Petersen, Hultman, Tollefsen. Primary hepatocytes from Arctic char (Salvelinus alpinus) as a relevant Arctic in vitro model for screening contaminants and environmental extracts
Volume 187, 141-152, 2017. ISSN 0166-445X.

The article has been published in final form by Elsevier at http://dx.doi.org/10.1016/j.aquatox.2017.03.023

Title: Primary hepatocytes from Arctic char (*Salvelinus alpinus*) as a relevant Arctic *in vitro* model for screening contaminants and environmental extracts.

Authors: Karina Petersen[1], Maria T. Hultman[1] and Knut Erik Tollefsen[1,2]

Affiliations: [1]Norwegian Institute for Water Research, Gaustadalleen 21, N-0349 Oslo, Norway. [2]Norwegian University of Life Sciences (NMBU), post Box 5003, N-1432 Ås, Norway

Corresponding authors: Knut Erik Tollefsen
Email: knut.erik.tollefsen@niva.no
and
Karina Petersen
Email: karina.petersen@niva.no

Abstract

Contaminants find their way to the Arctic through long-range atmospheric transport, transport via ocean currents, and through increased anthropogenic activity. Some of the typical pollutants reaching the Arctic (PAHs, PCBs) are known to induce cytochrome P450 1a (CYP1A) protein expression and ethoxyresorufin-O-deethylase (EROD) activity through the aryl hydrocarbon receptor (AhR). In addition, some endocrine disrupting chemicals (EDCs) such as estrogen mimics (xenoestrogens) have been documented in Arctic areas and may thus interfere with natural sexual development and reproduction. *In vitro* assays that are capable of detecting effects of such pollutants, covering multiple endpoints, are generally based on mammalian or temperate species and there are currently no well characterized cell-based *in vitro* assays for effect assessment from Arctic fish species. The present study aimed to develop a high-throughput and multi-endpoint *in vitro* assay from Arctic char (*Salvelinus alpinus*) to provide an non-animal (alternative) testing method for an ecologically-relevant Arctic species. A method for isolation and exposure of primary hepatocytes from Arctic char for studying the toxic effects and mode of action (MoA) of pollutants was applied and validated. The multi-versatility of the bioassay was assessed by classical biomarker responses such as cell viability (membrane integrity and metabolic activity), phase I detoxification (CYP1A protein expression, EROD activity) and estrogen receptor (ER) mediated vitellogenin (Vtg) protein

expression using a selection of model compounds, environmental pollutants and an environmental extract containing a complex mixture of pollutants. Primary hepatocytes from Arctic char were successfully isolated and culture conditions optimized to identify the most optimal assay conditions for covering multiple endpoints. The hepatocytes responded with concentration-dependent responses to all of the model compounds, most of the environmental pollutants and the environmental sample tested. The bioassay response and sensitivity of the hepatocytes from Arctic char differed slightly from closely related salmonid species, thus highlighting the need for developing *in vitro* assays relevant for Arctic species. The present multi-endpoint *in vitro* assay offer a highly versatile tool to screen potential effects of pollutants and complex samples relevant for Arctic exposure scenarios.

Key words: Arctic char, primary hepatocytes, *in vitro*, vitellogenin, CYP1A, EROD

1. Introduction

Contaminants find their way to the Arctic through long-range atmospheric transport, transport via ocean currents, and through increased anthropogenic activity in Arctic areas. Contaminants may also be distributed in the ecosystem by living organisms such as migration of fish and through guano from seabirds. Organisms in the Arctic environment are thus exposed to a number of contaminants and high concentrations of persistent organic pollutants (POPs) have been measured in some Arctic fish species (reviewed by Letcher et al., 2010). Surprisingly, higher concentrations of several groups of POPs were found in Greenland shark (*Somniosus microcephalus*) and Arctic char (*Salvelinus alpinus*) than in other species of fish considered in this study. Highest body burdens of POPs were found in the Greenland shark (Σ polychlorinated biphenyls (PCB) of 4400 ng/g lipid weight (lw), Σ chlordanes (CHL) of 1815 ng/g lw, and Σ dichlorodiphenyltrichloroethanes (DDT) of 7195 ng/g lw). High concentrations of POPs was also reported for Arctic char (ΣPCB of 2700 ng/g wet weight (ww), ΣCHL of 330-430 ng/g lw, and ΣDDT of 310-500 ng/g lw). In addition, local hot-spots of contamination have been demonstrated in areas such as Lake Ellasjøen at Bear Island (Norway), where high concentrations of organic halogenated compounds (OHCs) such as hexachlorobenzene, ΣCHLs (> 200 ng/g lw), mirex, ΣDDTs (1 585 ng/g lw) and ΣPCBs (>10 000 ng/g lw) have been demonstrated in resident populations of Arctic char (Bytingsvik et al., 2015; Evenset et al., 2004). These high levels of POPs might pose a problem to the fish as the dioxin-equivalents of

detected compounds was 8 times higher in Arctic char than the lowest observed effect concentration (LOEL) of dioxins in temperate salmonid fish (Bytingsvik et al., 2015).

Arctic char is a cold-water, Arctic species of the Salmonidae family, and is closely related to both Atlantic salmon and lake trout. The species has a wide spread distribution in the northern hemisphere and is the only native freshwater species found in the Arctic, sub-Arctic, alpine lakes and coastal waters. The Arctic char has been used in a few *in vivo* studies to investigate effects of contaminants (Aluru et al., 2004; Devaux et al., 2011; Jorgensen et al., 2001a, 2001b), studying amongst others, EROD activity and CYP1A protein expression (Jorgensen et al., 2001b). CYP1A is one of the most sensitive biomarkers for planar (chlorinated and non-chlorinated) hydrocarbons in fish (van der Oost et al., 2003) and like EROD activity, CYP1A gene and protein expression is mediated through transcriptional activation of the aryl hydrocarbon receptor (AhR). Known substrates for the AhR are dioxin-like compounds such as planar PCBs and polycyclic aromatic hydrocarbons (PAHs), POPs commonly found in arctic biota and environment (Bytingsvik et al., 2015; Evenset et al., 2004). POPs such as these have caused reproductive effects in fish at concentrations lower or similar to the ones detected in Arctic fish (Letcher et al., 2010). For instance, PCB levels < 500 ng/g ww in fish eggs is proposed to affect the survival after fertilization, and larvae exposed to PCB levels as low as 10–30 ng/g ww may suffer from reproductive dysfunctions later in life (reviewed by Letcher et al 2010). Some POPs are also known to or suspected to induce endocrine disruption (ED), including interference with estrogen receptor (ER)-mediated processes. A commonly used biomarker for exposure and effect of xenoestrogens is the induction of the ER-mediated production of vitellogenin (Vtg, egg yolk protein precursor). Vitellogenin protein and gene expression has been shown to be induced by a number of environmental pollutants including bisphenol A, nonylphenols, octylphenols and o,p'-DDE both *in vitro* and *in vivo* (Larsen et al., 2006; Park et al., 2003; Petersen and Tollefsen, 2011).

Although *in vivo* studies are regarded as the golden standard in ecotoxicological testing, *in vitro* bioassays have gained momentum as alternatives to resource demanding *in vivo* studies due to a high-throughput testing format, low sample volume requirement and multi-endpoint testing capability (Castaño et al., 2003; Schirmer, 2006). *In vitro* methods, such as the primary culture of fish cells, have been derived from various tissues and species and used in chemical toxicity screening and mechanistic studies (Avella et al., 1999; Björkblom et al., 2008; Ellesat et al., 2011; Farkas et al., 2011; Liebel et al., 2011; Segner, 1998; Tollefsen et al., 2003). Of the

different fish tissues, liver is the most commonly used donor organ for primary fish cell cultures. Primary fish hepatocytes retain native liver properties such as biotransformation, detoxification response, lipogenesis, and are estrogen responsive for up to 5-8 days in culture (Braunbeck and Storch 1992; Segner 1998; Tollefsen et al., 2003). Several toxicological relevant mechanisms have been demonstrated to be comparable to that observed *in vivo* (Hultman et al., 2015a).

There are currently no well characterized cell-based *in vitro* assays for effect assessment derived from Arctic fish species. In order to contribute to the understanding and assessment of pollutants on Arctic species, the present study aimed to develop and apply a method for isolation and exposure of primary hepatocytes from Arctic char to study potential effects of POPs. The multi-versatility of the bioassay was assessed by classical biomarker responses such as Vtg protein expression, EROD activity, CYP1A protein expression and cytotoxicity (cell membrane integrity and metabolic activity) using a suite of model compounds (17β-estradiol, copper sulphate and 2,3,7,8-tetrachlorodibenzo-p-dioxin), environmental contaminants (4-*tert*-octylphenol, bisphenol A, PCB126 and benzo(A)pyrene) and a complex environmental sample (extract of road maintenance water).

2. Materials and methods

2.1 Chemicals and environmental extract

The test chemicals 17β-estradiol (E2, ≥98%), benzo(a)pyrene (BAP), copper sulphate ($CuSO_4*5H_2O$), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), bisphenol A (BPA, 97%) and 4-*tert*-octylphenol (OP, 97%) were obtained from Sigma-Aldrich (St. Lois, MI, US) while 3,3',4,4',5-pentachlorobiphenyl (PCB 126) was purchased from Chiron AS (Trondheim, Norway). All chemicals, except $CuSO_4*5H_2O$ which was dissolved directly in the cell culture media before exposure, were dissolved in dimethylsulfoxide (DMSO) and stored at -20°C when not in use. An in-house environmental extract from road maintenance (tunnel wash) water from the Nordby tunnel (Ås, Norway) previously shown to induce EROD activity and CYP1A protein in primary rainbow trout hepatocytes (Petersen et al., 2016), and the extract of the corresponding control water, was stored at -20°C when not in use. The tested extract was considered to be representative for a highly complex polluted environmental sample (Meland et al., 2010, Petersen et al., 2016). The exposure concentration was expressed as a concentration ratio (CR) that referred to the extracted water to bioassay exposure concentration ratio. A CR

of 1 indicate that the nominal concentrations in exposure media corresponds to the concentrations in the water sample from which the extract was obtained. The final DMSO concentration in the exposure media was 0.1% for all chemicals and extract concentrations, except for the highest concentration of the extract (1% DMSO). Solvent controls of 0.1% and 1% DMSO was applied on each exposure plate and used as negative controls.

2.2 Arctic Char

Roe from Arctic Char (*Salvelinus alpinus*) were obtained from Tydalfisk (Løvøya, Tydal, Norway) and transported to the animal facilities at the Norwegian University of Life Sciences, NMBU (Ås, Norway) where they were hatched and reared. When reaching approximately 100 grams, fish were transported to the animal facilities at the University of Oslo (Oslo, Norway) where they were kept in tanks with a water temperature of 8±3°C, 100% oxygen saturation, pH 6.6 and a 12h light/12h dark cycle. The fish were fed daily with pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of the total body mass.

2.3 Isolation and exposure of Arctic Char hepatocytes

Prior to the exposure experiments, the isolation method developed for other salmonid fish (Tollefsen et al., 2003) was optimized for Arctic char by testing different types of collagenase. In brief, the fish (size 150-500 grams) were killed with a blow to the head and sexed by visual inspection of their gonads. Only juveniles (undeveloped gonads) or fish with male gonads were used. For optimal cell yield, the liver was perfused with a calcium free buffer (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, EGTA 26 µM, 0°C, 5 ml/min, 10-15 min) to remove the blood from the liver as described in Tollefsen et al., (2003). The liver was then perfused with the same buffer (5 ml/min, 10-15 min, 37°C) without EGTA and with added $CaCl_2$ (1.5 mM) and collagenase type VIII (Sigma-Aldrich, 0.3 mg/ml, different from the collagenase normally used for salmonids). The liver was transferred to a glass beaker on ice and dispersed in ice cold calcium free buffer supplemented with 0.1% w/v bovine serum albumin (BSA). The cell suspension was filtered first through a 250 µm nylon mesh and then through 100 µm nylon mesh before centrifugation at 500 rpm three times (4 min, 3 min and 3 min). Following the first centrifugation the supernatant was removed and the cells re-suspended in ice-cold calcium-free buffer supplemented with 0.1% w/v BSA. After the second and third centrifugation the cells were re-suspended in refrigerated serum-free L-15

medium containing L-glutamin (0.29 mg/ml), $NaHCO_3$ (4.5 mM), penicillin (100 units/ml), streptomycin (100 µg/L) and amphotericin (0.25 µg/ml). Cells were filtered through a 100 µM nylon mesh followed by assessment of the cell viability (>80%) using a Bürkner counting chamber and Trypan Blue (twice the volume of trypan blue as the volume of cell suspension). The cell suspension was thereafter diluted to the desired cell concentration, plated in 96-well Primaria™ microtiter plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) and left to acclimatize for 24h in a temperature regulated incubator at 4±2°C, 10°C and/or 15°C.

After 24h, 75µl culture media was removed from each well and 125 µl of exposure media with E2 (positive standard for estrogenic effects), TCDD (positive standard for EROD activity and CYP1A protein expression), and $CuSO_4$ (positive standard for cytotoxic effects) was added to determine the response of these model compounds. After 48h of exposure, cell media was removed and the cell plates were stored at -80°C for subsequent analysis of EROD activity and CYP1A protein. Plates determined for Vtg and cytotoxicity analysis were re-exposed after 48h by replacing 125 µl of the medium with freshly prepared exposure solutions and exposed for additional 48h (total exposure time 96h). At the end of the 96h exposure, the cell media was transferred to 96-well Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark), sealed with plate sealers (Nunc, Roskilde, Denmark) and stored at -80 for subsequent vitellogenin (Vtg) analysis. The remaining media was removed and the cells were subjected to cytotoxicity analysis.

Different exposure temperatures (4°C, 10°C and 15°C), exposure durations (24, 48 and 96h) and cell densities (750 000, 500 000, 250 000 and 100 000 cells/ml) were tested to identify optimal *in vitro* exposure and culture conditions for the different bioassay endpoints. To find the optimal temperature for exposure, cells from the same fish were divided into three batches, exposed to the same standards and incubated at 4±2°C, 10°C and 15°C. After finding the optimal temperature (i.e. the temperature that best balanced the environmental relevance and optimal bioassay conditions), four different concentrations of cells (100 000 cells/ml, 250 000 cells/ml, 500 000 cells/ml and 750 000 cells/ml) were tested to find the cell density that provided the best balance in terms of efficiency (high-throughput capability) and endpoint response (sensitivity, reproducibility and responsiveness).

After determining the optimal temperature and cell density, environmental pollutants (bisphenol A, 4-*tert*-octylphenol, PCB 126, benzo(a)pyrene), and a complex environmental

extract (tunnel wash water) assumed to display different mode of action (MoA) were tested individually to characterize the suitability of Arctic char hepatocytes as an *in vitro* screening assay.

2.4 Cytotoxicity assays

At the end of the 96h exposure period, metabolic activity and membrane integrity were determined essentially as described by Schreer et al. (2005) using the two probes Alamar blue (AB) and 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (CFDA-AM), respectively. The growth media was removed from the wells before the cells were incubated in 100 µl tris buffer (5 mM, pH 7.5) containing 5% AB and 4 µM CFDA-AM. Fluorescence was read after 30 min of incubation on an orbital shaker (100 rpm) in the dark (room temperature) at wavelength pairs of excitation and emission of 530-590 nm (AB) and 485-530 nm (CFDA-AM) using a Victor V^3 multilabel counter (PerkinElmer, Waltham, MA, USA). The results were normalized to the DMSO control (100% viability) and the highest concentration of $CuSO_4$ (10 mM) causing 100% cell death (0% viability).

2.5 EROD activity

The EROD activity was measured by incubating the cells with ethoxyresorufin (ER), a substrate for the CYP1A isoenzymes, which is enzymatically converted to resorufin (RR). The conversion of ER to RR is linear for at least 20 minutes and is monitored fluorometrically. In brief, the cell plates were thawed on ice, and incubated in 200 µl of 50 mM Tris buffer containing 0.1 M NaCl, 20 µM dicumarol, 2 µM ER, and 100 µM β-nicotinamide adenine dinucleotide phosphate (β-NADPH) for 15 min. Fluorescence was measured using a Victor V^3 multilabel counter (PerkinElmer, Waltham, MA, USA) with excitation and emission wavelength pair of 530 nm and 595 nm, respectively. The protein concentrations were measured with the Bradford method. The results were normalized to the response range (0-100%) using a negative (DMSO) and positive (0.3 or 3 nM TCDD) control.

2.6 CYP1A protein expression

The analysis of CYP1A protein expression was conducted essentially as described by Tollefsen et al. (2008). Following EROD analysis, the plates were frozen at -80 degrees and thawed again to produce a homogenous cell lysate. Once thawed, 40 µl from each well was

transferred to a new 96-well plate and diluted with 160 µl coating buffer (0.025 M Carbonate-bicarbonate). 100 µl of the diluted lysates and reference samples was transferred to 96-well Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark), sealed with plate seals (Nunc, Roskilde, Denmark) and incubated overnight in the dark at 4°C. The plates were washed three times with washing buffer (PBS added 0.05% Tween® 20) and incubated 1h in the dark (room temperature) with 200 µl blocking buffer (PBS with 2% BSA). After three washes with washing buffer, cells were incubated with 100 µl of the primary antibody polyclonal rabbit anti-fish CYP1A (CP-226, Biosense Laboratories, Bergen, Norway) diluted 1:1000 in 1% (w/v) BSA-PBS buffer at 37°C for 2 h. The CP-226 antibody is known to cross-react with CYP1A in liver samples from a wide variety of species, including rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), common carp (*Cyprinus carpio*), flounder (*Platichthys flesus*), sheepshead minnow (*Cyprinodon variegatus*) and gilthead bream (*Sparus aurata*) (Biosense Laboratories, product sheet for CP-226), and was therefore assumed to also cross-react with CYP1A in Arctic char. After three washes, 100 µl secondary antibody Goat-anti-Rabbit IgG conjugated with horseradish peroxidase (HRP, 1:3000, Bio-Rad, Hercules, CA, USA) was added and the plates were incubated at 37 °C for 2h. The plates were washed five times and 100 µl of the substrate for HRP (TMB plus2, Kem-En-Tech, Taastrup, Denmark) was added to each well. Plates were incubated for 15 min. and the reaction was stopped by adding 50 µl H_2SO_4 (1 M). The absorbance was measured by a VersaMax microplate reader (Molecular Devices LLC., Sunnyvale, CA, USA) at 450 nm and the results normalized to the response range (0-100%) using a negative (DMSO) and positive (0.3 or 3 nM TCDD) control.

2.7 Vitellogenin protein expression

The production of Vtg was measured by a semi-quantitative capture ELISA as described in Tollefsen et al. (2003). Plates were thawed before Vtg protein standards (Vtg from rainbow trout) were applied to empty wells. The Vtg standard was used to control that the assay performed as expected. Plates were left to incubate overnight in the dark at 4°C, then washed three times with washing buffer (PBS added 0.05% Tween® 20) and incubated 1h in the dark with 200 µl blocking buffer (PBS with 2% BSA). After three washes with washing buffer, cells were incubated with 100 µl of the primary antibody monoclonal mouse anti-salmon Vtg (BN-5, Biosense laboratories, Bergen, Norway) diluted 1:6000 in PBS buffer with 1% BSA at 37°C for 2h. The antibody BN-5 binds with high affinity to Vtg in plasma samples from Atlantic

salmon and cross-reacts with Vtg in plasma samples from Arctic char (Biosense laboratories, product sheet BN-5). After three washes, 100 µl secondary antibody goat-anti mouse IgG conjugated with horse radish peroxidase (HRP) was added and the plates were incubated at 37°C for 1h. The plates were then washed five times and 100 µl of the substrate for HRP (TMB plus2, Kem-En-Tech, Taastrup, Denmark) were added to each well. Plates were incubated for 15 min and the reaction was stopped by adding 50 µl H_2SO_4 (1 M). The absorbance was measured at 450 nm by a VersaMax microplate reader (Molecular Devices LLC., Sunnyvale, CA, USA) and the results normalized to the response range (0-100%) using a negative (DMSO) and positive (30 nM E2) control.

2.8 Data analysis

All data were assessed with Graphpad prism v6.01 software (GraphPad Software Inc., San Diego, CA, USA). Data were fitted with non-linear sigmoidal concentration-response curve with variable slope, with constraints for bottom (0) and top (100). Significant differences were identified by one way ANOVA using a p-value threshold of $p<0.05$.

3. Results

3.1 Isolation of arctic char hepatocytes

Viable hepatocytes were successfully isolated from Arctic char by use of a 2 step perfusion method with collagenase type VIII. The cell viability was generally above 80% (mean of 85±5 % based on 12 independent cell isolations) and a yield of 40-200 million cells per isolation was obtained. Microscopic inspection of perfused cells revealed a homogenous mono-layer of hepatocytes with an apparent high content of what looked like lipid vacuoles (supplementary figure S1). The unexposed primary hepatocytes were viable for at least 120h, determined by the trypan blue method. Use of collagenase type IV (Sigma-Aldrich), resulted in extensive cell disruption demonstrated by presence of a lipid layer after centrifugation and low cell yield, and could not be used for isolation of the Arctic char hepatocytes.

3.2 Influence of exposure conditions on endpoint responses

The exposure durations and sampling times of 48h for EROD and CYP1A analyses, and 96h with re-exposure after 48h for Vtg and cytotoxicity analyses were found to be appropriate to

obtain a clear concentration response of the model compounds. High quality (R^2 > 0.7) concentration-response curves (CRCs) for all endpoints were obtained when testing the model compounds (Fig. 1-3). In a preliminary study, cell viability of the primary Arctic char hepatocytes in culture media cultured at three different temperatures (8°C, 12°C and 15°C) was assessed daily over a period of 120 h by use of trypan blue and showed a viability > 80% at all time points for all temperatures (supplementary table S1). Screening of optimal incubation temperature (4, 10, 15°C) was performed using cells from one fish (Fig. 1). Cell density was more extensively evaluated, generally using 3-7 fish (depending on cell density), to identify both optimal assay sensitivity, reproducibility and robustness.

3.2.1 Influence of temperature and cell density on cytotoxicity

The incubation temperature affected the biomarker response of the exposed Arctic char hepatocytes, and the highest toxicity of the positive control $CuSO_4$ was observed at a temperature of 4±2°C (Fig 1). However, the responses (e.g. EC_{50} values) differed only by a factor of 1.3 (inhibition of metabolic activity) and 2.4 (loss of membrane integrity) between the different temperatures used in the study (Table S2). No clear coherence between the EC_{50} values and cell density was found for the two endpoints after exposure to $CuSO_4$ (Table S2).

3.2.2 Vtg protein expression

The E2-induced Vtg protein expression was also affected by the different exposure conditions used. The largest absolute Vtg induction was obtained at 15°C when reviewing raw data (Supplementary table S5). However, when normalizing data against the positive and negative controls, the EC_{50} values obtained at the different temperatures were within a factor of 2. The lowest EC_{50} value for Vtg protein expression was seen at 10°C (Fig 2, Table S1). Interestingly, cell density had a higher impact on the response than the exposure temperature. The EC_{50} value for Vtg protein expression declined with a factor of 11.5 from the highest to the lowest cell density (Table S1).

3.2.3 CYP1A protein expression and EROD activity

A concentration-dependent increase in hepatocyte CYP1A protein expression and EROD activity was observed after exposure to TCDD. In similarity with Vtg expression, the largest CYP1A protein expression was observed for non-normalized data at 15°C (supplementary table S5). Less variable EC_{50} values (within a factor of 2) for CYP1A protein expression and EROD

activity were achieved by normalizing the data against the positive and negative controls. The lowest EC_{50} value for both EROD activity and CYP1A protein expression was obtained at an exposure temperature of 10°C (Fig. 3, table S1). The EC_{50} for CYP1A protein expression generally declined with decreasing cell density, except for a higher EC_{50} at 250 000 cells/ml than for 500 000 cells/ml. The EC_{50} for CYP1A protein expression varied by a factor of 3.4 between the different cell densities (Table S1). The EC_{50} for EROD activity declined with decreasing cell density and varied by a factor of 2.2 between the highest (750 000 cells/ml) and lowest (100 000 cells/ml) cell density (Table S1).

3.3 Environmental pollutants

The temperature and cell density that best balanced the environmental relevance, optimal conditions for determining the endpoint measured and providing high-throughput (10°C and a cell density of 250 000 cells per ml) were used as standard exposure conditions for testing of the environmental pollutants BPA, OP, BAP and PCB126 (Fig. 4-6).

3.3.1 Cytotoxicity

The cell viability was expressed as membrane integrity and metabolic activity, displaying 100% viability in the media control and 0% viability in the positive control (0.1M $CuSO_4$) after 96h of exposure (Fig. 2). Complete cell death was confirmed by visual inspection (using microscope) of cells exposed to the positive control. In these wells, no intact cells were present. Full CRCs for inhibition of metabolic activity and loss of membrane integrity were obtained for both BPA (metabolic activity: EC_{50}= 9.2 µM, membrane integrity: EC_{50}= 57 µM) and OP (metabolic activity: EC_{50}= 22 µM, membrane integrity: EC_{50}= 32 µM), whereas only partial CRCs were observed for BAP (metabolic activity: 67% of solvent control and membrane integrity: 84% of solvent control at highest tested concentration) and PCB126 (metabolic activity: 71% of solvent control and membrane integrity: 96% of solvent control at highest tested concentration). Inhibition of metabolic activity was found to be 1.5–6.2 times more sensitive than loss of membrane integrity when comparing the obtained EC_{50} values for the compounds tested (Table 1).

3.3.2 Vitellogenin protein expression

A full CRC for Vtg protein expression was obtained for the model compound E2 (EC_{50} = 0.43 nM) after 96h of exposure, whereas only a small increase was observed for BPA (efficacy =

13% at 3 µM) and OP (efficacy = 22% at 10 µM), suggestively due to an increase in cytotoxicity at the highest concentrations tested.

3.3.3 CYP1A protein expression and EROD activity

Full CRCs for CYP1A protein expression and/or EROD activity were obtained for the positive control TCDD (CYP1A protein expression: EC_{50} = 0.597 nM, EROD activity: EC_{50} = 0.923 nM) after 48h of exposure. For the environmentally relevant compounds, both full (CYP1A protein expression) and partial (EROD activity) CRCs were obtained for PCB 126 (CYP1A protein expression: EC_{50} = 30 nM and efficacy = 120%, EROD activity: EC_{50} = 30 nM and efficacy = 50%) and BAP (CYP1A protein expression: EC_{50} = 350 nM and efficacy = 81%, EROD activity: EC_{50} = 475 nM and efficacy = 62%) (Fig. 6, Table 1). Induction of CYP1A protein expression and EROD activity was found to be equally sensitive when comparing the EC_{50} values obtained (within a factor of 1.5, Table 1).

3.4 Environmental extract

An extract of tunnel wash water was tested for cytotoxicity, Vtg protein expression, EROD activity and CYP1A protein expression (Fig. 7). The extract affected the cell viability shown by a reduction in both the membrane integrity and metabolic activity to 42% and 35% of solvent control, respectively. The $EC_{50}$was a CR of 7.4 for membrane integrity and of 5.7 for metabolic activity in the Arctic char hepatocytes. The extract of control water did not affect the cell viability of the Arctic char primary hepatocytes. No increase in the Vtg protein expression was observed for the tunnel wash water extract and control water extract at the tested CRs (up to 10 times concentrated from the original water sample). Both the EROD activity and CYP1A protein expression was induced by the tunnel wash water extract. At a CR $\geq$ 3, the EROD activity and CYP1A protein expression declined in parallel with the decline in metabolic activity and membrane integrity. A partial CRC was obtained for CYP1A with EC_{50} at a CR of 0.94 and an efficacy of 89%. The highest EROD activity was 38% of the positive control at a CR of 1.

4. Discussion

4.1 Isolation and exposure of Arctic char hepatocytes

The present study documents the development of a multi-endpoint *in vitro* bioassay using primary Arctic char hepatocytes and demonstrates the use for screening a suite of environmental pollutants and an ecologically-relevant complex mixture. A key feature for successful bioassay development as that described herein is the successful isolation of primary hepatic cells from live fish and the optimization of culturing and exposure conditions. In this study, high yield and quality isolation of primary Arctic char hepatocytes were obtained by the use of collagenase type VIII, whereas collagenase type IV, which is routinely used for isolation of rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) hepatocytes (Petersen and Tollefsen, 2011; Tollefsen et al., 2003) resulted in disrupted cells and low cell yield after centrifugation. The reason for this discrepancy is currently unknown, but presence of high content of lipid vacuoles or fat in the primary cells (supplementary, Fig. S1) can provide some explanation as similar challenges have been encountered when isolating Atlantic cod (*Gadus morhua*) hepatocytes due to their high fat content (Husøy et al., 1996). In contrast, type IV collagenase seemed to work well for the isolation of hepatocytes from different marine fish species such as plaice (*Pleuronectes platessa*), long rough dab (*Hippoglossiodes platessoides*) and Atlantic cod (Ellesat et al., 2011) and may indicate that other species-specific or methodological differences could account for low primary hepatocyte cell yields from fish. However, our results suggest that obstacles such as low yield and quality of isolated primary fish hepatocytes can be most effectively resolved by testing different types and batches of collagenase during the initial bioassay optimization steps.

In an optimal bioassay, the exposure temperature should be chosen to balance the environmental relevance, optimal conditions for determining the biomarker or effect endpoint measured and offer a high-throughput system. In general, protein synthesis increases with temperature in fish (Jankowsky et al., 1981), and higher level of Vtg mRNA have been observed in rainbow trout hepatocytes exposed at 18°C than at 14°C (Pawlowski et al., 2000). This is similar to the present study where higher raw data readings (absorbance and fold change) was observed with increasing incubation temperatures. Contrary to this, Tollefsen et al. (2003) observed that the Vtg protein production in primary hepatocytes from salmon was optimal at 12°C, whereas lower (8°C) and higher (16°C) temperatures produced sub-optimal CRCs. Thus the optimal temperature for a specific biomarker response seems both to be species and endpoint dependent,

and choice of exposure conditions potentially become a compromise between the two. The ultimate upper (23-24°C) and lower (0°C) incipient lethal temperatures for Arctic char clearly verify that this salmonid may successfully survive both temperate and Arctic conditions (Lyytikäinen and Jobling, 1998). However, low water temperatures (4-7°C) are required during the final stages of the reproductive cycle (Jobling et al., 1995), whilst the growth rates of juveniles peak at 12-14°C. Studies have shown relatively high growth rates even during periods with low water temperatures (Brännäs and Wiklund, 1992; Siikavuopio et al., 2009). Bioassay temperatures between 4°C and 10°C seem therefore to be most environmentally relevant for Arctic char, and agree well with the proposed optimal bioassay temperature of 10°C in the present study. The choice of temperature agrees very well with that used for studies with hepatocytes from plaice, long rough dab and Atlantic cod (10°C, Ellesat et al., 2011).

Another important factor affecting the biomarker response is the cell density used in the well of the microplates. Tollefsen et al. (2003) observed a density-dependent increase in Vtg production up to 500 000 cells/ml, whereas higher densities led to reductions in Vtg production in primary hepatocytes from salmon. In this study, a lower cell density than previously used for Atlantic cod (Ellesat et al., 2011; Søfteland et al., 2010), rainbow trout (Hultman et al., 2015b), plaice, and long rough dab (Ellesat et al., 2011) were found to best balance the response and efficiency (number of plates per million cells) of the Arctic char hepatocyte assay. Cell densities in a similar range as that used in the current study have previously been used to study Vtg induction in primary hepatocytes from *Oryzias latipes* and *Oreochromis mossambicus* (Kim and Takemura, 2003; Kordes et al., 2002; Riley et al., 2004, see supplementary Table S4 for more information). Reduction in cell density without compromising the sensitivity and responsiveness would render the assay more efficient in terms of the number of compounds/extract that could be tested and thus comply well with the 3R's (reduction, refinement, replacement) ambitions to reduce animal use for testing purposes.

The suggested optimal exposure conditions in this study using an incubation temperature of 10°C and exposure period of 48h for EROD and CYP1A activity and 96h for Vtg and cytotoxicity is similar to studies using primary hepatocyte cultures from other fish species (Ellesat et al., 2011; Petersen and Tollefsen, 2011; Petersen et al., 2016; Tollefsen et al., 2003). The conditions were chosen based on screening assays covering multiple endpoints. Thus

optimization for single endpoints and endpoints not investigated in this study might result in other recommendations.

4.2 Cell viability

Several assays have been employed to assess *in vitro* cell viability, including neutral red, propidium iodide, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT), release of preloaded radioactive label, leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH) and fluorescent dyes (reviewed by Schreer et al., 2005). The two probes used herein provide complementary information by differentiating between compounds targeting the (mitochondrial) metabolic processes (AB) and the more unspecific MOA associated with cellular disruption potentially reflecting narcosis or baseline toxicity (CFDA-AM) (Schreer et al., 2005).

Interestingly the EC_{50} for metabolic activity after exposure to BAP was 6 times lower than the EC_{50} for membrane integrity, which could indicate that BAP specifically target mitochondrial functions. This is supported by findings that exposure to BAP induced formation of reactive oxygen species (ROS) via CYP1A metabolism, resulting in harmful BAP diones (Farmen et al., 2010) which may cause cytotoxicity (full review see Verma et al., 2012) and potentially result in apoptosis (programmed cell death) at low concentrations and necrosis at high concentrations (Zacchino et al., 2013). No difference in the EC_{50} for metabolic activity and membrane integrity was observed after exposure to BPA in this study. Previous studies have shown that BPA elicit specific cytotoxicity in addition to its ER-agonistic properties, and it has been suggested that early cytotoxicity of BPA is mediated through activation of caspase-3 (Kaptaner and Kankaya, 2016), which plays a central role in apoptosis in fish (dos Santos et al., 2008). However, the present study did not investigate these underlying causes for cytotoxicity and although interesting will not be addressed in any detail herein.

The effect on metabolic activity after exposure to OP was highly similar to previous EC_{50} values reported for rainbow trout hepatocytes (table 2). The EC_{50} values for metabolic activity after exposure to BPA differed by a factor of 7, with Arctic char hepatocytes being more sensitive than rainbow trout hepatocytes. The lower sensitivity of rainbow trout hepatocytes may be due to temperature-dependent increase in biotransformation of the compounds, as previously reported *in vivo* (Buckman et al., 2007; Niimi and Palazzo, 1985), where the higher incubation

temperature used for rainbow trout hepatocytes could lead to higher level of biotransformation, and thus reducing internal cellular concentrations of the test compounds

4.3 Vitellogenin protein expression

Vtg gene and protein expression are commonly used biomarkers for environmental (xeno)estrogens in juvenile/male fish as it is mediated by the binding and transcriptional activation of the ER. The induction of Vtg expression has been proposed to be sensitive, reliable and easy to use in chemical screening and environmental monitoring (Bickley et al., 2009; Harman et al., 2010; Hultman et al., 2015b; Tollefsen et al., 2008) and acknowledged to be a good estrogenic biomarker due to fairly good knowledge of baseline data, low number of confounding factors and high toxicological significance (van der Oost et al., 2003). The ERα-isotype, which has been proposed to be the dominating estrogen responsive receptor in the fish liver, has retained its genomic structure and function across vertebrate species (Nelson and Habibi, 2013). Although the maximum level of Vtg varies among species (reviewed by Navas and Segner, 2006), *in vitro* induction of Vtg serves as a robust signal for estrogenic exposure. Furthermore, molecular and subcellular processes associated with the *in vitro* ER signaling pathway has been proposed to reflect *in vivo* bioactivity in other salmonid fish such as rainbow trout (Hultman et al., 2015a, 2015b).

The EC_{50} for Vtg protein expression in E2 exposed Arctic char hepatocytes was from 465 times lower to 4 times higher than that of E2 exposed primary rainbow trout hepatocytes, and 17 times higher than that reported in salmon (Table 1 and 2). The results indicate that the Arctic char hepatocytes have a similar sensitivity as that of rainbow trout hepatocytes, albeit slightly less sensitive than salmon. The Vtg protein expression induced by the two environmental contaminants OP and BPA were lower in primary hepatocytes from Arctic char than from rainbow trout (Petersen and Tollefsen, 2011) by displaying a 3.3 (BPA) and 1.9 (OP) fold higher maximum response (measured as % of positive control) in rainbow trout, respectively (Table 1 and 2). As the Arctic char hepatocytes contained high amount of lipid vacuoles, it can be hypothesized that this may lead to partition-induced restriction of the bioavailable fraction of the exposure chemicals available to interact with the intracellular ER-binding sites. However, a highly sensitive response was observed for E2 which has a $\log K_{OW}$ between those for OP and BPA, indicating that factors such as estrogenic potency, ER binding affinity (Rankouhi et al.,

2004), temperature dependent ER affinity (Petit et al., 1995) and biotransformation rate may also affect the Vtg response in fish.

4.4 EROD activity and CYP1A protein expression

In fish, the CYP1A subfamily is responsible for biotransformation of a myriad of xenobiotic compounds (PAHs, PCBs, dioxins, etc.) (Goksoyr and Forlin, 1992), and is one of the most sensitive biomarkers for planar (chlorinated) hydrocarbons used in environmental monitoring (van der Oost et al., 2003). The mechanism of AhR-mediated induction of CYP1A gene and protein expression and activation of EROD activity are well documented and display high sensitivity, good reliability and are easy to perform in multiple species (van der Oost et al., 2003). EROD activity appear to be the most sensitive catalytic assay for induction of the cytochrome P450 system in fish (Goksoyr and Forlin, 1992), and together with levels of CYP1A protein and mRNA, EROD activity may be used for exposure assessment and as an early-warning signal for potential harmful effects (van der Oost et al., 2003). The assay is typically conducted with liver tissue due to the high activity of biotransformation in this organ. Primary fish hepatocytes express stable levels of phase I and II enzymes (incl. CYP1A), which are induced after exposure to xenobiotics (Segner and Cravedi, 2001). The xenobiotic metabolite pattern in primary hepatocytes is generally similar to that observed *in vivo* (Segner and Cravedi, 2001), thus indicating primary hepatocytes' suitability for CYP1A protein expression and EROD activity analysis.

The EC_{50} for CYP1A protein production in Arctic char hepatocytes after exposure to TCDD was 14.6 times higher in the hepatocytes from Arctic char than rainbow trout (Petersen et al., 2016). The lower responsiveness towards TCDD might be due to compartmentalization of the highly hydrophobic TCDD ($logK_{OW}$ = 6.8) to passive lipid reservoirs inside the char hepatocytes, thus making it less bioavailable for the AhR-receptor and the activation of downstream events such as activation of CYP1A expression. The Atlantic cod liver which is also high in fat content has in several studies shown to be less responsive to AhR agonists than other fish species (Beyer et al., 1996; Goksøyr et al., 1996; Hektoen et al., 1994; Husøy et al., 1996). However, the current findings that both BAP and PCB 126 induce high levels of CYP1A protein expression in Arctic char hepatocytes (Table 1) highlights the assay suitability to study AhR mediated effects.

In the current study with Arctic char hepatocytes, the EC_{50} for EROD activity of TCDD was 37 times higher than in primary rainbow trout hepatocytes (Petersen et al., 2016), but only 1.5 lower than in primary tilapia hepatocytes cultured in media supplemented 5% FBS (Zhou et al., 2006). The EC_{50} for EROD induction after exposure to BAP was within the range previously observed for rainbow trout hepatocytes (Behrens et al., 2001; Scholz and Segner, 1999), and 6 times higher than in tilapia (Zhou et al., 2006). As the effects on EROD activity after exposure to BAP was within previous reported results for rainbow trout hepatocytes, it can be questioned whether the presence of lipid vacuoles in hepatocytes were of importance for differences in assay sensitivities as the hydrophobicity ($logK_{OW}$) of TCDD and BAP are fairly similar (log $K_{OW(TCDD)}$= 6.8, Log $K_{OW(BAP)}$= 6.13). It's therefore suggested that species-differences in cellular bioavailability, affinity and efficacy of these receptor-mediated responses may account for a substantial part of the difference in EC_{50} between the current study and previous reported results with other fish species. As expected, good correlation between EROD activity and CYP1A protein expression was obtained in the present study. The EC_{50} values from the two AhR-mediated responses differed by a factor of 1.5 after exposure to TCDD, was identical after exposure to PCB 126, and differed by a factor of 1.4 after exposure to BAP. Both endpoints were induced at non-cytotoxic concentrations. A higher efficacy was observed for CYP1A protein expression than EROD activity for BAP and PCB126. This is similar to *in vivo* findings where an increase in CYP1A protein level was observed without any alterations in EROD activity after intraperitoneal injections of *Sebastiscus marmoratus* with 10 mg/kg BAP (Wang et al 2008). However, both endpoints were significantly increased after 25 days of waterborne exposure to 1000 ng/L BAP (Wang et al., 2008). Thus, increased protein expression of CYP1A does not always correlate with increased EROD activity, and it has been suggested to include CYP1A protein expression to complement EROD activity for in toxicological assessments (Wang et al., 2008). In addition, both CYP1A expression and EROD activity might be necessary to avoid underestimation of effects in situations of co-exposure to CYP1A enzyme inhibitors in environmental samples (Celander et al., 2011).

4.5 Environmental samples

Pollutants in the environment occur as complex mixtures that vary with time and space. Such complex mixtures are likely to contain a large variety of compounds affecting similar and dissimilar endpoints. In some cases, compounds in a mixture can act additively, synergistically or antagonistically (Eaton and Gilbert, 2007) and even mask the effect of one another (Frische et al., 2009). A proper evaluation of environmental mixtures is of importance to elucidate the different types of combined effects that may occur (Petersen and Tollefsen, 2011, 2012).

Extracts from tunnel wash water contain a large number of anthropogenic contaminants, including PAHs and other organics such as organophosphates like tris-(2-chloro, 1-methylethyl)-phosphate (TCPP) and tributyl phosphate (TBP), compounds also found in the Arctic region (Bytingsvik et al., 2015; Letcher et al., 2010; Meland et al., 2010; Meland and Roseth, 2011). Although not directly comparable to an Arctic exposure scenario, the extract of tunnel wash water serves as an example of a complex environmental sample and is reported to cause cytotoxicity, induction of EROD activity and CYP1A protein expression in rainbow trout hepatocytes (Petersen et al., 2016). The Arctic char hepatocytes appeared to be slightly less sensitive in terms of measured metabolic activity (EC_{50} = CR of 5.7) than the rainbow trout hepatocytes (EC_{50} = CR of 4.3) and slightly more sensitive (2 fold) in terms of measured CYP1A protein expression when compared to the EC_{50} obtained from rainbow trout (Petersen et al., 2016). No induction of Vtg protein expression was observed, potentially due to high concentrations of AhR-agonists (i.e. PAHs) that has previously been proposed to mask the effect of ER agonists in fish through a nuclear receptor cross-talk between AhR and ER (Gräns et al., 2010; Mortensen et al., 2007; Petersen et al., 2016).

4.6 Environmental relevance

Increased anthropogenic activity in the Arctic regions is anticipated due to the rapid decline in Arctic sea ice which may offer new opportunities for economic activity like shipping, tourism, oil-drilling etc. With increased activity, local emission of pollutants may increase in addition to pollutants transferred by wind, ocean currents and organisms from temperate regions. It is therefore anticipated that Arctic species will be exposed to a wider range of pollutants at higher concentrations than they are today. In order to participate to a better understanding of

potential implications of increased exposure in the Arctic, development of relevant and feasible high-throughput *in vitro* methods from Arctic species is warranted.

The presented *in vitro* assays with Arctic char hepatocytes offer an environmentally relevant, highly versatile and high-throughput screening tool for potential effects of pollutants both individually and in complex environmental samples. Clear bioassay responses of model compounds, environmental pollutants and a complex environmental sample were obtained. The biomarker responses were easily detected at all temperatures, and all cell densities, showing the robustness and potential of the assay to be run at other conditions than those used herein. The Arctic char hepatocytes showed increase of AhR mediated effects (EROD activity and CYP1A protein expression) at ecologically-relevant environmental concentrations (e.g. CR<1), demonstrating the assays suitability to be used for effect screening of environmental extracts. Overall, the sensitivity of Arctic char hepatocytes compared to hepatocytes from other donor fish was compound- and endpoint-dependent, potentially due to species-specific differences in cellular absorption, distribution, metabolism and excretion (ADME) and/or ability to trigger the cellular responses studied.

Primary fish hepatocytes have proven versatile and our results suggests that hepatocytes from Arctic char generally respond similarly to cells from other fish species. Based on the current study, further optimization for other endpoints such as cellular energetics, metabolism, oxidative stress and cellular damage etc. may expand the versatility of the bioassay, and support assessing a larger number of toxic mechanism relevant for Arctic species.

5. Conclusion

Primary hepatocytes from Arctic char were successfully isolated and culture conditions optimized to cover multiple biomarker and effect endpoints in a high-throughput format. Culture conditions of 10°C, 250 000 cell/ml and 48h exposure for EROD and CYP1A analysis, and 96h exposure (with re-exposure after 48h) for cytotoxicity and Vtg analysis were used. The hepatocytes yielded concentration-dependent responses to the model compounds, environmental pollutants and the environmental sample tested. The bioassay response and sensitivity of the hepatocytes from Arctic char differed slightly from closely related salmonid species. The presented *in vitro* assays with Arctic char hepatocytes offer an environmentally

642 relevant and highly versatile tool to screen potential effects of pollutants and complex samples
643 relevant for Arctic exposure scenarios.

644

645 Aknowledgements

646 The work was funded by the Research Council of Norway (RCN) project 221373-Is the cocktail
647 effect of environmental contaminants a threat for Arctic fish populations? and the RCN project
648 196318-Non-animal (alternative) testing methods for REACH (AlterReach).

649

650 References

651 Aluru, N., Jorgensen, E.H., Maule, A.G., and Vijayan, M.M. (2004). PCB disruption of the
652 hypothalamus-pituitary-interrenal axis involves brain glucocorticoid receptor
653 downregulation in anadromous Arctic charr. Am. J. Physiol. - Regul. Integr. Comp.
654 Physiol. *287*, R787–R793.

655 Avella, M., Pärt, P., and Ehrenfeld, J. (1999). Regulation of Cl− secretion in seawater fish
656 (Dicentrarchus labrax) gill respiratory cells in primary culture. J. Physiol. *516*, 353–363.

657 Behrens, A., Schirmer, K., Bols, N.C., and Segner, H. (2001). Polycyclic aromatic
658 hydrocarbons as inducers of cytochrome P4501A enzyme activity in the rainbow trout
659 liver cell line, RTL-W1, and in primary cultures of rainbow trout hepatocytes. Environ.
660 Toxicol. Chem. *20*, 632–643.

661 Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skåre, J.U., and Goksøyr, A.
662 (1996). Contaminant accumulation and biomarker responses in flounder (Platichthys
663 flesus L.) and Atlantic cod (Gadus morhua L.) exposed by caging to polluted sediments
664 in Sørfjorden, Norway. Aquat. Toxicol. *36*, 75–98.

665 Bickley, L.K., Lange, A., Winter, M.J., and Tyler, C.R. (2009). Evaluation of a carp primary
666 hepatocyte culture system for screening chemicals for oestrogenic activity. Aquat.
667 Toxicol. 94, 195–203.

668 Björkblom, C., Salste, L., Katsiadaki, I., Wiklund, T., and Kronberg, L. (2008). Detection of
669 estrogenic activity in municipal wastewater effluent using primary cell cultures from
670 three-spined stickleback and chemical analysis. Chemosphere *73*, 1064–1070.

671 Brännäs, E., and Wiklund, B.-S. (1992). Low temperature growth potential of Arctic char and
672 rainbow trout. Nord. J. Freshw. Res. *67*, 77–81.
673 Braunbeck, T., and Storch, V. (1992). Senescence of hepatocytes isolated from rainbow trout
674 (Oncorhynchus mykiss) in primary culture. Protoplasma *170*, 138–159.
675 Buckman, A.H., Brown, S.B., Small, J., Muir, D.C.G., Parrott, J., Solomon, K.R., and Fisk,
676 A.T. (2007). Role of temperature and enzyme induction in the biotransformation of
677 polychlorinated biphenyls and bioformation of hydroxylated polychlorinated biphenyls
678 by rainbow trout (Oncorhynchus mykiss). Environ. Sci. Technol. 41, 3856–3863.
679 Bytingsvik, J., Frantzen, M., Götsch, A., Heimstad, E.S., Christensen, G., and Evenset, A.
680 (2015). Current status, between-year comparisons and maternal transfer of
681 organohalogenated compounds (OHCs) in Arctic char (Salvelinus alpinus) from
682 Bjørnøya, Svalbard (Norway). Sci. Total Environ. *521–522*, 421–430.
683 Castaño, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee,
684 L.E.J., Mothersill, C., Pärt, P., et al. (2003). The use of fish cells in ecotoxicology. The
685 report and recommendations of ECVAM Workshop 47. Altern. Lab. Anim. ATLA *31*,
686 317–351.
687 Celander, M.C. (2011). Cocktail effects on biomarker responses in fish. Aquat. Toxicol. 105,
688 72–77.
689 Eaton, D.L. and Gilbert, S.G. (2007). Principles of Toxicology. In Casarett and Doull's
690 Toxicology : The Basic Science of Poisons / Editor, Curtis D. Klaassen, (United States:
691 New York : McGraw-Hill Medical, 2008), p. 17.
692 Devaux, A., Fiat, L., Gillet, C., and Bony, S. (2011). Reproduction impairment following
693 paternal genotoxin exposure in brown trout (Salmo trutta) and Arctic charr (Salvelinus
694 alpinus). Aquat. Toxicol. *101*, 405–411.
695 Ellesat, K.S., Yazdani, M., Holth, T.F., and Hylland, K. (2011). Species-dependent sensitivity
696 to contaminants: An approach using primary hepatocyte cultures with three marine fish
697 species. Mar. Environ. Res. *72*, 216–224.
698 Evenset, A., Christensen, G.N., Skotvold, T., Fjeld, E., Schlabach, M., Wartena, E., and Gregor,
699 D. (2004). A comparison of organic contaminants in two high Arctic lake ecosystems,
700 Bjornoya (Bear Island), Norway. Sci. Total Environ. *318*, 125–141.
701 Farkas, J., Christian, P., Gallego-Urrea, J.A., Roos, N., Hassellöv, M., Tollefsen, K.E., and
702 Thomas, K.V. (2011). Uptake and effects of manufactured silver nanoparticles in
703 rainbow trout (Oncorhynchus mykiss) gill cells. Aquat. Toxicol. *101*, 117–125.

Frische, T., Faust, M., Meyer, W., and Backhaus, T. (2009). Toxic masking and synergistic modulation of the estrogenic activity of chemical mixtures in a yeast estrogen screen (YES). Environ. Sci. Pollut. Res. *16*, 593–603.

Goksoyr, A., and Forlin, L. (1992). The Cytochrome-P-450 System in Fish, Aquatic Toxicology and Environmental Monitoring. Aquat. Toxicol. *22*, 287–311.

Goksøyr, A., Beyer, J., Egaas, E., Grøsvik, B.E., Hylland, K., Sandvik, M., and Skaare, J.U. (1996). Biomarker responses in flounder (Platichthys flesus) and their use in pollution monitoring. Mar. Pollut. Bull. *33*, 36–45.

Gräns, J., Wassmur, B., and Celander, M.C. (2010). One-way inhibiting cross-talk between arylhydrocarbon receptor (AhR) and estrogen receptor (ER) signaling in primary cultures of rainbow trout hepatocytes. Aquat. Toxicol. 100, 263–270.

Harman, C., Farmen, E., and Tollefsen, K.E. (2010). Monitoring North Sea oil production discharges using passive sampling devices coupled with in vitro bioassay techniques. Journal of Environmental Monitoring 12, 1699–1708.

Hektoen, H., Bernhoft, A., Ingebrigtsen, K., Utne Skaare, J., and Goksøyr, A. (1994). Response of hepatic xenobiotic metabolizing enzymes in rainbow trout (Oncorhynchus mykiss) and cod (Gadus morhua) to 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD). Aquat. Toxicol. *28*, 97–106.

Hultman, M.T., Song, Y., and Tollefsen, K.E. (2015a). 17α-Ethinylestradiol (EE2) effect on global gene expression in primary rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol. *169*, 90–104.

Hultman, M.T., Rundberget, J.T., and Tollefsen, K.E. (2015b). Evaluation of the sensitivity, responsiveness and reproducibility of primary rainbow trout hepatocyte vitellogenin expression as a screening assay for estrogen mimics. Aquat. Toxicol. *159*, 233–244.

Husøy, A.-M., Myers, M.S., and Goksøyr, A. (1996). Cellular localization of cytochrome P450 (CYP1A) induction and histology in Atlantic cod (Gadus morhua L.) and European flounder (Platichthys flesus) after environmental exposure to contaminants by caging in Sørfjorden, Norway. Aquat. Toxicol. *36*, 53–74.

Jankowsky, H.D., Hotopp, W., and Vsiansky, P. (1981). Effects of assay and acclimation temperatures on incorporation of amino acids into protein of isolated hepatocytes from the european eel, Anguilla anguilla L. J. Therm. Biol. *6*, 201–208.

Jobling, M., Johnsen, H.K., Pettersen, G.W., and Henderson, R.J. (1995). Effect of temperature on reproductive development in Arctic charr, Salvelinus alpinus (L.). J. Therm. Biol. *20*, 157–165.

738 Jorgensen, E.H., Balm, P.H.M., Christiansen, J.S., Plotitsyna, N., and Ingebrigtsen, K. (2001a).
739 Influence of o â□™ p-DDD on the physiological response to stress in Arctic charr
740 (Salvelinus alpinus). Aquat. Toxicol. *54*, 179–193.
741 Jorgensen, E.H., Celander, M., Goksoyr, A., and Iwata, M. (2001b). The effect of stress on
742 toxicant-dependent cytochrome p450 enzyme responses in the Arctic charr (Salvelinus
743 alpinus). Environ. Toxicol. Chem. *20*, 2523–2529.
744 Kaptaner, B., and Kankaya, E. (2016). Caspase-3 Activation in Cytotoxicity of Isolated
745 Rainbow Trout (oncorhyncus Mykiss) Hepatocytes Induced by Bisphenol A. Fresenius
746 Environ. Bull. *25*, 1167–1174.
747 Kim, B.H., and Takemura, A. (2003). Culture conditions affect induction of vitellogenin
748 synthesis by estradiol-17β in primary cultures of tilapia hepatocytes. Comparative
749 Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 135, 231–
750 239.
751 Kordes, C., Rieber, E.., and Gutzeit, H.. (2002). An in vitro vitellogenin bioassay for
752 oestrogenic substances in the medaka (Oryzias latipes). Aquatic Toxicology 58, 151–
753 164.
754 Larsen, B.K., Bjornstad, A., Sundt, R.C., Taban, I.C., Pampanin, D.M., and Andersen, O.K.
755 (2006). Comparison of protein expression in plasma from nonylphenol and bisphenol
756 A-exposed Atlantic cod (Gadus morhua) and turbot (Scophthalmus maximus) by use of
757 SELDI-TOF. Aquat. Toxicol. *78*, S25–S33.
758 Letcher, R.J., Bustnes, J.O., Dietz, R., Jenssen, B.M., Jorgensen, E.H., Sonne, C., Verreault, J.,
759 Vijayan, M.M., and Gabrielsen, G.W. (2010). Exposure and effects assessment of
760 persistent organohalogen contaminants in arctic wildlife and fish. Sci. Total Environ.
761 *408*, 2995–3043.
762 Liebel, S., Oliveira Ribeiro, C.A., Silva, R.C., Ramsdorf, W.A., Cestari, M.M., Magalhães,
763 V.F., Garcia, J.R.E., Esquivel, B.M., and Filipak Neto, F. (2011). Cellular responses of
764 Prochilodus lineatus hepatocytes after cylindrospermopsin exposure. Toxicol. In Vitro
765 *25*, 1493–1500.
766 Lyytikäinen, T., and Jobling, M. (1998). The effect of temperature fluctuations on oxygen
767 consumption and ammonia excretion of underyearling Lake Inari Arctic charr. J. Fish
768 Biol. *52*, 1186–1198.
769 Meland, S., Heier, L.S., Salbu, B., Tollefsen, K.E., Farmen, E., and Rosseland, B.O. (2010).
770 Exposure of brown trout (Salmo trutta L.) to tunnel wash water runoff — Chemical
771 characterisation and biological impact. Sci. Total Environ. *408*, 2646–2656.

772 Meland, S. and Roseth, R. (2011). Organophosphorus compounds in road runoff.
773 Sedimentation and filtration as a mitigation strategy. In: Proceedings 2011 World
774 Congress on Engineering and Technology. Institute of Electrical and Electronics
775 Engineers, Inc, Shanghai, pp. 653-656.

776 Mortensen, A.S., Braathen, M., Sandvik, M., and Arukwe, A. (2007). Effects of hydroxy-
777 polychlorinated biphenyl (OH-PCB) congeners on the xenobiotic biotransformation
778 gene expression patterns in primary culture of Atlantic salmon (Salmo salar)
779 hepatocytes. Ecotox. Environ. Safe. 68, 351–360.

780 Navas, J.M., and Segner, H. (2006). Vitellogenin synthesis in primary cultures of fish liver cells
781 as endpoint for in vitro screening of the (anti)estrogenic activity of chemical substances.
782 Aquat. Toxicol. *80*, 1–22.

783 Nelson, E.R., and Habibi, H.R. (2013). Estrogen receptor function and regulation in fish and
784 other vertebrates. Gen. Comp. Endocrinol. *192*, 15–24.

785 Niimi, A., and Palazzo, V. (1985). Temperature effect on the elimination of pentachlorophenol,
786 hexachlorobenzene and mirex by rainbow trout (Salmo gairdneri). Water Research 19,
787 205–207.

788 Okoumassoun, L.-E., Averill-Bates, D., Gagné, F., Marion, M., and Denizeau, F. (2002).
789 Assessing the estrogenic potential of organochlorine pesticides in primary cultures of
790 male rainbow trout (Oncorhynchus mykiss) hepatocytes using vitellogenin as a
791 biomarker. Toxicology *178*, 193–207.

792 Olsen, C.M., Meussen-Elholm, E.T.M., Hongslo, J.K., Stenersen, J., and Tollefsen, K.-E.
793 (2005). Estrogenic effects of environmental chemicals: An interspecies comparison.
794 Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. *141*, 267–274.

795 van der Oost, R., Beyer, J., and Vermeulen, N.P.E. (2003). Fish bioaccumulation and
796 biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol.
797 *13*, 57–149.

798 Park, C.B., Na, O.S., Lee, Y.D., Takemura, A., Kim, B.H., Choi, Y.C., Baek, H.J., and Kim,
799 H.B. (2003). Induction of In Vitro Vitellogenin Synthesis by Bisphenol, Nonylphenol
800 and Octylphenol in Chinese Minnow (Phoxinus oxycephalus) Hepatocytes. Anim. Cells
801 Syst. *7*, 227–235.

802 Pawlowski, S., Islinger, M., Volkl, A., and Braunbeck, T. (2000). Temperature-dependent
803 vitellogenin-mRNA expression in primary cultures of rainbow trout (Oncorhynchus
804 mykiss) hepatocytes at 14 and 18 degrees C. Toxicol. In Vitro *14*, 531–540.

Petersen, K., Bæk, K., Grung, M., Meland, S., and Ranneklev, S.B. (2016). In vivo and in vitro effects of tunnel wash water and traffic related contaminants on aquatic organisms. Chemosphere 164, 363–371.

Petersen, K., and Tollefsen, K.E. (2011). Assessing combined toxicity of estrogen receptor agonists in a primary culture of rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol. *101*, 186–195.

Petersen, K., and Tollefsen, K.E. (2012). Combined effects of oestrogen receptor antagonists on in vitro vitellogenesis. Aquat. Toxicol. *112–113*, 46–53.

Petit, F., Valotaire, Y., and Pakdel, F. (1995). Differential functional activities of rainbow trout and human estrogen receptors expressed in the yeast Saccharomyces cerevisiae. European Journal of Biochemistry 233, 584–592.

Rankouhi, T.R., Sanderson, J.T., van Holsteijn, I., van Leeuwen, C., Vethaak, A.D., and van den Berg, M. (2004). Effects of natural and synthetic estrogens and various environmental contaminants on vitellogenesis in fish primary hepatocytes: comparison of bream (Abramis brama) and carp (Cyprinus carpio). Toxicol. Sci. 81, 90–102.

Riley, L.G., Hirano, T., and Grau, E.G. (2004). Estradiol-17β and dihydrotestosterone differentially regulate vitellogenin and insulin-like growth factor-I production in primary hepatocytes of the tilapia Oreochromis mossambicus. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 138, 177–186.

dos Santos, N.M.S., do Vale, A., Reis, M.I.R., and Silva, M.T. (2008). Fish and apoptosis: Molecules and pathways. Curr. Pharm. Des. *14*, 148–169.

Schirmer, K. (2006). Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. Toxicology *224*, 163–183.

Scholz, S., and Segner, H. (1999). Induction of CYP1A in primary cultures of rainbow trout (Oncorhynchus mykiss) liver cells: Concentration-response relationships of four model substances. Ecotoxicol. Environ. Saf. *43*, 252–260.

Schreer, A., Tinson, C., Sherry, J.P., and Schirmer, K. (2005). Application of Alamar blue/5-carboxyfluorescein diacetate acetoxymethyl ester as a noninvasive cell viability assay in primary hepatocytes from rainbow trout. Anal. Biochem. *344*, 76–85.

Segner, H. (1998). Isolation and primary culture of teleost hepatocytes. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. *120*, 71–81.

Segner, H., and Cravedi, J.P. (2001). Metabolic activity in primary cultures of fish hepatocytes. Atla-Altern. Lab. Anim. *29*, 251–257.

839 Siikavuopio, S.I., Skybakmoen, S., and Sæther, B.-S. (2009). Comparative growth study of
840 wild- and hatchery-produced Arctic charr (Salvelinus alpinus L.) in a coldwater
841 recirculation system. Aquac. Eng. *41*, 122–126.
842 Søfteland, L., Holen, E., and Olsvik, P.A. (2010). Toxicological application of primary
843 hepatocyte cell cultures of Atlantic cod (Gadus morhua) — Effects of BNF, PCDD and
844 Cd. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. *151*, 401–411.
845 Tollefsen, K.E., Mathisen, R., and Stenersen, J. (2003). Induction of vitellogenin synthesis in
846 an Atlantic salmon (Salmo salar) hepatocyte culture: a sensitive in vitro bioassay for the
847 oestrogenic and anti-oestrogenic activity of chemicals. Biomarkers *8*, 394–407.
848 Tollefsen, K.E., Bøyum, O., Finne, E.F., Fogelberg, O., Gregersen, I.K., Holth, T.F., Jensen,
849 M.H.S., and Thomas, K.V. (2008). Confounding factors affecting biological effects
850 measurements used in the Water Column monitoring surveys (NIVA).
851 Verma, N., Pink, M., Rettenmeier, A.W., and Schmitz-Spanke, S. (2012). Review on proteomic
852 analyses of benzo [a] pyrene toxicity. Proteomics 12, 1731–1755.
853 Wang, Y., Zheng, R., Zuo, Z., Chen, Y. and Wang, C. (2008). Relation of hepatic EROD
854 activity and cytochrome P4501A level in Sebastiscus marmoratus exposed to
855 benzo[a]pyrene. J. Environ. Sci. 20, 101–104.
856 Zacchino, V., Centoducati, G., Narracci, M., Selvaggi, M., and Santacroce, M.P. (2013). Effects
857 of benzo[a]pyrene on gilthead sea bream (Sparus aurata L.) hepatocytes exposed in vitro
858 to short and long term trials. Ital. J. Anim. Sci. *12*, e17.
859 Zhou, B., Liu, C., Wang, J., Lam, P.K.S., and Wu, R.S.S. (2006). Primary cultured cells as
860 sensitive in vitro model for assessment of toxicants-comparison to hepatocytes and gill
861 epithelia. Aquat. Toxicol. *80*, 109–118.

Highlights

- Primary hepatocytes were successfully isolated from Arctic char
- Bioassay optimized for determination of cytotoxicity and biomarkers (CYP1A, EROD and Vtg induction)
- Test compounds and environmental extract caused concentration-dependent responses for all endpoints
- Biological responses observed resemble that of hepatocytes from other species
- The assay offer a versatile, high-throughput, and relevant tool for assessment of Arctic exposure scenarios

Figure legends

Figure 1. Effects of temperature (top row, cell density 500 000 cells/ml) and cell density (bottom row, exposure temperature 10°C) on membrane integrity and metabolic activity of Arctic char (*Salvelinus alpinus*) hepatocytes exposed to copper sulphate. The data (mean ± SEM) was normalized between solvent control (DMSO: 100% viability) and positive control (0.01M copper sulphate, 0% viability. The lines represent non-linear curve fit to experimental data from 1 (top row) and 3-7 (bottom row) independent cell isolations.

Figure 2. Effects of temperature (top, cell density of 500 000 cells/ml) and cell density (bottom, exposure temperature of 10°C) on vitellogenin (Vtg) protein expression of Arctic char (*Salvelinus alpinus*) hepatocytes exposed to 17β-estradiol (E2). The data (mean ± SEM) was normalized between solvent control (DMSO, 0%) and positive control (30nM E2). The lines represent non-linear curve fit to experimental data from 1 (top) and 3-7 (bottom) independent cell isolations.

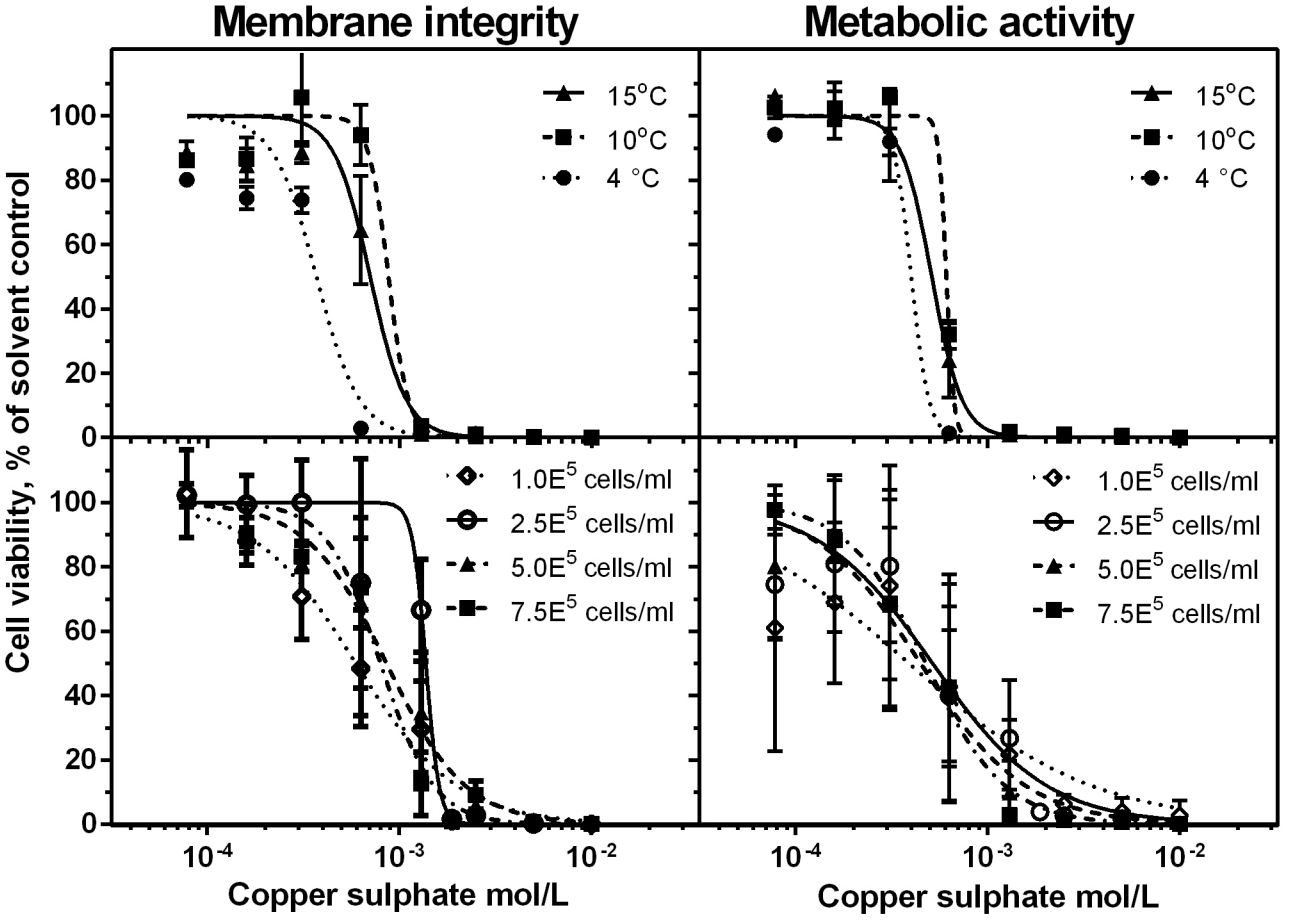
Figure 3. Effects of temperature (top, cell density 500 000 cells/ml) and cell density (bottom, exposure temperature 10°C) on ethoxyresorufin-O-deethylase (EROD) activity and cytochrome P450 1a (CYP1A) protein expression in Arctic char (*Salvelinus alpinus*) hepatocytes exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The data (mean ± SEM) was normalized between solvent control (DMSO, 0%) and positive control (0.3 or 3 nM TCDD, 100%). The lines represent non-linear curve fit to experimental data from 1 (top row and left bottom row) and 2-3 (right, bottom row) independent cell isolations.

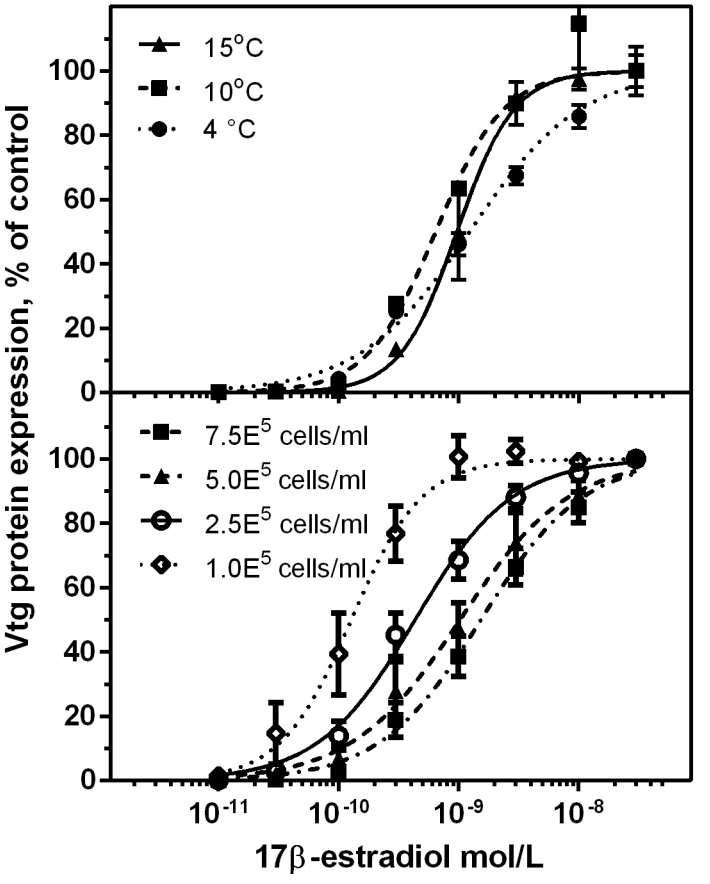
Figure 4. Membrane integrity and metabolic activity of Arctic char (*Salvelinus alpinus*) primary hepatocytes after exposure to environmental pollutants. The data (mean ± SEM) was normalized between solvent control (DMSO, 100%) and positive control (0.01 M copper sulphate, 0%). The lines represent non-linear curve fit to experimental data from 5-6 independent cell isolations.

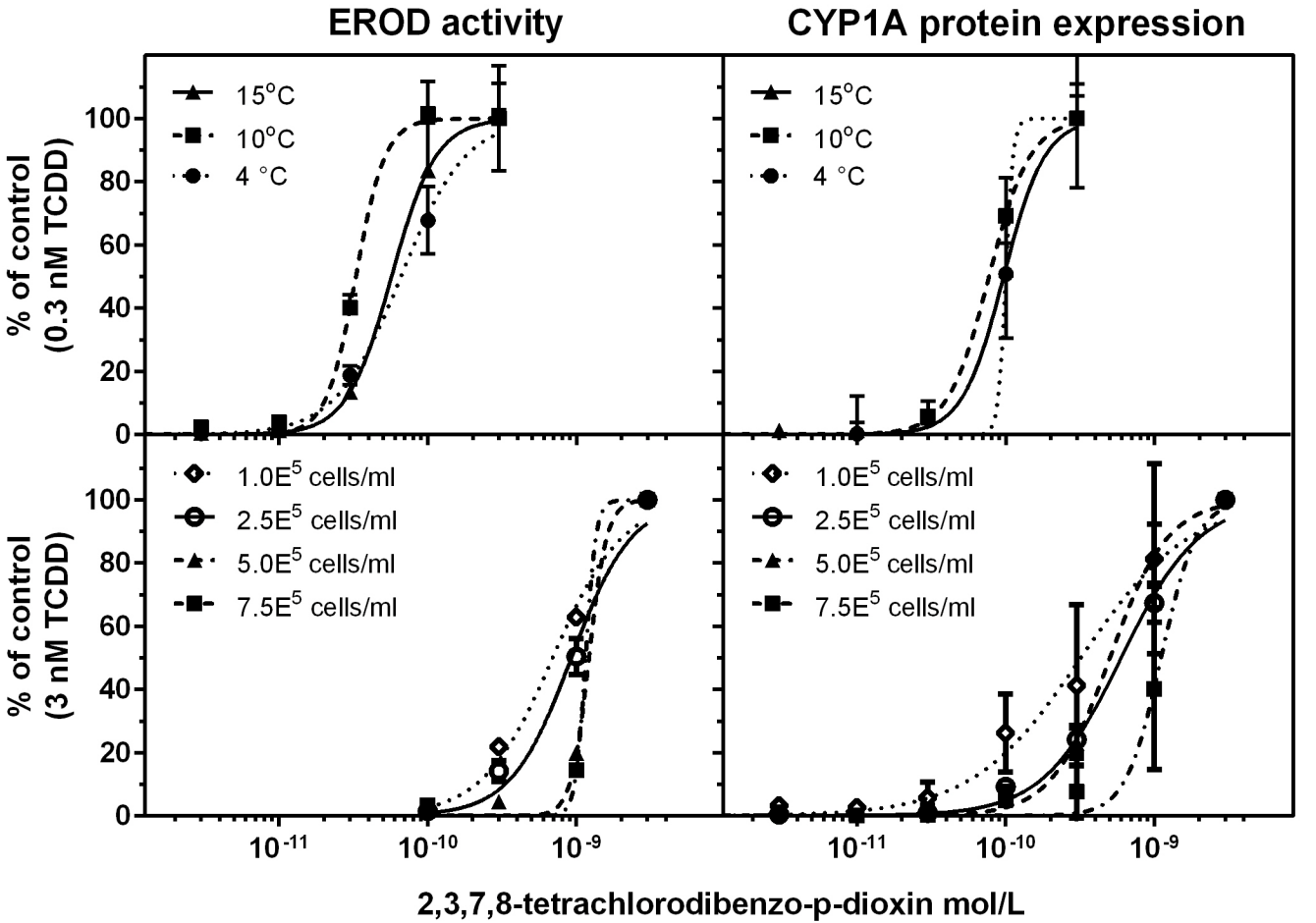
Figure 5. Relative vitellogenin (Vtg) protein expression (●) and metabolic activity (○) in Arctic char (*Salvelinus alpinus*) primary hepatocytes after exposure to environmental pollutants and 17β-estradiol (E2). The data (mean ± SEM) was normalized between solvent control (DMSO, 0%) and positive control (30 nM E2, 100%). The lines represent non-linear curve fit to experimental data from 3-5 independent cell isolations.

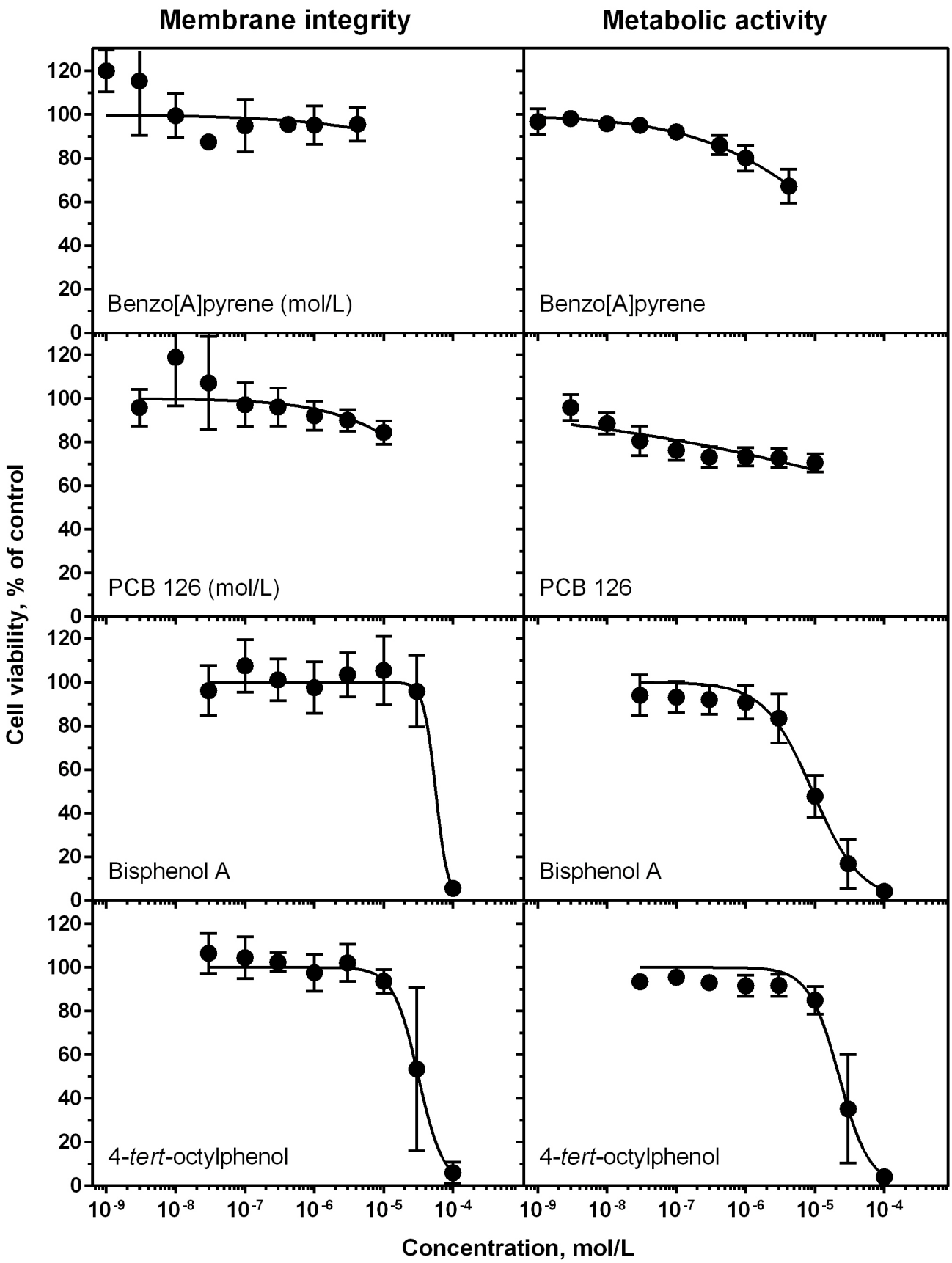
Figure 6. Relative ethoxyresorufin-O-deethylase (EROD) activity (●), cythocrome P450 1a protein expression (CYP1A) (●) and metabolic activity (◌) in Arctic char (*Salvelinus alpinus*) primary hepatocytes after exposure to environmental pollutants and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The data (mean ± SEM) was normalized between solvent control (DMSO, 0%) and positive control (3 nM TCDD, 100%). The lines represent non-linear curve fit to experimental data from 4-6 independent cell isolations.

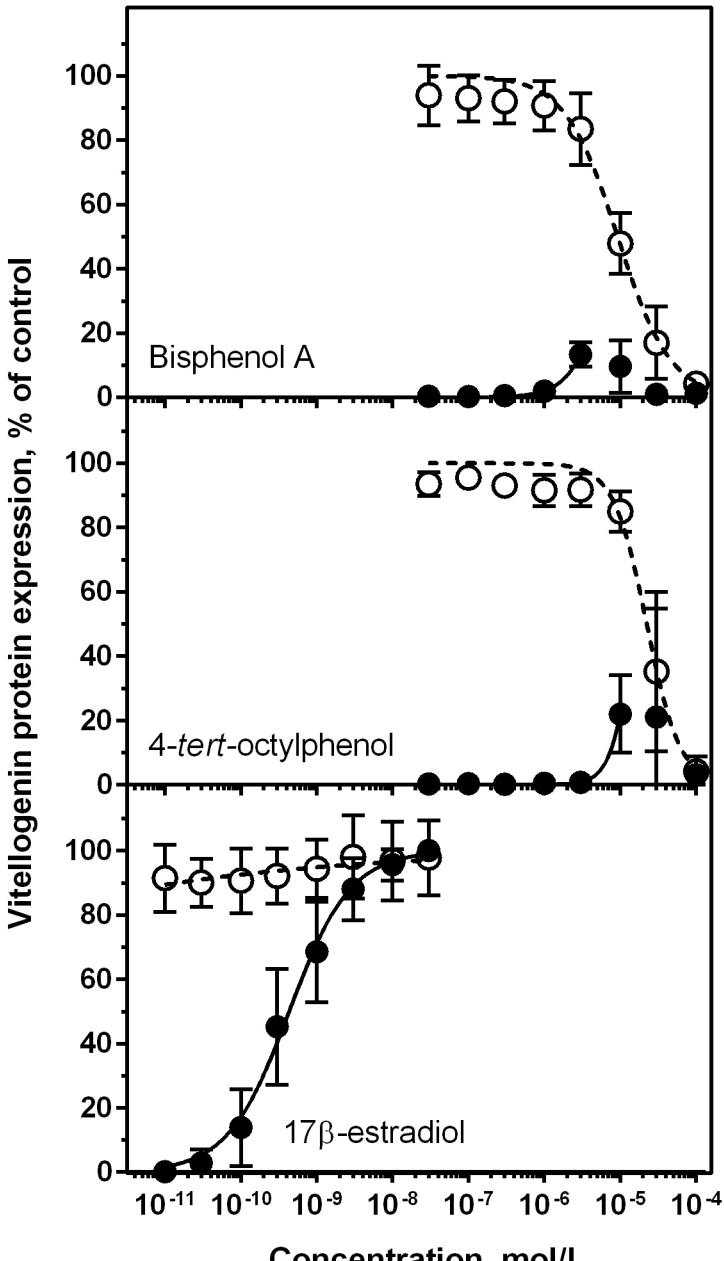
Figure 7. Relative expression of membrane integrity, metabolic activity, ethoxyresorufine-O-deethylase (EROD) activity and cytochrome P450 1a (CYP1A) protein expression in Arctic char (*Salvelinus alpinus*) hepatocytes after exposure to the tunnel wash water extract (●) and control water extract (○). The data (mean ± SEM) was normalized between solvent control (DMSO) and individual endpoint positive controls (0.01M $CuSO_4$; 3 nM TCDD). The lines represent non-linear curve fit to experimental data from 5 independent cell isolations.


Membrane integrity
Metabolic activity
Cell viability, % of solvent control
15°C
10°C
4 °C
1.0E5 cells/ml
2.5E5 cells/ml
5.0E5 cells/ml
7.5E5 cells/ml
Copper sulphate mol/L
Copper sulphate mol/L


15°C
10°C
4 °C
7.5E5 cells/ml
5.0E5 cells/ml
2.5E5 cells/ml
1.0E5 cells/ml
Vtg protein expression, % of control
0
20
40
60
80
100
10-11
10-10
10-9
10-8
17β-estradiol mol/L


EROD activity
CYP1A protein expression
15°C
10°C
4 °C
% of control
(0.3 nM TCDD)
1.0E5 cells/ml
2.5E5 cells/ml
5.0E5 cells/ml
7.5E5 cells/ml
% of control
(3 nM TCDD)
0
20
40
60
80
100
10-11
10-10
10-9
2,3,7,8-tetrachlorodibenzo-p-dioxin mol/L


Membrane integrity
Metabolic activity
Cell viability, % of control
Benzo[A]pyrene (mol/L)
Benzo[A]pyrene
PCB 126 (mol/L)
PCB 126
Bisphenol A
Bisphenol A
4-tert-octylphenol
4-tert-octylphenol
0
20
40
60
80
100
120
10-9
10-8
10-7
10-6
10-5
10-4
Concentration, mol/L


Vitellogenin protein expression, % of control
Bisphenol A
4-tert-octylphenol
17β-estradiol
Concentration, mol/L
0
20
40
60
80
100
10-11
10-10
10-9
10-8
10-7
10-6
10-5
10-4

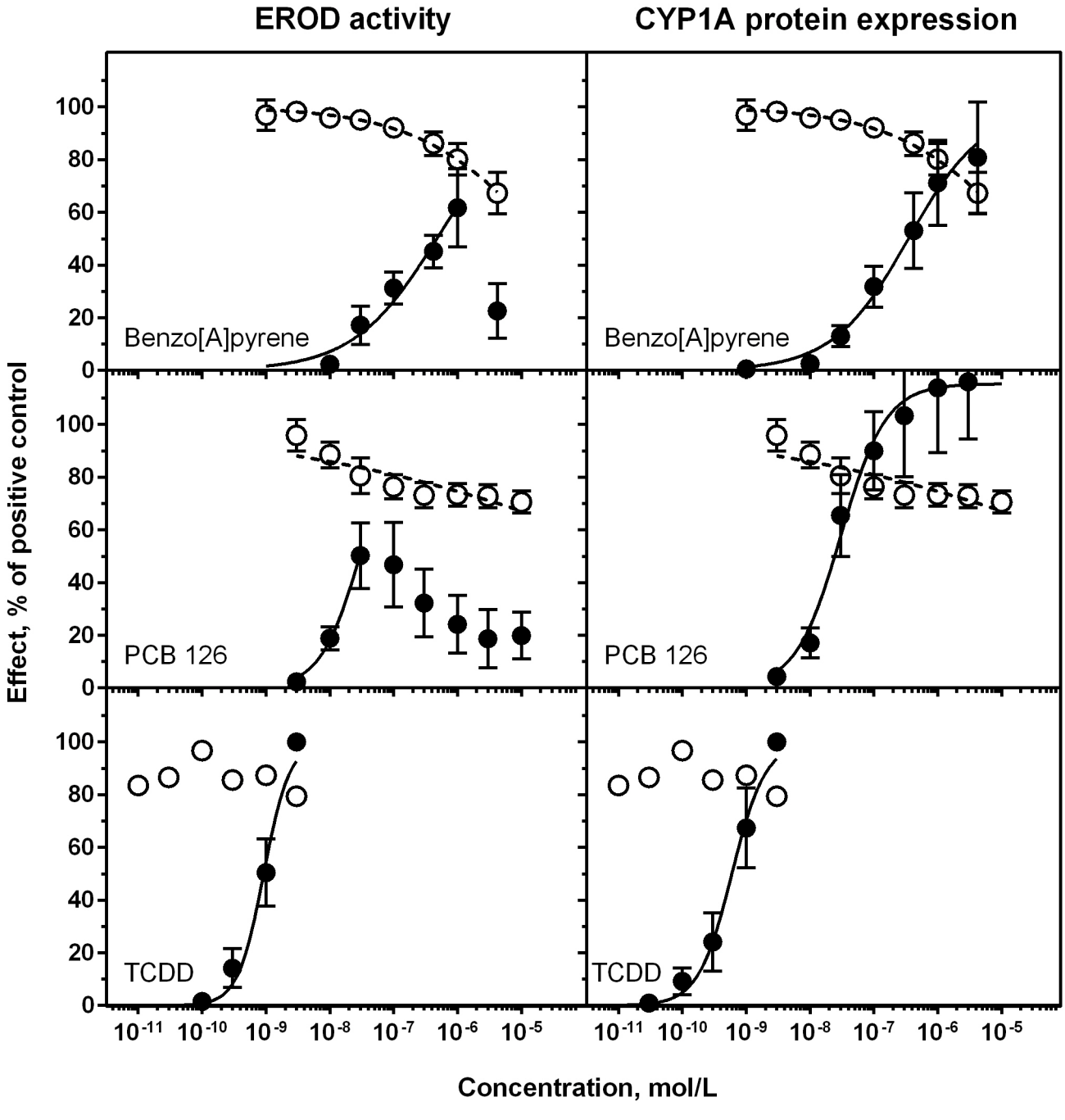

EROD activity
CYP1A protein expression
Benzo[A]pyrene
Benzo[A]pyrene
PCB 126
PCB 126
TCDD
TCDD
Effect, % of positive control
Concentration, mol/L
0
20
40
60
80
100
10-11
10-10
10-9
10-8
10-7
10-6
10-5

Table 1. Summary of results

Endpoint	**Compound**	**CAS**	**LogK_{OW}**	**EC_{50} (nM)**	**Efficacy (%)***
Membrane integrity	BAP	50-32-8	6.13	NR	84
	PCB 126	57465-28-8	6.98	NR	96
	BPA	80-05-7	3.32	57000	6
	OP	140-66-9	4.8	32000	6
Metabolic activity	BAP	50-32-8	6.13	NR	67
	PCB 126	57465-28-8	6.98	NR	71
	BPA	80-05-7	3.32	9200	4
	OP	140-66-9	4.8	22000	4
EROD activity	TCDD	1746-01-6	6.8	0.923	100
	PCB 126	57465-28-8	6.98	30	50
	BAP	50-32-8	6.13	475	62
CYP1A protein expression	TCDD	1746-01-6	6.8	0.597	100
	PCB 126	57465-28-8	6.98	30	120
	BAP	50-32-8	6.13	350	81
Vtg protein expression	E2	50-28-2	4.01	0.43	100
	BPA	80-05-7	3.32	NR	13
	OP	140-66-9	4.8	NR	22

* Highest effect relative to positive control, for membrane integrity and metabolic activity efficacy values are the cell viability obtained at the highest tested concentrations.

Table 2. Compiled data for other primary hepatocyte donor species than Arctic char .

Endpoint	Compound	CAS	$LogK_{OW}$	Primary hepatocyte donor species	EC50 (nM)	Efficacy (%)*	Reference
Metabolic activity	BPA	80-05-7	3.32	Rainbow trout	61000		Petersen and Tollefsen, 2011
	OP	140-66-9	4.8	Rainbow trout	24000		Petersen and Tollefsen, 2011
EROD activity	TCDD	1746-01-6	6.8	Rainbow trout	0.025	100	Petersen et al, unpublished
				Freshwater tilapia (*O. niloticus*)	1.4		Zhou et al., 2006
	BAP	50-32-8	6.13	Rainbow trout	92		Behrens et al., 2001
				Rainbow trout	922		Scholz and Segner, 1999
				Freshwater tilapia (*O. niloticus*)	81		Zhou et al., 2006
CYP1A protein expression	TCDD	1746-01-6	6.8	Rainbow trout	0.041	100	Petersen et al., 2016
Vtg protein expression	E2	50-28-2	4.01	Rainbow trout	0.63	100	Petersen and Tollefsen, 2011
				Rainbow trout	200		Okoumassoun et al., 2002
				Rainbow trout	0.1		Olsen et al., 2005
				Salmon (S*almo salar*)	0.026		Tollefsen et al., 2003
	BPA	80-05-7	3.32	Rainbow trout	13000^{+}	42.6	Petersen and Tollefsen, 2011
				Rainbow trout	3500		Olsen et al., 2005
	OP	140-66-9	4.8	Rainbow trout	14000	43.2	Petersen and Tollefsen, 2011
				Rainbow trout	3100		Olsen et al., 2005

* Highest effect relative to positive control, for membrane integrity and metabolic activity efficacy values are the cell viability obtained at the highest tested concentrations, $^{+}$extrapolated from concentration-response curve.