Toxicity of Nanoplastics for zebrafish embryos, what we know and where to go next

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ABSTRACT

Nanoplastics (NP) are an emerging threat to human health and there is a need to understand their toxicity. Zebrafish (ZF) is extensively used as a toxicology model due to its power to com-bine genetic, cellular, and whole organism endpoints. The present review integrates results regarding polystyrene NP effects on ZF embryo development. Study design was evaluated against NP effects. NP size, concentration, and exposure time did not affect organism responses (mortality, development, heart rate, locomotion) or cellular responses (gene expression, enzymes, metabolites). However, NP accumulation depended on size. Smaller NP can reach internal organs (brain, eyes, liver, pancreas, heart) but larger (>200 nm) accumulate mainly in gut, gills and skin. Locomotion and heart rate were commonly affected with hypoactivity and bradycardia being more prevalent. Effects on genetic/enzymatic/metabolic pathways were thoroughly analyzed. Immunity genes were generally upregulated whereas oxidative stress response genes varied. Central nervous system genes and visual related genes were generally downregulated. Results of genetic and enzymatic analyses coincided only for some genes/enzyme pairs. Reviewed studies provide a basis for understanding NP toxicity but results are hard to integrate. We propose key recommendations and future directions with regard to experimental design that may allow greater comparability across future studies.

Keywords: Toxicology; plastic; nanoparticles; polystyrene; Danio rerio

1. Introduction

Humans have been and are increasingly dependent on plastic products. In 2019 global plastic production reached 370 million tones (Plastics Europe, 2020) and in the wake of the Covid-19 pandemic this number is expected to rise due to increase use of disposable plastic, gloves and masks (Aragaw, 2020; Prata et al., 2020). By the end of 2018, overall plastic waste generated by humans reached 6900 MT (Letcher, 2020). This plastic reaches oceans, soils, landfills and even the atmosphere worldwide because only about 10% of it is recycled (Rhodes, 2018; Zhang et al., 2020b). The main categories of plastic are (in order of amount of waste produced) polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephtalate (PET), and polyurethane (PUR) (Letcher, 2020). Once it reaches natural landscapes, plastic is subject to mechanical and chemical degradation processes that result in a gradual decrease in size until micro or nanoparticles are created (Andrady, 2017). Microplastics (MP) are usually considered to be < 5 mm in size whereas the size of nanoplastics (NP) has not yet achieved a consensus with some considering it to be < 1000 nm and others < 100 nm (Gigault et al., 2018). Although MP have been detected in seafood, beer, and drinking water, among others (Devriese et al., 2015; Liebezeit and Liebezeit, 2014; Mason et al., 2018), detection of NP in food/water consumed by humans is more difficult and poses a technical challenge due to their nano-range sizes, different chemical characteristics, and variety of plastic types. Only a handful of studies have confirmed the presence of NP in seawater or snow (Davranche et al., 2020; Materić et al., 2020) and none so far in drinking water or food, although recently PS and PET nanoparticles were recovered from atmospheric samples (Xu et al., 2020).

In recent years MP and NP have been observed to be cause of toxic effects in aquatic (Chae and An, 2017; Wang et al., 2019) and terrestrial ecosystems (de Souza Machado et

al., 2018), with consequences ranging from disruption in metabolism, development, fertility, and mortality of both producers (i.e. algae, plants) and animal consumers. Humans can be exposed to NP through consumption of contaminated freshwater, marine, and terrestrial organisms, and also through drinking water, inhalation and dermal contact (Chang et al., 2020). Human health effects have also been recently reported (Lehner et al., 2019) and include accumulation in lungs, gastrointestinal and skin tissues, and cellular effects such as inflammatory responses and oxidative stress. In this context, there is an increasing demand for studies that evaluate toxic effects of NP on model organisms. These studies could elucidate not only genetic/molecular/metabolic responses but also changes in development, behavior and reproduction that could increase our knowledge of potential human toxicity mechanisms.

Zebrafish (*Danio rerio*) have been used for many years to study toxicological effects of chemicals and drugs (Gibert et al., 2013; Horzmann and Freeman, 2018) due to their *ex vivo* development which allows for easy xenobiotic exposure, and their high (~ 70%) genetic homology to humans (Howe et al., 2013). Zebrafish (ZF) embryos are translucent, have a short development time and all major body systems are formed by 72 h postfertilization (hpf) (Kimmel et al., 1995). In addition, they can be used to study pathways of toxicity from molecular initiating events to changes in whole organismal behavior and reproduction, and are currently part of high throughput strategies such as the Tox21 Program (Truong et al., 2014; Volz et al., 2015). Moreover, standard guidelines exist to study short term toxicity, and developmental abnormalities (OECD, TG 210, 212 & 236 (OECD, 1998; 2013a; 2013b), and the use of ZF embryos (up to independent feeding at 120 hpf) is in line with the 3 Rs principle by reducing the use of vertebrates in biomedical research (European Parliament, 2010; Strähle et al., 2012).

Due to this versatility, recent studies evaluating NP toxicity have mainly used ZF as a model organism. These studies have increased significantly since 2017 and include testing in adults, larvae, and embryos (Bhagat et al., 2020b). Polystyrene nanoplastic (PSNP) is used as a proxy for general NP toxicity due to its importance as plastic waste and because it is the only plastic type that is commercially available in different sizes and fluorescent labelling. However, in spite of increasing literature, our understanding of PSNP toxicity mechanisms and effects in ZF embryos are not clear. The main difficulties are related to the variation in size and concentration of PSNP used in these investigations, and also to the variety of endpoints studied. Thus, there is a need to review these results to better understand NP effects and possible toxicity pathways.

Therefore, the main objective of the present study is to perform a systematic review of available scientific literature in order to assess the current knowledge about organism (mortality, development, heart rate, locomotion) effects of < 1000 nm virgin PSNP (fluorescently dyed or not) on zebrafish embryo as a model. Our secondary objective was to integrate results on genetic/enzymatic/metabolic pathways affected by PSNP. In addition, the difficulties encountered due to varied study designs lead us to propose a series of recommendations to allow for greater comparability across future studies. Our conclusions could be incorporated into a global knowledge of adverse outcome pathway(s) for NP in ZF that can help understand their potential human toxicity.

2. Methods

2.1 Data collection

A systematic literature review was conducted in Google Scholar (https://scholar.google.com), Pubmed (https://pubmed.ncbi.nlm.nih.gov), Science Direct (https://www.sciencedirect.com), and Web of Science

(https://www.webofknowledge.com), using the terms “nanoplastic OR microplastic OR nanoparticle OR microparticle OR micro OR nano” AND “zebrafish OR zebra fish OR Danio rerio”. In addition, references of selected articles were also studied for possible hits. We found only 20 references that met the following requirements: 1) Studied toxic effects of polystyrene nanoparticles < 1000 nm in zebrafish embryos; 2) were written in English. We examined not only the articles but also their supplementary information for NP effects/no effects. Only two additional references (Ji et al., 2020; Walpitagama et al., 2019) were found that used other types of nanoplastics and these were kept out of the present review. It is important to note that in order to integrate results more accurately, the present analysis does not include works studying surface-modified NP, nor the synergistic/antagonistic effects of NP when combined with other contaminants such as metals or organic compounds. Articles available up to March 2021 were included.

2.2 Data analyses

The information analyzed include NP size (nm), shape and charge; exposure route and medium refreshment frequency; NP concentration (all units transformed to µg/ml); total exposure time (number of hours embryos were exposed); NP characterization and observation methods; endpoints with no toxic effects observed; and endpoints with observed effects. In addition study design aspects such as statistical analyses used, number of replicates, NP supplier and solvents were checked and the results can be found in the supplementary material. For NP size, if an article cited a catalog number for the PSNP, we have used the nominal size described by the supplier. If no catalog number is given, we have used the size given by the authors after their characterization. Please note that these two sometimes do not match (Pitt et al., 2018a) and in these cases, we have used supplier specifications. When within the same study different NP sizes/concentrations were analyzed, we considered data as separate “studies”.

Eight main endpoints were identified: mortality (all causes), hatching, development (malformations, total length and tail length), locomotion (hypoactivity and hyperactivity demonstrated by changes in velocity, distance moved and/or rotation angle), heart rate, accumulation (observation of NP particles in/around chorion and in ZF body by different techniques), genetic (individual genes/transcripts examined by PCR and transcriptome analysis), and enzyme/metabolic (individual enzymes/metabolites and metabolome analysis).

Potential general PSNP effects were evaluated by calculating the correlation of particle size, concentration, and exposure time with % positive effects per study. Percent positive effects was calculated numbering total endpoints studied and calculating the proportion of endpoints with positive effects. Mortality, hatching, development, heart rate and locomotion were counted each as one endpoint. Each gene transcript and/or each enzyme or metabolite studied on its own was counted as one endpoint, whereas if whole transcriptome and/or metabolome was studied, effects were counted as one endpoint.

In addition, the different observed endpoints have been analyzed for positive/negative effects taking concentration and NP size into consideration. We counted the number of studies in which each one was analyzed and calculated percent of studies in which endpoints were tested/not tested and observed/not observed. Effects on locomotion and cardiac toxicity have been studied separately to analyze hyperactivity/hypoactivity or tachycardia/bradycardia depending on NP size and concentration. NP accumulation was studied separately by analyzing organ localization depending on NP size and concentration, and by calculating percent of studies in which NP build-up was observed in each of the 14 organs cited by references studied.

2.3 Gene and enzyme/metabolic analyses

Genetic and enzyme/metabolite endpoints were analyzed by scanning and integrating results of articles considering similar gene transcripts, enzymes or metabolites. In our analysis, only gene transcripts and enzymes or metabolites that were analyzed individually and quantitatively were included. Affected genes enzymes or metabolites that were found by whole transcriptome/metabolome analyses were not included because they were not validated, i.e. qRT-PCR, protein expression levels, etc. The gene list was inputted into the Reactome analysis tool (https://reactome.org) to find pathways in which these genes are involved. On the other hand, metabolites were analyzed by inputting them into the String analysis tool (https://string-db.org). These analyses have been summarized in table format with counts of number of articles observing upregulation/downregulation or no effect of each gene transcript/metabolite. Percent alterations depicted in color codes were obtained by applying the following formula:

$$nU*(\%nU) - nD*(\%nD)$$

where nU/nD: number of articles in which gene/enzyme/metabolite was observed to be upregulated/downregulated; %nU/%nD: percent of articles with upregulation/downregulation with respect to total number of articles that studied this gene/enzyme/metabolite. This formula ensures more weight is given if more articles showed up/downregulation.

3. Results

A total of 20 articles dating from 2017 - 2021 were found that studied the effects of polystyrene nanoplastics < 1000 nm on zebrafish embryos. These articles have been subdivided into 50 studies (one study = one concentration/size combination; Table 1). Table 2 summarizes the principal aspects of these references and main effects found and the supplementary information includes a version with more detailed information on

study design (Table A.1). Articles in Table 2 have been ordered first by type of exposure, waterborne or injection, and second by size of particles used, from smaller to larger. Most authors exposed embryos through the medium (embryo water) but two (Brun et al., 2018; Zhang et al., 2020a) studied injection exposure as well. Therefore, their results have been considered as two separate works in the analyses. In addition, two articles studied only injection exposure (Sökmen et al., 2020; Veneman et al., 2017) and one studied maternal exposure (Pitt et al., 2018b). In general, studies varied greatly in their design and results observed, however some patterns were identified.

3.1 Study design

Polystyrene nanoparticles used by all studies were commercially available through different laboratory suppliers but the most common were ThermoFisher Scientific Co. (US) and Bangs Laboratories Inc. (US) (Table A.1). Most NP were negatively charged (Parenti et al., 2019) and all were spherical but size was quite varied, with particles ranging from 20 – 700 nm, although the preferred size was between 40 and 50 nm (Table 2 and Fig. 1a). A confusing aspect of NP size was found in many articles because a common commercial supplier cites different sizes for the same particle. For example, their NP PS02002 and FSDG001 have nominal diameters of 50 nm but mean diameters of 40 nm. Since studies cite either one or both in their work, and to avoid confusion, we have counted these as having 40 nm in size.

In the majority of cases fluorescently dyed NP were used, with red and green fluorophores being equally common (Table 2). Regarding their composition, NP stock solutions generally contained 0.1% of some type of surfactant and 0.05 – 0.09% of sodium azide as a preservative. NP working solutions were achieved by diluting stock solutions in different types of embryo culture water (Table A.1). The presence of additives was not always mentioned and they were usually not removed or controlled for. NP were

191 characterized by 90% of studies (Fig. 1c), mainly by DLS (dynamic light scattering,
192 measuring NP size, and charge: z potential), and followed by electron microscopy for
193 confirmation of size, size range, shape and agglomeration patterns. Usually size coincided
194 with vendor specifications and particles showed low agglomeration (Table A.1). Three
195 articles (Trevisan et al., 2020; Trevisan et al., 2019; Zhao et al., 2020) used vibrational
196 spectroscopy (FTIR or RAMAN) to confirm polystyrene composition of NP. No study
197 measured real exposure concentration in medium and only one (Chen et al., 2017)
198 measured PSNP percent recovery from larvae after exposure and found it to be between
199 75 – 120%.

200 Concentrations of PSNP used in waterborne assays varied by 5 orders of magnitude
201 (0.001 – 200 µg/ml) but the most frequent were 1 and 10 µg/ml (Table 2 and Fig. 1b).
202 About half the studies used only one concentration and the other half compared effects
203 between 2 – 4 concentrations. On the other hand, injection studies varied between 0.01 to
204 5 ng PSNP injected per embryo (Table 1). The studies also differed in exposure duration,
205 ranging from 12 – 162 hours (Table 1, Fig. 1d), and experiment starting time point (Table
206 2). However, around half the studies defined exposure windows between a few hours post
207 fertilization to 96 – 120 hpf (Fig. 1d) but they depended on endpoints studied (Table 2).
208 In waterborne experiments lasting for more than 24 h, medium renewal (MR) was
209 prevalent in 60% of the cases and it was usually done every 24 h although one study did
210 it every 48 h (Table 2).

211 3.2 *Study results*

212 There was a high variability in number of endpoints analyzed by different studies,
213 ranging from 2 (Lee et al. 2019) up to 18 (Brun et al. 2018) (Table A.2). Percent of
214 significant effects varied from 0 – 100%. Surprisingly, no correlation with particle size
215 or exposure time was found (Fig. 2a,b). A significant correlation with concentration was

only found if the highest exposure concentration (200 µg/ml) was included in the analysis (Fig. 2c). Since this concentration is unlikely to be found in nature (Gallego-Urrea et al., 2010; Lenz et al., 2016), a more realistic analysis was performed and found no correlation for the 0.001 – 20 µg/ml range (Fig. 2d).

This was confirmed when looking at individual studies that exposed embryos to different size NP (Pedersen et al., 2020; Van Pomeren et al., 2017; Zhang and Goss, 2020) and found little difference or contradictory results in the majority of endpoints (Table 2). For example, Zhang and Goss (2020) tested LC_{50} with two different sizes and found a lower LC_{50} for smaller NP (25 nm) than larger NP (100 nm). However, most endpoints were unaffected with both types of NP sizes. In addition, when studying gene expression, Pedersen et al. (Pedersen et al., 2020) found larger NP (200 nm NP) affected gene expression in much greater numbers than smaller NP (50 nm). Studies using one size NP, but more than one concentration, in the range of 0.001 to 10 µg/ml for different assays (Hu et al., 2020; Liu et al., 2021a; Pitt et al., 2018a; Zhang et al., 2020a), generally showed no difference in their results, except for accumulation/fluorescence which was increased with more concentration (Table 2). Nevertheless, when using a relatively high concentration of 200 µg/ml, Zhao et al. (2020) observed decrease hatching and body length (but no heart rate effects), compared with lower concentrations. No study tested the effect of exposure time as a variable for the same endpoints.

In general, the most common endpoints studied were mortality, development, NP accumulation, and hatching, followed by metabolites and gene expression. Heart rate and locomotion were the least studied (Fig. 3, Table A.3, Fig. A.1).

3.2.1 Mortality, hatching and development

Mortality and hatching were generally not affected by PSNP when ZF embryos were exposed to 0.0001 – 100 µg/ml. Mortality was the most frequent analyzed endpoint (82% of studies) but only one study (Sökmen et al., 2020) found that NP decreased embryo survival by 17% through the injection of 0.81 ng of 25 nm NP (Fig. 3a and Table 2). Only two studies (Brun et al., 2019; Zhang and Goss, 2020) calculated NP LC_{50} and found 50% ZF mortality at 75 µg/ml (25 nm NP) and 52 µg/ml (20 nm NP). However, Lee et al.(2009) found no effect on ZF survival at 100 µg/ml of 50 nm NP, and Duan et al. (2020) found no increase in mortality in ZF exposed to 100 nm NP at 200 µg/ml. No study calculated lowest observed adverse effect level (LOAEL) or no observed adverse effect level (NOAEL). When analyzed, hatching was found to be affected in only three of 32 studies (Fig. 3b, Table A.3). Two of them had a high 200 µg/ml waterborne concentration of 100 nm NP (Zhao et al. 2020 and Duan et al., 2020), and another with 0.81 ng of 25 nm NP injected (Sökmen et al., 2020). However, hatching was not affected with 2.6 ng of 42 nm NP injected per embryo (Zhang et al., 2020a).

On the other hand, ZF embryos exposed to PSNP sometimes exhibited increased rates of malformations. Development was studied in 78% of studies and 15.4% observed effects although without relation to concentration or particle size (Fig. 3c, Fig. A1, Table A.3). Of these, four were waterborne experiments (Brun et al., 2019; Chen et al., 2017; Zhang et al., 2020a), one an injection study (Sökmen et al., 2020) and one a transgenerational study (Pitt et al., 2018b) (Fig. 3c, Table A.3, Table 2). Brun et al. 2019 (20 µg/ml; 25 nm) and Pitt et al. 2018b (maternal transfer) found a decrease in swim bladder inflation rates with ZF exposure to NP although no difference was observed in bladder length/area in the first one (Table 2). Zhang et al. 2020a (0.5 µg/ml; 40 nm), and Chen et al. 2017 (1 µg/ml; 50 nm), observed higher rates of bent tail and decrease in body length respectively, but no difference in other abnormalities were found. In addition, an

injection study of 25 nm NP (0.81 ng/embryo) (Sökmen et al., 2020) showed the highest malformation rates, with problems in body axis, tail, yolk sac and pericardium. However, other studies with similar NP size/concentrations/exposure times did not observe any developmental effects (Fig. 3c, Table A.3).

3.2.2 Locomotion and heart rate

PSNP had deleterious effects on locomotion and heart rate although not always in the same manner (Figs. 3d,e and Fig. 4). ZF locomotion (distance travelled, velocity, rotation) and heart rate were studied in 46 and 34% of studies respectively and effects of NP exposure on both endpoints were similarly prevalent as ~ 50% of articles studying them found effects (Fig. 3d,e, Table A.3, Fig. A.1). Locomotion was studied with alternating light and dark periods and in general, affection occurred in the latter. In some studies, exposed larvae exhibited hyperactivity (13%) but in the majority (39%) locomotion was decreased (Fig. 4b, Table A.4, Fig. A.2). No clear trend in terms of NP size, concentration and/or exposure time was observed between works that observed both types of affected activity (Fig. 3d, Fig. 4b). Studies that observed ZF heart rate alterations observed tachycardia (6%) and bradycardia (41%) (Fig. 4a, Table A.4, Fig. A.2). In addition one study (Duan et al., 2020) detected both effects depending on exposure time (increased heart rate at 24 h and decreased at 48 h; Table 2). Moreover, 47% of studies did not observe any alterations with NP exposure and no relationship with NP size, concentration and/or exposure time was observed between articles that studied this endpoint (Fig. 3e and Table A.4).

3.2.3 Accumulation of NP

Generally, ZF embryos studied for NP accumulation exhibited a buildup of these particles in their bodies dependent on NP concentration. In addition, organ localization

sometimes depended on size. Fig. 3f shows that many studies (72%, Table A.3) considered PSNP accumulation and most of them (34 of 36) observed this effect, both in water exposed and injected embryos. There were three observation techniques used by researchers and the most common one was using fluorescently labeled NP and observing embryos under an optic microscope (75%), followed by electron microscopy of embryo sections (15%) and confocal microscopy (5%) (Table A.5). In most cases, whole embryo/larvae mounts were observed and all internal body parts were visible. The organs with more frequent NP accumulation were the gut (78%), yolk (67%) and brain (47%) whereas the least frequent were muscle and fascicle (3% each) (Fig. 5, Fig. A.3). There was no clear relation between NP size/exposure time and accumulation in specific organs when ZF were waterborne exposed to NP particles between 20 - 200 nm (Fig. 5, Table 2). However, articles that studied ZF exposed to larger size NP (> 200 nm) observed greater accumulation in gut, gills and skin than in embryo internal organs (Parenti et al., 2019; Van Pomeren et al., 2017) (Fig. 5). Injection/maternal exposure studies showed similar results for smaller particles but Veneman et al. (2017) observed 700 nm NP in gut, heart and blood vessels. In addition, Lee et al. (2019), using both fluorescence and electron microscopy, detected NP size differences in accumulation with fluorescent intensity being higher with 50 and 200 nm than 500 nm particles. In addition, they observed 50 nm NP in subcellular organelles such as mitochondria (Table 2).

In general, waterborne studies that considered accumulation with more than one concentration, observed a direct relationship between particles accumulated (i.e. fluorescence intensity) and concentration, in addition to greater number of organs affected (Hu et al., 2020; Pedersen et al., 2020; Pitt et al., 2018a; Fig. 5). However, the number of organs affected varied in different studies possibly due to differences in manipulation and/or technical equipment. In addition, within each study the maximum number of

affected organs was achieved between 0.5-1 μg/ml. In the case of the only injection study that used many concentrations (Zhang et al. 2020a), no differences were observed but their concentrations were in the same order of magnitude *vs.* waterborne studies that used up to 4 orders of magnitude (Table 1). Moreover, if particles were observed in the embryo's gut, there was a greater possibility of encountering them in adjacent organs such as liver, gall bladder, and pancreas (Fig. 5). On the other hand, when NP were observed in the brain, they were always observed in the yolk and/or gut as well (but not vice versa), whereas there was only a 50% chance of encountering them also in the eyes (Fig. 5).

3.2.4 Genetic, Enzyme and Metabolite changes

Genetic and enzymatic/metabolic changes due to NP exposure were considered in 42 and 34% of studies respectively and most encountered effects, irrespective of NP size/concentration (Fig. 3g, h; Table A.3, Fig. A.1). In order to unify putative molecular NP targets observed in different studies, we analyzed all specifically validated genes/enzymes/metabolites (see Methods section) and some interesting patterns were identified. We found 37 gene transcripts that were studied individually by authors, some of them only once and others in different research articles (Fig.6; information about gene names/functions can be found in Table A.6). In general, immunity and stress response related genes were the most studied. In the first category, the majority of genes (i.e. cytokines ccl20a, ifn-γ, il1-β) were observed to be upregulated. On the other hand, some oxidative stress response genes (i.e. gstp, gpx) were observed to be upregulated whereas others (i.e. gr, sod2) were downregulated in most studies. Central nervous system (CNS) genes (i.e. slc6a4, syn2-α, mbp) and visual related genes (i.e. opsins opn1mw1, opn1lw2) were downregulated in general. In addition, carbohydrate metabolism was also affected with some genes being upregulated (pck1, g6pca) and some downregulated (ldha, fgf21). Furthermore, three works have studied whole embryo transcriptome (Hu et al., 2020; Liu

et al., 2021a; Pedersen et al., 2020). Interestingly, Liu et al. (2021) found upregulation of genes related to antioxidant enzymes and affected genes in CNS and retinal development. Pedersen et al. (2020) also found downregulation of neural development genes and differentially expressed genes (some up and some downregulated) in carbohydrate metabolism. On the other hand, Hu et al. (2020) found the MAPK signaling pathway to be the most affected but they also observed differential expression of carbohydrate metabolism genes.

We found 16 articles that considered enzyme and metabolite responses to NP exposure, and most markers studied were responses to oxidative stress (Fig. 7; information about names/functions can be found in Table A.7). ROS were found to increase as a result of NP exposure in three studies whereas they were observed to decrease or not be affected in two other studies. On the other hand, some oxidative stress response enzymes/metabolites (i.e. CAT, GPx, GST, EROD, P-gp) were observed to be unaffected whereas others (i.e. GR, COX, GSH, Thiol) were downregulated. Interestingly, some authors studied oxidative stress enzymes whose gene transcripts had also been studied by other works. In some cases, results of both types of analyses coincided (no effect of NP on catalase and glutathione-s-transferase activities, downregulation of glutathione reductase) but in others results were contradictory (superoxide dismutase and glutathione peroxidase). CNS function enzymes/metabolites were rarely studied but one article found acetylcholinesterase (AchE) to be upregulated. No articles studied NP effects on neurotransmitters. Energy metabolism-related NADH was found to be downregulated by one study (Trevisan et al., 2019) (44 nm NP, 10 μg/ml) and upregulated in another (Trevisan et al., 2020) (44 nm NP, 1 μg/ml). In addition, mitochondrial ATP-linked oxygen consumption rate decreased only in the first one. Only Duan et al. (2020) studied effects of NP on metabolome, and found that metabolism of

essential unsaturated fatty acids (i.e. linoleic acid), and amino acids (i.e. alanine, aspartate, and glutamate) were the most affected.

4. Discussion

The study of nanoplastics toxicology is an emerging public health challenge. In this regard, the zebrafish model is a very promising *in vivo* tool as molecular and metabolic endpoints can be combined with whole organism effects such as development, heart rate, and behavior (Dooley and Zon, 2000). In addition, ZF have a great genetic homology to humans and thus results obtained from this model can be easily extrapolated (Howe et al., 2013). In the last 3-4 years, research examining toxic effects of NP in ZF embryos has increased significantly (Bhagat et al., 2020b). However, this field is just beginning and therefore it is important to establish common study parameters that help future investigators plan their experiments, harmonize criteria, and apply controls for possible confounding factors. In addition, up to date results need to be integrated in order to move forward in the direction of possible NP pathways of toxicity. The present review aims at achieving these goals by presenting results regarding the most relevant aspects of study design. In addition, we have analyzed thoroughly all the possible PSNP toxic effects, taking into consideration not only whole organism responses, but also genes, enzymes and metabolites that are differentially affected.

4.1 Study design considerations

The study of NP toxic effects in zebrafish embryos and other aquatic (Kögel et al., 2020; Shen et al., 2019) and terrestrial organisms (Lian et al., 2020), as well as in mammalian and cell models (Yong et al., 2020), has been achieved mainly by using commercially available polystyrene NP. Even though spherical engineered NP are not the same as irregularly shaped NP produced naturally in the environment (Li et al., 2020),

387 they provide a good model in which properties such as size and charge are controlled.
388 However, these commercial NP often contain sodium azide as a preservative and
389 surfactants (i.e. SDS) which can add toxicity (Heinlaan et al., 2020; Zhang and Goss,
390 2020) and are rarely controlled for. In addition, commercial PSNP are sometimes
391 bounded with fluorophore molecules for their detection in accumulation studies but
392 frequently these fluorescent NP are also used in toxicological analyses (Table 2). The
393 toxicity of fluorophores was not considered in any of the articles we found but other
394 studies have confirmed their leakage (Catarino et al., 2019) and toxicity (Alford et al.,
395 2009), although not in ZF nor for the exact dyes. These important confounding factors
396 should be controlled for.

397 NP characterization is important but was not always considered (Fig. 1c, Table A.1).
398 NP size confirmation by DLS and, if possible, also by electron microscopy is relevant in
399 order to confirm distributors' shape, size, and analyze aggregation patterns in embryo
400 medium used. Charge has been observed to affect toxicity, with cationic NP generally
401 having greater deleterial effects than anionic NP (Bhattacharjee et al., 2014; Jeon et al.,
402 2018; Ruenraroengsak and Tetley, 2015; Xia et al., 2008). Therefore, NP charge
403 characterization (i.e. z potential) is relevant, especially if non-commercial and
404 uncharacterized NP toxicity is tested. Spectroscopic techniques such as FTIR or RAMAN
405 have been used by some studies in order to confirm chemical characteristics of NP (Fig.
406 1c). These techniques, and others such as chromatography and mass spectrometry, will
407 become more relevant when testing toxicity of naturally occurring NP for which identity
408 and chemical composition need to be characterized (Jiménez-Lamana et al., 2020; Li et
409 al., 2020).

410 Most reviewed articles used waterborne concentrations ranging from 0.01 to 10
411 µg/ml and different size NP (most ~ 40 - 50 nm), but in the majority of cases, these sizes

412 and concentrations caused similar effects except for accumulation (Table 2, Fig. 3, Fig.
413 5). However, sometimes concentrations > 50 µg/ml caused mortality and higher rates of
414 sublethal effects (Brun et al., 2019; Duan et al., 2020; Zhang and Goss, 2020). Based on
415 the classical toxicology principle that the dose makes the poison (Frank and Ottoboni,
416 2011), measurement of environmental NP concentrations is key. Unfortunately, due to
417 technical difficulties, there is a lack of information on real-world NP concentrations, size
418 ranges, and composition (Gaylarde et al., 2020). Some studies (Liu et al., 2020; Qu et al.,
419 2019) testing NP toxicity in model organisms use predicted NP concentrations ranging
420 from 0.0001 – 0.001 µg/ml based on Lenz et al. (2016). However, we believe this is not
421 realistic since this estimation was calculated by extrapolation, using data on microplastic
422 concentrations, whereas NP concentration has been estimated to be up to 10^{14} higher
423 (Besseling et al., 2019). We found only one study (Gallego-Urrea et al., 2010) that
424 measured NP particles of 100 – 250 nm, in Scandinavian waters, counting a maximum of
425 10^{9} particles/ml. This would account for approximately 0.55 – 8.6 µg/ml, using
426 conversion factors of 1.8×10^{15} and 1.2×10^{14} beads/g for commercially available 100
427 nm and 250 nm PSNP respectively (Bangs Laboratories I. Ecuations Technote 206,
428 Indiana, US, 2018). These inferred values could be used (with caution) as reference for
429 more realistic exposure concentrations. Unfortunately, no study measured actual PSNP
430 concentrations in embryo water. This issue is relevant because of possible interactions
431 with culture plates and/or exposure medium components. Therefore, it is important to
432 measure actual NP concentration during studies or, if this is not possible, all available
433 precautions should be taken to avoid a decrease in exposure concentration that would
434 reduce study reliability (Moermond et al., 2016). Thus, medium renewal every 24 h is
435 recommended, as performed by many of the reviewed articles (Table 2).

Number of replicates used and experiment repetition varied greatly (Table A.1). In some cases, low number of embryos tested and/or large standard deviations made potentially significant NP effects appear as non-statistically significant (Chen et al., 2017; Parenti et al., 2019). In addition, care should be taken when repeating experiments several times with different ZF embryo cohorts as this population effect has been found to be important (Pitt et al., 2018a). Taking this into consideration, future studies should make an effort to calculate *a priori* minimal number of replicates needed, bearing in mind standard deviation of studied variables, effect size desired, and type I and type II errors established (Sokal and Rohlf, 1995). Moreover, the linearity or non-linearity of variables (i.e. fluorescence intensity is non-linear) should be taken into consideration, transforming variables appropriately, and making this information available in published articles. Only some of the presently reviewed articles mentioned variable transformation and/or use of non-parametric tests to analyze non-normally distributed variables (Brun et al., 2018; Chen et al., 2017; Parenti et al., 2019; Pedersen et al., 2020; Pitt et al., 2018a; Pitt et al., 2018b; Trevisan et al., 2020; Trevisan et al., 2019; Zhang and Goss, 2020) (Table A.1). These are important issues to consider so potential NP effects are not obscured.

4.1 NP effects

It was surprising to observe that differences in NP size had little effect on toxicity endpoints. However, this conclusion should be considered within the scope of this review, which only covered PSNP up to 1000 nm, with most studies testing toxicity endpoints using NP from 20 – 100 nm (Fig. 2a). When larger MP are compared to NP, size has been shown to affect toxicity in some cases by changing the magnitude of effects and/or endpoints affected. Only Duan et al. (2020) and Zhao et al. (2020) compared MP and NP effects on embryo development. Duan et al. (2020) observed that 100 nm NP inhibited ZF embryo hatching three times more than 160 μm MP. In contrast, Zhao et al. (2020)

found similar effects of 65 nm NP and 20 μm MP on thyroid function. Studies in adult ZF show size dependent effects (Gu et al., 2020) or similar effects when comparing NP to MP. For example, Lu et al. (2016) found similar liver toxicity when comparing 70 nm NP to 2 μm MP but larger gene expression effects of 200 nm NP *vs.* 20 - 90 μm MP (Lu et al., 2018). On the other hand, it is clear from data reported in this review as well as data on in vitro experiments (Walczak et al., 2015), that accumulation patterns differ depending on NP size, with ~200 nm being the limit for overcoming the chorion barrier (Lee et al., 2019). Nevertheless, other important barriers, such as the blood brain barrier: 1 nm (Jain, 2012), and nuclear pores: ~100 nm (Knockenhauer and Schwartz, 2016) have to be considered as well.

Regarding NP concentration, there is evidence that in ZF embryos it is directly related to accumulation (Hu et al., 2020; Pedersen et al., 2020; Pitt et al., 2018a), as has also been observed for *in vitro* systems (Shin et al., 2020). Particles can enter the embryo/larvae through the skin, gut and gills but the intestinal tract seems to be the most frequent mode of entrance, in some cases preceded by or together with yolk accumulation (Fig. 5). Particles of smaller size (< 200 nm) reach sensitive areas such as the brain/eyes or even subcellular organelles such as mitochondria, whereas larger particles stay in the gut, gills and skin. NP that enter into the gut can either move into adjacent organs such as liver/pancreas or travel through the blood stream (Geiser et al., 2005) to the brain and in some cases also to the eyes (Fig. 5). However, mortality or development was generally not affected by this accumulation (Table 2), and 4 weeks were enough for NP total elimination (Zhang et al., 2020a). Different mechanisms of entrance into cells have been observed in *in vitro* studies including phagocytosis, pinocytosis, and/or passive transport, depending on NP size (Kik et al., 2020; Sendra et al., 2021). In order to understand accumulation patterns and organ distribution, all these mechanisms should be considered.

In addition, the possibility of active membrane NP transporters, especially for crossing the blood brain barrier (Grabrucker et al., 2016) should not be ruled out. Moreover, when studying natural NP other factors such as shape and charge should be taken into account as they also affect accumulation (Jeon et al., 2018).

NP accumulation usually causes deleterious effects in zebrafish embryos (Table 2). Even though mortality was not generally observed, high NP doses (~ 50 – 200 μg/ml) did cause decrease embryo survival (Brun et al., 2019; Duan et al., 2020; Zhang and Goss, 2020). These doses, however, will probably not be encountered in the environment, as predicted by the few studies that address this issue (Gallego-Urrea et al., 2010), but they are nevertheless relevant to understand. Developmental effects were observed in about 30% of studies (NP 25 – 50 nm) addressing this. The most common alterations were in swim bladder, body size and tail shape which would alter organism fitness in the environment. However, aside from studies in ZF embryos, very few studies have addressed early developmental exposure of NP in other vertebrates such as mammals (except for Luo et al., 2019) and therefore the mechanisms by which NP may act as teratogens are not clear.

Alterations in locomotive activity of ZF embryos are used to study potential neurological effects of toxicants (Orger and de Polavieja, 2017; Selderslaghs et al., 2010). Fig. 4b shows that in ZF embryos PSNP has the capacity of being neurotoxic, altering locomotion in the majority of cases. However, it was surprising to find both hyperactivity and hypoactivity with no clear correlation to NP size/concentration or other parameters. Possible explanations for hyperactivity are alterations in carbohydrate metabolism (glucose could correct this effect: Brun et al., 2019) and cortisol metabolism (Faught and Vijayan, 2021), but also dysregulation of neuromotor control pathways (Pedersen et al., 2020). Hypoactivity was the most common locomotion effect but there is no clear

explanation for it. Authors have proposed different reasons such as oxidative stress and reduced acetylcholinesterase activity (Chen et al., 2017; Hu et al., 2020), metabolic effects and brain NP accumulation (Pitt et al., 2018a), as well as transcriptomic changes in visual and neural functions (Liu et al., 2021a). Although not observed in all studies, there is enough evidence so far to propose an alteration in locomotion because of PSNP exposure in ZF embryos. Moreover, experiments with PSNP-exposed ZF adults have also detected changes in complex behaviors such as shoaling, aggressiveness, predator avoidance, and feeding time (Mattsson et al., 2017; Sarasamma et al., 2020). However, none of the reviewed embryo exposure studies continues to the adult stage and therefore examined only distance or velocity in light and dark periods at 96 – 120 hpf (Table 2). While this is a very good neurobehavioral screening tool, other aspects of behavior and motor reflexes could also be evaluated by studying embryo startle or phototaxis responses, or studying effects on complex behaviors enhanced by chronic exposure. This should also be combined with observation techniques of NP localization in different neuronal cell types, and genetic/metabolic endpoints related to neurological development and function that can relate to human neurotoxicity (d'Amora and Giordani, 2018; Parng et al., 2007). In addition, it is important to consider certain experimental parameters that can alter ZF locomotion response to toxic substances, such as larvae age, plate size, and acclimation time prior to observations (MacPhail et al., 2009; Ogungbemi et al., 2019; Padilla et al., 2011).

Heart rate alterations were observed in over half of articles studying it but no correlation was found with particle size/concentration or exposure time. Bradycardia was the most common heart rate problem but tachycardia was also observed (Fig. 4a). A possible explanation for heart rate problems is pericardium NP accumulation, leading to interaction of particles with sarcomeres (Pitt et al., 2018a). However, a decreased heart

rate was observed by some authors (Duan et al., 2020; Hu et al., 2020; Pitt et al., 2018b) even though no accumulation in the heart area was noted. Activation of inflammatory pathways and generation of ROS have been observed to cause heart problems related to environmental toxicants (Simkhovich et al., 2008). Moreover, ZF cardiotoxicity has been linked to human cardiac effects (Milan et al., 2003; Park et al., 2013). Therefore, despite limited evidence, potential NP cardiotoxic effects should be considered, especially after long term exposure. Observation techniques should be used to study possible NP localization in cardiac cells/blood vessels in combination with analysis of genetic/metabolic endpoints related to cardiotoxicity. In addition, careful control of exposure medium oxygen content and temperature, as well as anesthetic (i.e. tricaine) concentration is recommended as these factors have been shown to alter ZF embryo heart rhythm (Barrionuevo and Burggren, 1999; Craig et al., 2006; Duan et al., 2020).

The study of gene expression, protein translation/function, and metabolite alterations are used in toxicology to evaluate molecular initiating and key events that lead to toxicity pathways and whole organism effects (Krewski et al., 2020). We found three works studying whole embryo transcriptome and one the metabolome of embryos exposed to PSNP. However, no studies so far have dealt with proteome alterations in ZF or *in vitro* studies (but see Liu et al., 2021b for alterations in *Daphnia*). Although these high content analyses are a good first approximation to unravel toxicity mechanisms, the validation (confirmation that altered gene expression relates to altered protein function) of specific transcripts/proteins/metabolites of putative altered pathways is also necessary (Krewski et al., 2020) since results from different studies varied (Table 2). Interestingly, our analysis of specific altered gene transcripts/enzymes/metabolites has revealed some important coincidences in the reviewed studies (Figs. 6-7). Specifically, we observed that PSNP can alter genes related to immunity, oxidative stress response, carbohydrate

metabolism, CNS function, and visual system (Fig. 6). Remarkably, this corresponds generally with altered pathways observed in whole transcriptome analyses. However, the metabolome study did not coincide with this finding, as the essential fatty acid pathway was the most affected (Duan et al., 2020). Moreover, our examination has revealed that in some cases, gene transcripts results are similar to results of works that study the enzymes they encode for, especially in the oxidative stress pathway (Figs. 6-7). However, in other cases effects were contradictory or not studied. Therefore, it is important to validate genetic transcription alterations by studying protein translation/function because altered gene expression does not always translate into protein malfunction/disease (Liu et al., 2016). In addition, there is now ample evidence that mRNA translation is conditioned by the presence of miRNAs (Gebert and MacRae, 2019) and lncRNA (Zhang et al., 2019). The effect of NP on miRNA/lncRNA expression and regulation could be a promising area for future research.

Even though much work remains to be done, our integrated analysis of NP effects on ZF embryos, together with genetic analyses in adult ZF (Gu et al., 2020; Umamaheswari et al., 2020) and other *in vivo* and *in vitr*o systems (Jeong and Choi, 2019; Prüst et al., 2020; Rubio et al., 2020) reveal a possible PSNP toxicity pathway. This would start with particles entering the cell through different processes (some known, some unknown, see above), accumulating in lysosomes (Brandts et al., 2020) and causing oxidative stress and immune responses that generate inflammation (Figs. 6-7). Further intrusion of NP into organelles such as mitochondria is possibly the reason for observed alterations in energy/carbohydrate metabolism (Trevisan et al., 2020; Trevisan et al., 2019). The localization and accumulation of NP in different organs such as heart, brain, liver, etc. (Fig. 5) could be responsible for specific behavioral-neurological/cardiac/lipid-metabolism alterations and this organ specific accumulation would depend on NP size,

586 concentration, exposure time, and other unknown biokinetic mechanisms. This last
587 conclusion is based on results of NP accumulation in different organs independently of
588 other factors studied (Tables 1 and Fig. 5). The main NP exposure routes would be
589 maternal/waterborne for embryos (Table 2), gastric/gills for adults (Lu et al., 2018;
590 Skjolding et al., 2017), and the intestinal tract could also be affected (and its microbiome)
591 by NP (Gu et al., 2020). However, other routes such as inhalation (terrestrial organisms)
592 or dermal and their effects on lungs and skin should not be ruled out (Chang et al., 2020).
593 Other affected organs could be the thyroid (Zhao et al., 2020; Zuo et al., 2020) and gonads
594 (Pitt et al., 2018b; Sarasamma et al., 2020) and thus NP could behave as endocrine
595 disruptors as well. Although endocrine disruption pathways have not been observed to be
596 altered in transcriptome analyses in ZF, it has been observed in rats (Amereh et al., 2020;
597 Amereh et al., 2019). Genotoxic and developmental effects are also possible (Poma et al.,
598 2019; Sökmen et al., 2020) although works regarding this issue are scarce (Rubio et al.,
599 2020) and our analysis shows that data on ZF embryo development is contradictory.

600 Although not reviewed in this article, future studies should also consider toxicity of
601 other common plastic types (Ji et al., 2020; Karami et al., 2017), including bioplastics (de
602 Oliveira et al., 2020; Zimmermann et al., 2020), environmentally produced NP (Fadare
603 et al., 2020; Natarajan et al., 2020), and the interaction of NP with other toxics present in
604 the environment (Bhagat et al., 2020a). Other plastics such as PET or PVC have rarely
605 been studied up to date because of less commercial availability in different sizes, and
606 fluorescently labelled, as PSNP. However, laboratory made NP could provide with a
607 partial solution to this problem (Ji et al., 2020).

608 Our analysis has focused on PSNP toxic effects on ZF embryos as a model for
609 potential human toxicity. The revised works have studied different endpoints, dealing
610 with cellular responses (oxidation pathway or gene expression alterations), organ

responses (heart rate) and organism responses (lethality, development, locomotion). However, very little information exists on NP biokinetics such as uptake, biodistribution, bioaccumulation, and excretion mechanisms. Since accumulation in different organs seems to be important to elucidate NP toxicity, it is essential to consider questions such as how/how much/how fast NP enters the brain or other organs and how/how much/how fast it is eliminated. Of course, this will all depend on NP type, size, and concentration and in this regard, it is of the upmost importance to evaluate NP concentrations in different environmental compartments. A blind combination of tests will not lead us to elucidate mechanisms of NP toxicity. Studies need to be better designed and combine the use of *in vivo* tools such as the zebrafish model with *in vitro* studies using human cells and *in silico* pharmacokinetic modeling in order to predict relevant NP human blood or tissue concentrations that could trigger toxicity pathways.

5. Conclusions

This review has summarized information on the toxicological effects of round PSNP to developing ZF embryos as a model of potential health risks to humans. We have found important methodological issues that can potentially affect result validation and/or interpretation. Therefore, we have the following recommendations:

- Design experiment carefully to evaluate number of replicates needed for each variable studied and consider cohorts.
- Design experiments with an adverse outcome pathway perspective.
- Avoid or at least control for the use of NP additives and fluorophores in toxicity assays.
- NP size and charge should be characterized using DLS and electron microscopy if possible.

- Environmental relevant concentrations should be used. Until we know better, around 0.55 – 8.6 µg/ml or 109 particles/ml.
- Test actual NP concentration and/or refresh medium every 24 h to maintain stable NP concentration.
- To evaluate organ/cellular localization, study accumulation by both fluorescence and electron microscopy if possible.
- Locomotion studies should include startle/phototaxis responses and complex behaviors when possible.
- Carefully control for oxygen and temperature when studying heart rate.
- Transcriptome/proteome/metabolome studies should be validated.

Some of these recommendations are general for any experimental bioassay design, and in these type of studies, we consider of key importance since they will lead to maximize study homogeneity and make results of ZF studies and *in vitro* studies more comparable. Results up to date show NP pose a threat for different organs through mechanisms of oxidative stress that trigger the immune system and through specific organ effects such as neurotoxicity, cardiotoxicity and energy metabolism disorders. However, there are many gaps in our knowledge and many questions remain unanswered, especially regarding areas less studied such as mechanisms of accumulation in different organs, metabolism and excretion, and NP effects on genotoxicity, teratogenesis or endocrine disruption. Furthermore, additional research is needed to evaluate toxicity of other types/shapes of NP and environmental NP both alone or in combination with other pollutants. We are confident that the ZF model is a robust instrument that integrates both molecular and cellular effects with whole organism responses, but future studies should

also combine this tool with human-derived cell cultures to accurately predict human toxicity of nanoplastics.

CRediT authorship contribution statement

Monica Torres-Ruiz: conceptualization, methodology, literature search, data analysis, original draft preparation, writing, review and artwork. **Antonio De la Vieja**: data analysis, artwork, writing, review, editing and funding. **Mercedes de Alba Gonzalez**: conceptualization, literature search, review and editing. **Marta Esteban Lopez**: writing, review and editing. **Argelia Castaño Calvo**: conceptualization and funding. **Ana Cañas Portilla**: conceptualization, writing, review, editing and funding. All authors have read and agreed to the published version of the manuscript.

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. Supplementary data

The following are available online at xxx: Table A.1: Complementary information about reviewed articles; Table A.2: Number of endpoints studied by each article used to calculate % effects; Table A.3: Results of endpoints studied by each article; Table A.4: Results of Locomotion and Heart Rate effects; Table A.5: NP observation techniques in ZF embryos; Table A.6: Full names and functions of validated gene transcripts used for analyses; Table A.7: Full names and functions of validated enzymes/metabolites used for analyses.

Figure captions

Fig. 1. Article design characteristics. Number of articles in percentages was represented versus key characteristics: NP sizes used in nm (a); NP concentrations used in µg/ml (b); NP characterizing techniques (S: size, Z.P.: zeta potential, M: Microscopy, V.S.: vibrational spectroscopy) (c); Total exposure time in hours (d).

Fig. 2. Correlation between percent positive effects for waterborne studies and particle size in nm (a, note x-axis break), exposure time in hours (b), or concentration used in µg/ml (c and d). Each dot represents a study. R = correlation coefficient, p = correlation significance, n = number of studies.

Fig. 3. Endpoints and studies in which effects were observed and not observed related to concentration (y-axes) and NP size (x-axis). Right y-axis: waterborne studies concentrations (open symbols); left y-axis: injected concentrations (solid symbols). Circles: no effect was observed for this endpoint; squares: effect was observed for this endpoint. Mortality (a), hatching (b), development (c), locomotion (d), heart rate (e), accumulation (f), genetic effects (g), and metabolic effects (h).

705 **Fig. 4**. Heart Rate (a, note y-axis break) and locomotion activity (b, note x-axis break)
706 effects of NP exposed ZF embryos plotted by concentration (y-axis) and NP size (x-axis).
707 Open circle: no effect was found; triangle: tachycardia/hyperactivity; inverted triangle:
708 bradycardia/hypoactivity; diamond: both tachycardia and bradycardia observed. Only
709 waterborne studies plotted/showed effects.

710 **Fig. 5.** Organs where NP accumulate in studies ordered by NP size (nm) and sub-ordered
711 by concentration [c]. Y: yolk; G: gut; P: pancreas; GB: gall bladder; L: liver; B: brain; E:
712 eye; H: heart; V: blood vessel; M: muscle; F: fascia; Gi: gills; S: skin. Red squares:
713 internal organ accumulation; orange squares: external accumulation. Light blue squares:
714 no accumulation. References in blue font: studies that used particles > 200 nm. Number
715 of organs in which NP accumulate (n). The bottom table depicts injected (I) and maternal
716 (M) studies.

717 **Fig. 6**. Analysis of validated gene transcripts studied by reviewed articles. Gene names
718 are depicted as inputted for analyses (some are human homologues). Gene names in
719 parentheses are identical to names used by authors. Numbers depict studies in which
720 effect was observed; n: total number of studies where gene transcript was considered.
721 Red: upregulated (U); green: downregulated (D); white: no effect observed (NE) or equal
722 number of studies observing up and downregulation.

723 **Fig. 7**. Analysis of validated enzymes/metabolites studied. Numbers depict studies in
724 which effect was observed; n: total number of studies where enzyme/metabolite was
725 considered. Red: upregulated (U); green: downregulated (D); white: no effect observed
726 (NE) or equal number of studies observing up and downregulation

727

728

729 **References**

730 Alford R, Simpson HM, Duberman J, Hill GC, Ogawa M, Regino C, et al. Toxicity of
731 organic fluorophores used in molecular imaging: literature review. Mol Imaging
732 2009; 8: 341-54.https://doi.org/10.2310/7290.2009.00031
733 Amereh F, Babaei M, Eslami A, Fazelipour S, Rafiee M. The emerging risk of exposure
734 to nano(micro)plastics on endocrine disturbance and reproductive toxicity: From
735 a hypothetical scenario to a global public health challenge. Environ Pollut 2020;
736 261: 114158.https://doi.org/10.1016/j.envpol.2020.114158
737 Amereh F, Eslami A, Fazelipour S, Rafiee M, Zibaii MI, Babaei M. Thyroid endocrine
738 status and biochemical stress responses in adult male Wistar rats chronically
739 exposed to pristine polystyrene nanoplastics. Toxicol Res (Camb) 2019; 8: 953-
740 963.https://doi.org/10.1039/C9TX00147F
741 Andrady AL. The plastic in microplastics: A review. Mar Pollut Bull 2017; 119: 12-
742 22.https://doi.org/10.1016/j.marpolbul.2017.01.082
743 Aragaw TA. Surgical face masks as a potential source for microplastic pollution in the
744 COVID-19 scenario. Mar Pollut Bull 2020; 159:
745 111517.http://doi.org/10.1016/j.marpolbul.2020.111517
746 Barrionuevo WR, Burggren WW. O2 consumption and heart rate in developing
747 zebrafish (Danio rerio): influence of temperature and ambient O2. Am J Physiol
748 1999; 276: R505-13.http://doi.org/10.1152/ajpregu.1999.276.2.R505
749 Besseling E, Redondo-Hasselerharm P, Foekema EM, Koelmans AA. Quantifying
750 ecological risks of aquatic micro- and nanoplastic. Critical Reviews in
751 Environmental Science and Technology 2019; 49: 32-
752 80.http://doi.org/10.1080/10643389.2018.1531688

753 Bhagat J, Nishimura N, Shimada Y. Toxicological interactions of
754 microplastics/nanoplastics and environmental contaminants: Current knowledge
755 and future perspectives. J Hazard Mater 2020a; 405:
756 123913.http://doi.org/10.1016/j.jhazmat.2020.123913
757 Bhagat J, Zang L, Nishimura N, Shimada Y. Zebrafish: An emerging model to study
758 microplastic and nanoplastic toxicity. Sci Total Environ 2020b; 728:
759 138707.http://doi.org/10.1016/j.scitotenv.2020.138707
760 Bhattacharjee S, Ershov D, Islam MA, Kämpfer AM, Maslowska KA, van der Gucht J,
761 et al. Role of membrane disturbance and oxidative stress in the mode of action
762 underlying the toxicity of differently charged polystyrene nanoparticles. RSC
763 Advances 2014; 4: 19321-19330.http://doi.org/10.1039/C3RA46869K
764 Brandts I, Barria C, Martins MA, Franco-Martinez L, Barreto A, Tvarijonaviciute A, et
765 al. Waterborne exposure of gilthead seabream (Sparus aurata) to
766 polymethylmethacrylate nanoplastics causes effects at cellular and molecular
767 levels. J Hazard Mater 2021; 403:
768 123590.http://doi.org.10.1016/j.jhazmat.2020.123590
769 Brandts I, Garcia-Ordoñez M, Tort L, Teles M, Roher N. Polystyrene nanoplastics
770 accumulate in ZFL cell lysosomes and in zebrafish larvae after acute exposure,
771 inducing a synergistic immune response in vitro without affecting larval survival
772 in vivo. Environmental Science: Nano 2020; 7: 2410-
773 2422.http://doi.org.10.1039/D0EN00553C
774 Brun NR, Koch BEV, Varela M, Peijnenburg WJGM, Spaink HP, Vijver MG.
775 Nanoparticles induce dermal and intestinal innate immune system responses in
776 zebrafish embryos. Environmental Science: Nano 2018; 5: 904-
777 916.http://doi.org/10.1039/c8en00002f

778 Brun NR, van Hage P, Hunting ER, Haramis AG, Vink SC, Vijver MG, et al.
779 Polystyrene nanoplastics disrupt glucose metabolism and cortisol levels with a
780 possible link to behavioural changes in larval zebrafish. Commun Biol 2019; 2:
781 1-9.http://doi.org/10.1038/s42003-019-0629-6
782 Catarino AI, Frutos A, Henry TB. Use of fluorescent-labelled nanoplastics (NPs) to
783 demonstrate NP absorption is inconclusive without adequate controls. Sci Total
784 Environ 2019; 670: 915-920.http://doi.org/10.1016/j.scitotenv.2019.03.194
785 Chae Y, An YJ. Effects of micro- and nanoplastics on aquatic ecosystems: Current
786 research trends and perspectives. Mar Pollut Bull 2017; 124: 624-
787 632.http://doi.org/10.1016/j.marpolbul.2017.01.070
788 Chang X, Xue Y, Li J, Zou L, Tang M. Potential health impact of environmental micro-
789 and nanoplastics pollution. J Appl Toxicol 2020; 40: 4-
790 15.http://doi.org/10.1002/jat.3915
791 Chen Q, Gundlach M, Yang S, Jiang J, Velki M, Yin D, et al. Quantitative investigation
792 of the mechanisms of microplastics and nanoplastics toward zebrafish larvae
793 locomotor activity. Sci Total Environ 2017; 584-585: 1022-
794 1031.http://doi.org/10.1016/j.scitotenv.2017.01.156
795 Craig MP, Gilday SD, Hove JR. Dose-dependent effects of chemical immobilization on
796 the heart rate of embryonic zebrafish. Lab Anim 2006; 35: 41-
797 7.http://doi.org/10.1038/laban1006-41
798 d'Amora M, Giordani S. The Utility of Zebrafish as a Model for Screening
799 Developmental Neurotoxicity. Frontiers in Neuroscience 2018; 12:
800 976.http://doi.org/10.3389/fnins.2018.00976

801 Davranche M, Lory C, Le Juge C, Blancho F, Dia A, Grassl B, et al. Nanoplastics on
802 the coast exposed to the North Atlantic Gyre: Evidence and traceability.
803 NanoImpact 2020; 20: 100262.https://doi.org/10.1016/j.impact.2020.100262
804 de Oliveira JPJ, Estrela FN, Rodrigues AS, Guimarães AT, Rocha T, Malafaia G.
805 Behavioral and biochemical consequences of Danio rerio larvae exposure to
806 polylactic acid bioplastic. Journal of hazardous materials 2020; 404 Pt A:
807 124152.https://doi.org/10.1016/j.jhazmat.2020.124152
808 de Souza Machado AA, Kloas W, Zarfl C, Hempel S, Rillig MC. Microplastics as an
809 emerging threat to terrestrial ecosystems. Global change biology 2018; 24:
810 1405-1416.https://doi.org/10.1111/gcb.14020
811 Devriese LI, van der Meulen MD, Maes T, Bekaert K, Paul-Pont I, Frere L, et al.
812 Microplastic contamination in brown shrimp (Crangon crangon, Linnaeus 1758)
813 from coastal waters of the Southern North Sea and Channel area. Mar Pollut
814 Bull 2015; 98: 179-87.https://doi.org/10.1016/j.marpolbul.2015.06.051
815 Dooley K, Zon LI. Zebrafish: a model system for the study of human disease. Current
816 Opinion in Genetics & Development 2000; 10: 252-
817 256.https://doi.org/10.1016/S0959-437X(00)00074-5
818 Duan Z, Duan X, Zhao S, Wang X, Wang J, Liu Y, et al. Barrier function of zebrafish
819 embryonic chorions against microplastics and nanoplastics and its impact on
820 embryo development. J Hazard Mater 2020; 395:
821 122621.https://doi.org/10.1016/j.jhazmat.2020.122621
822 European Parliament. Council, Directive 2010/63/EU of the European Parliament and
823 of the Council of 22 September 2010 on the protection of animals used for
824 scientific purposes. EU Oficial Journal 2010; 276: 20. https://eur-

lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF

Fadare OO, Wan B, Liu K, Yang Y, Zhao L, Guo L-H. Eco-Corona vs Protein Corona: Effects of Humic Substances on Corona Formation and Nanoplastic Particle Toxicity in Daphnia magna. Environmental Science & Technology 2020; 54: 8001-8009.https://doi.org/10.1021/acs.est.0c00615

Faught E, Vijayan M. Coordinated action of CRH and cortisol shapes acute stress-induced behavioural response in zebrafish. Neuroendocrinology 2021.https://doi.org/10.1159/000514778

Frank P, Ottoboni MA. The dose makes the poison: A plain-language guide to toxicology: John Wiley & Sons, 2011.http://doi.org/10.1002/9780470918449

Gallego-Urrea JA, Tuoriniemi J, Pallander T, Hassellöv M. Measurements of nanoparticle number concentrations and size distributions in contrasting aquatic environments using nanoparticle tracking analysis. Environmental Chemistry 2010; 7: 67-81.https://doi.org/10.1071/EN09114

Gaylarde CC, Neto JAB, da Fonseca EM. Nanoplastics in aquatic systems-are they more hazardous than microplastics? Environmental Pollution 2020: 115950.https://doi.org/10.1016/j.envpol.2020.115950

Gebert LF, MacRae IJ. Regulation of microRNA function in animals. Nature reviews Molecular cell biology 2019; 20: 21-37.http://doi.org/10.1038/s41580-018-0045-7

Geiser M, Rothen-Rutishauser B, Kapp N, Schürch S, Kreyling W, Schulz H, et al. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. Environmental health perspectives 2005; 113: 1555-1560.http://doi.org/10.1289/ehp.8006

Gibert Y, Trengove M, Ward A. Zebrafish as a genetic model in pre-clinical drug testing and screening. Current medicinal chemistry 2013; 20: 2458-2466.http://doi.org/10.2174/0929867311320190005

Gigault J, Ter Halle A, Baudrimont M, Pascal P-Y, Gauffre F, Phi T-L, et al. Current opinion: What is a nanoplastic? Environmental pollution 2018; 235: 1030-1034.https://doi.org/10.1016/j.envpol.2018.01.024

Grabrucker AM, Ruozi B, Belletti D, Pederzoli F, Forni F, Vandelli MA, et al. Nanoparticle transport across the blood brain barrier. Tissue barriers 2016; 4: e1153568.http://doi.org/10.1080/21688370.2016.1153568

Gu W, Liu S, Chen L, Liu Y, Gu C, Ren H-q, et al. Single-cell RNA sequencing reveals size-dependent effects of polystyrene microplastics on immune and secretory cell populations from Zebrafish intestines. Environmental Science & Technology 2020; 54: 3417-3427.https://doi.org/10.1016/j.envpol.2018.01.024

Heinlaan M, Kasemets K, Aruoja V, Blinova I, Bondarenko O, Lukjanova A, et al. Hazard evaluation of polystyrene nanoplastic with nine bioassays did not show particle-specific acute toxicity. Science of the Total Environment 2020; 707: 136073.https://doi.org/10.1016/j.scitotenv.2019.136073

Horzmann KA, Freeman JL. Making Waves: New Developments in Toxicology With the Zebrafish. Toxicol Sci 2018; 163: 5-12.http://doi.org/10.1093/toxsci/kfy044

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013; 496: 498-503.https://doi.org/10.1038/nature12111

Hu Q, Wang H, He C, Jin Y, Fu Z. Polystyrene nanoparticles trigger the activation of p38 MAPK and apoptosis via inducing oxidative stress in zebrafish and

874 macrophage cells. Environmental Pollution 2020; 269:
875 116075.http://doi.org/10.1016/j.envpol.2020.116075
876 Jain KK. Nanobiotechnology-based strategies for crossing the blood–brain barrier.
877 Nanomedicine 2012; 7: 1225-1233.http://doi.org/10.2217/nnm.12.86
878 Jeon S, Clavadetscher J, Lee D-K, Chankeshwara SV, Bradley M, Cho W-S. Surface
879 charge-dependent cellular uptake of polystyrene nanoparticles. Nanomaterials
880 2018; 8: 1028.http://doi.org/10.3390/nano8121028
881 Jeong J, Choi J. Adverse outcome pathways potentially related to hazard identification
882 of microplastics based on toxicity mechanisms. Chemosphere 2019; 231: 249-
883 255.https://doi.org/10.1016/j.chemosphere.2019.05.003
884 Ji Y, Wang C, Wang Y, Fu L, Man M, Chen L. Realistic polyethylene terephthalate
885 nanoplastics and the size-and surface coating-dependent toxicological impacts
886 on zebrafish embryos. Environmental Science: Nano 2020; 7: 2313-
887 2324.https://doi.org/10.1039/D0EN00464B
888 Jimenez-Lamana J, Marigliano L, Allouche J, Grassl B, Szpunar J, Reynaud S. A Novel
889 Strategy for the Detection and Quantification of Nanoplastics by Single Particle
890 Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Anal Chem 2020;
891 92: 11664-11672.http://doi.org/10.1021/acs.analchem.0c01536
892 Karami A, Groman DB, Wilson SP, Ismail P, Neela VK. Biomarker responses in
893 zebrafish (Danio rerio) larvae exposed to pristine low-density polyethylene
894 fragments. Environmental Pollution 2017; 223: 466-
895 475.https://doi.org/10.1016/j.envpol.2017.01.047
896 Kik K, Bukowska B, Sicińska P. Polystyrene nanoparticles: sources, occurrence in the
897 environment, distribution in tissues, accumulation and toxicity to various

organisms. Environmental Pollution 2020; 262: 114297.https://doi.org/10.1016/j.envpol.2020.114297

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203: 253-310.http://doi.org/10.1002/aja.1002030302

Knockenhauer KE, Schwartz TU. The nuclear pore complex as a flexible and dynamic gate. Cell 2016; 164: 1162-1171.http://doi.org/10.1016/j.cell.2016.01.034

Kögel T, Bjorøy Ø, Toto B, Bienfait AM, Sanden M. Micro-and nanoplastic toxicity on aquatic life: Determining factors. Science of the Total Environment 2020; 709: 136050.https://doi.org/10.1016/j.scitotenv.2019.136050

Krewski D, Andersen ME, Tyshenko MG, Krishnan K, Hartung T, Boekelheide K, et al. Toxicity testing in the 21st century: progress in the past decade and future perspectives. Archives of toxicology 2020; 94: 1-58.https://doi.org/10.1007/s00204-019-02613-4

Lee WS, Cho H-J, Kim E, Huh YH, Kim H-J, Kim B, et al. Bioaccumulation of polystyrene nanoplastics and their effect on the toxicity of Au ions in zebrafish embryos. Nanoscale 2019; 11: 3173-3185.https://doi.org/10.1039/C8NR09321K

Lehner R, Weder C, Petri-Fink A, Rothen-Rutishauser B. Emergence of Nanoplastic in the Environment and Possible Impact on Human Health. Environ Sci Technol 2019; 53: 1748-1765.http://doi.org/10.1021/acs.est.8b05512

Lenz R, Enders K, Nielsen TG. Microplastic exposure studies should be environmentally realistic. Proceedings of the National Academy of Sciences 2016; 113: E4121-E4122.https://doi.org/10.1073/pnas.1606615113

921 Letcher TM. Plastic Waste and Recycling: Environmental Impact, Societal Issues,
922 Prevention, and Solutions: Academic Press,
923 2020.https://doi.org/10.1016/C2018-0-01939-8
924 Li P, Li Q, Hao Z, Yu S, Liu J. Analytical methods and environmental processes of
925 nanoplastics. Journal of Environmental Sciences
926 2020.https://doi.org/10.1016/j.jes.2020.03.057
927 Lian J, Wu J, Xiong H, Zeb A, Yang T, Su X, et al. Impact of polystyrene nanoplastics
928 (PSNPs) on seed germination and seedling growth of wheat (Triticum aestivum
929 L.). Journal of hazardous materials 2020; 385:
930 121620.http://doi.org/10.1016/j.jhazmat.2019.121620
931 Liebezeit G, Liebezeit E. Synthetic particles as contaminants in German beers. Food
932 Additives & Contaminants: Part A 2014; 31: 1574-
933 1578.http://doi.org/10.1080/19440049.2014.945099
934 Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA
935 abundance. Cell 2016; 165: 535-550.https://doi.org/10.1016/j.cell.2016.03.014
936 Liu Y, Wang Y, Ling X, Yan Z, Wu D, Liu J, et al. Effects of Nanoplastics and Butyl
937 Methoxydibenzoylmethane on Early Zebrafish Embryos Identified by Single-
938 Cell RNA Sequencing. Environ Sci Technol 2021a; 55: 1885-
939 1896.http://doi.org/10.1021/acs.est.0c06479
940 Liu Z, Cai M, Wu D, Yu P, Jiao Y, Jiang Q, et al. Effects of nanoplastics at predicted
941 environmental concentration on Daphnia pulex after exposure through multiple
942 generations. 2020; 256: 113506.http://doi.org/10.1016/j.envpol.2019.113506
943 Liu Z, Li Y, Sepúlveda MS, Jiang Q, Jiao Y, Chen Q, et al. Development of an adverse
944 outcome pathway for nanoplastic toxicity in Daphnia pulex using proteomics.

945 Science of The Total Environment 2021b:
946 144249.https://doi.org/10.1016/j.scitotenv.2020.144249
947 Lu C, Kania PW, Buchmann K. Particle effects on fish gills: An immunogenetic
948 approach for rainbow trout and zebrafish. Aquaculture 2018; 484: 98-
949 104.https://doi.org/10.1016/j.aquaculture.2017.11.005
950 Lu Y, Zhang Y, Deng Y, Jiang W, Zhao Y, Geng J, et al. Uptake and accumulation of
951 polystyrene microplastics in zebrafish (Danio rerio) and toxic effects in liver.
952 Environmental science & technology 2016; 50: 4054-
953 4060.https://doi.org/10.1021/acs.est.6b00183
954 Luo T, Zhang Y, Wang C, Wang X, Zhou J, Shen M, et al. Maternal exposure to
955 different sizes of polystyrene microplastics during gestation causes metabolic
956 disorders in their offspring. Environmental Pollution 2019; 255:
957 113122.https://doi.org/10.1016/j.envpol.2019.113122
958 MacPhail RC, Brooks J, Hunter DL, Padnos B, Irons TD, Padilla S. Locomotion in
959 larval zebrafish: Influence of time of day, lighting and ethanol. Neurotoxicology
960 2009; 30: 52-8.http://doi.org/10.1016/j.neuro.2008.09.011
961 Mason SA, Welch VG, Neratko J. Synthetic Polymer Contamination in Bottled Water.
962 Front Chem 2018; 6: 407.http://doi.org/10.3389/fchem.2018.00407
963 Materic D, Kasper-Giebl A, Kau D, Anten M, Greilinger M, Ludewig E, et al. Micro-
964 and Nanoplastics in Alpine Snow: A New Method for Chemical Identification
965 and (Semi)Quantification in the Nanogram Range. Environ Sci Technol 2020;
966 54: 2353-2359.http://doi.org/10.1021/acs.est.9b07540
967 Mattsson K, Johnson EV, Malmendal A, Linse S, Hansson L-A, Cedervall T. Brain
968 damage and behavioural disorders in fish induced by plastic nanoparticles

delivered through the food chain. Scientific reports 2017; 7: 1-7.https://doi.org/10.1038/s41598-017-10813-0

Milan DJ, Peterson TA, Ruskin JN, Peterson RT, MacRae CA. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. Circulation 2003; 107: 1355-1358.http://doi.org/10.1161/01.cir.0000061912.88753.87

Moermond CTA, Kase R, Korkaric M, Ågerstrand M. CRED: Criteria for reporting and evaluating ecotoxicity data. Environmental Toxicology and Chemistry 2016; 35: 1297-1309.https://doi.org/10.1002/etc.3259

Natarajan L, Jenifer MA, Mukherjee A. Eco-Corona Formation on the Nanomaterials in the aquatic systems lessens their toxic impact: A comprehensive review. Environmental Research 2020: 110669.https://doi.org/10.1016/j.envres.2020.110669

Ogungbemi A, Leuthold D, Scholz S, Küster E. Hypo-or hyperactivity of zebrafish embryos provoked by neuroactive substances: a review on how experimental parameters impact the predictability of behavior changes. Environmental Sciences Europe 2019; 31: 1-26.http://doi.org/10.1186/s12302-019-0270-5

Organisation for Economic Co-operation and Development O. Fish, short. term toxicity test on embryo and sacfry stages (212). 0ECD Guidelines for the Testing of Chemicals (Paris), 2O07 1998; 1: 1-20. https://www.oecd-ilibrary.org/environment/test-no-212-fish-short-term-toxicity-test-on-embryo-and-sac-fry-stages_9789264070141-en

Organisation for Economic Co-operation and Development O. Fish embryo acute toxicity test (236). OECD guidelines for the testing of chemicals, section 2013a; 2: 1-22. https://www.oecd-ilibrary.org/environment/test-no-236-fish-embryo-acute-toxicity-fet-test_9789264203709-en

994 Organisation for Economic Co-operation and Development O. Fish, Early-life Stage
995 Toxicity Test (210), 2013b. https://www.oecd-ilibrary.org/environment/test-no-
996 210-fish-early-life-stage-toxicity-test_9789264070103-en
997 Orger MB, de Polavieja GG. Zebrafish behavior: opportunities and challenges. Annual
998 review of neuroscience 2017; 40: 125-147.http://doi.org/10.1146/annurev-neuro-
999 071714-033857
1000 Padilla S, Hunter D, Padnos B, Frady S, MacPhail R. Assessing locomotor activity in
1001 larval zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicology
1002 and teratology 2011; 33: 624-630.https://doi.org/10.1016/j.ntt.2011.08.005
1003 Parenti CC, Ghilardi A, Della Torre C, Magni S, Del Giacco L, Binelli A. Evaluation of
1004 the infiltration of polystyrene nanobeads in zebrafish embryo tissues after short-
1005 term exposure and the related biochemical and behavioural effects. Environ
1006 Pollut 2019; 254: 112947.http://doi.org/10.1016/j.envpol.2019.07.115
1007 Park MJ, Lee K-R, Shin D-S, Chun H-S, Kim C-H, Ahn S-H, et al. Predicted drug-
1008 induced bradycardia related cardio toxicity using a zebrafish in vivo model is
1009 highly correlated with results from in vitro tests. Toxicology letters 2013; 216:
1010 9-15.https://doi.org/10.1016/j.toxlet.2012.10.018
1011 Parng C, Roy NM, Ton C, Lin Y, McGrath P. Neurotoxicity assessment using zebrafish.
1012 Journal of pharmacological toxicological methods 2007; 55: 103-
1013 112.https://doi.org/10.1016/j.vascn.2006.04.004
1014 Plastics Europe. Plastics the Facts 2020.
1015 https://www.plasticseurope.org/en/resources/publications/4312-plastics-facts-
1016 2020 2020.Accesed February 2021.
1017 Pedersen AF, Meyer DN, Petriv AV, Soto AL, Shields JN, Akemann C, et al.
1018 Nanoplastics impact the zebrafish (Danio rerio) transcriptome: Associated

1019 developmental and neurobehavioral consequences. Environ Pollut 2020; 266:
1020 115090.http://doi.org/10.1016/j.envpol.2020.115090
1021 Pitt JA, Kozal JS, Jayasundara N, Massarsky A, Trevisan R, Geitner N, et al. Uptake,
1022 tissue distribution, and toxicity of polystyrene nanoparticles in developing
1023 zebrafish (Danio rerio). Aquat Toxicol 2018a; 194: 185-
1024 194.http://doi.org/10.1016/j.aquatox.2017.11.017
1025 Pitt JA, Trevisan R, Massarsky A, Kozal JS, Levin ED, Di Giulio RT. Maternal transfer
1026 of nanoplastics to offspring in zebrafish (Danio rerio): A case study with
1027 nanopolystyrene. Sci Total Environ 2018b; 643: 324-
1028 334.http://doi.org/10.1016/j.scitotenv.2018.06.186
1029 Poma A, Vecchiotti G, Colafarina S, Zarivi O, Aloisi M, Arrizza L, et al. In vitro
1030 genotoxicity of polystyrene nanoparticles on the human fibroblast Hs27 cell line.
1031 Nanomaterials 2019; 9: 1299.http://doi.org/10.3390/nano9091299
1032 Prata JC, Silva AL, Walker TR, Duarte AC, Rocha-Santos T. COVID-19 pandemic
1033 repercussions on the use and management of plastics. Environmental Science &
1034 Technology 2020; 54: 7760-7765.https://doi.org/10.1021/acs.est.0c02178
1035 Prüst M, Meijer J, Westerink RH. The plastic brain: neurotoxicity of micro-and
1036 nanoplastics. Particle fibre toxicology 2020; 17: 1-
1037 16.http://doi.org/10.1186/s12989-020-00358-y
1038 Qu M, Nida A, Kong Y, Du H, Xiao G, Wang D. Nanopolystyrene at predicted
1039 environmental concentration enhances microcystin-LR toxicity by inducing
1040 intestinal damage in Caenorhabditis elegans. Ecotoxicology environmental
1041 safety 2019; 183: 109568.https://doi.org/10.1016/j.ecoenv.2019.109568
1042 Rhodes CJ. Plastic pollution and potential solutions. Sci Prog 2018; 101: 207-
1043 260.http://doi.org/10.3184/003685018X15294876706211

1044 Rubio L, Marcos R, Hernández A. Potential adverse health effects of ingested micro-
1045 and nanoplastics on humans. Lessons learned from in vivo and in vitro
1046 mammalian models. Journal of Toxicology Environmental Health, Part B 2020;
1047 23: 51-68.http://doi.org/10.1080/10937404.2019.1700598
1048 Ruenraroengsak P, Tetley TD. Differential bioreactivity of neutral, cationic and anionic
1049 polystyrene nanoparticles with cells from the human alveolar compartment:
1050 robust response of alveolar type 1 epithelial cells. Particle and Fibre Toxicology
1051 2015; 12: 19.http://doi.org/10.1186/s12989-015-0091-7
1052 Sarasamma S, Audira G, Siregar P, Malhotra N, Lai Y-H, Liang S-T, et al. Nanoplastics
1053 cause neurobehavioral impairments, reproductive and oxidative damages, and
1054 biomarker responses in zebrafish: throwing up alarms of wide spread health risk
1055 of exposure. International journal of molecular sciences 2020; 21:
1056 1410.http://doi.org/10.3390/ijms21041410
1057 Selderslaghs IW, Hooyberghs J, De Coen W, Witters HE. Locomotor activity in
1058 zebrafish embryos: a new method to assess developmental neurotoxicity.
1059 Neurotoxicology teratology 2010; 32: 460-
1060 471.https://doi.org/10.1016/j.ntt.2010.03.002
1061 Sendra M, Pereiro P, Yeste MP, Mercado L, Figueras A, Novoa B. Size matters:
1062 Zebrafish (Danio rerio) as a model to study toxicity of nanoplastics from cells to
1063 the whole organism. Environmental Pollution 2021; 268:
1064 115769.https://doi.org/10.1016/j.envpol.2020.115769
1065 Shen M, Zhang Y, Zhu Y, Song B, Zeng G, Hu D, et al. Recent advances in
1066 toxicological research of nanoplastics in the environment: A review.
1067 Environmental pollution 2019; 252: 511-
1068 521.https://doi.org/10.1016/j.envpol.2019.05.102

1069 Shin H, Kwak M, Lee TG, Lee JY. Quantifying the level of nanoparticle uptake in
1070 mammalian cells using flow cytometry. Nanoscale 2020; 12: 15743-
1071 15751.https://doi.org/10.1039/D0NR01627F
1072 Simkhovich BZ, Kleinman MT, Kloner RA. Air pollution and cardiovascular injury:
1073 epidemiology, toxicology, and mechanisms. Journal of the American College of
1074 Cardiology 2008; 52: 719-726.https://doi.org/10.1016/j.jacc.2008.05.029
1075 Skjolding LM, Ašmonaitė G, Jølck RI, Andresen TL, Selck H, Baun A, et al. An
1076 assessment of the importance of exposure routes to the uptake and internal
1077 localisation of fluorescent nanoparticles in zebrafish (Danio rerio), using light
1078 sheet microscopy. 2017; 11: 351-
1079 359.https://doi.org/10.1080/17435390.2017.1306128
1080 Sokal RR, Rohlf FJ. Biometry: the principles and practice of statistics in biological
1081 research. New York: WH Freeman, 1995
1082 Sokmen TO, Sulukan E, Turkoglu M, Baran A, Ozkaraca M, Ceyhun SB. Polystyrene
1083 nanoplastics (20 nm) are able to bioaccumulate and cause oxidative DNA
1084 damages in the brain tissue of zebrafish embryo (Danio rerio). Neurotoxicology
1085 2020; 77: 51-59.http://doi.org/10.1016/j.neuro.2019.12.010
1086 Strähle U, Scholz S, Geisler R, Greiner P, Hollert H, Rastegar S, et al. Zebrafish
1087 embryos as an alternative to animal experiments--a commentary on the
1088 definition of the onset of protected life stages in animal welfare regulations.
1089 Reprod Toxicol 2012; 33: 128-32.http://doi.org/10.1016/j.reprotox.2011.06.121
1090 Trevisan R, Uzochukwu D, Di Giulio RT. PAH Sorption to Nanoplastics and the Trojan
1091 Horse Effect as Drivers of Mitochondrial Toxicity and PAH Localization in
1092 Zebrafish. Frontiers in Environmental Science 2020.
1093 https://doi.org/10.3389/fenvs.2020.00078

Trevisan R, Voy C, Chen S, Di Giulio RT. Nanoplastics Decrease the Toxicity of a Complex PAH Mixture but Impair Mitochondrial Energy Production in Developing Zebrafish. Environ Sci Technol 2019; 53: 8405-8415.http://doi.org/10.1021/acs.est.9b02003

Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL. Multidimensional in vivo hazard assessment using zebrafish. Toxicological Sciences 2014; 137: 212-233.http://doi.org/10.1093/toxsci/kft235

Umamaheswari S, Priyadarshinee S, Bhattacharjee M, Kadirvelu K, Ramesh M. Exposure to polystyrene microplastics induced gene modulated biological responses in zebrafish (Danio rerio). Chemosphere 2020: 128592.http://doi.org/10.1016/j.chemosphere.2020.128592

Van Pomeren M, Brun N, Peijnenburg W, Vijver M. Exploring uptake and biodistribution of polystyrene (nano) particles in zebrafish embryos at different developmental stages. Aquatic Toxicology 2017; 190: 40-45.http://doi.org/10.1016/j.aquatox.2017.06.017

Veneman WJ, Spaink HP, Brun NR, Bosker T, Vijver MG. Pathway analysis of systemic transcriptome responses to injected polystyrene particles in zebrafish larvae. Aquat Toxicol 2017; 190: 112-120.http://doi.org/10.1016/j.aquatox.2017.06.014

Volz DC, Hipszer RA, Leet JK, Raftery TD. Leveraging embryonic zebrafish to prioritize toxcast testing. Environmental Science & Technology Letters 2015; 2: 171-176.https://doi.org/10.1021/acs.estlett.5b00123

Walczak AP, Kramer E, Hendriksen PJ, Tromp P, Helsper JP, van der Zande M, et al. Translocation of differently sized and charged polystyrene nanoparticles in in

1118 vitro intestinal cell models of increasing complexity. Nanotoxicology 2015; 9:
1119 453-461.https://doi.org/10.3109/17435390.2014.944599
1120 Walpitagama M, Carve M, Douek AM, Trestrail C, Bai Y, Kaslin J, et al. Additives
1121 migrating from 3D-printed plastic induce developmental toxicity and neuro-
1122 behavioural alterations in early life zebrafish (Danio rerio). Aquat Toxicol 2019;
1123 213: 105227.http://doi.org/10.1016/j.aquatox.2019.105227
1124 Wang W, Gao H, Jin S, Li R, Na G. The ecotoxicological effects of microplastics on
1125 aquatic food web, from primary producer to human: A review. Ecotoxicol
1126 Environ Saf 2019; 173: 110-117.http://doi.org/10.1016/j.ecoenv.2019.01.113
1127 Xia T, Kovochich M, Liong M, Zink JI, Nel AE. Cationic polystyrene nanosphere
1128 toxicity depends on cell-specific endocytic and mitochondrial injury pathways.
1129 ACS nano 2008; 2: 85-96.https://doi.org/10.1021/nn700256c
1130 Xu G, Cheng H, Jones R, Feng Y, Gong K, Li K, et al. Surface-Enhanced Raman
1131 Spectroscopy Facilitates the Detection of Microplastics <1 mum in the
1132 Environment. Environ Sci Technol 2020; 54: 15594-
1133 15603.http://doi.org/10.1021/acs.est.0c02317
1134 Yong CQY, Valiyaveetill S, Tang BL, health p. Toxicity of microplastics and
1135 nanoplastics in mammalian systems. International journal of environmental
1136 research 2020; 17: 1509.https://doi.org/10.3390/ijerph17051509
1137 Zhang R, Silic MR, Schaber A, Wasel O, Freeman JL, Sepulveda MS. Exposure route
1138 affects the distribution and toxicity of polystyrene nanoplastics in zebrafish. Sci
1139 Total Environ 2020a; 724:
1140 138065.http://doi.org/10.1016/j.scitotenv.2020.138065

Zhang X, Wang W, Zhu W, Dong J, Cheng Y, Yin Z, et al. Mechanisms and Functions of Long Non-Coding RNAs at Multiple Regulatory Levels. International journal of molecular sciences 2019; 20: 5573.http://doi.org/10.3390/ijms20225573

Zhang Y, Goss GG. Potentiation of polycyclic aromatic hydrocarbon uptake in zebrafish embryos by nanoplastics. Environmental Science: Nano 2020; 7: 1730-1741.https://doi.org/10.1039/D0EN00163E

Zhang Y, Kang S, Allen S, Allen D, Gao T, Sillanpää M. Atmospheric microplastics: A review on current status and perspectives. Earth-Science Reviews 2020b; 203: 103118.https://doi.org/10.1016/j.earscirev.2020.103118

Zhao HJ, Xu JK, Yan ZH, Ren HQ, Zhang Y. Microplastics enhance the developmental toxicity of synthetic phenolic antioxidants by disturbing the thyroid function and metabolism in developing zebrafish. Environ Int 2020; 140: 105750.http://doi.org/10.1016/j.envint.2020.105750

Zimmermann L, Dombrowski A, Völker C, Wagner M. Are bioplastics and plant-based materials safer than conventional plastics? In vitro toxicity and chemical composition. Environment International 2020; 145: 106066.https://doi.org/10.1016/j.envint.2020.106066

Zuo J, Huo T, Du X, Yang Q, Wu Q, Shen J, et al. The joint effect of parental exposure to microcystin-LR and polystyrene nanoplastics on the growth of zebrafish offspring. Journal of Hazardous Materials 2020: 124677.https://doi.org/10.1016/j.jhazmat.2020.124677

Table 1. Revised articles subdivided into studies, each corresponding to one concentration/size. Concentration (µg/ml waterborne or ng injected); size (nm); total exposure time in hours (h). Shaded rows: Injected (I) or maternal (M) exposure studies.

	µg/ml	nm	h
Brun et al. 2018	10	25	120
Brun et al. 2019	20	25	48
Pitt et al. 2018a	0,1	40	162
	1	40	162
	10	40	162
Hu et al. 2020	0,1	40	115
	1	40	115
	10	40	115
Zhang et al. 2020a	0,5	40	95
	5	40	95
Trevisan et al. 2019	10	44	90
Trevisan et al. 2020	1	44	90
Chen et al. 2017	1	50	117
Lee et al. 2019	100	50	24
	100	200	24
	100	500	24
Zhao et al. 2020	0,02	100	164
	0,2	100	164
	2	100	164
	20	100	164
	200	100	164
	20	65	164
Duan et al. 2020	200	100	120
Liu et al. 2021	0,001	100	12
	0,01	100	12
	0,1	100	12
Paranti et al 2019	1	500	48
Zhang and Goss 2020	10	20	96
	10	500	96
Van Pomeren et al. 2017	50	25	48
	50	50	48
	5	250	48
	5	700	48
Liu et al. 2019	0,001	100	96
	1	100	96
Pedersen et al. 2020	0,01	50	114
	0,1	50	114
	1	50	114
	10	50	114
	0,01	200	114
	0,1	200	114
	1	200	114
	10	200	114
	ng	**nm**	**h**
Sökmen et al. 2020 I	0,81	25	116
Brun et al. 2018 I	0,01	25	24
Zhang et al. 2020a I	0,52	40	95
	1,56	40	95
	2,6	40	95
Veneman et al. 2017 I	5	700	72
Pitt et al. 2018b M	NA	40	138

Table 2. Summary of nanoplastic (NP) characteristics and concentration, exposure time, and analyzed effect of all articles reviewed[1].

Reference	NP Size (color), Shape, Route & Charge	NP concentration	Exposure time and medium renewal (MR)	Accumulation	Effects	No Effects
Brun et al. 2018	25 nm (red) Round Waterborne Anionic	10 µg/ml	Mortality, development 0-120 hpf Gene expression: 72-120 hpf MR: every 24h	- Internal exposure: gut - External exposure: skin (neuromasts, tail)	- Upregulated (UR) genes: il1β, irg1l, ccl20a, apoa2, aox5 - Downregulated (DR) genes: tnfα, trpv6, arrdc3	- Mortality, hatching rate, malformations - Unaffected genes (UG): socs3a, mucms1, try - No increase in neutrophil fluorescence - No difference in macrophages and il1β in situ
Brun et al. 2019	25 nm (green) Round Waterborne Anionic	20 µg/ml	72 - 120 hpf MR: every 24h	- Internal exposure: Gut, pancreas, gall bladder	- LC_{50} 25nm at 75 µg/ml; 100% mortality at 85 µg/ml - Malformations: swim bladder - Higher cortisol; lower glucose - UR genes: pck1, g6pca - DR genes: fgf21, ldha, slc6a4 - Larval hyperactivity in the dark	- Unaffected mortality - No difference in length or swim bladder area - UG: slc2a2 and cat
Pitt et al. 2018a	40 nm (green) Round Waterborne Anionic	0.1, 1, and 10 µg/ml	6 to 168 hpf MR:No	- Internal exposure: At 1 and 10 µg/ml yolk, brain, heart, gut, pancreas, liver, gall bladder. At 0.1 µg/ml gut, head and pancreas. - External exposure: chorion	**For all concentrations:** - Decreased heart rate - Hypoactive behavior in dark periods - Locomotion: Interaction of NP treatment and cohort in dark and light periods	**For all concentrations:** - Mortality, hatching, development - No difference in OCR (mitochondrial or not) - No difference in ECAR - No concentration response
Hu et al. 2020	40 nm (green and pristine) Round, Waterborne Anionic	0.1, 1, and 10 µg/ml	5 – 120 hpf MR: No	- Internal exposure: At 0.1 µg/ml gut, pancreas. At 1 and 10 µg/ml liver, gut, pancreas and gall bladder	-Decrease heart rate and locomotion (all concentrations) - Concentration response in genetic markers. More genes affected at highest concentrations: - UR genes at 1 µg/ml: ifn- γ - UR genes at 10 µg/ml: ifn- γ, il-6, tfn-α, il1β	-Mortality, hatching or development (all concentrations) - UG at 0.1 µg/ml: ifn- γ, il-6, tfn-α, il1β, sod1, sod2. - UG at 1 µg/ml: il-6, tfn-α, il1β, sod2.

					- DR genes at 0.1 µg/ml and 1 µg/ml: gstp, gr - DR genes at 1 µg/ml: sod 1 - DR genes at 10 µg/ml: gstp, gr, sod1, sod2. - Significant transcriptomic changes (only 10 µg/ml tested): Genes related to MAPK signaling pathway, carbohydrate metabolism, cell membrane biogenesis, immunity, endocytosis, and catalytic activity.	
Zhang et al. 2020a	40 nm (green) Round Waterborne Anionic	0.5 and 5 µg/ml	1 – 96 hpf MR: every 24h	- Internal exposure at all concentrations: eye, head, yolk, gut	- Significantly higher rates of bent tail (only 0.5 µg/ml tested) - Decreased larvae rotation (only at 5 µg/ml) - DR genes: sod2, gfap, opn1lw2, opn1mw1 (all concentrations)	- Mortality, hatching, length (all concentrations) - No difference in distance travelled or velocity (all concentrations) - UG sod1, mbp, syn2α, opn1sw2 (all concentrations) - No difference in jaw and heart abnormalities (only 0.5 µg/ml tested)
Trevisan et al. 2019	44 nm Round Waterborne Anionic	10 µg/ml	6 to 96 hpf MR: No	- Not studied	- Slight tail curve ($p = 0.07$) - Decreased ATP-linked OCR (50-65%) - Increased Proton leak OCR (6 fold) - Decreased mitochondrial coupling efficiency (50-60%) - Higher NADH at 96hpf	- Mortality and heart rate - No difference in EROD, blood vessel area, OCR (total basal, mitochondrial basal, non- mitochondrial basal, total maximal, mitochondrial maximal and mitochondrial spare capacity), and mitochondrial mass
Trevisan et al. 2020	44 nm Round Anionic	1 µg/ml	6 to 96 hpf MR: No	- Not studied	- Slight decrease in mitochondrial coupling efficiency ($p = 0.09$) - Lower NADH at 96hpf	- Mortality, hatching rate, malformations, heart rate - No difference in OCR (total basal, mitochondrial basal, non-mitochondrial basal, total maximal, mitochondrial maximal and mitochondrial spare capacity, ATP-linked and proton leak), and - No difference in CO_2 excretion

Chen et al. 2017	50 nm Round Anionic	1 µg/ml	3 to 120 hpf MR: every 24h	- Not studied	- Locomotor activity reduced - Decreased body length - Highly UR genes: gfap and a1tubulin - Small upregulation of: zfblue - Increased activity of AchE - Decreased GSH concentration	- Mortality or malformations - UG: zfrho - Unaffecterd enzymes: CAT and GPx
Lee et al. 2019	50 nm, (green and undyed) Round Waterborne Anionic	100 µg/ml	24 to 48 hpf	- Internal exposure: yolk, brain, retina, blood vessels, muscle, fascicles, spinal cord, CNS cells.	- Minor subcellular organelle damage with 50 nm NP (mitochondria). - Increased ROS - Increased mitochondrial ROS	- Mortality, malformations, hatching or cell death - UG: il1β and il-6
	200 nm (green and undyed) Round Waterborne Anionic	100 µg/ml Only accumulation and mortality tested	24 to 48 hpf	-Chorion and yolk.	NA	-Mortality
	500 nm (green and undyed) Round Waterborne Anionic	100 µg/ml Only accumulation and mortality tested	24 to 48 hpf	-Chorion and yolk. Less internal fluorescence than with 200 nm NP	NA	-Mortality
Zhao et al. 2020	100 nm (data in supplementary material)	0.02, 0.2, 2, 20 and 200 µg/ml	4 to 168 hpf	- Not studied	- Decreased hatching, body length (only at 200 µg/ml)	- Heart rate (all concentrations)
	65 nm (green) Round, dyed and pristine Waterborne Anionic	20 µg/ml	4 to 168 hpf Up to 12 dpf for vertebrae assay MR: every 24h	-Internal exposure: gut, pancreas	- Increase TSH - 37 changed metabolites from: amino acid, glycerophospholipid, and arachidonic acid groups.	- No effect on mortality, hatching, development and heart rate - No difference in T3 and T4 - Unaltered vertebrae
Duan et al. 2020	100 nm (red) Round, Waterborne	2 x 10^4 beads/ml = 200 µg/ml (author's	0-120 hpf	- Internal exposure: brain, gills, blood, liver, gut	- Delayed hatching by 12% - Heart rate increased at 24hpf but decreased at 48 hpf	- Mortality - Blood flow

	Anionic	personal communication)	Total: 24, 48, 60, 72 h depending on assay MR: No		- Reduction in melanin by 15% Metabolome affected: - Biosynthesis of essential fatty acids - Metabolism of alanine, aspartate and glutamate - Metabolism of taurine, nicotinate and nicotinamide	
Liu et al. 2021	100 nm Round Waterborne Anionic	Survival, heart rate, locomotion and RNA seq: 0.01 µg/ml Individual gene analysis: 0.001, 0.01, 0.1 µg/ml	Exposed: 2- 12hpf MR: No	- Not studied	- Increased heart rate (only tested at 0.01 µg/ml) - Decreased locomotion speed (only tested at 0.01 µg/ml) - UR genes: sod and gpx (all concentrations, concentration dependent increase); gst (all concentrations) - DR genes: dnmt3bb1, dnmt3bb2 (all concentrations, not concentration dependent) - Reduction of mesoderm cells (only tested at 0.01 µg/ml) - Increased abundance of neural mid cells (only tested at 0.01 µg/ml) - Transcriptome analysis: effect on brain and sensory system development, muscle development, altered notch pathway (only tested at 0.01 µg/ml)	- Mortality (only tested at 0.01 µg/ml) - UG: cat, dnmt1, dnmt3aa, cyp19aa, cyp19a1b (all concentrations) - No difference in abundance of endoderm, epidermal, neural anterior, neural posterior and neural crest cells (only tested at 0.01 µg/ml)
Parenti et al 2019	500 nm (red) Round Waterborne Neutral	1 µg/ml	72 to 120 hpf MR: every 24h	- Not studied	- NP observed in gut, gills and neuromast - Increased SOD - Decreased COX	-P-gp, GST, GPx, CAT, ROS not affected
Zhang and Goss 2020	20 nm (green) Round Waterborne Anionic	Assays: 10 µg/ml LC_{50}: 3.125 – 50 µg/ml	24 – 120 hpf Total: 96 h MR: every 48h	- Internal exposure: accumulation at 24 hpf: Chorion, yolk, eye, brain, and gut (only tested at 10 µg/ml). Other organs not distinguishable at this stage	- LC_{50} 20nm dialized 52 µg/ml, with NaN_3: 21.5 µg/ml.	-No difference in mortality, hatching or development (only tested at 10 µg/ml) - No difference in EROD (only tested at 10 µg/ml)

	500 nm (green) Round Waterborne Anionic	Assays: 10 µg/ml LC_{50}: 3.125 – 50 µg/ml	24 – 120 hpf Total: 96 h MR: every 48h	- Internal exposure: accumulation at 24 hpf: Chorion and yolk sac (only tested at 10 µg/ml). - Less accumulation than with 20 nm NP.	- LC_{50} 500 nm dialized > 100 µg/ml, with NaN_3: 78.3 µg/ml	-No difference in mortality, hatching or development (only tested at 10 µg/ml) - No difference in EROD (only tested at 10 µg/ml)
VanPomeren et al. 2017	25 nm (red) Round Waterborne Anionic	50 µg/ml	24-72 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: yolk, gut and ocular region (only for animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
	50 nm (red) Round Waterborne Anionic	50 µg/ml	0-48 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: yolk, gut and ocular region (only for animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
	250 nm (red) Round Waterborne Anionic	5 µg/ml	0-48 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: gut (only animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
	700 nm (red) Round Waterborne Anionic	5 µg/ml	0-48 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: gut (only animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
Liu et al. 2019	100 nm Round Waterborne Anionic	0.001 and 1 µg/ml	0 -96 hpf Total 96 h MR: No	- Not studied	- NP decreased ROS levels (at 0.001 µg/ml) - NP increased ROS levels (at 1 µg/ml)	- Mortality, hatching, development - No difference in SOD
Pedersen et al. 2020	50 nm (green) Round Waterborne Anionic	0.01, 0.1, 1 and 10 µg/ml Transcriptomics: 1 and 10 µg/ml	6 to 120 hpf Total: 114 h MR: every 24h	Accumulation concentration response: -No visible accumulation (0.01 µg/ml - Internal exposure: yolk and gut (0.1 µg/ml) - Internal exposure: yolk, gut, pancreas, liver, ocular and cranial regions (1 and 10 µg/ml)	- 2 genes affected in transcriptomic analysis (only at 10 µg/ml)	- Mortality, hatching, development (all concentrations) - Locomotion not affected (all concentrations)
	200 nm (green) Round Waterborne	0.01, 0.1, 1 and 10 µg/ml	6 to 120 hpf Total: 114 h MR: every 24h	Accumulation concentration response: -No visible accumulation (0.01 µg/ml - Internal exposure: yolk and gut (0.1 µg/ml)	- Hyperactive behavior in dark periods (only at 1 and 10 µg/ml; concentration response) - 734 genes affected (at 1 µg/ml)	- Mortality, hatching, development (all concentrations)

	Anionic	Transcriptomics: 1 and 10 µg/ml		- Internal exposure: yolk, gut, pancreas, liver, ocular and cranial regions (1 and 10 µg/ml)	- 864 genes affected (at 10 µg/ml) - Genes affected: Cardiovascular, skeletal and muscular, nervous and hepatic systems. Genes related to diseases: Cancer, development abnormalities, endocrine systems and neurological disorders, among others	
Sökmen et al. 2020	25 nm (red) Round Injection Anionic	0.81 ng injected/embryo	4 to 120 hpf Total: 116 h	- Internal exposure: accumulation of NP observed in yolk and brain by electron microscopy.	- Decreased survival (17%) and hatching rate (20%) - Increased malformations (curved body axis, tail, yolk sac and pericardial edema) - ROS detected throughout body, especially head - Cell death detected by Acridine Orange - DNA damage detection (presence of 8OHdG)	
Brun et al. 2018 I	25 nm (red) Round Injection Anionic	0.01 ng injected/embryo	Injected at 30 hpf Total: 24 h	- Not studied	- UR genes: il1β, socs3a, ccl20a - DR gene: trpv6	- No difference in tail neutrophil fluorescence - UG: tnfα, irg1l, mucms1, apoa2, aox5, try
Zhang et al. 2020a I	40 nm (green) Round Injection Anionic	0.52 ng, 1.56 ng and 2.6 ng injected/embryo	Injected at 0 hpf Total: 95h	- Internal exposure: eyes, head, yolk, gut (all concentrations)	- Downregulated genes: mbp, syn2α (only at 2.6 ng), opn1sw2, opn1lw2, opn1mw1 (all concentrations)	- Mortality, hatching, length (all concentrations) - No difference in bent tail, jaw and heart abnormalities (only 2.6 ng tested) - No difference in distance travelled, velocity or rotation (all concentrations) - Unaffected genes: sod1, sod2, gfap (all concentrations)
Veneman et al. 2017	700 nm (red) Round Injection Anionic	5 ng injected/embryo	1-120 hpf Injected at 1 hpf and 48 hpf Sampled at 72 and 120 hpf	- Internal exposure: yolk, blood stream and heart	- Significant transcriptomic changes: - Genes related to immunity (complement), nuclear receptors in lipid metabolism and toxicity, ACE pathway, and oxidative stress.	- Mortality, malformations

Pitt et al. 2018b	40 nm (green) Round Maternal Paternal Co-parental Anionic	Maternal, concentration unknown	6 to 144 hpf Total:138 h	- Internal exposure: yolk, gut, liver, pancreas, gall bladder	- Decreased heart rate - Uninflated swim bladders - Decreased Thiol and GR	- Mortality - No difference in locomotion - No difference in GPx, CAT or OCR

[1] NP size: Average diameter according to supplier. All NP in all studies were spherical. Color: color of fluorophore used. Transcript names are in lower case letters, enzymes, metabolites and processes in capital letters: ROS: reactive oxygen species , SOD: supeoxide distmutase, P-gp: p- glycoprotein, CAT:catalase, GPx: glutathione peroxidase, GST: glutathione-S-transferase, EROD: Ethoxyresorufin-O-deethylase, GR: Glutathione reductase, COX: cyclooxygenase, GSH: reduced form of glutathione; AchE: Acetylcholinesterase, TSH: Thyroid stimulating hormone, NADH: reduced nicotinamide adenine dinucleotide, T3: Triiodothyronine, T4: thyroxine, ACE: angiotensin converting enzyme, 8OHdG: 8-Oxo-2'-deoxyguanosine, OCR: oxygen consumption rate, ECAR: extracellular acidification rate; CNS: central nervous system. NA: Non-applicable, no other effect studied at this concentration/size

Table 2. Summary of nanoplastic (NP) characteristics and concentration, exposure time, and analyzed effect of all articles reviewed[1].

Reference	NP Size (color), Shape, Route & Charge	NP concentration	Exposure time and medium renewal (MR)	Accumulation	Effects	No Effects
Brun et al. 2018	25 nm (red) Round Waterborne Anionic	10 µg/ml	Mortality, development 0-120 hpf Gene expression: 72-120 hpf MR: every 24h	- Internal exposure: gut - External exposure: skin (neuromasts, tail)	- Upregulated (UR) genes: il1β, irg1l, ccl20a, apoa2, aox5 - Downregulated (DR) genes: tnfα, trpv6, arrdc3	- Mortality, hatching rate, malformations - Unaffected genes (UG): socs3a, mucms1, try - No increase in neutrophil fluorescence - No difference in macrophages and il1β in situ
Brun et al. 2019	25 nm (green) Round Waterborne Anionic	20 µg/ml	72 - 120 hpf MR: every 24h	- Internal exposure: Gut, pancreas, gall bladder	- LC_{50} 25nm at 75 µg/ml; 100% mortality at 85 µg/ml - Malformations: swim bladder - Higher cortisol; lower glucose - UR genes: pck1, g6pca - DR genes: fgf21, ldha, slc6a4 - Larval hyperactivity in the dark	- Unaffected mortality - No difference in length or swim bladder area - UG: slc2a2 and cat
Pitt et al. 2018a	40 nm (green) Round Waterborne Anionic	0.1, 1, and 10 µg/ml	6 to 168 hpf MR:No	- Internal exposure: At 1 and 10 µg/ml yolk, brain, heart, gut, pancreas, liver, gall bladder. At 0.1 µg/ml gut, head and pancreas. - External exposure: chorion	**For all concentrations:** - Decreased heart rate - Hypoactive behavior in dark periods - Locomotion: Interaction of NP treatment and cohort in dark and light periods	**For all concentrations:** - Mortality, hatching, development - No difference in OCR (mitochondrial or not) - No difference in ECAR - No concentration response
Hu et al. 2020	40 nm (green and pristine) Round, Waterborne Anionic	0.1, 1, and 10 µg/ml	5 – 120 hpf MR: No	- Internal exposure: At 0.1 µg/ml gut, pancreas. At 1 and 10 µg/ml liver, gut, pancreas and gall bladder	-Decrease heart rate and locomotion (all concentrations) - Concentration response in genetic markers. More genes affected at highest concentrations: - UR genes at 1 µg/ml: ifn- γ - UR genes at 10 µg/ml: ifn- γ, il-6, tfn-α, il1β	-Mortality, hatching or development (all concentrations) - UG at 0.1 µg/ml: ifn- γ, il-6, tfn-α, il1β, sod1, sod2. - UG at 1 µg/ml: il-6, tfn-α, il1β, sod2.

					- DR genes at 0.1 µg/ml and 1 µg/ml: gstp, gr - DR genes at 1 µg/ml: sod 1 - DR genes at 10 µg/ml: gstp, gr, sod1, sod2. - Significant transcriptomic changes (only 10 µg/ml tested): Genes related to MAPK signaling pathway, carbohydrate metabolism, cell membrane biogenesis, immunity, endocytosis, and catalytic activity.	
Zhang et al. 2020a	40 nm (green) Round Waterborne Anionic	0.5 and 5 µg/ml	1 – 96 hpf MR: every 24h	- Internal exposure at all concentrations: eye, head, yolk, gut	- Significantly higher rates of bent tail (only 0.5 µg/ml tested) - Decreased larvae rotation (only at 5 µg/ml) - DR genes: sod2, gfap, opn1lw2, opn1mw1 (all concentrations)	- Mortality, hatching, length (all concentrations) - No difference in distance travelled or velocity (all concentrations) - UG sod1, mbp, syn2α, opn1sw2 (all concentrations) - No difference in jaw and heart abnormalities (only 0.5 µg/ml tested)
Trevisan et al. 2019	44 nm Round Waterborne Anionic	10 µg/ml	6 to 96 hpf MR: No	- Not studied	- Slight tail curve ($p = 0.07$) - Decreased ATP-linked OCR (50-65%) - Increased Proton leak OCR (6 fold) - Decreased mitochondrial coupling efficiency (50-60%) - Higher NADH at 96hpf	- Mortality and heart rate - No difference in EROD, blood vessel area, OCR (total basal, mitochondrial basal, non- mitochondrial basal, total maximal, mitochondrial maximal and mitochondrial spare capacity), and mitochondrial mass
Trevisan et al. 2020	44 nm Round Anionic	1 µg/ml	6 to 96 hpf MR: No	- Not studied	- Slight decrease in mitochondrial coupling efficiency ($p = 0.09$) - Lower NADH at 96hpf	- Mortality, hatching rate, malformations, heart rate - No difference in OCR (total basal, mitochondrial basal, non-mitochondrial basal, total maximal, mitochondrial maximal and mitochondrial spare capacity, ATP-linked and proton leak), and - No difference in CO_2 excretion

Chen et al. 2017	50 nm Round Anionic	1 µg/ml	3 to 120 hpf MR: every 24h	- Not studied	- Locomotor activity reduced - Decreased body length - Highly UR genes: gfap and a1tubulin - Small upregulation of: zfblue - Increased activity of AchE - Decreased GSH concentration	- Mortality or malformations - UG: zfrho - Unaffecterd enzymes: CAT and GPx
Lee et al. 2019	50 nm, (green and undyed) Round Waterborne Anionic	100 µg/ml	24 to 48 hpf	- Internal exposure: yolk, brain, retina, blood vessels, muscle, fascicles, spinal cord, CNS cells.	- Minor subcellular organelle damage with 50 nm NP (mitochondria). - Increased ROS - Increased mitochondrial ROS	- Mortality, malformations, hatching or cell death - UG: il1β and il-6
	200 nm (green and undyed) Round Waterborne Anionic	100 µg/ml Only accumulation and mortality tested	24 to 48 hpf	-Chorion and yolk.	NA	-Mortality
	500 nm (green and undyed) Round Waterborne Anionic	100 µg/ml Only accumulation and mortality tested	24 to 48 hpf	-Chorion and yolk. Less internal fluorescence than with 200 nm NP	NA	-Mortality
Zhao et al. 2020	100 nm (data in supplementary material)	0.02, 0.2, 2, 20 and 200 µg/ml	4 to 168 hpf	- Not studied	- Decreased hatching, body length (only at 200 µg/ml)	- Heart rate (all concentrations)
	65 nm (green) Round, dyed and pristine Waterborne Anionic	20 µg/ml	4 to 168 hpf Up to 12 dpf for vertebrae assay MR: every 24h	-Internal exposure: gut, pancreas	- Increase TSH - 37 changed metabolites from: amino acid, glycerophospholipid, and arachidonic acid groups.	- No effect on mortality, hatching, development and heart rate - No difference in T3 and T4 - Unaltered vertebrae
Duan et al. 2020	100 nm (red) Round, Waterborne	2 x 10^4 beads/ml = 200 µg/ml (author's	0-120 hpf	- Internal exposure: brain, gills, blood, liver, gut	- Delayed hatching by 12% - Heart rate increased at 24hpf but decreased at 48 hpf	- Mortality - Blood flow

	Anionic	personal communication)	Total: 24, 48, 60, 72 h depending on assay MR: No		- Reduction in melanin by 15% Metabolome affected: - Biosynthesis of essential fatty acids - Metabolism of alanine, aspartate and glutamate - Metabolism of taurine, nicotinate and nicotinamide	
Liu et al. 2021	100 nm Round Waterborne Anionic	Survival, heart rate, locomotion and RNA seq: 0.01 µg/ml Individual gene analysis: 0.001, 0.01, 0.1 µg/ml	Exposed: 2- 12hpf MR: No	- Not studied	- Increased heart rate (only tested at 0.01 µg/ml) - Decreased locomotion speed (only tested at 0.01 µg/ml) - UR genes: sod and gpx (all concentrations, concentration dependent increase); gst (all concentrations) - DR genes: dnmt3bb1, dnmt3bb2 (all concentrations, not concentration dependent) - Reduction of mesoderm cells (only tested at 0.01 µg/ml) - Increased abundance of neural mid cells (only tested at 0.01 µg/ml) - Transcriptome analysis: effect on brain and sensory system development, muscle development, altered notch pathway (only tested at 0.01 µg/ml)	- Mortality (only tested at 0.01 µg/ml) - UG: cat, dnmt1, dnmt3aa, cyp19aa, cyp19a1b (all concentrations) - No difference in abundance of endoderm, epidermal, neural anterior, neural posterior and neural crest cells (only tested at 0.01 µg/ml)
Parenti et al 2019	500 nm (red) Round Waterborne Neutral	1 µg/ml	72 to 120 hpf MR: every 24h	- Not studied	- NP observed in gut, gills and neuromast - Increased SOD - Decreased COX	-P-gp, GST, GPx, CAT, ROS not affected
Zhang and Goss 2020	20 nm (green) Round Waterborne Anionic	Assays: 10 µg/ml LC_{50}: 3.125 – 50 µg/ml	24 – 120 hpf Total: 96 h MR: every 48h	- Internal exposure: accumulation at 24 hpf: Chorion, yolk, eye, brain, and gut (only tested at 10 µg/ml). Other organs not distinguishable at this stage	- LC_{50} 20nm dialized 52 µg/ml, with NaN_3: 21.5 µg/ml.	-No difference in mortality, hatching or development (only tested at 10 µg/ml) - No difference in EROD (only tested at 10 µg/ml)

	500 nm (green) Round Waterborne Anionic	Assays: 10 µg/ml LC_{50}: 3.125 – 50 µg/ml	24 – 120 hpf Total: 96 h MR: every 48h	- Internal exposure: accumulation at 24 hpf: Chorion and yolk sac (only tested at 10 µg/ml). - Less accumulation than with 20 nm NP.	- LC_{50} 500 nm dialized > 100 µg/ml, with NaN_3: 78.3 µg/ml	-No difference in mortality, hatching or development (only tested at 10 µg/ml) - No difference in EROD (only tested at 10 µg/ml)
VanPomeren et al. 2017	25 nm (red) Round Waterborne Anionic	50 µg/ml	24-72 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: yolk, gut and ocular region (only for animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
	50 nm (red) Round Waterborne Anionic	50 µg/ml	0-48 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: yolk, gut and ocular region (only for animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
	250 nm (red) Round Waterborne Anionic	5 µg/ml	0-48 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: gut (only animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
	700 nm (red) Round Waterborne Anionic	5 µg/ml	0-48 and 72-120 hpf Total: 48 h MR: every 24 h	Internal exposure: gut (only animals exposed from 72 – 120 hpf) External exposure: gills and skin	NA	- Mortality, malformations - Unaffected eye size
Liu et al. 2019	100 nm Round Waterborne Anionic	0.001 and 1 µg/ml	0 -96 hpf Total 96 h MR: No	- Not studied	- NP decreased ROS levels (at 0.001 µg/ml) - NP increased ROS levels (at 1 µg/ml)	- Mortality, hatching, development - No difference in SOD
Pedersen et al. 2020	50 nm (green) Round Waterborne Anionic	0.01, 0.1, 1 and 10 µg/ml Transcriptomics: 1 and 10 µg/ml	6 to 120 hpf Total: 114 h MR: every 24h	Accumulation concentration response: -No visible accumulation (0.01 µg/ml - Internal exposure: yolk and gut (0.1 µg/ml) - Internal exposure: yolk, gut, pancreas, liver, ocular and cranial regions (1 and 10 µg/ml)	- 2 genes affected in transcriptomic analysis (only at 10 µg/ml)	- Mortality, hatching, development (all concentrations) - Locomotion not affected (all concentrations)
	200 nm (green) Round Waterborne	0.01, 0.1, 1 and 10 µg/ml	6 to 120 hpf Total: 114 h MR: every 24h	Accumulation concentration response: -No visible accumulation (0.01 µg/ml - Internal exposure: yolk and gut (0.1 µg/ml)	- Hyperactive behavior in dark periods (only at 1 and 10 µg/ml; concentration response) - 734 genes affected (at 1 µg/ml)	- Mortality, hatching, development (all concentrations)

	Anionic	Transcriptomics: 1 and 10 µg/ml		- Internal exposure: yolk, gut, pancreas, liver, ocular and cranial regions (1 and 10 µg/ml)	- 864 genes affected (at 10 µg/ml) - Genes affected: Cardiovascular, skeletal and muscular, nervous and hepatic systems. Genes related to diseases: Cancer, development abnormalities, endocrine systems and neurological disorders, among others	
Sökmen et al. 2020	25 nm (red) Round Injection Anionic	0.81 ng injected/embryo	4 to 120 hpf Total: 116 h	- Internal exposure: accumulation of NP observed in yolk and brain by electron microscopy.	- Decreased survival (17%) and hatching rate (20%) - Increased malformations (curved body axis, tail, yolk sac and pericardial edema) - ROS detected throughout body, especially head - Cell death detected by Acridine Orange - DNA damage detection (presence of 8OHdG)	
Brun et al. 2018 I	25 nm (red) Round Injection Anionic	0.01 ng injected/embryo	Injected at 30 hpf Total: 24 h	- Not studied	- UR genes: il1β, socs3a, ccl20a - DR gene: trpv6	- No difference in tail neutrophil fluorescence - UG: tnfα, irg1l, mucms1, apoa2, aox5, try
Zhang et al. 2020a I	40 nm (green) Round Injection Anionic	0.52 ng, 1.56 ng and 2.6 ng injected/embryo	Injected at 0 hpf Total: 95h	- Internal exposure: eyes, head, yolk, gut (all concentrations)	- Downregulated genes: mbp, syn2α (only at 2.6 ng), opn1sw2, opn1lw2, opn1mw1 (all concentrations)	- Mortality, hatching, length (all concentrations) - No difference in bent tail, jaw and heart abnormalities (only 2.6 ng tested) - No difference in distance travelled, velocity or rotation (all concentrations) - Unaffected genes: sod1, sod2, gfap (all concentrations)
Veneman et al. 2017	700 nm (red) Round Injection Anionic	5 ng injected/embryo	1-120 hpf Injected at 1 hpf and 48 hpf Sampled at 72 and 120 hpf	- Internal exposure: yolk, blood stream and heart	- Significant transcriptomic changes: - Genes related to immunity (complement), nuclear receptors in lipid metabolism and toxicity, ACE pathway, and oxidative stress.	- Mortality, malformations

Pitt et al. 2018b	40 nm (green) Round Maternal Paternal Co-parental Anionic	Maternal, concentration unknown	6 to 144 hpf Total:138 h	- Internal exposure: yolk, gut, liver, pancreas, gall bladder	- Decreased heart rate - Uninflated swim bladders - Decreased Thiol and GR	- Mortality - No difference in locomotion - No difference in GPx, CAT or OCR

[1] NP size: Average diameter according to supplier. All NP in all studies were spherical. Color: color of fluorophore used. Transcript names are in lower case letters, enzymes, metabolites and processes in capital letters: ROS: reactive oxygen species , SOD: supeoxide distmutase, P-gp: p- glycoprotein, CAT:catalase, GPx: glutathione peroxidase, GST: glutathione-S-transferase, EROD: Ethoxyresorufin-O-deethylase, GR: Glutathione reductase, COX: cyclooxygenase, GSH: reduced form of glutathione; AchE: Acetylcholinesterase, TSH: Thyroid stimulating hormone, NADH: reduced nicotinamide adenine dinucleotide, T3: Triiodothyronine, T4: thyroxine, ACE: angiotensin converting enzyme, 8OHdG: 8-Oxo-2'-deoxyguanosine, OCR: oxygen consumption rate, ECAR: extracellular acidification rate; CNS: central nervous system. NA: Non-applicable, no other effect studied at this concentration/size

Figure 1

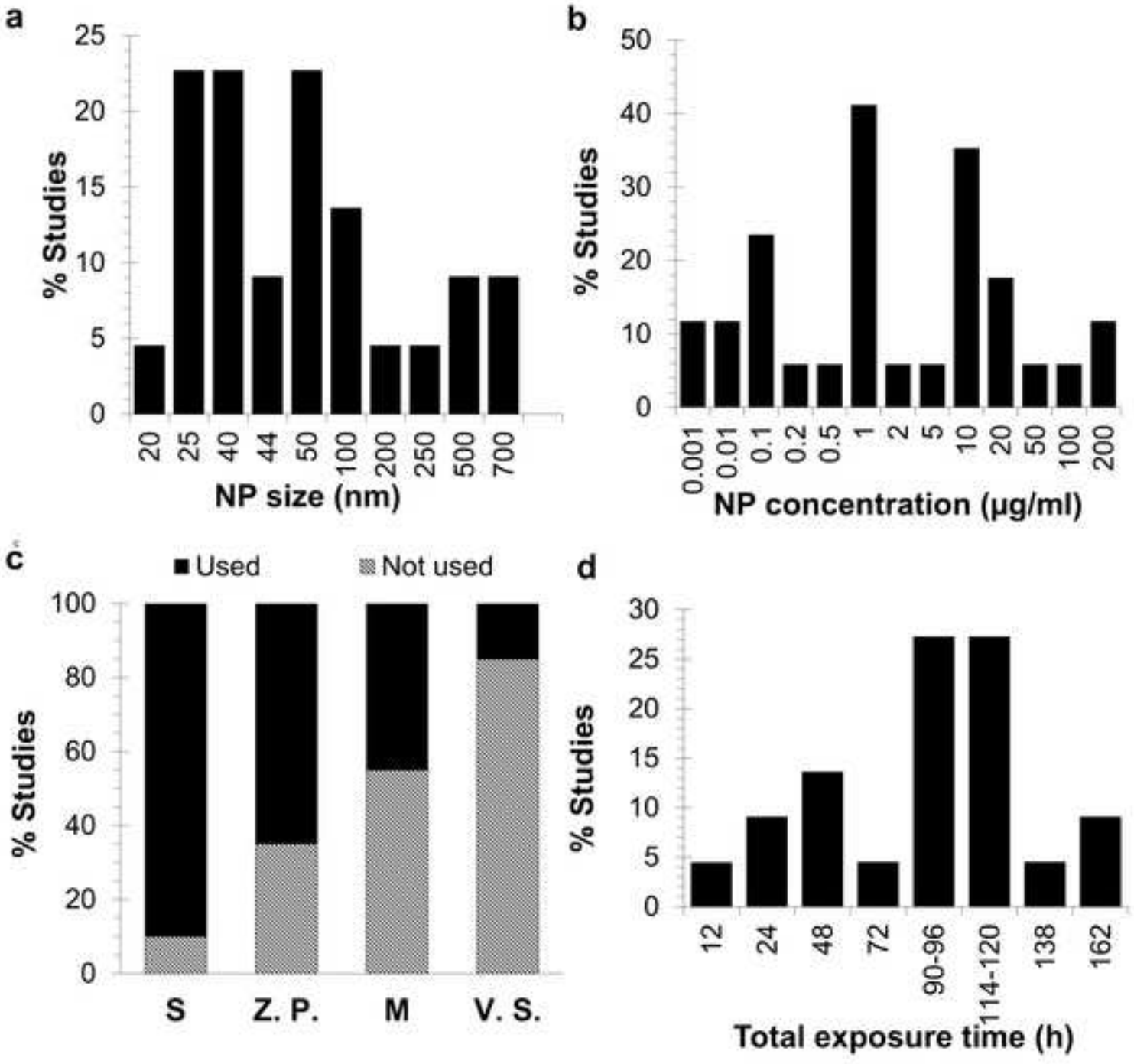


Figure 3

Click here to access/download;Figure;Torres-Ruiz M-Figure 3-138x140mm.TIF

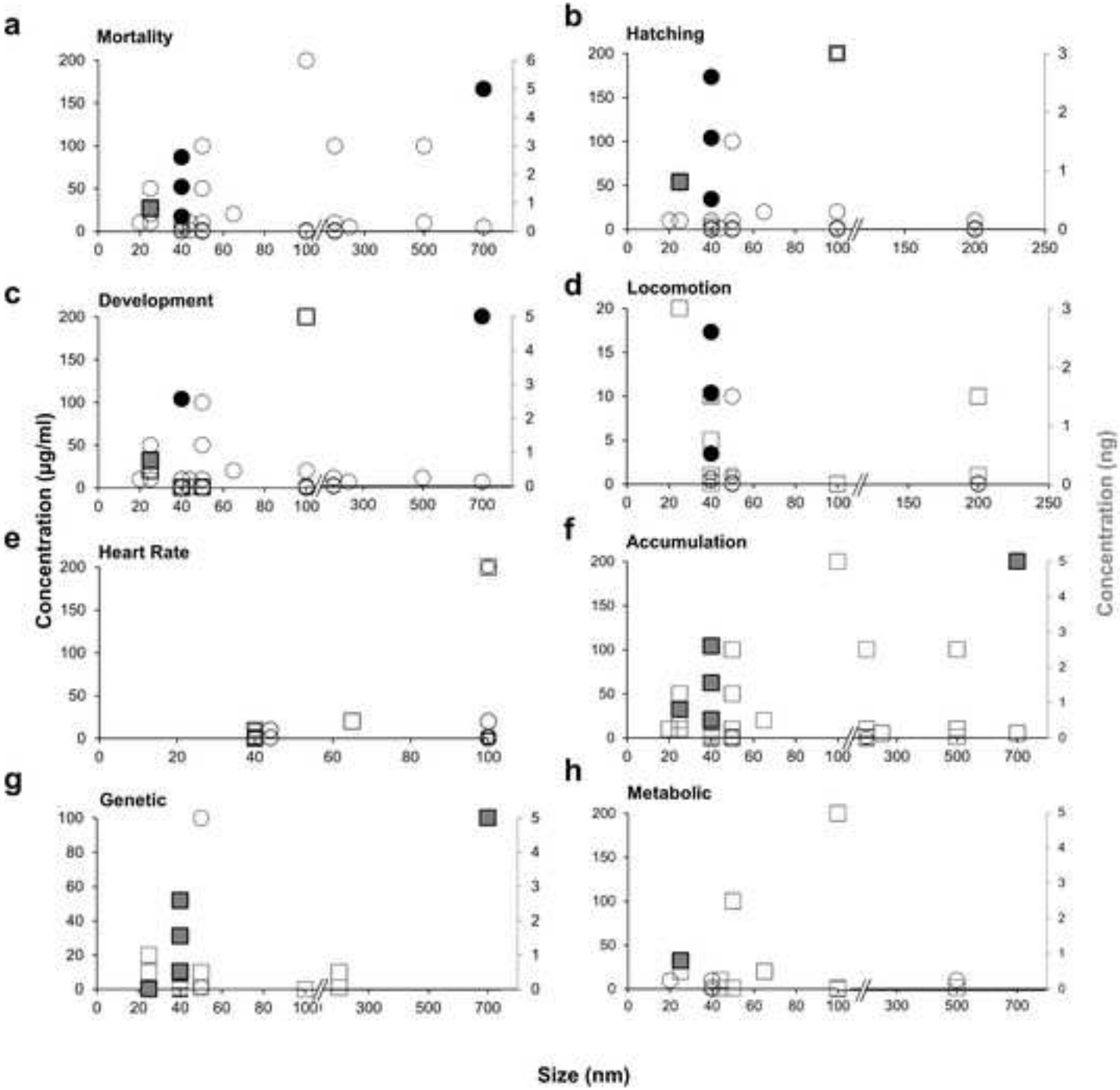


Figure 4

Click here to access/download;Figure;Torres-Ruiz M-Figure 4-130x90mm.TIF

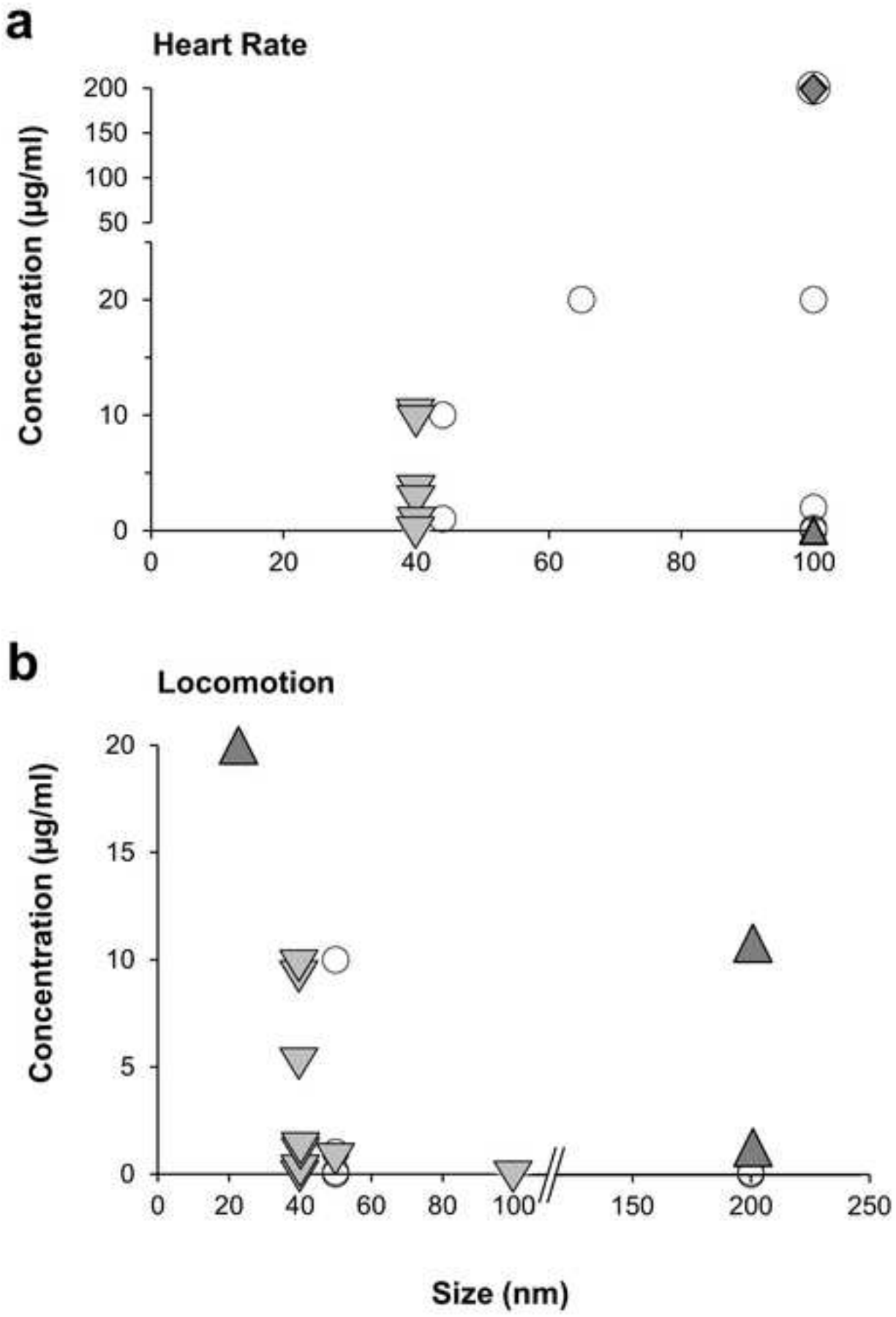


Figure 5

Click here to access/download;Figure;Torres-Ruiz M-Figure 5-115x90.TIF

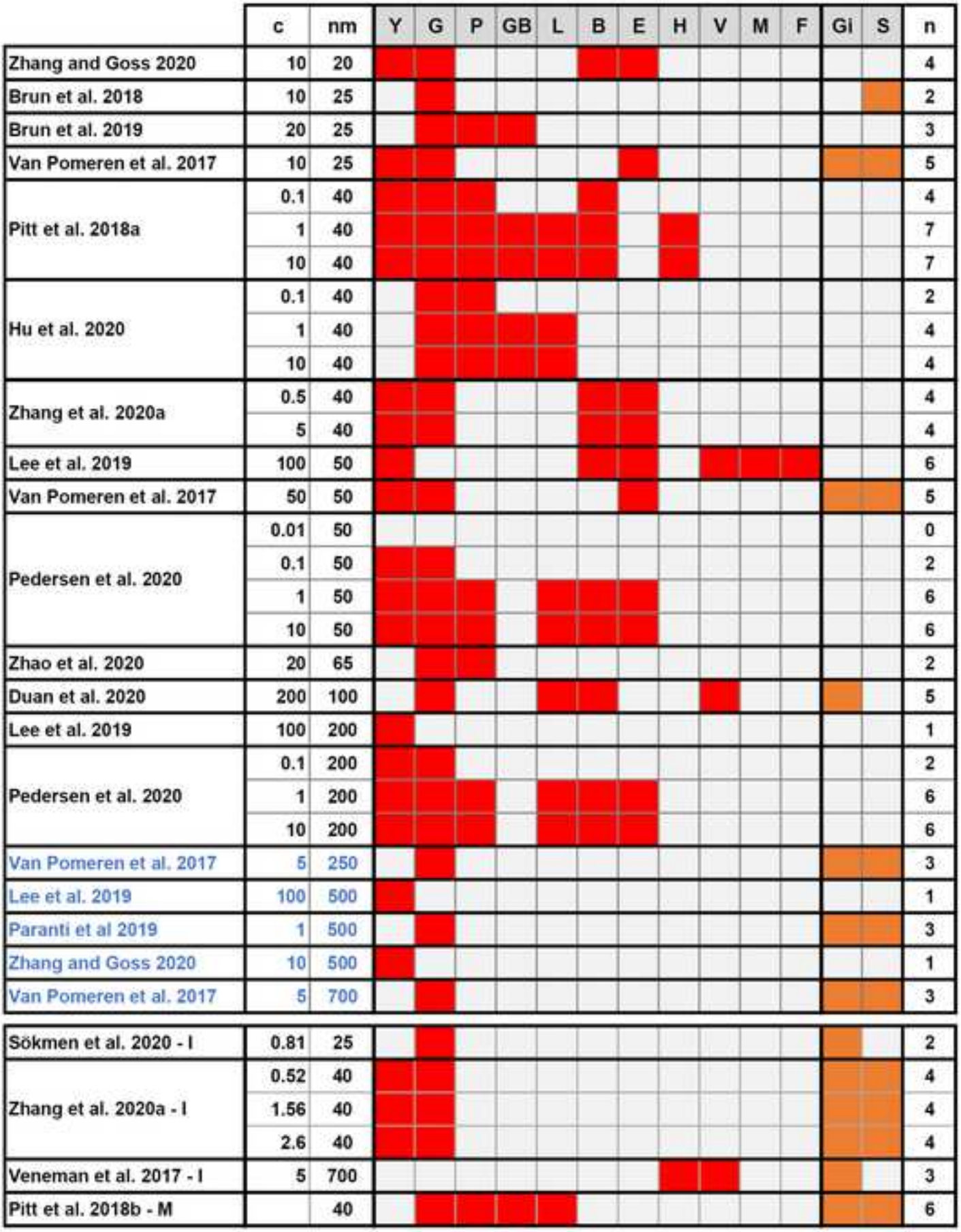


Figure 6

Click here to access/download;Figure;Torres-Ruiz M-Figure 6-169x90mm.TIF

Gene	U	NE	D	*n*	Classification
il1-β	3	1		*4*	Immunity
ccl20a	2			*2*	
ifn-γ	1			*1*	
il6	1	1		*2*	
socs3a	1	1		*2*	
mucms1		2		*2*	
tnf-α	1	1	1	*3*	
gpx	1			*1*	Response to stress
irg1l	1	1		*2*	
aox5	1	1		*2*	
gstp	1		1	*2*	
cat		2		*2*	
sod1	1	2	1	*4*	
gr			1	*1*	
sod2		1	2	*3*	
pck1	1			*1*	Carbohydrate metabolism
g6pca	1			*1*	
slc2a2		1		*1*	
fgf21			1	*1*	
ldha			1	*1*	
α-tubulin	1			*1*	Cytoskeleton
apoa2	1	1		*2*	Lipid metabolism
cyp19aa/a1b		1		*1*	Reproduction
try		2		*2*	Digestion
arrdc3a			1	*1*	G-prot mediated signalling
trpv6			2	*2*	Response to chemical
gfap	1	1	1	*3*	CNS process
mbp		1	1	*2*	
syn2-α		1	1	*2*	
slc6a4			1	*1*	
zfrho		1		*1*	Visual related genes
opn1sw2	1	1	1	*3*	
opn1lw2			2	*2*	
opn1mw1			2	*2*	
dnmt1		1		*1*	DNA methylation
dnmt3aa		1		*1*	
dnmt3bb1/2			1	*1*	

Figure 7

E or M	U	NE	D	*n*	Classification
ROS	3	1	1	*5*	Response to stress
SOD	1	2		*3*	
P-gp		1		*1*	
CAT		4		*4*	
GPx		3		*3*	
GST		1		*1*	
EROD		2		*2*	
GR			1	*1*	
COX			1	*1*	
GSH			1	*1*	
Thiol			1	*1*	
AchE	1			*1*	CNS function
TSH	1			*1*	Thyroid function
NADH	1		1	*2*	Energy metabolism
T3		1		*1*	Hormone
T4		1		*1*	Hormone

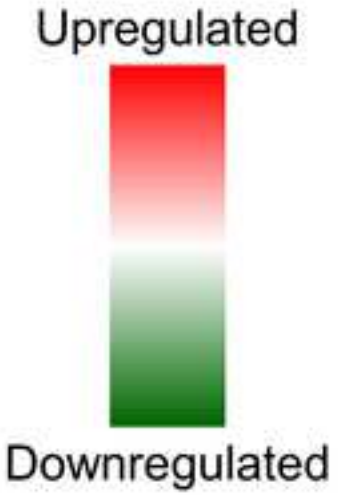
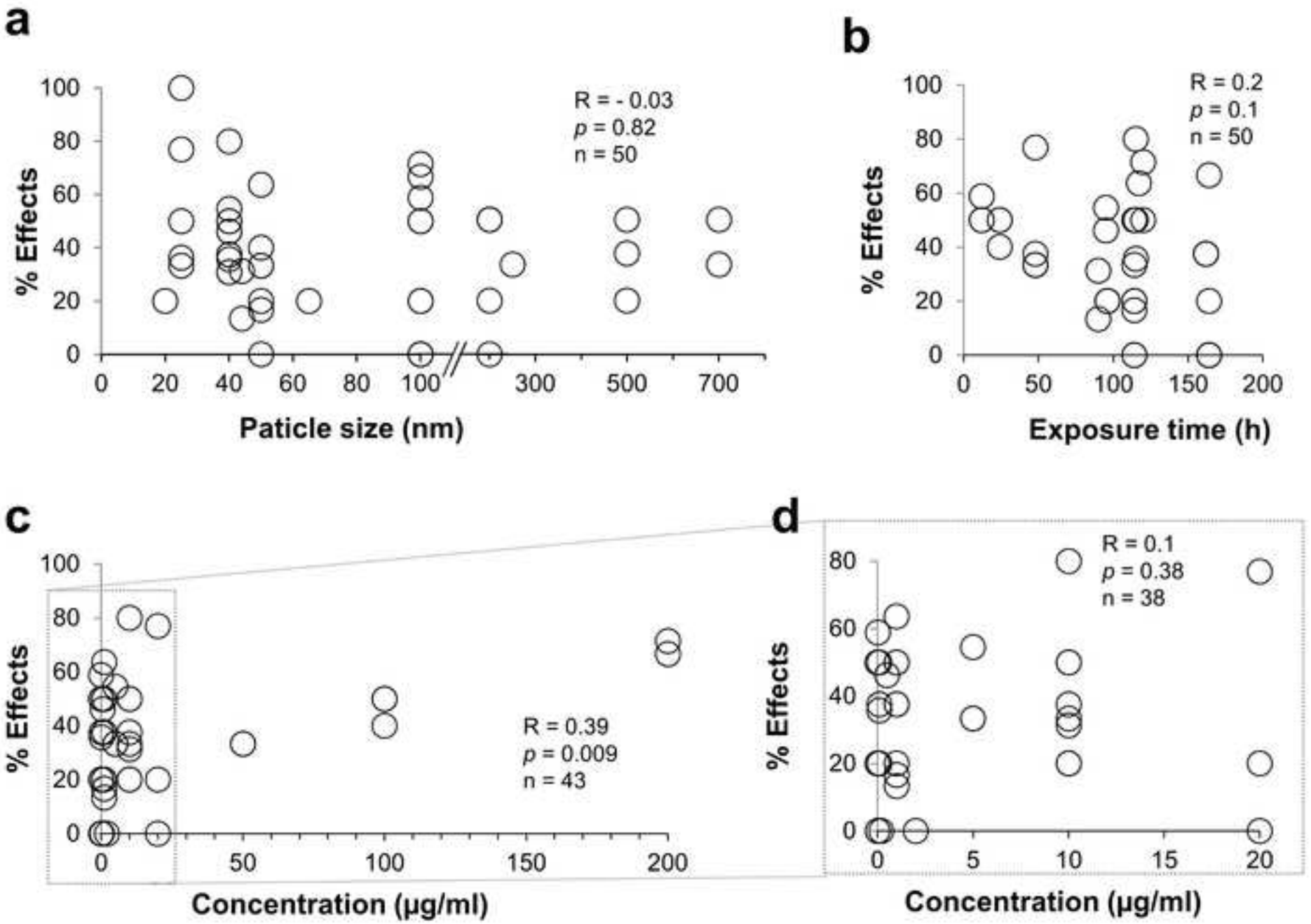


Figure 2



Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: