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Master's thesis

The toxicity profiles of variously sized micro- and nanoplastics in Schmidtea mediterranea

Stoffel Wirges

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Karen SMEETS

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ABSTRACT

Plastic litter exists in many forms, including tiny fractions called microplastics and nanoplastics (MNPs). Consequently, MNPs contaminate our soils, water bodies and sediments. Therefore, this project assessed the effects of exposure to various sizes of spherical MNPs, derived from polystyrene (PS), on (neuro)regeneration and stem cell dynamics in *Schmidtea mediterranea*. Regenerating worms were exposed to particles of 200 nm, 1 µm and 2 µm in diameter for seven days after amputation. General mitotic activity, stem cell acitivity, and neuroregeneration were assessed through H3P staining, Fluorescent in situ hybridization, and anti-synapsin. General regeneration was quantified by measuring the sizes of freshly regenerated body surface, called the blastema. The strongest adverse effects were present in brain development at middle and high concentrations. However, at low exposure levels, brain development was slightly promoted. Mitotic activity is significantly lowered at high concentrations of 200 nm and 1 µm particles, while the larger size (2 µm) showed no significant effects. Nevertheless, stem cell activity was significantly lowered after exposure to both 1 µm and 2 µm plastics. This research suggests that underlying mechanisms may exist that accelerate regeneration at low exposure levels, while high concentrations have adverse effects. Moreover, different toxicity profiles are addressed in 200 nm, 1 µm and 2 µm PS particle sizes, negatively impacting neuronal development and stem cell dynamics.

1. INTRODUCTION

Plastics have revolutionized our daily lives within the last few decades, as they are incredibly versatile materials (1). They are inexpensive, lightweight, strong, durable, corrosion-resistant, and have high thermal and electrical insulation properties (1). The diversity of polymers and the versatility of their properties facilitate the production of a wide variety of plastic products that bring technological advances, energy savings and numerous other societal benefits (1). Globally, plastic production exceeds 300 million tons every year. Due to their increased functionality associated with low production costs, plastic production is expected to vastly increase over the following decades. (2,3). Such excessive production entails the need to improve waste management (4). Currently, 30% of plastic waste is recycled, meaning that a massive amount of plastic waste is either incinerated or dispersed into the environment (2). An estimated 4.8 to 12.7 million metric tons of plastic enter the oceans annually, equivalent to a truckload of plastic every minute (5). Furthermore, marine-degradable plastics have a negligible market share and are not poised to make headway in the consumer plastics market (6).

Plastic litter is found in various sizes in the environment, including tiny fractions called microplastics and nanoplastics (MNPs). MNPs are collective terms to describe a mixture of plastic particles ranging from a few microns to several millimetres in diameter (7,8). Although universal definitions regarding MNPs sizes are currently lacking, microplastics are often described as

plastics smaller than 5 mm, whereas particles smaller than 100 nm are usually referred to as nanoplastics (9). The particles can originate from a variety of sources. Primary MNPs enter the environment directly as small particles. For example, these include particles deliberately manufactured for commercial uses or due to the release of rubber tire wear (8). In contrast, MNPs produced through the environmental degradation of larger debris into smaller pieces are described as secondary MNPs (10). The formation of secondary MNPs can result from reactions with UV light, making the material brittle (11). Moreover, after dispersion into the ocean, they could degrade due to mechanical friction with waves and interactions with marine organisms or bacteria (10).

Plastics of <5 mm in size have been documented in almost every open ocean and enclosed seas habitats, including beaches, surface waters, water columns, and the deep seafloor (8). Furthermore, they are detected in freshwater systems, including riverine beaches, sediments of rivers, lakes, and reservoirs (12-14). Although less studies are available compared to marine environments, it is mostly highlight that microplastics are ubiquitous and have comparable concentrations (15). Therefore, it is highly likely that organisms will encounter these emerging contaminants, resulting in potential ecotoxicological consequences. Accurate quantities of nanoplastics in the environment are unclear because there are no effective methods for the determination, quantification and assessment (16). *In vivo* studies have suggested that MNPs penetrate organisms via various routes, i.e. skin or respiratory tract, although most studies have found uptake of particles in the digestive tract (17,18). There are no pathways available for the breakdown of synthetic polymers (19). Instead, after ingestion, they are either egested or are accumulated within specific tissues like gills, gut, digestive gland and the circulatory system (20-23). Therefore, many organisms are contaminated by MNPs, including bivalves and fishes that are marketed for human consumption (24). This implies that the potential impacts are not limited to the ecosystem but also to food safety, as fish are an important protein source for humans (25). Additionally, the consumption of contaminated prey can lead to the transfer of MNPs, to higher trophic levels, a process called biomagnification (26). Recently, evidence has been reported that microplastics could reach the human food chain through these food web interactions (27). Nevertheless, the MNPs propagation pathways in complex food webs are yet to be identified to assess the risk of potential biomagnification accurately. Microplastics have also been detected in bottled and tap drinking water (28). As for nanoplastics in drinking water and food, there is no information available (28,29). In addition to diet, inhalation is a potential exposure method to MNPs in humans, as a result of airborne particles arising from sources like waste incineration, industrial emissions and particles released from traffic (30-32). However, the evidence base for both dietary and airborne microplastics exposures is inadequate to accurately estimate the human daily intake of MNPs (33). Albeit, microplastics were recently found in human intestines and for the first time in placental tissue (34).

The negative impacts of large plastic items, such as plastic bags, fishing nets and plastic fragments, on birds, turtles and marine mammals are well documented. The consequences of entanglement in the debris or ingestion of plastic items are considered to be the most harmful (35-37). In humans, it is generally believed that large plastic polymers are inert and are not absorbed by the intestinal system due to their size. Therefore, they are excreted and not metabolized (38). On the contrary, the adverse effects of MNPs are less well understood. The physicochemical properties of particles in the nanoscale are entirely different from bulk materials, including surface area composition, chemical composition, solubility and aggregation (39). Generally, as the particle size decreases, diffusion through membranes is facilitated, resulting in an increased uptake (40). Moreover, as the relative surface area increases, a higher proportion of atoms occupies the surface resulting in an increased reactivity with biological tissue (39,40). Considerable research has been conducted using laboratory experiments to quantify MNPs' physical and chemical impacts on organisms after exposure. In the freshwater species zebrafish, recent studies demonstrated molecular responses and histological alterations in gonads; oxidative stress and pro-inflammatory responses in the gut; negative impacts on survival rate; downregulation of genes associated with neuronal functioning,

neuron differentiation and axonogenesis. (41-43). In soil species *C. elegans*, studies reported neuronal damage and increased oxidative stress (44). In marine bivalves, MNP exposure induced neurotransmitters and gene expression alterations and aggravated neurotoxicity in combination with other toxicants (45). Information on whether the current studies translate to humans is lacking (33). Although, the adverse effects reported in different species imply the importance of fully understanding the degree of MNPs' environmental toxicity.

Developing and regenerating organisms are hypothesized to be more susceptible to environmental pollution (46). Development and regeneration show certain similarities, as they are both highly integrated processes, coordinating high proliferation and differentiation rates with programmed cell death (47). Injured organisms must have the regenerative capacity to restore tissue functions and morphology to survive (48). Moreover, the developing nervous system is qualitatively different and more vulnerable than the adult nervous system in animals and humans (49). Throughout an animal's lifetime, stem cells play an essential role in tissue development and regeneration (50). Mesenchymal stem cells have the regeneration ability to heal tissue injuries and repair organ functions through various mechanisms (51). In addition, mesenchymal stem cells can constantly self-renew and generate progeny. These progenies can undergo terminal differentiation to participate in body development (52). Only few studies have examined the potential interference of MNPs with tissue growth and regeneration and stem cell functions *in vivo*; in planarians, it was demonstrated that exposure resulted in a delayed regeneration, inhibition in proliferation and differentiation of stem cells and a decreased proportion of mitotic cells (46).

Planarians are flat, free-living organisms of the phylum Platyhelminthes or flatworms belonging to the order Tricladida. They are restricted mainly to marine and freshwater environments, where they are typical representatives of benthic communities (53). The freshwater planarian *Schmidtea mediterranea* has an excessive capacity to regenerate tissue due to a large pool of pluripotent stem cells called neoblasts; the planarian can regenerate the complete gut and nerve tissue (54). Regeneration can easily be induced by cutting the planarian into fragments. Then, freshly regenerated tissue is called the "blastema". The blastema lacks pigment, making it appear white, whereby the regeneration rate can be quantified (54). Therefore, as certain effects demonstrated in regeneration can estimate developmental effects due to the similarities of the mechanisms, planarians become the ideal *in vivo* model for ecotoxicological studies on the development of, e.g. central nervous system and stem cell dynamics (55). As a complete regeneration process of the nervous system takes approximately seven days, the duration of these experiments is relatively short, making the planarian a preferred choice over other developing animal models (54). Moreover, as planarians are invertebrates, they avoid any ethical debate (56). They are easily cultured in large quantities through binary fission and are susceptible to environmental pollution (57). Additionally, as planarians live in the benthic zone of water bodies, using their motile cilia to glide across the sediment surface, they frequently come into contact with deposited MNPs, providing a representative animal model for studying the effects of MNPs on aquatic organisms (58,59).

In this project, regenerating *Schmidtea mediterranea* will be exposed to variously sized spherical MNPs dissolved in an aqueous medium. Polystyrene (PS) microspheres were adopted as model particles to explore the effects of MNPs, as PS is one of the most produced polymers and is commonly found in the aquatic environment (60). Three sizes of pristine PS particles will be used; 200 nm, 1 µm and 2 µm (from here on, referred to as 200 nm PS, 1 µm PS and 2 µm PS). The objectives are to determine the potential adverse effects of MNPs on the general regeneration, neuroregeneration and cell-cycle dynamics. Furthermore, we will describe the differences in toxicity profiles of the different sizes.

2. EXPERIMENTAL PROCEDURES

2.1. Animal cultivation

This study used the asexual strain of the freshwater planarian species *Schmidtea mediterranea* as a model organism. Planarians were continuously kept in the dark in a cultivation room with a constant temperature of 20 °C. They are held in boxes filled with growth medium consisting of millipore water containing the following nutrients: NaCl (1.6 mM), CaCl2 (1 mM), MgSO4 (1 mM), MgCl2 (0.1 mM), KCl (0.1 mM), and NaHCO3 (1.2 mM). Every once week, animals were fed with veal liver. Before using them for experiments, all animals were starved for at least seven days.

2.2. Particle characterization

Commercially available non-fluorescent, carboxylated, PS plastics were purchased from Magsphere Inc (Pasadena, California). Transmission electron microscopy (TEM) analysis for size and form was performed as described in Verleysen et al. (61). The automated ParticleSizer software was applied to asses constituent particle dimensions, based on the protocol 'Measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer Software' (Nanodefine, 2016).

2.2. Planarian exposure

In this research, *Schmidtea mediterranea* was exposed to exposure media in 6-well plates with various concentrations of PS MNPs in different sizes and concentrations given in Table 2.1. MNPs were dissolved in the aforementioned growth medium, creating the exposure medium. First, particles were vortexed for 30 seconds, then sonicated for five minutes in a sonication bath at 37 kHz, and vortexed for 30 seconds again. Each well contained five adult regenerating worms and five milliliters of exposure medium. For every condition, two wells were made, giving each condition ten individual samples. The exposure duration was seven days. A control group was included for each exposure, consisting of growth medium without plastics. Exposure media were refreshed every two days or three days over the weekend. Regeneration was induced by transversally cutting an adult worm in two halves with an ethanol-sterilized blade above the pharynx (mouth part), creating a head and tail part.

2.3. General tissue regeneration

To quantify effects on general regeneration capacity, blastema growth was studied. Images were taken seven days after amputation with a Nikon SMZ600 camera mounted to a binocular loupe and using Nikon NIS Elements software. Blastema sizes were measured using ImageJ and are calculated as a size relative to the whole-body surface.

2.4. Neuroregeneration: anti-synapsin 3C11

To determine the effects on neurodevelopment, samples were immunostained with anti-synapsin 3C11, staining the ventral nerve chords and cephalic ganglia. During waiting steps, samples were always placed on a shaker at room temperature unless stated otherwise. Antibody dilutions are in 1% Bovine albumin serum (Sigma Aldrich) and PBST (1% BSA/PBST). Regenerated worms were transferred to a 24-well plate and killed in 2% HCL in millipore water on ice, washed in PBST (10 min.), and fixated in 4% formaldehyde in PBST (4% FA/PBST). After two rinses with PBST, the samples were bleached overnight under a lamp in an aluminum foil-covered box, in a bleaching solution containing 6% H_2O_2 in PBST. The next day, samples were washed in PBST twice for 10 min. and a blocking solution containing 1% bovine albumin serum (BSA) in PBST was added for four

Table 1: the rising concentrations used for the three differently sized particles used

MNP diameter	***Low exposure***	***Middle exposure***	***High exposure***
200 nm	0,1 µg/ml	10 µg/ml	20 µg/ml
1 µm	2,5 µg/ml	12,5 µg/ml	50 µg/ml
2 µm	1 µg/ml	10 µg/ml	20 µg/ml

hours at 4°C. After blocking the samples were washed in PBST (10 min.) and primary antibody (Mouse anti-synapsin 3C11: 1:50, Developmental Studies Hybridoma Bank, developed by Buchner E.) was added for 18-20 hours at 4°C. Next, samples were washed 10 x 10 min. in PBST at 4°C and 1% BSA/PBST was added for 1 hour. The second antibody (Alexafluor goat anti-mouse 488, 1:400, Invitrogen) was added for three hours whereafter they were washed 6 x 10 min. in PBST. After mounting in ImmuMount (Thermo Fisher Scientific), samples were imaged using a Nikon Ds-Ri2 camera placed on a Nikon eclipse i80 fluorescence microscope. During each experiment, imaging settings were unchanged. Brain widths were measured using Image J and were calculated as relative to the head width.

2.5. Stem cell dynamics: H3P

The mitotic activity of stem cells in reaction to MNPs exposure was determined by immunolabelling anti-phospho-histone H3 (H3P). The staining process is similar to staining Anti-synapsin 3C11 (crf. 2.4.1.). Differences are briefly described. After the animals are killed Fixation is done in Carnoy (60% MetOH + 30% chloroform + 10% acetic acid), bleaching is done in 6% H_2O_2/MetOH, whereafter they are rehydrated with 75%, 50% and 25% MetOH/PBST respectively for 10 min. each, and twice more in PBST for 10 min each. The primary antibody is rabbit anti-phospho histone H3 (1:600, Cell-Signalling) and the secondary antibody is Alexafluor goat anti-mouse 488 (1:400, Invitrogen). During mounting, there was no preference regarding the dorsal side or ventral side. Mitotic cells were counted using Image J and were calculated relative to body surface area.

2.6. Fluorescent in situ hybridization (FISH)

To perform FISH, the planarians were killed in 5% NAC and fixated in 4% FA/PBST, whereafter they were rinsed in PBST, 50% MetOH/PBST and 100% MetOH. After rehydrating the samples, they were incubated in 1x saline-sodium citrate (SSC) and bleached in a formamide bleaching solution containing 5% non-deionized formamide, 0.5% SSC and 1.2% H_2O_2. Then, samples were incubated in 1% SSC, rinsed in PBST, and incubated in a proteinase K (Thermo Fisher Scientific) solution (PBST, 10% sodium dodecyl sulfate, 10 µg/ml prot. K) for 5 minutes. Immediately after, the samples were incubated in 4% FA/PBST fixative and rinsed with PBST. This was followed by an incubation step in 50% prehyb/PBST, and in 100% prehyb. The latter was at 56 °C and for two hours. Then, the probe, being *smedwi-1* (forward primer: 5' GTGACGCAGAGAAACGGAAG 3', reverse primer: 5' TTGGATTAGCCCCATCTTTG 3', Reddien et al. 2005), with a concentration of 1 ng/µL solved in hyb was added to the samples which incubated overnight at 56 °C. The next day samples were rinsed in 2% SSC and in 0,2% SSC times, both four times for 20 minutes at 56°C. Then, they were rinsed in a TNTx buffer solution (0,1 M Tris, 0,15 M NaCl and 0,3% Triton X-100, pH = 7,5) twice and incubated in a blocking solution (5% horse serum and 0,5% Roche Western Blocking Reagent diluted in TNTx) for two hours. The secondary antibody being anti-DIG-POD was then added, diluted in blocking solution (1:500, Roche) and incubated overnight at 4°C. From here on samples were treated in the dark as much as possible. The last day, the samples were first rinsed, then washed six times for 20 minutes in TNTx. Then, a Tyramide signal amplification (TSA) mix was made by dissolving FAM fluorescein (1:500), 4-iodophenylboronic acid (1:100, Sigma-Aldrich) and H_2O_2 (0.003%) in TSA buffer (2 M NaCl and 0.1 M boric acid; pH = 8.5; sterilized) and added for 15 minutes. Then, samples were washed twice for five minutes, both in TNTx and PBST. Hoechst 33342 (ThermoFisher, nuclei) diluted in PBST (1:5000) was added for 3 hours at 4°C, wherafter samples were mounted in ImmuMount. Fluorescence intensity was measured using ImageJ and was standardized relative to body surface area.

2.7. statistical analyses

Statistical analyses was performed using the R studio statistical software. Data was tested for normality with Shapiro-Wilk. Homoscedasticity was assessed using Levene's test. When assumptions were not met (normality, homoscedasticity) the data was log-transformed. Oneway ANOVA was performed followed by a TUKEY post-hoc for multiple comparisons. P-values < 0,05 were considered significant.

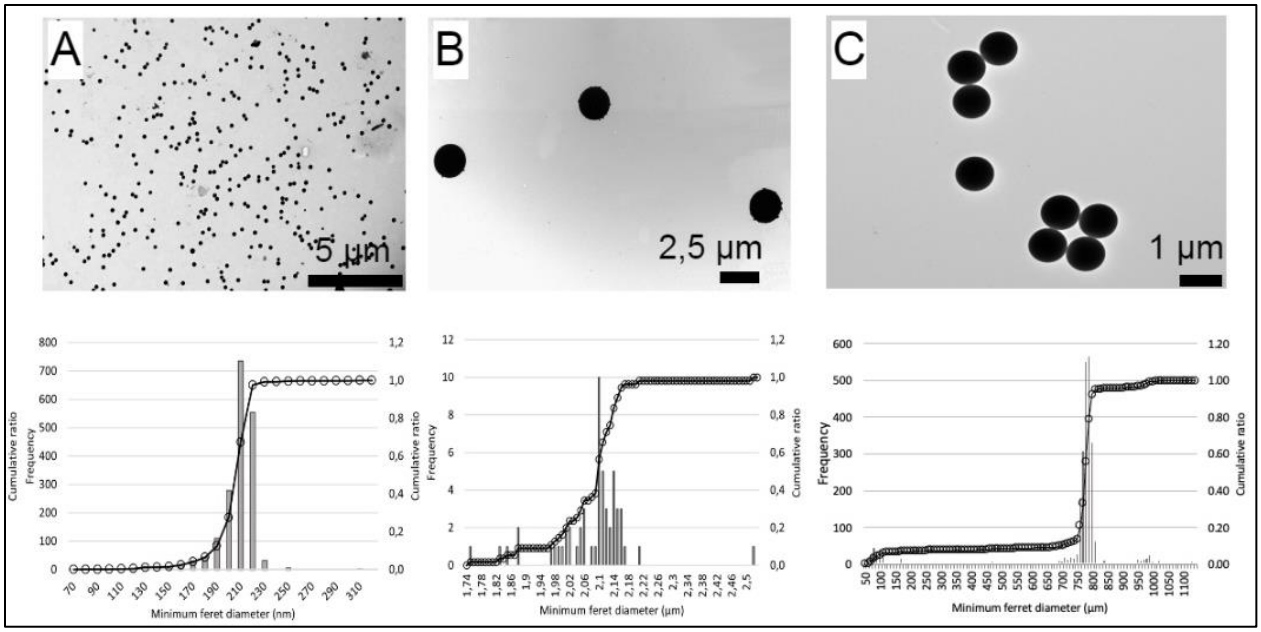

Figure 1: characterization of the PS MNPs by TEM. TEM images of (A) 215 µm PS, in this project referred to as 200 nm PS (scale bar = 5 µm), (B) 2 µm PS (scale bar = 2,5 µm), (C) 1 µm PS (scale bar = 1 µm). Histograms show the cumulative frequency distributions of particle minimum Feret diameters.

3. RESULTS

3.1. TEM analysis

TEM micrographs are shown in figure X. The minimum Feret diameter of constituent particles used confirmed the sizes and spherical shapes.

3.1. General regeneration

3.1.1. Blastema size

To quantify the effects of the different sizes of PS particles on general regeneration of *Schmidtea mediterranea*, the blastema sizes were measured. The average blastema sizes of each condition are given in figure 2.a. After seven days of exposure to the smallest PS particles used in this project, being 200 nm in diameter, showed no significant effects. However, in the head fragments, one trends is observed (p-value < 0,1). As the particle's concentration is introduced at the low level, the blastema size was slightly higher than at medium levels. As comparing with the control group however, no effects were observed. The samples exposed to PS particles of 1 µm in diameter showed no significant differences as well. Moreover, trends are not present in the data. Lastly, in the head fragments of the planarians exposed to 2 µm ¨PS particles, no significant differences were detected. In the tail fragments, the high exposure group had the lowest blastema surface, being significantly lower than the other groups at a significance level of 0,05.

3.1.2. Eye development

In the tail fragments, the ability of the sample to develop eyes in seven days was reported (fig 2.b.) Notably, after exposure to 200 nm PS particles the amount of tail fragments with developed eyes gradually lowered as the concentration raised. This was also the case with 1 µm PS exposure, however, the low exposure group had the fewest samples with eyes developed. Tail fragments exposed to 2 µm PS had the least amount of eyes developed at the lowest exposure group, with the highest exposure group being second. Both the control and the middle exposure group had a 100% eye developed ratio.

3.2. Neurotoxicity

3.2.1. Synorf

In order to determine the effects of PS MNPs exposure on neurodevelopment, the newly regenerated nervous system of *Schmidtea mediterranea* was studied after seven days after amputation. Furthermore, the ability to develop an anterior commissure (AC), which is the connection of the two brain lobes, was recorded. The relative

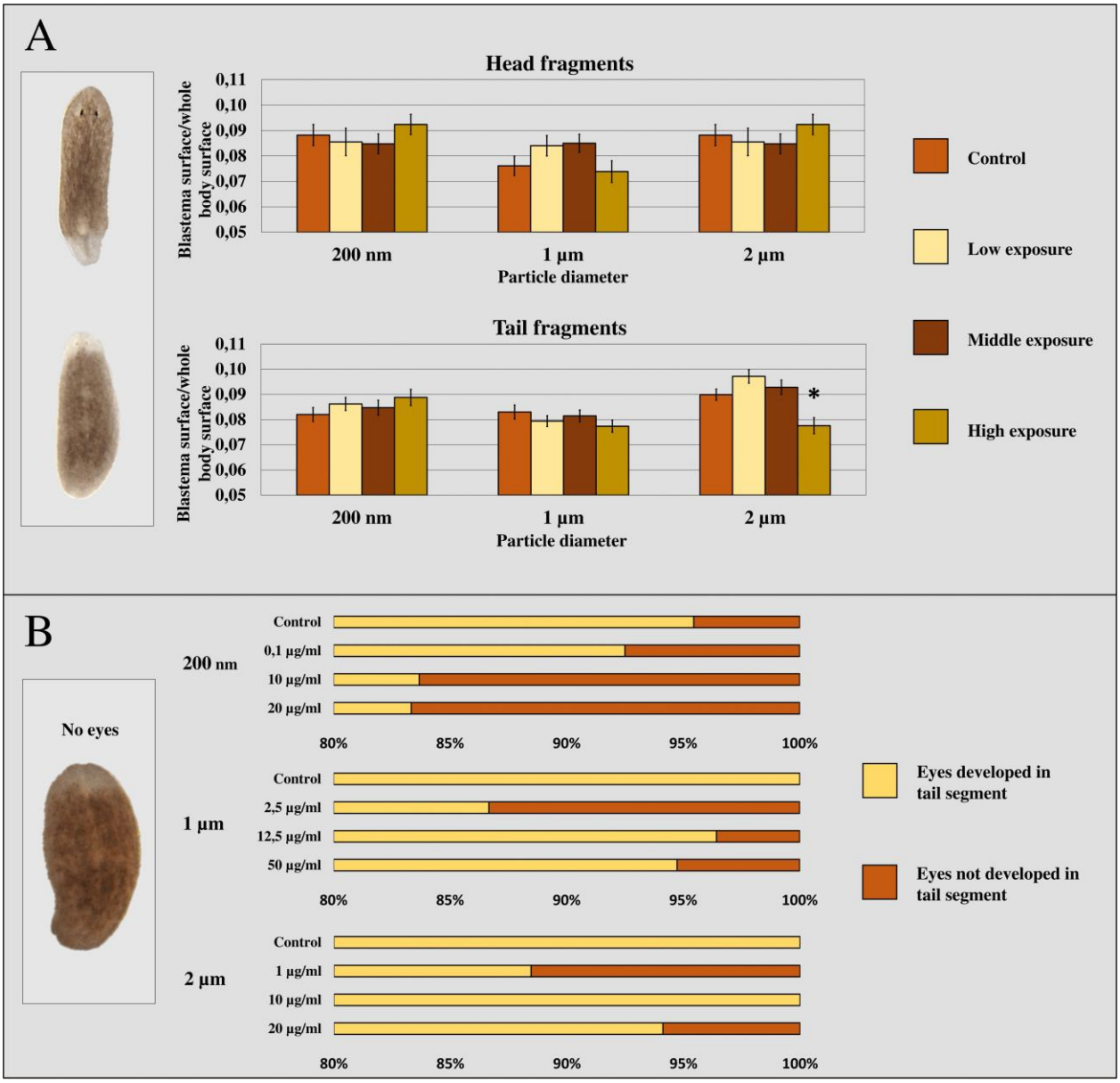

Figure 2: Phenotypic effects on general regeneration of regenerating *Schmidtea mediterranea* after seven days of exposure to PS MNPs. **A)** Histograms representing the relative blastema surface areas, calculated by dividing the blastema surface area by the whole sample's surface area. Upper graph shows the head fragments, lower graph shows the tail fragments. The Y axis shows the relative surface area. The X-axis is divided in three parts representing the diameters of the PS particles used, which is then further divided in the four groups of exposure levels. From left to right these are control, low, middle and high exposure. Exact concentrations are given in table 1. Conditions presented with an asterisk show a significant difference with respect to the control group at a 0,05 significance level. **B)** The eye development in tail fragment's blastema's. Yellow = no eyes present, brown = eyes not present. For both figures, the sample sizes for each particles sizeranged from 27 to 49.

brain widths and possible presence of ACs of each condition are given in figure 3. None of the three particle sizes showed a significant effect on the relative brain width. Notable observations in the AC will be described here. After 200 nm PS particle exposure the AC development ratio was slightly higher while exposed to low concentrations than in the control group. Meanwhile, the ratio in the group

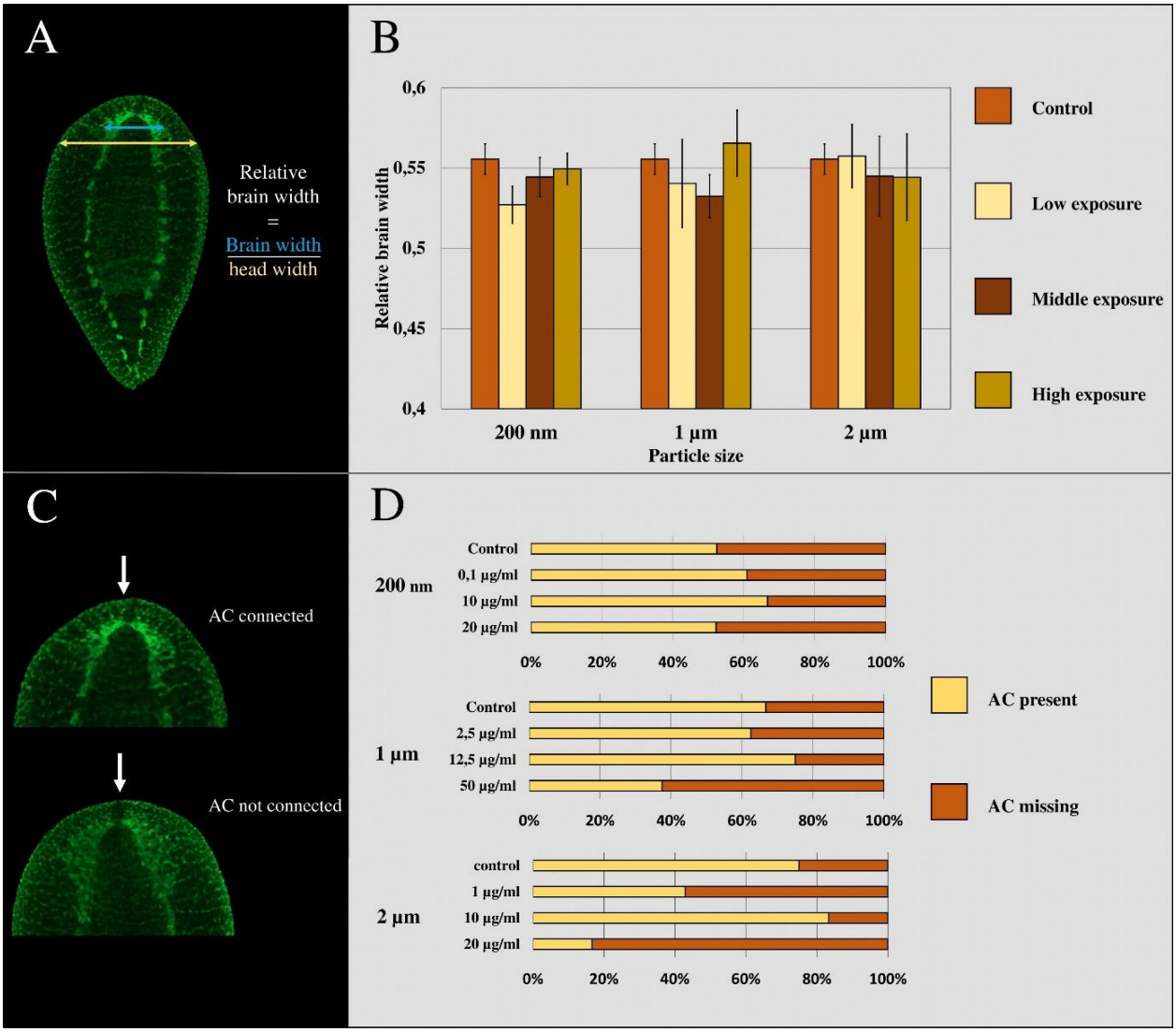

Figure 3: Toxicity on neurodevelopment in regenerating tail fragments of *Schmidtea mediterranea* after seven days of exposure to PS MNPs. **A)** Used method to calculate relative brain width. The blue line represents the brain width, the yellow line represents the head width. **B)** Histograms representing average relative brain widths of planarians in each condition. The Y-axis shows the relative brain width. The X-axis is identical in fig. 2.a. Exact concentrations are given in table 1. Error bars show a 95% confidence interval. Conditions presented with an asterisk show a significant difference with respect to the control group at a 0,05 significance level. **C)** Graphical representation of the presence (upper image) or the absence (lower image) of an AC of the brain lobes. AC = anterior commissure. **D)** The presence (yellow) or absence (brown) of AC of brain lobes in tail fragments of each condition. For both figures, the sample sizes for each particles size ranged between the following: 200 nm: 20-24; 1 µm: 7-9; 2 µm: 5-7.

exposed to middle concentrations was higher than with low concentration exposure. The samples exposed to high concentrations showed a similar ratio of AC presence as the control group. Exposure to 1 µm PS particles resulted in a decrease of the AC development ratio as the concentrations rised, with the exception of the middle exposure group. Here, the AC ratio raised slightly above control levels. In the planarians exposed to 2 µm PS, The AC development ratio gradually lowered as concentrations were raised, with again the exception of the middle exposure group. Once again, the AC ratio raised slightly above control levels.

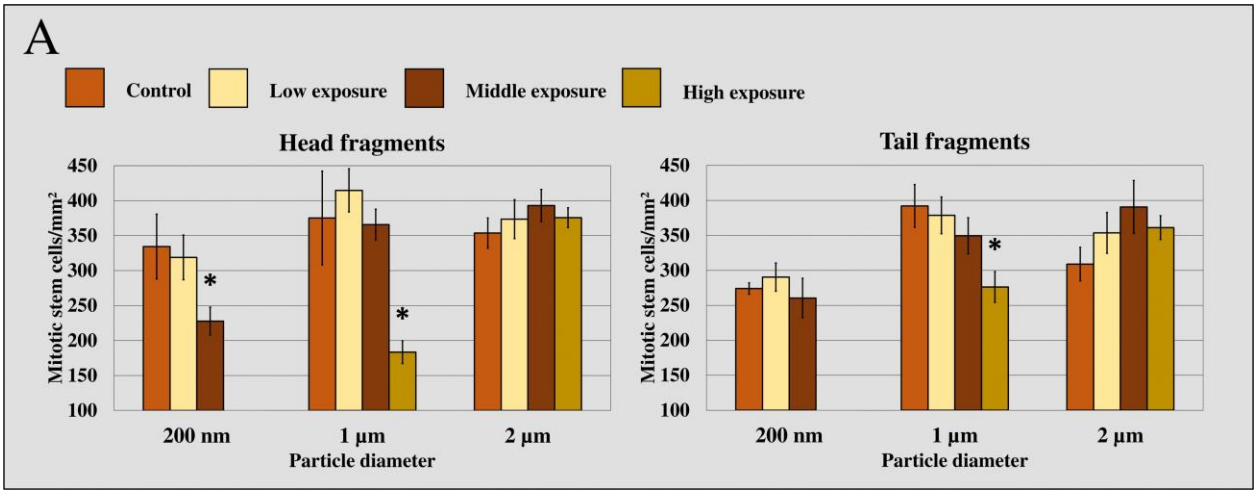

Figure 4: Histograms representing the average amount of neoblasts containing the phosphorylated histone H3P, meaning they are mitotically active, per mm². Mitotic activity in Head (left graph) and tail (right graph) fragments are shown. The Y-axis shows the average mitotic cells per mm². The X-axes are identical in fig. 2A. Exact concentrations are given in table 1; during the 200 nm experiment the concentrations were exceptionally control, 10 µg/ml, 15 µg/ml, 20 µg/ml. Error bars show a 95% confidence interval. Conditions presented with an asterisk show a significant difference in mitotically active cells per mm² with respect to the control group at a 0,05 significance level. The sample sizes for each particles size ranged between the following; 200 nm: 6-9, at the high exposure levels, no samples survived the regeneration period; 1 µm: 3-9; 2 µm: 6-9.

3.2. Stem cell dynamics

3.2.1. H3P

In order to quantify the effects of PS MNPs exposure on stem cell activity in *Schmidtea mediterranea*, mitotic neoblasts were stained with anti-histone-3 phosphorylated antibody (H3P) (62). The average amount of mitotic cells per square millimetres (mm²) of both head and tail fragments are given in figure 4. When exposed to 200 nm PS, the concentrations differed from the other concentrations used during the project. The concentrations were rather higher than before (10 µg/ml; 15 µg/ml; 20 µg/ml). In the head fragments, after exposure to 15 µg/ml, the mitotic activity was significantly lower than the control group ($p<0,05$). Then, at 20 µg/ml, there were no mitotically active cells were present. In the tail fragments, no significant differences in mitotic activity were detected. In this experiment, planarians exposed to 20 µg/ml did not survive the seven days of exposure period. The head fragments exposed to 1 µm PS, at the highest exposure level, there was a significant decrease in mitotic cells ($p<0,05$). Identically, in the tail fragments, the highest exposure group had a significantly lower activity than the control group. In the experiment where planarians were exposed to 2 µm PS, no differences were significant.

3.2.2. Fluorescent in situ hybridization

To further analyze the effects of PS MNPs exposure on mitotic activity, the expression of *smedwi-1* was measured, a gene which is expressed in the dividing adult stem cells of the planarian *Schmidtea mediterranea* (63) (fig 5). In both the head and tail parts, the planarians exposed to 200 nm PS showed no significant effects in stem cell activity at low, middle or high exposure. Exposure to 1 µm PS resulted in a significant decrease of stem cell activity in head fragments at middle exposure levels ($p<0,05$). At low exposure and high exposure, the expression levels were not affected. In the tail fragments, no significant differences were observed. After exposure to 2 µm PS the head fragments significantly lowered *smedwi-1* expression with middle concentration levels. At low and high exposure levels, the stem cell activity was unaltered. In the tail parts, the *smedwi-1* expression was not significantly affected.

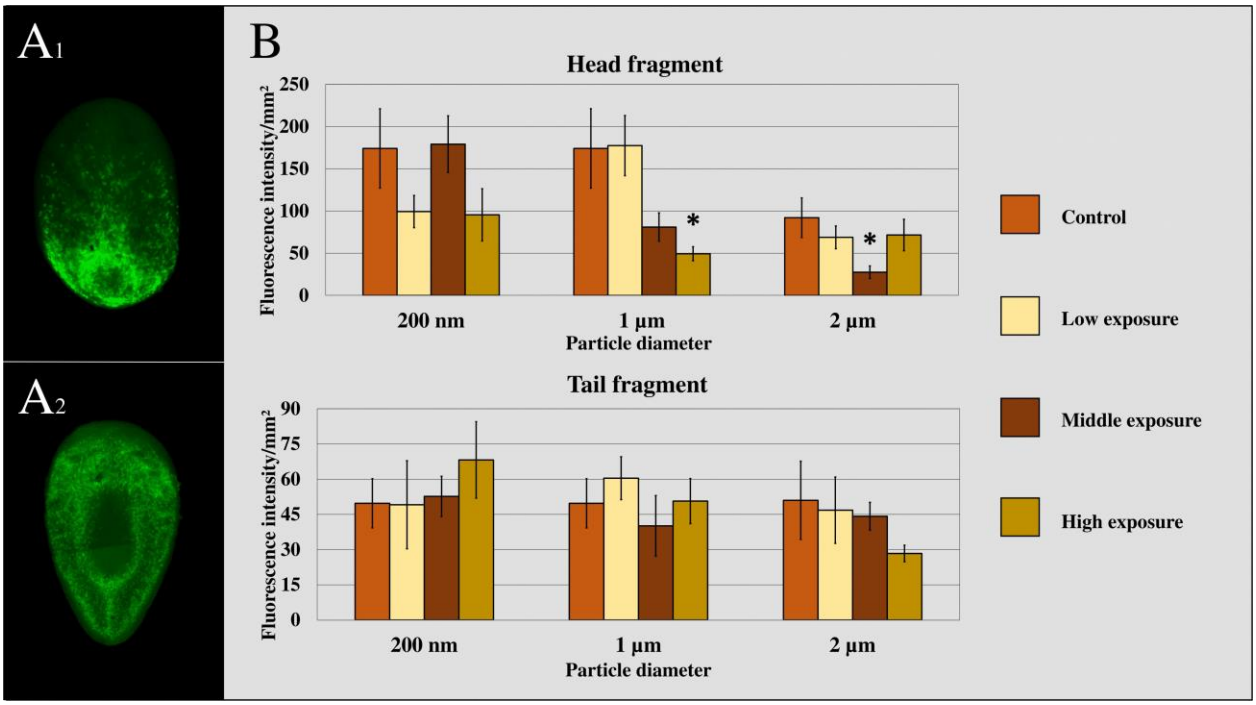

Figure 5: ***Smedwi-1* expression in regenerating *Schmidtea mediterranea* after seven days of exposure to PS MNPs**. **A)** Image of head (upper image) and tail (lower image) fragment with cells expressing proliferating stem cell marker *smedwi-1* stained through fluorescent *in situ* hybridization. **B)** Histograms representing the average fluorescence intensity, normalized through dividing by the surface area, of each condition. Head (upper graph) and tail (lower graph) fragments are shown. The Y-axes show the fluorescence intensity per mm², the X-axes are identical in fig. 1A. Exact concentrations are given in table 1. Error bars show a 95% confidence interval. Conditions presented with an asterisk show a significant difference with respect to the control group at a 0,05 significance level. The sample sizes for each particle size ranged from 4-9.

4. DISCUSSION

In this study, *Schmidtea mediterraea* was exposed to MNPs of three different sizes, 200 nm, 1 µm, and 2 µm. Neurodevelopment, cell-cycle dynamics, and general regeneration were comparatively investigated in the model organism. The results show that the particles exert an effect on the given parameters, but these are not always in line with the hypothesis. We hypothesized that the uptake of PS-derived MNPs, depending on their size, disturbs the neuroregeneration, cell-cycle dynamics and growth in regenerating *Schmidtea mediterranea.*

The most substantial effects of the PS particles were observed in the planarian's ability to develop an AC in the tail fragments, which is the connection between the two brain lobes. Exposure to the highest concentrations resulted in the lowest rate of successfully regenerated ACs after seven days of regeneration. This was the case in almost every particle size used, but the effect was not present in 200 nm PS. It is hypothesized that the AC formation was delayed. The experiment should be repeated with different timepoints to verify if the commissure is eventually formed after a longer regeneration period under the same conditions. The planarian brain, despite its simplicity, is structurally and molecularly similar to the mammalian brain, which signifies how neurodevelopmental toxicity in planarians is relevant to humans (64). To our knowledge, the effects of PS MNPs on the development of AC in *Schmidtea mediterranea* have not been reported before. However, recent findings in medaka fish showed a downregulation of the yippee-like a gene after exposure to 2 µm PS, an important gene in brain development, suggesting the neurodevelopmental delay after PS MNPs exposure (65). Similarly, the rate of planarians with visible eyes in the tail parts was lowered in exposed

groups compared to the control group. According to literature, eyes in regenerating tail parts are visible after seven days, suggesting a delay in eye development in our data (66).

As both eye development and AC development are indicators for neuronal development, these findings strengthen our hypothesis, stating that the exposure to MNPs disturbs the neuroregeneration in *Schmidtea mediterranea* (67). Neurodevelopmental toxicity is a topic that is on the march in other micro-and nanosized materials. Alumina nanoparticles induced neurodevelopmental toxicity in zebrafish (68). Furthermore, titanium dioxide nanoparticle affects motor behaviour, neurodevelopment and axonal growth in zebrafish larvae (69). Silver nanoparticles were also previously revealed to induce a substantial interference with tissue- and neuroregeneration in *Schmidtea mediterranea*, which was related to an altered stem cell cycle (70). Given the similarities between these chemically inert metal(oxide) nanoparticles and plastic particles, it is crucial to further assess the neurodevelopmental toxicity of MNPs (71).

While further exploring underlying mechanisms, adverse effects of MNP exposure were recorded on general mitotic activity, by screening for H3P-positive cells (72). After seven days of exposure, in the middle exposure concentration of 200 nm PS in the head parts, and the high exposure concentration of the 1 µm PS in head and tail parts, the amount of H3P-positive cells per mm² was significantly lowered. A lowered mitotic activity after exposure to similar particle sizes has recently been recorded before in the freshwater planarian *Dugesia japonica* (46). Notably, in the present study, exposure to the largest particle used, 2 µm, had no significant effects on the planarian's mitotic activity, contradicting recordings in literature on larger PS MNPs in *Dugesia japonica* (46). Although, in that study, the exposure was done through food mixtures. Therefore, we hypothesize that the exposure in an aqueous medium to 2 µm PS exerts different effects in planarians than exposure through food mixtures. It is remarkable that unlike the mitotic activity, our previously mentioned parameters, AC and eye development, were negatively influenced by 2 µm PS particles in this present study, suggesting different toxicity profiles of the used particle sizes.

Next, we identified changes in stem cell activity by quantifying the gene s*medwi-1* expression through FISH. In the exposure experiment with 200 nm PS, considerable variation was observed among the samples, resulting in substantial error bars, making us unable to detect significant influences. This variation is presumably caused by the general variation observed in particle toxicity and the biological variation present in organisms. However, seven days of exposure to 1 µm PS resulted in a significantly lower *smedwi-1* expression in the highest exposure group in the head parts, lying in line with recent findings in *Dugesia japonica* (46). Remarkably both lowered stem cell activity (*smedwi-1)* and lowered mitotic activity (H3P) were observed in 1 µm PS high exposure groups. Also, after exposure to 2 µm particles, there is a significantly lowered stem cell activity at the middle exposure group in the head parts. Nevertheless, after the exposure to 2 µm PS, there was a reduction in stem cell activity but no decrease in general mitotic activity, as discussed before. These results again imply a difference in the toxicity profiles between the smaller sized particles (1 µm PS and possibly 200 nm PS) and the larger sized particle (2 µm PS).

It should be noted that the H3P and *smedwi-1* experiments were only performed once per particle size during the project. Therefore, to make any conclusions, these experiments should be repeated. Furthermore, in the H3P experiment, the highest concentration of 200 nm PS had a sample size of only three. This condition was considered irrelevant.

The general expectation was that high exposure levels of PS MNPs had an adverse impact on the regeneration rate of *Schmidtea mediterranea*. This was recently demonstrated in *Dugesia japonica* by the findings of Gao et al., where exposure to three PS MNPs (100 nm, 1µm, and 10 µm PS) led to a gradual decrease in regeneration as the concentrations raised (46). The effects were present in both lower and higher concentrations used in our study. Note that the smallest diameter exerted the most significant negative effect (46). However, in this present study, exposure to 200 nm PS and 1

µm PS in both the head and tail fragments did not significantly affect the blastema size. 2 µm PS exposure showed no effects in the head fragments. A possible explanation we suggest is the agglomeration of smaller particles at higher concentrations. Agglomeration is a reversible weak physical adhesion of nanoparticles (40). When this occurs, nanoparticles' diameter, thus surface area, and associated reactivity are reduced (40). Despite sonicating our particles before adding them to the solution, these efforts may be inadequate to prevent the phenomenon. In the future, further investigation into PS nanomaterial (surface) chemistry and physics could give us further insights into this subject. The hydrodynamic diameter distributions of plastics suspensions, zeta potentials and polydispersity indices were evaluated by dynamic light scattering (73). On the contrary, exposure to the highest concentration of our largest particle used, 2 µm PS, resulted in a significant decrease in blastema size in the tail fragments, contradicting literature (46).

Regarding particle toxicity, the general hypothesis in literature states that the smaller particles result in an increased uptake due to facilitated membrane diffusion. Moreover, as the particle's relative surface area increases, there is an increased reactivity with biological tissue (39,40). In our present study, however, our largest used particles, 2 µm PS, resulted in a reduction in blastema growth, suggesting a different type of effect. Therefore, a plausible hypothesis we bring forth is the irritation of the dermal layer of the planarian by mechanical friction. In environmental stress conditions, the energy balance plays a critical role in setting limits for organisms' survival and tolerances (74). The adaptations of organisms under stressful conditions can involve a flexible allocation of energy resources or metabolic power (74). A possible hypothesis could be that the delayed blastema growth and neuronal development after exposure to 2 µm PS particles is not a result of reactivity within the planarian after uptake but instead involves an energy shift from regenerative capacity to the healing of the hypothesized dermal irritation. The observed combination of a decreased stem cell presence but unaltered mitotic activity could suggest an elevated rhabditids activity, large rod-shaped granules found in the epidermis of most Turbellarians (75). They are embedded in the epidermis and are discharged when the animals are irritated (76). However, these assumptions were made based on one experiment. This should be further investigated by repeating the procedures, followed by, for example, planarian fluorescent assay, which screens for possible acute skin irritants in flatworm models (77).

A trend in our data we can not ignore is the slight elevation of certain parameters at low concentrations. Interestingly, for all particle sizes, the results show that the rate of successful completed ACs was the highest in every condition at middle concentrations. Furthermore, however not significant, a similar phenomenon was a slightly increasing trend of mitotic activity and blastema size at low concentrations.

Hormesis is a concept that assumes that a low dose of a stressor agent can induce an adaptive response and strengthen certain biological responses. In contrast, higher doses could cause an adverse response (78). Recently, the phenomenon of hormesis has been increasingly documented in publications, and the hormetic model has been gradually developed into a fundamental dose-response model in toxicology (79). In recent years, reactive oxygen species (ROS) have emerged as key players in the initiation of the regenerative program in various mechanisms (80). Molecules such as O_2^-, H_2O_2, and OH^-, are by-products of the aerobic metabolism or enzymatic functions (81). ROS are highly reactive and have the ability to damage essential cellular components such as proteins, lipids, and nucleic acids (81). However, ROS has proven to be required for successful regeneration in various models, including tadpoles, where ROS production is responsible for cell proliferation and the activation of the Wnt/b-catenin pathway (82). ROS activates the JNK pathway in zebrafish to trigger the cell proliferation necessary to form the blastema (82,83). In planarians, after amputation, a rapid burst of ROS production occurs in the wound region (80). Studies showed that ROS inhibition disturbed a successful regeneration process in *Schmidtea mediterranea* (84). However, it is unknown whether ROS is required to initiate regeneration (84).

Exposure to different concentrations of PS MNPs can induce oxidative stress and activate redox-

sensitive signalling pathways. This phenomenon has recently been demonstrated in multiple organisms. These include mussels, *Artemia franciscana*, *Eriocheir sinensis*, and European Bass (85-88). Recently, Han et al. demonstrated that exposure in an aqueous medium to 1 µm PS induced significant changes in the levels of multiple antioxidants in planarians, suggesting that MNPs exposure induced oxidative stress (58). Therefore, the increase in AC development at low to middle concentrations of PS MNPs in the present study is explained by underlying mechanisms involving a redox imbalance induced by the particles. In that prospect, it is advised that in the future, a causal link between PS particles and ROS production, resulting in a hormetic effect, should be further investigated (58).

While measuring different developmental toxicity parameters, time is a factor that should not be overlooked. For the convenience of this project and standardization purposes, all planarians were exposed for a duration of seven days, meaning we only have information about one particular timepoint. Planarian regeneration is a global process involving an early response, axis establishment and morphogenesis (80). For further examination, we suggest investigating developmental activity at different time points. On the same note, most laboratory experiments have exposed organisms to relatively short-term acute exposures, but little is known about the long term effects (33). Given our demonstrated findings on neurodevelopment and cell cycle dynamics, long term exposure should be assessed in the future. During *in vivo* studies, diverse outcomes are to be expected. Repetition of experiments is essential due to the unpredictable variation in the results of particle toxicology and the biological diversity present in each organism. This could be solved by increasing the sample size. Therefore, in future studies, a possibility is to reduce the number of concentrations used to practically increase the number of planarians.

5. CONCLUSION

The toxicity profiles of variously sized polystyrene micro-and nanoparticles (200 nm, 1 µm and 2 µm in diameter) were compared. We hypothesize that smaller particles, being 200 nm and 1 µm, are taken up more readily, disrupting the redox balance. At higher concentrations, the effects of the smaller particles diminished. We suggest to further research on the possibility of agglomeration in these particles. In comparison, the 2 µm particles are hypothesized to induce a delayed development by skin irritation in the planarian model, resulting in a shift of the energy balance. This study enhanced our understanding of how the particles differ in behaviour in the planarian model *Schmidtea mediterranea*. Future risk assessment should continue to investigate the developmental effects, as it is also a fragile process in humans.

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