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

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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), polyacrylonitrile (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⁻¹.

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 DaphnoTest F magna kit (MicroBioTest Inc., Belgium).
- The biological material freshwater crustacean - *Daphnia magna*, 4th age, pre-feeding with Spirulina microalgae;
- Test condition: *Daphnia magna* exposure 48 hours, at 20-25°C, C, in dark, in absence (control) or presence of microplastics. Standard freshwater based on NaHCO₃, CaCl₂, MgSO₄, and KCl, pH 7.4±0.20, temperature 22±0.2°C, dissolved oxygen 6.8±0.54 mgO₂L⁻¹;
- The mortalities or immobilization effects of neontates, 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 40-48, 125 µm, and >125 µm;
  ii) polystyrene (PS), CAS 9003-53-6, spherical shape, colourless, density 1.05 g/ml, sizes of 20, 200, 430 µm, 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 1x10⁶ particles L⁻¹.

Microscopic analyses
- The microscopic analyses were performed using fluorescence inversion microscope Leica DMI6000B (brightfield (BF)) and fluorescence (FL). The images were acquired and processed with microscope software LAS V4.7.

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 digestive 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 tests, were suspected.

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