Preliminary study concerning the use of Nile Red staining for detecting microplastics in marine mussels

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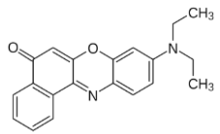
Introduction

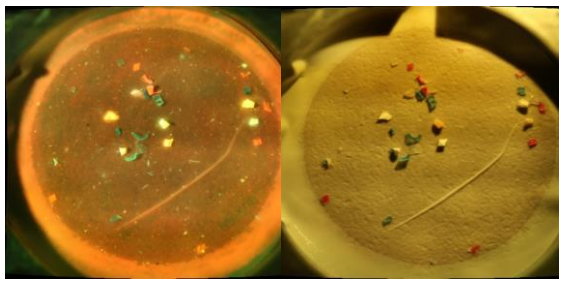
A fast, economic and standardized protocol is necessary for the correct enumeration and qualitative evaluation of microplastic polymers dispersed in the environment. Nile Red (NR) staining has been tested and proposed as an alternative or complementary method for quicker, more economical and routine analysis of microplastics (MPs) in biological samples [1]. NR is a lipophilic dye that exploits the hydrophobic properties of plastic which, once stained, may emit fluorescence if excited with certain wavelengths. A recognized problem of using NR staining for environmental samples is the presence of organic matter that would also be stained and therefore fluoresce, possibly leading to overestimation of MPs present [2]. The NR method has been used previously for sediments and water samples, together with an organic matter digestion step and/or density separation with saline solutions [3].

Aim

The aim of the present work is to evaluate the limits of using the NR staining method for the identification of MPs in marine mussels intended for human consumption.

Nile Red

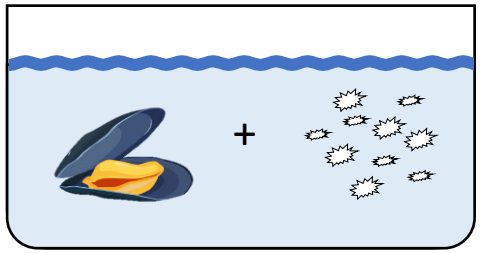




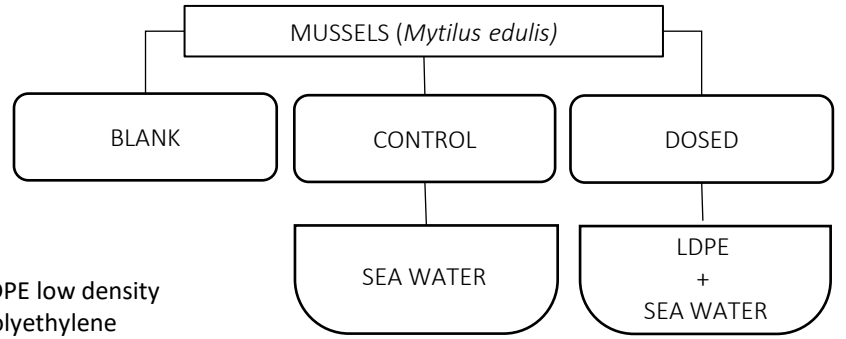
Photographs of a filter stained with NR containing MPs of different colors and polymers.

Methodology

Exposure experiment

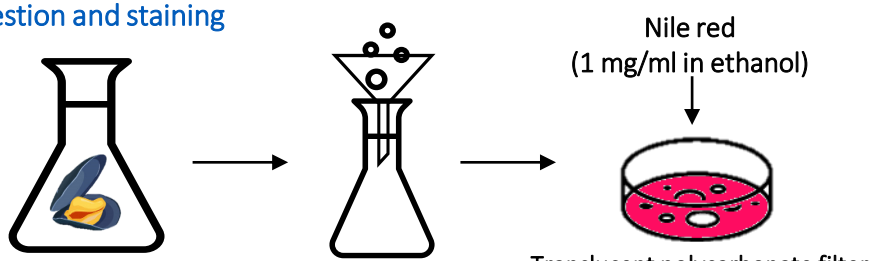


Mussels were exposed to LDPE particles < 250 µm (2 g) and between 500 and 710 µm (2 g) in a tank with 60 L of clean and filtered seawater.



LDPE low density polyethylene

Digestion and staining

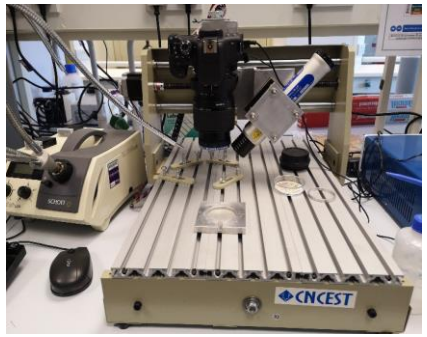


10% KOH [4]

Vacuum filtration

Translucent polycarbonate filter (10 µm pore-size)

Identification



A Canon EOS-760D camera, equipped with a macro-lens and an orange filter, was mounted on a milling machine which allowed automated movement in XYZ-axes. 30 photographs of each whole filter area were recorded under a blue-LED-light (420–470nm) and a white-light source. Autostitch software was used to generate single magnified blue- and white- light images of filters. The potential MPs were detected by evaluating their fluorescence, shape and appearance. Then, particles were analyzed under a dissection microscope and their polymer type confirmed by microscopy Fourier Transform Infrared spectroscopy (microFT-IR).

Validation

Recovery and particle size detection limits were evaluated by spiking known amounts of different size particles (from 63µm to 90µm; from 91µm to 125µm; from 126µm to 180µm; from 181µm to 355µm and from 356 to 510µm) onto blank mussels. Nine individual mussels were spiked for each size of LDPE particle. The potential MPs were detected by evaluating their fluorescence, shape and appearance. Then, particles were analyzed under a dissection microscope and their polymer type confirmed by microFT-IR.

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Results

Exposure experiment

Observing the blue-light images of the dosed samples, undigested organic residues of different shapes, colors and fluorescence interfered at various levels with the identification of the tested LDPE particles. Many aggregate fragments characterized by an intense yellow/green fluorescence were evident in the blue-filter images (Figure 1). Visual sorting of the corresponding filters under a dissection microscope showed potential LDPE particles mixed with organic residues. It was not possible to perform microFTIR on all the observed particles; therefore, 20 representative particles were selected for FTIR analysis which identified them as LDPE (Figure 2). Empirically, shape and size influenced the ability to recognize and distinguish fluorescent particles by the blue-filter images. Organic residues were characterized by soft and pale profile against a definite and solid appearance of the plastic particles. Furthermore, as size decreased, the plastic particles were less recognizable.

Validation

LDPE-particles of 180–355 µm were the smallest size tested where it was possible to distinguish plastic particles from organic residues in the spiked mussels (Figure 3). From these 9 mussels (each spiked with 20 LDPE particles), a total of 169 potential LDPE-particles were selected for microFTIR. MicroFTIR confirmed 165 of the 169 particles were LDPE-particles, with recovery rates of between 80 to 100 % per mussel.

Figure 2: IR spectrum of one of the suspected LDPE particles.

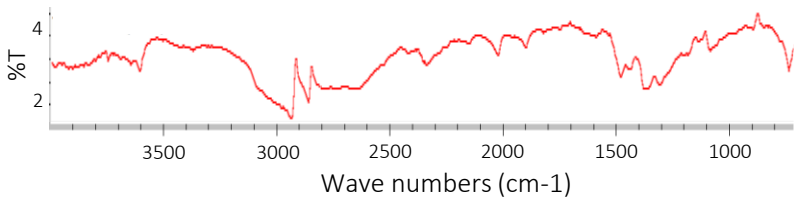


Figure 1: Blue (A) and white (B) light images of a mussel experimentally exposed to translucent LDPE microplastics

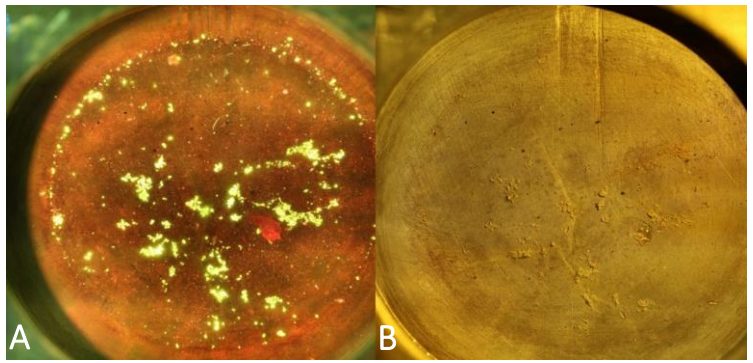
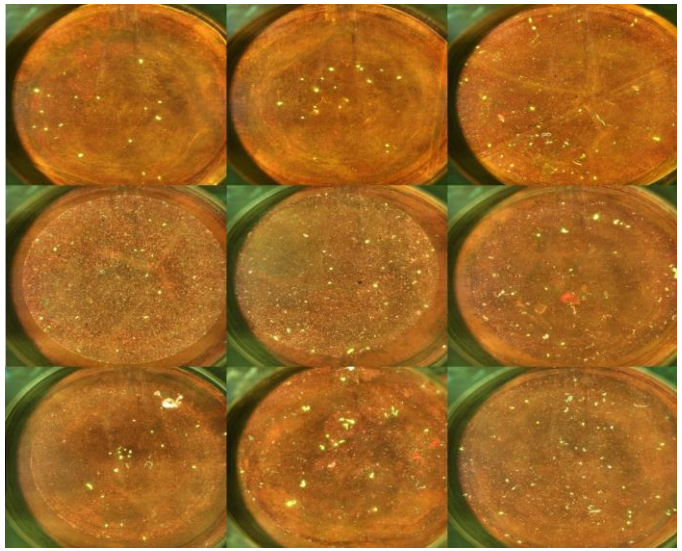


Figure 3: Blue light images of nine blank mussels spiked with twenty LDPE particles in the size range of 180-355 µm.



Discussion

The main limitation of the NR staining application for MPs research on biota is the persistence of organic residues. LDPE particles were easily detectable with the use of NR staining on its own but only if the digestion resulted in a fine and uniform degradation of the mussel tissue. In this regard, the achieved results showed a high variability. Even if the 10% KOH digestion allowed easy filtration of the digested samples through a 10 µm pore-size filter, the organic residues sometimes interfered with the identification of the MPs when using the NR-method only. However, using the implemented method in conjunction with studying the filter with a microscope gave increased efficiency. The NR staining processed samples, when observed in blue-light images, seems to be dependent on the plastic fragment size. A good recovery rate was obtained for LDPE particles > 180 µm, when the physical characteristics of the fluorescent fragments were more obvious, i.e. it was obvious from the size and shape that they were plastic particles. The fluorescence of the LDPE-particles was similar to some organic residues but dissimilar to other organic residues which had a different fluorescent color. Some organic residues did not fluoresce. Other digestive protocols, testing different polymers characterized by different colors, and the use of saline solutions for density separation of plastics fragments from organic residues should be investigated. Combining the NR method with a visual sorting of the filter may have some positive implication on the selection of particles to be subjected to the next step of chemical identification.

References

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