A new method for analyzing microplastic particle size distribution in marine environmental samples

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Abstract

Microplastics (MPs) are recognized as a global emerging threat to aquatic ecosystems and biodiversity worldwide. Though the number of publications and interest to the MP research have been increased rapidly, it is still hamper to compare the obtained data due to the usage of different methodologies in MP assay. Thus, there is an urgent need for a standardized approach to the procedures of MP quantification in order to produce comparative assessments. In this pilot study, the conventional NOAA protocol of MP extraction from seawater was combined with a simple and inexpensive method for analyzing shape and size spectrum of all MP particles making up the sample. A common flatbed scanner equipped with slide adapter was applied for image acquisition while MP dispersive properties (particle abundance, shape and size spectrum) were quantified using ImageJ software. Feret’s diameter and circularity (or roundness) appeared to be the most efficient shape descriptors for the particle analysis. The total silhouette area of MP particles was shown to produce a confident approximation of the MP overall mass. The first reliable estimates of MP concentrations in the Black Sea coastal waters (Sevastopol Bay) accounted for 0.6 to 7 items m$^{-3}$ and 6 to 750 µg m$^{-3}$ in terms of abundance and mass, respectively. No steady-state gradients have been revealed in MP distribution along the transect from the mouth of the bay to its corner. Inflow of MP to the bay waters and their transport along the bay seemed to be controlled by a complex combination of factors including rainfalls, wind regimes, currents and the Black river discharge.

Key words: Microplastics, pollution, marine, method, image acquisition, particle analysis, ImageJ, the Black Sea.

Introduction

MPs have become an emerging global environmental issue in the last few years due to their huge amount and ubiquity in the seas and oceans (do Sul, Costa, 2014; Rezania et al., 2018; Khatmullina and Chubarenko, 2019). At the current stage of MP pollution research, an extensive approach is prevailing, which is based on accumulating broad data on MP concentrations and masses in various marine water areas. Size spectra of MP particles drifted in the water column are still poorly studied despite the fact that urgent need of the
information about their dispersive properties is crucial not only for tracking and modeling in marine environments (Chubarenko et al., 2018), but also for predicting their effects on biota. The effect does depend on the particle size and, according to expanding experimental results, includes trophic processes (Egbeocha, et al., 2018), cell coagulation and planktonic aggregate formation (Alimi et al., 2018), and ecotoxicological effects (Anbumani and Kakkar, 2018).

Instead of tedious and time consuming size and shape analysis, the rapid and accurate technique is required for the further promising development in MP research. Therefore, we propose a simple and inexpensive method for analyzing shape and size of MP particles combined the NOAA protocol (Masura et al., 2015). This new approach was applied in a pilot study of assessment of MP pollution in Black Sea coastal waters.

Material and Methods

Sampling. MP samples were collected at four stations in Sevastopol bay, the Black Sea (Fig. 1), from January to May 2019 (16 samples in total). The neuston net has a rectangle design with the dimension of 1 x 0.5 m, and a mesh size is 300 µm. The net was submerged (0-25cm) in the sea surface, and towed for 20 min at a constant speed of ~2 knots. The flow meter was fitted in the mouth of the net, and used to measure the volume of water passing through the net during sampling. After sample collection, the net was washed and its content transferred to a pre-labeled glass container and kept in the ice box.

![Sampling sites in Sevastopol Bay, the Black Sea.](image)

Sample preparation. The collected samples were poured through a 5-mm sieve and concentrated in the 100-µm filtration funnel (the pore size smaller than 300 µm was used to minimize losses of fine particles and fibers). The materials retained on 5-mm sieve were discarded. The sample was rinsed with distilled water (to remove salts). The solids were washed from the funnel to a glass beaker to the final volume between 200 and 500 mL (depending on amount of solid material).

Wet peroxidation (WPO). 200-500 mL of 60% hydrogen peroxide was added to the wet sample to double its volume (30% H₂O₂ final concentration). If reaction appeared to have the potential to overflow the beaker, distilled water was added to slowdown the reaction. The mixture was left on a lab bench at room temperature till the end of the reaction (until no foam, bubbles, and organic residues obtained). The process could be fastened by stirring and heating (50-75°C on a hotplate) the solution. When foam disappeared, the solution was placed to a thermostat or a drying oven at +75°C. Usually, WPO took about 24 h. If natural organic material (like solid plant seeds, etc.) was still visible, hydrogen peroxide was added to continue the reaction.

Density separation. After WPO, the sample was washed from the beaker to the 100-µm filtration funnel with distilled water, rinsed carefully with 5 M NaCl solution, and washed from the funnel to the density separator with 5 M NaCl solution. The solids were allowed to settle overnight. The settled solids were drained from the separator and discarded. The floating solids were collected onto the 'storage’ filter
(100-µm nylon mesh), using a 45-mm standard filtration funnel. The sample was rinsed carefully with distilled water, dried at room temperature and kept in a clean Petri dish (Fig. 2, A, B).

**Figure 2.** A: Microplastic samples dried on the ‘storage’ filters (100-µm nylon mesh). B: Pure microplastics under an inverted microscope. C-D: Micrograph of a sample in Bogorov’s camera and its b/w image processed in an image editor. E: High-contrast scan image of a microplastic sample.

**Weight measurements.** 300-µm nylon mesh filter was weighted and placed to a standard filtration funnel (45-mm diam.). The sample was washed from the ‘storage’ filter to the funnel and collected onto the weighted nylon filter. The <300-µm fraction was trapped onto the ‘storage’ filter. The filter with >300-µm solids dried and its mass was measured using an analytical balance. The mass of the total solids (MPs) collected on the filter was calculated as the difference between the above two measurements.

**Microscopic examination.** MPs were washed from the ‘storage’ filter, resuspended in distilled water and poured into a Bogorov’s chamber (commonly used for zooplankton enumeration). Particles and fibers were distributed uniformly on the camera surface using a dissecting needle. Digital micrographs of all fields
of view were taken in transmitted light (all objects appeared as silhouettes) using an inverted microscope Nikon Eclipse TS100-F equipped with a camera (Ikegami ICD-848P) (Fig. 2, C). An image of a micrometer object was used for scale calibration.

**Scanning the sample.** The dry MP sample was rinsed off the ‘storage’ filter, resuspended in absolute alcohol and poured into a special glass cuvette placed on the glass platform of a flatbed scanner equipped with a slide adapter. Particles and fibers were distributed uniformly in the cuvette, using a dissecting needle. The material was scanned in the slide mode (transmitted light). A micrometer object was scanned additionally for scale calibration. Scanning resolution set to 2400 dpi was high enough to cover the particle size range between 0.3 and 5 mm.

**Counting and sizing particles and fibers in ImageJ.** Before the analysis, the micrographs and scans had to be processed (e.g. using Adobe Photoshop software) to obtain high-contrast black and white images of the sample, with the particles as black silhouettes on the white background (Fig. 2, D, E).

The processed images were then analyzed in ImageJ v.1.45s software (https://imagej.nih.gov, Rasband, 1997–2012). The procedure was included into 3 steps: (i) scale calibration (menu “Set Scale”), (ii) color mask (menu “Color Threshold…”), and (iii) particle analysis (menu “Analyze Particles”). In the result table, three shape descriptors were chosen for further analysis: Feret’s diameter (*Feret*), circularity (*Circ*) and area (*S*). *Feret* was a measure of maximum size of the particle (maximum distance between two points of the object boundary, as in Ferreira and Rasband, 2011) while *Circ* described its shape, varying between 1 for circle and 0 for thinnest fibers. The value of *Area* was used to approximate the particle mass.

In the result tables, the particles were sorted in descending order of *Feret* to exclude ones above 5 mm and below 0.3 mm and classified in the space of the two variables, *Feret* and *Circ* (*Feret*-*Circ* scatter). Fibers were usually characterized by extremely low *Circ* and a wide range of sizes (*Feret*). The largest plastic items were interpreted as fibers.

**Results**

Four shapes of MP particles were recognized and categorized as: rounded, irregular, elongated, and fibers. Their distribution patterns in a *Feret*-*Circ* scatter plot allowed us to separate the fibers cluster which occupied in the bottom of the plot (dashed line in Fig. 3).

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**Figure 3.** Distribution of the four types of MP particles (red – rounded, violet – irregular, blue – elongated, and green – fibers) in a Circularity-versus-Feret’s diameter plot (scatter). Dashed line represents the upper border of fibers.
The scatter plots obtained for the MP samples showed high variability in particle shape and size (Fig. 4). Their upper right corners (large rounded objects) were mostly free of the dots as the largest objects (1-5 mm) were represented predominantly by fibers and irregular particles. On the contrary, the smallest objects (300 to 400 µm) covered the entire spectrum of Circ, varying in shape from fibers to rounded particles. However, it should be noted that the density of dots in the plot was not always correspond to the MP abundance as different water volumes were filtered through the neuston net.

Figure 4. Circularity-versus-Feret’s diameter plots of the MP samples collected at four stations in Sevastopol bay (the Black Sea) in 2019. Dot numbers do not correspond to the MPs abundance in the water. Dashed line represents the upper border of fibers.
Figure 5. Distribution of MPs along Sevastopol bay (the Black Sea) in terms of average particle shape descriptors (left plots), overall abundance and weight of MP fragments, and percentage of fibers among them (right plots).
Presence of abundant large rounded particles in the samples (e.g. at St. 2 in March and late May, Fig. 4) and the ratio between fibers and particles were the most noticeable features in the scatter plots which sufficiently varied along the transect from the bay corner to its mouth. However, no patterns have been revealed in their spatial distribution, probably due to a combination of factors such as input and localization of MP pollution in the bay, current- and wind-driven MP transfer along the bay. The large particles were found at different locations in the bay and outside (Fig. 4), while a contribution of fibers to the MP abundance also varied (7 to 75%, Fig. 5, right plots) irrelevant to the location and time. Consequently, the «tracks» of the MP samples in coordinates of the particle shape descriptors (Fig. 5, left plots) were multidirectional and unpredictable, indicating a complexity of the mechanisms that control distribution and dynamics of MP in the coastal waters.

Over the study period, the abundance and weight of MP ranged 0.6-7 items m\(^{-3}\) and 6-750 µg m\(^{-3}\), respectively. The highest MP concentrations were obtained in the central part of the bay (St. 3) in January (7 items m\(^{-3}\)), and at the bay entrance (St. 1, 2) in late May (about 5.5 items m\(^{-3}\)) (Fig. 5). The peaks of MP mass and its corresponding abundance were not always coincide (Fig. 5), owing to variations in particle size. Though the abundance of MPs were high during winter, the mass of the particles were relatively low (75 µg m\(^{-3}\)) due to the presence of more small size particles. During the late spring, the abundance of large size MP particles (> 1 mm) was predominant, with highest weight of MP up to 0.76 mg m\(^{-3}\). Thus, the range of MP weight variability in the waters of Sevastopol Bay amounted to 3 orders of magnitude.

Overall weight of MP was shown to correlate significantly with the total silhouette area of the particles (Fig. 6). The link between the variables was well approximated by a power function, with the coefficient of determination of 0.84, and, consequently, the regression model could be used as an alternative way to estimate the MP weight.

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M = 0.259 \times S^{0.841}
\]

\[
R^2 = 0.84
\]

**Figure 6.** Weight of a MP sample (M) as a function of the total silhouette area (S) of the MP particles.

**Discussion**

The protocol described in this study proved to be efficient and simple for MP research, when compare to earlier methods mentioned in publications. Among a few known attempts to quantify MP size spectra, a hybrid approach was developed based on different methods of the sample visualization (micrographs or digital scans) and use of ZooScan (or ZooImage) software for counting and measuring MP fragments (Gilfillan et al., 2009; Pedrotti et al., 2016, 2018). The software was developed specially for semi-automated classification of zooplankton species (Irigoin et al., 2008; Bachiller et al., 2012; Medellin-Mora, Escribano, 2013) and, hence, could be applied to recognize and quantify MP fragments in the ‘mixed’ samples.
containing all sorts of objects, from plankton organisms to detritus, mineral particles and MP. The procedure is still quite complicated and time-consuming so, in our view, it is not reasonable to apply this kind of software to the MP extractions since the task of differentiating MP items from other objects is not posed in this case.

Another attempt to solve the problem was made recently (Lorenzo-Navarro et al., 2018) and consisted into: (i) image acquisition by a scanner; (ii) use of computer vision techniques for analyzing the acquired images; and (iii) use of machine learning techniques to develop accurate classifiers of the different types of MP particles. The authors used a flatbed scanner and a sample of pure MPs that was more or less equivalent to MP extraction from a natural seawater sample. However, the mathematical apparatus of the analysis and the procedures of the particle recognition and classification remained too bulky to be accepted as a standard method, at least at this stage of its development.

Thus, two alternative strategies can be chosen in developing the methodology of MP quantification in aquatic samples, based on either (i) MP recognition in a ‘complex’ sample (no MP extraction from the sample is needed), or (ii) MP particle enumeration and size analysis following the MP extraction procedure. The second approach does not demand a complex mathematical analysis (like artificial neural networks, learning algorithms, etc.) for MP fragment identification among other neuston particles and organisms. In our research, we have just preferred the second approach, simplifying both the post-extraction procedures, image acquisition (using a household flatbed scanner) and particle recognition and analysis (using free and simple ImageJ software). ImageJ was successfully applied in MP studies (e.g., Ballent et al., 2012) but mostly for particle size (Feret’s diameter) measurements. A combination of this variable with a shape descriptor like circularity is thought to empower the method as it provides additional information about dispersive properties of MP pollution. For all its simplicity, the Circ-vs-Feret scatter can serve as a ‘fingerprint’ of MP sample.

Our estimates of MP pollution in the Black Sea coastal waters (0.6 to 7 items m$^{-3}$) proved to be approximately at the center of the enormous range (eleven orders of magnitude) of the values that were obtained for different areas of the World Ocean, from 2 × 10$^{-7}$ items m$^{-3}$ in the Bering Sea (Day et al., 1990) to 4000 items m$^{-3}$ in the East China Sea, and even to 16000 items m$^{-3}$ in the coastal waters of South Korea (Song et al., 2014). MP pollution of the Black and Mediterranean Seas is still poorly studied. First regional data on distribution and composition of polymer materials in bottom sediments, surface samples and in the water column (Collignon et al., 2012; Ioakeimidis et al., 2014; Aytan et al., 2016; Öztok and Bat, 2017) have been obtained recently. In a few studies, the ratios were estimated between overall MP weight and biomass of zooplankton which potentially graze on MP particles and, thus, involve them in the pelagic food web (Collignon et al., 2012; Aytan et al., 2016). Measurements of concentrations of floating plastic throughout the Mediterranean Sea have allowed the conclusion to be drawn that this semi-enclosed basin can be regarded as an accumulation region of plastic debris (Cózar et al., 2015). Densities of MPs commonly observed in the Western part of the sea varied between 0.1 and 0.3 items m$^{-3}$ (Collignon et al., 2012; Faure et al., 2015; Pedrotti et al., 2016; Constant et al., 2018) while along the Israeli coast, their concentrations were an order of magnitude higher, about 7 items m$^{-3}$ (van der Hal et al., 2017), that is comparable to the accumulation zones in subtropical ocean gyres. Our estimates obtained for the Black Sea coastal waters correspond well with the above values.

In the southern Black Sea, the most common material type was ship paints (up to 55% and 54% in surface layer and water column, respectively) followed by fibres, hard plastic pieces and nylons (Öztokin, Bat, 2017). Their concentrations (between 2.3 and 24.5 items m$^{-3}$) were slightly higher than in Sevastopol Bays but of the same order of magnitude.

According to results of another research conducted in the same waters (Aytan et al., 2016), the revealed MP concentrations (about 10$^9$ items m$^{-3}$) were three orders of magnitude higher than in our study, that allowed the authors to define the Black Sea as a hotspot of MP pollution (Aytan et al., 2016). Nevertheless, these results raise many questions about the methods which were used to quantify MP pollution. In particular, the protocol did not include the procedures of wet peroxidation of organics and density separation of plastics. This may be a potential source of bias and cause a sufficient overestimation of MP concentrations in the water. It should be noted that before this study was made, none of the publications in the field of MP pollution of the Black Sea were based on confident and commonly used protocols of MP extraction. In this sense, our results can be considered the first and most accurate estimate of MP pollution of the Black Sea coastal waters.
Conclusions

1. A simple, inexpensive method has been proposed to perform a semi-automatic analysis of MP pollution of marine environments, using (i) a common flatbed scanner equipped with slide adapter for image acquisition, and (ii) ImageJ software for quantifying MP dispersive properties (particle abundance, shape and size spectrum).

2. The following particle shape descriptors have been found to be efficient in characterizing MP dispersive properties: Feret’s diameter, silhouette area and circularity (or roundness). Circularity versus Feret scatter plot may be considered as a ‘fingerprint’ of the MP sample.

3. The total silhouette area of MP particles can be used for a confident approximation of the MP overall mass.

4. In a pilot study of MP pollution of Sevastopol Bay, a standard method of MP extraction (including the procedures of wet peroxidation and density separation) and the new protocol of particle analysis have been applied to obtain the first confident estimates of MP concentrations in the Black Sea coastal waters, which accounted for 0.6 to 7 items m\(^{-3}\) and 6 to 750 µg m\(^{-3}\) in terms of abundance and mass, respectively.

5. No steady-state gradients have been revealed in MP distribution along the transect from the mouth of the bay to its corner. Inflow of MP to the bay waters and their transport along the bay seem to be controlled by a complex combination of factors including rainfalls, wind regimes, currents and the Black river discharge.

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