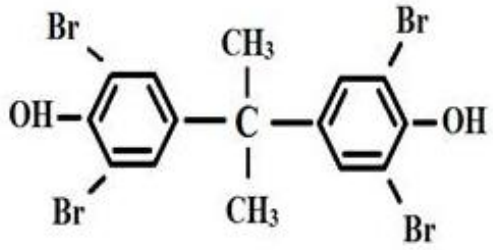

Figure 4: *Chemical structure of TBBPA.*

In experimental *in vivo* studies the toxicity of TBBPA is suggested to be low (Darnerud 2003). But rainbow trout exhibited irritation, twitching, erratic swimming, dark discoloration and labored respiration when exposed to TBBPA (de Wit 2002). TBBPA fed orally to mice and rats showed low or no effects on behavior, weight gain, mortality, organ abnormalities or hematology (de Wit 2002). A key concern with TBBPA is that it has a similar chemical structure to thyroxine (T4), and it has been shown to bind competitively, with high affinity to human transthyretin *in vitro* (Alaee et al. 2003). TBBPA has not shown to be mutagenic in studies with *Salmonella typhimurium* strains (Darnerud 2003). As far as I know there have not been performed any experiments to test if TBBPA is carcinogenic without being mutagenic.

1.3 Syrian hamster embryo cell transformation assay as a model

The SHE CTA was first developed by Berwald and Sachs in 1963 and 1964 respectively. The SHE CTA is one of several *in vitro* methods developed, which mimic some of the stages in cancer development (Vasseur & Lasne 2012). In risk assessment of chemical compounds it is important to investigate the carcinogenic potential of the substances (Corvi et al. 2012). Today the most used method is a two year rodent assay, and there is a need to develop methods to test chemical carcinogenicity without the use of animals. The Organization for Economic Co-operation (OECD) has therefore performed a pre-validation study on the SHE CTA and developed a test guide to secure reproducible results between laboratories (Vanparys et al. 2011).

SHE cells are isolated from 13 days old Syrian hamster embryos. They are primary cells and are therefore like "normal" cells, with a competent metabolic system and a finite lifespan, in contrast to cell lines, which are modified cells. SHE cells have a low spontaneous transformation rate and the transformation occurs within a few days of exposure. This makes this method much more time efficient than rodent assays which last for years. It is also much more cost efficient (Corvi et al. 2012; Maire et al. 2012a). The SHE CTA has shown to correspond 85% with rodent carcinogenic tests and is therefore a good method to investigate if a chemical should be studied further (Kamendulis et al. 2002).

In vitro transformed cells exhibit morphological changes related to neoplasia in cancer development. The phenomenon of morphological cell transformation involves changes in behavior and growth control of cultured cells, such as alteration of cell morphology, disorganized pattern of colony growth, and acquisition of anchorage independent growth (Combes *et al.*, 1999). Transformed cells have a lower cytoplasm to nucleus ratio compared to normal cells. They grow in criss-cross and can grow over feeder cells, whereas cells in normal colonies grow in a pattern, without stacking and tend avoid feeder cells.

In vitro cell transformation is, to date, the only well-established methodology which has the potential to detect both genotoxic and non-genotoxic carcinogenic compounds (Adler et al. 2011; Vanparys et al. 2011). The SHE CTA has been used to test several chemicals, among these PFOS, PFOA, Cyanide and BP-A have been demonstrated to be carcinogenic or to act as a tumor promoter in these cells (Jacquet et al. 2012a; Jacquet et al. 2012b; Kamendulis et al. 2002; Tsutsui et al. 1998). Many chemicals which can be carcinogenic can give negative

results in mutagenesis tests like Ames test and the mouse-lymphoma assay, whereas they may give positive results in the SHE CTA. The Mouse lymphoma assay detects mutagenic and clastogenic events at the thymidine kinase locus of L5178Y mouse lymphoma tk (+/-) cells. The Ames test also detects mutagenesis, but neither of these the tests detect non-genotoxic chemicals (Lloyd & Kidd 2012; Mortelmans & Zeiger 2000).

1.4 Reactive oxygen species

Reactive oxygen species are a group of short lived, oxygen containing, reactive molecules with one or more unpaired electrons (Alfadda & Sallam 2012; Yong et al. 2010). Examples are superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). ROS can be generated in many different organelles in the cell. The two organelles most intimately involved in their metabolism and production is the endoplasmic reticulum and the mitochondria (Liu et al. 2007). ROS can also be generated during irradiation by UV light, X-rays and by gamma rays. In addition exposure to POPs such as PCBs can increase the amount of ROS production in the cell (Brieger et al. 2012; Myhre et al. 2009).

ROS have several tasks in the body. They are linked to physiological processes and protective mechanisms, they can act as a second messenger in the cell, and have a role in immune defense and antibacterial action (Alfadda & Sallam 2012; Valko et al. 2006). ROS can also damage the cell. They can cause permanent structural changes in DNA, initiate lipid peroxidation and protein oxidation, and modulate activity of stress proteins and stress genes. In addition they can activate cytoplasmic and nuclear signal transduction pathways. ROS have also been thought to be involved in many diseases, including cardiovascular disorders, carcinogenesis, chronic inflammation, and neurodegenerative diseases (Yong et al. 2010). Insulin resistance, diabetes mellitus and atherosclerosis have also been linked to ROS production (Alfadda & Sallam 2012). ROS can lead to permanent modifications of genetic material associated with carcinogenesis. It induces damage involves single or double-stranded breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, which are all associated with cancer (Valko et al. 2006) .

1.5 Detection of reactive oxygen species

There are several types of ROS, and different techniques are used to detect them. To do a complete investigation of ROS production in cells, a variety of methods should be used, since none can detect all the different types of ROS. One of the techniques that may be used is the 2',7'-dichlorofluorescein (DCF) assay.

In the DCF assay the nonpolar, nonionic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) diffuse into the cell through the cell membrane where it is hydrolyzed to 2',7'-dichlorodihydrofluorescein (DCFH) by intercellular esterase activity. DCFH is non-fluorescent, but emits fluorescence when it is oxidized to DCF. The DCF assay detects the presence of various ROS, and is therefore often used as a general indicator of oxidative stress in cells (Wang & Joseph 1999). Some of the ROS the DCF assay is sensitive towards are $ONOO^-$, H_2O_2 in combination with cellular peroxidases, peroxidases alone and $\cdot OH$. It is however not suitable for measurement of HOCl or $O_2^{\cdot -}$. Some reactive oxidants will react with DCFH rapidly and almost immediately increase the DCF formation, while others need more time or higher concentrations to increase the formation of DCF (LeBel et al. 1992; Myhre et al. 2003).

1.6 Aim of study

The Syrian hamster embryo cell transformation assay has been used for decades as a screening system to detect both genotoxic and non-genotoxic compounds with carcinogenic potential. It is based on change in morphological growth of cell colonies when exposed to carcinogenic compounds, and has a high correspondence with *in vivo* tests. OECD has recently performed a pre-validation study and made a recommended protocol.

The perfluorinated compounds PFOS, PFOA and PFNA in addition to the brominated flame retardant TBBPA have all been proven to cause health effects. It has also been shown that they are not mutagenic. PFOS and PFOA have been tested in the SHE CTA, where PFOS gave transformation, and PFOA caused increased transformation in conjunction with Benzo-a-Pyrene (B[a]P). PFNA and TBBPA have not been tested to see if they are carcinogenic without being genotoxic. The perfluorinated compounds are found in relatively high concentrations in the environment and in humans.

The main objective of this study was to assess the carcinogenic potential of three different compounds, PFOS, PFNA and TBBPA, using the Syrian Hamster Embryo Cell Transformation Assay.

Secondary objectives were to investigate the toxicant's cytotoxicity and their effects on ROS production. The cytotoxicity was tested in the SHE CTA, and ROS production in a DCF assay.

2. Materials and methods

2.1 Syrian hamster embryo cell culture

The cells used in the current experiments were primary cells isolated from 13 days old Syrian hamster embryos. Four pregnant Syrian hamsters were ordered from Charles River, USA (New England, MA, USA). The isolation was performed according to the "Recommended protocol for the Syrian hamster embryo cell transformation assay" (Maire et al. 2012a). The mothers were euthanized by the use of CO_2 and decapitation. The pups were decapitated without the prior use of anesthesia. During the cell harvest the tissue was held on ice at all times, and the isolation was executed as sterile as possible. After isolation the cells were counted and frozen in vials in liquid nitrogen. This was all done before the experiments started.

2.2 Chemicals

Heptadecafluorooctanedulfonic acid potassium salt (PFOS) (purity ≥ 98.0 %), Perfluorooctanoic acid (PFOA) (purity > 96.0 %), Perfluorononanoic acid (PFNA) (purity > 97.0 %), and 3,3'5,5'-Tetrabromobisphenol A (TBBPA) (purity> 97.0 %) were all purchased in powder from Sigma-Aldrich® (St Louis, MO, USA).

2.3 Syrian hamster embryo cell transformation assay

The chemicals tested and described in this thesis are PFOS, PFNA and TBBPA. The toxicants were dissolved in Dimethyl Sulfoxide (DMSO) (Sigma Aldrich®), giving final concentrations in the medium from 0.1 nM to 1 mM in the dishes. The stocks were kept in the freezer (-20°C) between the experiments.

Since the toxic solutions were dissolved in DMSO the negative control should contain the same concentration of DMSO. The final DMSO concentration in each dish was 0.2 % after addition of feeder cells, target cells and exposure to toxicants. Benzo[A]Pyrene (B[a]P) (Sigma Aldrich ®), was used as the positive control. B[a]P was as the other toxicants dissolved in DMSO. The results were calculated relatively to the negative DMSO control. In

addition to dishes with positive and negative controls, there were also two dishes with feeder cells in each experiment to ensure that the feeder cells did not divide and form colonies.

Before the experiment started, medium was prepared and kept in the refrigerator for a few weeks. The medium consisted of DMEM powder (GIBCO®, Invitrogen, Paisley, UK) dissolved in MilliQ-water (Simplicity Water purification System, Millipore, Billerica, MA, USA), 20 % fetal bovine serum (FBS) (batch # 8180923, GIBCO®, Invitrogen), 1.5 g/L $NaHCO_3$ (Sigma Aldrich®) to adjust the pH and 2 µg/ml insulin (Sigma Aldrich®, stock solution 10 mg/ml in sterile water containing 1 % glacial acetic acid). All additives and medium were mixed and filtered through a 0.2 µm filter (Millipore) for sterilization.

Cryopreservation medium was made right before use, and consisted of complete medium (described over) and 15 % DMSO. The cryopreservation medium was kept on ice the whole time.

The SHE CTA can be performed at both pH 6.7 and pH 7.0. In the current experiments pH 7.0 was applied. It is a slight difference in the way the cell colonies grow depending on the pH, and therefore a difference in the colonies scored. A protocol using pH 7 was established in the laboratory of our collaborator, Edgar Rivedal, and it was therefore chosen to perform the current experiments at this pH. The cultivation of the SHE cells was carried out aseptically in a laminar air flow cabinet.

Primary culture of SHE cells were cultivated mainly corresponding to the "Recommended protocol for the Syrian hamster embryo cell transformation assay" (Maire et al. 2012a), with a few adaptations. On day one and day two respectively, the feeder and target cells were seeded onto the dish. On day three the cells were exposed to the toxicants and on day nine the medium was changed and the cells re-exposed. On the tenth day the cells were fixated, stained and left to air-dry. The procedure is showed step by step in *Figure 5*.

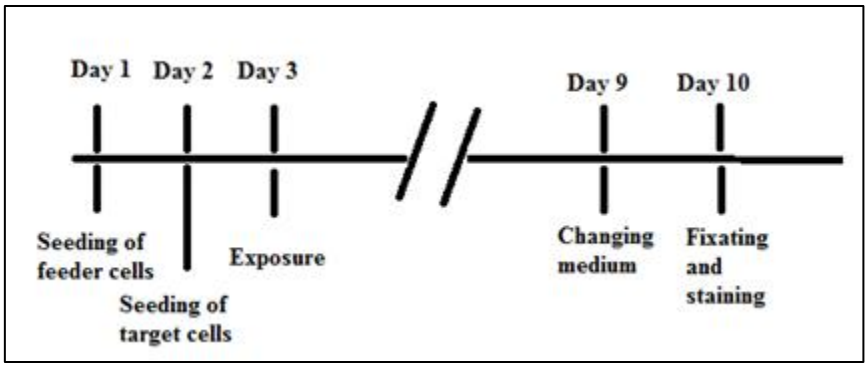

Figure 5: *Experimental procedure for the SHE CTA. The first day the feeder cells were seeded, the second day the target cells were seeded and on the third day the cells were exposed to the toxicants. The medium was changed on day nine, and on the tenth day the cells were stained and fixated.*

2.3.1 Development of the method

There are many parameters affecting the Syrian hamster embryo transformation assay. It is variability in how sensitive different SHE cell isolates are to morphological transformation. In addition there are differences in FBS lots' ability to support SHE cell growth and transformation (Kerckaert et al. 1996). Additionally the feeder cells can affect how well the target cells grow. Preliminary studies were first carried out testing four different batches of cell isolates (A, B, C and D) and three different batches of fetal bovine calf serum batch # 8180923 (GIBCO® Invitrogen), Lot no: A5011-2960 and Lot no: A55111-3462 (Thermo Fisher Scientific, Waltham, MA, USA), to find the most sensitive and growth effective combination. Cell isolate C and D were the cell isolates, which had the best growth. Batch D was chosen over batch C because it had almost 60 vials more of cells. FBS batch # 8180923 was chosen as it gave a good plating efficiency (PE), and it gave the densest cell colonies. The colonies in the two FBS batches from Fisher gave low PE and thin cell colonies. Later on another FBS batch from Invitrogen (batch # 1013153) was also tested, but it did not give any better results than the first batch from Invitrogen, and it was decided not to change batch.

Preliminary studies were also carried out to test varying percentages of FBS (10 %, 15 % and 20 %), varying concentrations of the positive control B[a]P, with and without an extra day of exposure, with and without insulin and with and without change of medium on day nine. Based on these studies the final medium contained 20 % FBS and insulin and was changed at day nine, to get rid of fibronectin, which accumulates in the medium and suppresses

transformation (Rivedal 1982). The method development was conducted with guidance from Edgar Rivedal at the Oslo University Hospital.

2.3.2 Preparation of feeder-cells

The feeder-cells were prepared in advance, and kept frozen in liquid nitrogen until used. Frozen vials of cryopreserved SHE cells were thawed rapidly in a 37°C water bath (Grant Instruments (Cambridge) Ltd, UK). The cell suspension was added gently to the side of a 100 mm tissue culture Falcon™ plastic dish (BD Bioscience, Bedford, MA) and mixed drop-wise with 15 ml cold medium (DMEM), to dilute the DMSO. The medium was changed with 10 ml fresh medium after 4-5 hours to remove the DMSO. The cells were then incubated in a cell incubator (Thermo Fisher Scientific, HERAcell 150i, CO2 Incubator) with 10 % CO_2, 37°C and humidified air, for 2-3 days to achieve 50-90 % confluence.

On the day of irradiation each plate was rinsed twice with 5 ml DPBS-CMF (-Ca^{2+}, - Mg^{2+}, GIBCO®, Invitrogen). After the PBS was removed, 2 ml of detachment solution (0.25 % Trypsin, GIBCO®, Invitrogen) was added to the cells. The detachment was observed in a light microscope (Leitz Labovert, Michigan, MI, USA) and the activity of the trypsin was stopped before the cells looked rounded, and were still a bit comma shaped. The cells were removed and added to 30 ml medium in a 50 ml Falcon™ tube (BD bioscience). The detachment procedure was repeated twice to get as many cells as possible. The 50 ml tube with the cells and the dissociation solution was centrifuged for 10 minutes at 300 xg, to remove the trypsin (Heraeus, Multifuge X1R, Thermo Fisher Scientific). The supernatant was removed and the pellet resuspended in 5 ml of cold medium and put on ice.

20 ml of cells were exposed to X-ray irradiation (5000 rads), for 10 minutes, in a Muller X-ray cabinet at the Oslo University Hospital, so that they were still viable, but no longer capable of replication. Viable cells were counted using an automated cell-counter (Invitrogen™, Countess ™, automated cell counter), and diluted to $3.3x10^6$ cells/vial in cryopreservation medium. They were placed at -80°C in a Styrofoam-box overnight, and then put in liquid nitrogen where they were kept until used. Two dishes with feeder-cells were seeded to check their viability and lack of ability to divide.

2.3.3 Dose-range finding test

Before running the SHE CTA a dose-range finding (DRF) test had to be carried out. The DRF test was done to decide, which concentrations of the different chemicals should be tested, and to decide how many cells should be seeded at the different concentrations. At least ten dishes were seeded per concentration tested; the number of target cells seeded corresponded to the number giving 25-45 colonies in the DMSO control. The definite doses for the transformation assay were based on the DRFs. The transformation experiments should include at least one dose, which had no cytotoxic effect, a high dose which caused an approximate 50 % reduction in RPE or relative colony size or density, and four concentrations in-between (Maire et al. 2012a). In the current experiments some lower concentrations were added to get closer to real-life exposures. In total 10 different concentrations of each substance were tested.

The DRF tests were also used to adjust target cell seeding number. An average of 25-45 colonies per dish should be obtained to consider the assay valid, and the number of target cells seeded must be adjusted according to the cytotoxicity of the toxic substances. In this experiment the seeding number was adjusted when the RPE was more than than 15 % lower than the DMSO control.

On day one, three vials of x-ray irradiated feeder cells were thawed and transferred into 15 ml tubes (BD bioscience) with 10 ml of cold fresh complete medium. The tubes were centrifuged at 300 xg for 10 minutes to eliminate DMSO, and the supernatant was removed. The pellets were resuspended and pooled from the tubes in 3 ml of medium. The cells were counted using an automated cell counter, and 17 000 cells/ml were seeded into 60 mm^2 plastic dishes (BD bioscience) in 3 ml fresh medium. The dishes were set in an incubation cabinet for 24 hours at 37°C and 10 % CO_2. A different batch of feeder-cells was used in the experiments with PFOS, than in the experiments with TBBPA and PFNA.

Also on the first day, one vial of frozen SHE cells, from batch D, was thawed. These cells were called target cells. The cells were added to a 25 cm^2 cell culture flask (BD bioscience) and mixed drop-wise with 10 ml of cold medium, to dilute the DMSO. The medium was removed and replaced by 5 ml of fresh medium after 4-5 hours. These cells were also left to incubate for 24 hours in the humidified cell incubator at 37°C and 10 % CO_2.

At the second day the target cells were seeded onto the dishes with the feeder cells. The medium was removed from the 25 cm^2 cell culture flask and the cells rinsed twice with 2 ml

DPBS-CMF. The DPBS-CMF was removed and 2 ml detachment solution (trypsin) was added to the flask. The extent of trypsinization was followed in the microscope and the cells were transferred to a 15 ml tube with 8 ml of cold medium, before they looked rounded, and still a bit comma shaped. The tube was centrifuged for 10 minutes at 300 xg, the supernatant removed and the pellet resuspended in 1.5 ml medium. The cells were counted using an automated cell counter, and seeded onto the 60 mm^2 plastic dishes in a concentration of 35 cells/ml in 3 ml medium. The cells were left to incubate for 24 hours in the incubator cabinet at 37°C 10 % CO_2.

On day three the cells were exposed to the toxicants. 2 ml of fresh medium was added to the dishes with 16 µl of the concentration of the toxicants, giving 2 µl/ml in the final solution of 8 ml. The dishes were then incubated for six more days at 37°C and 10 % CO_2.

The ninth day the medium was changed and the cells re-exposed. The medium was removed and replaced with 8 ml fresh complete medium with 2 µl/ml of each of the concentrations of the toxicants. Then the cells were incubated for another 24 hours.

On the tenth day the cells were fixed and stained. The fixation of the cells was done in a fume hood. The medium was aspirated and the dishes washed with 5 ml PBS (37°C). The PBS was then removed and the cells fixated with 5 ml 90 % ethanol (Kemetyl, Halden, Norway). After 10 minutes the ethanol was removed and the dish left to air-dry. Finally the cells were stained with 10 % Giemsa ((Sigma Aldrich®) mixed with distilled water) for 20 minutes, before the Giemsa was removed and the dishes were washed in running tap water. The dishes were then left to air-dry.

The colonies were counted in a stereomicroscope to decide the number of cells that had to be seeded to get about 35 colonies per dish in the following transformation assay, and to decide which concentrations should be used. The cytotoxicity of the compounds is shown by colony density, size and RPE (relative plating efficiency).

2.3.4 Transformation assay

The transformation assay was carried out as described above in the DRF test. In total 40 dishes were seeded per concentration over three different experiments. The number of cells seeded for each concentration was adjusted after the DRF test. For PFOS the concentrations

tested were between 0.1 nM and 250 µM. For PFNA the concentrations tested were between 10 nM and 550 µM, and for TBBPA the concentrations ranged between 0.1 nM and 185 µM.

The colonies were examined under a stereomicroscope for the scoring of transformed colonies. An example of normal and transformed colonies is displayed in *Figure 6*.

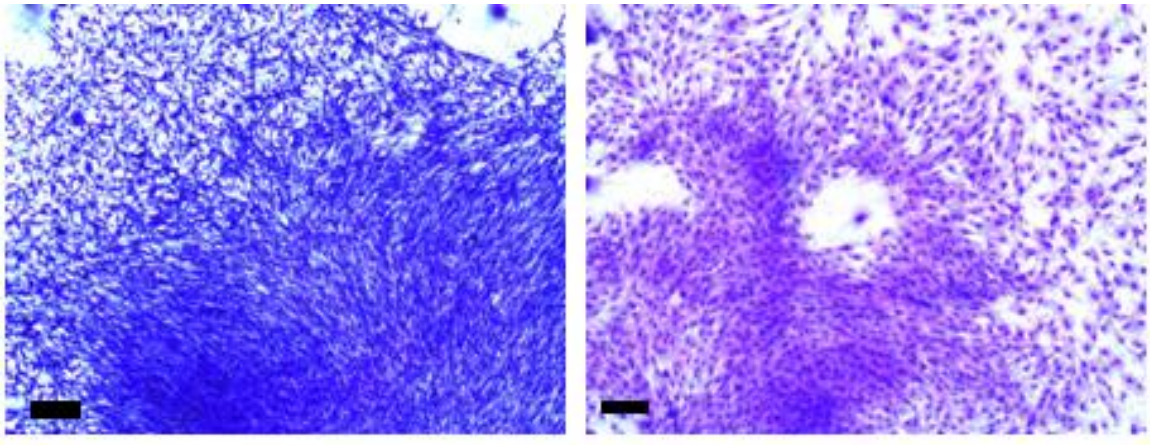

Figure 6: *Pictures of two Syrian hamster embryo cell colonies. To the left a transformed cell colony where the cells grow over the feeder cells, have a low cytoplasm to nucleus ratio and grow in criss-cross. To the right a normal cell colony where the cells are avoiding the feeder cells, have a high cytoplasm to nucleus ratio and grow in a pattern. Scale-bars equal approximately 175 µm. 4X magnification (Nikon ECLIPSE TS100, Tokyo, Japan).*

The scoring was performed blindly by two different persons. The photo catalogue for pH 7 was used as a guide (Maire et al. 2012b).

To evaluate the results from the SHE CTA several criteria have to be fulfilled. There have to be about thousand colonies of each concentration to make the test statistical significant and to determine the PE, RPE and MTF (morphological transformation frequency). The colony number in each dish has to be between 25-45 colonies, less than 25 giving false positive results, and above 45 giving false negative results. A colony has to have at least 50 cells to be counted. In addition is it required that the positive control B[a]P has a MTF over 0.6 % and that the negative control DMSO has a MTF lower than 0.6 %.

2.4 Detection of reactive oxygen species using the DCF assay

The DCF assay was used to investigate a potential increase in ROS production. The cells were exposed to triplicates of PFOS, PFOA and PFNA at concentrations ranging from 1 µM to 1

mM. The TBBPA was tested at concentrations ranging from 10 nM to 1 mM. Stock solutions of PFOS, PFOA, PFNA and TBBPA were prepared by dissolution in DMSO. The probe DCFH-DA was dissolved in methanol (Merck, Darmstadt, Germany).

The final DMSO concentration in each well should be 0.2 % after addition of cells, buffer and toxicants. DMSO is an antioxidant (Sanmartin-Suarez et al. 2011). It is therefore possible that it can decrease basal ROS levels in un-stimulated cells. DMSO controls with the same concentration of DMSO as in the experimental wells (0.2 %) were included in at least four different sites of the plate. This was done to correct for the suppressive effect of DMSO on ROS production. The subsequent calculation of relative fluorescence values were calculated as a percentage of the DMSO control. The perfluorinated compounds were tested together on one plate for comparison. The testing of TBBPA was done separately.

Before the experiments started Hank's Balanced Salt solution (HBSS) buffer (Gibco ® Invitrogen) was mixed. To make the 1X HBSS, 50 ml refrigerated HBSS (10X) was added to 450 ml of distilled water. 175 mg of $NaHCO_3$ was also added before the solution was sterile-filtered. The solution was kept refrigerated until the day of the experiment. At the day of cell-exposure 48.5 ml HBSS buffer was supplemented with 1 ml 1M Hepes buffer solution (Gibco ® Invitrogen) and 0.5 ml 0.5 M glucose (BDH, Poole, UK, dissolved in distilled water).

Before testing the ROS production after exposure to the chemicals each of the substances was tested for auto-fluorescence and to see if they would oxidize the probe by themselves. Different concentrations of the toxicants were tested in triplicates, with and without cells, in combinations with and without the probe. It was tried to both leave the mix of DCF and the toxicant on the plate during the reading and to take it off and wash with buffer before the reading. The probe was also tested alone both with and without aspirating and washing. All the steps from the addition of the DCFH-DA were done in the dark, since DCFH-DA is sensitive to light.

The Syrian embryo hamster cells used in the DCF experiments were from the same cell isolate as the one used in the SHE CTA (cell isolate D).

2.4.1 Method development of the DCF assay

Different concentrations of the probe; DCFH-DA (GIBCO ® Invitrogen), were tested, ranging from 10 µM to 100 µM, and it was decided to use a final concentration of 10 µM this

being the lowest concentration tested with a good effect. Different concentrations of the cells were also tested ranging from 10 000 cells/well to 30 000 cells/well. It was decided to use 25 000 cells per well, since this was the lowest concentration were the cells were confluent after 24 hours, giving the most equal amount of cells in each well and thus a consistent number of cells producing ROS. Two different positive controls were tested, H_2O_2 (Norsk Medisinal Depot, Oslo, Norway) and12-O-tetradecanoyl-phorbol-13-acetat (TPA) (Sigma-Aldrich®). H_2O_2 exposure resulted in between 150 % and 200 % increase in fluorescence relative to the DMSO control, while TPA gave a very small increase (*Figure 13*), and it was decided to use H_2O_2 as a positive control. Since the toxicants were dissolved in DMSO this was used as a negative control. In the tests there were also a blank well without cells or DCFH-DA to see if the buffer would auto-fluoresce and to remove any background noise. In addition a control well with cells and DCFH-DA, which was not exposed was added to the plate. Incubation from 15 minutes to 1 hour was tested, and it was decided to incubate for 30 minutes.

2.4.2 Measurement of ROS production after exposure to toxic chemicals

The assessment of ROS by the DCF assay was carried out mainly as described in Myhre et al (2009) and Reistad et al. (2005), with some modifications. The cells were thawed and seeded as described in the feeder cell preparation above, and then left to incubate for 2 days at 37°C and 10 % CO_2. On day three the cells were trypsinized, centrifuged and counted as described earlier. Then 250 µl medium with 100 000 cells/ml was added to each well in a 96 well optical black plate with transparent bottom and lid (Perkin Elmer, Waltham, MA), except for the blank were no cells were added. The cells were then incubated for 24 hours at 37°C and 10 % CO_2. After 24 hours the medium was removed and 250 µl HBSS buffer with 10 µM DCF was added to all the wells, except the blank, the plate were then incubated at 37°C. After 30 minutes the HBSS buffer with DCF was aspirated and the wells washed with 250 µl HBSS buffer. After removal of the buffer, 250 µl of HBSS buffer with the right concentration of toxicants was added to the wells. The concentrations tested were 1 mM, 500, 400, 300, 200, 100, 10 and 1 µM for the perfluorinated compounds, and 1 mM, 500, 250, 225, 200, 175, 150, 125, 100, 75, 50, 10 and 1 µM, 100, 10, 1 and 0.1 nM for TBBPA. The wells with the blank and the control cells were only added HBSS buffer. DCF fluorescence was subsequently measured every third minute for 180 minutes, giving 61 readings, in a microplate spectrofluorometer (Wallac VICTOR$^3{}_{TM}$ multilabel Counter, Perkin Elmer), at 37°C. The

energy of the lamp was set on full (65535), except for the TBBPA readings were it was set to 50000. The emission was too high to be detected when the lamp was on full energy after exposure to TBBPA. The lamp let out waves at 485 nm, and the emission filter picked up waves at 535 nm.

2.5 Calculations and statistical analysis

The statistical analyses were performed in Microsoft Excel 2010, GraphPad Prism 5 and R (64 bit). P-values less than 0.05 were considered significant.

In accordance with the recommended protocol, a one-sided Fisher's exact-test was used to evaluate the transformation results from the SHE CTA in R. This is a pairwise comparison of MTF relative to the vehicle control (DMSO control). A Chi-square test was also executed in GraphPad Prism 5 giving the same results (results not shown). Data from all three experiments were pooled for each concentration tested for analysis of treatment related effect (Custer et al. 2000a; LeBoeuf et al. 1996).

If only one concentration of the test substance shows a statistically significant response ($p < 0.05$), the Cochran-Armitage trend test for a positive dose-related response needs to be executed (Custer et al. 2000b; Maire et al. 2012a).

If the test substance did not get a statistically significant difference from the DMSO control, or got a MTF lower than 0.6 % it was considered non-transforming in the SHE CTA assay. A test substance was considered positive if an increase in MTF above 0.6 % was observed for at least two dose levels and were significantly different from the DMSO control or if a significant increase in MTF (above 0.6 %) was observed at a single dose level only, but with a general positive trend (Maire et al. 2012a).

PE, RPE and MTF were determined for all concentrations and controls as described below:

PE = (total amount of cells seeded/total number of colonies) * 100

RPE = (PE/PE of DMSO control) * 100

MTF = (total number of transformed colonies/RPE) * 100

The Wallac 1420 manager calculates the integral of the area under the curve (AUC) by the trapezium method. To evaluate the ROS production a One-way ANOVA test was used. The statistical analysis of the ROS production was made using the raw data, removing the background noise found in the blank. The graphs display the results as percent of the DMSO control. For comparison between the mean of the DMSO control and the means of each of the different experimental groups that were tested, a Dunnett's post hoc test was used (Motulsky 2007).

GraphPad Prism 5 does not perform the Bartlett's test in cases where there are fewer than four individual experimental values. There were only three individual experimental values in these experiments. It was therefore assumed that deviations from normality and homogeneity of variances between groups were small (Motulsky 2007).

3. Results

3.1 Syrian hamster embryo cell transformation assay

3.1.1 Method development

The pre-validation study testing the SHE CTA with SHE cells exposed to different concentrations of B[a]P with and without changing the medium at day nine, showed that changing the medium increased the MTF compared too not changing the medium. SHE cells exposed to 0.1 µg/ml and 0.5 µg/ml B[a]P with medium change got MTF of 1.89 % and 2.92 % respectively (*Table 1).*

Table 1. *Cell transformation results after exposure of SHE cells to B[a]P, testing with and without medium change. Morphological transformation frequency (MTF) in %. Negative control is 0.2 % DMSO.*

Test groups	Colonies scored	Transformed colonies	
Chemicals/concentrations	All	Transformed colonies	MTF (%)
0.2 % DMSO	350	0	0
0.1 µg/ml B[a]P	232	0	0
0.1 µg/ml with medium change	264	5	1.89
0.5 µg/ml B[a]P	248	0	0
0.5 µg/ml BaP with medium change	240	7	2.92
1 µg/ml B[a]P	248	1	0.4

The pre-validation study testing the SHE CTA with different adaptions to the FBS and with an extra day of growth showed that 20 % FBS and insulin in the medium increased the MTF in the SHE cells giving an MTF of 0.83 and 0.85 % respectively. Staining on day 11 gave a MTF of 0.46 %. All the tests were executed with a change of medium on day nine (*Table 2).*

Table 2. *Cell transformation results after exposure of SHE cells to B[a]P, testing percent of FBSs, addition of insulin and one extra day of exposure. All tests were performed with change of medium (MC). Morphological transformation frequency is showed in percent. The negative control is 0.2 % DMSO.*

Test groups	Colonies scored	Transformed colonies	
	Total	Transformed colonies	MTF (%)
15 % FBS, MC, DMSO control	516	0	0
15 % FBS, MC, 0.5 µM B[a]P	559	0	0
10 % FBS, MC, DMSO control	408	0	0
10 % FBS, MC, 0.5 µM B[a]P	546	0	0
20 % FBS, MC, DMSO control	147	0	0
20 % FBS, MC, 0.5 µM B[a]P	360	3	0.83
15 % FBS, MC, Insulin, DMSO control	126	0	0
15 % FBS, MC, Insulin, 0.5 µM B[a]P	351	3	0.85
15 % FBS, MC, Stain day 11, DMSO control	492	0	0
15 % FBS, MC, Stain day 11, 0.5 µM B[a]P	432	2	0.46

3.1.2 Cytotoxicity/Dose-range finding test

The cytotoxicity of the investigated compounds was calculated as a decrease in RPE relative to the DMSO control, in which the RPE was set to 100 %. Diagrams of the cytotoxicity of the compounds are shown in *Figure 7-9*.

PFOS

The dose range test performed to decide, which concentrations should be used, showed that the RPE was about 50 % after exposure to 250 µM PFOS (*Figure 7).* After the DRF test it was decided that the concentrations tested should be between 0.1 nM and 250 µM.

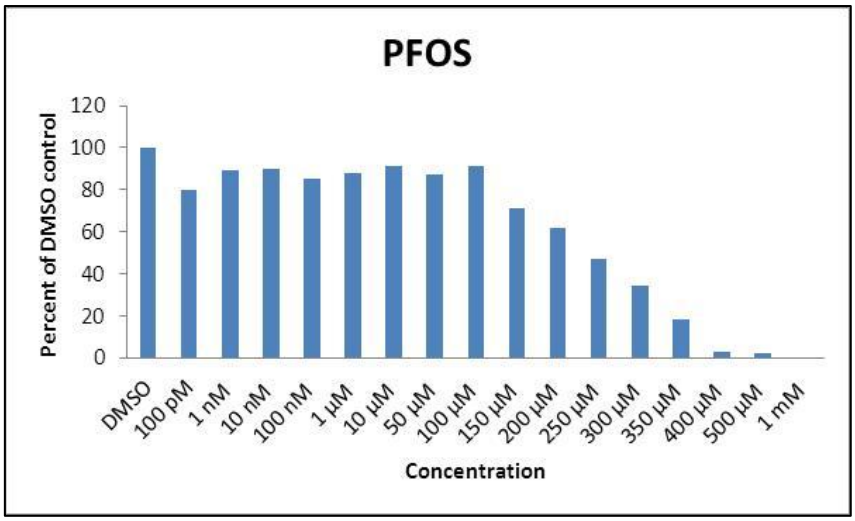

Figure 7: *Relative Plating Efficiency of SHE cells after exposure to PFOS in percent of the DMSO control.*

The DRF test was also done to be able to correct for a decrease in PE at the higher concentrations, so it was seeded enough cells to give the optimal amount of colonies (25-45). After the DRF experiment with PFOS the amount of cells seeded was adapted for the 150, 200 and 250 µM concentrations, seeding 36, 40 and 53 cells/ml respectively, in contrast to seeding 25 cells/ml for the other concentrations.

PFNA

The dose range test performed to decide, which concentrations should be used, showed that the RPE was about 50 % after exposure to 550 µM PFNA.

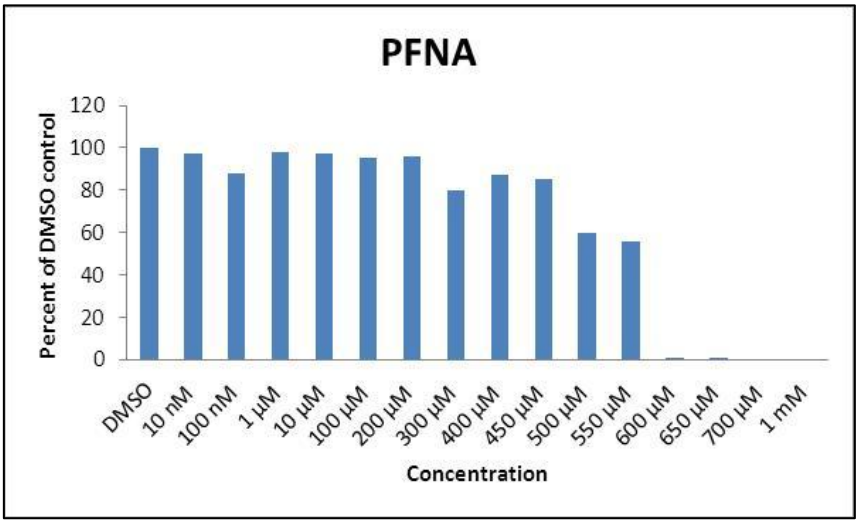

Figure 8: *Relative Plating Efficiency of SHE cells after exposure to PFNA in percent of the DMSO control.*

Based on the DRF test it was decided that the concentrations to be tested in the transformation assay for PFNA should be between 10 nM and 550 µM. The number of cells seeded in the exposure concentrations 450, 500 and 550 µM was adjusted, seeding 27, 33 and 41 cells/ml respectively, while for the less cytotoxic concentrations 25 cells/ml were seeded.

TBBPA

The dose range test performed to decide, which concentrations should be used, showed that after exposure to 185 µM TBBPA the RPE was closest to 50 %.

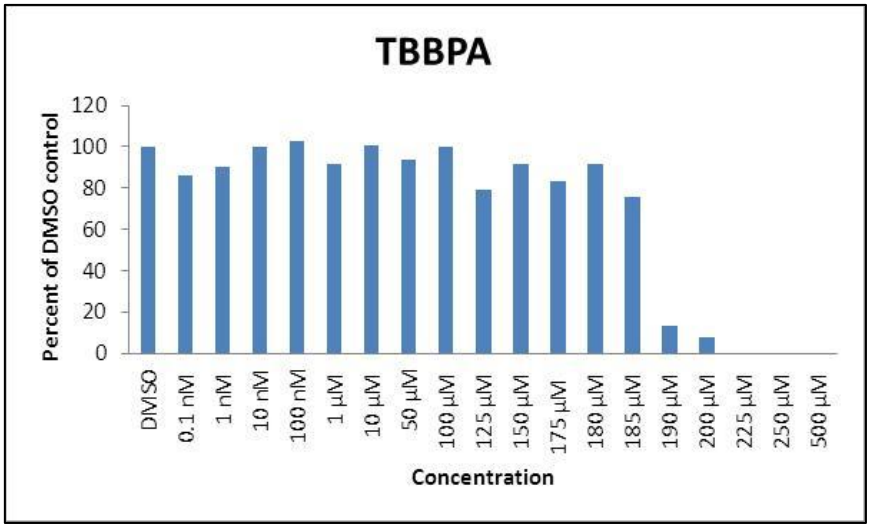

Figure 9: *Relative Plating Efficiency of SHE cells after exposure to TBBPA in percent of the DMSO control.*

Based on the DRF test the highest dose included in the transformation experiments was 185 µM TBBPA, and the lowest was 1 nM. It was decided to seed 35 cells/ml in 185 µM TBBPA, compared to 25 cells/ml in 3 ml for the rest of the dishes.

3.1.3 Transformation assay

PFOS

The transformation assay testing PFOSs carcinogenic potential found that none of the concentrations tested were significantly different from the negative control DMSO (One-sided Fisher exact test $p < 0.001$). Neither did B[a]P get significantly different values compared to the DMSO control, with a p-value of 0.097. Neither of the PFOS concentrations tested, nor 5 µg/ml B[a]P gave a MTF above 0.6 % (*Table 3).*

Table 3. *Cell transformation results after exposure of SHE cells to PFOS. Morphological transformation frequency (MTF in %), Relative plating efficiency (RPE in %) measured after seven days of exposure. Positive control was B[a]P.*

Test groups		Colonies scored			Transformed colonies	
Chemicals	Concentration	All	PE (%)	RPE (%)	Transformed colonies	MTF (%)
PFOS	0.1 nM PFOS:	1530	49	94	2	0.131
	1 nM PFOS:	1544	49	95	3	0.194
	10 nM PFOS:	1520	48	94	2	0.132
	100 nM PFOS:	1534	50	97	3	0.196
	1 µM PFOS:	1574	50	97	1	0.064
	10 µM PFOS:	1630	52	100	2	0.123
	100 µM PFOS:	1581	50	97	1	0.063
	150 µM	1564	35	68	1	0.064
	200 µM	1459	29	56	1	0.069
	250 µM	1465	22	42	1	0.068
BaP	5 µg/ml	1333	43	84	5	0.375
DMSO	0.2%	1624	52	100	1	0.062

PFNA

The transformation assay investigating PFNA showed that 500 µM PNFA and 5µg/ml B[a]P were significantly different from the negative control DMSO with $p= 2.2 * 10^{-16}$ and p= 0.0031 respectively (One-sided Fisher exact test). The same calculations performed on the rest of the concentrations did not give any results significantly different from the DMSO control. 500 µM PFNA, 1 µM and 5 µg/ml B[a]P gave MTF over 0.6 % with a MTF of 6.461, 0.637 and 1.085 % respectively (*Table 4)*. The DMSO control gave a MTF below 0.6 % with a MTF of 0.154 %

Table 4. *Cell transformation results after exposure of SHE cells to PFNA. Morphological transformation frequency (MTF in %), Relative plating efficiency (RPE in %) measured after seven days of exposure (**Significant different from DMSO control; P < 0.001). Positive control was B[a]P.*

Test groups		Colonies scored			Transformed colonies	
Chemicals	Concentration	All	PE (%)	RPE (%)	Transformed colonies	MTF (%)
PFNA	10 nM	1305	45	101	2	0.153
	100 nM	1299	44	99	7	0.539
	1 µM	1255	43	97	8	0.637
	10 µM	1285	44	99	1	0.078
	100 µM	1281	44	99	2	0.156
	200 µM	1176	40	90	2	0.170
	400 µM	1148	39	88	1	0.087
	450 µM	1216	38	86	2	0.164
**	500 µM	1362	42	95	88	6.461
	550 µM	855	18	41	4	0.468
B[a]P **	5 µg/ml	1198	41	92	13	1.085
DMSO	0.2 %	1298	44	100	2	0.154

TBBPA

The cell transformation assay testing TBBPA found that the concentrations 175 µM TBBPA, 185 µM TBBPA and 5 µg/ml B[a]P were significantly different from the negative control DMSO with p=8.19 $x10^{12}$, p= 2.2 *10^{-16} and p= 0.004956, respectively (One-sided Fisher exact test). The rest of the concentrations are not significantly different from the DMSO control according to the One-sided Fisher Exact test, and the DMSO control gave MTF below 0.6 % with a MTF of 0.468 (*Table 5).*

Table 5. *Cell transformation results after exposure of SHE cells to TBBPA. Morphological transformation frequency (MTF in %), plating efficiency (PE %) and relative plating efficiency (RPE in %) measured after seven days of exposure (** Significant different from DMSO control; p < 0.001). Positive control was B[a]P.*

Test groups		Colonies scored			Transformed colonies	
Chemicals	Concentration	All	PE (%)	RPE (%)	Transformed colonies	MTF (%)
TBBPA	0.1 nM	1377	46	107	2	0.145
	1 nM	1360	47	109	9	0.662
	10 nM	1351	47	109	4	0.296
	100 nM	1348	46	107	8	0.593
	1 µM	1341	46	106	4	0.298
	10 µM	1358	47	109	8	0.589
	100 µM	1403	45	105	7	0.499
	150 µM	1323	47	110	13	0.983
**	175 µM	956	36	83	47	4.916
**	185 µM	1393	28	66	98	7.035
B[a]P **	5 µg/ml	1215	40	93	20	1.645
DMSO	0.2%	1282	43	100	6	0.468

175 µM TBBPA, 185 µM TBBPA and 5 µg/ml gave a MTF over 0.6 %. The DMSO control gave a MTF below 0.6 %.

3.2 Detection of ROS production

3.2.1 Perfluorinated compounds

In the preliminary studies, it was found that the PFCs did not oxidize the DCFH-DA probe by themselves or auto-fluoresce (results not shown).

None of the three perfluorinated compounds were found to be significantly different from the DMSO control when analyzed with a one-way ANOVA and a Dunnett's post hoc test, whereas the positive control H_2O_2 was found to be significantly different from the DMSO control. Average relative fluorescence values for the different perfluorinated compounds are presented in *Figure 10, 11, and 12*.

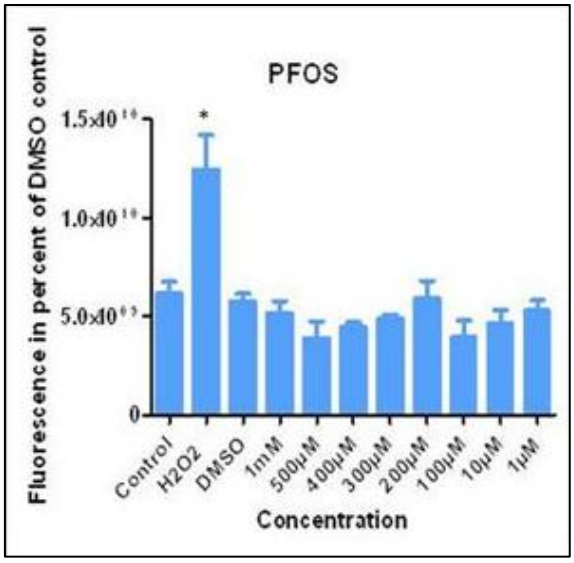

Figure 10: *Relative fluorescence values as a measure for ROS production in SHE cells after exposure to PFOS, in percent of DMSO control. The positive control is H_2O_2. (* Significantly different from DMSO control ($p < 0.001$)).*

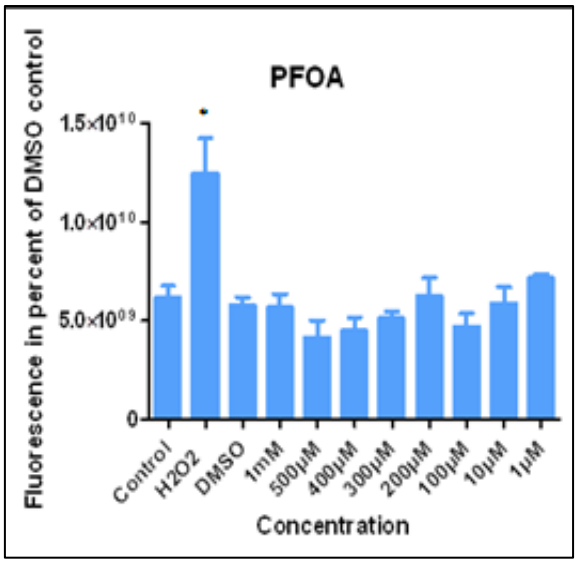

Figure 11: *Relative fluorescence as a measure of ROS production in SHE cells after exposure to PFOA in percent of the DMSO control. H_2O_2 is the positive control. (* Significant different from DMSO control ($p < 0.001$))*

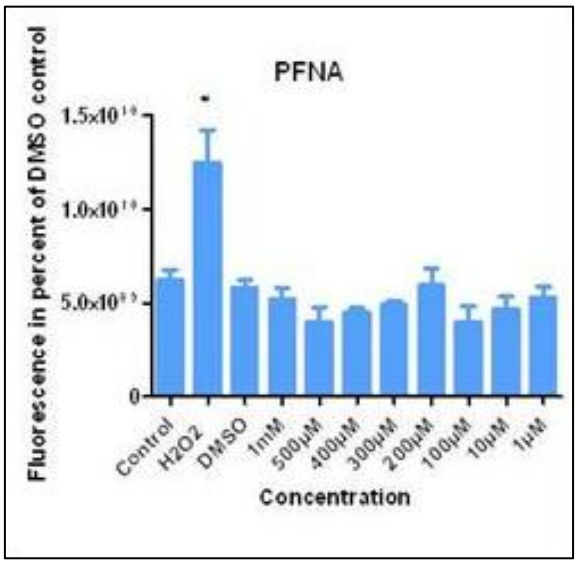

Figure 12. *Relative fluorescence values as a measure for ROS production in SHE cells after exposure to PFNA, in percent of the DMSO control. H_2O_2 is the positive control. (* Significantly different from DMSO control ($p < 0.001$))*

3.2.2 TBBPA

In the preliminary studies, when testing if TBBPA would auto-fluoresce or oxidize the probe by itself, it was found that TBBPA oxidized the probe. It was therefore not possible to detect increases in ROS production caused by TBBPA exposure using the DCF assay. The relative fluorescence for TBBPA with and without cells is presented in *Figure 13*.

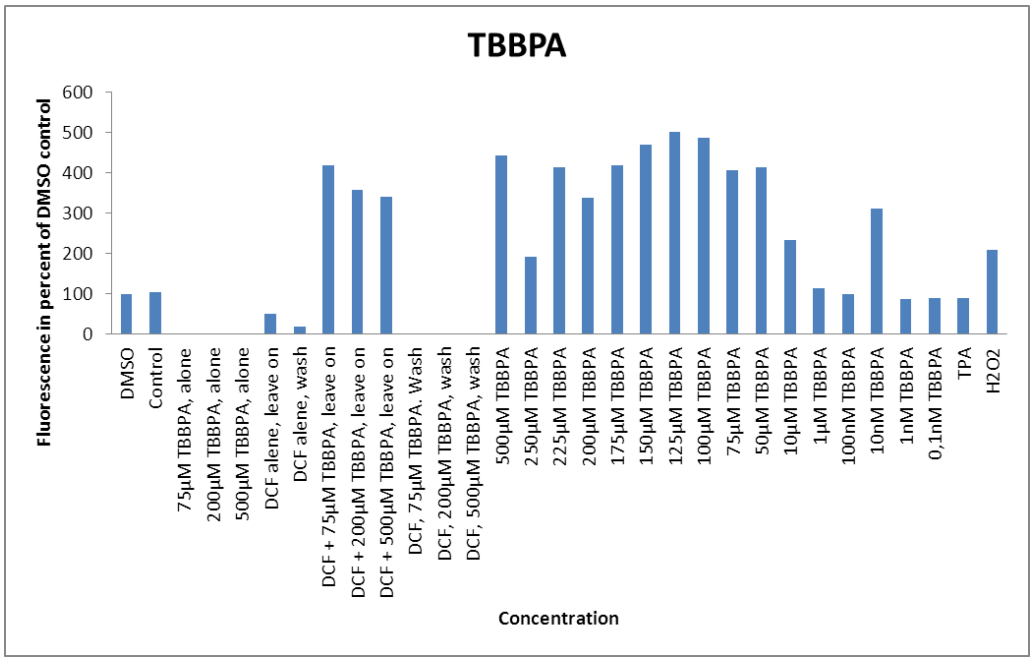

Figure 13: *Relative fluorescence as a measure of ROS production in SHE cells after exposure to TBBPA, in percent of the negative DMSO control, testing TBBPA for auto-fluorescence and if the toxicant would oxidize the probe DCFH-DA.* H_2O_2 *and TPA are positive controls.*

The concentrations of TBBPA tested with the probe DCFH-DA, without cells, that were left in the well during the reading gave values around 300 and 400 in percent of the negative control DMSO. It was about the same values as for the same concentration with cells.

4. Discussion

4.1 Syrian hamster embryo cell transformation assay

About 40 % of the chemicals which have been found to induce tumors in long-term animal experiments are devoid of gene mutation activity (Rivedal & Haddeland 1996; Tennant & Ashby 1991). It is therefore necessary to have methods detecting carcinogens which are not genotoxic in addition to the ones that are genotoxic. The SHE CTA is one of few *in vitro* assays which detect both genotoxic and non-genotoxic carcinogens (Adler et al. 2011).

4.1.1 Method development

There are a lot of factors influencing the capacity of SHE cells to transform. Different SHE cell isolates have different variability in sensitivity to morphological transformation. FBSs have different ability to support cell growth and transformation. In addition the feeder cells differ in their ability to support growth of target cells (Kerckaert et al. 1996). In the present study it was experienced that a change of feeder cell batch reduced PE in the PFNA and the TBBPA experiments as compared to the batch used in the PFOS experiments, it is also possible that it affected the transformation rate in the PFOS experiments.

The SHE cell isolates are from different hamsters and have different variability in sensitivity to morphological transformation. The FBS can contain different amounts of factors affecting the SHE cells growth and transformation. Three different FBS-lots were therefore tested and batch # 8180923 from Invitrogen was chosen as it gave the highest PE and the densest cell colonies. Another batch from Invitrogen was tested later on, but it did not give better results than the first batch, and it was decided not to change. Different percentages of the chosen FBS were also tested, 20 % FBS gave the best result in this experiment (*Table 2*).

Rivedal and Haddeland (1996) tested the role of serum in the SHE CTA, trying to characterize and partially purify protein factors in FBS. They found that one of the factors affecting the FBSs ability to support transformation is the amount of fibronectin in the medium (Rivedal & Haddeland 1996).The disorganized growth pattern of transformed cells has been shown to be restored after exposure to fibronectin (Rivedal 1982). To optimize the protocol in the present study it was decided to change medium on day nine to remove fibronectin, giving the cells a day with less inhibition. This was shown to be effective, giving a higher rate of transformation (*Table 1*).

On the background of preliminary tests it was chosen to add insulin to the medium to get thicker and more compact cell colonies. Insulin is an important growth factor, and it is thought to promote tumor development (Argiles & Lopez-Soriano 2001; Boyd 2003). The addition of insulin gave denser colonies, in addition to an increase in morphological transformation (*Table 2*).

Together these adjustments on FBS, insulin and medium change optimized the assay, giving the wanted type of colonies and a higher frequency of morphological transformation, than without the adaptions.

4.1.2 Cytotoxicity/Dose-range finding test

Based on the present study TBBPA reduces RPE to 50 % on lower concentrations than the perfluorinated compounds; 185 µM getting closest to 50 % *(Figure 9)*. PFOS halved the RPE with the lowest concentration of the perfluorinated compounds in these experiments; around 250 µM *(Figure 7)*. PFNA needed the highest concentration to get a 50 % reduction in RPE; around 550 µM *(Figure 8)*. It was not performed any cytotoxicity test on PFOA, since it was not tested in the SHE CTA in the current study. PFOA usually needs a higher concentration than PFOS to get a 50 % reduction in PE, such as in the experiments done by Florentine et al. (2011). In the SHE CTA experiments executed by Jacquet et al. (2012), the 50 % reduction in RPE was achieved between 300 µM and 370 µM.

In the transformation experiment at least one high concentration should be included, giving approximately 50 % reduction in RPE. The concentrations tested in the experiments were based on the DRF test. Several experiments were required to find a concentration giving a 50 % reduction in RPE after exposure to TBBPA. The cytotoxicity changed slightly between the experiments, and a few extra concentrations were tested in later experiments to get closer to a 50 % reduction in RPE, giving a less accurate number than if all the concentrations were tested in one experiment. In addition it is a small range were TBBPA changed from not reducing RPE to killing almost all the colonies.

Even though concentrations lower than the one not giving an effect on RPE was added to get closer to real life exposure concentrations, these concentrations are higher than the concentrations found in human serum. It is however important to pay attention to the uncertainties related to the extrapolation of effective doses *in vitro* to effective concentration in humans. Real life exposures in cell models are not really real life exposures, even though

the concentrations are the same. There are factors in the body *in vitro* studies cannot imitate. In addition the tests performed in this study only exposed for seven days, while in real life we are exposed to these toxicants on an everyday basis our entire life.

Although the SHE CTA is an *in vitro* method and the results cannot be directly extrapolated to *in vivo* studies, it can however give an indication of which chemicals should be further investigated. As earlier mentioned the SHE CTA corresponds 85 % to a two year rodent assay (Kamendulis et al. 2002).

4.1.3 Transformation assay

Accurate scoring of the colonies can be difficult and it is critical that this is executed correctly. It could be a certain degree of subjectivity in the scoring of each colony, and it is therefore vital that two people perform the scoring. It is also important that those doing the scoring go through some training, and use the photo catalogue as a guide. The scoring must be done blindly; the dishes are therefore coded before the scoring begins. Both within-laboratory and between laboratory reproducibility were assessed in the, European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM), pre-validation study (Vanparys et al. 2011). The study demonstrated that within-laboratory and between laboratories reproducibility were satisfactory for the SHE CTA. It was also considered to be satisfactory transferable between laboratories (Corvi et al. 2012).

PFOS

Exposure to PFOS did not give a statistically significant transformation rate in SHE cells in the current experiment. The positive control B[a]P did not get a MTF above 0.6 % and was therefore not significantly different from the DMSO control. The test is therefore not valid (*Table 3).* It is not possible to decide if PFOS causes transformation in SHE cells based on the present experiment.

Jacquet et al. (2012) have tested the carcinogenic potency of PFOS using the SHE CTA. They found that it caused transformation in cells when using the concentrations 3.7 and 0.37 µM giving a MTF of respectively 1.10 and 0.97 %.

PFOS is the perfluorinated compound found in highest concentration in the Norwegian population. It has been detected a concentration of 32 ng/ml in serum (Haug et al. 2010). In 2000-2001 concentrations between 800-950 µM PFOS were found in serum from

occupationally exposed workers in the USA. This is a very high number. Luckily the concentration has been reduced since 1995, and is hopefully still decreasing (Fromme et al. 2009). This shows that banning the production of a compound helps reduce its amount in the environment even though it is persistent.

Biological factors are important in this assay. The adaptions made to the protocol in the present study were carried out to make the assay as sensitive as possible. Still there were no significant results observed in the SHE cells after exposure to PFOS or B[a]P in the three studies performed. A difference in the feeder cell batch is a possible reason why this experiment did not get valid results, while the other two got high enough MTFs after B[a]P exposure. The change in feeder cell batch affected the PE, and it is possible that the second feeder cell batch in addition made the assay more sensitive to transformation.

PFNA

Exposure to PFNA led to a statistically significantly transformation rate in SHE cells for one of the concentrations. There have to be a significant difference from DMSO in two different concentrations for the assay to be considered positive, and these results are therefore inconclusive.

The one-sided Fisher exact test confirmed that 500 µM PFNA was significantly different from the negative control DMSO. The positive control B[a]P also got statistically significant values compared to the DMSO control. Exposure to 1 µM PFNA gave a MTF over 0.6 % (*Table 4)*, but the p-value was 0.06, above the limit of $p < 0.05$. All the dishes with 500 µM PFNA was exposed in the same experiment. This makes the results slightly uncertain, even though the rest of the results from that experiment do not differ much from the two other experiments.

Since only one concentration of PFNA was significantly different from the DMSO control a Cochran-Armitage trend test had to be performed. It did not detect a positive trend in the results after exposure of SHE cells to PFNA, and it is not possible to say if PFNA transform SHE cells.

500 µM PFNA gave 88 transformed colonies (*Table 4)*, while 550 µM gave only four. 550 µM was the highest concentration tested (*Figure 8*), and it is possible that a threshold value was reached. 550 µM PFNA gave thin colonies which were difficult to score. Some may have been excluded because of that. In addition too few colonies were scored at 550 µM (*Figure*

8). It is possible that this is because this concentration was too cytotoxic, and that the adjustment number of cells to be seeded was calculated too low.

There should therefore be performed another experiment on PNFA to clarify the original response. It is also possible to execute an experiment testing if PFNA act as a tumor promoter, like Jacquet et al. (2012) did with PFOA.

PFNA is the least investigated compound among the perfluorinated compounds used in this study. It is also the perfluorinated compound found at the lowest concentration in human serum in Norway, with a concentration of 1.1 ng/ml (Haug et al. 2010). People working with ski waxing are more exposed to PFNA than the rest of the population. A study testing a few professional ski waxers after the ski-season found that the median of the concentrations in the exposed group was 13 ng/ml (Freberg et al. 2010). This is more than ten times higher than the average concentration found in human serum in Norway.

PFNA is the perfluorinated compound, which increases the most in production among the PFCs investigated in the current study, since there already are restrictions on the use of the other two (Lau et al. 2007). Klif (Klima- og forurensningsdirektoratet) wants to increase the knowledge of PNFA and potential health risks associated with it. They have requested that more tests are executed to detect possible health effects, in case something should be done to reduce the amount of PFNA produced (Stefan et al. 2009). The results in this study shows that further studies should be performed to clarify the question about its carcinogenic potential.

The concentrations tested in present thesis were higher than what found in occupationally exposed workers, and the concentration giving significantly results is one of the highest tested. It is still within the limits of the test, which are set to 5 mg/ml or 10 mM, whichever is the lowest.

TBBPA

Exposure to TBBPA led to a statistically significant transformation frequency of the SHE cells in these experiments. TBBPA has not earlier been shown to be mutagenic (Darnerud 2003). The current experiment gives an indication that the carcinogenic potential of TBBPA should be further investigated.

The one-sided Fisher Exact test confirmed that both 175 and 185 µM TBBPA gave statistically significant results. The B[a]P was also significantly different from the DMSO

control. 150 µM TBBPA and 1 nM TBBPA had a MTF over 0.6 %, but they were not significantly different from the DMSO control, and are therefore not considered transformed. The rest of the concentrations gave a MTF lower than 0.6 %, had a high p-value, and were therefore not significantly different from the DMSO control. The transformation ratio was dose dependent, and the transformation frequency was only significant for the highest concentrations, which were cytotoxic (*Table 5, Figure 9*).

Since two concentrations were significantly different from the DMSO control and had a MTF over 0.6 %, in addition to the B[a]P giving a MTF above 0.6 %, this test was positive, and it is possible to say that TBBPA causes transformation in SHE cells in this study. Even though the concentrations giving transformation are high, they are much lower than the limit for this test, which as earlier mentioned, lies on 10 mM or 5 mg/ml, whichever is the lowest.

TBBPA is one of the most used BFRs. In a dismantling area for recycling of electronics, a concentration of 30 ng/m^3 TBBPA was found in the indoor air. Studies have also found presence of TBBPA in serum from workers in electronic recycling (Sjodin et al. 2003). Even though TBBPA has a half-life of only 2 days, the exposure may for some workers be constant if they inhale the contaminated air every day. The concentrations giving transformation in this assay was much higher than the concentrations these workers could get in their blood. But they are exposed for a much longer time than seven days.

4.2 Detection of ROS production

In the present study it was not found a significant increase in ROS production after exposure to PFOS, PFOA or PNFA (*Figure 10, 11 and 12)*. A previous study has shown that PFOS can increase ROS production by 1.25 fold and PFOA by 1.52 fold compared to unexposed cells, in a HepG2 cell line (Eriksen et al. 2010), while a study conducted by Florentin et al. (2011) found exposure by PFOS and PFOA to decrease ROS production in the same cell line. Both used the DCF assay to detect the ROS production. Eriksen et al. (2010) detected that PFNA exposure increased ROS production insignificantly, except after exposure to concentrations of 1 and 2 mM where the ROS production decreased. The absence of increased ROS production in these experiments makes it likely that it can be assumed that increased production of ROS is not the mechanism behind these perfluorinated compounds possible carcinogenic potential.

This support the assumption by Jacquet et al. (2012) saying ROS production is probably not the reason why PFOA causes transformation.

ROS production after exposure to TBBPA was not possible to measure with the method used in this study. TBBPA oxidized the probe by itself, even when there were no cells in the well. An increase between three and four fold was shown when TBBPA and DCFH-DA was left together in the well during the reading. Some of the concentrations of TBBPA with cells gave between four and five fold increases in ROS production in this experiment (*Figure 13*). Based on this experiment it cannot be said if ROS production is one of the mechanisms behind TBBPAs carcinogenic potential or not. TBBPA was found to give a concentration dependent increase in ROS production in cerebellar granule cells and in human neutrophil granulocytes (Reistad et al. 2005; Reistad et al. 2007). Reistad et al. (2005) also used the DCF assay, but the article does not say if they tested the compound for auto-fluoresce or to see if it oxidized the probe by itself.

It is possible that TBBPA increases ROS production, but it cannot be investigated using this assay. Perhaps an assay where the reduction of antioxidants is investigated could be used as an alternative method to examine TBBPAs potential to increase ROS production.

Even though I did not get any positive results from these experiments the positive control H_2O_2 showed that the assay worked, being the only exposure giving significant results.

One uncertainty to the accuracy of this assay is the constancy of the amount of cells in the wells. It was chosen to seed enough cells to get it confluent after 24 hours getting as close to similar conditions in each well at each experiment as possible. Unequal amount of cells can give a difference in fluorescence as a measure for ROS production, if fewer cells produce ROS.

In these experiments cells are exposed to single compounds. In real life humans are exposed to a mix of compounds, which may have both synergetic and antagonistic effects. It is therefore not possible to know how these compounds affect the body in real life based on these tests. It can however give an indication on what is interesting to investigate further.

4.3 Conclusion

This study was carried out to investigate the carcinogenic potential to PFOS, PFNA and TBBPA using the Syrian hamster embryo cell transformation assay. The results presented in this study show that the PFOS test did not give valid results because the morphological transformation after exposure to PFOS was too low. It is therefore not possible to say anything about the carcinogenic potential of PFOS. The results gave an indication of the carcinogenic potential of PNFA, but the results were inconclusive, and it should be performed a follow up experiment to clarify the answer presented in this study. TBBPA was shown to cause transformation in SHE cells with two concentrations significantly different from the DMSO control.

One of the secondary objectives was to investigate the cytotoxicity of the same three compounds. The results showed that TBBPA needed the lowest concentration to reduce the relative plating efficiency to 50 %, with a concentration of 185 µM getting the closest. 250 µM PFOS was required to do the same and 550 µM of PFNA. TBBPA had a narrow range were the cytotoxicity affected the PE, until that point was reached the size and density of the colonies were the only endpoints affected.

The last thing investigated in this thesis was the ability of PFOS, PFNA, PFOA and TBBPA to increase production of ROS in SHE cells. This was tested using the DCF assay. Based on the results presented in this study none of the perfluorinated compounds increased production of ROS in SHE cells. It was not possible to investigate if TBBPA increased ROS production because it oxidized the probe, and that made it impossible to know if the increase was because of ROS in the cells or if only the TBBPA oxidized the probe.

4.4 Future perspectives

Considering that PFNA did not give a clear answer it should be performed a follow up experiment to clarify the answer found in this study. It would also be interesting to test if it acts as a tumor promoter in a two stage transformation assay like PFOA did.

Further, non-genotoxic mechanisms could be investigated, since none of the compounds investigated in present study have proven to be genotoxic. Increase in ROS production is probably not a part of the mechanisms of the carcinogenic potential of the perfluorinated

compounds. It can however not be ruled out as a part of the mechanism when it comes to TBBPA, which oxidized the probe, like the positive control H_2O_2.

One possible mechanism that could be investigated is disturbance of gap junctions. When looking at the transformed colonies it is obvious that they grow in criss-cross and stacks. Normal cells would not grow like that and it is possible they do not “communicate” right with each other. Inhibition of gap junction can be a mechanism in cancer.

5. References

Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., Andersen, K. E., Angers-Loustau, A., Aptula, A., Bal-Price, A., et al. (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Arch Toxicol*, 85 (5): 367-485.

Alaee, M., Arias, P., Sjodin, A. & Bergman, A. (2003). An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. *Environ Int*, 29 (6): 683-689.

Alfadda, A. A. & Sallam, R. M. (2012). Reactive oxygen species in health and disease. *J Biomed Biotechnol*, 2012: 936486.

Argiles, J. M. & Lopez-Soriano, F. J. (2001). Insulin and cancer (Review). *Int J Oncol*, 18 (4): 683-687.

Boyd, D. B. (2003). Insulin and cancer. *Integr Cancer Ther*, 2 (4): 315-329.

Brieger, K., Schiavone, S., Miller, F. J., Jr. & Krause, K. H. (2012). Reactive oxygen species: from health to disease. *Swiss Medical Weekly*, 142: w13659.

Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., de Voogt, P., Jensen, A. A., Kannan, K., Mabury, S. A. & van Leeuwen, S. P. (2011). Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. *Integr Environ Assess Manag*, 7 (4): 513-541.

Calafat, A. M., Wong, L. Y., Kuklenyik, Z., Reidy, J. A. & Needham, L. L. (2007). Polyfluoroalkyl chemicals in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 2003-2004 and comparisons with NHANES 1999-2000. *Environ Health Perspect*, 115 (11): 1596-1602.

Corvi, R., Aardema, M. J., Gribaldo, L., Hayashi, M., Hoffmann, S., Schechtman, L. & Vanparys, P. (2012). ECVAM prevalidation study on in vitro cell transformation assays: general outline and conclusions of the study. *Mutat Res*, 744 (1): 12-9.

Custer, L., Gibson, D. P., Aardema, M. J. & LeBoeuf, R. A. (2000a). A refined protocol for conducting the low pH 6.7 Syrian hamster embryo (SHE) cell transformation assay. *Mutat Res*, 455 (1-2): 129-39.

Custer, L., Gibson, D. P., Aardema, M. J. & LeBoeuf, R. A. (2000b). A refined protocol for conducting the low pH 6.7 Syrian hamster embryo (SHE) cell transformation assay. *Mutat Res*, 455 (1-2): 129-139.

D'Hollander, W., de Voogt, P., De Coen, W. & Bervoets, L. (2010). Perfluorinated substances in human food and other sources of human exposure. *Rev Environ Contam Toxicol*, 208: 179-215.

Darnerud, P. O. (2003). Toxic effects of brominated flame retardants in man and in wildlife. *Environ Int*, 29 (6): 841-53.

de Wit, C. A. (2002). An overview of brominated flame retardants in the environment. *Chemosphere*, 46 (5): 583-624.

El-Shahawi, M. S., Hamza, A., Bashammakh, A. S. & Al-Saggaf, W. T. (2010). An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. *Talanta*, 80 (5): 1587-97.

Eriksen, K. T., Raaschou-Nielsen, O., Sorensen, M., Roursgaard, M., Loft, S. & Moller, P. (2010). Genotoxic potential of the perfluorinated chemicals PFOA, PFOS, PFBS, PFNA and PFHxA in human HepG2 cells. *Mutat Res*, 700 (1-2): 39-43.

Florentin, A., Deblonde, T., Diguio, N., Hautemaniere, A. & Hartemann, P. (2011). Impacts of two perfluorinated compounds (PFOS and PFOA) on human hepatoma cells: cytotoxicity but no genotoxicity? *Int J Hyg Environ Health*, 214 (6): 493-499.

Freberg, B. I., Haug, L. S., Olsen, R., Daae, H. L., Hersson, M., Thomsen, C., Thorud, S., Becher, G., Molander, P. & Ellingsen, D. G. (2010). Occupational exposure to airborne perfluorinated compounds during professional ski waxing. *Environ Sci Technol*, 44 (19): 7723-8.

Fromme, H., Tittlemier, S. A., Volkel, W., Wilhelm, M. & Twardella, D. (2009). Perfluorinated compounds--exposure assessment for the general population in Western countries. *Int J Hyg Environ Health*, 212 (3): 239-70.

Hardell, E., Carlberg, M., Nordstrom, M. & van Bavel, B. (2010). Time trends of persistent organic pollutants in Sweden during 1993-2007 and relation to age, gender, body mass index, breast-feeding and parity. *Sci Total Environ*, 408 (20): 4412-9.

Haug, L. S., Thomsen, C., Brantsaeter, A. L., Kvalem, H. E., Haugen, M., Becher, G., Alexander, J., Meltzer, H. M. & Knutsen, H. K. (2010). Diet and particularly seafood are major sources of perfluorinated compounds in humans. *Environ Int*, 36 (7): 772-8.

Jacquet, N., Maire, M. A., Landkocz, Y. & Vasseur, P. (2012a). Carcinogenic potency of perfluorooctane sulfonate (PFOS) on Syrian hamster embryo (SHE) cells. *Arch Toxicol*, 86 (2): 305-14.

Jacquet, N., Maire, M. A., Rast, C., Bonnard, M. & Vasseur, P. (2012b). Perfluorooctanoic acid (PFOA) acts as a tumor promoter on Syrian hamster embryo (SHE) cells. *Environmental Science and Pollution Research*, 19 (7): 2537-2549.

Kamendulis, L. M., Zhang, H. Z., Wang, Y. H. & Klaunig, J. E. (2002). Morphological transformation and oxidative stress induced by cyanide in Syrian hamster embryo (SHE) cells. *Toxicol Sci*, 68 (2): 437-443.

Kerckaert, G. A., Isfort, R. J., Carr, G. J., Aardema, M. J. & LeBoeuf, R. A. (1996). A comprehensive protocol for conducting the Syrian hamster embryo cell transformation assay at pH 6.70. *Mutat Res*, 356 (1): 65-84.

Kraugerud, M., Zimmer, K. E., Ropstad, E. & Verhaegen, S. (2011). Perfluorinated compounds differentially affect steroidogenesis and viability in the human adrenocortical carcinoma (H295R) in vitro cell assay. *Toxicol Lett*, 205 (1): 62-8.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A. & Seed, J. (2007). Perfluoroalkyl acids: a review of monitoring and toxicological findings. *Toxicol Sci*, 99 (2): 366-94.

LeBel, C., Ischiropoulos, H. & Bondy, S. (1992). Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol*: 227-231.

LeBoeuf, R. A., Kerckaert, G. A., Aardema, M. J., Gibson, D. P., Brauninger, R. & Isfort, R. J. (1996). The pH 6.7 Syrian hamster embryo cell transformation assay for assessing the carcinogenic potential of chemicals. *Mutat Res*, 356 (1): 85-127.

Liu, C., Yu, K., Shi, X., Wang, J., Lam, P. K., Wu, R. S. & Zhou, B. (2007). Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (Oreochromis niloticus). *Aquat Toxicol*, 82 (2): 135-43.

Lloyd, M. & Kidd, D. (2012). The mouse lymphoma assay. *Methods Mol Biol*, 817: 35-54.

Maire, M. A., Pant, K., Phrakonkham, P., Poth, A., Schwind, K. R., Rast, C., Bruce, S. W., Sly, J. E., Bohnenberger, S., Kunkelmann, T., et al. (2012a). Recommended protocol for the Syrian hamster embryo (SHE) cell transformation assay. *Mutat Res*, 744 (1): 76-81.

Maire, M. A., Rast, C. & Vasseur, P. (2012b). Photo catalogue for the classification of cell colonies in the Syrian hamster embryo (SHE) cell transformation assay at pH 7.0. *Mutat Res*, 744 (1): 97-110.

Melvin E. Andersen, John L. Butenhoff, Chang, S.-C., Farrar, D. G., Gerald L. Kennedy, J., Christopher Lau, Geary W. Olsen, Jennifer Seed, k. & Wallacekj, K. B. (2007). Perfluoroalkyl Acids and Related Chemistries—Toxicokinetics and Modes of Action. *Toxicol Sci*: 3-14.

Mortelmans, K. & Zeiger, E. (2000). The Ames Salmonella/microsome mutagenicity assay. *Mutat Res*, 455 (1-2): 29-60.

Motulsky, H. (2007). *GraphPad Prism 5.0 Statistics guide*: San Diego, GraphPad Software.

Myhre, O., Andersen, J. M., Aarnes, H. & Fonnum, F. (2003). Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem Pharmacol*, 65 (10): 1575-1582.

Myhre, O., Mariussen, E., Reistad, T., Voie, O. A., Aarnes, H. & Fonnum, F. (2009). Effects of polychlorinated biphenyls on the neutrophil NADPH oxidase system. *Toxicol Lett*, 187 (3): 144-8.

Nelson, J. W., Hatch, E. E. & Webster, T. F. (2010). Exposure to polyfluoroalkyl chemicals and cholesterol, body weight, and insulin resistance in the general U.S. population. *Environ Health Perspect*, 118 (2): 197-202.

Ohmori, K., Kudo, N., Katayama, K. & Kawashima, Y. (2003). Comparison of the toxicokinetics between perfluorocarboxylic acids with different carbon chain length. *Toxicology*, 184 (2-3): 135-140.

Olsen, G. W., Burris, J. M., Ehresman, D. J., Froehlich, J. W., Seacat, A. M., Butenhoff, J. L. & Zobel, L. R. (2007). Half-life of serum elimination of perfluorooctanesulfonate,perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. *Environ Health Perspect*, 115 (9): 1298-305.

Reistad, T., Mariussen, E. & Fonnum, F. (2005). The effect of a brominated flame retardant, tetrabromobisphenol-A, on free radical formation in human neutrophil granulocytes: the involvement of the MAP kinase pathway and protein kinase C. *Toxicol Sci*, 83 (1): 89-100.

Reistad, T., Mariussen, E., Ring, A. & Fonnum, F. (2007). In vitro toxicity of tetrabromobisphenol-A on cerebellar granule cells: cell death, free radical formation, calcium influx and extracellular glutamate. *Toxicol Sci*, 96 (2): 268-78.

Rivedal, E. (1982). Role of plasma fibronectin in the morphological transformation of hamster embryo cells. *Cancer Lett*, 17 (1): 9-17.

Rivedal, E. & Haddeland, U. (1996). Role of serum in the morphological transformation of Syrian hamster embryo cells: Characterization and partial purification of protein factors in foetal bovine serum. *Toxicol In Vitro*, 10 (2): 217-27.

Sanmartin-Suarez, C., Soto-Otero, R., Sanchez-Sellero, I. & Mendez-Alvarez, E. (2011). Antioxidant properties of dimethyl sulfoxide and its viability as a solvent in the evaluation of neuroprotective antioxidants. *J Pharmacol Toxicol Methods*, 63 (2): 209-15.

Sjodin, A., Patterson, D. G., Jr. & Bergman, A. (2003). A review on human exposure to brominated flame retardants--particularly polybrominated diphenyl ethers. *Environ Int*, 29 (6): 829-39.

Stefan, P., Sandra, R. & Elisabeth, O. (2009). *Survey of the extent of use and occurrence of PFNA (perfluorononanoic acid) in Norway*. Klif.no. Available at: http://www.klif.no/publikasjoner/2562/ta2562.pdf.

Tennant, R. & Ashby, J. (1991). Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. *Mutat Res*, 257 (3): 209-227.

Tsutsui, T., Tamura, Y., Yagi, E., Hasegawa, K., Takahashi, M., Maizumi, N., Yamaguchi, F. & Barrett, J. C. (1998). Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells. *Int J Cancer*, 75 (2): 290-4.

UN-ECE. *UN-ECE 2010. The 1998 Protocol on Persistent Organic Pollutants, Including the Amendments Adopted by the Parties on 18 December 2009*: United Nations Economic and Social Council Economic comission for Europe. Available at: http://www.unece.org/fileadmin/DAM/env/lrtap/full%20text/ece.eb.air.104.e.pdf:.

UNEP. *Stockholm Convention, about the convention*. Available at: http://chm.pops.int/Convention/tabid/54/Default.aspx.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact*, 160 (1): 1-40.

Vallack, H. W., Bakker, D. J., Brandt, I., Brostrom-Lunden, E., Brouwer, A., Bull, K. R., Gough, C., Guardans, R., Holoubek, I., Jansson, B., et al. (1998). Controlling persistent organic pollutants-what next? *Environ Toxicol Pharmacol*, 6 (3): 143-75.

Vanparys, P., Corvi, R., Aardema, M., Gribaldo, L., Hayashi, M., Hoffmann, S. & Schechtman, L. (2011). ECVAM prevalidation of three cell transformation assays. *ALTEX*, 28 (1): 56-9.

Vasseur, P. & Lasne, C. (2012). OECD Detailed Review Paper (DRP) number 31 on "Cell Transformation Assays for Detection of Chemical Carcinogens": main results and conclusions. *Mutat Res*, 744 (1): 8-11.

Wang, H. & Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader11Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable. *Free Radical Biology and Medicine*, 27 (5-6): 612-616.

Wania, F. & Mackay, D. (1999). The evolution of mass balance models of persistent organic pollutant fate in the environment. *Environ Pollut*, 100 (1-3): 223-40.

Watanabe, I. & Sakai, S. (2003). Environmental release and behavior of brominated flame retardants. *Environ Int*, 29 (6): 665-82.

Xiao-Zhong, H. & De-Cong, H. (2009). Effects of perfluorooctanoate and perfluorootane sulfonate exposure on hepatoma Hep G2 cells. *Toxicogenomics*.

Yong, Q., Alan, D., Rebecca, W., Steve, L., Valerie, B., Nancy, G. L., Xianglin, S., Val, V. & Vincent, C. (2010). Perfluorooctane sulfonate (PFOS) induces reactive oxygen species (ROS) production in human microvascular endothelial cells: role in endothelial permeability. *J Toxicol Environ Health*, 73.