Environment International 174 (2023) 107915

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Full length article

Establishment of killer whale (*Orcinus orca*) primary fibroblast cell cultures and their transcriptomic responses to pollutant exposure

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ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
In vitro
Cell culture
RNA-seq
Mixture
Persistent organic pollutant
Marine mammal

ABSTRACT

Populations of killer whale (*Orcinus orca*) contain some of the most polluted animals on Earth. Yet, the knowledge on effects of chemical pollutants is limited in this species. Cell cultures and *in vitro* exposure experiments are pertinent tools to study effects of pollutants in free-ranging marine mammals. To investigate transcriptional responses to pollutants in killer whale cells, we collected skin biopsies of killer whales from the Northern Norwegian fjords and successfully established primary fibroblast cell cultures from the dermis of 4 out of 5 of them. Cells from the individual with the highest cell yield were exposed to three different concentrations of a mixture of persistent organic pollutants (POPs) that reflects the composition of the 10 most abundant POPs found in Norwegian killer whales (*p,p'*-DDE, *trans*-nonachlor, PCB52, 99, 101, 118, 138, 153, 180, 187). Transcriptional responses of 13 selected target genes were studied using digital droplet PCR, and whole transcriptome responses were investigated utilizing RNA sequencing. Among the target genes analysed, *CYP1A1* was significantly downregulated in the cells exposed to medium (11.6 μM) and high (116 μM) concentrations of the pollutant mixture, while seven genes involved in endocrine functions showed a non-significant tendency to be upregulated at the highest exposure concentration. Bioinformatic analyses of RNA-seq data indicated that 13 and 43 genes were differentially expressed in the cells exposed to low and high concentrations of the mixture, respectively, in comparison to solvent control. Subsequent pathway and functional analyses of the differentially expressed genes indicated that the enriched pathways were mainly related to lipid metabolism, myogenesis and glucocorticoid receptor regulation. The current study results support previous correlative studies and provide cause-effect relationships, which is highly relevant for chemical and environmental management.

1. Introduction

"To understand and beat marine pollution" is listed as the first challenge in the UN Decade of Ocean Science program (UN Ocean Decade, 2021). Environmental pollutants from anthropogenic sources are ubiquitous in marine ecosystems and act as major stressors for marine biota (Ashraf, 2017). Persistent organic pollutants (POPs) is a generic term gathering groups of lipophilic chemicals that accumulate in lipophilic tissues of living organisms and magnify across food webs (Borgå et al., 2001; Fisk et al., 2001). POPs including organochlorine pesticides (OCPs; chlordanes (CHLs), dichlorodiphenyltrichloroethanes (DDTs) and its derivatives) and polychlorinated biphenyls (PCBs) were widely used in industry and agriculture over several decades (Alharbi et al., 2018). Due to their resistance to environmental degradation, these pollutants are transported to remote areas through the atmosphere and oceans (Wania and MacKay, 1996). Consumption of prey with bioaccumulated pollutants results in biomagnification of POPs in the food webs (Suedel et al., 1994). General effects of exposure to CHLs, DDTs

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https://doi.org/10.1016/j.envint.2023.107915
Received 21 December 2022; Received in revised form 27 March 2023; Accepted 31 March 2023
Available online 2 April 2023

and PCBs in vertebrates include endocrine and metabolic disruption, immune suppression, neurotoxicity, carcinogenesis, reproductive toxicity, and even increased risk of direct mortality (Dietz et al., 2019; Landrigan et al., 2020; Letcher et al., 2010). Levels of OCPs and PCBs are still prominent in the environment, even though the first regulations occurred by national bans in 1970s. In 2004, the Stockholm Convention on Persistent Organic Pollutants was put into force as a global treaty to regulate and eliminate the use and production of these chemicals (https://chm.pops.int/). Yet, legacy POPs, specifically OCPs and PCBs, still represent the most abundant compounds in the chemical cocktail that marine predators are exposed to, and at concentrations associated with serious individual and population health risks (Dietz et al., 2019). The ability of these chemicals to disrupt endocrine and metabolic systems is of particular concern, as the alteration of these systems may affect key physiological functions and processes, and consequently individual survival, reproduction, and population dynamics (Landrigan et al., 2020; Gore et al., 2015).

Populations of long-lived apex predators, such as killer whales (*Orcinus orca*), contain some of the most polluted animals on Earth (Jepson et al., 2016). Despite the numerous additional threats including direct anthropogenic disturbance, long history of hunting and live capturing, and prey depletion (Jourdain et al., 2019), this cosmopolitan species is listed as "data deficient" in the IUCN Red List (Reeves et al., 2017). The limited knowledge that we have so far suggests that current levels of pollutants are a risk for killer whales' health. Tissue extracts of contaminants suppressed various immune functions in killer whale lymphocytes at environmentally relevant concentrations (Desforges et al., 2017), whilst a follow-up study predicted that ΣPCB exposure could affect the viability of more than a half of world's killer whale populations over the next 100 years (Desforges et al., 2018). Levels of Σ_{136}PCBs measured in blubber were also related to increased transcript levels of genes involved in endocrine functions and detoxification in free-ranging killer whales from the Northeastern Pacific Ocean (Buckman et al., 2011). Furthermore, PCBs, DDTs and CHLs showed a potential to modulate the function of whale (including killer whale) nuclear receptors that play a key role in regulating energy metabolism, growth, development, and immune system (Lühmann et al., 2020). Nuclear receptors are transcription factors important for endocrine systems normally activated by endogenous compounds such as hormones and lipids.

Despite good overall knowledge about occurrences and concentrations of PCBs and OCPs in killer whales worldwide (Panti et al., 2022; Andvik et al., 2020; Desforges et al., 2018; Pedro et al., 2017), there is a lack of information about causal relationships between their exposure and adverse health effects, as well as a poor understanding of their modes of action. As conducting ecotoxicological research on whales is challenging due to ethical, technical, and legal issues, establishing alternative approaches with reduced impacts are needed (Fossi and Panti, 2018). This has also been requested by the International Whaling Commission (IWC, 2011). Establishment of cell cultures further used to perform *in vitro* exposure experiments are pertinent tools to study effects of pollutants in free-ranging marine mammals as they can provide information on cause-effect relationships and mechanistic understanding, which is important for management and conservation of marine wildlife and management of chemicals.

Fibroblasts have many advantages as a cell model as they are well studied, and cultures can be established from skin biopsies of free-ranging marine mammals. Fibroblast cultures have been established from several cetacean species, including pantropic spotted dolphin (*Stenella attenuata*) (Rajput et al., 2018), humpback whale (*Megaptera novaeangliae*) (Burkard et al., 2015), pygmy killer whale (*Feresa attenuata*) (Sun et al., 2018), white whale (*Delphinapterus leucas*) (Gauthier et al., 1998), finless porpoise (*Neophocaena asiaeorientalis* spp.) (Ochiai et al., 2020; Wang et al., 2011), striped dolphin (*Stenella coeruleoalba*), bottlenose dolphin (*Tursiops truncates*), fin whale (*Balaenoptera physalus*) (Fossi et al., 2006), killer whale (Marsili et al., 2012), sperm whale (*Physeter macrocephalus*) (Wise et al., 2011), and North Atlantic right whale (*Eubalaena glacialis*) (Godard et al., 2006). Despite this, our understanding of toxic responses in them is limited. Most studies have focused on responses following exposure to single contaminants, although cetaceans are exposed to a wide mixture of pollutants that may act additively or synergistically (Mori et al., 2006). Furthermore, most experiments on cetacean cells have used cytotoxicity, genotoxicity, immunotoxicity or transcript levels of genes involved in detoxification as responses, whereas our understanding on system-wide biological processes or molecular functions that may be affected by pollutant exposure in cetaceans still limits to responses to trace elements and a PCB metabolite (4′OH-CB72) (Ierardi et al., 2021; Ochiai et al., 2021; Pabuwal et al., 2013).

The main aim of this study was to establish a killer whale fibroblast cell culture and use it to characterize the transcriptional responses to an ecologically relevant mixture of POPs. Killer whale fibroblasts were exposed to a POP mixture that reflects the composition of 10 most abundant POPs found in blubber of Norwegian killer whales and was composed of eight PCBs, *p,p'*- dichlorodiphenyldichloroethylene (DDE) and *trans*-nonachlor. First, transcriptional responses of 13 target genes were studied using digital droplet PCR. The genes were nuclear receptors that may be modulated by these pollutants (Lühmann et al., 2020), and their target genes as well as genes involved in detoxification. Second, whole transcriptome responses to pollutant exposure were investigated utilizing RNA sequencing and subsequent bioinformatic analyses.

2. Materials and methods

2.1. Biopsy sampling and preparation

The killer whale samples were collected in November 2020 in the Lyngen and Kvænangen Fjord in Northern Norway from an open rigid-hull inflatable boat. The sampling was approved by the Norwegian Food Safety Authorities (Mattilsynet) under permit FOTS ID 24075. Skin/blubber biopsies (~40 mm long, 5 mm wide) from five killer whales were collected using a biopsy dart (Fig. 1A; Table A.1) that were shot from an Aerial Rocket Tag System launcher (LKARTS- Norway) targeting the body areas below the dorsal fin. The biopsies were immediately removed from the dart to separate skin, dermis, and blubber on a glass petri dish using disposable scalpels and cleaned scissors. All equipment used for sampling and handling of biopsy in the field was sterilized with 70% ethanol. Dermis i.e., the 1 cm dermal tissue part between skin and blubber (Fig. 1B) was directly placed in a 15 mL sterile plastic vial containing growth medium (GM) which consisted of 1× Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F-12), n-(2-hydroxyethyl) piperazine-n′-(2-ethanesulfonic acid) (HEPES), supplemented with 10% fetal bovine serum (FBS), 4 mM L-Glutamine, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, and 100 μg mL^{-1} Primocin (Invivogen, Toulouse, France). Samples were kept in a Styrofoam box with ice packs at ~4 °C until arrival to the laboratory and processed within 11–12.5 h from collection.

2.2. Establishment of primary cells

All biopsy and cell handling were conducted in sterile conditions. Establishment of killer whale fibroblast cell cultures was conducted following a protocol previously applied to humpback whale (Burkard et al., 2015), with some modifications. Prior to finely dissecting off dermis from the skin and blubber, each biopsy tissue piece was rinsed in 1× Dulbecco's Phosphate Buffered Saline (DPBS) with 100 μg mL^{-1} Primocin. The dermal tissue section was then cut into small pieces of ~1–3 mm^3, subsequently washed in a 3-step procedure with 1× DPBS. Between 5 and 10 dermis pieces were transferred to a 6-well plate and placed closely together in the center of each well. The pieces were

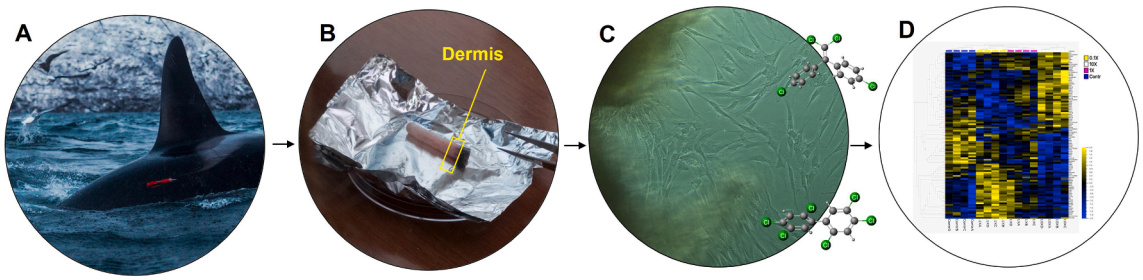

Fig. 1. Killer whale biopsies (A, B) were used to culture primary cells (C), which were exposed to pollutant mixtures followed by transcriptomic analyses (D).

covered by a sterile cover slip applied with a gentle pressure to ensure contact between tissue and plastic surface to facilitate fibroblast attachment and growth. One mL GM was carefully added to each well, and plates were incubated at 37 °C and 5% CO_2 (Sanyo MCO-19AIC (UV) CO_2 Incubator). After ~12 h, an additional 1 mL of GM was added to each well. Between 50 and 75% of the GM was changed every 2–3 days, and cell growth was closely monitored under microscope during the growth process.

After 13–14 days of incubation, the cells had grown into a monolayer and reached 90–95% confluency around the explants. The primary cells were isolated using trypsinization. First, the GM was removed, and wells were washed twice with 5 mL 1× DPBS containing 100 μg mL^{-1} Primocin. One mL of trypsin-EDTA (0.25%) was added to each well and removed after 30 s. The plates were incubated for 5 min at 37 °C and 5% CO_2. Each well of cells were resuspended in 2 × 3 mL of GM at 37 °C and filtered through a 70 μm sieve (Corning® cell strainer, Sigma) before seeded into a T75 cell flask with additional 4 mL of GM.

2.3. Cultivation and subculturing

During cultivation, 50–75% of the GM was changed every 2–3 days. The cells were split 1:3 or 1:2 when confluency reached 80–90% as described for the isolation of primary cells. A splitting ratio of 1:3 often resulted in too low cell densities and slow growth rate; thus, the ratio was increased to 1:2. The resuspended cells were seeded in new culture wells and flasks without filtering to prevent cell loss.

To examine cell viability and density, the resuspended cells were centrifuged at 200*g* for 4 min, the supernatant removed, and the cell pellet resuspended in fresh GM at 37 °C. Fifty μL of the resuspended cells were mixed with 50 μL of Trypan Blue staining solution (0.4%). This mixture was added to a hemocytometer where the cells were counted. The cell density was determined as cells number mL^{-1}.

To optimize the expansion of killer whale cells, which were generally growing slowly, different seeding densities, wells, flasks, and dishes were tested. In addition, elimination of the centrifugation step when splitting, different trypsinization methods and concentrations for splitting, increasing FBS concentration to 20% in the GM, and change of Primocin to Penicillin-Streptomycin was tested.

2.4. Cryopreservation and thawing

Killer whale cell cultures were cryopreserved at passage 1 (Table A.1). Cryopreservation of cells was proceeded following the splitting protocol, but the cell pellet was resuspended in a special cryopreservation solution (SolnIX-SK (CGX), Cryogenix, LLC, South Carolina, US (Baatz et al., 2014; Mancia et al., 2012)), or 90% FBS and 10% dimethyl sulfoxide (DMSO) with a final density of ~2.25 × 10^5 cells mL^{-1}. Cells were frozen in Corning® CoolCell™ LX Cell Freezing Container (Sigma-Aldrich), which enables slow freezing of samples by ~1 °C decrease per min (down to −80 °C) to ensure the most optimal freezing conditions (Pegg, 2007). Cryovials were afterwards transferred to liquid nitrogen for storage until further processing.

For thawing, frozen cells in cryovials were immersed in a 37 °C water bath and when fully thawed, the cells were immediately resuspended in 10–15 mL GM at 37 °C. The first vials were centrifuged before resuspension, but this step was dropped as cells reached a higher confluency more rapidly without centrifugation. Cells were seeded into a T25 or T75 flask at the density of ~3000 cells cm^{-2}, depending on given cell density of cryovial. GM was replaced after 24 h to remove all DMSO.

2.5. POP exposure

Cells from the individual that gave the highest cell yield (KW-20-11; Table A.1) were exposed to a POP mixture. The mixture consisted of 10 POPs (Table 1) that were the most abundant (in molarity) in killer whale blubber from Northern Norway (Blévin et al., unpublished). First, median blubber concentrations of 33 POPs (PCBs, OCPs and polybrominated diphenyl ethers) in pg g^{-1} wet weight that were measured in 23 killer whale blubber samples were converted to molar concentrations using molar weight of each compound (Table A.2). Emerging brominated flame retardants and phthalates were not considered as the concentrations of these compounds were below the detection limit or much lower than concentrations of PCBs and organochlorine pesticides (Andvik et al., 2021; Lippold et al., 2022; Blévin et al., unpublished). The molar concentrations were then ranked, and the 10 most abundant compounds were included in the mixture that reflected the concentrations and composition of these POPs in killer whale blubber (Table A.2; *p,p'*-DDE, *trans*-nonachlor, PCB52, 99, 101, 118, 138, 153, 180, 187). POP standards purchased from LGC standards and Merck/Sigma-Aldrich were dissolved in DMSO (Table A.3).

Cells of passage 1 (in total 7.6 × 10^5 cells) were seeded in T75 flask (~5000 cells cm-^{2}), cultured, and reseeded after 7 days when the cells had reached a confluency of 70–80 %. Cells from passages 3 and 4 were pooled and seeded in 12-well plates with a density of 7500 cells cm^{-2}. After settling for 48 h, with 100% medium change after 24 h, cells were exposed to three concentrations of the POP mixture as well as GM supplemented with 0.05% DMSO. The applied concentrations were 0.1×, 1× and 10× i.e., 1.16 μM, 11.57 μM and 115.7 μM, respectively, where 1× reflected median levels of the 10 POPs in killer whale blubber (Table 1). The exposure medium was discarded after 48 h, and cells were

Table 1
Composition of the POP mixture based on median concentrations of the 10 most abundant compounds in killer whales from Norwegian waters.

Compounds	Concentration 1× (μM)
p,p'-DDE	4.48
PCB-153	1.74
PCB-138	1.31
trans-nonachlor	0.99
PCB-180	0.64
PCB-101	0.52
PCB-52	0.52
PCB-99	0.48
PCB-187	0.45
PCB-118	0.44
Sum	11.57

washed in 100 μL 1× DPBS. Due to the limited number of cells obtained (discussed later), the experiment was performed once with four replicates of each treatment.

2.6. Isolation of RNA

RNA was isolated from the cells exposed to different concentrations of the POP mixture or solvent control using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the suppliers' protocol (Thermo Fisher Scientific, 2016). The RNA yield (ng μL^{-1}) and purity were determined using Thermo Scientific NanoDrop 2000 Spectrophotometer (Table A.4). Assessment of RNA integrity number (RIN = 9.39 ± 0.56, Table A.4) was conducted using RNA 6000 Nano Kit and the assay class Eukaryote Total RNA Nano for the Agilent 2100 Bioanalyzer following the protocol of the manufacturer (Agilent Technologies, Inc. 2001–2016, 2017, Waldbronn, Germany). The RIN value is used to assess the degradation of the RNA in the sample. RNA quality is further discussed in the Supporting Information.

2.7. Transcript levels of individual genes

Transcript levels of 13 genes of interest (Table A.5) were analyzed in the exposed and control killer whale cells. Two of the 14 samples (one control and one 10×) were not analyzed due to insufficient amount of RNA. The genes of interest included nuclear receptors that are modulated by POP exposure (Lühmann et al., 2020), their target genes and key detoxification enzymes or their transcription factors. The genes involved in various biological processes included aryl hydrocarbon receptor (*AHR*), cytochrome P450 1A (*CYP1A*), cytochrome P450 3A (*CYP3A*), cytochrome P450 4A (*CYP4A*), thyroid hormone receptor alpha (*THRA*), thyroid hormone receptor beta (*THRB*), glucocorticoid receptor (*GR*), peroxisome proliferator activated receptor alpha (*PPARA*), peroxisome proliferator activated receptor gamma (*PPARG*), fatty acid binding protein 4 (*FABP4*), estrogen receptor alpha (*ERA*), adiponectin (*ADIPOQ*), cluster of differentiation 36 (*CD36*) and the two housekeeping genes (HKG) were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and tyrosine 3-monooxygenase (*YWHAZ*) (Table A.5).

2.7.1. Primer design

Primers were designed using the online software Primer3 (v0.4.0) for all genes of interest on sequences derived from the annotated whole genome assembly available for *Orcinus orca* (GenBank: GCA_000331955.2). To further check primer specificity, the killer whale gene sequences were also blasted and aligned with sequences of other cetacean species (Table A.6). Primers were designed from different exons to avoid the co-amplification of genomic DNA if contamination was present (Table A.7). The absence of primer dimers and secondary structures was checked using the online tools: OligoEvaluator™ (Sigma) and Multiple Primer Analyzer (Thermofisher). The primers were purchased from Merck Sigma-Aldrich. The length of the amplified region for each gene was checked through standard PCR reactions (for details see Supporting Information) and electrophoresis on agarose gel with ethidium bromide staining. Primer efficiency was tested with cDNA serial dilution as a quality control (Fig. A.1).

2.7.2. Reverse transcription

Reverse transcription (RT) was performed using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. The starting amount of total RNA used for reverse transcription was 150 ng, according to the nanodrop RNA quantification provided. The kit used included a gDNA digestion step before the reverse transcription which reduced the risk of gDNA carryover.

2.7.3. Droplet digital polymerase chain reaction (ddPCR)

The ddPCR was performed following the protocol for the QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad). Each reaction was prepared in a total volume of 20 μL: 10 μL of EvaGreen Supermix (2×), 0.2 μL of each primer (10 μM), 5.1 μL of nuclease-free H_2O, and 4.5 μL of cDNA (1.875 ng μL^{-1}). No template control reactions were included for each gene analyzed. Droplets were generated using the QX200 Droplet Generator (Bio-Rad) and loaded in a 96-well plate. The PCR was performed in a SimpliAmp™ Thermal Cycler (Applied Biosystems) with the following parameters: enzyme activation for 5 min at 95 °C, 40 cycles of 30 sec denaturation at 95 °C, coupled annealing/extension for 1 min at 60 °C, and a last step of signal stabilization for 5 min at 4 °C.

Droplet's fluorescence was read using the QX200 Droplet Reader (Bio-Rad) and the results were analyzed with the software QX Manager (Bio-Rad). Only samples with at least 10 000 accepted droplets were considered for the analysis, and the fluorescence threshold was manually set to separate droplets with amplification from negative droplets. The software calculates the absolute value of cDNA concentration expressed in copies μL^{-1} for each sample and the relative Poisson interval of confidence. The results were normalized over HKG *GAPDH* to adjust for differences in material input, RNA degradation and correct for variation related to sample processing, for instance due either the RNA isolation or the reverse transcription from RNA to cDNA (Fleige and Pfaffl, 2006). *GAPDH* showed more stable transcript levels over the treatments than *YWHAZ* (Fig. A.2) and was thus chosen for normalization of the results. The normalized transcript levels of the GOIs showed similar tendencies as the quantified concentrations (Fig. A.3). *CYP3A* and *FABP4* were not detected and were thus not included in further data analyses.

2.7.4. Analyses of ddPCR data

The dataset of RNA quality and quantity and transcript levels of genes is available in the Norwegian Polar Data Centre repository (Routti et al., 2022). Data handling and statistical analysis was conducted using the software R version 4.0.3 (R Core Team, 2020). Transcriptomic responses to POP exposure were assessed as fold change relative to the mean of solvent control (DMSO). Linear models were used to assess transcriptional effects of exposure to the POP mixture in relation to the solvent control on the killer whale cells. The treatments including three concentrations of the POP mixture and the solvent control were included as independent factors in the model. The linear models were run separately for each gene of interest. Diagnostic plots of residuals were used to assess whether the data met the assumptions of linear models (Zuur et al., 2009) and the significance level was set at $\alpha \leq 0.05$ for gene expression.

2.8. RNA sequencing and bioinformatic analyses

RNA samples were submitted for sequencing to the Norwegian Sequencing Centre. TruSeq Stranded mRNA Library Prep solution (Illumina, USA) was used to prepare mRNA sequencing libraries from each of the 16 samples of 400 ng of RNA. Pooled libraries were subjected to paired end 150 bp sequencing using NovaSeq 6000 system with NovaSeq 6000 SP reagent kit (Illumina). PhiX library (Illumina) was used as spike-in for sequencing. The RNA-seq data has been uploaded to NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) database under BioProject PRJNA914516.

Low-quality reads and adapter sequences were trimmed using BBDuk (part of BBMap v34.56 toolkit; https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/) with following options: ktrim = r k = 23 mink = 11 hdist = 1 tbo tpe qtrim = r trimq = 15 maq = 15 minlen = 36 forcetrimright = 149. Clean and adapter removed reads were mapped to the published killer whale genome (NCBI GCF_000331955.2) using HISAT2 v2.1.0 with an option –rna-strandness RF (Kim et al., 2019). FeatureCounts v1.4.6-p1 (Liao et al., 2014) was used to count the fragments mapping to the genes (parameters: -p -s 2) (Table A.8). DESeq2 (Love et al., 2014) was used to detect differentially expressed genes between the individuals exposed to 0.1×, 1×, 10× concentrations of the POP mixture and solvent control, comparing all to all. The

differential expression results were visualized using SARTools (Varet et al., 2016) for quality control.

The list of differentially expressed genes (DEGs) was generated in differential expression analysis using DESeq2 method, with the DESeq2 cutoff values of multiple test adjusted p-value (pAdj) <0.05 and an additional fold-change cutoff of 1.5. Hierarchical clustering was performed using log2-transformed normalized RNA-seq read counts in Qlucore Omics Explorer version 3.4 (Qlucore AB, Lund, Sweden). The expression profile of the top genes (ANOVA, p-value <0.003, q-value <0.65) is presented as a heatmap (Fig. 3).

The list of DEGs (Table A.9) was used in pathway, gene ontology (GO) biological process and network enrichment analysis in Cytoscape (Shannon et al., 2003) using the ClueGo application (Bindea et al., 2009) and the ToppGene suite (Chen et al., 2009) using GO, KEGG, Wikipathway and Reactome databases based on human annotations. Bonferroni step-down correction (pAdj < 0.05) was applied to identify significantly enriched networks, pathways, and processes. Further, functional enrichment analysis of the DEGs was carried out using the ToppGene suite and the Benjamini–Hochberg procedure with a threshold of <0.05 was applied to control the false discovery rate.

3. Results and discussion

3.1. Establishment and cultivation of cell culture

Primary cells were successfully established from four out of five killer whale biopsies. The cultured cells were assumed to be fibroblasts based on visual observations of the large, elongated, spindle morphology of the cells and adherence to plastic (Fig. 1C). The first primary cells were observed 4–7 days after biopsy processing and a 90–95% confluent monolayer around the explants was observed after 13–14 days. The cell yield at P1 ranged from 7.8 to 11.4 × 10^5 (Table A.1) and the two highest yields were obtained from apparently young males. Establishment of humpback whale fibroblast cell cultures were only successful for two out of nine biopsies collected, where the cell culture which gave the highest passage number (>30) were from a youngling (Burkard et al., 2015).

Cells seeded at higher densities (>4000 cells cm^{-2}) showed higher abilities to proliferate to confluency than cells seeded at <3000 cells cm^{-2}, which did not proliferate sufficiently. The influence of density was more pronounced in cells at higher passages, which failed to proliferate when seeded at low densities. The cells' morphology also changed with increasing passage number. They increased in size and their distinct spindle-shape (Fig. 1C) turned into an undefined shape with many spikes and a wider cell body (Fig. A.5). After passage 5–7, the morphology of the cells had fully changed to large and smudgy cells, resembling senescent fibroblasts (Beck et al., 2020; Sanders et al., 2013). Accelerated cellular senescence may occur in response to stress exposure, where the cell's growth abilities are altered, and the cell is driven into permanent cell cycle arrest. Sherr & DePinho (2000) considered senescence to be a factor of either extrinsic or intrinsic stress including a literal "culture-shock" in response to changes in external environment (e.g., growth on plastic) or stress related to pollutant exposure.

3.2. Transcript levels of genes in cells exposed to the POP mixture

CYP1A1 was the only gene of the 11 genes quantified by ddPCR that was significantly affected by the POP mixture: its expression decreased with increasing concentration of the POP mixture and was significantly lower following 1× and 10× POP treatment compared to *CYP1A1* expression in control cells (Fig. 2; $p = 0.036$ and $p = 0.05$, respectively). The expression of *ADIPOQ*, *CYP4A*, *ERA*, *GR*, *PPARA*, *PPARG* and *THRA* tended to be upregulated in cells exposed to 10× POP, while *CD36* showed a tendency to be downregulated (Fig. 2). These differences were, however, not statistically significant due to high variation within the technical replicates (Fig. 2).

CYP1A, which is regulated by AHR, is an enzyme involved in biotransformation of planar aromatic hydrocarbons (Godard et al., 2004; Lewis et al., 1998; Teramitsu et al., 2000). The decrease of *CYP1A1* expression in killer whale fibroblasts exposed to the POP mixture may be related to antagonistic effects of non-planar PCBs on CYP1A1, as observed in murine B cells (Suh et al., 2003) Studies on human skin fibroblasts have also shown that *CYP1A* induction is low or even repressed after exposure to (planar) ligands of AHR, possibly due to

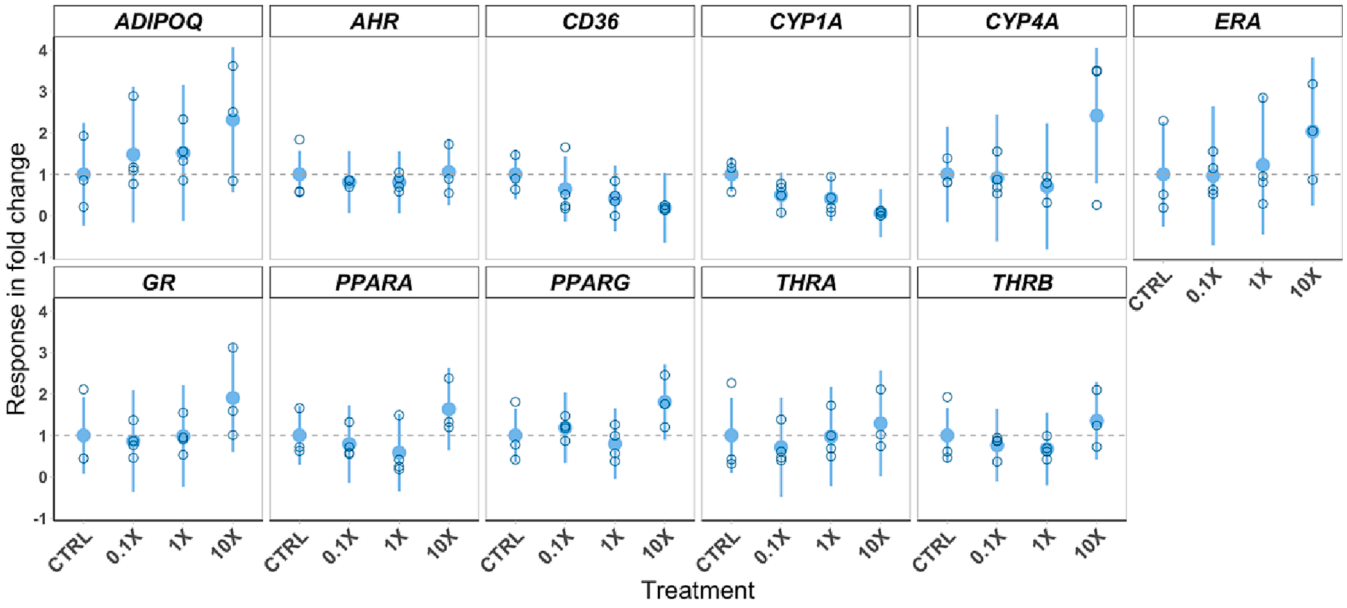

Fig. 2. ddPCR results for genes of interest of killer whale primary fibroblasts after exposure to different concentrations of a POP mixture (0.1×, 1× and 10×; n = 4, n = 3 and n = 4, respectively). 1× reflects median concentration present in blubber of killer whales sampled in Norway. Results are presented as fold change over solvent control (CTRL; n = 3), following normalization after expression of housekeeping gene *GAPDH*. Observations are presented as hollow circles, while estimates with the 95% confidence intervals derived from linear models are presented as point range. The results are considered significant if the confidence intervals do not cross the dashed line.

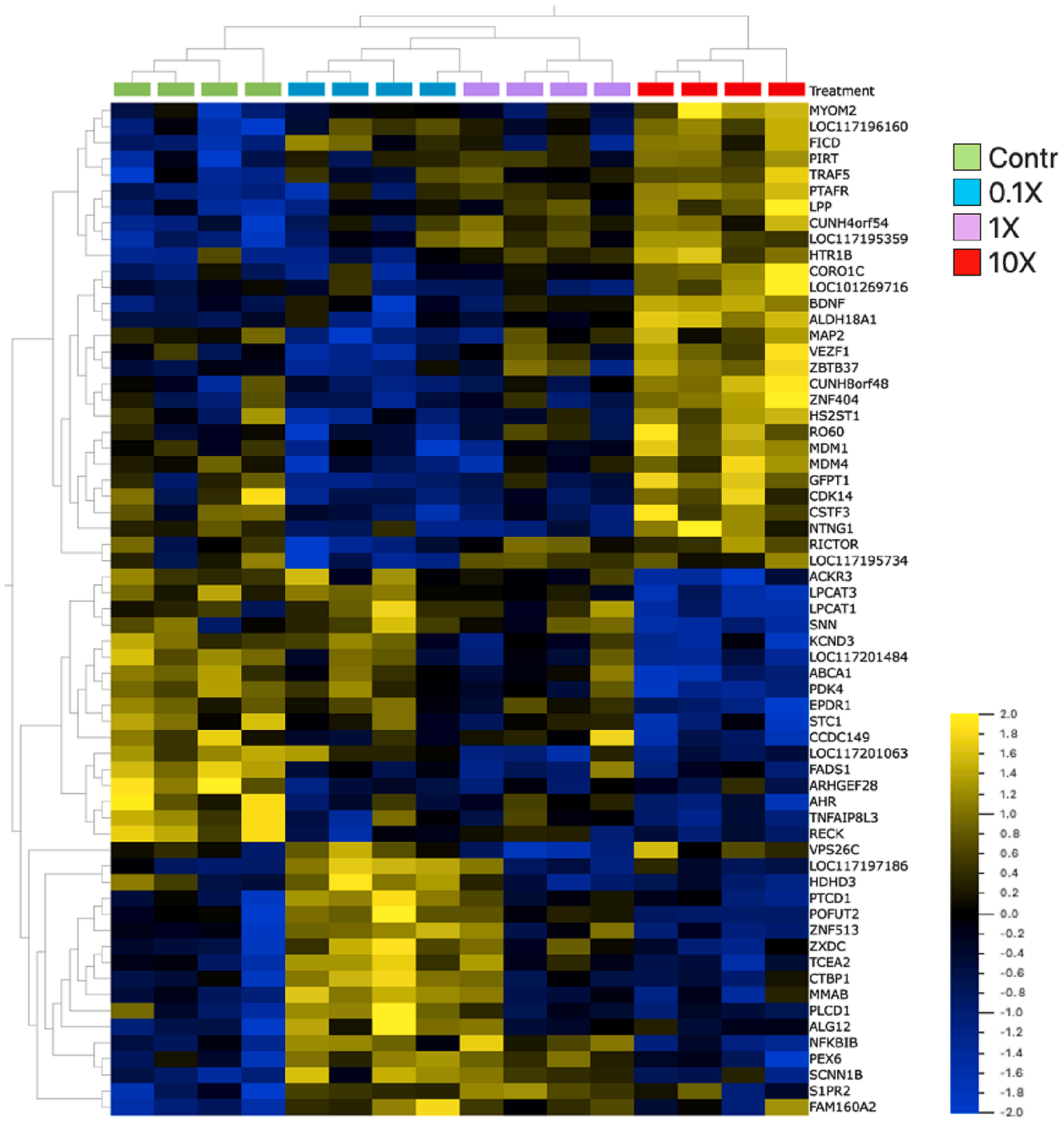

Fig. 3. Hierarchical clustering of the genes in killer whale primary fibroblasts exposed to different concentrations of a POP mixture (0.1×, 1×, 10× and DMSO control (Contr)). The heatmap shows the top discriminating genes based on multi-group comparison (ANOVA, Qlucore OMICs Explorer). Data represents normalized log2-transformed expression values (RNA-seq read counts). The heatmap shows relative expression levels relative to expression of each gene in the different samples. Yellow and blue ends represent highest and lowest relative expression, respectively. Rows and columns represent genes and samples, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activation of aryl hydrocarbon receptor repressor (AHRR) or other mechanisms (Tigges et al., 2013; Evans et al., 2008; Gradin et al., 1999). Furthermore, a study on killer whale fibroblasts found that the expression of CYP1A1 was lowest at the highest exposure concentration to Arochlor1260, *p,p'*-DDT and *p,p'*-DDE mixture in killer whale fibroblasts, although no clear dose–response was observed (Marsili et al., 2012). Field-based studies on false killer whale blubber (*Pseudorca crassidens*) and white whale liver have suggested that CYP1A1 expression may be suppressed in the most polluted individuals (Foltz et al., 2014; Wilson et al., 2005). However, several cetacean studies have also reported higher CYP1A levels in animals with higher tissue levels of pollutants (mainly ΣPCBs or planar PCBs) (Fossi et al., 2010; Noël et al., 2014; Panti et al., 2011; White et al., 1994; Wilson et al., 2005), while no significant correlation was found between *CYP1A* in the skin and levels of ΣPCBs or subgroups of PCBs in the blubber of Cuvier's beaked whale (*Ziphius cavirostris*) (Baini et al., 2020), or between *CYP1A1* levels in skin and ΣPCB and ΣDDT levels in blubber of low polluted (ΣPCBs: 0.42–3.97 ug g^{-1} lw) killer whales from Antarctica (Panti et al., 2022). The POP

mixture-mediated decrease in CYP1A1 expression is likely to decrease killer whale's ability to detoxify planar POPs. This is of particular concern to whales, which likely have a reduced ability to detoxify environmental pollutants due to evolutionary loss of central transcription factors (pregnane X receptor and constitutive androstane receptor) for enzymes involved in detoxification (Hecker et al., 2019).

PPARG, PPARA and their target genes *ADIPOQ* and *CYP4A*, respectively, tended to be upregulated in killer whale cells exposed to the highest concentration of the POP mixture. Our findings are in accordance with previous field-based studies, which indicated that the major regulator of adipogenesis, *PPARG,* and *ADIPOQ* expression increased with the exposure to ΣPCBs, and PCB-118, and *trans*-nonachlor, respectively in polar bear adipose tissue, and with ΣPOPs in ringed seal tissues (Castelli et al., 2014; Tartu et al., 2017). However, Buckman et al. (2011) did not report any relationships between *PPARG* and *ADIPOQ,* and ΣPCB exposure in killer whales. Our results are partly in line with an *in vitro* receptor activation studies, which reported both agonistic and antagonistic effects of several PCBs (including PCB-52, 101, 118, 138, 153), but mainly antagonistic effect of DDTs (including *p,p'*-DDE), on the transcriptional activity of fin whale PPARG (Lühmann et al., 2020), and agonistic effects of a PCB mixture Aroclor 1254 on polar bear PPARA (Routti et al., 2019). It was surprising that another target gene of *PPARG*, *CD36* (Zhou et al., 2008), tended to be downregulated in killer whale fibroblasts exposed to the POP mixture. However, the downregulation of *CD36* should be interpreted with caution, due to the low expression levels (Fig. A.3). Given the important role of PPARs in cell proliferation, survival, differentiation and metabolism (Feige et al., 2006), the results suggest that exposure to the POP mixture may interfere with these functions in killer whale fibroblasts.

ERA and *THRA* expression showed a tendency of upregulation in relation to increased exposure of the POP mixture, which is consistent, at least for PCBs, with findings of a correlative study on killer whales (Buckman et al., 2011). Other field-based studies have also shown a positive correlation of *THRA* and *ERA* expression with PCB levels in harbor seal blubber (Noël et al., 2017; Tabuchi et al., 2006), and with *ERA* levels in ringed seal liver (Brown et al., 2014). Disruptions of these nuclear receptors involved in regulation of estrogen and thyroid hormones may cause disorder in regulation of estrogenic dependent processes, such as growth and reproduction (Cooke et al., 2001; Matthews and Zacharewski, 2000), or impact development, metabolism, or cell differentiation, respectively (Yen, 2001). The indistinct response of *THRB* in this study is in accordance with previous studies (Buckman et al., 2011; Noël et al., 2014; Tabuchi et al., 2006).

The tendency of upregulation of *GR* following the POP mixture exposure in killer whale cells is consistent with previous correlative studies on ringed seals and harbor seals showing positive correlation between *GR* expression and blubber levels of PCBs (Brown et al., 2014; Noël et al., 2017). *GR* plays a significant role in stress and immune responses, and alterations in the signaling pathway of glucocorticoid may interfere with these vital functions (Odermatt et al., 2006).

3.3. Expression profile of top affected genes

Using RNA-sequencing we found 14 and 43 differentially expressed genes (DEGs), with the DESeq2 cutoff values (pAdj < 0.05) and an additional fold-change cutoff (greater than or equal to 1.5 for upregulated and less than or equal to 0.67 for downregulated genes) in the 0.1× and 10× concentration treatment groups, respectively (Table A.9). There were no common DEGs between the two groups. No DEGs were detected in the 1× concentration treatment group. Different sets of genes were, in general, differentially expressed in the 0.1× and the 10× groups (Fig. 3). However, dose response trends were apparent, and are shown in Fig. 3 for the strongest upregulated (the top cluster) and downregulated genes (the middle cluster) for the 10× concentration.

3.4. Pathway, gene ontology and network analysis

The combined list of DEGs from 0.1× and 10× concentrations (Table A.9) that was used in pathway, GO biological process, and network enrichment analysis indicated that the pathways affected by pollutant exposure were largely related to glucocorticoid receptor (GR), myogenesis, cellular response to drugs, Vitamin B12 and lipid metabolism (Fig. 4). Further functional enrichment analysis of the DEGs showed that many top GO biological process, molecular function and cellular component terms were related to muscle cell proliferation, structure, and function (Table A.10 A-C), which suggests that compounds in the POP mixture may promote fibrogenesis. The effects on muscle cell structure and function seem consistent with previous reports of fibrogenic effects of POPs (Das et al., 2006; Diamond et al., 2008). Indeed, ΣPCBs and *p,p'*-DDE have been associated with development of interfollicular fibrosis in harbor porpoise thyroids (Das et al, 2006).

Pathway analyses also indicated the GR pathway and lipid metabolic processes to be affected by the exposure to the POP mixture in killer whale cells (Fig. 4; Table A.10A). For example, one of the primary GR target genes, angiopoietin-like 4 (*ANGPTL4*), that is involved in triglyceride metabolism (Koliwad et al., 2009) was downregulated. This may be related to antagonistic effects of DDTs, PCB-138 and a whale POP mixture on transcriptional activity of whale GR (Lühmann et al., 2020). The receptor activation experiments were done with the ligand binding domain of fin whale GR, which is identical to killer whale GR (Lühmann et al., 2020). Upregulation of transcript levels of *GR* seen the ddPCR analyses may occur to compensate antagonistic effects of POPs on the transcriptional activity of the receptor. Another gene, that plays a role in fibroblast metabolism, pyruvate dehydrogenase lipoamide kinase isozyme 4 (*PDK4*) (Bolfer et al., 2020), was also downregulated in cells exposed to 10× concentration of the POP mixture (Table A.9).

The transcriptome analysis also showed a downregulation of *AHR* and its target gene *CYP1A1* involved in the pathways of nuclear receptor activity and ligand-activated transcription factor activity (Fig. 4). Downregulation of *CYP1A1* is consistent with the results from the ddPCR analysis (Fig. 3). Additionally, genes that inhibit apoptosis and enhance immune function (nuclear receptor subfamily 4 group A member 3 (*NR4A3*) (Shimizu et al., 2015), and interleukin 6 (Tanaka et al., 2014), TNF receptor associated factor 5 (*TNFRA5)* (Tada et al., 2001)) were downregulated in the cells exposed to 10× POP concentration (Table A.9). Enhanced apoptosis was also observed in fibroblasts of finless porpoises exposed to individual PCBs and DDTs (Ochiai et al., 2020).

3.5. Implications

This study successfully established a killer whale fibroblast cell culture, which enabled us to study responses to an environmentally relevant mixture of POPs at gene transcript level in killer whales, which is considered a "data deficient" (Reeves et al., 2017) keystone species in the ocean (Williams et al., 2004). The current study provides cause-effect relationships and support correlative studies on contaminant effects in killer whales and other marine mammals. Taken together, the results are highly relevant for environmental and chemical management. The establishment of the killer whale fibroblast cell cultures also provide possibilities to test toxic responses to emerging and legacy pollutants individually or in combination. However, the cultivation protocol of killer whale fibroblasts must be further optimized to improve cell proliferation, prevent early senescence, and thus allow the use of more replicates in toxicological testing to reduce variance and increase the power of the statistical analyses.

The application of ddPCR and RNA-seq indicated that the tested POP mixture interferes with transcription of genes involved in various biological processes in killer whale fibroblasts. The effects occurred at environmentally relevant concentrations, which corresponded to the concentrations at which PCBs affected the viability of toothed whale

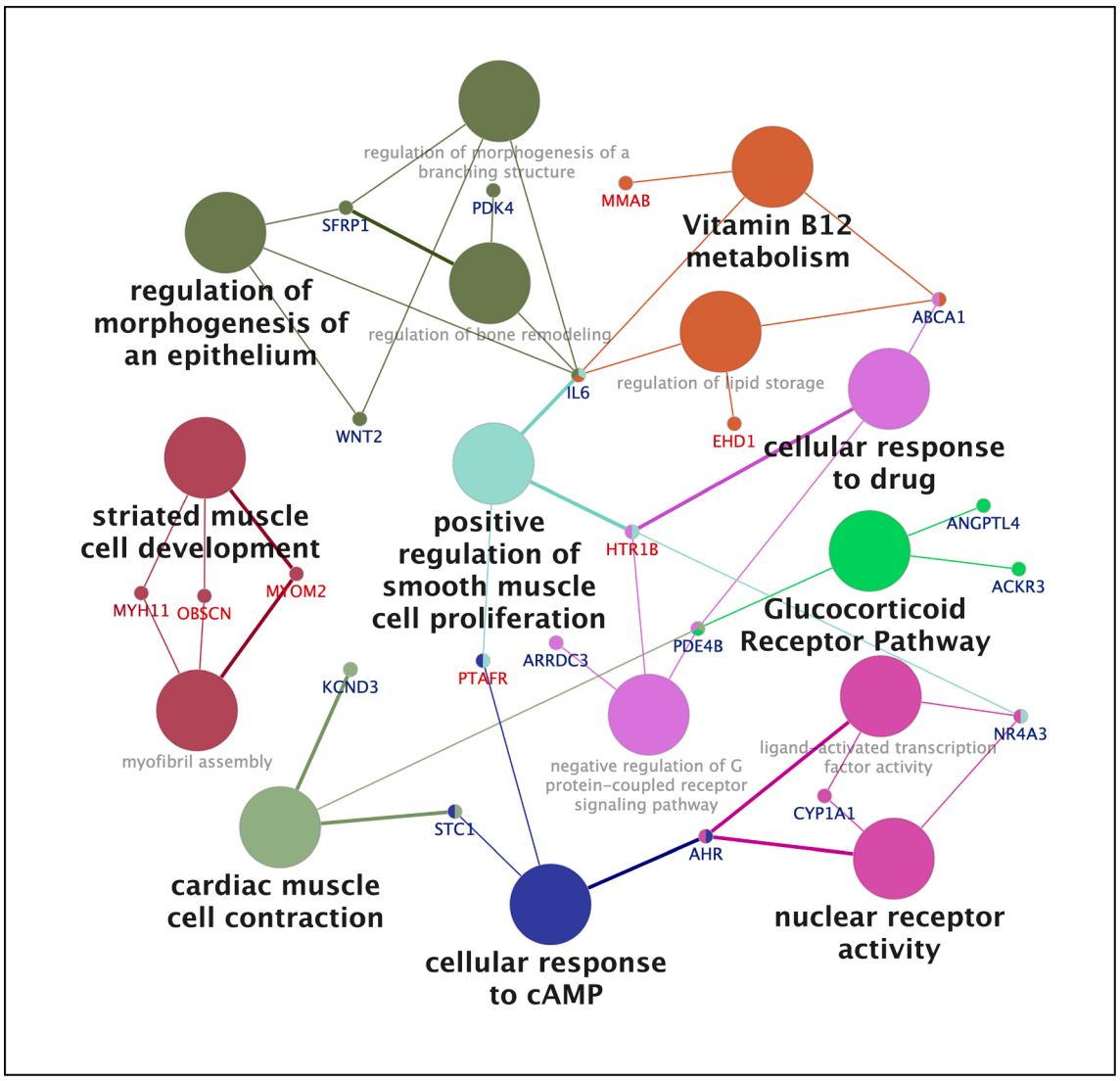

Fig. 4. Enriched pathways and networks visualized using ClueGo application in Cytoscape in killer whale primary fibroblasts exposed to a POP mixture. Significantly enriched networks generated using ClueGo with gene ontology biological process (GO BP), Wikipathway, KEGG and Reactome databases are shown. The label of the most significant term/node is highlighted in each group of functionally related pathway/GO BP terms. Functionally related nodes and edges share the same color. Gene symbols are shown in red, and blue color for up- and downregulated genes, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fibroblasts *in vitro* (Sun et al., 2022). The observed responses do not represent the full *in vivo* response as the results solely represent a snapshot response in one cell type among many. In the future, they could be applied for example in quantitative *in vitro* to *in vivo* extrapolation (QIVIVE; Yoon et al., 2015), which first would require a development of a physiologically based pharmacokinetic model for killer whales. It should also be recognized that none of the sampled individuals were "clean" of pollutants, due to the existing levels of pollutants in killer whales roaming the Norwegian coast (Andvik et al., 2020). The pollutants may already be interfering with cellular mechanisms prior to sampling, and the cells can thus not be concluded to represent a pristine baseline.

4. Conclusion

This study describes the establishment of cell cultures from killer whale biopsies, which enabled us to investigate transcriptomic responses to a mixture of the 10 most abundant POPs they are exposed to. Application of ddPCR and RNA-seq suggested that the POP mixture exposure led to altered transcription of a relatively small number of genes involved in various biological processes. In addition, the study established cause-effect relationships between environmentally relevant concentrations of pollutants and transcriptomic responses in a data-deficient toothed whale. Although the study gives insights into which biological pathways may be compromised by pollutant exposure in killer whale cells, toxic responses at individual level remain to be investigated by future studies. The fibroblast cultures established provide opportunities for testing toxic responses to emerging and legacy pollutants, individually or in mixture and other stressors. Both knowledge on toxic responses of current-used chemicals as well as mixture effects are highly needed for management and use of industrial chemicals.

CRediT authorship contribution statement

J. Bjørneset: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **P. Blévin:** Supervision, Writing – review & editing. **P.M. Bjørnstad:** Data curation, Writing – original draft. **R.A. Dalmo:** Resources, Methodology, Supervision. **A. Goksøyr:** Conceptualization, Resources, Writing – review & editing. **M. Harju:** Resources, Writing – review & editing. **G. Limonta:** Investigation, Writing –

original draft, Writing – review & editing. **C. Panti:** Investigation, Writing – original draft, Writing – review & editing. **A.H. Rikardsen:** Resources, Writing – review & editing. **A.Y.M. Sundaram:** Data curation, Writing – original draft. **F. Yadetie:** Writing – original draft, Writing – review & editing. **H. Routti:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The RNA-seq data in killer whale fibroblasts exposed to pollutants have been uploaded to NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) database under BioProject PRJNA914516. The data on quantity and quality of isolated RNA and transcript levels of selected genes in killer whale fibroblasts have been uploaded to Norwegian Polar Data Centre (https://doi.org/10.21334/npolar.2022.a6d075bb).

Acknowledgments

Emma Vogel helped with biopsy sampling. Morten Tryland, Eva Marie Breines, Demetri Spyropoulos, Alex West, Michael Burkard, Katharina Lühmann and Rhîan Gaenor Jacobsen provided helpful advice for cell culturing. Filipe Figueriedo assisted with the bioanalyzer, and Dhivya Borra Thiyagarajan helped with RNA isolation. Demetri Spyropoulos from the Medical University of South Caroline kindly provided the SolnIX cryopreservation solution for this study. Maria Cristina Fossi hosted the transcripts analysis in her laboratory and provided helpful advice. This study was funded by the "Hazardous Substances" flagship program (462/602019 and 602018 - H. Routti) and CLEAN program from the Fram Centre.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107915.

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