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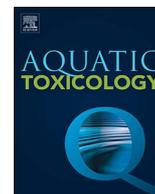
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Pathway analysis of systemic transcriptome responses to injected polystyrene particles in zebrafish larvae

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ABSTRACT

Microplastics are a contaminant of emergent concern in the environment, however, to date there is a limited understanding on their movement within organisms and the response of organisms. In the current study zebrafish embryos at different development stages were exposed to 700 nm fluorescent polystyrene (PS) particles and the response pathway after exposure was investigated using imaging and transcriptomics. Our results show limited spreading of particles within the larvae after injection during the blastula stage. This is in contrast to injection of PS particles in the yolk of 2-day old embryos, which resulted in redistribution of the PS particles throughout the bloodstream, and accumulation in the heart region. Although injection was local, the transcriptome profiling showed strong responses of zebrafish embryos exposed to PS particle, indicating a systemic response. We found several biological pathways activated which are related to an immune response in the PS exposed zebrafish larvae. Most notably the complement system was enriched as indicated by upregulation of genes in the alternative complement pathway (e.g. *cfhl3, cfhl4, cfb* and *c9*). The fact that complement pathway is activated indicates that plastic microparticles are integrated in immunological recognition processes. This was supported by fluorescence microscopy results, in which we observed co-localisation of neutrophils and macrophages around the PS particles. Identifying these key events can be a first building block to the development of an adverse outcome pathway (AOP). These data subsequently can be used within ecological and human risk assessment.

1. Introduction

As one of the first responses to the presence of xenobiotics, cells start transcribing RNA to maintain normal cellular function. Tracking this response using RNA-sequencing (RNAseq) techniques has made substantial contributions to our understanding of many biological processes triggered in organisms which are exposed to environmental contaminants. The pathways and processes activated and identified using RNAseq techniques are subsequently linked with adverse health outcomes, including development, adaptive evolution, host immune response, and stress response. The identification of target pathways of hazardous compounds allows for a better understanding of the molecular mechanisms underlying relevant adverse ecological outcomes making this area of research a primary focus in aquatic toxicology.

An excellent model organism to develop identify target pathways is

the zebrafish (*Danio rerio)* for a variety of reasons. They offer a mapped and annotated transcriptome together with a rich repertoire of genetic, molecular and cellular manipulation tools (Strähle et al., 2012). Using these tools, molecular results suggest that the zebrafish embryo is a suitable model for systematic testing of chemicals under the European REACH initiative (Lammer et al., 2009). For this reason they represent aquatic vertebrates in toxicological assessments (Scholz et al., 2008; Stegeman et al., 2010). Moreover, zebrafish are used to investigate and answer various biomedical and toxicological research questions, including research on cancer, infectious diseases and cardiovascular disease (Spaink et al., 2013; Strähle et al., 2012). Up to 80% of human disease related genes can be linked to a zebrafish orthologue (Howe et al., 2013) and therefore it holds a very strong position as a screening model in between cell or tissue culture systems and higher animal models. The conservancy in the molecular targets and commonalities of

effect pathways will assists in the extrapolation across different species of ecological relevance. This is especially relevant when studying emergent contaminants in the environment, for which there is limited understanding through which pathway organisms are adversely affected.

One such group of contaminants are microplastics (plastic particles < 5 mm; Andrady, 2011; Cole et al., 2011). Microplastics are intentionally added to cosmetic and household products (Chang, 2015; Fendall and Sewell, 2009) or can be formed as weathering products from larger pieces of plastics (Cole et al., 2011). As a result, there is a wide range in physico-chemical characteristics of microplastics. One of the most commonly used and produced plastics is polystyrene (PS), for example, in Europe the demand for PS in 2014 was roughly 3,5 million tons (PlasticsEurope, 2015). For this reason PS particles are frequently used as model particle to study the impact and uptake of microplastics in organisms (Besseling et al., 2013; Cole et al., 2013; Farrell and Nelson, 2013). To date, molecular responses induced by plastic particles in aquatic vertebrate species are unexplored, even though there is an urgent need to develop these pathways for highly relevant environmental compound groups such as the microplastics.

Since little is known on the frequency and consistency of uptake of particles across epithelial membranes (Skjolding et al., 2017), our study aimed to perform the first pathway analysis to investigate the effects of PS particles delivered inside zebrafish (*Danio rerio*) during early developmental stages. Our first objective was to understand *if* and to *what extent* migration and biodistribution of injected fluorescently labelled PS particles throughout zebrafish embryos occurred at different stages of development. Our second objective was to identify affected pathways and processes due to the presence of these particles using RNAseq. We determined biodistribution by imaging and conducted time series analyses on their transcriptomes. The transcriptomes were subsequently probed to seek pathways and processes activated, and link to potential adverse health outcomes.

2. Material and methods

2.1. Particle characterisation

Polystyrene (PS) particles (PS; nominal size 700 nm were purchased from Corpuscular Inc (103125-05,US). These particles are internally dyed with Nile Red to prevent dye leaching into aqueous media. The PS-particle solutions were prepared with purified water (Milli-Q) and sonicated before every use. Transmission electron microscopy (TEM, H-7500, Hitachi, Japan) was performed to verify particle size. The agglomeration behaviour at two time points (at 0, 24 h) of PS particles was tracked using dynamic light scattering (DLS). Also the zeta potential was determined, giving additional information of the agglomeration behaviour of PS particles in egg water.

2.2. Zebrafish husbandry

Zebrafish were handled in compliance with animal welfare regulations and maintained according to standard protocols (http://ZFIN.org). Mixed egg clutches from the wildtype ABxTL, *Tg(bactin:Hras-EGFP VU119)* (Cooper et al., 2005), *Tg(mpx:EGFP)* (Lawson and Weinstein, 2002a) or *Tg(mpeg1:EGFP)*[g122] (Ellett et al., 2011) strain were collected for the experiment. Fertilized eggs were selected at 2–8 cell stage and grown at 28 °C in egg water (60 µg mL^{-1} Instant ocean sea salt, Sera Marin). The egg water was refreshed every 24 h. All work was done under approval number of the 'Netherlands Food and

Consumer Product Safety Authority': 10612. As only early life stage zebrafish were used, no specific additional project authorization was needed.

2.3. Biodistribution of PS particles

The PS particles were injected in the yolk of the embryos to increase the likelihood of uptake within the zebrafish larvae. Previous research has shown that this injection technique results in reproducible exposure of injected materials (such as microbes or cancer cells) inside the zebrafish embryos and larvae (Spaink et al., 2013). Therefore we followed the protocols published in Nature Methods (Spaink et al., 2013) as done for the development of drugs for cancer and microbial related diseases. To determine the importance of developmental stage on particle migration two time points in development were chosen for injections in the yolk: the blastula stage (between 16 and 128 cell stage) and the long-pec stage (2 days post fertilization, dpf) as determined by morphological criteria (Kimmel et al., 1995). At each stage, 1 nL of 4% polyvinylpyrrolidone$_{40}$ (PVP$_{40}$; Calbiochem, US) containing 5 mg mL^{-1} of Nile red fluorescently labelled PS particles (103125-05, Corpuscular inc. US) were injected into the yolk sac of embryos (n = 150). Transmission electron microscopy (TEM; JEOL 1010; JEOL Ltd., Japan) analysis confirmed an average diameter of 710 ± 23 (SD) nm of these PS particles (Additional file 1). Injections were controlled using a Leica M50 stereomicroscope together with a FemtoJet microinjector (Eppendorf, Germany) and a micromanipulator with pulled and bevelled micro capillary needles. Control embryos (n = 50) were mock-injected with 1 nL of PVP$_{40}$ only.

Throughout the paper, for developmental stages within the chorion (until 2 dpf) the term "embryo" is used and for later stages the term "larva". Migration and biodistribution of injected PS particle in embryos and larvae were examined and imaged daily until 5 dpf using a fluorescence stereo microscope (MZ 205 FA, Leica, Germany). If not hatched at 3 dpf, embryos were manually dechorionaed for comparative imaging. To optimize visualization of the biodistribution of particles inside the embryos and larvae we used the *Tg(bactin:Hras-EGFP VU119)* strain which expresses membrane-targeted green fluorescent protein (GFP). Embryos and larvae were kept under anaesthesia (0.02% buffered 3-aminobenzoic acid ethyl ester; Tricaine, Sigma, The Netherlands) in egg water during imaging. The image processing package Fiji (Schindelin et al., 2012) was used for processing and analysis of the images.

2.4. Transcriptomic analyses

Based on the biodistribution data, injection at the long-pec stage (2 dpf) was chosen for RNAseq. ABxTL larvae (n = 150 embryos) in the long-pec stage were injected with 1 nL of 4% PVP$_{40}$ containing red fluorescently labelled PS particles into the yolk (as described above). Control embryos (n = 50) were mock-injected with 1 nL of PVP$_{40}$ only. Sampling was performed at 1 and 3 days post injection (dpi) aiming to find direct and late transcriptome responses. At 1 and 3 dpi, ten embryos or larvae, respectively, showing distribution of PS particles throughout the body were pooled and three samples per group collected (representing biological triplicates). For the mock-injection biological duplicates were used. All samples were snap frozen in liquid nitrogen, and stored at −80° C for RNA isolation later on.

RNA isolation was performed as described in Veneman et al. (2013). Briefly, a total of 3 µg of RNA was used to construct RNAseq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina Inc., San Diego, USA). In the manufacturer's instructions two

modifications were made. Firstly, in the adapter ligation step 1 μl instead of 2.5 μl adaptor was used. Secondly, in the library size selection step the library fragments were isolated with a double Ampure XP purification with a 0.7× beads to library ration. The resulting mRNAseq library was sequenced using an Illumina HiSeq2500 instrument according to the manufacturer's description with a read length of 2 × 50 nucleotides. Image analysis and base calling was done by the Illumina HCS version 2.2.38, RTA version 1.16.61.

2.5. Data analysis

The GeneTiles server (www.genetiles.com) was used for data processing, quantification and visualization of the RNAseq data as described in Veneman et al. (2014). Briefly, Bowtie2 (Langmead and Salzberg, 2012) was used to align the reads from the fastq file to the zebrafish genome (GRCz10). Reads were randomly distributed upon multiple hits (default settings) in the generated SAM file. Samtools (Li et al., 2009) converts and compress the SAM file into a BAM file. A python script containing HTseq and pysam (Anders et al., 2015) allowed read count within a gene for reads that partially fall within the gene exon and intron region. Statistical analysis was performed using DESeq. Reads were normalized using a scaling factor, computed as the median of the ratio, for each gene and of its read count of the geometric mean across samples. The variance and average of the measurement is compared to the control and expressed as a P value calculated by the dispersion per gene. The data has been deposited in the NCBI GEO database under accession number: GSE77755.

Three different open-source software packages were used for additional statistical analysis and/or pathway enrichment: Pathvisio 3 (Pico et al., 2008) to extract enriched biological pathways, STRING (Szklarczyk et al., 2015) to assess functional enrichment of protein-protein interactions and g:Profiler (Reimand et al., 2011, 2007) with the g:Cocoa function for functional profiling of differentially expressed of gene lists. For the complement pathway analysis the human (*Homo sapiens*) complement and coagulase cascades pathway from Wikipathways has been modified to zebrafish orthologues, since there are hitherto no complement genes annotated for zebrafish.

3. Results and discussion

3.1. Particle characteristics

The size and shape of PS particles were further confirmed by transmission electron microscopy (Fig. S1). No agglomeration of the particles over time were found (DLS results not shown), and also the zeta potential was stable and did not change over time.

3.2. Particle migration and distribution in blastula stage injected eggs

After injection of PS particles into the yolk of blastula stage (16–128 cell stage) embryos, more than 95% of the injected test organisms did not show signs of developmental defects. Most particles tended to stay at the yolk injection site, and in only 12.5% of the embryos at 5 dpi migration away from the injection site occurred. Specifically, when the yolk started to be depleted at 3–4 dpi, some larvae showed signs of accumulation of particles around the heart region and within the blood stream (Fig. 1). This is in line with previous results in our group, showing a low percentage (0–10%) of embryos with distributed micro sized (2–10 μm size range) PS particles after yolk injection (unpublished data). In comparison, in the blastula stage essential elements such as vitamins are transferred from the yolk to the developing embryo
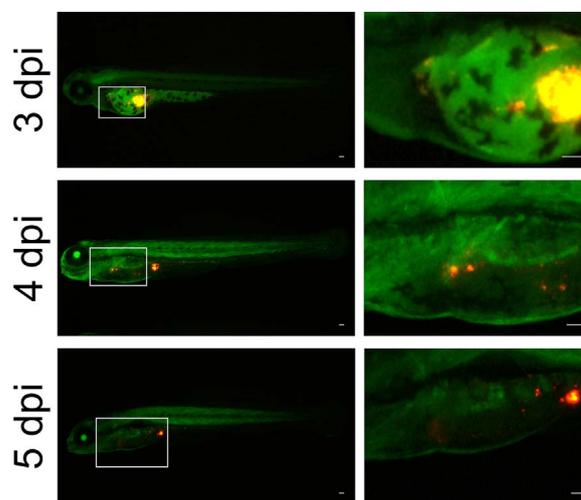

Fig. 1. Detailed overview of *Tg(bactin:Hras-EGFP VU119)* zebrafish larvae injected between 16 and 128 cell stage with red fluorescently labelled polystyrene (PS) particles. The regions of the images on the right are indicated by the white box on the left, showing the PS particles close to the heart region (3 dpi, 4 dpi, 5 dpi) or already migrated away from the yolk injection site (5 dpi). Scale bars are 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at a significant higher rate of 88% (Surai et al., 2014). In addition, various species of bacteria and some types of cancer cells are also much more effectively transferred from the yolk to the developing embryo (Spaink et al., 2013). Overall this indicates a rather low percentage of distribution of PS particles after injection at the blastula stage.

Waterborne exposure of zebrafish embryos and larvae to PS particles is an environmental relevant uptake path. Within this study we have not addressed this route, because previous studies reported for various different sizes (25–700 nm) PS particles that the internalization is low, estimated to be less than 10% (Van Pomeren et al. in press). The majority of the PS particles remained in the chorion or adsorbed to the zebrafish epithelium (both skin and gut epithelium) depending on their developmental stage. This < 10% absorption means that > 90% was not internalized, leading to variable internal concentrations which hampers identification of affected pathways and processes due to the presence of these particles. Dietary uptake of PS particles in fish is expected to be a factor 10 higher (Skjolding et al., 2017) but was beyond our current approach of short term (5 days) exposure. It is likely that waterborne exposure results in a more or less similar biodistribution especially over time.

3.3. Particle migration and distribution in 2 day old injected embryos

When injecting PS particles into the yolk of 2 dpf embryos, the percentage of larvae in which there was migration of microplastics to the bloodstream increased to 26.4% at 4 dpi (Fig. 2). At 2 dpf the embryos already have a vascular system developed and the yolk to embryo ratio is smaller than in earlier stages; both factors may explain the increased spreading of PS observed. In embryos with particle migration no uptake into tissues was observed, and particles remained within the vascular system. After injection, no damage was observed to the Duct of Cuvier, a vessel that spreads from the yolk sac towards the heart (Lawson and Weinstein, 2002b) and there was no indication that the PS particles leached into the Duct of Cuvier, indicating that PS
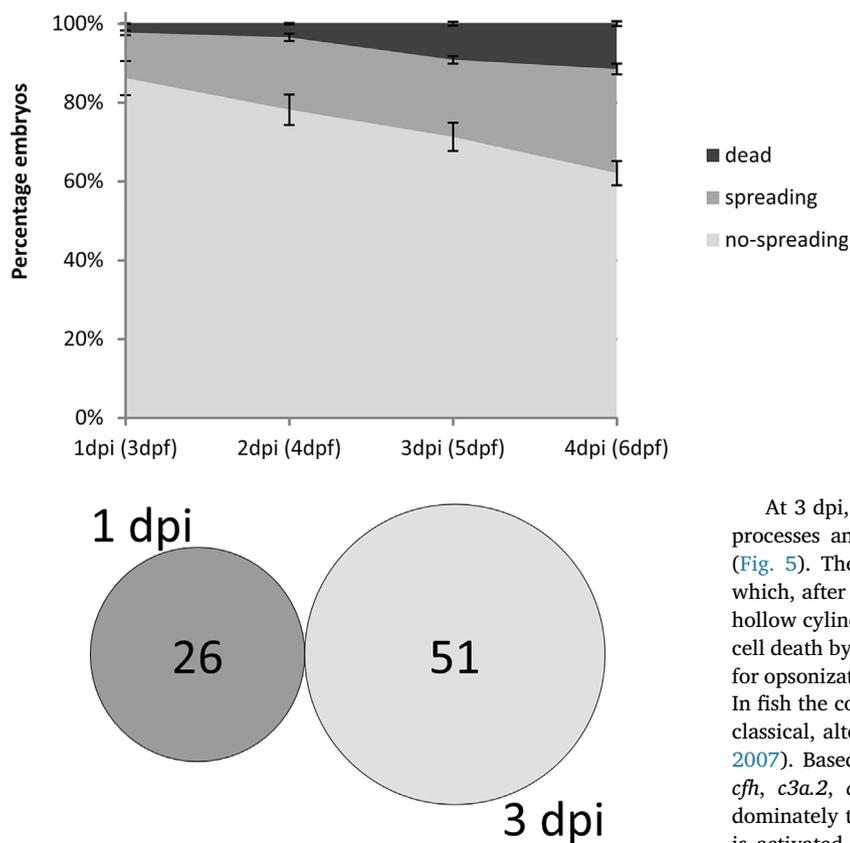
Fig. 3. No overlapping expression at different time points. Venn diagram showing the differentially expressed genes at 1 and 3 dpi in zebrafish embryos and larvae injected with polystyrene particles at 2 days post fertilization. (fc $> = 2$ or $< = -2$ and P value $< = 0.05$).

particles moved solely through the blood stream and got locally stuck in smaller vessels (as observed during live imaging).

3.4. Transcriptome analysis

Injection of PS particles caused significant changes in transcriptomic profiles of zebrafish larvae. At 1 dpi we found a group of 81 genes differentially expressed (fc: > 2 or < -2 and $P < 0.05$) in the PS particle injected embryos as compared to the non-injected group minus the mock control, while 55 genes were also differentially expressed in the mock-injected control group as compared to the non-injected control. This led to a group of 26 genes which were differentially expressed at 1 dpi in the PS particle injected embryos (Fig. 3). At 3 dpi 57 genes were differentially expressed in the PS particle injected larvae, while only 6 genes were differentially expressed in the mock-injected group, as compared to the non-injected. This resulted in 51 differentially expressed genes at 3 dpi (Fig. 3). This reduction between 1 and 3 dpi in differently expressed genes in the mock-injected larvae can be explained due to minor tissue damage associated with the injection (Cocchiaro and Rawls, 2013), resulting in increased differentially expressed genes in the mock-injected group at 1 dpi and subsequent recovery at 3 dpi. Interestingly, there were no overlapping particle-specific genes that were differentially expressed at 1 and 3 dpi. An overview off all differentially expressed genes grouped using STRING is shown in Fig. 4.

At 3 dpi, with reduced tissue damage expression, we found several processes and pathways enriched related to the complement system (Fig. 5). The complement system is composed of about 30 proteins, which, after activation by antigens, can cause either the formation of a hollow cylinder complex which can penetrate a pathogenic cell causing cell death by extracellular influx, or it results in coating of the pathogen for opsonization for phagocytic cell uptake (Jovanovic and Palic, 2012). In fish the complement system can be activated by three pathways: the classical, alternative or lectin pathways (Janeway et al., 2001; Whyte, 2007). Based on initial analysis using GeneTiles, genes including *cfb*, *cfh*, *c3a.2*, *c3a.3*, *c3a.6* and *c9* were induced, indicating that predominately the alternative pathway acts first. The alternative pathway is activated when there are pathogens without the presence of antibodies (Janeway et al., 2001; Whyte, 2007), which is the case for PS particles. Similar to our study, the complement system is activated in mice exposed to nanomedicines and carbon nanotubes (Moghimi et al., 2010). Interestingly we only observe upregulation of the alternative pathway, while with nanomedicines and carbon nanotubes both the classical and alternative pathways are activated (Moghimi et al., 2010). Our results show that although PS particles do not have any corresponding receptors and hence need to be tagged by the complement system to be cleared from the body, the particles are recognized by the immune system. Our results are supported with imaging data: there was an overlap of PS particles and neutrophils (Fig. 6) and macrophages (Fig. 7). In approximately 26% of the embryos the red PS particles overlap with neutrophils (green signal, MPX) and macrophages (green signal, MPEG) as shown in Figs. 6 and 7, respectively.

In the work by Zhang and Cui (2014) the current status of identification of the complement components in zebrafish based on the comparison with the mammalian system components is reported. It showed evidence that all the homologs of the mammalian complement fundamental components are present in zebrafish, as revealed by the phylogenetic analysis. Unfortunately this complement component pathway was not available for zebrafish pathway analysis which made comparison rather difficult. However 80% of human disease related genes can be linked to a zebrafish orthologue (Howe et al., 2013). Therefore we used the human pathway of complement and coagulase cascades (Wikipathways), and modified this to zebrafish orthologues. In our case, 8 zebrafish genes were linked to the human genes (Fig. 8: red squares). Since zebrafish at larval stage have no adaptive immune system (Meijer and Spaink, 2011), we only described the host response of the innate immune system. In vertebrates models and humans it was shown that the complement system is activated after exposure to lipid-based nanoparticles (Khanna et al., 2015). However, innate immune

source	term name **Gene Ontology (Biological process)**	term ID	n. of term genes	corrected p-value	1 dpi	3 dpi
BP	response to bacterium	GO:0009617	119	1.16e-02		4
BP	response to gonadotropin	GO:0034698	7	1.07e-02	2	
BP	cellular response to gonadotropin stimulus	GO:0071371	7	1.07e-02	2	
BP	multicellular organismal response to stress	GO:0033555	12	4.11e-02	2	
BP	response to organophosphorus	GO:0046683	12	3.37e-02	2	
BP	response to oxygen-containing compound	GO:1901700	180	8.47e-03		4
BP	response to purine-containing compound	GO:0014074	12	3.37e-02	2	
BP	response to cAMP	GO:0051591	12	3.37e-02	2	
BP	cellular response to lipid	GO:0071396	45	3.49e-02		3
BP	response to steroid hormone	GO:0048545	44	3.26e-02		3
BP	response to estrogen	GO:0043627	40	2.44e-02		3
BP	cellular response to organic cyclic compound	GO:0071407	61	1.25e-02		3
BP	cellular response to steroid hormone stimulus	GO:0071383	36	1.77e-02		3
BP	cellular response to estrogen stimulus	GO:0071391	33	1.35e-02		3
BP	cellular response to cAMP	GO:0071320	7	1.07e-02	2	

source	term name **Gene Ontology (Molecular function)**	term ID	n. of term genes	corrected p-value	1 dpi	3 dpi
MF	nucleic acid binding transcription factor activity	GO:0001071	389	1.26e-02	5	
MF	transcription factor activity, sequence-specific DNA binding	GO:0003700	389	1.26e-02	5	

source	term name **Human Phenotype Ontology**	term ID	n. of term genes	corrected p-value	1 dpi	3 dpi
hp	Nephritis	HP:0000123	33	1.02e-07		6
hp	Abnormality of the nephron	HP:0012575	148	2.93e-05		7
hp	Abnormality of the glomerulus	HP:0000095	102	2.12e-06		7
hp	Glomerulonephritis	HP:0000099	24	1.25e-08		6
hp	Membranoproliferative glomerulonephritis	HP:0000793	11	3.03e-04		3
hp	Renal insufficiency	HP:0000083	279	2.07e-02		6
hp	Recurrent bacterial infections	HP:0002718	83	8.66e-04		5
hp	Abnormality of humoral immunity	HP:0005368	158	4.63e-05		7
hp	Abnormality of complement system	HP:0005339	34	6.57e-10		7
hp	Complement deficiency	HP:0004431	28	1.45e-10		7
hp	Decreased serum complement C3	HP:0005421	7	6.44e-05		3

source	term name **Biological pathways (KEGG)**	term ID	n. of term genes	corrected p-value	1 dpi	3 dpi
ke	Phagosome	KEGG:04145	139	1.93e-02		3
ke	Degradation of aromatic compounds	KEGG:01220	4	4.52e-02		1

source	term name **Biological pathways (Reactome)**	term ID	n. of term genes	corrected p-value	1 dpi	3 dpi
re	Complement cascade	REAC:6298855	24	3.83e-09		5
re	Initial triggering of complement	REAC:6298854	13	1.09e-07		4
re	Alternative complement activation	REAC:6298921	6	2.60e-06		3
re	Activation of C3 and C5	REAC:6298856	6	2.60e-06		3
re	Regulation of Complement cascade	REAC:6299445	16	1.39e-07		4

Fig. 4. Functional profiling of differential expression. This image shows the functional profiling of the differentially expressed genes in zebrafish larvae injected with fluorescently labelled polystyrene particles at 1 and 3 dpi using the g:Cocao function of the g:Profiler software package (Reimand et al., 2011, 2007). (fc $>\ =$ 2 or $<\ =\ -2$ and P value $<\ =$ 0.05). The numbers in the boxes below 1 dpi and 3 dpi, respectively, indicate the enriched gene list, the colours intensity of red denotes the strength of enrichment.

responses to PS microparticles as we show here has to our knowledge not been documented yet. Uncontrolled complement activation likely leads to tissue damage and activation of down-stream inflammatory processes (Ricklin and Lambris 2013). However, the exact mechanism of how these complement proteins mediate nanotoxicity has not yet been elucidated (Zolnik et al., 2010).

The gene ontology shows several term IDs expressed at 1 and 3 dpi related to 'response', e.g. response to bacterium, gonadotropin, organophosphorus, oxygen-containing compound, purine-containing compound, steroid hormone and organic cyclic compound (Figs. 4 and 5). This indicates a broad host response against microplastic particles. Furthermore, using Pathvisio 3 we performed statistical analysis on the expression dataset in order to find enriched biological pathways at 1 and 3 dpi. At both days the pathway for nuclear receptors in lipid metabolism and toxicity was significantly enriched, and at 3 dpi the oxidative stress pathway (Table 1). Oxidative stress is a well-recognized mode of action of various nanoparticles (Manke et al., 2013; Sarkar et al., 2014). Our results indicate that even though spreading of particles is limited and local (based on the imaging pictures), this leads to innate immune responses of a systemic nature (based on imaging and transcriptomic data). Exposure to the PS particles enriched the complement system which showed that the immunological recognition process is active. These molecular initiating events and pathways have potentials to become early warning markers of long-term health
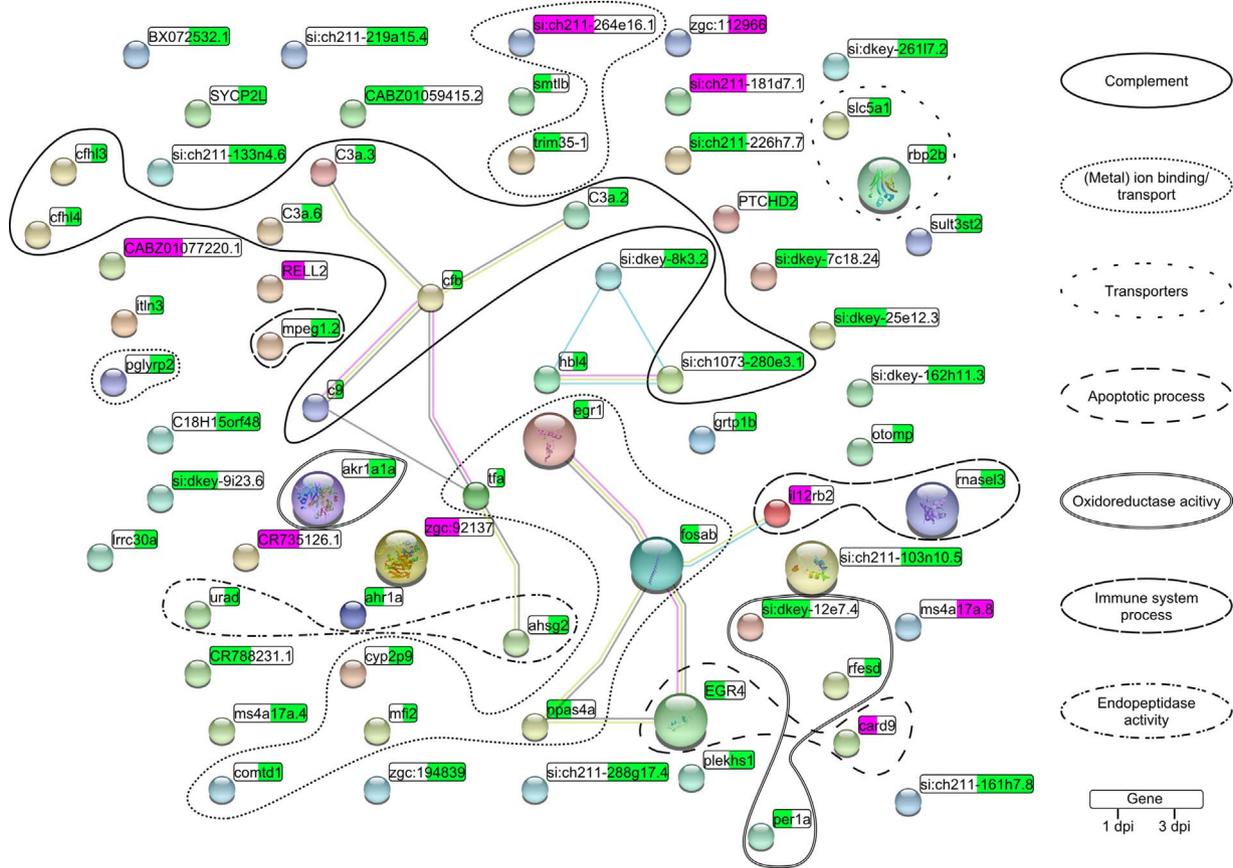
Fig. 5. Gene expression in zebrafish embryos and larvae after fluorescently labelled polystyrene (PS) particle injection visualized using STRING. The straight colored lines between the genes represent evidence for related genes. The enclosed groups (black lines) represent different functional ontology groups. RNAseq data is shown after 1 and 3 days post injection with PS particles. The green boxes represent up-regulation (fc > = 2) and the magenta boxes down-regulation (fc < = −2) with a P-value < = 0.05. The white boxes did not meet the criteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
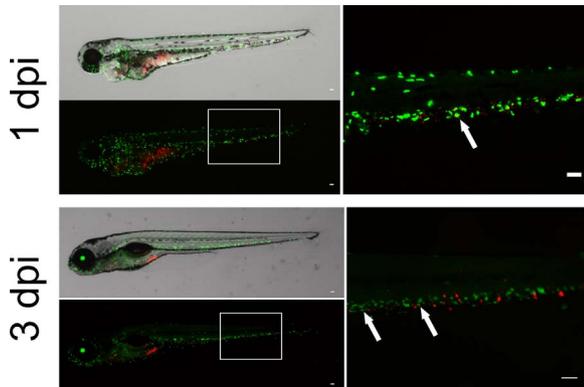

Fig. 6. Uptake of polystyrene particles by neutrophils. Overview of *Tg(mpx:EGFP)* zebrafish embryos injected at 2 dpf into the yolk with red fluorescently labelled polystyrene (PS) particles at 1 dpi and 3 dpi. These images represent the distributed PS particles away from the injection site (yolk) in the heart region and the tail area. The regions of the images on the right are indicated by the white box on the left. The overlap of the neutrophils (green) and PS particles (red) signal is indicated by the white arrows. Scale bars are 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Uptake of polystyrene particles by macrophages. Overview of *Tg(mpeg1:EGFP)*[g122] zebrafish embryos injected at 2 dpf into the yolk with fluorescently labelled polystyrene (PS) particles at 1 dpi and 3 dpi. These images represent the distributed PS particles away from the injection site (yolk) in the heart region and the tail area. The regions of the images on the right are indicated by the white box on the left. The overlap of the macrophages (green) and PS particles (red) signal is indicated by the white arrows. Scale bars are 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

impacts. Due to conservancy of certain effect pathways across species the pathways identified here can be of importance for different ecological levels. Within risk assessment, the identification of molecular key events (KE) and toxicological pathways are stepping stones to develop
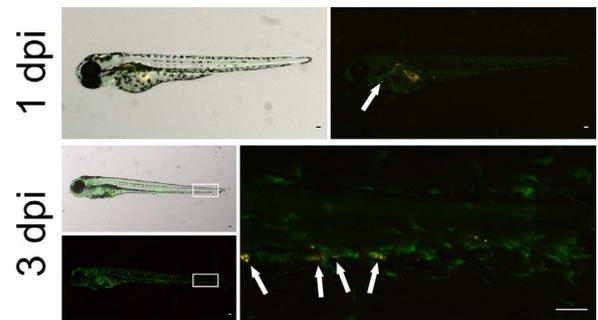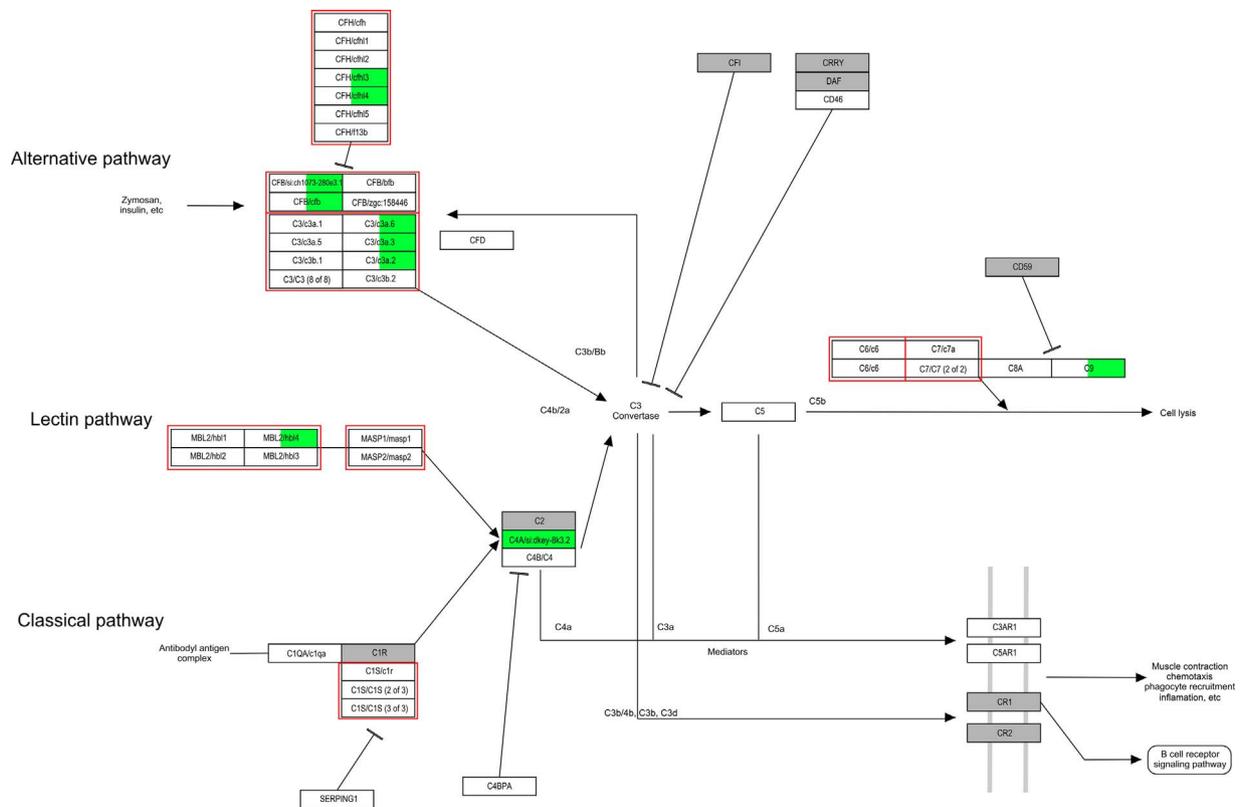
an Adverse Outcome Pathway (AOP) as initiated in 2012 under The Organization for Economic Co-operation and Development (OECD) guidelines. AOPs can be used for human and ecological risk assessment of drugs (Lee et al., 2015; Wink et al., 2014) or chemicals (Ankley et al., 2010; Ganguly et al., 2009).

Fig. 8. Complement component pathway. The human (*Homo sapiens*) complement and coagulase cascades pathway from Wikipathways has been modified to zebrafish orthologues. The red boxes around genes represent multiple orthologous between *Danio rerio* and *Homo sapiens*. The green boxes represent up-regulation (fc $> = 2$) and the magenta boxes down-regulation (fc $< = -2$) with a *P*-value $< = 0.05$. The white boxes did not meet the criteria, and grey boxes do not have a zebrafish orthologue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Biological pathway analysis with differentially expressed data using PathVisio 3. Positive (r): the number of genes on the pathway that pass the criterion. Measured (n): The number of genes on the pathway that were measured in the experiment. Total: The total number of genes on the pathway: The percentage of genes measured that passed the criterion (positive/measured). Z-score: The standard statistical test under the hypergeometric distribution. A high Z score indicates that the pathway has more genes that pass the criterion than would be expected by chance. Permuted p-value: A permutation test is performed to calculate permuted *p*-values. The data is permuted 1000 times and a rank is calculated of the actual Z score compared to the permuted Z Scores. Pathways with a permuted *p*-value $< = 0.05$ are significant. (Criterion: fc $> = 2$ or $< = -2$ and *P* value $< = 0.05$).

	Pathway	Positive (r)	Measured (n)	Total	%	Z Score	P-value (permuted)
1 dpi	Nuclear receptors in lipid metabolism and toxicity	4	12	26	33.33%	7.66	0
	Striated Muscle Contraction	4	21	30	19.05%	5.51	0.001
	ACE Inhibitor Pathway	2	6	12	33.33%	5.41	0.001
	Osteoclast	1	5	12	20.00%	2.83	0.019
	Regulation of Actin Cytoskeleton	5	84	113	5.95%	2.57	0.009

	Pathway	Positive (r)	Measured (n)	Total	%	Z Score	P-value (permuted)
3 dpi	ACE Inhibitor Pathway	2	6	12	33.33%	6.79	0
	Nuclear receptors in lipid metabolism and toxicity	2	12	26	16.67%	4.6	0.001
	Peptide GPCRs	3	34	41	8.82%	3.8	0.002
	Glycolysis and Gluconeogenesis	2	25	38	8.00%	2.89	0.015
	GPCRs, Class C Metabotropic glutamate, pheromone	1	8	11	12.50%	2.73	0.038
	Oxidative Stress	1	14	23	7.14%	1.88	0.028

4. Conclusions

Our results provide insights in the biodistribution and response pathways of zebrafish embryos and larvae exposed internally to PS particles. Although biodistribution of PS particles was relatively low, the transcriptome analysis showed systemic immune responses of zebrafish when exposed to PS particle. The transcriptomic data demonstrated that exposure to PS particles can result in activation of the complement system which indicates that PS particles are integrated in immunological recognition process. At all sampling time points, we found the nuclear receptors in lipid metabolism and toxicity pathway

significantly enriched, and the oxidative stress pathway only at 3 dpi significantly expressed. This data on key events (KEs) can be used to further investigate AOPs for microplastics, a group of relatively un-explored compounds. Such microplastics are widely distributed in aquatic environments, and therefore our results are highly relevant to environmental and human risk assessment of plastic particles.

Consent for publication

Not applicable.

Availability of data and material

All RNAseq data is submitted under GEO database accession number: GSE77755.

Reviewer access link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=yzineiuezjijfun&acc=GSE77755.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WJV designed and performed experiments, and analyzed the data. HPS and MGV conceived and supervised the study. WJV wrote the first version of the manuscript. All authors assisted with writing and editing of the manuscript and approved the final version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.06.014.

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