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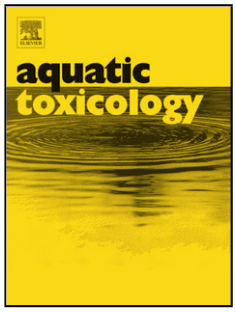


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# Establishment of the first humpback whale fibroblast cell lines and their application in chemical risk assessment

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## Highlights

We established and characterised the first humpback whale fibroblast cell lines.
Cell lines have a stable karyotype with 2n=44.
Exposure to *p,p'*-DDE resulted in a concentration-dependent loss of cell viability.
*p,p'*-DDE sensitivity differed considerably from human fibroblasts.
Exposure to a whale blubber extract showed higher sensitivity than to *p,p'*-DDE alone.

## Abstract

This paper reports the first successful derivation and characterization of humpback whale fibroblast cell lines. Primary fibroblasts were isolated from the dermal connective tissue of skin biopsies, cultured at 37°C and 5% $CO_2$ in the standard mammalian medium DMEM/F12 supplemented with 10% fetal bovine serum (FBS). Of nine initial biopsies, two cell lines were established from two different animals and designated HuWa1 and HuWa2. The cells have a stable karyotype with 2n = 44, which has commonly been observed in other baleen whale species. Cells were verified as being fibroblasts based on their spindle-shaped morphology, adherence to plastic and positive immunoreaction to vimentin. Population doubling time was determined to be ~41 h and cells were successfully cryopreserved and thawed. To date, HuWa1 cells have been propagated 30 times. Cells proliferate at the tested temperatures, 30, 33.5 and 37°C, but show the highest rate of proliferation at 37°C. Short-term exposure to para,para'-

dichlorodiphenyldichloroethylene (*p,p'*-DDE), a priority compound accumulating in humpback whales, resulted in a concentration-dependent loss of cell viability. The effective concentration which caused a 50% reduction in HuWa1 cell viability ($EC_{50}$ value) was approximately six times greater than the $EC_{50}$ value for the same chemical measured with human dermal fibroblasts. HuWa1 exposed to a natural, *p,p'*-DDE-containing, chemical mixture extracted from whale blubber showed distinctively higher sensitivity than to *p,p'*-DDE alone. Thus, we provide the first cytotoxicological data for humpback whales and with establishment of the HuWa cell lines, a unique *in vitro* model for the study of the whales' sensitivity and cellular response to chemicals and other environmental stressors.

**Keywords**: Megaptera novaeangliae; p,p,'-DDE; Antarctica; Persistent Organic Pollutants (POPs), karyotype, cell line characterization.

## Introduction

Humpback whales (*Megaptera novaeangliae*) belong to the family of baleen whales (*Balaenopteridae*). Southern hemisphere (SH) populations feed in the productive waters surrounding Antarctica, primarily on Antarctic krill (*Euphausia superba*). They build up large fat reserves during summer feeding to sustain their high energy needs and undertake long-term migration, associated with periods of voluntary fasting. Current anthropogenic threats to SH humpback whales include physical impacts, such as the resumption of whaling, net entanglements and boat strikes, as well as chemical pollution, such as exposure to persistent organic pollutants (POPs).

POPs are a group of man-made chemicals defined by their persistence, their toxicity, their potential for long range environmental transport and their tendency to bioaccumulate in organisms and bio-magnify along food chains. Historical or "legacy" POPs, such as organochlorine pesticides, have represented a major concern in environmental toxicology for decades. These POPs are semi-volatile and undergo temperature related long-range environmental transport, moving to progressively colder climates such as polar environments (Wania and Mackay, 1996). Cetaceans are among the organisms with the greatest potential for accumulating toxic levels of

POPs due to their long lifespan, their position at the top of the food web and their high proportion of body-fat, in which these lipophilic compounds accumulate.

The energy demanding annual migrations of SH humpback whales lead to a depletion of accumulated lipid reserves and serve to redistribute and concentrate the lipophilic POP burdens in remaining lipid stores. Average population levels of individual POPs in SH humpback whale blubber have been observed to increase by up to 50 times between early and late migration (Bengtson Nash et al., 2013). Recently characterized POP profiles of SH humpback blubber revealed appreciable levels of chemical residues including para,para'-dichlorodiphenyldichloroethylene (*p,p'-DDE*) (Bengtson Nash et al., 2013), the main metabolite of the well-known insecticide, dichlorodiphenyltrichloroethane (DDT).

Measuring the toxicological impact of POPs on wild populations of humpback whales is challenging. Adult stranding events are rare and timely collection of useful tissue samples is often impossible. Investigations on free ranging animals using non-lethal approaches represent a logistically challenging and cost intensive undertaking with limited tissue accessibility. Consequently, Australian policy, in line with the International Whaling Commission (IWC), has called for the development of improved *in vitro* models for cetacean chemical risk assessment (Australian Government, 2010, IWC, 2010).

To date, only a few studies have addressed chemical burdens in southern hemisphere humpback whales (Bengtson Nash et al., 2013) or biomarkers of chemical effects in the species as a whole (Bengtson Nash et al., 2014, Jauniaux et al., 2011, Waugh et al., 2011). In other cetacean species, a number of cell lines have been developed in order to assess viral impact (Cecil and Nigrelli, 1970, Nielsen et al., 1989, Kadoi et al., 1992) and the effects of contaminants (Wang and Pfeiffer, 2001, Fossi et al., 2006, Li Chen et al., 2009, Carvan et al., 1995, Spinsanti et al., 2008, Wise et al., 2010, Gauthier et al., 1999, Pine et al., 2007). Toxic effects in humpback whales are, however, still poorly understood and there are, to date, no established methods to study toxicological sensitivity. In order to bridge this information gap, better tools for deriving species-specific data are required. This study aims to develop and assess an *in vitro* toxicity approach for SH humpback whales using newly established fibroblast cell lines.

# Materials and methods

## Reagents

All chemicals were ordered from Sigma Aldrich (Castle Hill, Australia) and all cell culture ware, buffers and media from Life Technologies (Mulgrave, Australia), unless otherwise stated. Mycoplasma testing was done using the MycoAlert detection kit (Rockland, USA).

## Skin biopsy collection

Skin samples were obtained from healthy free-swimming individual humpback whales in Moreton Bay Marine Park, North Stradbroke Island (27° 26 S, 153° 34 E), categorized as breeding stock E1 by the IWC. Animals were sampled during their southward migration from northern breeding grounds to southern ocean feeding grounds. Samples were obtained with biopsy flotation darts (3.5 x 0.7cm) fired from a Paxarm™ air-rifle. Sampling was conducted under Griffith University Animal Research Ethics Committee (GU Ref No: ENV/17/10/AEC) approval. Biopsies consisted of skin and blubber, and were processed immediately following collection: a tissue piece of 1.5 cm × 0.7 cm interfacing epidermis and subcutaneous tissue was dissected and stored in complete sterile growth medium as described below. During the 6-8 h transport, the samples were stored on ice and upon arrival at the laboratory were immediately processed under sterile conditions.

## Processing of skin biopsies

Samples were processed according to the protocol of Whitworth et al. (2012). In brief, the tissue was rinsed with 1 x Dulbecco's Phosphate-Buffered Saline (DPBS) and separated into epidermis, dermis and blubber. The dermal tissue was cut into small sections (~1 $mm^3$) using a scalpel blade and tweezers. Approximately eight sections of tissue, covering about 0.5 $cm^2$, were transferred into a well of a 6-well tissue culture plate. Tissue fragments were covered with a sterile glass cover slip and light pressure applied to ensure tight contact to culture plates. A general fibroblast cell culture medium was chosen and cells were maintained at standard mammalian conditions at 37°C with 5% $CO_2$. Specifically, the medium is composed of DMEM/F12 (50:50 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12) containing 10 % Fetal Bovine Serum (FBS), 0.1 mM non–essential amino acids, 1 mM sodium pyruvate and 100 µg/ml Primocin

(InvivoGen, Toulouse, France). Tissue fragments were incubated in 3 ml medium per well with 50 % replacement every 48 h.

## Isolation of tissue fragments

After 32-38 days of incubation, primary cells were isolated from the residual tissue as follows: tissue fragments were washed 2 x with 5ml of 1 x DPBS, followed by incubation with 1 ml of trypsin (TrypLE™) for ~4 min. Thereafter, cells were dissociated from the well bottom and coverslip by pipetting 3 x 3 ml of medium and using a scraper blade. The resulting suspension was passed through a mechanical sieving unit (70 µm) and collected cells were centrifuged (200 x g, 4 min) before being re-seeded in T25 tissue culture flasks (Corning, Tewksbury, USA). Subsequent passaging was conducted similarly, washing with 5 ml of 1 x DPBS, incubation with 2 ml trypsin for ~4 min, neutralization by adding 5 ml medium and pipetting the resulting cell suspension into vials for centrifugation. Cells were re-suspended in fresh medium with a final splitting ratio of 1:3 or 1:2. After two passages, Primocin was replaced with 1% of 5.000 U/ml penicillin-streptomycin (P/S).

## Karyotype

Karyotyping was conducted with cells isolated from two different animals, i.e. in cells from HuWa1/passage 3 (P3) and of HuWa1 (P3) recovered after cryo-preservation. Cells were analyzed by Sullivan-Nicolaides Pathology (Taringa, Qld, Australia) using standard G- and C-banding procedures.

## Immunocytochemistry

Immunocytochemical staining was used for cell-type specific identification. In short, samples were fixed with 4% paraformaldehyde in DPBS for 20 min, permeabilised in 0.1% Triton X-100 in DPBS for 20 min, blocked with 4% goat serum in DPBS and incubated overnight with primary antibodies at 4°C. The following primary antibodies were applied; 1:500 monoclonal rabbit Vimentin (Abcam ab92547), 1:50 monoclonal mouse Cytokeratin AE1/AE3 (Dako M3515), 1:200 polyclonal rabbit Collagen I (Abcam ab292), 1:200 polyclonal rabbit Collagen IV (Abcam ab6586). Subsequently, samples were incubated with secondary antibodies: 1:1000 Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) and 1:1000 Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L). Nuclei were stained with DAPI before mounting under coverslips. Negative controls were

incubated with blocking buffer instead of primary antibody. CaCo-2 cells (Fogh, 1975) were used as a positive control for cytokeratin immunostaining.

## Growth kinetics

For growth characterization, cells were seeded in 24 well plates and fresh complete medium was added every two days. Seeded cells were grown under the following conditions: Incubation at 30, 33.5 and 37°C, FBS concentration of 0, 5, 10, 15, 20 % and supplementation of 0, 1, 3, 6 and 12 x additional Minimum Essential Medium (MEM) amino acid solution or MEM vitamin solution. The fluorescent dye Alamar Blue™ (AB) shows a linear correlation of fluorescence with cell number (Ganassin, 2000) and was used to calculate the cell number. To obtain an appropriate standard curve of AB fluorescent units vs. cell number, cells were seeded at different cell densities and fluorescence was measured after ten hours. The medium was discarded, cells were washed with DPBS and incubated for 30 min with 5% (v/v) AB in DPBS and fluorescence was measured at excitation/emission wavelengths of 530/595 nm using an Infinite M200 plate reader (Tecan, Switzerland). The obtained standard curve was used to calculate cell number:

(1) $Y= 0.02 x + 8.3$; $R^2 = 0.96$

with Y= AB fluorescence unit and x= seeded cell number/ml.

Population Doubling Time (PDT) was calculated from the log phase of the growth curve as

(2) $PDT = \log 2 / (\log N_2 - \log N_1)*t$

with $N_1$= cell number/ml at the beginning and $N_2$= cell number/ml at the end, with t= 96h.

## Cryopreservation and recovery

Approximately 1 x $10^6$ of HuWa1 cells (P2) were used for cryopreservation. Cells were washed with 5 ml of 1 x DPBS, trypsinized with 2 ml trypsin and centrifuged. The pellet was re-suspended in freezing medium containing 90% FBS and 10% dimethyl sulfoxide (DMSO). Cryotubes were frozen overnight to -80°C using an isopropyl alcohol freezing container and final storage was conducted in the vapor phase of liquid nitrogen. For recovery, frozen cells were rapidly thawed to room temperature and re-suspended in pre-warmed medium. Before plating in cell culture flasks, survival was measured by using the Trypan blue exclusion assay.

## p,p,'-DDE and humpback whale blubber extract preparation

Stock solutions were prepared by dissolving p,p,'-DDE directly in DMSO (≥99.9%) and sonicating 3 times for 5 min at 20°C. Working solutions were freshly prepared in DMSO and added to the cell culture system to achieve final concentrations ranging from 0.3 µg/l ($9.4x10^{-4}$ µM) to 2.4 mg/l (7.5 µM) and 0.25% solvent content.

For blubber extract preparation, blubber was obtained from a male adult humpback whale stranded on the Gold Coast (Queensland; Australia) in 2007. The blubber tissue had been wrapped in aluminum foil and stored in a zip-lock bag at -20°C for long-term storage. The blubber extract was prepared according to the extraction and clean-up method described in Bengtson Nash et al. (2013) without addition of internal standards. In brief, the tissue was homogenized and dried with mortar and pestle and anhydrous $Na_2SO_4$. The dried sample was extracted with cyclohexane:ethyl acetate (1:1) on a cold column and further cleaned by gel permeation chromatography, alumina oxide and silica columns. Finally the solvent was exchanged to 100 µl of DMSO. The blubber tissue had previously been analyzed for a range of POP compounds by high resolution-gas chromatography/high resolution mass spectrometry (HRGC/HRMS). The extract concentration is expressed as p,p,'-DDE equivalent. 467 $ng/g_{Blubber}$ p,p,'-DDE was measured and 1.3 g of blubber was extracted to a final stock solution of $6.05 \times 10^3$ µg/l (19.03 µM) p,p,'-DDE equivalent. For exposure, the stock solution was pre-warmed to room temperature and working solutions were prepared by diluting the stock 1:2, 1:20, 1:200 and 1:2000 in DMSO. The exposure procedure using the blubber extract was identical to that for the p,p,'-DDE stock solution except that the final DMSO content was 1%. The applied DMSO concentration did not affect cell viability compared to a non-DMSO control as quantified and described below.

## Cell viability

Cell viability upon exposure to p,p,'-DDE was assessed for HuWa1 (P8 - P16) cells and sub-cultured primary human fibroblast cells (HFb) (P5 - 8). HFb were obtained from the Australian Institute for Bioengineering and Technology (AIBN), The University of Queensland, (Brisbane; Australia). The primary cells from which this cell line was gained were isolated from the skin tissue of a healthy female donor. HFb were maintained in DMEM/high glucose containing 10% FBS, 0.1 mM non–essential amino acids, 1 mM sodium pyruvate, 6 mM L-glutamine and 1%

penicillin/ streptomycin. Cell viability upon exposure to blubber extract was measured in HuWa1 (P7) cells.

Cells were seeded at a density of 2 x $10^4$ cells/ml in 24 well plates and cultured for 48 h prior to treatment. HuWa1 and HFb exhibited plating efficiencies of about 85%. Thereafter, HuWa1 cells were exposed to *p,p'*-DDE or blubber extract for 24 h in DMEM/F12 without FBS. Exposure to HFb was conducted in DMEM high glucose without FBS. Minor metabolic differences following the use of DMEM/F12 and DMEM high glucose as exposure media cannot be excluded but are unlikely to play a major role for the applied time frame of 24 h. Response was assessed using the fluorescent dye, 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), indicating membrane integrity as described in Schirmer et al. (1997). Briefly, after treatment, exposure medium was discarded, cells were washed with DPBS and incubated for 30 min with 4 µM CFDA-AM in DPBS. Fluorescence was measured at excitation/emission wavelengths of 493/541 nm. The results were expressed as percentage of the solvent control, which was set to 100%. Concentration-response curves were fitted using non-linear regression (sigmoidal dose-response, variable slope, top= 100% and bottom= 0% constrained) and the concentrations or dilutions causing a 50% impact on cell viability ($EC_{50}$ values) calculated using GraphPad Prism 6 software.

### Statistical analysis

Data for *p,p,'*-DDE cell viability assessment is presented as the mean of three independent experiments and the error bars represent the standard deviation. The concentration-response curves were compared using the F-test and significant difference between the log$EC_{50}$ was tested. The analysis was done using GraphPad Prism 6 software.

## Results and discussion

### Cell culture establishment

A total of nine skin biopsies were obtained from free-swimming individual humpback whales (Tab.1). Biopsies included skin (epidermis, dermis) and the upper layer of subcutaneous tissue (blubber). All tissue fragments were explanted. The first single primary cells were observed after 14 days of culture as outgrowths from dermal samples. By day 25, some dermal fragments were surrounded by partly confluent monolayers of fibroblastic cells (Fig.1A-C). Cells were only observed from tissue fragments tightly fixed between the base of the well and the cover slip. No

cells grew in tissue fragments of dissected epidermal tissue or fat. The main cell type in dermal connective tissue lying between the epidermis and hypodermal fat are fibroblasts. The layer of connective tissue measured between 0.1 and 0.3 cm in depth and appeared slightly pink in colouration. These tissue fragments exhibited the highest capacity to grow spindle-shaped fibroblasts. However, in the connective tissue fragments 29S13 – 32S13, lipid droplets were present and only constrained cell growth was observed. We hypothesize that abundant lipid content may have inhibited proliferation of primary cells. Outgrowth occurred in five biopsy samples from three young animals (2x yearling, 1x calf) and two adults. The tissue of younger animals appeared to be softer and attached more readily to the plates. Overall, the cell yield was low. Of the five biopsies which showed primary cell outgrowth, only two lines were successfully isolated and, at the time of paper submission, passaged to P30 (2S13; designated HuWa1) and P10 (27S13; designated HuWa2), respectively. During passaging no signs of senescence, morphological variation or changes in growth behavior were observed. All subsequent experiments were carried out with HuWa1 except for karyotyping and growth rate determination, which were also conducted with HuWa2.

## Karyotype

Cells of HuWa1 (P3) in their metaphases were used for G- and C-banding (Fig. 2A). Cells exhibited diploid chromosome pairings with 2n=44 including 21 pairs of autosomes and 1 pair of sex chromosomes. HuWa1 were of male origin. The chromosome number is identical in HuWa2 cell (P7) (data not shown) and the C-banded chromosomes of *Megaptera novaeangliae* shown in O'Brien et al. (2006).

30 metaphase spreads of HuWa1 (P3) were fully karyotyped and about ~95% were diploid. Further C-banding indicated the presence of occasional tetraploidy in approximately 5% of cells examined with 2n=88 chromosomes (Fig. 2B). Tetraploidy in cetacean cells was also observed for blue whales (Arnason et al., 1985). HuWa1 cryopreserved cell cultures (P3) were analyzed for possible chromosomal rearrangements as a result of cryopreservation (Fig. 1C). The chromosomes did not display any visible rearrangements.

## Cell type identification

Two protein markers were selected to determine if isolated cells are indeed fibroblasts. Vimentin is an intermediate filament indicating mesenchymal origin such as endothelial cells, smooth muscle cells and fibroblasts. Therefore it is a typical and widely applied fibroblast marker (Richards et al., 1995, Sappino et al., 1990). Cytokeratin is an intermediate cytoskeleton filament produced by cells of epithelial origin. HuWa1 exhibited strong immunoreactivity to vimentin (Fig. 3A) and no immunoreactivity to cytokeratin (Fig. 3B). Although the technical control for cytokeratin was positive (Fig. 3B, inset), no humpback whale-specific positive control was available. However, cytokeratin has shown to be positively expressed in other cetacean species (Pine et al., 2004, Yu et al., 2005). These results therefore lend strong support that spindle-shaped HuWa1 cells are indeed fibroblasts. Collagen type I (Col I) is the abundant fibre-forming component in the extracellular matrix (Ricard-Blum and Ruggiero, 2005) and Collagen type IV (Col IV) is the abundant component of the basement membrane, both of which can be synthesized by fibroblasts. HuWa1 cells showed strong immunostaining for Col I (Fig. 3C) and weak immunostaining for Col IV (Fig. 3D).

## Recovery after cryopreservation

Cryopreservation enables long-term preservation and backup in case of morphological or genetic variations, senescence or contamination. Samples of passage two of HuWa1 cells were thawed to study their ability to recover from preservation. At the tested cell density of $8 \times 10^5$ cells/ml, ~90% were viable as assessed by Trypan blue staining. Further, cells regained their growth characteristics on continued culture (see below).

## Growth of HuWa1 and 2

Jin et al. (2013) reports the establishment of humpback dolphin fibroblast cultures and assessed 3-5 days of proliferation until cells reached confluency when passaged 1:3. The same study observed reduced proliferation starting from P10 and finally senescence with P17. Contrary to the dolphin fibroblasts, HuWa1 and HuWa2 reach confluency within ~16 days when split at 1:3. Growth properties of both HuWa cell lines are stable and no growth reduction or senescence was observed to date (P30). The generated growth curves (Fig. 4) indicate no major differences between HuWa1 (P9) and HuWa2 (P6). Population doubling time of HuWa1 was 41 h compared

to 31 h for human fibroblasts cultured in the same setup. HuWa1 cells that were cryopreserved and thawed exhibited slightly lower growth rates in passage one after thawing. However, after two passages, the growth rate returned to pre-cryopreservation levels.

## Influence of temperature, serum, vitamins and essential amino acids

Conventional mammalian cell culturing is conducted at 37°C and 5% $CO_2$ under standard conditions. Sperm whale (Wise et al., 2012) and right whale (Chen et al., 2009) fibroblasts are reported to be cultured at 33°C (doubling time of 36 h). Body temperature of humpback whales was estimated to be ~36°C (Morrison, 1962). The average habitat temperature of sampled SH humpback whales varies from tropical through to temperate and polar regions. The HuWa cell lines were derived from under-perfused tissue situated outside the thermoregulatory blubber. Therefore, we tested the temperature tolerance of HuWa1 cells. Cells proliferated at all tested temperatures (30, 33.5 and 37°C) and did not show any morphological variation. This indicates temperature tolerance down to at least 30°C. However, the cells grew in a temperature dependent manner and exhibited greater proliferation capacity at higher temperatures. At 37°C growth was most effective (Fig. 5A).

Serum, such as FBS, is one of the essential medium components providing nutrients, hormones and growth factors. Thus, HuWa1 cells were tested for their specific FBS requirements (Fig. 5B). In the absence of FBS, cell numbers decreased over time and senescence was observed by microscopy. In contrast, cell numbers increased in a serum concentration-dependent manner from 5 to 20 % of FBS.

Carvan et al. (1994) and Pine et al. (2004) used specific cetacean culture medium (CCM) enriched with additional vitamins and amino acids to grow epithelial cell lines. HuWa1 cells incubated with medium containing 0 – 10 fold the vitamins in MEM exhibited no difference in growth over time (Fig. 5C). Addition of essential amino acids, however, inhibited cell growth. From day six onwards, all concentrations of added amino acids led to decreased cell numbers compared to the control (Fig. 5D). This experiment demonstrates that HuWa1 cells do not require enriched CCM. The decrease in cell proliferation may be explained due to media requirements being specific for different cetacean cell types. In the case of HuWa1, high concentrations of single amino acids or a

change of the amino acid composition exceeding the optimum concentrations may lead to a decline of cell proliferation.

### Impact on cell viability of *p,p'*-DDE and blubber extract

Both HuWa1 and HFb were clearly impacted in their viability upon exposure for 24 h to the highest applied concentration of *p,p'*-DDE, which was 2400 µg/l (7.6 µM). However, the lowest observed effect concentration for HuWa1 cells was 1500 µg/l (4.7 µM), while for HFb was 120 µg/l (0.3 µM) (Fig. 6A). The calculated $EC_{50}$ value for HFb was about six-fold lower compared to HuWa1 (Fig. 6B). The hill slope of the dose response curves were -1.7 and -11.65 for HFb and HuWa1 cells, respectively. Varying hill slopes may indicate different species sensitivity or different mode of action (Rodier, 1994). These results suggest a species-specific response and point out that data cannot be transferred comprehensively between these two mammalian species. The average *p,p,'*-DDE concentration detected in the blubber of southward migrating humpback whales of 15.2 $ng/g_{blubber}$ (15.2 µg/l) (Bengtson Nash et al., 2013) did not appear to affect cell viability. In nature chemical exposure does not, however, occur to single chemicals in isolation, but to complex chemical mixtures. In order to test a more environmentally relevant exposure mixture, HuWa1 cells were finally exposed to a chemical extract from the blubber of a stranded humpback whale. The *p,p,'*-DDE content of this extract was measured previously and thus the extract concentrations are expressed as *p,p,'*-DDE equivalents. The concentration-response relationship of the POP extract was clearly shifted to the left compared to *p,p'*-DDE alone and the assessed $EC_{50}$ values were ~1880-times lower (Fig. 6). SH humpback whales accumulate potentially interacting mixtures of lipophilic chemicals including PCBs, DDTs, chlorobenzenes, chlordanes, cyclodienes and toxaphenes (Bengtson Nash et al., 2013). Elevated sensitivity may be due to the mixture of identified chemicals but also unidentified chemicals, which may elute in the same fraction but which have not been quantified in this individual, e.g. polybrominated diphenyl ethers. Therefore we presume that exposure to the chemical mixture also reflects additive, synergistic and/or antagonistic effects. Further toxicological studies are required and a wider range of chemicals with different modes of action needs to be tested to provide further evidence regarding responsiveness of this cell line and differences to other cetacean or human cell lines.

## Summary and Conclusion

We have established the first cell lines from humpback whales. Both HuWa1 and HuWa2 cells were identified as fibroblasts stemming from male individuals with a karyotype of 2n=44, which has commonly been observed in other baleen whale species e.g. blue whales. The sensitivity of the HuWa cells to a typical POP differs considerably from human fibroblasts and a natural POP mixture emphasizing the importance for species-specific toxicity evaluation and the role of *in vitro* methods to evaluate those. Future research will focus not only on toxicity aspects but also on the development of immortalised HuWa cell lines to enable long-term maintenance and use. The establishment of HuWa cell lines provides the unique opportunity to develop advanced approaches for an integrated toxicity risk assessment geared toward humpback whales with enhanced environmental relevance.

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**Figure Captions**

Figure 1: Phase contrast appearance of HuWa1 cells. (A) Primary cells growing out of connective tissue fragments, (B) detailed morphology of fibroblastic cells and (C) isolated primary cells forming a semi-confluent layer observed at the edge of a T75 cell culturing flask (P1). Mean size of HuWa1 varied from 50-200 µm in length and suspended cells exhibited an average diameter of 20-22 µm. The scale bar indicates 300 µm.
(2-column image; color)

Figure 2: Karyogram of Megaptera novaeangliae cell cultures. G-banded chromosomes of (A) HuWa1 (P3) show chromosome numbers 2n= 44 and male gender (arrow). (B) Combined G and C-banding of HuWa1 indicates tetraploidy in some cells and (C) cryopreserved HuWa1 (P3) show no evidence of chromosomal rearrangements.
(3-column image; color)

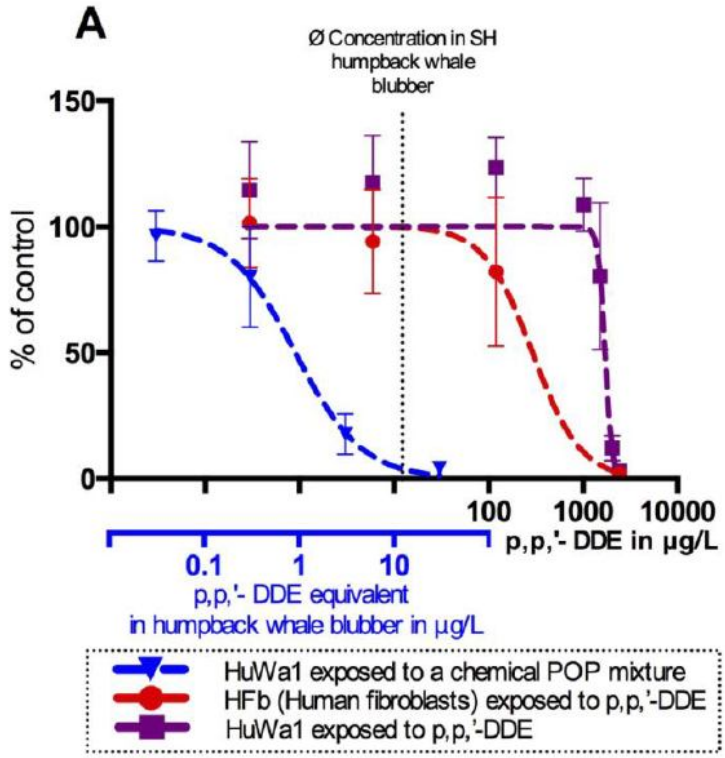
Figure 3: Assessment of selected protein markers in the HuWa1 cells. Cells exhibited positive expression for intracellular Vimentin (A, green), no reactivity of Cytokeratin (B), positive immunoreaction with Col I (C, red) and weak immunoreaction with Col IV (D, red). The inset in (B) shows CaCo-2 cells as technical positive control for Cytokeratin (green). Cell nuclei were counterstained with DAPI (blue). The scale bar indicates 50 µm.
(2-column image, color)

Figure 4: Growth curves of HuWa cell lines. HuWa1 and HuWa2 indicate similar growth properties and slightly reduced growth of cryopreserved HuWa1 (P3) for the first passage directly after thawing. Data represent the mean of three technical replicates and the error bars represent the SD.
(1-column image; color)

Figure 5: Assessment of the influence of varying temperatures (A), FBS concentrations (B), vitamin concentrations (C) and essential amino acids concentrations (D) on the proliferation capacity of HuWa1. Data represent the mean of three technical replicates and the error bars represent the SD.
(2-column image; color)

Figure 6: Cell viability assessment upon exposure to p,p,'-DDE and a POP mixture. (A) HuWa1 (P8-P15) and HFb (P5-8) exposed to p,p,'-DDE, and HuWa1 (P8) exposed to an extracted POP mixture, the latter of which is expressed as p,p,'-DDE equivalents (lower X-axes). Data for p,p,'-DDE is presented as the mean of three independent experiments and the error bars represent the standard deviation, whereas data for the POP mixture depict the mean of three technical replicates. The vertical dotted line indicates the average p,p,'-DDE concentration in the fat of southward migrating humpback whales (Bengtson Nash et al., 2013). (B) $EC_{50}$ values as assessed from corresponding concentration-response curves.
(1-column image; color)

*Figure 6*




**B**

| Substance | Species | $EC_{50}$ |
|---|---|---|
| p,p,'-DDE | HuWa1 | 1693 µg/L (3.32 µM)[a] |
| p,p,'-DDE | HFb | 290 µg/L (0.91 µM)[a] |
| POP extract | HuWa1 | 0.9 µg/L (0.003 µM) |

[a] *Significant difference between log$EC_{50}$ ($P < 0.05$)*

*Figure 5*

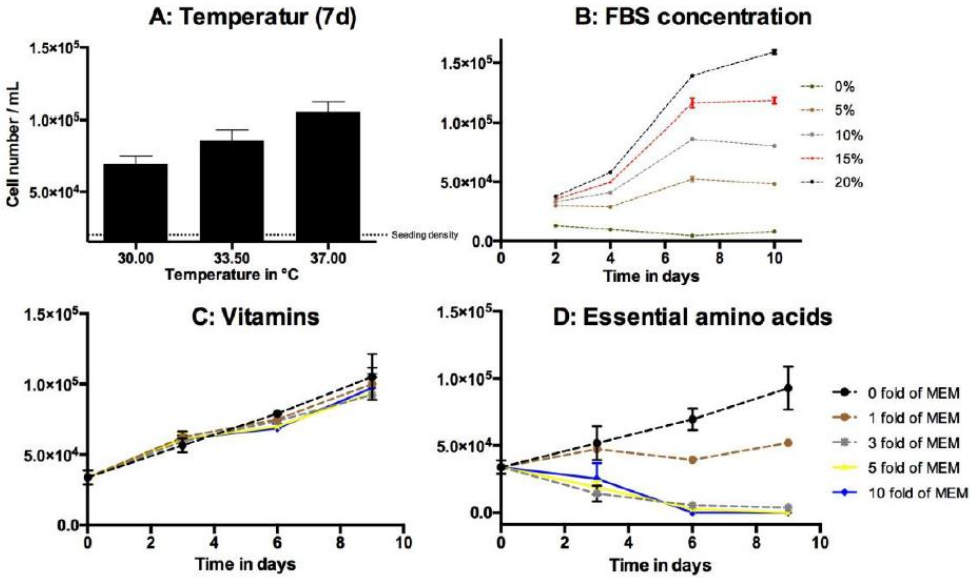


*Figure 3*

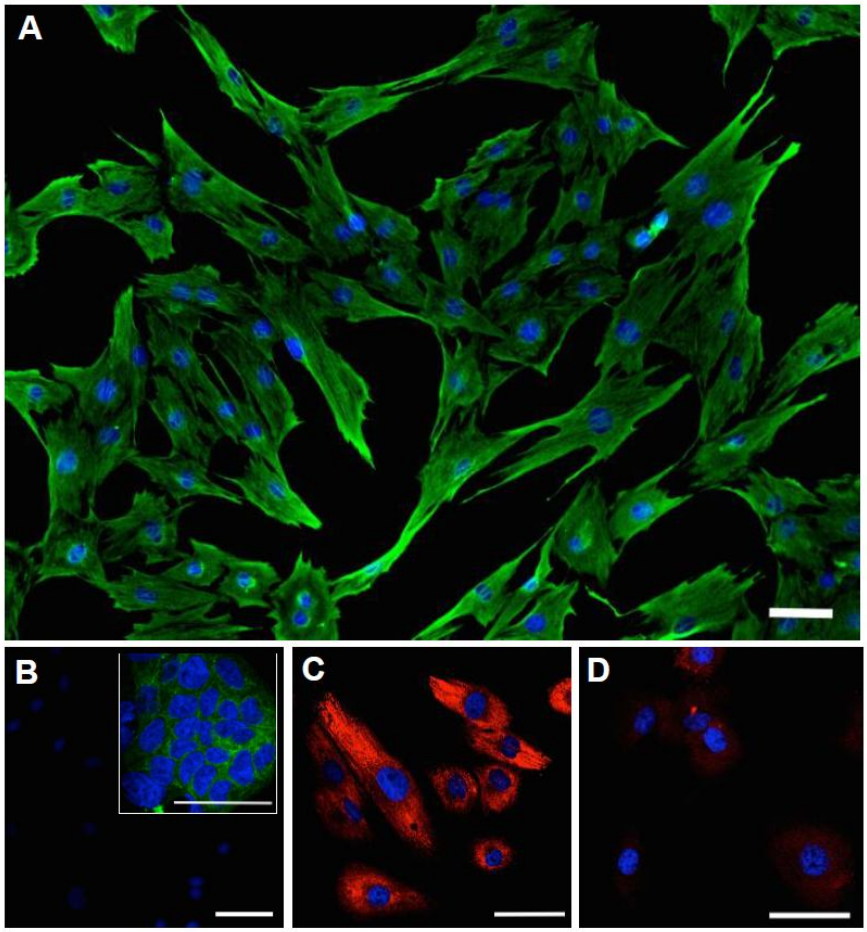



*Figure 4*

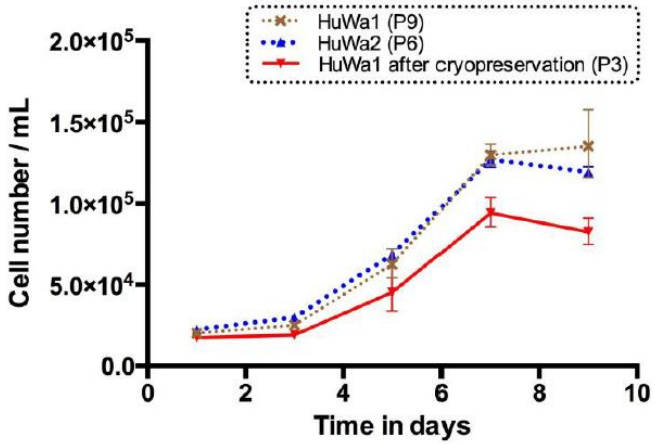


*Figure 1*

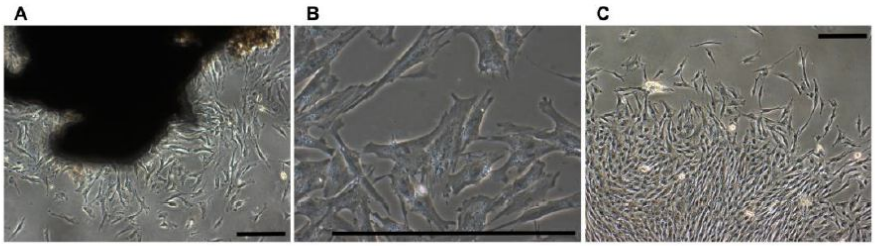
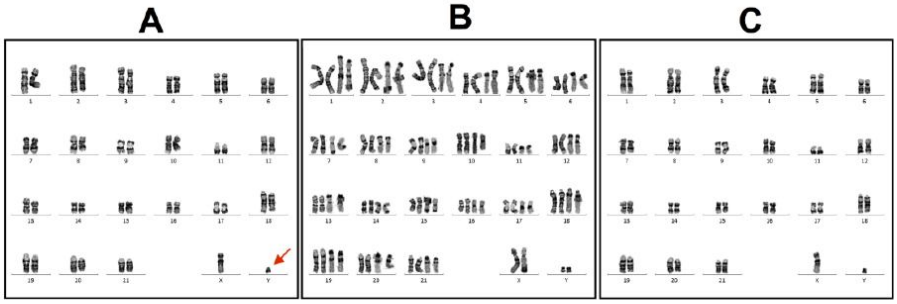



*Figure 2*




Tables

**Table 1.** Overview of sampled humpback whales and success of primary cell isolation.

| ID | Animal Age [a] | Transport (*t*) | Primary Cells [b,c] | Passage | Designation |
|---|---|---|---|---|---|
| 2S13 | Yearling (1-2 years) | 6 h | + | **> P 30** | HuWa1 |
| 15S13 | Adult (Female) | 8 h | - | n.a. | |
| 16S13 | Yearling (1-2 years) | 6 h | + | (P1) [e] | |
| 27S13 | Adult (Male) | 8 h | + | **> P 10** | HuWa2 |
| 28S13 | Calf (< 1year) | 8 h | + | (P1) [e] | |
| 29S13 | Adult (Male) | 7.5 h | - [d] | n.a. | |
| 30S13 | Adult (Male) | 7.5 h | - [d] | n.a. | |
| 31S13 | Adult (Male) | 7.5 h | + [d] | (P3) [e] | |
| 32S13 | Adult (Male) | 7.5 h | - [d] | n.a. | |

[a] The age and gender of biopsied animals was estimated by size and group composition of migrating cohorts

[b] + or - indicates the presence or absence of primary cells
[c] Primary cells were isolated after 32 to 38 days in culture
[d] Presence of abundant lipid droplets
[e] Non-successful passaging; at the number given in brackets cells stopped proliferation