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Establishment of killer whale cell cultures and their responses to pollutant exposure

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Cover photo: Killer whales (Orcinus orca) spotted during field work in Andenes, Norway in the beginning of April 2021. Photo: Juni Bjørneset.

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Abbreviations

3Rs	Replacement, Reduction and Refinement
ADIPOQ	*Adiponectin*
AHR	*Aryl Hydrocarbon Receptor*
CAR	*Constitutive androstane receptor*
CD36	*Cluster of Differentiation 36*
CFDA-AM	5-Carboxyfluorescein diacetate
CHL	Chlordanes
CYP1A1	*Cytochrome P450 1A1*
CYP3A	*Cyochrome P450 3A*
CYP4A	*Cytochrome P450 4A*
ddPCR	Droplet Digital Polymerase Chain Reaction
DDT	Dichlorodiphenyltrichloroethane
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Ham)
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
ERA	*Estrogen Receptor α*
FABP4	*Fatty Acid Binding Protein 4*
FBS	Fetal Bovine Serum
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
GM	Growth Medium
GR	*Glucocorticoid Receptor*
HCB	Hexachlorobenzene
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKG	Housekeeping Gene
HSF	Hooded Seal Skin Fibroblast
IL-10	*Interleukin 10*
IUCN	International Union for Conservation of Nature
IWC	International Whaling Commission
L-15 Medium	Leibovitz's L-15 Medium

L-Glut	L-Glutamine
MEM NEAA	MEM non-essential amino acids
MT1	*Metallothionein 1*
p,p'-DDE	Dichlorodiphenyldichloroethylene
pbASCs	Polar Bear Adipose tissue-derived stem cells
PCB	Polychlorinated biphenyl
Pen/Strep	Penicillin-Streptomycin
POP	Persistent Organic Pollutant
PPARA	*Peroxisome Proliferator-activated Receptor α*
PPARG	*Peroxisome Proliferator-activated Receptor γ*
P x	Passage number x
PXR	Pregnane X Receptor
RIN	RNA Integrity Number
TERT	Telomerase Reverse Transcriptase
THRA	*Thyroid Hormone Receptor α*
THRB	*Thyroid Hormone Receptor β*
TRizol	TRizol Reagent
Trypsin	Trypsin-EDTA Solution
YWHAZ	*Tyrosine 3-monooxygenase*

Abstract

The cosmopolitan apex predator killer whale (*Orcinus orca*) is one of the species worldwide with the highest levels of persistent organic pollutants (POPs) in their tissues. The knowledge of how POPs may affect the species is limited, likely due to the ethical, legal, and practical challenges of researching toxicology on free-ranging marine mammals. The use of *in vitro* models has proved to be a functional tool which may provide information of cellular responses to POPs exposure in marine mammals. Skin biopsies of killer whales were sampled from the Northern Norwegian fjords during aggregation of killer whales for foraging on spring spawning herring. Primary fibroblast-like cells were established from the dermis of 11 out of 13 killer whales sampled. Cytotoxic and gene transcript analysis were conducted on the fibroblast-like cells exposed to ecologically relevant concentrations of POPs, reflecting the 10 most abundant POPs found in Norwegian killer whales. The pollutants were applied at different concentrations to explore dose-dependent responses. Significant downregulation of the target gene *CYP1A* was observed at medium and highest exposure concentration, as well as non-significant tendencies of another downregulated gene (*CD36*) and seven upregulated genes (*ADIPOQ*, *CYP4A*, *ERA*, *GR*, *PPARA*, *PPARG* and *THRA*) at the highest concentration of POPs exposure. This study provides a successful establishment of killer whale fibroblast-like cells, as well as proven gene alterations of the cells exposed to POPs.

Keywords: Orcinus orca; fibroblast cell culture; persistent organic pollutants (POPs); toxicology; Polychlorinated biphenyl (PCB); Dichlorodiphenyldichloroethylene (DDE)

1 Introduction

Environmental pollutants from anthropogenic sources are ubiquitous in marine ecosystems and act as major stressors for marine biota (Ashraf, 2017). One of the most abundant groups of chemical pollutants is persistent organic pollutants (POPs), of which organochlorines (e.g. chlordanes (CHLs), dichlorodiphenyltrichloroethanes (DDTs) and polychlorinated biphenyls (PCBs)) are prevalent in wildlife (Arctic Monitoring and Assessment Programme, 2018). POPs were widely used in the industry over several decades and are resistant to environmental degradation (Alharbi et al., 2018). Owing to persistent properties, POPs have the capability to travel long distances, potentially affecting remote areas far away from their sources (Jones & De Voogt, 1999). Once deposited in marine ecosystems, POPs biomagnify across food webs and bioaccumulate in marine organisms. A lot of the pollutants taken up through diet are accumulated into the tissues of animals during their lifetime, as many species have a limited ability to metabolize POPs. Consumption of prey with bioaccumulated pollutants results in biomagnification of pollutants in the food web (Suedel et al., 1994). POPs are toxic and general effects of exposure 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). Both governmental and conservation organs have acknowledged the presence of POPs as one of the ocean's greatest challenges, and it has been addressed as a global concern (UN Ocean Decade, 2021). Levels of POPs are still prominent in the environment, even though the first regulations occurred by national bans in 1970s, and international regulations were commenced shortly after. In 2004, the Stockholm Convention on Persistent Organic Pollutants were put into force as a global treaty with the objectives to regulate and eliminate the use and production of POPs, and it is now signed by 185 parties (Stockholm Convention on POPs, http://chm.pops.int/). Toxicity of pollutants is challenging to study in wild animals. Nonetheless, for environmental management and chemical regulatory purposes it is essential to understand the consequences that environmental pollutants may exert in marine wildlife.

Killer whale (*Orcinus orca)* is a cosmopolitan apex predator of the marine environment. Despite the supremacy as a species in the ocean and function as a keystone species in many ecosystems, the killer whale is listed as "data deficient" in the IUCN Red List (Reeves et al., 2017). Like many other marine mammals, the killer whales were subject to long history of hunting and live capturing, and the worldwide population trend is still unknown (Reeves et

al., 2017). Additionally, they still face numerous threats such as direct anthropogenic disturbance, prey depletion and chemical pollution (Dietz et al., 2019; Jourdain et al., 2019). Killer whales are perhaps one of the most polluted species on the Earth (Jepson & Law, 2016; Letcher et al., 2010). As long-lived apex predators, they are subject to both bioaccumulation and biomagnification of pollutants. Furthermore, an evolutionary loss of *pregnane X receptor* (*PXR*) and *constitutive androstane receptor* (*CAR*), which are transcription factors for central detoxification enzymes, has reduced marine mammals' ability to detoxify environmental pollutants (Hecker et al., 2019).

Despite large differences in behavior, habitat, and diet within the species worldwide, the killer whales are still recognized as one species. These differences cause large variations of concentrations of pollutants. Killer whale ecotypes including marine mammals (e.g. seals) in their diet are exposed to significantly higher pollutant levels than those feeding mainly on fish (Andvik et al., 2020). There are also large individual variations; females transfer lipophilic pollutants through lactation to offspring, resulting in lower pollutant levels in lactating females than non-lactating individuals (Atkinson et al., 2019). It is currently widely discussed how PCBs and other POPs may affect killer whales at population level (Desforges et al. (2018) and related eLetters). Desforges et al. (2018) predicted a collapse of >50% of the world's killer whale populations induced by PCBs through effects on immune system and reproduction (Desforges et al., 2018). In that sense, one highly polluted pod of resident killer whales in the UK are already "doomed for extinction" due to severely reduced reproduction capabilities (Beck et al., 2014; Jepson et al., 2016).

The killer whale is the most polluted marine mammal species in the Norwegian Arctic (Blévin et al., in preparation). It was long believed that killer whales roaming the Norwegian coast solely fed on herring and therefore held lower PCB levels than other subpopulations (Jourdain et al., 2019). However, the killer whales have showed habits of prey switching, and the Norwegian seal-eating killer whales have now been suggested to hold PCB-levels above the threshold for immune and endocrine disruption resulting in health effects, derived from both laboratory rat studies and a feeding experiment on harbour seals (*Phoca vitulina*) (Andvik et al. 2020; Dietz et al. 2019). Nevertheless, exact physiological consequences and potential spill-over effects of POPs on population dynamics are still unknown. Despite good overall knowledge about occurrences and concentrations of POPs in killer whales worldwide (Desforges et al., 2018), there is still a lack of information about causal relationships between

POP exposure and adverse health effects, and poor understanding on modes of action of POPs. Nuclear receptors are transcription factors important for endocrine systems. They are normally activated by endogenous compounds such as hormones and lipids, but their activity can also be modulated POPs. For example, Lühmann et al. (2020) reported that POP modulate the transcriptional activity of the peroxisome proliferator-activated receptor γ (PPARG), glucocorticoid receptor (GR) and thyroid hormone receptor β (THRB) in fin whales (*Balaenoptera physalus*).

To date, a lot of the toxicological knowledge on marine mammals relies on correlative field studies (reviewed by Desforges et al., (2016) and Dietz et al., (2019)) and a handful of *in vitro* studies (e.g. Burkard et al., (2015), Lühmann et al., (2020), and Maner et al., (2019)). For instance, a study on a killer whale population from the Northeastern Pacific Ocean suggested an increase in transcript levels of *aryl hydrocarbon receptor* (*AHR*), t*hyroid hormone receptor α* (*THRA*), *estrogen receptor α* (*ERA*), *interleukin 10* (*IL-10*) and *metallothionein 1* (*MT1*) with increasing PCB concentrations in blubber (Buckman et al., 2011). Similarly, in a study conducted on a closely related species, the white whale (*Delphinapterus leucas*), Noël et al. (2014) showed positive correlations between *AHR*, *cytochrome P450 1A1* (*CYP1A1*) and PCB concentrations in blubber (Noël et al., 2014). While correlative approaches may provide valuable indications on potential adverse effects, they have several important inherent limitations: correlative field studies require huge sampling effort to get a decent sampling size which may be ethically discussed/raised, cause-effect relationships cannot be established, confounding factors may bias the results, and mechanistic insight to explain the observed responses is lacking.

One of the most important ethical aspects with animal research refers to the 3Rs principles – Replacement, Reduction and Refinement. The 3Rs were established to ensure and enhance animal welfare in science. Conducting research on whales is challenging due to ethical, technical, and legal issues. Thus, establishing alternative approaches with reduced impacts is needed and has even been requested by the International Whaling Commission (IWC, 2010). Establishment of cell lines further used to perform exposure experiments *in vitro* may provide highly valuable knowledge about toxicological responses in marine mammals as they can provide information on cause-effect relationships, which is important for management and conservation of marine wildlife. *In vitro* studies based on cells derived from a biopsy obtained from free ranging animals is a non-invasive tool. Fibroblasts have many advantages as a cell

model as they are well studied, and cultures can be established from skin biopsies of marine mammals. Fibroblast cultures have been established from several cetaceans including pantropic spotted dolphin (*Stenella attenuata*) (Rajput et al., 2018), humpback whale (*Megaptera novaeangliae*) (Burkard et al., 2015), pygmy killer whale (*Feresa attenuata*) (Yajing et al., 2018), white whale (Gauthier et al., 1998), Yangtze finless porpoise (*Neophocaena phocaenoides asiaeorientalis*) (Wang et al., 2011), striped dolphin (*Stenella coeruleoalba*), bottlenose dolphin (*Tursiops truncates*) and fin whale (Fossi et al., 2006), and striped dolphin (Spinsanti et al., 2008). In addition, they have been applied to study toxic responses of pollutants individually or in a mixture in different marine mammals (Burkard et al., 2015; Marsili et al., 2012). It is of high relevance to study responses of mixtures due to possible additive or synergetic effects, also known as cocktail effects, which would not be detectable if the cells were only exposed to single compounds. Due to a possibly huge interspecific variation of toxicity between closely related species (Boutros et al., 2008), a cell line of species of interest should be established.

The aim of this study was to establish a killer whale fibroblast cell line and use it to characterize the cytotoxic and transcriptional responses to POPs. Specifically, *in vitro* exposure studies were conducted on killer whale cells by exposing them to ecologically relevant concentrations of a POPs mixture reflecting the composition of the 10 most abundant POPs found in blubber of Norwegian killer whales. The pollutant mixture was applied at different concentrations to explore dose-dependent responses. This study was based on a developed methodology successfully applied to humpback whale for cell line establishment (Burkard et al., 2015), and aims to complement our understanding and use of methodologies applied over 10 years ago to establish cell cultures of several marine mammal species, including killer whale (Marsili et al., 2012).

2 Materials and method

2.1 Reagents

The reagents used in establishment of cell culture and following cell handling, RNA isolation and cytotoxicity testing are presented in Table 1 with supplier and product numbers, and information of storage and usage.

Table 1 *Overview of the reagents used in this study with the abbreviations used in parenthesis after reagent name. The supplier and CAS/product number is presented with additional information about storage and usage of the reagents.*

Reagent	Supplier	CAS/product number	Storage	Usage
Chloroform	Sigma-Aldrich	67-66-3	Fridge (+2°C to +8°C)	RNA isolation
5-Carboxyfluorescein diacetate (CFDA-AM)	Sigma-Aldrich	C4916-25MG	Freezer (-20°C)	Cytotoxicity assay
Dulbecco's Modified Eagle Medium - high glucose (DMEM high glucose)	Sigma-Aldrich	D5796 – 500 mL	Fridge (+ 2°C to +8°C, protect from direct light)	Medium for cell growth
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Ham) (DMEM/F12)	Thermofischer (Gibco)	11330-032 - 500 mL	Fridge (+ 2°C to +8°C, protect from direct light)	Medium for cell growth
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650-5X5ML	Room temperature	Freezing medium
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich	59331C-1000ML	Fridge (+2°C to +8°C)	Saline wash for biopsies and cell culture
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F9665-50ML	Freezer (-20°C)	Enhance cell growth
Isopropanol	Sigma-Aldrich	563935	Freezer (-20°C)	RNA isolation

Leibovitz's L-15 Medium (L-15 medium)	Gibco	11415-049	Fridge (+2°C to +8°C)	Medium for cell growth
L-Glutamine (L-Glut)	Sigma-Aldrich	G7513-20ML	Freezer (-20°C)	Essential amino acid and energy source for cells
MEM non-essential amino acids (MEM NEAA)	Thermofischer (Gibco)	11140-050 100 mL	Fridge (+2°C to +8°C)	Medium supplement to enhance cell viability and growth
Nuclease-free water	Qiagen	74004	Room temperature	RNA isolation
Resazurin sodium salt	Sigma-Aldrich	R7017-5G	Dark, room temperature	Cytotoxicity assay
Sodium Pyruvate	Sigma-Aldrich	S8636-100ML, CAS: 113-24-6	Fridge (+2°C to +8°C)	Carbohydrate source for cells
Soln IX	Made by Demetri D. Spyropoulos		Fridge (+2°C to +8°C)	Special made freezing medium
Penicillin-Streptomycin (Pen/Strep)	Sigma-Aldrich	P0781-100ML	Freezer (-20°C)	Antibiotic
Primocin™	InvivoGen	Ant-pm-1	Freezer (-20°C)	Antibiotic and antimycotic
Triton™ X-100 (Triton X-100)	Sigma-Aldrich	T8787-50ML	Room temperature	Control in cytotoxicity assay
TRizol Reagent (TRizol)	Thermofischer (Invitrogen™)	15596026	Fridge (+2°C to +8°C)	RNA isolation
Trypan Blue solution	Sigma-Aldrich	T8154-100ML	Room temperature	Cell counting
Trypsin-EDTA Solution (Trypsin)	Sigma-Aldrich	SLCC5608	Freezer (-20°C)	Cell dissociation reagent

2.2 Sample collection

Collection of killer whale samples was approved by the Norwegian Food Safety Authority and conducted by experienced and certified researchers, approval number: FOTS 24075: *Whalefeast - merking av kysthval utenfor Nord-Norge*. Fieldwork was conducted in November 2020 and April 2021 in the coastal area of Skjervøy, Lyngen and Andenes, located in Northern Norway. Biopsies from 13 killer whales (Table 2) were collected. Biopsies consisted of a small piece of skin/blubber (~4cm long), collected with a darting system shot from a rib boat with an air rifle. All individuals were mostly biopsied close to the base of the dorsal fin. Once collected, biopsies were immediately removed from the dart to separate skin, dermis, and blubber roughly on a glass petri dish using disposable scalpels and cleaned scissors. All equipment used for sampling and handling of biopsy in the field were sterilized with 70% ethanol. The dermis was used for cell culturing. Skin and blubber parts served for other research purposes (e.g. genetic analysis, contaminants, stable isotopes). Dermis, defined as the 1-1.5 cm tissue part between skin and blubber (Figure 2B), was directly placed in a sterile 15 mL plastic vial containing growth medium (GM; 1X DMEM/F-12, HEPES, supplemented with 10% FBS, 4 mM L-Glut, 1X MEM NEAA, 1 mM Sodium Pyruvate, and 100µg/mL Primocin (Table 1)). Samples were kept chilled in a styrofoam box (approx. 4°C) until arrival to a laboratory, and they were subsequently handled within 11-12.5 hours from sampling.

Table 2 *Overview of all killer whale samples collected for cell culturing with field ID given, date and location of sampling. Sex and age were challenging to determine, but they were based on visual observations during sampling. Additionally, information about whether the cells were cryopreserved or not, and if so in which passage, and cell yield at cryopreserved passage are included. Information of further thawing, cultivation, and additional comments are provided.*

ID	Date	Location Norway	Field age class	Field sex	Passage cryopreserved	Cell yield (10^4 cells) at cryopreserved passage	Thawed and cultivated	Comments
KW-11-01	11.11.2020	Skjervøy	Adult	M	P2	25.25		Rinsed in 10X DMSO by mistake
KW-11-02	11.11.2020	Skjervøy	Adult	M	P2	23.25		Rinsed in 10X DMSO by mistake
KW-11-03	11.11.2020	Skjervøy	Young	M	P2	7.25		Rinsed in 10X DMSO by mistake
KW-11-04	11.11.2020	Skjervøy	Young	M	Not cryopreserved	-		Rinsed in 10X DMSO by mistake
KW-11-05	11.11.2020	Skjervøy	Adult	F	P1	31		Rinsed in 10X DMSO by mistake Might be a young male
KW-11-06	11.11.2020	Skjervøy	Adult	F	P1	18.75		Rinsed in 10X DMSO by mistake
KW-11-07	11.11.2020	Skjervøy	Young	F	P1	29		Rinsed in 10X DMSO by mistake
KW-20-09	23.11.2020	Lyngen	Adult	F	P1	78	Yes 12/1, senescent at P5/P6	One vial frozen at P4 (T25 – $6.75*10^4$ cells)
KW-20-10	23.11.2020	Lyngen	Young	M	P1	101.25	Yes 5/3, senescent at P5-P7	One vial thawed early, one more used for exposure experiments
KW-20-11	23.11.2020	Lyngen	Young	M	P1	114	Yes	Used for exposure experiments
KW-20-12	23.11.2020	Lyngen	Young	M	P0 (primary cells frozen from well)	1.5		
KW-20-13	23.11.2020	Lyngen	Adult	F	P1	88.5	Yes 14/4, never settled – so died after thawing	
KW-21-01	30.04.2021	Andenes	Adult	M	Not cryopreserved		Did not reach higher passage than 1-2	Changed coverslips to mesh –good, Changed antibiotic after a while– not good → fungal infection.

2.3 Establishment of primary cells

All biopsy and cell handling were conducted in sterile conditions. Establishment of killer whale fibroblast-like 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 1X DPBS with 100 µg/mL Primocin. The dermal tissue section was then cut into small pieces of ~1-3 mm^3, subsequently washed in a 3-step procedure with 1X DPBS. KW-11-01 to -07 were accidentally rinsed and washed with 10X DPBS. Between 5 to 10 dermis pieces were transferred to a well of a 6-well plate and placed closely together in the center of each well. In this manner, the well of one 6-well plate were filled per biopsy. The pieces were covered by a sterile cover slip applied with a gentle pressure to ensure contact between tissue pieces and plastic surface to facilitate fibroblast attachment and growth. One mL growth medium (GM) was carefully added to each well, and plates were incubated at 37°C and in 5% CO_2 (Sanyo MCO-19AIC (UV) CO_2 Incubator). After ~12 hours, an additional 1 mL of GM was added to each well. Between 50-75% of the GM was changed every 2-3 days, and cell growth was closely monitored under microscope during the growth process. Issues with floating tissue pieces were experienced when adding 2 mL of GM to the wells too early and in wells where the tissue pieces not yet were attached to the plastic-surface. In those cases, GM was removed, tissue pieces were placed centrally again – and gently arrested by a bit harder pressure with multiple cover slips, and 1 ml of GM was then added.

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 trypsin. First, the GM was removed, and wells were washed twice with 5 mL 1X DPBS containing 100 µg/mL Primocin. One mL of trypsin-EDTA (0.25%) was added to each well and removed after 30 seconds. The plates were incubated for 5 min at 37°C and 5% CO_2. Each well of cells were resuspended in 2 x 3 mL of GM (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.

2.4 Cultivation and subculturing

Between 50-75% of the GM was changed every 2-3 days, and cells were split when confluency reached 80-90%. Splitting protocol was similar to the isolation of primary cells described in section 2.3, and normally occurred with a splitting ratio of 1:3. The resuspended cells were seeded in new culture wells and flasks without filtering to prevent cell loss (Table 7).

During seeding of these sub-cultured cells, to determine optimal seeding densities and size of culture dishes, wells, or flasks, killer whale cells were seeded in a variety of cell densities and wells and flasks (Table 7).

The normal splitting ratio of 1:3 often resulted in too low cell densities and slow growth. Thus, the slow growth was particularly a subject of further examination. To acquire specific cell densities for seeding, the resuspended cells were centrifuged at 200 x g for 4 minutes. The supernatant was removed, and the cell pellet was resuspended in fresh GM (at 37°C) with a known volume. In general, cell counting was done during centrifugation steps as the process likely can affect the cell viability. To examine cell viability, 50 µL of the resuspended cells in known volume 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/ml. Following, the number of cells were adjusted to desired seeding density and added to the culture well, plate or flask. All volumes of reagents were adjusted accordingly to the size of wells, flasks, and cell numbers (Table 7).

During cultivation of killer whale cells, problems related to slow growth and senescence were encountered. To overcome these technical challenges, several tests were run with different seeding densities (n= 23), wells (n = 4), flasks (n = 2), and dishes (n = 1) (Table 7). 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 Pen/Strep were tested. All these attempts were not convincing. Thus, another cell type was tested as a model cell for exposure experiments. Established hooded seal (*Cystophora cristata*) skin fibroblasts (HSFs; Table 3) were received from Alexander Christopher West (Lab of Arctic Chronobiology and Physiology, UiT). The HSFs were cultivated using similar procedures, but with another growth medium: DMEM high glucose supplemented with 20% FBS and 1% Pen/Strep (Table 1). The cultivation of HSFs was first done in parallel with testing Pen/Strep on the KW-21-01. Few days after changing

from Primocin to Pen/Strep in the GM, fungal infection was observed in the well. Therefore, the incubator was decontaminated, and all cells including the HSFs were discarded. In the second attempt, the HSFs were thawed at P4. They fully changed morphology to senescent looking cells at P5 and were discarded at P7. The level of CO_2 in the incubator was checked by measuring the pH of GM in empty culturing wells placed in the incubator. However, CO_2 levels were normal. The use of HSFs was not further considered. The experiments were continued on with killer whale cells with limited subculturing.

Table 3 *Information of hooded seal fibroblasts received from Alexander Christopher West (Lab of Arctic Chronobiology and Physiology, UiT) to use in this study. ID, passage when thawed, and highest passage achieved is presented with additional comments.*

ID	Passage when thawed	Highest passage	Comments
Seal skin fibroblast 1.1 / HSC 1	P4 (19/5)	P7	Fungal infection in incubator
Seal skin fibroblast 1.1 / HSC 2	P4 (6/9)	P7	Looked strange after P5, but used for preliminary cytotoxicity assay

2.5 Cryopreservation

Most killer whale cell cultures were cryopreserved at passage 1 (Table 2). Cryopreservation of cells was proceeded in the same way as the splitting protocol described in section 2.4, except for the last step of resuspension in fresh GM. In this step, the cell pellet was resuspended in 1-3 mL of freezing medium (either Soln IX, or 90% FBS + 10% DMSO) after centrifugation. The amount of freezing medium was determined according to the cell density, aiming to get ~ 2.25 x 10^5 cells/mL (i.e. 3000 cells x 75 cm^2) in each cryovial of 1 mL, and ≥ 3000 cells/cm^2 when later thawing cells and seeding one cryovial to a T75 flask. The density of 3000 cells/cm^2 was based on seeding density used for polar bear (*Ursus maritimus*) adipose-derived stem cells, with similar size and behavior as fibroblasts (Routti et al., 2016). Cell densities were checked by Trypan Blue staining and cells were frozen in Corning® CoolCell™ LX Cell Freezing Container (Sigma-Aldrich) in separate cryovials. The freezing container 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.

2.6 Thawing

The frozen cryovial was immersed in a 37°C water bath immediately after removing it from liquid nitrogen. When fully thawed, the cells were resuspended in 10-15 mL GM at 37°C. Thawing and resuspension in GM was done as fast as possible due to the high percentage of DMSO in the freezing medium, which is known to be harmful for live cells (Singh et al., 2017). The thawing protocol was modified through this study. The first cells KW-20-09, KW-20-10 (not the cryovial used for exposure experiment) and KW-20-13 were centrifuged after thawing. One of the cryovials of KW-20-11 was centrifuged and one was not after thawing, and it was noticed that the cells reached a higher confluency more rapidly when not centrifuged after thawing. KW-20-10 thawed for exposure study was not centrifuged. Cells were seeded directly into a T25 or T75 flask at the density of ~3000 cells/cm^2, depending on given cell density of cryovial (Table 7). GM was replaced after 24 hours to remove all DMSO.

2.7 Exposure to persistent organic pollutant (POP) mixture and experimental set up

As challenges with slow growth and senescence at higher passages were encountered, calculations based on number of cryopreserved killer whale cells were made to ensure sufficient cells to conduct the exposure experiments with limited subculturing. Thus, the *in vitro* exposure studies were conducted on cells from KW-20-10 and KW-20-11 owing to their large cell yield (Table 2).

The cells were thawed (section 2.6) in a 37°C water bath without centrifugation, and GM was replaced after 24 hours. The cryovial of KW-20-10 P1 (3.375 x 10^5 cells, ~ 4500 cells/cm^2) was plated in a T75 flask. After 7 days, the cells had grown to 80% confluency and were seeded for the experiment. The two cryovials of KW-20-11 P1 (3.8 x 10^5 cells each, ~ 5000 cells/cm^2) were pooled in one T75 flask, cultured, and reseeded after 7 days after reaching a confluency of 70-80%. KW-20-10 was used for cytotoxicity assay and for running preliminary testing for RNA-isolation with identical set up as the last exposure experiment, and KW-20-11 was used for droplet digital polymerase chain reaction (ddPCR) analysis and transcriptomics analysis (not a part of this thesis).

Killer whale fibroblasts were exposed to ecologically relevant concentrations of POPs, as found in Norwegian killer whales. Mixtures that reflect the composition and concentration range of the 10 most abundant POPs were prepared at Norwegian Institute for Air Research (NILU) based on available data (Blévin et al., in preparation) (Table 4). The persistent organic pollutants mixture (POP mixture) (50X) dissolved in DMSO was made by Mikael Harju at NILU. The standards were purchased from LGC standards and Merck/Sigma-Aldrich, Toronto Research chemicals, Dr. Ehrstorfe. The DMSO used in exposure studies was received from Mikael Harju, and was not the same as the one used for cryopreservation (Table 1).

Table 4 *Overview the composition of the POP mixture based on median concentration of the ten most abundant compoundsin killer whales from Norwegian waters. PCB: Polychlorinated biphenyl; DDE: Dichlorodiphenyldichloroethylene.*

Compounds	**Concentration 1X (µM)**
p,p'-DDE	4.477
PCB153	1.741
PCB138	1.310
trans-nonachlor	0.990
PCB180	0.637
PCB101	0.519
PCB52	0.519
PCB99	0.483
PCB187	0.451
PCB118	0.441
Total concentration	**11.569**

Table 5 *Overview of exposure concentrations of the treatments of POP mixture used in this study.*

Treatment concentrations	**µM**
Median concentration found in killer whale blubber (1X)	11.569
Concentration in killer whale POP mixture (50X)	578.443
20X	231.337
10X	115.689
5X	57.844
1X	11.569
0.1X	1.157
0.01X	0.116

For the cytotoxicity assay, cells from KW-20-10 (P3) were seeded in triplicates in a 96-well plate of a density of 6250 cells/cm^2. The concentrations of POP exposure were 20X, 10X, 5X, 1X, 0.1X and 0.01X (Table 5) dissolved in 0.1% DMSO. Negative controls included GM and GM supplemented with 0.1% DMSO, and GM with Triton X-100 (1%). Triton X-100 was used as positive control as it is known to be cytotoxic to mammalian cells (Dayeh et al., 2004).

Preliminary cytotoxic testing was done with cells from KW-20-10 (P3) to assess and practice the method of RNA-isolation as well as the experiment set up. One 12-well plate was seeded with 5840 cells/cm^2. Cells were seeded for chemical exposure to three concentrations (10X, 1X and 0.1X, Table 5) as well as the GM 0.1 % DMSO control, all in triplicates.

The exposure study for ddPCR analysis was conducted on cells from KW-20-11. Cells from passage 3 and 4 were pooled and seeded in 12-well plates with a density of 7500 cells/cm^2. Cells were exposed to three concentrations of POP mixture: 10X, 1X and 0.1X (Table 5) as well as GM supplemented with 0.05% DMSO, and all treatment included four technical replicates.

For all exposure studies, the cells were left to settle in the wells for 48 hours after seeding, with medium change (100%) after 24 hours. Following, cells were exposed to POP mixture or control medium for 48 hours, after which the exposure medium was discarded, and cells washed in 100 µL 1X DPBS before continuing with either cytotoxicity assay or isolation of RNA.

2.8 Cytotoxicity assay

Metabolic activity and membrane integrity of cells exposed to different concentrations of POPs mixture were assessed using the fluorescent dyes resazurin and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), respectively. One hundred µL sterile filtered (0.2 µm) of resazurin (0.15 mg/mL resazurin, 5%) and CDFA-AM (4mM, 0.1%) in 1X DPBS was added to each well, cells were incubated for 25 minutes at 37°C, 5% CO_2, and were protected from light. Fluorescence was measured at 530/595 nm (ex/em) for resazurin and 493/541 nm (ex/em) for CFDA-AM, with a plate reader (BioTek Synergy H1).

Preliminary testing of the cytotoxicity assay was done with similar methods as previously described on hooded seal cells at P7. However, most resazurin data were inconclusive ("OVERFLOW"), and most cells were senescent-looking before seeding for exposure experiment. The results are therefore not presented.

2.9 Isolation of RNA and quality check

Isolation of RNA from cells for gene transcript analysis was done with TRizol, according to the manufacturers protocol (Thermo Fisher Scientific, 2016). Briefly, the cells were washed with 1X DPBS (37°C) and 900 µL of TRizol was added to each well. Both a cell scraper and pipetting were used to homogenize the lysate. TRizol enables solubilization of cells and its component, and denaturation of proteins. Chloroform was used twice to separate the solution into three phases: 1) organic phase with protein, 2) the interface with DNA and 3) the aqueous phase with RNA (Rio et al., 2010). Isopropanol and ethanol were used to precipitate the RNA. Finally, each pellet was dissolved in 20 µL Nuclease-free water. The RNA yield (ng/µL) and purity was determined using Thermo Scientific NanoDrop 2000 Spectrophotometer. RNA purity was determined using the 260/280 and 260/230 ratios, which reflected the amount of contamination and presence of undesired organic compounds (e.g. chloroform), respectively (Thermofisher, 2021). Assessment of RNA integrity number (RIN) 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.

2.10 ddPCR

2.10.1 Genes of interest (GOI)

Transcript levels of 13 genes of interest (Table 6) were assessed in killer whale cells exposed to the POP mixture at different concentrations. These genes included *AHR, CYP1A, CYP3A, CYP4A, THRA, THRB, GR, PPARA, PPARG, FABP4, ERA, ADIPOQ, CD36,* and two housekeeping genes (HKG): *GAPDH* and *YWHAZ* (Table 6). The genes *ERA, GR, PPARA, PPARG, THRA*, and *THRB* belong to the nuclear receptor superfamily (Weikum et al., 2018). They are nuclear proteins which are activated by ligands with a high affinity to their ligand-

binding domains. They function as transcription factors that regulate expression of target genes (Table 6). Xenobiotic compounds, such as POPs, may interfere with the endogenous ligands, and bind to these ligand-binding domains (Lühmann et al., 2020). In this way, they may function as an agonist or antagonist by increasing or inhibiting gene expression (Matthews & Zacharewski, 2000; Weikum et al., 2018).

Table 6 *Genes of interest analyzed in this study, their abbreviations, and their major cellular involvement.*

Gene of interest	Abbreviation	Major involvement
Aryl hydrocarbon receptor	*AHR*	Detoxification (Rothhammer & Quintana, 2019; Stevens et al., 2009)
Cytochrome P450 1A	*CYP1A*	*AHR* target gene. Detoxification (Teramitsu et al., 2000)
Estrogen receptor alpha	*ERA*	Regulation of estrogenic responses (Matthews & Zacharewski, 2000)
Glucocorticoid receptor	*GR*	Immune system, regulation of inflammation, stress, metabolism, and reproductive and developmental physiology (Sapolsky et al., 2000)
Peroxisome proliferator activated receptor alpha	*PPARA*	Lipid metabolism, and detoxification, and energy metabolism (Feige et al., 2006; Shizu et al., 2013)
Cytochrome P450 4A	*CYP4A*	*PPARA* target gene. Lipid metabolism and detoxification (Ishibashi et al., 2008; Takeuchi et al., 2006)
Peroxisome proliferator activated receptor gamma	*PPARG*	Adipogenesis and inflammatory responses (Lefterova et al., 2014)
Adiponectin	*ADIPOQ*	*PPARG* target gene. Adipogenesis and inflammatory responses (Cristancho & Lazar, 2011; Ohashi et al., 2010)
Cluster of differentiation 36	*CD36*	*PPARG* target gene. Adipogenesis and inflammatory responses (Christiaens et al., 2012; He et al., 2011; Karunakaran et al., 2021)

Fatty acid binding protein 4	*FABP4*	*PPARG* target gene. Adipogenesis and inflammatory responses (Cristancho & Lazar, 2011; Furuhashi et al., 2014)
Thyroid hormone receptor alpha	*THRA*	Thyroid hormone action; including cell differentiation, development, and metabolism (Yen, 2001)
Thyroid hormone receptor beta	*THRB*	Thyroid hormone action; including cell differentiation, development, and metabolism (Yen, 2001)
Cytochrome P450 3A	*CYP3A*	Detoxification (Wilkinson, 1996)
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Housekeeping gene (Spinsanti et al., 2008)
Tyrosine 3-monooxygenase	*YWHAZ*	Housekeeping gene (Spinsanti et al., 2008)

2.10.2 Primer design

Primers were designed using the online software Primer3 (v. 0.4.0) for 13 genes of interest: *AHR, CYP1A, CYP3A, CYP4A, THRA, THRB, GR, PPARA, PPARG, FABP4, ERA, ADIPOQ*, *CD36*, and two housekeeping genes: *GAPDH* and *YWHAZ* (Table 6, Appendix Table A1). The primers were designed on sequences deriving from the fully annotated whole genome assembly (Genebank: GCA_000331955.2) for *Orcinus orca* available on The National Center for Biotechnology Information (NCBI) database. To further check primer specificity, the primer sequences were also blasted and aligned with sequences of other cetacean species (Appendix Table A1). The primers were designed against conserved cetacean regions. Two genes (*CYP3A* and *CYP4A*) were not present in the available annotation for the killer whale genome assembly. Thus, ortholog sequences available for other cetacean species (Appendix Table A1) was checked using BLAST against the killer whale genome assembly to retrieve the gDNA sequence on which the primers were designed. Primers were designed from different exons to avoid the co-amplification of genomic DNA if contamination is present. Primers sequences of 18-22 nucleotides were selected having a melting temperature around 64°C and with less than 2°C difference between forward and reverse oligos melting. The primers were also designed to amplify a region shorter than 200 base pairs for every gene, according to droplet digital PCR requirements. 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 (Appendix) and electrophoresis on agarose gel with ethidium bromide staining. Primer efficiency was tested with cDNA serial dilution as a quality control (Appendix Figure A1).

2.10.3 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 includes a gDNA digestion step before the reverse transcription which reduced the risk of gDNA carryover. The DNase reaction was performed in two steps in a SimpliAmp™ Thermal Cycler, DNA digestion for 5 min at 25°C and DNase inactivation for 5 min at 75°C. The thermal cycle for the reverse transcription was conducted as follows: priming for 5 min at 25°C, reverse transcription for 20 min at 46°C, RT inactivation for 1 min at 95 °C.

2.10.4 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 (2X), 0.2 µL of each primer (10 µM), 5.1 µL of nuclease-free H_2O, and 4.5 µL of cDNA diluted 1:4 (1.875 ng/µL). No template control reactions were included for each gene analyzed. Droplets were generated using 20 µL of reaction and 70 µL of the Droplet Generation Oil for EvaGreen (Bio-Rad) loaded in DG8 Cartridges (Bio-Rad) in the QX200 Droplet Generator (Bio-Rad). The droplets were then loaded in a 96-well plate and the PCR was performed in a SimpliAmp™ Thermal Cycler with the following thermal cycle 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 final 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 for each sample and the relative Poisson interval of confidence. Although the concentrations are absolute quantifications and consequently normalizing is not necessary, the results were normalized over HKG *GAPDH* to adjust for large sample variance and differences in material input, RNA degradation and correct for possible mistakes made, for instance in either the RNA isolation or the retro transcription from RNA to cDNA (Fleige & Pfaffl, 2006). *GAPDH* showed more stable transcript levels over the treatments than *YWHAZ* (Figure 1) and was thus chosen for normalization of the results. The normalized transcript levels of the GOIs showed similar tendencies as the quantified concentrations (Appendix Figure A2 and A3). *CYP3A* and *FABP4* were not detected and were thus not included in further data analyses.

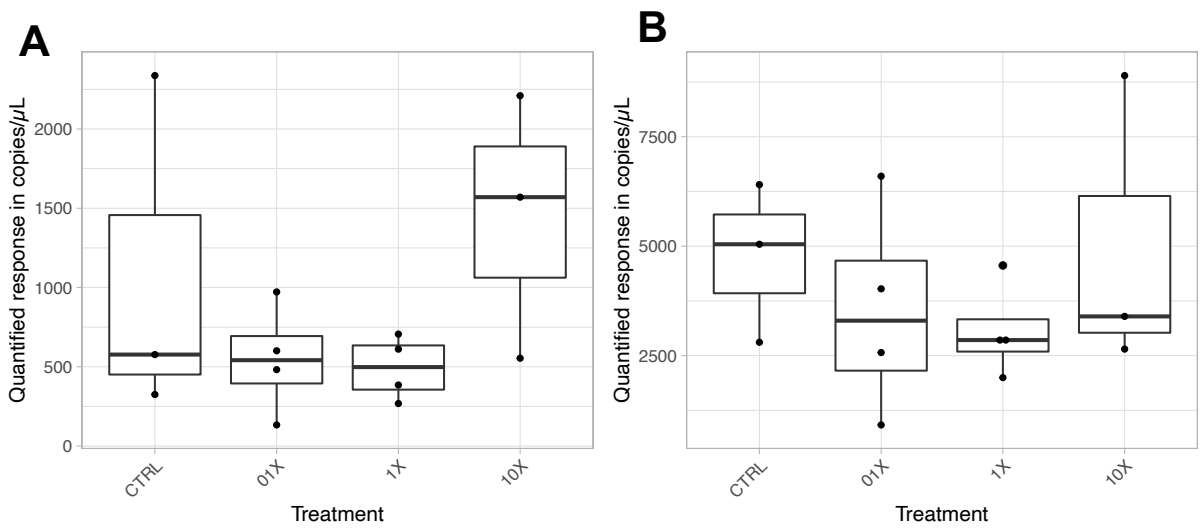

Figure 1 *Boxplot of ddPCR results from killer whale fibroblasts after exposure to different concentrations of POP mixture (01X, 1X, and 10X) and solvent control (CTRL). Results are presented, as the quantified response in copies/µL for the house keeping genes* ***A****) YWAHZ* ***B****) GAPDH. Points represent the observations.*

2.11 Data analysis

Data handling and statistical analysis was conducted using the software R version 4.0.3 (R Core Team, 2020). Cytotoxic effects (metabolic activity and membrane integrity) were assessed as fold change relative to the mean of solvent control (DMSO), where a decrease

indicates toxicity. Linear models were used to assess the cytotoxic effects of exposure to the POP mixtures in relation to the solvent control on the killer whale cells. The treatments including six concentrations of POP mixture (20X, 10X, 5X, 1X. 0.1X and 0.01X, Table 5), GM, Triton X-100, and the solvent control were included as independent factors in the model. The linear models were run separately for cytotoxic endpoints (metabolic activity and membrane integrity). One of the values for solvent control of the membrane integrity test was a ~45% lower (fluorescent read of 4915, compared to 8528 and 9264) than the two other replicates, thus categorized as an outlier and removed from the dataset. For gene expression, ddPCR data was normalized over the expression of HKG *GAPDH*. 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 POP mixture (10X, 1X and 0.1X, Table 5) 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 both cytotoxicity assay and gene expression.

3 Results and Discussion

3.1 Cell type identification

The culture cells were assumed to be fibroblasts based on visual observations of the large, elongated, spindle morphology of the cells (Figure 2A), the growth rates, adherence to plastic when cultured, and growth from the connective tissue in dermis (Figure 2B). However, the cells were not tested with specific fibroblast markers, such as immunoreaction to Vimentin (Boroda et al., 2020; Burkard et al., 2015; Richards et al., 1995; Yajing et al., 2018).

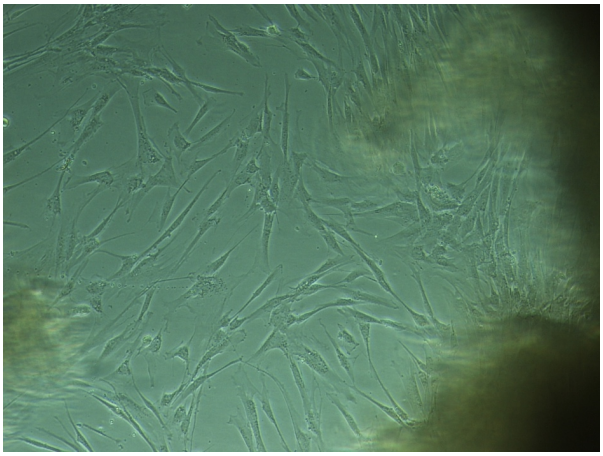

Figure 2A) *Micrograph of killer whale primary cells from tissue, derived from individual KW-21-01, taken on 22.04.2021. Tissue explant is seen at the right.*

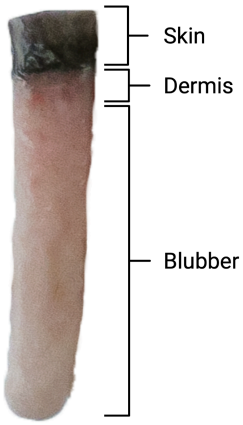

Figure 2B) *Presentation of a killer whale biopsy, with roughly separations of skin, dermis, and blubber.*

3.2 Establishment of primary cells

Primary cells were successfully established from 11 out of 13 killer whale biopsies. In four out of six biopsies washed with 1X DPBS, the first primary cells were observed 4-7 days after biopsy processing. The first cells from the 7 biopsies accidentally washed with 10X DPBS were not observed until day 12-13 after biopsy processing, which indicates a growth rate of approximately half of those washed with 1X DPBS. As it was not known how the exposure to such high DPBS concentration may affect the cells, these cultures were discarded from further experiments. A 90-95% confluent monolayer was observed around the explants washed with 1X DPBS after 13-14 days, which was earlier than humpback whale fibroblasts with the monolayer forming at day 25 (Burkard et al., 2015), pygmy killer whale at day 21 (Yajing et al., 2018), Yangtze finless porpoise at day 20 (Wang et al., 2011), and later that the pantropic

spotted dolphin which formed a monolayer at day 8 (Rajput et al., 2018). Primary cells from killer whales were proliferating faster than those from humpback whales for which the first primary cells were observed at day 14 (Burkard et al., 2015), compared to day 4-7 for the killer whale cells. The growth rate of primary cells from explant has been previously suggested to be dependent on the animal size (Savage et al., 2007), and a faster difference in proliferation rate was therefore expected for cells from killer whales which has a smaller body size than humpback whales, due to the size difference between the species. However, the results of the smaller species pygmy killer whale and Yangtze finless porpoise does not support this theory. The difference in number of days between biopsy processing and a formed monolayer and proliferation rates remains unknown, but sample and tissue handling and/or medium composition may contribute to this difference.

The cell yield at passage 1 (P1) ranged from 1.9 to 11.4 x 10^5 (Table 2), which is generally low as observed for humpback whales (Burkard et al., 2015). P1 cells in T75 flasks originating from KW-20-09, -10, -11 and -13 were cryopreserved on day 18-25, at a confluency level of 70-90%, and a total number ranging from 7.8 to 11.4 x 10^5 cells were cryopreserved from each biopsy, after growth in T75-flasks. The two highest cell yields were obtained from allegedly two young males. Establishment of humpback whale fibroblast cell lines were only successful for two out of nine biopsies collected, where the cells which they were able to passage the most (>P 30) were from a youngling (Burkard et al., 2015). This could indicate that there could be advantages of sampling younger animals for fibroblast establishment.

The cells from one of the six biopsies rinsed with 1X DPBS, KW-20-12, grew considerably slower than the others. The first primary cell was observed after 18 days after biopsy processing, and the cells never grew to a confluent monolayer. The cells were therefore directly frozen as P0 with a concentration of 1.5 x 10^4 cells. The cells from the last individual KW-21-01 were microscopically examined and the first primary cells were observed after 7 days from biopsy processing, like the other successfully established cell cultures. However, the cell proliferation rate hereafter decreased, and the primary cells could not be isolated from the tissue until day 22. This was twice as long as the four others successfully established cell cultures. Further subculturing of KW-21-01 was unsuccessful, and they were therefore discarded. It is difficult to speculate on the reasons for slow growth of cells from these two biopsies. They were both sampled from apparent healthy animals, there was no deviation in the methodology, and no contamination was detected.

3.3 Culturing of killer whale cells

The killer whale cells grew with limited consistency, which makes it challenging to conclude on specific proliferation rates and growth kinetics. Yet, some tendencies were observed; splitting ratio was kept at 1:3 or 1:2, to ensure a sufficient cell density, and cells were normally split every 7-8 days at the confluency level of 70-90%. The seeding density of 3000 cells/cm^2 seemed like a lower limit for killer whale cells. The cells seeded at <3000 cells/cm^2 densities were in general not thriving, and cells seeding at higher densities (<4000 cells/cm^2) showed higher abilities to proliferate to confluency. This is comparable to growth kinetics of white whale fibroblasts of which optimal seeding density was determined to be 5000 cells/cm^2, and cells seeded at 2000 cells/cm^2 or lower were unable to grow to confluency (Boroda et al., 2020).

The higher passages of the killer whale cells were more vulnerable to lower seeding densities and failed to proliferate to higher densities (Table 7). The cells' morphology was shifting as passages increased: The cell size was increasing, and the distinct fibroblast spindle-shape (Figure 3A) changed to a more undefined shape, with an increased number of spikes and a wider cell body (Figure 3B and C). The morphology of the cells had fully changed after passage 5-7 in all cell cultures to large and smudgy cells, resembling senescent fibroblasts (Beck et al., 2020; Sanders et al., 2013). Observed growth arrest after 5-7 passages occurred earlier than compared to established fibroblasts of other marine mammal species. Fibroblasts of pygmy killer whale and Yangtze finless porpoise were passaged 13 and 23 times, respectively, before experiencing growth arrest and morphological changes (Yajing et al., 2018), and the humpback whale fibroblasts were passaged 30 times without showing any signs of growth arrest or morphological changes (Burkard et al., 2015, 2019). 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) consider 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 induced telomere-shortening as a response to e.g. oxidative stress or exposure to pollutants (Sherr & DePinho, 2000). The killer whale cells were not verified as senescent, which could be done with a senescence-associated beta-galactosidase assay (Itahana et al., 2007). However, they were presumed senescent based on visual observations and growth arrest.

The growth of the cells did not seem to be largely affected by the different types of flasks, dishes, and wells, and their varying sizes, except that the cells had a high tendency to cluster in the center of smaller sized wells (≥12-well plate), which made them challenging to disperse evenly.

The L15 medium was tested on one 12-well of cells, without any improved growth; the cells stopped proliferating and the cell shape was altered to a senescent-looking cell after two days. Therefore, using L15 medium for establishment of killer whale primary cells is not recommended.

Due to limited growth consistency of the killer whale cells and limited knowledge within the area, it is challenging to suggest why they differed compared to other whale cell proliferation and growth as identical methods were used. General recommendations for future culturing of killer whale cells are keeping a seeding density of ≥ 4000 cells/cm^2, avoiding smaller sized wells and limiting the number of passages prior to running experiments.

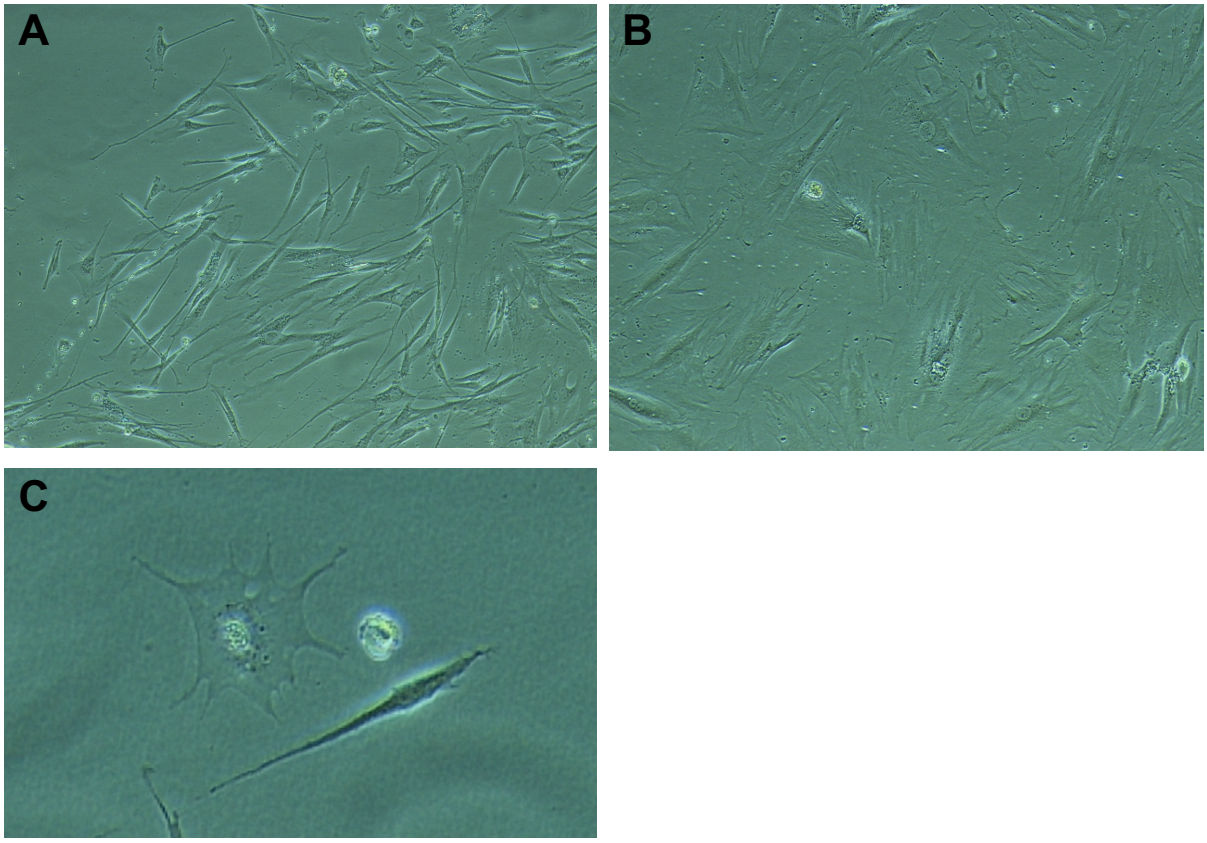

***Figure 3 A**) Primary cells of KW-21-01 observed in 10X objective of inverted microscopy. **B**) KW-20-10 passage 7 observed in 10X objective of inverted microscopy. **C**) One large smudgy cell resembling a senescent fibroblast, one dead shiny cell, and one normal cell resembling a healthy fibroblast, observed in 20X objective of inverted microscopy.*

Table 7 *Overview of dishes, culture plates and flasks tested and used in this study, and all volumes of trypsin, growth medium, and cell densities used, based on https://www.thermofisher.com/no/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html. Additionally, all cultivation experiments of the killer whale cells are presented with seeding densities tested, information of ID of the killer whale cells tested, and the passage seeded. Additional comments of culturing, including successful subculturing, confluency, or visual observations, are stated.*

	Surface area (cm^2)	Trypsin (mL)	GM (mL)	Seeding density tested ($cells/cm^2$)	ID and passage	Comment
Dishes						
10 cm	56.7	5	12	3000	KW-20-09 P3	Split at day 8 (70-90% confluency)
				1630 (1:3 from dish above)	KW-20-09 P4	Split at day 9 (70% confluency)
				660 (1:3 from dish above)	KW-20-09 P5	Did not reach over 30-40% confluency.
				1850	KW-20-09 P6	Did not reach over 30% confluency.
				3747	KW-20-13 P2	The cryopreserved cells never attached to the surface after thawing, thus died.
Wells						
6-well	9.6	1	1 - 3	3700	KW-20-10 P6	Senescent and/or dead looking at day 2.
				6000	KW-20-10 P6	Looked good, reseeded once more to P7, and looked senescent/dead 2-3 days after.
				6800	KW-21-01 P1	Isolated primary cells from tissue piece. Later pooled with other primary cells, unsuccessfully.
12-well	3.5	0.4 - 1	1 - 2	3000	KW-20-09 P3	Confluent at day 10-11
				2170	KW-20-09 P3	Confluent at day 10-11
				8500	KW-20-09 P4	Confluent at day 6-7
				20 000	KW-20-09 P4	Confluent at day 3
				3000	KW-20-10 P5	High confluency in the middle, split at day 9.
				3500		High confluency in the middle, split at day 9.

				4000		Confluent at day 7
				4500		Confluent at day 7
				5000		Confluent at day 7
				5500	KW-21-01 P1	Isolated primary cells from tissue pieces. Tested L-15 medium as GM, unsuccessfully.
24-well	1.9	0.2 - 0.3	0.5 - 1.0	8500	KW-20-09 P4	Confluent at day 6-7
				2200	KW-20-09 P5	Close to confluent in the center of the well, however not around the edges. Started dying before they were okay to split.
				3000	KW-20-09 P5	
				8500	KW-20-09 P5	
96-well	0.32	0.05 - 0.1	0.1 - 0.2	3000	KW-20-09 P3	Confluent at day 10-11
				8500	KW-20-09 P4	Confluent at day 5
				20 000	KW-20-09 P4	Confluent at day 3
Flasks						
T25	25	3	3 - 5	2200	KW-20-09 P4	For freezing – turned smudgy looking, but frozen at day 9 (80-90% confluency)
				1950	KW-20-09 P5	Split at day 10 (90% confluency - less around edges)
T75	75	5	8 - 15	3666	KW-20-09 P2	Unfrozen. Split after 7 days (80-90%)
				3333	KW-20-10 P2	Unfrozen. Split at day 4 (patches of 95% confluency, the rest were 40-70%)
				2133 (1:2 from flask above)	KW-20-10 P3	Two flasks with the same density. Became smudgy in shape after 4 days. Pooled these in one T75.
				NA	KW-20-10 P4	Hemocytometer was broken; thus, density assessment was not possible. Grew a lot better and was split 3 days later.

3500	KW-20-10 P5	Some smudgy cells and a little clumpy, thus reseeded in one T75.
4133	KW-20-10 P6	More smudgy cells. Resuspended once more, to P7 – however all turned senescent looking.

3.4 Cytotoxicity in cells exposed to POP mixture

Cytotoxicity in response to POP exposure experiments were conducted on cells from the individual with the second highest primary cell yield (KW-20-10, Table 2). The results indicated that metabolic activity of the cells measured by resazurin was decreased significantly by 10% ($p = 0.02$; Figure 4) when exposed to the POP mixture of 10X. Surprisingly, metabolic activity was also decreased in the control cells receiving only GM, while no other POP mixture concentrations led to a decrease in metabolic activity. It was also unexpected that the membrane integrity measured by CFDA-AM was higher in cells exposed with POP mixture compared to the solvent control (Figure 4). The decrease in fold change of cells exposed to Triton X-100 does however suggest that the assay functioned, for the positive control with known effect in CFDA-AM and resazurin tests. A gradual dose response for both CFDA-AM and resazurin tests with continuous lower fold change as concentrations of exposure to POPs increases was expected, as POP exposure have led to cytotoxic responses in humpback whale fibroblasts (Burkard et al., 2015) and killer whale and polar bear lymphocytes (Desforges et al., 2017). Burkard et al., (2015) compared the cytotoxicity of pollutants in humpback whale and human fibroblasts with CFDA-AM assays and observed that whale fibroblasts were less sensitive to *p,p'*-DDE, with the lowest effect concentration of 4.7 µM compared to 0.3 µM in human fibroblasts. In the POP mixture used in this study *p,p'*-DDE was the most abundant pollutant with the concentration of 4.47 µM, of a total of 11.57 µM at 1X. Hence, a higher response would be expected for the killer whale cells if it is assumed that killer whales and humpback whales have the same sensitivity. The different response in killer whale cells compared to humpback whale cells could indicate a lower sensitivity of killer whale cells to POPs, or a different mode of action of POPs between baleen and toothed whales. However, a more recent study conducted on the humpback whale fibroblast did not detect any significant effects of exposure to the POP hexachlorobenzene (HCB) (Maner et al., 2019), which is in accordance to the results of this study. A transient tendency of reduction of metabolic activity was measured after three hours of exposure, and

they speculate that this reaction could be linked to redistribution of energy as a stress response in the cell. Additionally, they observed a transient increase in membrane integrity, a tendency also seen in this study, however solely directly after seeding and link it to this (Maner et al., 2019).

The result of this study indicates a low cytotoxicity of the POP mixture for the killer whale cells. Nevertheless, the discrepancy in the results must be acknowledged, and the results must be interpreted with caution. The unexpected results may be related to viability of the cells with added DMSO (to achieve 0.1% DMSO), as some of these were partly detached from the well bottom and showed changes in morphology (Figure 5C). The cells exposed to 20X and only GM did not detach from the wells. Additionally, the lack of replicated experiments and high biological variation within the triplicates may have affected the results. Furthermore, changes in metabolic activity and membrane integrity may appear after the 48 hours of exposure.

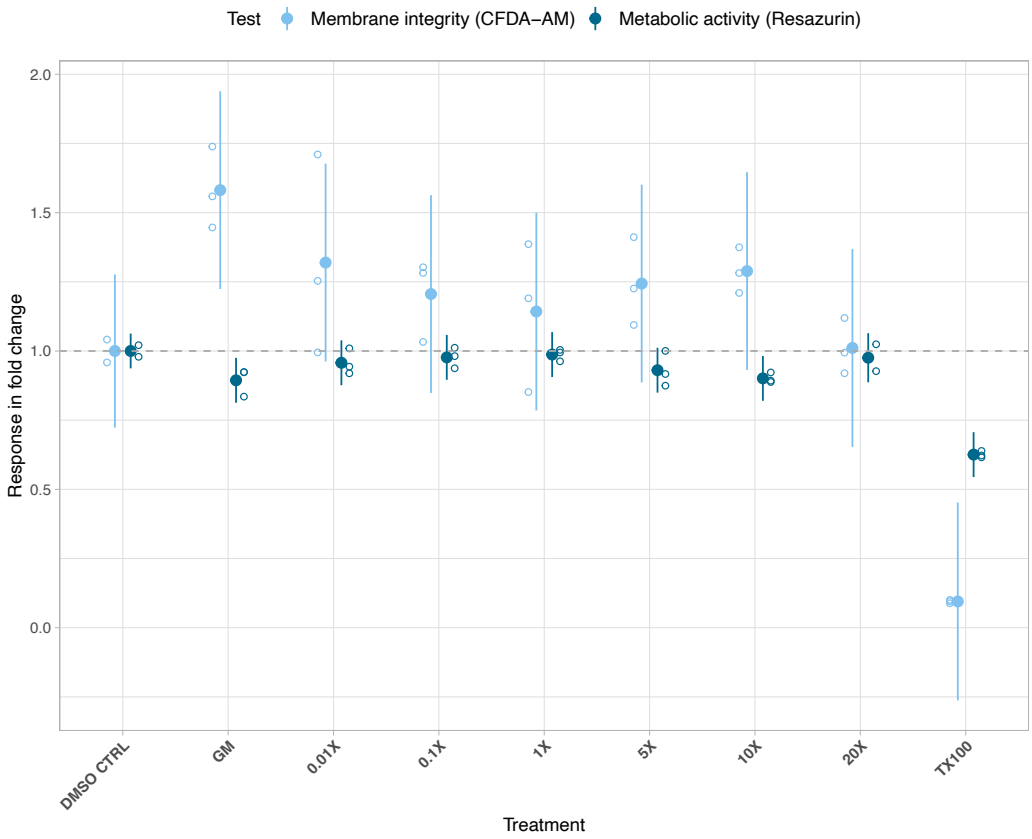

Figure 4 *Membrane integrity (CFDA-AM) and metabolic activity (resazurin) in killer whale fibroblasts after exposure to different concentrations of POP mixtures (0.01X, 0.1X, 1X, 5X, 10X, and 20X), solvent (DMSO CTRL), growth medium (GM) and Triton X-100 (TX100). 1X reflects median concentration present in killer whale blubber. Results presented as fold change over solvent control (DMSO CTRL). Observations are presented as hollow circles, while estimates with the 95% confidence intervals derived from linear models are presented as point range.*

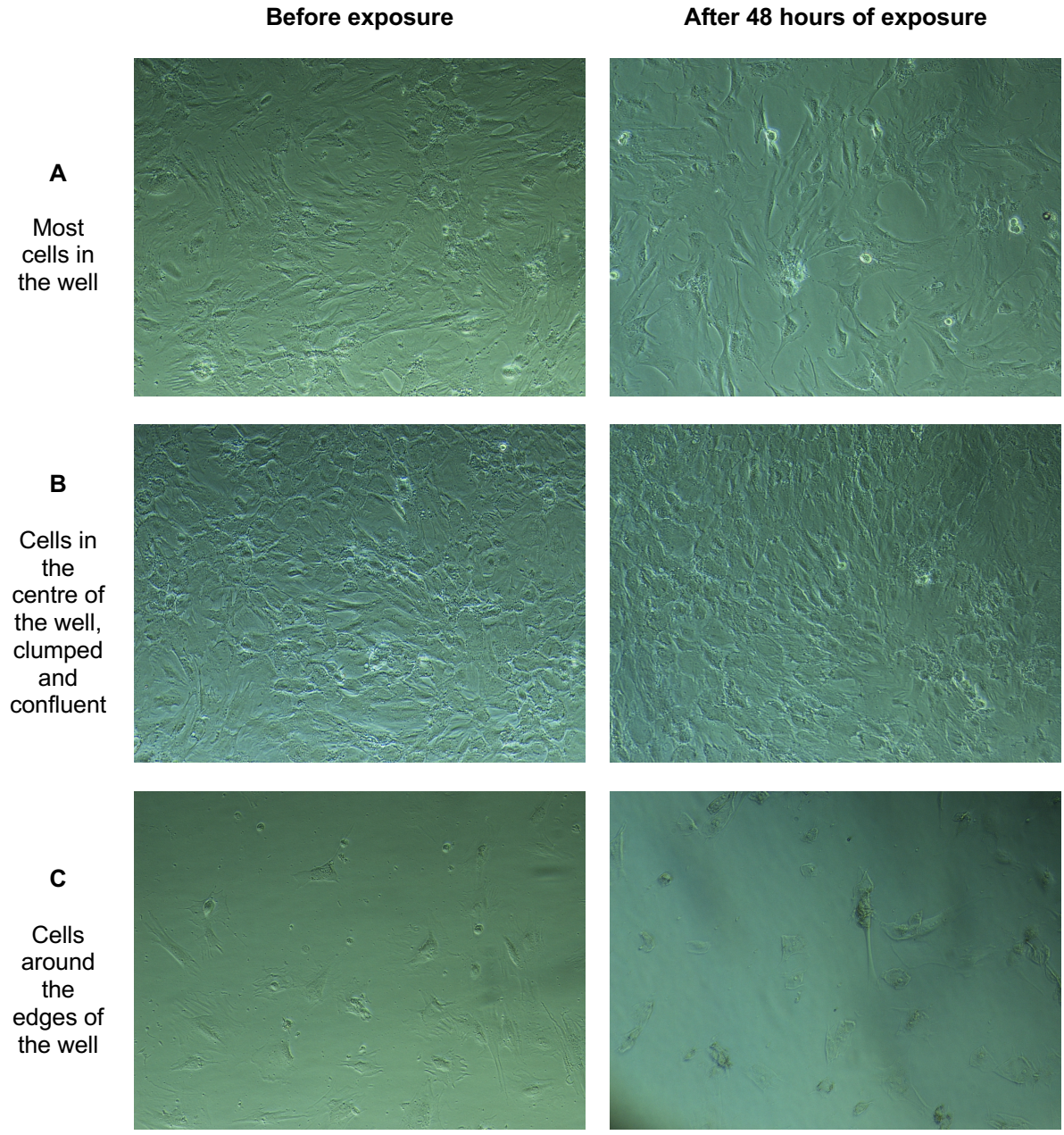

Figure 5 *KW-20-10 P3 in 12 well, directly before (left column) and after (right column) exposure to POP mixture at 10X for 48 hours. Most cells looked like A, however the cells in the center of the well looked like B, and the cells around the edges were less dense, and after exposure (C, right) possessed strange shapes where they looked partly detached, as they moved slightly when the plate was moved around. The before and after pictures are not taken at the exact same spot of the well but represents the general tendency.*

3.5 RNA quality

The RNA yield, purity and integrity (RIN) were better for KW-20-11 (Appendix Table A2) than for KW-20-10 (Appendix Table A3), thus KW-20-11 was used for further analysis. The purity of the RNA was assessed by the 260/280 and 260/230 ratios. The 260/280 ratio should

be around 2.0 and is considered low under 1.9 and high above 2.2. Low or high ratios may indicate undesired presence of protein, phenol, or other contaminants. In this study, the 260/280 ratio was on average 1.73 ± SD 0.09 (Appendix Table A2). The 260/230 ratio should ideally be 2.0-2.2 for transcript analysis. Lower or higher 260/230 ratio may indicate presence of undesired organic compounds, such as the phenolic reagent in TRizol which absorbs both at 230 and 270 nm, EDTA, or carbohydrates (Thermofisher, 2021). In this study, the 260/230 ratio was on average 0.26 ± SD 0.14 (Appendix Table A2). The reported deviations of RNA purity can have considerable impacts on the reported result. Specifically, contaminants may inhibit enzyme activity at the endpoint analysis and act as PCR inhibitors (Unger et al., 2019). A low RNA yield, seen in some of the samples, may also impact the absorbance ratios, as the background contamination has a greater impact on the ratios at lower RNA concentrations. However, the presence of RNA was confirmed by the Bioanalyzer traces and the 260/280 ratios from the Nanodrop. A minor degradation in the fast-region between the 5S- and 18S-region (Schroeder et al., 2006) was observed in most samples, however, the RIN values were generally acceptable. Contamination or degradation may be a result of error during sample processing, and may cause a reduction of the amplification (Gingrich et al., 2006). Nevertheless, it is difficult to estimate the possible effect contamination and/or degradation may have on the results of gene transcript levels in this study.

3.6 Transcript levels of genes

CYP1A was the only gene of the 11 genes quantified with a significant alteration of transcript levels following exposure to POP mixture. The expression (copies/µL) of *CYP1A* showed a decreasing dose-response tendency and was significantly lower following 1X and 10X POP treatment compared to control cells ($p = 0.036$ and $p = 0.05$, respectively). In general, there was quite high variation with respect to gene expression within the technical replicates (e.g. for *ADIPOQ*, *CYP4A*, *ERA*; Figure 6)). The expression of *ADIPOQ*, *CYP4A*, *ERA*, *GR*, *PPARA*, *PPARG* and *THRA* showed a non-significant tendency to be upregulated in cells exposed to 10X POP, while *CD36* showed a tendency to be down regulated (Figure 6, Appendix Figure A2 and A3).

CYP1A is a known biomarker of pollutant exposure as it is involved in detoxification (Foltz et al., 2014; Godard et al., 2004; Teramitsu et al., 2000). Killer whale fibroblasts showed a

significant decrease in expression - thus a negative dose-response when exposed to POPs. Although several studies on cetaceans have reported positive correlations between *CYP1A* levels and pollutant exposure (Fossi et al., 2010; Noël et al., 2014; Panti et al., 2011; White et al., 1994; Wilson et al., 2005), it has been suggested that *CYP1A1* expression may be suppressed in the most polluted individuals, seen in blubber of false killer whales (*Pseudorca crassidens*) (Foltz et al., 2014) and liver of white whale (Wilson et al., 2005). However, no significant correlation was found between *CYP1A* in the skin and PCB levels in the blubber of Cuvier's beaked whale (*Ziphius cavirostris*) (Baini et al., 2020), or *CYP1A* levels in killer whales skin in relation to POP levels in blubber (Panti et al., 2022). In addition, no clear induction response was seen of *CYP1A* expression in relation to organochlorine exposure in killer whale fibroblasts (Marsili et al., 2012). However, the expression of *CYP1A* was lowest at the highest exposure concentration, which could indicate a downregulation of *CYP1A*. The downregulation of *CYP1A* in this study may be caused by synergistic or additive effects of different substances in the POP mixture, which might not have been observed in single compound exposure setup. *CYP1A* is a target gene of *AHR*. However, *AHR* was not affected by POP exposure in this study, which may indicate that other mechanism may regulate *CYP1A* (Santes-Palacios et al., 2016).

Both *PPARA* and its target gene *CYP4A* tended to be upregulated in this study at the highest concentration of POPs. Exposure to PCB mixture Aroclor 1254 highly induced the transcriptional activity of polar bear PPARA expressed in COS7 cells (Routti et al., 2019). These results, together with prior knowledge of *PPARA* and *CYP4A* interactions (Feige et al., 2006; Takeuchi et al., 2006), suggest that POP exposure may induce an alteration in energy metabolism regulated by *PPARA* and *CYP4A*.

In the present study, there was a tendency of upregulation of both *PPARG* and its target gene *ADIPOQ*. These results are in accordance with the positive correlations seen between levels of *PPARG*, *ADIPOQ* and PCBs exposure in polar bear adipose tissue (Tartu et al., 2017). However, Buckman et al. (2011) did not report any relationships between these genes and pollutant exposure in killer whales. An *in vitro* study conducted on polar bears showed antagonistic effects of the PCB153 and *p,p*'-DDE on polar bear PPARG (Routti et al., 2016), and exposure to a synthetic POP mixture suppressed adipogenesis in polar bear adipose tissue-derived stem cells (pbASCs), supposedly due to the antagonistic effects of PPARG.

Another *in vitro* study on fin whale nuclear receptors reported both agonistic and antagonistic effects of PCBs, and mainly antagonistic effect of DTT, on the transcriptional activity of PPARG (Lühmann et al., 2020). Such *in vitro* studies as Lühmann et al. (2020) presented are receptor studies which only portray the ligand binding domains, and the effect of POPs exposure is not necessarily the same when compared to gene expression analysis. The main function of PPARG and the protein hormone ADIPOQ is related to lipid metabolism, but the expression of these genes in killer whale fibroblasts were low, and the tendency of upregulation should be interpreted with caution. It was surprising that *CD36* tended to be downregulated in killer whale fibroblasts exposed to POPs as *CD36* is a target gene of *PPARG* (Zhou et al., 2008). However, no relationship between levels of *CD36* and POPs were found in liver of ringed seals (*Pusa hispida*) (Castelli et al., 2014). The downregulation of *CD36* should too be interpreted with caution, due to the low expression levels which was one of the lowest in this study (Appendix Figure A2).

ERA and *THRA* showed a tendency of upregulation in this study in relation to increased POP mixture exposure, which is consistent with findings of correlative studies; A field study investigating correlations between PCB concentrations and transcript levels of 13 genes in killer whale blubber found five genes that were positively correlated with increasing PCB levels (Buckman et al., 2011), two of which were *ERA* and *THRA*. Other field studies have shown positive correlation of *THRA* and *ERA* expression with PCB levels in harbour seal blubber (Noël et al., 2017; Tabuchi et al., 2006), and with *ERA* levels in ringed seal liver (Brown et al., 2014). This supports the upregulated trends of *ERA* and *THRA* reported in the present study. 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 & 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, which have not seen any significant correlation between *THRB* and PCBs (Buckman et al., 2011; Noël et al., 2014; Tabuchi et al., 2006).

The tendency of upregulation of *GR* following POP exposure in killer whale cells is consistent with previous correlative studies on ringed seals and harbour seals showing

positive correlation between *GR* expression and blubber levels of PCBs (Brown et al., 2014; Noël et al., 2017). *GR* plays a central role in stress and immune responses, and alterations in the signaling pathway of glucocorticoid may interfere with these vital functions (Odermatt et al., 2006).

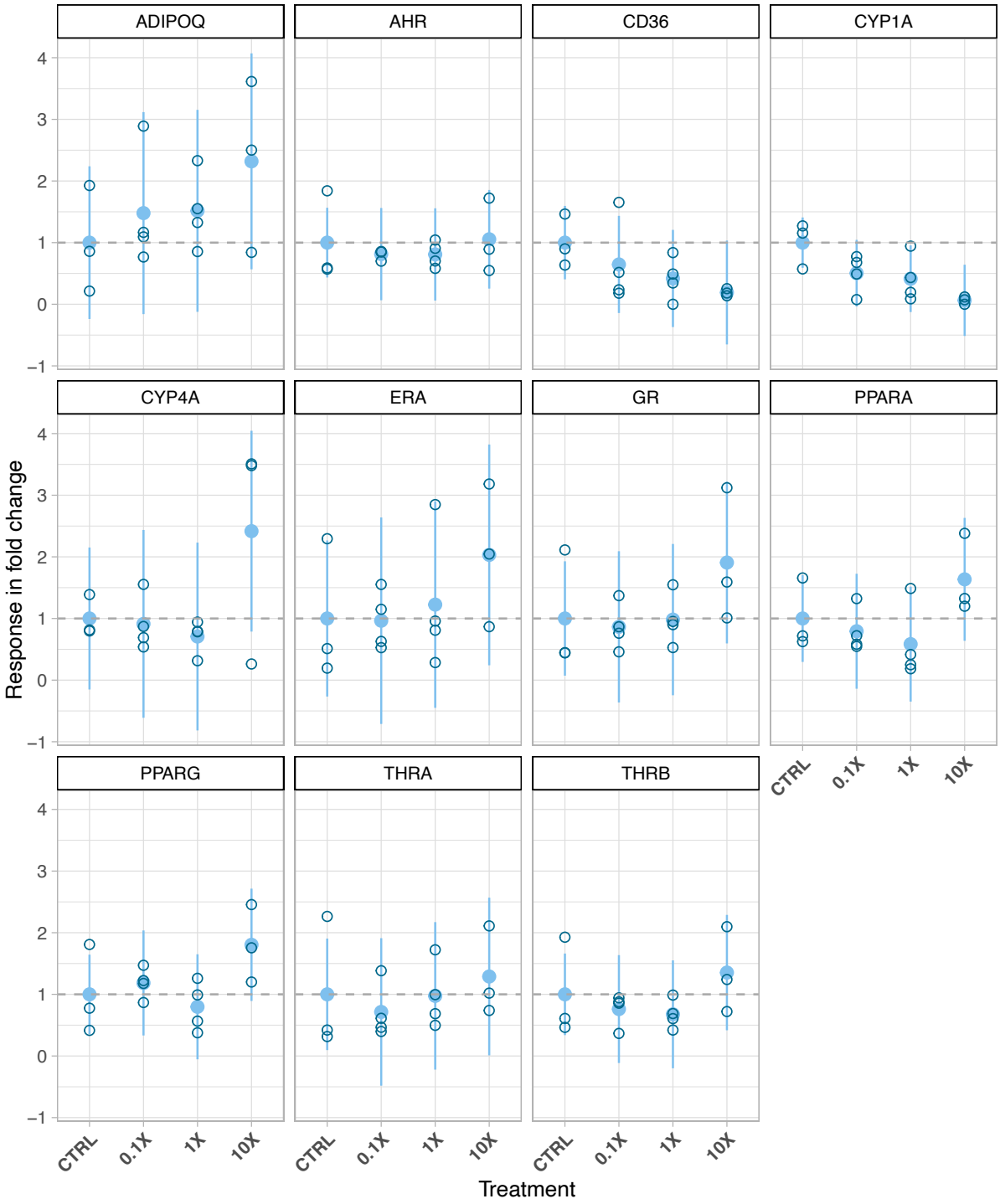

Figure 6 *ddPCR results for genes of interest of killer whale fibroblast-like cells after exposure to different concentrations of POP mixture (0.1X, 1X and 10X). 1X reflectes median concentration present in killer whale blubber. Results presented as fold change over solvent control (CTRL), following normalization after expression of housekeeping gene GAPDH. Observations are presented as hollow circles, while estimates with the 95% confidence intervals (CIs) derived from linear models are presented as point range. The results are considered significant if the confidence intervals do not cross the dashed line.*

3.7 Relevance, limitations, and prospects of this study

3.7.1 Relevance

Fibroblasts outgrown from skin biopsies have many advantages as model cells as they normally are easy to obtain from both live and newly diseased animals and to establish as a cell line if growth requirements are fulfilled. *In vitro* studies on fibroblasts from marine mammal biopsies are cost-effective and ethically sustainable as biopsies can be sampled from the animal with minimal harm, and the time spent distressing the animal is reduced to a minimum as no capturing is needed. An established cell line is a useful tool, which may be used for testing toxic responses to emerging and legacy pollutants individually or in mixture as well as 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. The cells may also be de-differentiated to stem cells and again differentiated to other cell types, to study functional or toxicological aspects without needing to harvest more tissues/cells from the animal.

This study successfully established a killer whale fibroblast-like cell culture, which enabled dose-response and cause-effect analyses of POP exposure in killer whale cells, assessed by cytotoxic and gene transcript responses. Killer whales are listed as “data deficient” in the IUCN Red List (Reeves et al., 2017), and this study contributes to provide knowledge on pollutants as stressors in a keystone species in the ocean (Williams et al., 2004), which otherwise is challenging to study. *In vitro* studies as this one also gives support to correlative studies, and additionally provide cause-effect responses.

3.7.2 Limitations

Technical difficulties were encountered during this study, especially problematic and time-consuming effort to cultivate the killer whale cells; this resulted in time constraints to carry out exposure studies and endpoint analysis in an optimal fashion (e.g. repetition of experiments). This should thus be recognized as a pilot study. The challenges of cultivation also led to exhaustion of the number of biopsies and cells, resulting in a low number of replicates in the exposure studies. The technical replicates displayed large biological

variances which resulted in large confidence intervals in the gene transcript analysis. Ideally, an increased number of replicates would counteract the large confidence interval. Due to lack of time, working material and cells the exposure experiments were only conducted once and should have been repeated.

The cultivation protocol for this cell line will need improvements. Improved growth and cultivation conditions would enable further studies possibly with reduced variances. Coating the cell culture plates with e.g. collagen (as done in Rajput et al. (2018) and Yajing et al. (2018)) and/or addition of fibroblast growth factors to growth medium (Aikawa, 2011) should be tested to improve cell proliferation.

This study suggested that POPs do have cytotoxic effects and do interfere with gene regulation in killer whales. This study, however, solely represents a snapshot response in one cell type among many. Different cell types will not necessarily respond in a similar way compared to the fibroblast-like cells, or in the same time frame. Additionally, other responses may be seen earlier or later, than 48 hours of exposure. Although this study provides indications about potential effects of POPs in killer whales, it is important to acknowledge that this study was conducted on cells – and the observed responses cannot and will not represent the full *in vivo* response. Due to this, and the limited number of genes examined, conclusions based on how environmental pollutants affect killer whales in a larger ecological context cannot be made. However, it may be the closest we now have come to understanding some of the responses occurring in the killer whales exposed to POPs.

It should also be recognized that none of the sampled individuals can be expected to be "clean" of pollutants, due to the existing levels of pollutants in killer whales roaming the Norwegian coast (Blévin et al., in preparation). The pollutants may already be interfering with cellular mechanisms prior to sampling, and the cells can thus not be concluded to be a fully clean baseline.

3.7.3 Future perspectives

Future research continuing the aims of this study is needed, and further investigation of the toxicological responses of killer whale cells to POPs should be conducted. Parts of the RNA isolated from cells of the exposure experiment used for the gene transcript analysis have been submitted for RNASeq analysis. The results of the RNASeq will likely provide a more complex and complete picture of the cell responses. Additionally, the cultivation method is being developed and tested for improved cell proliferation and preventing early senescence, by altering the growth medium, testing different culture plates, and incubating in a hypoxic chamber. Furthermore, a mycoplasma cell culture test should be conducted, to rule out contamination of mycoplasma as an affecting factor of the cell culture growth. Lastly, immortalizing the cell line by transfecting with e.g. telomerase reverse transcriptase (TERT) would be averting senescence and allow prolonged preservation of the cell line (Burkard et al., 2019), a desired ability when working with wild animals which are challenging to sample. However, it is important to note that increased number of passages and undergoing trypsin digestion may drive the biological properties of the cells further away from those of the original tissue and sampled animal (Li Chen et al., 2009).

4 Conclusion

This study describes the establishment of killer whale cell cultures assumed to be fibroblasts. This enabled an *in vitro* study of dose-response and cause-effect relationships between environmentally relevant concentrations of POPs, and gene transcript levels and cytotoxicity in the killer whale fibroblasts. *CYP1A* was the only of the 13 genes of interest that showed significantly altered (downregulated) transcript levels following POP exposure. Nevertheless, several of the genes related to endocrine systems showed non-significant tendencies to be upregulated following POP exposure. The measures of cytotoxicity, membrane integrity and metabolic activity, did not decrease in a dose-dependent manner. The results of the gene transcripts provide information on cause-effect responses, which give support to previous correlative studies, but due to high biological variation among the low number of replicates further research should be conducted to confirm the results of this study. The fibroblast cultures established in this study are useful tools, which may be used for testing toxic responses to emerging and legacy pollutants, individually or in mixture as well as other stressors. However, the culturing method should be improved to obtain a sufficient number of cells for further toxicological testing. Both knowledge on toxic responses of current-used chemicals as well as mixture effects are highly needed for management and use of industrial chemicals.

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Appendix

Primer design

Table A1 *Cetacean species and NCBI accession number of the sequences aligned for primer design.*

Symbol	Accession number	Species	Symbol	Accession number	Species
AhR	XM_004263467.3	*O. orca*	PPARα	XM_004279591.2	*O. orca*
	XM_019928248.2	*T.truncatus*		XM_019933140.2	*T. truncatus*
	XM_007100219.3	*P. macrocephalus*		XR_003680178.1	*P. macrocephalus*
CYP1A	XM_004276309.2	*O. orca*	PPARγ	XM_033414106.1	*O. orca*
	AY641536.1	*L. acutus*		XM_019944609.2	*T. truncatus*
	XM_007118237.3	*P. macrocephalus*		XM_007461547.1	*P. macrocephalus*
CYP3A	ANOL02021563.1	*O. orca*	FABP4	XM_004276170.3	*O. orca*
	XM_033839162.1	*T. truncatus*		XM_004323044.2	*T. truncatus*
	XM_032604774.1	*P. sinus*		XM_007125350.3	*P. macrocephalus*
CYP4A	ANOL02075116.1	*O. orca*	ADIPOQ	XM_004278474.3	*O. orca*
	AB109550.1	*B. acutorostrata*		XM_004330169.3	*T. truncatus*
				XM_028484721.1	*P. macrocephalus*
THRα	XM_033410899.1	*O. orca*	CD36	*XM_012534752.*	*O. orca*
	XM_033847960.1	*T. truncatus*		XM_028490140.1	*T. truncatus*
	XM_028486286.1	*P. macrocephalus*		XM_019940512.2	*P. macrocephalus*
THRβ	XM_033431958.1	*O. orca*	YWHAZ	XM_033438185.1	*O. orca*
	XM_033855256.1	*T. truncatus*	(HK)	XM_019925047.2	*T. truncatus*
	XM_028490309.1	*P. macrocephalus*		XM_028500466.1	*P. macrocephalus*
GR	XM_033406535.1	*O. orca*	GAPDH	XM_004279036.2	*O. orca*
	XM_033853352.1	*T. truncatus*	(HK)	XM_019925987.2	*T. truncatus*
	XM_024124771.2	*P. macrocephalus*		XM_007103683.3	*P. macrocephalus*

PCR method

PCR reactions of 25 µl were prepared using GoTaq® Flexi DNA Polymerase (Promega) as follows: 5 µl of 5x Green Flexi PCR Reaction Buffer, 2.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTP (deoxynucleotide mix 10 mM), 0.125 µl Go Taq DNA Polymerase (5 units/µL), 13.375 µl of nuclease-free water, 1.25 µl of each primer (10 µM), and 1 µl cDNA (25 ng/µL).

The thermal cycle was conducted in a SimpliAmp™ Thermal Cycler (Life Technologies) starting with enzyme activation for 3′ at 95 °C, followed by 35 cycles of: denaturation (30″ at 95 °C), annealing (30″ at 60 °C), extension (30″ 72 °C), and a final 7′ of polymerization at 72 °C. Electrophoresis of the samples was performed on 2% agarose gel with ethidium bromide staining. The products length was checked using a 100 bp DNA ladder (Promega).

Primer dilution series

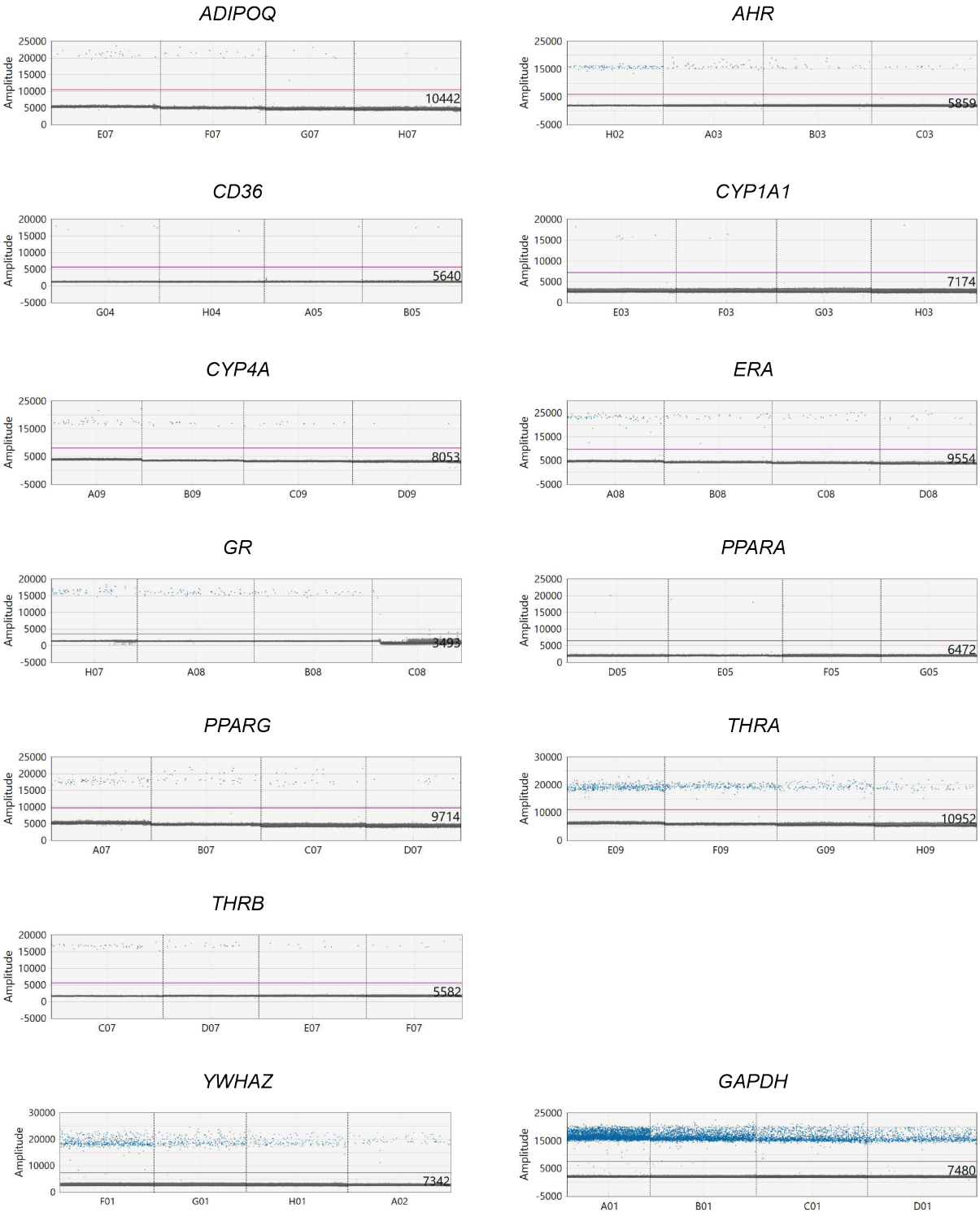

Figure A1 *ddPCR droplet graphs of cDNA serial dilutions run for primers of all genes of interest and housekeeping genes used in this study. The purple line indicates separation of positive and negative droplets.*

RNA quality results

Table A2 *Results from Nanodrop and Bioanalyzer chip analysis of isolated RNA of KW-20-11 cells, presenting RNA yield, purity and RNA Integrity Numbers (RIN).*

		Nanodrop data				Bioanalyzer Chip	Total RNA per sample (18 uL)
SAMPLE		**ng/uL**	**A260**	**260/230**	**260/280**	**RIN**	**ng**
10X	a	32.11	0.803	0.19	1.89	9.8	578
	b	33.51	0.838	0.27	1.86	9.8	603
	c	28.76	0.719	0.1	1.86	8.9	518
	d	107.25	2.681	0.22	1.68	10	1931
1X	a	144.95	3.624	0.34	1.69	8.7	2609
	b	67.96	1.699	0.14	1.73	9.7	1223
	c	81.45	2.036	0.17	1.62	9.1	1466
	d	177.37	4.434	0.38	1.7	8.1	3193
0.1X	a	191.38	4.784	0.46	1.71	N/A	3445
	b	121.61	3.04	0.24	1.64	9.8	2189
	c	113.12	2.828	0.24	1.66	9.9	2036
	d	50.41	1.26	0.3	1.64	9.6	907
Control DMSO	a	26.74	0.668	0.23	1.85	8.9	481
	b	88.52	2.213	0.19	1.74	9.6	1593
	c	33.24	0.831	0.07	1.64	N/A	598
	d	31.13	0.778	0.63	1.81	9.6	560
Mean		**83.09**	**2.08**	**0.26**	**1.73**	**9.39**	**1496**
SD		**54.84**	**1.37**	**0.14**	**0.09**	**0.56**	**987.1**

Table A3 *Results from Nanodrop and Bioanalyzer chip analysis of isolated RNA of KW-20-10 cells (test run), presenting RNA yield, purity and RNA Integrity Numbers (RIN), including mean and standard deviation (SD)..*

		Nanodrop data				Bioanalyzer Chip	Total RNA per sample (18 uL)
SAMPLE		**ng/uL**	**A260**	**260/230**	**260/280**	**RIN**	**ng**
10X	a	23.94	0.599	0.06	1.66	10	431
	b	17.8	0.445	0.7	1.68	9.5	320
	c	15.47	0.387	0.37	1.84	10	278
1X	a	14.48	0.362	0.84	1.89	9.4	261
	b	40.08	1.002	0.1	1.6	2.8	721
	c	38.97	0.974	0.1	1.59	10	701
0.1X	a	30.24	0.756	0.09	1.53	N/A	544
	b	17.8	0.445	0.07	1.74	5.7	320
	c	14.9	0.372	0.42	1.75	9.7	268
Control DMSO	a	7.89	0.197	0.26	1.83	N/A	142
	b	6.83	0.171	0.49	1.46	N/A	123
	c	9.64	0.241	0.64	2.1	5.3	174
Mean		**19.84**	**0.50**	**0.35**	**1.72**	**8.04**	**357.06**
SD		**11.27**	**0.28**	**0.28**	**0.18**	**2.71**	**202.87**

ddPCR results

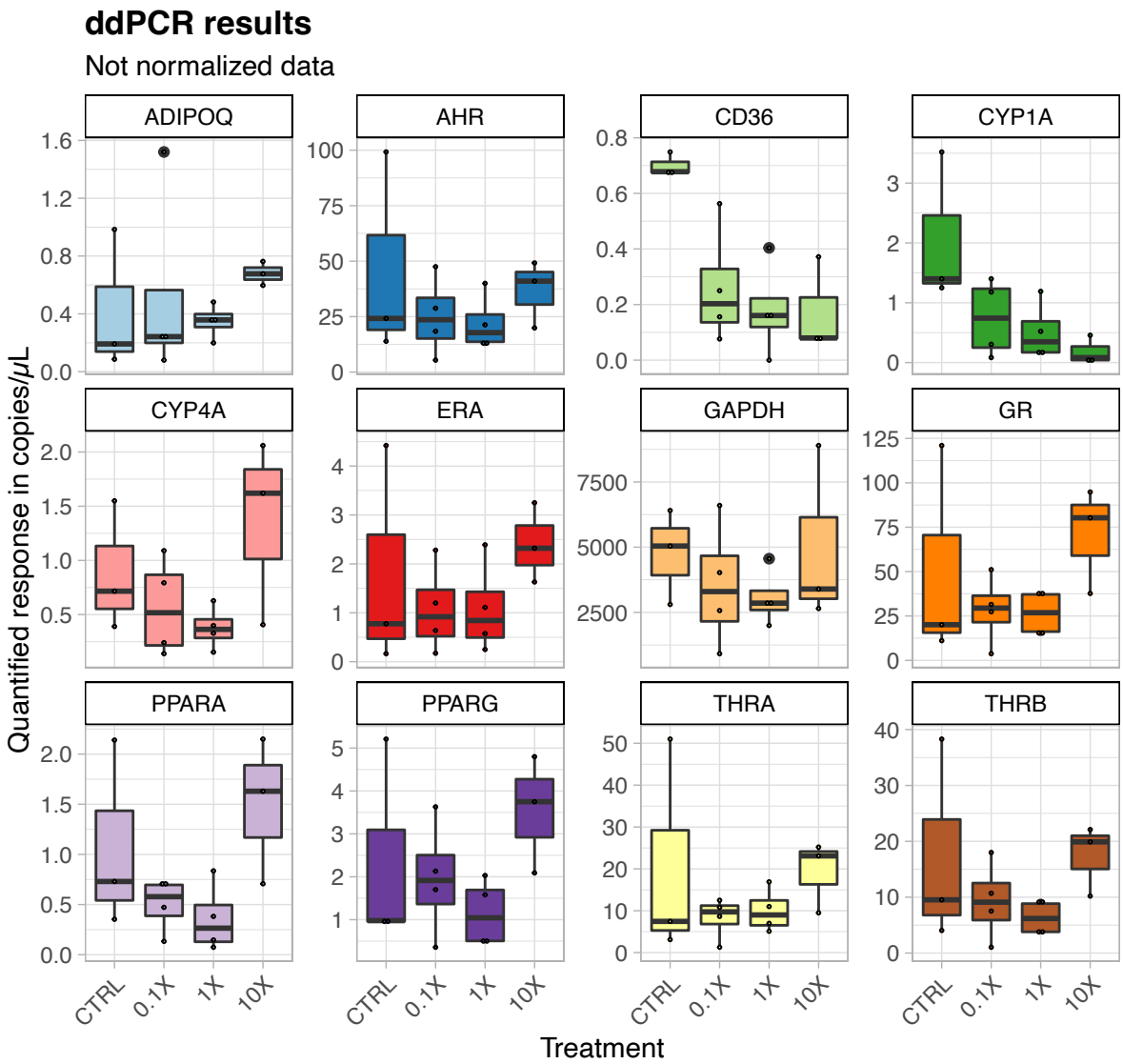

Figure A2 *ddPCR results for genes of interest of killer whale fibroblast-like cells after exposure to different concentrations of POP mixture (0.1X, 1X and 10X). 1X reflects median concentration present in killer whale blubber. Results presented as quantified response in copies/µL*

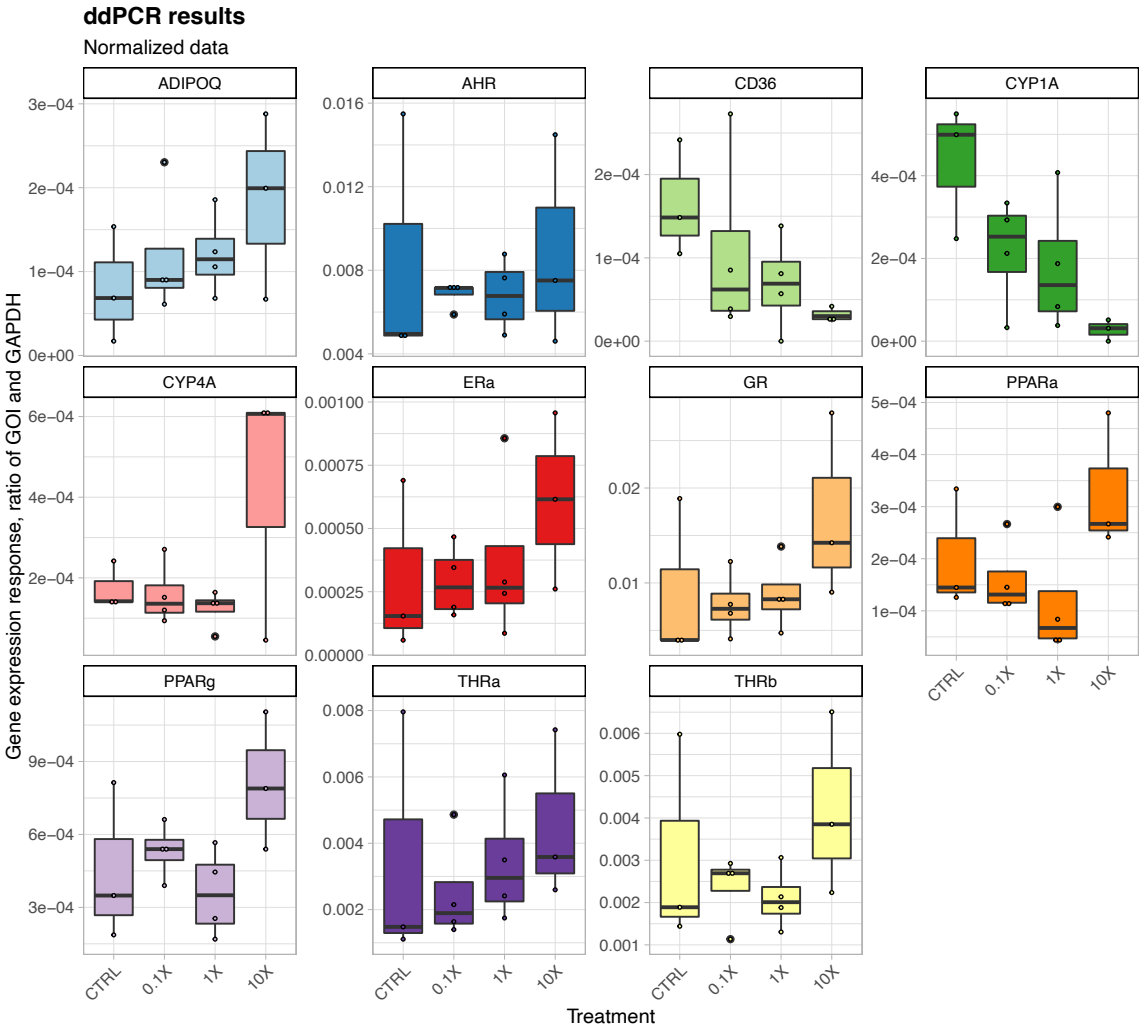

Figure A3 *ddPCR results for genes of interest of killer whale fibroblast-like cells after exposure to different concentrations of POP mixture (0.1X, 1X and 10X). 1X reflects median concentration present in killer whale blubber. Results presented as fold change over solvent control (CTRL), following normalization after expression of housekeeping gene GAPDH.*