Cover page

Title: **Characterizing cytotoxic and estrogenic activity of Arctic char tissue extracts in primary Arctic char hepatocytes**

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Characterizing cytotoxic and estrogenic activity of Arctic char tissue extracts in primary Arctic char hepatocytes

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Abstract

Contaminants from various anthropogenic activities find their way to the Arctic through long-range atmospheric transport, ocean currents and living organisms, (e.g. migrating fish or seabirds). Although levels of persistent organic pollutants in arctic fish are generally low, local hot-spots of contamination have been demonstrated in freshwater systems, such as Lake Ellasjøen at Bjørnøya (Bear Island, Norway). Higher concentrations of organic halogenated compounds (OHCs), and higher levels of cytochrome P450 and DNA-double strand breaks have been measured in Arctic char (*Salvelinus alpinus*) from this lake compared to fish from other lakes on Bjørnøya. Although several of the measured contaminants are potential endocrine disrupters, few studies have investigated the potential endocrine disruptive effects of the contaminant cocktail in this fish population. In this study we compared the toxic and estrogenic potency of the cocktail of pollutants in extracts of Arctic char livers from the contaminated Lake Ellasjøen with those from the less contaminated Lake Laksvatn at Bjørnøya to investigate if the contaminant cocktail in these fish populations could have cytotoxic and/or estrogenic effects *in vitro*. This was done by *in situ* sampling and contaminant extraction from

liver tissue, followed by chemical analysis and *in vitro* testing of the following contaminant extracts: F1-nonpolar OHCs, F2-polar pesticides and metabolites of OHCs, and F3-polar OHCs. Contaminant levels were highest in extracts from Ellasjøen fish. The F2 and F3 extracts from Lake Laksvatn and Lake Ellasjøen fish reduced the *in vitro* cell viability at a concentration ratio of 0.03 to 1 relative to tissue concentration in the Arctic char. Only the F3 liver extract from Ellasjøen fish increased the *in vitro* vitellogenin protein expression. Although compounds such as estrogenic OH-PCBs was quantified in the Ellasjøen F3 extracts, it remains to be determined which compounds were causing the estrogenic effect.

Key words: Primary hepatocytes; Arctic char; Vitellogenin; cytotoxicity; chemical analysis

1. Introduction

Organisms in the arctic are exposed to persistent organic pollutants (POPs) from long range-atmospheric transport and deposition, transport via ocean currents, as well as from local hot spots, such as military bases, oil extraction facilities and mining etc. Freshwater fish from certain arctic locations have been reported to contain high levels of POPs, with ∑polychlorinated biphenyls (PCBs) higher than 10 000 ng/g lipid weight in some cases (Bytingsvik et al., 2015; Christensen and Evenset, 2011; Evenset et al., 2004). Populations with higher risk of possible POP-mediated effects have been identified and among these is the Arctic char (*Salvelinus alpinus*) from Bjørnøya (Bear Island) (74°30′ N, 19°00′ E) (Letcher et al., 2010), and especially fish from Lake Ellasjøen. The pollutants generally occurring at high concentrations in these fish are PCBs, 2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p*-DDE), chlordanes, chlorobornanes (CHBs), polychlorinated naphthalenes (PCNs) and hexachlorobenzene (HCB) (Bytingsvik et al., 2015; Evenset et al., 2005, 2004). These contaminants are transferred to Lake Ellasjøen by guano from seabirds using the lake as a

resting area (Evenset et al., 2007). A between-lake comparison at Bjørnøya performed by Bytingsvik et al. (2015) showed that Arctic char muscle levels (lipid weight) of hexachlorobenzene (HCB), chlordanes (∑CHLs), mirex, dichlorodiphenyltrichloroethanes (∑DDTs) and ∑PCBs were 1.7, 3.1, 13.3, 28.1 and 35.6 times higher in fish from Lake Ellasjøen than in fish from the nearby Lake Laksvatn (12 km north of Ellasjøen), respectively. In Ellasjøen fish, highest concentrations were observed for ∑PCBs with concentrations > 10000 ng/g lipid weight in both muscle and ovary tissue (Bytingsvik et al., 2015). The higher contaminant concentrations in Arctic char from Lake Ellasjøen compared to Lake Laksvatn have been linked to higher levels of double DNA-strand breaks (Neerland, 2016), higher hepatic cytochrome P450(CYP)1A enzyme levels, lower glucocorticoid receptor (GR) protein expression, elevated heat shock proteins expression (Wiseman et al., 2011), and altered mRNA abundance of key genes related to the hypothalamic-pituitary-interrenal (HPI) axis functioning suggestive of endocrine disruptive effects (Jørgensen et al., 2017).

Over the last decade, legacy and emerging compounds have demonstrated a potential for endocrine disruptive effects in *in vitro* and *in vivo* studies on Arctic wildlife and fish (Letcher et al., 2010). Environmentally relevant compounds such as o,p′-DDT (Petersen and Tollefsen, 2011; Wojtowicz et al., 2007), *o,p′*-DDE and *p,p′*-DDE (Wojtowicz et al., 2007), and hydroxylated (OH-) PCBs (Andersson et al., 1999; Braathen et al., 2009; Carlson and Williams, 2001; Mortensen et al., 2007) display estrogenic effects in fish *in vitro* and/or *in vivo.* Furthermore, there are numerous reports that insecticides such as β-HCH, cis- and trans-chlordane, dieldrin, endosulfan, mirex, oxychlordane, toxaphenes and trans-nonachlor have reproductive and endocrine effects (for full review see Colborn et al., 1993). Several classic industrial chemicals detected in Arctic fish and wildlife, such as polychlorinated dibenzo-*p*-dioxins (PCDDs), have also been reported to have endocrine disruptive properties (Colborn et

al., 1993). Some industrial chemicals including polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBPA) and perfluorinated chemicals (PFCs) may interact with multiple endocrine piscine targets *in vitro* (Hamers et al., 2006, 2008; Harju et al., 2007; Jensen and Leffers, 2008; Liu et al., 2007; Morgado et al., 2007) and *in vivo* (Kuiper et al., 2007; Oakes et al., 2005).

Endocrine disruptive chemicals (EDCs) with an estrogenic mode of action (MoA) may affect the level of vitellogenin (Vtg) protein and/or messenger RNA (mRNA) in fish (Purdom et al., 1994; Sumpter and Jobling, 1995). Vitellogenin is an egg-yolk precursor protein produced in the liver of female fish during oogenesis, and is induced by increased levels of ovarian estrogens (Sumpter and Jobling, 1995). Once produced, Vtg is transported by the blood to the ovaries of female fish where it is incorporated into the growing oocytes (Mommsen and Walsh, 1988). Male and juvenile fish, which also have the gene coding for Vtg, do not normally express this gene due to low concentrations of circulating endogenous estrogens. Expression of Vtg gene and protein in male and juvenile fish has therefore become a suitable biomarker for (xeno)estrogenic compound exposure (Heppell et al., 1995; Mommsen and Walsh, 1988; Purdom et al., 1994). Synthesis of Vtg has also been used as a biomarker in primary cultures of hepatocytes from temperate fish such as common bream (*Abramis brama*), Siberian sturgeon (*Acipenser baeri*), Japanese eel (*Anguilla japonica*), channel catfish (*Ictalurus punctatus*), common carp (*cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), Mozambique tilapia (*Oreochromis mossambicus*) (reviewed by Navas and Segner, 2006), and Atlantic salmon (*Salmo salar*) (Tollefsen et al., 2003), and in the recently established multi-endpoint and high-throughput *in vitro* bioassay with Arctic char hepatocytes for screening single chemicals, complex mixtures, and environmental extracts (Petersen et al., 2017).

The population of Arctic char in lake Ellasjøen at Bjørnøya is known to be exposed to several different POPs, and endocrine disruptive effects have been suggested to occur in this population based on observations of altered mRNA abundance of key genes related to the HPI axis functioning (Jørgensen et al., 2017). It is therefore of interest to investigate whether the contaminant cocktail in the livers of spawning Arctic char can affect reproductive processes and cell viability. In this study the contaminant cocktail in the livers of spawning Arctic char from two different lakes (Ellasjøen and Laksvatn) on Bjørnøya were extracted and fractionated into three extracts; F1 - non-polar OHCs (PCBs, PBDEs and most of the nonpolar pesticides), F2 - polar pesticides and metabolites of OHCs ($MeSO_2$-PCBs/DDE), and F3 - polar OHCs (phenolics such as chlorinated phenols and hydroxylated metabolites of PCBs and PBDEs). The effect of these extracts on the *in vitro* cell viability and Vtg protein expression in primary Arctic char hepatocytes was investigated. The observed effects were compared to the detected chemicals present in the individual extracts to potentially identify candidate compounds contributing to the observed effects.

2. Materials and Methods

2.1. Chemicals

The chemicals used as positive controls, 17β-estradiol (E2, CAS 50-28-2, purity of ≥ 98%, positive control for Vtg protein expression), and copper sulphate ($CuSO_4 \times 5H_2O$, CAS 7758-99-8, positive control for cell viability), were obtained from Sigma-Aldrich (St. Lois, MI, US). $CuSO_4 \times 5H_2O$ was dissolved in fresh culture media on the day of exposure, whereas E2 was dissolved in dimethylsulfoxide (DMSO) and stored at -20°C when not in use. The solvents used for chemical analysis (n-hexane, dichloromethane, acetone, cyclohexane, methanol) were all

Suprasolv quality for Gas chromatography MS, and obtained from Merck (Darmstadt, Germany). All ^{13}C standards were obtained from Cambridge Isotop Laboratory, Inc. (Tewksbury, MA, US).

2.2. Sampling and extraction of Arctic char tissue

Female and male Arctic char was sampled from Lake Ellasjøen (n = 20) and Lake Laksvatn (n = 22) on Bjørnøya (74°30′ N, 19°00′ E), Svalbard (Norway) during the first two weeks of September 2012. Most individuals were maturing or mature at the time of sampling. Biometric data were recorded (supplementary table 1). Length (cm), body weight (g) and liver weight (g) were measured, and condition factor (CF: [body weight (g)/body length (cm)^3]x100) and liver-somatic index (LSI: [liver weight (g)/body weight (g)]x100) were calculated. Otoliths were collected for age determination. Liver tissue was weighed, wrapped in aluminum foil, and frozen at -20°C. Approximately 0.75 –5.9 g of liver from each fish were pooled and used for preparation of extracts. The extracts were produced from fish of both sexes and variable maturation status. All males were mature (running milt). The females reproductive stage was determined based on Sømme (1941) where fish in reproductive stages 1 to 3 are non-spawning fish (will not spawn during the year of sampling), fish in stages 4, 5 and 6 are spawning fish (will spawn during the year of sampling), and fish in stage 7 have recently spawned (supplementary table 1). The females used for liver extractions represented reproductive stages from 1 to 7.

All glass equipment was rinsed with solvents and burned for 8 h at 450°C before use. A total of 55 to 86 g of liver was homogenized in dry Na_2SO_4 in a glass kitchen mixer with stainless steel blades. The homogenate was placed in 1000 mL glass flasks and extracted with 200 mL of acetone/cyclohexane (two times with 1/3 v/v and once with 1/1 v/v) using shaking for 30

min on a shaking table and ultra-sonication for 10 min. Solvent extracts were decanted through a paper filter (Whatman Grade 1, diameter 185mm, Sigma-Aldrich, Darmstadt, Germany) placed in a glass funnel into a turbovap glass and concentrated in a Turbovap 500 (Biotage, Uppsala, Sweden) to a volume of 1 mL at 35°C, transferred to a 4 mL glass vial and further evaporated until dryness under a gentle stream of nitrogen until constant weight to determine lipid content. Sample cleanup was performed by adding the lipid extracts (diluted in n-Hexane) into a 30 cm long semipermeable membrane device (SPMD, EST-Lab, MO, USA) inside of 100 mL Pyrex cylindrical separator funnel with a PTFE stopcock (Sigma-Aldrich, Darmstadt, Germany) and collecting the acetone/n-Hexane extracts (two times with 1/3 v/v and two times with 1/1 v/v) every 24 h for 4 days. Solvent was evaporated in the Turbovap and further cleanup was achieved using a high performance liquid chromatography (HPLC) system utilizing gel permeation chromatography (Waters Envirogel GPC cleanup) using dichloromethane as eluent at a flow of 5 mL/min and collecting the fraction between 12.5 min and 25 min, which removes additional lipids from the samples. A final cleanup step was performed using a 300 mm x 20 mm ID glass column packed with activated florisil (450°C, 8h) and 2 g of Na_2SO_4 on top, fractionating the sample into three extracts (F1-F3). The fractionation of the samples was performed with an up scaled version of that used by Nøst et al., (2012) and Sandanger et al., (2004). The columns were washed with dichloromethane and conditioned with n-hexane and then 200 mL of 10% dichloromethane/n-hexane (v/v) was added to obtain the first fraction (F1) which contain neutral compounds such as PCBs, PBDEs and organochlorine pesticides, 250 mL of 10% aceton/n-hexane to obtain the second fraction (F2) containing polar pesticides and metabolites of OHCs like $MeSO_2$-PCBs/DDE, and 300 mL of 20% methanol/dichloromethane to obtain the third fraction (F3) containing polar OHCs like hydroxylated OH-PCBs/phenols. The second fraction was later cleaned from lipid residues on a column (same as above) packed with 2 g of Na_2SO_4, 10 g of 25% w/w sulfuric acid silica (silica activated at 600°C for 8 h) on

bottom, and 2 g of neutral active silica on top. The column was washed with dichloromethane and conditioned with n-hexane. Sample was diluted with a small amount of n-hexane and added on top of the column and eluted with 350 mL of dichloromethane. The third fraction was cleaned three times using liquid-liquid extraction with 50 mL concentrated sulphuric acid. All extracts (12 in total) were evaporated to 0.5 ml and transferred to the solvent dimethyl sulfoxide (DMSO) for *in vitro* testing. A procedural blank sample was fractionated in parallel to the tissue samples (producing the three procedural blanks F1 – F3) to correct for effects by methodologically introduced compounds.

2.3. Chemical analysis

An equivalent of 6 g liver of each extract was used for chemical analysis. ^{13}C-labeled standards of PCBs, PBDEs, new BFRs and pesticides (F1), surrogate standard $MeSO_2$-4Me-PCB120 (F2) and ^{13}C-OH-PCBs/PCP (F3) were added before chemical analysis. Further, extract F3 was derivatized using diazomethane before final cleanup on a solid phase extraction (SPE)-column with 25% sulfuric acid silica, concentrated and transferred to an analytical vial GC/MS vial with a 200 µL insert.

PCBs, DDTs and PBDEs were analyzed by GC-MS (Waters Quattro micro) in EI mode in single ion monitoring using a 30m x 0.25mmID DB5-MS column (Agilent) for PCBs and DDTs and a 15m x 0.18mmID RTx1-MS (Restek) for PBDEs. The pesticides, $MeSO_2$-PCBs/DDE and derivatized OH-PCBs/PCP were analyzed by GC/MS (Agilent 7890A/5975B MSD) in NCI mode in single ion monitoring, with methane as CI gas using a 30m x 0.25mmID DB5-MS column. OPFRs were analysed by LC–Q-TOF–MS after having spiked samples with internal standards, extracted in acetonitrile (1 ml) by repeated sonication and vortexing and cleaned up

the supernatant using ENVI-Carb graphitized carbon absorbent and glacial acetic acid. For further information on analysis see Nøst et al. (2012) and Sandanger et al. (2004).

2.4. Rearing of Arctic char for *in vitro* study

Fertilized roe of Arctic char (*Salvelinus alpinus*) (300-500g) were obtained from Tydalfisk (Løvøya, Tydal, Norway), transported to the animal facilities at the Norwegian University of Life Sciences, NMBU (Ås, Norway), where they were hatched and reared (< 100g) before they were transferred to the animal facilities at the University of Oslo. The fish were kept at the animal facilities at the University of Oslo in 1250 L circular, flow-through tanks, with a water temperature of 8 ± 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 the total biomass.

2.5. Isolation of primary hepatocytes

Arctic char were collected and terminated in November 2015 with a blow to the head and subjected to a two-step liver perfusion as described in Tollefsen et al. (2003) with minor modifications for Arctic char as described in Petersen et al. (2017). Blood was removed from the liver by perfusion with a calcium free buffer (NaCl 122 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, Na_2HPO_4 11mM, NaH_2PO_4 3.3 mM, $NaHCO_3$ 3.7 mM, EGTA 26 µM, 0°C) at 5 ml/min for 10-15 min. The liver tissue was perfused with the same buffer (37°C) now without EGTA and with added $CaCl_2$ (1.5 mM) and collagenase (0.3 mg/ml) type VIII at 5 ml/min for 10-15 min. The liver was excised and transferred to a glass beaker on ice containing calcium free buffer with 0.1% w/v bovine serum albumin (BSA) and gently stirred. The resulting cell suspension was filtered twice (250 µm and 100 µm sterile nylon mesh) before it was centrifuged three times

(4 min, 3 min, 3 min) at 500 rpm. Following the first centrifugation, the supernatant was removed and the cells re-suspended in calcium free buffer with 0.1% w/v BSA on ice. After the second and third centrifugation the cells were re-suspended in serum-free, refrigerated 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). After the last centrifugation, the cell suspension was filtered through a 100 µm nylon mesh, diluted to 250 000 cells per ml, plated (200 µl per well) in 96-well primaria™ plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) and left to acclimatize for 24 h at 10˚C in an incubator. Only cell isolations with ≥ 80% viable cells determined by the trypan blue method were used in the experiments. All extracts, blanks and controls were tested with cells from 3 to 4 independent cell isolations.

2.6. Exposure of primary hepatocytes

After 24 h of acclimatization, cells were exposed to positive controls and the different liver extracts from Arctic char from Ellasjøen and Laksvatn. A total of 12 liver extracts and three procedural blanks were produced (see Table 1). A dilution series was prepared for each extract resulting in 6 stock solutions. All stocks were diluted 1000 times in exposure media (DMSO concentration = 0.1%). In addition, the two highest stock concentrations were diluted 100 times in exposure media (DMSO = 1%) to obtain a total of 8 exposure concentrations of each extract) (Table 1). The exposure concentration is given as concentration ratio (CR), referring to the extracted tissue to exposure concentration. A CR of 1 indicate that the nominal concentrations in exposure media corresponds to the concentrations in the liver tissue. Contaminant concentrations in the extracts at CRs above or below 1 are concentrated (CR>1) and diluted (CR<1) compared to the original tissue concentrations. The procedural blanks were tested at the highest CR (1% DMSO) and 10 times diluted (0.1% DMSO) (Table 1). The hepatocytes were exposed for a total of 96 h with re-exposure after 48 h. At the end of the exposure period,

100 µl cell culture media from each well was transferred to 96-well Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark) and stored at -80˚C for subsequent Vtg analysis, whereas the cells were subjected to analysis for cell viability without further delay.

2.7. Cell viability

Cell viability was measured at the end of the exposure period essentially as described by Schreer et al. (2005) by use of the two probes Alamar blue (AB) and 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (CFDA-AM) for measuring the metabolic activity (AB) and membrane integrity (CFDA-AM). The probes are commonly used in combination to assess cytotoxicity. CFDA-AM is hydrolysed to the fluorescent 5-carboxyfluorescein (CF) by unspecific esterases (Schreer et al 2005) which is negatively correlated with cellular damage (Schirmer et al., 1997). After 96 h of exposure, exposure media was removed and cells were incubated in tris buffer (50 mM, pH 7.5, 100 µl per well) containing 5% AB and 4 µM CFDA-AM. After 30 min incubation in the dark on an orbital shaker (100 rpm), the fluorescence was read using Victor V^3 multilabel counter (Perkin Elmer, Waltham, MA, USA) with wavelength pairs of excitation and emission of 530-590 (AB) and 485-530 (CFDA-AM). The results were normalised between the negative control (solvent, DMSO = 100% viability) and positive control ($CuSO_4$ 10 mM = 0% viability).

2.8. Vitellogenin protein expression

Analysis of Vtg secreted to the growth media was performed with a capture ELISA essentially as described in Tollefsen et al. (2003). Plates containing 100 µl growth media sampled at the end of the exposure period were thawed for minimum 4 h in a refrigerator before 100 µl of standards were applied to assigned empty wells. The plates were left overnight (16 h) in the dark at 4˚C. The following day, the plates were washed three times with 250 µl washing buffer

(PBS with 0.05% Tween-20) and incubated with 200 µl blocking buffer (PBS with 2% BSA) in the dark at 20˚C for 1 h. After three washes with 250 µl washing buffer, 100 µl monoclonal mouse anti-salmon Vtg (BN-5, Biosense Laboratories, Bergen, Norway) diluted 1:6000 in incubation buffer (PBS with 1% BSA) was added to each well and the plates were incubated 2 h at 37˚C. The BN-5 antibody cross-reacts with Vtg in plasma from Arctic char Vtg (Biosence product no. V01402101, product description sheet). The plates were washed three times and 100 µl of the secondary antibody goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) diluted 1:6000 in incubation buffer was added to each well before the plates were incubated in the dark at 37˚C. After 1 h the plates were washed five times before 100 µl of enzyme substrate (TMB plus, KEMENTEC diagnostics, Taastrup, Denmark) was added to each well. After 15 min of incubation, the enzymatic reaction was stopped by adding 50 µl 1M H_2SO_4. The absorbance was measured at 450 nm using a VersaMax microplate reader (Molecular Devices LLC., Sunnyvale, CA, USA). The relative expression of Vtg was normalized between the negative (DMSO, 0%) and positive control (30 nM 17β-estradiol, 100% Vtg protein expression).

2.9. Data analysis

All data analyses were performed with Graphpad prism v6.01 software (GraphPad Software Inc., San Diego, CA, USA). The measured data for cell viability and Vtg were expressed as percent of induction/reduction between the solvent control (DMSO) and their individual positive controls. Data were fitted with non-linear sigmoidal concentration-response curve with variable slope and with constraints for bottom (0) and top (100). The data were checked for homogeneity of variance and normality, and statistics were performed using a one-way ANOVA with a Tukey post hoc test ($p<0.05$). Statistical differences in responses observed after exposure to the extracts and their corresponding procedural blanks were investigated to detect

responses caused by the contaminant load in the liver extracts. Statistical differences in the responses after exposure to extracts from the two different fish populations were also investigated.

3. Results

Liver extracts from Arctic char from a high- and low-contaminated lake at Bjørnøya were assessed for their contaminant contents, and for their ability to reduce cell viability and increase Vtg protein expression in Arctic char primary hepatocytes in 3-4 individual exposure experiments (Table 3).

3.1. Contaminants in liver extracts from Arctic char

The chemical analysis revealed that fish from Lake Ellasjøen contain higher levels of contaminants than fish from Lake Laksvatn (Table 2). The largest difference in contaminant concentrations was observed in the F1 extract. Highest concentrations were observed for the PCBs in the Ellasjøen F1 extract with ΣPCBs (709 000 pg/g ww) 108 times higher than in the Laksvatn F1 extract. A similar pattern was observed for ΣPBDEs (6580 pg/g ww) where the Ellasjøen extracts contained 120 times higher levels than the Laksvatn extracts. In this extract, only two compounds were found in higher concentrations in the Laksvatn extract; o,p,'-DDD and heptachlor, than the Ellasjøen extract. However, the concentration of heptachlor was lower in the Laksvatn extract than the procedural blank and the concentration of o,p,'-DDD was only two times higher in the Laksvatn than the Ellasjøen F1 extract, and ΣDDT was 32 times higher in extracts from Ellasjøen fish (27 900 pg/g ww) than Laksvatn fish (864 pg/g ww).

MESO_2-PCB concentrations above LOD were only detected in the F2 extract from Ellasjøen fish. Organophosphorus flame retardants (OPFRs), OH-PCBs and OH-PBDEs were detected in the F3 extracts. The concentration of TBEP, and the OH-PCBs were higher in the Ellasjøen liver extract than the Laksvatn liver extract, whereas the concentrations of the OH-BDEs (OH-BDE-68, OH-BDE-47/75 and OH-BDE-101 (<LOQ)) were higher in the Laksvatn liver extract than the Ellasjøen liver extract (Table 2). For several compounds, the concentration in the Laksvatn extracts (and in some cases in the Ellasjøen extracts) were below or similar to concentrations measured in the blank. The chemical results for these compounds should therefore be interpreted with caution.

3.2. In vitro effects of Arctic char liver extracts

Cell viability, measured as membrane integrity and metabolic activity, of the Arctic char hepatocytes was only slightly affected (>85% of DMSO control) at the highest tested CR by extract F1 of Arctic char liver from both fish populations of Ellasjøen and Laksvatn (Figure 1, Table 3). The slight decrease in cell viability was not significantly different from the F1 procedural blank.

Only the highest tested CR of the Ellasjøen F2-liver extract significantly reduced the metabolic activity compared to the F2 procedural blank. No significant reduction in the membrane integrity was observed for the F2-liver extracts. The F2 liver extracts reduced the metabolic activity in a concentration dependent manner with 50% reduction at a CR of 0.5 (Ellasjøen F2) and at a CR of 1.0 (Laksvatn F2). The Ellasjøen F2 had a significantly higher effect on the metabolic activity than the Laksvatn F2 in the CR range 0.3 – 1 (Figure 1, Table 3).

363 The F3-liver extract reduced both the membrane integrity and metabolic activity in a
364 concentration-dependent manner with a reduction to less than 10% of DMSO control at the
365 highest tested CR (CR=0.3). The 50% effect concentration (EC_{50}) for metabolic activity and
366 membrane integrity was a CR of 0.11 (metabolic activity) and of 0.15 (membrane integrity) for
367 the Ellasjøen extract, and a CR of 0.06 (metabolic activity) and 0.09 (membrane integrity) for
368 the Laksvatn extract. The F3 procedural blank did not reduce the metabolic activity or
369 membrane integrity compared to the DMSO control. A significant reduction in metabolic
370 activity was observed at a CR of 0.03 in Ellasjøen F3- and at a CR of 0.1 in Laksvatn F3-liver
371 extract when compared to the procedural blank F3. The Laksvatn F3-liver extract was
372 significantly more effective in reducing the metabolic activity of the primary Arctic char
373 hepatocytes than the Ellasjøen F3-liver extract, but only at a CR of 0.1 (Figure 1, Table 3).
374

375 The F3-liver extract from Ellasjøen fish significantly increased the production of Vtg at a CR
376 of 0.03 (Figure 1), with a maximum Vtg protein expression of 10% of positive control at a CR
377 of 0.1. The response of the Ellasjøen F3-liver extract on the Vtg protein expression was
378 significantly higher than the response of the Laksvatn F3-liver extract. No increased *in vitro*
379 Vtg protein expression was observed after exposure to any of the other liver extracts.
380

381 4. Discussion

382 Liver extracts from Arctic char from two different lakes at Bjørnøya were subjected to chemical
383 analysis and *in vitro* effect assessment using primary hepatocytes from Arctic char. Not
384 surprisingly, the highest levels of contaminants were found in liver extracts from Ellasjøen fish,
385 a population where high OHC concentrations have previously been reported (Bytingsvik et al.,
386 2015). The F2 and F3 extracts from both fish populations reduced the *in vitro* cell viability.

There was no clear difference between the lakes in terms of effects of the liver extracts on cell viability, but only the Ellasjøen F3-extract affected the Vtg protein expression.

The results from the chemical analyses were consistent with previously published studies on fish from these locations (Bytingsvik et al., 2015; Evenset et al., 2005, 2004). The chemical analysis indicates that the fish population in Ellasjøen is subjected to higher risk of biological effects than the Laksvatn fish population. A statement supported by previous observation of approximately 50 fold higher hepatic cytochrome P450(CYP)1A enzyme levels in Arctic char from Ellasjøen compared to those from another low-contaminated lake on Bjørnøya (Lake Øyangen) in addition to lower glucocorticoid receptor (GR) protein expression, elevated heat shock proteins expression (Wiseman et al. 2011), higher level of DNA double strand breaks in Lake Ellasjøen char (Neerland., 2016), higher liver *cyp1a* mRNA abundance, and altered mRNA abundance of key genes related to HPI axis functioning in the Lake Ellasjøen char compared to the Lake Laksvatn char (Jørgensen et al., 2017).

4.1. Effects on *in vitro* cell viability of Arctic char liver extracts

The lowest effect on cell viability was observed after exposure to the F1 extract from Ellasjøen and Laksvatn fish. This is interesting as these extracts (and especially F1 from Ellasjøen fish) contain high levels of OHCs like PCBs, PBDEs, DDTs and mirex. Moreover, there were large differences in concentration between the two lakes. The octanol-water partition coefficient (logKow) for several of these compounds are high (e.g. calculated LogKow for PCBs range from 3.76 for biphenyl to 8.26 for decachlorobiphenyl). This could have implications on the bioavailability of these contaminants in the *in vitro* assay due to potential compartmentalization

of highly hydrophobic contaminants to passive lipid reservoirs (lipid vacuoles) inside the isolated char hepatocytes.

Both F2 and F3 extracts reduced the cell viability at concentrations below and around original tissue concentrations, indicating that both fish populations are at risk for hepatotoxic effects. None of the analyzed compounds were detected in the Laksvatn F2 extract (all values < LOD). Since the F2 extract from both Ellasjøen and Laksvatn fish reduced the cell viability, endogenous compounds and/or environmental contaminants not included in the targeted chemical analysis likely mediate the effect.

The F3 extract from Laksvatn fish affected the cell viability at lower concentrations than the F3 extract from Ellasjøen. Of the contaminants measured in F3, concentrations of certain OH-BDEs (OH-BDE-68, OH-BDE-47/75 and OH-BDE-101) were higher in the Laksvatn than the Ellasjøen extract. Some of these compounds are known to have toxic effects. For example, OH-BDE-47/75 is cytotoxic in primary adult neural stem/progenitor cells from adult mice and is also more toxic than the parent compound BDE-47 (Li et al., 2013). Even though the concentrations measured in the extracts were lower than those known to cause an effect, the compounds might contribute to the observed effects on metabolic activity together with the other measured contaminants as well as contaminants not included in the targeted chemical analysis.

4.2. Effects on *in vitro* Vitellogenin protein expression of Arctic char extracts

Only the F3 extract of liver from Ellasjøen fish significantly increased *in vitro* Vtg protein expression compared to the procedural blank, suggesting that one or more compound(s) in this extract is estrogenic and may cause changes in the biological activity related to the estrogenic hormone system. A significant effect was observed at a CR of 0.03, which is well below the original tissue concentration (CR=1). It should also be noted that having a CR of 1 in the exposure media does not necessarily mean that the internal cell concentration will be the same. The internal cell concentration depends on the uptake from exposure media, and binding of compounds to the plastic wells, cell surface or other media components. Thus the actual internal exposure concentrations will most likely be lower than the original tissue concentration at a CR of 1.

The F3 extracts originates from tissue from female and male fish in different reproductive stages. High concentrations of the endogenous estrogen E2 were present in the chars' plasma (supplementary table 1), with higher concentrations measured in females from Ellasjøen than Laksvatn (Bytingsvik et al., in prep). Although the estrogen levels in the liver extracts were not measured, endogenous estrogens present in the liver samples will likely end up in F3 during the fractionation. Hence, endogenous estrogens may have contributed to the effect on the Vtg protein expression after exposure to the F3 liver extract from Ellasjøen.

The F3 extract also contains hydroxylated metabolites of PCBs, several of which are known to exert estrogenic (Braathen et al., 2009) and/or anti-estrogenic effects (Gustayson et al., 2015; Oh et al., 2007). Of the analysed compounds in the F3 extracts, concentrations of OH-PCBs (4-OH-PCB-107, 4-OH-PCB-146, 4-OH-PCB-163, and 4-OH-PCB-187) were found to be higher in the Ellasjøen liver extract than Laksvatn liver extract. Primary Atlantic salmon and rainbow

trout hepatocytes have previously shown a concentration-specific induction of *Vtg* mRNA when exposed to 4-OH-CB107, 4-OH-CB146, 4-OH-CB187, and 3-OH-CB138 (Braathen et al., 2009) and Vtg protein when exposed to 4-OH-PCB50, 4-OH-PCB30, 4-OH-PCB 72 and 4-OH-PCB 112 (Andersson et al., 1999). Increased plasma Vtg concentrations were also observed in rainbow trout dietary exposed to 4-hydroxy-2',4',6'-trichlorobiphenyl (OH-PCB30) and 4-hydroxy-2',3',4',5'-tetrachlorobipheny (OH-PCB-61) (Carlson and Williams, 2001). Although effects of endogenous estrogens cannot be ruled out, contribution to the observed increased Vtg protein expression by OH-PCBs detected in the F3-liver extract from Ellasjøen fish is likely as OH-PCBs are known to be estrogenic (Andersson et al., 1999; Braathen et al., 2009; Carlsson and Williams, 2001) and effects of estrogenic compounds in mixtures are known to be additive both *in vitro* and *in vivo* (Brian et al., 2005; Correia et al., 2007; Petersen and Tollefsen, 2011; Thorpe et al., 2001).

4.3. Environmental implications

Arctic char from Ellasjøen might be subjected to a total load of pollutants with a potential to disrupt estrogenic processes, ultimately leading to effects on reproduction if exposure occurs during sensitive developing stages (Bytingsvik et al., 2015; Letcher et al, 2010). This is further supported by altered mRNA abundances of key genes related to HPI axis functioning in the Ellasjøen char, suggestive of endocrine disruptive effects in this char population (Jørgensen et al., 2017). Dose-response relationships from *in vitro* and *in vivo* studies with temperate and Arctic species in a steady (high) nutritional state might not be relevant for Arctic animals that have fasting periods. Several studies have shown that fasting fish mobilize PCBs bound to lipid storages, potentially causing a larger effect at lower concentrations than in PCB-exposed fish that are still fed (Maule et al., 2005; Jørgensen et al. 2006). Fertilized fish eggs (Atlantic croacker, *Micropogonias undulates*) from PCB dosed adults (0.4 mg Aroclor 1254/ kg fish)

481 contained 0.66 µg/g egg (660 ng/g egg) PCB and showed reduced growth rate and impaired
482 startle response (McCarthy et al., 2003). From previous studies it seems that low PCB levels in
483 fish eggs from temperate species (<500 ng/g ww) affected embryonic and larval survival and
484 caused reproductive dysfunctions later in life at levels as low as 10–30 ng/g ww (reviewed by
485 Letcher et al., 2010). A negative correlation between lake trout (*S. namaycush*) egg PCB
486 concentration (124–314 ng/g w.w.) and egg and fry survival have also been reported (Mac and
487 Edsall, 1991). Interestingly, it was the extract containing OH-PCBs that showed estrogenic
488 effects in the present study. High levels of PCBs might lead to higher formation of OH-PCBs
489 through biotransformation, resulting in effects on the endocrine system as observed in the
490 present study, potentially affecting reproduction. Previous studies also support that early life
491 stages of fish are more vulnerable to pollutants than adult stages, which complicates the
492 determination of acceptable threshold concentration for contaminants in the environment
493 (reviewed by Letcher et al., 2010).

494

495 5. Conclusion

496 Arctic char from Lake Laksvatn and Lake Ellasjøen are exposed to a cocktail of pollutants, and
497 highest concentrations were generally found in liver extracts from Ellasjøen. The extracts
498 affected *in vitro* cell viability and Vtg protein expression. Cell viability was affected by F2 and
499 F3 from both fish populations. Potential contributors to the effects on cell viability of the F2
500 liver extracts could not be determined, whereas OH-BDEs may be partly responsible for the
501 observed effect on cell viability of the F3 liver extracts. Only the F3 liver extract from Ellasjøen
502 fish affected the Vtg protein expression, and although compounds such as estrogenic OH-PCBs
503 was quantified in the F3 extracts, it remains to be determined which compounds were causing
504 the estrogenic effect.

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Figure legends

Figure 1. Effects of Arctic char (*Salvelinus alpinus*) liver extracts F1-F3 on the cell viability (membrane integrity (□) and metabolic activity (○)) and Vitellogenin production (●) in Arctic char hepatocytes. N=3-4. The asterisk (*) indicate results significantly different ($p<0.05$) from procedural blanks (membrane integrity (□) and metabolic activity (○)). F1 contained neutral compounds such as PCBs, PBDEs and organochlorine pesticides, F2 contained $MeSO_2$-PCBs/DDE, and F3 contained hydroxylated (OH) PCBs/phenols.

Table 1. Overview of the tested liver extracts and the concentration ratio (CR) range used in the exposure studies with Arctic char hepatocytes.

Fish population	Extract	CR range
Ellasjøen	F1	0.0003-1
	F2	0.0003-1
	F3	0.0001-0.3
Laksvatn	F1	0.0003-1
	F2	0.0003-1
	F3	0.0001-0.3
Procedural blank	F1	0.1, 1
	F2	0.1, 1
	F3	0.03, 0.3

Table 2. Measured concentrations of different chemicals in the three extracts. Only compounds measured in concentrations above LOD are included and values are given in pg/g ww.

Compound	Abbreviation	Procedural blank	Ellasjøen	Laksvatn
Liver extract F1		pg/g ww	pg/g ww	pg/g ww
Hexachlorobenzene	HCB	9.3	1 084.7	415.8
Heptachlor		397.0	330.0	395.8
Oxychlordane	Oxy-CD	119.9	137.3	110.1
Cis-chlordane	c-CD	26.1	191.9	50.0
Trans-nonachlor	t-NC	<1.0	577.1	51.5
Cis-nonachlor	c-NC	10.9	229.4	36.9
Endosulfan I		11.3	125.7	26.3
Endosulfan sulfate		4.2	<0.4	<0.4
Mirex		8.2	1 545.0	28.0
1.1-Dichloro-2.2-bis(4-chlorophenyl)ethene	p.p-DDE	29.3	27 655.0	377.0
2-(2-Chlorophenyl)-2-(4-chlorophenyl)-1.1-dichloroethene	o.p-DDE	<2.2	39.1	<2.2
Mitotane	o.p-DDD	<61.0	253.0	487.0
Sum DDTs		**29.3**	**27 947.4**	**863.7**
2.2'.4-tribromodiphenylether	PBDE 17	<6.7	63.8	<6.7
2.4.4'-Tribromodiphenyl ether	PBDE 28	<7.6	139.3	<7.6
2.2'.4.5'-Tetrabromodiphenyl ether	PBDE 49	<10.7	98.3	<10.7
2.2'4.4'-Tetrabromodiphenyl ether	PBDE 47	<8.5	5 008.0	26.5
2.3'.4.4'-Tetrabromodiphenyl ether	PBDE 66	<13.6	39.4	<13.6
2.2'.4.4'.6-Pentabromodiphenyl ether	PBDE 100	<15.3	570.8	9.1
2.3'.4.4'.6-Pentabromodiphenyl ether	PBDE 119	<20.8	34.9	<20.8
2.2'.4.4'.5-Pentabromodiphenyl ether	PBDE 99	<19.5	386.8	19.1
2.2'.4.4'.5.6'-Hexabromodiphenyl ether	PBDE 154	<12.5	84.9	<12.5
2.2'.4.4'.5.5'-Hexabromodiphenyl ether	PBDE 153	<17.8	152.4	<17.8
sum PBDEs		**0.0**	**6 578.5**	**54.7**
BATE	BATE	<3.1	2.0 (<LOQ)	1.9 (<LOQ)
PBT	PBT	1.9	3.3 (<LOQ)	3.5 (<LOQ)
PBEB	PBEB	0.7	0.9 (<LOQ)	0.4 (<LOQ)
DPTE	DPTE	0.8	<0.5	1.3 (<LOQ)
BTBPE	BTBPE	2.8	<0.3	2.5 (<LOQ)
BEHTBP	BEHTBP	2.0	<1.3	2.3 (<LOQ)
2.2'.5-trichlorobiphenyl	PCB 18	<5.0	25.0	<5.0
2.4.4'- trichlorobiphenyl /2.4'.5- trichlorobiphenyl	PCB 28/31	3.7	640.5	47.9
2.2'.4.4'-tetrachlorobiphenyl/ 2.2'.4.5'-tetrachlorobiphenyl	PCB 47/49	<3.0	1 022.1	18.8
2.2'.5.5'- tetrachlorobiphenyl	PCB 52	16.4	158.1	41.0
2.3'.4.4'- tetrachlorobiphenyl	PCB 66	81.7	5 639.8	87.2
2.4.4'.5- tetrachlorobiphenyl	PCB 74	<2.0	3 538.3	<2.0
2.2'.4.4'.5-pentachlorobiphenyl	PCB 99	147.8	35 028.2	267.3
2.2'.4.5.5'- pentachlorobiphenyl	PCB 101	172.5	4276.2	245.1
2.3.3'.4.4'-Pentachlorobiphenyl	PCB 105	303.1	9854.0	168.7

2.3.4.4'.5- pentachlorobiphenyl / 2.3.3'.4'.5'-Pentachlorobiphenyl	PCB 114/122	14.2	1 189.2	<12.0
2.3'.4.4'.5- pentachlorobiphenyl	PCB 118	513.1	46 037.6	533.5
2.3'.4.4'.5'-Pentachlorobiphenyl	PCB 123	23.1	4 107.3	50.8
2.2'.3.3'.4.4'-Heachlorobiphenyl	PCB 128	105.3	10 106.8	126.7
2.2'.3.4.4'.5'- Heachlorobiphenyl	PCB 138	451.4	175 561.7	1 399.2
2.2'.3.4.5.5'- Heachlorobiphenyl	PCB 141	41.9	443.7	41.7
2.2'.3.4'.5'.6- Heachlorobiphenyl	PCB 149	95.5	1 384.8	112.7
2.2'.4.4'.5.5'-Hexachlorobiphenyl	PCB 153	271.8	268 701.7	1 791.1
2.3.3'.4.4'.5- Heachlorobiphenyl	PCB 156	82.4	8 551.9	107.3
2.3.3'.4.4'.5'- Heachlorobiphenyl	PCB 157	18.7	1 648.3	28.2
2.3'.4.4'.5.5'-Hexachlorobiphenyl	PCB 167	21.5	5 795.1	60.3
2.2'.3.3'.4.4'.5-Heptachlorobiphenyl	PCB 170	37.2	22 122.6	274.5
2.2'.3.4.4'.5.5'- Heptachlorobiphenyl	PCB 180	49.3	66 243.3	665.8
2.2'.3.4.4'.5'.6- Heptachlorobiphenyl	PCB 183	<4.0	12 154.5	123.7
2.2'.3.4'.5.5'.6- Heptachlorobiphenyl	PCB 187	<5.0	21 275.6	299.5
2.3.3'.4.4'.5.5'- Heptachlorobiphenyl	PCB 189	<5.0	612.3	8.9
2.2'.3.3'.4.4'.5.5'-Octachlorobiphenyl	PCB 194	<10.0	3 097.4	46.8
Sum PCBs		**2 450. 5**	**709 215.5**	**6 546.3**
Octachlorostyrene	OCS	<26.0	125.2	26.0
6-methoxy-2.2'.4.4'-tetrabromodiphenyl ether/6'-methoxy-2.4.4′.6-tetrabromodiphenyl ether	6-MeOBDE47/75	<9.0	330.0	<9.0
4'-methoxy-2.2'.4.5'-Tetrabromodiphenyl ether	4-MeOBDE49	20.0	54.0 (<LOQ)	<18.0
Liver extract F2		pg/g ww	pg/g ww	pg/g ww
4'-Methylsulfonyl-2.4.5.2'.5'-pentachlorobiphenyl	4MeSOPCB101	0.8	5.5	<0.8
4-MeSO2-2.2′.3.4′.5′.6-hexachlorobiphenyl	4MeSOPCB149	<0.9	13.8	<0.9
4-Methylsulfonyl-2.2'.3'.4'.5.5'-hexachlorobiphenyl	4MeSOPCB141	<0.8	3.2	<0.8
Liver extract F3		pg/g ww	pg/g ww	pg/g ww
triisobutyl phosphate	TiBP	420.0	200.0 (<LOQ)	300.0 (<LOQ)
tributyl phosphate	TBP	500.0	200.0 (< LOQ)	200.0 (< LOQ)
tris(2-chloroethyl)phosphate	TCEP	4280	4 260.0 (<LOQ)	4 380.0 (<LOQ)
tri(1-chloro-2-propyl)phosphate	TCPP	1760	1 500.0 (<LOQ)	1 720.0 (<LOQ)
tris(2-butoxyethyl)phosphate	TBEP	<87.0	420.0	240.0
Pentachlorophenol	PCP	5.9	53.3 (<LOQ)	53.3 (<LOQ)
4-Hydroxy-2'.3.3'.4'.5'-pentachlorobiphenyl	4-OH-PCB 107	0.9	7.8 (LOQ)	<2.6
4-Hydroxy-2.2'.3.4'.5.5'-hexachlorobiphenyl	4-OH-PCB 146	<0.1	4.4	<0.1
3-Hydroxy-2.2'.3.4.4'.5'-hexachlorobiphenyl	3-OH-PCB 138	0.1	1.3 (<LOQ)	<0.4
4-hydroxy- 2.2'.3.3'.4.5'-Hexachlorobiphenyl	4-OH-PCB 130	0.3	2.7 (<LOQ)	<0.9
4-Hydroxy-2.3.3'.4'.5.6-hexachlorobiphenyl	4-OH-PCB 163	0.1	1.1	<0.4
4-Hydroxy-2.2’.3.4’.5.5’.6-heptachlorobiphenyl	4-OH-PCB 187	0.1	5.6	<0.4
sum OH-PCB		**1.6**	**11.1**	**0.0**
2'-Hydroxy-2.3'.4.5'-tetrabromodiphenylether	2-OH-BDE68	<0.1	1.4	7.2
6-hydroxy-2.2'.4.4'-tetrabromodiphenyl ether /6'-hydroxy-2.4.4′.6-tetrabromodiphenyl ether	6-OH-BDE47/75	<0.1	1.6	3.7
4'-Hydroxy-2.2'.4.5.5'-pentabromodiphenyl ether	4-OH-BDE101	<0.1	<0.1	0.3 (<LOQ)

Table 3. Summary of results from the Arctic char (*Salvelinus alpinus*) hepatocytes exposed to extracts (F1-F3) of liver from Arctic char sampled in Laksvatn (low-contaminated lake) and Ellasjøen (high-contaminated lake) at Bjørnøya (Bear Island) in 2012. The table presents the no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), 10% and 50% effect concentration (EC_{10} and EC_{50}) given as the extracts concentration ratio (CR, ratio between concentration in the sampled tissue and exposure media) for the endpoints metabolic activity (Met. Act.) and vitellogenin protein expression (Vtg). The LOEC is the lowest CR causing a significant effect compared to the procedural blank ($p < 0.05$). F1 contained neutral compounds such as PCBs, PBDEs and organochlorine pesticides, F2 contained $MeSO_2$-PCBs/DDE, and F3 contained hydroxylated (OH) PCBs/phenols. Significant differences between corresponding extracts from the two fish populations are given by the CR at which the significant differences were observed with the lake from which the most effective extract was obtained in parenthesis.

Extract	**Endpoint**	**Ellasjøen**					**Laksvatn**					**Between lake comparison**
		NOEC CR	**LOEC CR**	**EC_{10} CR**	**EC_{50} (R^2)**	**Max effect (CR)**	**NOEC CR**	**LOEC CR**	**EC_{10} CR**	**EC_{50} (R^2)**	**Max effect (CR)**	
F1 liver	Met. Act.	1	na	na	na	90% (1)	1	na	0.030	na	na	ns
F2 liver	Met. Act.	0.3	1	0.074	0.499 (0.926)	35% (1)	1	na	0.14	0.985 (0.937)	51 % (1)	CR 0.3 and 1, (Ellasjøen)
F3 liver	Met. Act.	0.01	0.03	0.036	0.111 (0.927)	6% (0.3)	0.03	0.1	0.027	0.0599 (0.973)	3 % (0.3)	CR 0.1 (Laksvatn)
F1 liver	Vtg	1	na	na	na	0.3% (0.1)	1	na	na	na	0.1 (0.01)	ns
F2 liver	Vtg	1	na	na	na	0.6% (1)	1	na	na	na	0.2 % (1)	na
F3 liver	Vtg	0.01	0.03	na	na	10% (0.1)	0.3	na	na	na	0.15 % (0.003)	CR 0.03 (Ellasjøen)

Max effect: the lowest obtained metabolic activity or the highest obtained Vtg protein expression (% of control). The corresponding CR is given in parenthesis.
na: not applicable, no measurable effect observed, not possible to fit a concentration response curve
ns: not significant (one-way ANOVA with a Tukey post hoc test, $p > 0.05$)