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## A semi-automated Raman micro-spectroscopy method for morphological and chemical characterizations of microplastic litter

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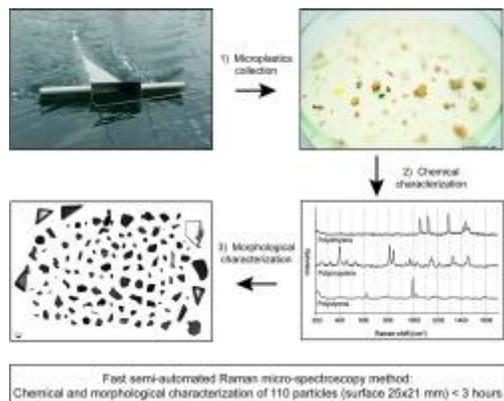
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### Abstract :

Every step of microplastic analysis (collection, extraction and characterization) is time-consuming, representing an obstacle to the implementation of large scale monitoring. This study proposes a semi-automated Raman micro-spectroscopy method coupled to static image analysis that allows the screening of a large quantity of microplastic in a time-effective way with minimal machine operator intervention. The method was validated using 103 particles collected at the sea surface spiked with 7 standard plastics: morphological and chemical characterization of particles was performed in < 3 h. The method was then applied to a larger environmental sample (n = 962 particles). The identification rate was 75% and significantly decreased as a function of particle size. Microplastics represented 71% of the identified particles and significant size differences were observed: polystyrene was mainly found in the 2–5 mm range (59%), polyethylene in the 1–2 mm range (40%) and polypropylene in the 0.335–1 mm range (42%).

## Graphical abstract



## Highlights

► Semi-automated micro-spectroscopy Raman method coupled to static image analysis ► Time-effective and reproducible method requiring minimum operator intervention ► High particles identification rate (> 70%) ► Fast morphological and chemical characterization of environmental particles ► Efficient method for microplastics monitoring in marine environment

**Keywords** : Microplastics, Raman micro-spectroscopy, Surface seawater, Morphology, Environmental monitoring, Automating

## 1. Introduction

For more than a decade, microplastic litter has been of concern to society and the scientific community, with increasing research effort dedicated to establishing the fate of microplastic litter and its impact on aquatic ecosystems. The upper size limit of microplastics was defined as 5 mm at the first International Microplastic Workshop organized by US National Oceanographic and Atmospheric Agency in 2008 (Arthur et al., 2009), while no standard boundary has yet been set for the lower size limit. A recent study led by Eriksen et al. (Eriksen et al., 2014) estimated that more than 5 trillion plastic pieces pollute the surface of the world's oceans, with microplastics accounting for 92.4% of the global particle count. These plastic debris is the result of the ever increasing global plastic production, from 1.7 in 1950 to 311 million tons in 2014 (PlasticsEurope, 2016). The subsequent release of plastic waste into the marine environment was estimated at 4.8 to 12.7 million tons per year (Jambeck et al., 2015). Microplastics are either classified as “primary” for the particles and fibers that are directly manufactured at the micrometric size (coming from a variety of sources including cosmetics, clothing, and industrial processes such as pre-production pellets and industrial abrasives) or as “secondary” when they result from the breakdown of larger plastic debris in the environment. The majority of marine plastics are considered to originate from land-based sources, introduced via many pathways: riverine inputs, waste water treatment plants, wind driven transport, marine based activities (Cole et al., 2011). Once in marine ecosystems, microplastics are efficiently transported across the oceans and seas and many studies have reported their presence in all compartments: waters, sediments and biota (Lusher, 2015; Wright et al., 2013).

As a consequence, an increasing number of studies are investigating microplastic concentration and distribution in coastal ecosystems in order to understand the extent as well as the hazard of this emerging pollution (Lusher, 2015). Currently several methodological limitations prevent accurate interpretation and comparison of environmental data (Filella, 2015). These mainly concern non-harmonized sampling methods leading to the inclusion or exclusion of certain types of microplastic (< 335  $\mu\text{m}$ , fibers, etc.), sample contamination, inconsistent units used to express data, lack of certificate reference material, standardization and adequate reporting, and problems with the accuracy and representativeness of samples. Regarding the latter, misidentification may occur when relying only on visual inspection under a dissection microscope to separate microplastic-like polymers from other organic and inorganic materials, and when analyzing only a fraction of the sampling. Both of these commonly occur due to time and cost constraints (Hidalgo-Ruz et al., 2012). The non-utilization of complementary methods to confirm the chemical composition of microplastic-like polymers may lead to high levels of misidentification, which has been estimated from 20% (Eriksen et al., 2013a) to 70% (Hidalgo-Ruz et al., 2012), with a negative correlation between successful identification and particle size (Filella 2015; Löder et Gerdts 2015). Indeed, a high probability of a misidentification exists when particle size is below 50  $\mu\text{m}$  (Hidalgo-Ruz et al., 2012; Lenz et al., 2015).

The most common techniques used to confirm the nature of micro-particle samples are infrared and Raman spectroscopies, which are both nondestructive (Rocha-Santos and Duarte, 2014). Raman and FTIR spectroscopies are complementary techniques, because molecular vibrations which are Raman inactive can be FTIR active and vice versa. However, Raman is able to achieve a better spatial resolution (down to 1  $\mu\text{m}$ ) than FTIR (10  $\mu\text{m}$ ) (Lenz et al., 2015). Raman spectroscopy involves the irradiation of the sample with a monochromatic laser source and determines vibrations that cause a change in the polarizability of the sample (Neha et al., 2014). The interaction of the laser light with the molecules and atoms of the sample results in differences in frequency of the backscattered light compared to the irradiating laser frequency (Imhof et al., 2012; Imhof et al., 2013). For both methods, the main disadvantage is the considerable time required for the sample analysis. As a consequence, particle recognition and identification are often carried out on a subsample, due to the extensive time that would be required to analyze the whole sample (Enders et al., 2015; Harrison et al., 2012; Song et al., 2015). To counteract this limitation, automated spectral mapping should be developed and used preferentially, as it could provide high throughput and thorough analysis of large sample sizes. The critical parameters required for the implementation of such automated scanning relate to the need to find an optimal combination of laser wavelengths that minimizes the interference between samples containing particles of a highly diverse nature (biological, inorganic, plastic), and to avoid background signals produced by the sample holder. Lastly, automatic data analysis is necessary for the data collection of other relevant information from microplastic litter, such as morphological characteristics, which is valuable for estimating its source (e.g. cosmetics, clothing, and industrial processes) and the fragmentation processes involved.

The aim of the present work was to develop, test the reproducibility and validate a time effective method using semi-automated scanning of particles to enable the thorough analysis of large quantities of environmental samples for microplastic characterization. The method involves a combination of static image analysis of particles with an automated Raman micro-spectroscopy analysis. The optimal combination between laser wavelength and sample holder was determined in order to enhance accurate particle detection and relevant Raman signal. The method was tested by analyzing a set of 103 particles collected at sea, spiked with 7 known reference polymers. Reproducibility was tested by comparing chemical characterization, shape and size data in three replications of the analysis. Finally, once validated, this method was applied to a larger sample (n=962 particles collected at sea) in order to test potential correlations between morphological features and the chemical nature of the particles.

## **2. Experimental Section**

### **2.1. Reference materials and particles collected at sea**

Fragments ( $2592 \pm 727 \mu\text{m}$ ; SI, table S1) of seven additive-free reference polymers supplied by GoodFellow (Lille, France) were used as spikes: polyethylene (PE), polypropylene (PP), polystyrene (PS), unplasticized polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyamide-6 (PA-6) and polyurethane (PUR). These 7 reference polymers were randomly positioned among 103 collected particles on a gold coated microscope slide prior to Raman micro-spectroscopy analysis. These 103 particles were randomly selected for the method validation from floating micro-particles collected in the Bay of Brest (Brittany, France) in September 2014 using a Manta trawl. The top ten centimeters of surface seawater was sampled by using a Manta trawl (0.6 x 0.16 x 2 m) with a 335  $\mu\text{m}$  mesh size. The net was rinsed with seawater to concentrate all floating material into the cod end. The sample was transferred into a 0.2 l sterile glass jar before laboratory analysis. Particles were sorted under a dissecting microscope (Leica Microsystems MZ6) and stored on a fiber glass filter at  $-20 \text{ }^\circ\text{C}$  (Frère et al., in revision).

### **2.2. Raman micro-spectroscopy method**

Measurements were carried out using a LabRAM HR800 Raman micro-spectrometer (Horiba Scientific), equipped with a Horiba Scientific ParticleFinder module for LabSpec6. This equipment provided easy and quick localization, counting and morphological characterization (size, area, perimeter, shape) of the 110 particles. Once all particles had been placed on a gold coated microscope slide, a video montage of the entire sample covering a large field of view of 25 x 21 mm area was captured with the aid of the motorized stage. From this, a single microscope image was obtained. The gold coated microscope slide was chosen as it avoids the signals generated by borosilicate glass or "unclean" non-gold microscope slides and does not contribute to the Raman signal. Moreover, for imaging, a reflective support allows better picture quality by lighting from above, rather than from below as with transparent microscope slides; this is especially true if plastics are translucent and / or very diffuse. Statistics of key 2D morphological features (minor and major size, particle area, diameter, and perimeter) and 2D shape descriptors (ellipse ratio and circularity) were calculated by the software for each particle.

After particle localization and morphological characterization, automated Raman analysis was performed. The ParticleFinder software module allowed automatic motorized stage positioning and analysis of particles. One spectrum was collected on the center of each particle. Parameters were selected to obtain a good compromise between spectra quality and time needed for the measurements: analyses were carried out using a 785 nm wavelength of a laser diode (Oxxius), as best fluorescence/signal ratio, and were performed using a 10-fold magnification objective (Olympus) with

numerical aperture of 0.25 and in a wavenumber range of 200 – 1700  $\text{cm}^{-1}$  with a spectral resolution of around 4  $\text{cm}^{-1}$  using 300 lines per millimeter grating. Spectra were collected with two accumulations of an integration time of 10 seconds for each particle. Spectral calibration was performed by zero-order correction of the used grating and with a crystalline silicon wafer using the known band at 520  $\text{cm}^{-1}$ . The chemical identification of particles was realized using commercial Raman libraries (KnowItAll Informatics Systems, Bio-Rad, Raman ID Expert) and the home database.

Raman micro-spectroscopy analysis was repeated 3 times in 3 different runs under the same conditions (R1 to R3) except for particle spatial location. Indeed, between each run, particles were randomly switched and were therefore analyzed at a different spatial location and at a different orientation on the gold coated microscope slide.

### **2.3. Application of the method to an environmental sample**

Once validated, the method presented here was applied to a larger set of floating micro-particles collected in the Bay of Brest (Brittany, France) in April and October 2014 using a Manta trawl and following the protocol detailed above in 2.1. All microplastic-like particles extracted from these samples (n=962 particles; fibers excluded) were subsequently analyzed for thorough morphological and chemical characterization. This chemical identification of particles was carried out using commercial Raman libraries and the home database as described above.

### **2.4. Data analysis**

To test the reproducibility of the method, a Chi-Square test and a Kruskal-Wallis test were applied to compare the proportion of observations in each chemical family and the mean of the morphological characteristics (minor and major size, particle area, diameter, perimeter, ellipse ratio and circularity) respectively between the 3 runs. All particles analyzed (n=110 for method validation and n=962 for method application) were assigned to one of three size classes, based on the major axis length: 0.335 – 1 mm range, 1 – 2 mm range and 2 – 5 mm range. For the environmental sample, a Chi-Square test was applied to compare the number of observations for each chemical family between the three size classes. Kruskal-Wallis tests followed by Bonferroni-Dunn post hoc tests were used to compare morphological characteristics between each chemical family identified in the environmental sample. All statistical analyses were conducted using the RStudio 0.99.491 platform (R Development Core Team, 2015). The significance level for all analysis was set at 95%.

## **3. Results and discussion**

### **3.1. Method validation**

#### **3.1.1. Chemical characterization**

All reference polymers were successfully identified for each of the three runs (Figure 1). Out of the 103 particles collected at sea, 74 - 75% of them were successfully identified and 71 - 72% of the particles were microplastics of different polymer nature (PE, PP, PS, PUR; Figure 2). The other identified particles (3 - 5%) corresponded to inorganic particles (calcium carbonate ( $\text{CaCO}_3$ ) and quartz ( $\text{SiO}_2$ )). The non-identified particles (25 - 26%) exhibited either spectra of a blue pigment (phthalocyanine blue, PB15) masking the signal of the polymer (5 - 6%), spectra with no identification in either the commercial libraries or the home database (4%), spectra exhibiting no signal (2 - 8%) or saturated signal (8 - 14%) (Figure 2). Saturated signals are mainly due to fluorescent molecules coming from pigments or environmental organic matter such as biofilm. It is noteworthy that the proportion of particles exhibiting saturated signal could potentially be reduced by wavelength modification or by applying a pre-treatment of the samples to remove the organic matter (e.g. biofilm, algae, etc.) covering the microplastics, as performed by Imhof et al. (2016). But these optional alternatives in the methodology are time consuming. The fraction of non-identified particles may originate from the presence of different compounds such as additives and/or dyes within the polymer matrix which may modify considerably the spectrum and thus complicate the identification of the particle (Imhof et al., 2016; Lenz et al., 2015; Van Cauwenberghe and Janssen, 2014). In the present study, only two dyes were identified: PB15 (blue dye) and titanium dioxide ( $\text{TiO}_2$ , white dye). While  $\text{TiO}_2$  did not prevent polymer identification (SI, figure S1), in some case the PB15 (blue dye) overlaid completely the polymer matrix. A chemometric approach, based on Moreau and Rinnert's (2015) work, was applied in order to separate the spectral sources in a mixture (polymers, additives, etc.) but it was not possible to reveal the polymer spectrum by subtracting the pigment spectrum due to its more intense Raman signal (Van Cauwenberghe et al., 2013b; Van Cauwenberghe and Janssen, 2014). No significant difference in particle identification success was noticed between the 3 runs (Chi-Square test, p-value > 0.05), emphasizing the robustness of the assay.

Some studies adapted the analytical parameters such as laser-set wavelength, integration times or magnifications for each particle to improve the rate of identification for particles with no or poor Raman signal quality (Collard et al., 2015; Enders et al., 2015). Similarly, Imhof et al. (2016) suggested increasing the acquisition time up to 100-500 sec per particle in order to distinguish the polymer spectrum from the pigment. However, these single particle optimizations result in a substantial increase of the processing time which is not appropriate for fast analysis of large sample set. The novelty in the present study is that analytical parameters were the same for all particles, which represents a time-saving improvement for microplastic characterization. Raman analysis took approximately 40 minutes for each run (surface analyzed: 25 x 21 mm; 110 particles) and did not need machine operator intervention as the analysis was automated once particles were detected with ParticleFinder software.

### **3.1.2. Morphological characterization**

The 110 particles (103+7) were successfully located and individually characterized using size and shape descriptors defined by ParticleFinder (Figure 3) and no significant difference was noticed between the 3 runs for each of the 7 descriptors (Kruskal-Wallis test, p-values > 0.05). Size and shape descriptors such as major and minor axis, perimeter, diameter, area, ellipse ratio and circularity (Table 1) allowed the thorough measurement of irregular objects, and particle positioning by the machine operator did not affect these measurements. Such morphological assessment is of relevance to better characterize microplastics because inadequate understanding of the particle size is probably behind the loss of particles in water sample analyses (Filella, 2015). Indeed, in most microplastics studies particles size are expressed in terms of diameter or longest dimension, which are clearly not sufficient to unequivocally inform on the particle size and shape, especially when considering irregular and fibrillar particles. Such thin/irregular particles may then slip through the net of the sampling device (for instance here a Manta trawl) especially for particles with size close to the lower size limit of the mesh. Some studies used a specific image analysis software to characterize single particle morphology, involving several manipulations of particles with a risk of particle loss and a great increase in the processing time (Dekiff et al., 2014; Enders et al., 2015; Imhof et al., 2016.). In the present study, about 2 hours were required to create the image of the 110 particles, but as it was automated it did not require operator time. Once the image was created, around 15 minutes were necessary to apply and adjust thresholds and morphological filtering operations to enhance particles localization prior to Raman analysis in order to be sure that all particles will be taken into account during the Raman spectra acquisition.

### **3.1.3. Outcomes of the complete analysis**

The results of the 3 runs validated the method for microplastics collected in surface water in the Bay of Brest with a size range between 0.335 and 5 mm. This methodology offers the possibility to analyze the morphological and chemical features of all micro-particles with the same device, thus avoiding any transfer or loss of particles, and in a time effective way. Indeed, less than 3 hours were required for the complete analysis of 110 particles (surface area analyzed: 25 x 21 mm) representing a real improvement in the time required for microplastics characterization.

Only two studies have published time efficient measurement using chemical mapping: either by reflectance (Tagg et al., 2015) or transmittance (Löder et al., 2015) micro-FTIR spectroscopy to identify microplastics in polymer-spiked wastewater and in marine environment samples (plankton and sediment), respectively. Depending on the instrumental parameters selected for the analysis of whole filters, the measurements in these studies were achieved in less than 9 h for the entire analysis of a membrane filter of 47 mm (Tagg et al., 2015) and in 10.75 h for a circular area of ~11 mm of an aluminum oxide filter of 25 mm (Löder et al., 2015). These methods were performed for microplastics < 500 µm. Our time-saving measurement method for microplastic analyses includes a wider range of

sizes (0.335 to 5 mm) and of different types (pellet, fragment and film), coupling polymer identification and morphological analyses.

### **3.2. Application of the methodology on large environmental sample**

#### **3.2.1. Visual sorting is not sufficient**

Out of the 962 particles collected in surface water, 75% were chemically characterized. Microplastics (PE 48%, PP 12%, PS 11%) represented 71% of the whole sample (Figure 4), similar to the 68% of microplastics found in environmental samples after micro-spectroscopy Raman analysis (Lenz et al., 2015). The 4% of identified particles remaining are quartz (2%) and carbonates (2%). The non-identified particles (25%) exhibited only PB15 dye spectrum (3%), spectra with no correspondence with databases (6%), or a signal that was either absent or saturated (16%) (Figure 4). Many published studies used visual sorting (Collignon et al., 2014; Eriksen et al., 2013b; Faure et al., 2012; Free et al., 2014; Ivar do Sul et al., 2013; Lima et al., 2014; McCormick et al., 2014; Van Cauwenberghe et al., 2013a). However our results clearly indicate that visual sorting may lead to misestimating of actual microplastic concentrations thus suggesting that a complementary analysis is needed to confirm the polymer nature of the particles. This was highlighted recently for both particles and fibres (Song et al., 2015) (Wesch et al., 2016). In addition, it is noteworthy that the proportion of identified particles significantly decreased with the decrease of the major axis (Chi-square test,  $p$ -value  $< 0.05$ ) (Figure 4), which is accordance with the results of Lenz et al. (Lenz et al., 2015). At the same time, the proportion of minerals (quartz and carbonates) increased as the size decreased. This emphasizes the difficulty in visually differentiating microplastics from other organic and inorganic particles in smaller size classes. The non-identified fraction in the smallest size class (0.335 – 1 mm) could be better identified with an adaptation of the microscope objective. Further study is needed to test the identification success rate with different sized objectives.

As the proposed method is time effective, a large sample size can be analyzed in a reasonable amount of time, which prevents the downsizing to subsamples as is done in most studies (Claessens et al., 2011; Cózar et al., 2014; Dekiff et al., 2014; Desforges et al., 2014; Free et al., 2014; Goldstein et al., 2013; Martins and Sobral, 2011; Reisser et al., 2013; Sadri and Thompson, 2014; Zhao et al., 2015). When only subsamples are analyzed, the ratio between the total number of visually identified microplastics and the part that could be confirmed by spectral analysis must be used to correct the microplastics concentration of the whole sample (Enders et al., 2015). However it is not necessarily accurate and most importantly, it is sometimes impossible especially when the analyzed subsample represents a fraction too small (Ballent et al., 2013) or when the distribution of sizes varies greatly within each subsample.

### 3.2.2. The utility of particle morphology description

The main properties that drive the fate of particles are size, shape and density. Indeed, they are known to influence the particles' buoyancy, sedimentation, biofouling and fragmentation processes (ter Halle et al., 2016). Hence, the assessment of these morphological parameters is of interest when studying microplastic contamination in the marine environment. For all the parameters tested (minor and major axis, perimeter, diameter, area, ratio ellipse and circularity) significant differences were observed in the distribution of quartz, PE, PP and PS particles (SI, table S2). For instance, the distribution of collected particles differed among the size (defined by the major axis) classes (SI, table S3; p-values < 0.05). Quartz was found exclusively in the smaller size classes: 13% in the 1 – 2 mm range and 87% in the 0.335 – 1 mm range. This may be due to their high density ( $d=2.63 \text{ g cm}^{-3}$ ) leading to the fact that bigger sand particles do not remain buoyant in the water column even in conditions of water mixing. On the other hand, PS particles were mainly found in the 2 – 5 mm range (60%). The disappearance or low proportion of PS in the small size classes (< 2 mm) in surface water may be related to physical processes such as wind blowing particles on shore, fragmentation processes and water mixing influencing sinking behavior. Rising or sinking velocity depends on the difference in density of the polymer (1.04 - 1.09  $\text{g cm}^{-3}$  for PS or 0.01 – 1.05  $\text{g cm}^{-3}$  for expanded PS) to seawater ( $\sim 1.027 \text{ g cm}^{-3}$ ), and on the size and shape of the object (Ballent et al., 2013). The model developed by Enders et al. (2015) showed that there is a fundamental difference between the vertical dispersion of small and large microplastics, with vertical distribution of microplastics increasing as their size decreases. These authors also showed that for particles collected in surface water, microplastics exhibiting a density lower than seawater were significantly larger (> 1 mm) than microplastics with higher density. This was not observed here as PS was found in higher proportion in the 1 – 5 mm range (94%, probably due to the presence of expanded PS) than PE (74%) and PP (58%) (SI, table S3). In addition, even though PE and PP were more evenly distributed among the three size classes, a significantly higher proportion of PP (42%) was observed in the smaller sizes (0.335 – 1 mm) compared to PE (26%) despite the fact that both polymers exhibit similar densities (0.90 - 0.92  $\text{g cm}^{-3}$  for PP vs. 0.91 - 0.95  $\text{g cm}^{-3}$  for PE) (SI, table S3). This difference in microplastic size distribution in surface water may be related to fragmentation or biological processes influencing their fate: the propensity to be incorporated into phytoplankton aggregates (Lagarde et al., 2016; Long et al., 2015) or into the food chain, as demonstrated in laboratory experiments (Cole et al., 2015; Farrell and Nelson, 2013), may differ according to the polymer type. In addition, it could be reasonable to speculate that the efficiency of particles collection using a Manta trawl may vary according to the particles size and shape. For instance Shaw et al. (1994) observed that the concentration of microplastics was lower as their size approaches the lower sampling limit and the authors hypothesized that this reduction could be due to a preferential removal of this size of particles by marine organisms. Alternatively, this could also be attributed to differences in hydrodynamics and particle movements (Ter Halle et al., 2016) or loss of

irregular shaped fragments through the net as suggested by Isobe et al. (2014). Further studies measuring morphological descriptors are of interest to address the specific size distribution of chemical families in various environments as well as the efficiency of the sampling methods for particles of different size and shape.

#### **4. Conclusion**

The assessment of microplastics in marine environment is a multi-step process (sampling, extraction, detection and quantification of microplastics), in which each step is time consuming. The high variability in chemical composition and morphology of microplastics represents a bottleneck for field and laboratory analysis while it is a real challenge for answering crucial questions about the sources and fate of microplastics in aquatic environments. The present study offers a reproducible and time effective method for rapid and thorough morphological and chemical characterization of microplastics using a semi-automated scanning of particles coupled to micro-Raman spectroscopy. The rapid analysis of large number of collected particles allows for an exhaustive assessment of large sample sizes and not only small subsamples. Until recently, most published studies using spectroscopy confirmatory methods did not necessarily provide the success rate of particle identification or the detailed proportions of true microplastics vs. other organic or inorganic particles. Yet this information is crucial for assessing the representativeness of the results as well as the efficiency of this type of analysis. To ensure reproducibility, comparability and representativeness of the spectroscopic analyses in the emerging field of microplastic research, standard operating procedures describing the way to sample, extract, conduct analyses and also report data should be implemented as was done for instance in the field of molecular biology for Polymerase Chain Reaction (PCR) analysis to assure a high degree of standardization (see the MIQE guidelines, Bustin et al., 2009).

Finally, the thorough assessment of particle morphological features of 962 particles collected at sea revealed significant differences in size/area distribution as a function of the chemical nature of the particle. These results raised questions about physical (fragmentation, sedimentation, water mixing) and biological processes (bioavailability, interactions with living cells) that may influence microplastic distribution in the first layers of the water column, and whether these influences could be polymer dependent. Further studies measuring these particle morphological features are encouraged for addressing the specific size distribution of the polymer families for various environments.

#### **5. Acknowledgements**

This study was partly funded by the MICRO EU Interreg-funded project MicroPlastics (MICRO 09-002-BE). We acknowledge I. Bihannic, M. Tardivel, K. Boukerma, F. Colas, V. Larat, S. Vergnole

and M. Cachera for helpful discussions and technical assistance. We thank E. Harney for his help in editing the English. L. Frère was funded by a joint Brest Métropole and Brest University grant.

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**Table 1:** Summary of morphological and shape descriptors of the 103 collected microplastics

Collected particles		Size descriptors				Shape descriptors		
		Area (mm <sup>2</sup> )	Diameter (mm)	Perimeter (mm)	Major axis (mm)	Minor axis (mm)	Ellipse ratio	Circularity
Replicate R1	Min	0.081	0.322	1.247	0.368	0.198	0.216	0.603
	Max	3.379	2.074	9.536	3.354	1.642	0.984	0.947
	Mean	0.577	0.786	3.255	1.051	0.646	0.669	0.812
	SD	0.561	0.344	1.513	0.513	0.294	0.173	0.075
Replicate R2	Min	0.061	0.279	1.021	0.312	0.172	0.216	0.603
	Max	2.420	1.755	6.639	2.066	1.664	0.984	0.947
	Mean	0.494	0.740	3.045	0.974	0.611	0.669	0.812
	SD	0.386	0.287	1.204	0.394	0.263	0.173	0.075
Replicate R3	Min	0.063	0.283	1.099	0.304	0.131	0.169	0.534
	Max	2.354	1.731	6.595	2.344	1.653	0.982	0.949
	Mean	0.497	0.745	3.070	0.985	0.614	0.672	0.817
	SD	0.378	0.280	1.229	0.403	0.255	0.169	0.080

## Figures and table

**Figure 1:** Spectra of the 7 reference polymers used, obtained by Raman analysis for 3 independent runs (R1 to R3). Intensity was normalized by the highest intensity peak.

**Figure 2:** Proportion of each polymer family for 3 independent measures (R1 to R3). (PUR = polyurethane, PA-6 = polyamide-6, PET = polyethylene terephthalate, uPVC = unplasticized polyvinyl chloride, PS = polystyrene, PP = polypropylene, PE = polyethylene).

**Figure 3:** Image of the montage of the 110 particles analyzed for method validation (picture of replicate R1).

**Figure 4:** Proportion of particles identified and not identified in seawater samples (n=962 collected particles) depending on size class (0.333-1/ 1-2/ 2-5 mm) (Chi-Square test, p-value < 0.05).

**Figure 1**

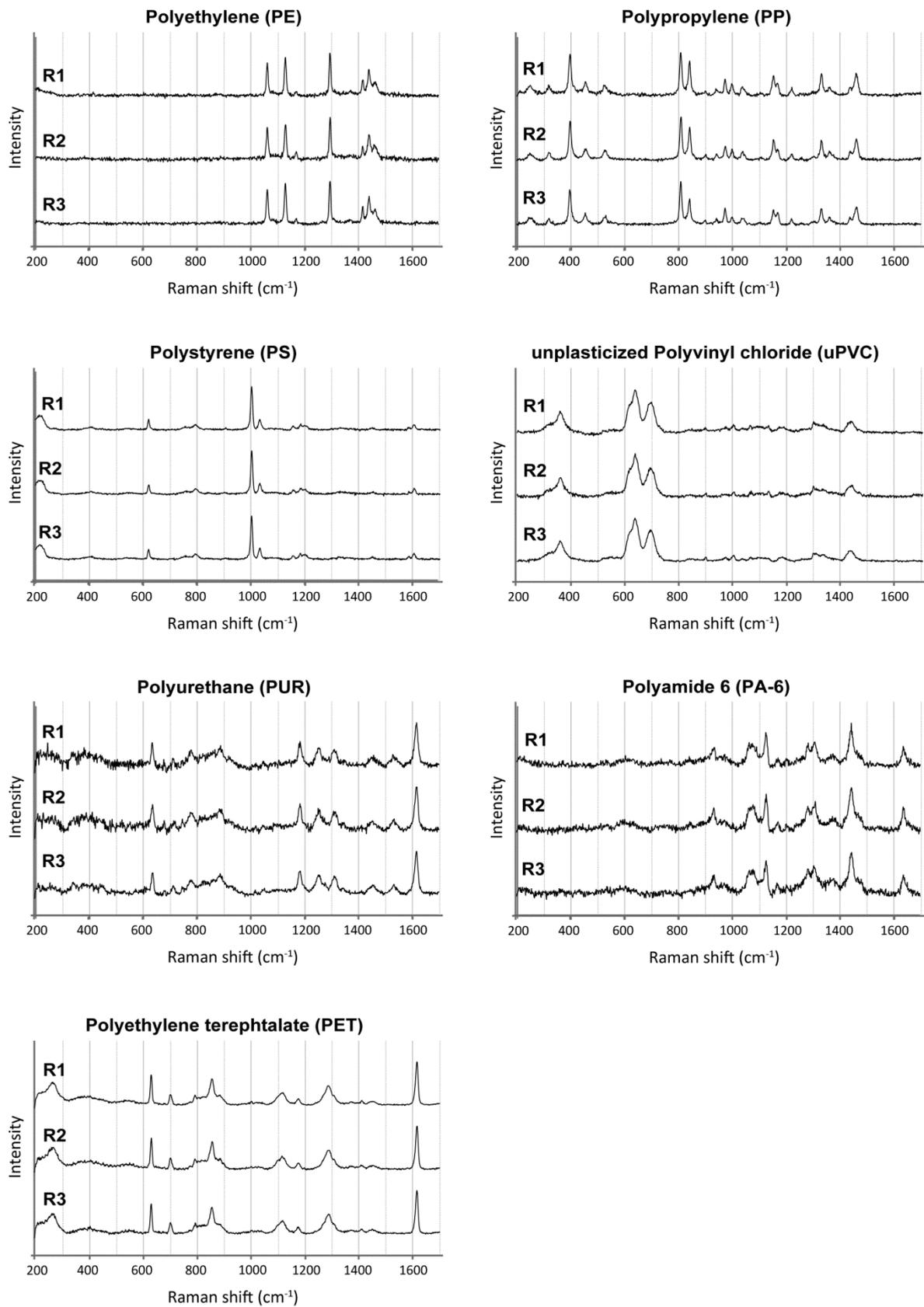


Figure 2

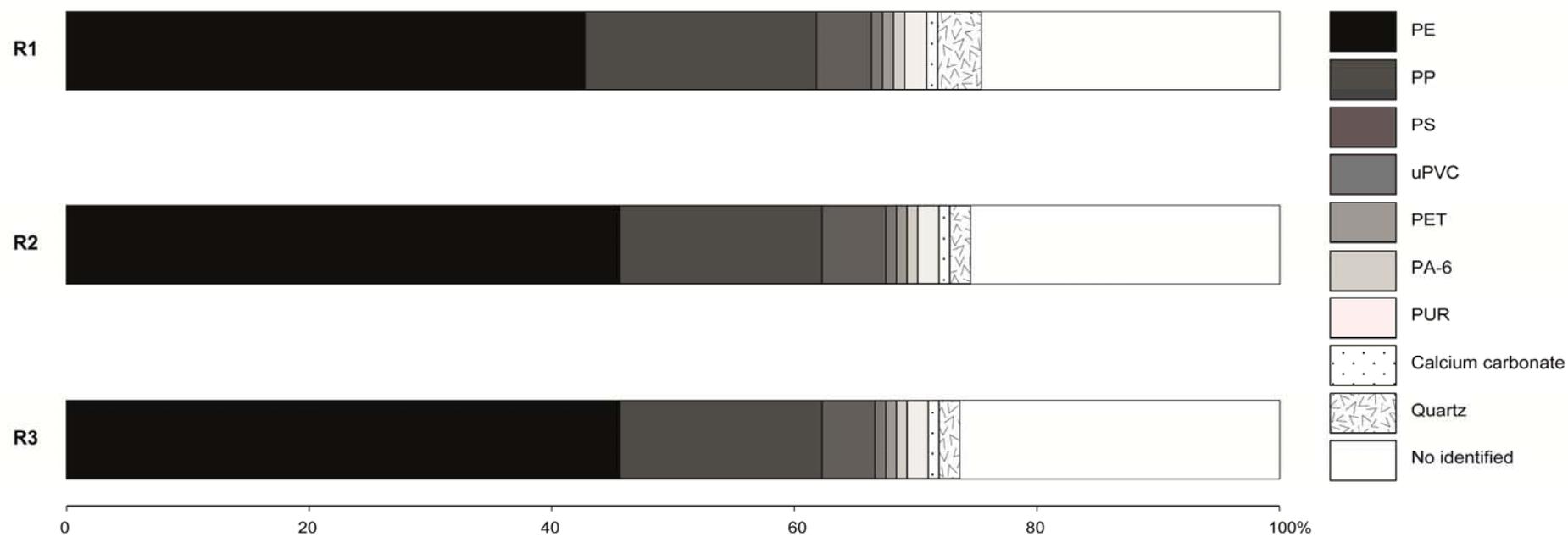
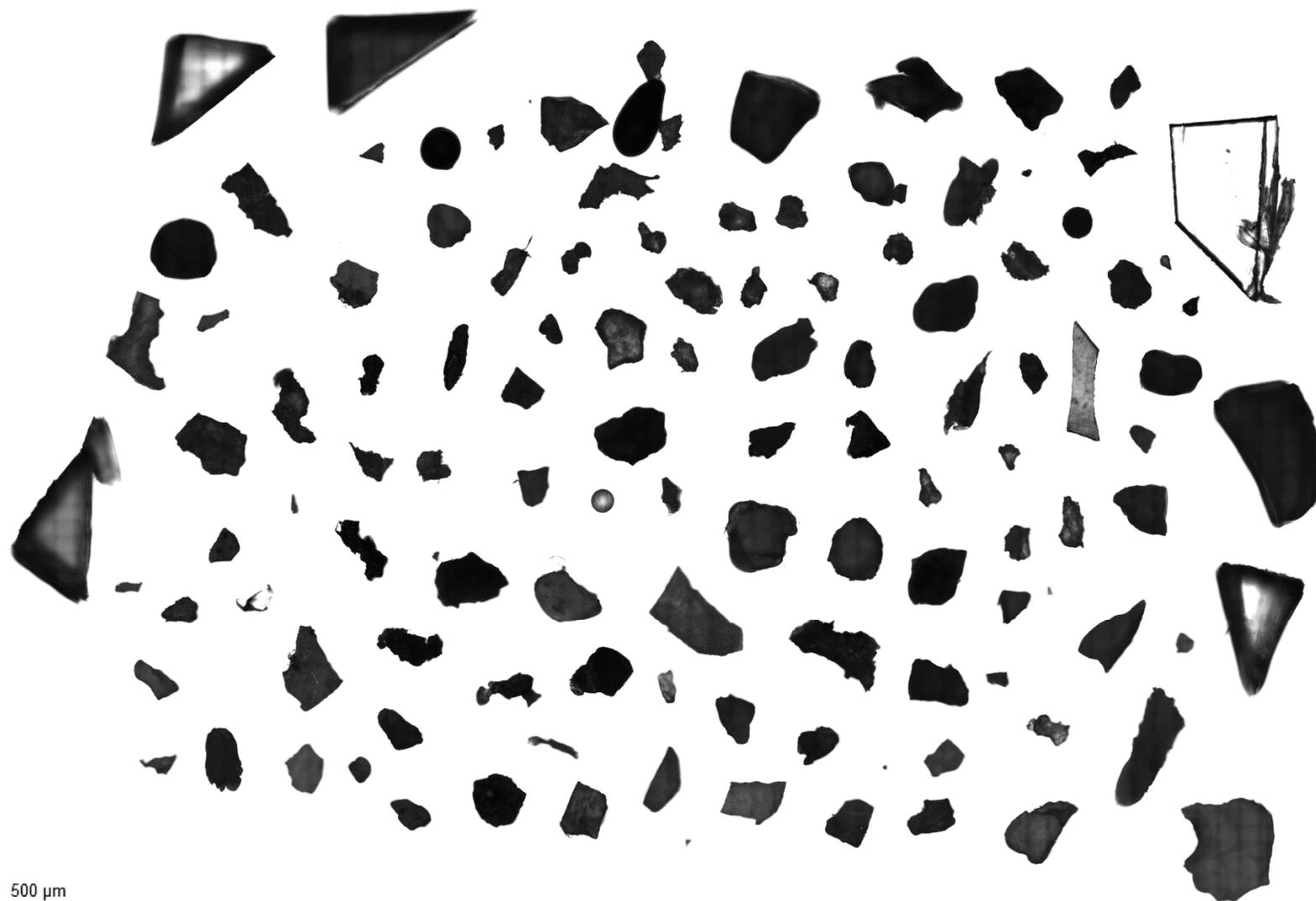
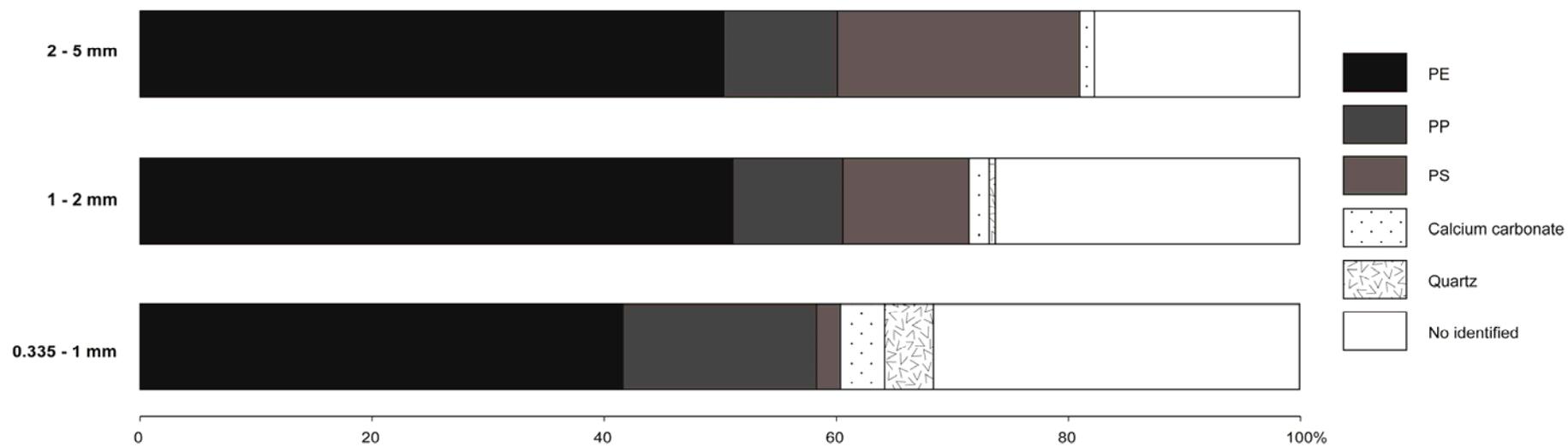


Figure 3



500 μm

Figure 4



# Supplementary information

## A semi-automated Raman micro-spectroscopy method for morphological and chemical characterizations of microplastic litter

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**Table S1:** Size of all reference polymers used as spikes.

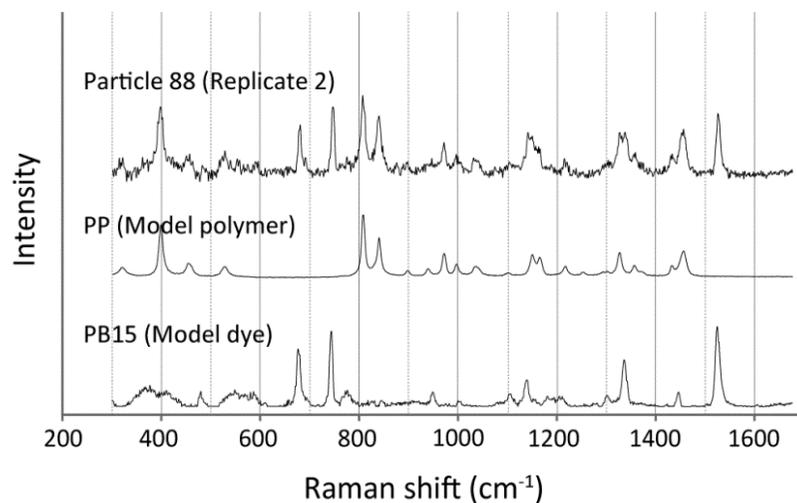
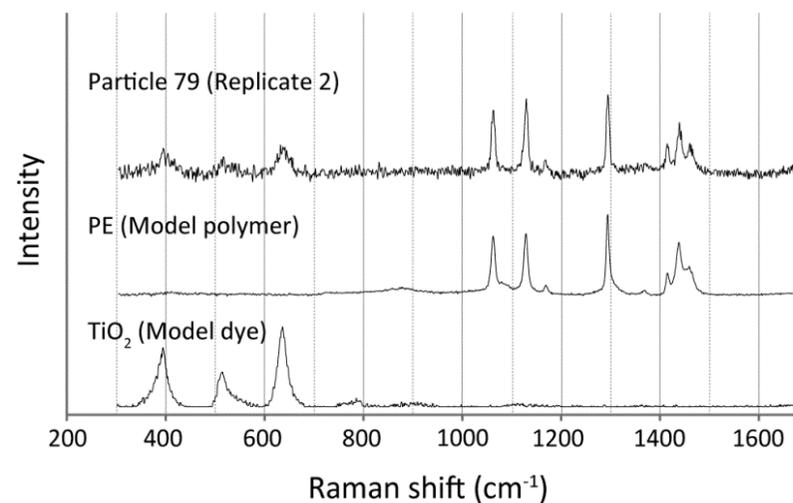
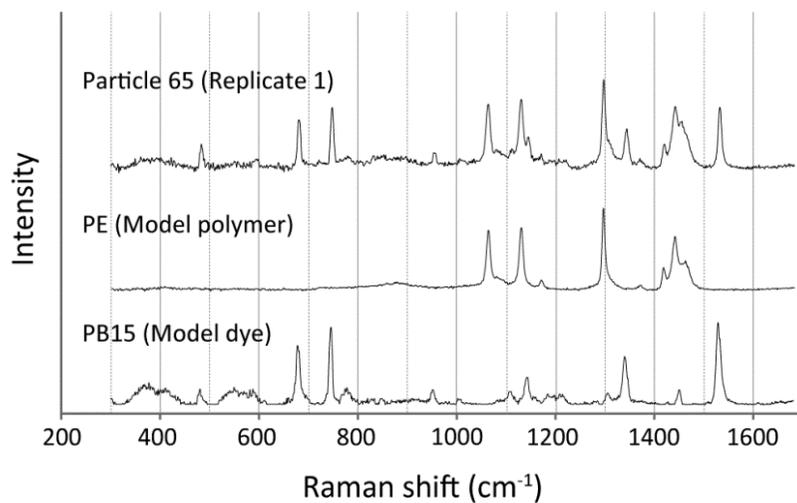
<b>Plastic references</b>	<b>Major axis (<math>\mu\text{m}</math>)</b>	<b>Minor axis (<math>\mu\text{m}</math>)</b>
PA-6	3312	1404
PE	3300	1304
PET	1221	890
PP	2923	1229
PS	2735	1617
PUR	2360	701
uPVC	2292	1421

**Table S2:** Kruskal-Wallis rank sum test to assess the difference in morphological features as a function of particle nature.

Morphological and shape features	p-values	degree of freedom	Posthoc Dunn test results
Major axis	$< 2.2 \cdot 10^{-16}$	4	PS $\neq$ PE, PP, Q, CaCo3 PE $\neq$ PP, PS, Q PP $\neq$ PE, PS, Q
Minor axis	0.1103	4	No significant difference
Diameter	$< 2.2 \cdot 10^{-16}$	4	PE $\neq$ PP, PS, Q PP $\neq$ PE, PS, Q PS $\neq$ PE, PP, Q, CaCo3
Perimeter	$3.3 \cdot 10^{-16}$	4	PE $\neq$ PS, Q, CaCo3 PP $\neq$ PS, Q PS $\neq$ PE, PP, Q, CaCo3
Area	$< 2.2 \cdot 10^{-16}$	4	PE $\neq$ PP, PS, Q, CaCo3 PP $\neq$ PE, PS, Q PS $\neq$ PE, PP, Q, CaCo3
Ratio Ellipse	0.1498	4	No significant difference
Circularity	$< 2.2 \cdot 10^{-16}$	4	PE $\neq$ PP, PS, Q, CaCo3 PP $\neq$ PE, PS, Q, CaCo3 PS $\neq$ PE, PP, Q, CaCo3

**Table S3:** Density ( $\text{g cm}^{-3}$ ) and size distribution (%) of collected particles in surface water (seawater density:  $\sim 1.027 \text{ g cm}^{-3}$ ).

Particle types	Density ( $\text{g cm}^{-3}$ ) (GESAMP 2015)	Size distribution in surface water (%)		
		0.335 - 1 mm	1 - 2 mm	2 - 5 mm
Polyethylene	0.91 - 0.95	26	40	34
Polypropylene	0.90 - 0.92	42	30	28
Polystyrene (expanded polystyrene)	1.04 - 1.09 (0.01 - 1.05)	5	35	59
Quartz	2.63	86	14	0
Calcium carbonates	2.70 - 2.90	52	29	19



**Figure S1:** Spectra of colored particles collected in the Bay of Brest, polymer and dye references obtained by Raman analysis (PE: polyethylene, PP: polypropylene, PB15: phthalocyanine 15, TiO<sub>2</sub>: Titanium dioxide). Each intensity was normalized by the highest intensity peak.