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Article

Oxidative Stress in Far Eastern Mussel *Mytilus trossulus* (Gould, 1850) Exposed to Combined Polystyrene Microspheres (μPSs) and CuO-Nanoparticles (CuO-NPs)

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Abstract: The ingress of nanoparticles of metal oxides and microfragments of synthetic polymers (microplastics) into a marine environment causes unpredictable consequences. The effects of such particles cannot be predicted due to a lack of ecotoxicological information. In this research, a series of laboratory experiments were conducted on the combined effects of CuO-nanoparticles (CuO-NPs) and polystyrene microspheres (μPSs) on the development of oxidative stress processes in the marine filter-feeder mollusk *Mytilus trossulus*. Biomarkers of oxidative stress, including the lysosome membrane stability of hematocytes (LMS), the index of antioxidant activity (IAA), the levels of malonaldehyde (MDA) and protein carbonyls (PCs), and DNA damage in digestive gland cells, were measured after 5 days of exposure. Based on a battery of biochemical markers, it was shown that oxidative stress was induced at varying degrees in the experimental mollusks when exposed to CuO-NPs and μPSs both separately and in combination. In contrast, the single-treatment effect on the lysosomal membrane was enhanced by the combined CuO-NPs and μPSs (from 77.14 ± 8.56 to 42 ± 4.26 min). In addition, exposure to both the compounds alone and in combination decreased the IAA (from 22.87 ± 1.25, to 19.55 ± 0.21, 10.73 ± 0.53, and 12.06 ± 1.62 nM/mg protein, respectively). The PC level significantly increased only after CuO-NP exposure (from 0.496 ± 0.02 to 0.838 ± 0.03 μM/mg protein). Furthermore, the results showed that the investigated particles, both alone and in combination, promoted DNA damage in digestive gland cells (from 2.02 ± 0.52 to 5.15 ± 0.37, 18.29 ± 2.14, and 10.72 ± 2.53%, respectively), indicating that these compounds are genotoxic. Overall, the results obtained suggest that oxidative stress is the leading factor in the negative effects of CuO-NPs and μPSs. Considering the exceptional role of genome integrity in the functioning of biological systems, the revealed damages in the DNA molecule structure should be attributed to the most important manifestations of the toxicity of these two forms of marine pollution.

Keywords: microplastics; nanoparticles; genotoxicity; bivalve; oxidative stress

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1. Introduction

A dramatic increase in the scale and diversity of forms of human industrial and domestic activities has led to the chronic pollution of marine ecosystems, not only quantitatively but also qualitatively. At present, apart from "traditional pollutants" (i.e., oil hydrocarbons, polycyclic hydrocarbons, surfactants, pesticides, heavy metals, etc.), products of nanotechnology, in particular, nanoparticles (NPs) of metal oxides and fragments of artificial polymers of various sizes and composition known as microplastics (MPs), are rapidly entering the marine environment.

Although the total level of NPs in the marine environment is currently very difficult to estimate [1,2] and concentrations of MPs rarely exceed a few μg/L, it must be assumed that marine organisms absorb them, accumulate them, and transport them through the food chain, threatening human health [3]. In addition, according to experimental data, NPs and MPs of artificial polymers of different natures are able to overcome biological barriers (in particular, biological membranes) and accumulate in the cells of hydrobionts of different organization levels [4,5], which threatens their survival and conservation as food resources for humans.

Recent data have demonstrated the potential ecotoxicological hazards of various NPs and MPs in marine environments [3,6–13]. At the same time, the accurate mechanisms of toxicity for both types of pollutants are far from being understood. There is reason to believe that the molecular basis for the identified physiological and biochemical shifts is the ability of NPs and MPs to induce the production of reactive oxygen species (ROSs), thereby causing oxidative stress that can destroy membranes, lipids, proteins, and DNA. Under experimental conditions, metal oxide NPs (CuO, TiO_2, and ZnO), have induced various sublethal effects in marine invertebrates at the molecular and biochemical levels, resulting from the development of oxidative stress and affecting the fertilization, larval development, immune responses, metabolism, growth, and survival of various marine organisms [4,9,14]. In addition, microplastic fragments have been shown to cause severe disturbances in the physiological processes of hydrobionts, including impaired enzyme secretion, decreased filtration rate, behavioral changes, decreased respiration, and reproductive function, even with short-term exposure. In addition, both nanoparticles and microplastics have affected the antioxidant (AO) system and the activity of various enzymes, induced the formation of ROSs and stress proteins, and stimulated such aspects of oxidative stress as lipid peroxidation (LPO), the destabilization of lysosome membranes, and inflammatory processes [5,7,15,16]. Such changes are an early step in the development of the whole set of biochemical events leading the organism to oxidative stress followed by death.

In a marine environment, especially in coastal areas, hydrobionts are exposed to complex mixtures of xenobiotics, where many of the components interact with each other to alter their bioavailability and toxicity to the organism [17]. These interactions in mixtures of xenobiotics can either reduce or increase toxicity compared to the individual components. Therefore, despite many publications revealing mechanisms of interaction for individual pollutant species with marine biota and some advances in this issue, the reality of the marine environment dictates the need to study the response of biological systems to the complex (combined) effects of pollutants.

Given the physico-chemical features of metal oxide NPs, in particular, CuO-NPs (propensity for aggregation, low water solubility, and sorption activity) [18] and polystyrene microspheres (μPSs; relative hydrophobicity and sorption activity) affecting bioavailability, there is inevitably a set of problems, which due to a lack of information, cannot be predicted yet. According to a number of authors, co-exposure to microplastics and heavy metals has led to significant changes in biochemical responses and is reflected in the physiological state of hydrobionts [17]. An analysis of experimental works shows that microplastic fragments are able to sorb on the surfaces of nanoparticles, heavy metals, and organic pollutants, affecting their toxicity [17,19]. In this regard, it is particularly relevant to study the effect in the marine environment of relatively stable CuO-NPs and μPSs on bivalve filter mollusks, which have the ability to concentrate in their tissues various xenobiotics. This property has made the *Mytilidae* mollusks unique models for studying the relationship of living organisms with xenobiotics of organic and inorganic origin, and they are widely used to monitor pollution in different areas of the world's oceans [20].

Previously, using the marine filter mollusk *Mytilus trossulus* as an example has shown that both types of priority pollutants, when exposed separately, have actively penetrated into the digestive system and have caused the enhanced destruction of nuclear DNA in mollusk cells [9,13]. Based on these considerations, a series of laboratory studies has been

carried out on the combined effects of CuO-NPs and μPSs on the development of oxidative stress processes in this mollusk.

Within the framework of the current problem, the aim of the present work is to investigate the toxic effects of μPSs and CuO-NPs and their combination on the bivalve *Mytilus trossulus* using the following oxidative stress biomarkers: lysosomal membrane stability (LMS), the genetic damage index of DNA molecules, integral antioxidant activity (IAA), malonodialdehyde (MDA) levels, and protein carbonyl content in tissue cells. This task is also interesting because it aims to apply oxidative stress indicators as adequate biomarkers in the assessment of coastal marine pollution.

2. Material and Methods

2.1. Description of the Experiment

Adults of *M. trossulus* (6.1 ± 0.9 cm in shell height) were selected from one cluster of one generation in the Peter the Great Gulf (the Sea of Japan, Russia). Before starting the experiment, the initial group of mussels was acclimatized for two days at 18–19 °C. After acclimatization, all the mollusks were divided into 4 groups of 30 specimens each. Three parallel 10 L aquariums were used for each group. Each had 10 mussels, with a stocking density of 1 mollusk per 1 L of sea water. The first "control" group was placed in a tank with water without any treatment. The second group was exposed to μPSs, the third group to CuO-NPs, and the fourth group to a combined exposure of PS microspheres and CuO-NPs.

The experiments were carried out under stable conditions (T 18.5 ± 0.5 °C; pH 8.2 ± 0.2; salinity 32.54 ± 0.24 psu; O_2 7.6 ± 0.4 mg/L, and photoperiod 16 h light: 8 h dark). The total duration of the experiment was 5 days. The water in the tanks was changed every 24 h. In order to ensure the contact of experimental animals with pollutants in the water column, we used intensive circulation with the help of active aeration. Constant, intensive aeration ensured the resuspension of MPs and NPs, as well as maintained a stable oxygen concentration in the water. During the acclimatization (2 days) and the experiment (5 days), the mussels were not fed. No *M. trossulus* mortality occurred during the experiment.

2.2. Preparation of Working Solutions

A solution of μPSs (diameter 0.9 μm, Tianjin BaseLine ChromTech Research Centre (China)) at a concentration of 10^5 pcs/L was used to prepare the MP working solution. Ultradispersed copper (II) oxide (CuO) (particle size < 50 nm and 29 m^2/g; Sigma-Aldrich Chemistry) was used to prepare the CuO-NP solution. The working solutions were prepared with distilled water. The concentration of NPs in the working solution was 20 μg/L. Before the nanoparticle solution was added to the seawater, the solution was stirred in a sapphire ultrasonic bath (44 kHz) for 30 min.

2.3. Comet Assay

To quantify the DNA damage in digestive gland cells, an alkaline version of a comet assay adapted for marine organisms [13] was applied. For this purpose, individual cells were isolated using an isotonic solution (500 mM NaCl, 12.5 mM KCl, 5 mM EDTA-Na2, and 20 mM Tris-HCl; pH 7.4). The working concentration was 10^5 cells/mL. Then, 50 μL of cell suspension was added to 100 μL of 1% low-melting-point agarose (LKB, Sweden) in 0.04 M phosphate buffer (pH 7.4) at 37 °C, mixed thoroughly, applied to a slide previously coated with 1% agarose solution for better adhesion, and covered with a coverslip. The sample was placed in the refrigerator for 3 min for agarose curing. The coverslip was carefully removed, and the slide was submerged in a lysis solution (2.5 M NaCl; 0.1 M EDTA-Na (1% Triton X-100 and 10% DMSO); 0.02 M Tris; pH 10) for 1 h in the dark at 4 °C. After washing with cold distilled water, the slides were placed in electrophoresis buffer (300 mM NaOH and 1 mM EDTA-Na2) and incubated for 40 min. Electrophoresis was performed at 2 V/cm for 15 min. After neutralization (0.4 M Tris-HCl; pH 7.4), the slides were stained with SYBR Green I.

In the control and experimental groups, counting of the comets was carried out for each mollusk (N = 30) containing at least 50 comets (n = 1500). The DNA comet was visualized and recorded using a fluorescence microscope (Zeiss, Axio Imager A1) equipped with an AxioCam MRc digital camera. CASP software v 1.2.2. (CASPLab, Wroclaw, Poland; https://casplab.com, accessed on 24 April 2022) was used to process the obtained digital images. This program allowed the calculation of different parameters of comets, indicating the degree of cellular DNA damage (% of DNA in the tail).

According to the degree of DNA molecule fragmentation, the obtained comets were differentiated into 5 classes (% DNA in the tail): C0 (<5%)—cells with minimal damage; C1 (5–20%)—cells with low 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 [13].

2.4. Lysosomal Membrane Stability

A cytochemical method [21] was used to assess the LMS. The method was based on the capture of neutral red dye by lysosomes, the retention time of which reflected the degree of membrane damage.

The hemolymphs of the mollusks were used to investigate the toxic effects of CuO-NPs and μPSs. Using a 1 mL syringe, the hemolymph (0.1 mL) was withdrawn from the anterior adductor muscle of each individual (n = 30) (ratio of hemolymph to filtered seawater in the syringe of 1:1). The incubation of the cells with dye (neutral red, >90% high purity, VWR International, LLC) was performed at 15 °C for 15 min. The stained preparations were visualized and recorded using an Axiostar plus microscope (×400 magnification) at 15 min and then at 30 min intervals (up to 180 min for healthy cells). In each case, the time of exposure of the specimen to the microscope lamp did not exceed 1 min. The results obtained were expressed in units of dye retention time for 50% of the cells in the preparation.

The following criteria were adopted to assess the physiological states of the organisms according to the LMS method: LMS $\geq$ 120 min—healthy; 120 min > LMS $\geq$ 50 min—in compensatory adaptation; and LMS < 50 min—in pathological state [21].

2.5. Integral Antioxidant Activity

Digestive gland tissues were used to determine antioxidant activity, as well as lipid and protein peroxidation. The dissected tissues from 10 individuals in 3 replicates (n = 30) in each group were frozen in liquid nitrogen and stored at −80 °C. The tissues were homogenized at 4 °C (0.1 M phosphate buffer, pH 7.0).

The determination of integral antioxidant activity (IAA) in the tissues was based on the ability of the cellular antioxidant system to recover the radical cation ABTS+ (2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonate), an oxidation reaction of ABTS+ by peroxyl, and alkoxyl radicals that result from thermal degradation of 2, 2-azobis (2 aminopropane) hydrochloride (ABAP) [22]. The reaction mixture was prepared in 0.1 M phosphate buffer (pH 7.0) and incubated at 37 °C. The measurements were made using a Shimadzu UV-2550 spectrophotometer with a thermostatted cell at a wavelength of 414 nm. The magnitude of activity was calculated with a calibration plot using trolox (6-hydroxy-2,5,7,8-tetramethylchloraman-2-carboxylic acid, Sigma Aldrich).

2.6. MDA Concentration

The concentration of MDA in the cells was determined using a colorimetric method [23]. The optical density was measured at 580 nm and 532 nm with a Shimadzu UV-2550 spectrophotometer. The molar extinction coefficient (E1M = 1.56×10^5) was used to calculate the concentration of MDA and was expressed in μM/g raw weight.

2.7. Carbonyl Concentration

The carbonyl groups of proteins in the tissue samples were determined with an alkaline method [24]. The optical density was measured with a Shimadzu UV-2550 spectrophotometer at 450 nm. The concentration of carbonyls was expressed in Mmol mg of protein

in 1 mL, taking into account the molar extinction coefficient for dinitrophenylhydrosine (DNPH) of 22,000 M-cm^{-1}.

The protein concentration in the supernatant was determined using modified Lowry method [25].

2.8. Statistical Analysis

The experiment results were processed with MS Excel and Statistica 10 software packages (StatSoft, Tulsa, OK, USA). For the data, nonparametric Kruskal–Wallis ANOVA followed by pairwise Mann–Whitney tests were performed. A difference of $p < 0.05$ was considered statistically significant.

2.9. Quality Assurance and Quality Control Assessment

A complete description of the Quality Assurance and Quality Control assessment (QA/QC) based on de Ruijter et al. [26] and a QA/QC evaluation score are provided in Supplementary Table S1.

3. Results

3.1. Lysosomal Membrane Stability

In the experimental groups of mussels exposed to μPSs and CuO-NPs separately, the LSM values in the hemocytes decreased by more than 15% and 30%, respectively, compared to the control group, and they were 65 ± 9.56 and 54.23 ± 4.63 min compared to 77.14 ± 8.56 min, respectively (Figure 1).

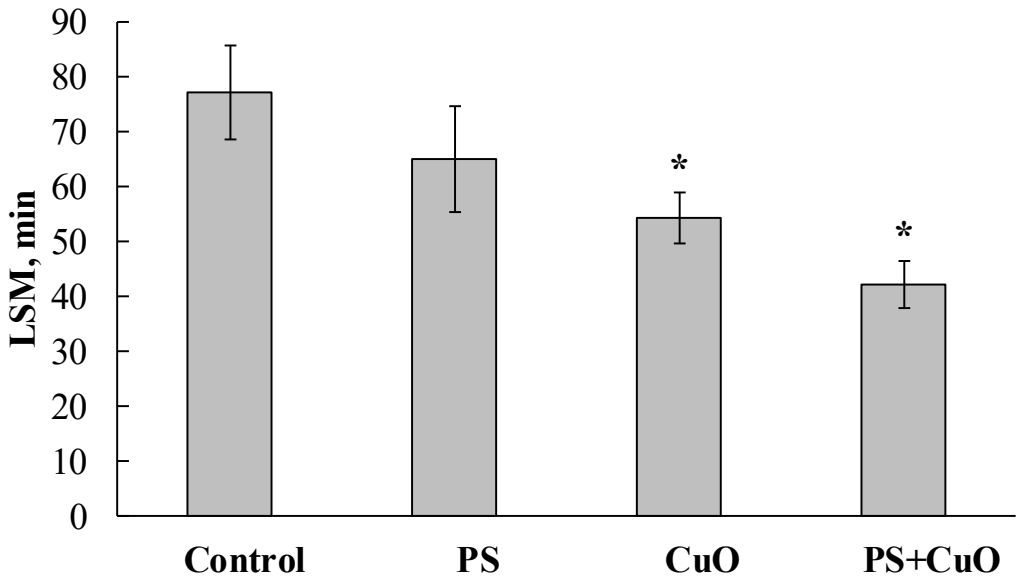

Figure 1. Changes in *M. trossulus* LMS when exposed to μPSs and CuO-NPs (mean ± standard deviation, n = 30); *—difference from control was significant at $p \leq 0.05$.

When the μPSs and NPs were combined, there was a summation effect, and the LSM was significantly reduced to 42 ± 4.26 min.

3.2. Integral Antiradical Activity

As a result of the experiments, it was found that, in all the experimental groups of mollusks, the level of IAA in the digestive gland cells decreased (Figure 2A), indicating suppression of the activity of the low-molecular-weight part of AO systems of mussels in the presence of the studied pollutants.

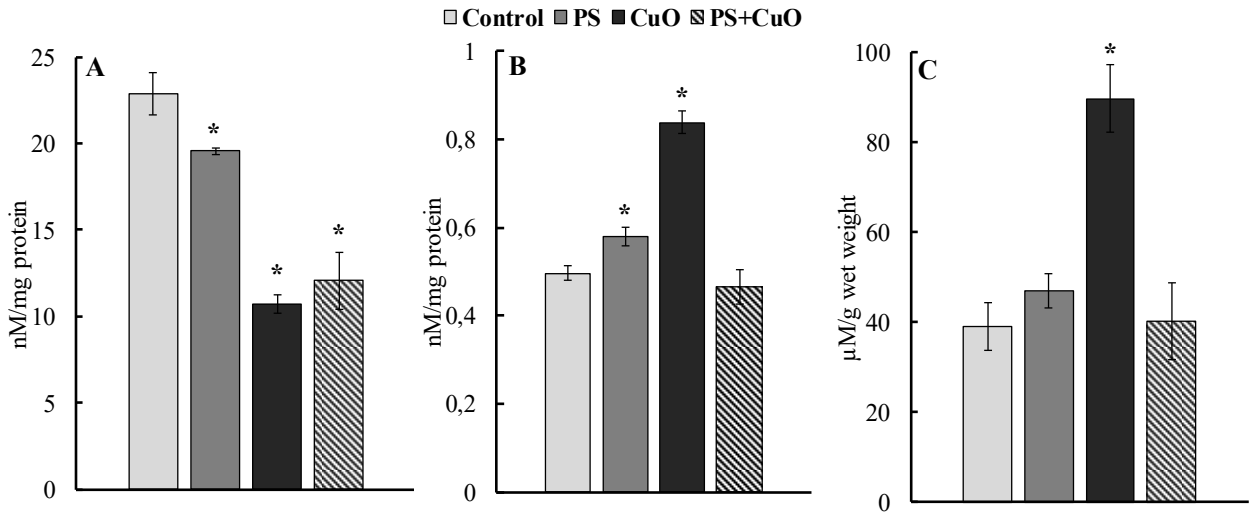

Figure 2. Changes in IAA (**A**), PC (**B**), and MDA (**C**) in *M. trossulus* tissues when exposed to μPSs and CuO (mean ± standard deviation, n = 30); *—difference from control is significant at $p \leq 0.05$.

While in mollusks exposed to μPS the IAA level in the digestive gland cells was significantly reduced by about 10% (from 22.87 ± 1.25 to 19.55 ± 0.21 nM/mg protein), when exposed to CuO-NPs, the IAA level decreased more than two-fold to 10.73 ± 0.53 nM/mg protein. This trend was maintained, and the IAA level also decreased significantly to 12.06 ± 1.62 nM/mg protein when the μPSs and CuO-NPs were cotreated (Figure 2A).

3.3. Carbonyl Concentration

The experiments revealed that μPSs had little effect on protein carbonyl (PC) formation in mussel tissues compared to the control group (0.578 ± 0.02 and 0.496 ± 0.02 μM/mg protein, respectively (Figure 2B)). After CuO-NP exposure, the PC level increased almost 1.5-fold to 0.838 ± 0.03 μM/mg protein. However, when MPs and NPs were exposed together, the levels of these products in the digestive glands of experimental mollusks did not differ from the controls at 0.465 ± 0.04 μM/mg protein.

3.4. MDA

Similar results were obtained when analyzing the content of MDA in the tissues of experimental mussels (Figure 2C). A sharp increase (more than two-fold, from 38.94 ± 5.24 to 89.74 ± 8.44 μM/g wet weight) in the MDA level was found in the digestive gland cells only in mollusks exposed to CuO-NPs. Exposure to μPSs alone and in combination had no significant effect on the MDA levels, which were 47.01 ± 7.45 and 40.17 ± 3.76, respectively.

3.5. Comet Assay

Using a comet analysis, convincing evidence was presented that exposure to MPs, CuO-NPs, and their combination caused genotoxic effects at varying degrees in the mussel *M. trossulus* (Figure 3).

Figure 3 shows the percentage of DNA in the "tail" of the comets, characterizing the degree of damage to the nuclear DNA molecule of digestive gland cells of the control and experimental mollusks. Common for all the experimental groups of mussels was a significant increase in the fraction of fragmented DNA migrating from the nucleus to the "tail" of the comet.

From the presented data, one can see that, in mussels exposed to μPSs, the level of DNA damage practically doubled in comparison with the control groups, from 2.02 ± 0.52 to 5.15 ± 0.37%. Exposure to CuO-NPs resulted in an almost nine-fold increase in DNA damage, amounting to 18.29 ± 2.14%. With joint exposure to μPSs and nanoparticles, the level of DNA fragmentation decreased but remained significantly high at 10.72 ± 2.53%.

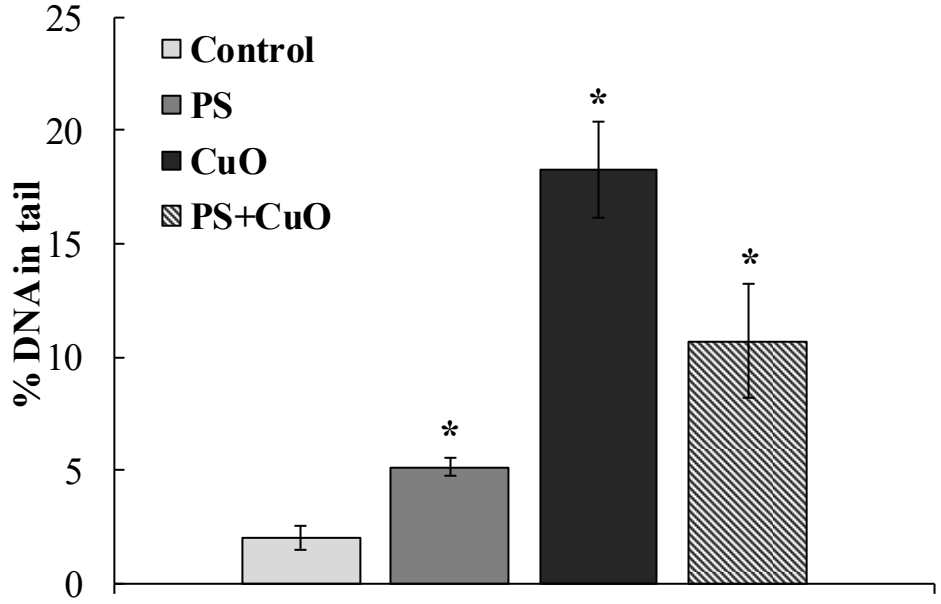

Figure 3. Assessment of digestive gland cell DNA damage from control and experimental groups (mean ± standard deviation; control: n = 30, experimental: n = 1500). *—difference from the control was significant ($p < 0.05$).

According to the comet classification [13] into classes based on determining the percent of DNA in the comet tail, the mollusk digestive cell comets prior to the experiments were exclusively of the C0 and C1 classes, which characterized the cells as undamaged and viable (Figure 4).

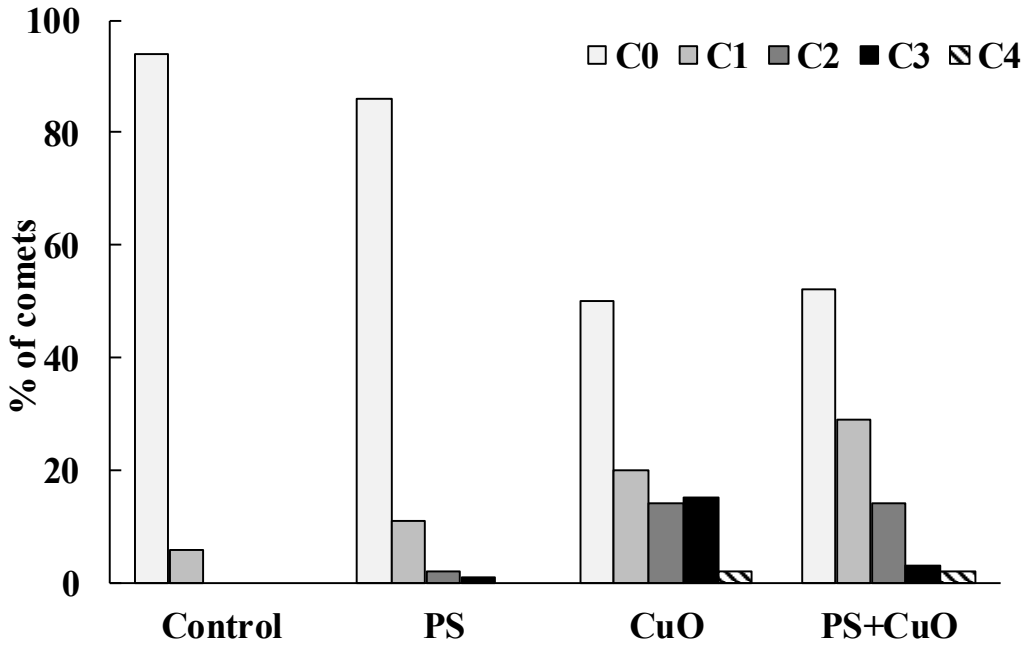

Figure 4. Cell gradation by DNA damage class in the digestive gland tissues of control and experimental groups (n = 30 and n = 1500, respectively).

The decrease in the proportion of C0- and C1-class comets and the appearance of comets from the C2-C4 classes was registered in all the experimental groups of mussels, which indicated an increased level of fragmentation of the nuclear DNA molecule.

Compared to the control group, the number of C0 and C1 comets decreased by more than 10% in the mussels exposed to μPSs (Figure 4), and comets with highly damaged DNA (C3 class) were observed. When the mollusks were exposed to CuO-NPs alone and together with MPs, the proportion of comets with undamaged or little damaged DNA decreased significantly (up to 70%), and comets with critical levels of DNA damage (class C4) corresponding to apoptotic and necrotic levels of decay were found.

4. Discussion

Despite the fact that the mechanisms of entry, accumulation, and localization of the main biochemical disturbances in biological systems when exposed to nanoparticles and fragments of MP have become the focus of intense study in recent years, the role of these pollutants in toxicity to hydrobionts requires additional research. In the interest of further development of this direction, it should be recognized that dangerous consequences of toxic properties depend not only on the level of pollution, but are also caused by the possibility of the interaction of individual toxicants with each other, which may significantly affect their bioavailability, accumulation, and mechanisms of toxicity. In this regard, the issue of the possible effects of joint exposure to MPs and NPs is important but relatively poorly studied.

Accounting for the unique sorption properties of these pollutants, the development of synergistic and antagonistic effects involving them seems most realistic, as confirmed by studies in recent years [5,18,27,28]. The combined exposure of fish to MP fragments and chemical contaminants has been shown to exacerbate the adverse effects caused by the components separately [18,28]. At the same time, the effects of mixtures of MPs with pollutants may vary depending on chemical composition. For example, plastic fragments altered the acute toxicity of Cr^{6+} ions [29] but did not affect the toxicity of Au nanoparticles [30]. Using an example of the marine filter clam *Mytilus edulis*, Avio and colleagues [5] revealed the ability of MPs to enhance the infiltration of pyrene into the digestive gland. It was also found that μPSs enhanced the negative effect of bromine-containing biphenyl ether (BDE-209) in the tissues of the bivalve mollusk *Chlamys farreri* [31]. However, in the case of combined exposure to MP fragments and mercury ions, antagonistic responses were observed in the mollusk *Corbicula fluminea* in such indicators as filtration rate, cholinesterase and glutathione-s-transferase enzyme activity, and LPO level [27].

A perfect example is the study of microalgae growth intensity in the presence of mixtures of different MPs and pollutants. Li and colleagues [32] observed an antagonistic response in the microalga *Chlorella pyrenoidosa* when it was exposed to low concentrations of polystyrene microgranules (μPSs) and dibutyl phthalate, but a synergistic response was observed at relatively high concentrations. When *C. pyrenoidosa* was exposed to μPSs and triphenyltin chloride in combination, a synergistic response was observed, whereas in the microalga *Skeletonema costatum*, the response to the same combination was antagonistic [33,34].

Based on the above examples, it is very likely that fragments of various MPs have a significant effect on the toxicity of accompanying chemical pollutants. In the similar few studies involving MPs and NPs, the same tendency can be traced. It has been experimentally shown that, separately, Au-NPs and MPs had little effect on the growth intensity of the marine microalga *Tetraselmis chuii*, but in mixture, they dramatically suppressed the growth of this microalga [35]. At the same time, there are quite convincing data showing that the toxicity of TiO_2 and CuO-nanoparticles to microalgae has been reduced in the presence of MPs [36,37]. In this case, the authors believe that the reason for the observed effect was the formation of heteroaggregates between metal oxide NPs and MP fragments, which reduced the interaction of nanoparticles with the surface of microalgae. Apparently, the formation of heteroaggregates did not affect their uptake by the marine nematode *Caenorhabditis elegans*, so PS NPs, even at low concentrations actually present in the environment, contributed to the toxicity of TiO_2-NPs [38].

According to generally accepted ideas, representatives of the family *Mytilidae*, to which, in particular, *Mytilus trossulus* used in our experiments belongs, are able to uptake and accumulate in tissues metal oxide NPs (TiO_2 and CuO) and MPs [4,10,13,39,40]. It is

believed that agglomerates of different sizes can enter the digestive systems of mollusks, move into hemolymph, and subsequently, enter hemocytes and cells of the digestive gland [5,39]. At the same time, NPs and MPs have a common pathway of penetration into cells through endocytosis, followed by deposition in lysosomes [5,27].

It was found that even short-term exposure to high-density polyethylene microgranules in mussels showed a significant increase in the membrane destabilization of lysosomes [4]. To a certain extent, the results of our studies in Figure 1 confirm this. Mussels exposed to CuO-NPs and μPSs separately showed a similar response, consisting of the destabilization of lysosomal membranes. Moreover, this effect was intensified when they were exposed together. When explaining the reasons for the synergism in the response, it should be taken into account that the leading factor in the oxidative destabilization of lysosome membranes is ROSs, which are generated by direct and indirect mechanisms [14]. In the case of CuO-NPs, ROSs generation can be initiated by Cu^{2+} ions, which are formed when nanoparticles dissolve in the acidic environment of lysosomes [41]. Given the chemical inertness of various polymers and PSs in particular, it can be assumed that the oxidative degradation of lysosome membranes is induced by both chemical and physical influences. This can be indirectly confirmed by the results of study [42], from which it follows that the physical adsorption of MPs on microalgae cells was accompanied by the suppression of photosynthesis and the generation of ROSs.

Although the specific mechanism of ROSs generation upon exposure to inert MPs is still unclear, several experimental studies in recent years have observed the development of oxidative stress in hydrobionts based on biomarkers [17,43]. This is apparently due to an imbalance in the antioxidant system. According to experimental data, exposure to a MP mixture (PE + PP) at concentrations present in the environment revealed changes in the antioxidant system in the tissues of *Mytilus* spp. [44]. Our results presented in Figure 2 are consistent with this view. Under the conditions of our experiments, CuO-NPs and μPSs significantly reduced the antiradical link of the antioxidant system when exposed to mussels separately. This effect was retained under the combined action of CuO-NPs and PSs.

The development of oxidative stress processes in mussels exposed to a mixture of pollutants was confirmed by the accumulation of protein oxidation products, such as PC in the digestive gland cells (Figure 2B). It is noteworthy that, when exposed to μPSs, these protein oxidation products did not accumulate, but in the presence of CuO-NPs, PC formation in digestive gland cells was sharply stimulated. In contrast to PC, the MDA level increased only when the mussels were exposed to CuO-NPs, but in experiments with PSs, the content of this lipid oxidation product did not differ from the control values (Figure 2C). Obviously, when analyzing this fact, it is reasonable to assume that MDA biotransformation enzymes are activated in the presence of μPSs.

CuO-NPs are known to exhibit genotoxic properties, causing breaks in the DNA molecules in cells of various organisms, including bacteria, mollusks, and fish, as well as human and mammalian cell cultures [9,45]. When explaining the causes of genotoxicity, it is worth paying attention to the ability of copper to generate ROSs. A review of the collected literature data provides evidence that MPs in environmentally realistic concentrations exhibit pronounced genotoxic properties when interacting with hydrobionts [13,46]. The DNA comet method has revealed damage in DNA molecules in the hemocytes of the mollusks *M. galloprovincialis* and *Scrobicularia plana* and in the shrimp *Neocaridina davidi* after exposure to MPs [5,19,47,48]. At the same time, in experiments with bivalve mollusks, there has been evidence of the absence of genotoxic damage in hemocytes after exposure to MPs [31,44,49,50]. It should be noted that the authors of the above papers in their studies have been limited to the analysis of the genotoxic properties of MPs on hemolymph cells, which are key components of the immune and detoxification systems. A distinctive aspect of our results presented in Figures 3 and 4 is that the fact of genome integrity destruction, indicating the genotoxic properties of μPSs and CuO-NPs, was demonstrated on the DNA of digestive gland cells directly involved in the accumulation of MPs [6]. The destruction

of the genome of this type of cell can consistently lead to pronounced and constantly increasing dysfunctions of digestive tissues with subsequent damage of activity up to the death of the organism.

To detect the genotoxic properties of MPs and NPs, we applied a DNA comet method, which, according to generally accepted opinion, is the most informative method of the cellular registration of nuclear DNA integrity, allowing the identification of cells with different degrees of genome damage at any given time. Moreover, this method allows the observation and evaluation of changes in the cell nucleus at early stages of genome destruction development. Based on this, we believe that, despite the relatively small degree of DNA destruction initiated by MPs, NPs, and their combination in our experiments, the risk of further spread of destructive oxidative processes with subsequent initiation of cell death mechanism remains. Although this assumption is hypothetical, there are nevertheless reasons to fear the direct and long-term consequences of MP and NP genotoxicity.

It is known that, in mussels, the bioaccumulation of MPs is mainly concentrated in the digestive gland and, to a lesser extent, in the gills and hemolymph [49,51,52]. It is likely that the response of digestive gland cells to exposure to NPs and MPs is due to features of the physiological and biochemical feeding and assimilation systems of dissimilar particle characteristic of filter mollusks and mussels in particular. The generalized results of the study of oxidative stress biomarkers indicated that short-term exposure to MPs, CuO-NPs, and their combinations caused serious changes in the antiradical link of the antioxidant system and in the destabilization of lysosome membranes and were accompanied by an increase in nuclear DNA degradation.

Given the exceptional role of lysosomes and the genome in the vital activity of biological systems, damage to these structures may be, chronologically, the earliest stage in the development of the entire set of biochemical events leading to toxic consequences. These research results provide a good basis for studying specific biomarkers of combined marine pollution by MPs and metal oxide NPs.

In conclusion, it should be noted that our studies were carried out under controlled laboratory conditions using concentrations of pollutants several times higher than those currently found in the environment. The use of high and unrealistic concentrations of potentially toxic substances in experimental practice provided an opportunity to understand the mechanisms of their negative impacts on organisms. Based on the data obtained, it was possible to predict long-term consequences in natural ecosystems under continuously increasing anthropogenic load. It also provided a basis for further research into the mechanisms by which MPs and NPs affect marine organisms at lower concentrations. In addition, the evidence for the adverse effects of MPs and NPs identified in this work is not exhaustive—it is more diverse—but its causes are poorly understood, although the importance of research in this area is undoubted, given that MPs and NPs are present in all parts of the ecosystem. Further research in this field should focus on the synergistic and antagonistic effects of pollutants, as well as the study of their environmentally realistic concentration exposure.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse10050707/s1, Table S1: Quality Assurance/Quality Control assessment, based on de Ruijter et al. (de Ruijter et al., 2020); Figure S1: Photo of used PS MPs; Figure S2: Spectra of used PS MPs.

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