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# Lost, but found with Nile red; a novel method to detect and quantify small microplastics (20 m1 mm) in environmental samples

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8 **Lost, but found with Nile red; a novel method to detect and quantify**  
9 **small microplastics (20 µm–1 mm) in environmental samples**

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22

23 **Abstract**

24 Marine plastic debris is a global environmental problem. Surveys have shown that plastic  
25 particles <5 mm in size, known as microplastics, are significantly more abundant in surface  
26 seawater and on shorelines than larger plastic particles. Nevertheless, quantification of  
27 microplastics in the environment is hampered by a lack of adequate high throughput methods  
28 to distinguish and quantify smaller size fractions (<1 mm), and this has probably resulted in  
29 an underestimation of actual microplastic concentrations. Here we present a protocol that  
30 allows high throughput detection and automated quantification of small microplastic particles  
31 (20–1000  $\mu\text{m}$ ) using the dye Nile red, fluorescence microscopy and image analysis software.  
32 This protocol has proven highly effective in the quantification of small polyethylene,  
33 polypropylene, polystyrene and nylon 6 particles, which frequently occur in the water  
34 column. Our preliminary results from sea surface tows show a power-law increase of small  
35 microplastics (*i.e.* <1 mm) with decreasing particle size. Hence, our data helps to resolve  
36 speculation on the ‘*apparent*’ loss of this fraction from surface waters. We consider that this  
37 method presents a step change in the ability to detect small microplastics by substituting the  
38 subjectivity of human visual sorting with a sensitive and semi-automated procedure.

39

## 40 **Introduction**

41 It has been estimated that mismanagement of plastic waste resulted in up to 12.7  
42 million tonnes of plastic entering the ocean in 2010 alone.<sup>1</sup> In the environment, plastics  
43 accumulate due to their recalcitrant nature, contaminating sediments and surface seawaters on  
44 a global scale.<sup>2,3</sup> In aquatic systems, polymer types with lower density than seawater have a  
45 higher transportability (*via* rivers<sup>4</sup> into marine coastal areas and oceanic gyres)<sup>5</sup> than higher  
46 density polymers, which tend to settle out.<sup>6-8</sup> Lower density plastics, such as polypropylene  
47 (PP), polyethylene (PE) and certain forms of polystyrene (PS) are frequently used as  
48 packaging materials<sup>9</sup> and hence, have a very short service life prior to disposal. These types of  
49 plastic are also more commonly found in environmental surveys.<sup>10,11</sup>

50 Eriksen *et al.*<sup>12</sup> estimated that about 5.25 trillion plastic fragments are currently  
51 floating on the ocean's surface. Extensive sampling of surface seawater and comparison  
52 across all size classes (>200 µm) has shown that plastic particles <5 mm are significantly  
53 more abundant than larger particles.<sup>5,12,13</sup> These plastic fragments (<5 mm) have been termed  
54 microplastics.<sup>14,15</sup> Marine microplastics are composed of two main types: (1) primary  
55 microplastics that stem directly from the source, such as microbeads contained in cosmetics,  
56 or fibres released during washing of synthetic garments,<sup>16</sup> and (2) secondary microplastics  
57 that are generated through macroplastic fragmentation, a break down process influenced by  
58 UV-irradiation, high temperatures and mechanical shear forces.<sup>17,18</sup> Morét-Ferguson *et al.*<sup>19</sup>,  
59 found that the average size of buoyant plastic particles in the Northern Atlantic and Caribbean  
60 had halved in size from an average 10 mm in the 1990s to 5 mm in the 2000s. The decrease in  
61 average size of plastic marine debris is of concern because the smaller synthetic polymers are  
62 ingested by relatively more organisms at the base of the marine food web.<sup>3</sup> Recent studies  
63 suggest that ingested particles can be transferred between trophic levels<sup>20,21</sup> and transport  
64 persistent organic pollutants.<sup>22</sup> The possible environmental effects of microplastics has led to  
65 growing public and media attention as well as policy measures to reduce inputs, such as  
66 banning the use of plastic microbeads in personal care products.<sup>23</sup> Besides these concerns and  
67 abatement measures, monitoring of marine litter is currently required in the EU under the

68 Marine Strategy Framework Directive (MSFD)<sup>24</sup> and it is therefore essential to have reliable,  
69 reproducible, rapid and inexpensive methods for quantification and monitoring of  
70 microplastic contamination in the environment.

71 Current methodology for quantification of environmental microplastic contamination  
72 is hampered by a lack of methods that are both sensitive and allow high throughput  
73 quantification. Commonly applied methods separate synthetic microparticles from non-  
74 synthetic materials *via* density separation and floatation techniques, before visually sorting  
75 the particles and finally confirming their identity with spectroscopy.<sup>25</sup> The data generated can  
76 result in an underestimate of small microplastics because of the visual step in the process.<sup>26</sup>  
77 Alternative, faster and less expensive protocols are of particular importance for routine  
78 monitoring of plastic contamination by regulatory bodies and the need for developing new  
79 methods has been clearly identified as a policy priority (MSFD).<sup>24</sup> Here, we adopt the  
80 sampling criteria proposed by the EU technical subgroup on marine litter,<sup>24</sup> who recommend  
81 two categories: *large* microplastics ranging from 5 mm–1 mm in size (visually recognizable)  
82 and *small* microplastics ranging from 1 mm–20 µm for which reliable quantification is still  
83 challenging.

84 In this study, we present the application of a fluorescence-based protocol using Nile  
85 red to detect and quantify small microplastics in environmental samples. This method is  
86 inexpensive, employs readily available equipment and can be semi-automated for high  
87 throughput sample analysis. The method requires a sample purification step, fluorescence  
88 microscopy (green fluorescence protein settings) and free image analysis software.

89 **Materials and Methods**

90

91 *Microplastic staining and quantification protocol validation using commercial synthetic*  
92 *polymers*

93

94 Nile red had been suggested as a tool to fluorescently label microplastics<sup>18,27</sup>, and its use was  
95 later demonstrated.<sup>28,29</sup> The dye is commonly dissolved in acetone,<sup>30</sup> but here methanol was  
96 chosen because common plastics are resistant to it. The fluorescence of Nile red is influenced  
97 by its concentration, which lies optimally between 0.1 and 2  $\mu\text{g mL}^{-1}$ ,<sup>30</sup> and higher  
98 concentrations lead to quenching.<sup>30</sup> Accordingly, the working solution for this study was  
99 prepared by dissolving Nile red (technical grade, N3013, Sigma-Aldrich) in methanol to a  
100 concentration of  $1\mu\text{g mL}^{-1}$ .

101 Staining efficacy and automated particle detection was tested on nine different  
102 polymer types: PE (powder, ~40–48  $\mu\text{m}$ , Sigma-Aldrich), poly(ethylene terephthalate) (PET,  
103 powder,  $\leq 300\ \mu\text{m}$ , GoodFellow), PVC (powder, ~80–148  $\mu\text{m}$ , Sigma-Aldrich), nylon 6  
104 (pellets, ~1 mm, Sigma-Aldrich), PP (pellets, ~7 mm, Sigma-Aldrich), PS (pellets, ~5 mm,  
105 Sigma-Aldrich), PC (fragment from panel, ~10 mm), polyurethane (PUR, pellets, ~3 mm,  
106 Sigma-Aldrich), and black tire rubber (fragment from bicycle tire, 7×4 mm). Nylon 6  
107 microplastics were prepared by heating the pellets and pulling them apart to produce thin  
108 fibres, which then were cut to microparticles (~63–91  $\mu\text{m}$ ) under a dissection microscope. PP  
109 pellets as well as black rubber were ground in liquid nitrogen with mortar and pestle to obtain  
110 small microplastic fragments (PP: ~20–130  $\mu\text{m}$ , black rubber: ~57–171  $\mu\text{m}$ ). PS and PC  
111 microparticles were obtained by sanding the pellets with a metal file and further cutting the  
112 obtained particles with a scalpel under a dissection microscope to the final sizes (PS: ~24–196  
113  $\mu\text{m}$ , PC: ~94–169  $\mu\text{m}$ ). PUR pellets were directly cut to size under a dissection microscope  
114 (~71–154  $\mu\text{m}$ ). Sizes of all microparticles produced in the laboratory were calculated from the  
115 square root of particle area, which was measured in ImageJ using brightfield microscope  
116 images. Ten particles of each polymer type were placed on separate clean PC track-etched

117 filter membranes (PCTE, 25 mm diameter, 10  $\mu\text{m}$  pore size, Whatman) to evaluate the  
118 efficiency of detection of our protocol. PCTE filters are optimal for two reasons: (1) their  
119 hydrophilic surface avoids Nile red background fluorescence and (2) translucent properties  
120 when exposed to methanol allow brightfield microscopy in addition to fluorescence  
121 microscopy. About 2–3 drops of Nile red solution were carefully added to cover each filter.  
122 Filters were placed on standard microscope slides, covered with cover slips and fixed with  
123 tape to avoid movement of the sample. The samples were then maintained for 10 minutes at  
124 60  $^{\circ}\text{C}$  in the dark.

125         Microscopic imaging was performed using a light microscope (Nikon Eclipse Ti)  
126 equipped with a widefield camera (Andor Zyla sCMOS) and a LED for fluorescence. We  
127 tested the fluorescence of the nine different polymers stained with Nile red on PCTE filters in  
128 green (excitation/emission 460/525 nm) and red (565/630 nm). Green fluorescence was  
129 chosen over red fluorescence because (1) synthetic polymers either fluoresced better in green  
130 (fig. S1 a-d) or fluorescence did not differ significantly (fig. S1 e), (2) natural contaminants  
131 fluoresced in red but not in green after hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) digestion (fig. S2, discussed  
132 below) and (3) background signal from the filter membrane was lower. Three types of whole  
133 filter images were obtained for each polymer type: red and green fluorescence, as well as  
134 brightfield, all at a magnification of 10 $\times$ . Exposure time for fluorescence was 30 ms at 30%  
135 LED strength.

136         Automated particle recognition and quantification based on the fluorescent images  
137 was performed in ImageJ (v1.50i). A macro was written to perform the following tasks: (1)  
138 set the scale, (2) subtract the background using a rolling ball radius of 1500 pixels, (3)  
139 convert images to 8bit, (4) adjust black and white thresholds using 29 and 175 as the lower  
140 and higher values of pixel brightness, and finally (5) quantify particles based on area (400 –  $\infty$   
141  $\mu\text{m}^2$ ). The size detection limit was set to 400  $\mu\text{m}^2$  to ensure that pores from the filter  
142 membrane (diameter = 10  $\mu\text{m}$ ) did not interfere in particle measurements.

143

144 *Validation of the pre-staining digestion protocol*

145

146 To prevent overestimation of synthetic particles in environmental samples, it is of critical  
147 importance that biogenic materials, such as lipids, chitin or wood lignin, which fluoresce  
148 when stained with Nile red (fig. S2 a, c), are eliminated or cease to fluoresce when stained.  
149 While digestion with nitric acid proved highly efficient at removing biogenic matter, its  
150 application is limited due to pH-sensitive polymers, such as PS particles, which melt together,  
151 or Nylon fibres, which are lost during the process.<sup>31</sup> A chemical alternative is given with  
152 H<sub>2</sub>O<sub>2</sub> treatments, against which common synthetic polymers are resistant.<sup>31,32</sup> Hence,  
153 digestion of biogenic material was performed as previously described by Claessens *et al.* with  
154 slight modifications.<sup>31</sup> Briefly, 20 ml of 30% H<sub>2</sub>O<sub>2</sub> was added to 250 mL Erlenmeyer flasks  
155 containing the filtered samples on PCTE filters, which were then kept at 60 °C for 1 h  
156 followed by a prolonged 7 h step at 100 °C. Following the digestion, PCTE filters were  
157 thoroughly rinsed with Milli-Q water and then removed. The remaining solution was filtered  
158 through a new PCTE filter rinsing all particles from the flask and filtering device with Milli-  
159 Q water. The new PCTE filter containing all collected material was stored in Petri dishes until  
160 Nile red analysis.

161 The effect of the H<sub>2</sub>O<sub>2</sub> digestion protocol on natural polymers was tested with the two  
162 most commonly occurring natural polymers: chitin (powder, Sigma-Aldrich) and wood lignin  
163 (below 1 mm in size; kindly provided by Prof. Tim Bugg and prepared according to  
164 literature).<sup>33</sup>

165

### 166 ***Validation of the fluorescent-staining protocol with environmental samples***

167

168 Net tow and beach sand samples were obtained in June 2016 to test our fluorescent-labelling  
169 method with environmental samples. Tow samples were collected from within Plymouth  
170 Sound, UK. Five consecutive tows of 15 min were undertaken with a manta net (0.50 m by  
171 0.15 m mouth, 300 µm mesh) at a ship speed of 4 knots. After each tow, the collected  
172 material was transferred into a container by rinsing the net and cod end with seawater. In the

173 laboratory, all material was pre-filtered through a 1 mm metallic mesh to eliminate large  
174 debris. Retained debris was thoroughly washed with Milli-Q water to extract all small  
175 microplastics. Retained debris was visually examined for plastic debris (fig. S3). The flow  
176 through containing plastic particles <1 mm was vacuum filtered through PCTE filters (47 mm  
177 diameter, 10 µm pore size, Whatman). All filters were placed into a 250 mL flask.

178 Sediment samples were collected from a beach at Bigbury (UK, 50°16'53N,  
179 3°53'42W) by transferring the top 1 cm layer of five 30 x 30 cm<sup>2</sup> plots with a metallic spoon  
180 into 500 mL glass bottles. Microplastics were extracted from sand samples according to the  
181 density-separation/floatation protocol described in Nuelle *et al.*<sup>34</sup> using NaCl (26% w/v)  
182 instead of NaI. The collected supernatant was vacuum filtered through PCTE filters, which  
183 were placed in a 250 mL flask. Flasks containing the PCTE filters from net tows and beach  
184 sand samples were stored at 60 °C during 24 h for desiccation. The H<sub>2</sub>O<sub>2</sub> digestion, staining  
185 and imaging was performed as described above.

186 Micro-Raman spectroscopy was used to verify the identity of the fluorescing and  
187 non-fluorescing particles found on the filters in order to ascertain the specificity of Nile red to  
188 only stain particles of synthetic origin. In total, 23 fields (23 × 1.8 mm<sup>2</sup>) from 6 different filter  
189 sections (4 sediment samples, 2 water samples) were imaged as described above. Raman  
190 spectra were acquired using an inVia Raman microscope (Renishaw). Raman shifted spectra  
191 were recorded using a 442 nm excitation laser in a range of 100 to 3500 waves cm<sup>-1</sup> and 10 s  
192 exposure time accumulating 20 scans. Particles were bleached during 5 min prior to spectrum  
193 acquisition as Nile red fluorescence interfered with the Raman signal. The baselines of  
194 Raman spectra were corrected in R (v3.2.3)<sup>35</sup> using the peak detection method from the  
195 baseline package<sup>36</sup> and then normalized.

196 To control for procedural contamination, Milli-Q water was processed in equal  
197 conditions as described above for environmental samples. To avoid lab contamination, lab  
198 coats were worn during all procedures, slides were washed with acetone, other glassware and  
199 filtering devices were thoroughly rinsed with Milli-Q water, pristine plasticware was used  
200 (see supplied protocol regarding required precautions during sample handling, such as

201 avoiding low quality pipette tips; fig S4), and Nile red staining solution was freshly filtered  
202 through 0.2  $\mu\text{m}$  filters.

203

#### 204 *Data analysis*

205

206 Automated quantification of fluorescing particles from tow and blank samples was performed  
207 in ImageJ using the macro described above. For each sample type, green fluorescence images  
208 (109 total) were randomly taken from 5 different filters. Two power-law models were fitted to  
209 the particle size distribution with `powerLaw`<sup>37</sup> in R. Different  $x_{\text{min}}$  values were used to  
210 estimate the scaling factor: either (1) the smallest particle present in the dataset or (2) an  
211 estimate at which the probability distributions of the particle size distribution and the best-fit  
212 power-law were most similar above  $x_{\text{min}}$ .<sup>38</sup> The latter discards particles below the estimated  
213  $x_{\text{min}}$  for which the power-law model is not valid. Testing for other distributions capable of  
214 explaining our data was done in accordance with Clauset *et al.*<sup>38</sup> Plotting was performed in R  
215 using the package `ggplot2`.<sup>39</sup>

216 A detailed protocol and the code for the macro to semi-automatically quantify  
217 fluorescent microplastics in ImageJ are available as supplementary materials.

## 218 **Results**

219

### 220 *Validation of the automated Nile red protocol using commercially available plastics*

221 *Fluorescence assisted counting.* Polymers PE, PP, PS, nylon 6, PC, PET, PVC and PUR  
222 fluoresced in green after staining with Nile red (fig. 1) demonstrating the utility of Nile red to  
223 detect and quantify small microplastics. Tire rubber did not fluoresce (fig. S1 f). Visual  
224 quantification can be performed directly under a microscope, but the implementation of a  
225 macro to automate counts allows high throughput counting as well as rapid measurement of  
226 the plastic particles. Here, fluorescence based automated detection of microplastics on PCTE  
227 membranes was 100 % for 4 polymer types (*i.e.* PE, PP, PS and nylon 6) as all 10 particles of  
228 each respective polymer were detected with ImageJ using 29 as the lower threshold for pixel  
229 brightness (fig. 1). The other 4 polymers (PUR, PC, PVC and PET) fluoresced weaker and a  
230 lower threshold value for pixel brightness (*i.e.* 9) was required to automatically detect all 10  
231 particles (fig. 1).

232 As fluorescence intensity varied with polymer type and thickness, the original setting  
233 for the pixel brightness threshold (*i.e.* 29) in our macro for ImageJ was optimized to capture  
234 all particles with strong fluorescence (*i.e.* PE, PP, PS and nylon 6), and to represent particle  
235 size accurately, using brightfield images as size references (figs. 1 & 2). As stated above,  
236 100% detection of PUR, PC, PET, and PVC was achieved by lowering the threshold value for  
237 pixel brightness. However, the adoption of the lower threshold (1) overestimated the size of  
238 more strongly fluorescing particles (*i.e.* PE, PP, PS and nylon 6 in fig. 2), (2) counted strongly  
239 fluorescent particles in very close proximity as one unique particle, and (3) so increased the  
240 risk of false positives (*i.e.* chitin in fig. 1; discussed below).

241

### 242 *Implementation of the fluorescent-staining protocol to environmental samples*

243

244 *Digestion of biogenic material.* Wood lignin fluoresces green and red when stained with Nile  
245 red (fig. S2 a). However, particles of this natural polymer, which are below 1 mm in size,

246 were completely eliminated after applying a 7-hour H<sub>2</sub>O<sub>2</sub> digestion protocol (fig. S2 b). As  
247 with wood lignin, chitin also fluoresces in green and red when stained with Nile red (fig S2  
248 c), but was not completely removed during the 7 hour H<sub>2</sub>O<sub>2</sub> treatment. Interestingly, after  
249 digestion, chitin showed a strong decrease in green fluorescence intensity (but not red  
250 fluorescence; fig. S2 d), possibly due to reduced hydrophobicity in response to oxidation. To  
251 test whether chitin would interfere with the detection and quantification of synthetic polymers  
252 in the green spectrum, we performed our protocol on a mix of chitin and PE. A stark  
253 distinction between PE particles and chitin was observed (fig S2 e–g) as the weak  
254 fluorescence given by chitin did not interfere when using our highly stringent macro settings  
255 (pixel brightness of 29), but chitin is detected to some extent when the settings are brought  
256 down (pixel brightness 9). This result highlights two issues that need to be considered when  
257 using this protocol: (1) Nile red strongly fluoresces under the GFP settings when staining  
258 highly hydrophobic plastics (such as PE, PP, PS) and, hence, green fluorescence should be  
259 used to eliminate background and inclusion of natural contaminants; and (2) reducing the  
260 sensitivity for the detection of less hydrophobic plastics (e.g. PC, PVC, PUR and PET) can  
261 come with a risk of including the detection of particles of natural origin.

262

263 *Detection and quantification of microplastics in environmental samples.* Here we isolated  
264 microplastics from environmental samples (*i.e.* beach sediment and sea surface) with  
265 saturated NaCl solutions and, hence, expected to extract plastics with densities  $\leq 1.2 \text{ g/cm}^3$   
266 (*e.g.* PE, PP, PS and nylon 6). We applied our Nile red staining protocol to discriminate small  
267 microplastic particles from other materials based on fluorescence (fig. 3) as well as to  
268 quantify and measure them. The automated ImageJ quantification of microplastics from the  
269 sea surface samples using stringent settings resulted in a total of 199 fluorescent particles,  
270 ranging between 20 – 338  $\mu\text{m}$  in size (*i.e.* particle size was obtained from the square root of  
271 the area measured for each individual particle; fig. 4). Neither of the power-law models  
272 describing the data could be dismissed ( $p_{x,\text{min}=20.02} = 0.72$  and  $p_{x,\text{min}=101.76} = 0.85$ ). The particle  
273 size distribution followed a power-law more closely for particles  $>101 \mu\text{m}$ , than if all data

274 were used, *i.e.* the smallest particle size = 20  $\mu\text{m}$  (see table S1 for statistical details). The  
275 calculated scaling factors were 2.13 for  $x_{\text{min}} = 20.02$  and 4.42 for  $x_{\text{min}} = 101.76$ .

276 Only one fluorescent particle was detected in our negative controls (*i.e.* processed  
277 Milli-Q water) demonstrating that laboratory contamination was minimal. It is of uttermost  
278 importance to include controls in order to assess the contamination acquired during the  
279 processing of samples.

280 For this protocol to be effectively applied to environmental samples we realise it is  
281 critical that *only* plastic particles should fluoresce and, hence, be quantified with the semi-  
282 automated process. Consequently, we scanned *via* Raman spectroscopy a total of 60  
283 fluorescing and non-fluorescing particles and found that all of the fluorescing particles ( $n =$   
284 37) were of synthetic origin, while all non-fluorescing particles ( $n = 23$ ) gave *non-plastic*  
285 Raman signatures (*e.g.* fig. 3). The environmental samples predominantly contained PP-type  
286 polymers (83.8%), although PE was also found (16.2%). The Raman spectra of the PP and PE  
287 particles contained slight variations in peak structure (fig. S5), which can occur in  
288 commercial polymer materials due to the inclusion of additional compounds and pigments or  
289 as a consequence of environmental weathering.<sup>40</sup>

## 290 Discussion

291

292 We present a fast, reliable and cost-effective method for detecting, quantifying and  
293 determining the size of small PE-, PP-, PS- and nylon 6 type microplastics (20  $\mu\text{m}$  – 1 mm)  
294 commonly present in sea surface samples.<sup>10,11,41</sup> This method uses the lipophilic dye Nile red  
295 to fluorescently label plastics and requires fluorescence microscopy to capture images at  
296 magnification 10 $\times$  prior to automated, image-based quantification in ImageJ using a macro  
297 (both protocol and macro are provided as supplementary information) enabling high  
298 throughput image analysis. Specific protocols for collecting and extracting microplastics from  
299 the environment were not in the scope of this work.

300 During the preparation of this manuscript, two studies were published<sup>28,42</sup> that  
301 reasserted our findings, demonstrating the effectiveness of Nile red to fluorescently label  
302 different types of commercially available synthetic polymers, such as the ones employed here,  
303 all of which fluoresce in the green spectrum (black rubber was not used in these previous  
304 studies and we show here that it does not fluoresce). Indeed, similarly to us, Shim *et al.*<sup>28</sup>  
305 concluded that green-yellow fluorescence (excitation/emission 450–490/515–565 nm)  
306 provided better particle recognition than red or blue fluorescence. Using a different light and  
307 filter set-up, Maes *et al.*<sup>42</sup> reported good fluorescence at longer wavelengths ranging from  
308 yellow to orange, depending on the polymer type. Such findings agree with previous reports  
309 about the behaviour of Nile red, which favours detection of strongly hydrophobic samples at  
310 short excitation/emission wavelengths (450–500/ $\leq$ 580 nm) compared to more neutral lipids,  
311 which should ideally be visualised at longer excitation wavelengths (*i.e.* red, 515–560/ $\geq$ 590  
312 nm).<sup>30</sup> For example, given that PE and PP are more hydrophobic than PET,<sup>43</sup> it is expected  
313 that the former will fluoresce more intensely at shorter wavelengths (*i.e.* green), while their  
314 fluorescence at longer wavelengths remains weak or even absent as we show in figure S1. We  
315 are therefore confident that the Nile red protocol we propose here is effective in detecting  
316 strongly hydrophobic plastics such as PE, PP, PS and nylon 6 through the use of GFP settings  
317 (green fluorescence), while preventing detection of contaminants, that would fluoresce at

318 longer wavelengths. We acknowledge the protocol's limitations for the less hydrophobic  
319 polymers PC, PUR, PET and PVC, which constituted about 25% of the European plastic  
320 demand in 2015.<sup>9</sup> These limitations can to some extent be overcome as suggested in the  
321 results section by increasing the sensitivity of the method (fig. 1), but this comes at a risk of  
322 overestimating the size of strongly fluorescent polymers (fig. 2) as well as incurring the  
323 possibility of false positives, such as chitin. It is also worth highlighting that all polymer types  
324 that fluoresced weakly in our study when stained with Nile red (PC, PUR, PET, PVC) are  
325 denser ( $\geq 1.2 \text{ g/cm}^3$ ) than the polymers that fluoresced more strongly (PE, PP, PS, nylon 6,  
326  $< 1.08 \text{ g/cm}^3$ ). Hence, the latter can be extracted using a saturated NaCl solution as done in  
327 this study, while denser polymers would require a higher density salt solution (e.g. NaI).

328         The successful application of a Nile red protocol to environmental samples relies on  
329 the efficient removal of biogenic particles that could be detected as false positives. As we  
330 show here, abundant natural polymers such as chitin and wood lignin fluoresced when stained  
331 with Nile red (fig. S2 a, c). Shim *et al.*<sup>28</sup> remained cautious on applying Nile red to quantify  
332 microplastics in environmental samples due to the risk of co-staining undigested biogenic  
333 material. We speculate that the problem they encountered resided in the weak digestion  
334 treatment they applied on their beach samples (*i.e. soaking* the filters with 35%  $\text{H}_2\text{O}_2$ ), which  
335 resulted in biogenic debris such as an amphipod carapace and plant parts still being present.  
336 In turn, Shim *et al.*<sup>28</sup> reported less such contamination for the neuston net samples, which  
337 were digested with the more aggressive Fenton reagent (including heating to  $75^\circ\text{C}$ ). Hence, a  
338 harsh digestion protocol such as the one we used here and which was previously suggested by  
339 Claessens *et al.*<sup>31</sup>, is required to prevent co-staining of natural organic polymers and  
340 confidently quantify Nile red-stained microplastics in environmental samples. Common  
341 plastics such as PE, PP, PS, PET and nylon 6 are resistant to  $\text{H}_2\text{O}_2$ , as demonstrated by Tagg  
342 *et al.*<sup>32</sup> during a 7-day exposure experiment, where no significant chemical changes were  
343 detected *via* FTIR, as opposed to alterations observed elsewhere that were induced by  
344 solvents such as acids and bases (e.g. HCl or NaOH).<sup>34,44</sup> In addition to the  $\text{H}_2\text{O}_2$  digestion, we  
345 propose to include a 1 mm mesh-size sieving step prior to the digestion to prevent the

346 inclusion of larger, hard-to-digest natural contaminants, such as amphipods or pieces of  
347 wood. If required, enzymatic digestion protocols could be implemented to digest biota-rich  
348 environmental samples.<sup>4,44</sup> Indeed, sample purification may further be optimized by  
349 combining digestion procedures with a density separation protocol, such as presented by  
350 Maes et al.<sup>42</sup> Nevertheless, our results show how the 30% H<sub>2</sub>O<sub>2</sub> digestion step used here was  
351 effective at preventing detection of small natural polymers (below 1 mm in size); wood lignin  
352 was completely degraded and chitin was no longer was detectable in ImageJ using green  
353 fluorescence images (fig S2). Furthermore, we successfully proved that all of the fluorescing  
354 particles from environmental samples assessed with micro-Raman spectroscopy (n = 37) were  
355 identified as synthetic plastic materials, whereas no non-fluorescing particles scanned (n =  
356 23) showed a synthetic polymer signature.

357 Other semi-automatable methods to detect and quantify small microplastics in  
358 environmental samples were recently developed.<sup>32,45,46</sup>

359 Chemical mapping *via* micro-FTIR was shown useful to detect and identify small  
360 microplastics directly on filters when combined with FPA detectors,<sup>32,45</sup> FPA detectors can  
361 record several thousand spectra simultaneously and plastics are then identified based on  
362 characteristic bands that are shared by synthetic polymers. However, access to such  
363 specialised pieces of equipment is not always possible, and the time required to image a  
364 whole filter membrane (10.75 h for a 25 mm diameter filter)<sup>45</sup> is significantly higher than  
365 when using the method in the present study *i.e.* 20 min.

366 A second semi-automatable approach used to detect small microplastics from  
367 environmental samples combined Micro-Raman spectroscopy with particle finding  
368 software.<sup>46</sup> The software provides geographical positions of the particles on a slide, and the  
369 particles are then scanned individually *via* a motorised stage. However, Frère *et al.*<sup>46</sup> did not  
370 apply this technique directly to the sample filter (such as in this study and others<sup>32,45</sup>). Instead,  
371 particles were visually pre-selected under a dissection microscope and then transferred onto a  
372 gold-coated microscope slide. It is therefore not yet clear whether this technique is also  
373 applicable to quantify small microplastics without potentially introducing visual bias.

374 While our Nile red staining method does not provide the chemical identity of the  
375 detected plastic particles (as achieved *via* FTIR and Raman), we present it as a sensitive, cost-  
376 effective and unbiased way of quantifying and measuring small PE, PP, PS and Nylon 6  
377 particles in environmental sample preparations to acquire large datasets with high statistical  
378 value. Ultimately, a fraction of pinpointed plastic particles should be identified *via* micro-  
379 Raman spectroscopy to obtain information on the diversity of polymer types within a sample.  
380 Moreover, despite having tested the most common potential natural contaminants, we  
381 advocate the use of micro-Raman spectroscopy on subsamples until full reliability of the  
382 method presented here has been evaluated.

383 Micro-Raman spectroscopy of plastic particles detected with Nile red showed that PP  
384 microparticles were more prevalent (83.8%) in our environmental samples than PE (16.2%).  
385 This is notable as PE is the most commonly produced polymer type<sup>47</sup> and literature highlights  
386 PE as the most abundant polymer on sea surfaces.<sup>48</sup> A recent study, however, found that this  
387 is only the case for large microplastics (>1 mm), as the smaller analysed size fraction (0.335 –  
388 1 mm) was dominated by PP (42%) rather than PE (26%).<sup>46</sup> Furthermore, the authors reported  
389 a lack of PS in size classes below <2 mm,<sup>46</sup> which resembles our findings. Curiously, PS was  
390 present in the fraction retained by the 1 mm sieve (fig. S3) but not found among particles  
391 assessed with Raman. Several non-exclusive hypotheses may explain these findings. For  
392 instance, fragmentation behaviours may differ with polymer type and shape. Particles have  
393 also been observed to adhere to organic matter, such as marine snow, and sink.<sup>49,50</sup> It is  
394 unclear, however, why small PE particles would more likely be incorporated into marine  
395 snow than PP particles, and further research is required to shed light on this interesting  
396 phenomenon.

397 Plastics in the environment are known to progressively fragment into smaller  
398 particles.<sup>2</sup> Based on the fragmentation pattern of three dimensional objects, it could be  
399 expected that the abundance of microplastic particles increases following a power-law with a  
400 factor of 3 as size decreases.<sup>5</sup> Contrary to this assumption, Cózar *et al.*<sup>5</sup> reported an intriguing  
401 loss in abundance of small microplastics after carrying out a global survey of sea surface

402 marine plastic debris. The expected correlation between size and fragment abundance was  
403 observed down to a particle size of 2 mm but, surprisingly, the abundance of microplastics  
404 sharply decreased for particles below 1 mm in size. This supported speculation regarding the  
405 substantial ‘missing’ fraction of marine plastic debris initiated in 2004,<sup>14</sup> and recently  
406 reviewed by Eriksen *et al.*<sup>51</sup> We believe that the extremely low incidence of small  
407 microplastics reported by Cózar *et al.*<sup>5</sup> may partly be ascribed to the methods employed in  
408 identifying and selecting particles, which were based on visual sorting under a dissecting  
409 microscope. In fact, in a study whose findings mirrored our own, Enders *et al.*<sup>52</sup> showed that  
410 small microplastics were indeed present in surface waters with increasing abundance as size  
411 decreased, and obtained a scaling factor of 1.96 for the size range of 10 – 110  $\mu\text{m}$ , close to  
412 one obtained in this study (*i.e.* scaling factor of 2.13). Further research is nevertheless  
413 required, as very little is known about the fragmentation pattern and particle behaviour of  
414 different polymer types in the marine environment.<sup>53</sup>

415         Here we suggest the use of a highly sensitive Nile red fluorescent staining method for  
416 identifying the smaller size range of lower density microplastics (<1 mm) commonly present  
417 in sea surface samples (*i.e.* PE, PP, PS and nylon 6). We acknowledge its limitations, but do  
418 not exclude its application, for less hydrophobic polymer types, a separation that coincides  
419 with higher polymer densities (>1.2  $\text{g}/\text{cm}^3$ ; fig. 1). Using this time- and cost-effective  
420 protocol to quantify and measure small microplastics allowed us to confirm that small  
421 microplastics are increasingly abundant with decreasing particle size in sea surface samples  
422 (fig. 4). This method therefore addresses the quantification uncertainties and provides an  
423 effective tool for rapid quantification of small microplastics by substituting the visual  
424 selection and quantification process with an automated process.

425

#### 426 **Supporting Information**

427 Five figures (green and red fluorescence, Nile red stained natural polymers, microplastics >1  
428 mm, contamination control, example Raman spectra of microplastics from environmental

429 samples), one table with statistical details, a sample preparation protocol and the code for the  
430 ImageJ script used to quantify fluorescent microplastic particles; all as noted in the text  
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432

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440 Robyn Wright for providing input throughout the study. The authors declare no conflict of  
441 interest.

442

443 **Author contributions**

444 G.E.C., J.C.-O. and M.I.G. conceived the study. G.E.C. designed and conducted the  
445 experimental work. R.C.T. provided expertise and advice on fieldwork, microplastic  
446 identification and gave access to the facilities. G.E.C. and J.C.-O. analysed the data and wrote  
447 the paper. M.I.G. and R.C.T. provided their expertise and reviewed the manuscript.

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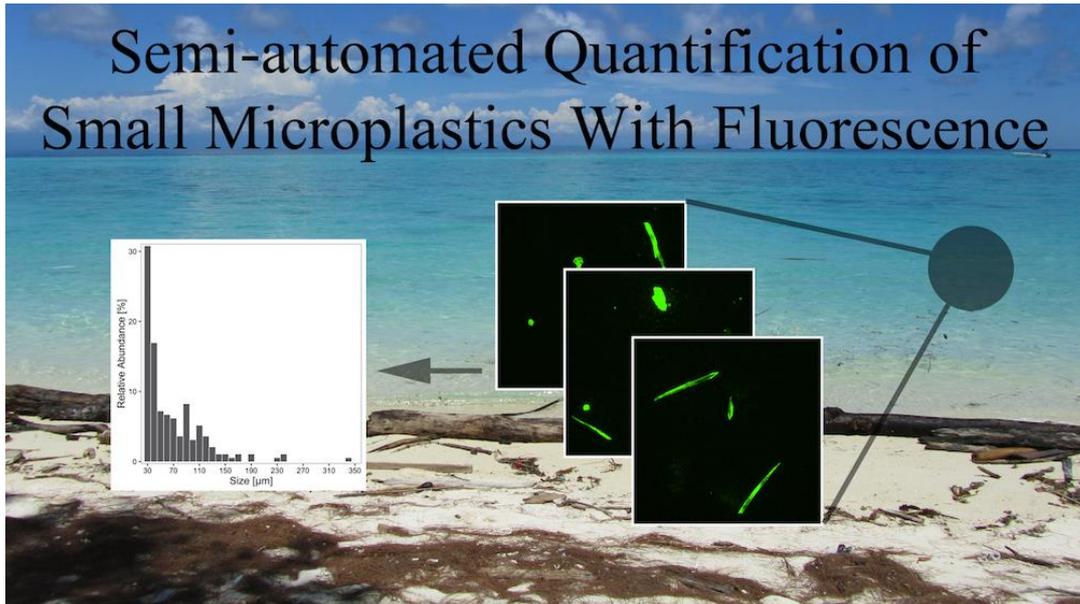
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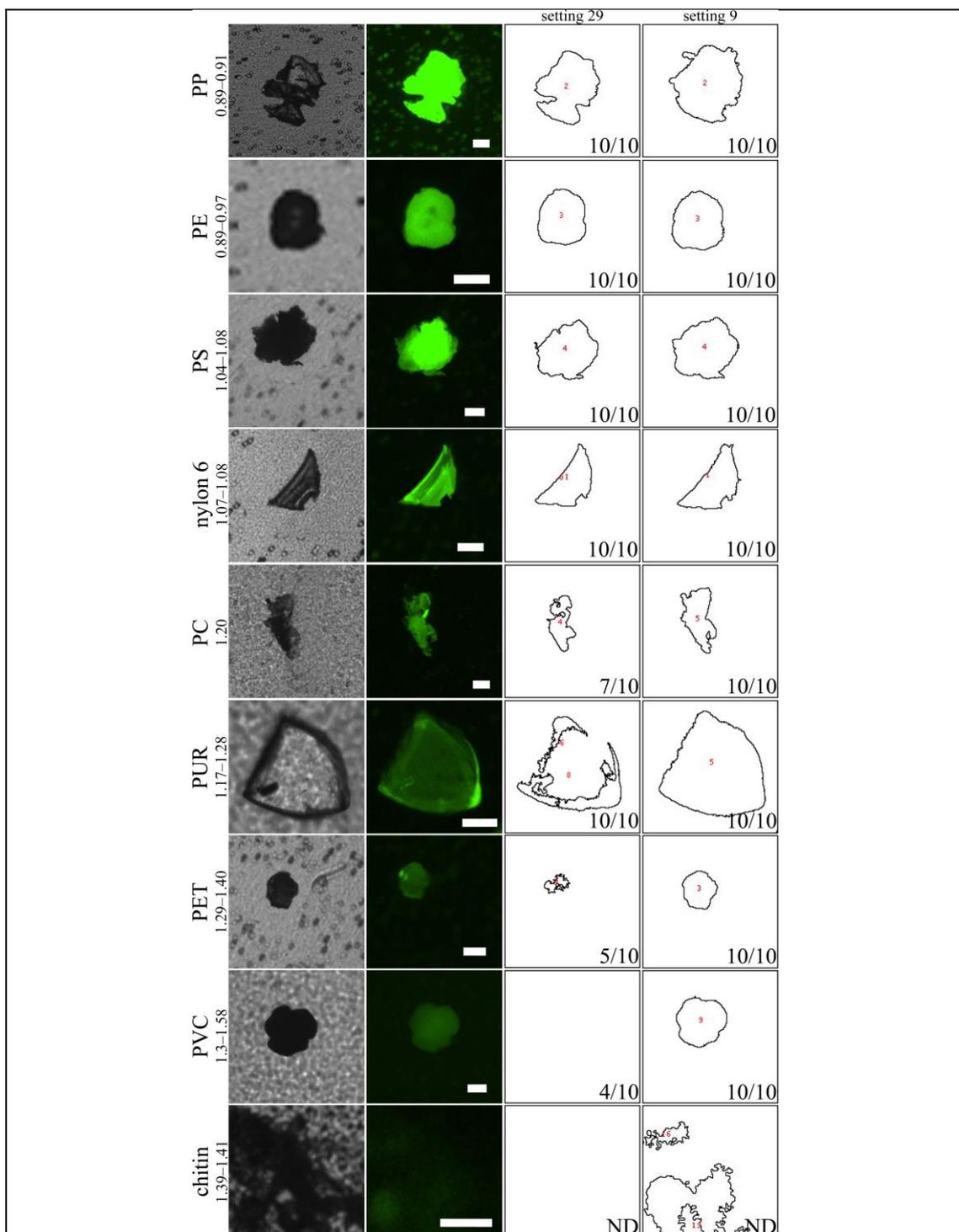
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# Semi-automated Quantification of Small Microplastics With Fluorescence

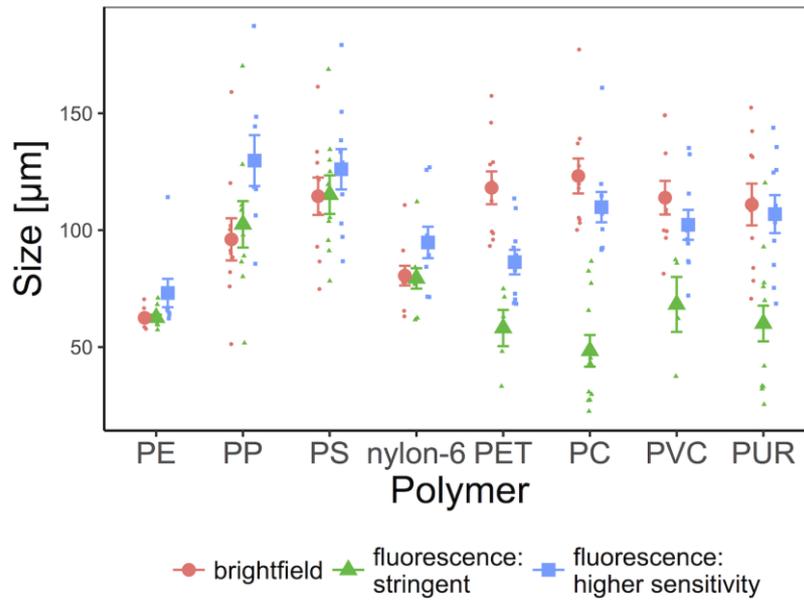


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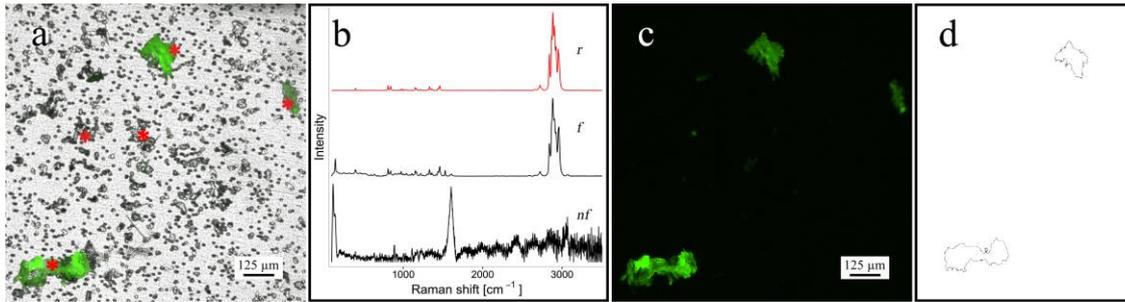
**Figure 1.** Microscope and ImageJ images of microparticles of different polymer types on PCTE filter membranes stained with Nile red. For each polymer, images show from left to right: a particle in brightfield, the same particle in green fluorescence (excitation/emission 460/525 nm), ImageJ rendition with stringent settings (setting 29) and ImageJ rendition with more sensitive settings (setting 9). Ratios in ImageJ renditions indicate the number of particles ( $n = 10$ ) detected with the respective setting and polymer; ND: not determined. Polymers are in descending order in accordance with increasing specific density ( $\text{g}/\text{cm}^3$ ), indicated below polymer name; PP: polypropylene, PE: polyethylene, PS: polystyrene, PC: polycarbonate, PUR: polyurethane, PET: poly(ethylene terephthalate), PVC: poly(vinyl carbonate). Scale = 50  $\mu\text{m}$ .





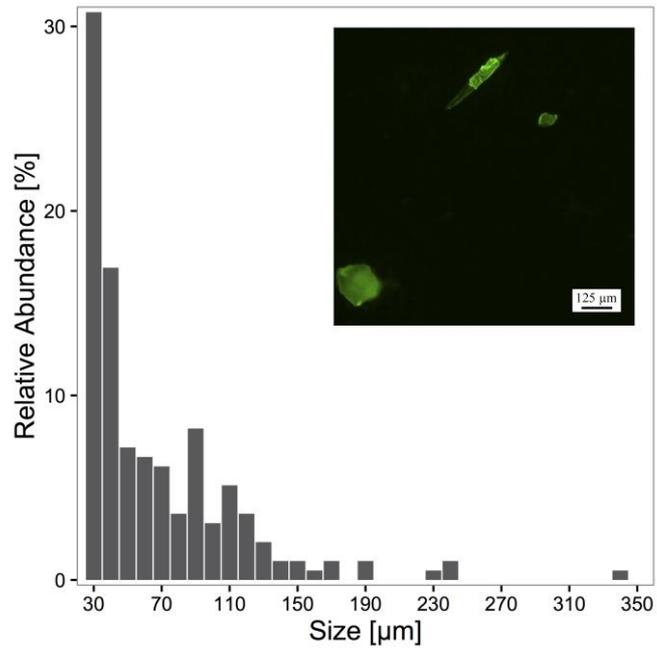
**Figure 2.** Mean size ( $\pm$  SE) comparison of microplastic particle ( $n = 10$  per polymer type) size measured in ImageJ using either brightfield images or green fluorescence images with our script. Note: *stringent* represents sizes measured with 29 as the lower threshold for pixel brightness and *higher sensitivity* corresponds to measurements generated with 9 as the lower threshold for pixel brightness. Size corresponds to the square root of particle area.

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**Figure 3.** Microscope images of processed sand samples demonstrating selective Nile red fluorescent staining of synthetic polymers with Raman spectra of scanned particles. (a) Composite image of excitation/emission 460/525 nm and brightfield. Asterisks indicate particles assessed via Raman-spectroscopy. (b) Normalised Raman spectra obtained from particles highlighted in image b. *r*: PP (Sigma-Aldrich) spectrum; *f*: typical spectrum of fluorescent particle in image b; *nf*: typical spectrum of non-fluorescing particle in image b. (c) Field shown in panel (a) using green fluorescence only. (d) ImageJ drawing depicting particles  $>400 \mu\text{m}^2$  that were quantified *via* our macro.

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**Figure 4.** Relative abundance of microplastic particle sizes ( $n_p = 199$ ) from all sea surface samples analysed via automated counting of fluorescent particles using 109 different microscope fields (one image shown as example). Size corresponds to the square root of the particle's area.

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