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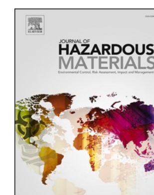

Plastic nanoparticles cause mild inflammation, disrupt metabolic pathways, change the gut microbiota and affect reproduction in zebrafish: A full generation multi-omics study

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A B S T R A C T

Plastic pollution has become a major concern on a global scale. The plastic is broken down into minuscule particles, which have an impact on the biosystems, however long-term impacts through an entire generation is largely unknown. Here, we present the first whole generation study exposing fish to a 500 nm polystyrene plastic particle at environmentally relevant concentrations. Short- and long-term adverse effects were investigated in the zebrafish model organism using a holistic multi-omics approach. The particles accumulated in the yolk sac of young larvae and short-term biological impacts included immune-relevant gene regulation related to inflammation and tolerance as well as disruption of metabolic processes, such as the fatty acid and lipid pathways. The long-term effects comprised gene regulations pointing towards skin and/or gill inflammation, dysbiosis of the gut microbiota, a tendency towards decreased condition factor in adult males as well as a lowered reproductive capability. From this study, it can be concluded that exposures to plastic nanoparticles have an impact on population as well as ecosystem level in fish and likely also in other vertebrates.

1. Introduction

The amount of plastic debris entering the oceans every year has been estimated to be between 4.8 and 12.7 million metric tons (Jambeck et al., 2015) and the amount is likely to increase. Once in the environment, the bigger sized plastic particles break down into smaller sized microparticles (MPs) (< 5 mm) that eventually degrade into fragments less than 1 μm, termed nanoparticles (NPs) (GESAMP, 2016; Guimarães et al., 2021; Estrela et al., 2021; Andrady, 2011). The term nanoparticle is still under debate (da Costa et al., 2016) and we chose to align to the definition by GESAMP (GESAMP, 2016). Especially the impact of NPs is gaining increased attention, due to the potentially harmful nature of these particles and their likely ubiquitous environmental distribution (Mitrano et al., 2021). However, most published studies have been conducted with concentrations far beyond those estimated in the environment. Realistic concentrations for plastic microparticles have been suggested to range from 1 μg/l or less to approximately 0.023 mg/l (Sussarellu et al., 2016; Lenz et al., 2016). For nanoparticles a low hypothetical concentration has been suggested to be 1 pg/l (Lenz et al., 2016), 34 ng/l in surface water measurements (Guimarães et al., 2021) and 15 μg/l as a high predicted environmental concentration (Al-Sid-Cheikh et al., 2018). In addition, most exposure studies assess the impact of plastic particles over a relatively short time span and only addressing a few specific endpoints. This is a key component of the major knowledge gaps concerning impact of NPs in more relevant environmental scenarios, especially concerning the continuous chronic exposure that

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aquatic organisms are subjected to during their lifetime (SAPEA SAfP-bEA, 2019) and the interlinkage of the biological processes that may be affected.

Of the few studies that have assessed long-term impact of plastic NPs and MPs Redondo-Hasselerharm et al. (2020) demonstrated an impact on benthic communities (decreased abundance of macroinvertebrates) after long-term exposure to NPs and MPs (Redondo-Hasselerharm et al., 2020). Studies have demonstrated an effect on fertility of *Daphnia* species (Cui et al., 2017; Kelpsiene et al., 2020), however, a study by Schür et al. (2020) showed that long-term impact of plastic MPs on *Daphnia magna* at lower concentrations did not manifest within the timeframe normally used for *D. magna* reproduction test (Schür et al., 2020). These studies exemplify potential long-term adverse effects but also demonstrate the major knowledge gap that exists concerning environmental impact on organisms under continuous chronic exposure of plastic NPs/MPs.

Zebrafish (*Danio rerio*) is an aquatic vertebrate extensively used to study the toxicity of plastic NPs and MPs (Bhagat et al., 2020). Research on zebrafish show that NPs and MPs can, depending on plastic size, cause physical damage after ingestion. The plastic ingestion can cause histopathological changes (Qiao et al., 2019a), including damage of mucosal lining (Lei et al., 2018; Qiao et al., 2019b), inflammation (Jin et al., 2018) and reduced feed intake (Mak et al., 2019) that in turn can decrease body weight (Zhao et al., 2020) and fecundity (Sarasamma et al., 2020). Plastic NPs have also been reported to alter other biochemical processes and induce dysbiosis in the gut microbiome (Qiao et al., 2019b; Jin et al., 2018; Wan et al., 2019), oxidative stress (Sarasamma et al., 2020; Sendra et al., 2021; Parenti et al., 2019), changes in metabolism (Qiao et al., 2019a), behavioral alterations (Parenti et al., 2019; Chen et al., 2017) and gene expression regulations (Limonta et al., 2019; Pedersen et al., 2020; Zhang et al., 2020). Several authors have reported that smaller sized plastic NPs penetrate the embryos and adult zebrafish and translocate into different organs (Kashiwada, 2006; Manabe et al., 2011; Pitt et al., 2018). Further, uptake of plastic is size-dependent with increased penetration of smaller particles (Sendra et al., 2021; Lee et al., 2019).

An increasing number of studies have examined the toxic effect of plastic on zebrafish using different polymer types with varying particle sizes. The majority of these consist of short-term exposure studies or longer exposure studies (about 21 days) aiming to establish chronic exposure scenarios that mimick real-life conditions. Impact on reproduction has been demonstrated in teleosts, for example in the form of histological changes in testes of marine medaka (Wang and Zhao, 2019), decreased fecundity (Zhu et al., 2020), and endocrine disruptions in Japanese medaka (Rochman et al., 2014), but has never, in any vertebrate, been documented with continuous exposures lasting throughout a whole generation.

The aim of the present study was to provide new insights and to generate new hypotheses into the short- and long-term impact of plastic NPs at environmentally relevant concentrations in zebrafish. We investigated the potential adverse effects of 500 nm polystyrene NPs during a whole generation time on different biological mechanisms, such as reproduction. Short-term (acute) adverse effects were investigated at 5 and 12 days, and long-term (chronic) adverse effects at 110 days following exposure. The biological effects and mechanisms were evaluated using a multi-omics approach from immune system biomarkers, global metabolomics and metabarcoding analyses.

2. Materials and methods

2.1. Ethics statement

The experiment was conducted in accordance with a permit from The Animal Experiments Inspectorate under the Danish Ministry of Environment and Food (Permit: 2017–15–0201–01301). During anesthesia and euthanization tricaine methanesulphonate (MS222, Sigma-Aldrich) was applied.

2.2. Brief overview of the experiment

Zebrafish were exposed to 500 nm plastic particles for 110 days in different concentrations (Fig. 1). Sampling was conducted to investigate the acute response during short-term exposure as well as long-term adverse effects of the particles at day 5–12 and 84–110, respectively. Short-term effects and uptake of the particles were evaluated with fluorescence stereo microscope imaging, gene expression studies and analyses of the metabolome. Long-term effects were investigated using microbiota analyses, gene expression, condition factor and measurements of reproductive success.

2.3. Fish

AB wildtype (WT) zebrafish were reared in a recirculated system (AquaSchwartz, Germany) with a light/dark cycle of 14/10 h, pH of 7.5, conductivity of 650 μS and a temperature of 27 °C. Breeding pairs were randomly set up in the evening before the experiment started. Fish started breeding the next morning when the lights were turned on, and within 2 h, eggs were collected and pooled.

Two transgenic (tg) zebrafish lines *Tg(MPX:eGFP)*^{i114} (Renshaw et al., 2006) and *Tg(MpegmCherry-CAAX)*$^{sh378/+}$ (Gray et al., 2011), reared in the same way as WT zebrafish were crossed to obtain double transgenic offspring with neutrophils emitting green fluorescence and macrophages emitting red fluorescence.

2.4. Feeding regime

From 5 days post fertilization (dpf) larvae received food 3 times per day. Throughout development and growth larvae were first fed with ZM-000 fry food, then ZM-100 and finally ZM-300 (ZM Fish food & Equipment, UK). From 14 dpf, one of the meals per day consisted of live artemia (JBL GmbH & Co, Germany).

2.5. Characterization of plastic NPs (500 nm)

Fluorescent blue polystyrene latex NPs with a diameter of 500 nm was obtained from Magsphere (Pasadena, California, USA) in a 2.5% W/V suspension. Particles were fluorescent in UV light with a wavelength of 400 nm. Hydrodynamic diameter (z-average) of plastic NPs was measured by dynamic light scattering (DLS) using Malvern Zetasizer Nano ZSP (zetasizer software 7.11, Malvern Instruments) at 27 °C (same temperature as exposures). Three measurements (each consisting of 10–12 measurements) were conducted for each sample. Further, DLS measurements were conducted to obtain *in situ* characterization of particle size in water from experiments or stock diluted in clean water to resemble the exposure conditions more accurately (compared to DLS of particles in MilliQ water). All samples were measured immediately after sample preparation and for measurements of particles in MilliQ water, also after 1 h.

2.6. Plastic NP medium for fish exposure

For the first 30 days of the experiment, conditioned zebrafish facility water was filtered with 0.22 μm filters, before making the different concentrations of the plastic particles. From 30–49 dpf, the facility water was filtered with 0.45 μm filters and subsequently, until the end of the experiment, facility water was run through a metal sieve (mesh size 63 μm). Filtering was conducted to eliminate or reduce bacterial load and remove feces and food debris. Different filters and water change volumes were used at different exposure stages to balance welfare of the fish and avoid extensive workload. The minimal volumes possible without affecting fish welfare were chosen and the volume increased with increasing fish size. Larger filter mesh sizes were used with

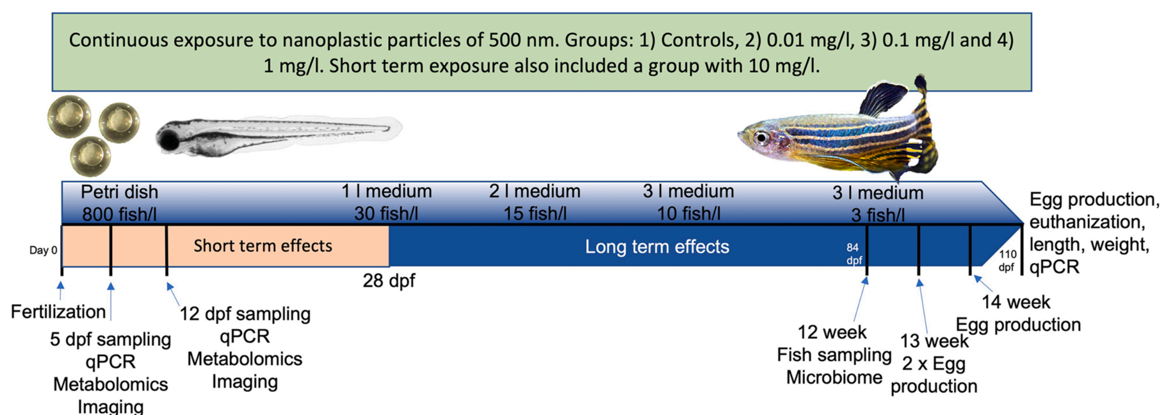


Fig. 1. Outline of the experimental setup. Zebrafish (*Danio rerio*) were exposed to 500 nm plastic particles for 110 days in different concentrations. Sampling was conducted to investigate short- and long-term adverse effects of the particles.

increasing volumes of water correlating well with larger fish being less sensitive than embryo and fry. The particles were not pre-treated before dilution.

2.7. Short-term exposure and sampling

Zebrafish embryos were exposed to 4 concentrations (0.01, 0.1, 1 and 10 mg/l) of plastic NPs in triplicates from fertilization until 12 dpf (Fig. 1). These exposure concentrations were chosen to cover the range of environmentally relevant concentrations, worst case scenarios at hot spots, and concentrations high enough to give significant responses to allow investigations on mechanisms of potential toxicity (Al-Sid-Cheikh et al., 2018; Huvet et al., 2016). The control group was exposed to only facility water. To obtain exposure concentrations, the particles were diluted in sterile filtered zebrafish facility water (0.22 µm filters). Fifteen eggs/embryos were kept in Petri dishes with 50 ml of the different exposure concentrations. All media were changed at day 2 following fertilization and 50% were changed daily from 2 to 12 dpf. Five larvae from each Petri dish were sampled at 5 and 12 dpf for qPCR analyzes and the same number of fish for metabolomic analyzes to investigate acute gene regulations and early adverse effects on the metabolome. For qPCR, 5 larvae/triplicate were transferred in medium to cryotubes and euthanized in an overdose of MS222 (500 mg/l). Subsequently the liquid was removed from the tube with a pipette and at least 5 x the volume of the fish was added in the form of RNAlater (Sigma-Aldrich). Samples were kept at 4 °C for 24 hrs and subsequently placed at − 20 °C until further processing. For metabolomic analyzes whole fish were euthanized by submerging in ice water and immediately transferred to − 80 °C.

2.8. Imaging

Five and 12 days after exposure, 2 samples, each consisting of 4 double tg larvae, were sampled for imaging to detect plastic NPs and innate immune cell responses inside the fish. Fish were anaesthetized using 150 mg/l MS222 and placed under a Zeiss fluorescence stereo microscope V8. White light was used to image the fish, GFP filters (excitation 488 nm) were used to image neutrophils (green), CY3 filters (excitation 545 nm) to image macrophages (red) and UV (excitation 400 nm) to image the plastic NPs (blue).

2.9. Long-term exposure and sampling

Fish were continuously exposed to plastic NPs from fertilization to adulthood for 110 days (≈ 3 ½ months) and then euthanized (Fig. 1). For the long-term study, 3 concentrations of NPs (0.01 mg/l, 0.1 mg/l, 1 mg/l) were applied in triplicates. The control group was exposed to only facility water. Fifty newly fertilized eggs were exposed to the different concentrations of plastic NPs in Petri dishes. After 2 days, all the media were changed, and the numbers of eggs reduced to 30/Petri dish. The following 7 days, 50% of the media were changed every day. From that point on, alongside the increasing size of the fish, the volume of media was gradually increased. At 28 dpf, each triplicate tank in every group held 1 l of medium of which 20% was changed every day. Eight days later, the volume was, over 5 days, slowly increased to 2 l/tank and subsequently 25% of the media was changed every day. From 62 dpf, fish from each triplicate tank were placed in 3 l of medium of which 700 ml were changed daily. From 72 dpf, 50% of the medium was changed until the end of the study at 110 dpf. At 84 dpf, 5 fish per triplicate tank (15 fish per concentration) were sampled to analyze the established gut microbiota in the adults. For this purpose, only the inner organs were sampled and kept in 1 x DNA/RNA Shield™ (Zymo research) until further processing. Zebrafish were euthanized in 300 mg/l methane sulfonate tricaine (MS222). From the remaining fish, 30 fish per concentration (3 x 10) were selected for breeding (we visually sorted them and aimed at equal numbers of males and females).

Three months after fertilization, reproduction analyzes began, and 5 breeding pairs were randomly chosen from the triplicate tanks at each concentration. The pairs were isolated in 2 l tanks with medium from their respective source tanks. The following morning, an empty box for pipette tips covered with a 3 mm mesh, was submerged in the tanks prior to the start of the light period. The fish were allowed to breed for 2 hrs before all breeding boxes were removed and eggs were collected, counted and placed in sterile filtered fish facility water. Analogous breeding experiments were conducted 3 times and the last breeding was conducted on the last day of the experiment (week 14). In the group exposed to 0.01 mg/l of the particles only 3 out of 30 fish were female and therefore only 3 pairs were set up for breeding in this group.

Ten fish per concentration were sampled for qPCR (5 fish from each of 2 tanks, which is standard procedure in our laboratory to create statistically solid data). The inner organ packet (intestine, liver, spleen, gall bladder) was aseptically removed from the fish and stored in RNAlater for 24 hrs at 4 °C and subsequently at − 20 °C until further processing.

2.10. qPCR of immune-relevant genes

RNA from the larvae was purified with GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Denmark) following the manufacturer's protocol. Subsequently the samples were treated with DNase (Thermo Fisher Scientific, Denmark) to remove DNA residuals. Finally, RNA was quality checked on a gel and concentrations were measured on a NanoDrop 2000 spectrophotometer (Saveen & Werner, Denmark). cDNA was synthesized on a T100 thermocycler (Biorad, Denmark) using Oligo d(T)16 primers and TaqMan® Reverse Transcription Reagents

(N8080234, Thermo Fischer Scientific, Denmark) as described in (Jørgensen et al., 2018). Primers and Taq-Man probe sequences for the 12 genes investigated are listed in Supplementary Table 1. Gene expression was measured with an AriaMx Real-Time PCR machine (G88^{30}A–^{04}R-010, AH diagnostics AS, Denmark). The 12.5 µl total volume reactions contained 6.25 µl Brilliant III Ultra-Fast QPCR Master Mix (600881, AH Diagnostics AS, Denmark), 2.5 µl cDNA, 1.0 µl primer-probe mixture (forward primer, 10 µM and reverse primer, 10 µM), Taq-Man probe (5 µM) and 2.75 µl RNase-free water (Invitrogen, Denmark). For every plate setup reverse transcriptase minus and no template controls were included. All qPCR assays exhibited efficiencies within 100 ± 5% and data were analyzed using a simplified $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Samples were considered significantly different when $p < 0.05$ (Student's *t*-test) between the control and each concentration (comparing 2 means) and regulation was more than 2-fold. The ΔΔCq values represent log-transformed folds (exponential data) and were used for statistical comparison using Student's *t*-test. β-*actin* was used as housekeeping gene to account for individual biological variance. When the Ct value exceeded 37 cycles values were set at 37 (Jorgensen et al., 2012). Thirty-seven cycles are theoretically the threshold number for detecting one intact gene from the template material and hence the highest reliable number.

2.11. Metabolomics

An untargeted metabolomics analysis workflow was applied using nano-liquid chromatography (LC)-Orbitrap HRMS/MS system (Poulsen et al., 2021). At 5 and 12 dpf, 5 animals were collected from each treatment group, transferred to a cryovial, and immediately stored at − 80 °C until extraction. Procedural blanks were included from the beginning and composite quality control (QC) samples were made for 5 and 12 dpf samples as well as for both timepoints collectively. Sample preparation and instrumental parameters are described in detail in the Supplementary information.

Compound Discoverer software version 3.2.0.421 (Thermo Scientific) was used for data processing (Bhattacharya, 1996) and analysis (for pipeline details see Supplementary information; section on metabolomics). As the metabolome is expected to change significantly between 5 and 12 dpf, the data analysis was performed separately for the 2 timepoints. Principal component analysis (PCA) and differential analysis by ANOVA (significance levels $p < 0.05$, Log2 Fold Change (FC)> 2) was also performed in Compound Discoverer. Putatively annotated compounds were based on comparisons of the experimental MS2-spectrum with in-house spectral libraries (ID Level 1) and the online spectral library, mzCloud, (ID Level 2) (Viant et al., 2019). Putatively characterized compound classes (ID Level 3) were based on assigned predicted composition and the plausible, tentative candidates to that composition. For instance, whether the compound was likely to naturally occur in zebrafish or could be related to NP exposure. Compounds that did not meet these criteria were assigned to level 4 and not incorporated in the functional analysis. The functional analysis was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2016), biocyc (Karp et al., 2019), and human metabolome database (HMDB) (Wishart et al., 2018).

2.12. Gut microbiota

A metabarcoding approach was used to characterize the gut microbiota community in a total of 15 zebrafish from each NP concentration. The whole gastrointestinal tract was carefully dissected out and used for DNA extraction. Part of the 16 S hypervariable V3-V4 region was amplified and sequenced using an Illumina MiSeq platform. Raw sequences were processed for standard quality parameters including trimming of sequences and removal of putative contaminant reads. We used the dada2 (Callahan et al., 2016) pipeline for quality filtering and trimming, inferring sequence variants, merging reads, removing chimeric sequences and ASV based taxonomy assignment at the genus level. Gut microbiota profiles were compared among the 3 exposure groups plus a control group, by considering both relative abundance of ASVs within each group, comparison of alpha diversity among the concentration groups as well as an individual based weighted UniFrac PCoA with all samples included. For further details on the methodology please refer to the Supplementary information.

2.13. Fish condition estimated by Fulton's condition factor

Fulton's condition factor (k) was calculated as:

$$\text{Fulton's condition factor} = \left(\frac{BM}{TL^3}\right) * 100$$

BM is the body mass and TL is the total length. Fulton's condition factor and embryo % survival data were tested with the non-parametric Kruskal-Wallis multiple comparison test with the Dunn's multiple comparisons test as post-test ($p < 0.05$).

3. Results

3.1. Characterization of plastic NPs (500 nm) in test media

DLS measurements confirmed that size of particles from the initial stock (diluted 100 x in MilliQ water) was as reported by supplier (z-average 501 nm, Polydispersity index, PDI: 0.011, Supplementary Figure 1). Measurements of NP particles in water collected at the test facility (i.e., particles in clean 63 µm filtered water at 0.01, 0.1 and 1 mg/l used in fish exposures (diluted 2 times)) revealed changes in particle counts and z-average over time indicating instability of suspension and possible aggregation, agglomeration, and particle coating with organic material. Furthermore, zetasizer software reported "poor data quality" for all these samples except at 1 mg/l, where PDI was high (z-average~ 600 nm and PDI 0.4) (See Supplementary Table 2).

3.2. Short-term effects

3.2.1. Imaging of plastic NPs and innate immune cells

Five-day old larvae showed an accumulation of the plastic NPs in the yolk sac (Fig. 2). At this time point the feeding of larvae had not started yet. Furthermore, locality specific appearances of macrophages and neutrophils were inspected visually, however, no obvious difference between the exposed and the control group was detected.

Images obtained at 12 dpf did not reveal any differences between the controls and the exposed fish regarding the number of macrophages, neutrophils, and NP accumulation, determined by visual inspection (Supplementary Figure 2).

3.2.2. Gene expression in larvae

qPCR data showed that the young larvae endured a mild inflammatory response when exposed to plastic NPs (Fig. 3). The classical inflammatory cytokines *il1β*, *il6* and *ifnγ1* were un-regulated or not expressed (Supplementary Table 3) whereas *tnfα* was significantly up-regulated in the 0.01 and the 0.1 mg/l group at 12 dpf (Fig. 3). The complement factor *c3a* and the transcription factor *nfκb* were un-regulated or not expressed at this stage and the acute phase protein serum amyloid A (*saa)* was significantly up-regulated at 5 dpf in the group exposed to the highest concentration of particles. The gene *foxp3*, associated with the immune-regulatory pathway and *tlr2*, a Toll-like receptor recognition molecules for pathogens, were up-regulated at 12 days after the exposure in the 0.01 and the 10 mg/l group, respectively. The anti-inflammatory cytokine *il10*, was significantly down-regulated at 5 dpf in the 0.01, 0.1 and 1 mg/l group and significantly up-regulated in 0.1 mg/l group at 12 dpf.

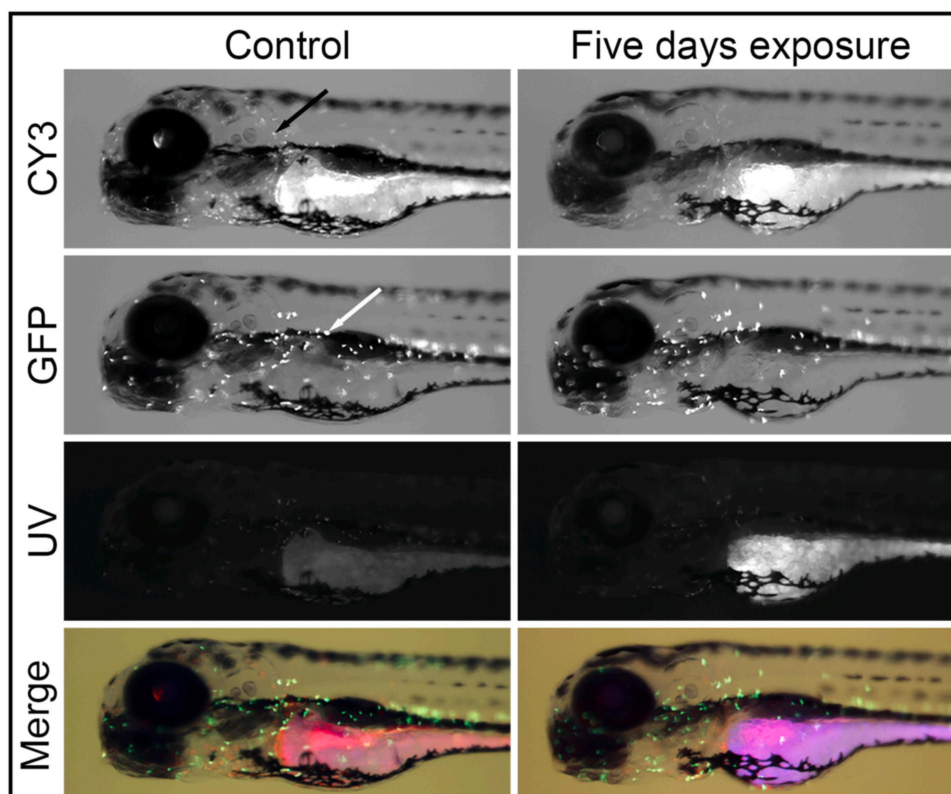


Fig. 2. Images visualizing innate immune cells and the plastic NPs in double transgenic zebrafish larvae 5 days after fertilization and continuous exposure to 10 mg/l polystyrene particles (500 nm). CY3 images capture the macrophages (red in overlay, black arrow), GFP the neutrophils (green in overlay, white arrow) and UV the plastic particles (purple in overlay). Images are merged with annotated colors.

3.2.3. Metabolic changes in larvae

In the untargeted metabolome analysis, a total of 4282 and 4218 metabolites were detected in 5 and 12 dpf larvae, respectively (Fig. 4). At 5 dpf the largest change in the metabolome, when compared to the control group, was observed in the larvae exposed to the intermediate concentration of 0.1 mg/l NPs (Fig. 4A, 4C). In the exposed 5 dpf larvae the concentration of 26 metabolites was significantly decreased and that of 126 metabolites increased. At 12 dpf a more monotonous trend was observed as the number of affected metabolites decreased with increasing concentration of NPs (Fig. 4C). The lowest concentration of 0.01 mg/l resulted in 198 significantly affected metabolites and the highest concentration of 10 mg/l in 157 affected metabolites (Fig. 4B-C).

Several of the compounds, for which a significantly decreased or increased concentration was found compared to controls, were also affected at other concentrations of NPs (Supplementary Figure 3). More specifically, after exposure to the different concentrations at 5 dpf, 45 compounds were significantly affected ($p < 0.05$, Log2 FC>2) in all 4 treatment groups and at 12 dpf, 40 compounds were significantly affected by all concentrations (Supplementary Figure 3). In total 276 and 369 different metabolites or isomers were significantly affected by at least 1 concentration at 5 and 12 dpf, respectively.

Annotation or putative identification of the affected compounds was possible for 64 (5 dpf) and 72 (12 dpf) metabolites. At 5 dpf, 17 of these could be assigned to pathways involved in fatty acid metabolism (Supplementary Table 4). Among them were 18-hydroxylinoleolate, involved in linoleic acid metabolism, and which was equally affected at 12 dpf (Fig. 4D-E). Several fatty acyls were also affected at 5 dpf (Supplementary Tables 4,5). Amongst those 2 caproleic acid isomers, of which the annotation was confirmed with in-house standards. When investigating the dose-response relationship for the compounds significantly affected at 5 dpf both monotonic and non-monotonic dose-response relationships (Lagarde et al., 2015) were observed (Fig. 4D, F, H). Some of them, such as 3-hydroxyoctanoylcarnitin displayed in Fig. 4F, confirmed the trend observed in the number of affected metabolites as the curve exhibited a u-shape with the largest difference from controls observed for the intermediate concentration of 0.1 mg/l.

At 12 dpf, the fatty acid metabolism was still found disrupted. Twenty-seven of the significantly affected compounds could be assigned to pathways involved in fatty acid metabolism (Supplementary Table 5). At both timepoints the curve takes the shape of a more classical dose-response relationship reaching a plateau. The dose-response plot for choline, which is involved in primary bile acid biosynthesis (Supplementary Table 5) and required to produce for instance the neurotransmitter acetylcholine and the choline phospholipids that are essential in cell membranes also followed a more classical dose-response relationship. Many of the compounds did however reflect the trend observed in the number of affected metabolites as most dose-response curves exhibited the largest relative difference from controls in the lowest exposure group and then approached the control levels. This is exemplified for the putatively characterized eicosanoid leukotriene B4 displayed in Fig. 4I. Leukotrienes are important inflammatory mediators.

3.3. Long-term effects

3.3.1. Fulton's condition factor in adult fish

Following 110 days of plastic NP exposure, k was estimated to establish the impact on the nutritional condition of the fish. There was no significant difference across all groups including both genders (Fig. 5A). When analyzing the males only, a significant difference was observed between the 0.01 and the 0.1 mg/l group (Fig. 5B), with the 0.1 mg/l group having the lowest k. The mean k was highest in the control group when considering both all fish and males only.

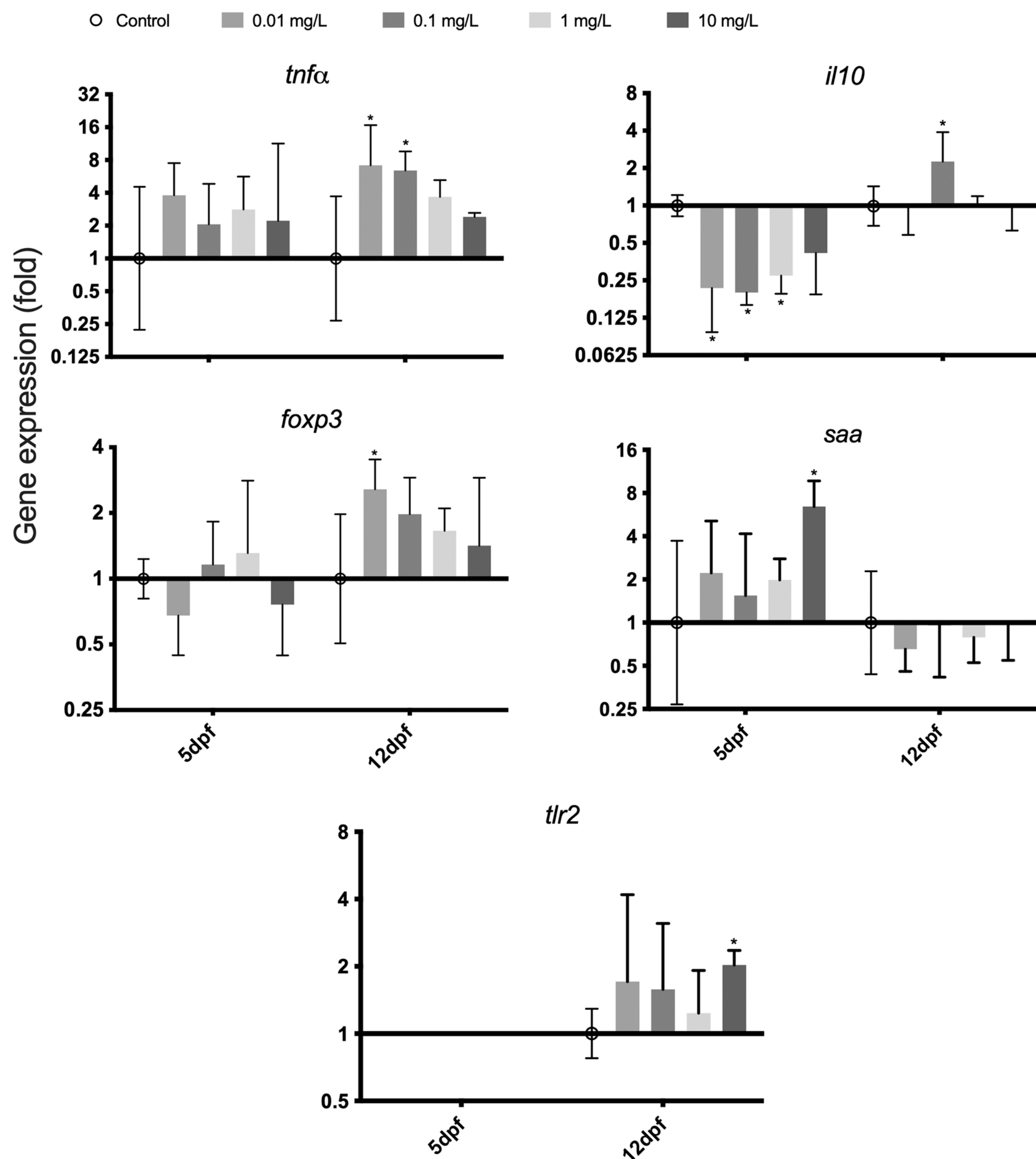


Fig. 3. Fold regulations of 5 genes analyzed 5 and 12 days post fertilization (dpf) in zebrafish larvae following continuous exposure to 500 nm polystyrene nanoparticles in concentrations of 0.01, 0.1, 1 and 10 mg/l. Significance between each group and the control is indicated by * ($p < 0.05$, Student's *t*-test). Error bars represent the geometric SD.

3.3.2. Adverse effects of NPs on reproduction

To investigate the ability to reproduce following long-term exposure to plastic NPs, 5 breeding pairs from each group were stimulated to spawn 4 times over 2 weeks. The 0.01 mg/l group only included 3 females and therefore only 3 breeding pairs were selected from this group. The mean number of eggs produced for each pair was highest in the control group however no significant difference was observed between fish exposed to different concentrations of plastics (Fig. 5C). Survival of the embryos was also recorded and while the control, the 0.01 and 1 mg/l groups had a similar survival rate, embryos from the 0.1 mg/l group had a significantly reduced survival percentage compared to the control group (Fig. 5D).

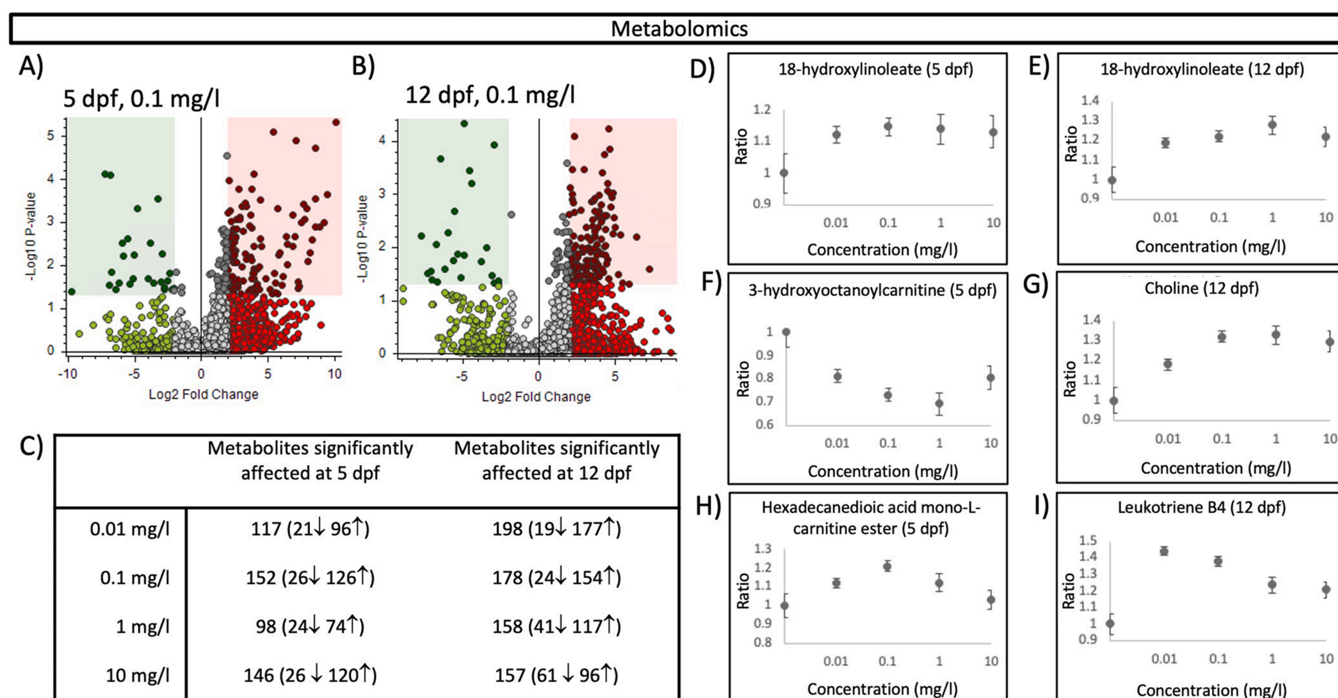
3.3.3. Gene expression in adult fish

The whole organ packet was analyzed for gene expression of 12 immune-relevant genes in adult zebrafish at 110 days after fertilization and continuous exposure to the plastic NPs. Only 2 genes were found to be differentially regulated (Student's *t*-test, $p < 0.05$) out of the panel of assays described in Supplementary Table 1. The genes encoding Foxp3 and Saa were significantly down- and up-regulated, respectively, in the fish exposed to the highest concentration (1 mg/l) of plastic NPs (Fig. 6).

3.3.4. Changes in the composition of the gut microbiota in adult fish

A total of 25 genera were detected in the gut samples, of which one, *Cetobacterium*, was the most dominant throughout the dataset (95.5% cumulative fraction abundance), followed by *Aeromonas* (3.5%), *ZOR006* (An unclassified *Firmicutes* sensu stricto, 0.4%) and *Fluviicola* (0.23%), all of which have previously been reported in zebrafish (Gaulke et al., 2019; Roeselers et al., 2011; Sharpton et al., 2021).

We observed differences in relative abundances of detected



	Metabolites significantly affected at 5 dpf	Metabolites significantly affected at 12 dpf
0.01 mg/l	117 (21↓ 96↑)	198 (19↓ 177↑)
0.1 mg/l	152 (26↓ 126↑)	178 (24↓ 154↑)
1 mg/l	98 (24↓ 74↑)	158 (41↓ 117↑)
10 mg/l	146 (26 ↓ 120↑)	157 (61 ↓ 96↑)

Fig. 4. Volcano plots showing down- and up-concentrated metabolites compared to control fish in A) 5 dpf and B) 12 dpf for the 0.1 mg/l group. The x-axis represents positive and negative log2-fold changes, respectively. Metabolites highlighted within the red and green fields differ significantly in their concentration when compared to control animals (ANOVA, $p < 0.05$, Log2 Fold Change (FC)> 2). C) Table showing the total amount of metabolites found in significantly different concentration compared to controls ($p < 0.05$, Log2 FC>2) with the amount of down and up concentrated compounds indicated in parentheses (↑↓). Dose-response plots showing the relative concentration of the metabolite as a function of the NP concentration for D) 18-hydroxylinoleate at 5 dpf and E) at 12 dpf, F) 3-hydroxyoctanoylcarnitin at 5 dpf, G) Choline at 12 dpf, H) Hexadecanedioic acid mono-L-carnitine ester at 5 dpf, I) Leukotriene B4 at 12 dpf. Data are presented relative to the control average (Control = 1).

microbiota groups at the phylum and genera levels (Fig. 7A-B). At the phylum level, a significantly higher relative abundance of Bacteroidetes, driven by the genus *Fluviicola* was observed between 0.1 mg/l and all other concentrations (p-adjusted=0.007 between 0.1 mg/l and control; Fig. 7A). At the genus level the relative abundance of *Macellibacteroides* abundance was significantly higher in the control than in 0.01 mg/l ($p = 1.301117 \times 10{-}3$, p-adjusted=0.025) and significantly higher in 0.1 mg/l than 0.01 mg/l ($p = 1.301117 \times 10{-}3$, p-adjusted=0.025; Fig. 7B). No other genera were significantly different from the control.

Zebrafish gut samples from the 0.1 mg/l group showed higher estimates of the genus richness and Shannon alpha diversity estimates although no significant differences could be detected (Fig. 7C-D). No significant differences in alpha diversities were observed among zebrafish from the different NP concentrations (Fig. 7C-D).

We did not observe any significant differences in the microbial composition among the different concentrations (Fig. 7E), although zebrafish from the 0.1 mg/l group showed a higher within-group variation among individuals suggesting a potential disruption of the natural composition. Three apparent outliers were removed from the PcoAs for visualization, and did not impact the results oberved. These apparent outliers mainly differed from the rest of the samples in the abundance of *Aeromonas* of which they had a higher abundance compared to the rest. In conclusion, the most significant observations from the gut microbiota analyses are; 1) no significant differences in diversity or composition of the gut microbiota were observed between the control and the group with the highest concentration of plastic NPs, 2) we did, however, observe significant effects for specific groups most clearly illustrated by i) the *Rhodobacter* genus only appearing in the 0.01 and 0.1 mg/l groups and that ii) Bacteroidetes was elevated in the gut microbiota of zebrafish exposed to 0.1 mg/l NPs.

4. Discussion

We have, for the first time, continuously exposed fish to plastic nanoparticles during a whole generation. Collectively, our results showed that all end-points investigated (gene expression, metabolomics, microbiota composition, fertility, condition factor and offspring survival) were moderately impacted by the particles at environmentally relevant concentrations (Sussarellu et al., 2016; Lenz et al., 2016; Al-Sid-Cheikh et al., 2018). Therefore, exposure throughout a whole generation adversely affect zebrafish and thereby likely also other fish and other vertebrates both on population and ecosystem level (Figs. 8 and 9).

There is a lack of tools for measuring nanoparticles in the environment. Some studies, however, suggest that the concentration of plastic nanoparticles might be magnitudes higher than plastic microparticles in a given environment (Al-Sid-Cheikh et al., 2018; Gaylarde et al., 2021; Besseling et al., 2019). It has furthermore been reported that extreme pollution may reach as much as 5.5 mg/l for plastic microparticles (Lasee et al., 2017), and that plastic pollution will increase further in the future (Besseling et al., 2019; Yin et al., 2019). Therefore, we suggest that the lower 0.01 and 0.1 mg/l concentrations used in this study are environmentally relevant with 0.1 mg/l in the high end.

4.1. Embryos and Larvae

4.1.1. Distribution of plastic particles in embryos

Plastic particles of 500 nm and up to 700 nm have been reported to diffuse through the chorion and into the eggs and embryos (Bhagat et al., 2020; Lee et al., 2019; Rawson et al., 2000; Duan et al., 2020). In the present study, particles were found in the yolk sac and immature intestine of 5-day old larvae. Plastic NPs will likely become coated and hetero- and homoaggregate in environmental media, such as freshwater

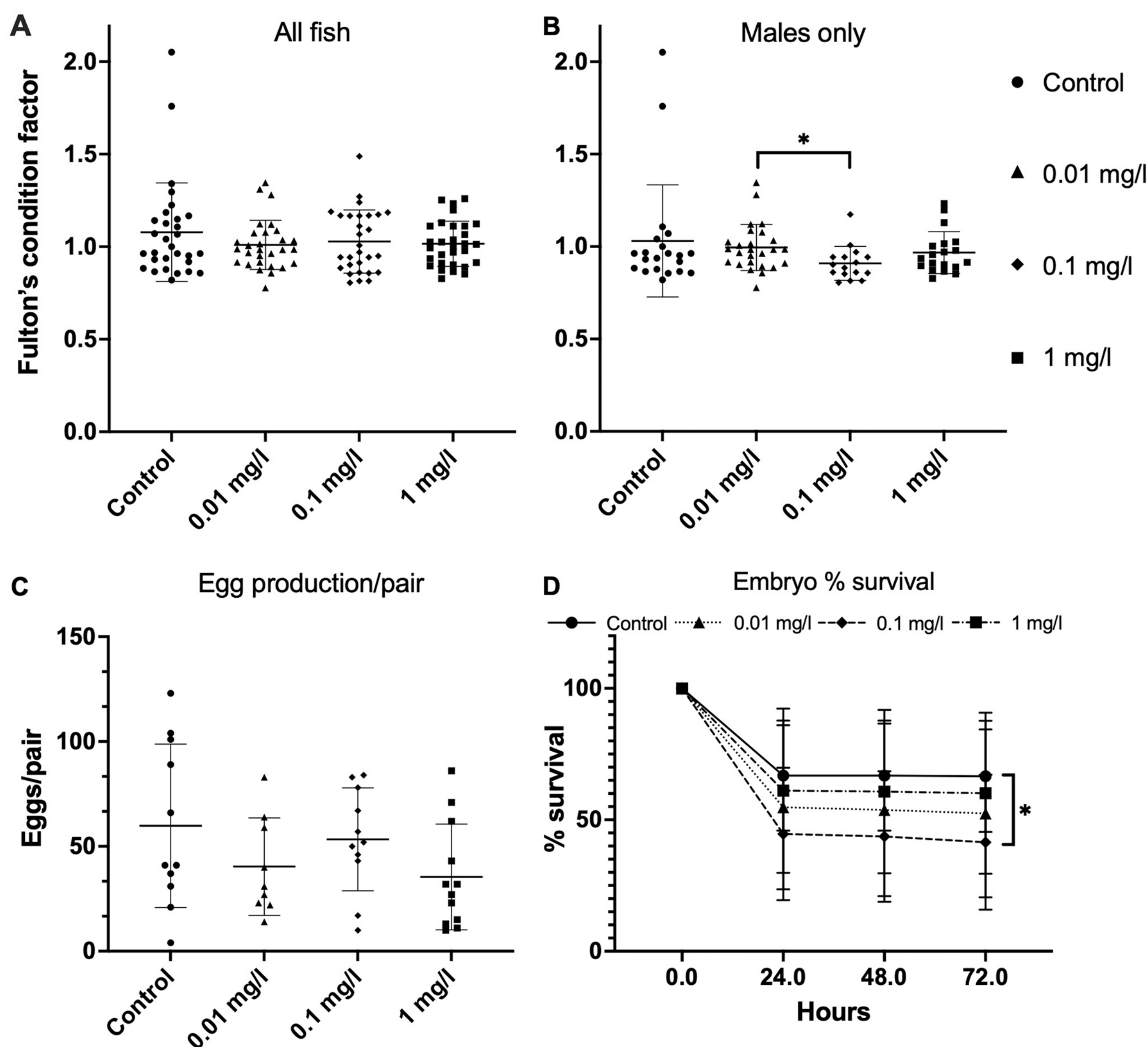


Fig. 5. Fulton's condition factor, reproduction, and embryo survival. Fulton's condition factor was calculated in all fish (A) and in males only (B) following 110 days of continuous exposure to 500 nm polystyrene NPs in concentrations of 0.01, 0.1 and 1 mg/l. Within the last 2 weeks of the experiment the fish were bred 4 times and the average number of eggs/pair is depicted (C) and survival of the embryos registered (D). * indicates significant difference ($p < 0.05$).

containing organic matter etc. These transformations cause changes in particle size and stability of suspension over time as seen for metal nanoparticles in zebrafish medium (Thit et al., 2017). We find it likely that the increase in hydrodynamic diameter (Supplementary Table 2) was a result of particle coating, agglomeration, and aggregation at the higher concentration of 1 mg/l, thus limiting diffusion across the chorion.

It is possible that the aggregation and agglomeration of particles was most pronounced in water from fish tanks at concentrations of 1 and 10 mg/l as sedimentation of particles was observed at the bottom of the Petri dish/tank. Since the larvae had not yet started to eat at 5 dpf the presence of NPs in the yolk sac may not only be assigned to ingestion of water through the mouth that opens 3 dpf (Wallace and Pack, 2003) but also because of diffusion through the egg chorion and/or active or passive uptake across skin and gills. Polystyrene has affinity to lipids and is known to become trapped in lipid-rich organs such as the yolk sac (Lee et al., 2019; Mattsson et al., 2015). The potential presence of NPs in 12-day old larvae, analyzed by imaging, was likely camouflaged in autofluorescence since autofluorescence of the mature intestines (Wallace and Pack, 2003) has increased significantly at this stage in life.

4.1.2. Induction of inflammation and tolerance

The immune system of the zebrafish larvae may recognize plastic NPs that are ingested or attached to the body surface (agglomerated or not) as foreign objects, which may trigger an inflammatory responses in the gut (Jin et al., 2018; Brun et al., 2018; Greven et al., 2016) and/or body surfaces like gills and skin (Limonta et al., 2019; Brun et al., 2018; Lu et al., 2018). Pro-inflammatory cytokines Il1β and Tnfα (Limonta et al., 2019; Lu et al., 2018; Brun et al., 2019; Qiang and Cheng, 2019) and genes related to the complement system (Veneman et al., 2017) are induced and these responses can be associated with oxidative stress, ROS production (Qiao et al., 2019a, 2019b; Sendra et al., 2021) and change in the gene expression of antioxidant genes (Qiao et al., 2019a; Sendra et al., 2021). The up-regulation of the pro-inflammatory cytokine *tnfα*, found in our study and the simultaneous down-regulation of the anti-inflammatory cytokine *il10* in the larvae indicate that an inflammatory response was initiated at the 2 lowest concentrations (0.01 and 0.1 mg/l) (Brugman et al., 2014; Jin et al., 2021; Zou and Secombes, 2016). Previously, *tnfα* has been shown to be up-regulated in the intestine of zebrafish adults after short-term exposure to polystyrene nanoparticles (Xie et al., 2021), in human lung epithelial cells and furthermore cause reproductive toxicity in mice (Jin et al., 2021; Xie et al., 2020; Xu et al., 2019). It was furthermore documented that polystyrene particles of 460 nm directly caused an elevation of TNFα in immune cells (Hwang et al., 2020). Inflammation, in our study, is supported by the up-regulation of the gene encoding the acute phase protein

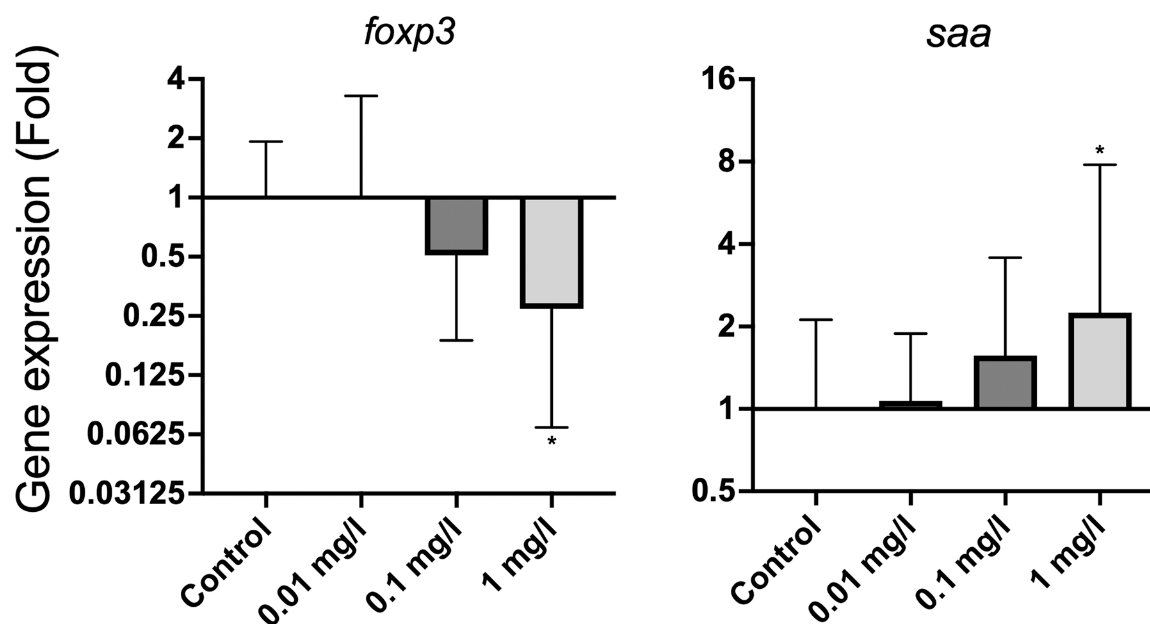


Fig. 6. Genes with significant regulations analyzed in adult zebrafish 110 days following fertilization (dpf) and continuous exposure to 500 nm polystyrene nanoparticles in concentrations of 0.01, 0.1 and 1 mg/l. Significance is indicated by * ($p < 0.05$, Student's *t*-test) and the bars represent the geometric SD.

Saa in the 10 mg/l concentration group. This acute phase protein may however have a dual role in inflammation since Saa produced in the intestine of zebrafish larvae in response to the gut microbiota, prevents excessive inflammation by suppressing pro-inflammatory markers and neutrophil bactericidal activity (Murdoch et al., 2019). The gene encoding the transcription factor Foxp3 that defines the development of regulatory T cells (Treg) was also up-regulated in larvae, which indicates that the regulatory T cell pathway dampening the inflammation and maintaining immune homeostasis was activated concurrently with the inflammatory response.

Toll-like-receptor Tlr2 that recognizes foreign pathogens is important in the initiation of innate immune responses. It is an important factor in the defense against mycobacterial infection (Hu et al., 2019) and respond to the gut microbiota in zebrafish (Koch et al., 2018). The *tlr2* expression was found to be up-regulated in zebrafish after wounding and a recent study showed that it was directly involved in controlling the macrophage and neutrophil migration pattern and speed to the site of injury (Hu et al., 2021). In addition, TLR2 also promoted the release of TNFα in mice (Xu et al., 2013). Therefore, the moderate up-regulation of the gene encoding Tlr2 induced in the larvae exposed to the highest concentration of NPs (10 mg/l) may have been induced by a direct mechanical damage by plastic NPs or by indirect changes in the microbiome. Gu et al. (2020) also found an increase of the *tlr2* gene in zebrafish after exposure to 100 nm and 200 μm PS particles (Gu et al., 2020) but not with 5 μm indicating that the activation of Tlr2 is dependent on particle size. Therefore, the increased size of our particles at the highest concentration may explain why only this group responds with an elevation of *tlr2*. The inverse dose-response relationship observed for *tnfα* and *foxp3* may indicate a level of tolerance at the high concentrations or that bigger sized particles are less harmful or taken up in lower quantities. A study on vaccinations of rainbow trout (*Oncorhynchus mykiss*) testing low and high doses of a vaccine found the low dose to induce tolerance (Marana et al., 2020). In the high dose, where fish acquired protection against the target pathogen, a significant down-regulation of *foxp3* was observed while fish vaccinated with the low dose were highly susceptible to the disease and *foxp3* in those fish remained unregulated. LeMoine et al. (2018) described how gene transcription increased immediately after exposure to plastic particles but diminished within 14 days of exposure (LeMoine et al., 2018), which also supports possible induction of tolerance against plastic MPs and NPs.

Uptake of polystyrene NPs (50 nm and 1 μm) via phagocytosis have been reported to activate migration of neutrophils and macrophages (Sendra et al., 2021). However, no obvious accumulation of neutrophils and macrophages near the yolk sac or the intestine was apparent in the present study, which indicates a lack or a weak local innate immune response following 5-12 days of exposure to the particles.

4.1.3. Adverse effects on the metabolism

Metabolomic analyzes in zebrafish have previously indicated significant changes in the metabolome after plastic NP/MP exposure. Changes in glucose, lipid- and energy metabolism were documented after the exposure of zebrafish larvae to PS particles (Wan et al., 2019) and similarly, glycolipid (Qiao et al., 2019a; Zhao et al., 2020), energy (Lu et al., 2016) and amino acid (Qiao et al., 2019b) metabolism disorders were found in the liver and gut of adult zebrafish following plastic particle exposure. Several metabolomic pathways were adversely impacted by the plastic NPs in the current study. The most significant results included a disruption of the biosynthesis of fatty acids and lipid metabolism as a result of the exposure in the young fish. Furthermore, the lower concentrations of 0.01 and 0.1 mg/l had a more significant influence on the zebrafish metabolome than the higher concentrations of 1 and 10 mg/l. We suggest that the decrease of affected metabolites with increasing concentration of plastic NPs at 12 dpf is because of either tolerance towards the plastic or because the particles agglomerate into bigger sized particles, which are less harmful and taken up in lower quantities when compared to smaller sized particles (Sendra et al., 2021).

We observed an inverse dose-response curve of both *tnfα*, *il10* and *foxp3* and affected metabolites in the larvae. An elevation of Tnfα can directly influence lipid metabolism (Chen et al., 2009; Lager et al., 2011) or indirectly via oxidative stress (Hassan et al., 2014) and the elevation of *tnfα* may therefore be a direct cause of metabolite interferences. The dose-response curve for leukotriene B4 and *tnfα* are also similar in our study and Fonseca et al. (2021) showed that these substances are interlinked in a systemic inflammatory response in a rat model via a spleen-liver axis (Fonseca et al., 2021) supporting our findings.

4.1.4. Short term effects lead to long term effects

It has been demonstrated that a short-term exposure of zebrafish to

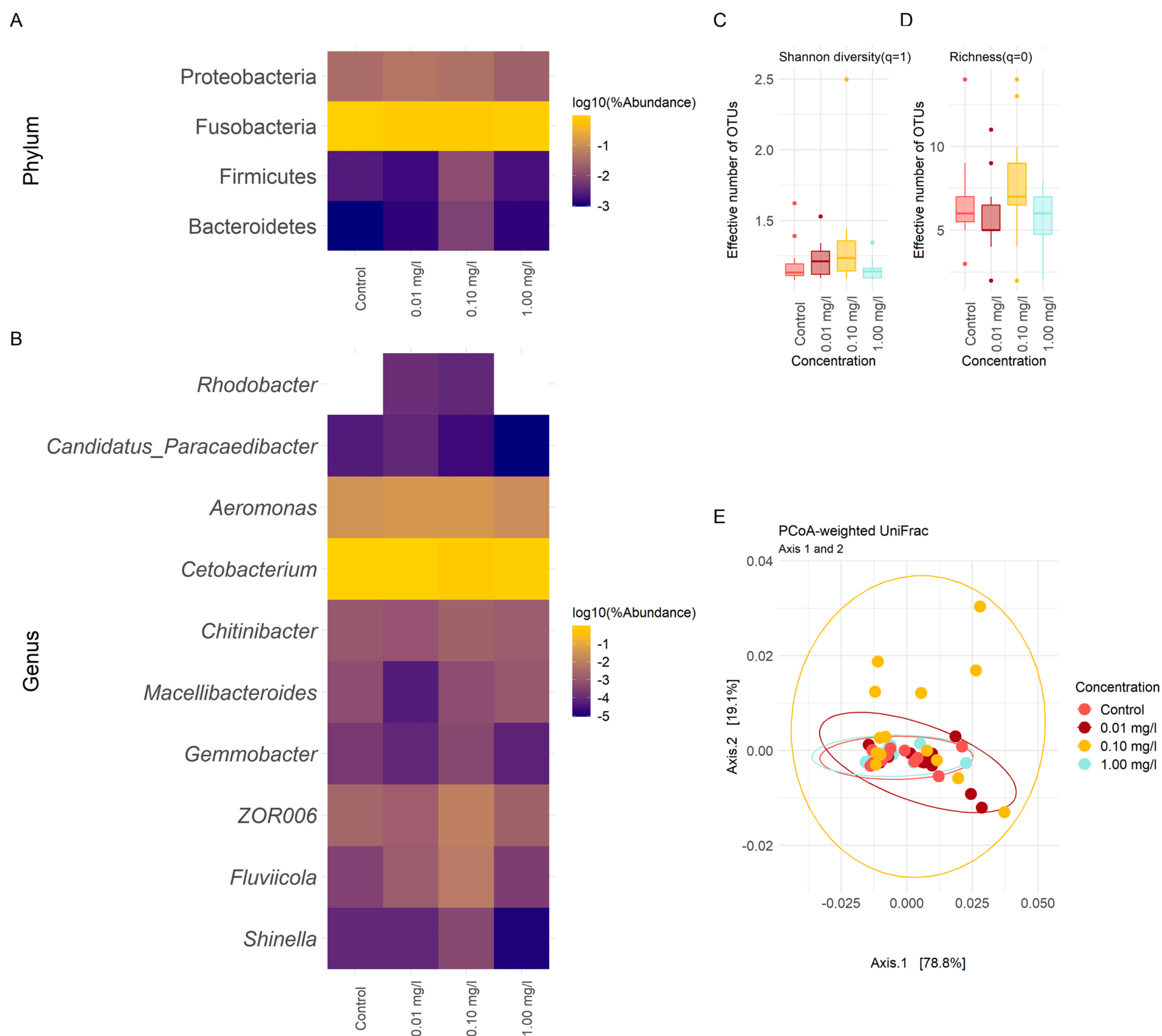


Fig. 7. Composition and diversity of gut microbiota in a control group and groups exposed to 0.01, 0.1 and 1 mg/l of plastic nanoparticles, respectively. A) and B) heatmaps of log10 transformed relative abundance of detected gut microbiota grouped by NP concentration. A) shows results for the top 4 phyla and B) shows results for the top ten genera observed in the samples. Alpha diversities of the gut microbiota are shown as the Shannon diversity (C) or as Richness (D). E) weigthed UniFrac PCoA of the gut microbial composition with colors depicting NP concentration.

different environmental stressors during the early phase of development can lead to persistent physiological alterations. including reproductive behavior of the exposed fish later in life (Chen et al., 2021). These alterations can even continue in the following generations without any further exposure (Anway et al., 2006; Knecht et al., 2017; Vera-Chang et al., 2018; Blanc et al., 2020). It has furthermore been shown that many environmental stressors act through the lipid metabolism pathway (Lee et al., 2018). As lipids are associated with energy homeostasis, structural development and other processes in zebrafish early development (Fraher et al., 2016), a disruption in lipid metabolism may contribute to abnormal development and subsequent adverse effects later in life. Lipid metabolism has also been linked to fertility and the reproductive system, which is one of the most sensitive systems and highly susceptible to environmental impacts (Negrovilar, 1993) in other organisms (Wan et al., 2020; Lobaccaro et al., 2012). We therefore suggest that the short-term adverse effects observed in this study: a mild chronic inflammatory state and/or tolerance together with altered lipid metabolism and biosynthesis, impact the gut- and overall health of the fish, resulting in long-term adverse effects. The health impact may include a reduction of the nutritional state which can lead to previously observed decreased growth in fish (Yin et al., 2019), and reduced reproductive output.

4.2. Adult zebrafish

4.2.1. Changes in gene expression

In the adult fish, the gene expression studies indicate the presence of a mild inflammatory reaction on peripheral surfaces of the fish exposed to the highest concentration of plastic NPs (1 mg/l). The *foxp3* down-regulation (i.e. reduced regulation of immune homeostasis and increase in inflammatory responses) and the moderate up-regulation of *saa*, which mostly is produced in the liver and subsequently relocate to target tissues or organs indicate that inflammation is taking place. Other genes encoding classical inflammatory cytokines, macrophages,

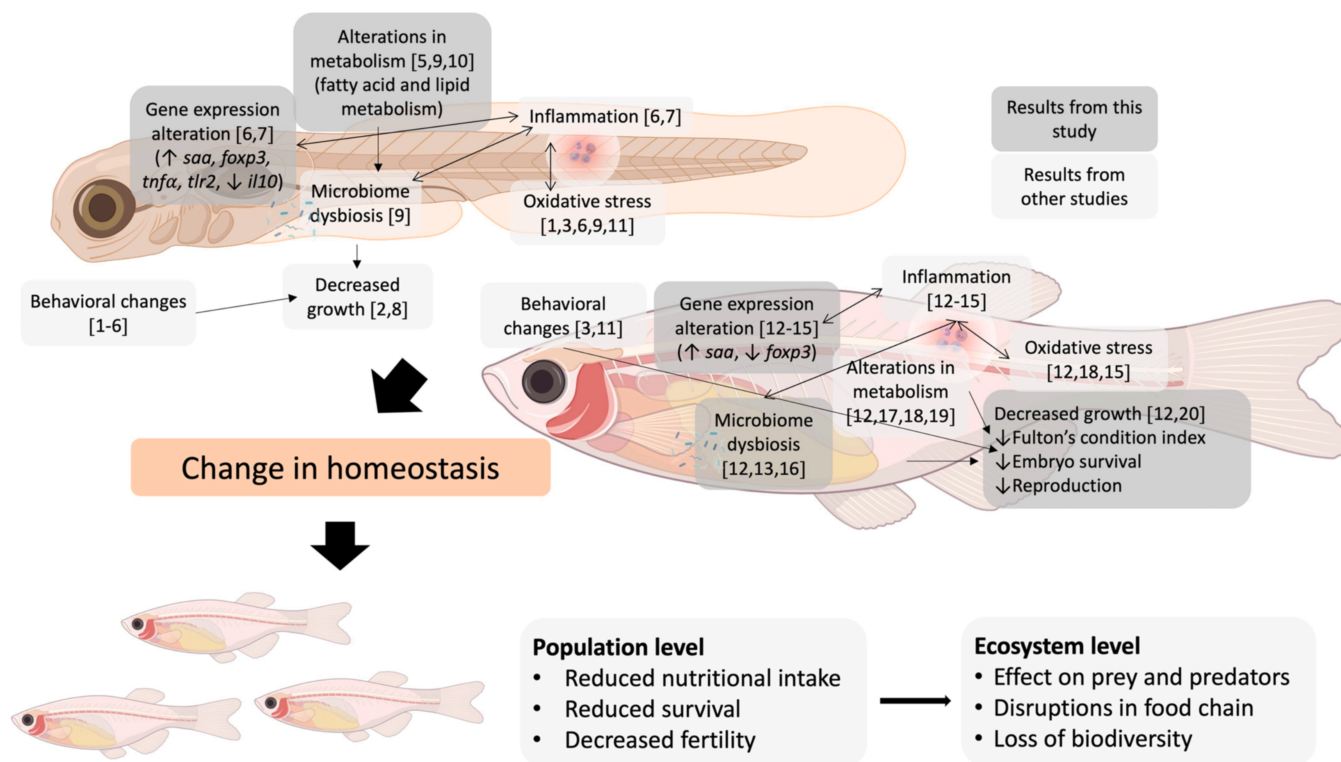


Fig. 8. Overview of processes induced by NPs and MPs in zebrafish larvae and adults and impact on population and ecosystem level. The figure is shown with transparent zebrafish only for visual effects. Created with Biorender.com. References: 1. (Parenti et al., 2019), 2. (Chen et al., 2017), 3. (Pedersen et al., 2020), 4. (Pitt et al., 2018), 5. (Brun et al., 2019), 6. (Qiang and Cheng, 2019), 7. (Brun et al., 2018), 8. (Zhang et al., 2020), 9. (Wan et al., 2019), 10. (Duan et al., 2020), 11. (Sendra et al., 2021), 12. (Qiao et al., 2019a), 13. (Jin et al., 2018), 14. (Lu et al., 2018), 15. (Lu et al., 2016), 16. (Qiao et al., 2019b), 17. (Zhao et al., 2020), 18. (Sarasamma et al., 2020), 19. (Limonta et al., 2019), 20. (Mak et al., 2019)

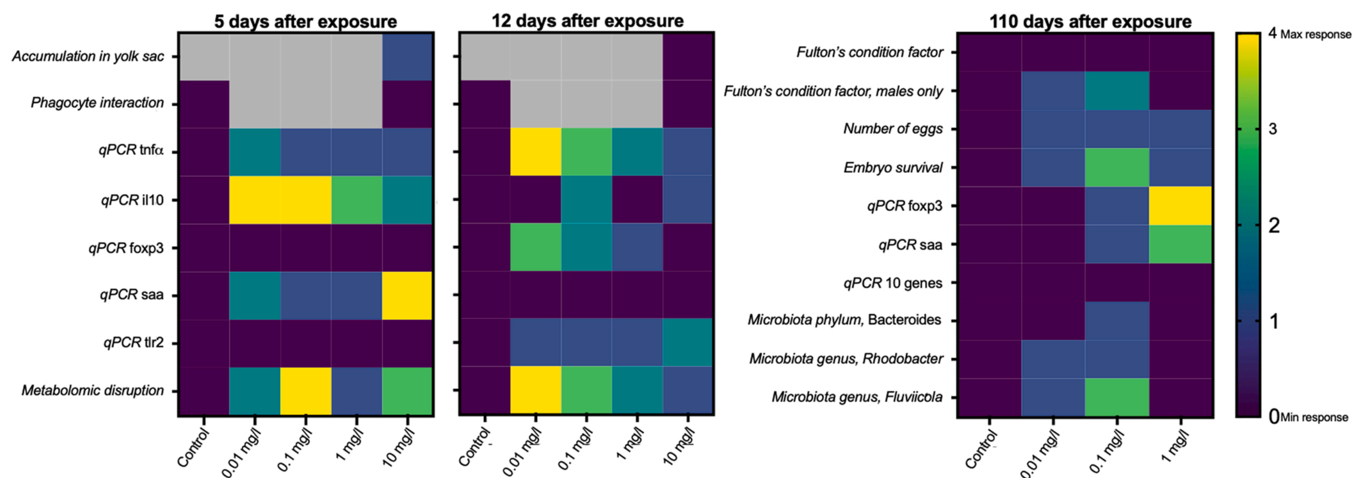


Fig. 9. Summary of the main findings, illustrating the impact of the tested plastic nanoparticle concentrations on the biological endpoints measured in the fish. Light gray indicated that the data was not determined. The color legend for the 3 heat maps is shown to the right and indicates minimum to maximum impact of the particles.

complement and transcription factors investigated here were unregulated in the organ packet indicating a local and not systemic or intestinal inflammation. Since there is also no difference between the 1 mg/l group and the control group regarding the gut microbiota it suggests that the inflammatory response may take place in peripheral tissues such as gills, skin, or the urogenital pore, which has been documented previously (Sugimoto et al., 2017; Kasheta et al., 2017). It has furthermore been demonstrated that T cells spontaneously can proliferate in skin and gills in the absence of *foxp3a*, which may lead to inflammation (Sugimoto et al., 2017). Li et al. (2020) described how plastic microparticle treatment induced a decrease in Treg cells and increased the microbiome diversity in mice (Li et al., 2020), and Gu et al. (2020) found that T cell numbers increased following PS exposure in zebrafish (Gu et al., 2020). These studies correlate well with our data obtained in zebrafish.

4.2.2. Plastic concentration correlates with changes in gut microbiota profiles

The microbiota colonizes the gut in zebrafish at hatching (Llewellyn et al., 2014) and establishes through the intricate combination of host genetics, immune system and environmental factors (Rawls et al., 2006). It plays an important role in host health and immune defense (Earley et al., 2018). The microbiota of many teleosts is composed to a high degree of Proteobacteria and Fusobacteria (Givens et al., 2015; Stagaman et al., 2020; Stephens et al., 2016) and in the case of zebrafish also Firmicutes (Roeselers et al., 2011). We see a much higher presence of Fusobacteria compared to many other zebrafish studies (Qiao et al., 2019a). However, it has been described how α-diversity decreases with age and that γ-proteobacteria are dominant throughout the life span with Fusobacteria developing into a major group in adults (Stephens et al., 2016). Housing conditions also have a great impact on the microbiome in zebrafish, hence the less common distribution may also be a result of laboratory and feed specific characteristics.

Plastic MPs and NPs have been shown to change the composition of zebrafish gut microbiota at the phylum level, however opposite scenarios have been described in different studies. Some papers describe a decrease of the abundance of Proteobacteria and an increase of Fusobacteria and Firmicutes (Qiao et al., 2019a; Jin et al., 2018; Wan et al., 2019) whereas a recent study documented an increase of Proteobacteria and a decrease in Fusobacteria, Firmicutes and Verrucomicrobiota following exposure to MPs or NPs (Xie et al., 2021). The essential information here is that the plastic particles changed the microbiota, which also was observed in this study even though only a moderate effect was observed. We suggest that the presence of plastic NPs in the gut may reduce energy intake from the feed that passes through the relatively simple intestine, thereby mimicking starvation. Starvation may explain the higher abundance of Bacteroides, which has been proposed to be more competitive compared to other bacteria during undernourishment and significantly less abundant in high fat diets (Méndez-Pérez et al., 2019; Clarke et al., 2012; Semova et al., 2012). However, an increased abundance of Bacteroides has also been associated with plastic materials (Oberbeckmann et al., 2016) and at the taxonomic level *Fluviicola* has specifically been associated with polyethylene terephthalate (Oberbeckmann et al., 2016) but is normally found in a wide range of water habitats (Bowman, 2020). Firmicutes thrive in zebrafish and mice exposed to high fat diets (Semova et al., 2012), and therefore the non-significant increase of this phylum in the 0.1 mg/l group is not in line with the "starvation" theory. However, Jin et al. (2018) also found an increase of Firmicutes following polystyrene exposure in zebrafish, which corroborates our results (Jin et al., 2018). Rhodobacter, which was only found in the fish exposed to 0.01 and 0.1 mg/l, has been identified on polystyrene plastispheres in several papers and may therefore be specifically driven by the polystyrene (Purohit et al., 2020; Barnier et al., 2020). Rhodobacter was not found in fish exposed to the highest concentration of NPs and we suggest that a lower amount of plastic was present in the gut due to agglomeration and precipitation of the plastic particles.

A mutual linkage between the immune system, the microbiota and the metabolome exist (Qiao et al., 2019a), and the plastic NPs may directly or indirectly affect all 3 systems and it is therefore challenging to decipher which system the NPs have the greatest impact on. Long-term inflammation or direct impact of plastic NPs will affect the microbiome and may cause dysbiosis and metabolic disorders (Qiao et al., 2019a) – or *vice versa* – an alteration of the microbiome caused by the plastic particles may cause inflammation and metabolic disturbances in the fish host (Clemente et al., 2012). NPs in the gut may therefore indirectly (via activation of the immune system and/or disturbance of the metabolic processes) or directly lead to an altered and dysfunctional gut microbiota.

4.2.3. Altered feeding behavior

Plastic NPs and MPs have also been shown to induce alterations in the behavior of fish which may influence their feeding performance (Mattsson et al., 2015; Brun et al., 2019). A gut full of plastic NPs may either give the sensation of being satiated and the fish would in that case reduce feeding or it may lead to decreased nutritional intake since the gut is full of non-nutritious "food" and the fish would crave more food and increase their feeding behavior. We observed that zebrafish from the 0.1 mg/l and the 1 mg/l groups were eating at a higher speed and seemed to have increased appetite compared to zebrafish from the control and the 0.01 mg/l groups (independent visual observations by 2 people), which is the opposite behavior compared to some findings in fish and mice (Mattsson et al., 2015; Jin et al., 2021). Since the condition factor had a tendency towards being lower in the 0.1 mg/l group the fish may have tried to compensate for a lower nutritional uptake by increased foraging.

4.2.4. Condition factor in adult fish

Metabolic disturbances were observed in the young fish, especially for the 0.1 mg/l group, and if these disruptions effect the fish into adulthood it may have led to the non-significant lower condition factor observed in males from that group (Qiao et al., 2019a; Karlsson et al., 2013). Small plastic particles have been shown to decrease the growth and condition factor of juvenile plantivorous (Critchell and Hoogenboom, 2018) and omnivorous fish (Mizraji et al., 2017) supporting our finding. Similarly, a study in male mice found that exposure to 4 and 10 μm polystyrene particles resulted in decreased weight (Jin et al., 2021). We chose to analyze males only because the prevalence of females was different among groups. Females often weigh more than males, and their weight furthermore depends on spawning condition. Thus, we expect that the absence of effects in females result from a variation in weight rather than a difference in response between the genders.

The 0.01 mg/l group only included 3 females, and we do not know if this skewness of gender is because of influence of the nanoparticles or because of human error sorting the fish according to gender. The metabolomic data from the larvae in this group showed a non-significant increase of tetrahydrocorticosterone indicating an elevated level of stress. Stress is known to skew the gender ratio towards more males (Ribas et al., 2017). This skewness probably does not affect the microbiota composition in zebrafish as described in previous studies (Roeselers et al., 2011; Cantas et al., 2012).

4.2.5. Limited documentation of plastic particle impact on fertility

There is a lack of information on the effects of plastic NPs/MPs on reproduction, especially concerning the long-term consequences on fertility and offspring survival. Pitt et al. (2018) showed that plastic NPs can be vertically transferred to the offspring, but the dietary plastic NP exposure for 7 days did not affect the reproductive success of zebrafish. However, maternally transferred plastic NPs did induce bradycardia (decreased heart rate) and delayed swim bladder inflation in the embryos (Pitt et al., 2018). Up-regulation of the gene encoding vitellogenin (*vtg1*) occurred in female zebrafish after exposure to plastic MPs indicating disruption of the oogenesis process (Mak et al., 2019). Relatively high concentrations of polystyrene microspheres (0.1 and 1 mg/l) increased reactive oxygen species (ROS) in male and female zebrafish gonads and caused apoptosis and histological changes in male zebrafish testes (Qiang and Cheng, 2021). In the current study we were able to document reduced embryo survival and a non-significant reduction of the number of eggs produced, which may be a result of decreased energy available for reproduction after plastic exposure.

5. Conclusion

We took advantage of the zebrafish model to record a range of health-related impacts induced by exposure to plastic nanoparticles during a whole generation. Exposure to the plastic NPs caused mild inflammation, changes in the metabolome and gut microbiota, a

tendency towards a lower body condition factor in males and lower survival of embryos produced by the exposed parents. Our results indicate that long-term effects may impact reproductive capabilities and potentially population dynamics; essential parameters that are often missed by short-term studies. It was demonstrated how studying zebrafish with its short generation time represents the ideal holistic model to investigate adverse impacts of plastic NPs in vertebrates. Adverse effects caused by exposure to plastic particles may furthermore not only be time dependent but also follow non-linear dose-response curves, where lower, often environmentally relevant exposure concentrations can be more harmful than more elevated concentrations.

The exposure concentrations used in this study ranged from environmentally relevant to magnitudes higher. We saw the highest adverse effect in the larvae with the lower concentrations (0.01 and 0.1 mg/l) and even if the 0.1 mg/l is in the higher end of the environmentally relevant concentrations it poses an environmental concern. It is expected that the concentration of plastic nanoparticles rises in the environment with time. Thus, with just a tenfold increase, there may be, according to our study, a long-term effect on the reproductive capacity on species population level leading ultimately to detrimental consequences not only for fish but for all aquatic organisms and top predators, disrupting food chains and negatively impacting biodiversity.

CRediT authorship contribution statement

Moonika Haahr Marana: Conceptualization, Data curation, Formal analyses, Methodology, Validation, Writing – original draft, Writing – review & editing. **Rikke Poulsen:** Conceptualization, Data curation, Formal analyses, Methodology, Writing – original draft, Software. **Eiríkur Andri Thormar:** Data curation, Formal analyses, Methodology, Writing – original draft, Software. **Cecilie Grønlund Clausen:** Data curation, Methodology, **Amalie Thit:** Conceptualization, Data curation, Formal analyses, Methodology, Writing – original draft, reviewing. **Heidi Mathiessen:** Formal analyses, Methodology. **Rzgar Jaafar:** Formal analyses, Methodology. **Rozalia Korbut:** Formal analyses, Methodology. **Anna Magdalene Brun Hansen:** Conceptualization, Formal analyses, Methodology. **Martin Hansen:** Conceptualization, Methodology, Resources, Software. **Morten Tønsberg Limborg:** Conceptualization, Methodology, Writing – original draft, Resources, Software, **Kristian Syberg:** Conceptualization, Writing – original draft, Resources, **Louise von Gersdorff Jørgensen:** Conceptualization, Data curation, Formal analyses, Methodology, Validation, Writing – original draft, Writing – review & editing, Resources,

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127705.

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