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Identification and quantification of microplastics using Nile Red staining

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ABSTRACT

We investigated the applicability of Nile Red (NR), a fluorescent dye, for microplastic analysis, and determined the optimal staining conditions. Five mg/L NR solution in *n*-hexane effectively stained plastics, and they were easily recognized in green fluorescence. The NR staining method was successfully applied to micro-sized polyethylene, polypropylene, polystyrene, polycarbonate, polyurethane, and poly(ethylene-vinyl acetate), except for polyvinylchloride, polyamide and polyester. The recovery rate of polyethylene (100–300 μm) spiked to pretreated natural sand was 98% in the NR stating method, which was not significantly ($p < 0.05$) different with FT-IR identification. The NR staining method was suitable for discriminating fragmented polypropylene particles from large numbers of sand particles in laboratory weathering test samples. The method is straightforward and quick for identifying and quantifying polymer particles in the laboratory controlled samples. Further studies, however, are necessary to investigate the application of NR staining to field samples with organic remnants.

1. Introduction

Microplastics have been recognized as important sources of marine contamination and in recent years there has been increasing concern about their levels in the environment (Shim and Thompson, 2015). They are widely distributed, from lakes to the open ocean (Imhof et al., 2013; Eriksen et al., 2014), in surface water and deep sea sediments (Song et al., 2015b; Woodall et al., 2014), and in various organisms through the trophic levels (Boerger et al., 2010; Murray and Cowie, 2011; Van Franeker et al., 2011; De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014). Levels of microplastics have been reported to have significantly increased over recent decades (Thompson et al., 2004; Claessens et al., 2011). The density of microplastics generally increases with decreasing their size in beaches and surface waters (Martins and Sobral, 2011; Lee et al., 2013; Cozar et al., 2014). However, the isolation and subsequent identification of smaller microplastics become more difficult.

Microplastics are usually visually identified with a microscope. However, the method has low reliability, especially for small, transparent and (or) fiber-type particles (Song et al., 2015a; Lenz et al., 2015). A Fourier Transform infrared (FT-IR) or Raman spectroscope equipped with a microscope has generally been used for chemical identification of micro-scale polymers, including qualitative confirmation of polymer types (Song et al., 2014; Van Cauwenberghe et al., 2013). It is theoretically possible to detect microplastics tens of microns in size using micro-FT-IR and Raman spectroscopes (Lenz et al., 2015), but repeated trials are often required to obtain reliable spectra of very small and weathered plastic particles. Both the FT-IR and Raman spectroscopy methods require expensive instruments and are time-consuming when a large number of microplastic particles are present on the filter paper. This is the case not only for environmental samples with complex matrices but also for quantification of controlled laboratory experimental samples. Therefore, it is becoming increasingly important to develop alternative methods to facilitate identification of microplastics for field monitoring and laboratory studies on the toxicity, accumulation, weathering, etc. of microplastics.

Plastics are comprised of hydrocarbons derived from petroleum, natural gas or biomass, and are hydrophobic. Lipophilic dyes can be used to visualize microplastics under a fluorescence microscope (Andrady, 2011). Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one; hereafter, NR), a fluorescent dye, has been used to stain neutral lipids in biological samples (Greenspan and Fowler, 1985) and can be used to stain synthetic polymers in polymer chemistry (Jee et al., 2009). NR is a hydrophobic fluorophore that specifically binds to neutral lipids and is strongly fluorescent only in the presence of a hydrophobic environment (Greenspan et al., 1985). We therefore decided to investigate the applicability of NR staining to the identification of microplastics among other interfering particles, as an alternative method to the conventional microscopic and spectroscopic identification of synthetic polymers.

We examined NR staining in 11 synthetic polymers. The NR carrying solvents, optimum NR concentration, and excitation and emission wavelengths for stained synthetic polymers were determined. In addition, the NR staining method was compared with the FT-IR spectroscopy

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method for the qualitative and/or quantitative identification of microplastics in the spiked and weathering test samples in the laboratory and samples collected in the field.

2. Materials and Methods

2.1. Solvent test

A solvent that does not degrade the black polycarbonate (PC) filter paper used for subsequent microscopic and FT-IR identification should be selected to dissolve and carry NR for the staining of microplastics. NR is highly soluble and strongly fluorescent in a wide range of organic solvents (Sitepu et al., 2012). A total of eight solvents – methanol, ethanol, ethyl acetate, acetonitrile, dichloromethane, toluene, *n*-Hexane, and cyclohexane, — were tested. A few drops of each solvent were added to black PC filter papers; the color change and deformation of shape of the filter papers were observed.

2.2. Excitation and emission wavelengths

For convenience in the subsequent analyses, three major common light filters generally used in a fluorescent microscope to identify stained microorganisms were tested. Each NR-stained synthetic polymer was tested with three excitation and emission wavelengths: blue (excitation wavelength (ex.): 365 nm; emission wavelength (em.): 445 nm), green-yellow (ex.: 450–490; em: 515–565 nm), and orange-red (ex.: 534–558; em.: > 590 nm) under a fluorescent microscope (Axio Scope A1, Zeiss, Jena, Germany).

2.3. Staining of different polymer types

NR (Sigma N-3013) was purchased as a dark purplish-red powder from Sigma-Aldrich (Seoul, Korea). Four micro-sized synthetic polymer particles – low-density polyethylene (LDPE; size range < 200 μm), high-density polyethylene (HDPE; 80–200 μm), polyurethane (PU; < 100 μm), and poly(ethylene-vinyl acetate) (PEVA; 100–400 μm) – were purchased from Abifor, Ltd. (Zürich, Switzerland). Pre-production resin pellets of polypropylene (PP), polyvinylchloride (PVC), and PC purchased from a local market in Busan, Republic of Korea were ground using sand paper to make micro-sized particles. Expanded polystyrene (EPS) and polyethylene-*tere*-phthalate (PET) were ground into powdered samples from spherules taken from EPS floats and PET bottles, respectively. Polyester (PES) and polyamide (PA) fiber samples were obtained by cutting a cloth and a stocking, respectively. The color of LDPE, HDPE, PP, PU, and PEVA were translucent, and PC, PES and PA were transparent, while EPS was white and PVC was black. Each polymer type was confirmed with a Fourier transform infrared (FT-IR) spectroscope equipped with a microscope (Thermo Nocolet 6700 and Continuμm, Thermo Scientific, Waltham, USA) in attenuated total reflection (ATR) mode (Song et al., 2014). Among the polymers tested, the NR staining conditions were examined first with the three common plastic debris types, LDPE, PP and EPS, and the method was subsequently applied to the other polymer types. NR staining of all the polymers did not affect their identification with FT-IR spectroscopy.

Powder from each polymer was placed on separate black PC filter papers and stained with 200 μL NR working solution. The particles and filter paper surface were washed with 100 μL *n*-hexane, and the filter papers were air-dried for about 5 min and examined under a fluorescent microscope. None of visible damage was detected in the plastics with *n*-hexane.

2.4. Recovery test

The NR staining method was tested with LDPE-spiked sand samples. Natural sand was collected from Heungnam Beach in Geoje, Korea and sieved to retain sand particles 63–1000 μm in size. Light particles including plastics mixed in the sand were removed by density separation with saturated NaCl solution, repeating the density separation until no more particles were visible. The cleaned sand was precombusted overnight at 450 °C to remove the remaining plastics and organic matter. Irregularly-shaped LDPE particles 0–200 μm in length were sieved to select particle sizes between 100 and 300 μm. Then, about 1 mg of sieved LDPE was spiked to 50 g of the pretreated sand in a 500-mL separation funnel and mixed by shaking for several minutes. Approximately 200 mL distilled water was added to the separation funnel, and the funnel was vigorously shaken by hand for 1 min and left to stand for 6 h to allow the sand to settle. The supernatant in the funnel was transferred to a pre-cleaned beaker, and the same separation procedure was repeated twice. The collected supernatant was filtered through black PC membrane filter paper (47-mm Ø, 1.2-μm pore size). The dried LDPE particles on the filter paper were stained using the above-described method. The stained LDPE particles were counted under a fluorescence microscope and collected to measure their total weight to calculate the LDPE recovery rate. The same samples were analyzed using a FT-IR spectroscope to compare the LDPE counts obtained from the two methods.

2.5. Staining of weathering test samples

PP pellets that had been exposed to ultraviolet light under a metal halide lamp in a chamber for 6 months were mixed with the pretreated natural sand in a 250-mL amber bottle using three replicates and tumbled in a roller mixer at 35 rpm for 2 months to determine the fragmentation of the PP to micro-sized particles. After 2 month of mechanical abrasion, the sand containing the PP particles was mixed thoroughly with 330 mL saturated NaCl solution, vigorously shaken by hand for 1 min, and then left to stand for 10 min. This process was repeated three times, and the supernatant was filtered using a black PC membrane filter (1.2 μm; 47-mm Ø). The filter was dried in a glass Petri dish at room temperature and stained with NR solution. Then, the occurrence and characteristics of staining of the PP particles with NR were determined.

2.6. Staining of field samples

Sand samples were collected from Heungnam Beach from a 0.5 × 0.5 m quadrat of 5-cm depth, using a stainless scoop, through a 1-mm sieve (Tyler sieve; Cisa, Barcelona, Spain). The sieved sand samples were density-separated with saturated NaCl solution using the above-described method, and the supernatants were collected in a 1-L beaker and left to stand until the overlying water appeared clear. The supernatant was filtered using a black PC membrane filter (1.2 μm; 47-mm Ø). The filter paper was soaked with hydrogen peroxide (H_2O_2) solution (35%) for 1 d to remove natural organic matter. Then, the filter was dried in a glass Petri dish at room temperature and stained with NR solution. The occurrence and characteristics of staining of the plastic particles with NR were qualitatively determined.

Floating microplastics were sampled at three stations along the southern coast of Korea using a Manta trawl (0.4 × 0.195-m rectangular opening, 3 m long; 330-μm mesh net) (Kang et al., 2015). After recovering the net, it was carefully washed from the outside with pre-filtered seawater into a cod-end bucket. The contents of the bucket were transferred to a 1-L sample bottle. In the laboratory, the 1-L sample was poured in a 0.3-mm stainless steel sieve and rinsed with distilled water to remove the salt. The sieved samples from each sieving were transferred into beakers using a spatula and minimally rinsed with distilled water. All samples were dried in a drying oven at 60 °C for 24 h. To remove the organic matter, the wet peroxide oxidation method was followed, using aqueous 0.05 M Fe(II) solution and 35% H_2O_2 (Masura et al., 2015). After oxidation of the natural organic material, the solution was transferred in a glass funnel to the density separation, adding NaCl and settled overnight. The supernatant was filtered using a PC