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Toxicity Evaluation Mechanisms of HDPE Nanoplastic on *Artemia* Nauplii: An Analysis Oxidative Stress, Morphology, and Ultrastructural Studies

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Abstract

Nanoplastics are produced abiotically and biotically from larger pieces of plastic. Although nanoplastic toxicity has received more attention recently, its biological effects have not been adequately investigated. In this study, the toxicity of nanoplastics (NPs) with an average size of <80 nm was carried out in the larvae of *Artemia* nauplii, an indicator organism of the aquatic environment, according to the OECD guideline 202 protocol. As a result, depending on exposure durations (24-96 h) and concentrations (50-300 µg/mL), the survival rate of nano-HDPE treated larvae was significantly reduced ($p < 0.05$). The larvae took up and internalized nano-HDPE at a concentration of 99.74 µg/mL, which is the calculated LC_{50} value. There was also a significant increase in biochemical markers in larvae at LC_{50} ($p < 0.05$). However, it was observed that this caused oxidative stress, cell membrane damage, limb loss and malformation in larvae treated with nano-HDPE.

Keywords: *Artemia salina*; nanoplastic; toxicity; oxidative stress; plastic

1. Introduction

The SAPEA (European Commission — the Science Advice for Policy by European Academies) has described nanoplastics as the most potentially dangerous type of marine litter. It is estimated that 51 trillion pieces of microplastics have accumulated on the ocean surface and the amount is increasing [1]. Nanoplastics (NPs) are formed by the breaking down of macro-and micro-plastics in various ways. The Scientific Committee on Emerging and Newly Identified Health Risks defined NPs as particles with at least one dimension between 1 and 100 nm [2]. NPs can occur as primary and secondary NPs [3]. Primary NPs are produced intentionally, while secondary NPs are produced unintentionally, that is, by the decomposition of larger plastic particles into smaller ones [3]. Regardless of how they are formed, it is very difficult to isolate these NPs from the aquatic environment. However, there is some experimental evidence indicating the potential for NPs to accumulate in the environment. For example, Koelmans et al., [4] reported that expanded polystyrene (EPS) disintegrated into micro- and nanosized fragments in experiments involving accelerated mechanical etching with glass beads and sand for a month. Several other laboratory studies have also demonstrated the presence of nanoscale plastics in water after exposing larger pieces of plastic to UV and visible radiation [5,6]. Nanoparticles are defined as having of a size and shape with one dimension ≤100 nm. For this reason, NPs also fall under the class of nanoparticles. Just like nanoparticle toxicity, nanoplastics toxicity should be demonstrated through experimental studies [7–10]. Despite the evidence that NPs form through abiotic and biotic degradation, there is still a significant lack of information about their bioaccumulation, environmental concentrations or toxicity. NPs are assumed to be abundant in aquatic environments as a result of physical and photodegradation of macro and microplastics in the environment [11,12]. NPs can have several

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different biological and/or toxic effects depending on their size, type, the particular organism affected, and the environment in which it occurs [13]. However, NPs may have various effects on tissues and organs after occurring in the environment and being ingested by organisms through oral, dermal and inhalation [14]. NPs are expected to have more toxic effects than bulk materials or microplastics because they are also nanoparticles. As a result of a small number of studies in line with this view, it has been established that NPs are more toxic than microplastics and that the smaller the nanoplastic size, the more toxic it has. For example, in one study, human gastric cancer cells were exposed to 500 nm polystyrene microplastics with different concentrations of 60 nm sized polystyrene NPs. Moreover, it has been demonstrated that high concentrations of polystyrene NPs reduce cell viability more and cause more reactive oxygen species formation and DNA damage than polystyrene microplastics at the same concentration [15]. Again, NPs have been noted to cross various biological barriers to target the heart, brain, liver and reproductive systems. In one study, they exposed developing *Danio rerio* to fluorescent polystyrene NPs and showed that they accumulated in the oviduct 24-h after fertilization and migrated to the gastrointestinal tract, liver, gallbladder, pancreas, brain and heart throughout development [16]. In another study, they stated that polystyrene NPs are not transported across the human intestine and placenta barrier, but are taken up and accumulated by cells in addition to also being weakly embryotoxic and non-genotoxic [17]. In recent years, the use of aquatic invertebrates such as *Artemia salina* (brine shrimp) as model organisms to detect the toxic effects of environmental pollutants has become widespread [44]. *A. salina* is a filter feeder and a species of zooplankton invertebrate in the marine ecosystem that is exposed to much more environmental pollutants than other aquatic organisms due to its extensive water filtering. It also, it plays an economic role as a protein source in the nutrition of fish fry and in the flow of energy in food chains [45,46]. *A. salina* is one of the most valuable test and model organisms among ecotoxicity tests, which was proposed by Michael and his colleagues in 1959 as a biological test organism for developing standard toxicology analyses and for the preliminary prediction of toxicity [45,47,48]. Today, A. salina lethality tests are widely preferred in research and applied toxicology due to a number of advantages, such as the absence of aseptic techniques and ethical approval, and the rapid, easy and low-cost nature of the analysesIn this study, the larvae of the aquatic organism *Artemia* nauplii were selected to investigate the intracellular settlement and toxicity of high-density polyethylene nanoplastics (nano-HDPE). The response of larvae to nano-HDPE was evaluated both by determining cell viability and by measuring biochemical enzymes in response to stress. This study makes a significant contribution to the toxicity assessment of nanoplastics derived from HDPE.This study was conducted in the laboratories of the Department of Molecular Biology and Genetics at Ordu University between 2023-2024.

2. Materials and Methods

Preparation and characterization of Nanoplastic

High density polyethylene (HDPE) pellets were used for nanoplastic (NP) production. For this, plastic pellets were reduced to micro size (approximately 200 μm) using a micro motor. Then, the micro-sized plastics were dehumidified in an oven at 60°C for 5 days. The plastics, whose moisture was removed, were crushed in a porcelain mortar. After this step, the size analysis of the plastics, which were thought to have reached nano-size, was performed with SEM. For SEM analysis, air-dried samples were coated with gold and then observed using SEM (SU-1510, Hitachi, Tokyo, Japan) in high vacuum mode (15 kV) and with 3 nm SE resolution. Then, the nanosized plastics were placed in ultrapure water and sonicated for 2-h to obtain individual nanoplastics. Functional groups of nanoplastics were characterized by Flourier transmission infrared Spectroscopy (FTIR) spectroscopy. The shape and structural morphology of nanoplastics were characterized by Transmission Electron Microscope (TEM jeol JEM-1220, JEOL, Japan) operating at 80 kV acceleration voltage after removing excess water and drying in air. The produced nanoplastics were kept in pure water to prevent aggregation and sonicated before each bioassay.

Bioassay organisms

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Artemia. salina cysts for the bioassay were obtained from a commercial company (Netakvaryum). To obtain instar I nauplii (24 and 48-h after hatching), 4 g of cysts were added to 1 L of artificial seawater and incubated for 24 h at 28˚C in a 16:8 light-dark cycle under continuous aeration conditions.

Short-term toxicity test

Acute toxicity testing was performed for 96-h of acute exposure in nauplii instar I according to the Organization for Economic Cooperation and Development, OECD 202 test guidelines [18]. The acute toxicity study involved administering five different test concentrations (50–300 µg/mL) of the nano-HDPE to nauplii after 4h of incubation. The control group was exposed to the test solution without NPs. The acute toxicity assay was performed in 24-well microplates containing 2 mL of test solution. The experiments were repeated three times, and each repetition consisted of 10 newly hatched nauplii. The microplate was kept in the climate chamber at 24±2°C, 12:12 light:dark photoperiod and 100 rpm shaking to prevent the nanoplastics from collapsing and to provide aeration. At the end of 96-h, dead and live *A. salina* were counted using a stereomicroscope [18].

Oxidative stress biomarkers

Artemia nauplii were collected to estimate the reactive oxygen species (ROS) produced after 24-h and the content of malondialdehyde (MDA) and antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT) after 96 h in larvae exposed to LC_{50} concentration of nano-HDPE. To prepare the enzymatic extracts, nauplii were washed several times with PBS (phosphate-buffered saline, pH − 7.2) to remove nano-HDPEs adhering to the control and treatment groups. Then, it was homogenized using a glass homogenizer (ILDAM, Turkey) in PBS buffer. After centrifuging the homogenized nauplii at 15,000 rpm for 15 minutes, the supernatant was gatgered and stored in a -20 °C refrigerator up to biochemical analysis.

Reactive oxygen species (ROS)

ROS activity estimation was performed according to the protocol reported by Ulm et al., [19]. Activity was determined using a non-fluorescent dye 2′,7′-dichloro dihydrofluorescein diacetate (DCFH-DA). Briefly, 10 µL of DCFH-DA dye was added onto 20 µL of supernatant and incubated at 37 °C for 25 minute in the obscurity. To see the fluorescence intensity of DCFH-DA, measurements were made using a UV–vis spectrophotometer (Shimadzu Brand, UVmini -1240 Model) at an excitation and emission wavelength of 485 and 530 nm, respectively.

Super Oxide Dismutase (SOD) Activity

SOD activity was performed according to the protocol reported by Ateş et al., [20]. 50 mM (1.3 mL) Na_2CO_3 buffer (pH 10), 96 mM (500 µL) NBT, 100 µL Triton X-100 and 20 mM hydroxylamine hydrochloride were suffixed to the supernatant. The final reaction blend was incubated for 20 min at 37 °C in the presence of light. Then, absorbance values were measured at 540 nm in UV-vis spectroscopy. The decrease at 540 nm absorbance is a measure of NBT reduction by the SOD enzyme.

Catalase (CAT) Activity

CAT activity was performed according to the protocol reported by Yilancıoğlu et al., [21]. The CAT enzyme reacts with hydrogen peroxide combined with ammonium molybdate to produce yellow color due to the production of hydrogen and water [22]. For CAT analysis, 2 mL hydrogen peroxide (H_2O_2) solution (10.8 mM) and 200 µL 50 mM PBS (pH 7.0) were added to 100 µL of the supernatant. This reaction mixture was measured in a UV–vis spectrophotometer at 240 nm at 3 min intervals. The reaction mixture without H_2O_2 was used as a reference.

Malondialdehyde (MDA) Activity

MDA activity was performed according to the protocol reported by Van Ye et al. [23]. In brief, 10 µL of butylated hydroxy toluene reagents (BHT), 0.25 mL of phosphoric acid reagent, and 0.25 mL of TBA reagent were suffixed to 0.25 mL of the supernatant. The obtained reaction blend was incubated at 90 °C 1 h and centrifuged after cooling at 13,000 rpm for 10 minutes to precipitate the suspended tissue. Afterwards, the reaction mixture was measured at 532 nm using UV–vis spectroscopy.

Ultrastructural analysis with TE

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TEM analysis was used to examine structural changes on Artemia nauplii exposed to nano-HDPE. Larvae were incubated in nano-containing medium at LC_{50} concentration (99.74 µg/mL) for 72-h and then the routine TEM procedure was treated. For this, suspensions containing larvae were fixed in 2.5% glutaraldehyde in 0.1µM PBS (phosphate-buffered saline) for 12 h at 4 °C. Then, they were washed three times in PBS and then incubated in 1% osmium tetroxide in the dark for 2-h. It was kept at room temperature for secondary fixation and washed again three times in PBS. The prepared samples were embedded in epoxy resin after being dehydrated with 30%–50%–70%–90%–96% and 100% graded ethanol series. Ultrathin sections of 60 nm thickness were cut with an ultramicrotome on 300-mesh copper grids (Leica Ultracut R).These ultrathin sections were then stained with lead citrate and uranyl acetate and analyzed with TEM (Hitachi HT7800) [24].

Lyophilization

Approximately 5 g of nauplii treated with nano-HDPE were homogenized in the homogenizer and then freeze-dried in the lyophilizer (Labconco, Freezone 2.5) for 72-h.

Morphological and behavioral analysis

As described above in the short-term toxicity test, nauplii that survived 96-h exposure to the LC_{50} (99.74 µg/mL) concentration of nano-HDPE were used to investigate behavioral and morphological differences. For this purpose, dead larvae were removed at the end of the experimental period and kept in the dark for a while to avoid stress. Later, morphological observations and swimming behaviors of the larvae taken into artificial seawater were carefully examined and recorded under a stereomicroscope. The same observations were made in untreated larvae for comparison. Images were then recorded with a digital camera (Nikon) fixed on the stereo microscope to demonstrate morphological changes.

Statistical analysis

Statistically significant differences between the means of groups exposed to different concentrations of nano-HDPEs were calculated using one-way analysis of variance (ANOVA) using Tukey's multiple comparison method. The Student's t-test was used for parallel comparison of groups. In all data analyses, the *p*-value was taken as 0.05. All experiments were performed independently of each other in at least three replicates, and the obtained information was recorded as the mean with standard deviations.

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. Characterization of Nano-HDPE

Size and surface properties of nano-HDPEs were determined via TEM micrographs (Figure 1a). The morphology of nano-HDPEs was spherical and their surfaces are smooth. The average diameter of nano-HDPEs was <80 nm (68 ± 11.5) (Figure 1b). FTIR spectra of nano-HDPEs were taken in the 600–4000 cm^{-1} region and are given in Figure 1c. The strongest peaks of nano-HDPEs were characteristically seen at wavelengths of 3834, 3741, 3278, 2924, 2854, 1643, 1535, 1317, 1234, 1064cm^{-1}. Characteristic peaks for HDPE are at 2854 and 2924 cm^{-1}, and this was attributed to CH stretching in the -CH_2- groups that arise in the first step degradation of polyethylene; At 1458 cm^{-1}, CH_2 groups have a CH bending band [25]. The source of the –C=O vibration at 1643 cm^{-1} is erucamide, which is used as a slip in film production [26]. It is possible to observe the same change due to Erucamide in the 3278 cm^{-1} NH_2 symmetric stress-stretch vibration [27]. The 1064 cm^{-1} wavelength is attributed to cyclic olefin copolymer (COC) ring vibration, the 1317 cm^{-1} wavelength is attributed to CH_3 bending vibration, 1458 cm^{-1} is attributed to -CH_2 – CH_2- stretching vibration [26].

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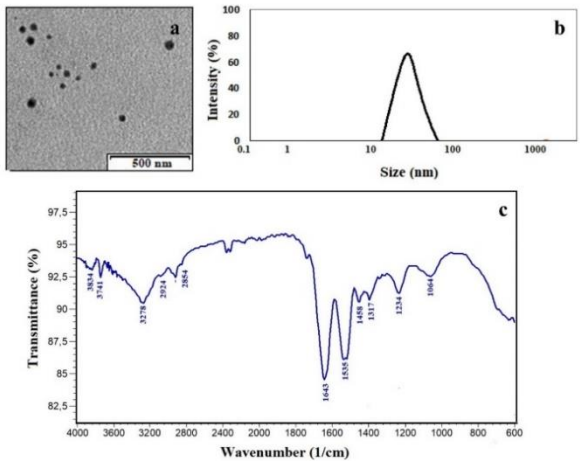

Figure 1. (a) TEM image of nano- HDPE, (b) size distribution curves of nano- HDPE with an average particle size of 80 nm, (c) FTIR spectra of nano-HDPE.

3.2. Effect of Nano-HDPE on Larval Survival and Morphology

Survival rate (viability) of Artemia nauplii exposed to nano-HDPEs at the different exposure durations (24-96 h) and concentrations (50-300 μg/mL) all caused significant decreases. The real reason for this decrease is that with increasing concentration of nano-HDPE, the more of it is ingested by nauplii and as a result, the survival rate decreases with different toxicity mechanisms induced (Figure 2). An acute toxicity assay was performed using 10 nauplii (triplicates) for each concentration group and untreated medium used as negative control. During the experiment, dead larvae were collected at the end of each exposure period and counted to calculate percent viability, including the negative control. In addition, larvae were exposed to the LC_{50} (99.74 μg/mL) of nano-HDPE for 96-h and the morphological changes in the larvae were observed (Figure 2a–e). Nano-HDPEs accumulated in the digestive system of larvae exposed to LC_{50} and various structural defects were observed. The most common structural defects in larvae are malformations such as abdominal curling (Figure 2c,d), abdomen protruding from the abdominal wall (Figure 2a,d,f), abdominal shortening (Figure 2e) and extremity loss (Figure 2a–e). However, when 24, 48, 72 and 96-h of exposure to nano-HDPE were considered separately, swimming speed decreased as the exposure time increased, and deaths were observed. It was noted that the swimming speed of the nauplii in the control group was faster and more stable than the exposed groups until the end of the experimental period (for 96-h). In addition, no deaths were observed in the untreated larvae after 96 h. Artemia nauplii mortality significantly increased with increasing concentrations of nano-HDPE short-term exposure ($p < 0.05$). Our results showed that Artemia nauplii were sensitive to nano-HDPE toxicity and this caused their death.

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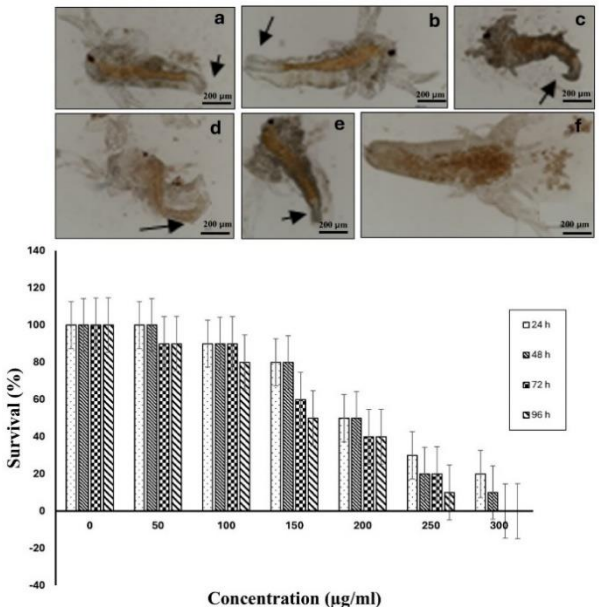

Figure 2. Survival rate of Artemia nauplii in an in vivo toxicity model after exposure to nano-HDPE for different time periods. Stereomicroscope images showing various malformations and extremity loss in exposed larvae (a–f).

3.3. Internalization of Nano-HDPE by Larvae

Artemia nauplii were exposed to a concentration of 99.74 µg/mL, which is the LC_{50} value calculated as a result of acute toxicity, for 96 h. Larvae were washed 3 times with PBS to remove nano-HDPE adhering to them after exposure. If washing is not done, misleading results may occur, especially in FTIR analysis. However, the washing process has no effect on TEM analysis, because the cells have already been examined by taking ultrathin sections. After washing, the larvae were lyophilized for FTIR analysis and FTIR spectra were taken in powder form. Figure 3 indicates the FTIR spectra of both larvae exposed to nano-HDPEs and nano-HDPEs. When both spectra are examined, it is seen that the characteristic peaks of nano-HDPEs are in the exposed larvae. This demonstrated that nano-HDPEs were ingested by the larvae but were not metabolized by the larvae.

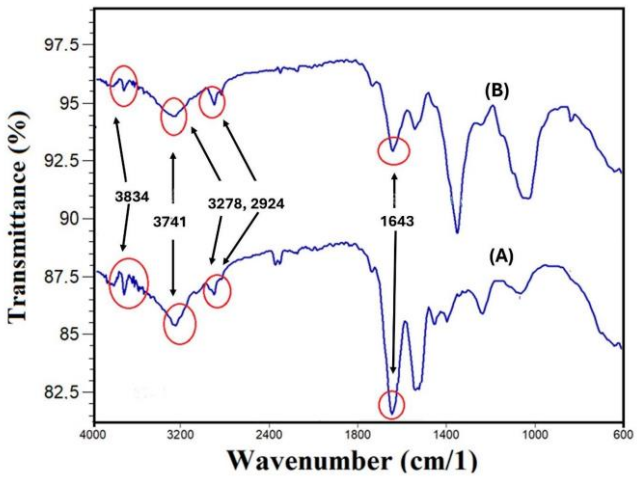

Figure 3. Fourier transform infrared spectroscopy (FTIR) spectra of (A) nano-HDPE at 100 mL concentration, (B) lyophilized larvae treated with nano-HDPE.

Once again TEM was used to verify nano-HDPE (<80 nm) internalization. Ultrastructural examination using TEM showed that nano-HDPEs were localized to the cell cytoplasm individually

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and in clusters. In TEM images of untreated larvae, the cells are well-circumscribed and there are no morphological anomalies (Figure 4a,c). In treated larvae, nano-HDPEs were seen individually in the cell and generally in clusters in the cytoplasm (Figure 4b,d,e). Again, cell contents leaked out in the apical parts of the processed cells, indicating that the cell membranes of the cells were seriously damaged due to nano-HDPEs. Moreover, the borders of these cells have an uneven wavy appearance (Figure 3b,d).

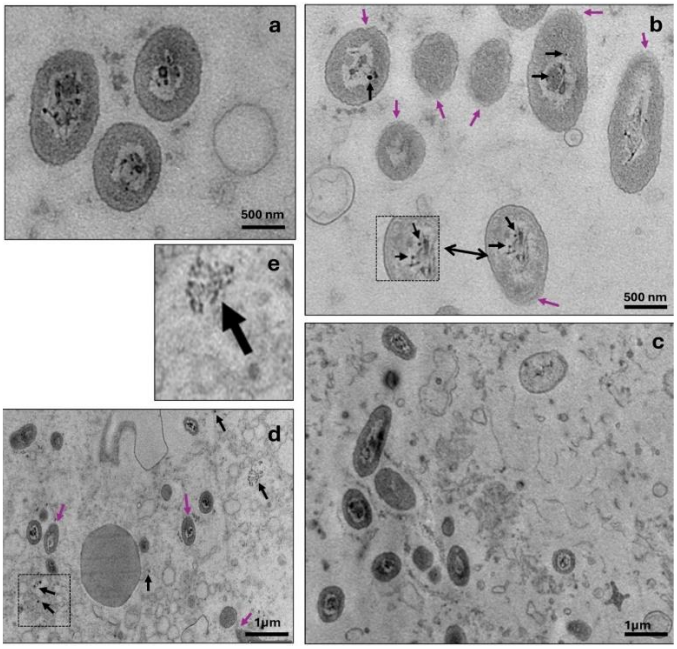

Figure 4. TEM Micrographs of the localization of nano-HDPE in treated *Artemia* nauplii. Untreated (control) larvae scale bar 500 nm (a), treated larvae scale bar 500 nm (b), untreated larvae scale bar 1 µm (c) treated larvae scale bar 1 µm (d), internal view of d (e). Black arrows indicate nano-HDPEs, purple arrows indicate cell damage.

3.4. Oxidative Stress Induction in Larvae

Oxidative stress induction is widely investigated in investigating the toxicity mechanism of xenobiotics. In the current study, the oxidative stress level of larvae exposed to nano-HDPEs at the LC_{50} for 96-h increased (Figure 5). A small amount of oxidative stress was also recorded in untreated larvae in both ROS measurement and fluorescence images (Figure 5a,c). However, this stress in untreated larvae is quite insignificant compared to treated larvae. Briefly, fluorescent images (green) of processed larvae indicated i intense stress generation throughout the entire body (Figure 5b). In addition, the oxidative stress level of treated larvae was significantly higher than that of untreated larvae ($p < 0.05$).

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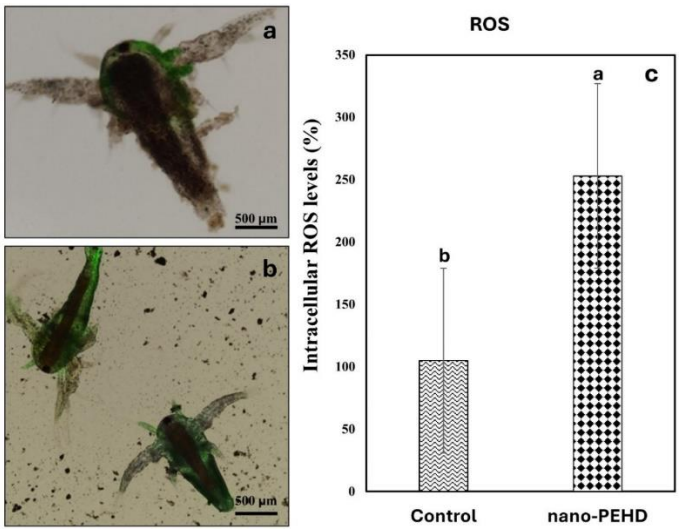

Figure 5. Fluorescence images and ROS generation graph obtained by evaluating intracellular ROS with DCFDA. Larvae untreated with nano-HDPE (a), Larvae treated with nano-HDPE at LC_{50} (b), Graph showing intracellular ROS levels (c).

3.5. MDA Content and Antioxidant Enzyme Activities in Larvae

Compared to untreated larvae, a significant increase in SOD and CAT activity with MDA content of larvae exposed to nano-HDPE was observed. The results showed that nano-HDPE exposure caused oxidative stress of larvae. However, the SOD, CAT activity and MDA content of nano-HDPE-treated larvae were significantly increased ($p < 0.05$) compared to untreated larvae (Figure 5).

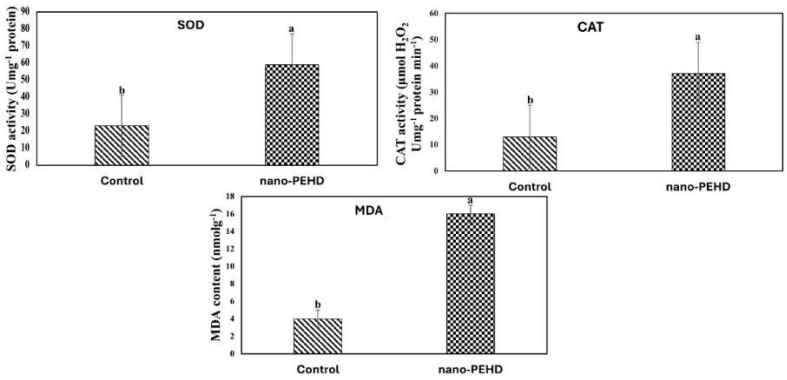

Figure 6. SOD, CAT antioxidant enzyme activities and MDA conten of *Artemia* nauplii treated and untreated with nano-HDPE at LC_{50} for 96-h. The data are from three independent biological replicates and values are mean ± SD. Letters (a,b) indicates significant differences $p < 0.05$.

4. Discussion

Many factors affect the toxicity of polyethylene plastics. The primary ones are their chemical structure, the type and amount of additives, and their affinity to persistent organic pollutants [28]. Thus far, polyethylene plastics have been found to have adverse effects on immunity and cell integrity, gene expression, inflammation, growth and reproduction in aquatic indicator organisms [29–31]. Polyethylene plastic waste is decomposed by biological and non-biological methods. Among the non-biological methods, mechanical processes, are used to break down polyethylene, into smaller particles by chemical or physical methods [32]. In the present study, high density polyethylene plastics were processed into nanoplastics using micromotor. The characterization of the nano-sized high density polyethylene plastics prepared in the present study gave the same FTIR spectra as the

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virgin plastic and the size was determined to be smaller than 80 nm (Figure 1). There are similar studies in which particles similar to micro- and nanoplastics in the environment were prepared for use in biological tests. One of these is the preparation of synthetic particles with geometric morphology very similar to the most preferred plastics such as polyethylene, polypropylene, polyvinyl chloride, polystyrene and polyethylene terephthalate and to the surrounding micro and nanoplastics (MPs/NPs) by the pin-on-disc method [33]. *Artemia salina* larvae (instar I, nauplii) were preferred in the LC_{50} acute toxicity test due to their sensitivity to environmental conditions. The LC_{50} value was calculated using SPSS analysis probit. LC_{50} is the value that indicates the concentration of toxic compounds that cause the death of the organism up to 50% and is a quantitative indicator of the toxicity degree of xenobiotics. Again, the LC_{50} relates to the total mortality of the tested animal rather than specific damage to organs. The simple digestive system of *Artemia* nauplii makes them useful for calculating LC_{50} in short-term tests [34]. The *A. salina* test system has been used successfully in numerous studies [35,36]. In the present study, the LC_{50} value of spherical high density polyethylene nanoplastics with an average size of < 80 nm was calculated as 99.74 µL/mL in the toxicity test for *Artemia* nauplii based on deaths after 96-h (Figure 2). The highest mortality of *Artemia* nauplii, up to nearly 100%, was obtained from the maximum concentration used (300 µg/mL). *Artemia* nauplii feed on particles ranging in size from 1 µm to 50 µm [37]. Therefore, nano-HDPEs with a size of <80 nm are easily ingested/taken by the nauplii. These nano-HDPEs taken up by the larvae were distributed individually and in aggregates within the cells and cytoplasm in TEM examination (Figure 4). In previous studies, *Artemia* nauplii has been reported to ingest/internalize nano- and microplastics of different sizes [38–40]. Moreover, in the FTIR analysis of nano- HDPE uptake by exposed lyophilized nauplii, the peak values of 3834, 374, 3278, 2924 and 1643 cm^{-1} belonged to high-density plastics. This indicates that nano-PEHDs were taken into the cells by the larvae but were not digested. Ingestion of microplastics by nauplii was verified by FTIR analysis. In Jeveyani et al., [22], ingestion of microplastics by *Artemia* nauplii was determined by FTIR analysis. In this study, they detected 2960, 2922, 1453, 1317, 895 cm^{-1} spectra of polypropylene microplastics in lyophilized larvae. In our current study, the survival rate of nauplii decreased significantly ($p < 0.05$) with increasing concentration of nano- HDPE and exposure time. Increasing the amount and duration of exposure caused the nauplii to ingest more nano- HDPE, which in turn reduced the survival rate by inducing one or more toxicity mechanisms. Stereo microscope images showed that nano- HDPE particles accumulated in the digestive tract of nauplii, causing extremityloss and malformation (Figure 3). Exposure to nano-HDPE for 96-h at LC_{50} decreased the swimming speed of the larvae and resulted in their deaths. Similar results have been reported in previous studies in aquatic organisms exposed to NPs. Ingestion of microplastics and nanoplastics reduces the survival rate of zooplanktonic organisms [41]. Polystyrene NPs increased toxicity in *Daphnia magna*, which is sensitive to nanoparticles due to being a filter feeder like the *Artemia* larvae [42]. Again, the survival rate of young freshwater shrimp *M. nipponense* exposed to polystyrene NPs decreased with increasing nanoplastic concentration [43]. As a result of exposure of *A. salina* to 50-100 µg/mL polypropylene microplastics for up to 48-h in the nauplii, meta-nauplii and juvenile life stages, swimming behavior was reduced compared to control [22]. All these studies are evidence of the fact that NPs are taken by organisms through nutrition, causing a decrease in survival rate. Today, there have been few studies on nanoplastic toxicity. In our study, we investigated the effects of short-term exposure (96-h) through feeding and respiration depending on the nano- HDPE size of the brine shrimp *Artemia* nauplii, a planktonic species of the marine trophic network, in multiple aspects. Oxidative stress and enzyme activities, which are toxicity mechanisms, were evaluated with some biochemical markers. In our study, the production of ROS and MDA, which are oxidative stress markers, increased in larvae exposed for 96-h at LC_{50} (99.74 µg/mL). Again, it activates the SOD and CAT antioxidant enzyme system, which is the first line of defense against oxidative stress. What is noteworthy here is that the amount of SOD and CAT decreased despite the increasing ROS and MDA levels. This indicates that the larvae do not show sufficient defense against the stress caused by nano- HDPE. As proof of this, TEM micrographs, ROS and stereomicroscope images obtained from ultrastructural examinations

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were produced and then evaluated. In these examinations, severe cell membrane damage was observed in the larvae, and this damage is confirmed by the increase in MDA level. What makes this situation even more interesting is that although the mortality rate is not high at the LC_{50}, serious toxicity due to oxidative stress occurs in the larvae. Extreme deaths were observed when the exposed nano- HDPE exceeded LC_{50}. These deaths were attributed to the organism's ability to withstand stress at the LC_{50} of nano-HDPE, but above this concentration to inadequate defense of the larvae against nano- HDPE. Another toxicity mechanism of nano-HDEP is the morphological evaluation of larvae. Nano-HDPE caused various malformations and extremity losses in larvae. Again, the decrease in swimming speed in larvae contributed to the death of the larvae. In this study, the increase in nano-HDPE concentration and the decrease in the survival rate of larvae depending on the exposure time is due to nano-HDPE causing multiple toxicity mechanisms. Similar results have been reported in other studies. In one of these studies, *A. salina* was exposed to polypropylene microplastics for 48-h at different life stages [38]. As a result, it was noted that this increased ROS production by causing changes in redox homeostasis in the organism, and caused behavioral changes and death with intestinal malformation by affecting biochemical biomarkers. In another study indicated that noted that polypropylene microplastic toxicity in *Artemia salina* changed homeostasis and swimming behavior in the larvae and caused death in larvae through high oxidative stress. They also noted that the larvae damaged the epithelial cells [22].

5. Conclusions

Artemia salina as a test organisms is a very useful indicator to investigate nanoplastic pollution in aquatic ecosystems in a short time and in a versatile way. With the data obtained from these tests, the degree of transport, accumulation and impact of nanoplastics on living things above trophic levels can be calculated. In the present study adds deep insight into nanoplastic toxicity. It fills an important knowledge gap, especially by demonstrating the damage it causes to cells. In addition, the current study is important in terms of ensuring comparability in future studies both in terms of trophic levels and the effect of plastic type and size on toxicity. nano-sized high-density polyethylene plastics were shown to cause toxicity in Artemia nauplii larvae by activating different toxicity mechanisms.

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Ethics Statement: Ethical approval was not required as zooplankton crustacean *Artemia salina* (Brine shrimp), is an invertebrate species. The experimental procedures were performed based on the principles of OECD Test Guideline 202, with modifications adapted for the *Artemia salina* model.

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Abbreviations

The following abbreviations are used in this manuscript:

NPs	Nanoplastics
nano-HDPE	High-density polyethylene nanoplastics
FTIR	Flourier transmission infrared Spectroscopy
OECD	Organization for Economic Cooperation and Development
MDA	Malondialdehyde

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ROS	Reactive oxygen species
CAT	Catalase
DCFH-DA	2′,7′-dichloro dihydrofluorescein diacetate
LC_{50}	Lethal concentration50%

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