

Quantification of microplastics by count, size and morphology in beverage containers using Nile Red and ImageJ

Shujuan Chen, Yue Li, Christopher Mawhorter and Saamon Legoski

ABSTRACT

Abundant evidence of microplastics (MP) found in the environment, and its toxicity effect in animals calls for human-related research. However, well-established quantitative controlled studies on the potential route of human exposure to MP are still sparse. MP count, size and morphology in 15 polylactic acid (PLA)-lined plastic cups and 15 PLA-lined paper cups were examined using Nile Red fluorescence tagging, microscopic photography, and morphology assessment and quantification based on ImageJ. In the plastic cups, the count and area of MP fibers were found to be significantly higher compared with blanks ($p < 0.05$), but not MP particles or total MP. In paper cups, count or area was not significantly different in terms of MP particle, MP fibers or total MP. No interesting trend was observed in the distribution regarding the size of MP particles or fibers. These results indicate that selected paper cups and plastic cups could be considered as safe beverage containers, but further research on the toxicological effects of MPs in different morphologies released from plastic cups on human health is needed.

Key words | beverage containers, microplastics, microscopy, morphology, quantification

HIGHLIGHTS

- This study examined microplastics being released from beverage containers quantitatively.
- This study improved previous methods of Nile Red fluorescence tagging, microscopic photography of full samples and automatic image processing.
- Introduced morphology and projection area of microplastics as new metrics.
- Results indicate that paper and plastic cups are not significant sources of microplastics.

INTRODUCTION

Microplastics (MPs) are water-insoluble, synthetic polymers of diverse shapes, with sizes ranging from 1 μm to 5 mm (Frias & Nash 2019). In recent years, MPs have become an emerging public health issue because a number of studies have shown that MPs are ubiquitous everywhere in our surrounding

environment such as shoreline and even glaciers (Ambrosini *et al.* 2019; Chouchene *et al.* 2019; Garcés-Ordóñez *et al.* 2019; He *et al.* 2019; Huang *et al.* 2019; Nabizadeh *et al.* 2019; Scopetani *et al.* 2019; Xu *et al.* 2019). The source of MPs can come from the breakdown of larger plastics, such as water bottles, but also from the use of synthetic polymers during the manufacturing process (US Department of Commerce 2020).

MP exposure can pose a threat to both single-cell and complex organisms, possibly including humans. Several

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doi: 10.2166/wh.2020.171

toxicity studies reveal MP to have adverse effects on the survival and reproductive success of aquatic organisms like juvenile fish and zebrafish. (Franzellitti *et al.* 2019; Mak *et al.* 2019; Naidoo & Glassom 2019) In particular, Mak *et al.* (2019) exposed zebrafish to different levels of MPs and found significant differences in how certain genes expressed themselves (e.g., phase 1 detoxification-related gene CYP 1a), as well as significantly higher levels of abnormal behaviors such as seizures after exposure (Mak *et al.* 2019). A study by Luo *et al.* (2019) of maternal exposure to MPs in mice found an increased risk of liver damage, metabolic disorder and gastrointestinal disruptions. Both first and second generations of offspring were found to be at increased risk of metabolic disorder, too (Luo *et al.* 2019). Also, heavy metals and some chemicals, such as phthalates, polybrominated diphenyl ethers and tetrabromobisphenol A, are typically present in MP, and many of these chemical additives, such as PFAS, leach out of the plastics after entering the environment (Dobaradaran *et al.* 2018; Gallo *et al.* 2018).

The abundance of MP in our environment and potentially negative health impacts has led researchers to study how humans take in MPs. For example, MPs were found in human consumables such as seafood (Smith *et al.* 2018), tea bags (Hernandez *et al.* 2019), bottle water (Mason *et al.* 2018), tap water, beer and sea salt (Kosuth *et al.* 2018). The detection of MPs in human stools also suggests MP consumption results in their transportation through, and contamination of the gastrointestinal system (Schwabl *et al.* 2019). These findings raised concern about the impact of MPs on human health and called for research in human toxicity (Smith *et al.* 2018). However, research on the human consumption of MP has focused primarily on where MP can be found, but rarely on contamination pathways. There are particularly insufficient studies about MP contamination from food packages and drink containers. Relatedly, well-established quantitative controlled studies with morphology information of MPs are still limited.

This study adds to a growing body of literature by determining whether MPs are released from the surfaces of different beverage containers, while quantifying and characterizing MPs in terms of their morphology. Our study adds to the evidence of human consumption of MPs with a study design of higher power, and provides an adapted

methodology for future studies related to MP quantification with lower detection limit and higher precision.

Based on the background knowledge above, we specified our study aims as the following: (1) to determine whether beverage containers are a significant source of microplastic exposure, (2) to determine whether different types of beverage containers have different MP levels by count and size, (3) to determine whether the results are modified by the morphology of MPs and (4) to determine whether there are distribution differences between different morphology of MPs.

MATERIALS AND METHODS

Study design

To meet our objectives, we included one experiment group of 15 plastic cups, one experiment group of 15 paper cups and one control group of 5 procedural blanks in our study design. Previous studies support using Nile Red fluorescence for distinguishing MP from non-polymer materials, and programs like ImageJ have been used to quantify MP using microscopic photography (microscopy; Maes *et al.* 2017; Dobaradaran *et al.* 2018; Mason *et al.* 2018; Prata *et al.* 2019; Akhbarzadeh *et al.* 2020). The recovery rate of spiked polyethylene (100–300 μm) was 98% in the Nile Red staining method as compared with 96% recovery rate in the Fourier-transform infrared spectroscopy (FTIR) method, which was not significantly ($p < 0.05$) different (Shim *et al.* 2016). We settled on a modified method that consists of Nile Red (Sigma-Aldrich 19123) fluorescence tagging, microscopy (OLYMPUS BX60PE, 10 \times zoom), followed by morphology assessment and quantification based on ImageJ and statistical analysis. Levels of MPs are reported in count and the sum of 2D projected area on the filter. Morphologies of MPs are dichotomized to particles and fibers. The whole experimental process is described briefly below, and detailed information is presented in the supplementary file.

Sample collection and processing

We examined polylactic acid (PLA)-lined paper cups and PLA-lined plastic cups, which were common beverage

containers (manufactured by NatureWorks) that were used and could be purchased in the New England area. Fifteen paper cups and 15 plastic cups were collected from a cafeteria in Harvard T.H. Chan School of Public Health.

To minimize potential contamination from external sources during the experiment, such as airborne fibers and particles, the whole experiment was processed in a fume hood, and the workplace as well as all glassware were sterilized using the standardized biochemistry laboratory protocol. The pre-filtered Milli-Q water was prepared by vacuum filtration of Milli-Q water with the same glass microfiber filter used to filter out MPs in each condition. Regular personal protection equipment was worn throughout the whole process.

To examine the contamination of MPs from the surface of the containers, 100 mL of pre-filtered Milli-Q water was added to all containers, followed by a slow, 1-min clockwise revolving motion in the fume hood under the laboratory temperature (23–25 °C). Afterwards, the filtered water was transferred to a clean beaker, dyed by Nile Red solution (dissolved with acetone) with a working concentration of 10 µg/mL, and incubated for 30 min. This concentration strikes a balance between fluorescent strength of dyed MP and reduction of ‘noise’ from Whatman filters that were used for filtration (Maes *et al.* 2017). Each beaker of incubated solution was then vacuum filtered through a dedicated glass microfiber filter. The processed filters were dried in the Petri dish for at least 24 h in the laminar fume hood.

To account for the potential contamination from ambient air, chemicals, glassware and other testing materials, we introduced five procedural blanks. In blank samples, we used clean beakers that were washed with pre-filtered Milli-Q water and went through the same procedure (rinsing, staining, vacuum filtration and incubation) identical to all the samples in the experiment groups. The level of microplastic contamination found in our procedural blanks suggests accountability of MP contamination from the laboratory environment.

Data collection

Using an Olympus BX-60 with zoom at 10×, we examined the fluorescent MP from the filters under an orange filter of wavelength ~529 nm and blue beam of 450–470 nm. Based on the result in Prata’s 2019 study, almost all polymers and

textiles (except nylon) showed strong fluorescence at 470 nm, thus it was considered appropriate to examine MPs on the filters under this wavelength (Prata *et al.* 2019). The reason we examined fluorescence at zoom 10× was due to the size of the present MPs in our samples. Different from marine samples which contain a higher number of MPs with larger size, MPs in our samples are much smaller, so we set a lower detection limit. To reduce overcounting or undercounting, well-etched glass slides were used to cover the top of the filters to provide a visual reference for photography and counting. The researcher started counting from the first row and went down through the whole filter following the navigation of the grids. A Nikon DSLR camera was attached to the microscope, and pictures were taken for every recognizable fluorescence substance regardless of the shape or size. Each full filter of the samples is photographed without partial selection, which reduces undercounting. When pictures were being taken, one researcher controlled the shutter and examined the area with the LED screen of the camera in real-time, while the other researcher examined the grid area with the microscope and made adjustments. A photo was taken if fluorescence was recognized by either researcher. Samples were randomly chosen from the pool, with the two researchers being blinded to the sample’s condition.

Data processing

Each recognized cluster of fluorescence was manually classified as either particles or fibers, based on the morphology of the fluorescence, in order to overcome miscounting issues that can arise when using a high threshold for MP classification by ImageJ. With ImageJ, any pixel that did not meet a set level of fluorescence shows up as white, while any pixel meeting the requirement shows up as black (Figure 1). This sometimes leads to fibrous MP being cut into multiple parts, which causes ImageJ to classify one fibrous MP as multiple MPs. We made duplications, separations and ‘blacked out’ parts of a photo prior to ImageJ analysis to separate fibrous MP from particles in a photograph, which prevented automated overcounting. This process was performed for every sample and was done independently by two researchers whose results of classification were compared and finalized through discussion. Both of

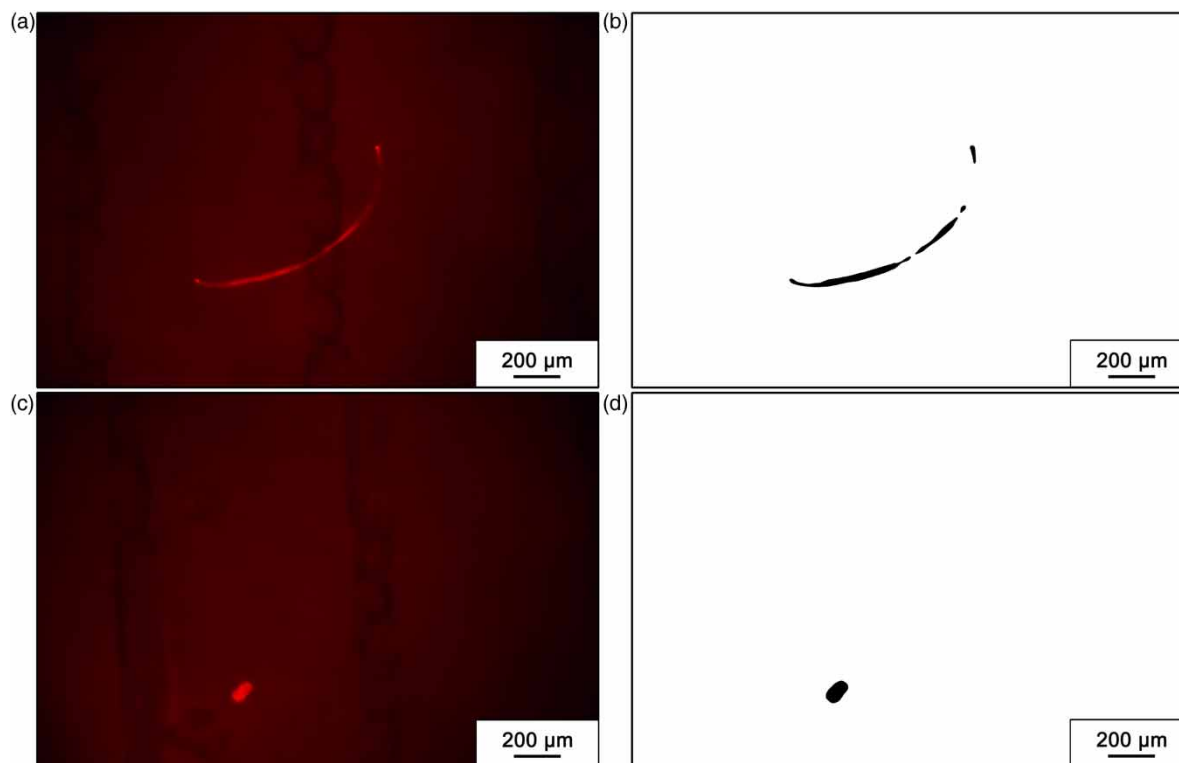


Figure 1 | A typical MP fiber (a) and particle (c) discovered under fluorescence microscope (10×) with processed image by ImageJ (b), (d).

them were blinded to the sample types. As a result, we obtained separated folders that contained photos with particles only or with individual fibers for each sample.

All manually separated photos were processed by ImageJ 1.52q. Image processing was based on the ImageJ script developed in the Prata *et al.* (2019) study, whose method was validated and showed good correlation between the number of MPs recognized by the researcher and the ImageJ script (Prata *et al.* 2019). Some modifications were made to fit our settings better: (1) images taken were $4,000 \times 6,000$ in pixel and the scale were set to 1.5×2.25 in millimeter in real size, (2) an absolute threshold of 222 was used to distinguish the fluorescence from the background instead of using a relative threshold with a default algorithm and (3) the addition of a ‘despeckle’ function and two ‘remove outliers’ functions with radius 15 and threshold at 40 for both ‘Bright’ and ‘Dark’ options. For each photo, an image with the recognized MPs in black on a white background and a csv file with information on the area, maximum Feret and other features for each detaching MP were given as output. Processed images were

reconfirmed by a researcher to identify and remedy any processing errors.

The modifications we made, which contain elements of manual separation and classification from previous studies, were based on the high prevalence of misclassifications between fibers and particles. Some thinner particles were recognized as fibers, while some very long fibers were surrounded by residual speckles that were recognized as particles. These misclassifications would have biased our count data in favor of a false-positive. The addition of two processing functions that smoothed the shape of the MPs and reduced sand-like specks eliminated our overestimation problem in ImageJ, but likely led to undercounting, too. In addition, the rationale for adopting an absolute threshold was that we needed a consistent standard that could apply to all photos. Our consistent laboratory environment and exposure across the three conditions ensured an equal standard when adopting the absolute threshold. The chosen threshold at 222 was optimally balanced between reducing false-positive rate from background noise and increasing recognition of MPs.

Statistical analysis

Data analysis was performed in R v3.5.1. The csv file for every image was read and summarized within samples for particles and fibers, respectively. Files that contained no result indicated that any fluorescence did not meet our absolute threshold, even if identified by the naked eye. Particle, fiber and total MP area of a sample were calculated by summing the area of the particles, fibers or both, respectively. For particle counts, row number in the csv file, which was a detached observation of MP, was summed up. Fiber count was measured by image count because each fiber had a dedicated image modified to remove other fluorescence. Particle area and fiber area were measured by summing up the area for all images within each sample. The feature used for particle inclusion was maximum Feret diameter, the maximum distance possible between any two parallel planes restricting the particle perpendicular to that direction. We included particles with maximum Feret diameter $\geq 7 \mu\text{m}$, which was the maximum Feret of the smallest recognized MP by human eye, under the microscope across all photos. For fiber inclusion, the threshold was area $> 0 \text{ mm}^2$. *T*-tests were performed to find any statistically significant differences in numbers and areas of MPs between different sample types.

RESULTS

All samples of 15 paper cups, 15 plastic cups and 5 blanks were successfully collected, processed and analyzed. During the data analysis process, 6,860 images were processed in total. On average, around 30 images per sample were originally mixed with fibers and particles, and around 6–7 images needed to be reprocessed due to misrecognition of background per sample by ImageJ. Figure 1 displays what a typical MP fiber (Figure 1(a)) and an MP particle (Figure 1(c)) look like under our settings. The vertical black lines indicated the etched lines by laser on the glass slide for navigation use. The right side of Figure 1 shows the processed image of the corresponding photo on the left by ImageJ (b,d). We could see some part of the fiber was of low fluorescence and not recognized by the software. This caused disconnection of the fiber in the processed image and thus supported our method of separating each fiber to an individual photo, and taking the sum of all black area as the fiber size rather than only processing counting.

Figures 2 and 3 display the means and standard errors from samples in each condition. The *p*-values from *t*-tests between plastic cup samples and paper cup samples compared with blanks in terms of each morphology of MP by counts and areas. The result of the total MP count in Figure 2

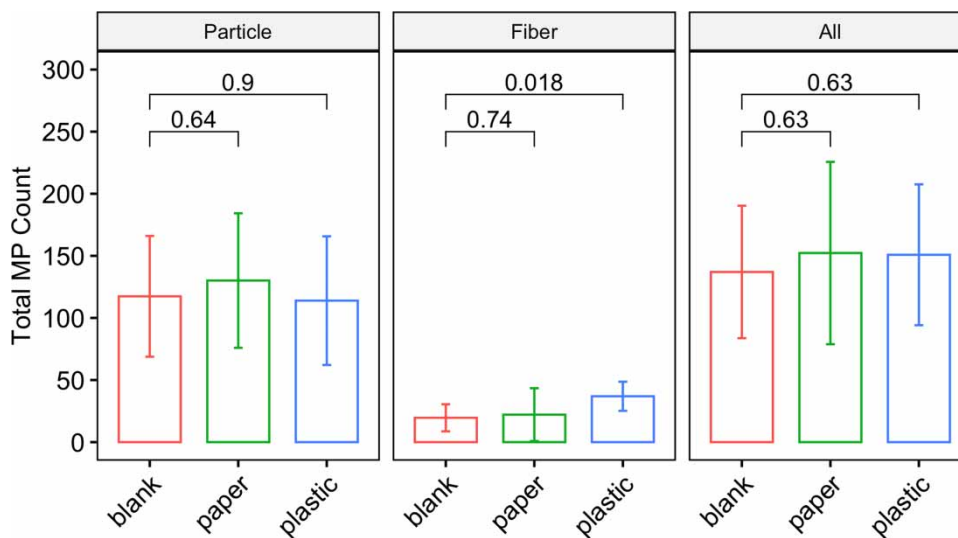


Figure 2 | Total MP count per sample across MP morphology and sample types. Note: Particle counts only include particles with maximum Feret diameter $\geq 7 \mu\text{m}$. Error bars are one standard deviation. Numbers at the top are *p*-values of *t*-test between blanks and paper cups or blanks and plastic cups.

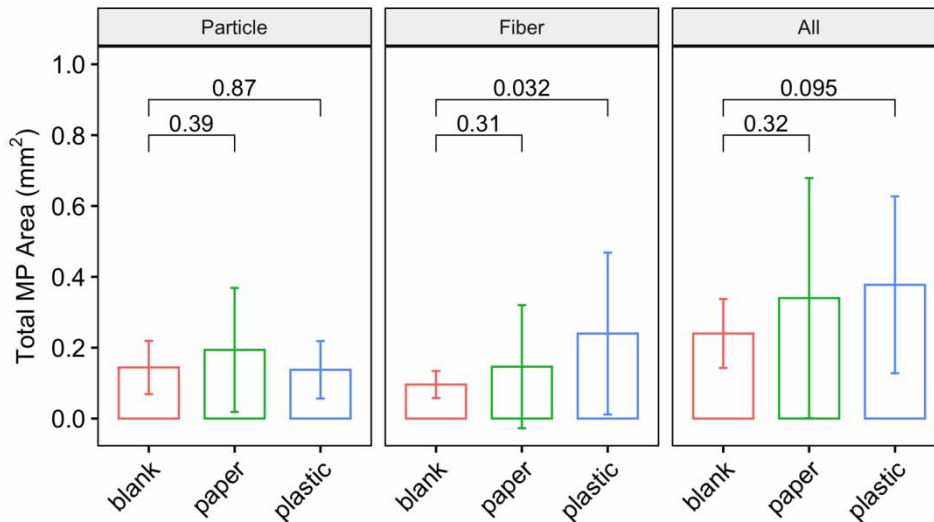


Figure 3 | Total MP area per sample across MP morphology and sample types. Note: Total MP particle areas only include particles with maximum Feret diameter $\geq 7 \mu\text{m}$. Error bars are one standard deviation. Numbers at the top are p -values of t -test between blanks and paper cups or blanks and plastic cups.

indicates that for MP particles, the numbers were quite similar between the three conditions. However, fiber count was significantly higher in plastic cups compared with blanks. The counts that included MP particles or combined numbers were not significantly different between the three conditions. Figure 3 displays the total MP areas across our three types of samples. We could see that the area of MP fibers from plastic cup samples was significantly higher than that of the blanks ($p = 0.032$). Total MP area in terms of the sum of particles and fibers was higher in both plastic and paper cups compared with the blanks, but was not significant with p -values of 0.095 and 0.320, respectively. We can, therefore, conclude that there was no significantly elevated level of count and area in plastic or paper cups in terms of MP particle or total MP. Nevertheless, MP fiber count and area were significantly higher when pre-filtered water was exposed to plastic cups, when compared with our blanks.

We also performed an analysis on the distribution of the size of MP in terms of particle maximum Feret diameter, particle area and fiber area. Density plots were made by merging information of all recognized particles and fibers across all samples within each sample type. However, the probability densities of log particle maximum Feret diameter (mm) of three sample types fall on the same curve and fit well. No significant differences were found in the

distribution of particle maximum Feret diameter (a), particle area (b) or fiber area (c) in the log scale as shown in Figure 4. These results indicate that the differences observed previously in counts and areas between sample types were independent to particle or fiber size distributions, and the differences exist in all levels of size of MPs. These results are also supported by comparing the particle median maximum Feret diameter (d), particle median area (e) and fiber median area (f) as shown in Figure 4. No significant difference was found in plastic cups or paper cups compared with blanks according to the result of t -tests.

DISCUSSION

Partially consistent with prior studies, MP fibers are found to be significantly elevated releasing from the surfaces of the PLA-lined plastic cups while being vibrating under the laboratory temperature (around 23°C). A related study demonstrated that steeping a single plastic teabag at brewing temperature (95°C) could release around 11.6 billion MP and 3.1 billion nano-plastics into a single cup of the beverage (Hernandez *et al.* 2019). Another study found that opening and closing a series of plastic bottles increased the number of microplastic particles (Winkler *et al.* 2019). However, the insignificant result of total MP in this study

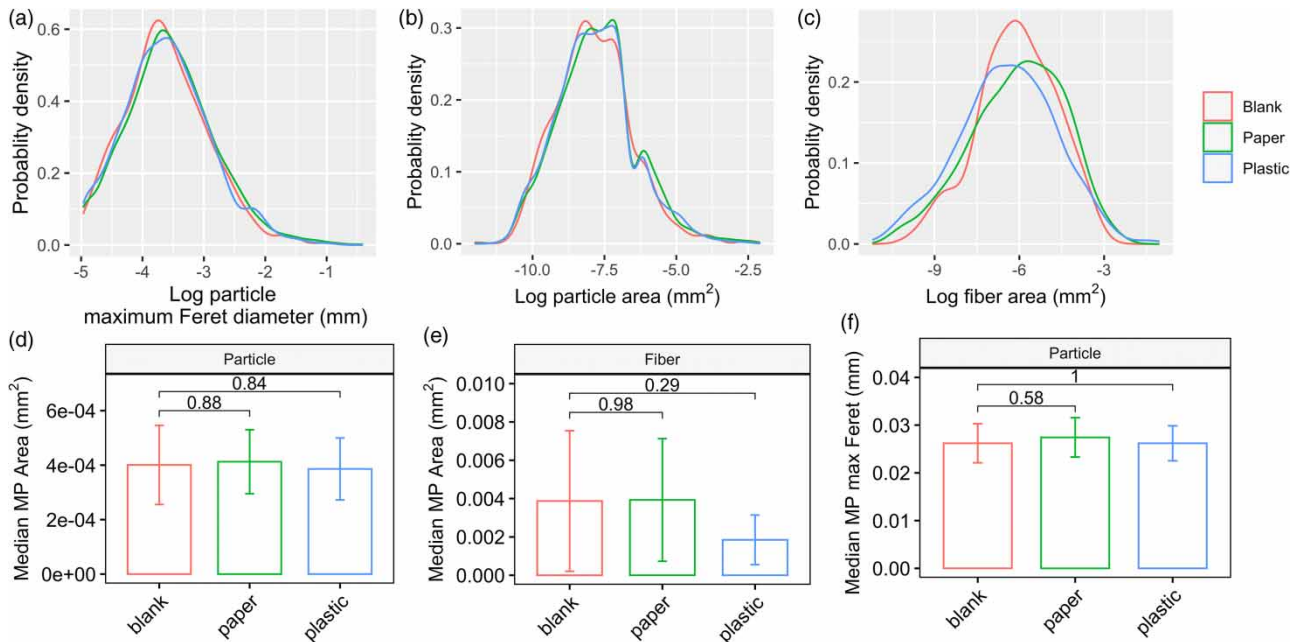


Figure 4 | Distribution of particle maximum Feret diameter (a), particle area (b) or fiber area (c) across all samples within the three sample types, and comparisons of particle median maximum Feret diameter (d), particle median area (e) and fiber median area (f). Note: Particle statistics only include particles with maximum Feret diameter $\geq 7 \mu\text{m}$. Error bars are one standard deviation. Numbers at the top are p -values of t -test between blanks and paper cups or blanks and plastic cups.

adds to the knowledge that PLA-lined plastic cups and paper cups are relatively safe containers under normal conditions compared to the background level of MP. Also, complementing those previous studies on contamination pathways, this is the first systematic and experimental study on MP being released from the surface of beverage containers quantitatively.

We improved upon previous techniques of fluorescent tagging with Nile Red (Prata *et al.* 2019) and automated counting (Maes *et al.* 2017) by using laser-etched glass slides as a reference to photograph the full filter without partial selection, which reduced undercounting. Though a comparison of quantification methods used in similar studies is listed in Table 1, we could see a tradeoff

Table 1 | Comparison of quantification methods used in similar studies

Study	This study	Prata <i>et al.</i> (2019)	Mason <i>et al.</i> (2018)	Maes <i>et al.</i> (2017)	Winkler <i>et al.</i> (2019)
Quantification method	Nile Red tagging, ImageJ	Nile Red tagging, ImageJ	Nile Red tagging, Galaxy Count	Nile Red tagging	SEM, ImageJ
Detection limit	7 μm (18 px)	50 μm (3 px)	6.5 μm (1 px)	5 μm (9 px)	3 μm
Accuracy	High	Low	Medium	Medium	Low
Speed	Low	High	Medium	Low	High
Cost	Low	Low	Low	Low	High
Count analysis	Yes	Yes	Yes	Yes	Yes
Size analysis	Yes	Yes	No	No	Yes
Morphological analysis	Yes	No result	No	No	Yes

between detection limit, analysis area and processing speed. This study demonstrated that a smaller limit of detection (7 μm) is compatible with automated counting for each filter, without the need for stitching images. The detection limit was set to 50 μm in Prata's study by allowing detection of fluorescence of over 3 px (Prata *et al.* 2019). In Mason's study, MPs were visualized as long as 1 px was recognized (Mason *et al.* 2018). The reason we did not use a minimum recognized pixel number to define our detection limit is that it would count detached pixels from the main particle that could not be eliminated through 'despeckle' function in ImageJ as independent particles, which would affect the accuracy of quantification greatly.

Additionally, Winkler *et al.* (2019) used scanning electron microscope (SEM) to obtain MP images on plastic bottle caps with very low detection limit but only scanned a very limited area and used extrapolation to estimate total MP, which may introduce uncertainties to the result. Also, Mason *et al.* separated the whole sample into four quadrants and Maes *et al.* further split the sample into a 9×6 array of images. These previous studies used random selection of the filter to estimate a rough count for MPs instead of considering the full filter (Cai *et al.* 2018; Mason *et al.* 2018; Xu *et al.* 2019). This study zoomed further into the sample resulting in lower detection limit and higher accuracy while sacrificing processing speed.

The addition of the morphology classification method is another contribution of this study and was rarely done by previous studies. The probability density distribution and median comparison of maximum Feret diameters, MP particle areas and MP fiber areas in this study indicate that MPs being released from our samples have a wide range of size. MPs examined under the microscope vary widely in length, and MPs of the same length may vary widely in width and shape, leading to a wide range of surface area. Given these facts, this study used the area of MPs to look at physiological differences in the absence of volume and mass measures. The size of MPs, which is linearly related to the projected area, might be correlated to their carrying ability and thus determine potential toxicity effects. The different morphologies suggest varying mechanisms of chemical attachments and interactions that make MP a stronger or weaker media carrier. For those reasons, we

suggest introducing area dimensions to MP characterizations in future research.

There are still several limitations of this study. First, we introduced modifications to previously validated methods, which may introduce chance for error or bias. Second, contamination from non-container sources persisted despite a stringent protocol. The protocol largely reduced the potential external contamination of MPs, especially due to pre-filtered Milli-Q water, and reinforced the importance of procedural blanks as a quality control measure in microplastic trials. Third, while this study demonstrated far less variance in MP count than previous, small-*n* studies on water bottles (Winkler *et al.* 2019), power calculations from preliminary samples in this study showed that larger sample sizes are better. Coupled with limited resources and enormous time required to process and analyze each sample, power was difficult to achieve, although this sample size was large enough to obtain meaningful results. Furthermore, identification of MPs using FTIR or Raman spectroscopy was not able to be performed due to the laboratory condition. Identifying the type of the stained polymers could support our results better, but it influences little and it is valid to draw a conclusion that both samples are insignificant sources of MPs. Overall, the precision of this study was balanced by the stringent experimental design and data analysis. Further development of methods, or greater resources and time, are needed to achieve the statistical power to definitively make claims about overall microplastic exposure from disposable drinkware.

Finally, this study points out that PLA-lined paper and plastic cups could be considered safe beverage containers. However, the elevated level of MP fibers released from PLA-lined plastic cups underscores the need to include PLA in the broader microplastic research agenda. Few studies exist specifically addressing PLA MPs, despite some early research on PLA in the environment indicating it may be more prone to producing MPs (Chen *et al.* 2020). As the regulatory and policy environment shifts toward 'renewable' and 'compostable' sources of raw material and disposable food contact utensils, PLA stands to command a greater market share. This indicates a greater share of pathways to human exposure to MP, such as more improper disposal of PLA products which increase their presence in the natural environment and thus in the food and water

sources on which humans rely. There are also indications that regardless of the manufacturing processes, ingested and food contact PLA may be a transport pathway for toxic and potentially harmful additives (Zimmermann *et al.* 2019).

Beyond the significant findings of MP fibers being released from the surfaces of PLA-lined plastic cups, the impacts of microplastic contamination on human health are still unknown. Also, the interaction between MP and chemicals in terms of MPs morphology is an interesting topic to explore. More research is needed to determine whether MP fiber contamination from plastic cups is derived from the manufacturing facilities or is coming off the containers themselves. These results call for further research on the toxicological effects of MPs in different morphologies on human health.

CONCLUSIONS

This study provides quantitative estimates to MPs releasing from the surface of PLA-lined beverage containers. The data showed PLA-lined paper cups are not significant sources of MPs. PLA-lined plastic cups were found to be significant sources only for MP fibers, but not MP particles or total MPs. These results indicate that selected paper cups and plastic cups could be considered as safe beverage containers. Further research on the toxicological effects of MPs in different morphologies released from plastic cups on human health is needed.

ACKNOWLEDGEMENTS

This project was greatly supported through technical advising and editing by Carmen Messerlian, HSPH Assistant Professor Environmental Reproductive, Perinatal and Pediatric Epidemiology, and Director of the Scientific Early Life Environmental Health & Development (SEED) Program; and by Jonathan Buonocore, HSPH Research Scientist at the Center for Climate, Health, and the Global Environment (C-CHANGE). Analysis was carried out in the Harvard Biogeochemistry of Global Contaminants Laboratory led by Dr Elsie Sunderland, Gordon McKay

Professor of Environmental Chemistry, Harvard John A. Paulson School of Engineering and Applied Sciences, and Department of Environmental Health, Harvard T.H. Chan School of Public Health. The authors gratefully acknowledge the support of members of the Laboratory, including Laboratory Manager Prentiss Balcom.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 27 July 2020; accepted in revised form 12 November 2020. Available online 8 December 2020