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Induction of CYP 1A enzyme activity and genotoxicity from ternary mixtures of produced water relvant compounds, evaluated by in vitro methods

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ABSTRACT

Produced water is a complex mixture discharged to sea in high volumes containing compounds at low concentrations. Compounds in mixtures can modify each other's expected toxic effect predicted from single exposure, and to obtain information about potential interactions is important. Carbazole is present in produced water and is suggested to contribute to produced waters potential of modifying cytochrome P450 (CYP) activity. Information on toxic effect of carbazole in relation to produced water is limited. Carbazole is included in this project to study its potential for modifying CYP 1A activity singly and in mixtures. The aromatic and phenolic fractions of produced water are significant contributors to toxicity and concentration of organic compounds in produced water. As representative compounds of these fractions, benzo(a)pyrene (BaP) and 2,5-dimethylphenol (DMP) are included in this master project.

Biotransformation of harmful compounds is often initiated by CYPs catalysing oxidation reactions. Modified CYP 1A protein or activity is an indication of interaction between a compound and the biological system. Biotransformation catalysed by CYP 1A can produce reactive oxygen species (ROS) and reactive metabolites. Increased contaminant load can deplete reduced glutathione (GSH) through increase in conjugation reactions. In addition to being an important conjugate, GSH is also an important antioxidant. With potential both to increase extent of DNA damage by increasing levels of ROS and producing reactive metabolites, and interfere with glutathione dependent defence protecting against oxidative stress, compounds interacting with CYP 1A and conjugation enzymes are suggested to contribute to DNA damage.

The aims of this master project were to determine carbazoles potential for modifying CYP 1A activity measured as ethoxyresorufin-*O*-deethylase (EROD) activity, to evaluate interaction in ternary mixtures with carbazole and two compounds representing fractions contributing to toxicity of produced water, and also to study correlation between biotransformation activity and genotoxicity by measuring DNA double strand breaks (DSB).

The aims were achieved by studying single compounds and ternary mixture *in vitro* in the continuous cell line PLHC-1. Concentrations of the three compounds in the ternary mixtures were varied using a statistical design. Results were analysed using partial least squares projection to latent structures (PLS). Concentrations of the compounds included in the design were determined from cytotoxicity results, EROD concentration effect curves for single compounds and concentrations measured in the marine environment.

It was hypothesized that compounds will modify EROD activity in PLHC-1 differently when present in ternary mixtures compared to single exposure. It was further suggested that ternary mixtures inducing high EROD activity also will enhance formation of DNA DSB in PLHC-1 cells. Carbazole was suggested to modify EROD activity induced by the other compounds.

Exposure of PLHC-1 to BaP singly significant induced EROD activity. Carbazole induced EROD activity slightly, and significant for the highest concentration in one replicate. An overall non-significant decrease in EROD activity was seen in PLHC-1 exposed to DMP. Exposure of PLHC-1 to ternary mixtures resulted in significant and positive PLS regression coefficient for both BaP and carbazole. The crossed term carbazole×DMP decreased EROD activity significantly. Squared terms for all three compounds were significant, equal and negative.

The results indicate that potential of carbazole at inducting CYP 1A is different alone compared to when it is present in mixture. Carbazole is suggested to contribute to EROD inducing potency in the ternary mixtures. Effect of carbazole on EROD activity is indicated to be dependent on co-exposed compounds. How EROD activity is modulated when exposed to DMP is suggested to be dependent on exposure conditions. The significant substantial square terms indicate that all three compounds interact with catalytic EROD activity at higher concentrations.

Catalytic EROD activity is presumed to be a good indication of potential interaction between compounds and biological systems. Determining extent of DNA damage by electrophoretic separation of DNA did not give consistent result. Electrophoretic separation of DNA from PLHC-1 is assumed to be more pertinent for determining genotoxicity of certain metals in PLHC-1. Statistical design and projection techniques are considered valuable tools when assessing toxicity of mixtures.

OPPSUMMERING

Produsert vann er en kompleks blanding som inneholder lave konsentrasjoner av mange forbindelser. Dette vandige avfallet fra oljeproduksjon slippes ut i det marine miljø i høye volum. Når forbindelser foreligger i blanding kan de ha en annen toksisk effekt enn de har alene. Det er derfor viktig å ha kunnskap om potentielle interaksjoner. Carbazol er tilstede i produsert vann og er foreslått som en av forbindelsene i produsert vann som har potensial for å endre cytokrom P450 (CYP) 1A aktivitet. Informasjon om toksiske effekter av carbazol i forbindelse med ekspoenering for produsert vann er begrenset. Carbazol er inkludert i denne masteroppgaven for å studere dens potensial for å modifisere CYP 1A aktivitet alene og som komponent i blanding. Fenol og aromatisk fraksjon er viktige bidragsytere til toksisitet. For å representere disse fraksjonen er benso(a)pyren (BaP) og 2,5-dimetylfenol (DMP) inkludert i studiet. Fenoler med kort hydrokarbon kjede er mest utbredt i produsert vann.

Biotransformasjon av skadelige forbindelser begynner ofte med en oksidativ reaksjon katalysert av CYP enzymer. Modifisert CYP 1A protein eller aktivitet er en indikasjon på interaksjon mellom forbindelser og et biologisk system. Biotransformasjon katalysert av CYP 1A kan produsere reaktive oksygen forbindelser (ROS) og reaktive metabolitter. Økt belastning for forurensende stoffer kan utarme redusert glutathion (GSH) gjennom økt bruk i konjugerinsreaksjoner. I tillegg til å være vikig i konjugeringsreaksjoner er GSH også en viktig antioksidant. Med potensial for å øke mengde skade på DNA ved økt oksidativt stress og å resultere i reaktive metabolitter, og ved samtidig å ha potensial til å forstyrre cellulært forsvar mot oksidativt stress kan forbindelser som interagerer med CYP 1A og konjugeringsreaksjoner bidra til økt forekomst av DNA skade.

Hensikten med dette masterprosjektet var å evaluere carbazols potensial for å påvirke CYP 1A aktivitet målt som ethoxyresorufin-*O*-deethylase (EROD) aktivitet, å evaluere interaksjon i tertiære blandinger med carbazol og to forbindelser som representerer fraksjoner som bidrar til toksisitet av produsert vann, og også å se på korrelasjon mellom biotransformasjon og gentoksistet målt som DNA-dobbelttrådbrudd (DNA DSB).

Dette ble gjort ved å studere forbindelser alene og i tertiære blaninger *in vitro* i den kontinuerlige cellelinja PLHC-1. Et statitisk forsøksdesign ble brukt for å bestemme konsentrasjonsmatrisen for de tertiære forbindelsene. Resultatene ble analysert med projeksjon til latente strukturer ved minste kvadraters metode (PLS). Konsentrasjon av forbindelsene inkludert i designet ble bestemt ved å se på cytotoksisitet og EROD konsentrasjons effekt kurver for enkle forbindelser. Målte konsentrasjoner i naturmiljøet ble også vurdert.

Det ble hypotetisert at forbindelser ville ha ulik effekt på EROD aktivitet avhengiga av om ekspoenringen skjedde i blanding eller ikke. Videre ble det foreslått at blandinger som induserer høyere EROD aktivitet ville resultere i høyere forekomst av DNA DSB. Carbazol ble foreslått å modifisere EROD aktivitet indusert av de andre forbindelsene.

Resultater viser at BaP induserte EROD aktivitet signifikant alene. Carbazol induserte EROD aktivitet i liten grad, men var signifikant for den høyeste konsentrasjonen i et replikat. En generell ikke-signifikant reduksjon i EROD aktivitet ble sett i PLHC-1 eksponert for DMP. Analyse av resultatene fra eksponering av PLHC-1 for tertiære blandinger med PLS regresjon ga positive signifikante PLS regresjonskoeffisienter for BaP og carbazol. Et interaksjonsledd, DMP×Carbazol, var signifikant negativ. Kvadratiske ledd for alle tre forbindelsene var signifikante, like og negative.

Resultatene indikerer at forbindelsene har ulik effekt når den er tilstede alene sammenlignet med blanding. Det antydes ut fra resultatene at carbazol kan ha potensial for å indusere EROD aktivitet når den er tilsted i tertiære blandinger. Effekt av carbazol på EROD aktivitet er indikert å være avhengig av andre forbindelser i blandingen. Hvordan EROD aktivitet moduleres etter eksponering for DMP antydes å være avhengig av eksponeringsforhold. De signifikante negative kvadratiske leddene sett for alle tre forbindelene tyder på at alle tre forbindelser interagerer med katalytisk EROD aktivitet ved høyere konsentrasjoner.

Katalytisk EROD aktivitet er antatt å være en god indikasjon på potensiell effekt av forbindelser i biologiske system. Elektroforetisk separasjon av DNA for å bestemme omfang av DNA skade ga ikke tilfredstillende resultat. Metoden antas å være bedre egnet for å bestemme genotoksisk potensial av metaller i PLHC-1. Statistisk design og PLS framtår som verdifulle verktøy for å evaluere toksisitet for blandinger.

ABRIVIATIONS

AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon nuclear translocator
APs	Alkyl phenols
BaP	Benzo(a)pyrene
BSA	Bovine serum albumin
BTEXs	Benzene Toluene Ethylbenzene Xylene
CAT	Catalase
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
DMP	2,5-dimethylphenol
DSB	Double strand break
EC_{50}	Median effective concentration
ELISA	Enzyme linked immunosorbent assay
ER	7-Ethoxyresorufin
EROD	Ethoxyresorufin-*O*-deethylase
GNMT	Glycine-N-methyltransferase
GSH	Reduced glutathione
GST	Glutathione transferase
GSSG	Oxidized glutathione
GPx	Glutathione peroxidase
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HR	Homologous recombination
K_{ow}	Octanol/water partition coefficient
L_{max}	Maximal molecular length
LMPA	Low melting point agarose
LV	Latent variables
MLR	Multiple linear regression
MML	Median molecular length
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NPDs	Naphtalene Phenanthrene and Dibenzothiophene
NSO-HET	Heterocyclic compounds containing nitrogen, sulphur or oxygen
O_2	Molecular oxygen
O_2^-	Superoxid radical
PAH	Polycyclic aromatic hydrocarbons
PAS	PER ARNT SIM
PCA	Principal components analysis
PCB	Polychlorinated biphenyl
PLHC-1	*Poeciliopsis lucida* hepatocellular carcinoma
PLS	Projection to latent structure by partial least squares

Q^2	Predicted variance
R^2	Explained variance
Rf	Relative front
RNSB/kb	Relative number of strand breaks per kilo base
ROS	Reactive oxygen species
RSD%	Per cent relative standard deviation
RTG-2	Rainbow trout gonadal cell line
SS	Total sum of squares
SS_{regr}	Sum of squares for regression model
SS_{resid}	Sum of squares for residuals
SS_{lof}	Sum of squares of lack of fit
SS_{pe}	Sum of squares of pure experimental error
TCDD	Tetrachlorodibenzodioxin

1 INTRODUCTION

1.1 Discharges to sea from oil production

The largest discharge to sea from oil production is of water with dispersed oil. This water is called produced water. Oil reservoirs contain varying amounts of water (OLF, 2010). Produced water is separated from the oil, processed and discharged to sea or injected back into reservoirs (Utvik, 1999). Permitted amount of dispersed oil in produced water discharged to sea at the Norwegian continental shelf is 30 mg/L (OLF, 2010). Figure 1 presents volumes of produced water discharged to sea and volumes injected back into reservoirs from 1999 to 2009.

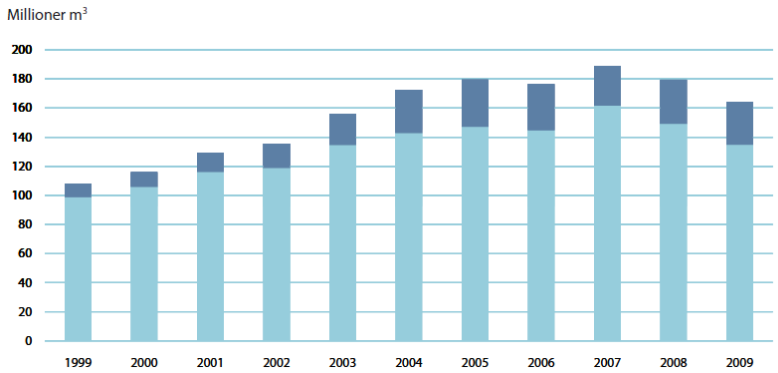

Figure 1.1. Volumes of produced water discharged to sea (light blue) and volumes of produced water injected back into reservoirs (dark blue) from 1999 to 2009 at the Norwegian Continental shelf (OLF, 2010).

1.2 Produced water is a complex mixture

Produced water is discharged to sea in high volumes and contains low amounts of dispersed oil and dissolved substances (OLF, 2010). Volumes of produced water are expected to increase as reservoirs get older (Thomas et al., 2004). Composition of produced water is field specific and complex (Utvik, 1999).

Compounds present in produced water include dispersed oil, dissolved hydrocarbons, organic acids, phenols, metals, and traces of chemicals added during extraction and production of oil. Dissolved hydrocarbons are dominated by the volatile aromatic fraction of the oil, by benzene, toluene, ethyl benzene and xylene (the BTEX compounds). Polycyclic compounds are dominated by naphthalene, phenanthrene and dibenzohiophenes (the NPDs) and their C1-C3 alkyl homologues. High molecular weight polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene (BaP) are present. Phenols are usually alkylated up to C7. Concentration of alkylated homologues of both the NPDs and phenols decreases with increasing alkylation

(Utvik, 1999). Environmental exposure to produced water is assumed to be fluctuating and low (Holth et al., 2008).

Produced water is a complex mixture (Utvik, 1999). Complex mixtures consist of numerous compounds and are not well characterized, neither quantitatively nor qualitatively, while simple mixtures have known composition and the total number of compounds present is ten or fewer (Groten, 2000). Components in mixtures can influence each other's toxicity (Cassee et al., 1998). Combined action of compounds in mixtures is discussed in Chapter 1.7.

1.3 Compounds present in produced water

Main contributors to toxicity of produced water are aromatic and phenolic fractions (Johnsen et al., 1994). In this project one representative compound for each of the two groups will be included in a simple mixture together with carbazole, for analyses of combined action.

1.3.1 Alkyl phenols

Present in oil, alkyl phenols (APs) distribute into the water phase of oil-water-systems (Bennett et al., 2007). General structure of APs is presented in Figure 1.2, together with structure of 2,5-dimethylphenol (DMP). The hydrocarbon chain of APs varies in length, degree of branching and position on the phenol ring (Solomons and Fryhle, 2004). Concentration of short-chain APs differ more between reservoirs than concentration of long-chain APs (Boitsov et al., 2007).

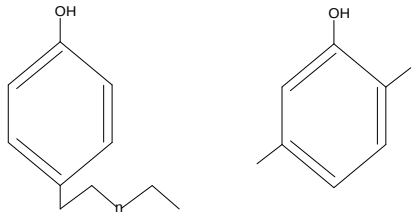

Figure 1.2. The general structure of alkyl phenol (left) and the structure of 2,5-dimethylphenol (right) (DMP).

Toxic effects seen after exposure to APs are likely chronic, since acute toxicity of APs is considered to be low (Boitsov et al., 2004). Toxicity of APs is dependent on molecular structure and degree of alkylation, generally increasing with increasing degree of alkylation (Jonsson et al., 2008). Long-chain APs have received attention because of their demonstrated potential to act as oestrogen mimics *in vitro* (Routledge and Sumpter, 1997) and *in vivo* (Nimrod and Benson, 1996; Arukwe et al., 2000; Arukwe et al., 2001). The C_6-C_8 para substituted tertiary APs are known as the most potent oestrogen mimics, but C_3-C_5 APs also show low oestrogenic activity. Phenol and C_1-C_3 APs constitute 95% of total AP concentration in produced water (Jonsson et al., 2008). Potential oestrogenic activity of produced water is assumed not to be caused by these APs, since *ortho*-substituted and short-

chain APs have little or no oestrogenic activity (Routledge and Sumpter, 1997; Thomas et al., 2004).

Exposure of two year old cod to a mixture of C_4 to C_7 APs at low concentrations changed levels of gene transcripts involved in xenobiotic biotransformation, oxidative stress defence, glucose metabolism and apoptosis. Gene transcripts encoding cytochrome P450 1A (CYP 1A) and sulfotransferase (SULT2) were up regulated. Gene transcripts encoding glutathione transferase (GSTs) were down regulated, while glutathione peroxidase transcripts (GPx4) were up regulated (Lie et al., 2009). Concomitant exposure of juvenile Atlantic cod to crude oil and a C_2 to C_5 AP mixture produced decreased CYP 1A protein expression and non-significant increased ethoxyresorufin-*O*-deethylase (EROD) activity compared to control individuals (Sturve et al., 2006). Exposure of first spawning cod to a C_4 to C_7 AP mixture resulted in a dose dependent increase in CYP 1A protein in male Atlantic cod after one and four weeks exposure to a mixture of C_4 to C_7 APs. No change was seen in CYP 1A activity. Exposure of female fish produced higher EROD activity and no change in CYP 1A protein. *In vitro* studies with pooled microsomes from fish treated with a potent inducer of CYP 1A activity showed efficient inhibition of induced activity (Hasselberg et al., 2004b). Combined exposure of cod to oil and a C_2 to C_5 AP mixture modulated oxidative stress parameters manifested as elevated levels of glutathione reductase (GR) and catalase (CAT), and an increase in per cent oxidized glutathione (GSSG) (Sturve et al., 2006). Modulation of total glutathione (GSH) levels and GR activity levels by C_4 to C_7 AP mixture observed in Atlantic cod was suggested to increase oxidative stress (Hasselberg et al., 2004a).

Decreased CYP 1A concentration or activity after exposure to APs can alter biotransformation of other xenobiotics and impair the cells ability to detoxify and excrete other compounds. Altered GSH status can increase cells susceptibility towards reactive oxygen species (ROS) (Sturve et al., 2006).

1.3.2 Carbazole

Carbazole and its alkyl and benzene derivatives are the main neutral nitrogen containing compounds in crude oil (Frolov et al., 1989). Structure of 9H-carbazole is shown in Fig. 1.4. Carbazole is present in produced water and is suggested to contribute to CYP 1A inducing potential of produced water (Ståle Johnsen, pers. comm.)

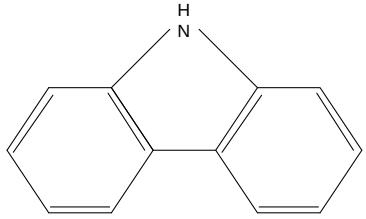

Figure 1.3. Structure of 9H-carbazole.

Combined effect assessment, fractionation procedures and chemical characterization have identified heterocyclic polycyclic compounds as potent inducers of CYP 1A activity (Brack

and Schirmer, 2003). Heterocyclic compounds containing nitrogen, sulphur or oxygen (NSO-HET) are expected to have similar affinities for the aryl hydrocarbon receptor (AhR) and similar CYP 1A inducing potencies as structurally similar PAHs (Jung et al., 2001). Azaarenes and NPAHs are shown to induce CYP 1A protein and activity. Potency of inducing CYP 1A activity expressed as $-\log EC_{50}$ correlated with the logarithm of the octanol water partition coefficient $\log K_{ow}$ and maximal molecular length of the compound (L_{max}) (Jung et al., 2001). Different AhR binding affinities for nitrated heterocyclic compounds can be explained by different net polarizability affected by position of nitration (Lofroth et al., 1984). Nitrated derivatives of BaP have higher affinities for AhR compared to BaP in affinity studies with rat (Lofroth et al., 1984).

Recently, carbazole has been reported to activate AhR in the DR-CALUX assay with the pGudLuc1.1 transfected rat hepatoma cell line H4IIE. Modulation of EROD activity was not observed (Hinger et al., 2011). Exposure of killifish to carbazole singly increased EROD activity twofold compared to control. When present in mixed exposure, carbazole inhibited EROD activity induced by a potent inducer. Half maximum inhibiting concentration (IC_{50}) was 4.6×10^{-3} mg/mL (Wassenberg et al., 2005).

Mechanism of induction of EROD activity by nitrated heterocyclic compounds, and also for some PAHs, is complex and is, as far as is known, not yet fully elucidated. Some compounds show poor correlation between AhR binding affinity and EROD inducing potency (Piskorska-Pliszczynska et al., 1986; Hinger et al., 2011).

1.3.3 Polycyclic aromatic hydrocarbons

Aromatic hydrocarbons have delocalized electrons in a six carbon ring system (Solomons and Fryhle, 2004). Compounds with two or more fused benzene rings are called PAHs (Staal et al., 2007). They are ubiquitous in the environment (Srogi, 2007) and constituents of crude oil (Monteiro et al., 2000). Presence of PAHs in marine environments can be due to both pyrogenic and petrogenic sources (Oliveira et al., 2009). Oil production at the Norwegian continental shelf results in discharges of both types (OLF, 2010). Petrogenic PAHs are presented in Figure 1.3.

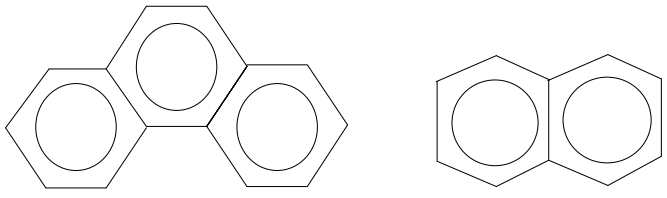

Figure 1.4. Structure of phenanthene (left) and naphthalene (right). Structure of BaP is presented in Fig. 1.5.

A concentration gradient of PAHs has been measured towards oil production facilities (Utvik, 1999). Accumulation of PAHs in sediments is seen, caused by resistance towards biological degradation and persistence in the environment (Oliveira et al., 2009).

There are numerous biological effects of PAHs, which include among others elevated levels of CYP 1A (Hylland et al., 2006), genotoxicity (Aas et al., 2000), deoxyribonucleic acid (DNA) adduct formation (Beyer et al., 2001), impairment of reproduction (Monteiro et al., 2000), altered antioxidant status (Sturve et al., 2006), and increased activity of GST (Hylland et al., 2006). Incorrect repair of PAH-DNA adducts can result in mutations (Staal et al., 2007).

Adverse effects of PAHs may be mediated via the AhR pathway (Hahn, 1998), which is responsive to planar aromatic compounds (Okey et al., 1994). Gene products regulated by AhR include CYPs, GST, glucuronosyl transferase and quinone oxireductase (Di Giulio and Newman, 2007). By altering levels of CYPs and conjugation enzymes PAHs can potentially interfere with endogenous processes (Monteiro et al., 2000). Low molecular weights PAHs are suggested to mediate toxicity independent of the AhR pathway (Incardona et al., 2005).

1.4 Bioavailability

Compounds discharged to the environment are subject to environmental processes, which together with emission patterns and physiochemical properties of the compounds determine distribution and concentration in biota (van der Oost et al., 2003). Water solubility and susceptibility to degradation largely determines fate of APs after discharge to sea. In the environment, APs have high mobility due to solubility in both polar and non-polar systems. Compounds with longer hydrocarbon chains have lower water solubility and are degraded more slowly (Boitsov et al., 2004). Low molecular PAHs are the most bioavailable PAHs (Holth et al., 2008). Increased mobility and bioavailability are expected for NSO-HETs compared to analogous PAHs (Hinger et al., 2011).

Important for exposure of marine organisms at lower trophic levels is the water soluble fraction of PAHs (Utvik and Johnsen, 1999). Uptake of PAHs to fish can be direct from water and via diet (Grung et al., 2009). Direct uptake of compounds from water is called bioconcentration while uptake of compounds from food is called biomagnification (van der Oost et al., 2003). Bioconcentration of APs increases with the length of the hydrocarbon chain (Mcleese et al., 1981) and is efficient for compounds with log K_{ow} below five (Sundt et al., 2009). It is necessary to establish a correlation between external exposure, internal dose and adverse effects for suspected harmful compounds present in the environment (van der Oost et al., 2003).

Close contact between water and blood over the gill epithelial membrane provide efficient uptake of water soluble compounds to fish (Castaño et al., 2003). Lipid soluble compounds can biomagnify and be found at high concentrations at higher ecosystem levels. Biomagnification is considered more important for total contaminant burden in large fish (van der Oost et al., 2003). Compounds with log K_{ow} between 5 and 8 are suggested to biomagnify in aquatic food webs (Kelly et al., 2007).

Fish have efficient metabolism and readily excrete short-chain APs (Sundt et al., 2009) and PAHs. Due to efficient biotransformation, PAHs are not expected to bioaccumulate (van der Oost et al., 2003; Hylland et al., 2009). Bioaccumulation encompass total uptake from both water and food and total elimination by both direct excretion and metabolism (van der Oost et al., 2003). Uptake, metabolism and elimination of APs are suggested to vary with compound, exposure route and fish species (Jonsson et al., 2008).

Sorption of compounds to sediment and suspended particles is determined by K_{ow} of the compound and by sediment characteristics, as content of organic matter, clay and moisture, and presence of oil or metals. Sorption influence partition of compounds discharged to receiving environment and determines bioavailability. Bioavailability is the fraction of compound that can be taken up in organisms' tissues over its lifetime (van der Oost et al., 2003).

1.5 Biotransformation

After initial absorption exogenous compounds are either excreted in parent form or biotransformed and excreted as metabolites (van der Oost et al., 2003). Biotransformation generally produces a less lipophilic metabolite, easier to excrete and less toxic than the parent compound (Goksoyr and Forlin, 1992). Biotransformation can result in formation of a more harmful compound (van der Oost et al., 2003).

Oxidation, hydrolysis and reduction reactions are usually first steps in biotransformation and introduces or unmasks functional groups (van der Oost et al., 2003). Biotransformation in fish is primarily initiated by oxidation reactions (Goksoyr and Forlin, 1992) catalysed by CYPs which are membrane bound heme-proteins predominantly located in endoplasmic reticulum of the liver. Oxidation is a cycle of reactions initiated when substrate binds to the enzyme. Substrate binding enable reduction of ferric iron (Fe^{3+}) to ferrous state (Fe^{2+}) by transfer of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase. Ferrous iron binds molecular oxygen (O_2). Interrupting the cycle at this stage produces and releases superoxide radical (O_2^-). By adding a proton and a second electron from either NADPH cytochrome P450 reductase or cytochrome b_5, $Fe^{2+}O_2$ is converted to $Fe^{2+}OOH$. Introducing a second proton cleaves the O-O bond yielding a substrate radical, which is oxidized by transfer of oxygen from the FeO^{3+} complex and released. Interrupting the cycle after addition of the second electron results in release of hydrogen peroxide (H_2O_2) (Parkinson and Ogilvie 2007). Uncoupling the catalytic cycle can contribute to toxicity of compounds since ROS are formed (van der Oost et al., 2003). Enzymes catalysing biotransformation of exogenous compounds are involved in metabolism of endogenous compounds (Goksoyr and Forlin, 1992).

Levels and activity of CYP 1A can be modulated by organic compounds *in vitro* (Hahn et al., 1993; Brüschweiler et al., 1996; Hahn et al., 1996; Jung et al., 2001) and *in vivo* (Hasselberg et al., 2004b; Sturve et al., 2006) via AhR (Hahn et al., 1993), which is a basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) transcription factor. Inactive AhR is primarily present in

cytosol (Hahn, 1998). Binding of agonist to AhR activates the receptor which dissociates from accessory proteins and combine with aryl hydrocarbon nuclear translocator (ARNT) via PAS domains (Gu et al., 2000). The complex consisting of AhR, ARNT and agonist interacts with xenobiotic response elements (XREs) in promoter regions of responding genes resulting in recruitment of co-activators and general transcription factors and transactivation (Safe, 2001). Increased level and activity of CYP 1A are observed after exposure to PAHs (Smeets et al., 1999; Fent and Bätscher, 2000), a mixture of C_4-C_7 APs (Hasselberg et al., 2004b), and NPAHs (Jung et al., 2001). Other CYPs are generally less responsive to exposure for exogenous compounds (Celander et al., 1993; Hasselberg et al., 2004b). Toxic responses seen after activation of AhR are epithelial changes, porphyria, liver damage, thymic involution, cancer, teratogenicity, severe wasting syndrome and death (van der Oost et al., 2003). Activation of the AhR pathway may affect other receptor controlled pathways by sequestering ARNT or other limiting factors (Safe, 2001), steric hindrance at promoter regions (Klinge et al., 1999), increased degradation of other receptors or signal transducers (Safe and McDougal, 2002). The crosstalk between the AhR pathway and the oestrogen receptor pathway is an example of this (Matthews and Gustafsson, 2006). A common perception of the endogenous role of AhR and what compound could be the endogenous ligand of AhR is not firmly established (Nguyen and Bradfield, 2008). Having similar binding affinity as the known potent CYP 1A agonist, tetrachlorodibenzodioxin (TCDD), 2-(1-H-indole-3-carbonyl)-thiazole-4-carboxylic acid methyl ester is suggested as a possible endogenous ligand for AhR (Henry et al., 2006).

Biotransformation proceeds with conjugation reactions attaching endogenous groups to functional groups introduced or exposed by hydrolysis, oxidation or reduction reactions (Jancova et al., 2010). The endogenous group is often large and polar, as for instance sugars or amino acids (Parkinson and Ogilvie, 2008). Conjugation reactions are sulphonation, glucoronidation, acetylation, methylation and conjugation with GSH and amino acids (Parkinson and Ogilvie, 2008). Glucoronic acid conjugates excreted via bile were identified as main excretion pathway after intramuscular injections of short- and medium-chain APs in Atlantic cod (Jonsson et al., 2008). Monohydroxy metabolites of carbazole have been detected in bile of rainbow trout after oral exposure (Hellou et al., 2002). Metabolites of PAHs in bile are found as hydroxylates or conjugated to sulfate, glucoronide or GSH (Grung et al., 2009). Levels of conjugation enzymes are suggested to be regulated via AhR (Bock et al., 1990) and also cofactors used in conjugation reactions are found to be responsive to exogenous compounds (Lindström-Seppä and Oikari, 1990).

In cellular defence GSH has several, potentially conflicting, roles (van der Oost et al., 2003). It protects the cell against oxidative stress as both a cofactor in reactions catalysed by GPx and a major non-enzymatic antioxidant, and it is also an important co-factor in conjugation reactions with electrophilic compounds (Forman et al., 2009). To maintain GSH:GSSG ratios GSSG is continuously reduced to GSH catalysed by GR. One GSSG is reduced to two GSH by oxidizing one molecule of NADPH (Halliwell and Gutteridge, 1989). *De novo* synthesis of GSH is induced by exposure to electrophilic compounds (Forman et al., 2009)

Responsiveness of AhR and induction of CYP are indicated to be dependent on cells thiol status, suggesting a crosstalk between the CYP system and GSH system (Otto et al., 1996; Otto et al., 1997)

1.6 Genotoxicity

Produced water relevant compounds are suggested to enhance amount of DNA damage direct and indirect, indirect through formation of ROS by enhanced CYP 1A activity and by affecting the glutathione dependent defence system (Halliwell and Gutteridge, 1989; van der Oost et al., 2003; Hasselberg et al., 2004a).

Damage to DNA can be caused by exogenous and endogenous agents or spontaneous due to intrinsic instability of DNA (Altieri et al., 2008). Recently, DNA double strand breaks (DSB) have been suggested to be due to failure of enzyme tethers during normal replication and repair (Bignold, 2009).

Oxidation of DNA by ROS is a major source of DNA damage. Derivatives of oxygen, O_2^-, hydroxyl radical ($OH^\bullet$) and H_2O_2 are examples of ROS. Damages identified after oxidation of DNA by ROS include single and double DNA strand breaks, purine, pyrimidine or deoxyribose modifications and DNA cross links (Valko et al., 2006). When ROS exceeds cellular antioxidant defence capacity the situation is termed oxidative stress (Altieri et al., 2008). Effects of oxidative stress are counteracted by action of antioxidants and antioxidant enzymes. Important non-enzymatic antioxidants are thiol antioxidants (Valko et al., 2006). The major thiol is GSH (van der Oost et al., 2003). Antioxidant enzymes are catalase, peroxidases and superoxide dismutases (Halliwell and Gutteridge, 1989).

Several processes can enhance oxidative stress (Halliwell and Gutteridge, 1989). Redox cycling produces O_2^- when a reduced reactive metabolite reacts with O_2. The process converts the metabolite back to the parent compound, contributing to oxidative stress and depleting redox potential in the cell by oxidizing one reductant per cycle (Halliwell and Gutteridge, 1989). Redox active compounds are aromatic diols, quinones, nitroaromatics and aromatic hydroxylamine (van der Oost et al., 2003). Uncoupling CYPs oxidative cycle produces O_2^- (Bondy and Naderi, 1994). By modulating quantity and activity of CYP 1A enzymes, compounds can indirectly enhance oxidative stress in a cell, as suggested for APs in Atlantic cod (Hasselberg et al., 2004a).

Reactive electrophilic compounds can interact with DNA and cause a variety of modifications and damages (Klaunig and Kamendulis, 2008). Potent DNA reactive compounds are assumed to be PAHs with bay regions, fjord regions or sterically hindered bay regions. Biotransformation of BaP yields more potent DNA reactive compounds and also redox active compounds, Fig. 1.5 (Baird et al., 2005). A general approach to study mechanisms and potential for compounds to cause genetic damage is to assess extent of DNA stand breaks (van der Oost et al., 2003).

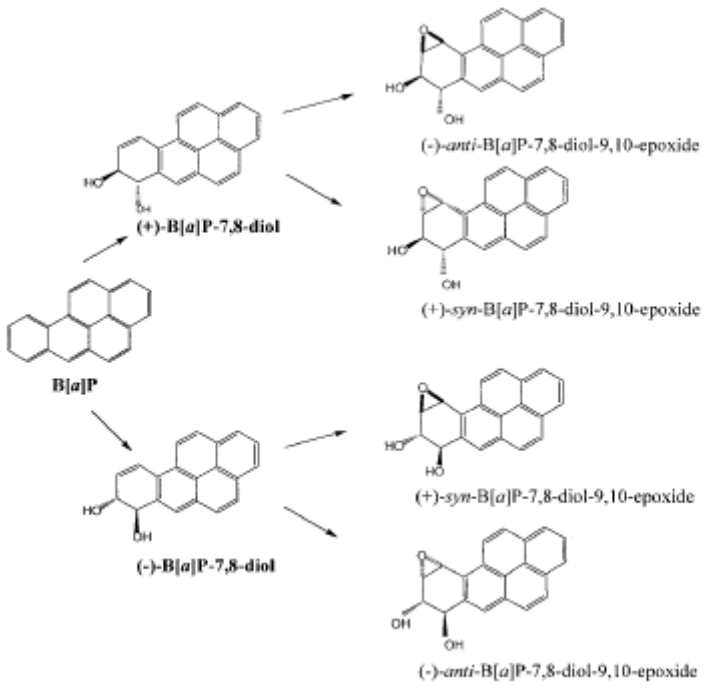

Figure 1.5. Biotransformation of BaP to diol epoxides. Biotransformation of PAHs is initiated by CYP 1A converting lipophilic PAHs to reactive electrophiles by adding an epoxide group. The electrophile intermediates can be conjugated and excreted. Biotransformation of PAHs may result in formation of DNA reactive PAH diol epoxides when the epoxide group is converted to diolhydro diol by epoxide hydrolase. Adding another epoxide to the compound catalysed by CYP 1A creates the electrophilic intermediate diol epoxide. The most potent DNA reactive compound is the BaP-7,8-diol-9,10-epoxide (Baird et al., 2005).

Repair mechanisms have evolved to protect DNA against damage. Most repair pathways are initiated by excision and resynthesis of the damaged area using the complementary strand as a template. Repair of minor DNA damages are usually processed via one of three pathways: The DNA base excision repair, the nucleotide excision repair (NER)PAH, or the general DNA mismatch repair. Two interconnected pathways repair DNA DSB; homologous recombination (HR) and non-homologous end joining (Altieri et al., 2008). Cells deficient in repair pathways are shown to be more sensitive towards damage induced by BaP-7,8-diol-9,10-epoxide compared to cells with intact repair mechanisms (Meschini et al., 2008).

1.7 *In vitro* studies

Harmful compounds can cause adverse effects on several levels of biological organization, ranging from molecular to ecosystem level. Potential toxic effect of compounds can be studied *in situ*, and in laboratories *in vivo* and *in vitro.* Cellular responses are usually the first manifestations of toxicity since the first interaction between compounds and biological systems is at the cellular or molecular level. Such interaction can be common across species (Fent, 2001). Identifying and understanding common and unique mechanisms of toxicity can enhance extrapolation of toxicity between species (Castaño et al., 2003). *In vitro* studies are valuable for examining cellular responses and as first line evaluation of compounds (Fent, 2001).

Continuous cell lines can be used in assays evaluating basal cytotoxicity of compounds and in assays studying specific endpoints like genotoxicity and CYP 1A induction (Babich et al., 1991; Fent, 2001). *In vitro* assays examining AhR mediated gene expression or CYP 1A enzyme activity are versatile when studying interaction in complex mixture of compounds with dioxin-like properties (Villeneuve et al., 2001).

Cytotoxicity *in vitro* correlates with *in vivo* acute fish toxicity (Babich et al., 1991). However, *in vitro* laboratory studies are limited when tissue-specific toxicity, adaptive responses and metabolic conversion are important. Effects seen in laboratory studies can be extrapolated to environmental exposure with restrictions (Fent, 2001). The success of extrapolating *in vitro* results will partly be determined by how well the cell line represents the tissue it is derived from (Babich and Borenfreund, 1991; Ryan and Hightower, 1994).

All fish cell lines are usually anchorage dependent and grow in monolayer (Babich and Borenfreund, 1991). The cell line used in this master project, PLHC-1 was initiated from a hepatocellular carcinoma. The original tumour was induced chemically in a desert topminnow (*Poeciliopsis lucida*) by using 7,12-dimethylbenz(a)anthracene. Cells of the cell line are epithelial-like and still maintain features of original liver functions (Ryan and Hightower, 1994), one of which is the CYP activity. The activity of CYPs is inducible (Babich et al., 1991) through binding of a ligand to the AhR (Hahn et al., 1993). The cells are metabolically active and can activate secondary toxicants (Babich et al., 1991). Very low levels of ER mRNA are detected in PLHC-1 (Fent, 2001).

1.8 Combined action in mixtures and analysis of mixtures

Simple similar action, simple dissimilar action and interaction are three basic concepts describing combined action of compounds in biological systems (Groten, 2000). Simple similar action is when each compound acts in the same way and by the same mode of action, only differing in their toxic potencies. Simple similar action is also called concentration or dose addition. Chemicals with simple dissimilar action have different modes of action and also possibly different target sites, and are not expected to influence the toxic effect of each other. This is also called response addition. Correlation of susceptibility of exposed biological systems to compounds with simple dissimilar action is described by complete negative correlation, complete positive correlation or no correlation (Groten, 2000).

Combined exposure to compounds resulting in stronger or weaker effect compared to what is expected based on additive combined effect, is known as interaction. Stronger effect can be caused by synergism, potentiation or supra-additive effects and weaker effect by antagonism, sub-additive effects or inhibition. The cause of interaction can be physiochemical or biological (Groten, 2000).

Complex mixture can be studied by several approaches including whole mixture analysis and component interaction analysis (Groten, 2000). Statistical designs are useful when studying

interaction between compounds in mixtures (Cassee et al., 1998), since carefully selected samples increase the chance of obtaining useful information from data. Statistical designs are screening designs or optimising designs. Screening designs are used when the purpose is to identify the most important variable, while optimising designs are used when variables are studied at more than two levels. Results from experiments using experimental design can be studied by analysis of variance (ANOVA), multiple linear regression (MLR) and by projection methods such as principal components analysis (PCA) and projection to latent structure by partial least squares (PLS) (Esbensen, 2000). Grouping responses and important variables can be done using PCA, while PLS correlates variation in a set of predictor variables with variation in response(s) (Feron and Groten, 2002).

1.9 Aims and hypothesis

Produced water is a complex mixture discharged to sea in high volumes containing compounds at low concentration. Combined exposure of compounds can modify predicted effect based on single exposure. To have knowledge about potential interactions in produced water is important. Carbazole is present in produced water and is suggested to contribute to CYP 1A inducing potential of produced water. Studies performed on toxicity of carbazole in relation to produced water are limited.

Increased or decreased EROD activity indicates interaction between compounds and biological system. Enhanced activity of biotransformation enzymes has potential to increase oxidative stress in organisms and produce active metabolites.

The aims of this master project are to determine carbazoles potential for modifying CYP 1A activity measured as ethoxyresorufin-*O*-deethylase (EROD) activity, to evaluate interaction in ternary mixtures with carbazole and two compounds representing fractions contributing to toxicity of produced water, and also to study correlation between biotransformation activity and genotoxicity by measuring DNA double strand breaks (DSB).

The aims are achieved by studying EROD activity *in vitro* in the continuous cell line PLHC-1 after single and combined exposure to the compounds. To best evaluate interaction a statistical design is used. Results are analysed with PLS. Compounds included are DMP, carbazole and BaP. Concentrations used in the design are determined by considering cytotoxicity results obtained with MTT cytotoxicity assay, EROD concentration effect curves for single compounds and environmentally relevant concentrations.

It is hypothesized that compounds will modify EROD activity different when present in ternary mixtures compared to single exposure. It is suggested that ternary mixtures inducing high EROD activity will produce a higher degree of DNA DSB in PLHC-1 cells. Carbazole is suggested to modify EROD activity induced by the other compounds.

2 MATERIALS AND METHODS

2.1 Cell line

Since effects of aquatic environmental contaminants are studied in this master project, a cell line from an aquatic organism, PLHC-1, was chosen as *in vitro* study system. Continuous cell lines have potential for numerous passages (Fryer and Lannan, 1994) and are valuable in long-term studies providing information about toxic compounds in the same biological system making direct comparison of compounds possible (Fent, 2001). Primary interactions between biota and compounds are at cellular levels making studies with cell cultures important (Fent, 2001). Cells from PLHC-1 is in previous considerations at our lab found better suited for evaluating aquatic contaminants than other fish cell lines, as RTG-2.

The origin of PLHC-1 is from a hepatic tumour formed in a desert topminnow (*Poeciliopsis lucida*) after multiple exposures to 7,12-dimethylbenz(a)anthracene (Ryan and Hightower, 1994). A positive trend between cytotoxicity *in vitro* in PLHC-1 and acute toxicity in fish *in vivo* has been reported. In initial evaluation and estimation of aquatic toxicity of chemicals PLHC-1 is seen as a valuable tool (Fent and Hunn, 1996). Cell cultures are a rapid, ethical, economical and scientifically beneficial test system for first line evaluation of the toxic effects of both single chemicals and mixtures of chemicals.

Important tissue-specific functions are still maintained in PLHC-1, as CYP activity (Ryan and Hightower, 1994) which is inducible through the aryl hydrocarbon receptor (AhR) (Hahn et al., 1993). Induction of stable CYP 1A is shown after exposure to PAHs and PLHC-1 cells are considered metabolically active cells (Brüschweiler et al., 1996).

2.2 Cultivating cells

2.2.1 Materials and chemicals

Materials, chemicals and solutions used when cultivating cells are presented below. Composition of the solutions is presented in Appendix 1.

Materials	Catalogue number	Producer
Autoclave	SX-700E	TOMY
Automated pipette, battery operated	4-00-031	Drummond scientific
Balance		Mettler AE 260 DeltaRange
Cell cultivating flasks, 75 cm^2	83.1813	Sarstedt
Centrifuge	3-10	Sigma
Centrifuge tubes	347856118559	Nunc
Cryo tubes 2 mL	479-0281	WR International
	3111	Forma Scientific

Incubator cabinet (CO_2 water jacketed incubator, 3111)		
Magnet stirrer	02075125	Gerhardt Bonn
Microscope	TS100	Nicon eclipse
Nitrogen container	HCB00B137	Thermolyne
pH-meter	PHM210	Radiometer Copenhagen
Pipettes		
200 µL	X51993J	Gilson
1000 µL	X52776K	Gilson
5000 µL	022492080	Eppendorf
Pipette tips		
200 µL	70.760.502	Sarstedt
1000 µL	70.762.100	Sarstedt
5000 µL	022492080	Eppendorf
Sterile hood	S-2010 1.2	Heto Holten AS
Sterile pipette tips		
25 mL	86.1685.001	Sarstedt

Chemical	Catalog number	Producer
Dimethylsulfoxide (DMSO, spectrophotometric grade)	1.02950	Merck
Disodiumhydrogenphosphate (Na_2HPO_4*$2H_2O$)	1.06580	Merck
Ethanol, 96%	24106	Sigma
Ethylenediaminetetraaceticacid disodium salt dihydrate (EDTA, 99+%)	E-5134	Sigma
Foetal Bovin Serum (FBS)	F3018	Sigma
Minimal Essential Medium (MEM)	M4655	Sigma
Penicilin-Streptomyocin	15070-063	Gibco
Potassiumchloride (KCl)	1.04936	Merck
Potassiumdihydrogenphosphate (KH_2PO_4)	1.04873	Merck
Sodiumchloride (NaCl)	S-3014	Sigma
Trypsin, 2.5%	15090-046	Gibco

Solutions
MEM-Growth Medium
Phosphate buffered saline (PBS)
Trypsin, 2.5% with 1% EDTA
EDTA

2.2.2 Procedure for cultivating cells

Cells from PLHC-1 were received from the American Type Culture Centre autumn 2006 and stored in liquid nitrogen. Cells were retrieved from liquid nitrogen 11.11.2009, briefly thawed, and transferred to a 75 cm^2 cell cultivating flask containing preheated growth medium (20 mL). Cells were grown in a humidified atmosphere (30 °C, 5% CO_2). Fresh room tempered medium was supplied every second day and 24 hours before starting an experiment.

Sub culturing was performed when cells were confluent. Growth medium was removed and the flask briefly rinsed with room tempered phosphate buffered saline (PBS) followed by four minutes incubation with 0.05% Trypsin (2.5% Trypsin with 1% EDTA) in PBS. Suspended

cells were transferred to centrifuge tubes and centrifuged for five minutes at 1188 rounds per minute (rpm). The supernatant was removed by gentle pouring and the cell pellet was suspended. Cells were centrifuged a second time (1188 rpm) for five minutes in PBS (10 mL). Supernatant was removed and the cells suspended in growth medium (1 mL), and the desired cell dilution(s) (between 25% and 12.5 %) were transferred to cultivating flask(s) with preheated growth medium (20 mL).

Procedure for preparing cells for storage in liquid nitrogen was performed as described above for sub culturing cells until suspending cell pellet after the second centrifugation. After removing the supernatant, the pellet was suspended in growth medium (0.5 mL) and transferred to a cryo tube with growth medium (0.5 mL, 10% DMSO). Cryo tubes containing cells were frozen (-80 °C, 1h) before they were transferred to liquid nitrogen. Cells were sub cultured approximately 30 times before a fresh batch was started.

2.3 MTT cytotoxicity assay

Cytotoxcity was asses using the MTT-assay according to Mosmann (1983). The MTT assay uses activity of various dehydrogenase enzymes which catalyse cleavage of a tetrazolium ring in the tetrazolium salt, 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt, as a measure of cell viability. Cleavage of the tetrazolium ring form insoluble formazan crystals. The formazan crystals can be solubilized in dimethyl sulfoxide (DMSO) and absorbance measured with a plate reader at 550 nanometer (nm).

2.3.1 Materials and chemicals

Materials, chemicals and solutions listed below were used. Composition of the solutions is given in Appendix 1.

Materials	Catalogue number	Producer
Costar sterile 96-well plate	3599	Corning Incorporated
Haemocytometer	Debt 0.1 mm	Brand
Multichannel pipette	3238643	Eppendorf
Spectrophotometric plate reader	354-00578	Labsystems
Sterile tubes		
15 mL	62.554.502	Sarstedt
50 mL	62.547.254	Sarstedt

Chemicals	Catalogue number	Producer
Thiazolyl Blue Tetrazolium Bromide (MTT, ~98%)	M2128	Sigma
2,5-dimethylphenol (99% GC-MS)*		
Benzo(a)pyrene (97% HPLC)	B-1760	Sigma
Carbazole (96%)		Aldrich
Phenanthrene (neat)	4-8564	Supelco

Solutions

MTT-solution
MTT/medium-solution
Test solution

* Analysed at the Department of chemistry, NTNU, by Rudolf Schmid

2.3.2 Procedure for the MTT assay

2.3.2.1 Linear area of formazan formation

Cytotoxic effect of compounds is more precisely determined if cell concentration is within the linear area of formazan formation. The linear area was determined by measuring formazan formation for a range of cell concentrations. Cells were treated as described for sub culturing. The last 1 mL growth medium containing suspended cells was diluted 1 to 10 (1:10) and concentration was determined by placing 30 µL of the 1:10 dilution on each of the two grids on a haemocytometer. Cells were counted in a defined area (0.0001 mL) on the grid. Counting was repeated three times on each grid. Cell concentration (cells/mL) in the 1:10 dilution was average value of the six parallels.

After determining the concentrations of the cell suspension, ten dilutions were made from the 1:10 dilution and seeded in six parallels on a 96 well plate. Edge wells were kept empty, but filled with medium or DMSO in order to avoid excess evaporation during incubation and edge effects when reading the result, respectively.

Plates were incubated for 96 hours. Fresh growth medium (200µL per well) was supplied after 24 hours to mimic exposure scenarios. After 96 hours incubation, growth medium was replaced with MTT/medium solution (200 µL). Plates were incubated for four hours before DMSO (200 µL) was added to stop the reaction and solubilize the crystals that had been formed. Absorbance was read at 550 nm with a plate reader. Results are presented as average absorbance ± standard deviation, cell concentration is concentration seeded on the first day. Well absorbance values that are ± two standard deviations from average values are considered to be outliers and removed from the average. These are marked with asterisk in Appendix 2.

2.3.2.2 MTT cytotoxicity assay

Cells were sub cultured (Chap. 2.2.2.) and cell concentration in the 1:10 dilution determined (Chap. 2.3.2.1.) as described above. Cells were seeded on a sterile 96-well plate at concentrations within the linear area of formazan formation. After incubation for 24 hours, cells were exposed to the test solution and incubated for 48 hours. At 96 hours after seeding MTT/medium solution (200 µL) was added, plates incubated for four hours and reaction stopped by adding DMSO (200 µL). Absorbance was read at 550 nm with a plate reader.

Cytotoxicity was determined by two or more independent experiments with six parallels for each concentration or mixture. Cytotoxicity is expressed as average per cent cell survival ±

standard deviation, in relation to the DMSO control. The cell survival for DMSO control is set to 100 %. The individual parallels with absorbance values ± two standard deviations from average values were considered to be outliers and were excluded from the average.

2.4 CATALYTIC EROD ACTIVITY

Measuring EROD-activity was performed as described in Fent and Bätscher (2000) with modifications and presented per mg protein using the BioRad protein assay solution as described in Bradford (1976). Procedure for both assays and modifications of EROD assay are described below.

2.4.1 Bradford total protein assay

To correct for variation in protein amount, EROD activity is presented per milligram protein.

2.4.1.1 Materials and chemicals

Materials, chemicals and solutions used when determining total protein are presented below. Total protein was determined on the same sample material as used for determining EROD activity, exposure conditions and harvesting is described below. Composition of the solution is given in Appendix 1.

Materials	Catalogue number	Producer
Spectrophotometric plate reader	354-00578	Labsystems
Unsterile 96-well plate	650-001	Greiner bio-one

Chemicals	Catalogue number	Producer
BioRad protein assay solutions	500-0006	BioRad
Bovine Serum Albumin (BSA)	B4287-56	SigmaAldrich

Solutions
BSA

2.4.1.2 Procedure for the Bradford assay

Unsterile 96-well plates were used when setting up the Bradford assay. A standard curve with known concentrations of protein was included in triplicate on each plate. First, BioRad protein assay solution (40 µL) was added to all wells. The standard curve was prepared, including points at 0, 5, 10, 15, 20, 30, 40 and 50 µg protein per mL. After adding the required amount of BSA, final volume of the wells was adjusted to 200 µL. The standard curve was completed before samples (4µL) were loaded in remaining wells in triplicate. Final volume was adjusted by adding milliQ-water (156 µL). Samples, BioRad protein assay solution and milliQ-water

were mixed with a multichannel pipette, and any bubbles formed were removed by using a hypodermic needle. The plate was incubated for five to 60 minutes before absorbance was read at 595 nm.

Amount of protein of a sample were average values three parallels. Parallel values were individually evaluated and disregarded from subsequent calculations if found deviating. These parallels are marked with an asterisk in Appendix 3.

2.4.2 EROD assay

EROD activity was determined by measuring fluorescence in intervals of 60s for 17 minutes at excitation and emission wavelengths of 530 and 590 nm, respectively. The substrate, 7-ethoxyresorufin (ER) is converted to resorufin by deethylation catalysed by CYP 1A enzymes. Fluorescence data is fitted to a resorufin standard curve for quantification, presented in Appendix 3.

2.4.2.1 Materials and chemicals

Materials, chemicals and solutions used in the EROD assay are presented below. Composition of the solutions is presented in Appendix 1.

Materials	Catalogue number	Producer
Eppendorf tubes		
1.5 mL	72.690.001	Sarstedt
0.5 mL	72.699	Sarstedt
Half area 96 well plate, unsterile	675096	Greiner bio-one
Multichannel pipette	3238643	Eppendorf
Plate reader	1010-1	Synergy HT
Costar Sterile 24-well plate	3524	Corning Incorporated

Chemicals	Catalogue number	Producer
2,5-dimethylphenol (99% GC-MS)		
7-ethoxy resorufin	E3763	Sigma
Bovine Serum Albumin (BSA)	B4287-56	SigmaAldrich
Benzo(a)pyrene (97% HPLC)	B-1760	SigmaAldrich
Carbazole (96%)		Aldrich
NADPH	N-7505	SigmaAldrich
Trizmabase	T6066	SigmaAldrich

* Analysed at the Department of chemistry, NTNU, by Rudolf Schmid

Solutions
Bovine Serum Albumin (BSA)
7-ethoxyresorufin
NADPH
Tris

2.4.2.2 Procedure for the EROD assay

Cells from a confluent cultivating flask were seeded in wells on a sterile 24 well plate. After incubation for 24 hours, growth medium was replaced with test solution (1 mL). Cells were incubated with test solution for eight hours. Test solution was removed and each well was rinsed with PBS (1 mL) before the plate was wrapped in aluminium foil and frozen (-80 °C). Plates were stored in the freezer until enzyme activity was determined. Length of freezing has previously been shown not to affect enzyme activity (Renate Haldsrud, pers. comm).

When assessing enzyme activity the plate was removed from the freezer, briefly thawed and tris (160 µL) was added to each well. The plate was wrapped in aluminium foil and frozen (-80 °C, 30-60 minutes). The plate was thawed (10 minutes) and tris with cell debris was transferred to autoclaved 0.5 mL eppendorf tubes. The tris solution was pipetted up and down forcefully in the well 5-10 times to loosen as much cell debris as possible. Eppendorf-tubes were kept on ice after the samples were transferred to the tubes.

Enzyme activity was measured by adding sample (50 µL) in two wells horizontally on a black half-area 96-well plate. The assay included four parallels of blanks with tris (50 µL). After samples were added, BSA (20 µL, 5.32 mg/mL in 50 mM tris, pH 7.8) was added to each well, then ER (80 µL, 4 µM in 50 mM tris, pH 7.8). The plates were incubated (25 °C for 15 minutes) with continuous shaking (30rpm), before NADPH (20 µL, 6.7 µM in 50 mM tris, pH 7.8) was added. Total volume in the wells was 170 µL.

The plate reader that was used to set up the standard curve used for quantification (µM resorufin) was also used for analyses in this master project. Results are blank corrected and presented as average values of three parallels ± standard deviation, as pmol resorufin per minute per mg protein. Values for EROD activity are negative for certain mixtures and concentrations. This is because low fluorescence values are outside the range of the standard curve. These values were not adjusted to zero values, since they represent a change in EROD activity relative to DMSO control. When interpreting negative values, DMSO control presented in figures are considered. For ternary mixtures, EROD activity after exposure to DMSO was -203 pmol resorufin/min/mg protein.

Three parallels for each concentration or mixture were included when determining EROD activity. Two and three parallel samples were included for each of these parallels when measuring fluorescence and total protein, respectively. Calculations were performed individually for the three parallels, while EROD activity is presented as average values of these.

Assessing EROD activity involves two procedures and several steps and is assumed to be sensitive to error. Fluorescence values are individually evaluated and disregarded from subsequent calculations if found deviating. These are marked with asterisk in Appendix 3. Parallel samples which are removed are marked with grey shade in Appendix 3. Three or more independent experiments were performed with each mixture or compound.

2.4.2.3 Optimization of protocol

The protocol was modified from a protocol presented by Fent and Bätscher (2000). Changes are listed sequentially as they were performed below and are illustrated in Fig. 2.1.

Fent and Bätscher 2000

Catalytic EROD activity was determined according to the original protocol except cells were seeded on 24 well plates and not 96 well plates as in the original protocol. Due to this change, cells had to be harvested as described above.

Incubation

Incubation for 15 minutes at room temperature as presented in the original protocol was changed and performed in an incubator at 25 °C to avoid fluctuating temperature conditions.

ER concentration

The area of a well on a 24 well plate is approximately six times bigger than the area of a well on a 96 well plate (2.3 cm^2 and 0.38 cm^2, respectively). Based on the size difference between wells and the amount of cell suspension added to each well when assessing enzyme activity, it was assumed that the amount of cell content present was approximately doubled compared to the original protocol. It was assumed that higher amount of enzymes required more ER, and the amount or ER added to each well was doubled.

NADPH concentration

As the enzyme activity increased after doubling the ER concentration, doubling of the NADPH concentration was evaluated.

Harvesting

Cells had to be properly lysed to get enough material for determining enzyme activity and total protein on the same sample material. Plates were in initial experiments freezed, thawed, and added tris before samples were collected. To improve cell lysis and detachment from well bottom, plates were frozen (-80 °C, 30 to 60 minutes) also after tris had been added.

Harvesting (2)

Based on the increase in enzyme activity observed after freezing plates with tris, it was examined if three sequential freezing/thawing cycles could increase cell lysis and amount of cell content in samples further.

Seeding density (70% and 50%)

It was contemplated if dense growth conditions could decrease enzyme activity in cells. This was assessed by seeding only 70% and 50% of the 1 mL cell suspension.

Eight hours exposure (8h)

Protein induction is a rapid response (Ryan and Hightower, 1994) and optimal exposure time for 10^{-4} mg/mL BaP has previously been shown to be eight hours (Smeets et al., 1999). The effect of a exposure time of eight hour was evaluated.

Reproducing 8h

The preliminary protocol was reproduced.

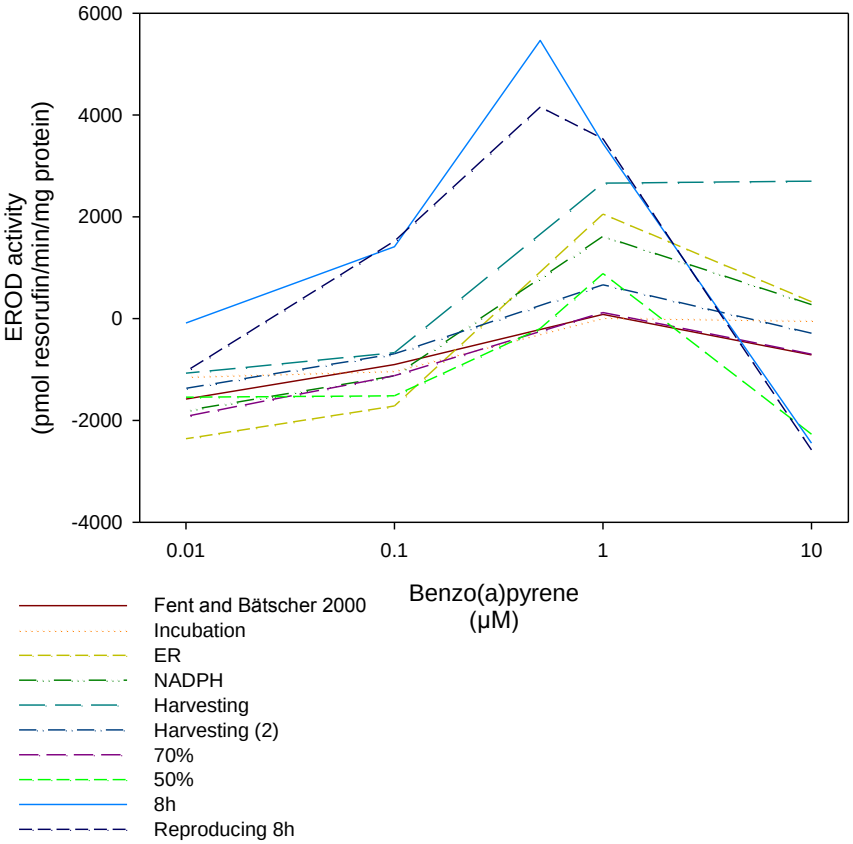

Figure 2.1. Sequential modifications of original protocol for EROD assay presented by Fent and Bätscher (2000). The EROD assay was first performed as described in the original protocol, but with changed growth conditions, Fent and Bätscher, 2000, then incubation, ER concentration, NADPH concentration cell lysis, seeding densities (70% and 50 %) and exposure conditions (8h) was changed sequentially. See text for details. Results obtained with the final protocol were reproduced (reproducing 8h).

2.5 GENOTOXICITY

Damage to DNA was assessed as DNA DSB by separating DNA from PLHC-1 on agarose gel electrophoretically as described in Theodrakis (1994) and Krøkje and co-workers (2006). Cells from PLHC-1 exposed to ternary mixtures were casted in low melting point agarose (LMPA), digested and DNA from the cells separated according to size on 0.6% agarose gel. A high degree of DNA DSB results in smaller DNA fragments that migrate further on the gel. After separation, DNA fragments were stained with ethidium bromide and visualized by fluorescence.

2.5.1 Materials and chemicals

Materials, chemicals and solutions used for gel electrophoresis are presented below. Composition of the solutions is presented in Appendix 1.

Materials	Product number	Producer
Biofuge fresco	75005510/01	Haraeus
Cast for gel		BioRad
Comb, 15 wells		BioRad
Electrophoreses power supply – EPS 200, GN003036	19-0200-00	Pharmacia-Biotech
Eppendorf tubes 1.5 mL	72.690.001	Sarstedt
GelDoc 2000		BioRad
Heat block (Dri Block DB-2D, FDB02DD)	117995-21	Techne
PowerPac Basic (300V, 4000 mA, 75W)	63S 28031	BioRad
Sterile 24 well plates		Corning Incorporated

Chemicals	Product number	Producer
Agarose for routine use	A9539	SigmaAldrich
Low melting point agarose	162-0019	BioRad
Active charcoal	C3014-2	SigmaAldrich
Boric acid (for electrophoresis, H_3BO_3)	B7901	SigmaAldrich
Ethidium bromide ($C_{21}H_{12}BrN_3$)	161-0433	BioRad
Lambda/Hind III marker 2	SM0101	Fermentas
Lambda DNA	SD0011	Fermentas
Loading dye × 6	R0611	Fermentas
Proteinase K (from *Tritirachium albumin*, 39 units/mg protein)	108K8610	SigmaAldrich
SDS (sodiumdodecylsulfat, $C_{12}H_{25}SO_4Na$)	L-3771	SigmaAldrich
Trismabase	T6066	SigmaAldrich

Solutions
Digestion buffer
Lambda-DNA marker
TBE-buffer
TE-buffer

2.5.2 Procedure for gel-electrophoresis

After sub culturing, 100 µL cell suspension was seeded per well. Cells were incubated for 24 hours before growth medium was replaced with test solutions and incubated for 48 hours with test solution. The cells were trypsinized (0.05 % trypsin in PBS) for three minutes. Cell solution was transferred to 1.5 mL eppendorf tubes and centrifuged (4000 rpm, two minutes). The pellet was suspended in TE-buffer (0.5 mL, 10 mM tris, 1 mM EDTA, pH 8, 30 °C) and LMPA (0.5 mL, 1%, 30 °C) was added. Cells suspended in TE-buffer/LMPA were transferred to plug moulds, 40 µL cell suspension/plug. Plugs were refrigerated (4 °C), transferred to 1.5 mL eppendorf tubes with digestion buffer (0.5 mL, 100 mM NaCl, 10 mM tris, 25 mM EDTA, 0.5% SDS, pH 8, added 1 mg/mL proteinase K) and incubated at 55 °C over night. Plugs were cooled to room temperature and placed in wells on a 0.6 % agarose gel (10 cm), which were sealed with 1% LMPA. Samples were run at 23 V (2.3 V/cm) for 12 hours together with a marker (1 µL λDNA marker solution, 4 µL 6 × loading dye and 15 µL distilled water). The standard used was λDNA and λ/HIND III marker 2. The marker was heated to 65 °C for five minutes and put straight on ice for three minutes and transferred to the gel after loading the samples. After separation, DNA fragments were stained with ethidium bromide (0.5 µg/mL) for two hours before DNA staining intensities were analysed with a BioRad GelDoc 2000. From DNA staining intensities, median molecular lengths (MML) were estimated. From MML relative numbers of strand breaks per kilobase pair (RNSB/kb) was calculated. The solution containing ethidium bromide was decontaminated with active charcoal.

2.5.3 Calculations

Estimated MML can indicate the extent of DNA DSB in cells (Theodorakis et al., 1994). By considering maximum DNA staining intensity MML can be found. After analyses results are presented as a graph with DNA staining intensities as a function of relative front (Rf) for each lane, Appendix 6. Related to migration distance, Rf is a relative number between zero and one. Position for maximum DNA staining intensity (DNA Max.) was estimated by accumulating all y-values for an interval along the x-axis and dividing the accumulated sum by two:

$$DNA\ Max. = \frac{1}{2}\sum_{i=1}^{n} y_i \Delta x \qquad (2.1)$$

The x-value for half the accumulated sum, is $Rf_{1/2}$. A standard curve is made for each gel relating known size of the fragments in the marker to their Rf. Using the standard curve, $Rf_{1/2}$ for samples are extrapolated to MML which is expressed in kilo base pairs (kb). Length of the DNA fragments (L_n) can be approximated from MML by (Ehmann and Lett, 1973):

$$L_n = 0.6 \times MML \qquad (2.2)$$

According to Freeman and co-workers (1986) RNSB are calculated from length of DNA fragments by:

$$RNSB = \frac{1}{L_{n\,(exposed)}} - \frac{1}{L_{n\,(carrier\ control)}} \qquad (2.3)$$

2.6 Compounds and concentrations

Carbazole is present in produced water and it is suggested to be a potential contributor to produced water toxicity (Ståle Johnsen, pers. comm). Information on toxic effect of carbazole in relation to produced water is limited. Carbazole is included in this project to study its potential for modifying EROD activity singly and in mixtures. It is important to have knowledge about interaction between compounds since mitigation measures aimed at reducing environmental risk of produced water discharge may be targeted on single compounds. Interaction is more common for high concentrations of compounds. The aromatic and phenolic fractions of produced water are significant contributors to toxicity of produced water (Johnsen et al., 1994; Utvik, 1999) and two compounds representing each of these fractions are included in this master project. The compounds selected as representative compounds are measured at high concentrations relative to other compounds in same fraction.

Compounds are included at concentrations that are environmentally realistic. Phenanthrene was included to represent aromatic fraction, while DMP was included to represent phenolic fraction. Phenanthrene is one of the NPDs which are dominating polycyclic compounds in produced water. The short-chain APs are less studied compared to the long-chain APs and are more relevant for produced water.

Phenanthrene was due to practical reasons replaced by BaP. Homogenous test solutions of phenanthrene could not be made at the desired concentrations in initial MTT cytotoxicity assay. The consequence was highly varible results. As phenanthrene, BaP is a PAH and it is detected in the marine environments (Utvik, 1999; Harman et al., 2009).

A statistical design was used to vary concentrations of the compounds in ternary mixtures systematically. Concentrations included in the design were chosen based on cytotoxicity (Fig. 3.2-3.4), concentration-effect curves for single compounds (Fig. 3.6-3.8) and measured concentrations of the compounds in marine environment, Appendix 4.

2.7 Statistical designs

Mathematical protocols for experimental designs are developed so that all variables influencing a system can be changed simultaneously without losing experimental control (Esbensen, 2000). Such experiments are more robust in terms of obtaining the correct information (Wold et al., 2001). The effect of changing one variable at the time is illustrated in Fig. 2.2.

Different types of statistical designs can be used depending on the purpose of the study. Screening designs identifies which variables are most important in modulating the measured response. Full and fractional factorial designs can be used for screening. In a full factorial design, all combinations of high and low are included. For three variables this can be illustrated as a cube where variables are varied from low to high in three directions(Esbensen, 2000). Experiments are placed in each of the eight corners of the cube. For a two level full

factorial design with k variables, 2^k experiments are generated. If many variables are present this can result in numerous experiments (Wold et al., 1986). Studies of a large number of variables can be performed with fewer experiments using fractionate factorial designs (Esbensen, 2000).

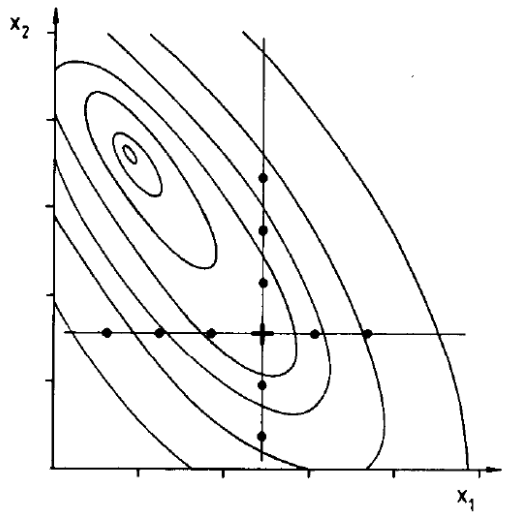

Figure 2.2. Ridge plot showing a process influenced by two process variables and a full factorial design. By changing one variable at the time the experiment identifies the ridge where the response is higher. The response is lowered again when the other variable is changed in steps. Highest response is identified as the +. The actual peak is missed (Wold et al., 1986).

Statistical designs including variables at more than two levels are Box-Behnken and Central Composite designs. The Box-Behnken design combines variables so that high levels of all variables are not present at the same time in any experiment. For a design with three variables this can be illustrated by placing experiments in the middle of all lines of a cube. The lines of the cube are the concentration range of the variables included from low to high (Esbensen, 2000).

A Central Composite design includes high levels of all variables. For a design with three variables it can be illustrated as a full factorial design with a sphere around with six experiments placed on the surface of the sphere extending from the middle of each face of the cube. Experiments placed on the sphere have a higher concentration of the variables than high concentration in the full factorial design. A variant of the Central Composite design is the Central Composite design Face Centred (CCF) (Esbensen, 2000). This design includes a full factorial design with experiments positioned on the face of the cube instead of outside the cube, Fig. 2.3. In this master project, a CCF was used to design 17 mixtures.

Composition of the 17 ternary mixtures with high, medium and low concentration of DMP, carbazole and BaP included in this project is presented in Table 2.1. Stock solutions were made using DMSO. Test solutions were made by adding stock solutions to growth medium. In all experiments DMSO control (0.1%) was included. Concentration of DMSO in test solutions was lower than 0.1% .

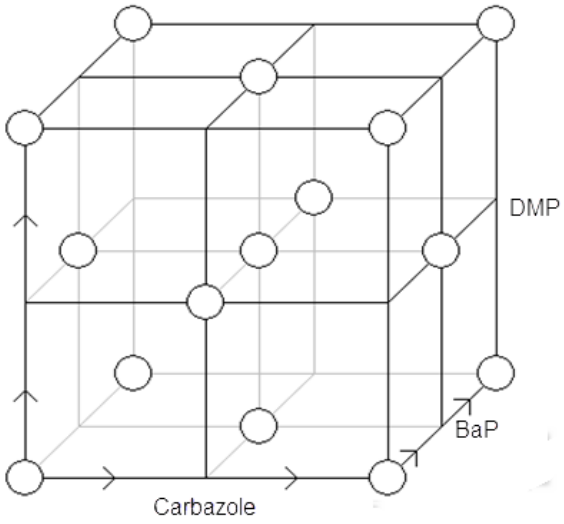

Figure 2.3. Central composite design face centred (CCF) with the compounds used in this project. Circles in the cube illustrates mixtures with high, medium or low concentration of benzo(a)pyrene (BaP), carbazole and 2,5-dimethylphenol (DMP). The design includes eight mixtures from the full factorial design, represented by the corners of the cube, six axial points positioned on the face of the cube and a triplicate centre point, resulting in total 17 mixtures.

Table 2.1. Ternary mixtures generated using CCF design combining high, medium and low concentrations of 2,5-dimethylphenol (DMP), carbazole and benzo(a)pyrene (BaP). In each individual experiment a DMSO control (0.1%) was included.

Mixture	Concentration (mg/mL)			% DMSO
	DMP	Carbazole	BaP	
1	1.2E-05	1.7E-06	1.0E-07	0.06
2	1.8E-02	1.7E-06	1.0E-07	0.07
3	1.2E-05	1.2E-03	1.0E-07	0.06
4	1.8E-02	1.2E-03	1.0E-07	0.06
5	1.2E-05	1.7E-06	1.3E-04	0.07
6	1.8E-02	1.7E-06	1.3E-04	0.08
7	1.2E-05	1.2E-03	1.3E-04	0.07
8	1.8E-02	1.2E-03	1.3E-04	0.07
9	1.2E-05	5.9E-04	6.3E-05	0.07
10	1.8E-02	5.9E-04	6.3E-05	0.08
11	9.2E-03	1.7E-06	6.3E-05	0.04
12	9.2E-03	1.2E-03	6.3E-05	0.04
13	9.2E-03	5.9E-04	1.0E-07	0.08
14	9.2E-03	5.9E-04	1.3E-04	0.09
15	9.2E-03	5.9E-04	6.3E-05	0.07
16	9.2E-03	5.9E-04	6.3E-05	0.07
17	9.2E-03	5.9E-04	6.3E-05	0.07

2.8 PLS regression

Results from experiments using experimental design can be analysed by projection techniques (Esbensen, 2000). In this master project, PLS is used. It is a regression tool, derived from MLR. The latter is sufficient as long as the x-variables are fairly few and uncorrelated. Data sets were x-variables are co-linear, noisy and numerous can be analysed with PLS. Modelling several responses simultaneously is also possible with PLS (Wold et al., 2001). In this project, the correlation between the x-matrix (the concentration of the compounds) and the y-matrix (the measured EROD activity) is found by using PLS regression (Kettaneh-Wold, 1992):

$$Y = F(X) + e \qquad (2.4)$$

A dataset analysed by PLS has two matrices, X and Y with N×K and N×M dimensions, respectively. Basic assumption of PLS regression is that the studied response is influenced by fewer underlying variables. The PLS regression model identifies these new variables called X-scores (t_a) which are estimates of latent variables (LVs). The X-scores predicts Y and models X and are linear combinations of the original variable (X_k) and the coefficients weights (W_{ka}^*) for all observations (i):

$$t_{ia} = \sum_k W_{ka}^* X_{ik} \qquad (2.5)$$

The estimates of LVs are few and orthogonal. The PLS regression model determines a new set of scores for the observations, t and u, corresponding to x- and y-values, respectively. The PLS regression weights are interpretations of these score, describing how the X-matrix and Y-matrix combine. The weights tell which variables are important for the response. Weight, scrores and loadings for both matrices are determined by the PLS algorithm. The estimates of LVs explains as much of the information in the y-response as possible, forming a new coordinate system, Fig 2.4. The number of estimated LVs in a model depends on predictive significance of each of the X-scores (Wold et al., 2001).

With good models the X-scores are good approximations of the X-matrix when multiplied with the loading vectors (P_{ak}), and X-residuals (e_{ia}) are small:

$$X_{ia} = \sum_a t_{ia} P_{ak} + e_{ia} \qquad (2.6)$$

The residuals for the x-matrix are the parts of the original data that is not used to generate the model. The x-matrix residuals are useful for identifying outliers which do not fit the model and points that deviate. X-scores are good predictors of the Y-matrix when combined with Y-loadings (c_a). Y-residuals (f_{im}) are small with good models:

$$y_{im} = \sum_a c_{ma} t_{ia} + f_{im} \qquad (2.7.)$$

This can also be written:

$$y_{im} = \sum_a c_{ma} \sum_k w^*_{ka}\, x_{ik} + f_{im} \quad (2.8)$$

Since X-scores are derived from coefficients weights and original variables.

The PLS regression coefficients (b_{mk}) are equal to:

$$b_{mk} = \sum_k c_{ma} w^*_{ka} \quad (2.9)$$

Prediction of y-values can hence be performed using the original x-variables and their PLS regression coefficients:

$$y_{im} = \sum_k b_{km} x_{ik} + f_{im} \quad (2.10)$$

The equation for a PLS model with two x-variables (x_1 and x_2) shows the relationship between original x-variables, interaction terms and PLS regression coefficients (Brakstad, 1992):

$$y = b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + f_{im} \quad (2.11)$$

If the relationship between the two predictor variables and the response is a linear relationship, then b_{11} and b_{22} is close to zero. If the relationship is quadratic these coefficients will be different from zero (Brakstad, 1992).

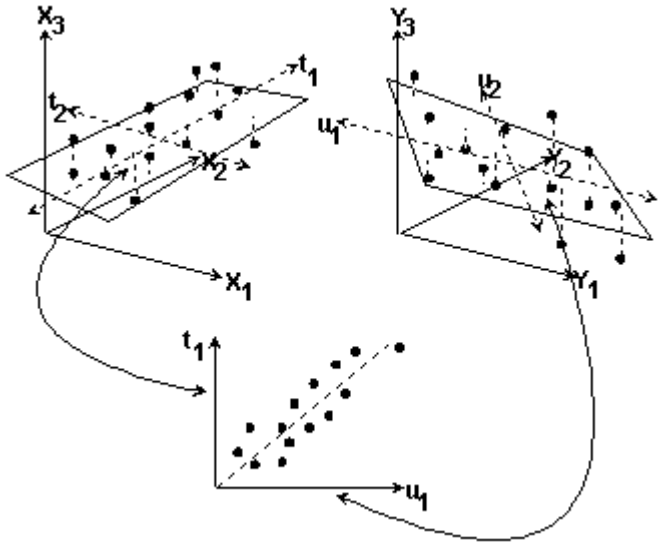

Figure 2.4. Geometric representation of PLS analyses. The x-matrix and y-matrix is visualized as points in the multidimensional space defined by the coordinate system. The PLS model is seen as new latent variables (LV) estimates. Number of LV estimates depends in predictive significance and determines number of dimensions of the hyper plane. Observations in the original matrices are given new coordinates in relation to the LV estimates (Wold et al., 2001; Umetrics, 2003).

Main contributors to the response and interactions between variables in a PLS model are visualized in a coefficient plots. The size of a coefficient represents the change in response as the factor is varied from zero to high. Other factors are kept at average values. The coefficients are generally scaled and centred. Contour plots or response surface plots show

predicted interaction between compounds based on the model. The observed predicted interaction is only valid for the concentration range tested (Umetrics, 2003).

2.8.1 Validating PLS models

Values of explained, R^2, and predicted, Q^2, variation are good estimates when evaluating PLS models (Lundstedt et al., 1998). How much of the total variation in the y-response the model explains is expressed as R^2:

$$R^2 = (SS - SS_{resid})/SS \quad (2.12)$$

SS is the sum of squares of total variation in a response corrected for the mean. Total SS include variation resulting from the regression model (SS_{reg}) and variation resulting from the residuals (SS_{resid}). A high degree of explained variance produces small residuals and larger R^2 (Lundstedt et al., 1998).

The fraction of total variation of the response that can be predicted by the model, Q^2, is obtained after cross validation by excluding about one sixth of the observations from the dataset and fitting the model using the remaining data. The excluded observations are predicted by the model generated with the remaining data. This is performed once for all observations. Deviation between predicted and observed values is calculated and squared deviations for all observations are summarized yielding predicted residual sum of squares (PRESS). Predicted variance can be written as:

$$Q^2 = (SS - PRESS)/SS \quad (2.13)$$

A good biological model have $R^2 > 0.7$ and $Q^2 > 0.4$ (Lundstedt et al., 1998).

The model used in this project was validated with respect to R^2, adjusted R^2 and Q^2, and also by considering plots showing normal probability of the residuals and observed versus predicted EROD activity.

Adjusted R^2 is considered when non-significant terms are removed from the model. When additional terms are added to the model they can be given an estimated coefficient of zero, which will not change or slightly increase R^2. Adjusted R^2 considers if added terms contribute to explanatory power of the model and is calculated using degrees of freedom. Adjusted R^2 are less or equal to R^2 (Erik Johansson pers. comm).

Normal probability of the residuals can be used to identify outliers and to assess normality of the residuals. The plot is generated by plotting cumulative probability against the standardized residuals, if the latter are normally distributed the curve is s-shaped. The y-axis is adjusted so that the s-curve formes a straight line describing the normal distribution (Lundstedt et al., 1998). If the residuals are not randomly distributed, systematic variation is present in the data which has not been included in the model.

Observed versus predicted plots of observations illustrates how a model predicts observations, and are generally sufficient to evaluate the predictive power of a model (Wold, 1996). In a good model observations are positioned close to the regression line. Plots showing values for observed versus predicted observations and normal probability can be used together with the replicate plot to detect outliers (Umetrics, 2003).

Statistical significance of a model is illustrated in the ANOVA plot. Total variation (SS) is sum of variation due to the regression model (SS_{regr}) and variation due to the residuals (SS_{resid}):

$$SS = SS_{regr} + SS_{resid} \quad (2.14)$$

The SS_{resid} can be further divided into sum of squares of lack of fit (SS_{lof}) and sum of squares of pure experimental error (SS_{pe}):

$$SS_{resid} = SS_{lof} + SS_{pe} \quad (2.15)$$

The SS_{regr} and the SS_{resid} is compared in the ANOVA plot compares. Models are significant at the chosen probability level (usually P = 0.05) if the standard deviation of SS_{regr} is larger than the standard deviation of SS_{resid} multiplied with by the square of the critical F ($RSD*\sqrt{F_{crit}}$) (Lundstedt et al., 1998).

The model used in this project had a significant lack of fit. For this model it was considered irrelevant since a good reproducibility, Fig. 3.9, can result in an artificial lack of fit (Erik Johanssen, Umetrics). Observed versus predicted plot, Fig. 3.12, illustrates that the model has good predictive power.

2.9 Statistical tests

Results from cytotoxicity testing of mixtures and EROD activity for all three individual compounds were tested for statistical significance. This was performed using SigmaPlot 12.0.1 and One Way ANOVA with Holm-Sidak post hoc test, with $p > 0.005$. Calculations were performed in Microsoft Excel 2010 for Windows 2010.

3 RESULTS

3.1 MTT cytotoxicity assay

Absorbance was measured for a range of cell concentrations in order to determine the linear area of formazan formation as a function of cell concentration. Results are illustrated in Figure 3.1, and are presented as absorbance at 550 nm as a function of cell concentrations. Raw data for absorbance is presented in Appendix 2.

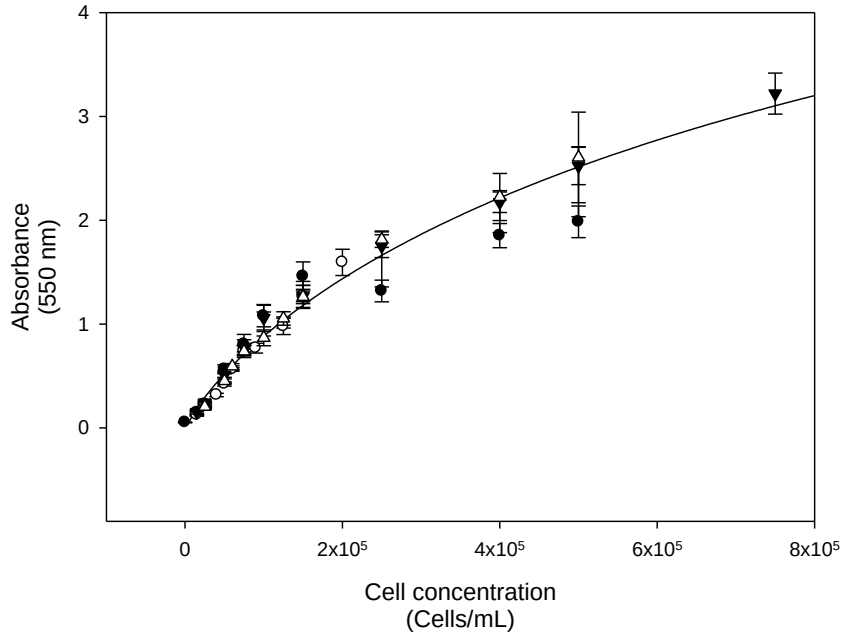

Figure 3.1. Absorbance (550 nm) as function of cell concentration (cells/mL). Independent experiments are marked with different symbols and are average values ± standard deviation, n=5-6. The graph is based on average absorbance at each of the cell concentrations tested, and is a sigmoid four parametric logistic curve, R^2=0.99.

Fig 3.1 indicates that the linear area of formazan formation was from zero to approximately 200 000 cells/mL, with increasing standard deviation as cell concentration increased.

Cytotoxicity was determined by exposing a cell concentration of 75 000 cells/mL to a concentration range of the compounds, or to the mixtures. Results are presented as per cent cell survival, DMSO control is 100% cell survival. Cytotoxicity of DMP, carbazole and BaP is illustrated in Fig. 3.2, 3.3 and 3.4, respectively. Highest non-cytotoxic concentration for DMP is approximately 0.02 mg/mL, and for BaP approximately 10^{-4} mg/mL. Carbazole has no apparent cytotoxic effect on PLHC-1 cells as evaluated with MTT assay.

Cytotoxicity of ternary mixtures is presented in Figure 3.5. The ternary mixtures have no apparent cytotoxic effects in PLHC-1. Two thirds of mixtures resulted in significant higher cell survival compared to DMSO control, mixtures 5, 6, 7, 8, 9, 10, 12, 13, 14, 16, 17. Cells exposed to mixtures 1, 4, 12 and 15 showed no difference in cell survival compared to DMSO

control. Mixtures 2 and 3 produced significant induced cell survival only in one replicate. Concentrations of compounds in the mixtures are presented in Table 2.1.

Raw data and per cent cell survival are presented in Appendix 2.

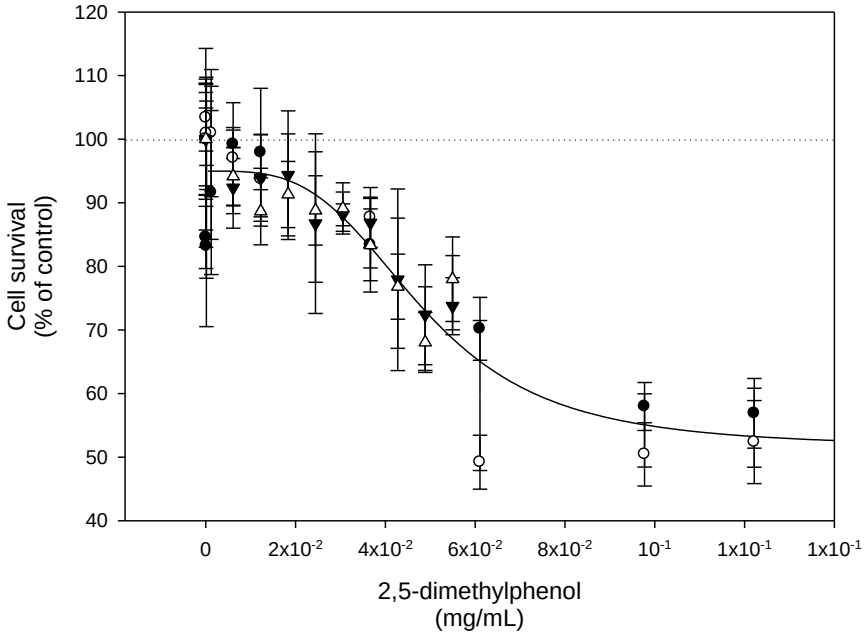

Figure 3.2. Cell survival in PLHC-1 cells presented as per cent of DMSO control after exposure to 2,5-dimethylphenol (1.2×10^{-1} – 1.2×10^{-5} mg/mL). Independent experiments are marked with different symbols and are average values of cell survival relative to average cell survival for DMSO control ± standard deviation, n=5-6. The dotted line is DMSO control which is 100% cell survival. The graph is based on average cell survival at each concentration tested, and is a sigmoid four parametric logistic curve with R^2=0.96

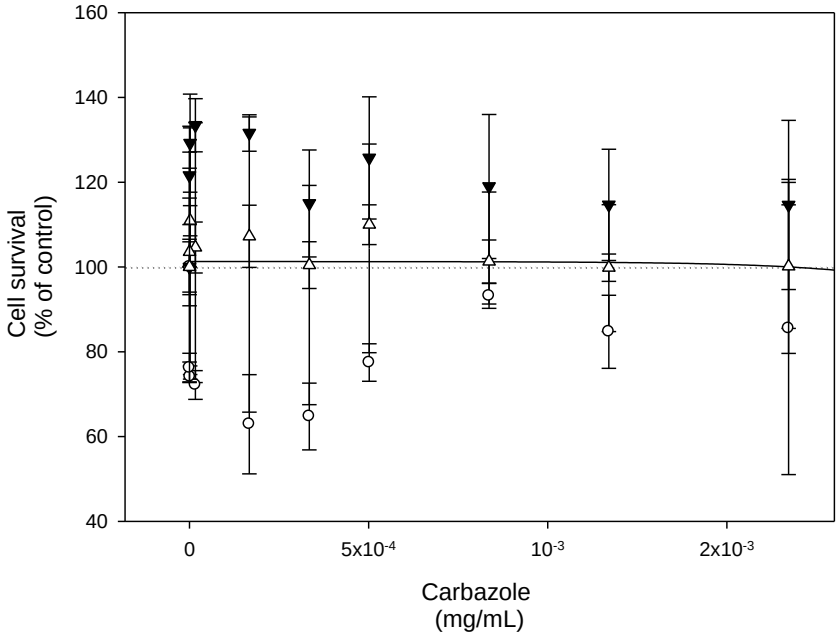

Figure 3.3. Cell survival in PLHC-1 cells presented as per cent of DMSO control after exposure to carbazole (1.7×10^{-3} – 1.7×10^{-7} mg/mL). Independent experiments are marked with different symbols and are average values of cell survival relative to average control values ± standard deviation, n=5-6. Control is 100% and indicated by the dotted line. The graph is based on average cell survival at each concentration tested, and is a sigmoid four parametric curve, R^2=0.013.

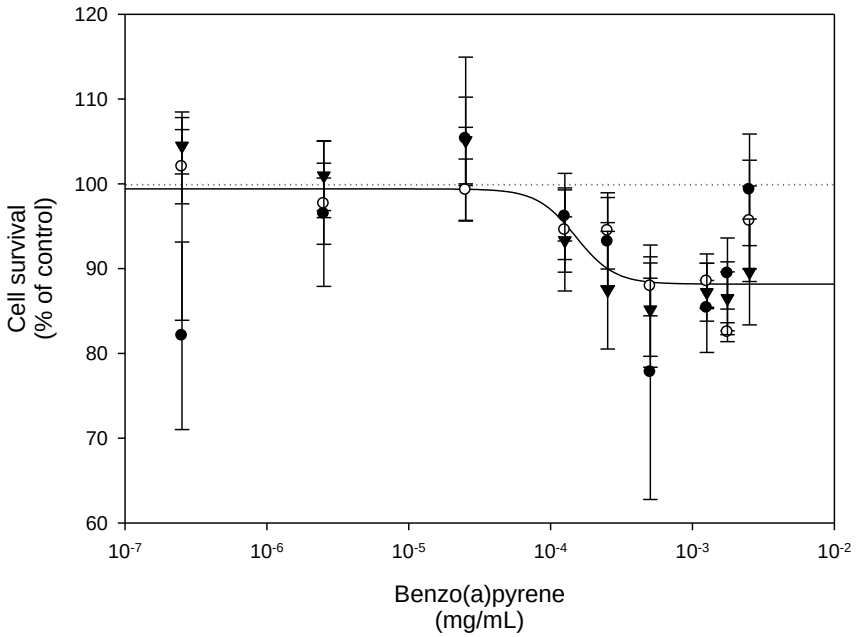

Figure 3.4. Cell survival in PLHC-1 cells presented as percent of DMSO control after exposure to benzo(a)pyrene (2.5×10^{-3} – 2.5×10^{-7} mg/mL). The x-axis is logarithmic. Independent experiments are marked with different symbols, and are average values of cell survival related to average control values ± standard deviation, n=5-6. Control is 100% and indicated by the dotted line. The graph is based on average cell survival at each concentration tested, and is a sigmoid four parametric curve, R^2=0.72.

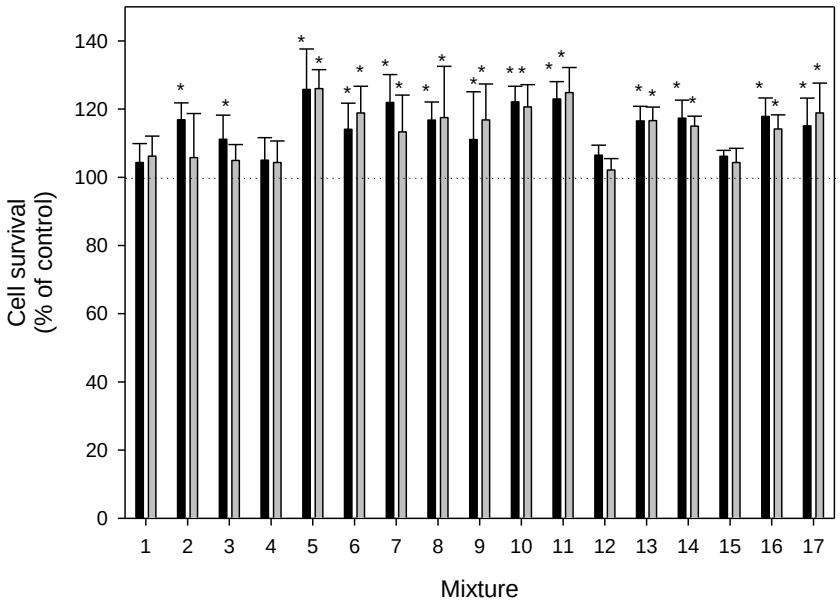

Figure 3.5. Cell survival in PLHC-1 cells presented as per cent of DMSO control after exposure to ternary mixtures. Two independent experiments were performed and are indicated in grey and black. Bars are average values of cell survival in relation to average cell survival in DMSO control ± standard deviation, n=5-6. The dotted line is DMSO control which is 100% cell survival. Mixtures resulting in significant higher per cent cell survival compared to average DMSO control are indicated with asterisk. Significance were tested with One Way ANOVA using Holm-Sidak post hoc test, $\alpha=0.05$ and $p<0.001$.

3.2 EROD assay

Results are presented as average values of three parallel samples as pmol product generated per minute per mg protein.

3.2.1 Concentration effect curves

Concentration-effect curves for DMP, carbazole and BaP are presented in Figure 3.6, 3.7 and 3.8, respectively. Raw data are presented in Appendix 3.

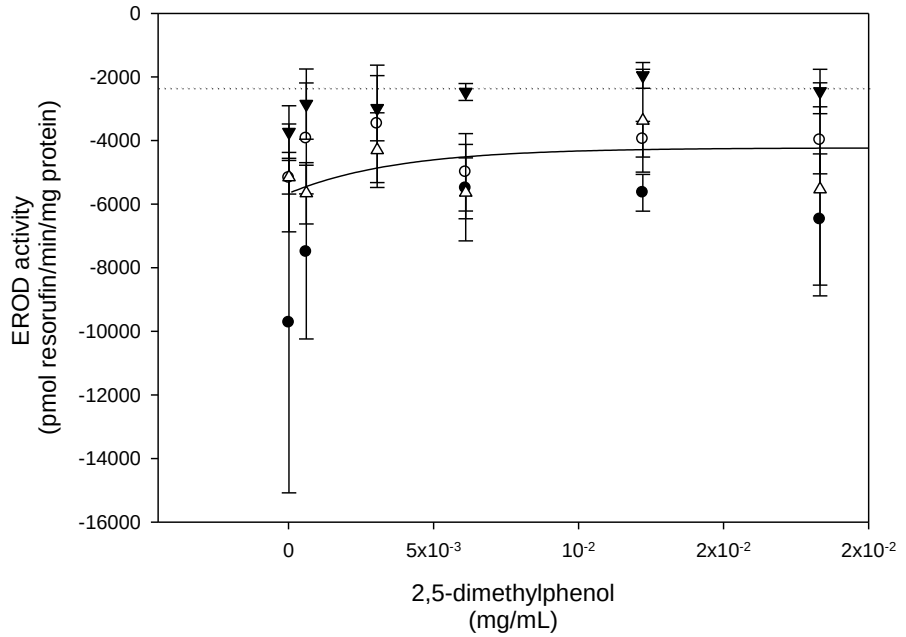

Figure 3.6. EROD activity (pmol resorufin/min/mg protein) in PLHC-1 after exposure to 2,5-dimethylphenol (1.8×10^{-2} – 1.2×10^{-5} mg/mL). Independent experiments are indicated with different markers and each point is average values ± standard deviation, n=2-3. None of the concentrations tested were significantly different from their respective DMSO control as tested with One Way ANOVA, α=0.05. The graph is based on average EROD activity at each concentration tested, n=3-4, and is exponential rise to maximum, single, three parametric curve, R^2=0.69. Average DMSO control is indicated with the dotted line.

Exposure of PLHC-1 to DMP caused overall non-significant reduced EROD activity compared to DMSO control. Variation was greater for some concentrations. Overall induced EROD activity was seen for carbazole, significant only for one replicate at the highest concentration tested, 1.2×10^{-3} mg/mL. Concentration-effect curve of EROD activity in PLHC-1 after exposure to BaP was bell shaped. Three concentrations showed significant induced EROD activity compared to respective DMSO control in all replicates. Highest activity was seen in cells exposed to 1.3×10^{-4} mg/mL BaP.

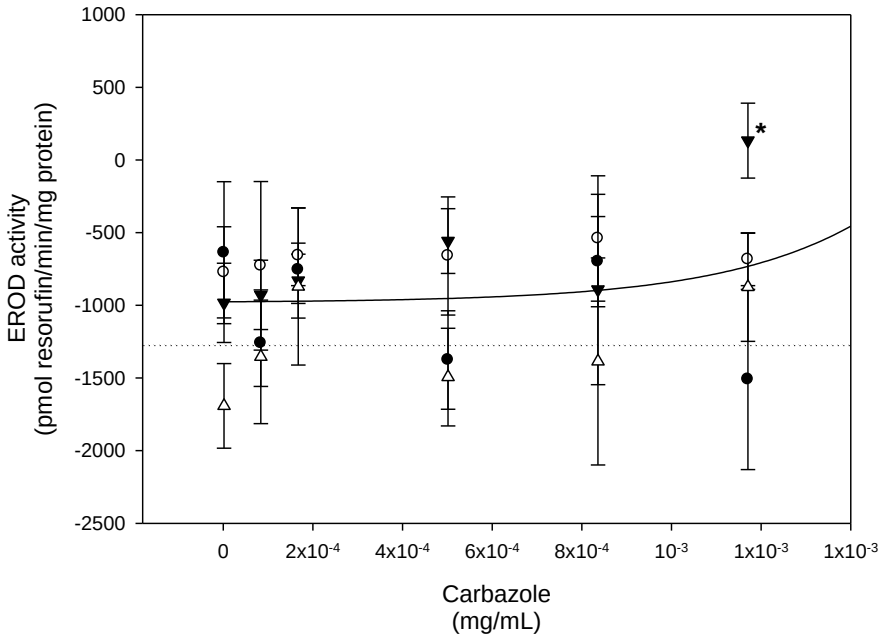

Figure 3.7. EROD activity in PLHC-1 presented as picomol resorufin per minute per mg protein (pmol resorufin/min/mg protein) after exposure to carbazole (1.2×10^{-3} – 1.7×10^{-6} mg/mL). Independent experiments are indicated with different markers and are average values ± standard deviation, n=3. One exposure group was found to be significant different from its respective DMSO control as tested with One Way Anova and Holm-Sidak post hoc test, α=0.05, p=0.005. This is indicated with an asterisk. The graph is based on average EROD activity at each concentration tested, n=4, and is exponential growth, single, three parametric, R^2=0.46. Average DMSO control is indicated with the dotted line.

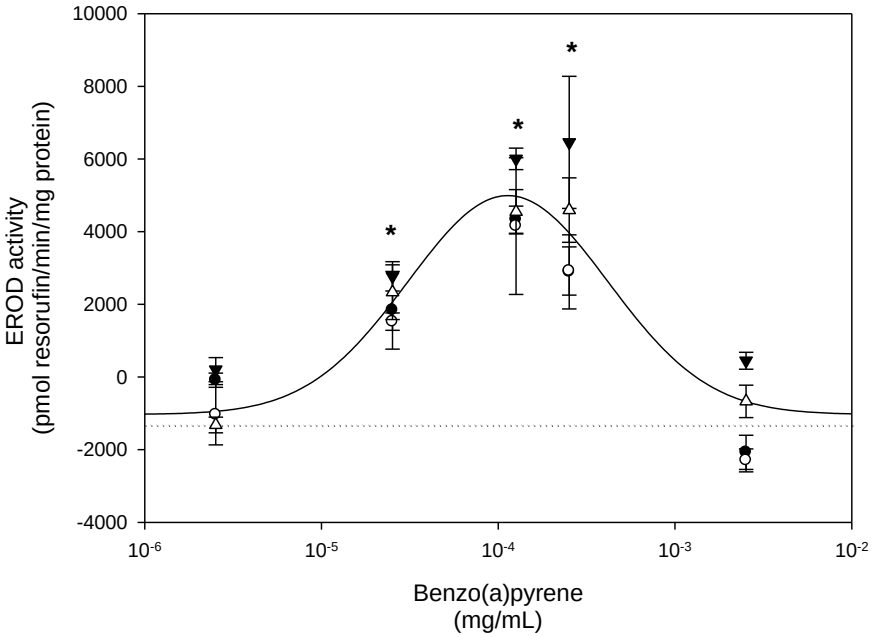

Figure 3.8. EROD activity in PLHC-1 presented as picomol resorufin per minute per mg protein (pmol resorufin/min/mg protein) after exposure to benzo(a)pyrene (2.5×10^{-3} – 2.5×10^{-6} mg/mL). The x-axis is logarithmic. Independent experiments are indicated with different markers and each point is average values ± standard deviation, n=2-3. Three exposure groups were significant different from their respective DMSO control in all experiments as tested with One Way Anova and Holm-Sidak post hoc test, α=0.05, p=<0.001. These are indicated with asterisks. The graph is based on average EROD activity at each concentration tested, and is a peak log normal four parametric curve, R^2=0.98. Average DMSO control is indicated with the dotted line.

3.2.2 Ternary mixtures and the PLS model

Five or six independent experiments were performed to determine catalytic EROD activity in PLHC-1 for the 17 ternary mixtures. The results were evaluated in excel and three replicates were used in PLS analyses using Modde 7.0 (Umetrics, Umeå, Sweden). Data on the PLS model are presented in Appendix. Replicates not included in the model are illustrated in column plots in Appendix 3, with raw data for the included replicates. The 17 mixtures were analysed in three groups; 1-6, 7-12 and 13-17, always including DMSO control (0.1%) with each analysis.

The model used was obtained after initially including all squared, crossed and interacting terms and then removing non-significant terms consecutively judging improvement by considering adjusted R^2. Appendix 5 includes tabulated values for R^2, adjusted R^2 and Q^2, together with number of LVs for each model investigated. The best model obtained excluded the two non-significant interactions, DMP×BaP and BaP×carbazole. The model have two LVs, $R^2 = 0.91$, adjusted $R^2 = 0.89$ and Q2 = 0.88.

A replicate plot is presented in Fig. 3.9. Variation between replicates is different for mixtures, but overall low. Lowest variation is seen for mixture 5, 7, 8, 11, while 1, 3, 4, 6, 12 and 14 show greater variation.

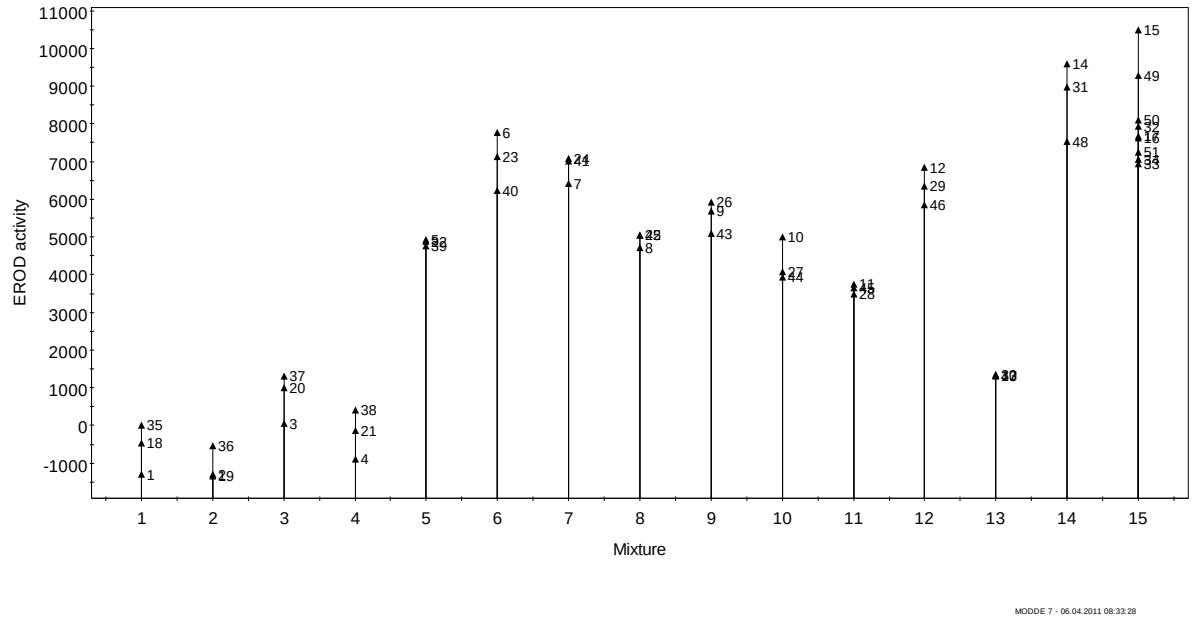

Figure 3.9. Replicate plot showing EROD activity (pmol resorufin/min/mg protein) in PLHC-1 after exposure ternary mixtures of high, low and medium concentrations of 2,5-dimethylphenol, carbazole and benzo(a)pyrene. Greater distance between markers indicates greater variation. Numbered markers are parallels in independent experiments: 1-17 the first individual experiment, 18-34 number two and 35-51 number three. Mixture 15 is mixtures for centre point. Composition of the mixtures is presented in Table 2.1.

Normal probability plot of the residuals is presented in Fig. 3.10 and Fig. 3.11 shows observed versus predicted values for EROD activity. Parallels 15 and 49 diverge from the line in Fig. 3.10 and 3.11 and are slightly higher than rest of the parallel samples for the center points in the replicate plot. These parallels were considered to be outliers and removed from the data set.

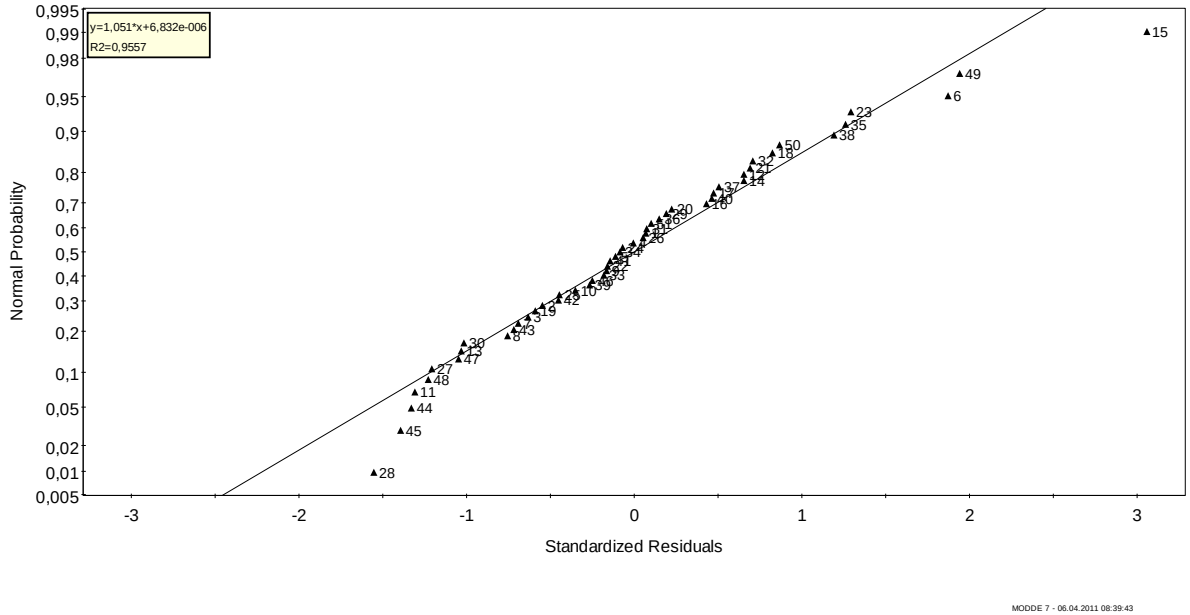

Figure 3.10. Normal probability plot for the model generated to study combined effects in mixtures with 2,5-dimethylphenol, carbazole and benzo(a)pyrene.

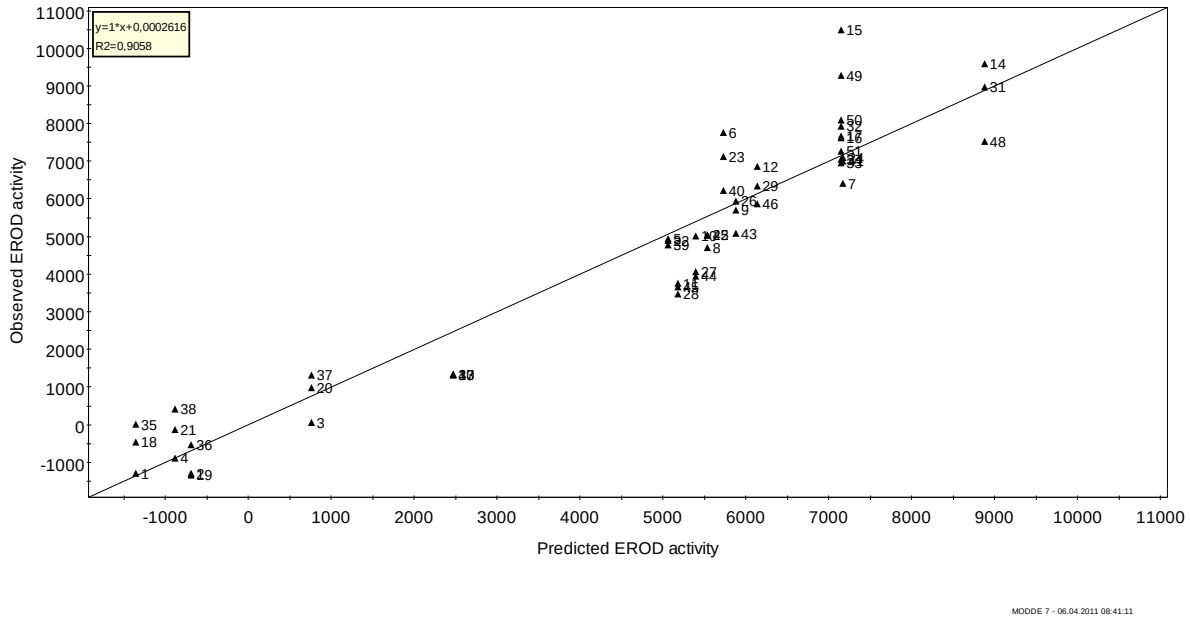

Figure 3.11. Predicted versus measured EROD activity (pmol/min/mg protein) using the best obtained model generated to study combined effect in ternary mixture with 2,5-dimethylphenol, carbazole and benzo(a)pyrene. Number 1-17 are parallel samples in the first individual experiment, 18-34 parallel samples in the second, and 35-51 in the third.

The new model have $R^2 = 0.93$, adjusted $R^2 = 0.92$ and $Q^2 = 0.90$ and two LVs. The first LV explains 80% of the variation and the second 12%. The model was significant at a 95%

confidence interval (Appendix 5). Lowest EROD activity after exposure to ternary mixtures was negative, -1 337 pmol resorufin/min/mg protein and caused by mixture 2, second replicate (19). Mixture 2 has high concentration of DMP and low of both carbazole and BaP (Table 2.1). Highest EROD activity, 9598 pmol resorufin/min/mg protein, was induced by mixture 14, first replicate (14). Mixture 14 has medium concentration of DMP and carbazole and high concentration of BaP. Predicted versus measured plot of values for EROD activity for the model used in this project is presented in Fig. 3.12.

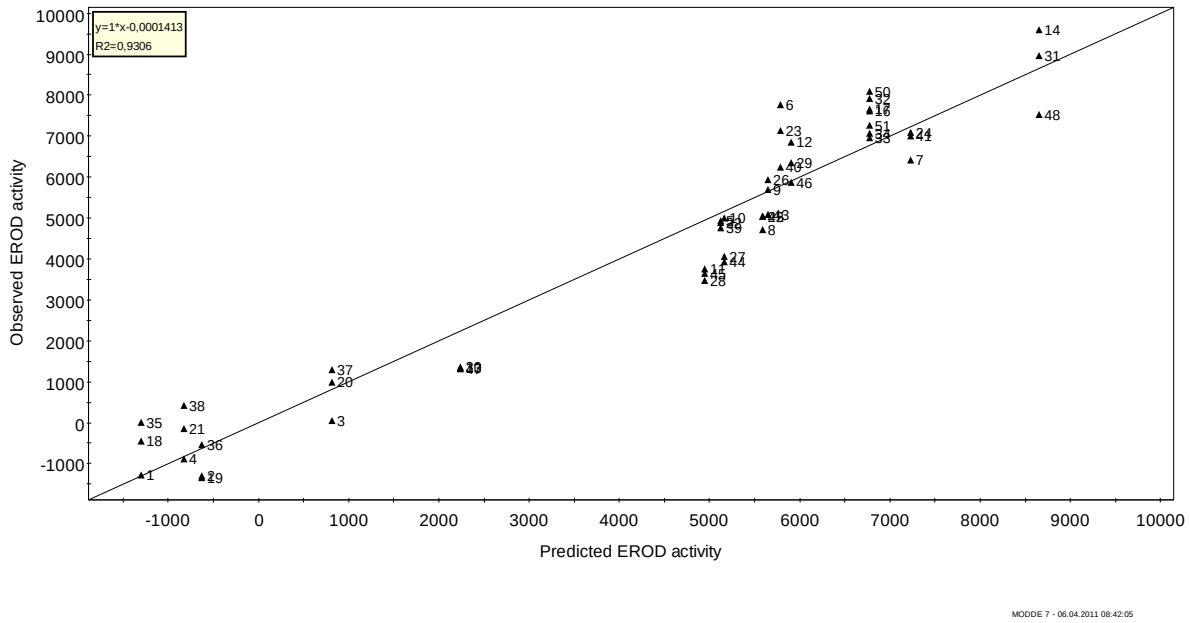

Figure 3.12. Predicted versus measured EROD activity (pmol/min/mg protein) from the PLS-model generated to study combined effects in ternary mixture 2,5-dimethylphenol, carbazole and benzo(a)pyrene. Number 1-17 are parallel samples in the first individual experiment, 18-34 parallel samples in the second, and 35-51 in the third.

Fig 3.13 is the coefficient plot for the PLS model. The model has seven PLS regression coefficients. The linear terms BaP ($p<0.001$) and carbazole ($p=0.006$) show a significant positive effect on EROD activity. The quadratic terms DMP^2, Car^2 and BaP^2 ($p<0.001$), together with the interaction term DMP×Car ($p=0.003$) show a significant negative effect on EROD activity. The linear term DMP ($p=0.15$) show non-significant negative effect on EROD activity.

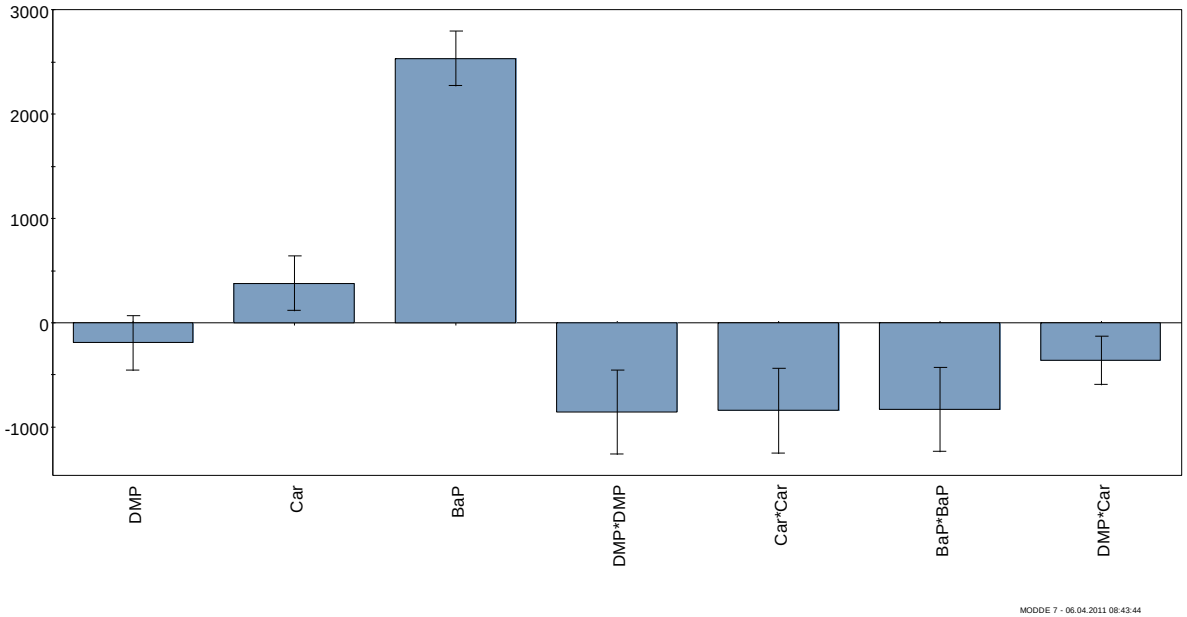

Figure 3.13. Scaled and centred PLS regression coefficients for a model of EROD activity in PLHC-1 exposed to ternary mixtures with 2,5-dimethylphenol (DMP), carbazole (car), and benzo(a)pyrene (BaP). Significant terms are the linear terms car and BaP, quadratic terms DMP^2, car^2, BaP^2, and also the interaction term DMP×Car.

The PLS regression model predicted combined effect of co-exposure to the compounds, visualized in 4D contour plots showing EROD activity after co-exposure of PLHC-1 to two compounds in three plots where the third compound is at low, medium and high concentration, from left to right in the figures. Predicted combined effect of BaP×Carbazole, DMP×BaP and carbazole×DMP are presented in Fig. 3.14, 3.15 and 3.16, respectively.

Fig 3.14 illustrates that both BaP and carbazole caused increased EROD activity when present in ternary mixtures. Co-exposure to the compounds caused higher EROD activity compared to single exposure. Co-exposure to BaP and DMP in ternary mixtures, Fig. 3.15, caused increased EROD activity for increasing concentrations of BaP, while DMP reduces EROD activity at higher concentrations. Fig. 3.16 shows that EROD activity was highest when both DMP and carbazole were present at medium concentrations.

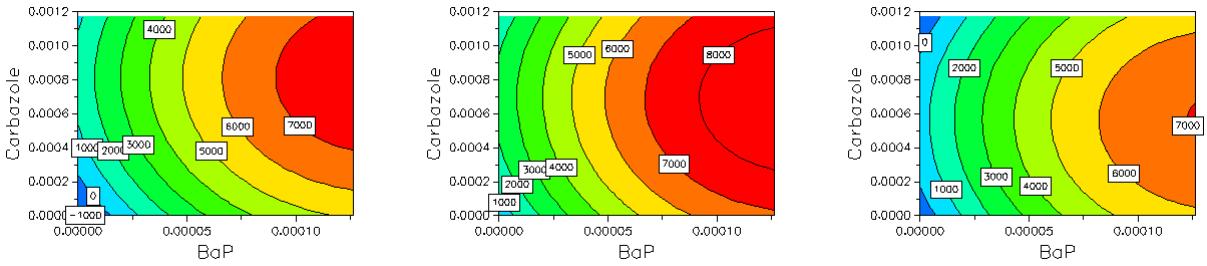

Figure 3.14. EROD activity (pmol resorufin/min/mg protein) predicted for co-exposure to carbazole and benzo(a)pyrene (BaP) (mg/mL) by a PLS model obtained after measuring EROD activity in PLHC-1 after exposure to ternary mixtures. DMP are set low (1.2×10^{-5} mg/mL), medium (9.3×10^{-3} mg/mL) and high (1.8×10^{-2} mg/mL) concentration from left to right.

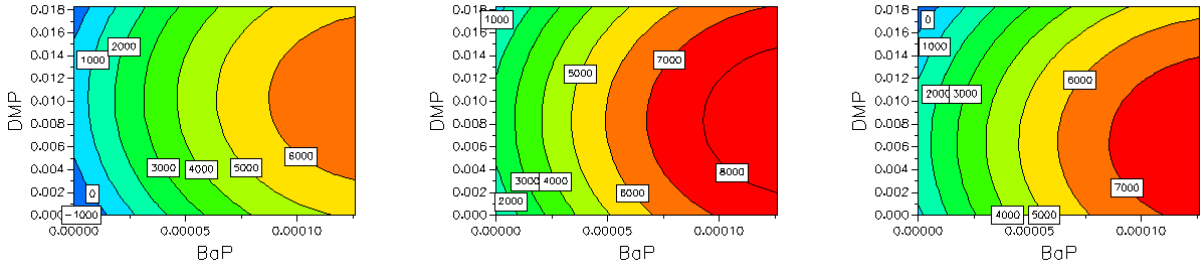

Figure 3.15. EROD activity (pmol resorufin/min/mg protein) predicted for co-exposure to 2,5-dimethylphenol (DMP) and benzo(a)pyrene (BaP) (mg/mL) by a PLS model obtained after measuring EROD activity in PLHC-1 after exposure to ternary mixtures. Carbazole are set to low (1.7×10^{-6} mg/mL), medium (5.9×10^{-4} mg/mL) and high (1.2×10^{-3} mg/mL) concentration from left to right.

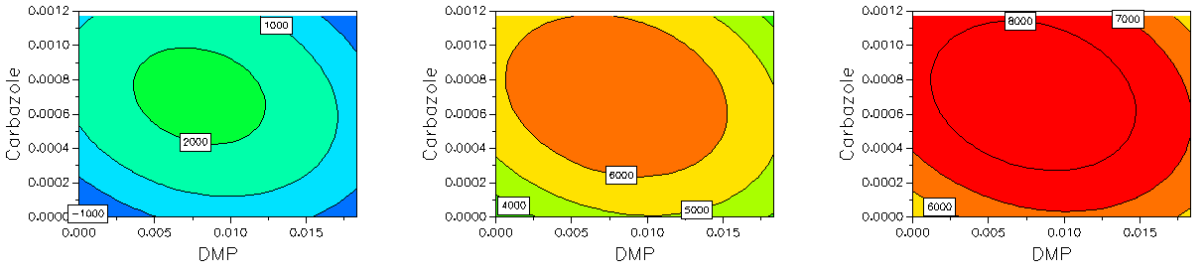

Figure 3.16. EROD activity (pmol resorufin/min/mg protein) predicted for co- exposure tocarbazole and 2,5-dimethylphenol (DMP) (mg/mL) by a PLS model obtained after measuring EROD activity in PLHC-1 after exposure to ternary mixtures. BaP are set to low (10^{-7} mg/mL), medium (6.3×10^{-5}) and high (1.3×10^{-4} mg/mL) concentration.

3.3 Genotoxicity of the ternary mixtures

DNA damage is presented as RNSB/kb. Extent of DNA damage was assessed by separating DNA on gel according to size. Median migration distance can be used to find RNSB/kb. Results from two independent experiments each with three parallels for the mixtures are illustrated in a column plot, Fig. 3.17. Calculated values are presented in Appendix 6, together with an illustration of the raw data. Raw data is not presented due to space limitations.

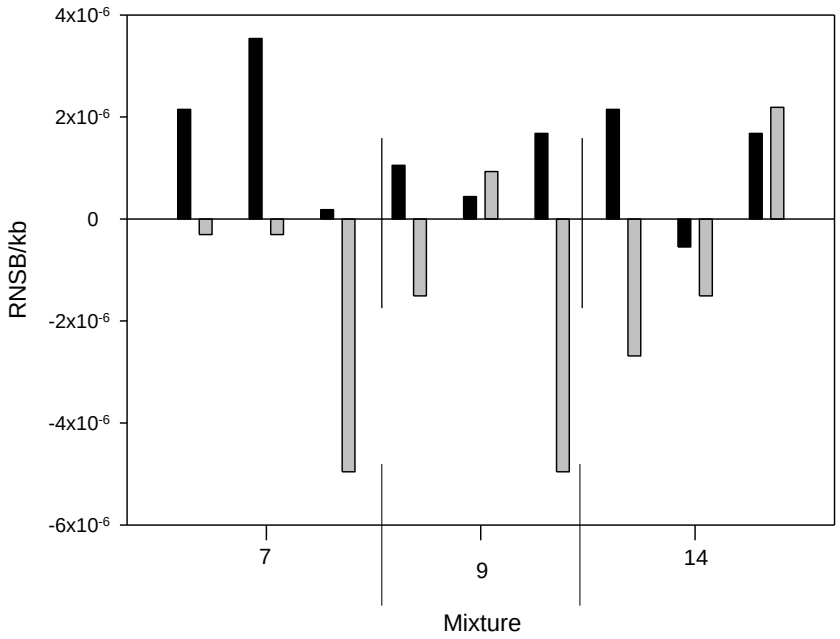

Figure 3.17. Relative number of DNA double strand breaks per kilo base pair (RNSB/kb) in PLHC-1 after exposure to three ternary mixtures, 7, 9 and 14. Two independent experiments were run with three parallels for each mixture. Independent experiments are indicated in black and grey and the parallel samples are presented as columns. Concentration of 2,5-dimethylphenol, carbazole and benzo(a)pyrene in the mixtures are presented in Table 2.1.

Fig. 3.17 shows substantial variation in in RNSB/kb. Analyses of DNA DSB were only performed with mixture 7, 9 and 14.

4 DISCUSSION

Results are discussed here in the same order as they are presented in Chapter 3. Cell concentration used in the MTT assay and cytotoxicity of single compounds and mixtures are discussed, as well as concentration-effect curves for EROD activity of single compounds. Optimising of the EROD protocol and confounding factors of EROD activity *in vitro* and *in vivo* are discussed. The statistical design and concentrations included in the design are discussed and the regression model is evaluated together with *in vitro* studies. Ternary mixtures are evaluated with respect to induced EROD activity and predicted interaction based on the regression model. Genotoxicity of the ternary mixtures is discussed before further work is suggested.

4.1 Cytotoxicity

4.1.1 Cell concentration

Cytotoxicity is more precisely determined using cell concentration within the linear area of formazan formation. In this master project the linear area was from zero to approximately 200 000 cells/mL for PLHC-1, Fig. 3.1. Higher cell concentrations showed increasing variation. A cell concentration of 75 000 cells/mL was used in further cytotoxicity testing. Cell concentration is the concentration initially seeded, determined by counting cells in grids on a haemocytometer.

The concentration determined in the MTT assay is assumed to be highly subjective since concentration was determined by counting cells manually. It was observed that cell concentration was dependent to considerable extent on the individual performing the experiment and also the cell line. The intention of the procedure is to identify optimal seeding density for further cytotoxicity testing, making consistent counting between experiments important. Non-consistent counting and a non-homogenous cell suspension can result in variability between experiments. The subjectivity of the determined cell concentration is suggested to be a problem if cell concentrations are compared between researchers.

Experiments with living systems are subject to variation, also highly controlled laboratory experiments. Non-homogeneous cell suspension of PLHC-1 was observed since the cells have a tendency of forming cell aggregates. Cell aggregates of PLHC-1 require vigorous shaking to disperse. Such treatment is assumed to significantly decrease cell survival. Homogeneity of cell suspension was fairly maintained by gently turning the tube containing cell suspension upside down a few times before distributing aliquots onto plates.

4.1.2 Cytotoxicity of single compounds

A concentration dependent increase in cytotoxicity in PLHC-1 exposed to DMP was observed from approximately 0.02 mg/mL, Fig. 3.2. Cells exposed to lower concentrations showed

slightly reduced cell survival compared to DMSO control. Results were variable between parallel samples and repeated experiments. Phenolic fraction of produced water was important contributor to acute toxicity determined by using the Microtox test (*Phosphobacterium phosphoreum*) (Johnsen et al., 1994). Lethality of APs to shrimp and salmon was seen to increase with increasing molecular weight and K_{ow} of compounds, with structure and position of the substituted carbon chain affecting toxicity. Concentration of C_4-AP causing 50% reduction in survival after 96 hours exposure was found to be 1.3×10^{-3} and 7.4×10^{-4} mg/mL for shrimp and salmon, respectively(Mcleese et al., 1981). Cytotoxicity of *p*-methyl phenol to a rainbow trout gonadal cell line (RTG-2) measured as inability to attach to growth surface were 2,85 mg/mL (Bols et al., 1985).

Defined cytotoxic effect was not seen in PLHC-1 after exposure to carbazole at the concentration range tested, Fig. 3.3. Variability between parallels and replicates was substantial. Neutral red assay with a rainbow trout liver cell line (RTL-W1) assessing cytotoxicity of carbazole showed no cytotoxic effect of concentrations as high as 0.01 mg/mL (Hinger et al., 2011), which is higher than the concentrations included in this master project. Studies with bacteria conclude that carbazole had no immediate toxic effect in the cells up to 2 mg/mL (Castorena et al., 2006). A sharp decline in neutral red uptake were seen for the NPAHs and azaarenes assessed by Jung and co-workers (2001) at concentrations approximately 15 times higher than concentrations tested in this master project.

Slight cytotoxicity was observed in PLHC-1 for BaP concentrations higher than approximately 10^{-4} mg/mL, Fig. 3.4. Variability between parallels and replicates was larger for certain concentrations. Previous studies of BaP using PLHC-1 and the neutral red assay with 24 hours incubation showed no cytotoxic effect for concentrations lower than 0.03 mg/mL (Fent and Bätscher, 2000). No observed effect concentrations obtained with Microtox test (*Vibrio fischeri*) for BaP was 2.3×10^{-3} and 0.019 mg/mL for 5 and 30 minutes exposure, respectively (Macken et al., 2008).

Variation between replicates and parallels in MTT cytotoxicity assay is suggested to be caused by non-homogenous cell suspension or variation in actual compound concentration. Significant loss of carbazole from solution is reported (Hinger et al., 2011). Loss of carbazole from test solutions could have resulted in variable exposure conditions in all assays performed in this master project. Concentration of compounds in test solution was not measured.

4.1.3 Cytotoxicity of ternary mixtures

Cytotoxic effects were not observed for the mixtures, Fig. 3.5, and approximately two thirds of the mixtures significantly induced cell survival. Induced cell survival after exposure to potentially harmful compounds can be due to hormesis. Maximum stimulatory response if hormesis is present is usually modest, at maximum 30 to 60%. Hormosis can be caused by direct stimulation or overcompensation. Proposed mechanisms for hormesis are numerous (Calabrese, 2008). A general mechanism is activated protective enzymatic pathways (Klaunig

and Kamendulis, 2008). True hormetic dose response cannot be established if high-dose inhibition is not detected (Calabrese, 2008).

4.2 EROD activity of single compounds, optimising the EROD protocol and confounding factors

Catalytic EROD activity is previously used as an approximation of CYP 1A activity in PLHC-1 and is assumed to be sufficient as a representation of available CYP 1A activity (Hahn et al., 1993).

4.2.1 Concentration effect curves

Overall DMP caused non-significant reduced EROD activity in PLHC-1 compared to DMSO, Fig. 3.6. Lower concentrations of DMP have higher variability compared to higher concentrations.

Long-chain APs are known to inhibit CYP 1A activity (Arukwe et al., 1997). Several mechanisms for interaction between AhR pathway and oestrogen receptor pathway are suggested (Brosens and Parker, 2003; Bemanian et al., 2004; Matthews and Gustafsson, 2006). Long-chain APs exhibit oestrogenic properties (Routledge and Sumpter, 1997; Arukwe et al., 2001) and likely inhibition of CYP 1A is via oestrogen receptor pathway (Andersson and Forlin, 1992). Oestrogenicity is assumed to require three or more carbon atoms in the hydrocarbon chain. Potent oestrogenic APs have the carbon chain placed in para position on an otherwise unhindered phenol ring (Routledge and Sumpter, 1997). Structure of DMP, Fig. 1.2, is suggested not to be consistent with oestrogenic activity.

One and four weeks exposure of Atlantic cod to a mixture of C_4 to C_7 APs resulted in dose dependent increase in CYP 1A content only in male fish and dose dependent increase in EROD activity only in female fish only after one week exposure (Hasselberg et al., 2004b). In cod orally exposed to the same mixture of C_4 to C_7 APs for 20 weeks, transcription of CYP 1A was up regulated (Lie et al., 2009). A small non-significant increase in cytotoxicity was observed for the concentration range of DMP included in the design.

Exposing PLHC-1 to a concentration range of carbazole induced EROD activity slightly. Enhanced activity was significant compared to DMSO control at highest concentration only for one replicate, Fig. 3.7. Heterocyclic compounds are suggested to contribute to increased CYP 1A activity observed for contaminated sediments *in vitro* (Brack and Schirmer, 2003). Azaarenes and NPAHs caused bell shaped induction of EROD activity and sigmoidal induction of CYP 1A protein. Potency of inducing EROD activity ($-\log EC_{50}$) of NPAHs and azaarenes correlated with log $K_{ow,}$ and L_{max}, respectively. Maximum enhanced EROD activity induced by these compounds was generally induced by concentrations between 10^{-6} and 10^{-5} M (Jung et al., 2001). This approximately corresponds to medium and high concentration of carbazole included in this master project

Molecular requirements describing relative affinity for AhR include electron acceptor capability, polarizability, planarity, steric fit and hydrophobicity (Mekenyan et al., 1996). Steric parameters are suggested more important for PAHs (Johnels et al., 1989), while for polychlorinated biphenyls (PCBs) co-planarity is important for steric fit and net polariziability for binding affinity. Polarizability should preferentially be distributed about a molecular length of 10.5 Å (McKinney and Singh, 1981). Position of nitration in nitrated compounds can affect polarizability and affinity for AhR (Lofroth et al., 1984). Carbazole is planar (Fig 1.3.) and has intermediate lipophilicity, log K_{ow} of 3.28 (Lassen and Carlsen, 1999). Nitrogen is a strongly electronegative atom (Solomons and Fryhle, 2004) and could result in polarization perpendicular to the length of the molecule. Common structural feature of PAHs with high AhR inducing potency is a four- to six-ring system containing fluoranthene or phenanthrene structures with an exposed bay region. Two and three ring un-substituted PAHs are generally not potent AhR inducers (Barron et al., 2004). And also for NSO-HETs the four and five ring compounds are suggested to be more potent compared to compounds with three rings at inducing EROD activity (Jung et al., 2001).

Inconsistency is seen for AhR affinity and potency of inducing CYP 1A activity (Piskorska-Pliszczynska et al., 1986; Hinger et al., 2011). Carbazole was showed to have affinity for the AhR in the DR-CALUX assay with H4IIE at concentrations of 0.167 mg/mL, but enhanced CYP 1A activity was not detected (Hinger et al., 2011).

Studies evaluating the EROD inducing potential of carbazole at concentrations relevant for produced water are not found. Carbazole is present in produced water and suggested to be a contributor to EROD inducing potential of produced water. Since carbazole is shown to have affinity for AhR (Hinger et al., 2011) it can be expected to have potential to modify CYP 1A activity. The affinity of carbazole for AhR was seen at concentrations higher than what is assumed to be relevant for produced water.

Competitive inhibition of EROD activity by NPAHs and azaarenes is seen (Jung et al., 2001) and cannot be excluded as the cause for the observed low EROD activity based on the results obtained in this master project.

Variability between parallels and individual experiments observed can be due to low efficiency and low potency of carbazole at inducing EROD activity as suggested by Hinger and co-workers (2011). Per cent relative standard deviation (RSD%) is a measure of experimental error. After exposure of PLHC-1 to BaP, RSD% for average values of EROD activity was ±35%. This is similar to RSD% obtained by Hinger and co-workers (2011). Experimental error is suggested not to be the cause for the observed variability between parallels and experiments in this master project. Significant loss of chemical reported for carbazole (Hinger et al., 2011) could contribute to variable exposure situations and variation observed. Hinger and co-workers suggests sorption or precipitation as possible causes for loss of chemical. Volatilization was also suggested, but was excluded since sealing the plates with

membrane did not prevent loss. Chemical reactions with other compounds present in test solution might also cause loss of chemical.

Low EROD activity measured after exposure to carbazole can be caused by sub-optimal exposure time. Exposure for 8 hours appears optimal for induction by BaP at the concentrations tested (results presented in this master project and Smeets et al. 2001), but can be different for carbazole. Co-exposure with DMP and BaP (Chapter 4.4) resulted in significant induction of EROD activity by carbazole.

Exposure of PLHC-1 to BaP produced a bell shaped concentration-effect curve, Fig. 3.8. Three of the concentrations tested resulted in significant increased EROD activity compared to DMSO control. Low and high concentration of BaP did not significantly alter EROD activity compared to control. The non-significantly increased cytotoxicity seen for highest exposure concentration of BaP is not assumed to reduce EROD activity.

Enhanced CYP 1A protein concentration and activity are previously seen for BaP (Smeets et al., 1999; Fent and Bätscher, 2000). Concentration-effect curves are bell shaped for EROD activity and sigmoidal for protein. Reduction in EROD activity at higher exposure concentrations is suggested to be caused by inhibition of catalytic activity by residual compound present in cells during activity assessment (Hahn et al., 1993; Brüschweiler et al., 1996; Hahn et al., 1996; Smeets et al., 1999; Fent and Bätscher, 2000). Competition was indeed confirmed to take place at the active site of the enzyme between dibenz(a,h)antracene and ER (Fent and Bätscher, 2000). Uncoupling of CYP 1A catalytic cycle is not detected after exposure to BaP (Schlezinger et al., 1999).

Induction of CYP 1A protein and activity after exposure to BaP is suggested to occur via an AhR independent pathway involving glycine-N-methyltransferase (GNMT) as a *trans*-acting transcription factor (Raha et al., 1995). High affinity of BaP for GNMT was observed in 14-day-old chick embryos (Raha et al., 1999). Studies with AhR deficient mice indicate that sustained transactivation of CYP 1A requires AhR (Foussat et al., 1998). The tetrameric form of the GNMT has methyltransferase activity, while the dimeric form binds PAHs. In humans GNMT sequesters BaP (Parkinson and Ogilvie, 2008).

4.2.2 Optimising the EROD protocol

Protocol for determining EROD activity was modified after a protocol presented by Fent and Bätscher (2000), Fig. 2.1. Cells were cultivated on 24 rather than 96 well plates to obtain sufficient quantities of material for analysing both EROD activity and total protein. It was considered more exact to do both assays on the same cell material. Wells on 24 well plates have well area about six times larger than 96 well plates. Suspension of cell content was necessary to determine EROD activity and total protein on same sample material. Previous experience, with the rat hepatoma cell line H4IIE, has shown that mechanical harvesting is sufficient for lysis of cells and suspension of cell content (Renate Haldsrund, pers. comm.) and was included in the protocol.

For EROD assay about one third of the cell suspension from each well on a 24 well plate was transferred to wells on a 96 well plate approximately doubling cell volume compared to Fent and Bätscher (2000). Double amount of ER resulted in higher EROD activity suggesting that substrate initially was a limiting factor. Double NADPH concentration showed no effect on EROD activity. Exogenous NADPH was found not to be a prerequisite for EROD activity in PLHC-1 (Hahn et al., 1996). The concentration of NADPH as described by Fent and Bätscher (2000) was still included in the protocol. The need for exogenous NADPH was not studied due to time limitations.

Harvesting was considered in two steps. First optimising involved a second freeze/thaw cycle of 30 to 60 minutes after adding 160 µL Tris to each well. This increased EROD activity suggested to be caused by more efficient lysis and detachment caused by crystals of Tris formed during freezing. The effect of three sequential freeze/thaw cycles was studied and was found to decrease EROD activity, suggested to be caused by increased degradation of enzyme.

Effect of a lower initial seeding density was considered by seeding 70% and 50% of the ordinarily seeded cell content. Dense growth conditions were contemplated to have an effect on enzyme activity. Less dense seeding did not enhance enzyme activity and dense seeding conditions are suggested not to have effect on enzyme activity.

Protein induction is considered to be a rapid response (Ryan and Hightower, 1994). Effect of shorter exposure time was studied. Temporal assessment of CYP 1A activity in PLHC-1 indicates that time for maximal activity is dependent on exposure compound and concentration. Exposure of PLHC-1 to 10^{-4} mg/mL BaP produced highest EROD activity after eight hours (Smeets et al., 1999). Changing the exposure time to eight hours increased enzyme activity and was included in the protocol. The preliminary protocol was verified with an identical performed experiment yielding similar response in EROD activity. This protocol was assumed adequate and was used when determining EROD activity.

The protocol presented by Jung and co-workers (2001) determines both EROD activity and total protein on the same plates as cells are seeded and appear as a good alternative providing less handling of sample by avoiding the harvesting step. Less handling is better when working with enzymes since enzyme activity is easily diminished. Number of steps in the deployed protocol can be reduced by assessing the requisite for NADPH, and also BSA which is included to preserve proteins, but found not to be of importance for EROD activity (Hahn et al., 1996).

4.2.3 Confounding factors

Catalytic activity of CYP 1A is regarded as both a biomarker of exposure to and effect of compounds (van der Oost et al., 2003). Induced CYP 1A activity is a transient, short time response and do not necessarily equate harmful effects *in vivo*. *In vivo* exposure to AhR

agonists are modulated by a range of confounding factors as age, sex, reproductive status and nutritional status (van der Oost et al., 2003; Hylland et al., 2009).

Discrepancy observed between CYP 1A catalytic activity and CYP 1A protein concentration (Hahn et al., 1993; Brüschweiler et al., 1996; Hahn et al., 1996; Smeets et al., 1999; Fent and Bätscher, 2000; Jung et al., 2001), is suggested to be due to competitive inhibition (Fent and Bätscher, 2000) or inactivation by formation of ROS at active site (Schlezinger et al., 1999). Comparing potency of two PCBs to TCDD at inducing EROD activity and CYP 1A protein showed substantially lower EROD activity at maximum for the two PCBs, but no difference in CYP 1A protein (Hahn et al., 1993). Incorrect assumptions about maximum induced EROD activity can underestimate EROD inducing potency of compounds.

Maximum EROD activity can be confounded by temporal response variations (Smeets et al. 1999 and results presented in this project), and optimum exposure time is dependent on exposure concentration. Three low concentrations (10^{-4}, 2.5×10^{-5} and 2.5×10^{-6} mg/mL) of BaP induced maximum EROD activity in five to eight hours (five, five and eight hours, respectively). Higher concentration induced increasing EROD activity until end of experiment at 48 hours (Smeets et al., 1999).

Temporal assessment of EROD activity could confirm that lack of activity of low activity are not due to unfavourable exposure time. Measureing protein and mRNA levels of CYP 1A in addition to EROD activity could reveal competitive inhibition. Non-competitive enzyme linked immunosorbent assay (ELISA) was suggested by Brüschweiler and co-workers (1996) as method for assessing CYP 1A protein levels. Real time, quantitative polymerase chain reaction (RT-qPCR) can be used to quantify mRNA levels of CYP 1A.

Modulated EROD activity is seen as a good indication of whether compounds have potential to interfere with biotransformation enzymes activity *in vitro*, and is indicated to precede potentially deleterious effects at higher levels of biological organization(van der Oost et al., 2003). A clear relationship is seen for tissue contaminant load, physiological status and EROD activity in Atlantic cod examined over an eight year period (Hylland et al., 2009).

4.3 The CCF design, the PLS model and *in vitro* studies

In this project interaction between DMP, carbazole and BaP was evaluated using CCF design and PLS regression modelling *in vitro*.

4.3.1 The CCF design and concentrations included

Modde 7 was used for generating both design and regression model. In Modde it is possible to choose between screening and response surface modelling (RSM). Screening identifies important variables contributing to the measured response. Detailed modelling using quadratic and cubic terms is possible using RSM (Umetrics, 2003). Important contributor was assumed to be BaP. Interacting effects were considered interesting to investigate.

When selecting RSM as purpose, it is possible to choose from five different designs in Modde. First recommended is Central Composite Face (CCF) design, while Box Behnken is recommended as second. Box Behnken design has fewer mixtures compared to CCF, 15 vs. 17, respectively, including a triplicate centre point for both, and is the economic alternative (Esbensen, 2000). Box Behnken design does not include experiments with all three compounds at high concentrations. It is important to have knowledge about interaction at high exposure concentrations. The preferred design alternative was CCF with three centre points. Centre points are included to investigate if non- linearity is present and to verify reproducibility (Esbensen, 2000).

Concentrations included in the design were based on cytotoxicity, EROD concentration effect curves of single compounds and environmental concentrations. High concentration of DMP was set to 0.018 mg/mL, which were the highest non cytotoxic concentration. Low concentration for DMP was set to 1.2×10^{-5} mg/mL. The concentrations included for DMP are environmentally realistic (Appendix 4). High concentration of carbazole was set to 1.2×10^{-3} mg/mL. High concentration was set to highest possible exposure concentration limited by aqueous solubility. Low concentration was set to 1.7×10^{-6} mg/mL. Reports of carbazole concentration in produced water are not found. High concentration of BaP was set to 1.3×10^{-4} mg/mL. This concentration induced highest EROD activity of the concentrations tested (Fig. 3.7). The high concentration chosen is within the same ranges as concentration of BaP used in others studies (Smeets et al., 1999; Fent and Bätscher, 2000). Low concentration was set to 10^{-7} and is environmentally realistic (Utvik, 1999).

4.3.2 The PLS model

In this project a PLS model was used to detect interaction between compounds. Fit of a model is sufficiently evaluated by considering values for R^2 and Q^2 (Lundstedt et al., 1998). The best model obtained had R^2 of 0.93 and Q^2 of 0.90. Two non-significant interaction terms were removed from this model. When removing non-significant terms adjusted R^2 was considered. The best obtained model had adjusted R^2 of 0.92. Biological models are considered acceptable with R^2 above 0.7 and Q^2 above 0.4 (Lundstedt et al., 1998). Explained and predicted variance, R^2 and Q^2, for the model obtained is better than the values suggested as acceptable indicating good explanatory and predicative power of the model. The model has two LVs.

Normal probability plot of the residuals was also used when evaluating the model. Residuals are variance not explained by the model (Esbensen, 2000). All points in Fig. 3.10 align along the regression line indicating that no important, systematic variation is excluded from the model.

Parallels 15 and 49 were considered to be deviating and removed from the model based on the observed versus predicted plot, Fig. 3.11, and Fig. 3.10. Observed versus predicted plot illustrates the predictive power of a model and parallels should fall close to the regression line

for good models. This is seen in Fig. 3.12. The model was considered good based on R^2, Q^2 and observed versus predicted plots.

4.3.3 *In vitro* studies for evaluating environmental effects

Environmental exposure of fish to produced water is assumed to involve myriads of compounds in various stages of degradation with varying composition between different locations (Utvik, 1999; Holth et al., 2008). Harmful compounds may have markedly different time of onset for toxic effect, some requiring long term, chronic exposure before effects are manifested (Calabrese, 1995). The sequential order of exposure may affect toxicity of compounds. Exposure of PLHC-1 to PCB 126 for 24 hours and then BaP for four hours increased metabolism of BaP and formation of RNA adducts compared to exposure to BaP singly (Smeets et al., 1999). Laboratory interaction studies can be limited by exposure regimes administering compounds simultaneously (Calabrese, 1995). Toxic effects observed *in vitro* can in *in vivo* environmental exposure be modulated by toxicokinetic factors affecting absorption and distribution of toxic compounds and by several natural stressors (Fent, 2001). Toxic effects of a compound are not manifested unless the active compound is transported to the site of toxic action at concentrations sufficient to produce a response over the duration of time required (Eaton and Gilbert, 2008).

The PLHC-1 cell line is derived from a tropical fish species. Assessing effects in a cell line from a temperate species is considered more relevant for produced water exposure. Results obtained with the cell line is assumed possible to transfer to cellular mechanics in more temperate species, since interaction between potentially harmful compounds and biological systems initiates at the molecular levels. Cellular effects are assumed, to a certain degree, to be universal across species (Fent, 2001; Castaño et al., 2003). Since interspecies differences exist, it was investigated if a cod cell line was obtainable and could be used. As far as is known, a stable continuous cod cell line is not developed.

Cells from PLHC-1 are assumed to be a good system for examining the relative CYP 1A inducing potency of aromatic hydrocarbons in fish (Hahn et al., 1993). Good correlation between toxicity *in vitro* and acute toxicity *in vivo* is observed for PLHC-1. Studies performed with this cell line are suggested to be a good approximation to environmental studies (Fent and Bätscher, 2000). The cell line is used in several studies for evaluating interaction between compounds (Fent and Bätscher, 2000; Jung et al., 2001).

4.4 Combined action in ternary mixtures

4.4.1 Combined action measured as EROD activity

Catalytic EROD activity in PLHC-1 after exposure to the ternary mixtures was significantly induced by the potent agonist BaP, which was approximately six times more potent than carbazole also inducing EROD activity significantly, Fig. 3.13. Regression coefficients describe the relationship between variation in a set of predictor variables and variation in the

response in a PLS model (Esbensen, 2000). Size of the coefficients illustrates change in response when factors are varied from 0 to 1, while the other factors are kept at average value. Coefficients are considered significant when confidence intervals do not cross zero (Umetrics, 2003). Significant enhanced EROD activity after exposure to BaP was expected based on single exposure (Fig. 3.8).

Significant induced EROD activity caused by carbazole appear conflicting to concentration-effect curves for EROD activity after exposure to carbazole singly (Fig. 3.7). The significant square terms modulating EROD activity negatively indicates non-linearity in the model. The one significant interaction term in the model, DMP×Carbazole, showed negative modulation of EROD activity. Interaction with DMP is suggested not to be the explanation of differently modulated EROD activity after combined exposure compared to single exposure to carbazole.

Predicted co-exposure of carbazole and BaP, Fig. 3.14, showed increasing EROD activity with increasing concentration of carbazole. Increased EROD activity after co-exposure to BaP and carbazole is not large compared to exposure to the compounds singly. Maximum induced EROD activity after exposure to BaP and carbazole singly caused a positive change of approximately 6 500 and 500 pmol resorufin/min/mg protein, respectively, compared to DMSO control. Maximum induced EROD activity after co-exposure at low concentration of DMP was predicted to 7000 pmol resorufin/min/mg protein (Fig. 3.14, left plot). Binary mixtures of PAHs have shown additive combined effects (Fent and Bätscher, 2000). Also binary mixtures of PAHs with either NPAHs or azaarenes show additive combined effect (Jung et al., 2001). Simple similar action or simple dissimilar action can result in additive combined effects, which would imply either same mechanism of action or different mechanisms with negative correlation or no correlation of susceptibility for the assessed compounds (Cassee et al., 1998). Different mechanisms of EROD induction could be suggested for carbazole and BaP since carbazole is shown to bind AhR (Hinger et al., 2011) while BaP is suggested to induce EROD activity via GNMT (Raha et al., 1999). Nature of additive combined effect can be studied by comparing EC_{50} for single exposure to EC_{50} of combined exposure as was done by Fent and Bätscher (2000) and Jung and co-workers (2001).

Reduced EROD activity was predicted at high concentrations of co-exposure, Fig. 3.14. Competitive inhibition has been observed for binary mixtures of PAHs (Fent and Bätscher, 2000), NPAHs and PAHs and also for azaarenes and PAHs (Jung et al., 2001) and is suggested to be the cause for the predicted decrease. By suggesting competitive inhibition at higher concentrations it is suggested that both carbazole and BaP interacts with CYP 1A. Monohydroxy metabolites of carbazole have been detected in bile of rainbow trout orally fed olive oil containing carbazole (Hellou et al., 2002), suggesting oxidative biotransformation of carbazole. In bacteria carbazole is metabolized by oxidases forming monohydroxy metabolites (Resnick et al., 1993; Shindo et al., 2001; Waldau et al., 2009). The discrepancy between EROD inducing potency for carbazole singly and in the model is suggested to be explained by the presence of an EROD inducing metabolite formed by catalytic action of

CYP 1A. Induced CYP 1A by BaP could enhance formation of the suggested carbazole metabolite in the ternary mixtures.

Carbazole was reported to inhibit EROD activity induced by a potent AhR agonist in embryo of killifish. The inhibition was assumed to be non-competitive based on kinetic analysis (Wassenberg et al., 2005). Non-competitive inhibition of enzymes occurs when substrate and inhibitor binds simultaneously to the enzyme at different binding sites (Berg et al., 2007). Enzyme inhibition can be studied by varying the substrate concentration.

The discrepancy between effects of carbazole singly compared to mixed exposure can be studied further by adding additional mixtures to the design. Mixtures with only two components present could affirm that the coefficient for carbazole is due to effects of carbazole, and not BaP.

Co-exposure to carbazole and DMP in ternary mixtures significantly inhibited EROD activity, Fig. 3.13, while DMP alone had a negative, non-significant effect. Negative interaction coefficients are suggested to be caused by either response addition with positive correlation or antagonism. Predicted interactions between the two compounds, Fig. 3.16, show similarity with isobolograms displaying antagonistic action between two compounds (Eide, 2002). Interaction between compounds is usually seen at high concentrations and the mechanism behind may be of biological or physiochemical nature. Chemical reactions between compounds could yield more or less toxic compounds compared to parent compound (Calabrese, 1995; Eide, 2002). Low concentration interaction usually is interaction in toxicokinetic phase (Eide, 2002). In *in vitro* studies either toxicokinetic interaction affecting biotransformation processes or chemical interaction are suggested as likely interactions and cause for the predicted interaction. Adding additional mixtures to the design could also contribute to understanding interaction between DMP and carbazole.

In all three figures predicting combined exposure, Fig. 3.14, 3.15 and 3.16, EROD activity was higher for medium concentrations and lower for low and high concentrations of DMP. Fig. 3.14 and 3.15 are similar in reversed order and are suggested to reflect the combined effect of DMP and carbazole. The effect of APs on EROD activity appear to be dependent on several factors as the specific compound studied, whether the study is *in vitro* or *in vivo*, sex, reproductive stage, route of exposure and if exposure is mixed or not.

Squared terms for all three compounds showed significant equal reduction of EROD activity, indicating that higher concentrations of DMP will interfere with EROD activity. Based on equal size of the square terms it is suggested that the general high contaminant load in the ternary mixtures interfere with EROD activity.

4.4.2 Combined action measured as genotoxicity

Exposure to produced water relevant compounds has effect on oxidative status in biological systems (Sturve et al., 2006; Bohne-Kjersem et al., 2009; Lie et al., 2009) and the GSH

dependent defence system (Hasselberg et al., 2004a). Produced water relevant compounds modulate CYP 1A activity (Goksoyr and Forlin, 1992; Aas et al., 2000; Hasselberg et al., 2004b; Hylland et al., 2009). Catalytic activity of CYP 1A enhance ROS (Halliwell and Gutteridge, 1989; Bondy and Naderi, 1994; Zangar et al., 2004) and form reactive metabolites (Baird et al., 2005). Increased use of GSH in conjugation reactions is suggested to interfere with the GSH dependent defence against oxidative stress. Enzymes catalysing conjugation reactions are regulated via nuclear receptors (Bock and Kohle, 2004). Increased EROD activity was found after exposure of PLHC-1 to ternary mixtures (Fig. 3.9) and it was considered relevant to assess DNA damage.

Clear modulation of DNA DSB was not seen in PLHC-1 exposed to three of the ternary mixtures, Fig. 3.17. These mixtures induced highest EROD activity and all have medium and high concentration of BaP. Based on this they were assumed to have high potency of inducing DNA damage. When these mixtures did not produce consistent trend in DNA DSB it was decided not to test the remaining mixtures. The method for determining DNA damage was not attempted improved due to time limitations.

Separation of DNA from PLHC-1 on agarose gel has previously been successfully used to determine DNA damage potential of ternary mixtures with cadmium, copper, nickel and zink using the same protocol as used in this project. Direct and indirect processes can result in DNA DSB and several DNA repair systems are involved in repairing DNA strand breaks. Genotoxic effect of metals can be due to inhibition of DNA repair (Dally and Hartwig, 1997). Higher proportion of CA and SCE were seen in cells with non-functioning NRE and HR (Meschini et al., 2008).

After exposure of PLHC-1 to 2.5×10^{-5} mg/mL BaP DNA adduct formation were found to be bell shaped with maximum amount of adducts detected after 12 hours exposure. Reduced amount of DNA DSB at longer exposure time was assumed to be due to enhanced activity of DNA repair mechanisms. Oxidative status was not affected and intrinsic GST activity suggested sufficient to detoxify activated BaP. Increasing RNA adducts concomitant with BaP concentration and concurrent with EROD activity were seen, and was proposed to be a better marker for DNA damage induced by reactive compounds (Smeets et al., 1999).

5 SUMMARY AND CONCLUSION

The aims of this master project were to determine carbazoles potential for modifying CYP 1A activity measured as EROD activity, to evaluate interaction in ternary mixtures with carbazole and two compounds representing fractions contributing to toxicity of produced water, and also to study correlation between biotransformation activity and genotoxicity by measuring DNA DSB.

It was hypothesized that compounds will modify EROD activity in PLHC-1 differently when present in ternary mixtures compared to single exposure. It was suggested that ternary mixtures inducing high EROD activity also will enhance formation of DNA DSB in PLHC-1 cells. Carbazole was suggested to modify EROD activity induced by the other compounds.

Exposure to carbazole modified EROD activity in PLHC-1 differently when exposed singly compared to exposure in ternary mixtures. Exposure in ternary mixtures resulted in significant induced EROD activity, while single exposure was significant only for one replicate at highest concentration. The difference can be suggested to be caused by an active metabolite of carbazole formed in oxidation catalyzed by CYPs. The significant, large, negative PLS regression coefficient for squared carbazole suggested interplay between carbazole and CYPs. Furthermore, BaP and carbazole can be suggested to induce EROD activity through different paths. Induction of CYPs by BaP is previously suggested to be via GNMT.

One interaction term was significant in the PLS regression model, DMP×Carbazole, which reduced EROD activity. The interaction between these compounds was suggested to be caused by toxicokinetic interaction affecting biotransformation enzymes or chemical interaction. How EROD activity is modified after exposure to DMP is suggested to be dependent on exposure conditions.

Carbazole was included in this project to study its potential for modifying CYP 1A activity singly and in mixtures. The results obtained suggest that carbazole contributes to EROD inducing potency in the ternary mixtures. Effect of carbazole on EROD activity is indicated to be dependent on co-exposed compounds, and how additional compounds present in produced water may affect the effect of carbazole are so far not known. Literature on concentration of carbazole in produced water and marine environment is not found. Environmental concentrations are likely to be lower than the highest concentration included and the results presented here are suggested to be relevant for effect of carbazole singly in the marine environment.

Further studies on carbazoles potential of interacting with CYP 1A is needed. Non-competitive ELISA or rt-qPCR are suggested as supplementary methods for obtaining information on protein or mRNA levels of CYP 1A in PLHC-1. Catalytic EROD activity after exposure to carbazole should also be evaluated as a function of time.

Catalytic EROD activity is presumed to be a good indication interplay between a compound and a biological systems. Determining extent of DNA damage by separating DNA electrophoretically did not give consistent result and the method is suggested to be more pertinent for determining genotoxicity of certain metals in PLHC-1. Statistical design and projection techniques are considered valuable tools when assessing toxicity of mixtures.

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APPENDICES

APPENDIX 1. SOLUTIONS

1. Cultivating cells

PBS, 1L, pH 7.4, autoclaved
- 8 g NaCl
- 0.2 g KCl
- 1.44 g Na_2HPO_4
- 0.24 g KH_2PO_4

2.5% Trypsin with 1% EDTA
- 1.07 g EDTA
- 20 mL 2.5% Trypsin.

Growth medium, 500 mL
- 500 mL Mimimal Essential Medium, Eagle's
- 25 mL FBS
- 5 mL Pencilin-streptomyocin

70 % Ethanol, 1L
- 730 mL 96% ethanol
- 270 mL distilled water

EDTA 0.5 M, 0.5 L, pH 8.0
- 93.5 g EDTA

2. MTT-assay

MTT-solution
- 0.005 g MTT salt
- 1 mL PBS

MTT/Medium-solution, adjusted for number of wells
- 20 µL MTT-solution
- 180 µL growth medium

3. EROD-assay

TRIS, 50 mM, 0.2 L, pH 7.8
1.2114 g
Distilled water

BSA 5.32 mg/mL, pH 7.8
106.4 mg BSA
20 mL 50 mM Tris

ER, 1000 µM,
1 mg ER
4.14 mL DMSO.

NADPH, pH 7.8
25 mg NADPH
1 mL 50 mM TRIS

4. Bradford-assay

BSA 1mg/mL
1 mg BSA
Autoclaved milli-Q-water

5. Gel Electrophoresis

10% SDS, 100 mL
10 g SDS
Distilled water

Digestion buffer, 200 mL, pH 8
1.1688 g NaCl
0.2423 g TRIS
10 mL 0.5 M EDTA
10 mL 10% SDS

Lambda-marker solution, 200 µL
100 µL λ/HIND III (50 µg)
37 µL λ-DNA (11 µg)
63 µL TE-buffer

5×TBE-buffer, 1 L, pH 8

54 g Trisbase
27.5 g H_3BO_3
20 mL 0.5 M EDTA

TE- buffer, 500 mL, pH 8

0.6057 g Tris
1 mL 0.5 M EDTA

EDTA 0.5 M, 0.5 L, pH 8

93.5 g EDTA

APPENDIX 2. MTT CYTOTOXICITY ASSAY

Raw data and calculated average absorbance values for linear area for formazan formation for MTT assay in PLHC-1 are presented in Table 1. Raw data and calculated average percent cell survival in PLHC-1 are presented after exposure to concentration range of DMP, carbazole, BaP and mixtures in Table 2, 3, 4 and 5. Absorbance was measured at 550 nm with a plate reader. Values greater, smaller or equal to average values ± 2 standard deviations were considered outliers and disregarded. Individual experiments are marked with date.

Average percent cell survival was calculated using equation 1:

$$\%\ Cell\ survival = \frac{1}{n}\sum_{\forall parallels,i}\frac{x_i}{Av} \times 100 \quad (1)$$

x_i = values of absorbance of parallels
n = number of parallels
Av. = average absorbance of DMSO control treated cells

Table 1.

Friday 17.09.10

Conc. (cells/mL)		500000	400000	250000	150000	100000	75000	50000	25000	15000	0
Parallells	B	2.253	1.937	1.348	1.216	1.114	0.741	0.592	0.229	0.120	0.053
	C	1.855	1.922	1.411	1.538	0.959	0.837	0.559	0.205	0.128	0.051
	D	1.968	1.859	1.305	1.481	1.123	0.884	0.608	0.236	0.133	0.050
	E	1.911	1.843	1.366	1.577	1.239	0.797	0.587	0.253	0.147	0.048
	F	2.059	1.919	1.361	1.373	1.044	0.915	0.553	0.253	0.157	0.053
	G	1.861	1.628	1.117	1.566	0.981	0.656	0.488	0.184	0.191	0.060
Av. absorbance		1.985	1.851	1.318	1.459	1.077	0.805	0.565	0.227	0.146	0.053
Std. dev.		0.15	0.12	0.10	0.14	0.10	0.10	0.04	0.03	0.03	0.00

Thursday 23.09.10

Conc. (cells/mL)		200000	150000	125000	90000	75000	60000	50000	40000	25000	15000
Parallells	B	1.698	1.279	0.966	0.723	0.764	0.553	0.398	0.306	0.257	0.106
	C	1.565	1.171	0.863	0.727	0.673	0.586	0.417	0.326	0.221	0.124
	D	1.762	1.302	1.011	0.804	0.749	0.552	0.400	0.327	0.197	0.114
	E	1.440	1.209	1.045	0.828	0.773	0.581	0.448	0.338	0.205	0.142
	F	1.629	1.264	1.064	0.752	0.798	0.555	0.434	0.295	0.163	0.127
	G	1.470	1.366	0.913	0,183*	0.698	0.545	0.437	0.307	0.206	0.133
Av. absorbance		1.594	1.265	0.977	0.767	0.743	0.562	0.422	0.317	0.208	0.124
Std. dev.		0.13	0.07	0.08	0.05	0.05	0.02	0.02	0.02	0.03	0.01

Friday 24.09.10

Conc. (cells/mL)		750000	500000	400000	250000	150000	100000	75000	50000	25000	15000
Parallells	B	3.053	2.290	2.124	1.597	1.155	0.819	0.708	0.522	0.229	0.136
	C	3.162	2.385	2.130	1.646	1.215	1.042	0.662	0.471	0.209	0.129
	D	3.044	2.681	2.253	1.850	1.376	1.092	0.838	0.503	0.246	0.139
	E	3.406	2.622	2.140	1.857	1.259	1.145	0.773	0.524	0.238	0.165
	F	3.129	2.732	2.329	1.818	1.415	1.192	0.781	0.530	0.275	0.167
	G	3.521	2.422	2.062	1.732	1.180	1.055	0.869	0.500	0.264	0.168
Av. absorbance		3.219	2.522	2.173	1.754	1.267	1.058	0.772	0.508	0.244	0.151
Std. dev.		0.20	0.18	0.10	0.12	0.11	0.13	0.08	0.02	0.02	0.02

Saturday 25.09.10

Conc. (cells/mL)		500000	400000	250000	150000	125000	100000	75000	60000	50000	25000
Parallells	B	2.447	2.253	1.741	1.290	1,549*	0.940	0.727	0.560	0.457	0.218
	C	3.493	2.653	1.819	1.353	1.142	0.912	0.724	0.610	0.441	0.194
	D	2.414	2.145	1.713	1.249	1.051	0.870	0.737	0.633	0.462	0.224
	E	2.454	2.177	1.916	1.398	0.974	0.892	0.753	0.579	0.422	0.200
	F	2.358	1.995	1.856	1.179	1.013	0.722	0.826	0.597	0,698*	0.195
	G	2.461	2.117	1.830	1.098	1.087	0.865	0.645	0.568	0.473	0.189
Av. absorbance		2.605	2.223	1.813	1.261	1.053	0.867	0.735	0.591	0.451	0.203
Std. dev.		0.44	0.23	0.07	0.11	0.07	0.08	0.06	0.03	0.02	0.01

* = values greater, smaller or equal to average absorbance ±2 std.dev.

Table 2.

Tuesday 26.10.10

2,5-dimethyl phenol	µM	1000	800	500	300	100	50	10	1	0.1	0
	mg/mL	1.2E-01	9.8E-02	6.1E-02	3.7E-02	1.2E-02	6.1E-03	1.2E-03	1.2E-04	1.2E-05	0.0
Absorbance (550 nm)	B	0.409	0.422	0.500	0.576	0.752	0.688	0.704	0.610	0.589	0.647
	C	0.428	0.445	0.453	0.556	0.681	0.718	0.594	0.413	0.560	0.641
	D	0.430	0.393	0.460	0.523	0.692	0.701	0.670	0.679	0.647	0.808
	E	0.416	0.376	0.533	0.589	0.643	0.682	0.691	0.591	0.583	0.675
	F	0.353	0.381	0.466	0.633	0.570	0.674	0.475	0.596	0,279*	0.718
	G	0.344	0.408	0.524	0.612	0.758	0.687	0.699	0.592	0.569	0.695
Av. % Cell survival		57	58	70	83	98	99	92	83	85	100
% Std. dev.		5	4	5	6	10	2	13	13	5	9

Thursday 4.11.10

2,5-dimethyl phenol	µM	1000	800	500	300	100	50	10	1	0.1	0
	mg/mL	1.2E-01	9.8E-02	6.1E-02	3.7E-02	1.2E-02	6.1E-03	1.2E-03	1.2E-04	1.2E-05	0
Absorbance (550 nm)	B	0.430	0.413	0.380	0.660	0.655	0.713	0.614	0.695	0.703	0.566
	C	0.359	0.364	0.358	0.587	0.639	0.711	0.765	0.737	0.677	0.706
	D	0.326	0.327	0.335	0.600	0.668	0.719	0.754	0.596	0.681	0.695
	E	0.359	0.320	0.348	0.620	0,604*	0.676	0.761	0.774	0.733	0.721
	F	0.307	0.333	0.292	0.618	0.647	0.559	0.682	0.722	0.770	0.721
	G	0.398	0.342	0.335	0.565	0.642	0.660	0.626	0.676	0.738	0.753
Av. % Cell survival		52	50	49	88	94	97	101	101	103	100
% Std. dev.		7	5	4	5	2	9	10	9	5	9

Thursday 02.12.10 A

2,5-dimethyl phenol	µM	450	400	350	300	250	200	150	100	50	0
	mg/mL	5.5E-02	4.9E-02	4.3E-02	3.7E-02	3.1E-02	2.4E-02	1.8E-02	1.2E-02	6.1E-03	0
(550 Absorbance nm)	B	0.560	0.550	0.599	0.710	0.709	0.754	0.717	0.738	0.711	0.797
	C	0.615	0.568	0.554	0.697	0.727	0.800	0.719	0.704	0.714	0.624
	D	0.612	0.574	0.562	0.693	0.725	0.691	0.745	0.800	0.775	0.812
	E	0.648	0.559	0.618	0.695	0,802*	0.728	0.800	0.746	0.778	0.842
	F	0.574	0.619	0.660	0.740	0.734	0.501	0.761	0.818	0.751	0.948
	G	0.650	0.722	0.872	0.774	0.748	0.829	0.939	0.853	0.853	0.939
Av. % Cell survival		74	72	78	87	88	87	94	94	92	100
% Std. dev.		4	8	14	4	2	14	10	7	6	14

Thursday 02.12.10 B

2,5-dimethyl phenol	µM	450	400	350	300	250	200	150	100	50	0
	mg/mL	5.5E-02	4.9E-02	4.3E-02	3.7E-02	3.1E-02	2.4E-02	1.8E-02	1.2E-02	6.1E-03	0
Absorbance (550 nm)	B	0.717	0.595	0.651	0.759	0.777	0.798	0.745	0.821	0.760	0.779
	C	0.604	0.528	0.648	0.755	0.728	0.705	0.775	0.747	0.754	0.867
	D	0.642	0.525	0.617	0.692	0.771	0.709	0.823	0.721	0.759	0.826
	E	0.694	0.566	0.592	0.592	0.710	0.743	0.768	0.692	0.805	0.875
	F	0.573	0.569	0.626	0.707	0.706	0.700	0.704	0.743	0.791	0.753
	G	0.680	0.628	0.717	0.673	0.776	0.797	1,155*	0.721	0.852	0.914
Av. % Cell survival		78	68	77	83	89	89	91	89	94	100
% Std. dev.		7	5	5	7	4	5	5	5	5	7

* = values greater, smaller or equal to average absorbance ±2 std.dev.

Table 3.

Thursday 28.10.10

Carbazole	(µM)	1000	800	500	300	100	50	10	1	0.1	0
	(mg/mL)	1.7E-01	1.3E-01	8.4E-02	5.0E-02	1.7E-02	8.4E-03	1.7E-03	1.7E-04	1.7E-05	0.0E+00
Absorbance (550 nm)	B	0.343	0.318	0.318	0.320	0.561	0.350	0.680	0.604	0.592	0.521
	C	0.347	0.292	0.299	0.352	0.628	0.364	0.626	0.516	0.653	0.545
	D	0.366	0.343	0.310	0.364	0.579	0.607	0.574	0.593	0.540	0.466
	E	0.368	0.297	0.306	0.380	0.589	0.699	0.610	0.623	0.552	0.453
	F	0.339	0.319	0.328	0.391	0.650	0.411	0.582	0.632	0.708	0.570
	G	0.365	0.307	0.349	0.387	0.607	0.346	0.681	0.669	0.573	0.666
Av. % Cell survival		66	58	59	68	112	86	117	113	112	100
% Std. dev.		2	3	3	5	6	28	9	10	12	14

Thursday 11.11.10

Carbazole	(µM)	10	7	5	3	2	1	0.1	0.01	0.001	0
	(mg/mL)	1.7E-03	1.2E-03	8.4E-04	5.0E-04	3.3E-04	1.7E-04	1.7E-05	1.7E-06	1.7E-07	0.0E+00
Absorbance (550 nm)	B	0.102	0.485	0.488	0.407	0.327	0.332	0.413	0.405	0.416	0.585
	C	0.544	0.462	0,403*	0.444	0.278	0.318	0.369	0.400	0.381	0.567
	D	0.499	0.503	0.494	0.403	0.346	0.361	0.386	0,353*	0.399	0.522
	E	0.596	0.403	0.529	0.388	0.383	0.233	0.373	0.397	0.422	0.551
	F	0.595	0.399	0.501	0.448	0.386	0.381	0.391	0.400	0.431	0.501
	G	0.439	0.498	0.508	0.424	0.381	0.417	0.410	0.402	0.424	0.520
Av. % Cell survival		85	85	93	77	65	63	72	74	76	100
% Std. dev.		34	9	3	4	8	12	3	1	3	6

Thursday 02.12.10 A

Carbazole	(µM)	10	7	5	3	2	1	0.1	0.01	0.001	0
	(mg/mL)	1.7E-03	1.2E-03	8.4E-04	5.0E-04	3.3E-04	1.7E-04	1.7E-05	1.7E-06	1.7E-07	0.0E+00
Absorbance (550 nm)	B	0.748	0.696	0.646	0.593	0.708	0.757	0.755	0.743	0.627	0.606
	C	0.780	0.685	0.692	0.757	0.679	0.793	0.773	0.707	0.714	0.706
	D	0.657	0.531	0.803	0.742	0.718	0.766	0.786	0.708	0.716	0.441
	E	0.733	0.718	0.772	0.749	0.552	0.780	0.785	0.751	0.692	0.691
	F	0.726	0.753	0.546	0.819	0.676	0.780	0.862	0.851	0.769	0.362
	G	0.455	0.717	0.796	0.836	0.779	0.830	0.810	0.861	0.830	0.770
Av. % Cell survival		115	115	119	126	115	132	133	129	122	100
% Std. dev.		20	13	17	14	13	4	6	12	12	27

Thursday 02.12.10 B

Carbazole	(µM)	10	7	5	3	2	1	0.1	0.01	0.001	0
	(mg/mL)	1.7E-03	1.2E-03	8.4E-04	5.0E-04	3.3E-04	1.7E-04	1.7E-05	1.7E-06	1.7E-07	0.0E+00
Absorbance (550 nm)	B	0.694	0.671	0.653	0.737	0.674	0.774	0.660	0.782	0.751	0.627
	C	0.785	0.682	0.670	0.773	0.676	0.676	0.658	0.717	0.709	0.627
	D	0.679	0.655	0.639	0.723	0.694	0.729	0.757	0.723	0.685	0.681
	E	0.403	0.703	0.728	0.728	0.713	0.769	0.715	0.759	0.534	0.723
	F	0.741	0,555*	0.711	0.699	0.606	0.651	0.697	0.748	0.733	0.657
	G	0.743	0.649	0.690	0.782	0.694	0.732	0.738	0.751	0.771	0.724
Av. % Cell survival		100	100	101	110	100	107	105	111	104	100
% Std. dev.		21	3	5	5	6	7	6	4	13	7

* = values greater, smaller or equal to average absorbance ±2 std.dev.

Table 4.

Thursday 02.12.10 A

Benzo(a) pyrene	(µM)	10	7	5	2	1	0.5	0.1	0.01	0.001	0
	(mg/mL)	2.5E-03	1.8E-03	1.3E-03	5.0E-04	2.5E-04	1.3E-04	2.5E-05	2.5E-06	2.5E-07	0
Absorbance (550 nm)	B	0.306	0.319	0.273	0.298	0.328	0.341	0.341	0.315	0.298	0.338
	C	0.303	0.280	0.298	0.288	0.318	0.314	0.350	0.327	0.280	0.334
	D	0.329	0.286	0.274	0.160	0.294	0.292	0.356	0.279	0.209	0.337
	E	0.328	0.300	0.301	0.268	0.310	0.328	0.380	0.311	0.306	0.368
	F	0.332	0.289	0.285	0.260	0.280	0.309	0.288	0.308	0.246	0.288
	G	0.363	0.292	0.255	0.262	0.310	0.315	0.365	0.365	0.282	0.310
Av. % Cell survival		99	89	85	78	93	96	105	96	82	100
% Std. dev.		7	4	5	15	5	5	10	9	11	8

Thursday 02.12.10 B

Benzo(a) pyrene	(µM)	10	7	5	2	1	0.5	0.1	0.01	0.001	0
	(mg/mL)	2.5E-03	1.8E-03	1.3E-03	5.0E-04	2.5E-04	1.3E-04	2.5E-05	2.5E-06	2.5E-07	0
Absorbance (550 nm)	B	0.364	0.278	0.285	0.291	0.323	0.296	0.342	0.344	0.349	0.303
	C	0.288	0,287*	0.295	0.303	0.305	0.310	0.318	0.319	0.324	0.402
	D	0.328	0.280	0.297	0.296	0.312	0.331	0.325	0.314	0.338	0.289
	E	0.319	0.286	0.298	0.319	0.307	0.313	0.345	0.320	0.360	0.333
	F	0.325	0.276	0.313	0.285	0.331	0.338	0.350	0.335	0.364	0.340
	G	0.322	0.279	0.313	0.295	0.344	0.336	0.340	0.355	0.341	0.368
Av. % Cell survival		96	82	89	88	94	95	99	98	102	100
% Std. dev.		7	1	3	3	4	5	4	5	4	12

Thursday 09.12.10 B

Benzo(a) pyrene	(µM)	10	7	5	2	1	0.5	0.1	0.01	0.001	0
	(mg/mL)	2.5E-03	1.8E-03	1.3E-03	5.0E-04	2.5E-04	1.3E-04	2.5E-05	2.5E-06	2.5E-07	0
Absorbance (550 nm)	B	0.324	0.305	0.299	0.309	0.322	0.348	0.347	0.368	0.349	0.340
	C	0.330	0.302	0.294	0.281	0.276	0.311	0.355	0.364	0.354	0.357
	D	0.327	0.316	0.316	0.272	0.273	0.313	0.364	0.339	0.370	0.336
	E	0.327	0.288	0.291	0.324	0.326	0.301	0.367	0.353	0.372	0.360
	F	0.287	0.282	0.308	0.293	0.320	0.332	0.371	0.332	0.380	0.347
	G	0.283	0.320	0.320	0.306	0.316	0.351	0.399	0.360	0.365	0.356
Av. % Cell survival		90	86	87	85	87	93	105	101	104	100
% Std. dev.		6	4	3	6	7	6	5	4	3	3

* = values greater, smaller or equal to average absorbance ±2 std.dev.

Table 5.

Sunday 06.03.10 Mix. 1-9 (1)

Mixture		1	2	3	4	5	6	7	8	9	0
Absorbance (550 nm)	B	0.606	0.637	0.580	0.615	0.632	0.655	0.687	0.705	0.600	0.680
	C	0.585	0.697	0.625	0.633	0.663	0.602	0.656	0.676	0.645	0.543
	D	0.579	0.687	0.665	0.550	0.787	0.667	0.734	0.627	0.625	0.572
	E	0.605	0.672	0.689	0.594	0.782	0.658	0.675	0.678	0.549	0.528
	F	0.670	0.725	0.660	0.634	0.765	0.683	0.750	0.706	0.694	0.595
	G	0.618	0.685	0.684	0.661	0.786	0.740	0.779	0.708	0.786	0.593
Av. % Cell survival		104	117	111	105	126	114	122	117	111	100
% Std. dev.		6	5	7	7	12	8	8	5	14	9

Sunday 06.03.10 Mix. 1-9 (2)

Mixture		1	2	3	4	5	6	7	8	9	0
Absorbance (550 nm)	B	0.546	0.575	0.548	0.540	0.690	0.594	0.540	0.505	0.590	0.488
	C	0.552	0.613	0.561	0.534	0.649	0.629	3,747*	0.630	0.608	0.555
	D	0.599	0.635	0.597	0.607	0.688	0.689	0.669	0.673	0.592	0.564
	E	0.613	0.622	0.602	0.585	0.716	0.706	0.697	0.693	0.691	0.517
	F	0.622	0.632	0.615	0.593	0.735	0.693	0.628	0.659	0.692	0.610
	G	0.607	0.448	0.573	0.618	0.721	0.649	0.612	0.755	0.720	0.598
Av. % Cell survival		106	106	105	104	126	119	113	117	117	100
% Std. dev.		6	13	5	6	6	8	11	15	11	8

Sunday 06.03.10 Mix. 10-17 (1)

Mixture		10	11	12	13	14	15	16	17	0	0
Absorbance (550 nm)	B	0.633	0.640	0.573	0.648	0.645	0.595	0.669	0.686	0.632	0.598
	C	0.698	0.695	0.580	0.655	0.686	0.595	0.613	0.557	0.644	0.553
	D	0.690	0.677	0.609	0.623	0.631	0.585	0.629	0.650	0.615	0.512
	E	0.702	0.726	0.615	0.650	0.648	0.592	0.682	0.664	0.625	0.538
	F	0.695	0.699	0.598	0.638	0.627	0.584	0.674	0.639	0.677	0.635
	G	0.679	0.688	0.598	0.695	0.699	0.611	0.686	0.666	0.713	0.519
Av. % Cell survival		122	123	106	117	117	106	118	115	116	100
% Std. dev.		5	5	3	4	5	2	5	8	7	9

Sunday 06.03.10 Mix. 10-17 (2)

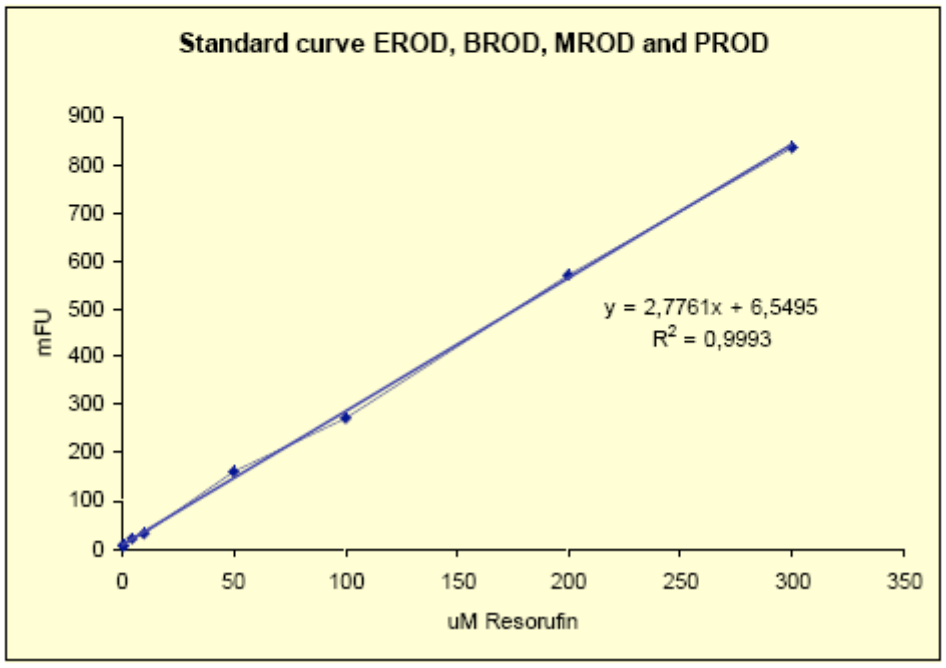
Mixture		10	11	12	13	14	15	16	17	0	0
Absorbance (550 nm)	B	0.608	0.645	0.549	0.648	0.646	0.573	0.602	0.585	0.601	0.529
	C	0.657	0.700	0.549	0.658	0.631	0.588	0.610	0.640	0.561	0.571
	D	0.707	0.712	0.580	0.655	0.639	0.619	0.650	0.644	0.527	0.506
	E	0.695	0.736	0.596	0.677	0.665	0.566	0.642	0.697	0.610	0.581
	F	0.662	0.641	0.570	0.636	0.635	0.579	0.661	0.670	0.606	0.544
	G	0.691	0.726	0.559	0.612	0.616	0.551	0.639	0.724	0.645	0.601
Av. % Cell survival		121	125	102	117	115	104	114	119	107	100
% Std. dev.		6	7	3	4	3	4	4	9	7	6

* = values greater, smaller or equal to average absorbance ±2 std.dev

APPENDIX 3. EROD ASSAY

Standard curve

Resorufin uM	M530/590	M530/590	M530/590	Average mFU
0,5	6	6	6	6
1	7	7	7	7
5	21	21	20	21
10	32	31	31	31
50	154	160	165	160
100	269	271	277	272
200	595	576	540	570
300	818	849	839	835



Standard curve relating fluorescence (mFU, y-axis) to amount of resorufin (µM, x-axis). The equation presented in the figure was used when calculating amount of resorufin (Anne Mortensen and Martine Horn Gjernes).

Calculated EROD activity single compounds

Calculated average EROD activities are presented in Table 1, 2 and 3 for 2,5-diemthylphenol (DMP), carbazole and benzo(a)pyrene (BaP), respectively. Fluorescence (mFU) and total protein (µg/mL) measured in PLHC-1 exposed are presented in Table 4, 5 and 6, for DMP, carbazole and BaP, respectively. Calculation of resorufin was performed according to the equation given in the standard curve:

$$Resorufin\ (\mu M) = \frac{Fluorescence\ (mFU) - 6{,}5495}{2.7761} \quad (1)$$

Table 1.

DMP (µM)	DMP (mg/mL)	F 28.01 (1)		F 28.01 (2)		Tu 01.02		W 09.02		AVERAGE	
		EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev
150	1.8E-02	-6482	2064	-3991	1053	-2456	698	-5536	3351	-4616	1769
100	1.2E-02	-5644	578	-3959	560	-1951	406	-3376	1618	-3732	1528
50	6.1E-03	-5503	955	-4999	1215	-2472	267	-5637	1517	-4653	1479
25	3.1E-03	-9502*		-3477	1847	-2983	1027	-4303	1174	-3587	667
5	6.1E-04	-7508	2731	-3934	1745	-2853	1103	-5658	965	-4988	2038
0.1	1.2E-05	-9727	5352	-5176	1696	-3733	826	-5155	527	-4688	2609
DMSO	0	-6876*	223	-2993	1920	-1889	35	-2143	36	-2342	578
Medium	0	-6608	2215	-4215	1044	-1621	497	-2235	796	-3670	2250

*Values not presented/included

Table 2.

Carbazole (µM)	Carbazole (mg/mL)	Thursday 20.01		Thursday 27.01		Monday 31.01		Monday 7.02		AVERAGE	
		EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev
7	1.2E-03	-1510	620	-684	180	133	258	-875	374	-734	677
5	8.4E-04	-700	310	-540	431	-892	655	-1386	712	-879	367
3	5.0E-04	-1376	339	-661	406	-558	223	-1494	336	-1022	481
1	1.7E-04	-756	109	-658	329	-830	258	-871	540	-779	94
0.5	8.4E-05	-1261	297	-728	580	-928	239	-1354	460	-1068	291
0.01	1.7E-06	-638	488	-773	314	-983	272	-1692	291	-1022	469
DMSO	0	-923	533	-970	266	-1260	313	-1929	267	-1271	463
Medium	0	-405	278	-899	468	-1049	307	-1770	526	-1031	564

Table 3.

BaP (µM)	BaP (mg/mL)	M 17.01		Th 20.01		Th 27.01		W 02.02		AVERAGE	
		EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev
10	2.5E-03	-2074	470	-2294	317	447	234	-672	446	-1149	1283
1	2.5E-04	2893	1020	2917	663	6457	1818	4594	886	4215	1694
0.5	1.3E-04	4330	375	4154	1882	6003	296	4546	611	4758	845
0.1	2.5E-05	1837	1069	1523	240	2770	403	2333	754	2116	549
0.01	2.5E-06	-88	197	-1039	831	203	332	-1319	216	-561	733
DMSO	0	-1076	831	-2334	809	-905	345	-976	251	-1323	678
Medium	0	-1208	545	-2125	628	-942	429	-1327	155	-1401	509

Raw data single compounds

Table 3.

DMP	ID	F 28.01 (1)					F 28.01 (2)					Ti 01.02					O 09.02				
		mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)		
Medium	1	44	47	91	104	58	51	47	202	246	148	45	51	431	459	384	52	51	403	414	435
	2	45	46	87	108	98	45	47	145	209	118	52	50	386	417	344	48	50	267	320	311
	3	48	49	53*	143	110	47	47	197	221	178	52	57	474	500	325	55	43	242	268	231
DMSO	4	45	50	86	55	108	43	46	192	175	191	50	46	454	479	435	54	48	242	315	273
	5	42	47	113	109	122	49	51	211	231	159	49	50	443	434	365	50	52	260	311	279
	6	46	44	0	0	0	44	32	169	227	191	49	50	391	414	392	41	44	0	0	0
150 µM	7	41	46	48*	92	117	44	50	188	253	174	46	44	378	366	350	43	45	36*	152	104
	8	45	51	10	41	0	44	45	191	231	127	45	46	304	382	380	45	43	169	107	174
	9	47	50	70*	104	7*	44	52	275	75*	202	51	53	366	398	356	53	54	274	244	0*
100 µM	10	46	47	79	126	0*	46	48	228	199	201	49	47	389	434	399	48	53	348	483	317
	11	46	49	74	144	52*	43	49	201	196	155	48	48	380	412	354	46	54	172	206	170
	12	45	45	97	183	50*	46	49	228	233	177	51	52	416	458	427	49	39	250	254	132
50 µM	13	47	47	54*	145	80	45	47	223	231	192	47	46	330	420	325	38	44	206	188	148
	14	45	47	0*	105	0*	47	46	143	114	0*	46	49	334	381	315	44	40	41	0	0
	15	49	50	92	124	89	45	47	218	170	145	47	52	401	335	329	106*	41	261	306	230
25µM	16	44	43	34	0	45	47	51	154	128	111	43	47	197	299	269	46	44	317	325	232
	17	117	26	112	100	11	51	56	252	266	284	60*	46	377	367	311	50	44	247	205	184
	18	48	36	109	85	36	49	47	183	211	155	50	51	374	327	328	50	49	64*	31*	123
5 µM	19	49	45	0*	72	0*	44	49	54*	186	109	47	53	334	372	297	47	42	10	0	94
	20	46	42	0*	84	0*	48	49	311	373	321	45	43	263	312	228	45	47	237	175	134
	21	47	49	115	137	60*	46	46	211	220	170	50	48	356	342	379	46	44	199	109	150
0,1 µM	22	45	43	64	41	0*	48	47	110	0	0	44	43	216	243	273	51	53	0	0	0
	23	50	49	0*	101	0*	48	45	161	171	131	44	48	356	326	283	50	48	173	0*	129
	24	44	49	0*	87	51	50	48	185	213	180	45	46	335	263	271	49	48	184	107	118
Blank	B	49	51				52	52				65	50				51	60			
	B	50	52				52	57				49	58				54	52			

*Values not included in calculations

123 Parallels not included in average

Table 4.

Carbazole	ID	Thursday 20.01					Thursday 27.01 (1)					Monday 31.01					Monday 7.02				
		mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)		
Medium	1	39	48	348	334	322	44	49	601	551	601	45	48	560	553	574	54	54	534	516	224
	2	43	50	366	322	303	52	52	590	587	577	48	47	538	523	532	48	53	540	483	510
	3	41	48	150*	335	0*	45	44	553	555	460	41	42	579	564	599	47	42	493	500	485
DMSO	4	39	51	355	378	301	52	46	580	594	594	43	45	554	538	430	48	49	552	562	527
	5	41	43	311	329	307	48	46	531	590	553	41	43	549	501	468	46	46	520	460	475
	6	35	40	406	450	376	45	43	575	632	542	46	48	540	522	482	48	49	482	500	484
10 µM	7	42	41	251	342	250	47	48	556	620	586	60	54	512	495	402	60	59	459	619	468
	8	44	38	285	387	340	47	49	645	809	648	59	55	478	493	486	55	53	481	438	512
	9	37	40	211	0*	260	52	52	530	496	355	56	51	461	532	455	48	63	528	557	527
1 µM	10	37	48	234	279	339	58	52	704	616	530	50	46	393	470	425	58	59	531	577	539
	11	41	45	351	217	422	54	45	708	615	581	46	46	308	422	345	49	49	476	621	378
	12	49	42	234	156	386	49	43	719	622	589	49	57	550	603	491	51	46	513	529	509
0,5 µM	13	41	42	238	226	343	47	43	640	616	566	50	49	572	668	532	55	52	548	543	543
	14	38	42	249	184	304	49	50	701	583	608	44	46	776	785	734	47	53	457	498	433
	15	43	40	306	268	346	52	53	803	1078*	636	54	48	664	721	672	47	53	543	595	458
0,1 µM	16	41	47	270	228	338	47	47	640	503	522	45	56	460	481	401	53	49	543	562	437
	17	41	43	371	347	337	48	53	463	502	449	45	49	610	612	568	56	61	499	616	600
	18	45	40	406	380	373	55	51	538	540	554	47	43	548	574	512	62	53	622	626	676
0,01 µM	19	39	39	316	334	308	45	42	516	564	538	48	46	570	581	551	56	55	496	420	518
	20	42	42	323	376	277	55	48	575	594	755	39	49	538	563	554	56	53	493	463	490
	21	41	39	327	368	308	55	49	618	584	652	51	43	653	639	631	47	48	503	501	564
Medium	22	45	36	368	294	338	48	44	506	579	509	47	47	423	437	367	48	52	536	500	521
	23	49	41	324	378	322	54	49	483	460	521	48	45	488	504	379	48	47	470	488	502
	24	42	48	380	356	357	49	50	584	527	668	47	53	450	493	372	53	51	460	446	548
Blank	B	38	38				47	49				47	50				60	59			
	B	45	41				48	53				51	45				52	57			

*Values not included in calculations

Table 5.

BaP	ID	M 17.01					To 20.01					To 27.01 (2)					O 02.02				
		mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)			mFU		Total prot. (µg/mL)		
Medium	1	50	51	407	448	334	50	54	44	338	132	58	53	648	617	627	41	49	383	366	400
	2	58	54	506	545	416	57	52	159	328	207	59	62	660	624	606	46	47	440	386	377
	3	45	59	675	696	502	53	58	312	444	402	60	68	656	708	634	48	44	382	369	319
DMSO	4	63	59	468	448	422	51	46	348	412	313	65	58	638	695	665	46	52	383	368	324
	5	52	56	380	545	312	53	60	320	378	357	65	61	654	640	614	44	47	423	411	409
	6	48	53	390	462	341	55	47	300	340	232	56	57	625	630	437*	48	50	383	403	372
10 µM	7	50	53	447	469	380	55	53	246	273	209	72	75	613	593	564	45	49	357	389	357
	8	45	45	427	504	427	57	55	182	388	170	76	79	670	712	623	50	55	362	216	313
	9	44	46	17*	489	412	54	57	283	281	227	79	66	630	759	594	49	53	349	367	355
1 µM	10	91	101*	437	462	216*	83	102*	299	292	354	174	168	712	786	679	89	89	410	413	406
	11	77	74	465	398	434	79	79	293	321	300	118	122	554	676	551	80	41	313	359	286
	12	83	79	399	443	344	80	73	326	332	313	125	141	673	716	664	83	73	390	450	390
0,5 µM	13	87	103*	383	373	310	87	89	311	302	262	103	87	603	647	614	87	83	380	408	339
	14	108*	85	405	333	306	90	94	319	268	324	127	133	616	679	614	81	72	358	373	337
	15	96	97	417	460	450	71	70	165	193	186	146	154	765	832	775	88	85	404	453	403
0,1 µM	16	67	64	265	371	405	72	68	289	222	277	103	93	571	638	536	65	61	319	328	266
	17	74	81	401	384	292	76	67	242	218	271	104	89	700	691	736	57	54	452	413	349
	18	68	73	521	455	418	76	71	317	411	333	120	97	824	759	759	69	68	266	346	314
0,01 µM	19	64	61	355	468	411	68	59	262	332	302	78	67	655	640	665	46	48	303	312	234
	20	58	67	576	541	557	61	61	222	270	144	77	74	688	689	688	48	48	303	286	242
	21	64	56	447	467	429	60	54	236	303	200	68	68	729	781	683	50	46	343	353	319
Blank	B	59	55				57	60				57	66				49	49			
	B	55	54				56	59				68	62				45	47			

*Values not included in calculations

123 Parallels not included in average

Calculated EROD values mixtures

Calculated average EROD activities are presented in Table 6 for all 17 mixtures, DMSO control and medium control. Fluorescence (mFU) and total protein (µg/mL) measured in PLHC-1 exposed to mixtures 1-6, mixtures 7-12 and mixtures 13-17 are presented in Table 7, 9 and 10, respectively. Mixtures 6 were measure for two parallels separately and raw data from these are presented in Table 8. Fluorescence was measured as two parallels, total protein as three parallels and three parallel samples were run for each concentration tested. Calculation of resorufin was performed according to equation 1.

Table 6.

	1		2		3	
Mixture	EROD activity	Std dev	EROD activity	Std dev	EROD activity	Std dev
1	-1278.0	279.9	-456.1	373.3	21.8	452.8
2	-1287.7	475.0	-1337.2	223.2	-527.8	185.9
3	63.8	218.5	998.2	464.5	1307.6	225.9
4	-888.9	90.3	-130.5	334.5	418.0	354.7
5	4939.7	370.5	4887.9	1014.9	4769.5	848.1
6	7771.3	341.9	7136.6	593.1	6234.1	534.5
7	6419.3	2370.6	7096.6	461.7	7013.8	671.7
8	4709.7	2560.4	5044.3	468.1	5039.1	514.4
9	5698.7	1280.3	5936.7	163.4	5095.5	324.0
10	5008.4	281.4	4074.9		3938.7	423.0
11	3750.4	2159.0	3480.0	250.5	3656.0	105.5
12	6852.6	1355.1	6344.2	623.4	5865.8	1262.3
13	1335.8	395.4	1353.8	32.3	1320.1	604.8
14	9598.4	167.9	8967.3	433.8	7540.2	904.9
15	10491.8	621.2	7927.0	487.5	9276	84.9
16	7625.4	663.9	6955.4	864.4	8099.9	457.6
17	7671.4	569.7	7062.2	453.9	7261.6	239.6
DMSO	4.7	172.7	6.9	111.2	-879.4	262.3
Medium	-600.6	669.1	-221.1	453.6	-20.5	590.6

Table 7.

Mix. 1-6	ID	1					2					3				
		mFU		Total protein (µg/mL)			mFU		Total protein (µg/mL)			mFU		Total protein (µg/mL)		
1	1	42	44	271	347	295	43	42	265	352	332	44	44	381	428	423
	2	40	44	335	426	407	41	40	370	405	359	44	45	421	497	480
	3	45	45	305	402	343	39	36	423	363	414	40	37	439	440	484
2	4	43	45	442	505	474	37	36	508*	321	230	38	36	433	471	445
	5	41	45	345	405	381	37	37	313	409	307	40	39	497	502	475
	6	42	42	164*	292	174*	39	36	258	202	113*	38	38	460	498	491
3	7	52	47	238	321	303	46	44	0*	112	0*	56	50	525	572	594
	8	48	53	350	392	373	46	44	182	273	255	53	52	521	529	547
	9	49	51	232	350	340	48	49	206	297	198	56	55	510	520	526
4	10	47	43	83*	266	200	43	43	189	287	241	48	43	410	447	416
	11	46	47	263	396	288	43	39	194	326	221	44	41	446	445	452
	12	46	45	302	359	317	44	43	0*	213	0*	49	46	398	448	463
5	13	71	73	226	337	267	66	62	290	355	280	73	74	460	546	500
	14	70	68	0*	246	175	61	62	247	282	232	75	74	365	403	406
	15	77	75	341	347	306	70	69	298	309	196	83	76	373	455	432
6	16											84	85	407	435	338
	17											82	84	419	368	366
	18											80	47	428	381	425
DMSO	19	42	44	220	315	257	37	37	313	305	162*	38	37	386	315	0*
	20	38	37	318	377	353	38	37	272	242	200	41	41	436	440	437
	21	48	45	530	556	520	40	40	270	349	0*	41	39	427	430	417
Blank	22	41	41				33	32				37	32			
	23	47	44				39	39				36	35			
	24	46	45				38	38				37	37			

*Values not included in calculations

Table 8.

Mix. 6	mFU		Total protein (µg/mL)		
6	105	98	285	358	367
	88	86	247	250	328
	101	96	303	216*	392
6	92	93	315	387	356
	108*	97	400	316	335
	118	105	391	352	386
DMSO	62	55	312	287	330
	58	53	160	210	210
	55	52	298	400	306
Blank	45	43			
	46	46			
	50	47			
	52	49			

*Values not included in calculations
123 Parallels not included in average

Table 9.

Mix. 7-12	ID	1 mFU		1 Total protein (µg/mL)			2 mFU		2 Total protein (µg/mL)			3 mFU		3 Total protein (µg/mL)		
7	1	71	69	57*	227	193	110	109	414	455	446	93	95	394.7	424.1	385.8
	2	61	55	305	360	310	88	89	381	457	422	95	93	397.7	455.1	458.3
	3	56	52	64*	94*	152	94	80	333	392	388	86	84	388.3	448.6	361.9
8	4	55	54	253	313	274	73	74	367	395	377	61	61	276.4	327.8	286.7
	5	57	60	56*	332	185	72	76	406	439	441	78	72	342.5	453.7	433
	6	58	54	0*	111	0*	81	81	388	460	453	74	73	322.4	391.7	298.6
9	7	70*	60	162	168	76	78	74	419	422	418	75	69	283.2	367.4	422*
	8	69*	52	111	137	126	88	85	434	490	478	72	69	307.6	366.5	428*
	9	66	67	0*	134	0*	84	84	402	447	288*	73	71	469.7	473.4	387.6
10	10	58	63	266	264	184	54	57	290	401*	295	94	89	418.4	478.5	404.8
	11	60	57	73*	189	72*	74	69	463	491	374	72	64	363.3	453.9	243*
	12	60	59	208	241	181	58	57	347	470*	339	73	76	422	494.6	417.9
11	13	51	51	123	179	0*	64	57	303	403	347	68	64	378.4	440.8	323.8
	14	56	58	135	171	147	61	63	259	436	349	64	51	0	0	0
	15	46	48	116	244	161	68	64	351	439	235*	67	67	363.7	444.3	338.6
12	16	57	46	115	200	197	78	77	302	371	287	74	62	292	306	0*
	17	58	61	149	134	0*	75	79	377	330	380	82	81	359.6	345.1	322.4
	18	69	62	213	153	55*	98	87	401	406	454	70	30	348.5	397.9	291.8
DMSO	19	33	36	218	183	99*	35	38	307	389	262	41	38	356.6	406.7	300.4
	20	38	40	192	125	82*	37	42	375	414	389	41	42	387.6	360.3	116.3
	21	40	39	157	136	0*	35	35	365	455	499	41	41	494.8	491.8	366.2
Blank	22	40	38				28	26				35	35			
	23	36	34				39	38				40	38			
	24	32	34				41	41				40	35			

*Values not included in calculations

123 Parallels not included in average

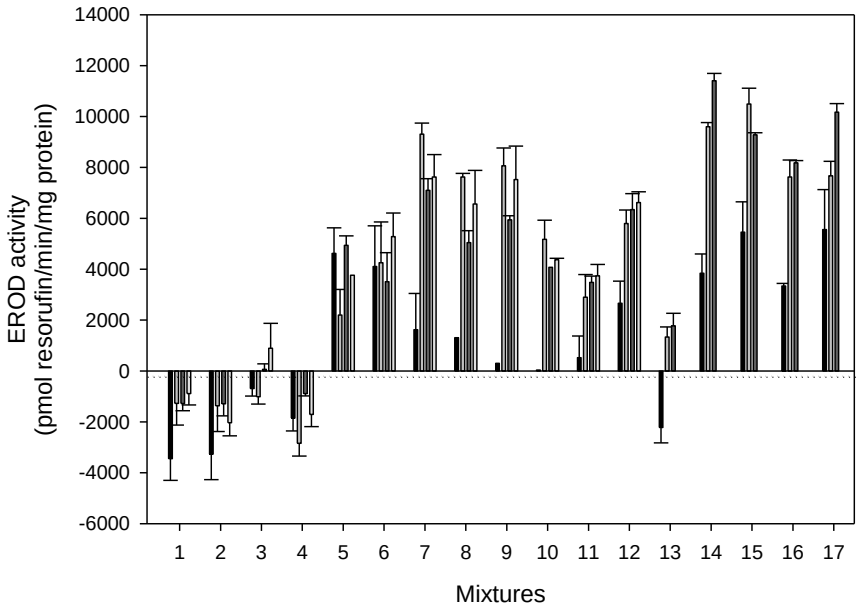
Table 10.

Mix. 13-17	ID	1					2					3				
		mFU		Total protein (µg/mL)			mFU		Total protein (µg/mL)			mFU		Total protein (µg/mL)		
13	1	53	51	347	405	380	63	61	561	588	601	56	58	330	344	320
	2	52	47	399	464	433	59	64	566	588	588	53	52	331	337	351
	3	44	41	197	0*	0*	52	48	499	508	554	50	48	470	492	436
14	4	117	100	427	466	395	131	141	554	665	661	87	80	295	311	320
	5	106	102	383	406	382	136	130	529	601	583	88	90	390	332	353
	6	111	103	306	358	0*	142	136	565	590	611	89	95	337	306	309
15	7	112	93*	400	455	423	120	124	598	594	715*	78	76	299	342	308
	8	113	108	362	419	369	130	127	559	622	694	73	70	253	318	305
	9	95	90	498	457	461	129	126	538	620	548	77	72	313	349	333
16	10	96	91	397	405	424	118	103	591	647	603	91	87	302	300	63
	11	92	79	327	339	302	160	148	835	908*	801	86	77	303	377	389
	12	84	79	305	392	0*	119	113	572	634	596	98	91	376	382	351
17	13	95	90	399	460	438	121	108	523	544	522	94	94	338	419	382
	14	95	88	416	391	390	121	108	567	588	621	87	85	306	364	301
	15	108	104	460	513	446	115	111	575	571	584	124	130	442	539	544
DMSO	16	40	45	389	433	405	48	48	491	544	467	43	40	328	329	324
	17	45	41	387	414	472	51	49	649	562	589	44	45	321	288	312
	18	41	41	332	343	0*	49	49	449	527	493	43	42	337	382	315
Medium	19	39	36	204	0*	0*	43	41	586	584	545	47	40	317	362	382
	20	41	39	403	378	370	50	50	618	549	632	48	50	226	275	253
	21	43	40	333	270	0*	50	47	530	639	580	51	47	324	318	288
Blank	22	33	32				46	41				44	43			
	23	34	36				44	41				44	44			
	24	37	41				42	40				35	36			

*Values not included in calculations

123 Parallels not included in average

Replicates not included in the PLS model



Catalytic EROD activity was determine for five to six replicates for each mixture. Replicates not used in the PLS regression model are illustrated here. Raw data are not presented.

APPENDIX 4. CONCENTRATION OF PRODUCED WATER RELEVANT COMPOUNDS

Table 1.

Compound (group)	Concentration (mg/mL)	Locality	Refrence
C2-alkylphenols	0.51	Brage	Utvik 1999
"	0.75	Oseberg F	"
"	0.95	Oseberg C	"
"	0.41	Troll	"
2,5-DMP	0.11	Oseberg C	Boitsov et al., 2004
"	0.02	Troll B	Boitsov et al., 2007
"	0.10	Statfjord A	"
"	0.07	Statfjord C	"
"	0.08	Åsgård B	"
"	0.01	Grane	"
"	0.15	Oseberg C	"
"	0.03	Heidrun	"
"	0.04	Snorre A	"
"	0.02	Gullfaks B	"
"	0.10	Statfjord C	"
"	0.02	Oseberg C	"
AVERAGE	0.21		

Table 2.

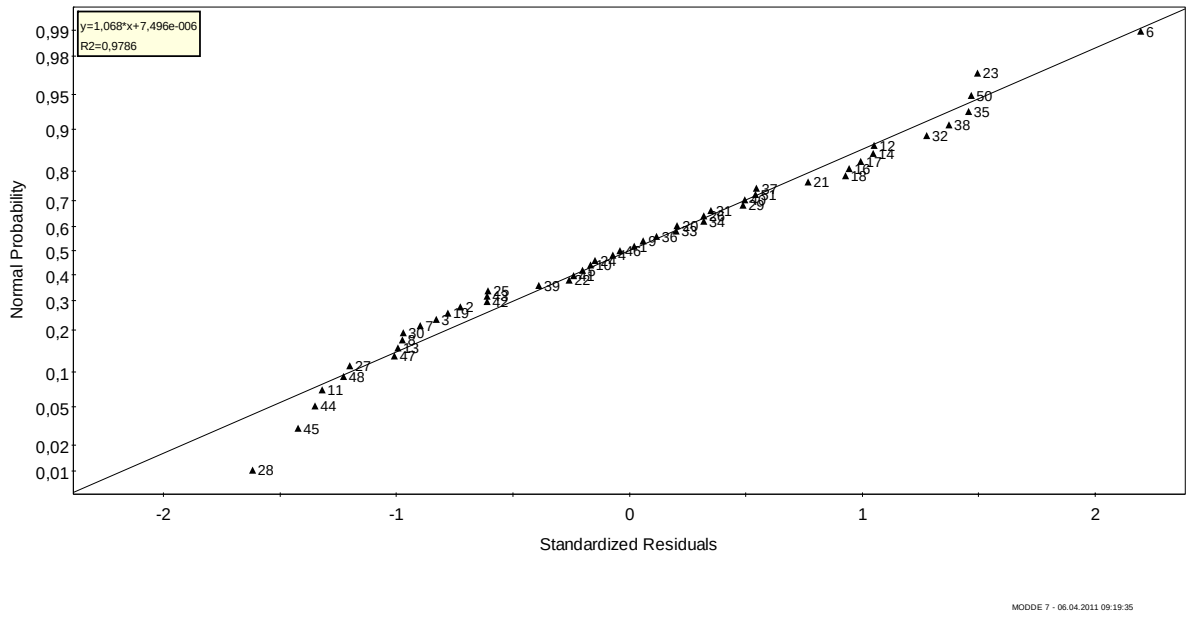
Compound (group)	Concentration (mg/mL)	Locality	Refrence
BaP	0.20	Brage	Utvik, 1999
"	0.10	Oseberg C	"
4/5 ring PAH	0.003	Statfjord A	Neff et al., 2006
"	0.004	Statfjord B	"
"	0.001	Statfjord C	"
"	0.001	Gullfaks A	"
"	0.002	Gullfaks B	"
"	0.001	Gullfaks C	"
"	0.002	Veslefrikk	"
"	0.000	Troll A	"
"	0.005	Troll B	"
"	0.005	Snorre TLP	"
"	0.006	Oseberg C	"
"	0.003	Oseberg FS	"
"	0.006	Brage	"
"	0.001	Ekofisk 2/4 K	"
"	0.001	Ekofisk 2/4 J	"
"	0.001	Eldfisk 2/7 B	"
"	0.001	Eldfisk 2/7 A	"
"	0.000	Tor	"
"	0.000	Valhall	"
"	0.005	Ula	"
"	0.001	Gyda	"
AVERAGE	0.015		

APPENDIX 5. THE PLS MODELL

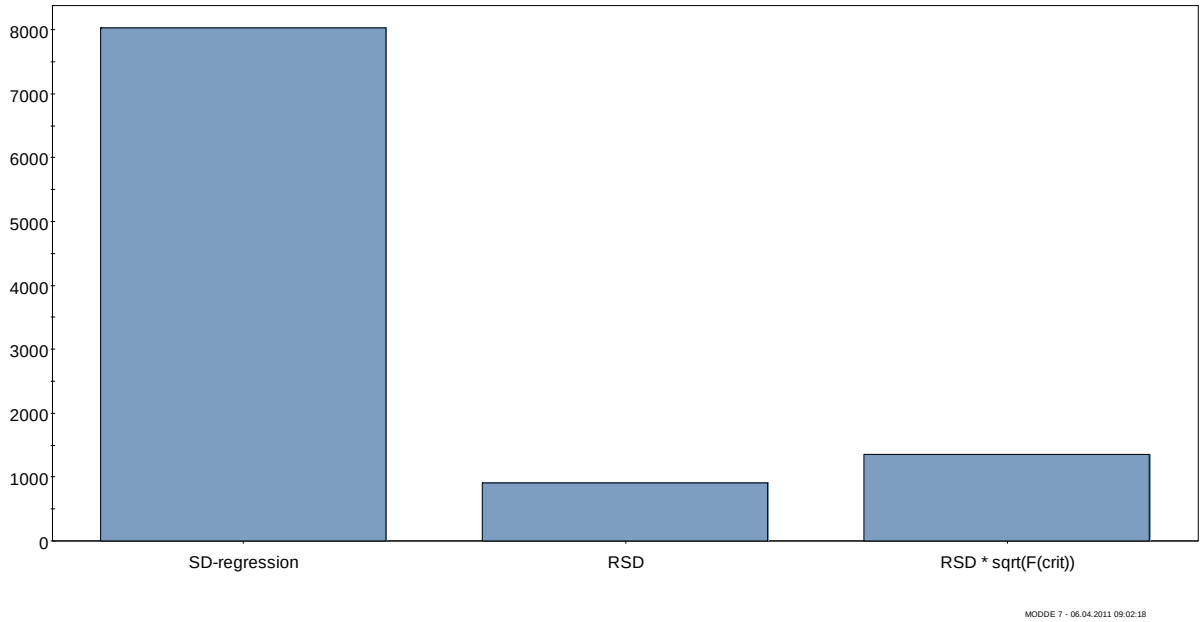
Tabulated values for the generated models as insignificant terms are excluded sequentially from the model.

Terms included in the model	R^2	Adjusted R^2	Q^2	LVs
All interaction terms	0.91	0.89	0.88	2
All interaction terms -Car×BaP	0.91	0.89	0.88	2
All interaction terms -Car×BaP and -DMP×Bap	0.91	0.89	0.88	2

R^2 = explained variance
Adjusted R^2 = R^2 adjusted for number of explanatory terms
Q^2 = predicted variance
LVs = number of latent variables



Normal probability plot of the residuals for the utilized PLS model with no non-significant terms and parallel 15 and 49 excluded.



ANOVA plot compares standard deviation of the regression with standard deviation of the residuals, which represent noise present in observations. Good models should have standard deviations of regression (SD-regression) much larger than standard deviation of the residuals multiplied with its upper confidence level (RSD×sqrt(F_{crit}). The model used was significant at a 5% confidence level.

APPENDIX 6. GEL ELECTROPHORESIS

Relative front (Rf), median molecular length (MML), length of DNA fragments (L_n) and relative number of strand breaks per kilo base DNA (RNSB/kb) in PLHC after exposure to ternary mixtures of produced water relevant compounds. Exposure and lane (Exp./lane) are marked to the left in the table, L= DNA ladder, D= DMSO control, 7,9 and 14 = mixtures tested. Calculated values for first and second replicate are presented in Table 1 and 2, repectively, raw data are illustrated below

Table 1.

Tuesday 08.03.10

Exp./ lane		Rf	MML	Ln	Average Ln	RNSB/kb	Average RNSB/kb	Std. dev.
L	1	0.620						
D	2	0.417	55640	33384.0				
	3	0.414	57179	34307.4	33494.4			
7	4	0.426	52072	31243.2		2.2E-06		
	5	0.429	49910	29946.0		3.5E-06		
	6	0.417	55484	33290.4		1.8E-07	2.0E-06	1.7E-06
9	7	0.420	53923	32353.8		1.1E-06		
	8	0.418	55016	33009.6		4.4E-07		
	9	0.423	52853	31711.8		1.7E-06	1.1E-06	6.2E-07
14	10	0.424	52072	31243.2		2.2E-06		
	11	0.414	56867	34120.2		-5.5E-07		
	12	0.423	52853	31711.8		1.7E-06	1.1E-06	1.4E-06
D	13	0.416	56554	33932.4				
	14	0.421	53923	32353.8				
L	15	0.544						

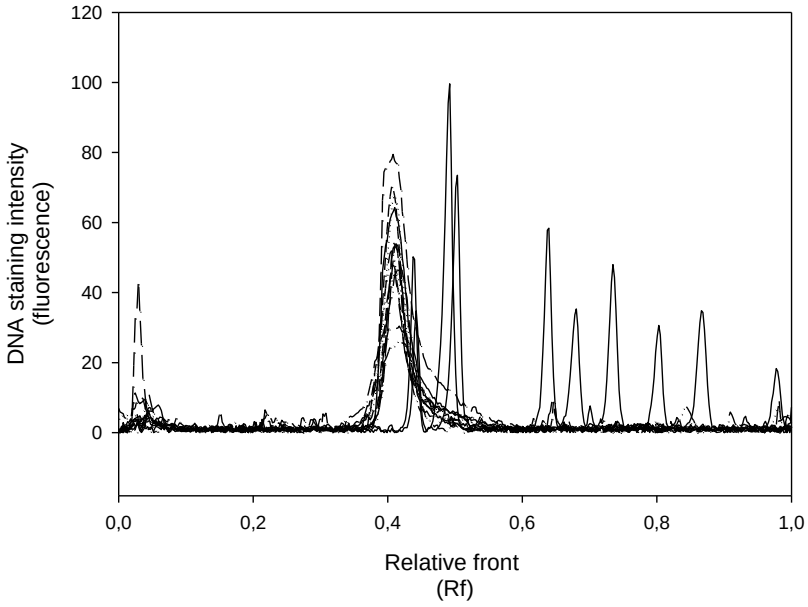


Table 2.

Saturday 19.03.10

Exp./ lane		Rf	MML	Ln	Average LN	RNSB/kb	Average RNSB/kb	Std. dev.
L	1	0.600	13782	8269.2				
D	2	0.580	15690	9414.0				
	3	0.570	17045	10227.0	9733.5			
	4	0.577	16271	9762.6		-3.1E-07		
7	5	0.576	16271	9762.6		-3.1E-07		
	6	0.569	17045	10227.0		-5.0E-06	-1.9E-06	2.7E-06
	7	0.574	16464	9878.4		-1.5E-06		
9	8	0.573	16077	9646.2		9.3E-07		
	9	0.573	17045	10227.0		-5.0E-06	-1.8E-06	3.0E-06
	10	0.575	16658	9994.8		-2.7E-06		
14	11	0.578	16464	9878.4		-1.5E-06		
	12	0.579	15884	9530.4		2.2E-06	-6.7E-07	2.5E-06
D	13	0.583	15884	9530.4				
	14	0.579	16271	9762.6				
L	15	0.599	13587	8152.2