An interlaboratory comparison exercise for the determination of microplastics in standard sample bottles

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A B S T R A C T

An interlaboratory comparison exercise was conducted to assess the consistency of microplastic quantification across several laboratories. The test samples were prepared by mixing one liter seawater free of plastics, microplastics made from polypropylene, high- and low-density polyethylene, and artificial particles in two plastic bottles, and analyzed concurrently in 12 experienced laboratories around the world. The minimum requirements to quantify microplastics were examined by comparing actual numbers of microplastics in these sample bottles with numbers measured in each laboratory. The uncertainty was due to pervasive errors derived from inaccuracies in measuring sizes and/or misidentification of microplastics, including both false recognition and overlooking. The size distribution of microplastics should be smoothed using a running mean with a length of > 0.5 mm to reduce uncertainty to less than ± 20%. The number of microplastics < 1 mm was underestimated by 20% even when using the best practice for measuring microplastics in laboratories.

1. Introduction

Recently, protocols for field surveys and subsequent analyses of microplastic abundance have been developed by several organizations (e.g., Desforges et al., 2014; Galgani et al., 2015; Cózar et al., 2014; Masura et al., 2015; Michida et al., 2019). However, these procedures have not yet been optimized and harmonized sufficiently because microplastics are a novel terrestrial and aquatic property that we have to monitor. A critical step in the processing of microplastic samples is the identification of plastic debris from plankton and other organic/
inorganic debris. The picking of microplastics from the seawater samples may include the digestion of organic matter, density separation or be just based on the expertise of the observer (Hidalgo-Ruz et al., 2012). On the other hand, the verification of the plastic nature of the selected particles is based on their surface properties (i.e. hardness, color, shape, and hydrophobicity; Hidalgo-Ruz et al., 2012; Masura et al., 2015; Crichton et al., 2017) or, at best, on spectrometric analyses (e.g. Fourier transform infrared spectrometer [FTIR], Raman method, and pyrolysis) of a sample subset especially for tiny fragments (< 100 μm; Galgani et al., 2013 or < 1 mm; Hidalgo-Ruz et al., 2012: the methods in these early papers have not been extensively tested), due to the long time still consuming these kind of analyses (Shim et al., 2017; Serranti et al., 2018). Variation in sample processing prevents researchers from reliably comparing and synthesizing microplastic abundances. Simultaneously, protocols should meet the minimum requirements for reliable microplastic analysis and should be tested and optimized between a number of different researchers.

A research project was initiated to compare protocols for surveying pelagic microplastics in 2017. As part of this project, the present study involved an interlaboratory comparison (ILC) exercise to provide error estimates for measuring microplastic abundances in the laboratory. The present study investigated both human errors and errors that might be caused by inappropriate choices in the procedures. Two “standard sample bottles”, in which man-made microplastics along with natural particles were combined to mimic field samples, were sent to each of 12 laboratories around the world. Particle counts in each 0.1-mm size class were reported by these laboratories for comparison with the actual numbers of man-made microplastics. The objectives of this exercise were to provide an error estimate for the procedures adopted in each laboratory and to find the most appropriate method for measuring microplastic abundance in seawater samples.

2. Material and method

2.1. Preparation of standard sample bottles

Three types of man-made microplastics with maximal Feret diameters (hereinafter referred to as “sizes”) ranging from 0.4 mm to 5.7 mm were manufactured for this ILC exercise. First, irregularly shaped plastic fragments with sizes longer than 1.0 mm were created from plastic sheets (Table S1). These irregularly shaped microplastics were created using two metallic molds in which microplastics representing those collected from offshore waters around Japan (stations in Isobe et al., 2015) were reproduced (Fig. S1). Secondly, microplastics < 1 mm were reproduced as rhombic fragments (Fig. S2) by cutting plastic sheets (Table S1) due to the technical difficulty of making such small pieces in a metallic mold like that used for irregularly shaped fragments. Thirdly, fibrous microplastics of various lengths (Fig. S3) were produced from plastic fibers with four different diameters (Table S1). All three types of man-made microplastics and number of particles for each 0.1-mm size interval were recorded. In order to represent pelagic microplastics in the ocean, the polymer types of man-made microplastics were polypropylene, low-density polyethylene, and high-density polyethylene. They are commonly found in pelagic studies due to their density being lower than that of seawater (e.g., Hidalgo-Ruz et al., 2012; Cózar et al., 2014; Isobe et al., 2014; Enders et al., 2015; Zhang et al., 2017; Shim et al., 2018; Song et al., 2018). Furthermore, the colors of the man-made microplastics were chosen to represent those observed in field surveys (e.g., Eriksen et al., 2013; Lusher et al., 2014; Lusher et al., 2015; Table S1).

Two standard sample bottles were prepared for each laboratory participating in the ILC exercise (Fig. 1). The man-made microplastics described above were divided into two treatments with relatively small (257 pieces) and large numbers (397 pieces) (Table S2), and were mixed into 1-L polyethylene bottles containing seawater free of microplastics. Filtered seawater was prepared first using sands, followed by a 10-μm filter twice, and finally a 1-μm filter before filling the sample bottles, and thereafter the absence of plastic fragments was confirmed by both naked eyes and a stereoscope. In addition to the man-made microplastics, artificial suspended sediments were also added to the sample bottles. To produce the natural particles, we mechanically fragmented wood chips, bivalve shells, crab shells, eggshells, and cultured zooplankton (Artemia) free of plastic fragments into similar sizes as the microplastics (Fig. S4). The sizes of these natural particles were measured using sieves with 0.5-, 1-, 2-, and 4-mm mesh sizes. The natural particles were thereafter divided into two treatments with relatively small (1000 pieces) and large numbers (3000 pieces) for the two bottles (Table S3). The standard sample bottle containing small numbers of both man-made microplastics and suspended sediments was referred to as Sample 1, whereas the standard sample bottle containing large numbers of man-made microplastics and suspended sediments was referred to as Sample 2. These samples therefore represented seawater samples collected in sediment-poor and sediment-rich waters respectively. The number ratios between the man-made microplastics and suspended sediments in the Samples 1 and 2 were 30% and 13%, respectively, which were commonly observed in the actual oceans; Table S4 provides the weight ratios between microplastics and suspended sediments collected concurrently around Japan using a neuston net. As in the field surveys, 5% formalin was injected into 1 L of seawater in each bottle.

2.2. Measurement of microplastic abundance in 12 laboratories

The standard sample bottles were sent to 12 laboratories, which
Table 1
Procedures for extracting microplastics in each laboratory.

| Lab.   | Filtration | Chemical treatment to remove organic matter | Density separation | Fractionation from seawater samples | Size measurement | Plastic identification |
|--------|------------|--------------------------------------------|-------------------|-----------------------------------|-----------------|-----------------------|
| A      | Sieving & suction | − | − | NE & S | I | FTIR; k |
| B      | Sieving | Fe(II) & H_2O_2 | NaCl | NE & S | I | FTIR |
| C      | Sieving | − | NaCl | S | I | FTIR |
| D      | Sieving | Fe(II) & H_2O_2 | NaCl | NE & S | I | R' |
| E      | Sieving & suction | − | S | − | I | − |
| F      | Suction | − | − | NE & S | I | − |
| G      | Sieving | KOH | Gravity Separation | NE & S | I | FTIR |
| H      | Sieving & suction | C' & KOH | − | − | − | − |
| I      | Sieving | Fe(II) & H_2O_2 | LiM | NE & S | Microscope | µFTIR |
| J      | Sieving | − | − | NE & S | V' | − | & R |
| K      | Sieving | − | − | S | − | − |
| L      | Sieving | H_2O_2 | NaCl | NE & S | Siieving | − |

* See Table S5 for the detailed description.
* See Table S6 for the detailed description.
* See Table S7 for the detailed description.
* Not used
* Corolase enzyme
* Lithium metatungstate solution
* Extraction with the naked eye using tweezers
* Extraction under a stereomicroscope
* Stereomicroscope and image processing software
* Vernier caliper
* Fourier transform infrared spectrometer
* Raman spectrometer

adopted different procedures to analyse the samples (Table 1). The specific names and affiliations of the researchers are not of particular relevance and, therefore, are hereafter referred to as laboratories A–L.

First, to extract particulate matter from the seawater samples, sieving and/or suction (vacuum filtration) were conducted following protocols used in each laboratory (Table S5).

Second, the extracted particulate matter was treated with chemical reagents such as hydrogen peroxide to remove organic matter, following the protocols outlined in Table S6. This chemical treatment was not conducted by some laboratories (A, C, E, F, J, and K in Table 1) in order to simplify the extraction procedure. Some laboratories that focus on transport of pelagic microplastics in the environment may avoid removing organic matter from seawater samples, which would exclude microplastics inside marine organisms in seawater samples.

Third, to efficiently extract microplastics density separation was used. Here, lightweight particles were separated from particulate matter by floating them in relatively dense solvents (B–D, I, and L in Table S7). In laboratory G, lightweight particles were separated using gravity by leaving the sample in a cylinder to stand over 24 h (Table S7).

Fourth, suspected microplastics were extracted using tweezers from particulate matter with the naked eye or under a stereomicroscope.

Fifth, sizes (δ) of microplastics were measured and sorted into classes of 0.1 mm. Laboratories A–J met this requirement using a stereomicroscope in conjunction with image processing software, such as ImageJ; a stereomicroscope installed on the µFTIR; and a Vernier caliper. Laboratory K (L) extracted microplastics in specific size ranges by sieving and recorded the numbers of fragments with δ < 1 mm and 1 < δ < 5 mm (δ < 5 mm).

Sixth, plastic materials (or polymer types) were distinguished from non-plastic materials through spectroscopy, using FTIR, µFTIR, or Raman method (Table 1). Some laboratories distinguished plastic materials visually based on properties such as color, luster, shape (regular and/or straight edges), and a lack of cellular or organic structures (e.g., Hidalgo-Ruz et al., 2012). Laboratory J received four bottles and examined suspected microplastics with and without spectrometry for comparison.

3. Results

The size distribution was approximately, but not exactly, reproduced in the 10 laboratories (A–J) where the man-made microplastics were counted in 0.1-mm classes, although the numbers counted by different laboratories were highly scattered (Fig. S5; Table S8). Furthermore, microplastics were counted in size classes for which man-made microplastics were absent (e.g., < 0.4 mm), probably due to fragmentation during sample processing and inaccurate measurement of sizes. To the best of our knowledge, it was difficult to observe any effects of hydrogen peroxide on the generated plastic fragments, because it would react only with biological matters on the plastic surface. In fact, the relative uncertainty evaluated as (N − N_0)/N_0 × 100%, where N is a number of microplastics counted in each laboratory, and N_0 is the actual number of microplastics, varied among size classes with a standard deviation of around ± 50%, although relative uncertainty averaged over all sizes was nearly null (Fig. 2b: note that Fig. 2a was created using the means and actual numbers shown in the lower panels in Fig. S5). The standard deviation was reduced to 10–20% when the size distribution was smoothed using a running mean with a length of 0.5 (1.0) mm, as shown in Fig. 2b.(c). The relative uncertainty averaged over the entire size spectrum was underestimated by < 10% in the smoothed size distributions.

The relative uncertainty averaged over each 1-mm interval, which was computed to reduce fluctuations in the size distribution, clearly shows that the number of microplastics was underestimated by > 20%, especially for sizes smaller than 1 mm (Fig. 3a). The differences in uncertainty between particles < 1 mm and larger sizes were statistically significant, as supported by a t-test with 99% confidence limit (95% between 2 and 3 mm). The standard deviation for plastics < 1 mm was much larger than those for larger particle sizes. The difference in standard deviation between particles < 1 mm and larger sizes was statistically significant, as indicated by an F-test with 99% confidence limit.

We next examined the relative importance of each procedure in Table 1 for accurately measuring microplastic abundance. The identification and confirmation of plastic materials (i.e., polymer types) using vibrational spectroscopy is especially critical for microplastics smaller than 2 mm (Fig. 4a). The relative uncertainty averaged over each 1-mm
interval and standard deviations in this size range both differed significantly between laboratories with (A–E, G, I, J) and without (F, H, J) FTIRs. The numbers of microplastics smaller than 2 mm were overestimated in the absence of FTIR analysis, probably due to false recognition of tiny fragments as plastics. The standard deviation was reduced significantly when using FTIR (Fig. 4a and b), suggesting that plastic materials were accurately identified by these laboratories, even for microplastics smaller than 2 mm. In other words, plastic materials could be identified for microplastics with sizes larger than 2 mm irrespective of the usage of spectrometry. A comparison only among those laboratories using FTIR showed a statistically significant difference in relative uncertainty between laboratories with (B, D, G, I) and without (A, C, E, J) chemical treatment (Fig. 4c), as well as between laboratories with (B, C, D, G, I) and without (A, E, J) density separation (Fig. 4d), for microplastics smaller than 1 mm. The relative uncertainty was reduced from 40% to 20% with chemical treatment and/or density separation.

However, the present ILC exercise was not able to determine the relative importance of chemical treatment and density separation, as the laboratories conducting chemical treatment generally overlapped those that used density separation. A comparison only among laboratories using both FTIR and density separation suggested an insignificant difference in relative uncertainty between laboratories with (B, D, G, I) and without (C) chemical treatment over the entire size range (Fig. 4e). However, the number of laboratories without chemical treatment (but with both FTIR and density separation) was only one (two standard sample bottles), and therefore it was impossible to conclude whether chemical treatment or density separation is more important to measure microplastics accurately.

4. Discussion and conclusions

The size interval required for measuring microplastics in this ILC
exercise was 0.1 mm. At this accuracy level, the fluctuations in relative uncertainty reached to ± 50%, irrespective of size, as shown in Fig. 2a. These erroneous measurements were unlikely to be caused by the absence of FTIR, chemical treatment, or density separation, as the uncertainty was generally independent of these procedures for microplastics larger than 1–2 mm (Fig. 4). Therefore, it is reasonable to consider that these fluctuations were partly caused by human error in measuring the sizes of microplastics. For instance, observers using ImageJ determined the maximum length of a plastic fragment visually on a monitor display through trial and error. When the magnifying power was 40–50 on the monitor display to use ImageJ, a length of 0.1 mm corresponded to only around 20 pixels (or a few mm on the display), which could be observed differently by different observers. Magnifying power to measure particles should be 100 or higher to measurements accuracy < 0.1 mm. To obtain a size distribution of microplastics collected in the ocean, the size distribution should be smoothed using a running mean with a length of > 0.5 mm to reduce uncertainty to less than ± 20% (Fig. 2b and c).

Accurate measurement without spectrometry is possible for microplastics > 2 mm. In the absence of spectroscopy, the particle count is overestimated for microplastics < 2 mm, because false recognition of non-plastic materials as plastics is likely to occur. The large standard deviation obtained without spectroscopy suggested that false recognition might be reduced by developing skills for observers to distinguish tiny plastic fragments from natural materials based on their surface properties. Nonetheless, spectroscopy apparently allowed accurate identification of microplastics at sizes < 2 mm, regardless of the skills of the observer. Thus, we recommend that identification of microplastics in fragment < 2 mm requires spectroscopy. Meanwhile, our data suggest that microplastics > 2 mm can be identified through public participation in scientific research and educational programs without expensive FTIR or Raman analysis if the properties of the plastics are described well in protocols (i.e., hardness, color, shape, and hydrophobicity; Hidalgo-Ruz et al., 2012; Masura et al., 2015; Crichton et al., 2017).

Chemical treatment and density separation are used to avoid underestimating the particle count for microplastics < 1 mm. In general, these procedures are employed to shorten the time needed to extract microplastic fragments from seawater samples. Observers become more likely to miss tiny plastic fragments < 1 mm as the operation time is lengthened. Microplastics collected in the actual ocean are usually covered with biofilms, and therefore the chemical treatment to remove organic matter would be more useful than in this ILC exercise. Our recommendation is that chemical treatment and/or density separation should be included in protocols for measuring microplastics, especially for sizes < 1 mm. However, some laboratories that focus on transport of pelagic microplastics in the environment may avoid removing organic matter from seawater samples, which would exclude microplastics inside marine organisms in seawater samples.

In general, increases of handling and treatment in analysing microplastics might result in a risk of sample loss such as particle sticking to labware, spills, and so forth. Nevertheless, the best practice identified in the present ILC exercise was a combination of spectrometry and short operating time to avoid human error (e.g., chemical treatment and/or density separation), especially for microplastics < 1–2 mm. Four laboratories met the criteria of this ILC exercise and provided relative uncertainty values (Fig. 3b), which were compared with the total uncertainty derived from all laboratories (Fig. 3a). The differences in the relative uncertainty between Fig. 3a and b were insignificant (supported by t-test) even for microplastics < 1 mm, probably because an erroneous estimate of the size of smaller microplastics occurred, either through overestimation or underestimation (Fig. 4). Nonetheless, a significant difference in the standard deviation for the size range < 1 mm demonstrates that the best practice leads to stable measurement irrespective of the skill level of the observer. Notably, the number of microplastics < 1 mm was underestimated by 20% even when using the best practice for measuring microplastics in laboratories.

The recommendations and notes for counting microplastics in the laboratory are summarized in Table 2, where the filtration, chemical treatment, density separation, fractionation from seawater samples, size measurement, and plastic identification of microplastics are itemized. Hopefully, our exercise is followed by exercises by other experts to improve these recommendations and notes. In a first step, the present exercise compared the microplastic measurements including both human errors and errors generated by methods. However, different methods conducted in one laboratory will provide error estimates and their propagation in a method, while an exercise with a single method in different laboratories will provide human errors.

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