Toxicity of organic compounds from unresolved complex mixtures (UCMs) to primary fish hepatocytes

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Abstract

Many environmental matrices contaminated with organic pollutants derived from crude oil or degraded petroleum contain mixtures so complex that they are typically unresolved by conventional analytical techniques such as gas chromatography. The resulting chromatographic features have become known as 'humps' or unresolved complex mixtures (UCMs). These UCMs often dominate the organic contaminants of polluted environmental samples: for example, in oil sands produced water up to 150 mg L^{-1} of 'naphthenic acids' appear as UCMs when examined by gas chromatography as the esters. In oil-contaminated mussels, aromatic hydrocarbon UCMs may comprise almost all of the total toxic hydrocarbons, with over 7000 µg g^{-1} dry weight reported in some samples. Over the last 25 years, efforts to resolve and thus identify, or at least to produce average structures, for some UCM components, have proved fruitful. Numerous non-polar UCM hydrocarbons and more polar UCM acids have been identified, then synthesised or purchased from commercial suppliers. As UCMs has been proposed to represent a risk to aquatic organisms, the need for assessment of the ecotoxicological effects and characterisation of the mode of action (MoA) of these environmental pollutants has arisen. In the present study, several chemicals with structures typical of those found in some UCMs, were assessed for their potential to disrupt membrane integrity, inhibit metabolic activity, activate the aryl hydrocarbon receptor (AhR), and activate the estrogen receptor (ER) in primary rainbow trout hepatocytes (*Oncorhynchus mykiss*). These endpoints were determined in order to screen for common toxic modes of action (MoA) in this diverse group of chemicals. The results from the *in vitro* screening indicated that of the endpoints tested, the predominant toxic MoA was cytotoxicity. EC_{50} values for cytotoxicity were obtained for 16 compounds and ranged from 77 µM - 24 mM, whereof aliphatic monocyclic acids, monoaromatic acids, polycyclic monoaromatic acids and alkylnaphthalenes were the most toxic.

The observed cytotoxicity of the chemicals correlated well with the hydrophobicity (LogK_{OW}) suggesting that the toxicity was predominantly due to a non-specific MoA. Interestingly, two compounds induced the ER-mediated production of vitellogenin (Vtg) and six compounds induced the AhR-mediated Ethoxyresorufin-O-deethylase (EROD) enzymatic activity to >20% of the positive control; by doing so suggesting that they may act as ER or AhR agonists in fish. The heterogeneous group of 'UCM compounds' tested exhibited multiple MoA that may potentially cause adverse effects in fish. Additional studies to determine if these compounds may cause adverse effects *in vivo* at environmentally relevant concentrations, may be warranted to identify if such compounds are indeed of potential environmental concern.

Keywords: Naphthenic acids; in vitro; cytotoxicity; EROD; vitellogenin; AhR agonist; ER agonist; xenoestrogen.

1 Introduction

Many environmental matrices (e.g. water, sediments, biota) contaminated with organic pollutants derived from crude oil or degraded or refined petroleum, such as some lubricating oils, contain mixtures so complex that they are typically unresolved by conventional analytical techniques such as gas chromatography. The resulting gas chromatographic features have become known as 'humps' or unresolved complex mixtures (UCMs), a term first introduced to describe the hydrocarbons found in sewage discharge waters from a large municipal sewage treatment plant, surface sediments and hard shell clams (Farrington and Quinn, 1973). These authors later attributed the UCM features of their chromatograms to contamination from biodegraded oils and/or lubricating oils, following comparison with data for biodegraded reservoir crude oils of the Bell

Creek Formation on the border of Montana and Wyoming (reviewed by Farrington and Quinn, 2015). Typical UCM hydrocarbon contents are dependent on the samples assessed and the analytical approaches used, but can range from a few % to as much as 97% of total hydrocarbons in sediments and polluted bivalves (reviewed by Booth, 2004; Booth et al.,2007).

Over the following four decades, efforts to resolve chromatographically and thus identify individual compounds in UCMs (Frysinger et al., 2003; Gros et al., 2014), or to use degradative methods to produce 'average' structures for some UCM components (Gough and Rowland, 1990; Thomas, 1995; Warton et al., 1999) have proved at least partially fruitful, for the hydrocarbons. The advent of multidimensional GC coupled to modern mass spectrometers (e.g. GCxGC-MS) has also led to partial resolution (usually following derivatisation) even of UCMs of more polar petroleum constituents, such as petroleum acids (e.g. naphthenic acids (NAs), Bowman et al., 2014; Swigert et al., 2015). Such components become more quantitatively important following biodegradation of petroleum, e.g. following oil spills (Ruddy et al., 2014), or in oil sands processing (Brown and Ulrich, 2015).

Numerous examples of non-polar UCM hydrocarbons and more polar UCM acids and hetero compounds, have been synthesised or purchased from commercial suppliers (Rowland et al., 2011b; Smith et al., 2001; West et al., 2014). Compounds such as NAs display acute toxicity (LC50) in fish at concentrations (25-75 mg/l) that is typically reported for NA mixtures in oil sands process-affected water (OSPW) (Scott et al., 2008;Dokholyan and Magomedov, 1984), whereas a range of modes of action (MOAs) including cytotoxicity, genotoxicity, immunotoxicity, and endocrine disruption has been proposed (Lacaze et al., 2014; Leclair et al., 2013, 2015; Thomas et al., 2009; Tollefsen et al. 2012). The prospect of ecotoxicological testing and characterisation of the toxic MoA of these UCM-type components has thus arisen. In this way a proper assessment of

their potential for causing adverse effects under ecologically relevant exposure scenarios might be made. One of the methods suitable for screening the toxicity and MoA of UCM compounds is the use of *in vitro* bioassays. These bioassays offer a high-throughput and multi-endpoint testing capacity. Primary hepatocytes from fish has been demonstrated to be a highly versatile multi-endpoint screening assay by testing a suite of endpoints ranging from assessment of cytotoxicity to characterisation of their molecular MoA (Finne et al., 2007; Hultman et al., 2015a, 2015b; Petersen and Tollefsen, 2011; Tollefsen et al., 2008a). These analyses include determination of potential estrogen receptor (ER) agonists and antagonists by measuring their binding to and activation/inhibition of ER and downstream processes such as the ER-mediated production of the egg-yolk precursor protein vitellogenin (Vtg) (Petersen and Tollefsen, 2011, 2012; Tollefsen et al., 2008b). Furthermore, determination of aryl hydrocarbon receptor (AhR) activity by cytochrome P450-dependent monooxygenase mediated 7-ethoxyresorufin-O-deethylase (EROD) enzyme activity has been used to characterise the ”dioxin-like” activity of single chemicals and complex mixtures (Melbye et al., 2009; Segner and Cravedi, 2000). In addition, primary hepatocytes have been applied in the screening of the cytotoxicity of single NAs and multi-compound NA mixtures (Tollefsen et al., 2012). As the current knowledge of the toxicity of UCMs is limited, conducting multi-endpoint screening of relevant UCM components is warranted.

The objective of the present study was therefore to screen a number of UCM-related chemicals for their cytotoxic, ‘dioxin-like’ and estrogenic properties in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes to screen for common toxic MoA. The chemicals tested were chosen to secure a broad chemical applicability domain by including chemicals with large differences in hydrophobicity (e.g. $\text{Log}K_{OW}$) and chemical structure.

2 Materials and methods

2.1 Chemicals

Twenty-two substances (Table 1) were chosen for the study on basis of demonstrated or anticipated relevance as UCM components. The alkynaphthalenes, 1-adamantanecarboxylic acid, 3-cyclohexylpentanoic acid, (1R,3S)-(+)-camphoric acid, 1,3,5-trimethyl-cyclohexane-1,3,5-tricarboxylic acid, 4,5,6,7-tetrahydro-1-benzothiophene-2-carboxylic acid, 4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid, 3-methylbenzo[b]thiophene-2-acetic acid and benzo[b]thiophene-3-acetic acid were obtained from Sigma, whereas 4-(4'-t-butylphenyl)butanoic acid was obtained from Molport (Riga, Latvia). The additional tested compounds were synthesised at Plymouth University, England (Sturt, 2001; Smith, 2002). Chemicals used as positive controls, such as 17β-estradiol (E2, ≥98%, CAS: 50-28-2) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 10 µg/mL in toluene, CAS: 1746-01-6) were obtained from Sigma–Aldrich (St. Lois, MI, US), while $CuSO_4 \times 5H_2O$ (CAS: 7758-99-8) was purchased from VWR (Merck, Darmstadt, Germany). All compounds and standards, with exception of $CuSO_4$ which was spiked into the growth media, were dissolved in dimethylsulfoxide, DMSO (99%, Sigma-Aldrich) and stored in the dark at -20°C until use.

2.2 Rainbow trout

Juvenile rainbow trout, *O. mykiss* (size 200-500 g) were purchased from Valdres Ørretoppdrett (Valdres, Norway) and kept at the Institute of Biology at the University of Oslo (Norway) for a minimum of 4 weeks prior to the first experiment. The water parameters were 9 ± 3˚C, 100 %

oxygen saturation, pH 6.6 and a 12 h light/12 h dark cycle. The fish were fed daily with pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5 % of total body mass.

2.3 Isolation and exposure of hepatocytes

Primary rainbow trout hepatocytes were obtained by a 2-step perfusion of livers from juvenile fish as described in Tollefsen et al. (2003). Cell viability was determined with the trypan blue exclusion test and isolations with ≥85 % viability were diluted to 500 000 cells/ml and plated into 96-well Primaria^{TM} plates, 200 µl/well (Falcon, Becton Dickinson Labware, Oxnard, CA, USA). Cells were incubated at 15˚C for 24 h prior to replacement of half the volume of media with media containing the solvent control (DMSO, 0.1-1%, v/v) or increasing concentrations of the test chemicals or standards. Each chemical was tested at eight concentrations in a dilution series with a dilution factor of three except for $CuSO_4$ where a dilution factor of two was used. A minimum of three (n=3-4) independent exposure experiments, each including three technical replicates (three wells), were performed per test chemical.

To characterise the MoA, cytotoxicity (96 h), induction of Vtg protein expression (96 h) and EROD activity (48 h) were determined in the hepatocytes to obtain information about acute toxicity, the estrogenic potential and “dioxin-like” activity, respectively. Solvent control and positive controls (E2 for Vtg, TCDD for EROD, and $CuSO_4$ for cytotoxicity) were included on appropriate plates in each exposure study and were used to calculate the relative responses of the different chemicals (see below for details).

After 48 h exposure, media was removed from cells targeted for EROD analysis, where upon the cells were frozen and stored at -80ºC until further analysis. Cells to be targeted for cytotoxicity and Vtg analysis were re-exposed for additional 48 h. At the end of the 96 h exposure period, 100 µL growth media from each well was transferred to Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark), sealed with sealing tape (Nunc, Roskilde, Denmark) and frozen at -80˚C for subsequent analysis of Vtg. The cytotoxicity was measured directly in the microplate wells at the end of the exposure period.

2.4 Cytotoxicity determination

Cytotoxic effects were measured as decrease in metabolic activity and/or membrane integrity essentially as described by Schreer et al. (2005) using the two probes: Alamar blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM). The AB probe is reduced to a fluorescent form of the probe by cellular oxidoreductases and the mitochondrial electron transport chain once taken up in cells (Goegan et al., 1995; Page et a., 1993). The CFDA-AM probe is in its original form virtually non-fluorescent, but is converted to a measurable fluorescent product by intracellular esterase activity (O'Connor et al., 1991). For both probes, a reduction in fluorescence is considered a measure of cell damage by interference with metabolic activity (AB) or membrane integrity (CFDA-AM). In brief, the exposure media was replaced with Tris buffer (50 mM, pH 7.5) containing 5% AB and 4 µM CFDA-AM after 96 h exposure. Plates were incubated in the dark at room temperature on an orbital shaker (100 rpm, 30 min) and fluorometric readings were performed with a Victor V^3 multilabel counter (Perkin Elmer, Waltham, MA, USA) using excitation and emission wavelength pairs of 530-590 (AB) and 485-530 (CFDA-AM). The cell

viability was expressed relative to the solvent control (100% cell viability) and the positive control exposed to 0.01 M $CuSO_4$ (maximum loss of viability, 0%).

2.5 Ethoxyresorufin-O-deethylase (EROD) activity analysis

EROD activity was determined directly in the microplate wells by the cellular conversion of ethoxyresorufin (ER), a substrate for the CYP1A isoenzymes, to resorufin (RR) essentially as described in Tollefsen et al. (2006). In brief, the microplates containing cells were thawed on ice before being incubated for 15 minutes in 50 mM Tris buffer containing 0.1 M NaCl, 20 µM dicumarol, 2 µM ER, 100 µM beta-NADPH (200 µl pr. well). Fluorescence was measured with excitation and emission wavelength pair of 530 nm and 595 nm, respectively. The protein concentration was measured with the Bradford method, using bovine gamma-globulin (Bio-Rad, Hercules, CA, USA) as protein standard. The cellular EROD activity was expressed relative to the solvent control (no induction of EROD activity, 0%) and the positive control exposed to 0.3 nM TCDD (maximum EROD induction, 100%) after normalisation to protein content.

2.6 Vitellogenin analysis

Vitellogenin was measured directly in the growth media according to the method described by Tollefsen et al. (2003). Plates containing cell culture media collected after 96 h exposure were thawed for a minimum 4 h at 4˚C before 100 µl standards (rainbow trout Vtg) was applied to assigned (empty) wells and the plates were further incubated overnight (16 h) in the dark at 4˚C. Vitellogenin capture ELISA was performed with the monoclonal mouse anti-salmon Vtg (BN-5, Biosense Laboratories, Bergen, Norway) and the secondary antibody goat anti-mouse IgG (Bio-

Rad, Hercules, CA, USA) both diluted 1:6000 in PBS containing 1 % BSA. After the final washing step, a HRP enzyme substrate (TMB plus, KEMENTEC diagnostics, Taastrup, Denmark) was added to initiate the color development and the reaction was stopped after 15 min by addition of 50 µl H_2SO_4 (1 M). The colour absorbance was measured at 450 nm using a Thermomax microplate reader (Molecular Devices, USA). The cellular Vtg secretion to the medium was expressed relative to the solvent control (no induction of Vtg production, 0%) and the positive control exposed to 30 nM 17-β-estradiol (maximum induction of Vtg production, 100%).

2.7 Data analysis

The measured endpoints were all expressed relative to the negative and positive controls as previously described. The responses of the tested compounds were analysed with GraphPad Prism v6.01 software (GraphPad Software Inc., San Diego, CA, USA). All concentrations were log(10) transformed and a sigmoidal concentration-response curve (CRC) with variable slope was fitted to the experimental data (expressed relative to the solvent control and positive control) using the equation 1. As the responses were expressed relative to the negative and positive controls, the bottom and top values for the CRC were constrained to 0 and 100 (%), respectively.

$$Y = Bottom + \frac{(Top-Bottom)}{(1+10\text{^}((Log\ EC50-X)*Slope))} \quad (1)$$

The quality of the fit was determined and R^2 values > 0.7 were considered indicative of a good model fit (Lundstedt et al., 1998). The EC_{50} and EC_{10} values derived from the CRCs were used to establish fixed effect concentrations and assess the potency of the different test chemicals. Where the observed response did not reach a 50% reduction or induction, the modeled CRCs were used

to estimate the effect concentrations. In cases where the EC50 could not be determined due to partial CRCs but exposure resulted in a >20% response compared to control, these data were also reported.

A principal component analysis (PCA) was applied to identify correlations between hydrophobicity ($LogK_{ow}$), chemical grouping (see Table 2), predicted baseline toxicity in fish ($LC_{50,}$ see effect and MoA predictions below) and the observed cytotoxicity to primary rainbow trout hepatocytes (EC_{50}). Computations were performed using XLSTAT2015® with a p-value <0.05 considered to be statistically significant. Variables identified as relevant for the present study were further analysed by linear regression and correlation analysis. The linear regression analysis was performed using GraphPad Prism v6.01 software (GraphPad Software Inc., San Diego, CA, USA), reporting the regression line's Goodness of fit, followed by a Pearson correlation analysis (R^2). The analysed data was log(10)-transformed prior to the regression analysis.

2.8 Lethality and MoA predictions

Effect concentrations (LC_{50}) for baseline acute toxicity (lethality) in fish (96 h) using the Ecological Structure Activity Relationships (ECOSAR) were predicted on basis of quantitative structure activity relationship (QSAR) models using the Chemprop ver. 6.1 software (UFZ Department of Ecological Chemistry 2015. ChemProp 6.1 http://www.ufz.de/ecochem/chemprop).

3 Results

All test chemicals were screened for cytotoxicity and induction of Vtg production and EROD activity. All endpoint analysis yielded high quality concentration response curves ($R^2 > 0.91$) for the positive controls. Due to the steep hillslope, the fit for metabolic activity and membrane integrity after exposure to $CuSO_4$ was ambiguous with an EC_{10} for both endpoints of ~ 1.1 mM and EC_{50} for both endpoints of ~1.2 mM. The EC_{10} and EC_{50} for Vtg production after exposure to E2 was 0.066 nM and 0.49 nM, respectively. The EC_{10} and EC_{50} for EROD activity after exposure to TCDD was 3.3 pM and 11 pM, respectively. No changes in cytotoxicity was observed after exposure to the tested concentrations of E2 or TCDD. The solvent DMSO did not cause any significant changes in any of the parameters tested compared to non-exposed cells.

3.1 Observed and predicted toxicity

The predicted LC_{50s} for baseline toxicity and evaluation of whether these were within the applicability domain of the QSAR model in fish are given in Table 2. The predicted LC_{50s} for fish varied by more than five orders of magnitude and ranged from 37 nM to 12 mM for the compounds investigated. The compounds with highest predicted toxicity to fish were the polycyclic monoaromatic acids, whereas the monocyclic di-acid and monocyclic tri-acid had the lowest predicted toxicities of those tested. The chemicals which were predicted as least toxic of the 22 compounds were camphoric acid and 1,3,5-tricarboxylic acid.

Concentration-dependent reductions in metabolic activity and membrane integrity in exposed primary hepatocytes occurred at fairly similar concentrations for most chemicals (Table 2). The inhibitory effects on metabolic activity seemed to be slightly greater than loss of membrane

integrity in most cases (Figure 1), and led to the use of metabolic activity as the most sensitive and relevant parameter for developing CRCs. Of the 22 compounds assayed, 20 reduced the metabolic activity to less than 80% of the control. High quality CRCs with R^2 >0.7 were obtained for 16 of these compounds. Based on EC_{50} extrapolations (ranging from 0.077 to 24 mM for metabolic activity), the order of potency was 4-(4-n-BCH)BA > 4-(4-s-BCH)BA > 7-CH-1-iAT > 4-(4-i-BCH)BA > 2-E-6-MN > 4-(4-s-BPh)BA > 6-CHT > 4-(4-t-BPh)BA > (i-BPh)PA > 4-(4-n-BPh)BA > 4-(4-i-BPh)BA > 3-MB[b]Thio-2-AA > B[b]Thio-3-AA > BThio-2-CA > 1-ACA > 1,3,5-tricarboxylic acid (Table 2).

The most cytotoxic compounds belonged to aliphatic monocyclic acids, alkylnaphthalenes, polycyclic monoaromatic acids and monoaromatic acids. Low cytotoxicity was generally associated with aliphatic monocyclic acids, monocyclic di-acids and thiophenic acids. Alkylnaphthalenes exhibited both low and high cytotoxicity, thus illustrating that toxicity was compound-specific in some cases.

For six of the tested compounds, no EC_{50} could be predicted due to lack of toxicity at the concentrations tested (<1 mM). The ECOSAR predicted toxicity (LC_{50}) of these six compounds to fish ranged from about 20 µM to 5 mM (Table 2). The predicted LC_{50} values for *in vivo* fish toxicity were typically around 1.2 to 20 times lower than the experimental EC_{50} for cytotoxicity in the primary hepatocytes, with some exceptions (e.g. 7-CH-1-IAT: predicted LC_{50} 2560 times lower than EC_{50} for metabolic activity).

3.2 EROD activity

The induction of EROD activity, indicative of the activation of the AhR-mediated or 'dioxin like' effects of chemicals, was analysed after 48 h exposure to the 22 compounds investigated. Of these, 6 compounds (3-MB[b]Thio-2-AA, B[b]Thio-3-AA, B[b]Thio-3-CA, 7-CH-1-iAT, 4-(4-n-BPh)BA, and 4-(4-t-BPh)BA) induced the EROD activity to more than 20% of the positive control (Figure 2). The compounds shown to induce the EROD activity belonged to the chemical groups of monoaromatic thiophenic alkanoic and carboxylic acids, polycylic monoaromatic hydrocarbons and monoaromatic acids (Table 2). However, the other monoaromatic thiophenic carboxylic acids, polycylic monoaromatic hydrocarbons and monoaromatic acids did not induce any EROD activity, thus indicating that the activity was compound-specific. However, reduced EROD-activity at high concentrations was associated with cytotoxicity (>20%) for many of the compounds (Figure 1). The EC_{10} values for EROD induction could only be obtained for two compounds (R^2 >0.7); 3-MB[b]Thio-2-AA (EC_{10} = 0.19 mM) and B[b]Thio-3-AA (EC_{10} = 0,095 mM), both belonging to the group of monoaromatic thiophenic alkanoic acids (Table 2).

3.3 Vitellogenin induction

The secretion of Vtg, a measure of activation of the ER and cellular production of Vtg, was assessed for all 22 investigated compounds. Of the compounds tested, the two compounds 2,6-DMN and 6-CHT induced more than 20% Vtg production compared to the positive control (Figure 3). Only 6-CHT displayed a clear concentration-dependent response up to 0.1 mM, where a sharp reduction in Vtg production due to cytotoxicity occurred. Hepatocytes exposed to 2,6-DMN did not elicit a

full Vtg concentration response curve, probably due to solubility issues at the two highest tested concentrations. Full overview of the experimental data is presented in Table 2.

3.4 Principal Component Analysis (PCA)

The PCA analysis represented 90.3% (PCA1:61.7%; PCA2:28.6%) of the total variance (Figure 4), where PCA 1 clearly separated endpoints of observed and predicted toxicity from hydrophobicity ($LogK_{ow}$) and chemical grouping. The results demonstrated a very strong correlation between observed and predicted toxicity ($p \leq 0.0001$), whereas a moderate correlation was observed between the $LogK_{ow}$ and chemical grouping ($p \leq 0.0001$). The overall PCA demonstrated a negative correlation between the compounds $LogK_{ow}$ and their toxicity (cytotoxicity and predicted baseline toxicity in fish). Subsequent linear regression and correlation analysis verified that the observed cytotoxicity (EC_{50}) and the predicted baseline toxicity (LC_{50}) was positively correlated ($R^2=0.729$, $p \leq 0.0001$). Only 7-CH-1-IAT displayed a large discrepancy between observed and predicted toxicity. Moreover, the observed EC_{50} ($R^2=0.626$, $p \leq 0.0003$) and the predicted baseline toxicity (LC50) to fish ($R^2=0.930$, $p \leq 0.0001$) was negatively correlated with $LogK_{ow}$. The toxicity ratio (i.e. ratio between observed EC50 and predicted LC50) displayed a positive correlation with $LogK_{ow}$ ($R^2=0.736$, $p \leq 0.0001$).

4 Discussion

Accidental oil spills and legal or accidental discharges of processed or refined petroleum fractions may result in exposures of fish to various UCMs of compounds, both polar (e.g. NA) and non-polar (e.g. hydrocarbons) (Conly et al., 2002; Headley and McMartin, 2004; Swigert et al., 2015).

Quantitatively, UCMs can represent almost all of the total toxic hydrocarbons in environmental samples. For example, polluted mussels around the UK coasts contained up to 125 µg g^{-1} (dry weight) aromatic UCM hydrocarbons which accounted for the reduction of Scope for Growth (a measure of 'health') of the animals (Booth et al., 2007). Mussels from the entrance to Cape Town Harbour, South Africa contained over 7000 µg g^{-1} dry weight UCMs (reviewed by Booth, 2004). Similarly, nearly all of the approximately 150 mg L^{-1} toxic NA in oil sands process waters appear as UCMs when examined by GC as methyl or trimethylsilyl or similar, esters (reviewed by Clemente and Fedorak, 2005). Despite the dominance of complex mixtures of pollutants unresolved by GC or GC-MS (Farrington and Quinn, 1973; 2015) in many petroleum-contaminated samples (e.g. Frysinger et al., 2003) and the known toxicity of some of these UCMs to aquatic organisms such as bivalves (Booth et al., 2007, 2008; Donkin et al., 2003; Rowland et al., 2001; Scarlett et al., 2011) and bacteria (Jones et al., 2011), few studies have investigated the toxicity of individual UCM-related chemicals to fish. This is partly due to a historic lack of proper identification of UCM-compounds by GC-MS and the limited availability of pure compounds for testing from commercial sources. However, a number of aliphatic, alicyclic and aromatic UCM-related hydrocarbons and several monocyclic, aromatic and alicyclic and hetero-containing aromatic acids identified in UCMs by GCxGC-MS, have been successfully synthesised or made available from commercial vendors (Rowland et al., 2011a, 2011c, 2011d; Scarlett et al., 2011; Smith et al., 2001; Tran et al., 2010; West et al., 2014; Wraige, 1997). Such compounds are designated as 'UCM-related' herein since the structures of some of the hydrocarbons have only been deduced following degradative studies and are therefore only considered 'average' or 'model' structures (Smith et al., 2001; Sturt, 2001; Thomas, 1995), whilst those of the acids have been more firmly identified, usually by comparison of the mass spectra and GCxGC retention times with those of authentic compounds (Rowland et al., 2011d; West et al., 2014). This study is therefore one of

the first to characterise the toxicity and MoA of a heterogenous set of UCM-related compounds to fish cells *in vitro* by assessing their cytotoxic, AhR agonistic and ER agonistic potency in primary rainbow trout hepatocytes.

4.1 Cytotoxicity and baseline toxicity

Several *in vitro* and *in vivo* studies have reported various toxic effects of NA mixtures, which are typically revealed as UCMs by GC-MS analysis of derivatives (e.g. oil sands process water-derived and commercial NA) including embryo deformities, mortality, reduced immune response, endocrine disruption, oxidative stress, necrosis, and cell death (a comprehensive review is given by Chao Li, 2014; Bartlett et al., 2017; Marentette et al., 2015a, b; 2017; Wang et al., 2015a,b). The present study demonstrated that 20 out of the 22 tested compounds were indeed cytotoxic for the primary rainbow trout hepatocytes, by reducing the metabolic ability by more than 20%. Considerable differences in cytotoxicity were observed for the different groups of compounds tested (EC_{50}: 0.077 to 24 mM), however. Overall, EC_{50} values were obtained for a total of 16 of the 22 compounds tested, a majority being monoaromatic acids, at concentrations generally around 0.1-0.6 mM, with some exceptions (Table 2). Only 2 of the 22 compounds (camphoric acid and 4-DBThio-BA) were non-toxic at the concentrations tested, and suggest that they should not be expected to be acutely toxic to fish (Table 2). Scarlett et al. (2012) predicted that *in vivo* baseline toxicity of polycyclic acids containing a single aromatic ring would be the most toxic NAs, with LC_{50} values typically around 1 µM. These predictions agree with the current experimental findings as the polycyclic monoaromatic acids were among the most toxic compounds (EC_{50}= 0.12-0.24 mM), only exceeded by some aliphatic monocyclic acids such as 4-(4'-n-butylcyclohexyl)- and 4-(4'-s-butylcyclohexyl) butanoic acid (EC_{50}= 0.077-0.097 mM). Although no EC_{50} was obtained for

aliphatic monocyclic acid 3-CHPA in the present study, reduction in cell viability by more than 20% at the highest exposure concentrations suggest that this chemical is only weakly toxic to fish cells.

Different UCMs and UCM components such as NAs may be toxic to fish cells by causing cellular swelling, affecting the cell membranes, metabolic activity, and disruption of mitochondrial and lysosomal integrity (Tollefsen et al., 2012; Chao Li, 2014). Although the MoA has not been studied in detail, increase in the membrane fluidity by disruption of the cell membrane lipid bilayer leading to cell death has been proposed for narcotic chemicals (Chao Li, et al., 2014). The present study measured cytotoxicity as disruption of membrane integrity and the metabolic activity dependent on oxidoreductase and the mitochondrial electron transport chain, and the results were fairly consistent between the two endpoints tested. Metabolic activity seemed to be slightly more sensitive than disruption of membrane integrity, however. It is expected that effects on the mitochondria (i.e. perturbations of energy homeostasis) occur prior to disruption of the membrane (cell death), although the actual difference in threshold has not been properly established (Tollefsen et al. 2008; Tollefsen et al. 2012). The probe AB have shown to be more sensitive than CFDA-AM in other studies potentially due to methodological issues (higher background values) and/or the MoA of the tested compounds (Dayeh et al., 2002; Schreer et al., 2005). Also, higher background values has previously been associated with cell media residues in the microplate well upon addition of the CFDA-AM probe, which can lead to a variable and potentially less sensitive fluorescent signal (Schreer et al., 2005).

4.2 Induction of EROD activity

Ethoxyresurofin-O-deethylase (EROD) is a well-established biomarker for the catalytic measurement of the AhR-mediated induction of cytochrome P450 1A detoxification activity and associated with 'dioxin-like' properties of several chemicals (Whyte et al., 2000). The EROD activity is a measure of activation of the AhR and may serve as an early warning marker for adverse effects such as immunotoxicity, histopathological lesions and mortality in fish (Whyte et al., 2000). The present study reports novel findings of induction of EROD activity after exposure to individual UCM-relevant compounds. Few studies have investigated the effect on AhR gene expression and AhR-activity of UCM-related compounds and OSPW extracts containing UCMs such as NAs (Knag et al., 2013; Marentette et al., 2017). However, effects typical of 'dioxin-like' exposure have been observed in early life stages of fathead minnow exposed to OSPW; albeit no significant increase in the AhR-mediated induction of CYP1A transcripts was observed (He et al., 2012). In agreement, no statistical difference in EROD activity was observed in rainbow trout exposed to different pond waters and extracts of NA at low milligram per liter (between 1 mg/L and 8 mg/L) concentrations (Leclair et al., 2013). However, increased CYP1A activity was observed in fish exposed in South Bison Pond, Canada, a tailings pond containing aged un-extracted oil sands material, compared to fish exposed in a demonstration pond and reference lake (Arens et al., 2015). In addition, increased CYP1A1 gene expression was observed in Walleye (*Sander vitreus*) embryos exposed to naphthenic fraction components (Marentette et al., 2017). The EROD activity in the present study was mainly associated with monoaromatic thiophenic alkanoic acids, polycylcic monoaromatic acids and monoaromatic acids, whereof the latter are known substrates for CYP1A2 in humans (Scarlett et al., 2012). Five of the six EROD-active compounds induced concentration-dependent activity at non-cytotoxic concentrations. Interestingly, the extrapolated EC_{50} values for EROD activity were in some cases higher than the EC_{10} for cytotoxicity, thus introducing the

possibility that acute toxicity masked the EROD activity at high concentrations. Apart from sharing some structural similarities with AhR agonists, the tested compounds are structurally dissimilar to typical EROD inducers such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD), polychlorinated biphenyls (PCBs) and multi-ring PAHs (Whyte et al., 2000), thus suggesting that the compounds tested herein might contain non-typical and novel groups of AhR agonists. The role and extent of UCM components as potential AhR agonists in fish is still unknown, and may require further attention to assess whether interaction with the AhR may cause adverse effects in fish.

4.3 Induction of Vtg production

Induction of Vtg has been associated with adverse endocrine-mediated effects in fish and mammals such as feminization and reduced growth, reproductive success, and fecundity, (Colborn et al., 1993; Janošek et al., 2006; Sumpter and Jobling, 1995). Model ER agonists such as 17β-estradiol, 17α-ethinylestradiol and bisphenol A all have specific structural properties that allow them to bind to the steroid hormone binding pocket and induce conformational changes that activate transcription of the ER and its downstream targets (O'Malley and Tsai, 1992). Several (mono)aromatic acids related to UCMs, OSPW fractions and NAs have been suggested to be weak estrogens (He et al., 2012; Scarlett et al., 2012; Wang et al., 2015). A selection of the UCM-related compounds tested in the present study was therefore anticipated to be at least partial ER agonists, as having structural resemblance to NAs proposed to interact with the ER (Scarlett et al., 2012). The hydrocarbon 2,6-DMN, which is easily resolved and identified in petroleum mixtures by GC-MS, is not considered an UCM compound *per se.* Nevertheless, it is structurally similar to many of the hundreds of unresolved branched alkylnaphthalenes present in UCMs and which are toxic to mussels (Scarlett et al., 2011). Unfortunately, the more UCM representative branched

alkylnaphthalenes, which have also been synthesized (Scarlett et al., 2011), was not available for the assay conducted herein. However, both, 2,6-DMN and the 'model' UCM compound 6-CHT (Sturt, 2001) induced Vtg by more than 20% from control levels in the present study. Most of the tested compounds did not elicit any Vtg induction, thus suggesting that they are either not active, display too low affinity to activate the ER or are acting as ER antagonists at the concentrations tested. Groups of NAs (e.g. (mono)aromatic acids) have previously been predicted to be ER agonists (Scarlett et al., 2012) or assumed to be responsible for estrogenic effects of UCM related extract fractions (Reinardy et al., 2013). However, the estrogenic activity of these NAs might be highly variable as even small changes to the chemical structures of an ER agonist may cause substantial modulation of their affinity and ability to activate the ER (Labaree et al., 2003, Sippl, 2002). The prediction of the UCM components and NAs as estrogens solely based on their structural resemblance to steroidal acids may therefore be unreliable, and may require testing in suitable bioassays. Interestingly, several studies have demonstrated that commercially available mixtures (OSPW, oil sand (OS), produced water (PW)) and single naphthenic acids may induce transcriptional and translation changes of ERα/β2, Vtg and Cyp19b in modified yeast strains with human ER (YES), primary rainbow trout hepatocytes, and zebrafish (*Danio rerio*) embryos (Gagné et al., 2012; Thomas et al., 2004; Wang et al., 2015a,b). In addition, mixtures and fractions of NAs and PW have displayed not only ER agonistic properties, but also displayed androgen- and ER antagonistic properties *in vitro* (Thomas et al., 2004, 2009; Leclair et al., 2015). There are however uncertainties when comparing single fractionated compounds from UCMs e.g. NAs and complex mixtures of OSPW, OS and PW as the presence of other estrogen mimicking compounds than NAs (e.g. phenols) may account or contribute collectively to the estrogenicity of these complex mixtures.

4.4 Relationship between physico-chemical properties and toxicity

The PCA showed a strong positive correlation between the predicted and observed base line toxicity, suggestive of a good compliance between the *in vitro* data and the predicted *in vivo* base line toxicity. Furthermore, there was an overall strong correlation between relative *in vitro* and *in vivo* toxicity, and the compounds physico-chemical properties (Figures 4 and 5). However, large differences (<3 orders of magnitude) between the observed and predicted base line toxicity demonstrated that primary hepatocytes exhibit somewhat limited applicability as an alternative screening tool for the acute toxicity for highly hydrophobic compounds such as those found in UCMs. Clear correlations between *in vitro* (R^2=-0.685) and in predicted *in vivo* (R^2=-0.634) toxicity and Log K_{ow} were observed, and cohere well with observations for synthesized UCM hydrocarbons elsewhere (Smith et al., 2001). As observed in the present study, pronounced deviation between *in vitro* (EC_{50}) and *in vivo* (LC_{50}) toxicity for compounds with specific MoA and high Log K_{ow} have been reported in fish (Fent and Hunn, 1996; Segner and Lenz, 1993; Castano et al., 1996). The deviations may partly be due to the reduced bioavailability of hydrophobic compounds in the *in vitro* assays, as plastic-well surface interaction (Schreiber et al., 2008), interaction with proteins and other cell media components (Groothuis et al., 2015; Hestermann et al., 2000; Riedl and Altenburger, 2007) may reduce the free concentrations of hydrophobic compounds. Such assay-related factors have previously been reported to underestimate the toxicity of compounds with Log K_{ow}>3 when reported as nominal concentrations (Groothuis et al., 2015; Heringa et al., 2004) and seems to cohere well with observations that the hepatocyte assay underestimated the toxicity of compounds in this LogK_{ow} range (Figures 4 and 5). Predictions of chemical mass balance in *in vitro* systems have been proposed to improve such

in vitro to *in vivo* extrapolations for neutral organic compounds (Armitage et al., 2014), but no models are currently developed for ionizing compounds such as NAs and potentially also other compounds present in UCMs. Until such models are developed, *in vitro* to *in vivo* predictions would benefit from larger reliance on analytical verification of exposure concentrations of hydrophobic compounds or compounds with properties being challenging to test in *in vitro* bioassays.

4.5 Environmental implications

UCMs are known to contain large numbers of chemicals which may vary both in composition and concentrations in the environment, depending largely on nearby sources and fate in the environment. Several of the 'UCM-related' compounds tested herein are representative of previously identified chemical groups and components in mixtures of crude oil (Smith, 2002) and OSPW (Rowland et al., 2012). Several UCMs associated with oil and OSPW cause toxicity to benthic organisms, and when combined with other compounds (e.g. PAHs) may even increase their bioavailability (Du et al., 2012). One group of highly bioavailable UCMs are the NAs, which have been proposed to remain mainly in the water phase instead of partitioning to soils or sediments (Headley and McMartin, 2004). Reports of OSPW concentrations as high as 110 mg/L of mixtures of NAs in tailing pond waters (Scott et al., 2008) and experimental LC_{50}-values as low as 25-75 mg/L for ecologically relevant fish species (Dokholyan and Magomedov, 1984), suggest that certain groups of UCMs such as NAs may be a risk to fish. The hydrocarbons tested herein were used as proxies to environmentally relevant UCMs (Warton et al., 1999), analogues of those identified in UCMs by GCxGC-MS (Booth et al., 2008; Scarlett et al., 2011, Rowland et al., 2011c) or belonging to chemical classes known to occur in the UCMs of OSPW (Barrow et al., 2010;

Clemente and Fedorak, 2005; Grewer et al., 2010; Headley and McMartin, 2004; Madill et al., 2001; Rogers et al., 2002; Rowland et al., 2011b, 2011c, 2011e; West et al., 2014). Albeit the model compounds tested herein predominantly represent different aliphatic monoaromatic thiophenic alkanoic acids, polycylic monoaromatic acids and monoaromatic acids and were displaying low acute toxicity, some of them caused sub-lethal effects such as induction of CYP1A-mediated EROD activity and induction of the estrogenic biomarker Vtg at micromolar concentrations. In similarity to the present work, significant increase of phase I detoxification, antioxidant enzymes and premature hatching in fish eggs and embryos (He et al., 2012) were associated with exposure to OSPW, demonstrating some of the potential ecological impacts that UCM compounds may have. The present data suggest that UCM compounds display multiple MoA, and efforts taken elsewhere suggest that similar compounds may also be genotoxic (Dissanayake et al., 2016; Lacaze et al., 2014), immunotoxic (Leclair et al., 2013) and modulate steroidal receptor activity (e.g. androgen- and thyroid receptor) (Leclair et al., 2015; Thomas et al., 2009). Although many of the effects observed occurred at exposure concentrations in the µM to mM range, the high hydrophobicity of many of these compounds may have underestimated the toxicity due to bioassay-specific artefacts and thus limited the ability to perform reliable *in vitro* to *in vivo* extrapolations. Despite this, the present study indicates that most of these chemicals are cytotoxic, and some of them may also cause toxicity by multiple MoA. Knowing that UCM-relevant compounds occur in complex mixtures and may cause combined toxicity in an additive manner (Tollefsen et al., 2012), suggests that assessments of the total toxicity of ecologically-relevant mixtures of UCM-related compounds in relevant *in vivo* models are still highly warranted. This applies also to other sources of UCM, as complex multiple-component UCMs, and in particular, the water-soluble fraction of UCMs has been demonstrated to account for a large portion of crude oil toxicity in a similar bioassay system as that used herein (Melby et al. 2009).

5 Conclusion

The present study screened a high number of 'UCM-related' compounds for their ability to cause cytotoxicity, estrogenicity and induction of AhR-mediated EROD activity. The study indicated that the compounds predominantly caused cytotoxicity, likely by narcosis, with EC_{50} values ranging from 77 µM to 24 mM. A few compounds also exhibited specific MoA such as estrogenicity and 'dioxin-like' effects at micromolar concentrations. Even though the environmental concentrations of the individual compounds in UCMs might be below those documented in this study, multiple UCM compounds may cause combined toxicity that cannot be predicted on basis of the individual compounds alone. Discrepancies between observed *in vitro* and predicted *in vivo* EC-values suggested that these compounds represent challenges associated with *in vitro* bioassays' exposure (dosing) strategies and exposure documentation, and not related to the sensitivity of the cells themselves. The UCM-related compounds include highly diverse groups of chemicals with a potential for a wide range of effects on exposed wild life. Thus, further identification of UCM-related compounds and characterisation of the toxicity these compounds cause are still considered of high relevance.

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Figure legends

Figure 1. Metabolic activity (●) and membrane integrity (○) as measures for cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 96 h to a selection of compounds associated with the Unresolved Complex Mixture. The data (mean ± standard deviation) represent three independent cell isolation and exposure studies.

Figure 2. Ethoxyresorufin-O-deethylase (EROD) activity (columns) and metabolic activity (○) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to Unresolved Complex Mixture (UCM)-related compounds for 48 h and 96 h respectively. The data (mean ± standard deviation) consists of a minimum of three individual cell isolation and exposure studies.

Figure 3. Vitellogenin (Vtg) (columns) and metabolic activity (○) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 96 h to the estrogen receptor (ER) agonistic Unresolved Complex Mixture (UCM) compounds 2,6-dimethylnaphthalene and 6-cyclohexyltetralin. The data (mean ± standard deviation) consists of a minimum of three exposure studies.

Figure 4. Principal component analysis (PCA) of measured cytotoxicity at 50% effect concentration (EC50) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes and predicted acute toxicity at EC50 fish when exposed to UCM-related compounds.

Figure 5. A comparative linear regression and correlation analysis of observed cytotoxicity at 50% effect concentration (EC50), predicted lethal concentration (LC50) in fish, water solubility and Log $K_{octanol\text{-}water}$ (ow) of the tested compounds The analysis consist of single factor (Predicted LC50 vs. observed EC50 vs. Log Kow) and more complex composite factors (Ratio between observed and predicted toxicity vs. Log Kow). The observed cytotoxicity data (mean ± standard deviation) consists of a minimum of three exposure studies measuring the metabolic activity and is expressed as percentage of a positive control (0.01 M $CuSO_4$), towards the solvent control (DMSO). No statistically verified outliers were identified in the data set when applying Grubb's outlier-test, however visually suspected outliers was marked with a gray ring. The predicted acute fish toxicity data was obtained from Chemprop. The gray ring depicts an apparent visual outlier.

Fig. 1

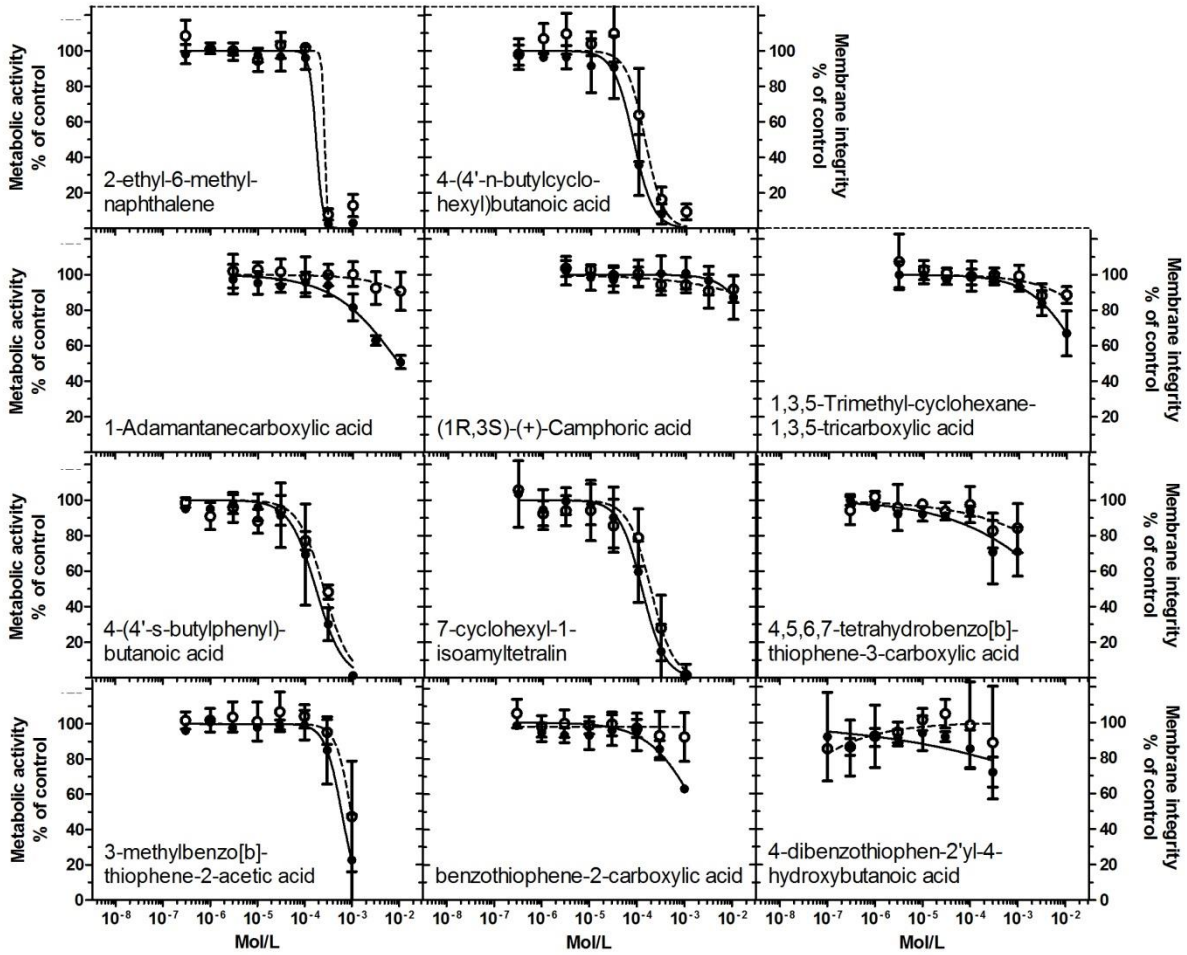

Metabolic activity % of control
Membrane integrity % of control
2-ethyl-6-methyl-naphthalene
4-(4'-n-butylcyclo-hexyl)butanoic acid
1-Adamantanecarboxylic acid
(1R,3S)-(+)-Camphoric acid
1,3,5-Trimethyl-cyclohexane-1,3,5-tricarboxylic acid
4-(4'-s-butylphenyl)-butanoic acid
7-cyclohexyl-1-isoamyltetralin
4,5,6,7-tetrahydrobenzo[b]-thiophene-3-carboxylic acid
3-methylbenzo[b]-thiophene-2-acetic acid
benzothiophene-2-carboxylic acid
4-dibenzothiophen-2'yl-4-hydroxybutanoic acid
Mol/L

Fig. 2

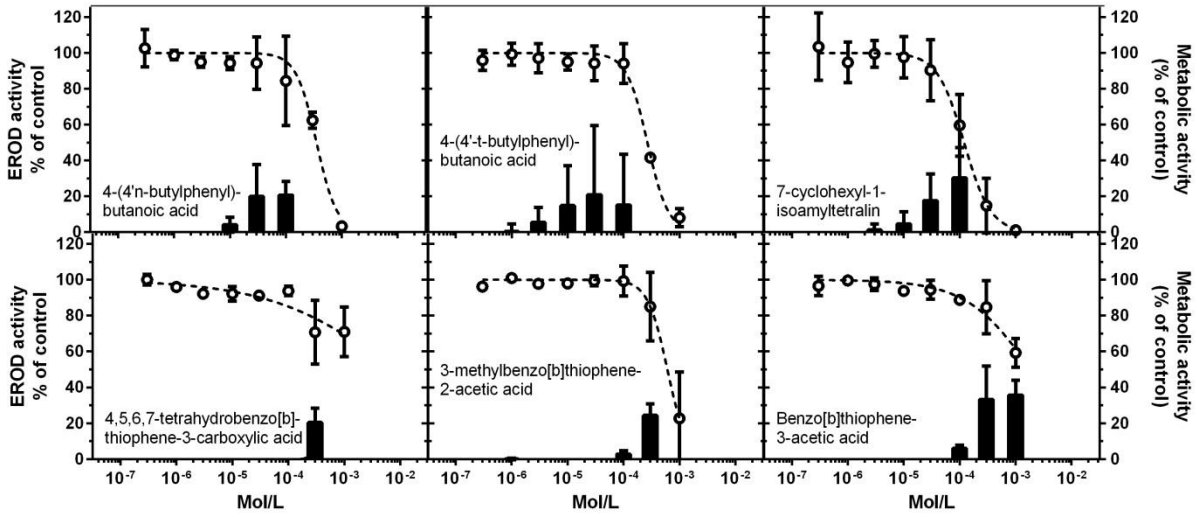

EROD activity
% of control
Metabolic activity
(% of control)
4-(4'n-butylphenyl)-
butanoic acid
4-(4'-t-butylphenyl)-
butanoic acid
7-cyclohexyl-1-
isoamyltetralin
4,5,6,7-tetrahydrobenzo[b]-
thiophene-3-carboxylic acid
3-methylbenzo[b]thiophene-
2-acetic acid
Benzo[b]thiophene-
3-acetic acid
Mol/L

Fig. 3

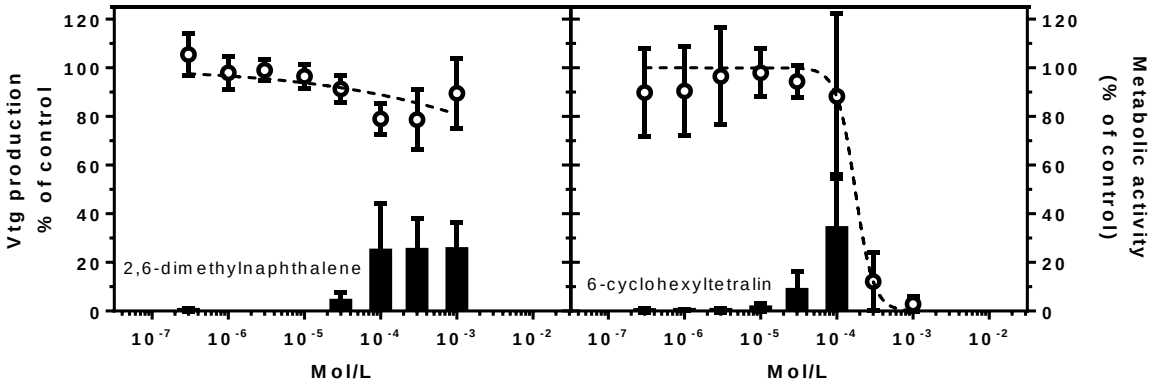

Vtg production
% of control
Metabolic activity
(% of control)
2,6-dimethylnaphthalene
6-cyclohexyltetralin
120
100
80
60
40
20
0
10 -7
10 -6
10 -5
10 -4
10 -3
10 -2
Mol/L

Fig. 4

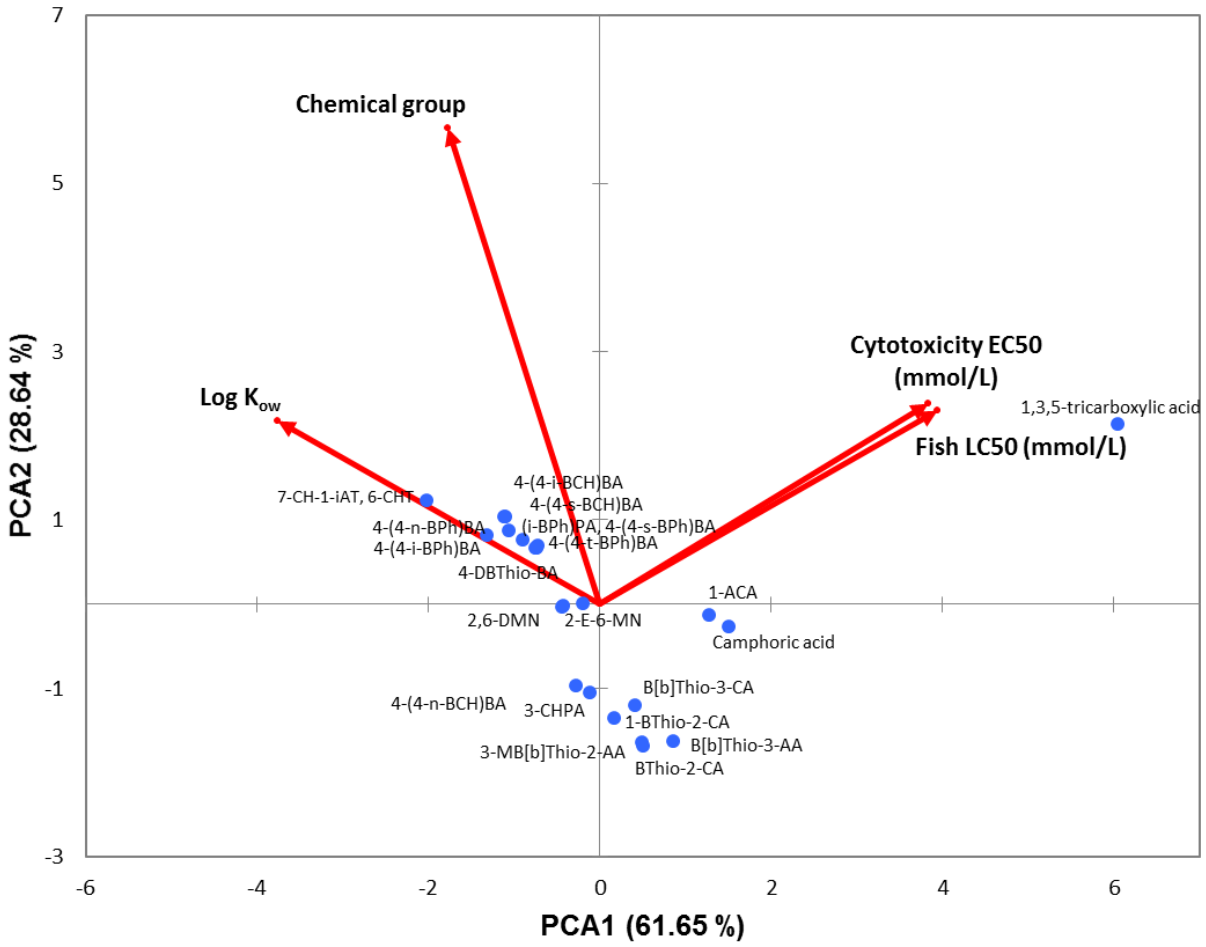

Chemical group
Log K_{ow}
Cytotoxicity EC50 (mmol/L)
Fish LC50 (mmol/L)
1,3,5-tricarboxylic acid
7-CH-1-iAT, 6-CHT
4-(4-i-BCH)BA
4-(4-s-BCH)BA
4-(4-n-BPh)BA
(i-BPh)PA, 4-(4-s-BPh)BA
4-(4-i-BPh)BA
4-(4-t-BPh)BA
4-DBThio-BA
1-ACA
2,6-DMN
2-E-6-MN
Camphoric acid
B[b]Thio-3-CA
4-(4-n-BCH)BA
3-CHPA
1-BThio-2-CA
3-MB[b]Thio-2-AA
B[b]Thio-3-AA
BThio-2-CA
PCA2 (28.64 %)
PCA1 (61.65 %)
7
5
3
1
-1
-3
-6
-4
-2
0
2
4
6

Fig. 5

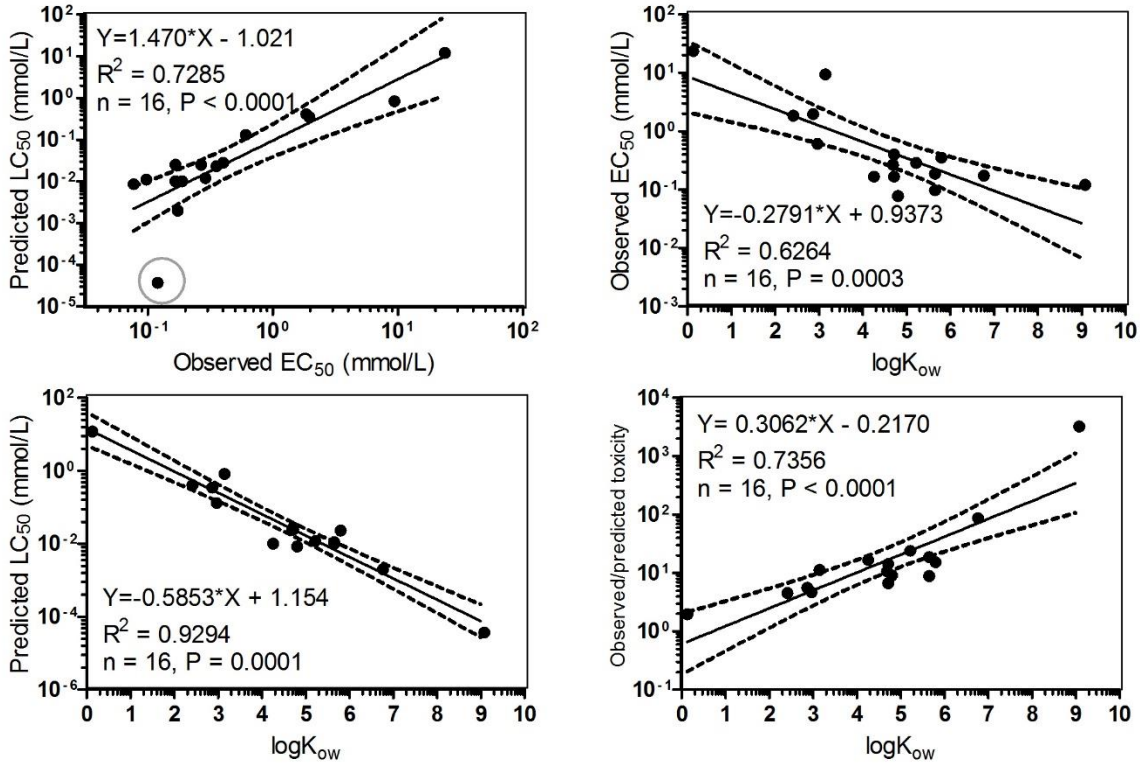
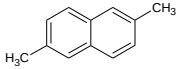
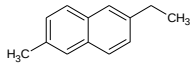
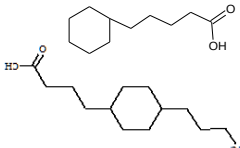
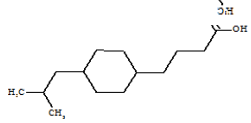
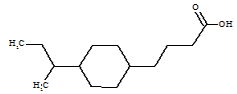
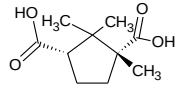
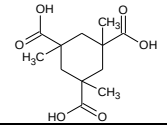
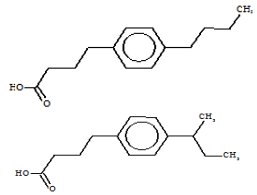
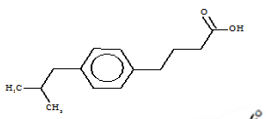
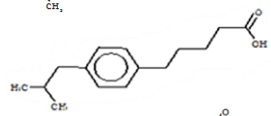
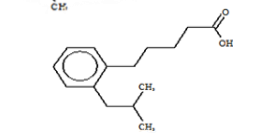
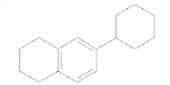
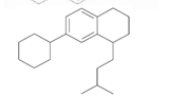
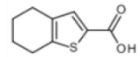
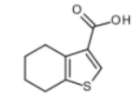
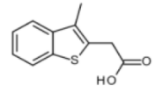
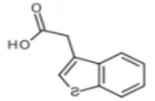
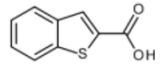
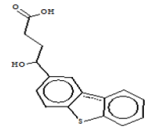

Y=1.470*X - 1.021
R2 = 0.7285
n = 16, P < 0.0001
Predicted LC50 (mmol/L)
Observed EC50 (mmol/L)
Y=-0.2791*X + 0.9373
R2 = 0.6264
n = 16, P = 0.0003
Observed EC50 (mmol/L)
logKow
Y=-0.5853*X + 1.154
R2 = 0.9294
n = 16, P = 0.0001
Predicted LC50 (mmol/L)
logKow
Y= 0.3062*X - 0.2170
R2 = 0.7356
n = 16, P < 0.0001
Observed/predicted toxicity
logKow

Table 1. Chemicals tested in a multi-endpoint rainbow trout (*Oncorhynchus mykiss*) hepatocyte assay. The chemical name, abbreviation, CAS RN, molecular weight (MW), SMILES, logK_{OW} and chemical structure are shown for all the tested compounds.

Chemical name	Abbreviation	CAS RN[a]	MW	SMILES	LogKoW	Chemical structure
Aliphatic naphthalenes						
2,6-dimethylnaphthalene	2,6-DMN	581-42-0	156.23	Cc1ccc2cc(C)ccc2c1	4.26 [b]	
2-ethyl-6-methylnaphthalene	2-E-6-MN	7372-86-3	170.26	Cc1ccc2cc(ccc2c1)CC	4.26 [b]	
Aliphatic monocyclic acids						
3-cyclohexylpentanoic acid	3-CHPA	13126-82-4, 5456-30-4, 5962-88-9	184.28	O=C(O)CCCCC1CCCCC1	4.32[c]	
4-(4'-n-butylcyclohexyl)butanoic acid	4-(4-n-BCH)BA	NR	226.36	C(=O)(O)CCCC1CCC(CCCC)CC1	4.81 [c]	
4-(4'-i-butylcyclohexyl)butanoic acid	4-(4-i-BCH)BA	NR	226.36	C(=O)(O)CCCC1CCC(CC(C)C)CC1	5.65 [c]	
4-(4'-s-butylcyclohexyl)butanoic acid	4-(4-s-BCH)BA	NR	226.36	C(=O)(O)CCCC1CCC(C(C)CC)CC1	5.65 [c]	
Aliphatic tricyclic acid						
1-adamantanecarboxylic acid	1-ACA	828-51-3	180.25	O=C(O)C12CC3CC(C1)CC(C2)C3	3.15 [c]	
Monocyclic di-acid						
(1R,3S)-(+)-camphoric acid	Camphoric acid	124-83-4	200.24	C[C@]1(CC[C@H](C(=O)O)C1(C)C)C(=O)O	1.78 [c]	
Monocyclic tri-acid						
1,3,5-trimethyl-cyclohexane-1,3,5-tricarboxylic acid	1,3,5-tricarboxylic acid	118514-35-5	258.27	OC(=O)C1(C)CC(C)(CC(C)(C1)C(=O)O)C(=O)O	0.13 [c]	
Monoaromatic acids						
4-(4'n-butylphenyl)butanoic acid	4-(4-n-BPh)BA	NR	220.31	c1(CCCC)ccc(CCCC(=O)O)cc1	5.8 [c]	

4-(4'-i-butylphenyl)butanoic acid	4-(4-i-BPh)BA	NR	220.31	c1(CCCC(=O)O)ccc(CC(C)C)cc1	4.72 [c]	

Chemical name	Abbreviation	CAS RN[a]	MW	SMILES	LogKoW	Chemical structure
4-(4'-s-butylphenyl)butanoic acid	4-(4-s-BPh)BA	NR	220.31	c1(C(C)CC)ccc(CCCC(=O)O)cc1	4.72 [c]	
4-(4'-t-butylphenyl)butanoic acid	4-(4-t-BPh)BA	24475-36-3	220.31	C(C)(C)(C)c1ccc(CCCC(=O)O)cc1	4.69 [c]	
(iso-butylphenyl)pentanoic acid	(i-BPh)PA	NR	234.34	c1(CCCCC(=O)O)ccc(CC(C)C)cc1 c1(CCCCC(=O)O)cc(CC(C)C)ccc1 c1(CCCCC(=O)O)c(CC(C)C)cccc1	5.22 [c]	
Polycyclic monoaromatic hydrocarbons						
6-cyclohexyltetralin	6-CHT	NR	214.35	C3CCC(c2ccc1CCCCc1c2)CC3	6.77[b]	
7-cyclohexyl-1-isoamyltetralin	7-CH-1-IAT	NR	284.49	c12c(C(CCC(C)C)CCC1)cc(C1CCCCC1)cc2	9.08[b]	
Monocyclic thiophenic carboxylic acid						
4,5,6,7-tetrahydro-1-benzothiophene-2-carboxylic acid	1-Bthio-2-CA	40133-07-1	182.24	c1c2c(sc1C(=O)O)CCCC2	3.66 [c]	
4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid	B[b]Thio-3-CA	19156-54-8	182.24	c1c(c2c(s1)CCCC2)C(=O)O	3.66 [c]	
Thiophenic alkanoic acids						
3-methylbenzo[b]thiophene-2-acetic acid	3-MB[b]Thio-2-AA	1505-52-8	206.26	Cc1c2ccccc2sc1CC(=O)O	2.97 [c]	
benzo[b]thiophene-3-acetic acid	B[b]Thio-3-AA	1131-09-5	192.23	c1ccc2c(c1)c(cs2)CC(=O)O	2.42 [c]	
Monoaromatic thiophenic carboxylic acid						
benzothiophene-2-carboxylic acid	BThio-2-CA	6314-28-9, 527-72-0, 900791-89-1	178.21	c1ccc2c(c1)cc(s2)C(=O)O	2.87 [c]	
Diaromatic thiophenic alkanoic acid						

4-dibenzothiophen-2'yl-4-hydroxybutanoic acid	4-DBThio-BA	NR	286.35	C1=CC=C2C(=C1)C3=C(S2)C=CC(=C3)C(CCC(=O)O)O	3.29[b]	

[a]RN numbers obtained from chemspider (http://www.chemspider.com); [b]Predicted with ECOSAR (http://www.epa.gov/oppt/newchems/tools/21ecosar.htm); [c]Predicted with AlogPS (http://www.vcclab.org/lab/); NR – Not reported.

Table 2. Cytotoxic, estrogenic (vitellogenin induction) and dioxin-like (EROD activity) potency of compounds associated with Unresolved Complex Mixtures (UCMs). The data represent the predicted baseline toxicity in fish (LC_{50}), and experimental data (EC_{10}, EC_{50} and 20% efficiency) for cytotoxicity (96 h), estrogenic (96 h) and dioxin-like (48 h) activity in the rainbow trout (*Oncorhynchus mykiss*) hepatocytes. The data was derived from minimum 3 independent studies.

Compound	Predicted values	Experimental values							
	Fish toxicity (96h)	Cytotoxicity (96h)			EROD activity (48h)			Vtg (96h)	
	LC_{50}, mM [a]	EC_{50} (95% CI), mM [b]	EC_{10}, mM	>20% effect [c]	EC_{50} (95% CI), mM	EC_{10}, mM	≥ 20% Effect	EC_{50} (95% CI), mM	≥ 20% Effect
Aliphatic naphthalenes									
2,6-dimethylnaphthalene	0.021	n.a.	n.a.	YES	n.a.	n.a.	NO	63	YES
2-ethyl-6-methylnaphthalene	0.010	0.17 (0.14 - 0.21) [d]	0.12 [d]	YES	n.a.	n.a.	NO	na.	NO
Aliphatic monocyclic acids									
3-cyclohexylpentanoic acid	0.095	n.a.	n.a.	YES	n.a.	n.a.	NO	n.a.	NO
4-(4′-n-butylcyclohexyl)butanoic acid	0.0085	0.077 (0.063 – 0.096) [d]	0.027	YES	n.a.	n.a.	NO	n.a.	NO
4-(4′-i-butylcyclohexyl)butanoic acid	0.010[k]	0.13 (0.098-0.18) [d]	0.026[d]	YES	n.a.	n.a.	NO	n.a.	NO
4-(4′-s-butylcyclohexyl)butanoic acid	0.011 [k]	0.097 (0.082 – 0.12) [d]	0.030 [d]	YES	n.a.	n.a.	NO	n.a.	NO
Aliphatic tricyclic acid									
1-adamantanecarboxylic acid	0.83[l]	9.4 (6.7-13)[d,j]	0.29	YES	n.a.	n.a.	NO	n.a.	NO
Monocyclic di-acid									
(1R, 3S)-(+)-Camphoric acid	4.9	n.[d].	n.[d].	NO	n.a.	n.a.	NO	n.a.	NO
Monocyclic tri-acid									
1,3,5-trimethyl-cyclohexane-1,3,5-tricarboxylic acid	12[l]	24 (14-40)[d,j]	1.6[d]	YES	n.a.	n.a.	NO	n.a.	NO
Monoaromatic acids									
4-(4′-n-butylphenyl)butanoic acid	0.023	0.35 (0.29 – 0.43) [d]	0.14 [d]	YES	0.55	n.a.	YES	n.a.	NO
4-(4′-i-butylphenyl)butanoic acid	0.028	0.40 (0.31 – 0.52) [d]	0.079 [d]	YES	n.a.	n.a.	NO	n.a.	NO
4-(4′-s-butylphenyl butanoic acid)	0.025	0.17 (0.13 – 0.22) [d]	0.040 [d]	YES	n.a.	n.a.	NO	n.a.	NO
4-(4′-t-butylphenyl)butanoic acid	0.025	0.27 (0.23 – 0.31) [d]	0.11 [d]	YES	n.a.	n.a.	YES	n.a.	NO
(iso-butylphenyl)pentanoic acid	0.012[k]	0.29 (0.26 – 0.32) [d]	0.12 [d]	YES	n.a.	n.a.	NO	n.a.	NO
Polycyclic monoaromatic acid									
6-cyclohexyltetralin	0.0020 [k]	0.17 (0.12-0.24) [d]	0.095 [d]	YES	n.a.	n.a.	NO	0.16	YES
7-cyclohexyl-1-isoamyltetralin	$3.7E^{-5}$ [k]	0.12 (0.091 – 0.16) [d]	0.036 [d]	YES	0.26	n.a.	YES	n.a.	NO
Monocyclic thiophenic carboxylic acid									
4,5,6,7-Tetrahydro-1-benzothiophene-2-carboxylic acid	0.20	n.a.	n.a.	YES	n.a.	n.a.	NO	n.a.	NO
4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid	0.27	n.a.	n.a.	YES	n.a.	n.a.	YES	n.a.	NO
Monoaromatic thiophenic alkanoic acid									
3-methylbenzo[b]thiophene-2-acetic acid	0.13	0.61 (0.48 - 0.77)	0.25	YES	0.50 (0.39 - 0.64) [e,f]	0.19	YES	n.a.	NO
Benzo[b]thiophene-3-acetic acid	0.41	1.9 (1.1 - 3.1) [e]	0.10	YES	1.6 (0.7-3.5)	0.095	YES	n.a.	NO
Monoaromatic thiophenic carboxylic acid									
Benzothiophene-2-carboxylic acid	0.35[l]	2.0 (1.1-3.6)[d,j]	0.15	YES	n.a.[f]	n.a.	NO	n.a.	NO
Diaromatic thiophenic alkanoic acid									
4-dibenzothiophen-2′yl-4-hydroxybutanoic acid	0.057 [l]	n.a.	n.a.	NO	n.a.	n.a.	NO	n.a.	NO

a – 50% lethal concentration. b – 10 or 50% effect concentration. c – 20% or higher effect is observed in measured endpoint. d- above predicted water solubility. e – predicted, effect concentration predicted by the concentration-response curve at higher than tested concentrations. f – potential masking by cytotoxicity. g- Predicted by ECOSAR. h- Predicted with ALOGPS based on LogKow. i- (Smith et al., 2001). j - outside the tested concentration range, estimated value. n.a - not applicable, compound tested but no value achieved. k- outside logKoW range of model. l-higher than the water solubility stated in ECOSAR. Not possible to fit a concentration-response curve with $R^2 \geq 0.7$.

Highlights:

- UCMs cause multiple mode of action in fish liver cells
- Most UCM compounds caused cytotoxicity
- Some UCM components were ER and AhR agonists
- In vitro toxicity could not fully explain predicted *in vivo* bioactivity