Journal of Marine Science and Engineering

Article

Genotoxicity of Polystyrene (PS) Microspheres in Short-Term Exposure to Gametes of the Sand Dollar *Scaphechinus mirabilis* (Agassiz, 1864) (Echinodermata, Echinoidea)

Andrey Alexandrovich Mazur [1], Viktor Pavlovich Chelomin [1], Elena Vladimirovna Zhuravel [2], Sergey Petrovich Kukla [1,*], Valentina Vladimirovna Slobodskova [1] and Nadezda Vladimirovna Dovzhenko [1]

[1] Il'ichev Pacific Oceanological Institute, Far Eastern Branch, Russian Academy of Sciences, 690041 Vladivostok, Russia; mazur.aa@poi.dvo.ru (A.A.M.); chelomin.vp@poi.dvo.ru (V.P.C.); slobodskova@list.ru (V.V.S.); doreme_07@mail.ru (N.V.D.)

[2] Department of Ecology, Far Eastern Federal University, 690922 Vladivostok, Russia; zhrvl@rambler.ru

* Correspondence: kukla.sp@mail.ru

Abstract: Microplastic pollution appears to be one of the major environmental problems in the world today, and researchers have been paying special attention to the study of the impact of microplastics on biota. In this article, we studied the short-term effects of polystyrene micro-spheres on genome integrity using the gametes of the *Scaphechinus mirabilis* sand dollar with the comet assay method. This highly sensitive method allowed us to identify the level of genome damage in both gametes before and after short-term exposure to PS microparticles. It was shown that primary polystyrene microspheres at concentrations of 10^4, 10^5, and 10^6 particles/L had a genotoxic effect during short-term exposure to the sperm of the sand dollar *S. mirabilis*, which was expressed as a significant increase in sperm DNA damage. The highest percentage of DNA damage (more than 20%) was detected in spermatozoa exposed for 1 h in water containing 105 microspheres of plastic per 1 L. Additionally, at all concentrations of microplastic studied in the experiment, the genetic damage index (GDI) values in spermatozoa exceeded the control level. However, regardless of the level of DNA damage, spermatozoa retained the ability to fertilise eggs with up to 97% efficiency. We must acknowledge that the genotoxic property of microplastic against sperm to some extent predicts the development of long-term adverse effects of environmental significance.

Keywords: microplastic; PS; sand dollar; *Scaphechinus mirabilis*; genotoxicity; gametes

Citation: Mazur, A.A.; Chelomin, V.P.; Zhuravel, E.V.; Kukla, S.P.; Slobodskova, V.V.; Dovzhenko, N.V. Genotoxicity of Polystyrene (PS) Microspheres in Short-Term Exposure to Gametes of the Sand Dollar *Scaphechinus mirabilis* (Agassiz, 1864) (Echinodermata, Echinoidea). *J. Mar. Sci. Eng.* **2021**, *9*, 1088. https://doi.org/10.3390/jmse9101088

Academic Editors: Michele Arienzo and Luciano Ferrara

Received: 16 September 2021
Accepted: 1 October 2021
Published: 5 October 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

At present, the pollution of the world's oceans with microplastics is a global environmental problem. Small synthetic polymer particles easily penetrate aquatic organisms and pose a serious threat to their life. According to Eriksen et al. [1], more than 5 trillion microscale particles of synthetic polymers are present in the world's oceans, with about 2 million tons of plastic released into the marine environment each year. In coastal waters, microplastic concentrations are estimated to range from 3 to 102,000 particles per m^3 [2].

Planktonic and benthic marine organisms are most susceptible to negative impacts from microplastic because the vast majority of polymer particles released into the marine environment are either in the water column or in the bottom layer [3]. In addition, relatively recent studies have shown that in addition to the potential physical hazards from blockage or damage to the digestive system when hydrobionts ingest microplastic, such particles also pose hazards due to exposure to chemicals used in polymer production [4]. The literature on the genotoxic properties of synthetic polymers is still scarce and contradictory. Studies have shown both an increase in DNA damage when exposed to microplastics [5,6] and the absence of genotoxic damage [7,8] in invertebrate cells. However, despite the wide range of physiological and biochemical effects initiated by polymeric microparticles upon

penetration into hydrobiont tissues and organs presented in the literature, the mechanisms of microplastic toxicity to marine organisms, including their early development stages, are still largely unclear [9,10].

In modern ecotoxicology, gametes, embryos, and larvae of various marine invertebrate species—in particular sea urchins—are among the most common biological models used to test the toxicity of various types of pollution. This is due to the generally accepted view in the literature that early developmental stages are more sensitive than adults and represent a critical period in the life cycle of an organism [11–14].

Additionally, gametic and larval aquatic invertebrates are plankton and, unlike actively swimming organisms, cannot avoid polluted water masses. In doing so, they are directly exposed to a wide range of chemicals present in the environment, including particles of microplastics [13,15].

Accordingly, the aim of this work was to investigate the potential risk of polystyrene microspheres on genome integrity (genotoxicity) and the subsequent effect on the fertilisation capacity of gametes using contaminant-sensitive gametes of the *S. mirabilis* sand dollar.

2. Materials and Methods

For the experiment, mature individuals of the *S. mirabilis* sand dollar were collected in Vostok Bay from a depth of 4–4.5 m. After being delivered to the laboratory, the animals were acclimatized at 18–19 °C for 2 days. Then, gametes of *S. mirabilis* were obtained by stimulating spawning with a 0.5 M potassium chloride solution. Eggs were treated according to a standard technique [16]. Sperm was collected immediately before the experiment and diluted with filtered and sterilized seawater. Then, control fertilisation was performed to check the quality of the germ cells; eggs with a fertilisation rate below 95% were not used in the experiment.

The micro-PS stock solution, with a concentration of 5.0% *w/v* suspended in deionized water, was procured from Baseline Chromtech Research Centre, China. This micro-PS stock had a nominal bead size of 0.9 μm (standard deviation—0.0264; distribution coefficient—0.0120). The micro-PS stock solution was used to prepare the microplastic suspensions to be tested. Suspensions containing 10^4, 10^5, and 10^6 microspheres/L were prepared. Before starting the experiment, the prepared solutions were placed in a Sapphire ultrasonic bath for 30 min to prevent the aggregation of the microplastics. To determine the working microsphere concentrations of the solutions, they were counted in a Goryaev chamber with 3 repetitions.

Two versions of the experiment were carried out. In experiment 1, the spermatozoa were kept for 1 h in the tested solutions. In experiment 2, eggs were exposed to the test solutions [17]. The ratio of spermatozoa to eggs in all experiments was 200:1. Fertilisation was then carried out in pure, sterile water, and the proportion of formed zygotes was counted after 20 min. The effect of microplastics was visually assessed by the formation of fertilisation membrane. Counting was performed in 4 parental pairs (n = 4), each in 4 parallels (n = 16) containing at least 100 zygotes.

After exposure, the degree of DNA molecule damage was assessed in *S. mirabilis* sperm and egg cells using an alkaline version of the DNA comet assay described previously [18]. This method is based on the ability of a single cell DNA immersed in low-melting agarose to distribute itself in a constant electric field according to the degree of fragmentation. As a result, fragments of damaged DNA molecules show increased migration from the nucleus, forming a "comet tail", whose length and a fraction of DNA are related to the degree of DNA damage in the cell. Under alkaline assay conditions, these parameters indicate an imbalance between the generation levels of damaged DNA sites (including single- and double-stranded breaks) and their repair by the repair system. Counting was performed in 4 parental pairs in two parallels; in each parallel, counts were made in duplicates (n = 16) containing at least 50 comets (n = 800).

The DNA comets were visualised and registered using a scanning fluorescence microscope AxioImager A1 (Carl Zeiss, Oberkochen, Germany) equipped with a AxioCam MRc digital camera (Carl Zeiss, Oberkochen, Germany). Digital images were processed using the V 1.2.2. CASP program (https://casplab.com (accessed on 5 October 2021) University of Wroclaw, Wroclaw, Poland), which can be used to make calculations of various parameters of comets indicating the DNA damage level (Figure 1). The genetic damage index (GDI) was calculated as an indicator of genotoxic effects. For this, the comets were divided into 5 classes depending on the degree of DNA molecule fragmentation (%DNA in the tail): C0 (<5%), cells with minimal damage; C1 (5–20%), cells with the low level of damage; C2 (20–40%), cells with medium damage; C3 (40–75%), cells with high DNA damage; and C4 (>75%), cells with very high DNA damage [19]. Based on the number of comets attributed to each class, the GDI was calculated as follows: GDI = (C1 + 2 × C2 + 3 × C3 + 4 × C4)/(C0 + C1 + C2 +C3 + C4) [20].

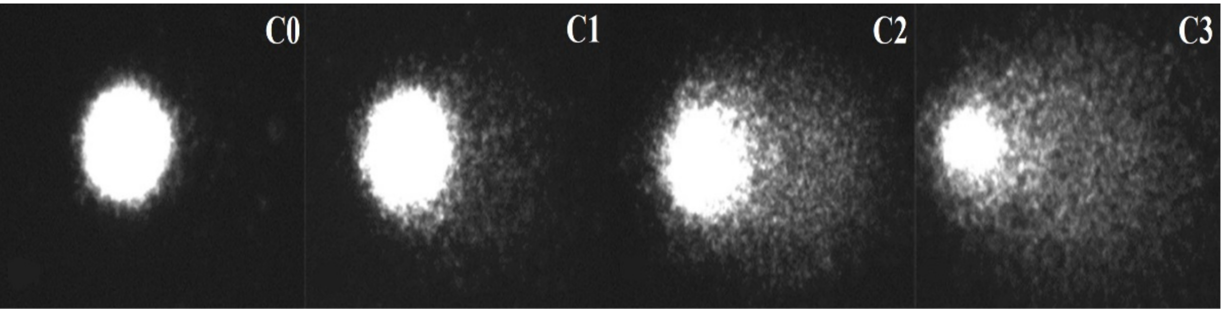

Figure 1. Comet images of the sperm S. mirabilis for different DNA damage classes.

The results of the experiment were processed with the MS Excel and Statistica 10 software packages (StatSoft, Tulsa, OK, USA). The assumptions of normality and homogeneity were assessed using Levene's and Shapiro–Wilk's tests, respectively. Data on the DNA content in the tail of the comet and fertilisation did not achieve normality, and nonparametric tests of variance Kruskal–Wallis ANOVA followed by pair-wise Mann–Whitney tests were conducted.

3. Results

In this study, we obtained data on the genotoxicity of polystyrene microspheres to the sperm and egg cells of the *S. mirabilis* (Table 1). When sand dollar eggs were briefly exposed to the investigated concentrations of microplastic, no significant difference was found in the percentage of damaged DNA compared to the control values. However, statistically significant differences were found in the percentage of sperm DNA damage from controls when exposed to water with the addition of all investigated concentrations of polystyrene microspheres.

Table 1. Assessment of DNA damage (mean ± standard deviation, N = 16; n = 800).

Concentration (Microspheres/L)	%DNA in tail		GDI	
	Sperm Cells	Egg Cells	Sperm Cells	Egg Cells
Control	10.89 ^{a} ± 0.72	5.51 ± 0.57	0.87	0.47
10^4	14.06 ^{b} ± 1.03	4.86 ± 0.62	1.06	0.34
10^5	20.17 ^{c} ± 0.81	4.15 ± 0.49	1.43	0.28
10^6	19.11 ^{c} ± 0.90	5.17 ± 0.51	1.26	0.37

Letters denote values significantly different from each other ($p < 0.05$).

The highest percentage of DNA damage (more than 20%) was detected in sperm exposed for 1 h in suspension containing 105 microspheres per 1 L.

Based on the classification proposed by Collins and colleagues [19], sperm from the control group more often formed comets that belong to classes C0 and C1, which are characteristic of undamaged and low-damaged viable cells, respectively. In addition, when sperm were exposed to microplastic, we observed an increase in the number of class 2 comets, characterized by medium damage, and the appearance of class 3 comets, characterized by a high level of damage (Figure 2).

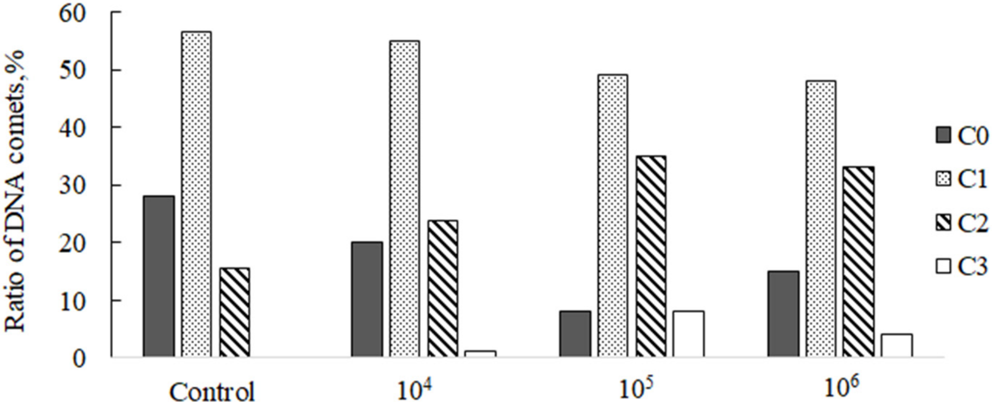

Figure 2. Induction of DNA strand breaks, represented as a percentage of comet damage classes in spermatozoa from control and experimental groups (N = 16; n = 800).

In addition to determining the percentage of DNA in the comet tail, the genetic damage index was calculated (Table 1). At all concentrations of microplastic studied in the experiment, the GDI values in sperm exceeded the control level. It is worth noting that at a concentration of 10^5 particles of microplastic/L, the GDI value in the sperm reached a value of 1.43, indicating a pronounced genotoxic effect of the studied toxicant.

In addition, we obtained data on the effect of the studied microplastic on the fertilising ability of sand dollar gametes. The experiment evaluating the effect of polystyrene microspheres on sperm and egg cells showed no significant effects on both sea urchin sperm motility and the fertilisation process itself (Figure 3).

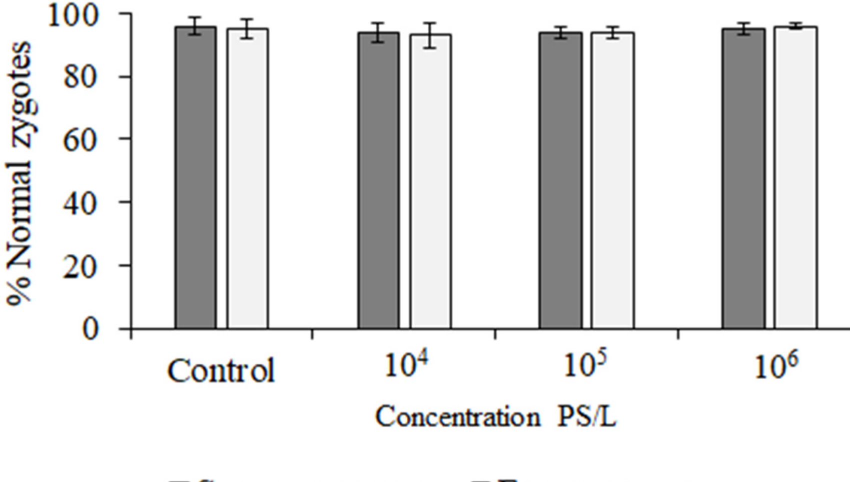

Figure 3. Effect of polystyrene microspheres on fertilisation success when exposed to *S. mirabilis* gametes (mean ± standard deviation, N = 16; n = 1600).

The percentage of normal zygotes formed after the exposure of gametes to water with the tested concentrations of primary microplastic was not significantly different from the control values in any of the cases (Supplementary File S1).

4. Discussion

Sea urchins have external fertilisation, and during spawning, their gametes are directly released into the seawater and exposed to a wide range of factors, including microplastic particles present in the environment [4,9].

Our experiments, in part, replicated the environmental conditions in terms of the interaction between plastic microparticles and sea urchin gametes. However, our studies were also carried out under controlled laboratory conditions where exposure to other concomitant stressors unique to the marine environment was minimized.

We used PS microparticle concentrations that, although many times lower than concentrations commonly used in ecotoxicological experiments and having embryotoxic effects [10,21], were nevertheless significantly higher than plastic microparticle concentrations recorded in the most polluted locations of the world's oceans [22]. This approach, belonging to the category of acute experiments, is able to identify the most vulnerable cell structures to show the associated mechanisms of toxicity. Moreover, the analytical tools available in current ecotoxicological research cannot identify the biological effects occurring at low concentrations of perturbing factors.

In our work, we applied the comet assay, which is widely used in ecotoxicological studies as a sensitive tool to assess DNA damage in individual eukaryotic cells [23,24]. This molecular approach is estimated to be tens of times (35–50) more sensitive than any biomarker used to assess the degree of toxicity at the organismal level (such as viability, reproduction, and growth) [25].

This method allowed us to detect the level of genome damage in both gamete species before and after short-term exposure to PS microparticles (Table 1). The presence of a small percentage of nuclear DNA fragmentation in untreated gametes can be explained by the accumulation of alkali-labile sites and/or single- and double-stranded breaks during oogenesis [26].

In experiments with sand dollar *S. mirabilis* sperm, the percent of DNA in the comet "tail" significantly increased depending on the microplastic concentration, indicating damage to the integrity of the genome in these cells. For presentation purposes, the obtained experimental data are presented in the form of a diagram characterizing the distribution of cells according to the degree of DNA damage of nuclei at intervals of 3% (Figure 4).

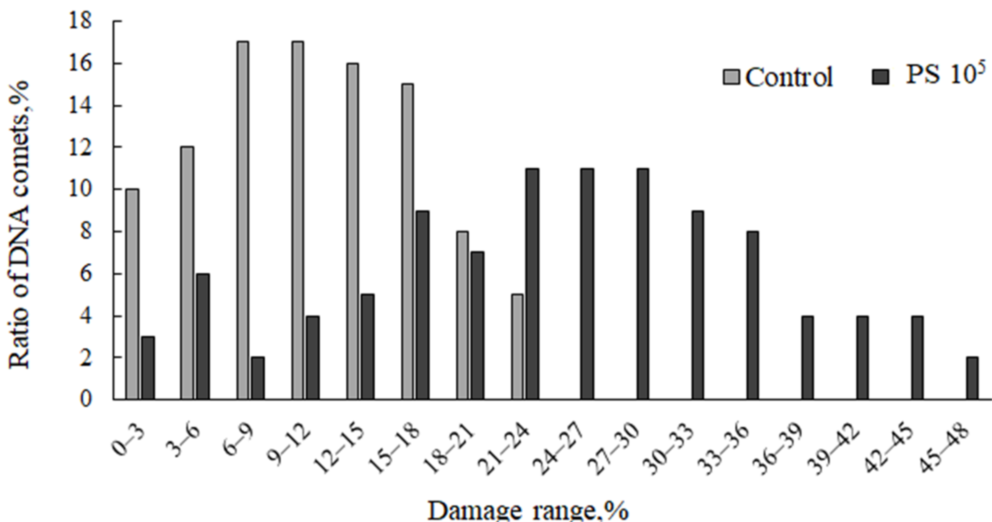

Figure 4. Distribution of DNA comet at 3% damage interval in sperm (N = 16; n = 800).

In controls, the highest number of cells had a 6–18% damage range, with only 5% of cells showing the highest DNA content in the comet tail (21–24%). Upon short-term exposure to polystyrene microspheres, about one-third of the total number of spermatozoa had DNA damage in the range of 21–33%. In addition, about 10% of the cells formed

comets with damage above 40%, which is characteristic of significant genotoxic exposure (Supplementary File S1).

Literature on the genotoxic properties of synthetic polymers is still scarce. The first evidence of genotoxicity of plastic microparticles, based on the comet assay, was presented in experimental studies using marine bivalve molluscs. The haemocytes of the mussel *Mytilus galloprovincialis* and *Scrobicularia plana* were found to have increased DNA strand breaks after the uptake of plastic microparticles PS and polyethene (PE) [5,6]. In chronic experiments, Gonzalez-Soto et al. [27] observed a biphasic response of mussel haemocyte genome to PS microparticle uptake: at the beginning of the experiment (7 days), DNA damage levels decreased compared to controls, but at the end of the experiment (26 days), the authors recorded a sharp increase in genome destruction. According to experimental data [28], when *Neocaridina davidi* shrimps were exposed to PS microspheres for 24 h, DNA molecule damage and a significant increase in comet tail length compared to control values were observed. In another example, Trifuoggi et al. [15] showed that the exposure of the sea urchin *Sphaerechinus granularis* to PS and polymethyl methacrylate (PMMA) microparticles at early developmental stages leads to a significant increase in cytogenetic abnormalities, expressed as mitotic aberrations and developmental defects.

At the same time, sufficiently convincing data have been obtained in experiments with bivalve molluscs, the scallop *Chlamys farreri* [7], and freshwater *Dreissena polymorpha* [8] indicating the absence of genotoxic damage in haemocytes after exposure to PS microparticles.

These contradictory data are not particularly surprising since the model experiments were performed under different conditions, including differences in the type, shape, and concentration of plastic microparticles; temperature; and duration of the experiments.

A distinctive feature of our results is that genome integrity destruction, indicating the genotoxic properties of PS microparticles, was demonstrated on the nuclear DNA of gametes. In addition, when discussing the obtained results, attention should be paid to the fact that, under equal conditions of exposure of male and female gametes to PS microparticles, the genome of sperm cells was more sensitive than that of eggs (Table 1).

It is likely that such significant differences in the response of sand dollar gametes to exposure to PS microparticles are due not only to specific physiological and biochemical systems and the efficiency of defence systems but also to differences in the mechanisms of genome stability retention. It is well known that sperm, unlike somatic cells or eggs, are potentially more susceptible to damage from environmental contamination by substances with genotoxic properties, as sperm are thought to have a limited capacity for DNA repair and antioxidant protection [29,30]. In *S. mirabilis*, among other protective mechanisms, the eggs are surrounded by an outer hyaline shell with pigment granules 100 μm thick [31], which may also prevent microsphere penetration into the egg and increase its resistance.

The results of our experiments showed that regardless of the level of DNA damage, sperm retained the ability to fertilize eggs with up to 97% efficiency (Figure 3). Based on the obtained data, it can be assumed that the level of biochemical shifts induced by polystyrene microparticles is enough to cause DNA damage in sperm but not enough to affect their fertilisation ability. It follows that sperm genome integrity is not critical for fertilisation, at least in this sand dollar species. There is valid experimental evidence to support this notion. For example, short-term exposure to genotoxic agents such as benz[a]pyrene and diuron on the sperm of the bivalve mussel and oyster resulted in significant and dose-dependent DNA damage, but the sperm retained their fertilizing capacity [29,32]. Additionally, in other studies, despite high levels of DNA damage in fish sperm induced by exposure to genotoxic agents such as methylmethanesulfonate (MMS) and diuron, fertilisation success was maintained at high levels [33,34].

In another study [3], the exposure of sea urchin *Paracentrotus lividus* sperm to PS and PMMA microparticles revealed spermiotoxic effects that were in turn accompanied by a slight decrease in fertilisation success.

The inconsistency in the available data on the effects of microplastics on different aquatic organisms is probably related to differences in interspecific sensitivity to such particles.

The mechanisms underlying the genotoxic effects of microparticles of synthetic polymers, particularly PS, are not clear. Unlike chemical compounds that penetrate the cell and directly or indirectly initiate genome destruction processes, PS microparticles are chemically inert, which significantly complicates the interpretation of the obtained results. Most researchers, in explaining the reasons for the possible genotoxic effect of microplastic, have drawn attention to the ability of such particles to induce the increased in-cell generation of reactive oxygen species (ROS), thereby causing oxidative stress. The activation of the antioxidant system has been found in model experiments examining the effects of microplastics on hydrobionts of various trophic levels, including larvae of the Pacific oyster *Crassostrea gigas* [35], the rotifer *Brachionus koreanus* [36], and the water flea *Daphnia magna* [37]. Highly reactive radicals are thought to be the main cause of oxidative damage and chain breaks of the DNA molecule [5,6].

The toxicity of plastic microparticles may be because synthetic polymers may contain low molecular weight fragments of mono- and oligomers, catalysts, synthetic stabilisers, and a wide range of specific chemical additives (phthalates, bisphenol A, polychlorinated biphenyls (PCBs), stabilisers, flame retardants, and pigments) that are, in turn, compounds with genotoxic characteristics [4,9].

In the case of sperm, in the absence of literature data indicating the penetration of 50 μm microparticles into the cell, a different assumption can be made. Given the relatively hydrophobic nature of polymers and PS, in particular, it is conceivable that microparticles adsorbing to various hydrophobic sites on the outer sperm membrane may, to some extent, disorganize the receptor-signalling system and provoke an outbreak of oxidative stress. As shown earlier, sea urchin spermatozoa can generate at least two types of ROS: H_2O_2 and O_2 [26].

To fully identify and evaluate the effects of plastic microparticles on male and female germ cells, longer experiments involving subsequent developmental stages are required. However, even based on the results obtained from the short-term contacts of gametes and microplastic, we believe it is necessary to emphasize the following points.

The induction of DNA damage in sperm cells indicates the inability of sea urchin sperm defence mechanisms to protect DNA from polystyrene microparticles. Accordingly, fragmented DNA strands not repaired in haploid mature spermatozoa, if successfully fertilized, are transferred to the zygotes and participate in the formation of the next generation genome. The contribution of these lesions to the egg genome can vary, and, in the case of small lesions, there is a possibility that they can already be repaired at the zygotic stage.

Considering that DNA controls the sequential development of all physiological, biochemical, and morphological processes, significant damage to the integrity of the genome can affect the normal course of these processes up to the appearance of anomalies and developmental arrest, followed by the death of the embryos. This reasoning is consistent with researchers [38] who suggest that the accumulation of damage in sperm DNA is a major cause of embryotoxicity by many chemical pollutants. Lewis and Galloway [29] found in two marine invertebrate species that, despite successful fertilisation, a significant percentage of embryos derived from sperm with induced DNA damage exhibited severe malformations later in development.

5. Conclusions

In our work, we studied the genotoxicity of polystyrene microspheres during the short-term exposure of *S. mirabilis* sand dollar gametes. Statistically significant differences were found in the percentage of sperm DNA damage compared to the control when exposed to water with the addition of all investigated concentrations of polystyrene microspheres. In experiments with *S. mirabilis* spermatozoa, the percentage of DNA in the 'tail' of the comet significantly increased depending on the microsphere concentration, indicating

genome integrity damage in these cells; however, regardless of the level of DNA damage, spermatozoa retained the ability to fertilise eggs at up to 97% efficiency.

We must acknowledge that the genotoxic property of microplastics against sperm to some extent predicts the development of long-term adverse effects of environmental significance. Although we used concentrations of microplastic in excess of actual concentrations in all experiments, we find the results particularly relevant. Undoubtedly, given the enormous scale of global artificial polymer production, the total amount of microplastic particles in the marine environment will rapidly increase due to the continuous fragmentation of larger plastic products.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmse9101088/s1, Supplementary File S1: Comet assay primary data.

Author Contributions: Conceptualization, V.P.C.; Data curation, E.V.Z. and V.V.S.; Formal analysis, S.P.K. and V.V.S.; Funding acquisition, V.P.C.; Investigation, N.V.D.; Methodology, A.A.M. and E.V.Z.; Project administration, V.P.C. and E.V.Z.; Software, S.P.K.; Supervision, V.P.C. and N.V.D.; Validation, V.V.S.; Visualization, A.A.M.; Writing—original draft, A.A.M. and V.P.C.; Writing—review & editing, V.P.C. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported by the State assignment for research work of V.I. Il'ichev Pacific Oceanological Institute, FEB RAS (No. 121-21500052-9).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Eriksen, M.; Lebreton, L.C.M.; Carson, H.S.; Thiel, M.; Moore, C.J.; Borerro, J.C.; Galgani, F.; Ryan, P.G.; Reisser, J. Plastic pollution in the world's Oceans: More than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PLoS ONE* **2014**, *9*, 111913. [CrossRef] [PubMed]
2. Doyle, M.J.; Watson, W.; Bowlin, N.M.; Sheavly, S.B. Plastic particles in coastal pelagic ecosystems of the Northeast Pacific ocean. *Mar. Environ. Res.* **2011**, *71*, 41–52. [CrossRef]
3. Thomas, P.J.; Oral, R.; Pagano, G.; Tez, S.; Toscanesi, M.; Ranieri, P.; Trifuoggi, M.; Lyons, D.M. Mild toxicity of polystyrene and polymethylmethacrylate microplastics in *Paracentrotus lividus* early life stages. *Mar. Environ. Res.* **2020**, *161*, 105132. [CrossRef]
4. Lithner, D.; Larsson ADave, G. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Sci. Total Environ.* **2011**, *409*, 3309–3324. [CrossRef] [PubMed]
5. Avio, C.G.; Gorbi, S.; Milan, M.; Benedetti, M.; Fattorini, D.; Errico, G.; Pauletto, M.; Bargelloni, L.; Regoli, F. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environ. Pollut.* **2015**, *198*, 211–222. [CrossRef]
6. Ribeiro, F.; Garcia, A.R.; Pereira, B.P.; Fonseca, M.; Mestre, N.C.; Fonseca, T.G.; Ilharco, L.M.; Bebianno, M.J. Microplastics effects in *Scrobicularia plana*. *Mar. Pollut. Bull.* **2017**, *122*, 379–391. [CrossRef]
7. Xia, B.; Zhang, J.; Zhao, X.; Feng, J.; Teng, Y.; Chen, B.; Sun, X.; Zhu, L.; Sun, X.; Qu, K. Polystyrene microplastics increase uptake, elimination and cytotoxicity of decabromodiphenyl ether (BDE-209) in the marine scallop *Chlamys farreri*. *Environ. Pollut.* **2020**, *258*, 113657. [CrossRef] [PubMed]
8. Magni, S.; Gagné, F.; André, C.; Della Torre, C.; Auclair, J.; Hanana, H.; Parenti, C.C.; Bonasoro, F.; Binelli, A. Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). *Sci. Total Environ.* **2018**, *631–632*, 778–788. [CrossRef]
9. Hamlin, H.J.; Marciano, K.; Downs, C.A. Migration of nonylphenol from food-grade plastic is toxic to the coralreef fish species *Pseudochromis fridmani*. *Chemosphere* **2015**, *139*, 223–228. [CrossRef]
10. Martinez-Gomez, C.; León, V.M.; Calles, S.; Gomáriz-Olcina, M.; Vethaak, A.D. The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. *Mar. Environ. Res.* **2017**, *130*, 69–76. [CrossRef]
11. Dinnel, P.A.; Stober, Q.J.; Crumley, S.C.; Nakatani, R.E. Development of a sperm cell toxicity test for marine water. *Aquat. Toxicol. Haz. Asses.* **1982**, *1*, 82–98. [CrossRef]
12. Kobayashi, N. Marine ecotoxicological testing with echinoderms. *Ecotoxicological Test. Mar. Environ.* **1984**, *1*, 341–356.
13. Nobre, C.R.; Santana, M.F.M.; Maluf, A.; Cortez, F.S.; Cesar, A.; Pereira, C.D.; Turra, A. Assessment of microplastic toxicity to embryonic development of the sea urchin *Lytechinus variegatus* (Echinodermata: Echinoidea). *Mar. Pollut. Bull.* **2015**, *92*, 99–104. [CrossRef]

14. Mazur, A.A.; Zhuravel, E.V.; Slobodskova, V.V.; Mazur, M.A.; Kukla, S.P.; Chelomin, V.P. Waterborne exposure of adult sand dollar, *Scaphechinus mirabilis* (Agassiz, 1864), to zinc ions and zinc oxide nanoparticles affects early development of its offspring. *Water Air Soil Pollut.* **2020**, *231*, 115. [CrossRef]
15. Trifuoggi, M.; Pagano, G.; Oral, R.; Pavičić-Hamer, D.; Burić, P.; Kovačić, I.; Siciliano, A.; Toscanesi, M.; Thomas, P.J.; Paduano, L.; et al. Microplastic-induced damage in early embryonal development of sea urchin *Sphaerechinus granularis*. *Environ. Res.* **2019**, *179*, 108815. [CrossRef] [PubMed]
16. Buznikov, G.N.; Podmarev, V.K. The sea urchins *Strongylocentrotus droebachiensis*, *S. nudus*, and *S. intermedius*. In *Ob'ekty biologii razvitiya (Objects of Developmental Biology)*; Nauka: Moscow, Russia, 1975; pp. 188–216.
17. Bekova, N.V.; Zhuravel, E.V.; Khristoforova, N.K. Effects of desalination and the detergent sodium dodecylsulphate on the early development of the sand dollar Scaphechinus mirabilis. *Russ. J. Mar. Biol.* **2004**, *30*, 175–182. [CrossRef]
18. Slobodskova, V.V.; Kukla, S.P.; Chelomin, V.P. An analysis of the quality of the marine environment based on determination of the genotoxicity of DNA in the gill cells of the Yesso Scallop *Mizuhopecten yessoensis* (Jay, 1856). *Russ. J. Mar. Biol.* **2015**, *41*, 495–498. [CrossRef]
19. Collins, A.R.; Kumar, A.; Dhawam, A.; Stone, V.; Dusinska, M. Mechanisms of genotoxicity. Review of recent in vitro and in vivo studies with engineered nanoparticles. *Nanotoxicology* **2013**, *52*, 1–70. [CrossRef]
20. Cavas, T.; Konen, S. In vivo genotoxicity testing of the amnesic shellfish poison (domonic acid) in piscine erythrocytes using the micronucleus test and the comet assay. *Aquat. Toxicol.* **2008**, *90*, 154–159. [CrossRef]
21. Balbi, T.; Camisassi, G.; Montagna, M.; Fabbri, R.; Franzellitti, S.; Carbone, C. Impact of cationic polystyrene nanoparticles (PS-NH2) on early embryo development of *Mytilus galloprovincialis*: Effects on shell formation. *Chemosphere* **2017**, *186*, 1–9. [CrossRef]
22. Paul-Pont, I.; Tallec, K.; Gonzalez-Fernandez, C.; Lambert, C.; Vincent, D.; Mazurais, D.; Zambonino-Infante, J.L.; Brotons, G.; Lagarde, F.; Fabioux, C.; et al. Constraints and priorities for conducting experimental exposures of marine organisms to microplastics. *Front. Mar. Sci.* **2018**, *252*, 1–22. [CrossRef]
23. Jha, A.N. Ecotoxicological applications and significance of the comet assay. *Mutagenesis* **2008**, *23*, 207–221. [CrossRef] [PubMed]
24. Istomina, A.; Chelomin, V.; Kukla, S.; Zvyagintsev, A.; Karpenko, A.; Slinko, E.; Dovzhenko, N.; Slobodskova, V.; Kolosova, L. Copper effect on the biomarker state of the *Mizuhopecten yessoensis* tissues in the prespawning period. *Environ. Toxicol. Pharmacol.* **2019**, *70*, 103189. [CrossRef] [PubMed]
25. Smith, M.A.; Fernandez-Triana, J.; Roughley, R.; Hebert, D.N. DNA barcode accumulation curves for understudied taxa and areas. *Mol. Ecol. Resour.* **2009**, *9*, 208–216. [CrossRef] [PubMed]
26. Kazama, M.; Hino, A. Sea urchin spermatozoa generate at least two reactive oxygen species; the type of reactive oxygen species changes under different conditions. *Mol. Reprod. Dev.* **2012**, *79*, 283–295. [CrossRef] [PubMed]
27. Gonzalez-Soto, N.; Hatfield, J.; Katsumiti, A.; Duroudier, N.; Lacave, J.M.; Bilbao, E.; Orbea, A.; Navarro, E.; Cajaraville, M.P. Impacts of dietary exposure to different sized polystyrene microplastics alone and with sorbed benzo[a]pyrene on biomarkers and whole organism responses in mussels *Mytilus galloprovincialis*. *Sci Total Environ.* **2019**, *684*, 548–566. [CrossRef] [PubMed]
28. Berber, A.A. Genotoxic evaluation of polystyrene microplastic. *SAUJS.* **2019**, *23*, 358–367. [CrossRef]
29. Lewis, C.; Galloway, T.S. Genotoxic damage in Polychaetes: A study of species and cell-type sensitivities. *Mutat. Res. Genet. Toxicol. Environ. Mutat.* **2008**, *654*, 69–75. [CrossRef] [PubMed]
30. Lacaze, E.; Geffard, O.; Goyet, D.; Bony, S.; Devaux, A. Linking genotoxic responses in Gammarus fossarum germ cells with reproduction impairment, using the Comet assay. *Environ. Res.* **2011**, *111*, 626–634. [CrossRef] [PubMed]
31. Dautov, S.S.; Kashenko, S.D. Development of the sand dollar Scaphechinus mirabilis. *Russ. J. Mar. Biol.* **2008**, *34*, 415–420. [CrossRef]
32. Akcha, F.; Spagnol, C.; Rouxel, J. Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos. *Aquat. Toxicol.* **2012**, *106–107*, 104–113. [CrossRef] [PubMed]
33. Santos, R.; Palos-Ladeiro, M.; Besnard, A.; Porcher, J.M.; Bony, S.; Sanchez, W.; Devaux, A. Relationship between DNA damage in sperm after ex vivo exposure and abnormal embryo development in the progeny of the three-spined stickleback. *Reprod. Toxicol.* **2013**, *36*, 6–11. [CrossRef] [PubMed]
34. Devaux, Y.; Zangrando, J.; Schroen, B.; Creemers, E.E.; Pedrazzini, T.; Chang, C.P.; Dorn, G.W.; Thum, T.; Heymans, S. Cardiolinc Network. Long noncoding RNAs in cardiac development and ageing. *Nat. Rev. Cardiol.* **2015**, *12*, 415–425. [CrossRef] [PubMed]
35. Cole, M.; Galloway, T.S. Ingestion of nanoplastics and microplastics by pacific oyster larvae. *Environ. Sci. Technol.* **2015**, *49*, 14625–14632. [CrossRef] [PubMed]
36. Jeong, C.B.; Won, E.J.; Kang, H.M.; Lee, M.C.; Hwang, D.S.; Hwang, U.; Zhou, B.; Souissi, S.; Lee, S.J.; Lee, J.S. Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). *Environ. Sci. Technol.* **2016**, *50*, 8849–8857. [CrossRef]
37. Imhof, H.K.; Rusek, J.; Thiel, M.; Wolinska, J.; Laforsch, C. Do microplastic particles affect *Daphnia magna* at the morphological, life history and molecular level? *PLoS ONE* **2017**, *12*, 0187590. [CrossRef]
38. Chatel, A.; Bruneau, M.; Lièvre, C.; Goupil, A.; Mouneyrac, C. Spermatozoa: A relevant biological target for genotoxicity assessment of contaminants in the estuarine bivalve *Scrobicularia plana*. *Mar. Pollut. Bull.* **2017**, *116*, 488–490. [CrossRef] [PubMed]