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Inexpensive adaptations of basic microscopes for the identification of microplastic contamination using polarization and Nile Red fluorescence detection.

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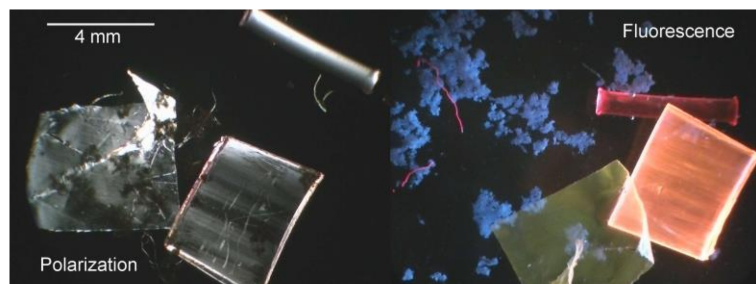
ABSTRACT

Microplastic contamination of the environment is a major concern but detecting such contamination presents a challenge, particularly for particles <0.1 mm in size. Community scientists and students who participated in a plankton monitoring program and routinely examined samples of seawater using bright-field microscopy, often found fragments and filaments that were likely of anthropogenic origin. While in some cases this was clear from their structure and color, the origin of many particles remained questionable. To address this problem, optical components were added to microscopes for polarization and fluorescence detection. Plastics often show birefringence under crossed-polarizers due to alignment of the polymer chains within. They also bind Nile Red dye to give green, yellow or red fluorescence emission, depending on the hydrophobicity of the polymer. Fluorescence excitation was achieved using a focused blue LED flashlight mounted externally to a microscope and emission was detected through a yellow plexiglass or gel filter. The required optical components cost a few tens of dollars and can be applied to any stereo (dissecting) microscope and most compound microscopes. These modifications were tested by community college students who set up their own microscopes and reported the presence of microplastics in the majority of their plankton samples. The equipment was

30 also used at outreach events, where the observation of glowing microplastic particles suspended in natural water samples made an immediate impression with the public and demonstrated the magnitude of the environmental problem.

GRAPHICAL ABSTRACT



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KEYWORDS

First-Year Undergraduate, Environmental Chemistry, Multidisciplinary, Outreach, Hands-On Learning, Consumer Chemistry, Dyes, Instrumental Methods, Fluorescence spectroscopy, UV-Vis Spectroscopy.

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INTRODUCTION

The pervasive nature of plastic pollution in the environment is of major concern with both known and unknown consequences[1,2]. Macroplastics may entangle or choke larger marine fauna, while

45 microplastics (fragments < 5 mm) may enter the food chain via plankton and filter feeders and ultimately be taken up by humans[3,4]. While the World Health Organization has yet to identify any direct consequences of the ingestion of microplastics by humans, they conclude more research is needed. As a result there is much effort into assessing the nature and abundance of microplastics in the environment[5,6], as well as instigating legislation and grass-roots projects directed at the reduction

50 of single-use plastics. The plastics industry is also responding by developing materials that are more readily decomposable and exploring the fluorescence bar-coding of plastics to enable automated sorting at recycling centers[7].

Research laboratories generally use spectroscopic techniques such as infra-red and Raman microscopy or pyrolytic mass spectrometry to identify signature chemical groups diagnostic of a

55 variety of plastic polymers[8-10]. However, these techniques are generally unavailable to undergraduate students and community scientists who may rely on visual inspection using bright-field microscopy to identify microplastics[11,12]. While this approach may be satisfactory for millimeter-sized particles, it becomes increasingly challenging to distinguish sub-millimeter particles from those derived from living material, such as fragments of plant cell walls. Polarization[13,14] and fluorescence microscopy[5,15-17] can
60 provide an accessible means to identify smaller microplastic particles. As described in this paper, these techniques can be made widely available through the inexpensive modification of standard bright-field microscopes.

When examined under crossed-polarizers, many plastics appear bright and often display colored patterns due to birefringence. Birefringence, which refers to different refractive indices along the
65 different axes of an object, arises from the alignment of polymer molecules during extrusion or molding in the manufacture of most plastics. While natural materials usually show some optical effect on transmitted polarized light due to the inherent asymmetry of the molecules within, only in highly ordered structures do these molecular effects add up to give bright images under crossed polarizers. For example, false-positive results are given by hair. Nevertheless, polarization provides a quick way of
70 scanning a sample for microplastics which usually standout against other detritus.

Staining with the lipophilic dye, Nile Red (9-(diethylamino)-5H-benzo[α]phenoxazin-5-one) has also attracted attention for identifying microplastics[5,15-17]. Nile Red is used-by cell biologists to stain lipid membranes and fat stores[18], and shows strong solvatochromic effects[19]. This sensitivity to the local polarity is also reflected in the emission wavelength when Nile Red is bound to microplastics, shifting
75 from red to green with increasing hydrophobicity[16]. Not surprisingly, Nile Red can show false positives when used for microplastic detection because it stains lipids within living or degraded cells. Staining is therefore normally combined with a digestion step to remove natural organic matter[15] or floatation[16].

Methods to construct microscopes capable of fluorescence detection have been noted in this journal[20,21], based on the epifluorescence optics employed in research-grade microscopes. In these
80 microscopes, a dichroic mirror separates the excitation and emission light which is transmitted through a common objective lens. However, the cost of a dichroic mirror can exceed that of some bright-field microscopes and may be beyond the budget of a class investigation. Here we use an

inexpensive blue LED flashlight mounted externally to the microscope as an excitation source, with an optical filter to block the scattered excitation light. We compare several arrangements suited to different microscopes.

This project was inspired by a monitoring program involving volunteers from the community, schools, and colleges who collect plankton samples from sites along the central coast of California. Since June 2018, over 500 samples have been collected. Fibers and microplastics were often noted as present in samples but the identification was not done with any degree of certainty. It became clear that methods were required to accommodate community scientists who did not necessarily have laboratory facilities for analysis.

MATERIALS AND METHODS

As the protocol was initially developed for NOAA's Monterey Bay National Marine Sanctuary Community Plankton Science Monitoring Program, the requirements for materials and instrumentation were restricted to items that could be obtained at local stores or via the internet (Supporting Information I). The Nile Red dye was the only item that required purchase through an institution (MP Biochemicals via VWR Ltd). A stock solution of 1 mg/mL in methanol was prepared. Alternatively, Nile Red was dissolved in 91% isopropyl alcohol (rubbing alcohol) available from a local pharmacy. Plankton samples were collected from the Santa Cruz, CA wharf using five 3 m vertical tows with a 25 cm diameter mouth, 20 μm mesh plankton net. This procedure potentially concentrates the particles about 2,000 fold in the 400 mL cod end (Supporting Information II). For examination under a stereo (dissecting) microscope, about 6 mL of the plankton sample from the cod end was placed in a 55 mm diameter *glass* Petri dish and 1 drop (≈ 20 μL) of the stock Nile Red was added. A plastic fragment (e.g. clear polythene bag) or fiber (e.g. cigarette filter) of the order of a millimeter in size and of recognizable shape was often added at this point as a control for the staining procedure and microscope alignment. The stained sample was incubated for 1 hour, during which time the plankton species were identified using bright-field microscopy as part of the standard monitoring procedure. After this time, the sample was examined using polarization and fluorescence optics in a search for microplastics. Although Nile Red also stains some natural material, especially zooplankton,

a digestion step with H_2O_2 [15] or Fenton's reagent[11] was not included because this would require access to a fume hood (see Results and Discussion). A detailed protocol is provided as Supporting Information I and is summarized in a video[22].

Microscopy

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Several bright-field microscope brands and types were tested for their suitability for adaptation for polarization and fluorescence detection. We consider that any stereo and most compound microscopes operating up to 100x magnification can be adapted. Figure 1 shows the arrangement of components for a stereo microscope adaptation that allows rapid switching between bright-field, polarization and

120 fluorescence modes. Variations of this arrangement are outlined below.

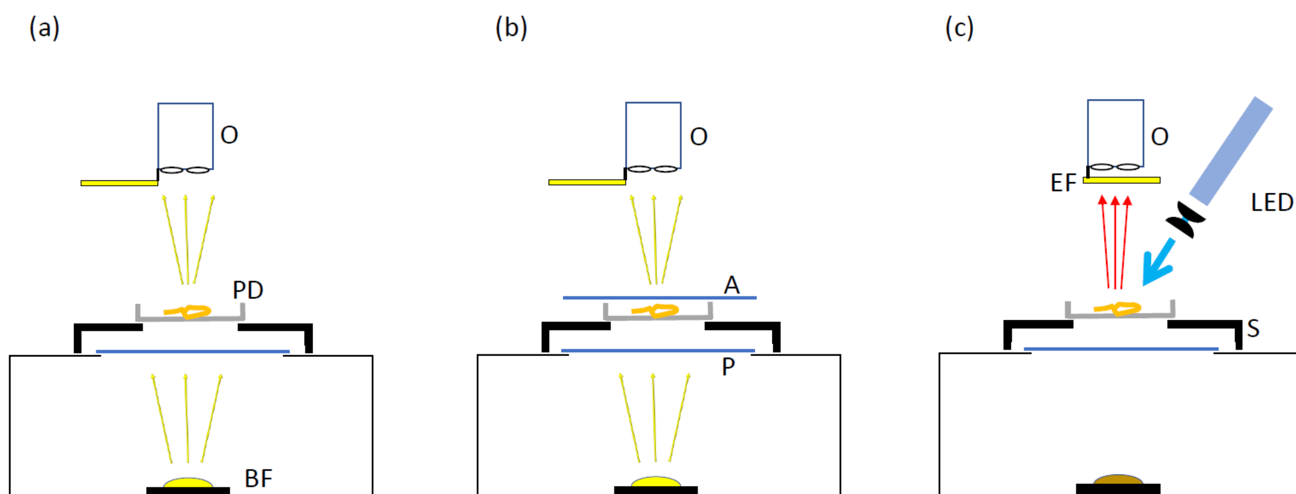

Figure 1. Schematic of a stereo microscope configuration for (a) bright-field illumination, (b) polarization and (c) fluorescence detection. O = microscope stereo objective lenses, PD = Petri dish with sample, S = spacer, BF = Built-in bright-field illuminator, P = polarizer, A = Analyzer (second crossed-polarizer), EF = emission filter on magnetic hinge, LED = blue excitation flashlight. Note the bottom polarizer, P and spacer,

125 S can be left in place for all three modes. Switching between modes is achieved by selecting the light source and adding the analyzer, A or swinging the emission filter, EF in front of the objective lenses.

Bright-field microscopy was used to initially scan the sample for plankton and potential anthropogenic material (Figure 1a). For this purpose, it is advantageous to minimize the condenser

130 aperture to maximize contrast and reveal potential internal components. Typically, samples were first viewed at low magnification (10x to 30x) to explore a large field of view, and then suspect microplastics were examined at higher magnification to check for the lack of internal structure. Strongly-colored plastic fragments and fibers suppressed Nile Red fluorescence emission[23] and reduced the brightness

under crossed-polarizers, therefore bright-field observation remained an important part of the

135 protocol.

Polarization microscopy

Several polarization options were explored: An 8 x 8 cm polarizing film (Artec Co., Ltd.) was placed under the *glass* Petri dish on the microscope stage and a second film (the analyzer) rested on top of

140 the dish (Figure 1b). This arrangement allowed for rapid interchange between the different illumination modes. Alternatively, linear polarizing filters or circular polarizers designed for photography were used which give better quality images, are less prone to scratching and come in a rotatable threaded mount. Note that most camera filters sold today are circular polarizers, because linear polarizers affect the light metering system of digital cameras. Circular polarizers can be used provided they are in the

145 correct orientation relative to the sample (the quarter wavelength surfaces of both polarizers should point away from sample). In one configuration tested, a polarizer was placed on the stage of a stereo microscope, and the glass Petri dish placed on top of a 9.5 cm diameter spacer made from a gelato jar lid (Talenti) with a 2 cm hole in its center. A 37 mm threaded circular polarizer (Vivitar) was screwed into a 43-37 mm stepping ring adapter which was glued to the objective housing. This option allows

150 exchange with 37 mm diameter fluorescence filters (cf. Figure 2b). In all cases, the analyzer was rotated to the crossed position to minimize the background transmitted light.

The same polarizers may be used with a compound microscope and are straight forward to mount for models which contain a removable ocular head. In such instruments there is usually room to rest one polarizer on the light source with the second inserted into the body of the microscope. If this

155 option is unavailable, then a small circle can be cut from a polarizing film and temporarily inserted between the objective lens and the turret or within the eyepiece. Note that some microscopes contain a glass diffuser plate in the condenser, which depolarizes light by multiple scattering and this plate needs to be removed. These polarizer adaptations have been demonstrated in a video[24].

Fluorescence microscopy

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A blue (450 nm) LED flashlight (Wayllshine) was used as an excitation source. Important characteristics include a single LED for efficient optical coupling to the sample (Supporting information II), an output of >100 lumens and a focusable beam. At closest focus, the flashlight illuminated an area of 4 cm square which is larger than the field-of-view of a stereo microscope

165 operating at ≥30x. For more efficient coupling, another lens taken from an identical LED flashlight was fixed to the existing lens using electrical tape which allowed the LED chip to be focused to a 2 mm square. The illumination area was adjusted to match the sample field-of-view by varying the distance of the flashlight from the sample (typically about 5 cm). The flashlight was held in position at an angle of 40° to 60° using a clamp attached to a table-top tripod (Figures 1c, 2a). The sample was placed on a

170 spacer (9.5 cm diameter gelato jar lid with a 2 cm drilled hole) to reduce the reflection from the ground-glass plate of the microscope stage but still allow the sample to be illuminated from beneath for bright-field and polarization illumination.

The same blue LED flashlight was used with a compound microscope for objectives up to 10x which have sufficient working distance to allow the excitation beam to illuminate the sample. More

175 efficient optical coupling was obtained using a 5 mW 445 nm laser slide pointer pen (Laserland store). However, a laser pointer introduces an additional potential hazard and may not be suitable in some settings. Further details are provided in Supporting Information III.

Scattered 450 nm excitation light was blocked with a yellow or orange filter mounted in front of the objective lenses of a stereo microscope. Several inexpensive filter arrangements were tested, the choice

180 being dependent on the size and shape of microscope housing (Supporting Information III). Threaded optical filters designed for photography blocked the excitation light better than gel film (Supporting Information Figure SIII-2). The former were mounted using a filter stepping ring glued to the objective turret (Figure 2 b). Gel filters were cut to size and mounted on a small magnet, allowing quicker interchange of the filter position (Figure 2 c-d). In the case of compound microscopes, the emission

185 filter was located within the body of the microscope, requiring the removal of the ocular head (Figure SIII-1c). Videos showing the assembly of the microscope for fluorescence detection are available[25,26]. Samples were photographed using a smart phone held above the eyepiece or a JVC camcorder mounted via an Eagle Optics digiscope eyepiece.

(a)

(b)

(c)

(d)

Figure 2. (a) Use of a blue (450 nm) flashlight to excite fluorescence in a sample viewed under a stereo microscope. In this set-up the flashlight was powered from the rechargeable batteries (2 x 1.5 V) within the microscope. Alternatively, a single 3.2 V rechargeable 14500 battery can be used in the flashlight itself. (b) 37 mm yellow emission filter (EF) mounted beneath the objective lenses via a stepping ring (R) glued to the housing. (c) Alternative arrangement using a filter cut from a yellow gel sheet and mounted between two magnets. (d) Filter rotated away to allow bright-field illumination. Tape was adhered to one end of the filter for handling.

HAZARDS

The Nile Red safety data sheet indicates no specific hazards, but Nile Red has not been fully tested for toxicity. Stock solutions at 1 mg/mL were made up by a technically-trained person wearing gloves and a lab coat. The LED light source may dazzle if viewed directly but it does not pose the danger of a laser source. Any laser pen used should be first tested for output (<5 mW) using a power meter to check it does not exceed the class 3R or 3A laser standard (Supporting Information III).

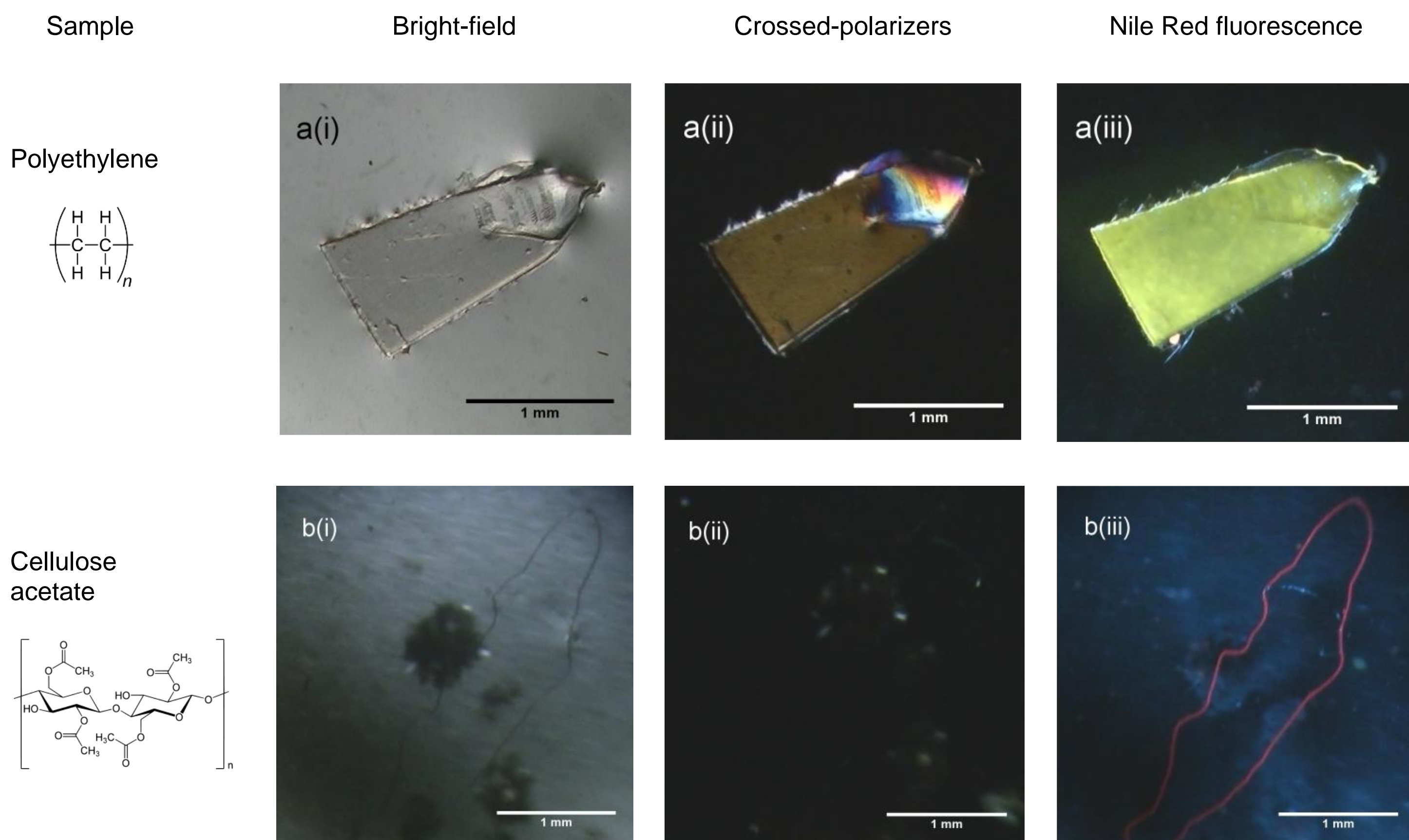
RESULTS AND DISCUSSION

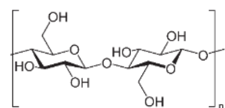
As a check on the performance of the polarization and fluorescence adaptations to a bright-field microscope, samples of plastic and other anthropogenic material were added to seawater samples and examined using the protocols described in the Materials and Methods section (Figures 3 and 4). These control samples were cut to characteristic shape and size so that they could be distinguished from intrinsic microplastic contamination. Some plastic material displayed color fringes under crossed-polarizers (Figure 3a(i)). This property reflects the wavelength dependence of refraction[13,14] and depends on both chemical and physical characteristics of the sample, such a degree of polymer chain alignment and sample thickness (Supporting Information II). Similar to a previous report[16], hydrophobic plastics, such as polyethylene, showed yellow-green emission in the presence of Nile Red (Figure 3a(iii)), while more hydrophilic plastics, containing ester or amide bonds, showed red emission (Figure 3b(iii)). Native cellulose fails to fluoresce but appears blue due to breakthrough of scattered light (Figure 3c(iii).

Nylon fishing line (a polyamide) was strongly birefringent when viewed under crossed-polarizers (Figure 4). This property indicates that it is necessary to rotate the Petri dish by >10° when observing a sample under crossed-polarizers to make sure a fiber is not oriented at its null position. In practice, this was rarely crucial because most contaminant fibers were curved or contorted, so that the fiber axis was observed over a range of angles relative to the polarizing filters. Nylon fishing line stained red with Nile Red but because of its strong scattering properties, a bluish tinge dominated the image when viewed with the yellow plexiglass or gel filter having breakthrough transmission (Supporting Information III). However, the red staining was clearer at the ragged ends where the nylon was thinner and the surface area to volume ratio was greater. In contrast, cellulose acetate fibers from cigarette filters were only weakly birefringent (Figure 3b(ii)). They were, however, rapidly stained red along their length (Figure 3c(iii)). Cigarette filters are designed to be porous to trap materials in the smoke and the polymer molecules within are presumably not as well aligned as the polyamide molecules within nylon. On the other hand, non-acetylated cellulose fibers from paper tissues appeared bright under crossed-polarizers but showed very weak fluorescence with Nile Red (Figure 3c).

These characteristics of cellulose are of practical significance for two reasons. In our initial studies bright fibers were frequently seen under crossed-polarizers which we thought might be plastic.

235 Subsequently, we noted they did not stain with Nile Red and now believe they arose from paper tissues used to dry the Petri dish. Other potential sources of contamination are identified in Supporting Information I. Natural fibers of plant origin (i.e. predominantly cellulose-based) were commonly found in our seawater samples and also failed to stain with Nile Red. Usually some kind of cellular structure could be observed within them, confirming their natural origin, but lack of Nile Red staining helped to 240 confirm their identity. On the other hand, zooplankton and some dinoflagellates stained rapidly with Nile Red, often revealing internal lipid stores (Figure 5d). Many of these specimens were actively mobile and so easily distinguished from microplastics.





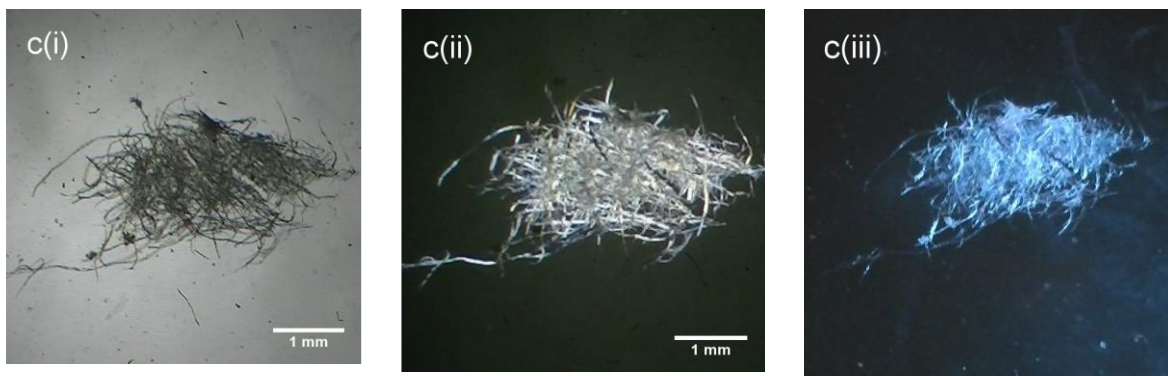
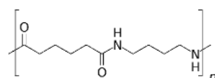


Figure 3. Examples of known plastic and other anthropogenic materials used to test the procedures. (a) polyethylene from a plastic bag (b) cellulose acetate fiber from a cigarette filter and (c) cellulose fibers from a paper tissue viewed under (i) bright-field, (ii) crossed-polarizers and (iii) fluorescence microscopy following Nile Red staining, using a 450 nm LED flashlight and plexiglass yellow filter. Note the cellulose fibers viewed with fluorescence is dominated by the light scatter signal that bleeds through the emission filter.

Diatoms did not stain significantly with Nile Red, but some weak red fluorescence was observed from their chlorophyll, particularly when a focused 445 nm laser pen was used for excitation. This emission was restricted to patches corresponding to the chloroplasts and was readily distinguished from the more even staining of plastic particles with Nile Red. Previous investigations of algae used Nile Red staining to estimate their lipid content[27]. However, these studies required a solvent, such as 20% dimethylsulfoxide, to permeate the cells, without which staining was low[16].



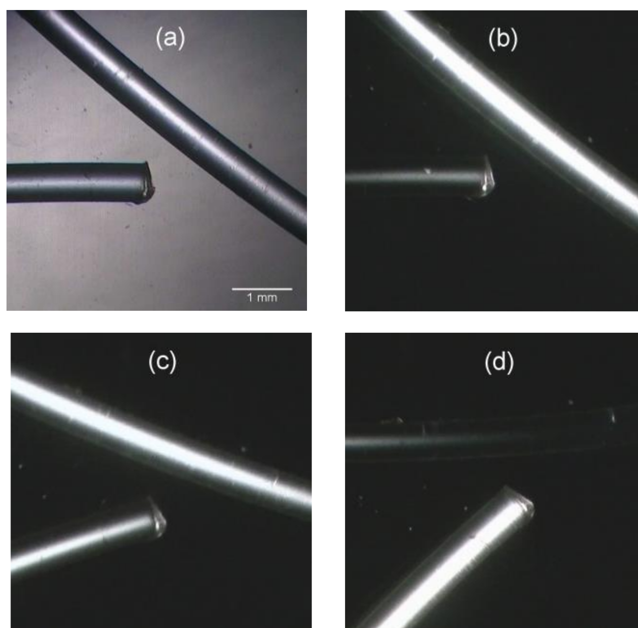
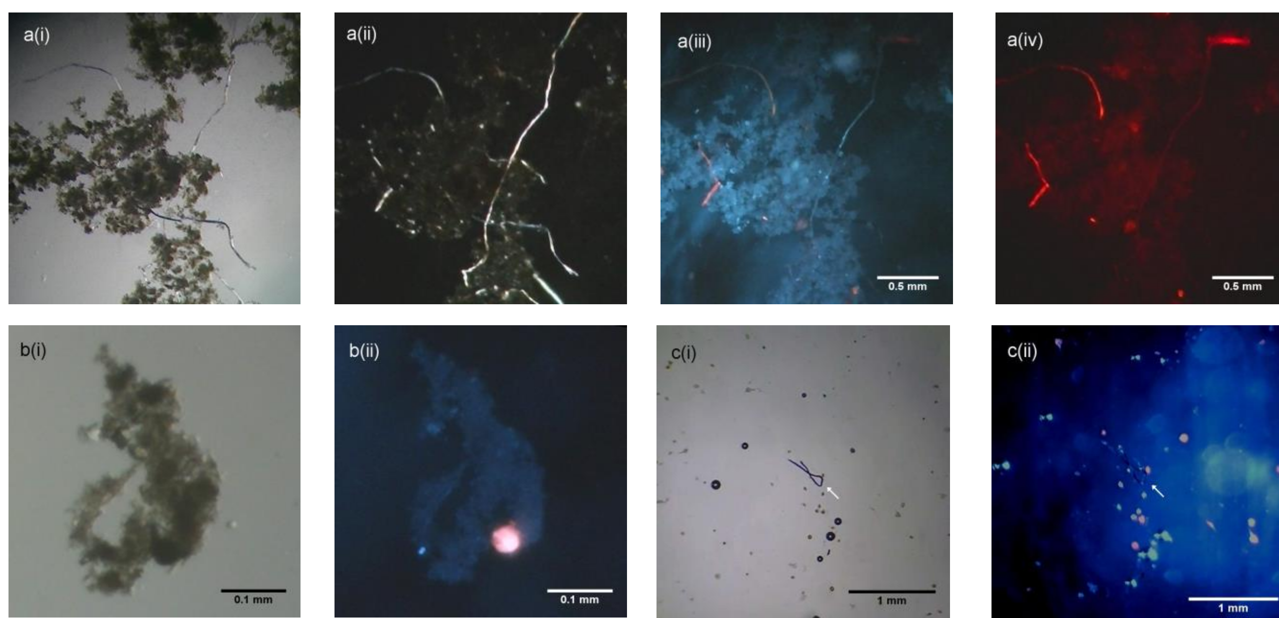


Figure 4. Two fragments of Nylon (polyamide) fishing line positioned at about 45° to each other viewed under; (a) bright-field and (b-d) crossed-polarizers with different rotations of the sample relative to the crossed-polarizers to demonstrate birefringence. The image was brightest when a fiber axis was at 45°, but dark when horizontal or vertical in the image plane.

260 In previous studies, strong oxidants were used to destroy natural organic material so as to remove false positive results with Nile Red staining[11,15]. We chose to avoid this step due to our primary focus on developing a protocol suitable for community scientists and students with limited chemistry facilities. We also reasoned that unless the digestion procedure approached 100% efficiency, partially-degraded organisms that would have been identifiable in their living state, would add to false-positive 265 readings. Enrichment of microplastics to aid analysis is often achieved by floatation in concentrated salt solutions[11, 16]. We tested this option using household salt (sodium chloride) but found it offered little advantage for our seawater samples that usually contained little sediment (Supporting Information I).

Having established the polarization and fluorescence characteristics of known plastics, samples of 270 seawater were then investigated for intrinsic microplastics. Fibers were the most frequently observed form of contamination and often found floating on the surface of the sample. Figure 5a-c shows some representative particles that we consider likely of anthropogenic origin, while Figure 5d shows the staining of lipid stores within a motile Nauplius.



275 Figure 5. Identification of putative microplastics in seawater (a) fibers amongst detritus showing strong polarization and Nile Red fluorescence. (i) bright-field, (ii) crossed-polarizers, (iii) fluorescence imaged though yellow plexiglass filter, (iv) fluorescence imaged through orange plexiglass filter. (b) potential plastic microbead trapped in detritus (i) bright-field, (ii) fluorescence through yellow plexiglass filter. (c) dark blue fiber, marked with arrow, in (i) bright-field, (ii) fluorescence through yellow plexiglass filter; emission is suppressed by blue pigment. (d) Zooplankton (Nauplius) showing lipid stores stained with Nile Red (i) bright-field, (ii) fluorescence + low level bright-field, (iii) fluorescence only
280 through yellow filter. Panels (c) and (d) demonstrate the need for bright-field observation.

Student feedback

The identification of microplastics using crossed-polarizers and Nile Red fluorescence received
285 positive feedback in community science projects and at public outreach events. The protocol was incorporated into a plankton monitoring course run by a local community college. We trained one teaching assistant in the assembly of a microscope who subsequently trained four other teaching assistants who helped run the course in the following semester. These assistants set up four microscopes independently and demonstrated the procedure to about 20 students using protocols
290 based on Supporting Information I. During the course, we presented background information on the physical principles behind fluorescence and polarization. In a course questionnaire, the students were unanimous in reporting that polarization and fluorescence detection were helpful to identify microplastics in seawater samples with more confidence than using bright-field microscopy alone (Supporting information IV). The data were reported in terms of presence or absence of microplastics
295 in the plankton monitoring database. They found microplastic in 86% of their 10 mL samples (n=36) collected over a period of 6 months. With some assumptions, this observation provides an estimate of an average of about 100 microplastic particles per cubic meter of the original seawater (Supporting Information II).

CONCLUSION

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A blue (450 nm) LED flashlight provides an inexpensive and efficient excitation source for fluorescence microscopy at magnifications of 10 to 60 times. While at higher magnifications a LED source can be used, the higher radiant intensity (flux/solid angle) of a laser pointer provides increased sensitivity over small fields-of-view. We have used Nile Red fluorescence staining as a screen for
305 microplastics in water samples collected for monitoring plankton. Observation through crossed-polarizers helps confirm the identity of some microplastics from their birefringence. The project proved successful for community scientists, school and college students who had access to standard bright-field microscopes, but not to advanced imaging facilities or a chemistry laboratory. The procedures involved could also provide contemporary examples to introduce topics such as spectroscopy, optics,
310 polymer chemistry and quantitative analysis to advanced undergraduates, where more background theory may be incorporated (see Supporting Information II).

While this article was being prepared, Scircle and Cizdziel[28] published a study, designed for an undergraduate laboratory class, using Nile Red to determine microplastics in bottled water. They used a home-built fluorescence microscope based a published design[29] that incorporated a dichroic mirror
315 and filters which cost ten times more than our adaptation. Their multi LED excitation source cost 200 times more than our flashlight and could not be efficiently optically coupled to the sample (Supporting Information II). Scircle and Cizdziel[28] relied exclusively on Nile Red fluorescence to identify microplastics. Stanton et al.[23] considered that such reliance overestimated the microplastic particle count, including those of previous bottled water analyses[17]. They proposed using 4′,6-diamidino-2-
320 phenylindole (DAPI) as a co-stain with Nile Red, to identify material of natural origin containing DNA and thereby help reduce false positives. We checked our experimental set-up (Figure 2a) using a uv LED flashlight (Wollcocer blacklight) as an excitation source and could see DAPI stained nuclei in a test sample (onion skin) and confirmed DAPI did not stain the test plastics (Supporting information III). However, the procedure required the use of research-grade emission filters to prevent cross-talk
325 between the DAPI and Nile Red fluorescence, which may be beyond the budget of community science projects. Nevertheless, our findings demonstrate the general applicability of our optical design for the detection of fluorescence at other excitation and emission wavelengths.

ASSOCIATED CONTENT

330 Supporting Information

The Supporting Information is available on the ACS Publications website at DOI:

10.1021/acs.jchemed.0c00518

I Microplastics identification protocols (docx)

II Underlying principles and tutorial discussion topics (docx)

335 III Supplementary methods(docx)

IV Student feedback (docx)

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