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SIMI

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, 57-73 Drumul Podu Dambovitei, district 6, 060652, Bucharest, Romania, Department of Pollution Control, Biotests – Biological Analyses Laboratory

THE GLOBAL PROBLEM OF PLASTIC

- Plastic is detected in all environmental factors, including food and drinking water sources.
- Plastic is not assimilated by the environment through decomposition and/or biodegradation processes.
- The use of disposable plastic materials / products after COVID-19 pandemic has intensified the plastic problem worldwide. 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).
- Microplastics are the fragments of synthetic polymers (plastic) with impact on living organisms, posing risks, including to human health.
- Due to the diverse types and sizes of polymers present in the environment, the detection and evaluation of their effects is still a challenging issue.
- PE and PS are the most commonly used polymers and they are, therefore, predominantly detected in both marine and freshwater aquatic environments.
- Microplastics concentrations in the international waters was reported as 0 - 20 mg/L; 0-10.000 particles /L

AIMS OF THE STUDY

Firstly, Red Nile-stained PE and PS were microscopic visualized. Secondly, the effects of PE and PS, in different particle sizes (PE 40-48, 125, >125 µm, and PS 20, 200, 430 µm) on *Daphnia magna* acute tests were evaluated through microscopic analysis.

MATERIALS AND METHODS

Toxicity test

- Acute toxicity test was done in accordance with OECD 202 and *Daphtoxkit F magna kit* (MicroBioTest Inc., Belgium);
- The biological material freshwater crustacean - *Daphnia magna*, 48h age, pre-feeding with Spirulina microalgae;
- Test condition: *Daphnia magna* exposure 48 hours, at 20-25°C, in dark, in absence (control) or presence of microplastics. Standard freshwater based on NaHCO3, CaCl2, MgSO4, and KCl, pH 7.74±0.20, temperature 22.4±0.2°C, dissolved oxygen 6.85±0.54 mgO2/l;
- The mortalities or immobilization effects of neonates, and the behaviour of actively swimming organisms were observed.

Tested materials

- polymers types (Sigma-Aldrich):

i) polyethylene (PE), CAS 9002-88-4, irregular shape, white colour, density 0.92 g/ml, sizes of 40-48µm, 125 µm, and >125 µm;
ii) polystyrene (PS), CAS 9003-53-6, spherical shape, colourless, density 1.05 g/ml, sizes of 20, 200, 430µm, suspended in a 10% aqueous solution.

Staining:
Prior testing, PE and PS particles were stained with Nile Red (10 µg/ml stock solution in acetone) for 24 hours.

Tested concentrations PE - 1, 10, 50 mg/L and PS - 1, 10, 100 mg/L of each size. It was estimated a density of 0 to $1x10^6$ particles /L.

Microscopic analyses
The microscopic analyses were performed using fluorescence inversion microscope Leica DMi8 in brightfield (BF) and fluorescence (FL). The images were acquired and processed with microscope software LAS V4.7.

RESULTS

- The microplastics visualization in the tested solutions were emphasized through Red Nile staining. Thereby, the presence of PE and PS in different sizes was highlighted. **Figures 1-4** showed the presence of unstained (Fig.1) and stained (Fig.2) PE of sizes 40-48, 125, >125 µm in unregulated shapes. Due to its spherical shape, PS was easier observed. 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.

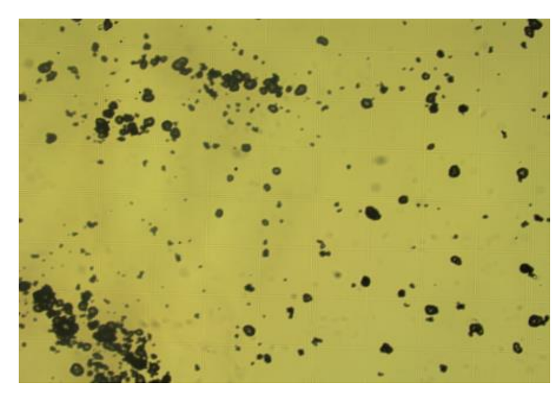

Fig. 1 PE 40-48µm, BF, 10x

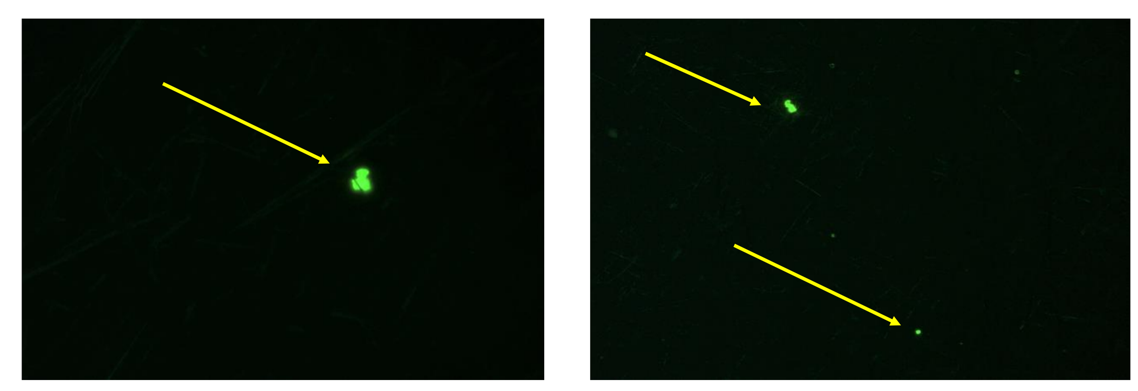

Fig. 2 PE 40-48 µm- unregulated shape (Nile Red stained), FL, 10x

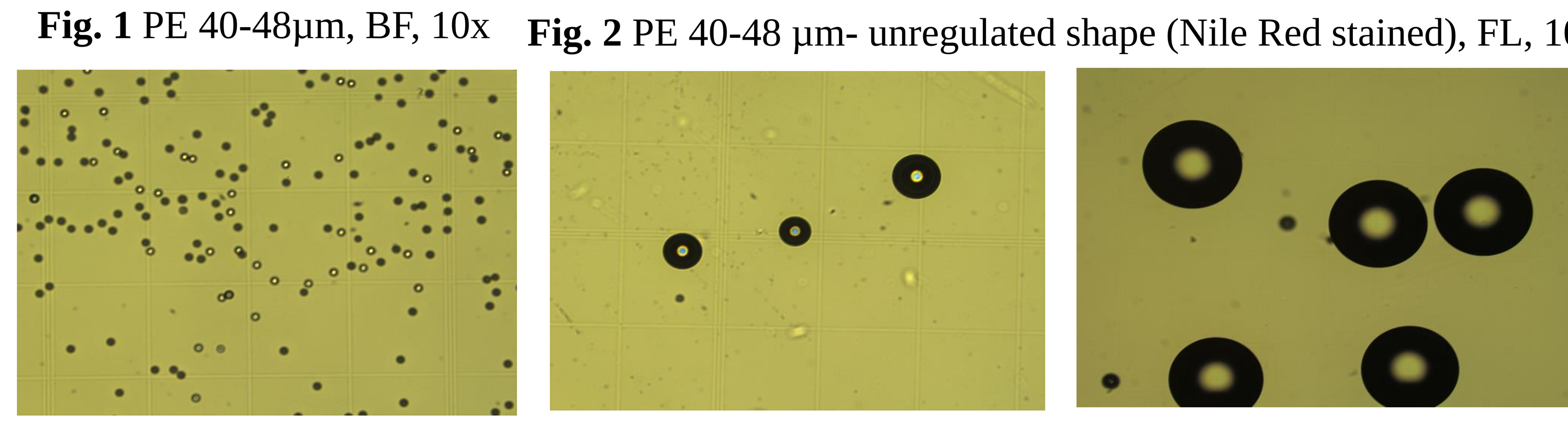

Fig. 3 PS 20, 200, 430 µm - spherical shape, BF, 10x

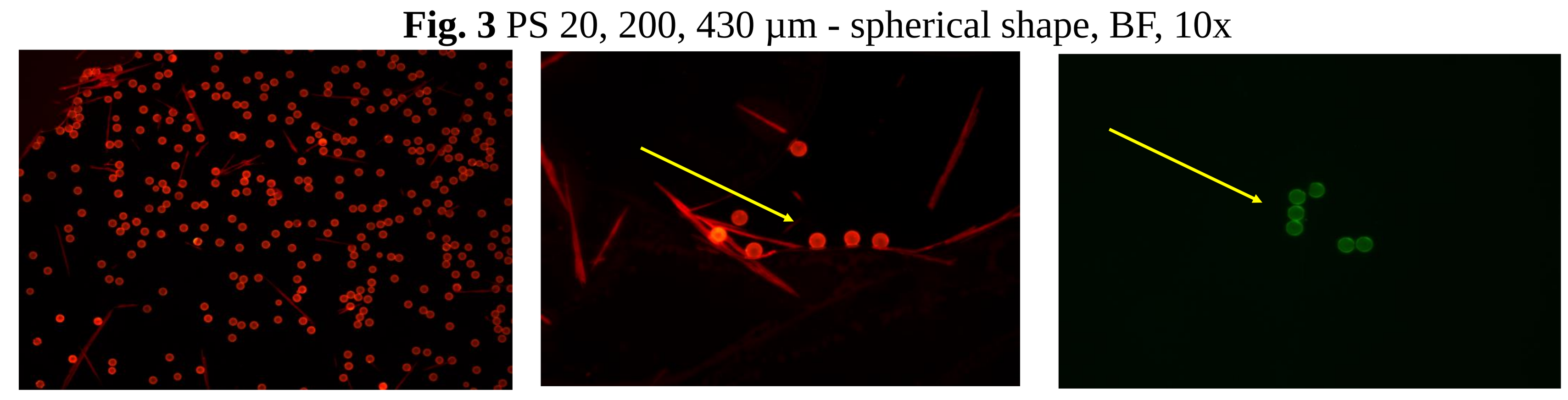

Fig. 4 PS 20 µm-Red Nile stained in testing media, FL, 20x

- Tested microplastics (both PE and PS) did not induce lethal or immobilizing effects on *Daphnia magna* at concentrations ranging from 1 to 50 or 100 mg/L during 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 of freshwater crustacean *Daphnia magna* (Fig.6), in comparison to control organisms (Fig. 5).

CONCLUSIONS

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, explained by the nature of polymer type. PS have a polar surface that influences dye adhesion and fluorescence detection. The effects of Red Nile-stained PE and PS, tested in different particle sizes (PE 40-48, 125, >125 µm, and PS 20, 200, 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. Red Nile staining allowed the microscopic visualization of PS and their entry pathways into the Daphnia digestive tract. 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, were suspected.

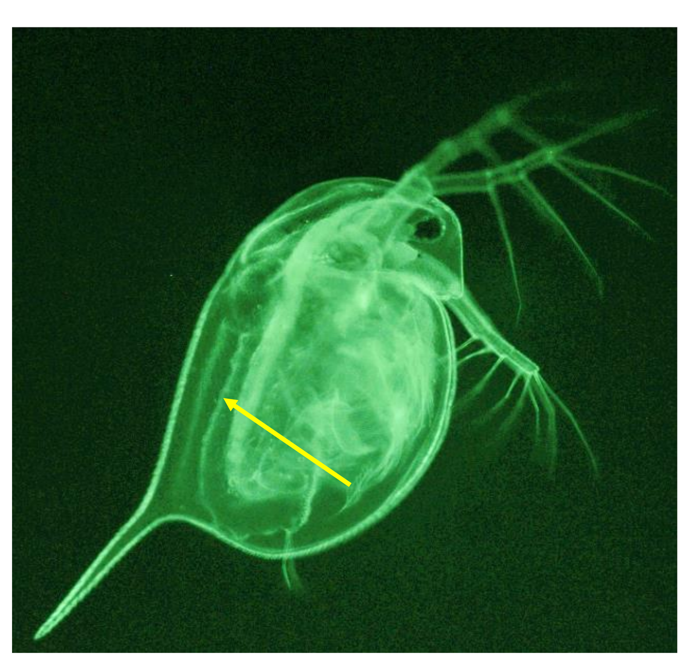

Fig. 5 *Daphnia magna* – control, FL, 20x

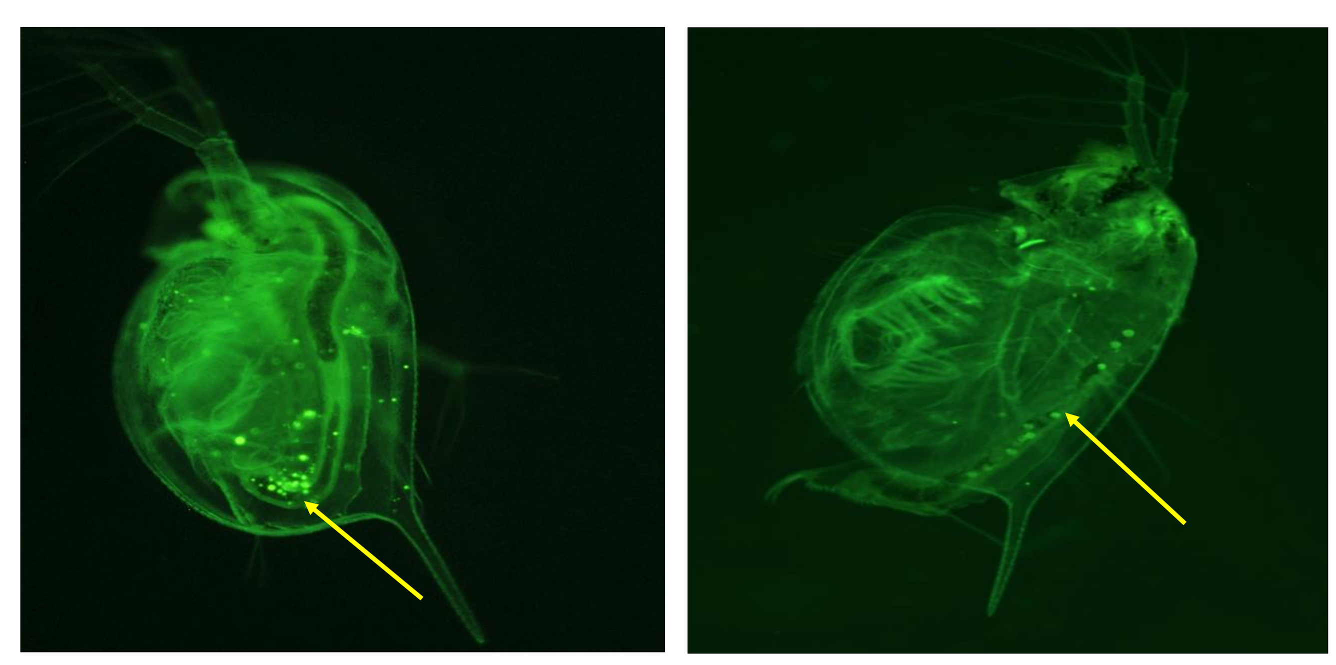

Fig. 6 *Daphnia magna* – presence of PS 20 µm in digestive tract

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MicroPlasFish

Stefania GHEORGHE, PhD
57-73 Drumul Podu Dambovitei, district 6, 060652, Bucharest, Romania
E-mail: stefania.gheorghe@incdecoind.ro
Phone: 40314100377 int. 242
Mobile: 40768593537

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