Exploring the efficacy of Nile red in microplastic quantification: a co-staining approach

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Abstract

The presence of microplastic particles (<5 mm) in the environment has generated considerable concern across public, political, and scientific platforms. However, the diversity of microplastics that persist in the environment poses complex analytical challenges for our understanding of their prevalence. The use of the dye Nile red to quantify microplastics is increasingly common. However, its use in microplastic analysis rarely accounts for its affinity with the breadth of particles that occur in environmental samples. Here we examine Nile red's ability to stain a variety of microplastic particles and common natural and anthropogenic particles found in environmental samples. To better constrain microplastic estimates using Nile red, we test the co-application of a second stain that binds to biological material, 4',6-diamidino-2-phenylindole (DAPI). We test the potential inflation of microplastic estimates using Nile red alone by applying this co-staining approach to samples of water and freshwater. The use of Nile red dye alone resulted in a maximum 100% overestimation of microplastic particles. These findings are of particular significance for the public dissemination of findings from an emotive field of study.

1. Introduction

The prevalence of microplastic particles (pieces of plastic <5 mm) across marine, freshwater, and atmospheric systems has captured the attention of scientists, politicians, and members of the public worldwide. These particles are known to exert a variety of environmental pressures on organisms[1,2]. Accurate quantification of microplastic particles in environmental samples is fundamental to our understanding of their environmental fate and prevalence. However, at present our understanding of microplastic distributions across these systems is hindered by inconsistencies in the isolation and identification of microplastic particles[3].

Microplastic quantification regularly adopts a time-consuming tiered approach of visual identification followed by confirmatory, often spectroscopic, particle characterization using techniques including Fourier Transform Infrared (FTIR)[4]. However, the visual preselection of particles is subject to size-dependent levels of error[5], and both the visual and spectroscopic characterization of microplastic particles requires a degree of specialist knowledge[6].

The lipophilic fluorescent dye Nile red has recently emerged as a rapid, more accessible, and less subjective technique for microplastic quantification. Nile red has quantified microplastics in samples from aquatic[7-9], sedimentary[10-12], and biological[13] environments. It has also quantified microplastics in bottled water[14], the findings of which are highly relevant to human health[15]. However, though its affinity with biological material has been found to vary, even following hydrogen peroxide (H_2O_2) treatment to remove it[16-18], Nile red may also stain some biological particles.

Here we explore the limitations of Nile red in microplastic quantification. We assess the variability of Nile red staining using plastics of different polymers and colors. We also highlight the extent to which Nile red stains biological material using a fluorescent dye that almost exclusively stains biological materials, DAPI[19], which binds to adenine-thymine rich regions of DNA[20]. River water and drinking water samples are analyzed using this co-

staining approach to assess the accuracy of Nile red microplastic counts in samples of environmental and public importance.

2. Materials and methods

2.1 Sample Preparation

2.1.1 Microplastic fragment and textile fiber production

Microplastic particles were generated from items of polypropylene (PP), high-density polyethylene (HDPE), expanded polystyrene (EPS), and polyvinyl chloride (PVC) using a utility knife. Natural (cotton, wool, silk), regenerated (rayon), and microplastic (hereafter synthetic) (polyester, polyamide, acrylic) textile fibers were pulled from garments woven from 100% of each fiber type using tweezers. The colors of the materials studied is detailed in table S1

Erni-Cassola et al.[17], treated polyethylene and polypropylene microplastic particles with a 7 hour 30% H_2O_2 treatment at 100°C. The prepared particles were therefore placed in a 15 ml polypropylene centrifuge tube with 5 ml of distilled water, and 5 ml of 30% H_2O_2. Samples were heated to 80°C to avoid excessive thermal decomposition of the H_2O_2, and were left covered for 8 hours. No bubbles were observed following the addition of H_2O_2, indicating that these particles did not react with the H_2O_2.

2.1.2 Freshwater samples

In order to quantify the extent to which biological particles might be stained by Nile red in environmental samples, three samples of river water were collected from the River Soar, UK, in line with a previous microplastic pollution study[6]. Briefly, 30 L of water was concentrated onto a metal sieve with a pore size of 63 µm. Material retained on this sieve was transferred into a 200 ml glass sample bottle with an aluminum-lined plastic lid for transportation to the laboratory. In the laboratory, each sample was treated with 50 ml of 30% H_2O_2 and was gradually heated to 75°C over 4–5 h. After being left to cool overnight,

these samples were filtered onto 0.45 µm mixed cellulose ester gridded filter papers (Whatman ME 25/41) using glass vacuum filtration apparatus.

To prevent sample contamination, the metal sieve and all glassware was thoroughly rinsed using distilled water prior to sample collection and filtration, and samples were covered with aluminum foil at all times except for during the transfer of samples to the vacuum filtration apparatus.

2.1.3 Drinking water samples

The quantification of microplastic particles in drinking water was assessed using tap water and five types of drinking water purchased from major UK supermarkets (three single use plastic bottles of still water, one single use plastic bottle of sparkling water, and one can of still water). Each of the six samples was 500 ml. All 500 ml of each sample was vacuum filtered following the same contamination controls and vacuum filtration procedure as the river water samples. Drinking water samples were not treated with H_2O_2 to allow for comparison with previous the Nile red staining of bottled water samples that did not treat samples by Mason et al.[14].

2.2 Staining procedure

To the 15 ml centrifuge tubes containing known particles in a 10 ml 1:1 solution of distilled water and H_2O_2, Nile red dissolved in acetone, and DAPI dissolved in water, were added to yield dye concentrations of 10 µg ml^{-1} and 0.5 µg ml^{-1} respectively. The samples were then left in the dark for 30 minutes before being vacuum filtered 0.8 µm Nuclepore black Track-Etch Membrane filter papers (Whatman 110659).

To the filtered river and drinking water samples, 10 ml of distilled water was added to the vacuum filtration apparatus after the samples had been filtered but before the filter papers were removed. To this, Nile red and DAPI were added to each sample to yield the same respective concentrations as stated above. The samples were then left in the dark for 30 minutes before filtering the remaining liquid. All filtered papers were transferred to microscope slides and analyzed immediately.

2.3 Visualization

Particle counts were conducted manually at 40x and 100x (total) magnification using a light microscope with a mercury vapor fluorescence illumination attachment (Euromex iScope, Euromex Microscopen B.V., Arnhem, The Netherlands). Nile red staining was observed in green fluorescence (excitation wavelength: 430-490 nm, emission wavelength: 510-560 nm) and DAPI staining was observed in blue fluorescence (excitation wavelength: 355-405 nm, emission wavelength: 420-480 nm).

For river and drinking water samples concentrated onto gridded filter papers, it was possible to standardize particle analysis across all samples. The same ten cells of the filter paper were analyzed for each sample (Figure S1). All cells were analyzed at 40x magnification, the lowest magnification possible given the configuration of the microscope. For each sample, two cells were also analyzed at 100X magnification. Only fluorescent particles with a clearly defined edge were counted.

2.4 Autofluorescence

Fluorescent dyes are not always the source of particle fluorescence. Some materials and organisms will fluoresce under certain wavelengths of light due to autofluorescence. The autofluorescence of a sample can be determined by observing it under the wavelengths of light specific to the dye being used without exposing samples to said dye. Autofluorescence in the wavelength regions for Nile red (green) and DAPI (blue) was determined in this manner for all known plastic particles and natural textile fibers, as well as a replicate for each filtered sample of drinking water and the materials used for the caps and bodies of the drinking water samples.

3. Results and discussion

3.1. Nile red staining of plastic fragments and fibers

The validation of Nile red as a technique for microplastic analysis has predominantly used white and translucent particles[17,21], and its ability to stain synthetic textile fibers has also

been found to be limited[18]. Nile red will also not stain some microplastic particles, including tire rubber[22,23]. In green fluorescence only light blue wool and white PVC exhibited autofluorescence, and in blue fluorescence, autofluorescence was noted in white cotton, light blue wool, red and grey polyamide, and polypropylene (Table S1).

Following H_2O_2 treatment, all white and transparent microplastic fragments were uniformly stained across their surface by Nile red (Figure 1). However, the staining of colored fragments was not uniform. Brown HDPE and black PP were stained only around their edges and, though all colored fragments fluoresced in some way at 100x magnification, their fluorescence at 40x magnification was less clear (Figure 1). Where staining is uneven, automated microplastic enumeration, using software such as ImageJ[17], could lead to particle overestimation (Figure 1; Table S1).

The color of microplastic fragments identified by Nile red staining is not always reported[9-11]. However, where it has been reported, proportions of white and colorless particles have been as high as 95%[24]. We show here that Nile red does not reliably stain all plastic particles, and that the presence of plastic dyes effects their affinity with Nile red. Numerous dyes can be used to stain plastics similar colors, and so it is possible that Nile red's affinity with particles of the same color and polymer will vary with the dye used. Whilst it is not possible to speculate how much the use of Nile red has previously incorrectly quantified microplastics of different colors, it is possible that lighter colored particles have dominated previous microplastic studies that use Nile red alone.

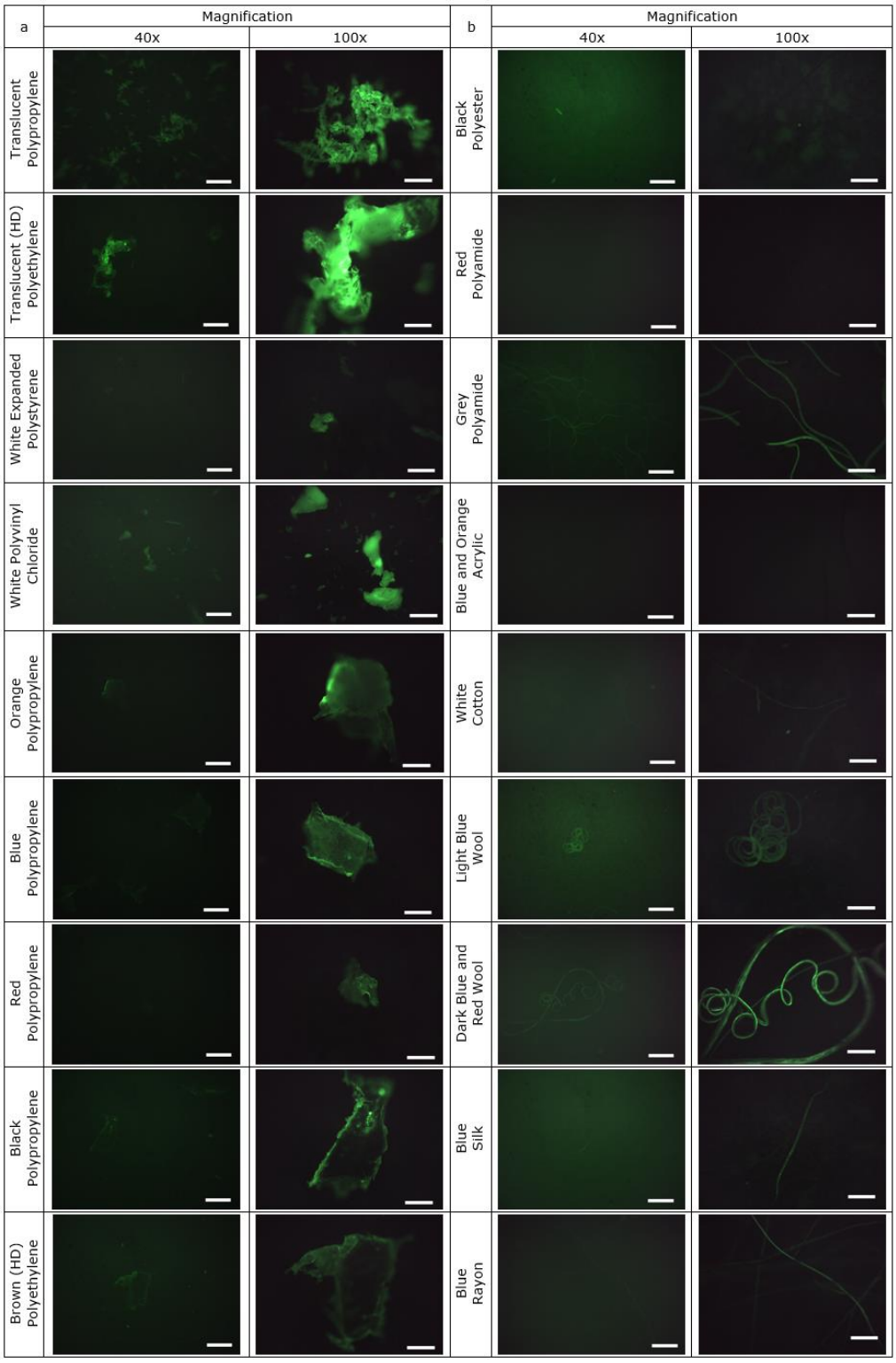


Figure 1: Nile red staining of H_2O_2 treated microplastic fragments of known polymers, and known synthetic and non-synthetic textile fibers. Colors denote those of the particles in the field of view imaged. Scale bars represent 500 µm and 200 µm for 40x and 100x magnification respectively. Images of white PVC and light blue wool are a result of autofluorescence, not staining (Table S1).

Of the fibers assessed in the present study, grey polyamide fibers and some, but not all, orange acrylic fibers were stained by Nile red, however, Nile red did not stain black polyester, blue acrylic or red polyamide fibers (Figure 1). That no part of these synthetic textile fibers was stained indicates that the uneven staining of brown HDPE and black PP fragments may be due to the thinning, and therefore lightening, of fragment edges during their production.

Moreover, Erni-Cassola et al.[17] state that following H_2O_2 treatment natural particles do not fluoresce in green, and Wiggin and Holland[18] report that natural and regenerated textile fibers are not stained by Nile red. However, here we show that even after H_2O_2 treatment, cotton, wool, silk, and rayon, all exhibited varying levels of fluorescence (Figure 1). A basic understanding of textile fiber morphology can go some way to differentiating between natural and synthetic textile fibres[6], but this can be a time consuming exercise, negating one of the main benefits of Nile red in microplastic analysis.

The fluorescence of wool and rayon fibers after Nile red staining is particularly limiting due to the morphological similarities they share with many synthetic textile fibers, possessing largely uniform diameters similar to that of many synthetic textile fibres[6]. Moreover, the smooth surface of rayon fibers, made from the extrusion of regenerated cellulose, bears a particularly close resemblance to synthetic textile fibers, which are also extruded[25]. These findings, question the efficacy of Nile red's application in the analysis of textile fibers all together. As expected, DAPI stained all natural fibers.

Though we identify plastic polymers of different colors that are not stained by Nile red, it is beyond the scope of this study to quantify the extent to which particle counts will be underestimated given the heterogeneity of polymers and colors of microplastic particles that persist in the environment.

3.2. Nile red and DAPI staining of natural particles in treated freshwater samples

Though reportedly negligible[16], Nile red has been shown to stain natural, lipid containing, particles in environmental samples[17,21]. Even after H_2O_2 treatment, material stained by

both Nile red and DAPI was abundant in samples of river water (Figure 2). Because H_2O_2 naturally occurs in aquatic environments, eukaryotic phytoplankton such as dinoflagellates produce peroxidase enzymes in order to counteract its damaging effects[26], which include cell lysis[27]. They are therefore resistant to H_2O_2[27]. These organisms can be identified by eye as with other biological indicators such as pollen, however, their presence in samples stained with Nile red could inflate particle abundance where analysts are not appropriately trained, or where automated particle counts are conducted.

Though plastics of some colors autofluoresced in the blue wavelengths of light used to observe DAPI fluorescence, the majority did not. It was therefore possible to estimate the extent to which Nile red could overestimate microplastic counts using DAPI to identify particles of biological origin that are stained by Nile red.

Nile red significantly overestimated microplastic abundance in two of the three river water samples (Table S2). The median Nile red overestimation of microplastic abundance in river water was 48.4% (ranging from 10.8% to 66.67%) at 40x magnification. At 100x magnification this rose to 54.5% (ranging from 37.5% to 58.8%). Furthermore, 95% of fibers at 40x magnification, and 100% of fibers at 100x magnification fluoresced with both Nile red and DAPI. Particle counts for all samples are detailed in Table S2. Though particles stained by both Nile red and DAPI could have been autofluorescing (Table S1), it is unlikely that this accounted for all of the fluorescent particles. For example, though it autofluoresced under blue light, polyamide resins accounted for approximately just 2% of total plastic resin demand across the European Union in 2017[29].

Across the river samples, multiple dark colored fragments exhibited inconsistent Nile red staining, and non-plastic particles were found to fluoresce (Figure S2). The results here cannot provide a universal estimate of false positive rates in environmental samples; however, they do illustrate considerable limitations to the use of a method of microplastic quantification that has not been appropriately validated.

3.3. Microplastic particles in drinking water

A small number of studies have identified microplastic particles in bottled water[14,30,31]. Of these, Mason et al.[14] relied solely on the use of Nile red in their analyses of particles <100 µm, reporting mean concentrations of 325 microplastic particles per liter of water. This finding influenced a World Health Organization review of bottled water[32] and garnered international media coverage. However, repeating this analysis using this co-staining approach resulted in considerable levels of error (Figure 2, Table S2).

Assessment of the materials from which the drinking water containers were made (detailed in table S3) showed that none of the cap materials exhibited autofluorescence. This included a polypropylene cap that was a different shade of blue to that detailed in section 3.1 (Table S3). However, the translucent Polyethylene Terephthalate (PET) from which the bodies of the four plastic bottled drinking water samples were made did exhibit both green and blue autofluorescence. Despite this, the presence of autofluorescent particles across the unstained filtered samples of drinking water was negligible (Table S4).

In the six samples of drinking water to which Nile red and DAPI were added, Nile red significantly overestimated microplastic abundance in five samples (Table S2). Across these samples, Nile red's median microplastic overestimation was 66.7% (ranging from 40% to 100%) (Table S2), placing considerable doubt on the results reported by Mason et al.[14] for particles <100 µm. Moreover, given the absence of autofluorescing particles in the unstained samples of drinking water, this study indicates that PET water bottles are not a source of potentially microplastic fluorescing particles in bottled water.

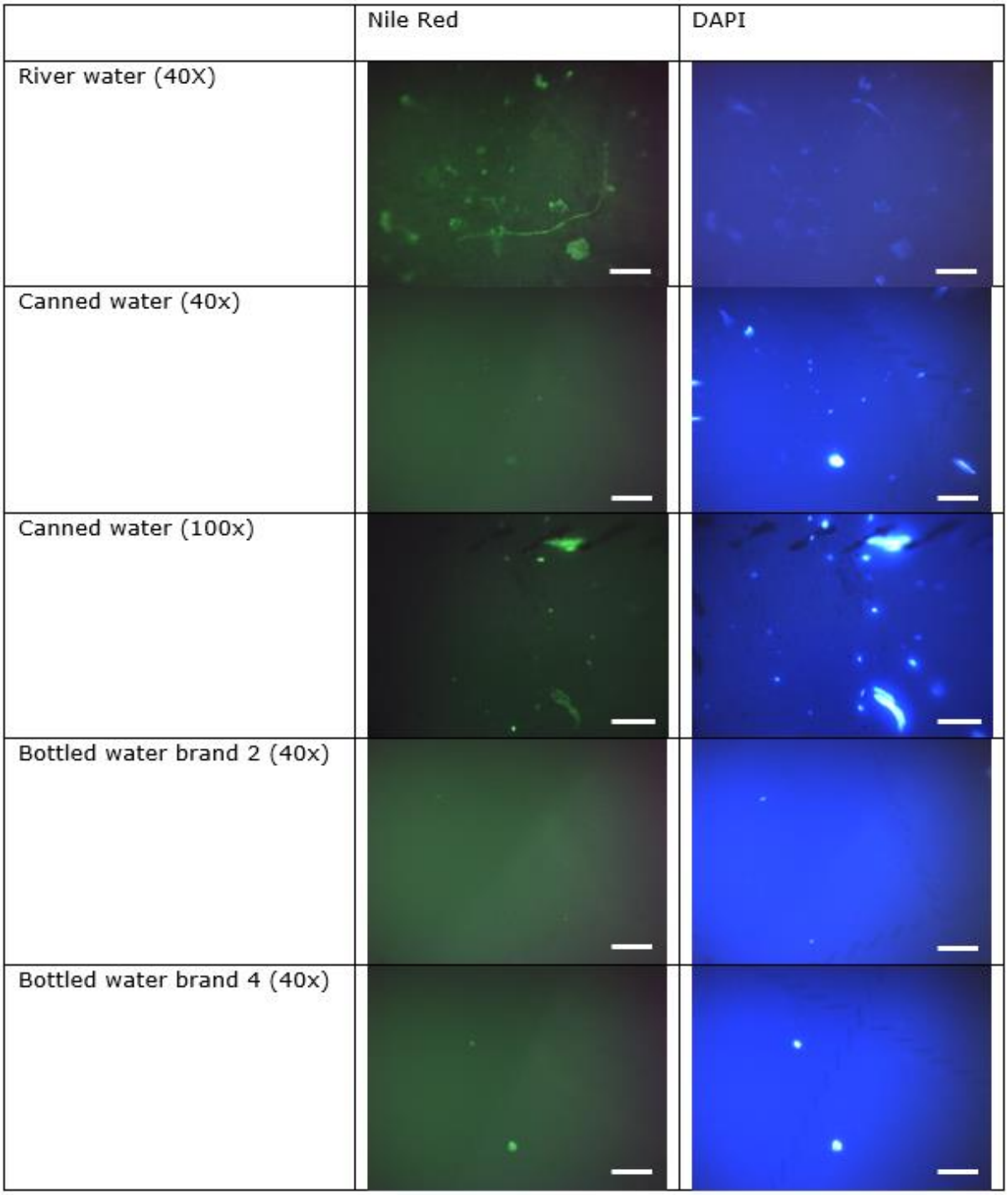


Figure 2: Nile red and DAPI co-staining of particles in river and drinking water illustrating the extent of possible false positives using Nile red alone. Each column shows the same field of view, as seen with the corresponding dyes. Scale bars represent 500 µm and 200 µm for 40x and 100x magnification respectively.

3.4. The future use of Nile red in microplastic analysis

Accurate particle characterization underpins our understanding of the spatial and temporal distribution of microplastic pollution. This informs policy and influences both industry and public opinion. As Nile red's application to microplastic research has made global headlines, there is growing need for rigorous assessment of its application to microplastic quantification. Whilst Nile red's ability to mark certain types of microplastic particle has been repeatedly demonstrated, its validation has not accounted for the breadth of microplastic colors and polymers that are known to pollute the environment. Furthermore, we show here that the use of H_2O_2 does not effectively remove common biological material that can also be stained by Nile red. It is beyond the scope of this study to provide an exhaustive audit of Nile red's ability to identify different plastic types, and assess the breadth of natural particles that may give rise to false positives in different environmental matrices. Nevertheless, the extent of errors found in environmental, and drinking water samples are indicative of the potential magnitude of such errors. As such, reliance on this approach in future studies should be discouraged without further development.

4. Acknowledgements

Stanton was supported by the University of Nottingham Sir Francis Hill Scholarship during this research.

5. Supporting Information Available

Two figures (illustrating the counting technique used and the variety of particles stained by Nile red and DAPI in environmental samples), and four tables (information about the known particles used, particle counts and results of statistical tests for river water and drinking water samples, autofluorescence of drinking water container materials, and autofluorescence of drinking water controls samples) are provided in the supporting information associated with this publication. This information is available free of charge via the internet at http://pubs.acs.org/journal/estlcu.

6. References

(1) Wright, S.L.; Thompson, R.C; Galloway, T.S. The physical impacts of microplastics on marine organisms: a review. *Environmental Pollution* **2013**, 178, 483-492.

(2) Eerkes-Medrano, D.; Thompson, R.C.; Aldridge, D.C. Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. *Water Research* **2015**, 75, 63-82.

(3) Shim, W.J.; Hong, S.H.; and Eo, S.E. Identification methods in microplastic analysis: a review. *Analytical Methods* **2017**, 9 (9), 1384-1391.

(4) Harrison, J.P.; Ojeda, J.J.; Romero-González, M.E. The applicability of reflectance micro-Fourier-transform infrared spectroscopy for the detection of synthetic microplastics in marine sediments. *Science of the Total Environment* **2012**, 416, 455-463.

(5) Lenz, R.; Enders, K.; Stedmon, C.A.; Mackenzie, D.M.; Nielsen, T.G. A critical assessment of visual identification of marine microplastic using Raman spectroscopy for analysis improvement. *Marine Pollution Bulletin* **2015**, 100 (1), 82-91.

(6) Stanton, T.; Johnson, M.; Nathanail, P.; MacNaughtan, W.; Gomes, R.L. Freshwater and airborne textile fiber populations are dominated by 'natural', not microplastic, fibers. *Science of The Total Environment* **2019**, 666, 377-389.

(7) Maes, T.; Jessop, R.; Wellner, N.; Haupt, K.; Mayes, A.G. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile red. *Scientific Reports* **2017**, 7, 44501.

(8) Desforges, J.P.W.; Galbraith, M.; Dangerfield, N.; Ross, P.S. Widespread distribution of microplastics in subsurface seawater in the NE Pacific Ocean. *Marine Pollution Bulletin* **2014**, 79 (1-2), 94-99.

(9) Fischer, E.K.; Paglialonga, L.; Czech, E.; Tamminga, M. Microplastic pollution in lakes and lake shoreline sediments–a case study on Lake Bolsena and Lake Chiusi (central Italy). *Environmental Pollution* **2016**, 213, 648-657.

(10) Tamminga, M.; Hengstmann, E.; Fischer, E.K. Microplastic analysis in the South Funen Archipelago, Baltic Sea, implementing manta trawling and bulk sampling. *Marine Pollution Bulletin* **2018**, 128, 601-608.

(11) Hengstmann, E.; Tamminga, M.; vom Bruch, C.; Fischer, E.K. Microplastic in beach sediments of the Isle of Rügen (Baltic Sea)-Implementing a novel glass elutriation column. *Marine Pollution Bulletin* **2018**, 126, 263-274.

(12) Wang, Z.; Su, B.; Xu, X.; Di, D.; Huang, H.; Mei, K.; Dahlgren, R.A.; Zhang, M.; Shang, X. Preferential accumulation of small (< 300 μm) microplastics in the sediments of a coastal plain river network in eastern China. *Water Research* **2018**, 144, 393-401.

(13) Tiwari, M.; Rathod, T.D.; Ajmal, P.Y.; Bhangare, R.C.; Sahu, S.K. Distribution and characterization of microplastics in beach sand from three different Indian coastal environments. *Marine Pollution Bulletin* **2019**, 140, 262-273.

(14) Catarino, A.I.; Macchia, V.; Sanderson, W.G.; Thompson, R.C.; Henry, T.B. Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibers fallout during a meal. *Environmental Pollution* **2018**, 237, 675-684.

(15) Mason, S.A.; Welch, V.G.; Neratko, J. Synthetic polymer contamination in bottled water. *Frontiers in Chemistry* **2018**, 6.

(16) Koelmans, A.A.; Nor, N.H.M.; Hermsen, E.; Kooi, M.; Mintenig, S.M.; De France, J. Microplastics in freshwaters and drinking water: Critical review and assessment of data quality. *Water Research* **2019**, 155, 410-422

(17) Erni-Cassola, G.; Gibson, M.I.; Thompson, R.C.; and Christie-Oleza, J.A. Lost, but found with Nile red: a novel method for detecting and quantifying small microplastics (1 mm to 20 μm) in environmental samples. *Environmental Science & Technology* **2017**, 51(23), 13641-13648.

(18) Wiggin, K.J.; Holland, E.B. Validation and application of cost and time effective methods for the detection of 3–500 μm sized microplastics in the urban marine and estuarine environments surrounding Long Beach, California. *Marine Pollution Bulletin* **2019**, 143, 152-162.

(19) Porter, K.G.; Feig, Y.S. The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* **1980**, 25 (5), 943-948.

(20) Kapuscinski, J. DAPI: a DNA-specific fluorescent probe. *Biotechnic & Histochemistry* **1995**, 70 (5), 220-233.

(21) Shim, W.J.; Song, Y.K.; Hong, S.H.; Jang, M. Identification and quantification of microplastics using Nile red staining. *Marine Pollution Bulletin* **2016**, 113(1-2), pp.469-476.

(22) Araujo, C.F.; Nolasco, M.M.; Ribeiro, A.M.; Ribeiro-Claro, P.J. Identification of microplastics using Raman spectroscopy: latest developments and future prospects. *Water Research* **2018**, 142, 426-440.

(23) Hitchcock, J.N.; Mitrovic, S.M. Microplastic pollution in estuaries across a gradient of human impact. *Environmental Pollution* **2019**, 247, 457-466.

(24) Dobaradaran, S.; Schmidt, T.C.; Nabipour, I.; Khajeahmadi, N.; Tajbakhsh, S.; Saeedi, R.; Mohammadi, M.J.; Keshtkar, M.; Khorsand, M.; Ghasemi, F.F. Characterization of plastic debris and association of metals with microplastics in coastline sediment along the Persian Gulf. *Waste Management* **2018**, 78, 649-658.

(25) Greaves, P.H.; Saville, B.P. *Microscopy of textile fibers. Volume 32 of Royal Microscopical Society Microscopy Handbooks.* BIOS Scientific, Oxford **1995**

(26) Matthijs, H.C.; Visser, P.M.; Reeze, B.; Meeuse, J.; Slot, P.C.; Wijn, G.; Talens, R.; Huisman, J. Selective suppression of harmful cyanobacteria in an entire lake with hydrogen peroxide. *Water Research* **2012**, 46 (5), 1460-1472.

(27) Burson, A.; Matthijs, H.C.; de Bruijne, W.; Talens, R.; Hoogenboom, R.; Gerssen, A.; Visser, P.M.; Stomp, M.; Steur, K.; van Scheppingen, Y.; Huisman, J. Termination of a toxic Alexandrium bloom with hydrogen peroxide. *Harmful Algae* **2014**, 31, 125-135.

(28) Hidalgo-Ruz, V.; Gutow, L.; Thompson, R.C.; and Thiel, M. Microplastics in the marine environment: a review of the methods used for identification and quantification. *Environmental Science & Technology* **2012**, 46 (6), 3060-3075.

(29) Plastics Europe. *Plastics – The Facts 2018*. Brussels, Belgium **2018**

(30) Oßmann, B.E.; Sarau, G.; Holtmannspötter, H.; Pischetsrieder, M.; Christiansen, S.H.; Dicke, W. Small-sized microplastics and pigmented particles in bottled mineral water. *Water Research* **2018**, 141, 307-316.

(31) Schymanski, D.; Goldbeck, C.; Humpf, H.U.; Fürst, P. Analysis of microplastics in water by micro-Raman spectroscopy: release of plastic particles from different packaging into mineral water. *Water Research* **2018**, 129, 154-162.

(32) Readfearn, G. WHO launches health review after microplastics found in 90% of bottled water. *Guardian Online* **2018** https://www.theguardian.com/environment/2018/mar/15/microplastics-found-in-more-than-90-of-bottled-water-study-says, [accessed 19/05/19].

Supplementary Material

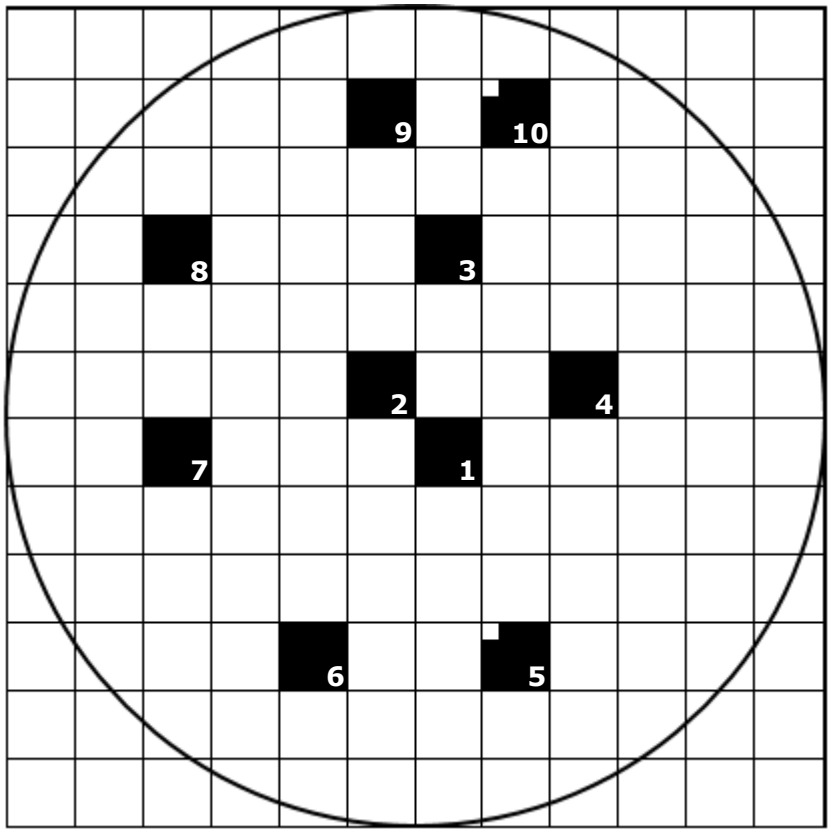


Figure S1: The cells on the gridded filter papers that were observed at 40 x magnification in each sample. The two cells with the white square within them were also observed at 100x magnification.

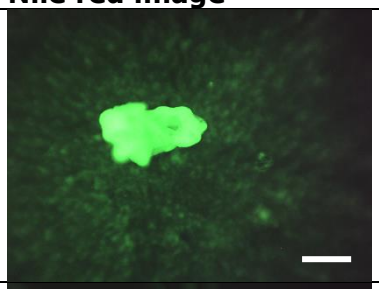
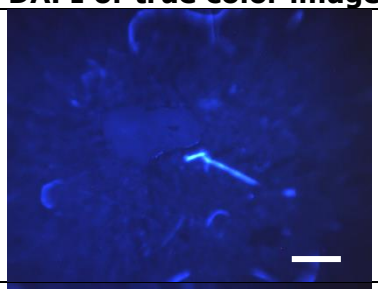
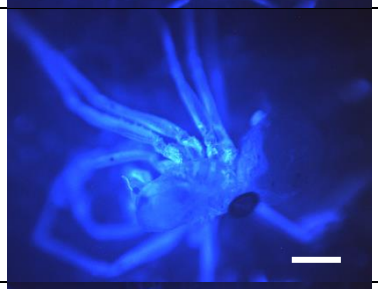
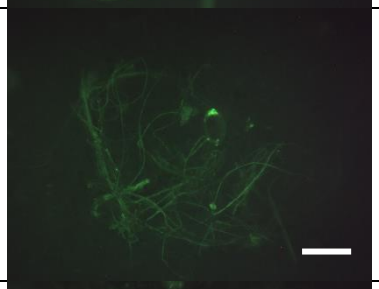
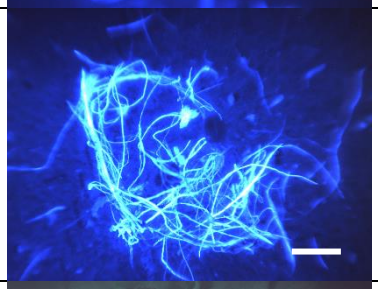
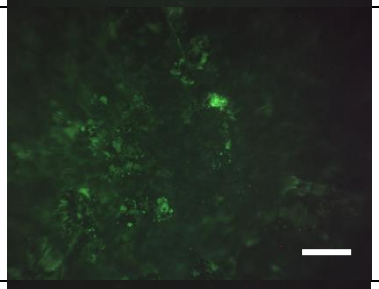
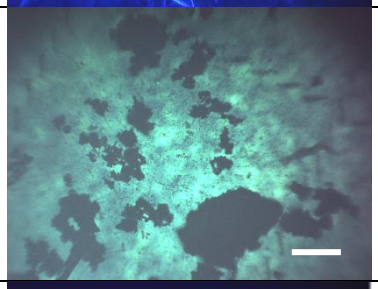
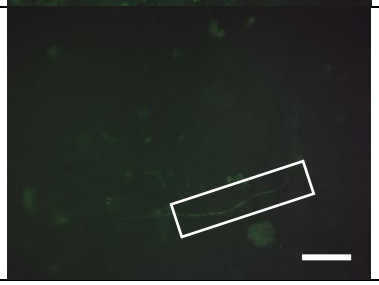
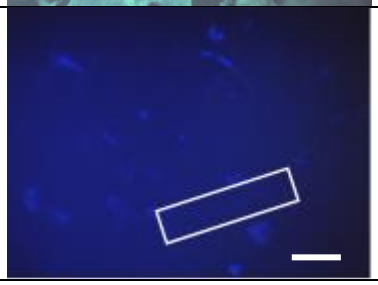
Particle	Nile red image	DAPI or true color image
Possible microplastic particle		
Invertebrate		
Bundle of fibers		
Black particles exhibiting uneven staining		
Natural textile fiber		

Figure S2: Nile Red and DAPI images of natural and possible microplastic particles stained in samples of river water. All images taken at 40x magnification and the scale bar represents 500 µm

Table S1: Table of known plastic particles and natural and synthetic fibers, detailing their color, autofluorescence at the green and blue wavelengths used to assess Nile red and DAPI staining respectively (see section 2.3), and affinity with Nile Red and DAPI fluorescent stains where autofluoresence was not observed.

Polymer	Color	Autofluorescence		Nile red fluorescence?	DAPI fluorescence?
		Green	Blue		
PP	Transparent	No	No	Yes	No
	Orange	No	No	Yes	No
	Blue	No	Yes	Yes	-
	Red	No	No	Yes	No
	Black	No	No	Yes - at edges only	No
HDPE	Translucent	No	No	Yes	No
	Brown	No	No	Yes - at edges only	No
EPS	White	No	No	Yes	No
PVC	White	Yes	No	-	No
Polyester	Black	No	No	No	No
Polyamide	Red	No	Yes	No	-
	Grey	No	Yes	Yes	-
Acrylic	Blue	No	No	No	No
	Orange	No	No	Yes	No
Cotton	White	No	No	Yes	Yes
Wool	Light blue	Yes	Yes	-	-
	Dark blue	No	No	Yes	Yes
Silk	Blue	No	No	Yes	Yes
Rayon	Blue	No	No	Yes	Yes

Table S2: Number of particles that fluoresced with Nile Red and DAPI at 40x and 100x magnification across ten and two cells of the filter area respectively (Figure S1). Significance was calculated using a paired sample t-test for counts at 40x magnification, in which the mean particle counts across these ten grid cells was compared for particles that fluoresced with Nile red, and particles that fluoresced with Nile Red but not DAPI (i.e. suspected microplastics). The paired samples T test could not be calculated at 100x magnification due to only two grid cells being quantified at this magnification.

Sample	Magnification	Particles that fluoresced with Nile Red	Particles that fluoresced with both Nile Red and DAPI	Suspected MPs (particles that only fluoresce with Nile red)	Significance (p=)
River water 1	40	33	22	11	0.003
River water 1	100	17	10	7	
River water 2	40	31	15	16	0.002
River water 2	100	11	6	5	
River water 3	40	74	8	68	0.087
River water 3	100	16	6	10	
Bottled water 1	40	14	12	2	0.005
Bottled water 1	100	14	13	1	
Bottled water 2	40	3	2	1	0.168
Bottled water 2	100	5	3	2	
Bottled water 3	40	9	9	0	0.019
Bottled water 3	100	11	8	3	
Bottled water 4	40	15	6	9	0.024
Bottled water 4	100	18	10	8	
Canned water	40	28	13	15	0.001
Canned water	100	70	57	13	
Tap water	40	24	18	6	0.001
Tap water	100	20	12	8	

Table S3: The material, color, and autofluorescence of different parts of the containers of drinking water samples. The lid of bottled water 4 had two parts.

Sample		Material	Color	Autofluorescence	
				Green	Blue
Bottled water 1	**Lid**	HDPE	Light blue	N	N
	Body	PET	Transparent	Y	Y
Bottled water 2	**Lid**	HDPE	Green	N	N
	Body	PET	Transparent	Y	Y
Bottled water 3	**Lid**	HDPE	Dark blue	N	N
	Body	PET	Transparent	Y	Y
Bottled water 4	**Lid inner**	HDPE	White	N	N
	Lid outer	PP	Dark blue	N	N
	Body	PET	Transparent	Y	Y
Canned water	**Lid**	Polypropylene	Black	N	N
	Body	Aluminium	N/A		

Table S4: Abundance of autofluorescing particles across the six drinking water samples.

Sample	Magnification	Autofluorescence	
		Green	Blue
Bottled water 1	40	0	5
Bottled water 1	100	0	1
Bottled water 2	40	0	5
Bottled water 2	100	0	0
Bottled water 3	40	0	0
Bottled water 3	100	0	0
Bottled water 4	40	0	2
Bottled water 4	100	0	2
Canned water	40	2	4
Canned water	100	1	2
Tap water	40	0	8
Tap water	100	0	3