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Fluorescence photobleaching of microplastics: A cautionary tale

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ABSTRACT

Fluorescence microscopy is an important step in visual identification of microplastics and is used to highlight white and transparent plastics that are otherwise easily missed or misidentified. Investigators using fluorescence must proceed with caution, however, as fluorescence photobleaching can significantly reduce the fluorescence output of samples within experimentally relevant time frames. We report on the photobleaching rate and subsequent lack of fluorescence recovery of five common plastics. Our results reveal statistically different photobleaching rates across plastic types. In the best-case scenario of low illumination intensity and a robust plastic, initial fluorescence intensity decayed by 10% in just 11(3) s and by 33% in 230(40) s. In all cases, fluorescence failed to recover more than 13(8)% in 3 h. These results indicate that significant bleaching can occur while searching a sample for plastics to identify and that the lack of recovery can compromise samples for further study.

1. Introduction

Microplastics – plastics smaller than 5 mm in size (Barnes et al., 2009) – are a recognized environmental hazard and their presence in our waterways is of growing concern. In order to quantify the effects of microplastics, researchers must first determine their prevalence in the environment. Logically, then, the separation and identification of microplastics in bulk samples are of utmost importance. The most popular method for separation and identification is a combination of density filtration (Thompson et al., 2004; Imhof et al., 2012) and visual identification (Hidalgo-Ruz et al., 2012). Often, researchers then use FT-IR (Thompson et al., 2004; Vianello et al., 2013; Frias et al., 2014) or Raman spectroscopy (Murray and Cowie, 2011; Cole et al., 2013; Lenz et al., 2015) to specifically determine polymer type, though many studies are conducted without spectroscopic methods due to time and cost (Qiu et al., 2016; Song et al., 2015). Visual identification is fast and effective for large plastics, but requires greater time and expertise as plastic size diminishes. Even with the aid of a stereomicroscope, visual identification is difficult for plastics smaller than 1 mm (Song et al., 2015; Hidalgo-Ruz et al., 2012). As well, white and transparent plastics, which can be the majority colors in bulk samples (Di and Wang, 2018; Martin et al., 2017; Reisser et al., 2013), are particularly difficult to distinguish (Noren, 2008).

These difficulties have led researchers to search for ways to enhance visual identification. One novel solution is to selectively stain microplastics with Nile Red, a dye that adsorbs to the surface of plastics and fluoresces under blue light (Maes et al., 2017). This technique allows for detection of plastics down to single microns in size via image capture and analysis. This method is effective, but like the spectroscopic methods, adds both time and cost to the identification process. Rather than add a fluorescent dye, researchers can identify microplastics based on the plastic's innate ability to fluoresce. Guidelines for the visual identification of microplastics were established by Noren (2008). Noren et al. suggest that white and transparent plastics be examined using fluorescence microscopy to verify that they are non-organic. This step reduces mis- or non-detection of microplastics and can lower the size detection threshold when coupled with imaging and image analysis.

An important, and previously unexplored, caveat to using fluorescence microscopy to study microplastics is the susceptibility of microplastics to photobleaching. Photobleaching is a photochemical process that breaks down fluorescent molecules and results in a reduction in the fluorescence output of an object under prolonged exposure to illumination at the excitation wavelength (Lichtman and Conchello, 2005). This loss in fluorescence can cause the edges of a sample, or even an entire sample, to reduce in intensity below the detectable limit of the imaging device and is rarely recoverable. In this study, we investigated the photobleaching rate for five of the six major types of plastics designated as recyclable by the Society of the Plastics Industry. Our results show that significant photobleaching can occur over experimentally-relevant time frames but can be mitigated by minimizing both the illumination time and intensity.

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2. Materials and methods

Representative objects from five of the seven plastic recycling categories were chosen for this study. Only transparent and white plastics were selected due to their prevalence and because they are particularly difficult to detect by visual inspection. The objects included a water bottle (polyethylene terphthalate, category 1, transparent), milk jug (high density polyethylene, category 2, transparent), PVC pipe (polyvinyl chloride, category 3, white), Glad brand food storage container (polypropylene, category 5, transparent), and packing peanuts (polystyrene, category 6, white). We omitted category 4, low density polyethylene, because it has the same underlying chemical structure as high density polyethylene and preliminary results showed insignificant variation in photobleaching for the two plastic types. We also omitted category 7, which is a catchall category with no single defined plastic type.

For the hard plastics, "micro"-sized pieces (< 1 mm) were produced by rubbing each object vigorously with a metal file, while the Styrofoam packing peanuts were shaved with a razor blade to produce small pieces. The resulting microplastics were brushed onto glass slides (one plastic type per slide), covered with no. 1.5 cover slips, and sealed with nail polish. Samples were imaged on an inverted microscope (IX51, Olympus) using a 10 × air immersion lens (UPLFLN10X, Olympus) and an EMCCD camera (iXon Ultra 987, Andor).

Plastics chosen for imaging were approximately 50 to 150 μm in diameter. Plastics of this size were large enough to easily discern in the field of view and yet small enough to also meet the important constraint that the majority of the plastic was in focus in the z-plane. Smaller plastics, down to 5 μm in diameter, could be detected but were not analyzed because these plastics encompassed fewer pixels and were thus more difficult to process. Samples were photobleached by exposure to 405 nm light produced by a solid state laser (DL-405-015, Crystalaser) at either 5.7(4) mW/m^2 or 61(4) mW/m^2 for 30 min while being imaged in a kinetic series at a frequency of 1/10 Hz. The excitation beam was then shuttered and opened again briefly (< 2 s per frame) to take single frames at 2 min, 5 min, 10 min, 20 min, 40 min, 90 min and 180 min post-bleach. Three bleach-recovery trials were completed for each plastic and laser intensity combination according to this process. Two to four additional bleach trials were conducted for each plastic and laser intensity combination, with the samples exposed to the excitation beam for 10 min while a kinetic series of images was taken at a frequency of 1 Hz. These trials provided more detailed information about the early bleaching behavior and reduced the uncertainty of our results.

Images were processed using NIH ImageJ (Waband, 1997-2016) to determine the average pixel intensity in a region of interest (ROI) within the borders of each fluorescing microplastic. The ROI for each microplastic was user-defined to encompass the majority of the bright interior of the plastic. The average intensity of each image in the kinetic series was then normalized to the first frame and the result plotted as a function of time. Finally, the normalized intensity data for each plastic was fitted to a double exponential decay and the times at the intersections of this curve with normalized average intensity values of 90%, 75%, 67% and 50% were determined. (See supplemental materials for details.) The uncertainty in final bleaching times arising from the choice of ROI was found to be negligible ($< 1\%$) compared to the overall uncertainty arising from variation among samples within the same recycle category.

3. Results

The goal of this paper is to provide investigators with constraints within which to work to prevent significant fluorescence photobleaching of plastics such that samples remain viable for study. Two significant contributors to photobleaching are the intensity of the illumination light at the sample and the duration of a sample's exposure to illumination at the fluorescence excitation wavelength. To demonstrate the effect of illumination intensity on photobleaching, bleach-recovery curves were taken for all plastics at both a "high" – 61(4) mW/mm^2– and "low" – 5.7(4) mW/mm^2– intensity setting. Both settings produced fluorescence from all plastic types that was comfortable to the eye (at the start of the experiment) when the samples were viewed through the microscope eyepiece. All plastics photobleached rapidly at both illumination intensities and recovered their fluorescence slowly and incompletely.

"Micro"-sized plastics produced from representative objects from five of the plastic recycling categories were exposed to 405 nm laser light to induce fluorescence photobleaching over a prolonged period. The extent of photobleaching and subsequent fluorescence recovery were visualized by taking a kinetic series of images during the laser exposure, followed by a series of six single frames taken over the first three hours after the excitation beam was shuttered. The average intensity of a region of interest within the boundary of a fluorescing plastic was then calculated for each image, normalized to the first image, and plotted as a function of time (in minutes).

Representative bleach-recovery curves for PETE (category 1) at both illumination intensities can be seen in Fig. 1. The shape of the photobleaching curves indicates a dominant fast decay at early times and a subsequent slow decay toward a final, minimum normalized intensity. This behavior was verified by fitting all curves to a double exponential decay (see supplementary materials). For all trials, at both high and low intensity, the fluorescence intensity failed to recover more than 13(8)% of the lost normalized intensity over the first three hours post-bleach. There were no significant differences evident among recovery percentages at the 3- h time point post-bleach for different plastic types or between plastics bleached at high versus low intensity. These results are confirmed by both overlap of standard error between groups and by high p-values ($\gg 0.05$) using analysis of variance (for plastic type) and t-test (for intensity level). Data taken at 24 h post-bleach indicate little potential for full recovery of fluorescence emission.

To quantify the speed of photobleaching, we used the kinetic series of images taken of each sample to calculate the time at which the normalized average pixel intensity of an ROI within a fluorescing plastic had decayed to 90%, 75%, 67% and 50% of its initial normalized intensity. Our results at high illumination intensity are shown in Fig. 2. Photobleaching occurs rapidly in the first minute of exposure to the excitation beam. Samples of all plastic types were reduced to 90% of

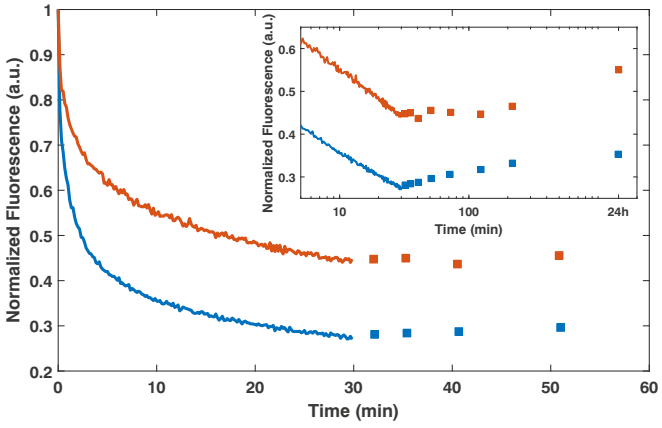

Fig. 1. Representative fluorescence intensity decay curves (solid lines) as a function of time (in minutes) and recovery points (square markers) for PETE (category 1) taken at high (deeper blue curve) and low (shallower orange curve) illumination intensity. The curves exhibit a bi-exponential decay, with a short-lived fast decay and a longer, slow decay. The recovery is highlighted in the inset, which has a logarithmic time axis. The plastics recover 8.5% (high) and 3.2% (low) of the lost fluorescence intensity over the first 3 h, and an additional 2.9% (high) and 15% (low) by the 24 h point post-bleach.