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OXIC EVALUATION OF POLYSTYRENE MICROP

Ahmet Ali Berber*

ABSTRACT

Pollution of the aquatic environment by microplastic could be having a massive impact on marine life. As far as the dimensions of the microplastics decrease, the negative effects are also increasing. In this study, the effects of 1 µm diameter polystyrene microplastics (PSMs) on *Daphnia magna* and *Neocaridina davidi* were investigated. The acute toxicity test was conducted on *Daphnia magna*. According to the test LC_{50} value was calculated as 808.97 µg/mL. According to genotoxic evaluation on *Neocaridina davidi* with single cell gel electrophoresis (Comet), tail length, tail intensity and tail moment were increased by PSMs compared to the control.

Keywords : *Daphnia magna*, *Neocaridina davidi*, microplastic, genotoxicity, polystyrene

INTRODUCTION

Rapid population growth supports the increase of industrial, urban and agricultural activities. Unfortunately, these activities pollute our water resources. The main problem is that many hazardous wastes are given uncontrolled and illegally to receiving ecosystems such as river beds, lakes and seas. The most common waste in marine ecosystems among the hazardous wastes is undoubtedly the plastics. With more than 20 types of plastics, which are dangerous to the environment apart from the partial positive effects in medical applications, the production amount has been increasing day by day. It is estimated that in 2020 world plastic production will reach approximately 540 million tons [1].

Plastics can form microplastics by separating smaller pieces. Similarly, polyester fibers from textile products, plastic bags such as bags, packaging can also reach the seas in the form of polyethylene particles. They are also directly reach the aquatic ecosystem from cosmetic products.

Due to the ingestion of plastics by marine animals or wrapping these creatures more than 140.000 marine animals have been reported to die each year since the 1990s [2].

Eunomia [3] stated in his report that how much and where the plastics came from to the marine environment. According to this report; 9 million tons of terrestrial plastic, 0.5 million tons of plastic from inland water (streams etc.), 1.75 million tons of plastic from ships and fishery sector (marine waste) enters the marine system every year. In addition, in the related report it is stated that seas are polluted by 0.95 million tons

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of microplastic and 70 kg of plastic deposits per square kilometer on the seabed each year.

Shrimps are a livestock group that is a major part of the aquaculture sector, forming a large part of the crustaceans and classified in the Decapoda of the Crustacea class. The size of them is very variable, ranging from a few mm to 35 cm. The delicious taste of the meat and the high nutritional value make it possible to increase the commercial value and to find buyers in world markets at high prices every time. For this reason, most countries have shown significant improvements in the operation of stocks more efficiently and in hunting and aquaculture. Shrimps live especially in muddy, sandy-muddy or rocky rifts [4]. This life form of shrimps plays an active role in the micro-plastics found in sediment in the way they think of food and take it into the body. Microplastics / plastics can be involved in the food chain by incorporating microplastics / plastics into the digestive system of the shrimp in the lower strand of the food chain.

Polystyrene microplastics have negative effects on living systems. This theory has been proved scientifically given by the studies carried out in relation to this. As a matter of fact, in this study about the effects of study of polystyrene (~ 70 nm) to *Scenedesmus obliquus* algae cultures which exposed 0,22-103 mg / L for 5 days. It was found that especially at concentrations of 30 mg/L and higher, there was a decrease in the growth and chlorophyll density in algae [5]. These algae were then used as nutrients in Daphnia magna culture and indicated that algae caused malformations in both body shapes and diminished body size and reproduction in Daphnia.

On the other hand, microplastics tend to adsorb Persistent Organic Pollutants (POPs). In a study of this issue, Avio et al [6] reported that polystyrene, polyethylene and pyrene, which can adsorb them, caused genetic damage to *Mytilus galloprovincialis*.

In this study, an acute immobilization test on *Daphnia magna* was conducted and it was aimed to investigated whether polystyrene microplastics (1 μm diameter) produced any genetic damage on cherry shrimp (*Neocaridina davidi*) DNA.

MATERIALS AND METHODS

Polystyrene Microsphere

In our study, polystyrene microspheres used as the application material were supplied from Sigma (Neustadt an der Weinstraße, Germany) (d = 1.05 g/mL, diameter = 1μm, 89904-5ML-F).

Daphnia magna Acute Immobilization Test

Ovarian individuals were firstly removed from *Daphnia magna* fed with *Saccharomyces* and *Spirulina* in the laboratory and taken to a separate container. After ovulation, the offspring were used in experiments. During the experiments, no feeding was done to the offspring. The experiments were carried out according to the test protocol of the Organization for Economic Cooperation and Development (Test No. 202, *Daphnia sp*., Acute Immobilization Test) [7].

For each petri dish in which the experiments were made, the polystyrene microplastics were added at concentrations of 50, 100, 200, 400, 800, 1600 and 3200 µg/mL. For each petri dish, 20 mL of application solution and 5 *Daphnia magna* were placed. This application was made with 20 individuals as 4 replicates for each concentration. During the experiment the temperature was kept at 20 ± 2 °C and constant light cycle (14 h light–10 h dark) was used. The LC50 value was found by calculating the *Daphnia*, which is the dead and immobile (non-swimming for 15 seconds) in the examinations after 48h exposure.

Comet (Single Cell Gel Electrophoresis) Assay

20 shrimps under 1 week were placed in 1000 mL beakers and exposed to PS microplastics at 200, 400, 800 and 1600 µg / mL for 24 hours. At the end of the period hemolenf extraction was done with glass spheres. In this case, 2 ml PBS (Biochrom, Cat No: L1825) solution was used as a buffer. Shrimps were placed in eppendorf tubes as 2 mL/10 individuals and whole body homogenization was performed on the vortex device for 1 minute. After the homogenization, the tubes were centrifuged for 2 minutes at 1000 rpm. After the centrifugation, 150 µl of pellet was sampled and placed in a separate eppendorf tube. 150µl of Low Melting Agar (Applichem, Cat No: 9012-36-6) was rapidly mixed with hemolymph and spread over the slides previously coated with agar (Applichem, Cat No: 9012-36-6). Then it is covered with 24x60 mm coverslip and was held in a sealed box for 20-25 minutes at +4 °C.

At the end of the period, the lamellae on the slide were removed and treated with lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA) in the dark (1 hour or 16 hours at +4 degrees). After the lysis, the slides were placed in the buffer in the electrophoresis tank and allowed to stand for 20 minutes. Electrophoresis was done at 30 V 300mA for 20 minutes at the end of the period. After electrophoresis, the slides were rinsed with neutralization buffer (0.4 M Tris, pH=7.5). After all the procedures, 50 µl of EtBr (Applichem, cat no. 1239-45-8) was added to each slide and covered with lamellae.

Three different parameters were evaluated in the preparations; tail length, tail moment and tail intensity (%) of DNA. For this evaluation, 100 cells were examined at each concentration and all these studies were performed with fluorescence microscope (BAB Research Microscope, with filter of 546 nm and a barrier filter of 590 nm at 400X) and image analysis systems (BS200 ProP; BAB Imaging System, Ankara, Turkey).

Dose Selection

To examine the genotoxic effect of polystyrene microspheres on shrimps, LC_{50} value on *Daphnia magna* was determined by acute immobilization test. As a result, the LC50 value was 808.97 µg / mL according to the probit analysis. Concentrations of 1600, 800, 400 and 200 µg/mL were applied to the shrimp for genetic damage detection.

Statistical Evaluation

Statistical evaluation of *Daphnia magna* immobilization and shrimp comet assay results

was performed with IBM SPSS Statistics v22 software.

RESULTS

Daphnia magna Acute Immobilization Test

The 24 and 48 hour acute toxicity test results of Daphnia magna are presented in table 1.

Period (hour) Doses (µg / mL) Affected Individual Total Individual Affected (%)

Table 1. Acute toxicity test results of Daphnia magna

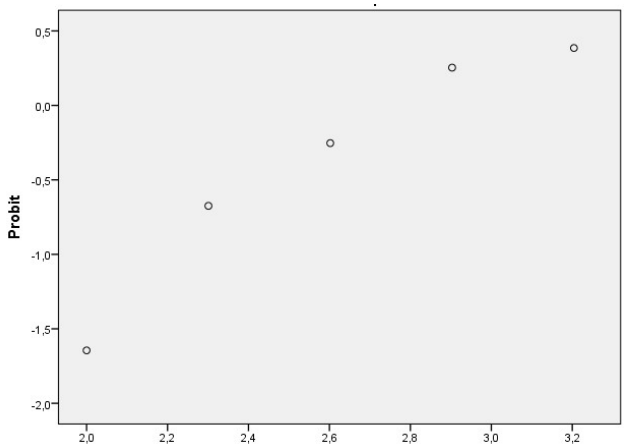
Test substances	Period (hour)	Doses (µg/mL)	No. of Affected Individuals	No. of Total Individuals	Affected (%)
Control	24	0	1	20	5
PSM	24	50	0	20	0
		100	0	20	0
		200	2	20	10
		400	6	20	30
		800	6	20	30
		1600	7	20	35
		3200	12	20	60
Control	48	0	0	20	0
PSM	48	50	0	20	0
		100	1	20	5
		200	5	20	25
		400	8	20	40
		800	12	20	60
		1600	13	20	65
		3200	20	20	100

It was observed that at the highest concentration of 48 hours application, 3200 µg / mL, all living organisms were affected by this application concentration (%100). At other concentrations, it was 65% at 1600 µg/mL, 60% at 800 µg/mL, 40% at 400 µg/mL, 25% at 200 µg/mL and 5% at 100 µg/mL but no effect was observed at the smallest concentration of 50 µg/mL. While some of the Daphnia individuals died, some immobile were considered as affected individuals (According to OECD test 202, Daphnia species that remain immobile for 15 seconds are considered immobilized).

Similarly, in 24 hour application, no effect was detected at the lowest concentrations of 50 and 100 µg/mL, while a sensitivity of 10% was determined at a concentration of 200 µg/mL. At other concentrations, 30% at 400 and 800 µg/mL, 35% at 1600 µg/mL and 60% at 3200 µg/mL respectively were observed. When the control groups were examined, only 1 individual was affected in the 24-hour application. This corresponds to an influence rate of 5%.

According to OECD test 202, if the 10% mortality rate is observed in the control groups of studies, the study should be repeated. The work continued because our rate was below 10%. According to the obtained data, the LC value was calculated according to the probit analysis and found to be 826.04 µg/mL for 48 hours.

The relationship between the probit rates of the 48-hour exposure rates of concentrations as a result of the application was presented in Graph 1.



Graph 1. Relationship between application concentrations and probit of 48 hour exposure rates of Daphnia magna

Shrimp (*Neocaridina davidi*) Comet Test

In this test, the genotoxic effect of polystyrene microspheres (1 µm) on *Neocaridina davidi* was tested by the comet (single cell gel electrophoresis) assay method.

After LC50 calculation on *Daphnia magna*, concentrations of 200, 400, 800 and 1600 µg / mL were selected for the comet test.

For each concentration, 100 comet cells were examined and evaluated in three different parameters (tail length, tail moment and tail intensity) (Table 2).

Table 2. Genetic damage frequencies of 1µm polystyrene microparticles on *Neocaridina davidi*

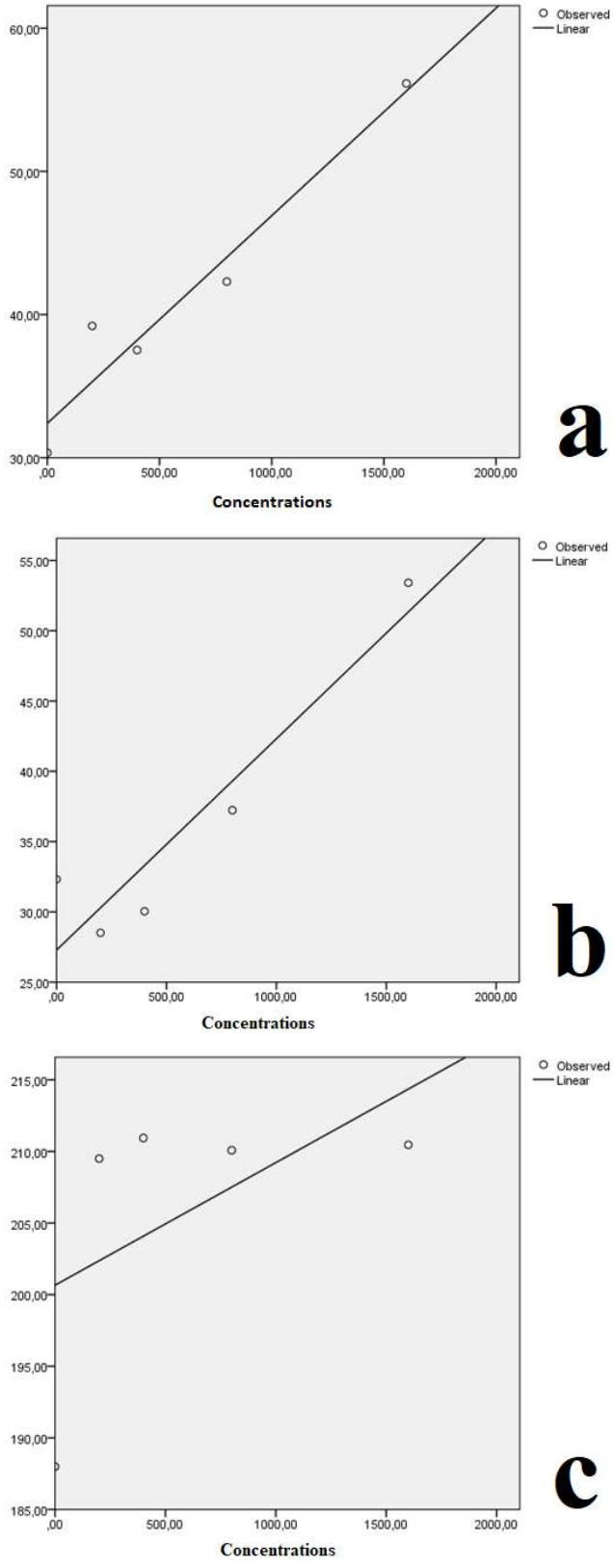
Concentrations (µg/mL)	Tail Length (µm)	Tail Moment	TailIntensity (%)
Control	30,85±2,90	32,32±3,29	187,99±2,43
200	29,21±2,89	28,51±3,04	209,50±1,99**
400	37,52±4,51	30,04±4,38	210,94±1,79**
800	42,30±4,67*	37,23±4,56*	210,08±1,41**
1600	56,14±5,13**	53,41±5,10*	210,46±3,32**

* Statistically significant compared to control p <0,05
** Statistically significant compared to control p <0.001 (t-test)

As a result of polystyrene microsphere exposure tail length increased with all doses except the smallest concentration (200 µg / mL) compared to the control. Statistically, tail length increased significantly at concentrations of 800 µg/mL and 1600 µg/mL compared to the control ($p < 0.05$, $p < 0.001$, respectively). When the increase in tail length was assessed according to doses, the increase was dose dependent (r=0,97) (Graph 2-a). Consequently, polystyrene microspheres cause to increase the length of comet tail (except 200 µg / mL) resulting in genotoxic damage.

The comet tail moment was lower than the control at two small doses (200 and 400 µg/mL), different from the tail length. However, the other two doses (800 and 1600 µg/mL) were found to be higher than the control. This increase in application doses was also statistically significant according to the control ($p < 0.05$). It has been found that there was a strong correlation between the tail moment and the application concentrations, (r=0,94) (Graph 2-b).

When assessed in terms of tail intensity, it was determined that all application doses cause a significant increase the tail intensity compared to the control. This increase was dose depended. (r=0,54) (Graph2-c).



Graphic 2. (a); dose-dependent regression graph of tail length (r=0,97), (b); dose-dependent regression graph of tail moment (r=0,94), (c); dose-dependent regression graph of tail intensity (r=0,54).

DISCUSSION

According to the terrifying predictions made about plastics, about 10% of the produced plastics enter our oceans [8,9]. For this reason, plastic is a common pollutant of aquatic ecosystems. In addition, microplastics can be directly incorporated into the aquatic ecosystem, polyester fibrils from textile products and cosmetic products. Microplastics passively float in the oceans under the influence of physical currents and can also be seen in sediments. As many aquatic organisms think they are food sources, they are involved in the food chain. It has been proven that many aquatic organisms have been ingested microplastics as a food, such as Copepod, Euphausiacea (krill) [10], amphipoda, mussel, oyster [11-13], fish [14] and whale [15].

Furthermore, many organisms have been detected they eat microplastic as a food such as algae [5], plankton [16], cnidaria [17], echinoderms [18, 19], polychaeta [20], crustaceans (21-23) and Molluscs [24, 25].

Microplastics, which are a common concern for potential toxic effects, have become important contaminants today. Therefore, studies on microplastics used by aquatic biota need to be increased. The harmful effects of microplastics in the aquatic environment have recently started to be discussed and researched recently. Besseling et al [5] evaluated reproduction and chlorophyll density of *Scenedesmus obliquus* exposed to

polystyrene (~70 nm) at the concentrations of 0.22-103 mg / L for 5 days and the researchers found that there were decrease in terms of reproduction and chlorophyll density. Later, when these algae were used as nutrients in *Daphnia magna* culture, malformations in body shapes of Daphnia, decrease in body size and reproduction were detected. In our study, it was found that polystyrene microplastics were perceived as nutrients and included in their systems when given to *Neocaridina davidi* culture media, as in the above mentioned studies. PSM also increased the frequency of damage in genetic structure.

When microplastics are taken by aquatic organisms, they have important effects on their physiological functions such as respiration, nutrition, growth, reproduction [20]. In addition, microplastics may adsorb persistent organic pollutants (POPs). Scientific studies have shown that microplastics contaminated by POPs are taken as nutrients and then the POPs are transferred to aquatic organisms that ingest the microplastics. The effects of both microplastic and POP + microplastic combination on neurotransmission, energy production and oxidative metabolism have been demonstrated [26-29].

Avio et al [28] showed that pyrene (PR) contamination of polystyrene (PS) microparticles increased with dose and time. In addition, in the same study, the toxicological evaluation of both contaminating (PS + PR) and pure microplastic (PS) effects on *Mytilus galloprovincialis* was performed. According to the results of the research, PR accumulation was detected in Mytilus tissues besides microplastics. According to the results of the researchers, *Mytilus galloprovincialis* observed PR accumulation in different tissues of the body except microplastics. Also, toxicological evaluation with comet assay and micronucleus test on *Mytilus galloprovincialis* was conducted by applying PS and PS+PR at a size less than 100 μm. In the comet test, PS showed significant damage to mussel DNA. Unlike the comet test, in the micronucleus test in which the nuclear abnormality was evaluated, only statistically significant differences were found in PS + PR applications compared to the control. These test results clearly showed that polystyrene microplastics had a negative effect on DNA. As a matter of fact, in our comet experiment, it was shown that polystyrene microspheres of 1 μm size significantly increased the DNA tail length, tail moment and tail density compared to the control. If we look at the microplastic dimensions used in our work and in the study of Avio et al [32], it is unlikely that a material of this size (10-100 μm) will reach the DNA through the cell membrane and cause such genotoxic damage. The hypothesis that should be considered here is that microplastics may increase the production of reactive oxygen species (ROS) and the increased amount of ROS may cause breakage of DNA strands. Intracellular ROS levels were evaluated by Jeong et al [30] in *Brachionus koreanus* exposed to PS microplastics of sizes 0.05, 0.5 and

6 µm for 24 hours. According to the obtained results, the level of ROS increased in inverse proportion to the particle sizes. This result shows that the increase of microplastic and reactive oxygen species may lead to the eventual DNA damage.

As a result, there is intensive microplastic pollution both in the world and in our aquatic ecosystems. When the above-mentioned studies and the results of our work are evaluated, the harmful effects of microplastics on living organisms are obvious. In addition, the increasing frequency of the appearance of plastics undoubtedly leads to great concern.

SUGGESTIONS

Microplastics are taken by many aquatic creatures as if they were part of the food chain. This induces microplastics to enter the food chain and cause some of the living organisms to be directly affected and some of them to be indirectly affected. As mentioned in the Eunomia [3] report, about 1.75 million tons of plastic waste enters the marine systems each year from the maritime transport and fishing industry. Likewise, 9.5 million tons of inland water and terrestrial plastic waste enter the marine ecosystem.

Marine paints (16 thousand tons), cosmetics (35 thousand tons), road and construction paints (210 thousand tons), textiles (190 thousand tons), pellets (230 thousand tons) and vehicle tires (270 thousand tons) [3], which make up the microplastic source in particular, cause a different composition in the sea sediment.

There may be a need for improvements or sanctions in government policies relevant to the issue in order to eliminate or at least minimize this negative situation. The suggestions in this regard can be as follows

- Taking necessary measures by local authorities to prevent pollution of plastic by about 2 tons of plastic pollution per square kilometer in coast with coastal protection regulations
- Control of filtration systems of all kinds of factories, facilities, enterprises or equipments such as textile, industry which will produce microfibril in their wastes (or/and support for R&D for filtration systems)
- Encouraging companies to recycle, especially on plastics or ensuring that companies adopt a recycling.
- Reducing the use of packaging used during shopping such as plastic bags, pouches.
- Consciousness of people (especially cosmetic products containing microplastic, reusing of plastic bags or packages and environmental sensitivity).

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