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Polystyrene Nanoplastic Exposure Adversely Affects Survivability of Zebrafish Larvae

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ABSTRACT

The amount of plastics in the environment has continuously increased since the successful synthesis of plastic from fossil fuels. Because of its high durability and stability, plastic has become a necessity in our modern life. However, these plastics break down into small pieces called Nanoparticles (NPs), and are not degraded. Several studies have shown that NPs are toxic to life forms because they could accumulate within living organisms, interfering with their physiological processes. Aquatic lives are more exposed to NP pollution, particularly polystyrene (PS), which is the major ingredient of Styrofoam, as most NP eventually end up in the ocean. However, the effects of NPs on marine vertebrates have not been fully explained yet. I have used zebrafish larvae as a model to assess the effects of NPs on the physiology of aquatic vertebrates. Using fluorescently labeled polystyrene (PS) nanoparticles (PSNPs), I found that sustained exposure of PSNPs to zebrafish larvae had negative effects on the physiology. PSNPs appear to accumulate in diverse regions of the zebrafish larvae, including the gills, eyes, and more importantly, the alimentary canal. Furthermore, sustained exposure to PS nanoparticles led to increased mortality in zebrafish larvae, potentially due to the accumulation in the alimentary canal. Considering that zebrafish share similar physiological characteristics and anatomical features with other commercially important species of fish, my data can be extended to estimate the potential physiological effects of PSNPs on aquatic vertebrates.

Introduction

Plastics are synthetic materials that are made up of high molecular compounds derived from fossil fuels. Due to its durability and plasticity, plastics have been used in various industries. The types of plastics that are mostly used in industrial production are polyethylene(PE), polystyrene(PS), polyvinyl chloride (PVC), polyamides(PA), and polyurethane. In 2021, more than 390 million metric tons of plastics were produced worldwide, and 90 percent of them were fossil fuel based (Plastics Europe, 2022). However, the waste of these products flows to the ocean and sea, causing serious harm to marine organisms, which is a serious issue these days. In aquatic environments, PS, is the most critical pollutant, as PS is the primary ingredient for expanded polystyrene foam, often referred to as Styrofoam which comprises the majority of marine debris (Ahrendt et al., 2023; Hipfner et al., 2018).

The effects of PSNPs exposure have been studied using both invertebrates and vertebrates, including Daphnia and zebrafish (Aljaibachi et al., 2020; Pitt et al., 2018; Torres-Ruiz et al., 2021). The research has shown that PSNPs exposure reduces the nutrition and health of Daphnia, shortening the lifespan of the Daphnia (Aljaibachi and Callaghan, 2018). Similarly, PSNPs exposure to zebrafish led to behavioral changes and increased mortality (Torres-Ruiz et al., 2021), suggesting that PSNPs can affect both invertebrates and vertebrates. More importantly, zebrafish that consumed PS-exposed Daphnia develop similar symptoms as zebrafish directly exposed to PSNPs, suggesting that PSNPs can pass through different species within the food chains (Chae et al., 2018; Siddiqui et al., 2023). While informative, previous research has two main limitations. First,

these researches tend to use an excess amount of PSNPs. Even though it provided a clear result of how acute exposure to highly concentrated PSNPs affected aquatic lives, the findings failed to provide an extensive picture of the effects of PSNPs within the environments. Second, the duration of PSNP exposure may not be relevant to the life cycle of aquatic life, in particular, for aquatic vertebrates. Since most of the study exposed PSNPs to fully grown fish or embryos, it failed to explain the effects on the vertebrate's transition phase. As the juvenile stage of fish is critical for their growth as well as fecundity, it is important to expand current research on this important stage.

I have utilized zebrafish as a model to overcome these limitations and fill up the current gap in our understanding of the effects of PSNPs exposure on marine vertebrates. The reason why I utilized zebrafish as a model is because first, zebrafish is an ideal model to investigate the effects of PSNPs on aquatic vertebrates as it offers unique benefits as a well-established model organism for genetic and developmental studies (Torres-Ruiz et al., 2021). As the zebrafish is the first aquatic vertebrate of which the genome has been completely sequenced, zebrafish could be used to identify specific target genes upon PSNP exposure, as well as its anatomical features have been fully annotated and analyzed against humans (Gore et al., 2018; Sprague et al., 2006). Second, individuals with identical genetic backgrounds can be obtained in a controlled laboratory setting, which was the best option for me (Gore et al., 2018). Therefore, using zebrafish juvenile, I wish to fill up the gap in the current understanding of how PSNP exposure affects aquatic vertebrates, and establish an aquatic model for assessing the effects of chronic PSNP exposure.

Hypotheses

I hypothesized that 1) PSNPs may accumulate within a restricted area of the zebrafish juvenile as it may interact with a specific cell type to attach to the zebrafish juvenile, and 2) chronic exposure to PSNPs adversely affects the physiology of zebrafish juvenile.

Statement of Purpose

The increase in plastic waste threatens the survival of aquatic life. While the effects of plastic exposure on early developmental stages or adult stage have been well-established, the effects of plastic exposure during the juvenile stage remains unknown yet. Therefore, the main purpose of this study is to clarify the effects of plastic waste exposure on the juvenile stage of aquatic vertebrates using zebrafish (Danio rerio) as a model.

Materials

Wild type Danio rerio embryos
Embryonic media
Fluorescently labeled polystyrene beads
Pronase
Micropipette
Tricaine
0.33mM of $MgSO_4 \cdot 7H_2O$
10L of distilled water
Microscope with camera
12 well plates
Buffered embryo media
Brine shrimp powder
Fluorescence microscope
0.33mM of $CaCl_2 \cdot 2H_2O$
0.1% of Methylene blue
50 U/mL penicillin

Methods

Preparation of Zebrafish Juvenile

Zebrafish embryos were obtained by mating the wild type (AB strain) as previously reported (Sprague et al., 2006). Embryos were synchronized 6 hours post-fertilization to ensure their developmental stages were identical. Dead embryos and any debris were removed to minimize the infection. Embryos were raised in the embryo media, containing 0.33mM of CaCl2•2H2O, 0.33mM of MgSO4•7H2O, 0.1% of Methylene blue, and 10L of distilled water (Sprague et al., 2006). To inhibit bacterial growth, 50 U/mL penicillin was added to the media. At 24 hours post-fertilization, embryos were treated with Pronase to remove the chorion as previously reported (Sprague et al., 2006). Embryos were submerged with embryo media containing Pronase for 30 minutes at room temperature and subsequently washed five times to remove the remaining Pronase. Subsequently, zebrafish embryos were allowed to develop into juveniles for 10 days. Since the zebrafish larvae started to digest external food sources 5 days after fertilization, fine brown shrimp powder was provided to zebrafish two times per day. Extra brine shrimp powder was removed by micropipette to minimize the bacteria grown on the media.

Treatment with Fluorescently Labeled Polystyrene Beads

The zebrafish juvenile at 5 days post fertilization were exposed to fluorescently labeled polystyrene beads. Two different concentrations of fluorescently labeled polystyrene beads, 0.1μM (100 mg/L) and 0.2μM (200 mg/L), were used to treat zebrafish juveniles. The number of dead juveniles was counted every day to increase the accuracy of the experiment.

Assessing The Phenotypes of Zebrafish Larvae

At 7 days post fertilization, after 2 days of treatment with fluorescently labeled polystyrene beads, zebrafish juveniles were anesthetized by Tricaine solution and examined with fluorescent stereomicroscope. Zebrafish larvae were assessed by 1) their body pigmentation, 2) their body length, 3) their total fluorescence within the body, 4) and their any obvious morphological defects such as a bent body or lack of eye development, and behavioral defects such as paralysis were assessed. Embryos showing such defects were divided by the total number of individuals to calculate the prevalence of each defect, and converted as a percentage for comparison.

Calculating The Survivability of Zebrafish Larvae Upon Long-Term Exposure of PSNPs

To assess the effects of PSNPs on survival of zebrafish juveniles, those at 5 days post-fertilization were treated with either control media or PSNP-containing media for 5 days. To examine the concentration dependent responses, two different doses of PSNP-containing media was used. Every day, the number of dead zebrafish juveniles in each well of 12-well plate were counted. The number of dead zebrafish juveniles was divided by the initial number of zebrafish juveniles in the 12-well plate to calculate the survivability for each condition.

Results

Zebrafish Larvae Can Absorb Fluorescently Labeled Polystyrene Beads

To determine whether zebrafish larvae can absorb the PSNPs, the zebrafish of 5 days post fertilization were exposed to different concentrations of fluorescently labeled PSNPs (Figures 1A to 1C). After 2 days of exposure, those of 7-day post fertilization seemed to have remaining fluorescently labeled PSNPs even after five times of washing process (Figures 1D). This implies that fluorescently labeled PSNPs can accumulate within the zebrafish juvenile. Additionally, as a result of researching the location where the PSNPs were accumulated, the surface of the body appeared to be the primary location for PSNP deposition, especially in the gill area. Therefore, it seems that the PSNPs attach to the specific type of body part and cell, which, in particular, is the surface of the body coated with protective mucus. In addition to the surface, retina, and gill, I also found strong fluorescence within the intestine and stomach of the juvenile zebrafish, assuming that fish may ingest polystyrene beads.

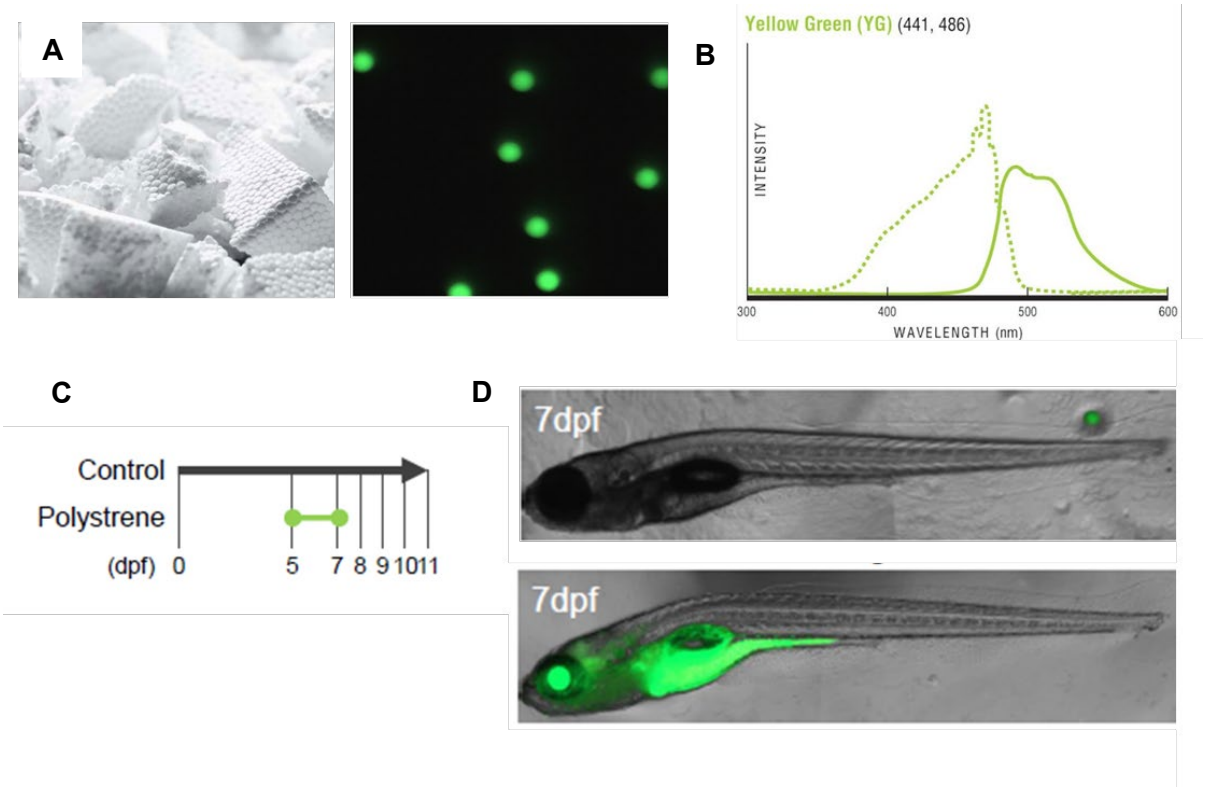

Figure 1. Presence of fluorescently labeled PS nanoparticles in zebrafish juvenile. Photomicrographs of polystyrene beads used in the experiments taken under the fluorescent stereomicroscope (A), and the absorption curve of the fluorescent PSNPs used in the experiments (B). Zebrafish juveniles were treated with PSNPs from 5 days post-fertilization to 7 days post-fertilization (C), when photomicrographs of embryos exposed to PSNPs were taken under either light or fluorescent stereomicroscopes. Fluorescent PSNPs were deposited on the surface of the zebrafish juveniles, such as retina, skin, and gills. In addition, fluorescent PSNPs were present in the alimentary canal.

PS Exposure Increases the Morphological Defects in Zebrafish Juvenile

To measure the effects of PSNP exposure on the zebrafish larvae, I have first measured the body length and pigmentation of the zebrafish juvenile at 7 days post-fertilization. Compared to the mock-treated zebrafish juveniles, those treated with PSNPs showed shortened body length and reduction of pigmentation (Figure 2). This could be related to malnutrition, as accumulated PSNPs in digestive systems disrupt the digestion of food. For instance, the body length of the PSNP-treated zebrafish juveniles appeared to be only 70 percent of the non-treated control zebrafish juveniles. Similarly, the coverage of pigmentation, which could serve as an indication for melanocyte development, was significantly reduced in those treated with PSNPs which means that PSNPs potentially have adverse effects on the zebrafish juveniles (Figure 2). In addition, PSNP exposure significantly increased the morphological abnormalities accompanied with changes in their motilities. For instance, PSNP exposure drastically increased the number of zebrafish juveniles with bent bodies. Similarly, the touch responses in the treated zebrafish juveniles attenuated to be reduced as the number of non-motile zebrafish juveniles was higher in PSNP-exposed zebrafish juveniles than in mock-treated zebrafish juveniles. Therefore, it appears that PSNPs exerted harmful effects on zebrafish juveniles.

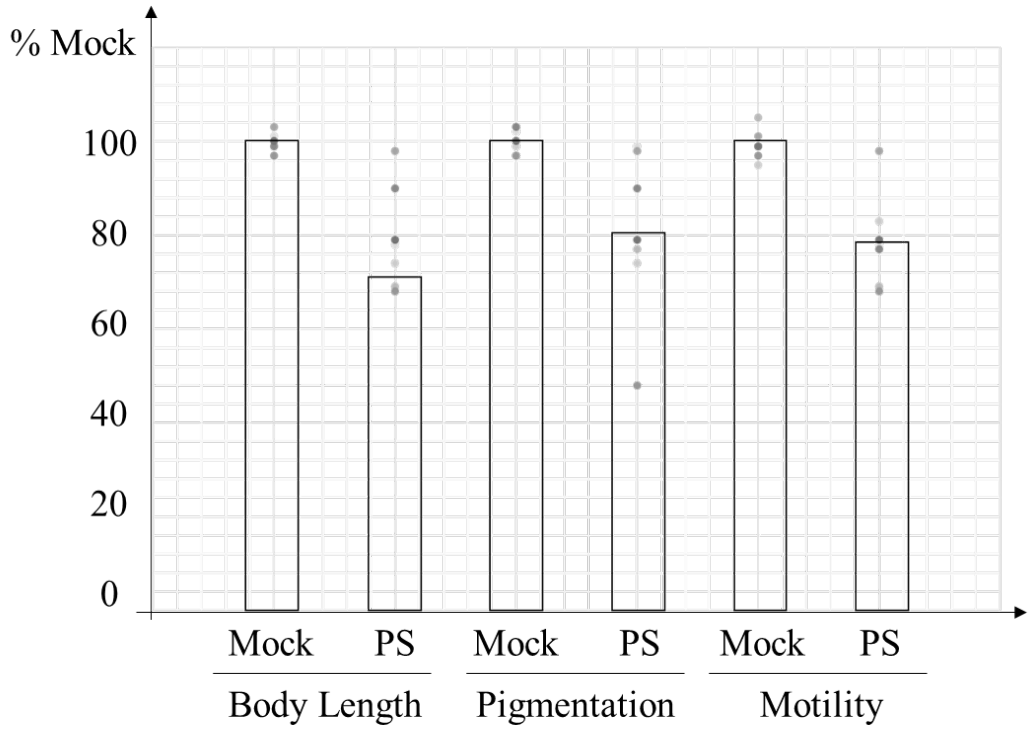

Figure 2. Effects of PS nanoparticles exposure on zebrafish juveniles. Zebrafish larvae at 5 days post-fertilization were treated with control media or PSNP-containing media and their morphology and motility were assessed at 7 days post-fertilization. In zebrafish juveniles exposed to PSNP-containing media had significantly reduced body length. In addition, the coverage of pigmentation was decreased and motility was reduced in these juveniles.

PS Exposure Decreased the Survivability of Zebrafish Juvenile

To see the toxic effects of PSNP exposure on zebrafish juveniles, I have calculated the survival rate of zebrafish juveniles over 5 days up to 10 days post-fertilization. While there were no noticeable differences in survivability between mock-treated and PSNP-exposed groups early on, the number of dead zebrafish juveniles increased over time in PSNP-exposed groups. For instance, nearly all zebrafish juveniles within the mock-treated group survived. However, in zebrafish treated with 0.1um, only 60 percent survived from that group, and in the group treated with 0.2 um of PSNPs was even lower. The toxic effects of PSNPs became apparent 2 days post-treatment, at 7 days post-fertilization. Therefore, it appears that the PSNPs exposure has harmful effects and eventually decreased the survivability rate in zebrafish juvenile. Moreover, as the concentration of PSNPs increases, the survivability rate also decreases, suggesting that the decreased survivability rate observed in PSNP-exposed zebrafish juveniles is due to the toxicity of polystyrene beads rather than other factors.

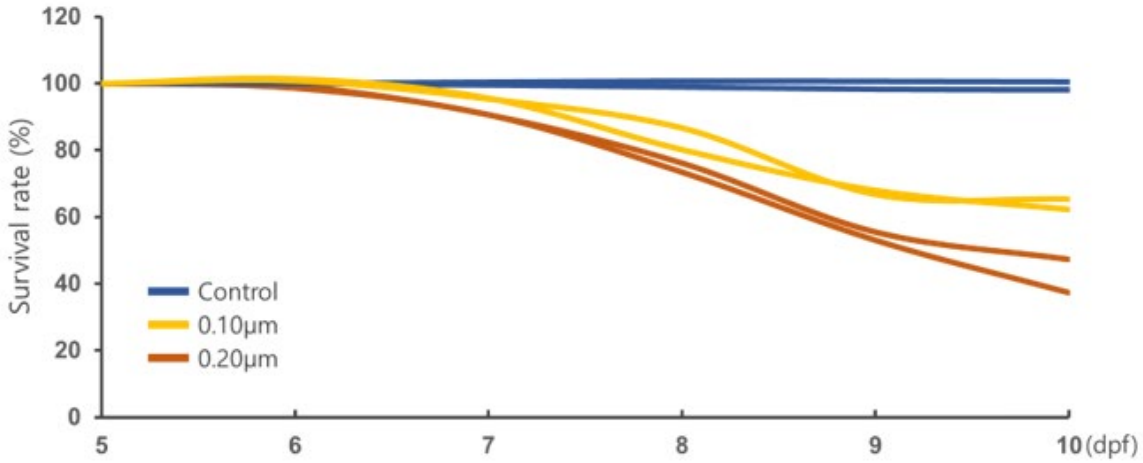

Figure 3. Survivability of zebrafish juveniles upon PS exposure. The survivability of zebrafish juveniles upon PSNP exposure was assessed. Zebrafish juveniles at 5 days post-fertilization was exposed to either control media or PSNP-containing media, and the percentage of free-swimming zebrafish juveniles per condition was calculated every day. As the concentration of PSNP increases, the survivability of zebrafish embryos decreased. Two independent sets were used for each condition.

Discussion

The hypothesis that PSNPs may accumulate within a restricted area of the zebrafish juvenile as it may interact with a specific cell type to attach to the zebrafish juvenile had to be rejected. Based on the experiments, it appears that PSNPs are attached to various regions within zebrafish juveniles, including internal organs such as the alimentary canal. Considering that PSNP deposition is mostly found on the face toward the outside of the body of zebrafish juveniles, it was not the evidence that PSNPs were attached to specific cell types. Considering the nature of the tissues that PSNPs were accumulated, it appears that PSNPs are largely absorbed by the epithelial cells, the flat-shaped cells lining the surface of the tissues where PSNPs were found. For instance, surface of alimentary canal as well as the integument of zebrafish juveniles are covered with epithelial cells (Warga

and Stainier, 2002). Therefore, it is tempting to speculate that PSNPs are recognized by a specific type of surface protein which is enriched in epithelial cells.

Based on my results, the second hypothesis that sustained exposure to PSNPs negatively affects the physiology and behavior of zebrafish juvenile cannot be rejected, as PSNP-exposed zebrafish juveniles tended to have short body, less pigmentation, and decreased touch responses. Since body length and degree of pigmentation could be used as surrogate measures for the developmental stage in zebrafish (Sprague et al., 2006), it appears that PSNP exposure either delayed the development of zebrafish juvenile or did harmful effects on the physiology of zebrafish juvenile. Moreover, PSNP exposure significantly decreased the survivability of the juvenile zebrafish.

My findings suggest that exposure to the PSNPs could be toxic to marine vertebrate juveniles. As the world production of plastics increases every year, and it flows to the marine ecosystem, it is understandable that PSNPs in aquatic environments will continue to increase and harm marine vertebrates, and other commercially important fishes. More importantly, as PSNPs appear to be transmitted through the food web, it is likely that the accumulation of PSNPs could end up in humans because we eat fishes caught from those marine environments. Therefore, reducing nanoplastic pollution is not only important for preservation of the environment, but also critical to reduce the potential risk of human exposure.

Conclusion

I have shown that PSNPs, which are prevalently present in the aquatic environments, exert harmful effects on zebrafish juveniles. The fluorescent PSNPs appear to be accumulated on the surface regions of the affected zebrafish juveniles. Most notably, the PSNPs were present in the alimentary canal along with eyes, skin, and gills. Exposure to PSNPs led to morphological and behavioral defects in zebrafish juveniles, indicating the toxic effects of PSNPs on developing aquatic vertebrates. Moreover, persistent exposure of PSNPs for 5 days significantly decreased the survivability of zebrafish juveniles in a dose-dependent manner. The higher concentration of PSNPs appears to be more toxic to zebrafish juveniles than the lower concentration of PSNPs. Considering that zebrafish is a widely used aquatic vertebrate model, it is likely that other aquatic vertebrates may display similar responses toward PSNP-exposure.

Limitations and Future Research

One of the most significant limitations of my research was the sample size. While it was better to have more sample size to obtain more accurate and precise results, the limited capacity, lab facility, and limited resources. Therefore, my research here should be taken as a pilot experiment for a larger scale operation. In addition, the criteria used for my analyses were limited. While it was possible to measure the body size, pigmentation of zebrafish juveniles, there were practical limitations in the delicate measure of organ development. Organ or tissue specific fluorescent transgenic lines are required for those measurements; we couldn't use those transgenic lines in conjunction with PSNPs. Therefore, it is necessary to scale up the sample size and include more measurements to accurately depict the effects of PSNP exposure in future.

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References

Ahrendt, C., Galban-Malagon, C., Gomez, V., Torres, M., Mattar, C., DeCoite, M., Guida, Y., Pribylova, P. and Pozo, K. (2023). Marine debris and associated organic pollutants in surface waters of Chiloe in the Northern Chilean Patagonia (42 degrees -44 degrees S). *Mar Pollut Bull* 187, 114558.

Aljaibachi, R. and Callaghan, A. (2018). Impact of polystyrene microplastics on Daphnia magna mortality and reproduction in relation to food availability. *PeerJ* 6, e4601.

Aljaibachi, R., Laird, W. B., Stevens, F. and Callaghan, A. (2020). Impacts of polystyrene microplastics on Daphnia magna: A laboratory and a mesocosm study. *Sci Total Environ* 705, 135800.

Chae, Y., Kim, D., Kim, S. W. and An, Y. J. (2018). Trophic transfer and individual impact of nano-sized polystyrene in a four-species freshwater food chain. *Sci Rep* 8, 284.

Gore, A. V., Pillay, L. M., Venero Galanternik, M. and Weinstein, B. M. (2018). The zebrafish: A fintastic model for hematopoietic development and disease. *Wiley Interdiscip Rev Dev Biol* 7, e312.

Hipfner, J. M., Lok, E. K., Jardine, C., Studholme, K. R., Belette Lebeau, A. C., Wright, K. G., Trefry, S. A., Drever, M. C. and Jones, G. (2018). Beach-cast debris surveys on Triangle Island, British Columbia, Canada indicate the timing of arrival of 2011 Tohoku tsunami debris in North America. *Mar Pollut Bull* 136, 407-413.

Pitt, J. A., Kozal, J. S., Jayasundara, N., Massarsky, A., Trevisan, R., Geitner, N., Wiesner, M., Levin, E. D. and Di Giulio, R. T. (2018). Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (Danio rerio). *Aquat Toxicol* 194, 185-194.

Plastics Europe (2022). Plastics-The Facts 2022.

Siddiqui, S. A., Singh, S., Bahmid, N. A., Shyu, D. J. H., Dominguez, R., Lorenzo, J. M., Pereira, J. A. M. and Camara, J. S. (2023). Polystyrene microplastic particles in the food chain: Characteristics and toxicity - A review. *Sci Total Environ* 892, 164531.

Sprague, J., Bayraktaroglu, L., Clements, D., Conlin, T., Fashena, D., Frazer, K., Haendel, M., Howe, D. G., Mani, P., Ramachandran, S., et al. (2006). The Zebrafish Information Network: the zebrafish model organism database. *Nucleic Acids Res* 34, D581-585.

Torres-Ruiz, M., De la Vieja, A., de Alba Gonzalez, M., Esteban Lopez, M., Castano Calvo, A. and Canas Portilla, A. I. (2021). Toxicity of nanoplastics for zebrafish embryos, what we know and where to go next. *Sci Total Environ* 797, 149125.

Warga, R. M. and Stainier, D. Y. (2002). The guts of endoderm formation. *Results Probl Cell Differ* 40, 28-47.