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Confounding factors affecting biological effects measurements used in the Water Column monitoring surveys

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Abstract

A combination of effects-directed analysis of produced water from Statfjord C and verification studies using in vitro biomarker studies with single chemicals were used to determine whether the simultaneous presence of inducing and inhibiting compounds from produced water may confound biomarker responses (CYP1A, EROD, vitellogenin) in the water column monitoring (WCM) surveys. Assessment of cytotoxicity and DNA damage was additionally determined to link the findings to more adverse effects. The results from the studies show that compounds in produced water itself may cause both induction and inhibition of biomarker responses such as CYP1A, EROD and vitellogenin as well as cytotoxicity and DNA damage. Dilution and modification of produced water in the receiving water column do rapidly alter the composition of the discharge in the recipient. Estimation of predicted environmental concentrations (PECs) using different data sets from Statfjord B for 2001 and 2007 showed that PECs for produced water compounds were lower than the predicted no effect concentrations (PNECs) for the biomarker responses in three out of the four data sets. One data set based on SPMD data from the BECPELAG water monitoring Workshop in 2001 resulted in PECs that were in the same range as the PNEC for induction of EROD (and CYP1A) as well as inhibition of vitellogenin production at the closest caging station (500 m station) at Statfjord B, however. Given the uncertainty in the extrapolation of in vitro reponses to in vivo bioactivity and the limited data used to derive PECs suggest that more thorough studies should be conducted to determine whether environmentally realistic concentrations of produced water components may cause such effects.

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Confounding factors affecting biological effects measurements used in the Water Column monitoring surveys

Report to OLF

Preface

The present study has been performed by the Section for Ecotoxicology and Risk Assessment at the Norwegian Insitute for Water Research (NIVA) under the auspices of the Water Column Monitoring survey 2007. The work was funded by Oljeindustriens Landsforening (OLF) and key oil production companies operating on the Norwegian sector of the North Sea. The authors are grateful to Tor Fadnes (StatoilHydro) and the personnel at Statfjord C for supplying the produced water.

Oslo, 18 April 2008

Knut-Erik Tollefsen

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Summary

The present study, using effects-directed analysis of produced water and verification studies using in vitro assessment of single compounds showed that produced water is a highly complex mixture capable of causing induction and inhibition of CYP1A, EROD and Vtg as well as DNA damage. Results suggest that EROD induction was predominantly caused by 1-, 2-, 4- and 5+ ring polycyclic aromatic compounds, as well as compounds contained in more polar HPLC fractions with similar chromatographic properties to alkylphenols. Induction of CYP1A protein expression was predominantly caused by produced water and compounds co-eluting with organic acids. Vitellogenin induction was primarily due to compounds found in the same HPLC fraction as alkylphenols. The inhibiting activity of produced water components on EROD and CYP1A was associated with 4-ring aromatics and polar compounds (compounds with similar physico-chemical properties to aromatic acids and alkylphenols), whereas the inhibition of Vtg production was due to 1- and 2-ring aromatics and more polar compounds (compounds with similar physico-chemical properties to aromatic acids and alkylphenols). The genotoxic activity of produced water was mainly due to compounds being highly hydrophobic (5+ ring aromatics) and polar (compounds with similar physico-chemical properties to aromatic acids and alkylphenols). Verification studies using single compounds confirmed that EROD and CYP1A induction was due to 4- and 5+ PAHs and vitellogenin induction by alkylphenols. The inhibiting activity of EROD and CYP1A was predominantly due to 3-, 4- and 5-ring PAHs as well as alkylphenols such as 4-*t*-butylphenol. The inhibition of Vtg production was associated with presence of 4- and 5+ ring PAHs. Estimation of predicted environmental concentrations (PECs) using different data sets from Statfjord B for 2001 and 2007 showed that PECs for produced water compounds were lower than the predicted no effect concentrations (PNECs) for the biomarker responses in three out of the four data sets. One data set based on SPMD data from the BECPELAG water monitoring Workshop in 2001 resulted in PECs that were in the same range as the PNEC for induction of EROD (and CYP1A) as well as inhibition of vitellogenin production at the closest caging station (500 m station) at Statfjord B, however. Given the uncertainty in the extrapolation of in vitro reponses to in vivo bioactivity and the limited data used to derive PECs suggest that more thorough studies should be conducted to determine whether environmentally realistic concentrations of produced water components may cause such effects.

1. Introduction

The Norwegian offshore Water Column Monitoring (WCM) surveys have reported that the biological effects observed in several monitoring endpoints such as the induction of EROD, vitellogenin (Vtg) and glutathion-s-transferase (GST) do not follow a clear distance-response relationship. For instance, EROD activity and vitellogenin (Vtg) induction in fish, which were core endpoints in the water column surveys, are equal or lower in caged fish closest to the discharge source than farther away in the surveys performed in 2003 and 2004 (too few data points were available in WCM survey 2006). One possible explanation for this discrepancy may be the non-optimal exposure of cages close to the production platform as a consequence of the uneven distribution and settling of the discharge plume as well as possible deviations from the modelled dispersion of the discharge plume. It has also been pointed out that common produced water components such as PAHs, alkylphenols, and organic acids may affect multiple mechanisms of effect (Tollefsen *et al.*, 2006; Tollefsen, 2007; Tollefsen *et al.*, 2007). For instance, studies document that both branched and normal alkylphenol isomers from C1 to C9 are toxic and cause sub-lethal effects such as binding and activation of the fish estrogen receptor (ER) as well as interfere with the binding of natural steroids to specific sex-steroid binding proteins (Tollefsen, 2007; Tollefsen *et al.*, 2008; Tollefsen *et al.*, In press). However, in vitro fish bioassay data suggest that some normal chained isomers are not estrogenic by activating the ER-mediated production of the vitellogenin. There is therefore a possibility that these compounds are also acting as anti-estrogens (Tollefsen *et al.*, In press). The potential for the presence of both estrogenic and anti-estrogenic effects are in agreement with a recent report of estrogenic effects in male and apparent anti-estrogenic effects in female cod exposed to mixtures of C4-C7 alkylphenols (Meier *et al.*, 2007). A reduction in circulating levels of female Vtg at the closest cage to the Statfjord oil production in the 2004 WCM survey seems to be in agreement with the findings from Meier et al. (Meier *et al.*, 2007), although the mechanism of anti-estrogenic effects has not been properly elucidated.

Another scenario is that chemicals may induce effects in one endpoint whereas inhibiting other toxicity endpoints that are normally affected by totally different compounds. For instance, weakly estrogenic alkylphenols and low molecular PAHs have been proposed to inhibit the CYP1A-mediated induction of EROD activity (Navas and Segner, 2000; Hasselberg *et al.*, 2004; Hasselberg *et al.*, 2005), whereas EROD inducers such as high molecular weight PAHs are able to inhibit the estrogenic activity of endogenous estrogens (Navas and Segner, 2000). It has been verified by in vitro gene expression studies with fish that the cellular response to individual model compounds does not predict the toxicity when exposed in an equi-molar synthetic mixture (Finne *et al.*, 2007), thus introducing the possibility that one or several groups of compounds in complex mixtures may affect the results of other endpoints.

A major limitation in most studies reporting modulatory effects on both endocrine and biotransformation (e.g. EROD) endpoints is that the mechanisms of action is not thoroughly

documented and more importantly that typical produced water components such as low molecular alkylphenols, PAHs and organic acids have not been properly studied. It can therefore be expected that complex effluents such as produced water that contain a mixture of compounds arising from both the reservoir and additives used in oil production may differentially affect the effect and biomarker endpoints used in the WCM surveys. Since many of the compounds discharged to the recipient do not have high persistence and are rapidly diluted, inhibiting effects should be expected to occur primarily close to the production platform. A key issue in the interpretation of data from the WCMS is therefore to answer whether the low sub-lethal toxicity determined at the closest caging stations are due to the inhibiting properties of produced water chemicals or whether the lack of biological responses were due to concentrations of compounds being below the threshold for effect. Thorough mechanistic studies, investigating the modulation effects of common produced water components may provide insight into better understanding of this complex issue.

2. Objective

The objective of the current study was to reveal the potential biological mechanisms behind the lack of consistency between the distance from the discharge source and observed biological responses in caged animals during the WCM surveys. The key question to be answered is whether some compounds common to produced water may mask the biological effects of other compounds and therefore lead to the absence of effects in biomarker responses. In vitro studies on selected mechanisms such as vitellogenin and EROD induction will be performed in combination with DNA damage and cytotoxicity to determine whether co-exposures to multiple produced water components may affect the biological responses used in WCM surveys.

3. Rationale of methods and approaches

A comprehensive in vitro screening of eight crude fractions of produced water (separating aliphatic, aromatic and polar compounds) and confirmative studies using produced water components such as naphthalenes, low molecular PAHs, high molecular PAHs, carbazoles, alkylphenols, organic acids and oil field chemicals (e.g. production chemicals) were conducted to determine whether produced water compounds may affect typical WCM survey endpoints such as EROD, CYP1A, and Vtg production and DNA damage (genotoxicity). The endpoints that were studied include the induction and inhibition (inhibition) of CYP1A and CYP1A-mediated EROD induction and estrogen receptor-mediated Vtg production in an in vitro bioassay based on rainbow trout liver cells (hepatocytes). Rainbow trout, which is an intensely studied species with considerable literature on biological effects, may provide the same biological endpoints and use essentially the same methods as that deployed in the WCM surveys of cod and is thus proposed as a surrogate experimental model. Although some

differences exist between the two species such as fat-storage strategies that may affect the accumulation of lipid-soluble compounds, no data is to our knowledge available to clearly indicating that there are substantial differences in sensitivity to produced water compounds. The resulting effect concentrations obtained from in vitro studies were used to calculate theoretical no-effect concentrations (NOECs) by Quantitative Structure Activity (QSAR) model estimates for bioconcentration factors.

The chromatographic separation of oil components has been common practice in oil characterisation studies for decades (Tissot and Welte, 1984). Typically oil is separated into groups of compounds with a common chemical structure. More recently these chromatographic separation techniques have become increasingly used in toxicity characterisation studies of complex mixtures. The use of chromatographic techniques in combination with bioassays to measure the toxicity of the resulting fractions and direct the fractionation procedure is termed effects-directed analysis (EDA). EDA in its complete form is a powerful approach in identifying the toxic components in complex environmental mixtures (Figure 1). The technique is particularly suited to complex mixtures of hydrocarbons since many of the chromatographic techniques required to successfully simplify the complex mixtures of hydrocarbons have previously been developed. EDA and EDA type procedures have been used to great success in identifying a range of toxic oil components; for example the toxicity of oil components (Barron *et al.*, 1999) and the toxicity characterisation of produced waters (Thomas *et al.*, 2004). One of the major advantages of this approach is that it allows different classes of oil/produced water components to be separated and the effects they exert as a compound class tested. This also allows for a better understanding of the overall effects that different oil components exerts.

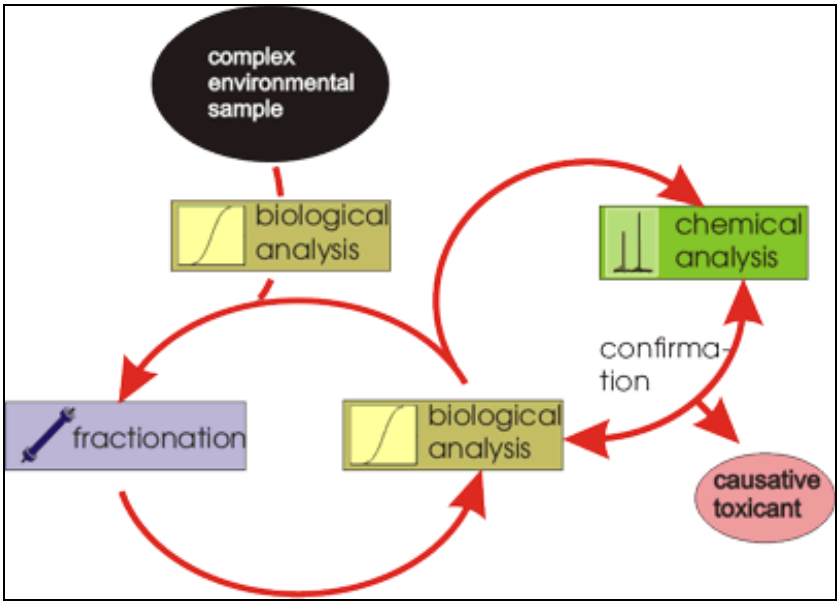

Figure 1 *Schematic of an effect-directed analysis (EDA) procedure*

4. Materials and Methods

4.1 Chemicals

The test chemicals (purity≥96%, Annex 1) were diluted in anhydrous ultra pure DMSO (99.9 %, Sigma-Aldrich) or directly in growth media ($CuSO_4$) prior to use. Chemicals in DMSO were stored in the dark at -20 °C until use.

4.2 Produced water

Produced water samples were collected in solvent rinsed stainless steel transport containers from the Statfjord C oil production platform. The samples, which were all taken after the treatment processes to remove oil and clean the process water, represented the last sample point before discharge to sea. Samples were shipped by the fastest available transport route, stored at 4 °C upon arrival and extracted within 24 h.

Samples (approximately 30 L) were extracted using the method described in Thomas et al. (2001). Briefly, the samples were passed, using compressed nitrogen, at a flow rate of 60 ml min^{-1} through a glass wool filter to trap oil droplets and particulate matter, whereas the water soluble fraction (WSF) of produced water was extracted by pre-conditioned SPE columns containing the resin ENV+ (1 g, IST, Hengoed, UK) and octadecylsilane (C18, 5 g; IST, Hengoed, UK). After the entire sample had passed through the columns the columns were washed with 2 x 6 ml of distilled water, dried using nitrogen, wrapped in hexane-rinsed foil and stored at -80°C. The glass wool was dried in a clean fume hood, wrapped in hexane rinsed aluminium foil and stored at -80°C. A control sample was obtained by extracting seawater from the North Sea.

Glass wool and SPE columns were stored for a maximum of 2 weeks before being defrosted, eluted with 2 x 5 ml methanol (HPLC grade, Rathburn, Walkerburn, Scotland) and 2 x 5 ml cyclohexane (Ultra resi-analyzed, J,T,Baker, Deventer, Holland). The resulting extracts were pooled, reduced in volume and subjected to solvent change into ultra-pure dimethylsulfoxide, DMSO (purity: 99.8%, Sigma.Aldrich, St. Louis, MI, USA). The DMSO extracts were stored for maximum 2 months at -20 °C until use for embryotoxicity testing.

4.3 Fractionation and characterisation of different fractions

The produced water sample was fractionated into the following fractions:

- Aliphatics
- 1-ring aromatics
- 2-ring aromatics

- 3-ring aromatics
- 4-ring aromatics
- 5+ ring aromatics
- Alkylphenols
- Aromatic and aliphatic acids

This was achieved through a mixture of open column and semi-preparative high performance liquid chromatography (HPLC) using the scheme presented in Figure 2.

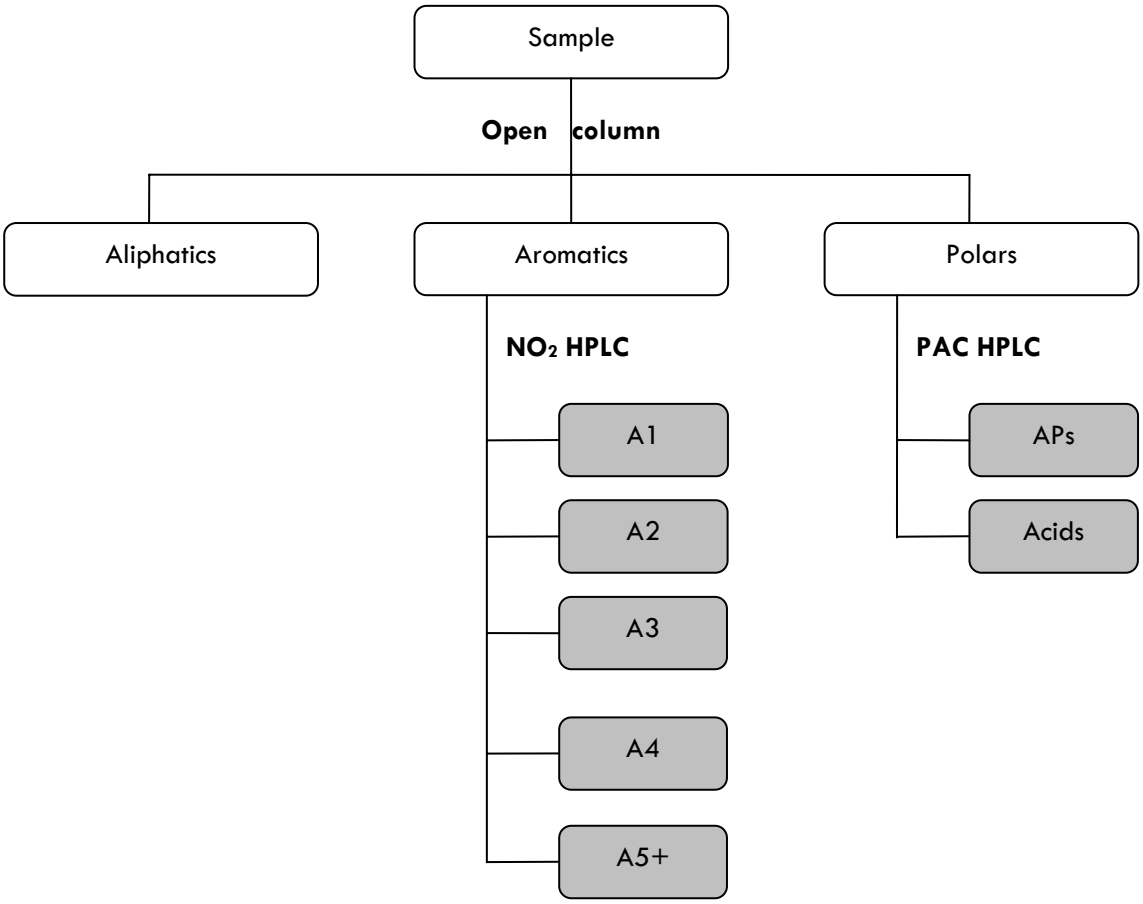

Figure 2. Schematic of sample fractionation procedure

4.4 Open column chromatography

The produced water extract was first fractionated into aliphatic, aromatic and polar fractions by open column liquid solid chromatography. A column was packed with a slurry of silica gel (60-100 mesh, 5% H2O-SiO2; 40g) under alumina (grade 1-neutral; 1.5% H2O-Al2O3; 20g). The produced water extract was applied in cyclohexane (2 ml) to the top of the column and the column eluted with hexane

(150 ml), dichloromethane (200 ml) and methanol (200 ml) to provide aliphatic, aromatic and polar fractions (Figure 2). Solvent transfer to methanol was conducted on all samples prior to testing in the fish bioassay.

4.5 High-performance liquid chromatography

4.5.1 Ring-size separation of aromatics

Ring-size separation of the aromatic fraction was performed essentially as described by as described by Thomas et al. (2006) by HPLC using a Nitro (NO_2)-bonded silica column that separates aromatic compounds and compounds with double bonds. The Nucleosil nitrophenylpropyl silica semi-preparative HPLC column (25 cm x 10 mm x 5 µm; Phenomenex, Cheshire, UK) was fitted with a SecurityGuard™ column (Silica, 5 cm x 10 mm x 10 µm; Phenomenex, Cheshire, UK) and used a mobile phase of hexane (HPLC grade) and DCM (HPLC grade) at a flow rate of 5 ml min^{-1}. Gradient elution was used over 40 min; starting with isocratic elution for 5 min with hexane, followed by a 20 min gradient of 100 % hexane to 5 % DCM which was held for 5 min, followed by a 5 min gradient of 5 % DCM to 100 % DCM, and ending with a 100 % DCM flush for 5 min. Forty 5 ml fine fractions were collected at 5 min intervals. Following injection of standards (tolulene, naphthalene, phenanthrene, pyrene, benzo[a]pyrene and perylene), the fractions were taken as follows:

A1 (1-ring aromatics)	0-7 min
A2 (2-ring aromatics)	7-15.5 min
A3 (3-ring aromatics)	15.5-19.5 min
A4 (4-ring aromatics)	19.5-25 min
A5+ (5+ ring aromatics)	25-50 min

4.5.2 Isolation of alkylphenols and acids

The polar open column fraction was further fractionated by HPLC using a cyano-aino (PAC) bonded silica semi-preparative HPLC column (25 cm x 10 mm x 5 µm; Phenomenex, Cheshire, UK) fitted with a silica guard column (5 cm x 10 mm x 5 µm) at a flow rate of 5 ml min^{-1}, with a UV detector (210 nm) using HPLC grade hexane, dichloromethane (DCM) and iso-propanol as a mobile phase. Gradient elution was used over 50 min. The hexane was pumped isocratically for a period of 5 min, followed by a gradient of 100% hexane to 100% DCM for 15 min, followed by a gradient of 100% DCM to 100% isopropanol, ending with a 15 min 100% iso-propanol flush. Thirty 5 ml fine fractions were collected at one-minute intervals. Following injection of standards (C1-5 alkylphenols and Fluka naphthenic acids mixture), the fractions were taken as follows:

Alkylphenols	0-27 min
Acids	27-50 min

4.6 Fish in vitro bioassay

4.6.1 Fish

200-500 g immature male rainbow trout (*Oncorhynchus mykiss*), obtained from Killi Oppdrettsanlegg (Dombås, Norway), were kept in tanks at the Institute of Biology, University of Oslo (Norway), at a water temperature of 8-10 ºC, oxygen saturation of approximately 100 % and pH 6.6. The fish were fed daily with commercial fish pellets (EWOS, Bergen, Norway) in amounts corresponding to 0.5% of total body mass until isolation of the liver cells. The tanks received artificial illumination (100 lux) for 12 h/day.

4.6.2 Hepatocyte isolation, culturing and exposure

Hepatocytes were isolated, cultured and exposed essentially as described by Tollefsen et al. (2003). At sampling, fish was killed by a blow to the head and the liver was perfused *in situ* (10 ml/min, 10 min) with a calcium-free solution containing: NaCl (122 mM); KCl (4.8 mM); $MgSO_4$ (1.2 mM); Na_2HPO_4 (11 mM); NaH_2PO_4 (3.3 mM); $NaHCO_3$ (3.7 mM) and EGTA (26 uM) at 2 °C, until all blood had been washed out. The liver was then perfused for 10-15 min. with the same buffer, now containing $CaCl_2$ (1.5 mM) instead of EGTA, and 0.3 mg/ml collagenase (Type IV, Sigma-Aldrich) at 37°C to maximize the enzymatic tissue digestion. The liver was then removed and the cells dispersed in ice-cold calcium free buffer (EGTA-buffer) containing 0.1 % (w/v) BSA (Sigma-Aldrich). The cell suspension was filtered through 250 μm and then a 100 μm nylon mesh and centrifuged at 50 g for 3 min at 4 °C. The cells were washed with ice cold EGTA-buffer three times before finally to be resuspended in serum-free L-15 medium containing L-glutamine (0.29 mg/mL), $NaHCO_3$ (4.5 mM), penicillin (100 Units/mL), streptomycin (100 μg/L) and amphotericin (0.25 μg/mL), all supplied by Cambrex (East Rutherford, NJ, USA). After viability and cell yield were measured by the trypan blue exclusion test, the cells were diluted to 0.5 million cells/ml and seeded out at a density of 0.1 million cells/well in 96 wells primaria® plates (Falcon, Becton Dickingson Labware, Oxnard, Ca, USA). Only cell preparations with viability of 90 % or more were used for the experiments. All glassware and instruments were autoclaved before use and solutions were sterilised by filtration (0.22 μm).

The cells were cultured for one day in unspiked growth medium prior to replacement of half the initial volume by medium containing the vehicle DMSO (final concentration <1 % v/v), different concentrations of test compounds in triplicate either alone to determine agonistic acitivity or in

combination with the natural estrogen E2 (30 nM) or TCDD (20 pM) to determine the antagonistic activity. Cells targeted for EROD and CYP1A analysis were subjected to cytotoxicity determination after 48h exposure and cells frozen for subsequent analysis of EROD and CYP1A levels. Cells targeted for Vtg analysis was re-exposed for additional two-days, media removed and frozen at –80 °C for subsequent analysis of Vtg, and cytotoxicity determined on the cells. Cells targeted for DNA damage by the COMET method were exposed 2h, the media removed and the cells analysed directly for cytoxicity and DNA damage by the COMET assay. Figure 3 depict the the experiemtnal design used for determining the agonistic and antagonistic activity of single chemicals, produced water and produced water fractions.

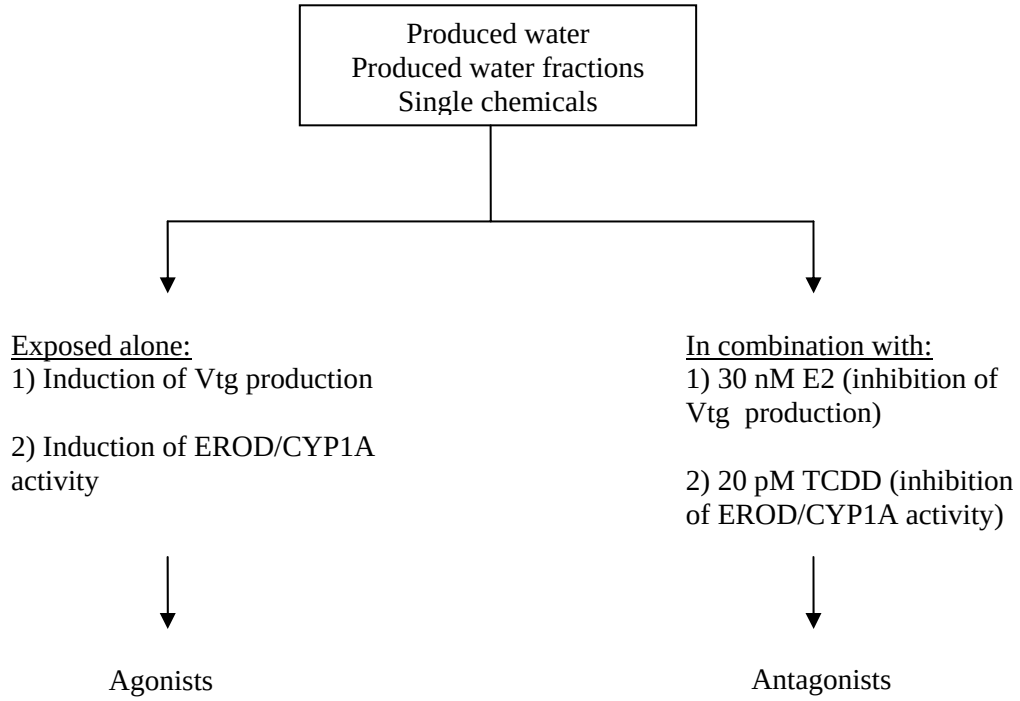

Figure 3. Schematic representation of the experimental approach used. Fish cells were exposed either alone to produced water, produced water frations or single chemicals to determine agonistic activity or in combination with a low concentration of the natural estrogen 17β-estradiol (E2, 20 nM) or to the model CYP1A inducer TCDD (30 pM). Controls in the different studies were either the solvent (DMSO or methanol) for determining agonistic activity or the model compound (30 nM E2 or 20 pM TCDD) for studies determining antagonistic activity.

4.6.3 Vitellogenin analysis

Vitellogenin was measured by a capture ELISA essentially as described by Tollefsen *et al.* (2003). In brief, microwell plates containing 75 µl growth media were thawed on ice, 75 µl internal Vtg assay

standards were added selected empty wells, and the plates incubated overnight in a humid atmosphere (4°C). Non-specific binding was blocked with 200 µl of 2 % (w/v) bovine serum albumin (Sigma-Aldrich) in PBS (2.5 mM Na_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, pH 7.2) for 1 hour (20 °C). For protein detection, 100 µl of a monoclonal mouse anti-salmon vtg, BN-5 (1:6000, Biosense Laboratories, Bergen, Norway) diluted in 1 % (w/v) BSA-PBS was incubated in the wells for 2 h. (37 °C). For enzymatic detection, 100 µl of a secondary antibody towards mouse IgG (1: 6000, Bio-Rad, Hercules, CA, USA) was incubated for 1 hour (37 °C), followed by incubation with 100 µl Trimethyl blue as the enzymatic substrate (20 °C). The reaction was stopped by adding 50 µl 2M H_2SO_4 and absorbance measured at 450 nm. Between all incubation steps the plates were washed three times with PBS added 0.05 % (w/v) Tween-20 except prior to the addition of substrate where the plates were washed five times.

4.6.4 7-ethoxyresorufin *O*-deethylase (EROD) activity

The EROD activity was measured directly in the cell culture essentially as described by (Ganassin *et al.*, 2000). Briefly, exposure media was removed from wells and exchanged with 100 µl of 50 mM Tris buffer containing 0.1 M NaCl, 829 nM of 7-ethoxyresorufin and 20 µM dicumarol (Sigma-Aldrich) and incubated on an orbital shaker for 15 minutes in the dark at room temperature. Fluorescence was thereafter measured using the excitation and emission wavelength of 530 nm and 590 nm, respectively. EROD activity was normalised to protein concentration as measured by the Bradford method (Bradford, 1976) using Bovine Serum Albumin as protein standard.

4.6.5 CYP1A analysis

CYP1A protein levels were measured by a capture ELISA using specific polyclonal antibodies. In brief, microwell plates containing cells were thawed on ice, 100 µl internal CYP1A assay standard were added selected empty wells, and the plates incubated overnight in a humid atmosphere (4°C). Non-specific binding was blocked with 200 µl of 2 % (w/v) bovine serum albumin (Sigma-Aldrich) in PBS (2.5 mM Na_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, pH 7.2) for 1 hour (20 °C). For protein detection, 100 µl of a polyclonal rabbit anti-fish CYP1A, CP-226 (1:6000, Biosense Laboratories, Bergen, Norway) diluted in 1 % (w/v) BSA-PBS was incubated in the wells for 2 h. (37 °C). For enzymatic detection, 100 µl of a secondary antibody towards rabbit IgG (1: 9000, Bio-Rad, Hercules, CA, USA) was incubated for 1 hour (37 °C), followed by incubation with 100 µl Trimethyl blue as enzymatic substrate (20 °C). The reaction was stopped by adding 50 µl 2M H_2SO_4 and absorbance measured at 450 nm. Between all incubation steps the plates were washed three times with PBS added 0.05 % (w/v) Tween-20 except prior to the addition of substrate where the plates were washed five times.

4.6.6 Cytotoxicity

Metabolic inhibition and loss of cell membrane integrity was assessed as cytotoxic effects directly in the cell culture wells using the fluorescent probes Alamar blue, AB (metabolic activity) and 5-carboxyfluorescein diacetate acetoxymethyl ester, CFDA-AM (membrane integrity) essentially as described by Tollefsen et al. (Tollefsen *et al.*, 2008). In essence, exposure media was removed from the wells and exchanged with 100 µl of Tris buffer (50 mM, pH 7.5) containing 5 % AB (BioSource international, Camarillo, CA, USA) and 4 µM CFDA-AM (Molecular Probes, Eugene, Oregon, USA). The cells were incubated for 30 minutes in the dark (20 °C) and the concentrations of the fluorescent metabolites of Alamar blue and CFDA-AM was measured simultaneously using the wavelength pairs of 530-590 nm and 485-530 nm (excitation-emission), respectively. The viability of the cells was determined on basis of the fluorescence of cells exposed to the solvent control DMSO (no effect) and the maximum toxicity obtained for $CuSO_4$ (10 mM). Only data for AB will be presented in this report due to interference of fluorescent compounds such as certain PAHs with the endpoint CFDA-AM.

4.6.7 Analysis of DNA damage by the COMET assay

Cells were then analysed for DNA strand breaks using the comet assay (single-cell gel electrophoresis), essentially as described in Olsen et al. (2003), with some modifications (Brunborg G, personal communication). In short, 60 ul of 0.63% agarose containing 10,000 cells were added to GelBond® films (Cambrex, ME, USA). Following lysis, electrophoresis (1.3 V/cm, 20 minutes) and neutralisation, films were fixed in ethanol and dried. For scoring, films were rehydrated and stained with SYBR Gold (InVitrogen, CA, USA; 0.1 ul/ml, 20 minutes) and examined in an Olympus BX51 fluorescence microscope, using the Perceptives Instruments Comet Assay IV software (Haverhill, UK). Resulting comets were scored and tail moment, tail length, head width and length were calculated.

4.7 Data treatment and statistical analysis.

The agonistic activity of the different samples, measured as an induction of CYP1A, EROD and Vtg were expressed relative to the solvent control (methanol or DMSO) representing the minimum induction level and the maximum induction level obtained for cells exposed to the model compounds (positive controls) E2 (100 nM) and TCDD (100 pM), respectively. In these studies the cells were exposed separately for the different test samples but expressed relative to cells exposed to the positive controls. The antagonistic activity, which was determined as the inhibition of CYP1A, EROD and Vtg, was expressed relative to the sub-maximal induction by the model compound E2 (30 nM) and

TCDD (20 pM), respectively. Cytotoxicity was determined as metabolic inhibition and was expresses relative to the toxicity by 10 mM $CuS0_4$ used as a positive control. Non-linear Hill (logistic) regression and statistical analyses were performed using GraphPad Prism 4.0 (GraphPad software Inc. San Diego, California, USA). Statistical differences between groups were determined by Anova, followed by a Bonferroni post test. Groups were considered significantly different from control at the $p<0.05$ level. The lowest observed effect concentration (LOEC) was determined as the lowest concentration causing a significant increase in the parameters measured.

5. Results and discussion

5.1 Fractionation and characterisation of different fractions

Produced water components were fractionated into eight fractions of known chemical composition. Ring-size separation of the aromatic fraction produced a UV chromatogram that showed the sample to be dominated by 2, 3 and 5+ aromatic ring compounds (Figure 4). Figure 5 shows the separation of alkylphenols and acids. The performance of the column fractionation for produced water compounds was not confirmed by in-depth chemical analysis, but reproducible fractionation of standards (see section 4.5. for details) were observed during method optimization. Thorough characterisation of fraction composition to confirm successful fractionation of compounds should be considered performed as follow up studies, however.

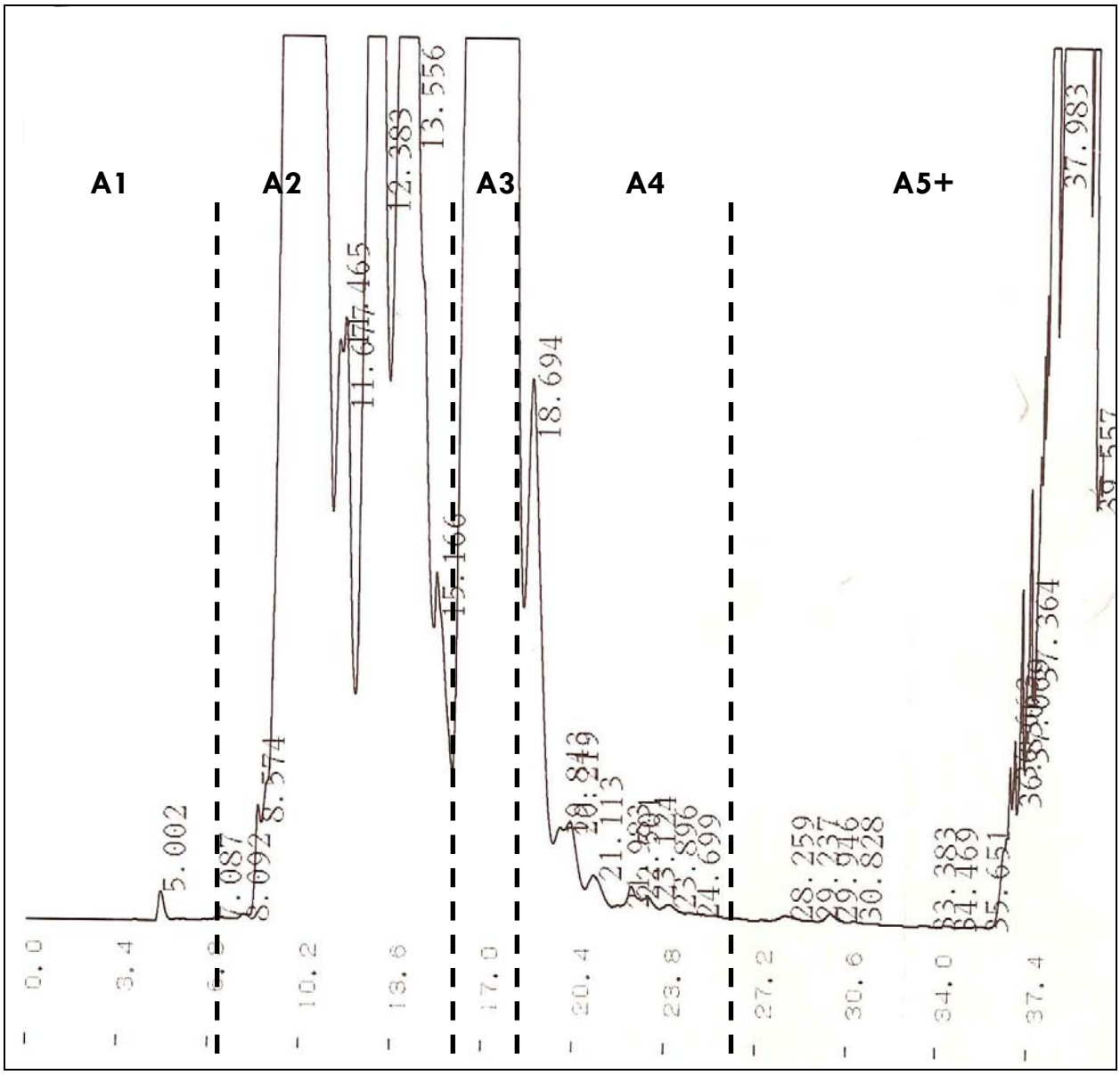

Figure 4. Ring-size separation (A1: 1-ring aromatics, A2: 2-ring aromatics, A3: 3-ring aromatics, A4: 4-ring aromatics, A5+: 5-ring aromatics and higher molecular aromatics) by normal phase HPLC.

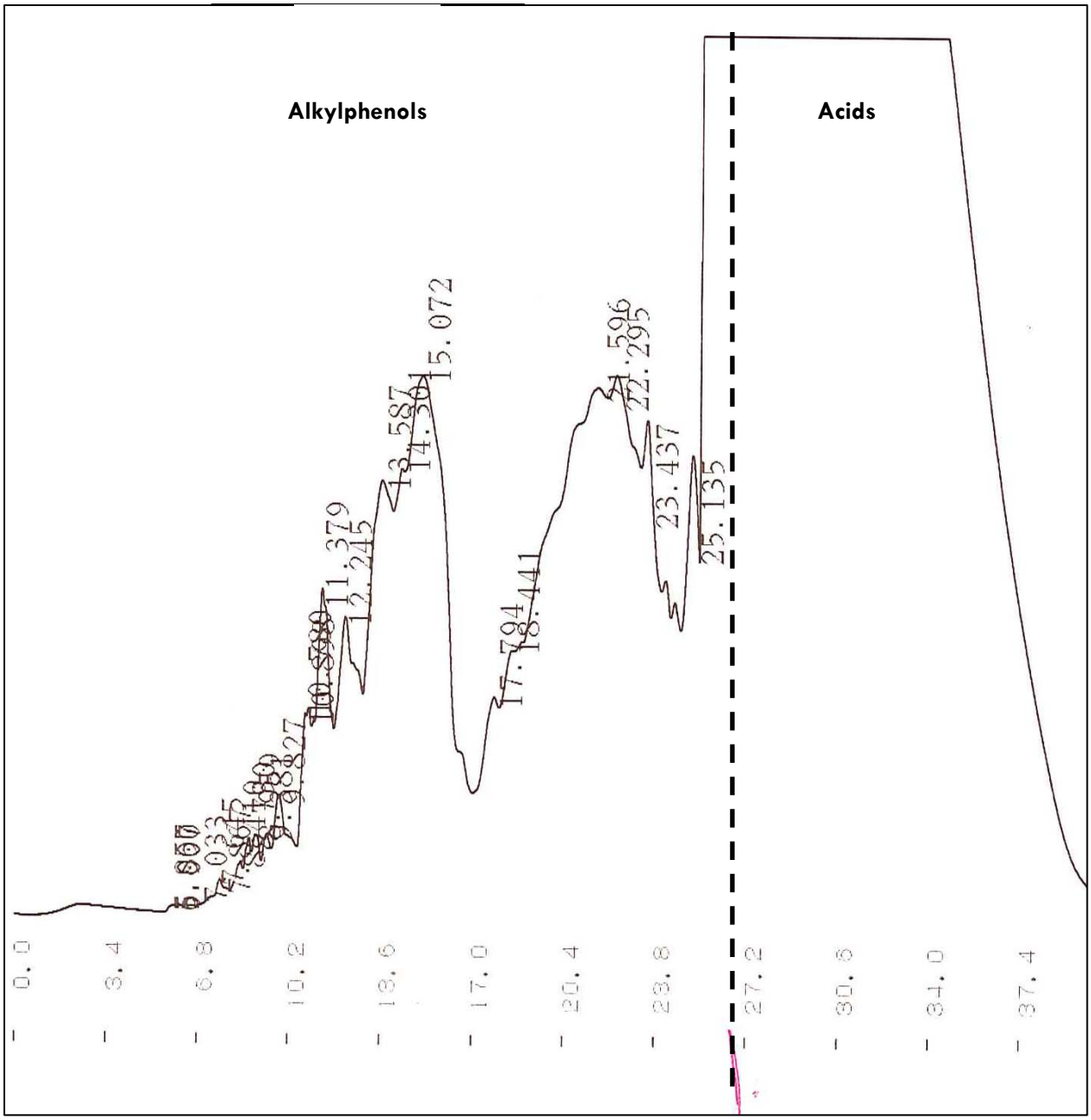

Figure 5. Separation of alkylphenols from acids by normal phase HPLC

5.2 Fish in vitro bioassays

Produced water and fractions of produced water were tested for the ability to induce and inhibit CYP1A, EROD and Vtg as well as causing DNA damage in a primary culture of rainbow trout hepatocytes. Cytotoxicity, measured as inhibition of metabolic activity, was tested in parallel in order to determine whether observed reduction in bioactivity was caused by acute toxicity to the cells. Verification studies were conducted with single model compounds representing napthalenes, PAHs, alkylphenols and selected production chemicals.

5.2.1 EROD induction

Solid phase extracts of produced water caused induction of EROD activity at the lowest concentration tested and an apparent inhibition of EROD activity was evident at higher concentrations (fig. 6). EROD induction was also observed for 1-, 2-, 4-, 5-ring aromatics, as well as for fractions containing alkylphenols. The largest relative induction was observed for 5+-ring aromatics, whereas 4-ring aromatics were apparently causing induction at the lowest concentrations, typically causing induction at concentrations being present in produced water or weakly up-concentrated samples. Surprisingly, 1-ring aromatics, which contained only low amount of organic material, were able to induce EROD acitivity. The fractions representing 3-ring aromatics, acids and aliphatics were not able to induce EROD activity at any of the concentrations tested. Some cytotoxicity was observed for the produced water extract and fractions containing alkylphenols, which partly explained the reduction of EROD activity at the highest concentrations tested.

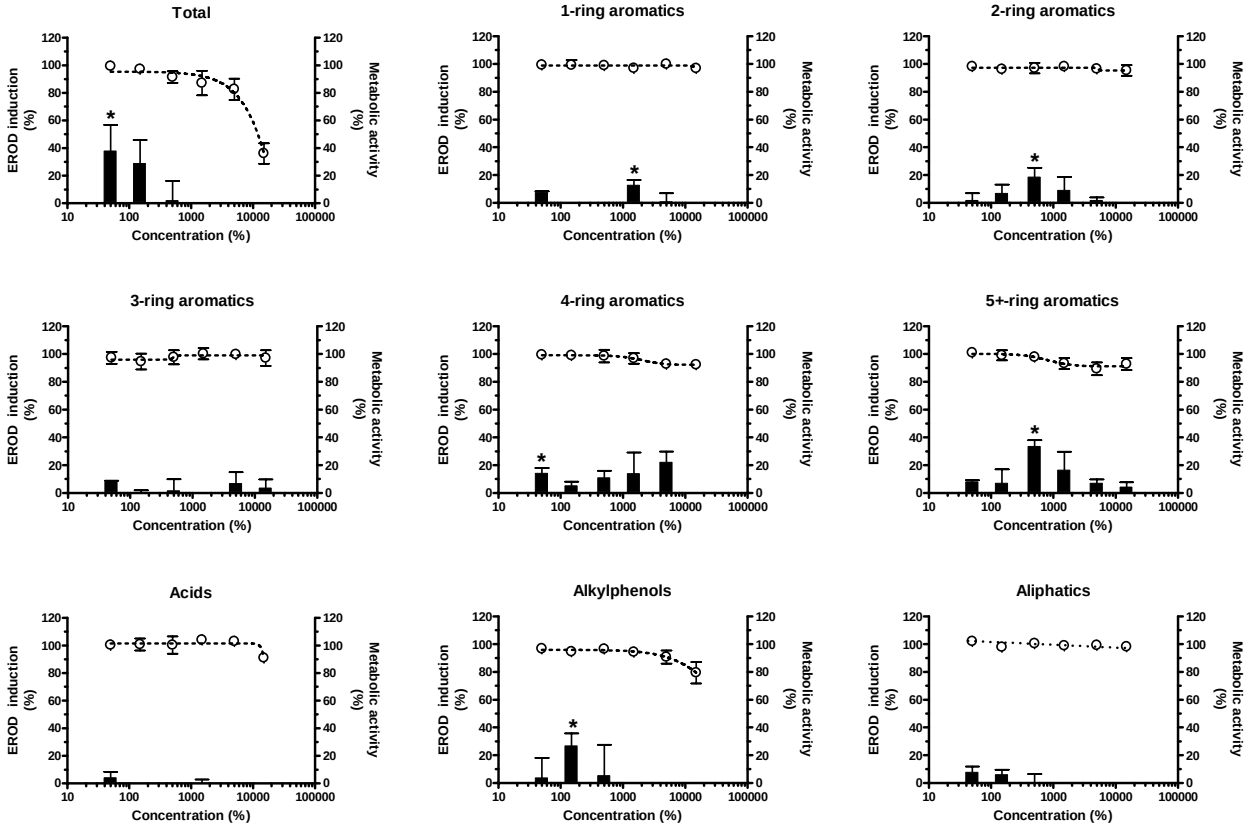

Figure 6. Induction of EROD activity by different concentrations (100%: undiluted produced water) of produced water (Total) and resulting fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics. The results (Mean±SEM) depict the cellular EROD activity expressed relative to cells exposed to 100 pM TCDD as positive control (bars). Cytotoxicity was measured as the depression of metabolic activity and expressed relative to the solvent control methanol (open circles). Asterisks denote level of significance compared to the solvent control.

The EROD inducing capability was determined for a selection of naphtahalenes, PAHs, alkylphenols and two production chemicals to determine whether typical produced water components were able to cause the observed effects. Napthalene (fig. 7), 2- and 3-ring PAHs (results not shown) and production chemicals were typically not able to cause significant induction of EROD activity, whereas 4- and 5 ring PAHs such as Benzo[*a*]anthracene, chrysene and Benzo[*a*]pyrene were potent EROD inducers causing induction at low micromolar concentrations (fig. 7). Interestingly, the bioactivity of the 4- and 5+-ring PAHs exhibited an inverted U-shaped curve that could not be explained by cytotoxicity limiting the cellular response, but rather indicating that these compounds act as EROD inducers at low concentrations and as EROD inhibitors at high concentrations. Alkylphenols were not able to induce EROD activity at the concentrations tested (results not shown) and could therefore not explain the bioactivity found in the alkylphenol fractions of produced water. The finding that

alkylphenols do not induce CYP1A–mediated EROD activity are in good agreement with findings reported by Hasselberg et al. (2004). The presence of unknown polar organics with EROD inducing properties that co-eluted with the alkylphenols during the HPLC fractionation procedure may explain the bioactivity observed in this fraction. Further chemical characterisation and possibly fractionation would be required to confirm and identify these compounds.

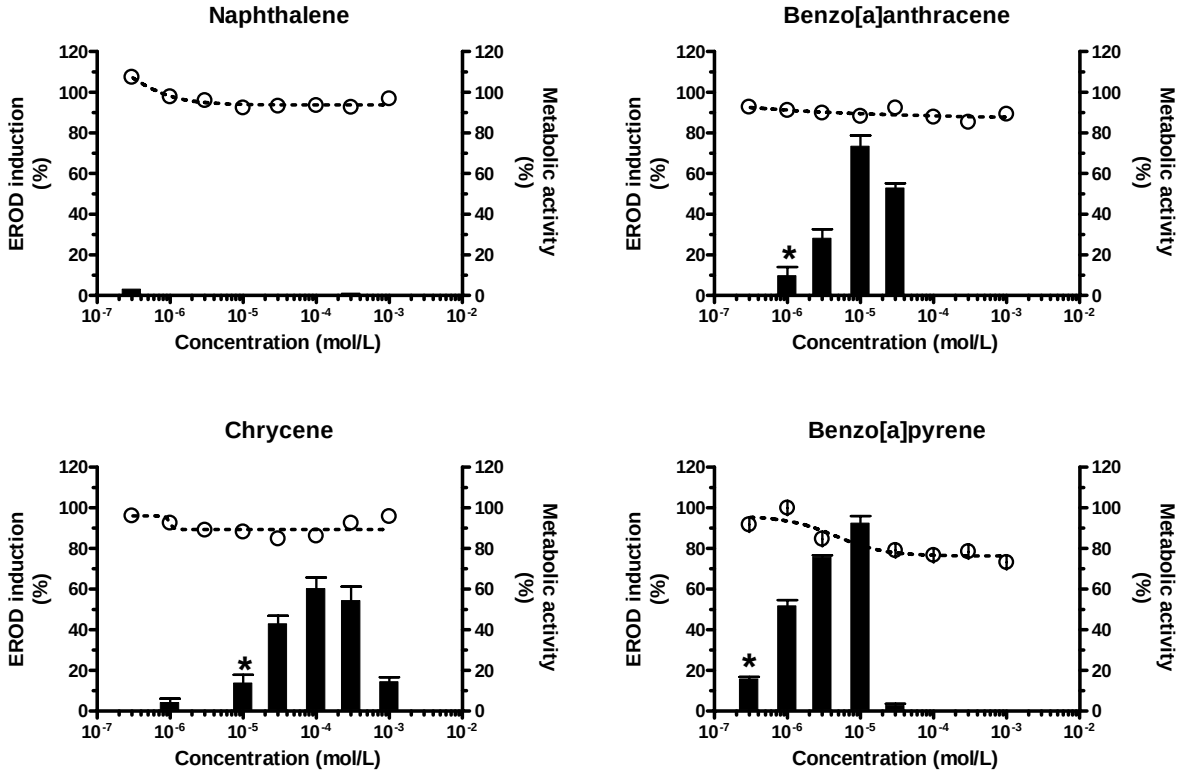

Figure 7. Induction of EROD activity by representatives for 2-ring PAHs (naphthalene), 4-ring PAHs (benzo[a]anthracene and chrysene) and 5+PAHs (benzo[a]pyrene). The results (Mean±SEM) depict the cellular EROD activity expressed relative to cells exposed to 100 pM TCDD as positive control (bars). Cytotoxicity was measured as the depression of metabolic activity and expressed relative to the solvent control DMSO (open circles). Asterisks denote level of significance compared to the solvent control.

5.2.2 CYP1A induction

Produced water and the organic acid fraction of produced water caused a concentration-dependent increase in CYP1A levels in the rainbow trout hepatocytes at 50-150 fold concentrated samples (compared to the original produced water sample), whereas none of the other fractions caused induction of CYP1A protein levels at the concentrations tested (fig. 8). Cytotoxicity clearly limited the response for the produced water sample, but did not limit any cellular response to the other fractions. Interestingly, produced water and several of the fractions (i.e. 1-, 2-, 4- 5+ PAH and alkylphenols) being able to induce EROD activity (fig. 7) did not affect CYP1A expression at the concentrations

tested (fig. 8), thus suggesting that EROD is the more sensitive and/or robust endpoint to typical produced water components when exposed in complex mixtures. Although CYP1A induction may be less sensitive for induction, CYP1A protein expression seems to be less affected than EROD activity to inhibition at high concentrations of produced water.

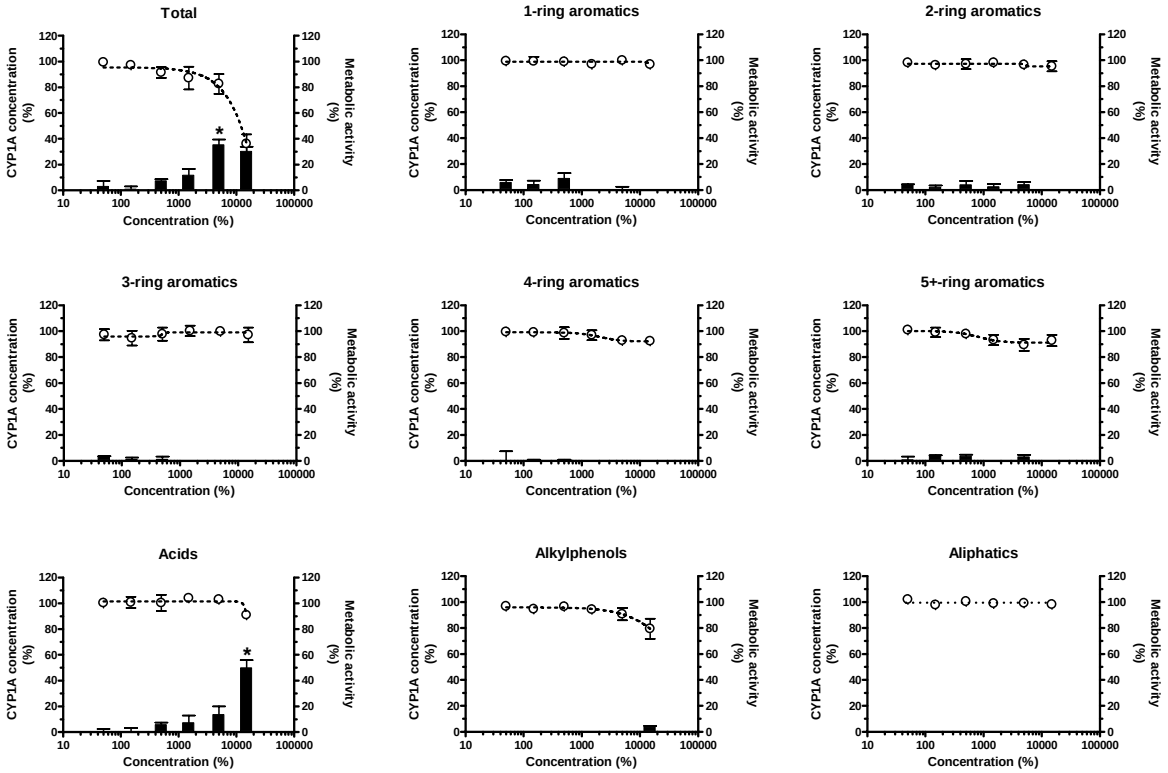

Figure 8. Induction of CYP1A by different concentrations (100%: undiluted produced water) of produced water (Total) and resulting fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics. The results (Mean±SEM) depict the cellular CYP1A level expressed relative to cells exposed to 100 pM TCDD as positive control (bars). Cytotoxicity was measured as the depression of metabolic activity and expressed relative to the solvent control methanol (open circles). Asterisks denote level of significance compared to the solvent control.

Assessment of the CYP1A inducing properties of common produced water compounds verify that compounds causing induction of CYP1A lso cause induction of EROD activity (fig. 9). As seen for EROD, 4- and 5+ ring PAHs such as benzo[*a*]anthracene, chrysene and benzo[*a*]pyrene were potent CYP1A inducers at micromolar concentrations and high concentrations of both benzo[*a*]anthracene and benzo[*a*]pyrene displayed partial agonistic acitivity (fig. 9). These findings are in agreement with findings elsewhere that large molecular weight PAHs are potent CYP1A and EROD inducers (Scholz and Segner, 1999; Behrens *et al.*, 2001), but contrast the lack of CYP1A induction by produced water

fractions puportingly containing these compounds (fig. 8). One reason for the discrepancy observed may be that the concentrations in the individual fractions were not sufficient to cause induction of CYP1A, or that the complex mixtures occurring in produced water may differentially affect CYP1A and EROD induction. In general, napthalene (fig. 9), low molecular PAHs, alkylphenols and the two production chemicals were poor CYP1A inducers, although certain compounds such as carbazole and methylantracene did cause small elevation of CYP1A levels at high micromolar concentrations (results not shown). No clear induction of CYP1A was observed for the production chemicals tested.

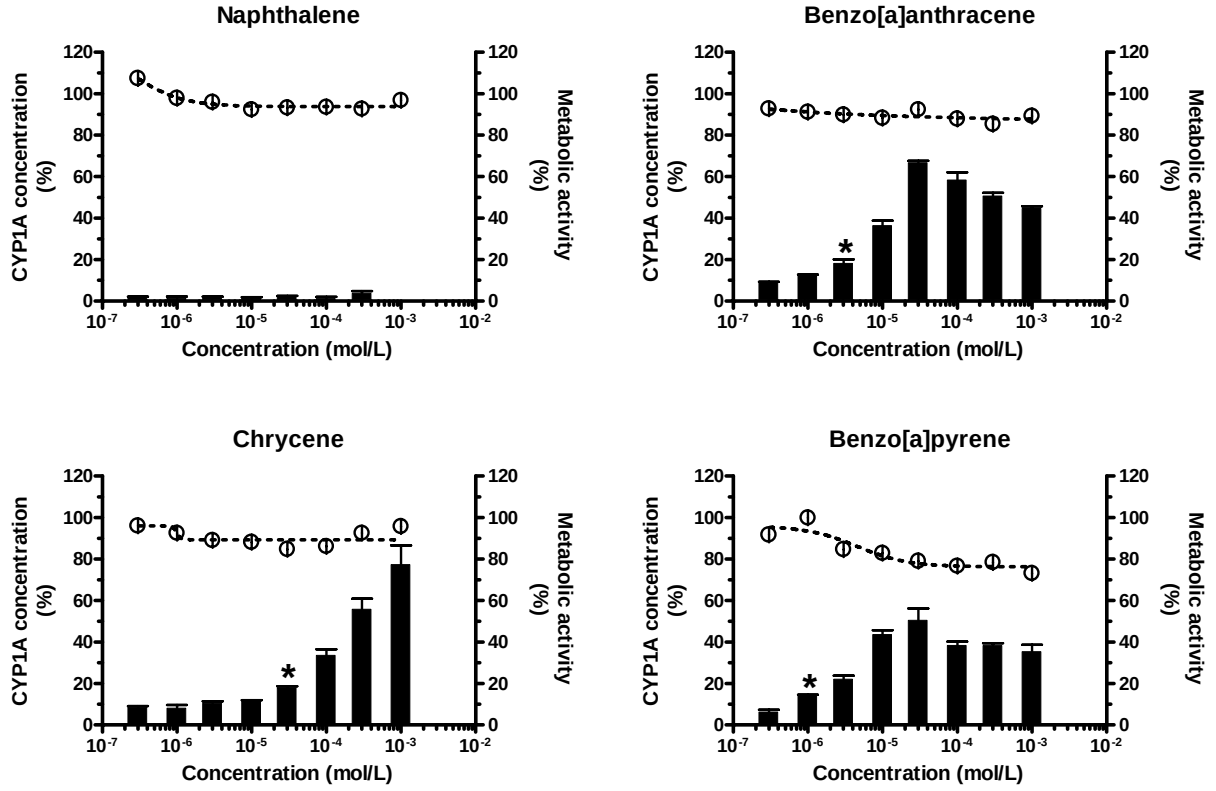

Figure 9. Induction of CYP1A by representatives for 2-ring PAHs (naphthalene), 4-ring PAHs (Benzo[a]anthracene and chrysene) and 5+PAHs (Benzo[a]pyrene). The results (Mean±SEM) depict the cellular CYP1A level expressed relative to cells exposed to 100 pM TCDD as positive control (bars). Cytotoxicity was measured as the depression of metabolic activity and expressed relative to the solvent control DMSO (open circles). Asterisks denote level of significance compared to the solvent control.

5.2.3 Vitellogenin induction

Exposure to SPE extracts of produced water and the alkylphenol fraction caused a statistically significant increase in the estrogenic biomarker vitellogenin at high sample concentrations, typically 50-150 times more concentrated that the original produced water sample (fig. 10). The Vtg induction typically occurred at concentrations being either weakly cytotoxicity or very close to the cytotoxic

concentrations, thus suggesting a narrow window between sub-lethal toxicity and cytoxicity as seen for alkylphenols elsewhere (Tollefsen *et al.*, In press). No significant Vtg induction was observed for the other fractions.

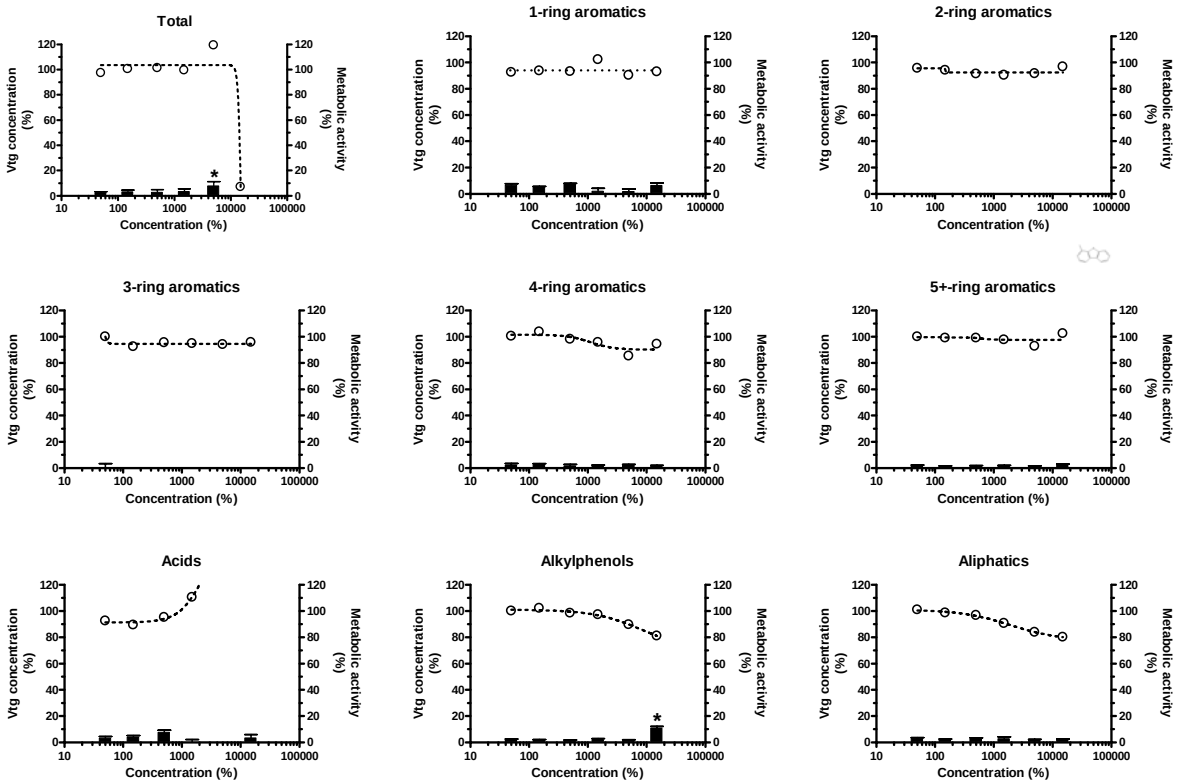

Figure 10. Induction of vitellogenin (vtg) production by different concentrations (100%: undiluted produced water) of produced water (Total) and resulting fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics. The results (Mean±SEM) depict the cellular Vtg production relative to cells exposed to 100 nM E2 as positive control (bars). Cytotoxicity was measured as the depression of metabolic activity and expressed relative to the solvent control methanol (open circles). Asterisks denote level of significance compared to the solvent control.

Alkylphenols have previously been proposed to cause the estrogenicity of produced water (Thomas *et al.*, 2004, 2004) and the estrogenicity of this diverse group of compounds has been demonstrated in the rainbow trout hepatocyte model (Tollefsen *et al.*, In press). Although most APs may act as estrogens when present in mixtures (Tollefsen *et al.*, In press), only low molecular weight APs are present in high enough concentrations in produced water to cause induction alone. APs such as C3-C5 are the most probable candidates for the estrogenicity observed in produced water and these compounds were estrogenic at low micromolar concentrations (fig. 11). In vitro studies with rainbow trout cells and homogenates of hepatic estrogen receptors confirm that moderate molecular weight APs are among the most estrogenic APs, and finding that contradict previous studies with recombinant in vitro bioassays with human estrogen receptors (Routledge and Sumpter, 1997).

Naphthalenes, low molecular PAHs and the two production chemicals were not able to cause consistent Vtg induction at the concentrations tested (results not shown).

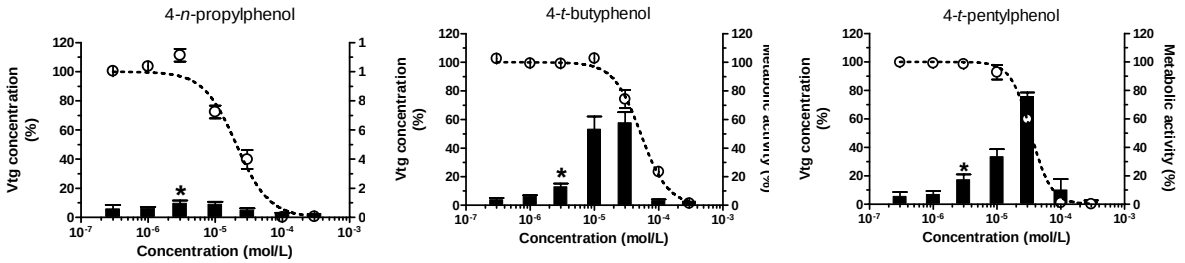

Figure 11. Induction of vitellogenin (vtg) production by 4-n-propylphenol, 4-t-butylphenol and 4-t-pentylphenol. The results (Mean±SEM) depict the cellular Vtg production relative to cells exposed to 100 nM E2 as positive control (bars). Cytotoxicity was measured as the depression of metabolic activity and expressed relative to the solvent control DMSO (open circles). Asterisks denote level of significance compared to the solvent control. Data is compiled from Tollefsen et al. (In press).

5.2.4 EROD inhibition

The ability to inhibit cellular EROD activity was determined in co-exposures with a sub-maximum response concentration of the potent inducer TCDD. Produced water, 4-ring aromatics, the organic acid and alkylphenol fraction of produced water caused a concentration-dependent inhibition of the EROD activity in the fish liver cells. An apparent inhibition in EROD activity was also observed for the 5+ ring aromatic fraction, although not statistically significant. Inhibition for most compounds occurred at compound concentrations corresponding to concentrated produced water (0.5-5000 times concentrated) and the cellular responses were not clearly limited by cytotoxicity in most cases except for high concentrations of the alkylphenolic fraction. Although the observed inhibition by the alkylphenol fraction may be argued to be caused by cytotoxicity at high concentrations, studies with the estrogenic 4-*t*-butylphenol suggest that this compound may inhibit EROD activity at micromolar concentrations. This findings agree well with data from Hasselberg et al. (2004) reporting that mid-sized alkylphenols are inhibitors of CYP1A-mediated EROD activity. Studies with compounds such as coumarin, benzo[*a*]anthracene and benzo[*a*]pyrene confirm that both low molecular weight and high molecular weight PAHs are also able to inhibit the EROD activity (fig. 13). The two production chemicals were not causing any inhibition of EROD activity at concentrations that were non-toxic to the cells.

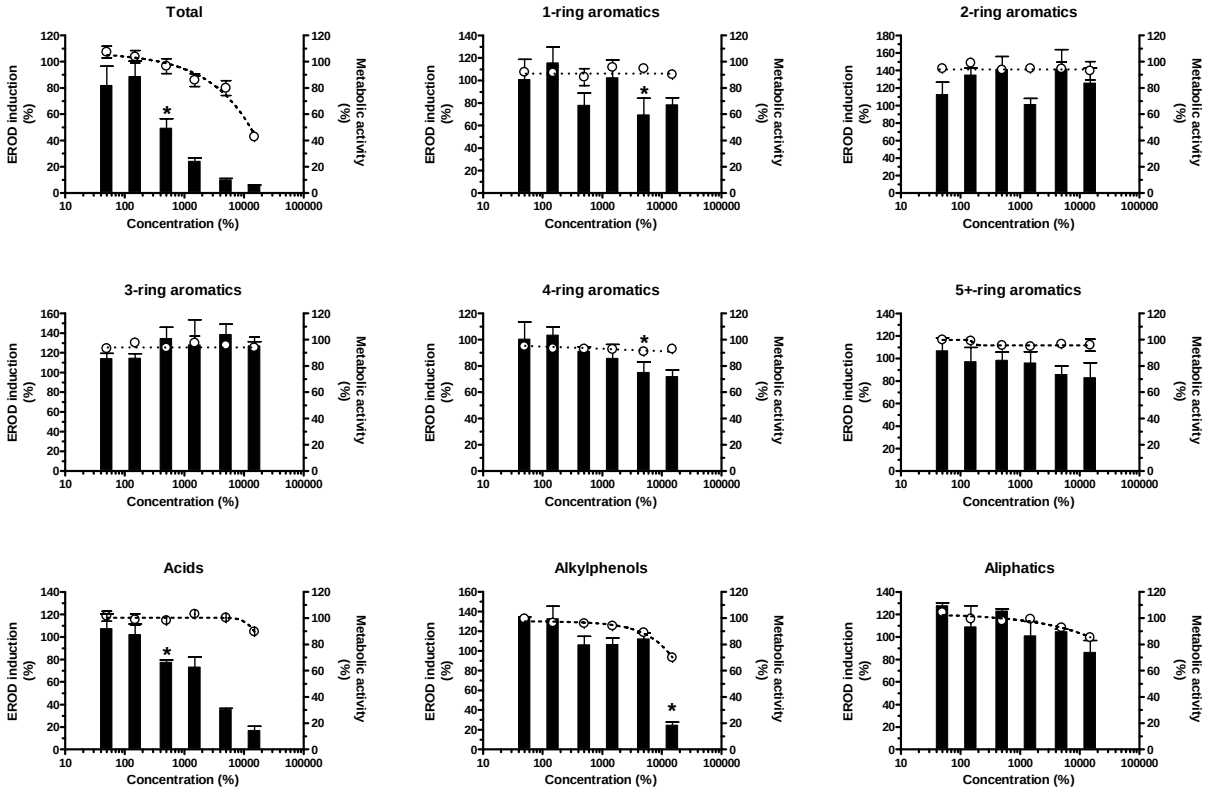

Figure 12. Inhibition of cellular EROD activity by different concentrations (100%: undiluted produced water)of produced water (Total) and resulting fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics when co-exposed to 20 pM TCDD (induction control). The results (Mean±SEM) depict the cellular EROD activity (bars) and cytotoxicity measured as metabolic inhibition (open circles) expressed relative to the activity of the induction control alone. Asterisks denote level of significance compared to cells exposed to the induction control.

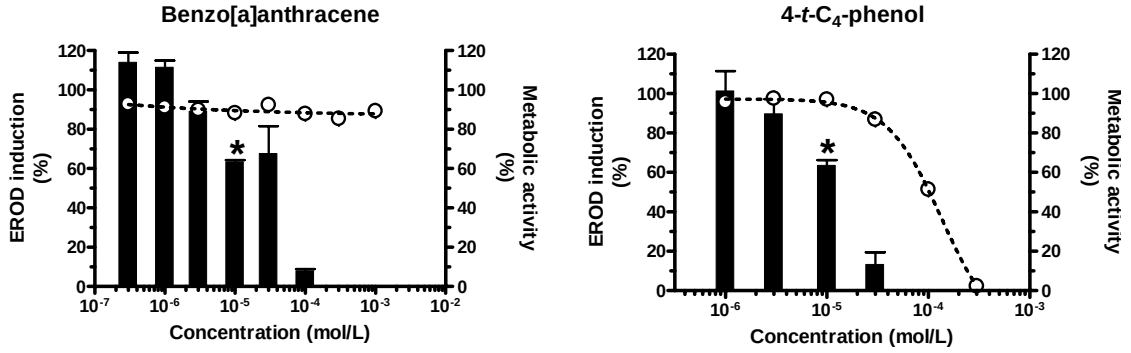

Figure 13. Inhibition of cellular EROD activity by representatives of 4-ring PAHs (benzo[a]anthracene) and alkylphenols (4-t-butylphenol) when co-exposed to 20 pM TCDD (induction control). The results (Mean±SEM) depict the cellular EROD activity (bars) and cytotoxicity measured as metabolic inhibition (open circles) expressed relative to the activity of the induction control alone. Asterisks denote level of significance compared to cells exposed to the induction control.

5.2.5 CYP1A inhibition

Inhibition of CYP1A protein expression was determined on the same cell population as that studying inhibition of EROD activity. The results from these studies show that produced water as well as the 1-ring aromatic, organic acid, alkylphenol and the aliphatic fraction caused inhibition of CYP1A protein expression (fig. 14). The inhibition occurred at fairly similar concentrations as that causing inhibition of EROD activity, although some differences were observed. Produced water and the acid fraction were less efficient in inhibiting CYP1A activity than EROD activity, whereas the alkylphenol fraction was most efficient in inhibiting CYP1A expression compared to EROD activity. The aliphatic fraction was only able to inhibit CYP1A induction, whereas 4-ring aromatics were only able to inhibit EROD activity. 1-ring aromatics were in both cases only able to inhibit CYP1A and EROD activity when samples were highly concentrated. Cytotoxicity were observed at high concentrations of produced water, the alkylphenol and the aliphatic fraction, and may have caused some of the inhibition in CYP1A protein expression observed, especially in the case of the produced water sample and fractions representing alkylphenols and aliphatics. The limited window of bioactivity compared to cytotoxicity was verified in studies with 4-*t*-butylphenol (fig. 15), thus indicating that inhibition of CYP1A activity barely preceded any cytotoxic effects. Studies with coumarin, benzo[a]anthracene show that both low molecular and high molecular PAHs may inhibit CYP1A protein expression (fig. 15). The two production chemicals did not cause any inhibition of CYP1A expression at concentrations that were non-toxic to the cells.

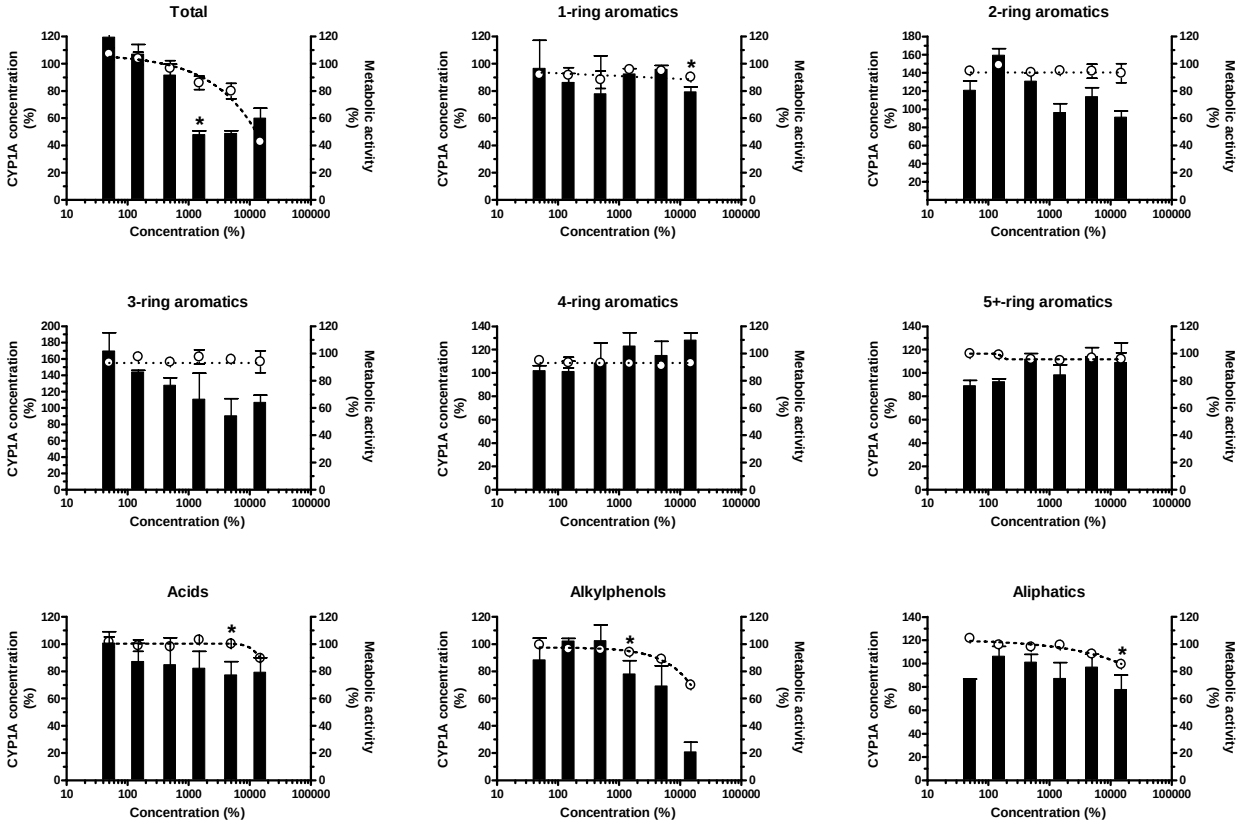

Figure 14. Inhibition of CYP1A by different concentrations (100%: undiluted produced water) of produced water (Total) and resulting fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics when co-exposed to 20 pM TCDD (induction control). The results (Mean±SEM) depict the cellular CYP1A levels (bars) and cytotoxicity measured as metabolic inhibition (open circles) expressed relative to the activity of the induction control alone. Asterisks denote level of significance compared to cells exposed to the induction control.

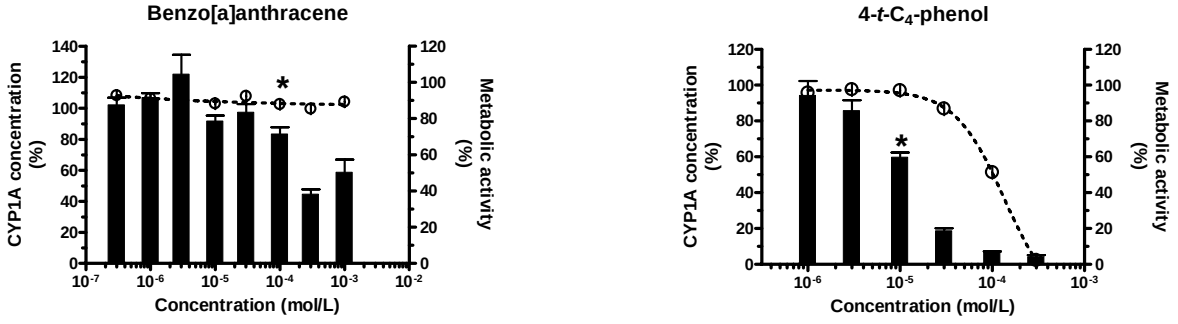

Figure 15. Inhibition of CYP1A by representatives of 4-ring aromatics (benzo[a]anthracene) and alkylphenols (4-t-butylphenol) when co-exposed to 20 pM TCDD (induction control). The results (Mean±SEM) depict the cellular CYP1A levels (bars) and cytotoxicity measured as metabolic inhibition (open circles) expressed relative to the activity of the induction control alone. Asterisks denote level of significance compared to cells exposed to the induction control.

5.2.6 Vitellogenin inhibition

Hepatic induction of vitellogenin production was found to be inhibited by produced water and fractions representing 1-ring, 2-ring, organic acids, alkylphenols and aliphatic compounds (fig. 16). Inhibition of Vtg protein expression occurred in a concentration-dependent manner and was most evident in the produced water sample. Only minor inhibiting potential was observed for 1-ring, 2-ring and aliphatic fractions, whereas more pronounced effects were observed for the acid and alkylphenolic fraction. Verification studies with coumarin, Benzo[*a*]anthracene and Benzo[*a*]pyrene show that all of these compounds were able to inhibit Vtg induction in a dose-dependent manner (fig. 17). Although these compounds were cytotoxic to the cells at micromolar concentrations, they were efficient inhibitors at concentrations being 3-100 times lower than that causing cytotoxicity. Exposure to alkylphenols and the two production chemicals did not cause any clear inhibition of vitellogenin production, although some inhibition of in vitro Vtg production has previously been demonstrated for the alkylphenols (Tollefsen *et al.*, In press).

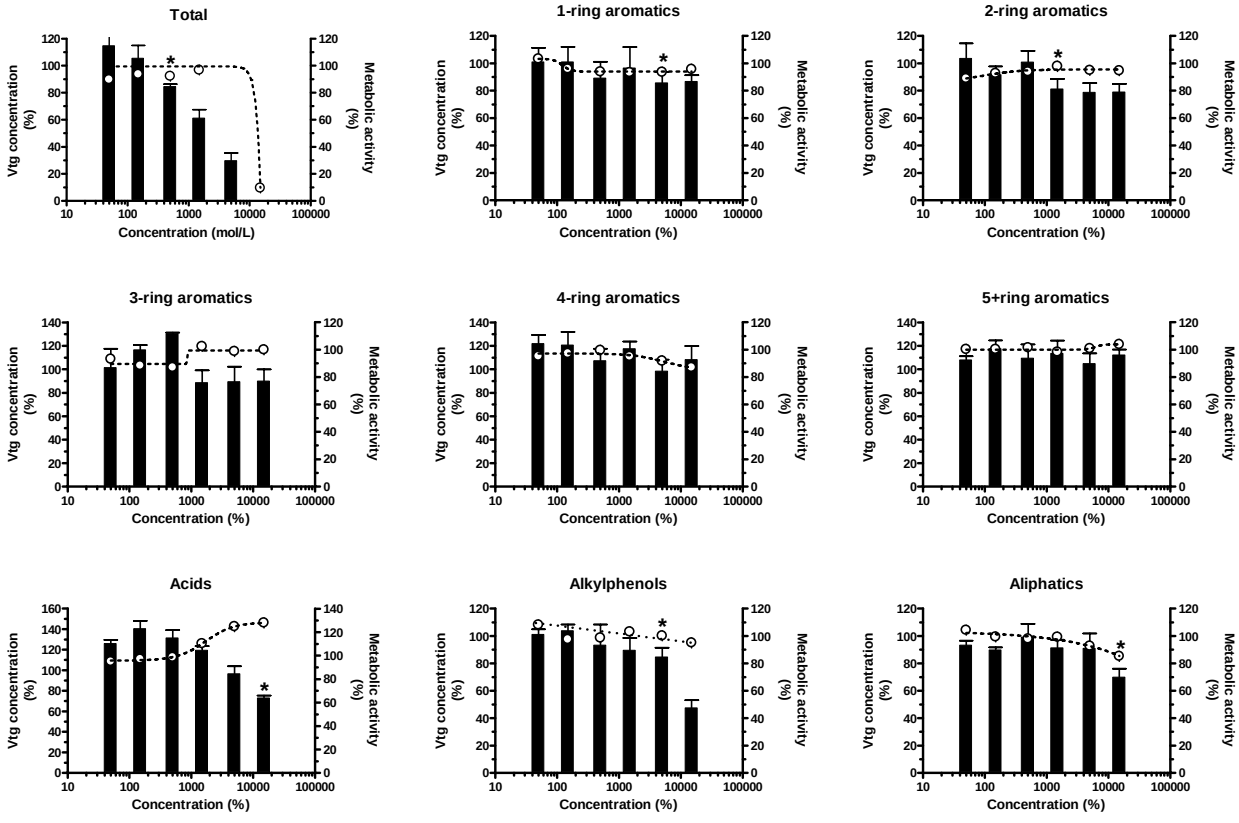

Figure 16. Inhibition of vitellogenin (Vtg) production by different concentrations (100%: undiluted produced water) of produced water (Total) and resulting fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics when co-exposed to 17β-estradiol (E2). The results (Mean±SEM) depict the cellular Vtg production (bars) and cytotoxicity measured as metabolic inhibition (open circles) expressed relative to the activity of 30 nM E2 (induction control) alone. Asterisks denote level of significance compared to cells exposed to the

induction control. Asterisks denote level of significance compared to cells exposed to 30 nM E2 in methanol (induction control).

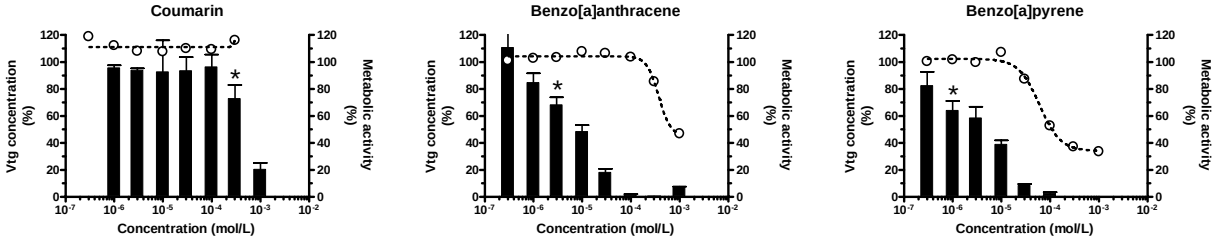

Figure 17. Inhibition of vitellogenin (Vtg) production by 2-ring aromatics (coumarin), 4-ring aromatics (Benzo[a]anthracene) , 5+-ring aromatics (Benzo[a]pyrene) when co-exposed to 17β-estradiol (E2). The results (Mean±SEM) depict the cellular Vtg production (bars) and cytotoxicity measured as metabolic inhibition (open circles) expressed relative to the activity of 30 nM E2 (induction control) alone. Asterisks denote level of significance compared to cells exposed to the induction control. Asterisks denote level of significance compared to cells exposed to 30 nM E2 in DMSO (induction control).

5.2.7 DNA damage

The DNA damaging potential of produced water and produced water fractions was determined by the COMET method at a fixed concentration corresponding to produced water concentrated 500 times. Results from these analyses show that cells exposed to the positive control and fractions representing 5-ring aromatics and acids consistently caused DNA damage to the cells. Produced water and fractions representing alkylphenols and 1-ring aromatics gave non-conclusive results, some COMET parameters clearly showing damage whereas others did not. Endpoint-specific variation in DNA damage assessment by the COMET method have previously been reported (Schnurstein and Braunbeck, 2001), and have outlined the importance to report multiple COMET parameters. Higher prevalence of damage in fish cells exposed to large molecular weight aromatics such as PAHs have previously been reported (Mitchelmore and Chipman, 1998), but DNA damaging properties of polar organics co-eluting with acids have been poorly characterised. The concentrations tested did not cause any cytotoxicity to the cells, thus did not negatively affected the assessment of DNA damage. Exposure to the positive control NQO

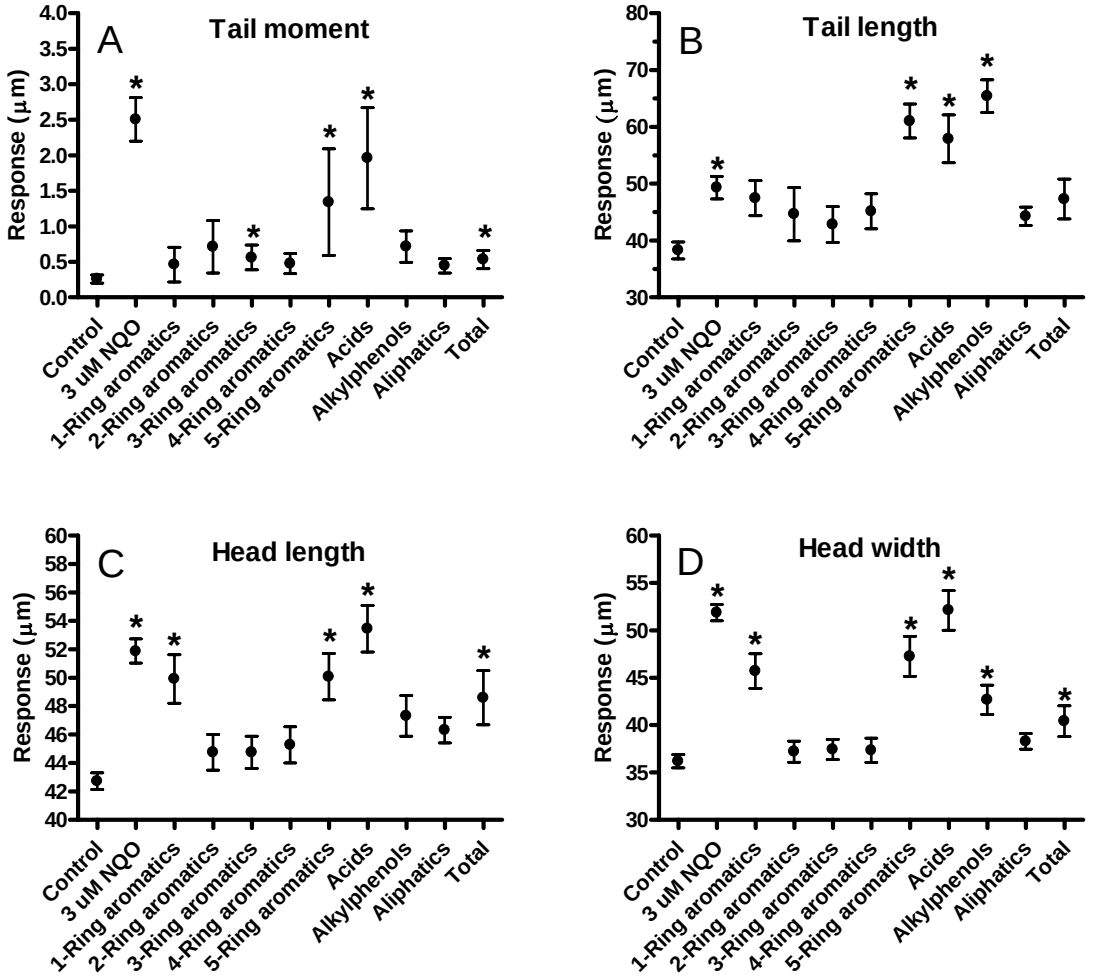

Figure 18. DNA damage in rainbow trout hepatocytes after 2h exposure to the positive control 4-nitroquinoline-1-oxide (NQO), 500 times concentrated produced water (Total) and resulting fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics. The results (Mean±SEM) depict the (A) tail moment, (B) tail length, (C) head Length and (D) head with when analysed by the COMET method. Asterisks denote level of significance compared to the solvent carrier methanol which was used as control.

5.2.8 No observed effect concentration (NOECs)

NOECs were determined as the highest concentration not causing any significant effects compared to the control groups in the in vitro experiments with fish hepatocytes. The results from these analysis show that produced water from Statfjord C were clearly the most toxic compound to the cells, determined by the ability to interfere with one or more endpoints, closely followed by alkylphenols and acids. The aromatic fractions were largely associated with EROD induction (and to a lesser degree inhibition) and DNA damage (only 5+-ring aromatics), whereas the alkylphenol fraction was associated with EROD and Vtg induction (and inhibition) as well as CYP1A and EROD inhibition. The acid fraction, which is poorly characterised and studied, caused a variety of effects including CYP1A induction and DNA damage as well as inhibition of EROD, CYP1A, Vtg cellular production. The 3-ring aromatics and aliphatics were not causing any consistent results at the concentrations tested in the fish bioassay. Although it is amply demonstrated that the produced water composition (Utvik, 1999) and toxicity (Tollefsen, pers. comm.) vary between different oil and gas production platforms, it should be expected that the measured NOECs may only provide indication of the toxic potential by produced waters from other sources, however.

Table 2. No observed effect concentration (NOEC) of produced water (Total) and isolated fractions of produced water compared to their fractions of 1-ring aromatics, 2-ring aromatics, 3-ring aromatics, 4-ring aromatics, 5+-ring aromatics, acids and aliphatics. NOEC values depict the concentration of sample representing a given concentration of produced water (100%: undiluted produced water).

	NOEC (%)						
	Induction			**Inhibition**			**DNA damage**
Compounds	**EROD**	**CYP1A**	**Vtg**	**EROD**	**CYP1A**	**Vtg**	
Total	<50	5000	1000	150	500	150	5000
1-ring aromatics	500	>15000	>15000	>15000	5000	1500	>5000
2 ring aromatics	150	>15000	>15000	>15000	>15000	1500	>5000
3 ring aromatics	>15000	>15000	>15000	>15000	>15000	>15000	>5000
4 ring aromatics	<50	>15000	>15000	5000	>15000	>15000	>5000
5+ ring aromatics	150	>15000	>15000	>15000	>15000	>15000	5000
Acids	>15000	5000	>15000	150	1500	5000	5000
Alkylphenols	50	>15000	5000	5000	500	1500	(5000)
Aliphatics	>15000	>15000	>15000	>15000	>15000	5000	>5000

Determination of NOECs for some of the individual compounds tested, representing the most toxic fractions from produced water, show that small molecular aromatic compounds such as naphthalene (2-ring aromatics) did not cause any cellular effects at the concentrations tested (table 3). Other 2-ring aromatics such as coumarin were fairly potent inhibitors of EROD, CYP1A and vitellogenin induction. Larger aromatics such as chrycene, benzo[*a*]anthracene and benzo[*a*]pyrene displayed a biphasic effect pattern by being EROD and CYP1A inducers at low concentrations, but EROD inhibitors at higher concentrations. Benzo[*a*]anthracene and benzo[*a*]pyrene also caused inhibition of CYP1A, although at higher concentrations than that causing inhibition of EROD acitivity. Both benzo[a]anthracene and benzo[a]pyrene, but not chrysene, were potent inhibitors of Vtg production at low micromolar concentrations. Alkylphenols, exemplified by 4-*t*-butylphenol, were largely associated with Vtg induction, but also with the inhibition of both EROD and CYP1A at low micromolar concentrations. Unfortunately, lack of good standards for assessing the effects of organics acids limited our ability to study this group of compounds. NOECs for individual compounds are presented in annex 2.

Table 3. No observed effect concentration (NOEC) of selected produced water components.

NOEC (µmol/L)

	Induction			Inhibition		
Compound	**EROD**	**CYP1A**	**Vtg**	**EROD**	**CYP1A**	**Vtg**
Naphthalene	>1000	>1000	>1000	>1000	>1000	>1000
Coumarin	>1000	>1000	>1000	10	3	100
Benzo[a]anthracene	0.3	1	>1000	3	30	1
Chrycene	3	10	>1000	300	>1000	>1000
Benzo[a]pyrene	<0.3	0.3	>1000	30	1000	0.3
4-*t*-butylphenol	>300	>300	1	3	3	>300

NOEC (mg/L)

	Induction			Inhibition		
Compound	**EROD**	**CYP1A**	**Vtg**	**EROD**	**CYP1A**	**Vtg**
Naphthalene	>128	>128	>128	>128	>128	>128
Coumarin	>146	>146	>146	1.5	0.44	14.6
Benzo[a]anthracene	0.07	0.23	>228	0.69	6.9	0.23
Chrycene	0.69	2.28	>228	68.5	>228	>228
Benzo[a]pyrene	<0.08	0.08	>252	7.6	>252	0.08
4-*t*-butylphenol	>50	>50	0.15	0.45	0.45	>50

5.2.9 Predicted environmental concentrations.

The composition of produced water is highly complex, but fairly well characterised for a number of compound groups. Determination of concentrations of compounds in the Sea water recipient, and at stations being relevant for the WCM surveys, is rather poorly described in literature. A study using measured produced water concentrations of key compounds and predictions made on basis of deployment of SPMDs, mussels and dispersion modelling provided some indications on concentrations of compound groups at the closest caging station at the Statfjord BECPELAG water column Monitoring in 2001 (Utvik and Gärtner, 2006) and was used to derive Predicted Environmental Concentrations (PECs) for a range of compounds (table 4). Although produced water is highly variable in composition, the use of data for Statfjord B may provide some theoretical insight into potential PECs for produced waters discharged to sea from the Statfjord area. For comparison purposes it has been assumed that the concentrations of produced water components are fairly similar at Statfjord B and C, however.

Table 4. Measured produced water, calculated seawater concentrations and Predicted Environmental Concentration (PEC) of organic produced water components at Statfjord B. Data compiled from Utvik and Gärtner (2006).

	Concentration (ng/L)				
Compound group	**Produced water**	**500 m (SPMD[1])**	**500 m (Mussels[2])**	**500 m (Dream [3])**	**PEC 500 m (Median)**
Naphthalenes	1.8×10^6	461	6.4	147	147
2-3 ring PAHs	1.8×10^5	95	3.5	4.6	4.6
4-5 ring PAHs	4.2×10^3	5.5	0.61	0.16	0.61
C0-C3 phenols	3.7×10^6	-	-	-	527[4]
C4-C5 phenols	1.8×10^5	-	-	-	154[4]
C6-C9 phenols	2.0×10^3	-	-	-	0.25[4]
Acids	-	-	-	-	-
Aliphatics	2.0×10^7	-	-	-	2818[4]

[1]Water concentration estimated by SPMD, [2]Water concentration estimated from mussel concentrations, [3]Water concentration estimated by dispersion modelling using DREAM, [4] calculated on the basis of the median dilution factor of 8163 for naphthalenes, 2-3 ring PAHs and 4-5 ring PAHs.

5.2.10 Predicted no effect concentrations.

In vitro methods have the advantage of rapidly screen for potential toxicological effects and provide mechanistic understanding for how chemicals and complex mixtures such as produced water may act in an organism. Extrapolation of in vitro response data to in vivo bioactivity is however required to determine whether sufficient concentrations can be obtained in the target organs (such as the liver) to cause effects in fish exposed to produced water components. The NOECs derived for cellular endpoints in this study were therefore used to calculate predicted no observed effect concentrations (PNECs) for fish (in vivo) by using estimated bioconcentration factors for individual produced water compounds. In essence, NOECs from in vitro studies were assumed to represent the NOEC of the target tissues in fish and bioconcentration factors were used to back-calculate to water concentrations that should not be expected to cause effects (PNECs). Although being a clearly theoretical and non-traditionbal approach to the construction of PNECs, the resulting data should provide a rough estimate on whether to expect effects under relevant exposure situations.

The present approach predicted that exposure to individual compounds at concentrations present in undiluted produced water may cause induction of EROD (benzo[*a*]anthracene, chrycene, benzo[*a*]pyrene), CYP1A (benzo[*a*]anthracene, chrycene, benzo[*a*]pyrene), vitellogenin (4-*t*-butylphenol). Concentrations of individual compounds in produced water should also be sufficient to inhibit the induction of EROD activity (benzo[*a*]anthracene, benzo[*a*]pyrene and 4-*t*-butylphenol), CYP1A (coumarin, benzo[*a*]anthracene, 4-*t*-butylphenol) and vitellogenin (benzo[*a*]anthracene and benzo[*a*]pyrene). Since concentrations are expected to quickly reduced some distance away from the production platform, few effects are to be expected at station farther than some hundred meters away from the discharge point based on the predictions made for PECs during the BECPELAG field trial at Statfjord in 2001 (Utvik and Gärtner, 2006) (table 5). In fact, calculations based on SPMDs, caged mussels and dispersion modelling suggest that the median PEC for the present groups of compounds are about ten fold to several orders of magnitude lower than the experimentally derived PNECs. The largest PEC/PNEC ratio was identified for induction of EROD and CYP1A (benzo[*a*]anthracene and benzo[*a*]pyrene), induction of Vtg (4-*t*-butylphenol), inhibition of EROD and CYP1A (4-*t*-butylphenol) and inhibition of Vtg (benzo[*a*]anthracene and benzo[*a*]pyrene). Similar assessment using calculated PEC data derived from use of deployed SPMDs during the BECPELAG workshop in 2001, as a worst case modelling scenario for the Statfjord field, suggest that compound-specific PECs for the 500 m station were in the same range as the PNECs for induction of EROD and CYP1A (benzo[*a*]anthracene, chrycene, benzo[*a*]pyrene) and for inhibition of Vtg (benzo[*a*]pyrene), whilst being 5-20 fold lower than the PNECs for Vtg induction (4-t-butylphenol), EROD induction (Chrycene) and EROD inhibition (benzo[*a*]anthracene and benzo[*a*]pyrene). It should thus be expected that induction as well as inhibition of biomarker reponses, most notably induction of EROD

and CYP1A as well as inhibition of vtg induction, may have occured at the closest caging station (500 m) during the WCMS performed under the BECPELAG 2001 workshop. Emission concentrations for individual compounds taken in 2007 for Statfjord C (naphthalene: 278 ug/L, chrycene: 0.12 ug/L, benzo[*a*]anthracene: 50 ng/L, benzo[*a*]pyrene: 20 ng/L, C4-C5 alkylphenols: 98 ug/L; Lars Petter Myhre, pers. comm.) were in general lower than those reported by Utvik and Gärtner (2006) thus suggesting that few, if any effects should be expected on basis of reported produced water concentrations for single compounds.

In the studies with concentrated samples of either produced water or fractions of produced water only concentrated samples (0.5-50 times concentrated relative to produced water) were sufficiently enriched by bioactive compounds to cause effects in vitro. If assuming that bioconcentration factors summarised in table 5 are representative for the compounds in produced water and individual fractions, no biological effects should be expected at produced water dilutions larger than 6000-12000 times for EROD induction, whereas inhibiting effects of EROD, CYP1A and Vtg induction were occurring at even 10 to 100 times higher concentrations than this. For comparison, prediction of median dilution factors for naphthalenes, 2-3 ring PAHs and 4-5 ring PAHs were about 8100, thus verifying that biological effects in general should only be expected to occur in the close proximity of the discharge points at the Statfjord. The present data from assessment of individual compounds, produced water samples and resulting produced water fractions are in agreement with the lack of of clear biological effects in fish caged at the closest stations during the WCM surveys at Statfjord (Hylland *et al.*, 2008). A slight increase in Vtg induction in male (2001 survey) and decrease in female (2004 survey) Atlantic cod was however reported, proposed to be caused by the simultaneous presence of estrogens and antiestrogens (Hylland *et al.*, 2008). Our studies suggest that high molecular weight PAHs such as benzo[*a*]pyrene may in fact have inhibited Vtg induction during this WCM survey, but that prediction of PNECs based on the concentration of these compounds in produced water from Statfjord B in 2007 are insufficient to cause such effects. In the same studies in 2001, no clear induction of CYP1A or EROD was identified; an effect partially ascribed to the presence of small molecular weight PAHs inhibiting EROD activity (Förlin and Hylland, 2006). Our studies confirm that small molecular weight PAHs and certain alkylphenols are able to inhibit both EROD and CYP1A at high concentrations (table 3), but there remains uncertainty to whether the PEC for the closest station at Statfjord B during the WCM survey in 2001 and similar PEC prediction for 2007 were sufficient to cause such effects. This uncertainty is mainly related to the lack of proper models to extrapolate in vitro cellular responses to in vivo bioactivity and the lack of consistent PECs for the areas of interest.

Table 5. Produced water concentrations (PW) at Statfjord B, predicted environmental concentrations (PEC), estimated bioconcentration factors (BCFs) and the predicted no effect concentrations (PNEC) for selected model compounds and produced water components. Bioconcentration factors were calculated by the BCFWIN package of EPI suite (http://www.epa.gov/oppt/exposure/pubs/episuite.htm), whereas the PNEC were calculated based on in vitro NOECs and the predicted BCFs.

				PNEC (ng/L)					
				Induction			Inhibition		
	PW (ng/L)	PEC[1] (ng/L)	BCF	EROD	CYP1A	Vtg	EROD	CYP1A	Vtg
Naphthalene	$1.8x10^6$	147 (6.4-461)	69	$>1.9x10^6$	$>1.9x10^6$	$>1.9x10^6$	$>1.9x10^6$	$>1.9x10^6$	$>1.9x10^6$
Coumarin	$1.8x10^5$	4.6 (3.5-95)	2.3	$>6.2x10^7$	$>6.2x10^7$	$>6.2x10^7$	$6.2x10^5$	$1.9x10^5$	$6.2x10^6$
Benzo[a]anthracene	$2.0x10^3$	0.61 (0.2-5.5)	5495	12	42	$>4.2x10^4$	120	$1.2x10^3$	42
Chrysene	$4.0x10^3$	0.61 (0.2-5.5)	5888	120	390	$>3.9x10^4$	$1.2x10^4$	$>3.9x10^4$	$>3.9x10^4$
Benzo[a]pyrene	$4.0x10^3$	0.61 (0.2-5.5)	10471	<7.2	7.2	$>2.4x10^4$	720	$>2.4x10^4$	7.2
4-*t*-butylphenol	$2.3x10^7$	154	71	$>7.0\ x10^5$	$>7.0\ x10^5$	$2.1x10^3$	$6.3x10^3$	$6.3x10^3$	$>7.0\ x10^5$

[1] **Median (range),**

6. Future work

The present work has investigated the bioactivity of produced water and individual fractions and screened a high number of compounds for their ability to cause induction and suppression of biomarker responses in fish. The studies have identified several groups of compounds being biologically relevant, where some are rather well characterised compounds such as PAHs and alkylphenols. Additional chemical characterisation and possibly further bioassay-directed fractionation of produced water will yield further information on the detailed composition of the fractions and the active substances present. In addition, some compounds such as organic acids are poorly characterised in terms of chemical composition, environmental occurrence, and ecotoxicological effects. This applies in particular to complex mixtures of compounds such as naphthenic acids, which have recently been identified as potential contributors to the toxicity of UCMs (Hogstad *et al.*, 2007). Since the current approach utilises in vitro methods and predictions to in vivo levels, more thorough studies may provide additional confirmation about the robustness of the biomarker responses used during the WCM surveys. More thorough modelling to predict the potential biological effects based on single chemicals and mixtures of such may provide better predictive models for future use in WCM surveys.

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8. Annex

Annex 1 List of compounds tested

Model compounds	**Naphthalenes PAHs**		**Alkylphenols**
17β-estradiol	Carbazole	Benzo[b]fluoranthene	4-methylphenol
Phenol	9-Methylcarbazole	Benzo[k]fluoranthene	4-ethylphenol
TCDD	Coumarin	Benzo[a]pyrene	4-*n*-propylphenol
	Quinoline	Benzo[g,h,i]perylene	4-*t*-butylphenol
	2,6-Dimethylquinoline	Indeno[1,2,3-cd]pyrene	4-*n*-butylphenol
	Naphthalene	Fluorene	4-*t*-pentylphenol
	2-methylnaphthalene	Dibenzothiophene	4-*n*-pentylphenol
	2,6-dimethylnaphthalene	4-methyldibenzothiophene	4-*n*-hexylphenol
	Anthracene	4-ethyldibenzothiophene	4-*n*-heptylphenol
	2-methylanthracene	Pyrene	4-*t*-octylphenol
	9,10-dimethylanthracene	Phenanthrene	4-*n*-octylphenol
	Acenaphtene	9-methylphenanthrene	4-*n*-nonylphenol
	Fluoranthene	9-ethylphenanthrene	**Production chemicals**
	Benzo[a]anthracene	9-n-propylphenanthrene	CRW85365
	Chrysene		CRO82307

Annex 2 In vitro No Observed Effect Concentrations (NOECs) for the single compounds tested.

	NOEC (umol/L)							
	Induction			Inhibition			Cytotoxicity	
Compound	EROD	CYP1A	Vtg	EROD	CYP1A	Vtg	48h (EROD/CYP1A)	96 h (Vtg)
Carbazole	>1000	>1000	>1000	30	30	30	100	300
9-Methylcarbazole	>1000	1000	>1000	100	>1000	>1000	>1000	>1000
Coumarin	>1000	>1000	>1000	10	3	100	>1000	>1000
Quinoline	>1000	>1000	>1000	30	300	100	>1000	>1000
2,6-Dimethylquinoline	>1000	>1000	>1000	100	300	100	300	300
Naphthalene	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
2-methylnaphthalene	>1000	>1000	100	1000	>1000	>1000	>1000	>1000
2,6-dimethylnaphthalene	>1000	>1000	100	1000	300	>1000	>1000	>1000
Anthracene	>1000	>1000	>1000	1000	>1000	>1000	>1000	>1000
2-methylanthracene	>100	>100	>100	30	>100	30	>100	>100
9,10-dimethylanthracene	>1000	>1000	>1000	300	1000	300	>1000	>1000
Acenaphtene	>1000	>1000	100	1000	>1000	>1000	>1000	>1000
Fluoranthene	>1000	30	>1000	1000	100	30	300	300
Benzo[a]anthracene	0.3	1	>1000	3	30	1	>1000	100
Chrysene	3	10	>1000	300	>1000	>1000	>1000	>1000
Benzo[b]fluoranthene	<0.3	<0.3	>1000	1	>1000	0.3	>1000	10
Benzo[k]fluoranthene	<0.3	0.3	>1000	30	>1000	3	>1000	>1000
Benzo[a]pyrene	<0.3	0.3	>1000	30	1000	0.3	>1000	100
Benzo[g,h,i]perylene	>1000	>1000	>1000	30	300	>1000	>1000	>1000
Indeno[1,2,3-cd]pyrene	0.3	0.3	>1000	10	100	0.3	>1000	100
Fluorene	>1000	>1000	30	1000	300	300	1000	300
Dibenzothiophene	>1000	>1000	30	30	300	>1000	300	300
4-methyldibenzothiophene	>1000	>1000	10	300	>1000	>1000	>1000	300
4-ethyldibenzothiophene	>1000	>1000	30	1000	>1000	>1000	>1000	>1000
Pyrene	>1000	>1000	>1000	100	100	100	100	100
Phenanthrene	>1000	>1000	300	300	300	>1000	1000	300
9-methylphenanthrene	>1000	>1000	>1000	100	>1000	>1000	1000	300
9-ethylphenanthrene	>1000	>1000	>1000	1000	>1000	100	>1000	100
9-n-propylphenanthrene	>1000	100	>1000	100	300	>1000	300	300
4-methylphenol	>300	>300	>300	10	100	>300	300	100
4-ethylphenol	>300	>300	>300	10	10	>300	30	10
4-*n*-propylphenol	>300	>300	1	3	1	>300	30	3
4-*t*-butylphenol	>300	>300	1	3	3	>300	100	10
4-*n*-butylphenol	>300	>300	1	10	3	>300	30	10
4-*t*-pentylphenol	>300	>300	1	3	30	>300	100	10
4-*n*-pentylphenol	>300	>300	3	10	10	>300	100	10
4-*n*-hexylphenol	>300	>300	>300	3	3	>300	100	10
4-*n*-heptylphenol	>300	>300	>300	3	100	>300	300	10
4-*t*-octylphenol	>300	>300	0.3	30	30	>300	100	10
4-*n*-octylphenol	>300	>300	10	100	100	>300	100	10
4-*n*-nonylphenol	>300	>300	10	100	30	>300	300	10
CRW85365 [1]	-	-	-	-	-	-	-	-
CRO82307 [1]	-	-	-	-	-	-	-	-

[1] Molar concentration not available due to being a mixture with unknown composition.

NOEC (µmol/L)

	Induction			Inhibition		
Compound	**EROD**	**CYP1A**	**Vtg**	**EROD**	**CYP1A**	**Vtg**
Naphthalene	>1000	>1000	>1000	>1000	>1000	>1000
Coumarin	>1000	>1000	>1000	10	3	100
Benzo[a]anthracene	0.3	1	>1000	3	30	1
Chrycene	3	10	>1000	300	>1000	>1000
Benzo[a]pyrene	<0.3	0.3	>1000	30	1000	0.3
4-*t*-butylphenol	>1000	>1000	1	3	3	>1000