Primary cell cultures from deceased harbor porpoises to study effects of polystyrene nanoplastics on gene expression

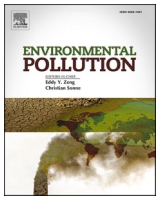
Roelen, B.A.J.; Roest, H.P.; Coenen Morales, I.; Meirer, F.; IJsseldijk, L.L.; Bosker, T.; Laan, L.J.W. van der

Environmental Pollution 383 (2025) 126805

Contents lists available at ScienceDirect

Environmental Pollution

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



Primary cell cultures from deceased harbor porpoises to study effects of polystyrene nanoplastics on gene expression☆

Bernard A.J. Roelen [a,*], Henk P. Roest [b], Immelie Coenen Morales [a,d], Florian Meirer [c], Lonneke L. IJsseldijk [d], Thijs Bosker [e], Luc J.W. van der Laan [b,**]

[a] Department Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[b] Department of Surgery, Erasmus MC Transplant Institute, University Medical Center Rotterdam, the Netherlands
[c] Inorganic Chemistry & Catalysis, Institute for Sustainable and Circular Chemistry, Faculty of Science, Utrecht University, Utrecht, the Netherlands
[d] Division of Pathology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[e] Institute of Environmental Sciences, Leiden University, Leiden, the Netherlands

ARTICLE INFO

Keywords:
Microplastics
Nanoplastics
MNPs
Polystyrene
Cetacean
In vitro
Cell culture
Gene expression
RNA sequencing
Fibroblasts
Exposome
Environmental toxicity

ABSTRACT

Micro- and Nanoplastic particles (MNPs) are abundantly present in the environment including in our oceans. Whale species (cetaceans) occupy the top of the food chain and get exposed to MNPs via their diet. Therefor cetaceans are considered sentinel species to investigate health effects of MNPs in mammals. The aim of this study is to understand how these particles affect cellular function of marine mammals. For this harbor porpoise tissues were isolated *post mortem* and cells were cultured *in vitro*. Cells were subsequently exposed to fluorescently-labelled polystyrene MNPs of 1000, 200, and 50 nm diameter. After exposure the cells were examined by confocal laser scanning microscopy and RNA sequencing. Plastic particles, even of 1000 nm diameter were observed inside the cells after 24 h of exposure. Gene expression changes were observed after plastic particle exposure in pathways of various biological processes including signaling and metabolism. In conclusion, this study shows that exposure to MNPs induces cellular responses and changes in gene expression in fibroblast-like cells from a sentinel marine mammal. Comparative analyses between human, cetacean and fish show common as well as species-specific gene responses which could be relevant to further study the health impact of MNPs.

1. Introduction

Marine plastic pollution poses a significant concern, with an estimated 3200 kilotons of plastic litter present in the oceans in the year 2020, and an annual input of additional 500 kilotons (Kaandorp et al., 2023). An estimated 60–80 % of debris found in the marine environment is plastic waste (MacLeod et al., 2021; European Commission, 2018). The biodegradability of most plastics is very limited, but large plastic pieces can over time fragment into micro and nanoplastic particles (MNPs). The size of microplastics ranges from 5000 to 1 μm; plastic particles smaller than 1000 nm are termed nanoplastics (Weber et al., 2022; Lu et al., 2024). Due to their small size MNPs are easily ingested by marine life, both via direct and indirect, via the food, exposure (Zantis et al., 2021). As an integral part of the food chain it very likely that MNP impact human health via aquatic organisms.

Whale species (cetaceans) occupy a prominent position in the food chain and are sentinel species, thereby serving as indicators of ocean health. Marine mammals are frequently exposed to MNPs, with estimates ranging from 200,000 to 10 million particles per day in filter feeders (Zantis et al., 2021) and 150 particles per day in delphinids (Dool and Bosker, 2022). Recently a study found that small cetaceans can also inhale MNPs (Dziobak et al., 2024). Importantly, these estimates are based on the presence of relative large microplastics, while very little is known about nanoplastics, which are more prominent in the marine environment (Cuningham et al., 2023). Although exposure of cetaceans to MNPs is well documented, effect testing is challenging, as most marine mammals are protected species. Notably, *in vitro* cell cultures offer a prospective avenue to examine the effect of exposure to anthropogenic pollutants on cellular and tissue function, which can be used to predict impacts on animal health (Borod et al., 2020; Ochiai

☆ This paper has been recommended for acceptance by Jung-Hwan Kwon.

* Corresponding author.
** Corresponding author.
E-mail addresses: B.A.J.Roelen@uu.nl (B.A.J. Roelen), l.vanderlaan@erasmusmc.nl (L.J.W. van der Laan).

https://doi.org/10.1016/j.envpol.2025.126805
Received 14 May 2025; Received in revised form 21 June 2025; Accepted 8 July 2025
Available online 9 July 2025

et al., 2020). It has been described that cultured cells of well-known cell lines such as Caco-2 and HT-29 can passively take up MNPs of different sizes. Cytotoxicity has been observed in cell lines exposed to MNPS, particularly with regard to oxidative stress and membrane. In addition, it has been suggested that higher dose can lead to higher cellular uptake damage (Banerjee and Shelver, 2021 and references therein).

Here we investigated the impact of MNPs on cultured cells of the harbor porpoise (*Phocoena phocoena*). The harbor porpoise is a small cetacean species that is native to waters of the northern hemisphere, including the North Sea. In this study, harbor porpoise cells obtained from stranded animals were collected *post mortem* and cultured *in vitro*. First, we highlight our procedure to successfully obtain viable cells from deceased porpoises. Next, we exposed cultured cells to different sizes of labelled polystyrene MNPs and examined for uptake and changes in gene expression. The findings indicate that even the largest of MNPs (1000 nm diameter) can enter the cytoplasm of cells and change gene expression patterns. To our knowledge this is the first study on cetacean cell lines that investigates the impact of NMPs on cetacean cells, providing an important method that will allow to study impacts of NMPs on cetacean health. Comparative analyses between cetacean and humans may provide new insights into the health impact of MNPs in humans as well (van der Laan et al., 2023)

2. Materials and methods

2.1. Tissue collection

Between September 2021 and June 2022, harbor porpoises that stranded dead or died shortly after stranding on the Dutch coast were transported to the Division of Pathology of the Faculty of Veterinary Medicine at Utrecht University as part of the ongoing Stranding Research Program (IJsseldijk et al., 2022). Upon arrival, the animals were examined externally and internally to identify their health status and cause of death, following internationally standardized guidelines and procedures (IJsseldijk, 2019). Tissue pieces of different origins were excised using a sterile scalpel, and transferred to a sterile 15 mL tube containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco; Grand Island NY, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco) and 1 % AA (Antibiotic-Antimycotic; Gibco). Alternatively, tissue pieces were collected in University of Wisconsin (UW) organ preservation solution (Viaspan, Duramed Pharm Inc. Pomona NY, USA) if longer storage periods (several days) were required. For information on the animals, including sex, age and cause of death see Supplementary Table 1.

Maximally 2 h after collection, the tissue pieces were placed in Petri dishes in a biosafety cabinet under sterile conditions and cut into approximately 1 mm^3 pieces using sterile disposable scalpel blades. Pieces were placed in 6-well plates (Primaria™ 6-well Clear Multiwell Plate; Corning, Corning NY, USA) with 3 mL culture medium, which consisted of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco), supplemented with 10 % fetal bovine serum (FBS; Gibco) and 1 % AA (Gibco) and placed in a humidified incubator at 37 °C and 5 % CO_2. The medium was refreshed daily for two weeks. After this period, medium was refreshed every other day. The wells were examined daily with a reversed light microscope for cellular outgrowth. Cells recovered from harvested tissues were cryopreserved in Cryostor (Merck, Amsterdam, the Netherlands) or 90 % FBS and 10 % DMSO using a Mr. Frosty (Merck) according to manufacturer's recommendations and banked for future research at −196 °C in vapor phase nitrogen.

For thawing, frozen cells in cryovials were kept for approximately 1 min at 37 °C in a water bath, after which the cells were resuspended in 10 mL culture medium. After centrifugation for 5 min at 163 g the pellet was resuspended in 3 mL DMEM/F12 medium (Gibco) with 10 % FBS, 1 % AA (Gibco), and seeded in 6 well plates.

2.2. MNP exposure

Lung cells (adult females national reference numbers: UT1919 and UT1924) and diaphragm cells (neonate male UT1934) were cultured in 8 well-chamber slides (Nunc Lab-Tek Chamber Slide System 177445; Thermo Scientific, Waltham MA, USA) in DMEM/F12 medium (Gibco) with 10 % FBS, 1 % AA (Gibco), in a humidified incubator at 37 °C and 5 % CO_2. Fluorescein-labelled Yellow Green (YG) polystyrene (latex) nanoplastic particles (abbreviated as YG-NP) with 50 nm (Polybead 17149-10), 200 nm (Polybead 09834-10) and 1000 nm (Polybead 17154-10) diameter sizes were used (Fluoresbrite Microparticles; Polysciences Europe; Hirschberg an der Bergstrasse, Germany).

Cells at 40 % confluency were exposed to the particles for 20 h at either 50 or 100 μg/mL. Thereafter, cells were washed 2x with Hanks' Balanced Salt Solution (HBSS; Gibco; Grand Island, NY, USA), and fixed with 4 % paraformaldehyde for 15 min at room temperature. Subsequently, cells were stained with 1 μg/mL Hoechst 33342 (ab228551; Abcam, Cambridge, UK) for 5 min, again washed 2x with HBBS, permeabilized with 0.1 % Triton X-100 and stained with 0.25 μg/mL Phalloidin-568 (Invitrogen; Waltham MA, USA) for 15 min followed by two final washes with HBSS. Cells were sealed in mounting medium (Fluorsave Reagent 3769661; Millipore, Burlington MA, USA) and covered with microscope slides. Imaging took place using confocal laser scanning microscopy with a Leica SPE-II system at 405 nm (Hoechst), 635 nm (Phalloidin) and 488 nm (YG-NP).

2.3. RNA isolation and sequencing

Cells were lysed in buffer RLT (Qiagen; Germantown MA, USA) and stored at −80 °C until further use. RNA extraction was performed with an RNeasy minikit (Qiagen) according to the manufacturer's instructions. RNA was eluded with 2x 100 μL water, pooled and lyophilized for 2h in a sublimator (Zirbus, Tiel, the Netherlands). Subsequently RNA was dissolved in 15 μL RNase free water and examined for purity and concentration on a Bio-analyzer (Agilent; Santa Clara CA, USA). RNA was sequenced and data were uploaded to Galaxy Web platform (Goecks and Nekrutenko, 2010). Reads were mapped using HISAT2 (version 2.2.1) against the Vaquita (*Phocoena sinus*) genome file GCF_008692025.1_mPhoSin1.pri_genomic.fna.gz (Morin et al., 2021). Mapped reads were annotated against the Phocoena_sinus_annotation_release_100 and converted to counts using FeatureCounts (version 2.0.1). DESEQ2 (version 2.11.40.7) was used to identify differentially expressed genes. Genes with an adjusted p-value <0.05 (Benjamini--Hochberg FDR correction) were considered significant. Transcriptome analysis was performed using the GSEA platform for gene set enrichment Subramanian et al., (2005) (https://www.gsea-msigdb.org/gsea/index.jsp); and ShinyGO for pathway analysis (Ge et al., 2020; https://bioinformatics.sdstate.edu/go). After normalization for the count depth using the median of ratios method, the Wald test was used for hypothesis testing between two groups. Correction for multiple testing was performed using the Benjamini and Hochberg method (Love et al., 2014). Raw data are available from the Gene Expression Repository (GEO) at NCBI (accession number GSE289215).

3. Results

3.1. Establishing harbor porpoise primary cell cultures

To understand the viability of cells from various tissues of deceased harbor porpoises, an initial phase involved the collection of diverse tissue types from 24 animals, which were subsequently mechanically minced and cultured *in vitro*. Wells were examined daily by light microscopy, and despite the presence of antibiotics and antifungals, growth of bacteria and fungi was frequently observed. Any wells showing bacteria or fungal culture were promptly discarded. For skin tissue, liberated and substrate-attached cells were observed at, on

average 11 ± 11.9 (SD) days after the start of culture (n = 10). Skeletal muscle cells (Fig. 1A) were observed after 11.7 ± 9.4 days (n = 10); cells from the diaphragm (Fig. 1B) after 8.6 ± 2.3 days (n = 14), liver cells after 23 ± 13.2 days (n = 3); intestinal tissue after 7.9 ± 2.0 days (n = 9); cells from lung tissue after 8.3 ± 4.1 days (n = 10). The timing of cells liberating from the tissues seemed influenced by the specific individual rather than the tissue type, and is, therefore, most likely due to time between death of the animal and obtaining the tissue pieces. Indeed, tissues from several specimens were unsuccessful in generating viable cultured cells.

Viable cells were obtained from a variety of tissues collected, including lung, liver, skin, small intestine, skeletal muscle, and diaphragm. However, there were also tissues, such as heart, testis, fat, tendon, ovary, and bone marrow that did not result in any viable cultured cells. Tissues directly collected in UW organ preservation solution could be stored at 4 °C for up to 6 days before culture and still give rise to viable cells, but cells liberated from tissues that had been stored in UW solution only appeared after 10 days or more (data not shown).

Cells were most likely fibroblast type, as defined by the spindle shape and parallel linear actin filaments (Jalal et al., 2019). In addition, transcriptional activity as determined by RNAseq experiments, here presented, clearly show that both Vimentin (VIM) and αSMA (ACTA2) are amongst the genes with the highest expression levels (7th and 47th, respectively, of 19508 annotated genes. In addition, other genes that are related to the formation of connective tissue, like Fibronectin (FN), and Collagen (COL1A1, COL1A3) are ranked highest on gene expression.

Culturing the tissue pieces on wells coated with Collagen type I did not lead to faster liberation of the cells, although cells cultured on collagen appeared to exhibit enhanced proliferation as indicated by the time the cells reached confluency after passaging (not shown). Regardless of the tissue type, the cells originally displayed a spindle like morphology (Fig. 1A and B), with several prominent nucleoli in the nucleus. After several passages, the majority of the cells lost their spindle shapes, enlarged their surface area and obtained a fibroblast like morphology (Fig. 1C).

3.2. Cellular uptake of fluorescent polystyrene nanoplastic particles

In order to determine the potential uptake of MNPs by harbor porpoise cells, cultured fibroblast-like lung and skeletal muscle cells were exposed to yellow green fluorescent polystyrene MNPs (YG-NP) of different sizes (50 nm, 200 nm, and 1000 nm), at concentrations of 50 μg/mL or 100 μg/mL. An exposure time of 20 h was selected considering the potential increase of particle size due to adsorption of proteins and lipids from the culture medium, which could occur after 24 h (Revel et al., 2021). Lung and skeletal muscle-derived fibroblast-like cells from both male and female animals were exposed to the MNPs and analyzed for cellular uptake, with no differences observed between tissue types or sexes. Confocal laser scanning microscopy revealed the presence of YG-NPs within the lung-derived fibroblast-like cells, particularly evident for the 200 and 1000 nm-sized particles (Fig. 2). No differences were observed between the different concentrations of 50 and 100 μg/mL, prompting the subsequent use of 50 μg/mL in follow-up experiments. The particles appeared to be localized around the nucleus, indicating retrograde transport from endosomes to the *trans*-Golgi network (Bonifacino and Rojas, 2006). On occasion, particles were observed that appeared to be within the nucleus. However, further investigation is needed to confirm their actual presence within the nucleus. Where exposure to 200 and 1000 nm particles clearly showed the presence of MNPs intracellularly (Fig. 2B and C), detection of uptake of the smaller, 50 nm YG-NPs, was more challenging. Only subtle fluorescence, slightly above background levels, was observed, both in the cytoplasm as at the location of the nucleus (Fig. 3). Signal was constrained by the resolution limitations of confocal laser scanning microscopy.

3.3. Change in gene expression in response to polystyrene MNPs

To examine the effect of polystyrene YG-NP internalization on gene expression, two batches of primary lung-derived fibroblast-like cells were analyzed by bulk RNA sequencing after 20 h exposure to 50 μg/mL of 50 nm, 200 nm and 1000 nm size YG-NPs. Principal Component Analysis (PCA) of the whole transcriptome showed that exposure to 50 nm and 200 nm particles cluster together with the untreated (control) samples in the first dimension, but that RNA profiles after exposure to 1000 nm diverge from that cluster (Fig. 4A). This observation is substantiated by the non-hierarchical sample-to-sample distances where the 1000 nm samples as well as sample 200 nm are the least divergent with each other as they are to the other samples (control, 50 nm and 200 nm) (Fig. 4B). To identify genes that were commonly differentially expressed between the 6 possible combinations, DESeq2 analysis was applied on the raw counts obtained after mapping the RNAseq output on the Vaquita (*Phocoena sinus*) genome, currently the sequenced genome most closely related to that of the harbor porpoise. Filtering for differentially expressed (DE) genes (Padj<0.05) clearly shows that there were only a limited number of commonly DE genes between 50 nm and 200 nm, 50 nm and control, and 200 nm and control (3, 23, and 26 respectively) while exposure to 1000 nm YG-NPs resulted in much higher number of commonly DE genes when compared to 200 nm, control, and 50 nm (134, 142, and 155, respectively) (Fig. 4C). Of these DE genes, 49 genes are differentially expressed in all three comparisons (Fig. 4C).

As the number of DE genes were limited between 50 nm and 200 nm, 50 nm and control, and 200 nm and control, we focused on the effect of 1000 nm MNP exposure in comparison to untreated fibroblasts. When the set of 142 DE genes (for the full list see Supplemental Table S2) was analyzed for their presence and involvement in various molecular functions (Fig. 5A), cellular components (Fig. 5B), and biological processes (Fig. 5C), a wide range of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa et al., 2021) were identified that show significant differences (Fig. 5D). These data indicate that exposure of harbor porpoise cells to YG-NP affects cellular pathways essential for normal homeostasis.

To identify putative regulatory factors that can play a role in the

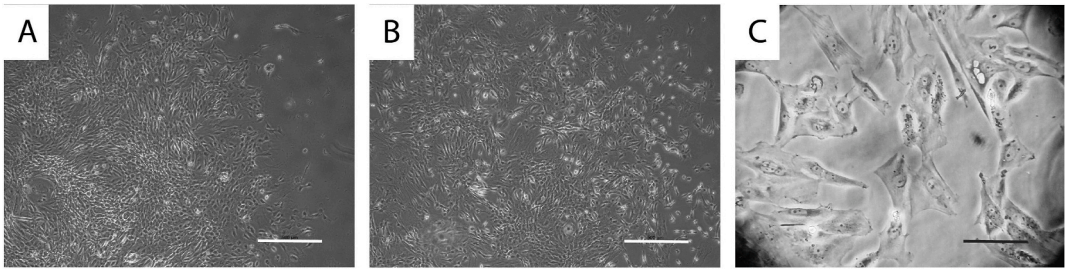


Fig. 1. Photomicrographs of cultured harbor porpoise cells. (A) Skeletal muscle, freshly liberated cells without passage, (B) Diaphragm-derived cells, freshly liberated cells without passage, displayed a spindle like morphology. (C) Skeletal muscle-derived cells after 5 passages obtaining a fibroblast-like morphology. Scale bars represent 500 μm (A,B) and 100 μm (C).

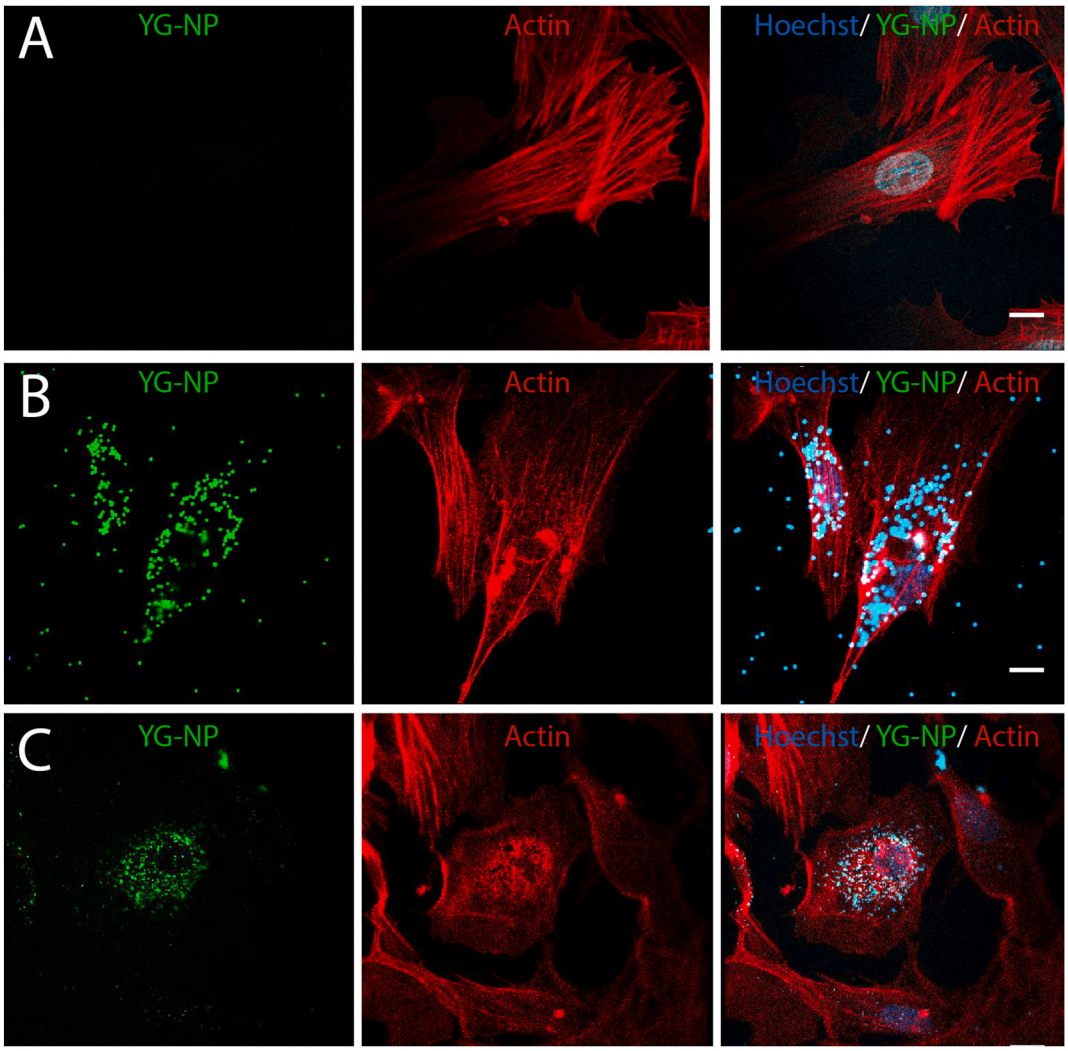


Fig. 2. Uptake of Yellow Green fluorescein-labelled polystyrene nanoplastic particles (YG-NP) by harbor porpoise lung-derived fibroblast-like cells. (A) Untreated control cells, and after incubation with 1000 nm (B) or 200 nm (C) YG-NP at concentration 50 μg/mL. Blue-green = nanoplastic particles, red = actin cytoskeleton (phalloidin), blue = DNA/Nucleic acid (Hoechst). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

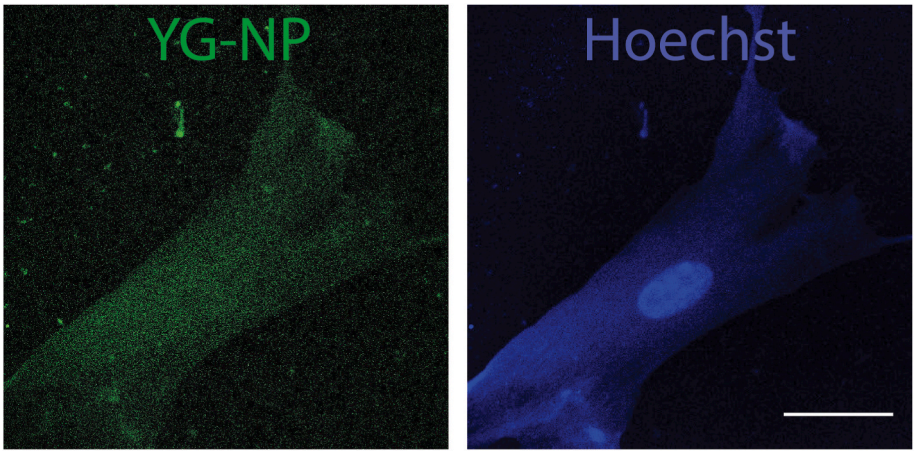


Fig. 3. Uptake of 50 nm Yellow Green fluorescein-labelled polystyrene nanoplastic particles (YG-NP; 50 μg/mL). High magnification image shows YG-NP is present throughout the cell, while Hoechst (blue) staining indicates the nucleus. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

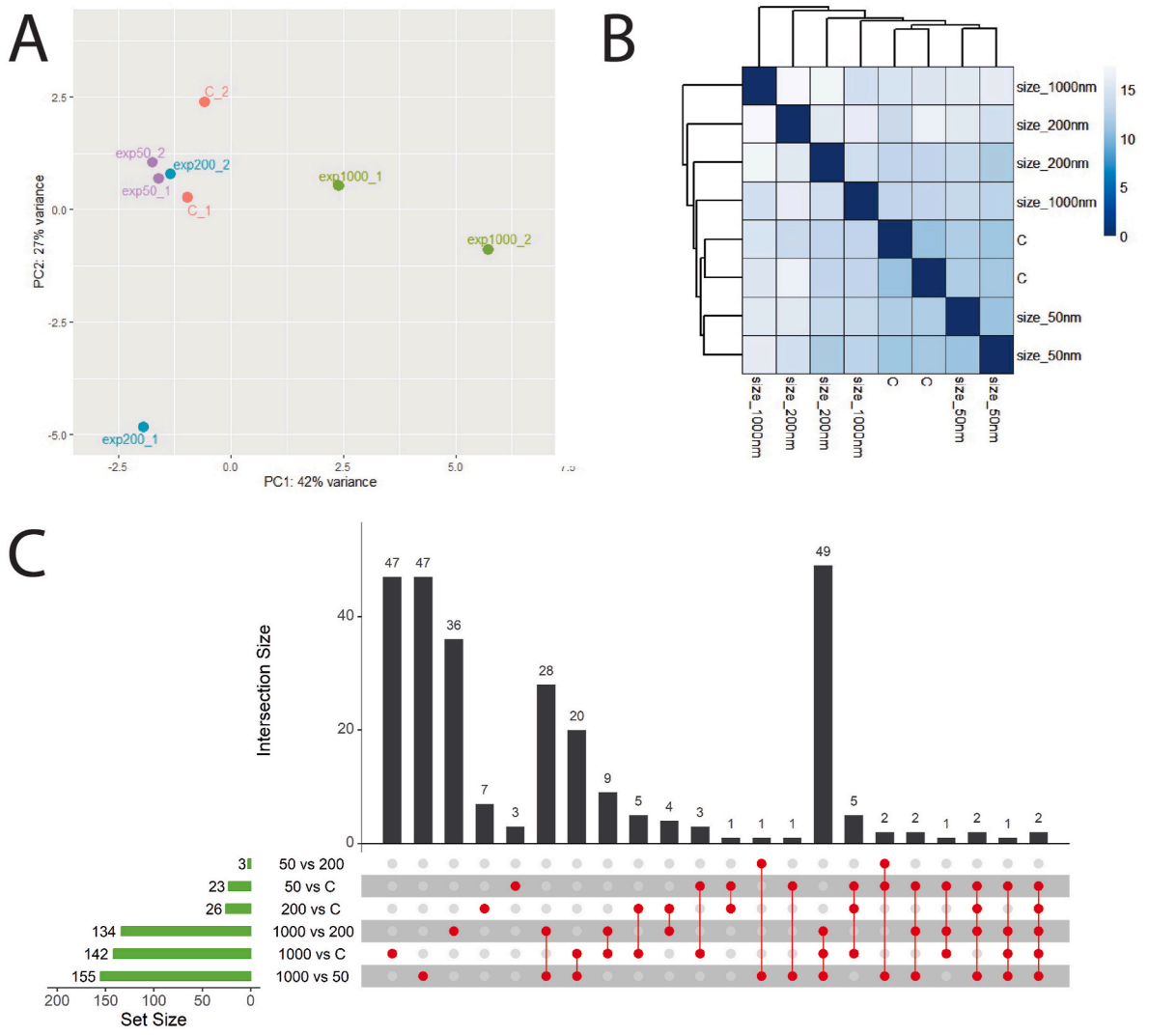


Fig. 4. Characteristics of gene expression profiles of cells before and after YG-NP exposure. (A) Principal Component Analysis, (B) heatmap of the sample-to-sample distance matrix (with clustering) based on the normalized counts, and (C) visualization of the intersection of all six differentially expressed (DE) gene sets obtained after DESeq2 analysis. Red connected dots mark the number of genes that are common for the indicated comparisons on the left. Cells exposed to 1000 nm GY-NP showed most changes in gene expression compared to other conditions. Genes with a FDR <0.05 are considered significantly, differentially expressed. C = Control before treatment and exp50, exp200, exp1000 = cells after treatment with 50 nm, 200 nm and 1000 nm YG-NP, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ecotoxicological consequences of NMP treatment, we performed a search using the significantly differentially expressed genes for the 1000 nm particles against all other three condition (no particles, 50 nm particles, and 200 nm particles) using the online transcription factor analysis tool ChEA3 containing approximately 1700 putative transcription factors and their targets (Keenan et al., 2019). Out of a total of 89 common transcription factors, three transcription factors were identified that made the top 10 for all three comparisons: ATF3, GLMP, and NFE2L2. ATF3 is a member of the CREB protein family of transcription factors and functions as a rapid responder to cellular stress, participating on proapoptotic pathways. NFE2L2 is a transcription factor that is involved in cellular defense against oxidative and electrophilic stress via genes that contain antioxidant response elements (ARE) in their promoter region. GLMP (formerly known as NCU-G1) encodes a lysosomal membrane protein. Although the transcriptional function of this protein is yet not clarified, it was shown that inactivation of this gene resulted in oxidative stress and increased fibrosis and hepatic cell death (Kong et al., 2014) Combining these individual characteristics suggests that stress response is the major driver in cellular coping with, especially, 1000 nm NMPs.

One of the KEGG pathways that clearly stands out is that of the Terpenoid Backbone Biosynthesis. Three enzymes that are active in the mevalonate pathway are significantly up-regulated; 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1; 2.3.3.10), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) 1.1.1.34, and isopentenyl-diphosphate delta isomerase 1 (IDI1; 5.3.3.2) (Supplemental Fig. 1A). All three enzymes are also up-regulated in cells exposed to 1000 nm YG-NPs compared to 50 and 200 nm YG-NPs (data not shown). In addition to these differentially expressed genes, acetyl-CoA acetyltransferase 1 (ACAT1; 2.3.1.9) and mevalonate diphosphate decarboxylase (MVD; 4.1.1.3) are also expressed at a higher, however not significant, level (Supplemental Fig. 1). That this pathway might be affected by the exposure to 1000 nm polystyrene YG-NPs is also supported by the gene set enrichment analysis, where it is shown that all genes tend to be ranked higher in the list of identified genes. This pathway functions upstream of the ubiquinone and terpenoid-quinone pathway. In human cells, metabolites of this pathway were shown to be dysregulated in response to polystyrene MNP exposure (Wang et al., 2022).

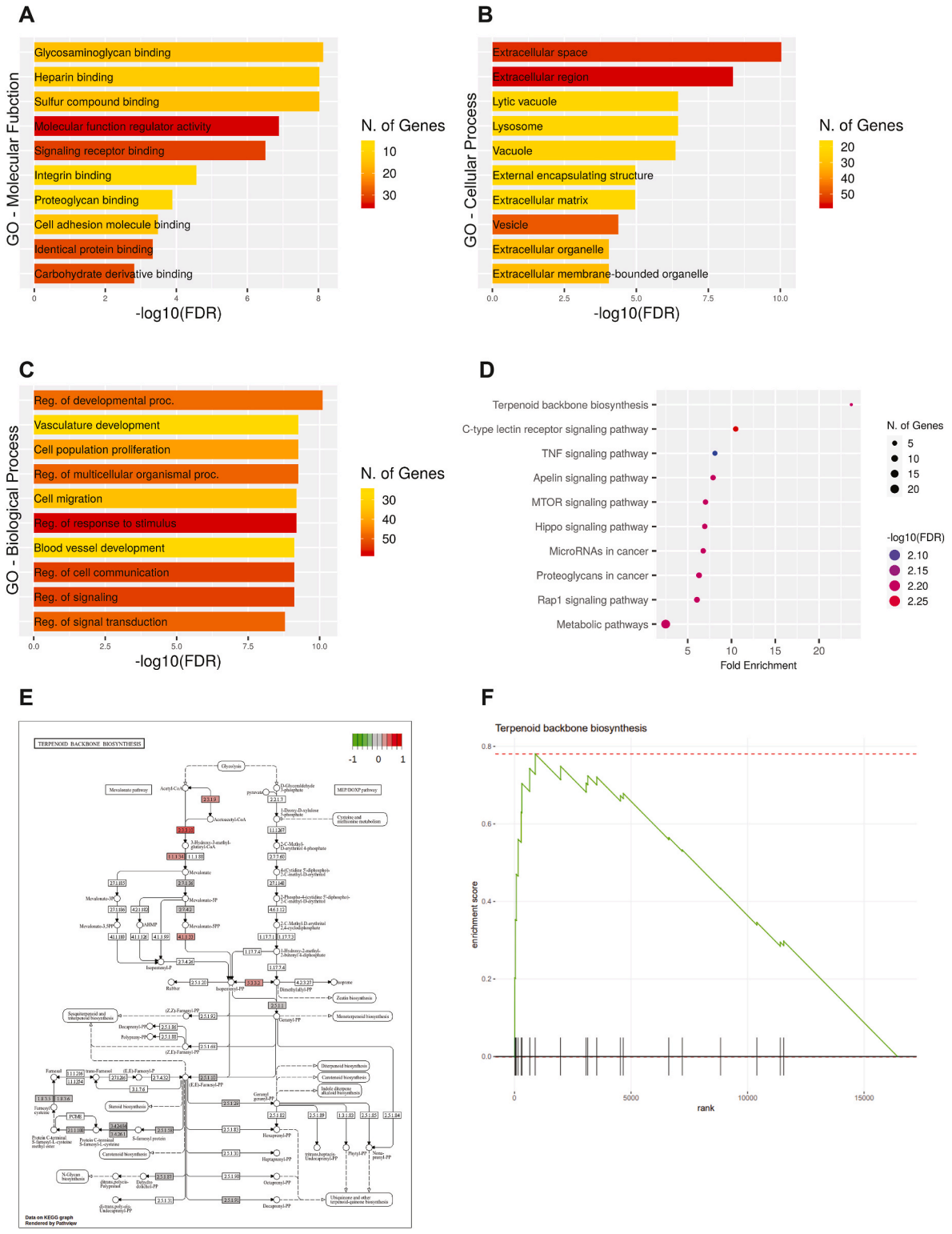


Fig. 5. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of differentially expressed genes before and after exposure to 1000 nm YG-NP. (A) Top 10 GO – Molecular function analysis, (B) GO – Cellular component, and (C) GO – Biological process gene sets significantly enriched after exposure to 1000 nm YG-NP.(D) KEGG pathway enrichment analysis using the same set and calculated using the ShinyGO 0.80 platform. Highest enrichment was observed for the terpenoid backbone biosynthesis pathway. (E) Graphical representation of the terpenoid backbone biosynthesis pathway (hsa00900 pathview representation). Scale depicts –log2FC of expression. Red indicates up-regulation of that specific gene in the exposed cells. (F) Gene Set Enrichment Analysis comprising 23 genes. NES = Normalized enrichment score. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Species-specific and common gene expression pathways affected by polystyrene MNPs

Exposure to polystyrene MNP, both i*n vivo* and *in vitro,* have been shown to affect a plethora of cellular processes (Yin et al., 2023a; Yin et al., 2023b). Several studies using next-generation sequencing revealed the complex biological effects of polystyrene MNPs *in vitro* and *in vivo,* including inflammation, oxidative stress, and cellular dysfunction. Whether or how different species respond differently to polystyrene MNP exposure has not been extensively investigated. Therefore we comparted the polystyrene MNP-induced gene pathways of our harbor porpoise fibroblast-like cells to gene pathways reported in human skin fibroblasts (Stojkovic et al., 2023) and zebrafish liver fibroblasts (stellate cells) (Deng et al., 2023). We identified 142 genes being differentially expressed upon exposure to 1000 nm PS-NPs ($p_{adj} < 0.05$) as compared to non-exposed fibroblasts (Supplementary Table S3).

Analyses of KEGG pathways using ShinyGO resulted in 29 significantly up- or down-regulated pathways in harbor porpoise cells (Fig. 6). Differentially expressed genes from human fibroblasts were identified by extracting all unique genes from the supporting information of Stojkovic et al., (2023). This resulted in a list of 785 genes differentially expressed between cells treated with 50 nm polystyrene NPs and non-treated controls; 238 up- or down-regulated KEGG pathways were identified (Fig. 6). These two lists were compared with the significantly different pathways identified in zebrafish (*Danio rerio*, Deng et al., 2023). The Venn diagram visualizing the relationship between these three datasets is showing common and unique, species- or environment-specific, pathways. Harbor porpoise fibroblast-like cells share 21 out of 29 KEGG pathways and 3 pathways with human and zebrafish, respectively. This leaves 5 pathways that might be unique for mammals in a marine environment. This comparative analyses between human, cetacean and fish show common as well as species-specific gene responses which could be relevant to further study the health impact of MNPs.

4. Discussion

Marine mammals are sentinels for ocean health, yet conducting toxicity studies on these animals is restricted due to ethical and logistical constraints, and the fact that most species are protected. Here we have successfully developed *in vitro* cell culture techniques using cells from deceased harbor porpoises. This non-invasive method could, in the future, enable studies to assess and better understand the impact of pollutants on this important group of animals. While cells from cetaceans have been successfully cultured, they were predominantly obtained from skin biopsies of free-ranging animals. Despite being a significant advancement, the logistical challenges of obtaining tissue samples from free-ranging cetaceans are complex, invasive and primarily limited to sampling skin tissue (Burkard et al., 2015).

Significantly, primary cells have a limited ability to proliferate, necessitating either recurrent sampling or genetic modification for the establishment of immortal cell lines (Tashiro et al., 2024; Hosen et al., 2023). The cultivation of primary cells sourced from deceased stranded animals would bypass these issues as it enables the propagation of cells from multiple animals and various tissues. A drawback may arise from the animals being deceased, potentially compromising cell viability and facilitating bacterial or fungal overgrowth. The cause of death of the animals might also influence cell viability. Herein, we demonstrate the successful cultivation of viable cells from deceased animals without bacterial or fungal contamination. Moreover, the use of UW solution for tissue preservation permits samples to be stored for several days before initiating *in vitro* culture.

Although the harbor porpoise is the most frequently stranded cetacean across the North Sea, other species such as Orca (*Orcinus orca*) and Sowerby's beaked whale (*Mesoplodon bidens*) occasionally strand and indeed we also successfully established cell cultures from these species (data not shown).

This highlights that cells from a variety of tissues could be cultured from stranded animals. The exact nature of the cells has not be established; based on morphology and plastic adherence they were primarily classified as fibroblasts. No difference was noted in the cells obtained and cultured from male or female animals. The morphology changed

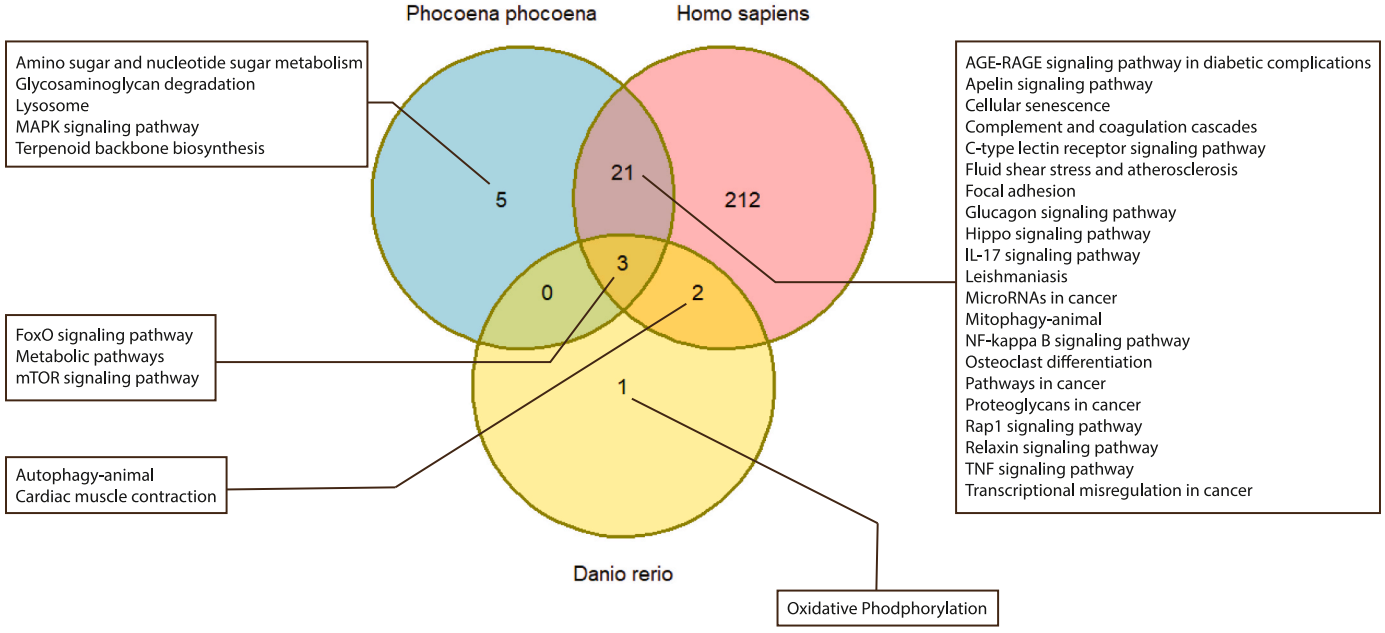


Fig. 6. Species-specific differences in genes expression after exposure to 1000 nm polystyrene YG-NP. Venn diagram presenting the common and unique gene set enrichment (KEGG) pathways of differentially expressed genes from *Phocoena phocoena* fibroblasts (this study), *Homo sapiens* fibroblasts (Stojkovic et al., 2023) and *Dario rerio* stellate cells (Deng et al., 2023), identified after exposure to polystyrene nanoplastics. Groups of (limited numbers of) individual and overlapping pathways are listed in squares.

however with increasing passage number, with cells occupying a larger surface and increased cell doubling time. It is expected that these cells eventually become senescent. Early passage number cells are therefore preferred for studies such as impact of environmental compounds.

Exposure of cultured harbor porpoise cells to MNPs revealed their uptake by the cells, even those particles with a diameter of 1000 nm. The mechanism of uptake, whether active or passive, remains unknown. However, the cellular distribution suggests retrograde transport from endosomes to the *trans*-Golgi network (Bonifacino and Rojas, 2006). The fate of the particles within the cells over time is currently unclear; prolonged culturing of the cells in the absence of nanoparticles following short-term exposure could offer further insights. Moreover, exposure in the presence of inhibitors such as those targeting clathrin-mediated endocytosis could help discern whether cellular uptake is active and, if so, through which mechanism.

Gene expression analysis revealed up- and downregulation of various genes, with 6 genes showing upregulation after exposure to all three sizes of MNPs. This observation emphasizes the unique consequences that size differences can invoke upon exposure. Intriguingly, exposure of zebrafish to 1000 nm sized polystyrene MNPs revealed altered KEGG pathways of, among others, the 'autophagy-animal' and 'mTOR signaling pathway' in hepatic stellate and epithelial cells (Deng et al., 2023). Accordingly, in the harbor porpoise cells exposed to 1000 nm MNPs, the 'mtorc1 signaling' and 'oxidative phosphorylation' were the most frequent gene enrichment pathways. How activation of these pathways would affect harbor tissue lungs cells remains to be investigated. Although the KEGG database is designed as a computer representation of the biological system, comprising most of the known characteristics on cellular metabolism and function, some organisms are less represented or incomplete. Especially data on more rare species which are not considered model systems are lacking. In addition, the lack of in depth data on the zebrafish fibroblasts hampered a more thorough and detailed analysis of our data with other aquatic organisms. We should, therefore, consider our interpretation in a more general approach, merely showing the fact that there are differences in KEGG pathways identified per species.

It is expected that MNPs can disrupt membrane integrity and accumulate in organelles like lysosomes and mitochondria. Inside cells, they may generate ROS, leading to oxidative stress and damage to proteins, lipids, and DNA. In addition as demonstrated here gene expression patterns are altered by exposure to MNPs. These combined effects may impair cell function, promote inflammation, and trigger apoptosis or necrosis. It is expected that cells of different species respond differently, for instance by differences in cell membrane composition, but also differences in immune responses and antioxidant enzyme systems.

Upon exposure to fine particles smaller than 2.5 μm diameter, human bronchial epithelial cells showed a significant upregulation of IL24 expression, echoing findings described in this study (Liu et al., 2022). These lung cells underwent autophagy, likely due to the heightened IL24 expression. Furthermore, IL24 expression has been linked to inflammatory diseases (Mitamura et al., 2020). Harbor porpoise cells exposed to NPs also exhibited increased IL24 expression across all three tested sizes, indicating direct cytotoxicity and thus harmful effects induced by NP pollution.

To conclude: this is the first study to successfully establish cell lines of stranded cetaceans. This approach allows us to study the potential adverse effects of pollutants (such as MNPs) in a non-invasive way. We show that exposure to MNPs impacts harbor porpoise cells, which highlight the risk these particles pose to marine ecosystems. As sentinel species, harbor porpoises can serve as indicators of ecosystem health and the extent of anthropogenic disturbances such as plastic pollution. Disruption of cellular function and viability due to NPs could signal broader ecological repercussions, including reduced population resilience and altered trophic dynamics. Moreover, the transfer of NPs and associated contaminants through the marine food web can amplify the exposure risks for other marine organisms, including commercially important fish species and apex predators.

CRediT authorship contribution statement

Bernard A.J. Roelen: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Henk P. Roest:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Immelie Coenen Morales:** Writing – review & editing, Visualization, Data curation. **Florian Meirer:** Writing – review & editing, Resources, Methodology. **Lonneke L. IJsseldijk:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation. **Thijs Bosker:** Writing – review & editing, Methodology. **Luc J.W. van der Laan:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization.

Funding statement

Necropsies of harbor porpoises in The Netherlands are commissioned by the Ministry of Agriculture, Fisheries, Food Security and Nature, and since 2016 embedded under the Legal Research Tasks Nature & Environment unit of Wageningen UR (project reference numbers 140000353, WOT-04-009-045).

Declaration of competing interest

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

Acknowledgement

We sincerely thank the Dutch stranding network volunteers and organizations for reporting and retrieving deceased harbor porpoises for our research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2025.126805.

Data availability

Data will be made available on request.

References

Banerjee, A., Shelver, W.L., 2021. Micro- and nanoplastic induced cellular toxicity in mammals: a review. Sci. Total Environ. 755 (Pt 2), 142518. https://doi.org/10.1016/j.scitotenv.2020.142518.

Bonifacino, J.S., Rojas, R., 2006. Retrograde transport from endosomes to the trans-Golgi network. Nat. Rev. Mol. Cell Biol. 7, 568–579. https://doi.org/10.1038/NRM1985.

Burkard, M., Whitworth, D., Schirmer, K., Nash, S.B., 2015. Establishment of the first humpback whale fibroblast cell lines and their application in chemical risk assessment. Aquat. Toxicol. 167, 240–247. https://doi.org/10.1016/j.aquatox.2015.08.005.

Deng, J., Zeng, X., Li, J., Luo, L., Yang, Y., Luan, T., 2023. Single-cell transcriptomic analysis reveals heterogeneity of the patterns of responsive genes and cell communications in liver cell populations of zebrafish exposed to polystyrene nanoplastics. Sci. Total Environ. 889, 164082. https://doi.org/10.1016/j.scitotenv.2023.164082.

Dool, T., Bosker, T., 2022. Predicted microplastic uptake through trophic transfer by the short-beaked common dolphin (Delphinus delphis) and common bottlenose dolphin (Tursiops truncatus) in the Northeast Atlantic Ocean and Mediterranean Sea. Mar. Pollut. Bull. 180, 113745. https://doi.org/10.1016/j.marpolbul.2022.113745.

Dziobak, M.K., Fahlman, A., Wells, R.S., Takeshita, R., Smith, C., Gray, A., Weinstein, J., Hart, L.B., 2024. First evidence of microplastic inhalation among free-ranging small cetaceans. PLoS One 19 (10), e0309377.

European Commission, 2018. Single-Use Plastics: New EU Rules to Reduce Marine Litter. European Commission. https://ec.europa.eu/commission/presscorner/detail/en/MEMO_18_3909.

Ge, S.X., Jung, D., Yao, R., 2020. ShinyGO: a graphical gene-set enrichment tool for animals and plants. Bioinformatics 36 (8), 2628–2629. https://doi.org/10.1093/bioinformatics/btz931.

Goecks, J., Nekrutenko, A., 2010. TaylorJ & the galaxy team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome Biol. 11 (8), R86. https://doi.org/10.1186/gb-2010-11-8-r86.

Hosen, M.H.A., Sykes, A.M., Wood, S.A., Leusch, F.D.L., Whitworth, D.J., Bengtson Nash, S.M., 2023. Novel use of cell profiling technology to visualize mitochondrial responses of humpback whale fibroblasts to chemical exposure. Environ. Sci. Technol. 57 (24), 8975–8982.

IJsseldijk, L.L., 2019. Best practice on cetacean post mortem investigation and tissue sampling. In: Brownlow, A.C., Mazzariol, S. (Eds.), Joint ACCOBAMS and ASCOBANS Document. https://doi.org/10.31219/osf.io/zh4ra.

IJsseldijk, L., Leopold, M., Begeman, L., Kik, M., Wiersma, L., Morell, M., Bravo Rebolledo, E.L., Jauniaux, T., Heesterbeek, H., Gröne, A., 2022. Pathological findings in stranded harbor porpoises (Phocoena phocoena) with special focus on anthropogenic causes. Front. Mar. Sci. 9, 1–19. https://doi.org/10.3389/fmars.2022.997388. Article 997388.

Jalal, S., Shi, S., Acharya, V., Huang, R.Y., Viasnoff, V., Bershadsky, A.D., Tee, Y.H., 2019. Actin cytoskeleton self-organization in single epithelial cells and fibroblasts under isotropic confinement. J. Cell Sci. 132 (5), jcs220780. https://doi.org/10.1242/jcs.220780.

Kaandorp, M.L.A., Lobelle, D., Kehl, C., Dijkstra, H.A., van Sebille, E., 2023. Global mass of buoyant marine plastics dominated by large long-lived debris. Nat. Geosci. 16, 689–694.

Kanehisa, M., Furumichi, M., Sato, Y., Ishiguro-Watanabe, M., Tanabe, M., 2021. KEGG: integrating viruses and cellular organisms. Nucleic Acids Res. 49 (D1), D545–D551. https://doi.org/10.1093/nar/gkaa970.

Keenan, A.B., Torre, D., Lachmann, A., Leong, A.K., Wojciechowicz, M.L., Utti, V., Jagodnik, K.M., Kropiwnicki, E., Wang, Z., Ma'ayan, A., 2019. ChEA3: transcription factor enrichment analysis by orthogonal omics integration. Nucleic Acids Res. 47 (W1), W212–W224. https://doi.org/10.1093/nar/gkz446.

Kong, X.Y., Nesset, C.K., Damme, M., Løberg, E.M., Lübke, T., Mæhlen, J., Andersson, K. B., Lorenzo, P.I., Roos, N., Thoresen, G.H., Rustan, A.C., Kase, E.T., Eskild, W., 2014. Loss of lysosomal membrane protein NCU-G1 in mice results in spontaneous liver fibrosis with accumulation of lipofuscin and iron in Kupffer cells. Dis. Model. Mech. 7 (3), 351–362. https://doi.org/10.1242/dmm.014050.

Liu, Y., He, X., Liu, J., Zhang, L., Xiong, A., Wang, J., Liu, S., Jiang, M., Luo, L., Xiong, Y., Li, G., 2022. Transcriptome analysis identifies IL24 as an autophagy modulator in PM2.5 caused lung dysfunction. Ecotoxicol. Environ. Saf. 244, 114039. https://doi.org/10.1016/j.ecoenv.2022.114039.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15 (12), 550. https://doi.org/10.1186/s13059-014-0550-8.

Lu, H., Hou, L., Zhang, Y., Guo, T., Wang, Y., Xing, M., 2024. Polystyrene microplastics mediate cell cycle arrest, apoptosis, and autophagy in the G2/M phase through ROS in grass carp kidney cells. Environ. Toxicol. 39 (4), 1923–1935. https://doi.org/10.1002/tox.24068. Epub 2023 Dec 8. PMID: 38064284.

MacLeod, M., Arp, H.P.H., Tekman, M.B., Jahnke, A., 2021. The global threat from plastic pollution. Science 373 (6550), 61–65. https://doi.org/10.1126/science.abg5433.

Mitamura, Y., Nunomura, S., Furue, M., Izuhara, K., 2020. IL-24: a new player in the pathogenesis of pro-inflammatory and allergic skin diseases. Allergol. Int. 69 (3), 405–411.

Morin, P.A., Archer, F.I., Avila, C.D., Balacco, J.R., Bukhman, Y.V., Chow, W., Fedrigo, O., Formenti, G., Fronczek, J.A., Fungtammasan, A., Gulland, F.M.D., Haase, B., Peter Heide-Jorgensen, M., Houck, M.L., Howe, K., Misuraca, A.C., Mountcastle, J., Musser, W., Paez, S., Pelan, S., Phillippy, A., Rhie, A., Robinson, J., Rojas-Bracho, L., Rowles, T.K., Ryder, O.A., Smith, C.R., Stevenson, S., Taylor, B.L., Teilmann, J., Torrance, J., Wells, R.S., Westgate, A.J., Jarvis, E.D., 2021. Reference genome and demographic history of the most endangered marine mammal, the vaquita. Mol Ecol Resour 21 (4), 1008–1020. https://doi.org/10.1111/1755-0998.13284.

Ochiai, M., Kurihara, N., Hirano, M., Nakata, A., Iwata, H., 2020. In vitro cytotoxicity and risk assessments of environmental pollutants using fibroblasts of a stranded finless porpoise (Neophocaena asiaeorientalis). Environ. Sci. Technol. 54 (11), 6832–6841. https://doi.org/10.1021/acs.est.9b07471.

Revel, M., Roman, C., Châtel, A., 2021. Is cell culture a suitable tool for the evaluation of micro- and nanoplastics ecotoxicity? Ecotoxicology 30 (3), 421–430. https://doi.org/10.1007/s10646-021-02355-z.

Stojkovic, M., Guzmán, Francisco Manuel Ortuño, Han, Dongjun, Stojkovic, Petra, Dopazo, Joaquin, Stankovic, Konstantina M., 2023. Polystyrene nanoplastics affect transcriptomic and epigenomic signatures of human fibroblasts and derived induced pluripotent stem cells: implications for human health. Environ. Pollut. 320, 120849. https://doi.org/10.1016/j.envpol.2022.120849.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA 102 (43), 15545–15550. https://doi.org/10.1073/pnas.0506580102.

Tashiro, K., Hikobe, K., Segawa, T., Suzuki, M., Maeda, K., Itou, T., 2024. Establishment and characterization of a novel lung cell line derived from the common bottlenose dolphin. In Vitro Cell. Dev. Biol. Anim. 60 (1), 98–105.

van der Laan, L.J.W., Bosker, T., Peijnenburg, W.J.G., 2023. Deciphering potential implications of dietary microplastics for human health. Nat. Rev. Gastroenterol. Hepatol. 20 (6), 340–341.

Wang, H., Shi, X., Gao, Y., Zhang, X., Zhao, H., Wang, L., Zhang, X., Chen, R., 2022. Polystyrene nanoplastics induce profound metabolic shift in human cells as revealed by integrated proteomic and metabolomic analysis. Environ. Int. 166, 107349. https://doi.org/10.1016/j.envint.2022.107349.

Weber, A., Schwiebs, A., Solhaug, H., Stenvik, J., Nilsen, A.M., Wagner, M., Relja, B., Radeke, H.H., 2022. Nanoplastics affect the inflammatory cytokine release by primary human monocytes and dendritic cells. Environ. Int. 163, 107173. https://doi.org/10.1016/j.envint.2022.107173.

Yin, K., Wang, D., Zhang, Y., Lu, H., Wang, Y., Xing, M., 2023a. Dose-effect of polystyrene microplastics on digestive toxicity in chickens (Gallus gallus): Multi-omics reveals critical role of gut-liver axis. J. Adv. Res. 52, 3–18. https://doi.org/10.1016/j.jare.2022.10.015. Epub 2022 Nov 9. PMID: 36334886; PMCID: PMC10555772.

Yin, K., Wang, D., Zhang, Y., Lu, H., Hou, L., Guo, T., Zhao, H., Xing, M., 2023b. Polystyrene microplastics promote liver inflammation by inducing the formation of macrophages extracellular traps. J. Hazard Mater. 452, 131236. https://doi.org/10.1016/j.jhazmat.2023.131236. Epub 2023 Mar 20. PMID: 36958159.

Zantis, L.J., Carroll, E.L., Nelms, S.E., Bosker, T., 2021. Marine mammals and microplastics: a systematic review and call for standardisation. Environ. Pollut. 269, 116142. https://doi.org/10.1016/j.envpol.2020.116142.