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Nile red staining of polyethylene and polystyrene in *Daphnia magna* tests

STEFANIA GHEORGHE, CATALINA STOICA*, ANCA MARIA HARABAGIU

National Research and Development Institute for Industrial Ecology-ECOIND Bucharest, 57-73 Drumul Podul Dambovitei, district 6, 060652, Bucharest, Romania
 # Corresponding author (e-mail): catalina.stoica@incdecoind.ro

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Abstract
Microplastics pollution represents a stringent global issue. Their accumulation in the aquatic environment remains complicated due to the abundance, distribution, and resulting environmental effects they induce. Recently, assessing the toxicity level on aquatic organisms has gained scientific interest. Due to the diverse types and sizes of polymers present in the environment, the detection and evaluation of their effects are still a challenging issue. Polyethylene (PE) and polystyrene (PS) are the most commonly used polymers and are therefore predominantly detected in both marine and freshwater aquatic environments. This paper aims to evaluate the effects of Red Nile-stained PE and PS, tested in different particle sizes (PE 40 µm ÷48 µm, 12 µm 5, higher than 125 µm, and PS 20 µm, 200 µm, 430 µm) on Daphnia magna, and highlight them through microscopic analysis. Acute toxicity tests conducted over a 48-hours exposure did not reveal significant toxicity effects in terms of mortalities compared to the controls. Red Nile staining allowed the visualization of the shapes and sizes of the tested microplastics and their entry pathways into the Daphnia bodies. PS of 20 µm size was detected in the digestive tract of Daphnia, indicating as primary pathway of entry into the body of aquatic organisms. No acute toxic effects were recorded as a result of direct exposure to PE and PS particles. However, sub lethal effects such as feeding and growth disturbances, which could affect organisms in the long term, are suspected.

Keywords: *polyethylene, polystyrene, Daphnia magna, Red Nile*

INTRODUCTION

The anthropogenic activities and pandemic situations led to an increase demand for plastic products easily to use and disposal. In particular, plastic elimination contributed to an environmental omnipresent stress. Because plastic materials require a long time for degradation or biodegradation (up to hundreds of years), they could affect the environment and its ecosystems in multiple ways. It is estimated that the COVID-19 pandemic caused an increase in the consumption of single-use plastic materials up to 300% higher compared to the pre-pandemic period [1]. These type of situations had a major influence on environmental protection strategies regarding the reduction of plastic waste pollution. Most of the polymers of mass consumption and medical use are based on polymers such as polypropylene (PP), polystyrene (PS), polyethylene (PE), polycarbonate (PC), polyvinyl chloride (PVC).

These types of polymers have determined the increase of the incidence of microplastics (MPs) and nanoplastics in the aquatic environment. Microplastics consists mainly by fragments, granules, fibers, plastic strips with sizes between 5 mm and 1 µm, while nanoparticles have an irregular shape with a size between 1 to 1000 nm. Another classification of microplastics is the one by their source. Primary microplastics are produced especially for industrial, textile, cosmetic activities, while

secondary microplastics result from the fragmentation of larger pieces of plastic under the pressure of natural environmental conditions [2].
The effects of microplastics are studied more and more regarding a variety of aspects such as: the influence of their size and shape [3]; direct or indirect exposure in various laboratory conditions and in various concentrations [4, 5]; association with other pollutants [6]; detection, identification and distribution among the compartments of the aquatic environment [7]; translocation and bioaccumulation in various food sources [8].
Most of the ecotoxicological studies on the impact of microplastics have been carried out in marine water, while the studies in freshwater resources are limited. The freshwater resources are ones of the target paths of MPs reaching marine environment.
It is considered that these pollutants favored by their shape and size cause negative effects on planktonic and benthic freshwater organisms. Some studies have shown that PS can cause sub-lethal toxic effects such as genotoxicity and oxidative stress [4].
The literature specifies a size of microplastics of the PS type from 1 to 500 μm in surface water [9] and a concentration of 5 ng/L to 13 ng/L [10]. PE was found in size lower than 100 μm to higher than 300 μm and concentration ranging 0.389 μg/L ± 0.377 μg/L [11].
Actually, the identification and characterization of microplastics from real environmental samples is carried out through a combination of physical (microscopy) and chemical (spectroscopy) methods, each of them having advantages and limitations both in terms of errors, costs and time consuming. The fluorescent staining of microplastics using dyes such as Red Nile (usually used in histopathology investigations) allows a good time-management assessment of the MPs detection in water and biota samples using fluorescence microscopy. The interaction between the dye and the polymer depends on its chemical characteristics, which facilitate fluorescent-based detection. Semi-automatic applications such as "Plastic Detection Model" and "Polymer identification Model" which used Red Nile staining, were successful use in 92.7% of microplastics detection and for 80% of microplastics identification [12].
The aims of the paper were: *i)* to visualize microscopically the Red Nile-stained PE and PS in different particle sizes and *ii)* to evaluate the effects of Red Nile-stained PE and PS in different particle sizes (PE 40 μm ÷48 μm, 125 μm, higher than 125 μm, and PS 20 μm, 200 μm, 430 μm) on *Daphnia magna*.

EXPERIMENTAL PART

The acute toxicity test was carried out in accordance with both OECD 202 method and SR EN ISO 6341:2013, respectively, using *Daphtoxkit F magna kit* (MicroBioTest Inc., Belgium). The biological material was the species *Daphnia magna* in the form of ephippia (resistance eggs - protected within a chitinous capsule called ephippium, which allows for long-term storage without losing viability and can, under specific environmental conditions, develop into neonates used in toxicity tests within 3 days). The standard freshwater components included $NaHCO_3$, $CaCl_2$, $MgSO_4$, and KCl. Before use the standard freshwater was aerated for 15 minutes and then used for activating *Daphnia magna* eggs or preparing test solution. The physical and chemical parameters of the standard freshwater were pH 7.74±0.20, temperature 22.4±0.2ºC, dissolved oxygen 6.85±0.54 mgO_2/L.
The age of the neonates was 48 hours from hatching. Prior to testing, *Daphnia magna* neonates were fed using a pre-feeding procedure of 2 hours with Spirulina microalgae (MicroBioTest Inc., Belgium). The test was conducted for 24 to 48 hours in testing plates, utilizing the *Daphnia magna* neonates uniform in size and age. A temperature-controlled incubator set at 20÷25ºC (Aqua Lytic TC 1355, MicroBioTest Inc., Belgium), light table and standard laboratory glassware were used. In parallel with the PE-PS tests, control tests were also set up using the standard freshwater without microplastics.
The results recording was carried out after 24 and 48 hours of incubation by observing the plate under illuminated conditions and read the mortalities or immobilization effects of neonates, and the behaviour of actively swimming organisms. Finally, the number of immobile neonates was

calculated for each tested concentration, along with the mean and the recorded percentage effect. Randomly, some *Daphnia magna* species were collected for microscopy examination in order to visualize the ingestion of microplastics.

The tested materials were polymers of the following types: ***i)*** polyethylene (PE) irregular shape, CAS 9002-88-4, white colour, density 0.92 g/mL, in sizes of 40 µm ÷48 µm, 125 µm, and higher than 125 µm; and ***ii)*** polystyrene (PS) spherical shape, CAS 9003-53-6, colourless, density 1.05 g/mL, in sizes of 20 µm, 200 µm, 430 µm, suspended in a 10% aqueous suspension. All tested polymers were provided by Sigma-Aldrich, Saint Louis, USA. Prior to testing, the PE and PS particles were labelled by staining with Nile Red for microscopy from Sigma –Aldrich (10 µg/mL solution in acetone) for 24 hours, followed by two consecutive washing steps, first step with phosphate buffer saline (PBS, 1x) and second step with deionized water.

PE of 1 mg/L, 10 mg/L, 50 mg/L and PS of 1 mg/L, 10 mg/L, 100 mg/L for each size were the tested concentrations. A range of 0 to $1x10^6$ particles/L was estimated. According with other studies [4], MPs concentrations from $1x10^{-5}$ particles/L to $1x10^5$ particles/L were estimated.

The microscopic analyses were performed using fluorescence inversion microscope Leica DMi8 in bright field and fluorescence. The images were acquired and processed using microscope software LAS V4.7.

RESULTS AND DISCUSSION

Microscopic visualization of microplastics - Nile Red staining

The principle of Red Nile staining is based on the solvatochromic nature, whose emission spectrum shifts depending on polarity of its environment. According to this the microplastics can be classified into polar and hydrophobic based on polymer characteristics. Their interactions with the dye were detected through fluorescence. Gabriel Erni-Cassola (2017) suggest that Nile Red method is a highly sensitive method for detection smaller size microplastics such as PE or PS from environment samples [13].

The visualization of microplastics in the tested solutions were emphasized through Red Nile staining. Thereby, the presence of PE and PS in different sizes was highlighted. Figures 1-3 shows the PE of sizes 40 µm ÷ 48 µm, 125 µm, higher than 125 µm in unregulated shapes, not stained with Nile Red. In Figures 4 and 5, Nile Red-stained PE of size 40 µm÷48 µm is presented - displaying irregular shapes (stained in green).

The Figures 6 to 8 shows PS of sizes 20 µm, 200 µm, 430 µm appeared as unmarked perfectly spherical particles. In the Figures 9 and 10, PS spheres of 20 µm size are displayed, marked with Nile Red staining.

Due to its spherical shape, PS is easier to be highlighted. PE, with its irregular shape, could be easily confusing with other particle types within a complex matrix. It can also be observed that the size of PE particles can vary, despite being from the same product batch. A weaker staining of PE compared to PS was observed. This can be explained by the fact that PE has a non-polar surface and PS a polar surface that influences dye adhesion and fluorescence detection.

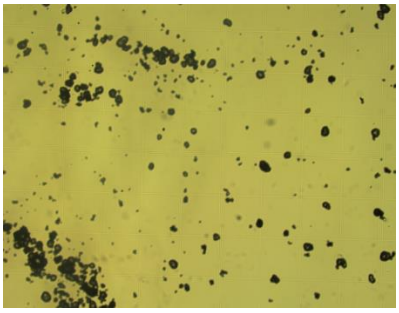

Fig. 1 PE 40 µm ÷48 µm

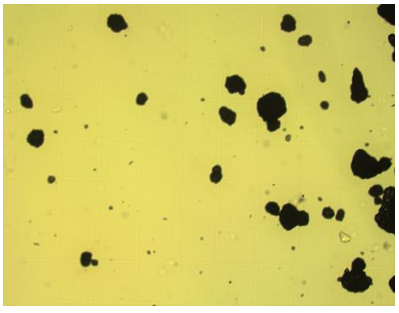

Fig. 2 PE 125 µm

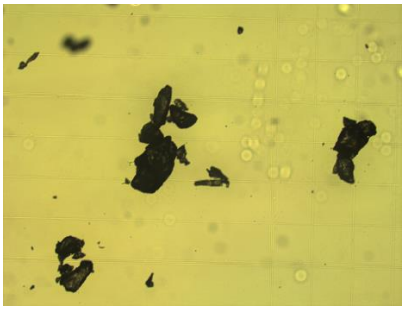

Fig. 3 PE >125 µm

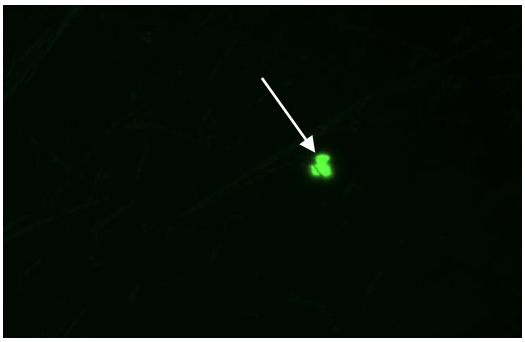

Fig. 4 PE 40 µm ÷48 µm – unregulated shape (Nile Red stained)

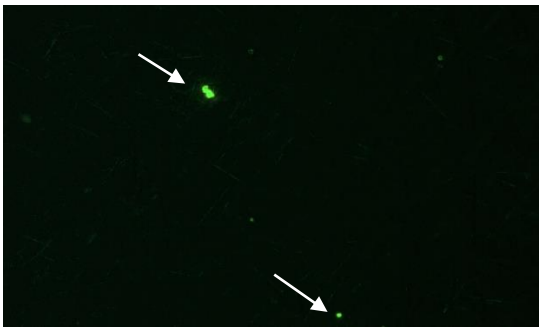

Fig. 5 PE 40 µm ÷48 µm - unregulated shape (Nile Red stained)

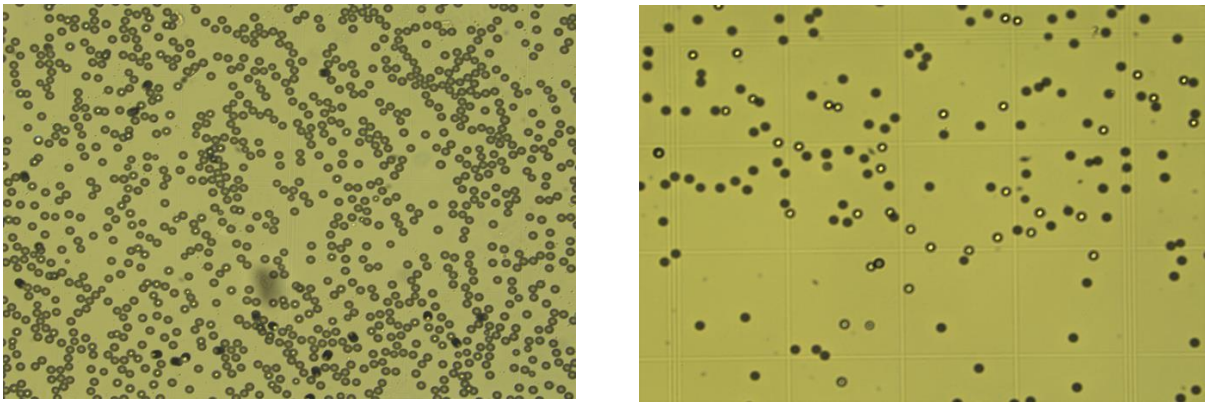

Fig. 6 PS 20 µm - spherical shape

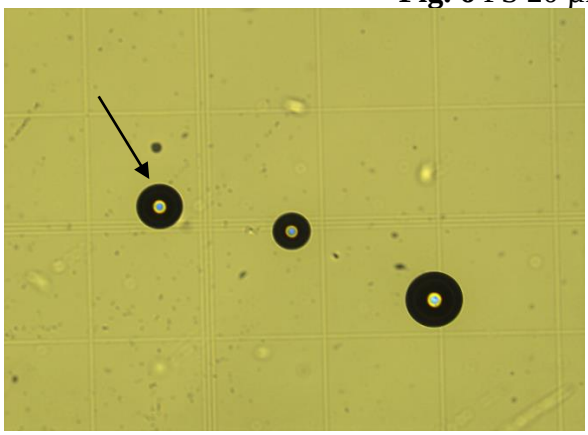

Fig.7 PS 200 µm

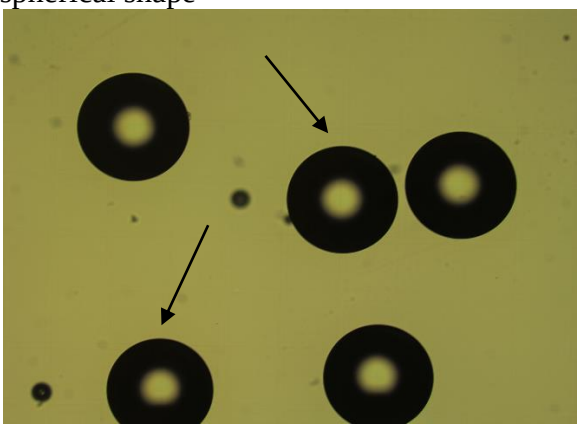

Fig.8 PS 430 µm

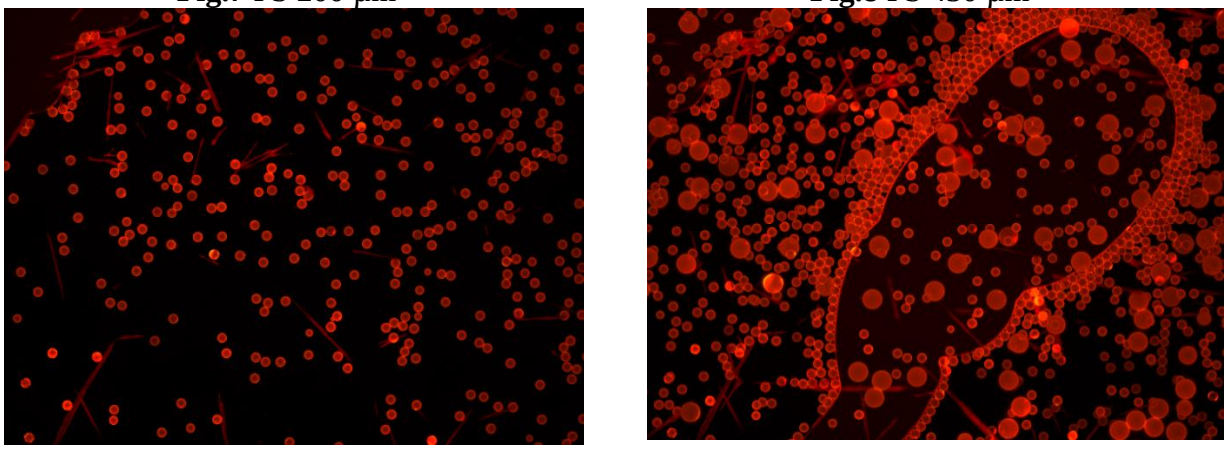

Fig. 9 PS 20 µm (Red Nile stained) – spherical shape

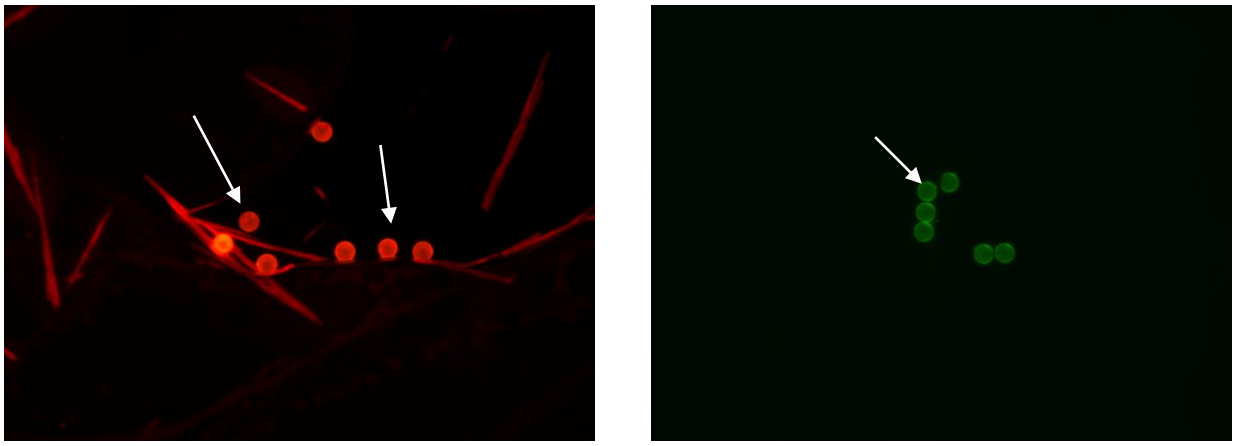

Fig. 10 PS 20 µm-Red Nile stained in testing media
(micelle of colorant)

Effects on Daphnia magna

The parameters investigated during the test indicate suitable conditions for the survival of the *Daphnia magna* species. No abnormal changes were observed in the determined values in the test solutions compared to the control solutions. In the exposure time, the control solutions (standard freshwater) registered pH 7.44 ÷ 7.60, dissolved oxygen 5.18÷5.67 mgO_2/L, temperature 21.4 ÷22.9ºC. The microplastics solutions showed pH 7.32÷7.55, dissolved oxygen 5.40÷5.75 mgO_2/L, temperature 21.2÷22.8ºC.

From method validity standpoint, the acute toxicity test was conducted on Daphnia planktonic crustaceans in the laboratory conditions which met all the criteria specified in the standardized working methodology: the dissolved oxygen concentration at the end of the test in control groups was ≥3 mg/L, and the immobilization percentage ranged from 0 to 5%. The EC50 value (24h) estimated for the reference substance (potassium dichromate) was 0.78 mg/L (Certified value for the used *Daphnia magna* - 0.80 mg/L), value within the acceptable range outlined by ISO 6341: 0.6 mg/L÷2.1 mg/L.

D. magna are planktonic crustaceans that belong to the phylum Arthropoda, Brachiopoda class which are feed on small, suspended particles in the water. The *D. magna* collect particles that are transferred into the food groove by special setae. Although the feeding apparatus is so efficient that even bacteria can be collected, the food is usually made up of planktonic algae. *D. magna* usually consume particles from around 1 µm up to 50 µm [14].

Tested microplastics did not induce lethal or immobilizing effects on *D. magna* at concentrations ranging from 1 mg/L to 50 mg/L PE and respectively 1 mg/L to 100 mg/L PS, during the 48-hour exposure period.

Our results are far from the estimated concentrations in the environment and therefore acute exposure does not cause negative effects on the environment. Microscopic examination revealed the presence of PS 20 µm particles in the digestive tract. Figures 13÷16 highlight the presence of spherical PS particles in the digestive system of daphnia, in comparison to control organisms (Figures 11, 12), indicating the primary route of polymer ingestion by aquatic organisms. Despite the absence of mortality within 48 hours, the presence of microplastics in the digestive tract can lead to satiety and finally the obstruction of organism's feeding capability, potentially leading to mortality over time. Some authors highlighted that prolonged exposure of *D. magna* to small concentrations of microplastics determines their random ingestion while swimming and feeding. According to other studies, *D. magna* has the ability to ingest particles below 70 µm suspended in the water mass without distinguishing between size and quality. Once ingested, it can cause effects such as disruption of the nutrient absorption mechanism, growth, development and reproduction processes [14]. This notification regarding the filling of the digestive tract with microplastics such as PE (up to 63 µm to 75 µm) [15] or PS (1 µm to 10 µm) [16] were observed during long-term exposure tests.

Also in our study, the microscopic analysis highlighted the presence of 20 µm PS in the digestive tract of the *D. magna*, regardless of the concentration in which these types of microplastics were tested.

The ingestion of microplastics by planktonic organisms considered primary producers can lead to bioaccumulation effects in the trophic chain with an important impact on food resources and on human and animal health [17]. For example, PE, PS, PP were identified in bivalve molluscs 0.15 ÷ 0.20 particles/g (size 43 µm to 4720 µm), crustaceans 0.5 ÷ 3.3 particles per individual (size 7 µm to 5000 µm), fish 28÷7527 particles / fish (size µm ≤25 to 2000 µm) [18÷20]. In addition, sub-lethal effects such as oxidative stress and alteration of the genetic material evident in studies of intoxication of *Daphnia magna* with polystyrene in dimensions of 1 µm or in concentrations of ordinary µg/l cannot be neglected [4].

The ingestion or simple transit of microplastics through the body of organisms can cause negative effects on the energy metabolism of individuals. The first effect is that of satiety, thus modifying the normal feeding behaviour and sometimes it can increase the filtering activity [8].

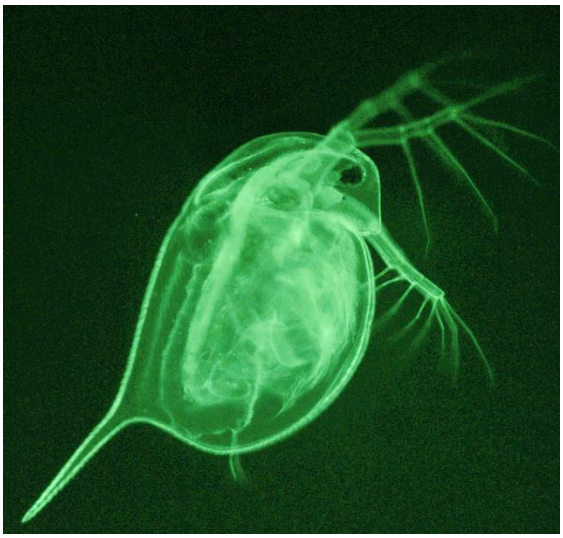

Fig. 11 *Daphnia magna* - control

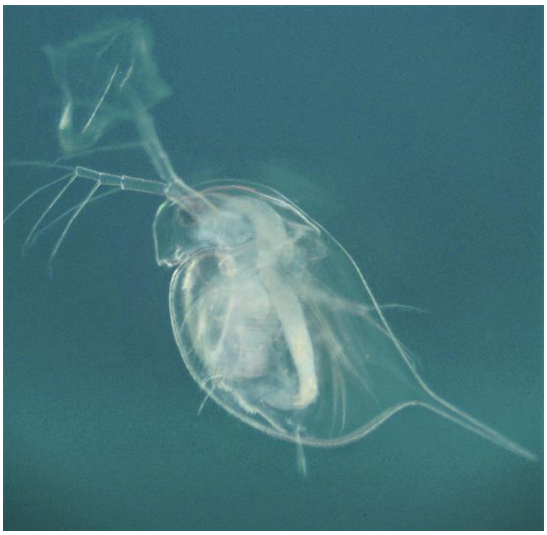

Fig. 12 *Daphnia magna* – control

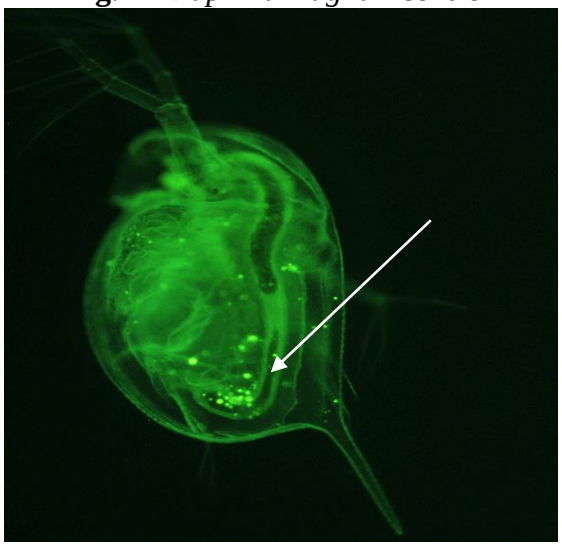

Fig. 13 *Daphnia magna* – presence of PS 20 µm in digestive tube

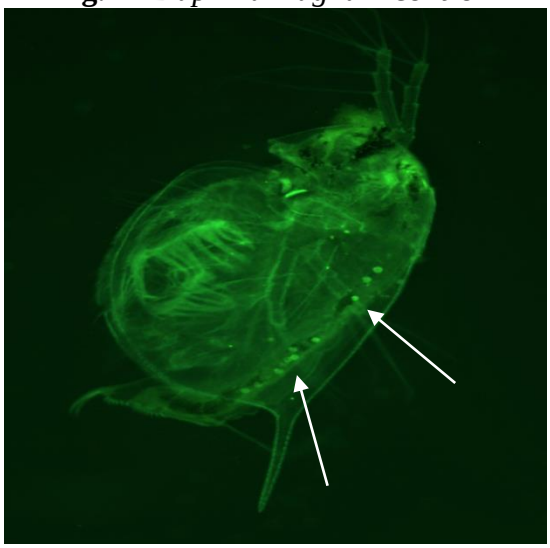

Fig. 14 *Daphnia magna* – presence of PS 20 µm in digestive tube

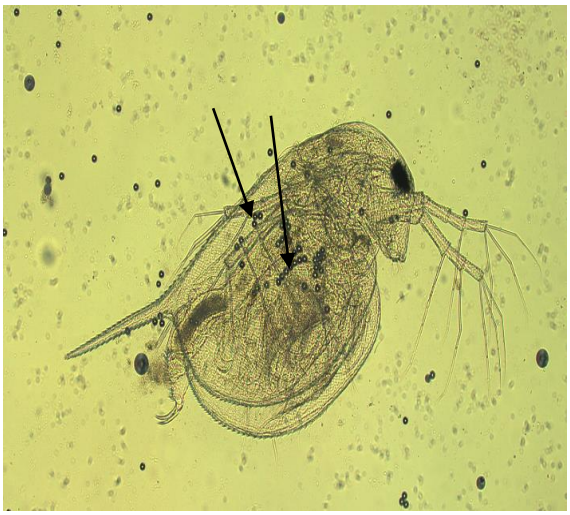

Fig. 15 *Daphnia magna* – presence of PS 20 µm in digestive tube

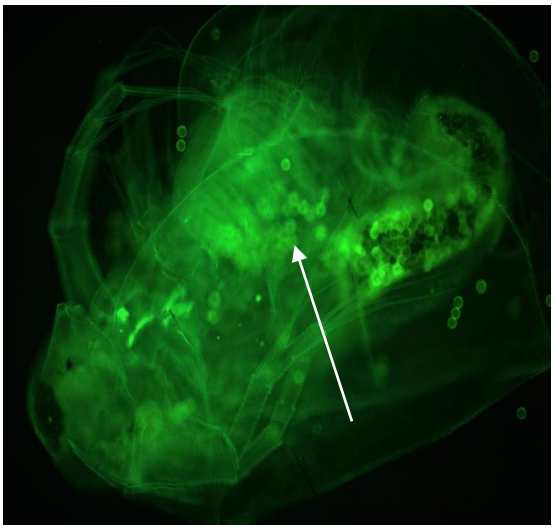

Fig. 16 *Daphnia magna* – presence of PS 20 µm in digestive tube

CONCLUSIONS

The effects of Red Nile-stained PE and PS, tested in different particle sizes (PE 40 µm ÷ 48 µm, 125 µm, higher than 125 µm, and PS 20 µm, 200 µm, 430 µm) on *Daphnia magna* were evaluated. Acute toxicity tests conducted over a 48-hours exposure did not reveal toxicity effects in terms of mortalities compared to the controls.

Synthetic PE and PS in different sizes were highlighted by Red Nile staining using fluorescence microscopy. PS was easier to be highlighted compared to PE due to the regular shape (spherical shape). A weaker staining of PE compared to PS was observed (data is not shown), explained by the nature of polymer type. PS have a polar surface that influences dye adhesion and fluorescence detection. Red Nile staining allowed the microscopic visualization of PS and their entry pathways into the Daphnia bodies. PS of 20 µm size was detected in the digestive tract of Daphnia, indicating as primary pathway of entry into the body of aquatic organisms. Even that no acute toxic effects were recorded as a result of direct exposure to PE and PS particles, sub lethal effects such as feeding and growth disturbances in chronic test, are suspected.

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