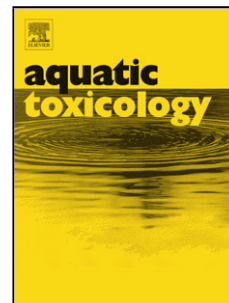


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Exploring uptake and biodistribution of polystyrene (nano)particles in zebrafish embryos at different developmental stages

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Highlights

- Only uptake (across epidermis) observed for particles smaller than 50 nm
- Small particles were detected to have biodistribution and even ended up in the eye
- Uptake of particles via chorion and epidermis is marginal
- Uptake should be monitored to provide more in depth toxicity information

Abstract

In ecotoxicology, it is continuously questioned whether (nano)particle exposure results in particle uptake and subsequent biodistribution or if particles adsorb to the epithelial layer only. To contribute to answering this question, we investigated different uptake routes in zebrafish embryos and how they affect particle uptake into organs and within whole organisms. This is addressed by exposing three different life stages of the zebrafish embryo in order to cover the following exposure routes: via chorion

and dermal exposure; dermal exposure; oral and dermal exposure. How different nanoparticle sizes affect uptake routes was assessed by using polystyrene particles of 25, 50, 250 and 700 nm.

In our experimental study, we showed that particle uptake in biota is restricted to oral exposure, whereas the dermal route resulted in adsorption to the epidermis and gills only. Ingestion followed by biodistribution was observed for the tested particles of 25 and 50 nm. The particles spread through the body and eventually accumulated in specific organs and tissues such as the eyes. Particles larger than 50 nm were predominantly adsorbed onto the intestinal tract and outer epidermis of zebrafish embryos. Embryos exposed to particles via both epidermis and intestine showed highest uptake and eventually accumulated particles in the eye, whereas uptake of particles via the chorion and epidermis resulted in marginal uptake. Organ uptake and internal distribution should be monitored more closely to provide more in depth information of the toxicity of particles.

Abbreviations

DLS: Dynamic light scattering; hpf: hours post fertilization; NP: Nanoparticle; PSP: Polystyrene particle; SD: Standard deviation; SM: Supplementary material; TEM: Transmission electron microscopy.

Keywords: *fluorescent polystyrene particles; oral exposure; fish embryos; uptake route; dermal exposure*

1. Introduction

Nano- and microparticles of varying sizes are increasingly detected in aquatic ecosystems (Gottschalk et al., 2013). Once in the environment, biota are exposed to particles and may subsequently be adversely affected, although field studies about these effects are scarce and complicated to perform (Schaumann et al., 2015). To assess the origin of the effects from small particles, a better understanding of uptake routes and internalization is required.

Particles can enter organisms via various ways: via the epidermis and/or gills (dermal exposure), via the gastrointestinal tract (oral exposure) or via inhalation. This latter exposure route is only applicable for organisms with lungs, whereas the first two apply to all multicellular organisms. To our knowledge, it is still unknown whether in *in vivo* exposures particles cross one of these barriers and which barrier they cross most effectively. Forced uptake routes via injection directly in the blood system (Fenaroli et al., 2014) or in muscles (Speshock et al., 2016) as used e.g. in cancer research, can lead to metallic particle distribution through the whole body. This knowledge about biodistribution emphasizes

the importance of understanding the factors that determine whether or not (metallic and polystyrene) particles cross the cell membrane barrier of the epithelial layer.

Studies linking waterborne exposure of whole organisms to target organs and biodistribution report that particles in the GI tract are common (Kashiwada et al. 2006). Particles are not only taken up from the surrounding medium, but can also accumulate from the food. Based on their experimental data, Kalman et al. (2015) even stated that in freshwater exposures, uptake of metallic nanoparticles (MNPs) via ingestion of exposed food can have major effects on higher organisms and induces more severe effects than via waterborne exposure. This was also argued by Jackson et al. (2012) when the amphipod *Leptocheirus plumulosus* fed on Cd/Se quantum dot exposed algae showed more severe lethal effects of Cd/Se quantum dots as compared to waterborne exposure. Both Cederval et al. (2012) and Mattsson et al. (2015) exposed fish to polystyrene nanoparticles (PS NPs) by feeding them *Daphnia magna*, which in turn were fed NP-contaminated algae. These studies showed that polystyrene NPs administered via the food altered brain texture and water content indicating biodistribution towards the brain. Also for TiO₂ NPs and carbon nanotubes it was found that accumulation occurred within multiple consumer species (Yeo and Nam, 2013).

The route of particle uptake and subsequent target organ are hitherto uncertain, and especially the impact of exposure route on uptake is to be further substantiated. In this study, we investigated if polystyrene (nano)particles adsorb to the intestine or epidermis, or if the particles are taken up and internalized. Thereby we explore if this is related to different sizes of PSPs. We defined internalization as absorption, thus cellular uptake of particles. Uptake is defined as particles being found within the organs and/or tissue of the organism. The word 'biodistribution' is used to describe the process of particles trafficking through cells and organisms. Ingestion is not considered uptake inside the body, only presence in the GI tract.

We investigated the major uptake routes that determine uptake into organs and biodistribution within whole organisms. In this experiment we use the development of zebrafish embryos to obtain 3 different uptake routes: uptake via chorion and epidermis; uptake via epidermis; and uptake via epidermis and intestine due to ingestion of the particles, to answer the following research questions:

(1) Is the exposure route important for (nano)particle uptake in zebrafish embryos and is this influenced by size? And (2) does the uptake route determine the target organ?

For the first research question, we hypothesize that oral uptake plays an important role, since uptake over an internal mucosal membrane occurs faster than over an intact dermal epithelial membrane. On the other hand, the chance of particle penetration is proportional to the surface area of

the epidermis. Based on previous observations (Kashiwada et al., 2006; Rosenkranz and Chaudhry, 2009; Manabe et al., 2011) we hypothesize that uptake of particles increases with decreasing particle size.

The second research question is based on previously detected target organs of particles in fish. Particles that enter the blood stream via the intestine pass the hepatic portal vein and are often detected in the liver (Connolly et al., 2016; Jang et al., 2014; Kashiwada, 2006; Pan et al., 2013). Therefore we hypothesize that particles taken up via oral exposure target the liver, whereas particles taken up via dermal exposure might target other organs.

2. Materials and Methods

2.1 Preparation of particle suspensions

Fluorescent polystyrene particles (PSPs) of 25 and 50 nm in H₂O were purchased from ThermoFisher Scientific (Catalog number R25 and R50 resp.; Waltham, USA) and particles of 250 and 700 nm in H₂O were purchased from Corpuscular Inc. (Catalog number 103127-05 and 103129-05 resp.; Cold Spring, USA), both with a density of 1.05 g/cm³. Exposure solutions were prepared by adding the purchased stock solutions to egg water (60 µg/ml Instant Ocean Sea Salt, Sera GmbH, Heinsberg, Germany). Immediately before exposure, the solutions were freshly prepared and sonicated for 10 min using an ultrasonic water bath (USC200T, VWR, Amsterdam, The Netherlands).

2.2 Physicochemical characterization

Transmission electron microscopy (TEM; JEOL 1010, JEOL Ltd., Tokyo, Japan) was used to characterize the size and morphology of the PSPs after 1 hour of incubation in egg water. Due to the chemical properties of polystyrene, the contrast of especially the 25 nm PSP TEM images was limited. Dynamic light scattering (DLS) assessments were performed on a Zetasizer Nano-ZS instrument (Malvern Instruments Ltd, Malvern, UK) to detect the size distribution and zeta-potential of PSP suspensions in egg water at 0 h and 24 h.

2.3 Experimental setup

2.3.1 Zebrafish husbandry

Zebrafish were handled as described by animal welfare regulations and maintained according to standard protocols (<http://ZFIN.org>). Adult zebrafish were maintained at 25 ± 0.5 °C in a 14 h light : 10 h dark cycle. Fertilized zebrafish eggs were obtained from an AB/TL wild-type zebrafish.

2.3.2 Exposure of zebrafish embryo life stages to PSPs

In order to test the importance of different uptake routes, three different exposure starting points were tested. Developing zebrafish embryos undergo different stages: at first the embryo is protected by the chorion, followed by the second stage in which the mouth of the hatched embryo is closed until the third stage in which the morphogenesis is completed and oral uptake and excretion are fully functioning. This provides the opportunity to test the three uptake routes as mentioned in the introduction. This can also be seen in Table 1. Zebrafish embryos were exposed in 24-well plates and an exposure regime of 48 hours was maintained within each group (n=10). Particle suspensions and egg water were renewed every 24 hours. Particle concentrations were selected based on pilot tests (data not shown) and are given in Table 1. The concentration selected showed clear visibility of the particles without observations of negative effects in the embryos. Temperature was maintained at 28 ± 0.8 °C during the experiments.

2.3.3 Microscopy

Prior to the experiments, pilot studies were executed with a 2 photon confocal laser microscope and a stereo fluorescent microscope (Supplementary material, SM) in order to determine the most suitable method for visualizing adsorbed, ingested or biodistributed particles. Since the visualization of the 2-photon confocal laser microscopy was limited to a few cell layers hampering NP tracking, the experiment was conducted with a stereo fluorescent microscope capturing the whole embryo.

Zebrafish embryos were examined and imaged daily during the exposure to check fitness (malformations and mortality) using a fluorescence stereo microscope (M205 FA, Leica). During the final examination, embryos were rinsed three times with egg water and kept under anesthesia (0,02% Tricaine, Sigma) in egg water. Fitness was not affected during this inspection. Embryos exposed until 48 hours post fertilization (hpf) were manually dechorionated prior to examination (Henn and Braunbeck, 2011).

2.3.4 Eye size measurement

From exposure regime 3, a minimum of 4 zebrafish embryos were imaged per exposure group using the fluorescence stereo microscope. Eye width and length were measured using the image processing package Fiji (Schindelin et al., 2012).

2.4 Statistical analysis

Significance ($p < 0.05$) for effects on eye-size among the different treatments was tested using a one way ANOVA with the SPSS 23 software package. Results are given as mean \pm standard deviation (SD).

3. Results

3.1 Physico-chemical characterization of polystyrene particles

TEM images showing the size of the PSPs after 1 h of incubation in egg water are given in Figure 1. All PSPs were spherical, and only small clusters of less than 4 particles were detected in the samples.

According to the TEM pictures, actual and nominal sizes of the particles at T0 deviated less than 2-5%. The 25 nm particles had an actual average size of ca. 27 nm. The average size of the 50 nm NPs was measured to be 50 nm. The 250 and 700 nm particles had a diameter of 217 and 727 nm, respectively. From the DLS measurements (Table 2) it can be concluded that the 25 and 700 nm particles behaved slightly differently in the exposure medium of the zebrafish embryos as compared to the behavior of the 50 and 250 nm particles. The 25 nm NPs tended to form agglomerates over time. Suspensions of the 50 and 250 nm particles appeared stable, whereas the suspensions of the 700 nm PSPs were found to agglomerate directly after preparation. The clusters of PSPs formed declined in size over time. It is also apparent that the 25 and 50 nm NPs were present in clusters of a few particles within the samples at both T0 and T24, whereas the DLS measurements of the 250 and 700 nm PSPs indicated that only single particles were present after 24 hour. It should be noted that due to the fluorescence properties of the particles, the DLS measurements might be less accurate than for non-fluorescent particles. The zeta potential measurements indicated that the 25 and 50 nm particles were negatively charged and maintained the zeta potential over time. In contrast, 250 nm particles became less negatively charged over time, and the 700 nm particles became more negatively charged. These zeta potentials indicate that the 25 and 50 nm particles will form agglomerates within 24 h, whereas the 250 and 700 nm particles will remain a stable particle at 24 h which is also shown in the DLS results.

3.2 Uptake and biodistribution of PSPs

Uptake and biodistribution of the differently sized PSPs were monitored after 48 h of exposure starting at three different time points (based upon hpf), of which representative pictures are shown in Figure 2. Per group, all embryos ($n=10$) showed the same degree of uptake. The embryos exposed directly after fertilization (regime 1) had no particles taken up. In fact, all NPs were adsorbed to the chorion. The embryos exposed after 24 hpf (regime 2) displayed particle adsorption to the epidermis and no uptake

of PSPs was observed (Figure 2). As can be seen from Figure 2, uptake of PSPs was observed only when the exposure started at 72 hpf (third exposure regime, at which oral and dermal exposure is possible). The smallest particles of 25 and 50 nm were taken up by the embryo between 72 and 120 hpf, as shown by their accumulation in the eye (Figure 3). The 250 and 700 nm PSPs were found only in the digestive tract and absorbed to the gills of the exposed zebrafish embryos and were not found in the eye.

3.3 Eye size measurement

The eye size of embryos in which PSPs were present in the eye was compared with the eye size of embryos in which PSPs were absent in the eyes. No significant difference in eye size was found between any of the treatments and the control (see Figure 4). Thus, the PSPs present in the eye did not affect ocular development.

4. Discussion

In this contribution, the first question we aimed to answer is whether the exposure route is important for the uptake of particles. Differentiation between uptake routes in nanotoxicology is a yet unexplored avenue of research and only a few studies distinguish oral uptake from other uptake routes. Since exposure via food (here defined as oral exposure) also resulted in metallic particles in the gills (Connolly et al., 2016), the difference between oral exposure and full body exposure becomes undistinguishable. Irrespective of the exposure route, only particles adsorbed in the intestine were further biodistributed (Connolly et al., 2016; Jang et al., 2014; Kashiwada, 2006; Pan et al., 2013), suggesting that the epithelial layer is an important route for particle uptake and biodistribution. Our results underline the importance of the oral exposure route and its impacts on internalization of particles. When embryos were exposed solely via the dermal route, PSPs adsorbed on the epidermis and gills. However, no PSPs were detected inside the embryos, indicating that oral exposure is the most important route for PSP uptake in zebrafish. Subsequently this means that embryonic stages after 72 hpf are most suited for studying internalization and uptake of particles from waterborne exposures. The results indicate that especially for higher organisms, oral exposure is the major route of uptake and source for subsequent internal distribution of particles.

Besides the exposure route, other factors are found to be important for particle uptake. Most commonly, size is reported to influence uptake, with a restricted particle size of maximal 50 nm (Bisesi et al., 2014; Manabe et al., 2011). This is confirmed by the results of our experimental study as only the tested particles equal to or smaller than 50 nm were taken up into the embryos and particles equal or larger than 250 nm were not found to be taken up in the eye of embryos. The larger particles (≥ 250 nm)

were not able to penetrate body tissue in any of the exposure scenarios. Even more, large particles cannot cross the epidermis-barrier or intestine lining and are also often too large to be consumed by the organisms (Fent et al., 2010; Ronneberger, 1998; Van Hoecke et al., 2013). Here we show that particles of up to 700 nm can be ingested by zebrafish embryos.

Not only size, but also other factors can influence uptake of particles, such as the surface characteristics of the particle (Albanese et al., 2012; Fröhlich, 2012). In our experiments, the negative surface charge of the PSPs might have prevented uptake via the relatively large pore channels (500 to 700 nm, Lee et al., 2007) of the chorion, since the chorion showed to be an effective barrier to all sizes of our PSPs. Also the chorion of the Japanese medaka (*Oryzias latipes*) is capable of protecting recently fertilized embryos from intruding polystyrene NPs smaller than 50 nm (Kashiwada, 2006). Also other studies found only size-dependent effects after hatching, with no effects prior to hatching (Lee et al., 2007). Surface charge of the PSPs might have resulted in large adsorption to the chorion. It can be speculated that transport of particles over the chorion occurs only when the chorion has reached its adsorption maximum (maximum strength to bind particles). Another possibility is that large clusters of particles, which were adsorbed to the chorion, hampered passage through the pore channels. Yet, at a later stage of development, while still being protected by the chorion, particles were found internalized in the medaka (Kashiwada, 2006). Since medaka embryos have a longer developmental time whilst still being in the chorion (hatching around 216 hpf ;Iwamatsu, 2004) than zebrafish embryos (hatching around 72 hpf), exposure over a longer time period might allow NPs to cross the more slowly thinning chorion and to enter the organism.

The second question we aimed to answer in this article is whether the uptake route influences the target organ. In our study we show an exposure route dependent effect on uptake. Our tested particles larger than 50 nm were not taken up, even though they were found to distribute through the intestine when the mouth opens. In contrast, accumulation of small PSPs (≤ 50 nm) in the eyes was observed. This was only observed after exposure of embryos via the dermal + oral route (while the mouth was open), whereas this accumulation was not observed when the mouth was still closed i.e. under conditions of dermal uptake only. Although particles might have been taken up via the maturing epidermis of the zebrafish, the exposure route is most likely via the gastrointestinal tract as direct dermal exposure induced no uptake of particles.

Particles tend to accumulate in specific organs after uptake (Handy et al., 2008; Zhu et al., 2012). In general, most particles are found to accumulate in the liver of fish (Connolly et al., 2016; Jang et al., 2014; Kashiwada, 2006; Pan et al., 2013). This observation is according to expectation, since the liver is

primarily used to clear the body from toxicants. Other organs are also targeted by particles, since particles were found to be distributed throughout the whole body – including the brain – once the particles have entered the blood stream or lymphatic system of vertebrates (Jung et al., 2014; Kang et al., 2015; Reisch and Klymchenko, 2016). In our experiments, particle exposure via the intestine and epidermis led to accumulation in the eye of the zebrafish. It is not clear if transport occurred via the blood stream or via the lymphatic system, or that uptake occurred after epidermal exposure, since there were no particles detected in either system. Over time, the free PSPs might be further distributed through the body at a later stage of development and accumulate in other organs such as the brain. This distribution of particles was found for other particles, and for instance Ag NPs were located in both the eye and the brain of zebrafish embryos (Lee et al., 2012). However, for metallic NPs that dissolve in aquatic and biological media it is hard to distinguish between the biodistribution of the NPs and the ions. Lee et al. (2012) focused at the signal of the particles, but often total internal Ag concentrations are measured with no distinction between ions and particles (Jang et al., 2014). Not only particles shedding off ions, but also stable NPs were found to be distributed within an organism. For instance presence of polystyrene NPs altered the structure of the brain from adult rainbow trout and subsequently influenced their behavior (Mattsson et al., 2015) indicating that NPs not only have short term effects, but also long term behavioral effects (Wang et al., 2016).

An important part in understanding the significance of uptake and internalization of PSPs are the biodistribution and elimination possibilities (Vijver et al., 2006). Investigation of clearing mechanisms in zebrafish embryos is limited due to short assay time span of 120 h. However, in adult zebrafish most particles are excreted from the body via the liver, spleen and gall bladder (Boyle et al., 2013; Jung et al., 2014; Kashiwada, 2006; Zhao et al., 2011). Excretion from cells and subsequently from tissues occurs at a much slower rate than uptake, which can occur within half an hour (particles found intracellularly; Fang et al., 2011). For carbon quantum dots, the fastest excretion time (in which all of the particles were cleared from the body) of internalized particles was 56 hours (Kang et al., 2015). Thus it can be expected that once accumulated, PSP may be excreted again. However, if this is actually the case and in what time span remains to be investigated.

5. Conclusions

The exposure route influences the uptake and target organ of particles in zebrafish embryos. The three different uptake routes we tested (chorion and epidermis; epidermis; epidermis and intestine via ingestion) provided insight in the contribution of uptake routes to actual uptake. Our data suggest that

the predominant uptake route of PSPs was the oral route, while dermal uptake only marginally contributed to uptake and subsequent biodistribution. Therefore, the time window between 72 and 120 hpf is of importance in zebrafish embryo exposure. The main physicochemical factor accounting for uptake, internalization and further distribution was particle size. Particles with a diameter of 25 and 50 nm were the largest particles found to be taken up in the eye. Accumulation in the eye might either originate from outer epidermal exposure or from uptake through the intestinal epidermis and subsequent internal biodistribution. Organ uptake and internal distribution should be monitored more closely to provide more in depth information of the toxicity of particles. As a future perspective, we suggest that measurements of the biodistribution and depuration of NPs may become a useful sub-lethal endpoint. Simultaneous examination of biodistribution and depuration endpoints will provide insight in the bioaccumulation capacity of particles by biota.

Additional file 1: Supplemental material.docx

Competing interests: The authors declare that they have no competing interest.

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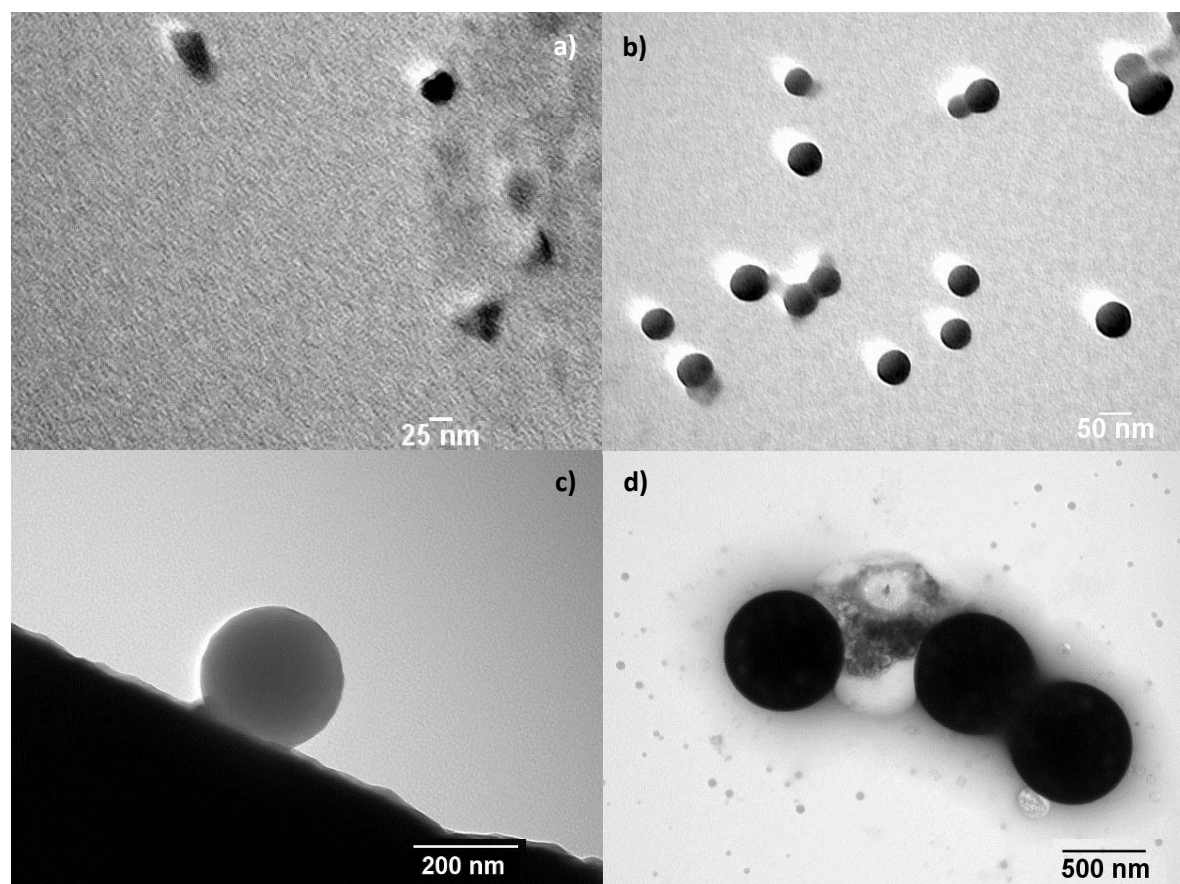


Figure 1. TEM image of a) 25 nm, b) 50 nm, c) 250 and d) 700 nm PSPs.

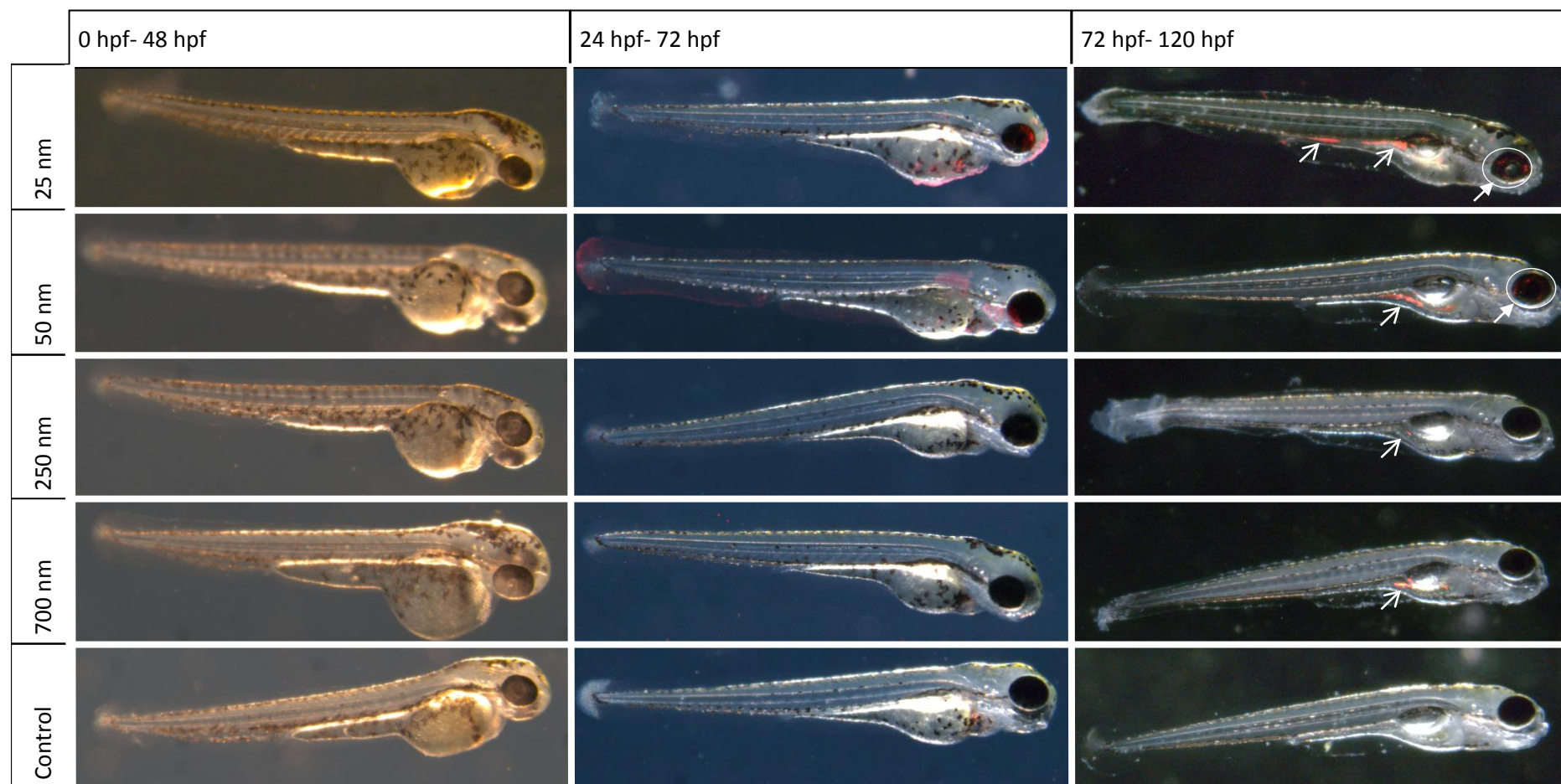


Figure 2. Images of fluorescent PSPs in zebrafish embryos. For each of the four PSPs of different size, a representative picture of each group after the exposure regime is given. Open arrows show PSPs in the digestive tract, closed arrows indicate PSPs in the eye of the zebrafish

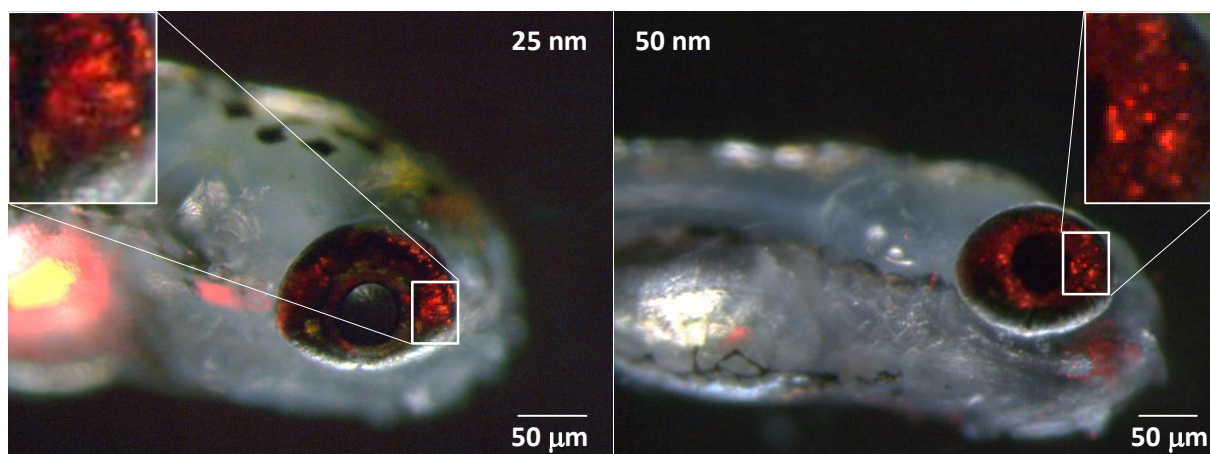


Figure 3. Images of fluorescent PSPs in the eye of zebrafish embryos after exposure to 25 nm and 50 nm PSPs exposed from 72 till 120 hpf. The inset is providing further details using 10 times magnification.

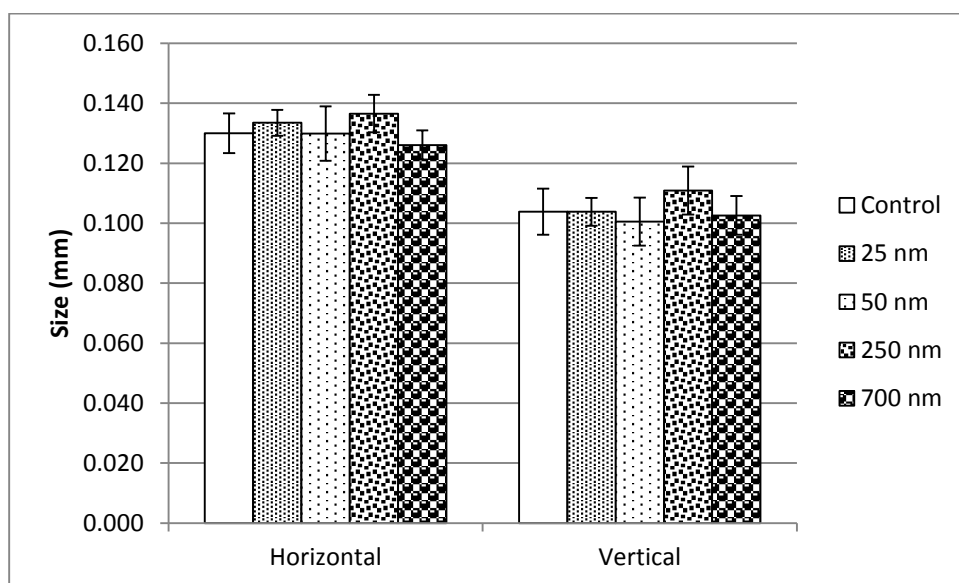


Figure 4. Eye size measurement of embryos exposed to different sized PSPs from 72 till 120 hpf (n=4). Vertical and horizontal refer to the measurement direction.

Table 1. Overview of the different exposure regimes indicating the age of the embryos expressed in hours post fertilization (hpf), the targeted uptake routes and nominal concentrations used. 'Dechorionated' indicates that the embryos were manually dechorionated before exposure.

Regime	Developmental stage (hpf)	Uptake route	Nominal concentration (mg/L)	
			25 / 50 nm	250/700 nm
1	0 - 48	Chorion and dermal uptake	25	5
2	24 - 72 (dechorionated)	Dermal uptake	50	5
3	72 - 120	Oral and dermal uptake	50	5

Table 2. Overview of the zeta potential and size distribution after 0 and 24 h of exposure. In case of size distribution, the two dominant peaks in size are given with the first peak representing the highest number of counts.

Particle	Zeta potential (mV \pm SD)		Size distribution by DLS (nm \pm SD)			
			0 h		24 h	
	0 h	24 h	Peak one	Peak two	Peak one	Peak two
25 nm	-5.1 \pm 2.0	-6.1 \pm 1.1	38.1 \pm 7.1..	134.8 \pm 53.9....	125 \pm 5.4 ..	32.0 \pm 1.0
50 nm	-15.8 \pm 5.2..	-19.5 \pm 1.5..	70.6 \pm 18.1	3408.3 \pm 2952.0	67.0 \pm 1.7	5338.3 \pm 118.4
250 nm	-20 \pm 1.4.....	-9.7 \pm 2.9	298.4 \pm 24.7..	-	277.7 \pm 13.6	-
700 nm	-17.4 \pm 8.9..	-31.9 \pm 2.2..	943.6 \pm 108.2	-	735 \pm 57.6...	-