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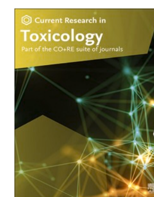

Research Paper

Reproductive cytotoxic and genotoxic impact of polystyrene microplastic on *Paracentrotus lividus* spermatozoa

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ABSTRACT

In recent decades, industrialization, intensive agriculture, and urban development have severely impacted marine environments, compromising the health of aquatic and terrestrial organisms. Inadequate disposal results in hundreds of tons of plastic products released annually into the environment, which degrade into microplastics (MPs), posing health risks due to their ability to biomagnify and bioaccumulate. Among these, polystyrene MPs (PS-MPs) are significant pollutants in marine ecosystems, widely studied for their reproductive toxicological effects. This research aimed to evaluate the reproductive cytotoxic and genotoxic effects of PS-MPs on sea urchin (*Paracentrotus lividus*) spermatozoa *in vitro*. Results showed that PS-MPs significantly reduced sperm viability and motility without altering morphology, and induced sperm DNA fragmentation mediated by reactive oxygen species production. Furthermore, head-to-head agglutination of the spermatozoa was observed exclusively in the sample treated with the plastic agents, indicating the ability of microplastics to adhere to the surface of sperm cells and form aggregates with microplastics on other sperm cells, thereby impeding movement and reducing reproductive potential. These findings suggest that PS-MPs can adversely affect the quality of sea urchin sperm, potentially impacting reproductive events.

Introduction

Environmental pollution, resulting from human activities, poses significant threats to terrestrial atmospheric and aquatic ecosystems. It is recognized as a major challenge of our time and the leading environmental contributor to global disease, causing around 9 million premature deaths annually (Landrigan et al., 2018).

Pollution of aquatic environments, often overlooked, has significant direct and indirect impacts on human health (Landrigan et al., 2020). Around 90 % of pollutants are carried by rivers to the sea, compounding the pollution already present due to the high percentage of people living in coastal areas (Morroni et al., 2023). Water pollution is a complex mix of chemicals, biological materials, and waste, including plastics, petroleum products, toxic metals, pharmaceuticals, pesticides, and sewage. These pollutants exhibit varying concentrations across different regions and seasons, further complicating the issue (Landrigan et al., 2020).

Among the several contaminants present in the environment, plastic is a major concern due to its widespread use and significant presence in the environment. It is composed of organic polymers and additives like bisphenols, phthalates, and flame retardants (Jiang et al., 2020), leading some to dub this era as the “age of plastic” (Thompson et al., 2009). Its affordability and versatile properties have made it a ubiquitous material in everyday items such as packaging, cosmetics, electronics, and furniture (Hirt and Body-Malapel, 2020).

Global plastic production has exceeded 380 million tons annually, highlighting its status as an essential commodity (Paço et al., 2017). However, their cheap and disposable nature often leads to them being used and discarded unnecessarily. In fact, of the million tons of plastic waste produced each year, only 60 % is collected (Skoczinski et al., 2021). In turn, the collected plastic waste may not be disposed of correctly and, therefore, inevitably reaches the environment, where it is broken down into smaller fragments, through various physical–chemical processes such as atmospheric agents, ultraviolet radiation (UV) and human activities (Song et al., 2017). In this case, based on size, these particles are divided into macro- (>2.5 cm), meso- (0.5–2.5 cm), micro- (MP = 1 μm–5 mm) and nano- (NPs < 1 μm) plastics (Dissanayake et al., 2022).

Microplastics are attracting the attention of researchers, due to their widespread abundance and distribution and their impact on natural ecosystems, as well as the risks to human health. MPs are estimated to

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account for 92.4 % of marine plastic debris and MP pollution is widely distributed. In fact, they have been detected in a vast number of environmental matrices, such as water (Xu et al., 2020), soil (Ding et al., 2020) and air (Wright et al., 2020), but also in regions Antarctic (Bessa et al., 2019) and Arctic (Almeda et al., 2021).

The human body can meet MPs through three main routes: the ingestion of food contaminated with microplastics, the inhalation of microplastics in the air and the dermal contact of these particles, contained in products, fabrics, or dust. Of these, oral ingestion is the main route of human exposure to MPs (Prata et al., 2020). In fact, microplastics have been detected in several foods, including seafood (Daniel et al., 2021), vegetables and fruit (Oliveri Conti et al., 2020). The effects of polystyrene microplastics (PS-MPs), one of the most widespread plastics (Menon et al., 2023), on marine organisms and terrestrial mammals have been well documented and mainly include impacts on the reproductive system (Sharifinia et al., 2020). The reproductive toxicity and genotoxicity of MPs raises concerns also for human fertility, particularly considering the recent discovery of plastic micro fragments in human seminal fluid (Montano et al., 2023).

In multicellular animals, spermatogenesis is a multistage, sequential process resulting in mature male germ cells. It involves mitotic and meiotic divisions of spermatocytes, followed by spermiogenesis, leading to the formation of mature spermatozoa with condensed chromatin, an acrosome, and a flagellum (Nishimura and L'Hernault, 2017; Hermo et al., 2010). While variations exist among species, comparative research suggests commonalities in this process. However, differences in reproductive modes between species with external and internal fertilization result in varying effects of environmental stressors on gamete function and behavior (Gallo et al., 2020).

Research on the genotoxic and cytotoxic effects of microplastics (MPs) on fertility has primarily focused on aquatic organisms, with both *in vivo* and *in vitro* analyses conducted. A notable study by Qiang and Cheng (2021) investigated the impact of polystyrene microplastics on the reproductive organs of zebrafish (*Danio rerio*). The findings revealed a significant increase in reactive oxygen species (ROS) in both male and female gonads, accompanied by elevated levels of apoptosis via p53-mediated pathways. Additionally, histological alterations, including a remarkable decrease in the thickness of the testicular basement membrane, were observed.

Several other studies have demonstrated the detrimental effects MPs on reproductive functionality across different trophic levels. For instance, Sussarellu et al. (2016) found that oysters exposed to PS-MPs experienced reduced sperm count, diameter, and velocity, along with anomalies in fertilization and diminished larval yield and development. Furthermore, researchers have highlighted the ability of MPs to accumulate in reproductive organs, leading to alterations in normal functionality. Shi et al. (2022) showed that MPs decrease fertilization rates in oysters by impairing sperm swimming performance and reducing ATP production and cell viability, resulting in fewer successful gamete collisions. Additionally, the deposition of MPs in the testicular tissue of male river shrimp induces oxidative stress, disrupting the quality of testicular germ cells and causing imbalances in sex hormones. Again, studies on aquatic vertebrate organisms, such as zebrafish embryos, have revealed delayed larval development and structural defects due to MPs exposure, including spine and tail deformities, as well as peripheral microcirculatory dysfunction (De Marco et al., 2022; Park and Kim, 2022).

The most significant effects of toxic substances on fertilization capacity and embryonic development mainly involve the creation of an oxidative environment that damages sperm DNA (Rocco et al., 2023). In physiological conditions the main process for preserving sperm DNA integrity involves remodeling sperm chromatin, which condenses and stabilizes significantly by replacing histones with protamines in humans (Okada, 2022; Török and Gornik, 2018). However, organisms such as echinoid sea urchins have a unique sperm-specific histone family (spH2B) without protamines. This family includes an elongated N-terminal tail with multiple repeats of an SP(K/R)(K/R) sequence, known as the SPKK motif, binding to A/T-rich DNA sequences. Following fertilization, serine phosphorylation on the SPKK motif enhances chromatin accessibility, preventing DNA binding and enabling transcription (Török et al., 2023; Poccia and Green, 1992). However, an external event could disrupt these processes, leading to the formation of spermatozoa with not completely intact DNA (Mottola et al., 2022).

From a careful investigation of the data present in the literature, the total absence of experiments on the effects of microplastics on sea urchin spermatozoa was observed; on the other hand, many scientific studies have been highlighted which ascertain the cytotoxic and genotoxic properties of PS-MPs.

Considering that *Paracentrotus lividus* has been proposed as an interesting non-mammalian model to evaluate human health risks caused by the presence of different contaminants in the marine environment and as a promising alternative organism in line with the principle of replacement, reduction and refinement of animals (3R) for use in regulatory tests (Pinsino and Alijagic, 2019), the aim of this research was to evaluate, the *in vitro* cytotoxic and genotoxic ability of PS-MPs on *P. lividus* spermatozoa highlighting any alterations in seminal parameters, production of reactive oxygen species (ROS), alterations in the genetic material detected in term of sperm DNA fragmentation.

Materials and methods

Chemical

In the study polystyrene microplastics with a standard size of 1.0 µm and a specific weight of 1.05 g/cm^3 were used, with a solid content of 2 % by weight. They were purchased as a concentrated aqueous solution 21.000 mg/L, containing approximately 3.43 x 10^{10} particles/mL, from the pharmaceutical company Sigma-Aldrich (St. Louis, USA; product code 72.938; batch number BCCH6977; purity grade analytical standard).

Sea urchins collection

The sea urchins *Paracentrotus lividus* (Lamarck 1816) were collected in the Gulf of Naples (Tyrrhenian Sea), from a place not private or protected in any way, according to the Italian legislation of the Merchant Navy (DPR 1639/68, 19/09/ 1980 confirmed by Legislative Decree 9/ 01/2012n.4). The field studies did not involve endangered or protected species. All animal procedures conformed to European Union guidelines (Directive 609/86).

After collection, the sea urchin specimens were transported to the Molecular Cytogenetics laboratory of the Department of Pharmaceutical Biological Environmental Sciences and Technologies within 1 h and maintained in tanks with circulating sea water at 18 °C. During the entire breeding period, the animals were fed *Ulva lactuca and Posidonia oceanica*. After acclimatization for 10 days in tanks with circulating sea water (temperature: 18 ± 1 °C, salinity: 38 ± 1, dissolved O2: 7 mg/L, pH: 8.1), the samples were transferred to 5 L tanks with filtered sea water (FSW), maintaining the same breeding conditions.

Sea urchins sperm exposure

To obtain a large number of gametes to perform all tests in triplicate, and for enhanced data robustness, sperm deposition, was induced in 8 *P. lividus* specimens generating an osmotic shock by injecting a 0.5 M KCl (Carlo Erba, Milan, Italy; CAS N. 7447–40-7) solution through the peristomal membrane. Then, semen was collected dry from each male, directly from the gonopores or gonadal pores, using an automatic pipette and stored pooled, undiluted, in a sterile tube placed on ice at 4 °C (Adams et al., 2019).

Centrifugation at 2000 revolutions per minute (rpm) for 1 min was applied to the semen samples and spermatozoa were isolated.

Subsequently, the cellular pellet from each specimens was diluted in 1 ml of FSW, and then treated *in vitro* as follows: 7 aliquots were treated with 50 μg/mL of PS-MPs for 30 min; 7 aliquots were treated with 1 μl/mL of hydrogen peroxide (H_2O_2), a known genotoxic agent and used as a positive control; 5 untreated aliquots were used as a negative control. Once the exposure was completed, centrifugation was performed at 2000 rpm for 1 min and sperm cells collected. Finally, the pellet of spermatozoa of each experimental groups was suspended again in a volume equal to 1 mL of FSW to then be subjected to sperm parameters evaluation, cytotoxicity and genotoxicity tests.

Sperm parameters evaluation

The viability of the spermatozoa was assessed using the eosin test (Sigma-Aldrich, CAS N. 15086–94-9; Lin et al., 1998) also called the Williams Pollack test, which highlights the cellular integrity of the spermatozoa. This method involves the preparation of slides, previously washed in methanol (WWR Chemicals, CAS 67–56-1; Milan, Italy) with 10 μL of spermatozoa suspended in FSW, to which 10 μL of 5 % eosin Y were added and, finally, covered with a coverslip. The slides were immediately observed under an optical microscope (Carl Zeiss, Germany) at 40X magnification to perform a count of viable cells which have not incorporated the dye versus dead cells which will appear colourful. At least 200 spermatozoa were counted in 5 different observation fields.

The morphological evaluation is based on the distinction between typical and atypical shapes. Atypias may affect the head, neck, and tail and generally include: macrocephalic, microcephalic, elongated heads, pointed heads, double heads, angulated necks, immatures, twisted tails, double tails, and other forms (WHO, 2001).

Three slides were prepared for each experimental group by depositing 10 μL of suspended spermatozoa in FSW and subsequently stained with an equal quantity of Papanicolaou staining solution (Sigma-Aldrich, CAT. HT40432) to then be observed under the optical microscope with 40X magnification.

The evaluation of motility was performed by the same expert operator by a Makler counting chamber. For each replicate, 10 μL of sperm sample was placed on a glass slide and covered with a coverslip. Sperm were classified into motile (progressive and non-progressive) and non-motile (Ignoto et al., 2023). At least 200 spermatozoa from each experimental group were considered in 5 different observation fields of three slides.

Sperm DNA fragmentation analysis

The sperm DNA Fragmentation Analysis was conducted as previously described form human samples (Iovine at al., 2021). Three slides were prepared for the portion tested with the PS-MPs, for the positive control and for the negative control groups. 10 μl of each aliquot of spermatozoa (2 x 10^6 cell/mL) were smear on the slides and then left to dry at room temperature. After this step, the slides were placed in 4 % fixative paraformaldehyde (Carlo Erba, CND: W0103705) for 1 h at room temperature, then the slides were incubated in the permeabiliser solution (0.1 % sodium citrate, 0.1 % Triton X-100). Following a rinse in 1X PBS the slides were subjected ready to reaction mix of *In Situ* Cell Death Detection Kit (Roche Diagnostics, Germany, REF 11684795910) consisting of 25 μl of Label Solution and 5 μl of enzyme as reported in the manufacturer's instructions. The slides were then placed in a humid chamber for 1 h in the dark. After enzymatic reaction the coverslips were removed and the slides were immersed in DAPI solution containing 20X SSC, stock DAPI (4,6-diamidin-2-phenylindole; Sigma-Aldrich, CAS N. 28718–90-3) and distilled H_2O. Finally, 100 μl of DABCO solution (Tris HCl 0.02 %, Glycerol 90 %, DABCO (1,4-diazabicyclo[2.2.2]octane) 2.2 %; Sigma-Aldrich)) is placed on each individual slide and the coverslip was placed on top.

The slides thus obtained were then carefully observed under an epifluorescence microscope (Nikon ECLIPSE – E600) equipped with a DAPI and FITC (fluorescein isothiocyanate) with 100X magnification.

The result will be the observation of blue or green cells nuclei, depending respectively on the presence (green) or absence (blue) of fragmented DNA. Damage was measured numerically through the determination of the Sperm DNA fragmentation (SDF) index, which consists of the ratio of green nuclei to the total blue nuclei per 100.

Sperm reactive oxygen species detection

For determination of reactive oxygen species three slides were prepared for the portion of sperm tested with the PS-MPs, with H_2O_2 and for the negative controls. 20 μL of sperm suspension in FSW of each experimental groups were diluted in 1 mL of 0.2 % NBT solution (Sigma-Aldrich, CAS N. 298–83-9) and incubated at 18 °C for 15 min. After centrifuging at 2000 rpm for 1 min, 1 mL of could 1X PBS was added. Subsequently, a series of three centrifuge was performed at 1500 rpm for 5 min to allow purification of the cellular samples. At the end of the last washing procedure, the pellet was suspended in 200 μL of 1X PBS and 100 μL of cell solution was deposited on the glass slides. After air dried, the slides were fixed with 100 % methanol (WWR Chemicals, CAS 67–56-1) for 1 min and then rinsed with highly purified water. To highlight the cells, Safranin (Sigma-Aldrich, CAS N. 477–73-6) was used as a counterstain. The slides were air dried and then observed under the optical microscope with 40X magnification. The result of this test was expressed as a percentage of reactive oxygen species (%ROS), i.e. the ratio between the number of cells presenting ROS and the total number of cells per 100.

Statistical analysis

Differences in percentage of cellular viability, motility, morphology, %SDF and %ROS, between experimental groups were expressed as mean ± SD. *U* test (Mann-Witney-Wilcox test) and *t*-test, as nonparametric tests, were used for statistical evaluation using GraphPad Prism 10 (GraphPad Software Inc., San Diego, CA, USA). Results were considered statistically significant when $p \leq 0.05$.

Results

Sea urchin sperm parameters

The percentage of viable sperm cells decreased after PS-MPs exposure compared to the negative controls. After 30 min the percentage of viability in sperm cells exposed to 50 μg/mL PS-MPs and in untreated cells was 20.3 ± 1.27 and 32.0 ± 1.70 respectively; however, the viability in the PS-MPs exposed sample was higher than the positive control (14.2 % ± 2.55).

No statistically significant alteration in morphology was highlighted after treatment with 50 μg/mL PS-MPs. In the treated sample a presence of normal forms of 22.3 % ± 0.85 was found, compared to the negative control (23 % ± 0.71); instead, after 30 min of H_2O_2 exposure an alteration in normal morphology was observed (14.2 % ± 3.25). Furthermore, following 30 min of treatment with PS-MPs, agglutination of head-to-head spermatozoa was observed, not found in the negative control and in positive control (Fig. 1).

The samples exposed to 50 μg/mL PS-MPs for 30 min showed a percentage of sperm motility equal to 9.0 % ± 0.99, compared to a value of 30.7 % ± 1.27 of sperm motility found in the negative controls. The sperm motility was more reduced after 30 min of H_2O_2 treatment (6.9 % ± 1.70). (Table 1).

Sea urchin sperm DNA fragmentation

The TUNEL test shows DNA fragmentation in sea urchin spermatozoa. A statistically significant increase in the sperm DNA fragmentation

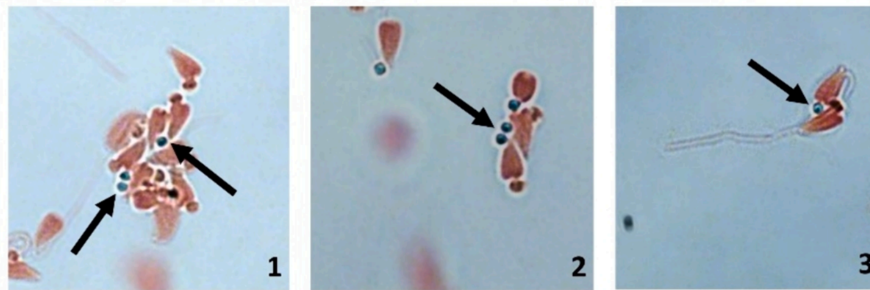

Fig. 1. Three representative images (1, 2, 3) obtained by optical microscope (Carl Zeiss, Germany) at 40X magnification showing sea urchin (*P. lividus*) sperm agglutination after a 30-minute treatment with 50 μg/mL of PS-MPs. The presence of microparticles between sperm heads is also visible (black arrows).

Table 1
Mean percentage ± SD of viable, motile and normal sea urchin (*P. lividus*) spermatozoa after 30 min of treatment with 50 μg/mL PS-MPs and 1 μL/mL H_2O_2. *$p \leq 0.05$.

Sperm parametres	Negative Control	50 μg/mL PS-MPs	1 μL/mL H_2O_2
Viability (%)	3.0 ± 1.70	20.3 ± 0.27*	14.2 ± 2.55*
Motility (%)	30.7 ± 1.27	9.0 ± 0.99*	6.9 ± 1,70*
Morphology (%)	**23.0 ± 0.71**	**22.3 ± 0.85**	**14.2 ± 0.25***

(SDF) index in the sample treated for 30 min with 50 μg/mL PS-MPs. The plastic micro-fragments were identified as responsible for a sperm DNA fragmentation equal to 18.7 % ± 0.85, compared to the negative control, where the %SDF was found to be around 3.4 % ± 0.42. Instead, in the positive control groups the percentage of sperm DNA damage detected was 22 % ± 0.57. (Fig. 2).

Sea urchin sperm reactive oxygen species

The results of the NBT assay suggest that 30 min of 50 μg/mL PS-MPs exposure induces a statistically significant increase of ROS in *P. lividus* spermatozoa, as 15.6 % ± 0.57 treated spermatozoa showed the formazan reaction, compared to a value of 2.4 % ± 0.42 formazan reaction observed in untreated groups. Additionally, a percentage of 19.5 % ± 0.85 sperm cells with the presence of formazan reaction was observed in positive control groups (Fig. 3).

Discussion

Environmental health is significantly compromised by numerous pollutants, such as heavy metals, plastic products, pesticides, chemicals, and pharmaceuticals, which are generated by anthropogenic activities (Landrigan et al., 2020). Although they have been recognized as responsible for the imbalance of all natural habitats, the main concern regarding these environmental contaminants is that they represent a serious insult to animal and human health. Plastic products have become an essential commodity in everyday life. However, they are designated as prominent environmental pollutants, capable of entering the food chain and reaching humans (Krause et al., 2021) interfering with organs functionality included the reproductive system (Blackburn and Green, 2022). Plastic materials are subject to chemical-physical processes, which lead to their fragmentation into microplastics (MP). Among the multitude of plastic materials, polystyrene (PS) stands out which, thanks to its versatility and inert nature, is used in a large variety of commercial products (Marquez et al., 2023).

Scientific studies have established that PS-MPs affect the reproductive capacity of various aquatic and terrestrial organisms. In this case, the induced effects are thinning of the basal membrane of the testicle, destruction of the blood-testis barrier, oxidative stress, apoptosis, reduction in sperm count and motility and increase in abnormal spermatozoa number (Qiang and Cheng, 2021; Hou et al., 2022; Sussarellu et al., 2016; Jin et al., 2021).

Furthermore, literature data have confirmed the ability of PS-MPs to determine alterations in the growth and morphology of larvae and embryos of the *P. lividus* species (Martínez-Gómez et al., 2017), however there is no study on the effects of PS-MPs in *P. lividus* spermatozoa. Most

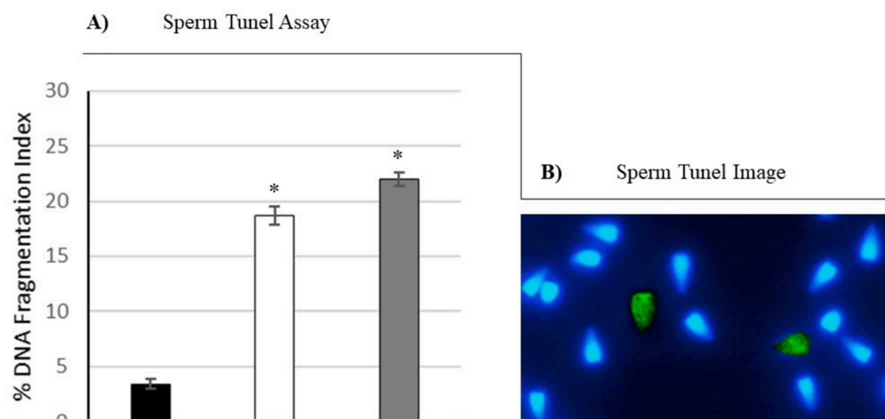

Fig. 2. A) Percentage of Sperm DNA Fragmentation (SDF) in sea urchin (*P. lividus*) spermatozoa obtained by TUNEL Assay. Black bar: untreated spermatozoa; white bar: spermatozoa after 30 min of treatment with 50 μg/mL PS-MPs; grey bar: spermatozoa after 30 min of treatment with 1 μL/mL H_2O_2. **B)** Representative image of sea urchin (*P. lividus*) spermatozoa after treatment with 50 μg/mL PS-MPs obtained by TUNEL technique. In blue (DAPI filter) spermatozoa with intact DNA, in green (FITC filter) spermatozoa with fragmented DNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

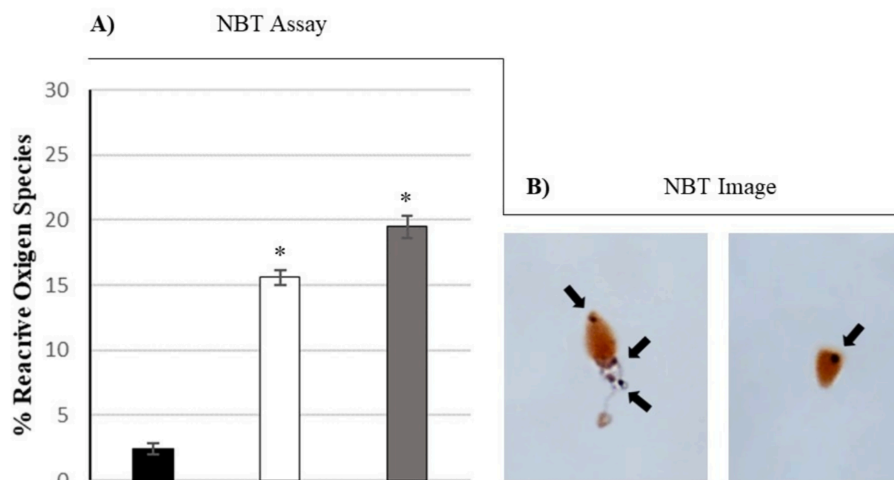

Fig. 3. **A)** Percentage of Nitro-Blue Tetrazolium chloride (NBT) in sea urchin (*P. lividus*) spermatozoa. Black bar: untreated spermatozoa; white bar: spermatozoa after 30 min of treatment with 50 μg/mL PS-MPs; grey bar: spermatozoa after 30 min of treatment with 1 μL/mL H_2O_2. **B)** Representative image of ROS highlighted by NBT (arrows) in *P. lividus* spermatozoa after 30 min of treatment with 50 μg/mL PS-MPs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

marine invertebrate organisms, including *P. lividus* release gametes into the marine environment, to allow the formation of a new individual. Therefore, the quality of spermatozoa depends greatly on the quality of the environment in which they are released. Considering the lack of data in the literature, the aim of this research was to evaluate the cytotoxicity and genotoxicity of polystyrene microplastics on *P. lividus* spermatozoa. The study evaluated cytotoxicity in *P. lividus* spermatozoa using microscopic analysis of seminal fluid, assessing viability, motility, and morphology. Sperm DNA fragmentation (%SDF) was determined via the TUNEL technique to assess genome integrity, while intracellular reactive oxygen species were measured using the NBT test. Given the unknown toxicity level of PS-MPs on *P. lividus* spermatozoa, experiments were conducted with a single exposure time (30 min) and concentration (50 μg/mL), selected based on relevant literature concerning exposure of gametes to similar substances (Pikula et al., 2020). The results obtained are perfectly in agreement with the data already present in the literature, in which polystyrene microplastics are classified as polluting and toxic substances, capable of causing damage to various organisms (Yang et al., 2023).

The analysis of seminal parameters indicates a concurrent decrease in sperm vitality and motility alongside elevated %SDF following PS-MPs treatment. Additionally, the NBT test demonstrated a PS-MPs toxic action mediated by oxidative stress. The alteration in sperm parameters and the increase in SDF are presumably the consequence of the genotoxicity of PS-MPs. The mechanism of action is not very clear, but it is known that they can generate an inflammatory-oxidative environment, which leads to the generation of genotoxic oxidant species that trigger apoptosis (Dubey et al., 2022). However, the genomic damage caused by PS-MPs could also be of a direct nature given that, thanks to their small size, they can penetrate cell membranes by endocytosis (Liu et al., 2021) and probably reach and damage the nucleus. In fact, studies demonstrate that PS-MPs can be internalized by cells both through endocytosis and through the diffusion mechanism (Han and Ryu, 2022).

As for the initiation of an inflammatory event, substantial support can be inferred from the study conducted by Li and colleagues (2020), wherein the intestinal microbiota of a C57BL/6 mouse model was exposed to MP-PS. The research revealed that mice fed with microplastics exhibited noticeable inflammation in the intestine (colon and duodenum) along with increased expression of the innate immune receptor TLR4, the AP-1 protein which triggers the pro-inflammatory transcription factor c-Jun, and the interferon regulatory factor IRF5. A second plausible hypothesis that could lead to the reduction of sperm motility and vitality is that of the formation of reactive oxygen species (ROS), which induce oxidative stress and could indirectly result in modifications of the genome or damage to DNA. A plausible finding is proposed in the work of Dong and collaborators (2021), in which exposure to PS-MPs resulted in the formation of ROS causing oxidative damage to human lung epithelial BEAS-2B cells. It has been demonstrated that the ROS produced induce an up-regulation of the p53 gene transcription which, in turn, determines the activation of casp3b. Activated casp3b promotes transcription of gadd45ba resulting in DNA damage and apoptosis (Xie et al., 2020). However, DNA fragmentation does not always evolve into apoptosis, not infrequently cells with altered DNA escape cellular control mechanisms by inheriting mutations which in the case of germ cells translate into the transfer of genetic defects to offspring (Sengupta et al., 2023). Epigenetic changes with transgenerational effects are the most worrying repercussions, in fact, environmental disorders determine changes in the maturation of sperm chromatin by modifying gene transcription without modifying the DNA sequence through epigenetic processes (Delbès et al., 2010; Lombó et al., 2019). This poses even more concerns on transgenerational effects of paternal PS-MPs exposure.

In addition to these results, following treatment with PS-MPs, sperm agglutination was observed. Sperm agglutination generally occurs in cases of immunological infertility. This condition is characterized by the presence, within the seminal fluid, of anti-sperm antibodies which, recognizing them as foreign, bind to them and, therefore, act as a bridge in the bond between two spermatozoa (Leathersich and Hart, 2022). In this research, the possibility of immunological infertility was excluded, as the sperm agglutinates were absent in the untreated samples and were detected exclusively after treatment with microplastics. Therefore, it is assumed that agglutination is caused by the presence of PS-MPs which, thanks to their own characteristics and the environment in which they are found, adhere to the sperm cell membrane, resulting in the formation of an aggregate of spermatozoa. In fact, recent literature studies have highlighted that the intrinsic chemical-physical characteristics of microplastics grant them an aggregation capacity, generally influenced by the ionic strength and electrolytic valence of the surrounding medium (Shams et al., 2020).

Therefore, in agreement with those reported in the literature conducted on other experimental models, PS-MPs affect *P. lividus* male fertility through their cytotoxic and genotoxic action on spermatozoa. In particular, the data obtained provide new insights into the deleterious effects of PS-MPs on marine life, revealing the vulnerability of *P. lividus*

spermatozoa to such compounds. It is therefore possible to conclude that environment exposure to PS-MPs can represent a risk to exposed organism's fertility creating an oxidative environment that affects the normal structure of sperm chromatin with consequent DNA fragmentation and alteration in normal seminal parameters.

However, further studies will certainly be necessary to confirm the data obtained and the damage hypotheses advanced. More precisely, it will be essential to verify, with more advanced tools i.e. spectroscopic analytical techniques (UV–Vis and FT-IR), Scanning Electron Microscopy (SEM), and with higher concentrations and at longer exposure times the ability of PS-MPs to aggregate together, bind to the sperm membrane and form bridges between spermatozoa, blocking movement, as well as to cross membranes and induce intracellular damage.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Adams, N.L., Heyland, A., Rice, L.L., Foltz, K.R., 2019. Procuring animals and culturing of eggs and embryos. Methods Cell Biol. 150, 3–46. https://doi.org/10.1016/bs.mcb.2018.11.006.

Almeda, R., Rodriguez-Torres, R., Rist, S., Winding, M.H.S., Stief, P., Hansen, B.H., et al., 2021. Microplastics do not increase bioaccumulation of petroleum hydrocarbons in Arctic zooplankton but trigger feeding suppression under co-exposure conditions. Sci. Total Environ. 751, 141264 https://doi.org/10.1016/j.scitotenv.2020.141264.

Bessa, F., Ratcliffe, N., Otero, V., Sobral, P., Marques, J.C., Waluda, C.M., et al., 2019. Microplastics in gentoo penguins from the Antarctic region. Sci. Rep. 9 (1), 14191. https://doi.org/10.1038/s41598-019-50621-2.

Blackburn, K., Green, D., 2022. The potential effects of microplastics on human health: What is known and what is unknown. Ambio 51 (3), 518–530. https://doi.org/10.1007/s13280-021-01589-9.

Daniel, D.B., Ashraf, P.M., Thomas, S.N., Thomson, K.T., 2021. Microplastics in the edible tissues of shellfishes sold for human consumption. Chemosphere 264 (Pt 2), 128554. https://doi.org/10.1016/j.chemosphere.2020.128554.

De Marco, G., Conti, G.O., Giannetto, A., Cappello, T., Galati, M., Iaria, C., et al., 2022. Embryotoxicity of polystyrene microplastics in zebrafish Daniorerio. Environ. Res. 208, 112552 https://doi.org/10.1016/j.envres.2021.112552.

Delbès, G., Hales, B.F., Robaire, B., 2010. Toxicants and human sperm chromatin integrity. Mol. Hum. Reprod. 16 (1), 14–22. https://doi.org/10.1093/molehr/gap087.

Ding, J., Zhu, D., Wang, H.T., Lassen, S.B., Chen, Q.L., Li, G., et al., 2020. Dysbiosis in the Gut Microbiota of Soil Fauna Explains the Toxicity of Tire Tread Particles. Environ. Sci. Tech. 54 (12), 7450–7460. https://doi.org/10.1021/acs.est.0c00917.

Dissanayake, P.D., Kim, S., Sarkar, B., Oleszczuk, P., Sang, M.K., Haque, M.N., et al., 2022. Effects of microplastics on the terrestrial environment: A critical review. Environ. Res. 209, 112734 https://doi.org/10.1016/j.envres.2022.112734.

Dong, C.D., Chen, C.W., Chen, Y.C., Chen, H.H., Lee, J.S., Lin, C.H., 2020. Polystyrene microplastic particles: In vitro pulmonary toxicity assessment. J. Hazard. Mater. 385, 121575 https://doi.org/10.1016/j.jhazmat.2019.121575.

Dubey, I., Khan, S., Kushwaha, S., 2022. Developmental and reproductive toxic effects of exposure to microplastics: A review of associated signaling pathways. Frontiers in Toxicology 4, 901798. https://doi.org/10.3389/ftox.2022.901798.

Gallo, A., Boni, R., Tosti, E., 2020. Gamete quality in a multistressor environment. Environ 138, 105627. https://doi.org/10.1016/j.envint.2020.105627. Epub 2020 Mar 6 PMID: 32151884.

Han, S.W., Ryu, K.Y., 2022. Increased clearance of non-biodegradable polystyrene nanoplastics by exocytosis through inhibition of retrograde intracellular transport. J. Hazard. Mater. 439, 129576 https://doi.org/10.1016/j.jhazmat.2022.129576.

Hermo, L., Pelletier, R.M., Cyr, D.G., Smith, C.E., 2010. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. Microsc Res Tech 73 (4), 241–278. https://doi.org/10.1002/jemt.20783. PMID: 19941293.

Hirt, N., Body-Malapel, M., 2020. Immunotoxicity and intestinal effects of nano- and microplastics: a review of the literature. Part. Fibre Toxicol. 17 (1), 57. https://doi.org/10.1186/s12989-020-00387-7.

Hou, L., Wang, D., Yin, K., Zhang, Y., Lu, H., Guo, T., et al., 2022. Polystyrene microplastics induce apoptosis in chicken testis via crosstalk between NF-κB and Nrf2 pathways. Comparative Biochemistry and Physiology. Toxicology & Pharmacology : CBP 262, 109444. https://doi.org/10.1016/j.cbpc.2022.109444.

Ignoto, S., Pecoraro, R., Scalisi, E.M., Contino, M., Ferruggia, G., Indelicato, S., et al., 2023. Spermiotoxicity of Nano-TiO2 Compounds in the Sea Urchin Paracentrotus lividus (Lamarck, 1816): Considerations on Water Remediation. Journal of Marine Science and Engineering 11 (2), 380. https://doi.org/10.3390/jmse11020380.

Iovine, C., Mottola, F., Santonastaso, M., Finelli, R., Agarwal, A., Rocco, L., 2021. In vitro ameliorative effects of ellagic acid on vitality, motility and DNA quality in human spermatozoa. Mol. Reprod. Dev 88 (2), 167–174. https://doi.org/10.1002/mrd.23455.

Jiang, B., Kauffman, A.E., Li, L., McFee, W., Cai, B., Weinstein, J., et al., 2020. Health impacts of environmental contamination of micro- and nanoplastics: a review. Environ. Health Prev. Med. 25 (1), 29. https://doi.org/10.1186/s12199-020-00870-9.

Jin, H., Ma, T., Sha, X., Liu, Z., Zhou, Y., Meng, X., et al., 2021. Polystyrene microplastics induced male reproductive toxicity in mice. J. Hazard. Mater. 401, 123430 https://doi.org/10.1016/j.jhazmat.2020.123430.

Krause, S., Baranov, V., Nel, H. A., Drummond, J. D., Kukkola, A., Hoellein, T., et al. (2021). Gathering at the top? Environmental controls of microplastic uptake and biomagnification in freshwater food webs. Environmental pollution (Barking, Essex : 1987), 268(Pt A), 115750. https://doi.org/10.1016/j.envpol.2020.115750.

Landrigan, P.J., Stegeman, J.J., Fleming, L.E., Allemand, D., Anderson, D.M., Backer, L. C., et al., 2020. Human Health and Ocean Pollution. Ann. Glob. Health 86 (1), 151. https://doi.org/10.5334/aogh.2831.

Landrigan, P.J., Raps, H., Cropper, M., Bald, C., Brunner, M., Canonizado, E.M., et al., 2023. The Minderoo-Monaco Commission on Plastics and Human Health. Ann. Glob. Health 89 (1), 23. https://doi.org/10.5334/aogh.4056.

Leathersich, S., Hart, R.J., 2022. Immune infertility in men. Fertil. Steril. 117 (6), 1121–1131. https://doi.org/10.1016/j.fertnstert.2022.02.010.

Li, B., Ding, Y., Cheng, X., Sheng, D., Xu, Z., Rong, Q., et al., 2020. Polyethylene microplastics affect the distribution of gut microbiota and inflammation development in mice. Chemosphere 244, 125492. https://doi.org/10.1016/j.chemosphere.2019.125492.

Lin, M.H., Morshedi, M., Srisombut, C., Nassar, A., Oehninger, S., 1998. Plasma membrane integrity of cryopreserved human sperm: an investigation of the results of the hypoosmotic swelling test, the water test, and eosin-Y staining. Fertil. Steril. 70 (6), 1148–1155. https://doi.org/10.1016/s0015-0282(98)00351-3.

Liu, L., Xu, K., Zhang, B., Ye, Y., Zhang, Q., Jiang, W., 2021. Cellular internalization and release of polystyrene microplastics and nanoplastics. The Science of the Total Environment 779, 146523. https://doi.org/10.1016/j.scitotenv.2021.146523.

Lombó, M., Fernández-Díez, C., González-Rojo, S., Herráez, M.P., 2019. Genetic and epigenetic alterations induced by bisphenol A exposure during different periods of spermatogenesis: from spermatozoa to the progeny. Sci. Rep. 9 (1), 18029. https://doi.org/10.1038/s41598-019-54368-8.

Marquez, C., Martin, C., Linares, N., De Vos, D., 2023. Catalytic routes towards polystyrene recycling. Mater. Horiz. 10 (5), 1625–1640. https://doi.org/10.1039/d2mh01215d.

Martínez-Gómez, C., León, V.M., Calles, S., Gomáriz-Olcina, M., Vethaak, A.D., 2017. The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. Mar. Environ. Res. 130, 69–76. https://doi.org/10.1016/j.marenvres.2017.06.016.

Menon, V., Sharma, S., Gupta, S., Ghosal, A., Nadda, A.K., Jose, R., et al., 2023. Prevalence and implications of microplastics in potable water system: An update. Chemosphere 317, 137848. https://doi.org/10.1016/j.chemosphere.2023.137848.

Montano, L., Giorgini, E., Notarstefano, V., Notari, T., Ricciardi, M., Piscopo, M., et al., 2023. Raman Microspectroscopy evidence of microplastics in human semen. Sci. Total Environ. 901, 165922 https://doi.org/10.1016/j.scitotenv.2023.165922.

Morroni, L., Rakaj, A., Grosso, L., Flori, G., Fianchini, A., Pellegrini, D., et al. (2023). Echinoderm larvae as bioindicators for the assessment of marine pollution: Sea urchin and sea cucumber responsiveness and future perspectives. Environmental pollution (Barking, Essex : 1987), 335, 122285. https://doi.org/10.1016/j.envpol.2023.122285.

Mottola, F., Iovine, C., Carannante, M., Santonastaso, M., Rocco, L., 2022. In Vitro Combination of Ascorbic and Ellagic Acids in Sperm Oxidative Damage Inhibition. Int. J. Mol. Sci. 23 (23), 14751. https://doi.org/10.3390/ijms232314751.

Nishimura, H., L'Hernault, S.W., 2017. Spermatogenesis. Curr Biol 27 (18), R988–R994. https://doi.org/10.1016/j.cub.2017.07.067. PMID: 28950090.

Okada, Y., 2022. Sperm chromatin structure: Insights from in vitro to in situ experiments. Curr Opin Cell Biol 75, 102075. https://doi.org/10.1016/j.ceb.2022.102075. Epub 2022 Mar 25 PMID: 35344802.

Oliveri Conti, G., Ferrante, M., Banni, M., Favara, C., Nicolosi, I., Cristaldi, A., et al., 2020. Micro- and nano-plastics in edible fruit and vegetables. In: The First Diet Risks Assessment for the General Population, 187. Environmental research, p. 109677. https://doi.org/10.1016/j.envres.2020.109677.

Paço, A., Duarte, K., da Costa, J.P., Santos, P.S.M., Pereira, R., Pereira, M.E., et al., 2017. Biodegradation of polyethylene microplastics by the marine fungus Zalerion maritimum. Sci. Total Environ. 586, 10–15. https://doi.org/10.1016/j.scitotenv.2017.02.017.

Park, S.H., Kim, K., 2022. Microplastics induced developmental toxicity with microcirculation dysfunction in zebrafish embryos. Chemosphere 286 (Pt 3), 131868. https://doi.org/10.1016/j.chemosphere.2021.131868.

Pikula, K., Zakharenko, A., Chaika, V., Em, I., Nikitina, A., Avtomonov, E., et al., 2020. Toxicity of Carbon, Silicon, and Metal-Based Nanoparticles to Sea Urchin Strongylocentrotus Intermedius. Nanomaterials (basel, Switzerland) 10 (9), 1825. https://doi.org/10.3390/nano10091825.

Pinsino, A., Alijagic, A., 2019. Sea urchin Paracentrotus lividus immune cells in culture: formulation of the appropriate harvesting and culture media and maintenance conditions. Biology Open 8 (3), bio039289. https://doi.org/10.1242/bio.039289.

Poccia, D.L., Green, G.R., 1992. Packaging and unpackaging the sea urchin sperm genome. Trends Biochem Sci 17 (6), 223–227. https://doi.org/10.1016/0968-0004(92)90382-j. PMID: 1502725; PMCID: PMC7135322.

Prata, J.C., da Costa, J.P., Lopes, I., Duarte, A.C., Rocha-Santos, T., 2020. Environmental exposure to microplastics: An overview on possible human health effects. Sci. Total Environ. 702, 134455 https://doi.org/10.1016/j.scitotenv.2019.134455.

Qiang, L., Cheng, J., 2021. Exposure to polystyrene microplastics impairs gonads of zebrafish (Danio rerio). Chemosphere 263, 128161. https://doi.org/10.1016/j.chemosphere.2020.128161.

Rocco, L., Mottola, F., Roychoudhury, S., 2023. Editorial: DNA damage and repair in reproductive and embryo cells. Front. Cell Dev. Biol. 11, 1274341. https://doi.org/10.3389/fcell.2023.1274341.

Sengupta, P., Dutta, S., Liew, F.F., Dhawan, V., Das, B., Mottola, F., et al., 2023. Environmental and Genetic Traffic in the Journey from Sperm to Offspring. Biomolecules 13 (12), 1759. https://doi.org/10.3390/biom13121759.

Shams, M., Alam, I., Chowdhury, I., 2020. Aggregation and stability of nanoscale plastics in aquatic environment. Water Res. 171, 115401 https://doi.org/10.1016/j.watres.2019.115401.

Sharifinia, M., Bahmanbeigloo, Z.A., Keshavarzifard, M., Khanjani, M.H., Lyons, B.P., 2020. Microplastic pollution as a grand challenge in marine research: A closer look at their adverse impacts on the immune and reproductive systems. Ecotoxicol. Environ. Saf. 204, 111109 https://doi.org/10.1016/j.ecoenv.2020.111109.

Shi, W., Sun, S., Han, Y., Tang, Y., Zhou, W., Zhang, W., et al., 2022. Microplastics hamper the fertilization success of a broadcast spawning bivalve through reducing gamete collision and gamete fusion efficiency. Aquatic Toxicology (amsterdam, Netherlands) 242, 106049. https://doi.org/10.1016/j.aquatox.2021.106049.

Skoczinski, P., Krause, L., Raschka, A., Dammer, L., Carus, M., 2021. Current status and future development of plastics: Solutions for a circular economy and limitations of environmental degradation. Methods Enzymol. 648, 1–26. https://doi.org/10.1016/bs.mie.2020.11.001.

Song, Y.K., Hong, S.H., Jang, M., Han, G.M., Jung, S.W., Shim, W.J., 2017. Combined effects of uv exposure duration and mechanical abrasion on microplastic fragmentation by polymer type. Environ. Sci. Tech. 51 (8), 4368–4376. https://doi.org/10.1021/acs.est.6b06155.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M.E., et al., 2016. Oyster reproduction is affected by exposure to polystyrene microplastics. PNAS 113 (9), 2430–2435. https://doi.org/10.1073/pnas.1519019113.

Thompson, R.C., Swan, S.H., Moore, C.J., vom Saal, F.S., 2009. Our plastic age. Philos. Trans. R. Soc. Lond.Series b, Biological Sciences 364 (1526), 1973–1976. https://doi.org/10.1098/rstb.2009.0054.

A. Török S.G. Gornik Sperm nuclear basic proteins of marine invertebrates Results Probl Cell Differ 65 2028 15 32 10.1007/978-3-319-92486-1_2 PMID: 30083913.

Török A, Browne MJG, Vilar JC, Patwal I, DuBuc TQ, Febrimarsa, Atcheson E, Frank U, Gornik SG, Flaus A. (2023). Hydrozoan sperm-specific SPKK motif-containing histone H2B variants stabilise chromatin with limited compaction. Development, 1; 150(1):dev201058. doi: 10.1242/dev.201058. Epub 2023 Jan 12. PMID: 36633190; PMCID: PMC9903204.

World Health Organization, 2001. Manuale di laboratorio della WHO per l'esame del liquido seminale umano e dell'interazione tra spermatozoi e muco cervicale [Laboratory manual of the WHO for the examination of human semen and sperm-cervical mucus interaction]. Annali Dell'istituto Superiore Di Sanita 37 (1). I–123.

Wright, S.L., Ulke, J., Font, A., Chan, K.L.A., Kelly, F.J., 2020. Atmospheric microplastic deposition in an urban environment and an evaluation of transport. Environ. Int. 136, 105411 https://doi.org/10.1016/j.envint.2019.105411.

Xie, X., Deng, T., Duan, J., Xie, J., Yuan, J., Chen, M., 2020. Exposure to polystyrene microplastics causes reproductive toxicity through oxidative stress and activation of the p38 MAPK signaling pathway. Ecotoxicol. Environ. Saf. 190, 110133 https://doi.org/10.1016/j.ecoenv.2019.110133.

Xu, S., Ma, J., Ji, R., Pan, K., Miao, A.J., 2020. Microplastics in aquatic environments: Occurrence, accumulation, and biological effects. Sci. Total Environ. 703, 134699 https://doi.org/10.1016/j.scitotenv.2019.134699.

Yang, S., Li, M., Kong, R.Y.C., Li, L., Li, R., Chen, J., et al., 2023. Reproductive toxicity of micro- and nanoplastics. Environ. Int. 177, 108002 https://doi.org/10.1016/j.envint.2023.108002.