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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Polystyrene nanoplastic uptake in nervous tissue delays dopaminergic neurodevelopment in Schmidtea mediterranea

Dario Geebelen

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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Polystyrene nanoplastic uptake in nervous tissue delays dopaminergic neurodevelopment in *Schmidtea mediterranea*

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*Running title: *Polystyrene nanoplastics delay neurodevelopment*

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ABSTRACT

Polystyrene micro- and nanoplastics (PS-MNPs) can be released into freshwater environments through wastewater effluents and plastic debris fragmentation, raising concerns about their impact on various aquatic organisms. Sediments, in particular, can act as a sink, accumulating plastic fragments and potentially exposing benthic organisms to high particle concentrations. Nevertheless, the current risk assessment lacks in-depth knowledge about the potential neurotoxic effects of PS-MNPs on both benthic invertebrates and developing organisms. In the present study, the freshwater planarian *Schmidtea mediterranea* was used to investigate polystyrene uptake and its impact on neurodevelopment. Specifically, adult and regenerating planarians were exposed to freshwater medium containing different-sized (2 µm, 1 µm, 200 nm and 50 nm) PS-MNPs (20 mg/L). Potential behavioural changes were assessed through planarian locomotive velocity (pLMV) assays. Immunohistochemistry (anti-synapsin) was used to determine particle uptake in nervous tissue. Moreover, fluorescence *in situ* hybridization (FISH) was used to evaluate potential changes in dopaminergic *(Th)* and serotonergic (*SERT*) neuronal populations. Results show that PS-MNP exposure did not affect pLMV motility scores. Mucus production significantly increased after exposure to PS-NPs (200 nm). PS-NPs (200 nm) were also detected in planarian nervous tissue, while PS-MPs (2 µm and 1 µm) were mainly absent. PS-NP exposure (200 nm and 50 nm) delayed dopaminergic neurodevelopment in regenerating organisms, while serotonergic cells were unaffected. Present findings highlight the adverse impact of PS-NPs on dopaminergic neurodevelopment in the freshwater planarian *S. mediterranea*. Future research should compare the neurotoxic potential of other plastic particle types during development.

INTRODUCTION

In 2021, global plastic production was estimated to exceed 390 million tonnes (Mt) and is projected to double by 2050 (1, 2). Approximately 5% of this amount is attributed to polystyrene (PS) plastic production (2). PS is among the most commonly used plastics in various food storage components, including food containers, packing foam, cups, and plates (3, 4). Its widespread use can be attributed to PS being a translucent thermoplastic polymer favoured for its low production cost, high tensile strength, versatility, and durability (4, 5). However, PS plastic can undergo various ageing processes, such as mechanical abrasion, chemical photo-oxidation, and biological degradation, ultimately leading to its fragmentation (6). As a result, they can break down into secondary microplastics (MPs; 0.1 µm - 5 mm) and nanoplastics (NPs; < 100 nm) (7, 8). In contrast, primary micro- and nanoplastics (MNPs) are intentionally manufactured and found in several products, like cosmetics and toothpaste (9). Nevertheless, PS-MNPs can be introduced into the environment through land-based litter and wastewater effluents, highlighting the need for proper waste management (9, 10).

MPs have been detected globally in all environmental compartments, including freshwater and riverine systems (11). In European rivers, measured MP surface water concentrations ranged from 0.03 to 187,000 particles/m^3, varying with the location and time of sampling (12). Conversely, sediments are also considered an important long-term sink for MPs, showing higher concentrations compared to surface waters (13, 14). In European river sediments, levels up to 72,400 particles kg^{-1} were reported (12, 15). Recent studies conducted in Flanders (Belgium) showed that concentrations in surface waters and sediments reached up to 4.8 MP L^{-1} and 9,558 MP kg^{-1} dry weight, respectively, with PS among the most commonly found polymers (16). As a result, (aquatic) organisms may get exposed to PS-MNPs, raising widespread concerns regarding the impact on ecosystems and human health (17, 18). For instance, animals may get exposed to these particles by ingesting contaminated water or food, inhaling polluted air, or through dermal contact (19). Consequently, PS-MNPs may accumulate in various tissues and organs, such as the gut and liver, ultimately leading to their integration into the food chain (5, 19). Nevertheless, thorough risk assessment requires data from all ecosystem compartments, including organisms living in the (epi)-benthic zone (20).

Previous studies have shown that PS-MNPs can adversely affect benthic fauna, inducing abnormal behaviour, physiological changes, and developmental toxicity (21, 22, 23). To illustrate, freshwater clams (*Corbicula fluminea*) exposed to PS-NPs (80 nm; 0.1, 1 and 5 mg/L) accumulated particles in the gill, stomach, and intestine, resulting in intestinal inflammation and liver damage (24). Neurotoxicity has also been reported in zebrafish (*Danio rerio*) and nematodes (*Caenorhabditis elegans*) (25). In zebrafish, PS-NP exposure (70 nm; 0.5, 1.5, and 5 mg/L) induced alterations in circadian rhythm locomotion activity and neurotransmitter levels, such as acetylcholine, dopamine, and serotonin (26). Nematodes (*C. elegans*) chronically exposed to UV-aged PS-MPs (± 1 µm; 0.1-100 µg/L) exhibited changes in behaviour and significant neurodegeneration of glutamatergic, serotonergic, and dopaminergic neurons (27). Dopaminergic and serotonergic neurons are essential in regulating behaviour (28, 29). Dysregulation in both neurotransmitters due to MNP exposure could therefore lead to abnormal behaviour, disturbing growth and development (30, 31). Nonetheless, assessing the neurotoxic potential of PS-MNPs remains challenging due to species-specific sensitivities, variations in the physiochemical characteristics of the particles, and variable experimental conditions (32, 33). Furthermore, limited knowledge is available on the neurotoxic effects of PS-MNPs in benthic freshwater invertebrates, especially regarding their impact on dopaminergic and serotonergic neurons.

This study used the freshwater planarian *Schmidtea mediterranea* to investigate PS-MNP uptake and its impact on neurodevelopment during regeneration. Planarians are an emerging animal model commonly used for developmental neurotoxicology (34). They possess an exceptional regenerative capacity due to the high amount (30%) of stem cells called neoblasts (35, 36). This allows them to regenerate their entire body, including the central nervous system (CNS), within approximately seven days (37). Morphological and behavioural effects in planarians can also be linked to changes at the cellular and molecular levels. This may provide insight into the differential impact of PS-MNPs across different stages of neurological development and regeneration (38). Moreover, many genes and core pathways related to the CNS that are highly conserved in planaria can also be found in humans (39). To illustrate, the planarian brain consists of various distinct neuronal populations, including serotonergic and dopaminergic neuronal networks, which can produce neurotransmitters similar to those of the mammalian brain (40). This makes freshwater planaria an attractive non-vertebrate animal model, in line with the 3R (Replacement, Reduction and Refinement) and NAM (New Approach Methodologies) principles, useful for chemical risk and hazard assessment (41, 42, 43). In this study, the impact of PS-MNPs was investigated on planarian behaviour, particle uptake in neuronal tissue, and development of serotonergic/dopaminergic neural populations during regeneration. These findings provide data on PS uptake and developmental neurotoxicity in freshwater benthic planaria, which can be useful for the risk assessment of PS-MNPs.

EXPERIMENTAL PROCEDURES

2.1 Materials

Red fluorescent and non-fluorescent carboxylated (-COOH) polystyrene beads were used, both purchased from Magsphere Inc. (Pasadena, CA, USA). This study utilised four different particle sizes (2 µm, 1 µm, 200 nm, and 50 nm). Similar-sized particles have been used in prior neurotoxicological research involving aquatic animals (33). Stock suspensions (1000 mg/L) in MilliQ were provided by Mrs. Julie Tytgat, prepared as described in Saenen et al. (2023) (44). The stock suspensions were vortexed for 30 seconds and diluted in planarian cultivation medium to achieve the desired concentrations for exposure.

2.2 Planarian cultivation

An asexual strain of the freshwater planarian *Schmidtea mediterranea* was maintained in freshwater medium consisting of 1.6 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, and 1.2 mM $NaHCO_3$ in milliQ water (45). The worms were kept in the dark at a constant temperature of 20 °C and were fed veal liver once a week. Before PS-MNP exposure, similar-sized worms (3-6 mm) were starved for at least seven days to avoid food-related influences on measurements.

2.3 Planarian exposure and experimental setup

To evaluate PS uptake and its impact on the neurodevelopment of *Schmidtea mediterranea*, planarians were exposed to freshwater media containing 20 mg/L PS-MNPs. Although this does not correspond to concentrations found within freshwater, it could fall within the concentration ranges detected in sediments (12, 13, 16). Previous range-finding experiments by the research group have also shown that this concentration can induce sublethal adverse effects in *S. mediterranea*. Moreover, several studies used similar concentrations to explore toxicity mechanisms in aquatic organisms, including zebrafish (*Danio rerio*) and water fleas (*Daphnia magna*) (46, 47). During the experiments, planarians were exposed in six-well plates, with five animals per well, to either five mL of freshwater medium (control) or diluted PS-MP/NP solutions. Every two to three days, the worms were transferred to new plates containing freshly prepared control or exposure solutions. Research was conducted on adult or regenerating planarians. In the case developing worms were studied, adult planarians were cut transversally anterior to the pharynx to obtain a regenerating head and tail fragment just before exposure. Depending on the experiments, worms were exposed for three, four, five or seven days (Fig. 1). At these designated time points, tests were performed to assess planarian behaviour, PS uptake in neuronal tissue, and neurodevelopment. On the seventh day, planarian locomotive velocity (pLMV) assays were used on developing organisms to assess potential abnormalities in motility. Moreover, mucus production of adult planarians was measured on the third day using a Wheat Germ Agglutinin (WGA) staining. On the fourth and seventh day, immunohistochemistry was performed to stain the nervous system (anti-synapsin) of regenerating planarians for particle uptake assessment. Fluorescence *in situ* hybridisation (FISH) was used to visualise dopaminergic (*Th*) and serotonergic (*SERT*) neurons at five and seven days post-amputation (DPA).

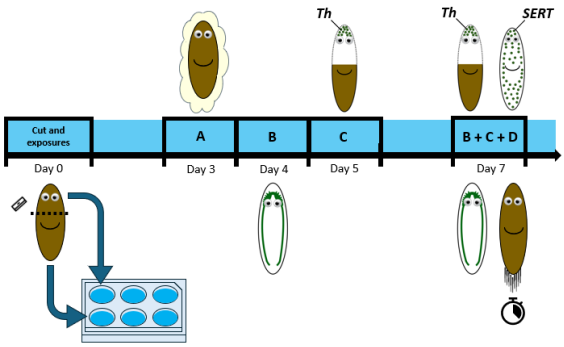

Fig. 1: Timeline of experimental setup. Experiments were performed on adult and regenerating worms. Worms were exposed to 20 mg/L PS-MNPs (2 µm, 1 µm, 200 nm and 50 nm) at day zero. **(A)** On day three, mucus measurements were performed on adult worms using a Wheat Germ Agglutinin (WGA) staining. **(B)** PS-MNPs were localised in the nervous tissue of regenerating tails via immunohistochemistry (anti-synapsin) on the fifth and seventh day. **(C)** Dopaminergic (*Th*) and serotonergic (*SERT*) neurons were visualised in regenerating tails after five and seven days post-amputation (DPA). **(D)** On the seventh day, planarian locomotive velocity assays (pLMV) were used to assess behavioural abnormalities in regenerating worms.

2.4 Planarian behaviour assessment

Behavioural tests were conducted on regenerating fragments up to seven days after exposure, as planarians can fully regenerate their central nervous system within this timeframe, making behavioural changes more easily discernible. Specifically, planarian motility was examined with a two-minute planarian locomotive velocity (pLMV) assay (48, 49). Briefly, a Petri dish containing freshwater medium was placed on a 0.5 cm grid. Afterwards, planarian velocity was measured as the cumulative number of lines crossed or recrossed by a planarian tail within two min, including 20 seconds of acclimatisation.

2.5 Mucus production measurements

The mucus production was measured from animals that were exposed to either control or 20 mg/L non-fluorescent PS-MNPs (1 µm and 200 nm) for three days. Mucus extraction and staining with Wheat Germ Agglutinin (WGA) (Alexa FluorTM 488 conjugate of WGA; Thermo Fisher Scientific, dilution 1:250) were performed as described in Leynen et al. (2024) (48). Fluorescence intensity was assessed at 519 nm with a spectrophotometer (FLUOstar Omega, BMG Labtech). For each condition, four measurements were performed from one independent experiment.

2.6 Whole mount immunohistochemistry: synorf

To determine the localisation of PS-MNPs within the central nervous system (CNS) of *S. mediterranea*, immunostaining was carried out using Mouse Anti-Synapsin 3C11 (Developmental Studies Hybridoma Bank, 1:50) (50). Alexa 488-conjugated goat anti-mouse (Molecular Probes, dilution 1:400) was used as the secondary antibody. Staining of the CNS by whole-mount immunohistochemistry was performed as described by Pirotte et al. (2015) (45). Confocal laser-scanning microscopy with an LSM900 (CLSM, Zeis, Zaventem, Belgium), mounted on an Axio Observer Z1/7 (Zeis, Zaventem, Belgium), was used for imaging. Regenerating tails exposed to fluorescent-labelled PS-MNPs (2 µm, 1 µm and 200 nm) for four or seven days were examined. Two independent experiments were conducted with at least eight worms per condition. Particle uptake was assessed by investigating their presence within or near the cephalic ganglia (CG) and ventral nerve cords (VNC) via z-stack imaging, specifically through orthogonal views at a magnification of 20x. The number of particles was counted in images of the VNC from planarians exposed to PS-MPs (2 µm and 1 µm). Mean percentages were calculated for particles present in, near, and outside the VNC.

2.7 Fluorescence in situ hybridization (FISH)

Whole-mount fluorescence *in situ* hybridisation (FISH) was used to visualise dopaminergic (*Th*) and serotonergic (*SERT*) neurons. The FISH protocol was performed as described by Leynen et al. (2019) (51). Expression patterns for *Th* were investigated in regenerating tail fragments after exposure to non-fluorescent PS-MNPs for five and seven days. To visualise *SERT* expression, regenerating tail and head fragments were exposed only for seven days. Confocal microscopy (LSM900, Zeiss) was used for imaging. The number of *Th*-positive cells in each tail was counted manually to calculate the average cell amount per condition. For *SERT* expression in heads and tails, cells were counted and normalized against the body surface using ImageJ (version 1.54g). During the study, four experiments with *Th* and two experiments with *SERT* were carried out, with at least ten worms per exposure condition.

2.8 Statistical analysis

Statistical analyses were performed with R statistical software, version 4.2.1 (Rstudio Inc., Boston, USA). Grubbs' test was performed using GraphPad (GraphPad Software, 2024, California, USA) to test for outliers. The assumptions of normality and homogeneity were checked with a Shapiro-Wilk test and Levene's test, respectively. If the assumptions were not met, the data were transformed (log, square root, 1/x and e^x). Next, one-way ANOVA was performed and subgroups were compared using Dunnett's post hoc tests. In cases where previous assumptions of normality and homoscedasticity were still not met, a non-parametric Kruskal-Wallis test with pairwise Wilcoxon rank-sum test was utilised. The Fisher exact test was used to check for the significance of binomial data (presence/absence of particles within neuronal tissue). All tests were carried out on a 5% significance level.

RESULTS

3.1 PS-MNPs do not affect planarian motility

The results from the planarian locomotive velocity tests can be found in (Fig. 2). No significant difference was observed between the mean motility scores from the control group (M: 6.4; SD: 0.76) and the regenerating tails exposed to 20 mg/L PS-MPs of 2 µm (M: 6.2; SD: 0.71; p = 0.981) and 1 µm (M: 5.8; SD: 0.65; p = 0.813) in size after seven days. Similarly, no significant difference was observed between the control group and the animals exposed to PS-NPs of 200 nm (M: 8.3; SD: 0.97; p = 0.362) and 50 nm (M: 9.3; SD: 0.96; p = 0.120).

3.2 PS-NPs increase mucus production

Mucus production from adult planarians was assessed via WGA staining and fluorescence intensity (FI) measurements (519 nm) after three days of exposure to PS-MNPs (1 µm and 200 nm). The mean FI results are shown in (Fig. 3). A significant increase in the production of mucus was observed after exposure to 20 mg/L PS-NPs of 200 nm in size (M: 665.8; SD: 69.4; p = 0.01). No significant difference in mucus production was observed in worms exposed to 1 µm PS-MPs (M: 275.8; SD: 33.6; p = 0.08).

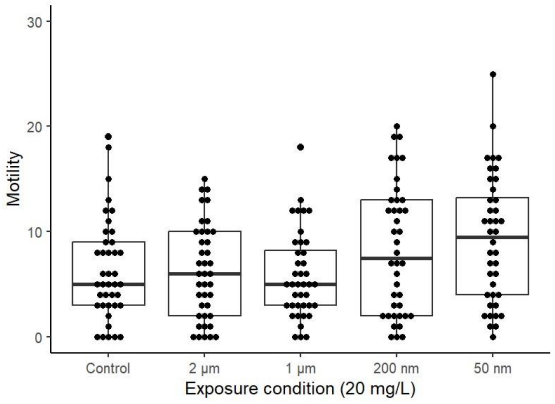

Fig. 2: Distribution of motility scores after exposure to PS-MNPs. Planarian locomotive velocity (pLMV) assays were performed on regenerating tails exposed to freshwater medium (Control) and 20 mg/L PS-MNPs (2 µm, 1 µm, 200 nm or 50 nm) for seven days. Motility scores are calculated as the cumulative number of lines crossed or recrossed by a planarian tail within two min, including 20 seconds of acclimatisation. Results were pooled from four independent experiments, each with a total number of ten tails per condition. Individual scores are represented as black dots.

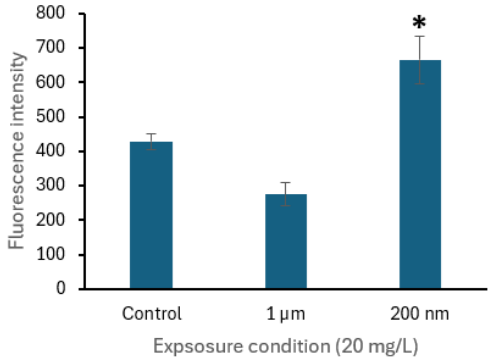

Fig. 3: Effect of PS-MNP exposure on mucus production. Adult planarians were exposed to freshwater medium (Control) and 20 mg/L PS-MNPs (1 µm and 200 nm) for three days. Mucus was extracted and stained with Wheat Germ Agglutinin (WGA). Fluorescence intensity was assessed at 519 nm with a spectrophotometer. One experiment was performed with four technical replicates. Statistical significance relative to the control is indicated by * ($p \leq 0.05$).

3.3 Uptake of PS-MNPs in neuronal tissue

Confocal imaging and orthogonal projections of nervous system structures, stained with anti-synapsin, revealed the presence of PS in the cephalic ganglia (CG) and ventral nerve cords (VNC) of regenerating planarians exposed to 20 mg/L fluorescently labelled PS-MNPs. Specifically, PS-NPs (200 nm) were detected within the CG and VNC at seven DPA (Fig. 4A). PS-MPs (2 µm and 1 µm) were mostly not found within the nervous tissue (NT) at four and seven DPA. PS-MPs of 2 µm could be found near the VNC, while 1 µm PS-MPs were both within and near the VNC (Fig. 4B). Moreover, no significant difference was observed between the uptake of both particles at seven DPA (p = 0.058).

3.4 PS-NPs delay neuroregeneration

The impact of PS-MNPs on dopaminergic and serotonergic neurons was investigated by

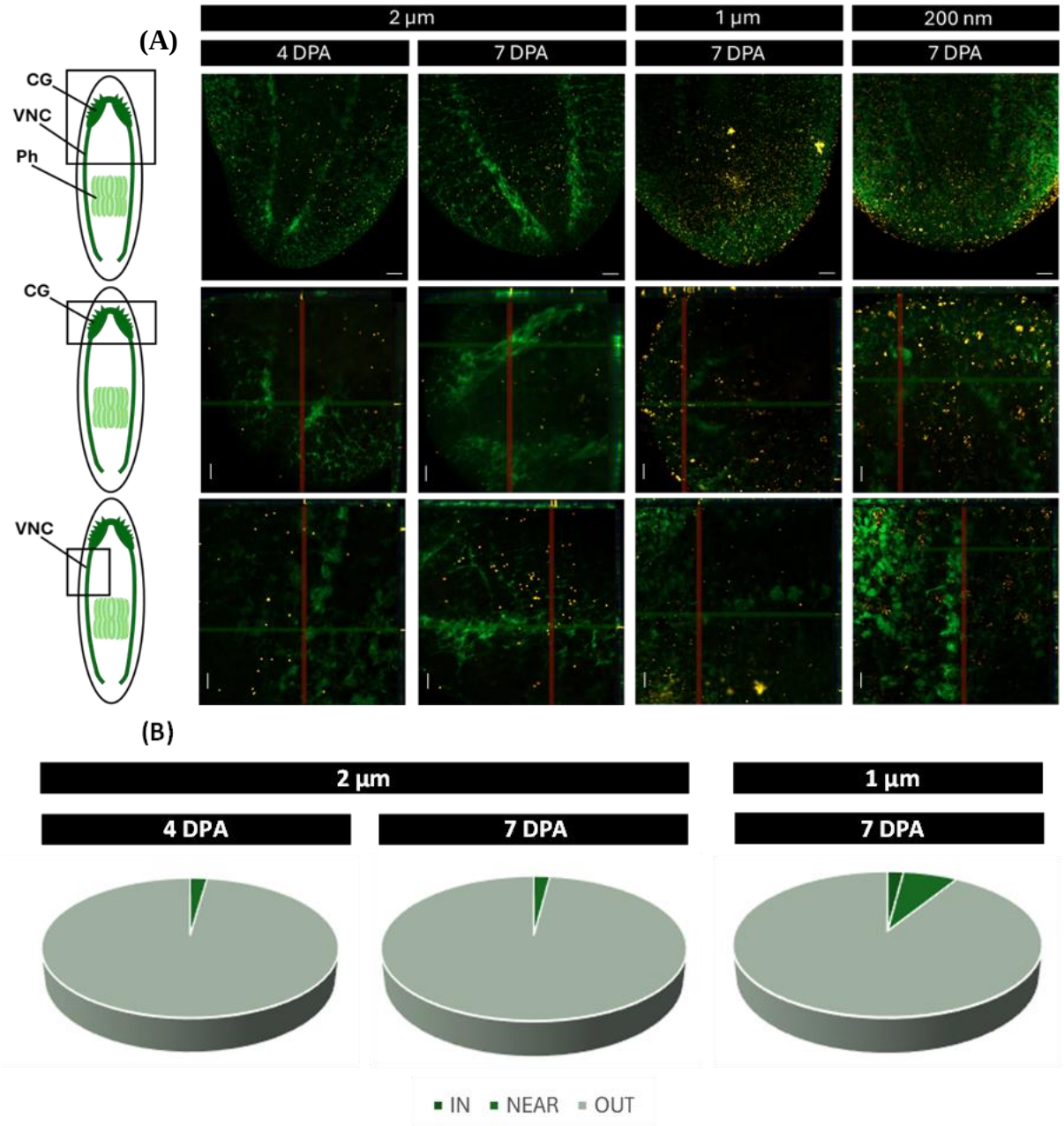

Fig. 4: PS-MNP uptake in central nervous system during regeneration. (A) Images of nervous system structures in regenerating tails exposed to 20 mg/L PS-MPs (2 µm or 1 µm) and 20 mg/L PS-NPs (200 nm) at 4 DPA or 7 DPA. The left side of the figure specifies the nervous structures stained with anti-synapsin, focusing on the cephalic ganglia (CG) and ventral nerve cords (VNC). The first row contains images of the head region (scale bars = 50 µm), while the second and third rows are orthogonal images of the CG and VNC (scale bars = 20 µm), respectively. Fluorescently labelled PS-MNPs are visualised as yellow dots. **(B)** The proportion of regenerating tails containing PS-MPs in, near and outside (OUT) the nervous tissue (NT) of the VNC at 4 DPA and 7 DPA. The number of PS-MPs was manually counted within five different orthogonal pictures for each condition from a single independent experiment. Results were relativized to 100 particles and the mean was calculated. Statistical significance between 2 µm and 1 µm at 7 DPA was assessed using the Fisher exact test ($p \leq 0.05$). Abbreviations: Ph, pharynx

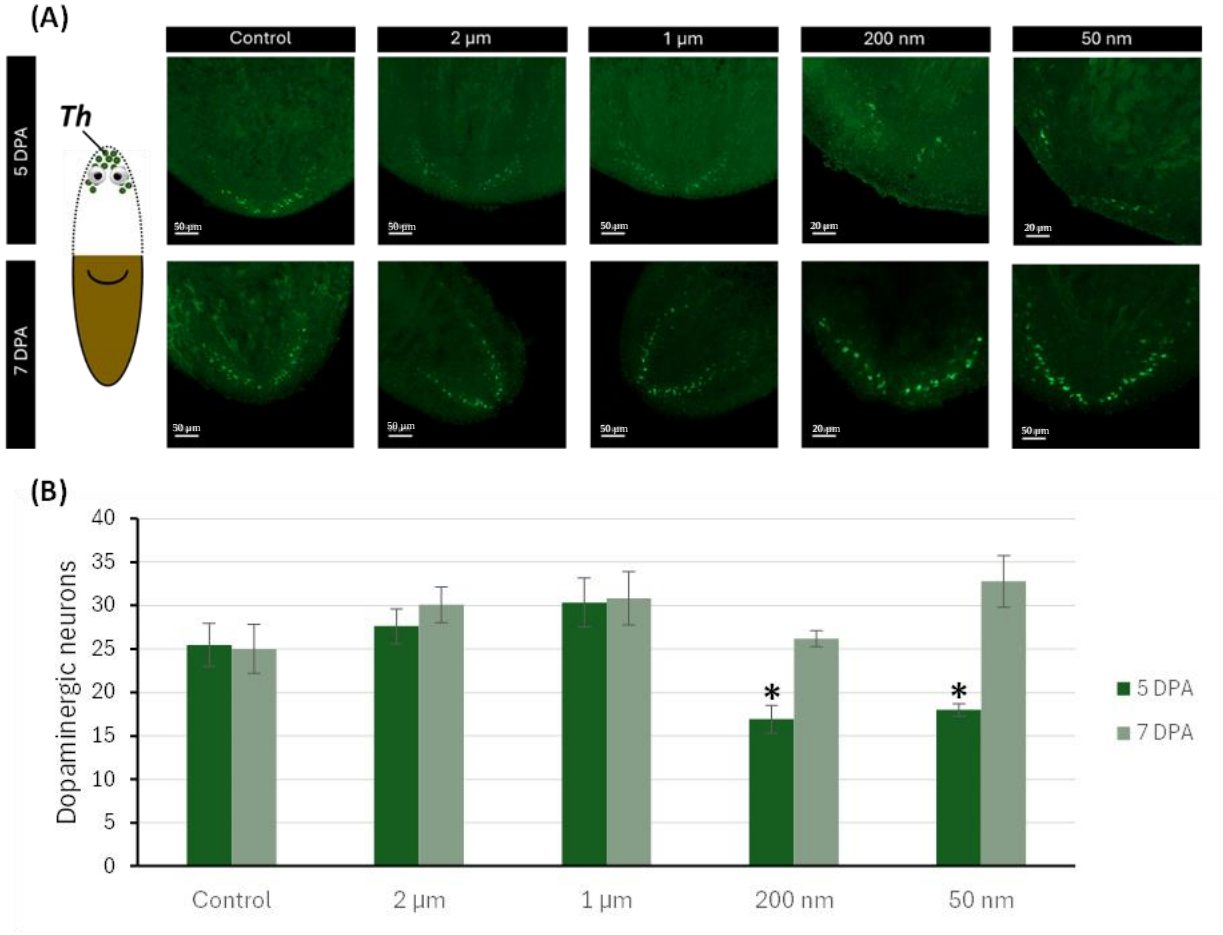

Fig. 5: Effects on regeneration of dopaminergic neurons after PS-MNP exposure. (A) Planarians were cut and exposed to freshwater medium (Control) and 20 mg/L PS-MNPs (2 µm, 1 µm, 200 nm or 50 nm) for five days (top images) or seven days (bottom images). *Tyrosine hydroxylase (Th)*-positive cells were visualised in the head region of regenerating tails. **(B)** Barplot representing the mean number of *Th*-positive cells (dopaminergic neurons) ± SD in the head region of regenerating tails. Pooled results of three independent experiments with a total of ≥ 11 replicates per condition at 5 DPA and one experiment at 7 DPA with ≥ 7 replicates per condition. Statistical significance relative to the control is indicated by: * ($p \leq 0.05$).

visualising the expression patterns for tyrosine hydroxylase (*Th*) and the serotonin transporter (*SERT*), respectively. Expression patterns were visualised in regenerating tails and heads after exposure to 20 mg/L PS-MNPs at five and seven DPA. For dopaminergic neurons, a size-dependent decrease in the number of *Th*-positive cells in regenerating tails at five DPA was observed (Fig. 5A). Specifically, significant differences were observed between the control group and animals exposed to PS-NPs with a diameter of 200 nm ($p = 0.032$) and 50 nm ($p = 0.0004$) (Fig. 5B). At seven DPA, no significant effects were observed between the controls and the exposures, implying a delay in dopaminergic neuron regeneration. For serotonergic neurons, regenerating tails showed no significant differences ($p = 0.18$) in the number of *SERT*-positive cells between the controls and exposures at seven DPA (Fig. 6). Similarly, no effects were detected in the regenerating heads between the controls and the exposures.

DISCUSSION

Polystyrene micro- and nanoplastics (PS-MNPs) can be released into freshwater compartments, raising widespread concerns regarding their impact on various aquatic organisms (3). Freshwater sediments, in particular, may accumulate these particles, acting as a sink and potentially exposing benthic organisms to high concentrations (13, 14).

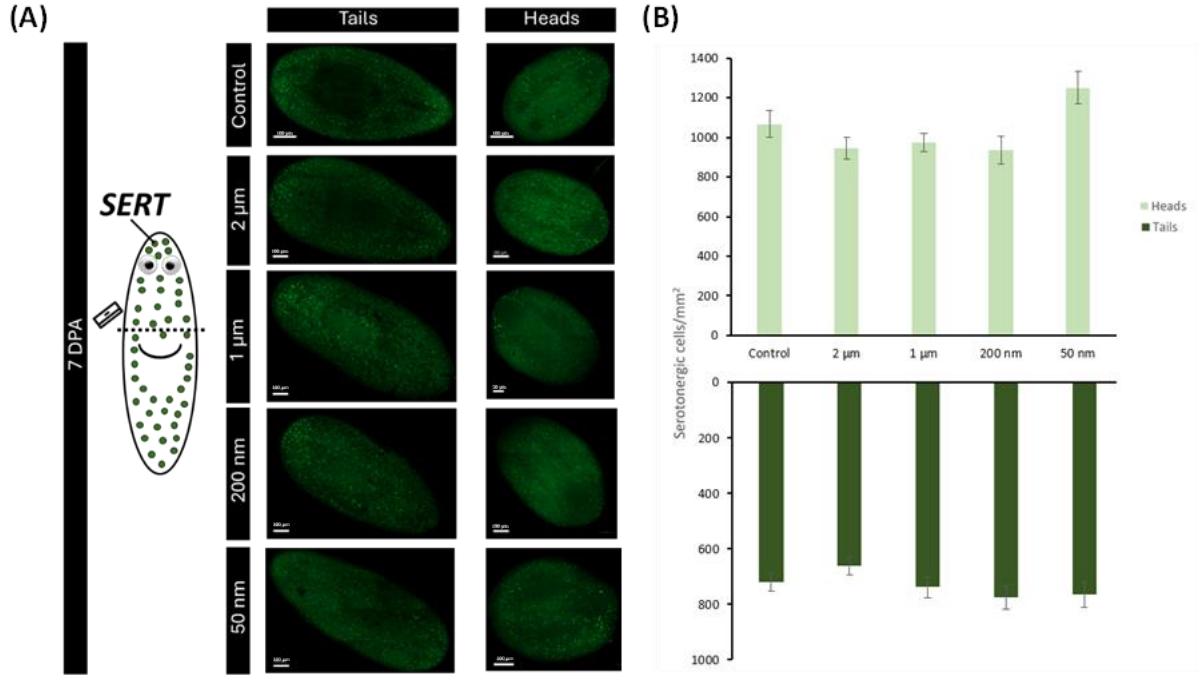

Fig. 6: Effect on regeneration of serotonergic neurons after PS-MNP exposure. (A) Planarians were cut with tail (left images) and head (right images) fragments separately exposed to freshwater medium (Control) and 20 mg/L PS-MNPs (2 µm, 1 µm, 200 nm or 50 nm) for seven days. Cells expressing the *serotonin transporter* (*SERT*) were visualised via confocal imaging. **(B)** Barplot representing the mean number of *SERT*-positive cells normalized against the body surface (serotonergic cells/mm^2) ± SD in regenerating head (top plot) and tail (bottom plot) fragments. Pooled results of two independent experiments with a total of 16 tail fragments and 13 head fragments per condition at 7 DPA. Statistical significance relative to control is indicated by: * ($p \leq 0.05$).

Nevertheless, the current risk assessment lacks a comprehensive understanding of the impacts of PS-MNPs on benthic invertebrates, especially regarding neurodevelopment. Neurotoxicity can be induced due to changes in dopaminergic and serotonergic neuronal populations, ultimately leading to abnormal behaviour (27). For this reason, the current study investigates the neurotoxic effects of PS-MNPs on the freshwater epibenthic invertebrate *S. mediterranea*. Specifically, the impact of PS-MNPs was investigated on planarian behaviour, particle uptake, and the development of serotonergic and dopaminergic neurons in regenerating planarians. During this study, animals were exposed to 20 mg/L carboxylated (-COOH) polystyrene beads with four different particle sizes (2 µm, 1 µm, 200 nm, and 50 nm). Similar-sized particles were used in previous neurotoxicological research involving aquatic animals with the present concentration falling within the ranges detected in freshwater sediments (16, 33).

Abnormal planarian behaviour due to particle exposure can serve as an indicator of effects on the central nervous system (CNS) (39). The behaviour of regenerating tails exposed to PS-MNPs was assessed at seven DPA by a planarian locomotive velocity (pLMV) assay (49). Planarian motility scores of regenerating tails showed no significant differences between the control and exposure groups (Fig. 2). Similar results were observed in the freshwater planarian *Girardia tigrina*, with no effects on planarian locomotor velocity after exposure to 1-10 mg/L PS-NPs (426 nm ± 175 nm) for 10 days (52). However, variation in motility scores from animals exposed to PS-NPs with a diameter of 200 nm and 50 nm was greater compared to other exposure conditions. This might imply that PS-NPs induce different effects on individuals within the same exposure group, resulting in potential aberrant behaviour. Planarian locomotion has been shown to be affected by contaminants such as herbicides, attributed to potential changes in the CNS, ultimately leading to neurotoxicity (53).

However, disruption in planarian locomotion can also be displayed in the amount of mucus that is produced when exposed to harmful stimuli (54).

Planarians secrete mucus for protection and locomotion (55). Current results have shown a significant increase in mucus production in adult worms after three days of exposure to 200 nm PS-NPs (Fig. 3). Planarians can increase mucus secretion as a defence mechanism to environmental stimuli, resulting in a thicker protective coat and stronger adhesion to the substrate (54, 55, 56). In zebrafish, exposure to PS-MNPs (50 µm and 100 nm) resulted in continuous mucus secretion, with mucus adsorbing more particles, damaging the intestine and gill (57). *In vitro* research with intestinal cell models has also shown a significant decrease in the translocation of neutral PS-NPs (50 nm) through mucus, while the translocation of similar-sized carboxylated NPs increased (58). The MNPs' charge is an important physicochemical trait that can influence their adsorption and translocation in living organisms (59). Carboxylated PS-NPs have a negative charge and may not aggregate heavily in mucus, but can still adhere as single particles to the mucus network (60). Moreover, carboxylated PS-NPs can adsorb biomolecules from their environment or biological fluids and tissues, forming a protein corona which can affect their surface charge and uptake (61). To summarise, PS-NP exposure increased mucus production as a potential defence mechanism to trap particles in the mucus network and avoid epithelial uptake (48). However, there is still a risk that PS could be taken up through ingestion (52).

Once taken up, PS-MNPs may target the CNS and accumulate in nervous tissue (NT) (62). Confocal imaging and orthogonal projections of nervous system structures stained with anti-synapsin revealed the presence of PS-MNPs in the NT of developing tails at seven DPA (Fig. 4A). Specifically, PS-NPs of 200 nm were detected in the cephalic ganglia (CG) and ventral nerve cords (VNC). Moreover, larger particles (2 µm and 1 µm) were mostly absent from the VNC (Fig. 4B). Experiments with *C. elegans* showed that PS-NPs smaller than 200 nm in size could directly penetrate and integrate into cell membranes, causing neurotoxicity (63). Larger particles (> 200 nm) cannot be taken up directly, but may cause nerve damage indirectly via other processes (33). This size-dependent uptake was also observed in studies with other freshwater organisms. In red tilapia, exposure to 100 µg/L PS-MNPs (300 nm, 5 µm, 70-90 µm) for 14 days resulted in particle accumulation in various tissues, including the gut, liver, and brain (64). In another study with zebrafish larvae (*D. rerio*), PS-NPs (100 and 500 nm) were found in the brains at 48 hours post-fertilisation, while particles with a diameter of 1000 nm were not detected in the larval brains (65). Further differences can be explained by the variations in feeding behaviour (filter vs deposit feeders), exposure route (water vs feeding), exposure concentration, and exposure time (33, 66).

Focussing on neurogenesis, PS-NPs (200 nm and 50 nm) also significantly decreased the number of dopaminergic neurons in developing tails at five DPA (Fig. 5). However, no changes were observed at seven DPA, implying a delay in regeneration. Dopamine (DA) synthesis is catalysed by the rate-limiting enzyme tyrosine hydroxylase (Th), converting tyrosine into l-3,4-dihydroxyphenylalanine (l-DOPA) (67). This enzyme is highly conserved between vertebrates and invertebrates, with DA playing an important role in planarian locomotion coordination (68). The delay in dopaminergic neuron regeneration at five DPA potentially arose due to damage induced by the PS-NPs present in the CNS, altering *Th* expression. Consequently, dysregulation in DA could be associated with the increased variation in previous motility scores, indicating potential abnormal behaviour. In mice, oral exposure to 0.25-250 mg/kg body weight PS-NPs (50 nm) resulted in Parkinson's disease-like neuro-degeneration, caused by various metabolic and physiological alterations, including dopaminergic neuron loss (69). Moreover, PS-NP (70 nm) exposure in zebrafish (*D. rerio*) induced behavioural alterations and down-regulation in the expression levels of neurotransmitters, including DA and serotonin, after seven days (26). In the current study, PS-MNP exposure did not alter serotonergic neurodevelopment in regenerating tails and heads at seven DPA (Fig. 6). Serotonin (5-HT) plays an essential function in planarians, such as regulating thermotaxis, eye pigmentation, and muscular function (70). The biosynthesis of 5-HT is regulated by the rate-limiting enzyme

tryptophan hydroxylase (TPH), an important serotonin biomarker (71). Cells involved with 5-HT recycling were visualised by looking at the serotonin transporter (*SERT*) expression. SERT is an important protein that is essential in the termination and regulation of 5-HT signalling (72). Changes in SERT availability can cause disturbances in serotonin transport, altering cognitive and muscular function (73). Future studies should examine the expression patterns of neurotransmitters and transporters involved with behaviour at different time points during neurodevelopment.

This study has several strengths. First, the impact of PS-MNP exposure on planarian behaviour could be linked to nervous tissue uptake and altered neuronal populations. This can provide an improved understanding of how these particles can influence cellular and molecular pathways to induce neurotoxicity. Second, multiple independent experiments were conducted to account for the variability in particle distribution in planarian medium within and between experiments. This can improve the robustness of statistical analyses, resulting in more reproducible and reliable results.

This study also comes with several limitations. First, the sample size in several experiments is small, reducing the power of the study and increasing the margin of error. Limitations in data were caused by technical errors and unexpected occurrences, such as planarians exhibiting a phenotype before exposure. Second, some analyses could not be performed due to methodological limitations. To illustrate, confocal imaging could only be performed with a resolution up to 120 nm. Therefore, PS-NPs with a diameter of 50 nm could not be visualised separately in the planarian nervous system. Finally, the impact of PS-MNPs on nervous system uptake and neuronal subpopulations was investigated in *S. mediterranea*, but mechanisms underlying potential toxicity were not studied. Future research should focus on the underlying molecular mechanisms that cause the neurotoxic effects of PS-MNPs in benthic planarians.

CONCLUSION

In summary, present findings show that PS-MNPs can induce adverse neuro-developmental effects in the epibenthic organism *S. mediterranea*. Mucus production was increased in adult organisms, creating a protective coat against these particles. PS-NPs (200 nm) were taken up and detected within the CNS. In developing organisms, PS-NPs (200 nm and 50 nm) significantly reduced the number of *Th*-positive cells, indicating a delay in dopaminergic neuroregeneration. Overall, this study reveals that PS-MNPs can interfere with neuroregeneration during development. Future research should examine the impact of PS-MNPs on other neuronal populations and focus on the underlying molecular mechanisms to provide more objective evidence.

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