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TITLE:

Sampling, Identification and Characterization of Microplastics Release from Polypropylene Baby Feeding Bottle during Daily Use

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KEYWORDS:

Microplastics, plastic product, daily use, baby feeding bottle, hot water, polypropylene

SUMMARY:

This study detailed a reliable and cost-effective protocol for microplastics collection and detection from the daily use of plastic products.

ABSTRACT:

Microplastics (MPs) are becoming a global concern due to the potential risk to human health. Case studies of plastic products (i.e., plastic single-use cups and kettles) indicate that MP release during daily use can be extremely high. Precisely determining the MP release level is a crucial step to identify and quantify the exposure source and assess/control the corresponding risks stemming from this exposure. Though protocols for measuring MP levels in marine or freshwater

has been well developed, the conditions experienced by household plastic products can vary widely. Many plastic products are exposed to frequent high temperatures (up to 100 °C) and are cooled back to room temperature during daily use. It is therefore crucial to develop a sampling protocol that mimics the actual daily-use scenario for each particular product. This study focused on widely used polypropylene-based baby feeding bottles to develop a cost-effective protocol for MP release studies of many plastic products. The protocol developed here enables: 1) prevention of the potential contamination during sampling and detection; 2) realistic implementation of daily-use scenarios and accurate collection of the MPs released from baby feeding bottles based on WHO guidelines; and 3) cost-effective chemical determination and physical topography mapping of MPs released from baby feeding bottles. Based on this protocol, the recovery percentage using standard polystyrene MP (diameter of 2 µm) was 92.4-101.2% while the detected size was around 102.2% of the designed size. The protocol detailed here provides a reliable and cost-effective method for MP sample preparation and detection, which can substantially benefit future studies of MP release from plastic products.

INTRODUCTION:

Most types of plastics are non-biodegradable but can break down into small pieces due to chemical and physical processes such as oxidation and mechanical friction[1,2]. Plastic pieces smaller than 5 mm are classified as microplastics (MPs). MPs are ubiquitous and found in almost every corner in the world. They have become a global concern due to the potential risk to humans and wildlife[3,4]. To date, significant accumulations of MPs have been found in fish, birds, insects[5,6] as well as mammals (mouse, in the gut, kidney and liver[7,8]). Studies found that the exposure and accumulation of MPs can damage the lipid metabolism of mice[7,8]. A risk assessment focusing on fish found that sub-micron MPs can penetrate the blood-to-brain barrier and cause brain damage[9]. It should be noted that to date all MP risk results have been obtained from animal studies while the specific risk to human health is still unknown.

In the last 2 years, concerns about the MP threat to human health substantially increased with the confirmation of the levels of human exposure to MPs. The accumulation of MPs has been found in the human colon[10], the placenta of pregnant women[11] and adult stool[12]. A precise determination of MP release levels is crucial to identify exposure sources, assess the health risk and evaluate the efficiency of any potential control measures. In the last few years, some case studies reported that daily-use plastics (i.e., the plastic kettle[13] and single-use cups[14]) can release extremely high quantities of MPs. For example, disposable paper cups (with interiors laminated with polyethylene-PE or copolymer films), released approximately 250 micron-sized MPs and 102 million sub-micron-sized particles into each milliliter of liquid following exposure to 85-90 °C hot water[14]. A study of polypropylene (PP) food containers reported that up to 7.6 mg of plastic particles is released from the container during a single use[15]. Even higher levels were recorded from teabags made from polyethylene terephthalate (PET) and nylon, which released approximately 11.6 billion MPs and 3.1 billion nano-sized MPs into a single cup (10 mL) of the beverage[16]. Given that these daily-use plastic products are designed for food and beverage preparation, the release of high quantities of MPs is likely and their consumption is a potential threat to human health.

Studies on MP release from household plastic products (i.e., the plastic kettle[13] and single-use cups[14]) are at an early stage, but it is expected that this topic will receive increasing attention from researchers and the general public. The methods required in these studies are significantly different from those used in room temperature marine or freshwater studies where well-established protocols already exist[17]. In contrast, studies involving the daily-use of household plastic products involves much higher temperature (up to 100 °C), with in many cases repeated cycling back to room temperature. Previous studies pointed out that plastics in contact with hot water can release millions of MPs[16,18]. In addition, the daily-use of plastic products may over time change the properties of the plastic itself. It is therefore crucial to develop a sampling protocol that accurately mimics the most common daily-use scenarios. The detection of micro-sized particles is another major challenge. Previous studies pointed out that MPs release from plastic products are smaller than 20 µm[16,19,20]. Detection of these types of MPs requires the use of smooth membrane filters with small pore size. In addition, it is necessary to distinguish MPs from possible contaminants captured by the filter. High sensitivity Raman spectroscopy is used for chemical composition analysis, which has the advantage of avoiding the need for high laser power that is known to easily destroy small particles[20]. Hence, the protocol must combine contamination-free handling procedures with the use of optimal membrane filters and for a characterization method that allows fast and accurate MP identification.

The study reported here focused on the PP-based baby feeding bottle (BFB), one of the most commonly used plastic products in daily life. It was found that a high number of MPs are released from plastic BFB during formula preparation[18]. For further study of MP release from daily plastics, the sample preparation and detection method for BFB is detailed here. During sample preparation, the standard formula-preparation process (cleaning, sterilizing and mixing) recommended by the WHO[21] was carefully followed. By designing the protocols around the WHO guidelines, we ensured that the MP release from BFBs mimicked the baby formula preparation process used by parents. The filter process was designed to accurately collect the MPs released from BFBs. For the chemical identification of MPs, the working conditions for Raman spectroscopy were optimized to obtain clean and easily identified spectra of MPs, while at the same time avoiding the possibility of burning the target particles. Finally, the optimum test procedure and applied force to allow accurate 3-dimensional topography mapping of MPs using atomic force microscopy (AFM) was developed. The protocol (**Figure 1**) detailed here provides a reliable and cost-effective method for MP sample preparation and detection, which can substantially benefit future studies of plastic products.

PROTOCOL:

1. Hot water preparation

1.1) For all hardware that comes into contact with the samples, use clean glass made of borosilicate 3.3 to prevent any potential contamination. Thoroughly clean all the glassware.

Caution: Pre-existing scratches or imperfection spots on glassware can release particles during the heating and shaking process. We suggest that users check the glassware and avoid the use of

the scratched glassware. Our comparison of glassware made of different glasses (such as soda-lime and borosilicate) showed that borosilicate 3.3 releases the lowest quantity of glass particles (can be screened for by Raman spectroscopy), and we recommend the use of borosilicate 3.3 glassware in all tests.

1.2) Pour 360 mL of DI water into a glass beaker. Cover the beaker with a clean glass disk. Then move it into a brand-new microwave oven and heat for 2.5 minutes at full oven power. After gently shaking to remove any potential temperature gradients due to uneven heating, the temperature of the water inside of the beaker is 70 °C and ready for sample preparation.

1.3) Prepare 95 °C water for BFB sterilization by pouring 1 L of DI water in glassware and heating in a microwave oven for 14 minutes.

Caution: Never use plastic kettles to prepare hot water. The plastic kettle itself releases millions of MPs into the hot water during the boiling process[13].

2. MP release during formula preparation

NOTE: Carefully following the standard formula-preparation process (cleaning, sterilizing and mixing) recommended by the WHO[21], the MPs released from BFBs during formula preparation is mimicked in the following 3 steps.

2.1) Collect brand-new BFB products from pharmacy stores and clean them thoroughly after removing the product from its packaging. Wash each BFB using detergent water (repeat 3 times at room temperature-RT) and distilled water (repeated 3 times, RT). Finally, rinse the BFB 3 times using DI water at RT.

Caution: Do not clean the BFB using sonication. Though sonication is widely used in laboratories for mixing and cleaning, the sonication of BFB can severely damage the bottle surface and cause MP release from PP products within 1 minute.

2.2) Soak the BFB in 95 °C DI water (section 1.3) to sterilize the bottle. To avoid the floating of the BFB, slightly press the exterior of the BFB using a stainless-steel tweezer and ensure that the whole bottle body immerses in the water.

2.2.1. After 5 minutes, take out the bottle and move it to a clean glass disk. During the air-drying step, invert the bottle on the glass disk until there is no evidence of droplets.

2.3) Pour 180 mL of hot DI water (70 °C, from Section 1.2, corresponding to WHO guidelines) into the air-dried bottle. Then cover the bottle immediately using a glass Petri dish and place it into a shaking bed.

2.3.1. To simulate the formula mixing process, shake the bottle at a speed of 180 rpm for 60 seconds. After shaking, move the bottle to a clean glass plate and allow it to cool down.

3. Sample preparation for MP identification and quantification

3.1) Sonicate and thoroughly rinse all parts of the glass filter (diameter of 25 mm, glass funnel, fritted glass support base and receiver flask) using DI water.

3.1.1. Place a piece of gold-coated polycarbonate-PC membrane filter (pore size of 0.8 µm, Au coating layer thickness of 40 nm) in the middle of glass base.

3.1.2. Assemble the glass funnel and stainless-steel clamp to fix the membrane filter. Finally connect the assembled glass filter to a vacuum pump (**Figure 2**).

Caution: To ensure that the membrane smoothly sticks onto on the surface of the glass base it is important to keep the glass base wet. If necessary, 1-2 drops of DI water should be dropped on the surface of the glass base before placing down the membrane filter.

3.2) Carefully mix the cooled water sample in the BFB (from Section 2.3), and then transfer a certain amount of the water sample to the glass funnel using a glass pipette. Switch on the vacuum pump to allow the water sample to filter through the membrane filter slowly.

3.2.1. After filtering, wash the interior of the glass funnel using DI water to ensure that there are no particles sticking on the funnel.

Caution: To avoid the overlap of the particles on the surface of the membrane filter, it is important to carefully choose the correct volume of water that is passed through the filter. BFBs release large number of particles, so that 3-5 membrane filters are needed to filter the entire volume of the water sample.

3.3) Disconnect the vacuum pump and disassemble the glass filter. Then carefully take out the membrane filter using a stainless-steel tweezer and move it to a clean cover glass. Fix the membrane filter on the cover glass using a small piece of paper tape. Immediately store the sample in a clean glass Petri dish.

4. Sample preparation for AFM topography characterization

4.1) Prepare a clean silicon wafer. Drop a 50 µL water sample (from Section 2.3) on the surface of the silicon wafer and dry it in an oven at a temperature of around 103 °C. Repeat this process if the MP level in the water sample is low.

4.2) After 1 hour of drying, move the wafer to a clean glass Petri dish and allow it to cool down in a desiccator.

4.3) After the wafer has cooled, store the sample in a dry place.

5. MP identification and quantification using Raman spectroscopy

5.1) Calibrate the Raman system using a zero-order correction and a silicon wafer. Ensure that the peak location of silicon wafer is at 520.7 cm^{-1} and that peak intensity is higher than 6000 a.u. when the laser intensity is at 100%.

5.2) Set up the parameters of the Raman system to obtain high signal-to-noise MP spectra while avoiding the burning of MPs. Set the system as follows: 532 nm excitation laser, remove cosmic ray, laser intensity of 10% (laser power of 0.18 mW), spectral resolution of 1.5 cm^{-1}, exposure time of 10-20 seconds, accumulations of 10-40 times and spectral range of 200-3200 cm^{-1}. **Figure 3** showed typical spectra of MPs with accumulation times from 1 s to 400 s.

Caution: Do not test particles using 100% laser directly to avoid the rapid-burning (can be burned in 1 minute if the particle is small). Use low intensity (10-50%) to conduct the test first.

5.3) Place the filter sample (from Section 3.3) in the middle of the Raman sample stage. Choose four representative spots (2 spots are in the middle area while other 2 spots are close to the edge of working area, **Figure 3C**) on the membrane filter to conduct the test (total test area around 1.5 mm^2).

5.4) Observe and photograph the particles on the surface of the membrane filter using an optical microscope (100x) followed by chemical identification using Raman spectroscopy.

5.4.1. Compare the Raman spectrum obtained to the reference standard PP spectra (from bulk material of BFB and previous publication[22]).

5.4.2. Determine the particles' chemical property using the intensive peaks in the range of 2780-2980, 1400-1640 and 709-850 cm^{-1}, corresponding to the stretching vibrations of $CH/CH_2/CH_3$ and C-C groups associated with polymer materials (**Figure 3**).

5.5) Analyze the size and quantity of the identified MPs using ImageJ.

5.5.1. Obtain the MPs concentration in the water sample based on the tested area, total working area (227 mm^2) and the known filtered sample volume.

5.5.2. Classify the confirmed MPs into 5 groups in terms of the size: 0.8-5 µm, 5-20 µm, 20-50 µm, 50-100 µm and > 100 µm.

5.5.3. Finally, determine the MPs quantity in one liter of water sample based on the filtered sample volume, number of MPs recorded and tested area of the membrane filter.

6. MP topographic characterization using AFM

6.1) Equip the AFM system (NT-MDT) with a tapping mode probe. Calibrate the system using

a step height standard (SHS). Set up the system within optimal work conditions: the scan rate is 1 Hz, the scan size is 10-50 µm, the tuning frequency is around 160 kHz, and the scan line is 512 pixels.

6.2) Fix the silicon wafer (from Section 4.3) on the AFM sample stage. Observe and photograph the target particles on the surface of the silicon wafer, followed by chemical identification using the method in Section 5.

6.3) Switch the system to AFM mode (the Raman spectroscopy and AFM are assembled in one system) and test the topography of identified MPs.

6.4) Analyze the 3d data using Gwyddion 2.54 software. Use the option of *profile* to obtain the particle dimensions and average heights while *3D view* to obtain 3D structure.

REPRESENTATIVE RESULTS:

To validate this protocol, the water sample was prepared by adding standard polystyrene microplastic spheres (a diameter of 2.0 ± 0.1 µm) to DI water. The MP quantity added corresponded to 4,500,000 particles/L, which is similar to the MP release level from BFBs. Following protocol sections 2-3, the MPs were successfully collected (**Figure 4A**) and the recovery rate was 92.4-101.2%. This recovery rate is comparable to a previous study on MPs[23]. Using ImageJ, the detected diameter of standard MPs was 2.04±0.08 µm (where ± represents standard error of the mean value), which is around 102.2% of the designed size (2.0 ± 0.1 µm). Meanwhile, the potential interference from other types of MPs, such as PP and PE, was also tested for but none was found in these standard PS water samples. Hence, the developed protocol avoids contamination and is a reliable test of MP release from BFBs.

This protocol was used to test the MP release from eight popular BFB products. **Figure 4B** showed the typical MPs collected on the surface of the membrane filter. During the chemical determination using Raman spectroscopy (**Figure 3**), the peaks in the range of 2830-2970 cm^{-1} became more and more significant with the increased accumulation time. These peaks reflect the stretching vibrations of $CH/CH_2/CH_3$ groups, which can be used to identify MPs. A high number of MPs were released during the use of BFBs. The MPs levels ranged from 1.31 million to 16.20 million particles per liter (**Figure 5**). This result is 3-5 orders of magnitude higher than the previously reported levels of MPs in drinking water[24]. It is evident that the babies likely experience high levels of MPs exposure.

Figure 6 shows the typical topography maps of MPs recorded using protocol sections 1, 2, 4 and 6. For large MPs of around 8 µm in lateral size (P1 in **Figure 6**), the average thickness is 0.82 µm. For smaller MPs around 3 µm in lateral size (P2 in **Figure 6**), the thickness is close to 0.25 µm. In general, the thickness of the MPs released from BFB is around a tenth of the lateral size. It is also noticeable that the surface texture of MPs is rich with nano-sized bumps and valleys, which can substantially increase their absorption capacity. Previous studies found that MPs are effective carriers for pollutants, such as pesticides[25,26]. The observed topography of the MPs found here is likely an important contributor to the high carrying capacity of MPs.

FIGURE AND TABLE LEGENDS:

Figure 1: The diagram of sample preparation and test.

Figure 2: Assembly of the glass filter and pump. 1-cooled water sample in BFB; 2-assembled glass filter; 3-glass transfer pipette; 4-vacuum pump; 5- reciprocal shaker.

Figure 3: Typical Raman spectra for MPs determination. (A) The Raman spectra of a bulk piece from BFB, membrane filter and MPs on the membrane filter, respectively. **(B)** The Raman spectra of one potential MP with different acquisition time (1 s, 10 s, 100 s, 400 s). **(C)** The representative spots tested. The total diameter of filter membrane is 25 mm in diameter with a 17 mm diameter real working area. The four white boxes indicate full representative spots for Raman testing. 2 spots are in the middle area while the other 2 spots are close to the edge of the working area. In total, the tested area of the four spots is 1.5 mm^2.

Figure 4: Typical optical image of standard PS MPs and MPs release from BFB, respectively. (A) The optical image of standard PS MPs. The particle inside of the red box was confirmed as typical PS MP. **(B)** The optical image of MP release from BFBs. The particle inside the red box was confirmed as a typical MP.

Figure 5: Quantity of MPs released from plastic BFB products. 8 popular products were chosen at the study. The error bar indicates the standard error of the mean value.

Figure 6: Typical 3D image of MPs release from BFB. (A) AFM image of typical MPs release from BFB. **(B)** Extracted cross-section profiles of the MPs. **(C)** The 3D topographic image of the released MPs.

DISCUSSION:

Though the study of MPs in marine and freshwater has been widely reported and the relevant standard protocol has been developed[17], the study of daily-use plastic products is an important emerging research area. The different environmental conditions experienced by household plastic products means that extra care and efforts are needed to obtain reliable results. The study protocol must be consistent with the real daily use scenarios. For example, sonication is widely used in lab-tests to clean samples. However, it was found that 1 minute sonication can severely damage the BFB's surface, resulting in levels of MP release an order of magnitude higher. Similar polymer breakage due to sonication was also reported previously[27], which indicates that sonication is not a suitable cleaning method for plastic sample preparation in MP studies.

In addition, potential contamination sources must be identified and eliminated. Kettles are widely used to prepare hot water, which is necessary for the BFB test. However, a single boil can generate up to 30 million particles per liter in a plastic kettle[13]. Microwave ovens are a non-contact method to prepare hot water once care is taken to eliminate local heating. For filtration, a glass transfer pipette is recommended rather than the plastic one (usually made of PP). For brand-new PP products, it has been reported that a high quantity of MPs is attached to the

surface due to the manufacturing process [15] so care must be taken to properly clean all products before testing begins. In summary, the researcher must be vigilant to avoid any procedure that can adversely influence the measured levels of MP release from BFBs.

It should be noted that the protocol cannot account for all types of MP release. Due to the use of a filter with a 0.8 µm pore size, nanoparticles smaller than 0.8 µm are beyond the scope of this method. In addition, individual parents might not follow the WHO guidelines on which the protocol is based so that in real life the MPs level in prepared formula could be significantly different from that reported here.

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DISCLOSURES:

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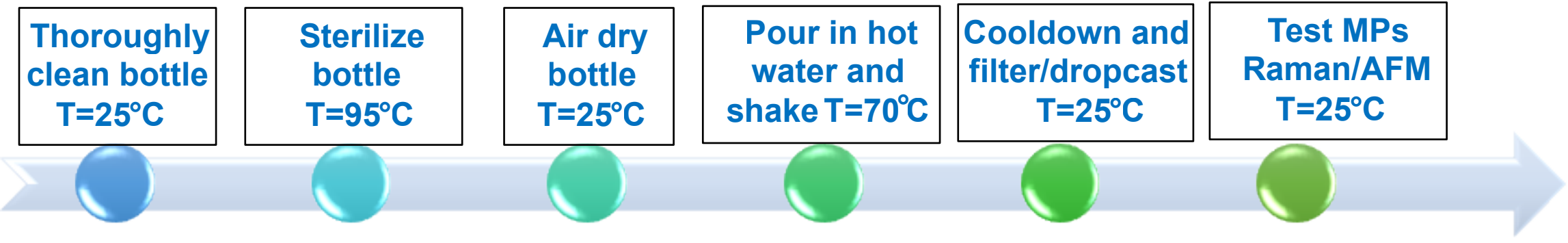

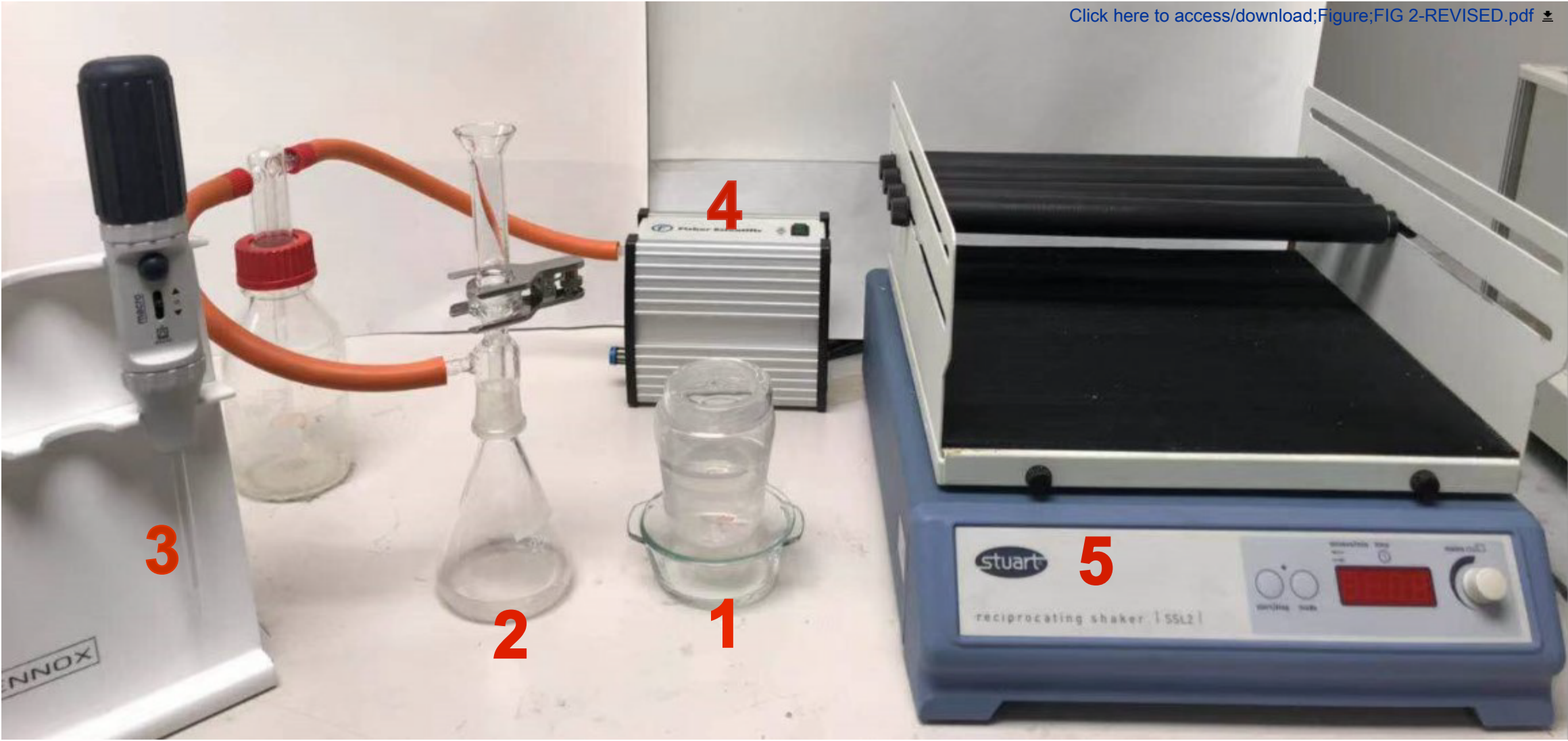

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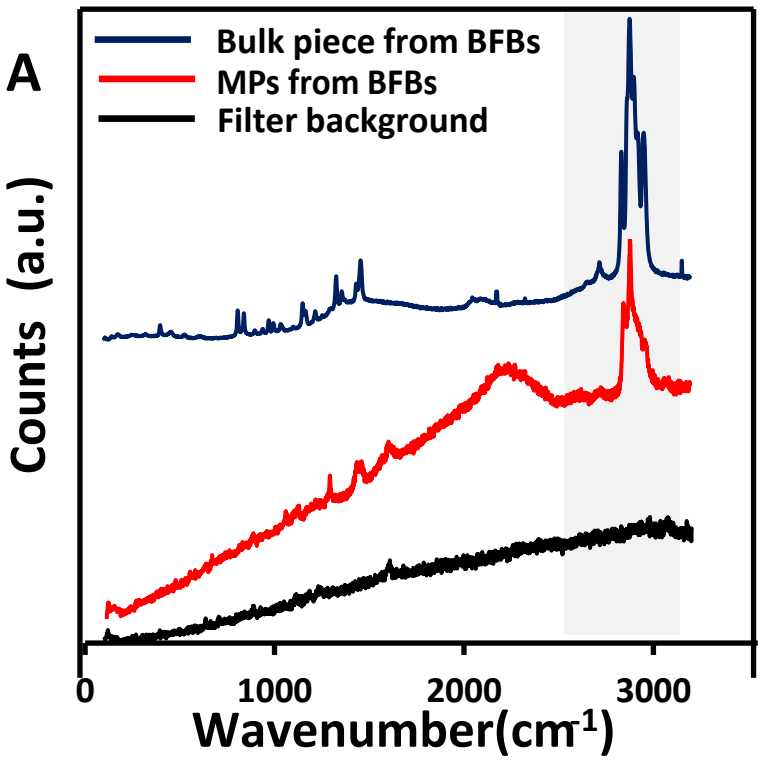

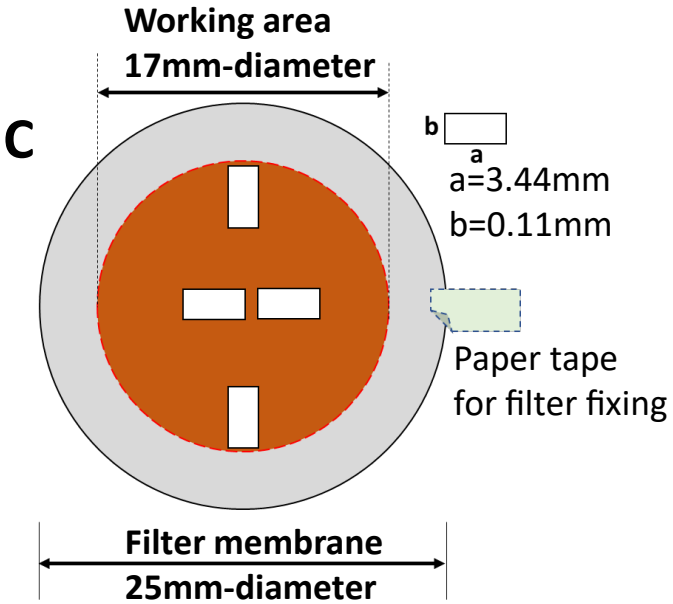

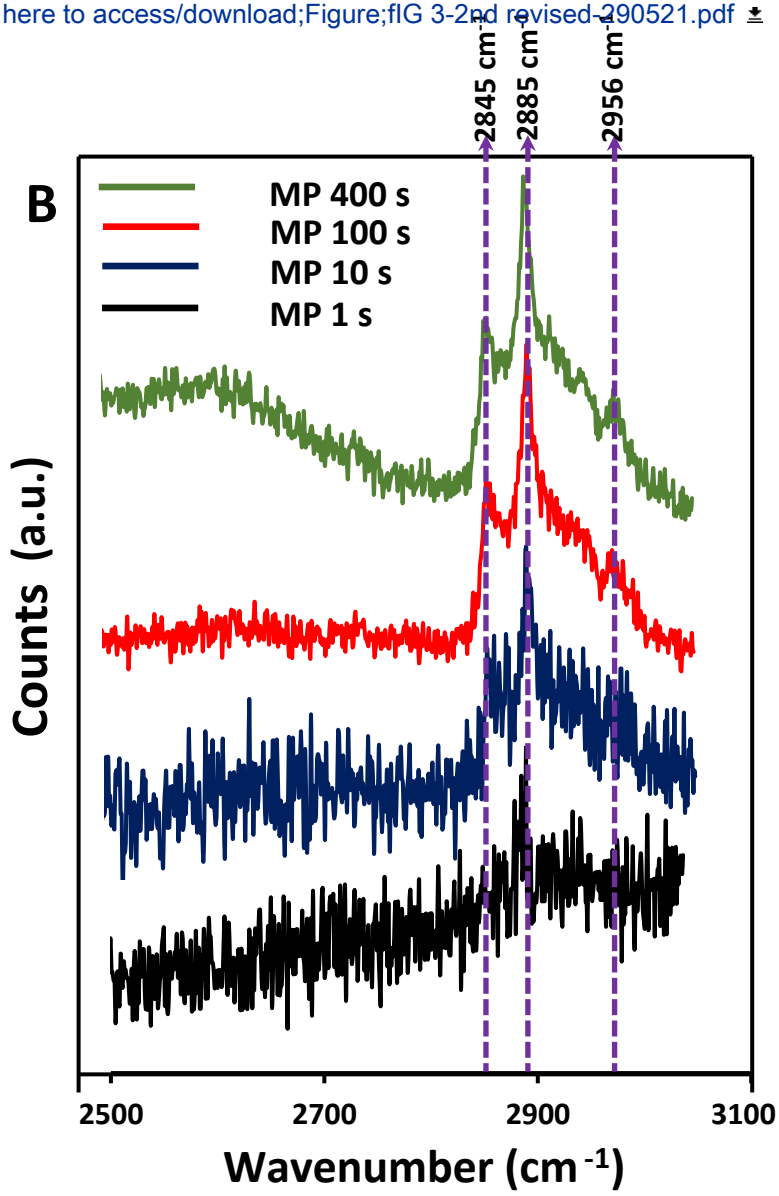

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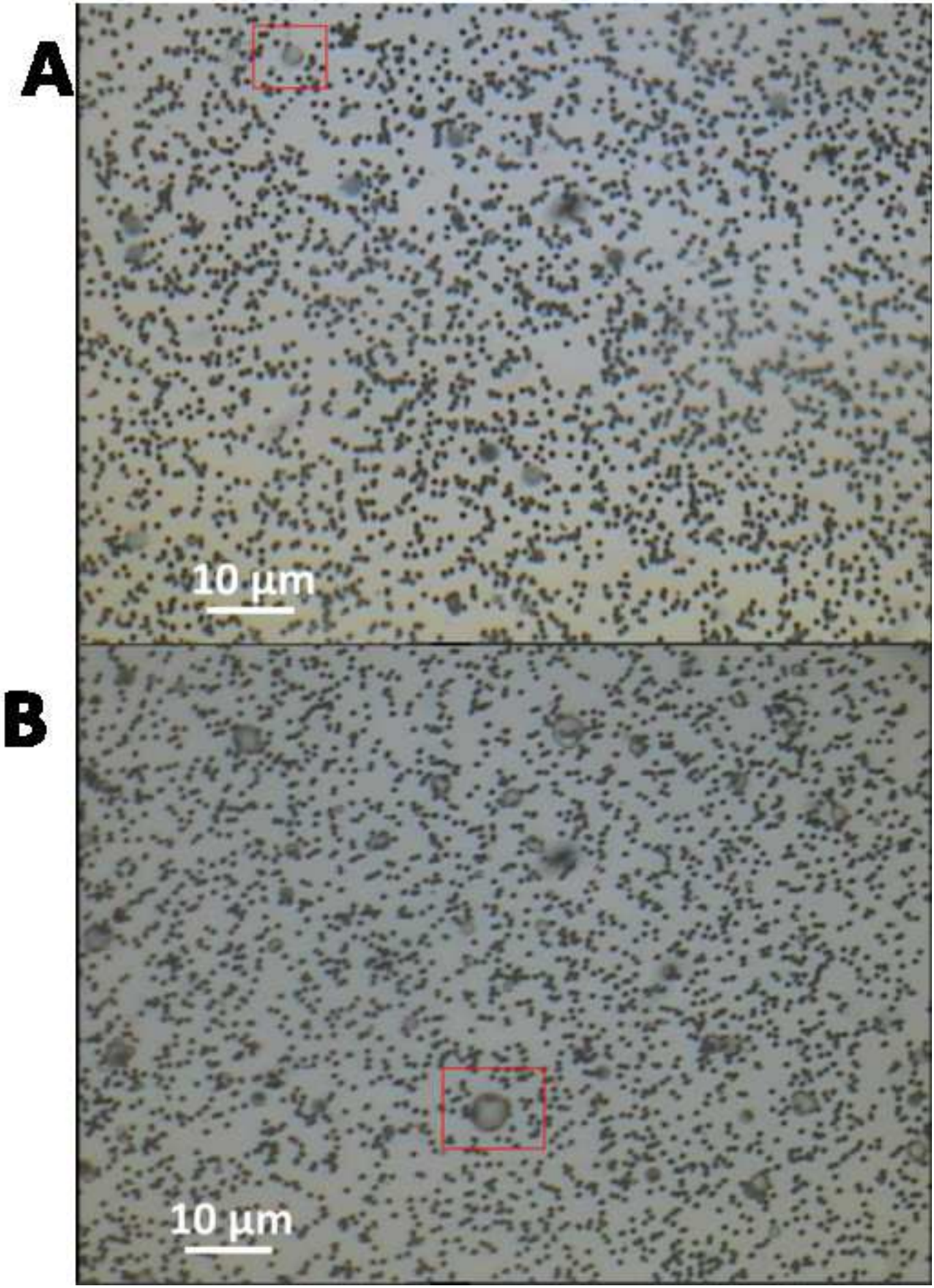

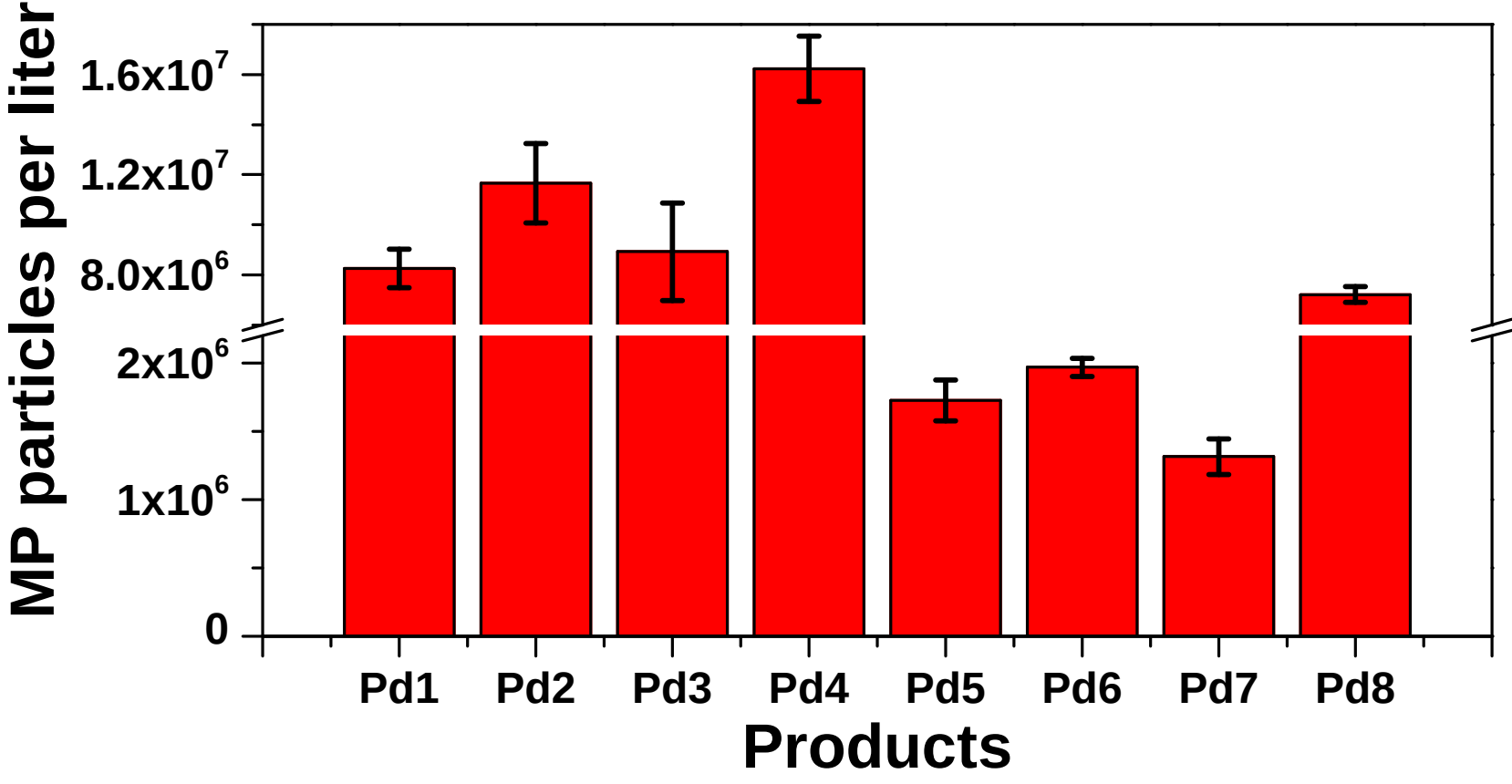

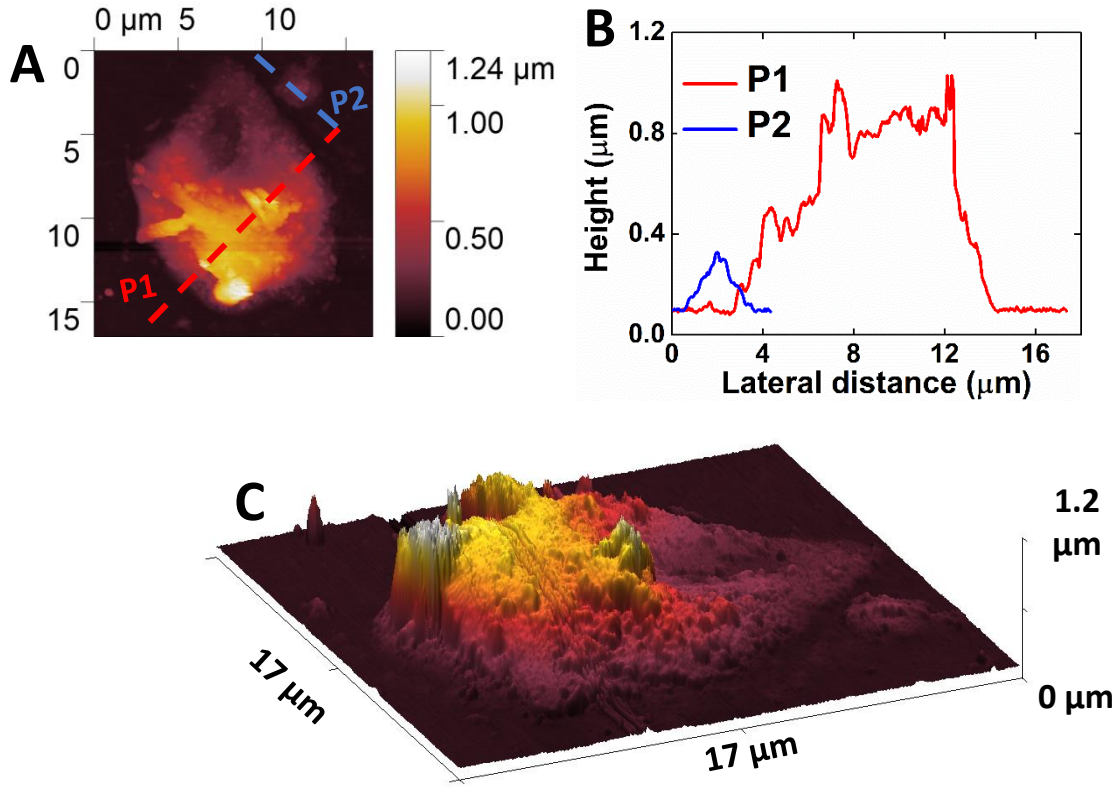

Responses to Editor and Referees

We would like to thank the editor and the reviewers for their review of our manuscript. We agree with most of the comments and the manuscript have been revised accordingly. In our repose to Reviewer 1 we hope that our detailed explanation can help to eliminate any misunderstandings that may have arisen from our original submission. All changes are highlighted in grey and can be found in the word document (grey marked-manuscript JOVE.doc). Our responses to the specific comments of the editor and reviewers are summarized below.

Responses to Editor

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: As suggested, all authors have carefully read through the paper and ensured there are no spelling or grammar errors.

Changes to be made by the Author(s) regarding the video:

- 0:48 - Consider using a fade/cross-dissolve rather than a hard cut here to convey the passage of time within the same action
  Response: Thanks for the suggestion. We have inserted a cross-dissolve to make the before and after shots connected more natural.

- 0:52 - a color filter or profile seem to change here, please check if this was an export, color correction, or editing issue and make any necessary adjustments
- 1:32 - a color filter or profile changes again here. please check if this was an export, color correction, or editing issue and make any necessary adjustments
  Response: Thanks for reminding us. We recalibrated the colors of these two shots to make the whole video uniform in color.

- 1:52 - please use a fade/cross-dissolve rather than a hard cut here to convey the passage of time within the same action
  Response: Thanks for the suggestion. We have inserted a cross-dissolve to make the before and after shots connected more natural.

- 2:05 - there is a hard transition here that reframes the shot of the setup and then immediately blurs and dissolves into the next step. Please consider removing this extra angle, as the presence of a brief clip with no corresponding narration is disorienting to viewers.

Response: Thanks for your suggestion. We deleted this unnecessary scene to make this step more easily understood by the audience.

- 2:22 - a color filter or profile changes again here. please check if this was an export, color correction, or editing issue and make any necessary adjustments
  Response: Thanks for reminding us. We recalibrated the colors of these two shots to make the whole video uniform in color.

- 2:26 - the cut here is disorienting because the liquid is at a visibly different level in the wide shot than it is when it cuts in close. Consider excising the footage 2:26-2:30 in order to make the cut more seamless. Similarly, consider removing the clip that plays 2:31-2:32; such a rapid but seemingly unmotivated edit adding another angle of the same setup and action is disorienting to viewers.
  Response: Thanks for the comments, we revised the clips accordingly. We removed the close-ups of 2:26-2:30 and 2:31-2:32 to avoid confusing the audience and make the experiment operation clearer.

- 2:38 - please use a fade/cross-dissolve rather than a hard cut here to convey the passage of time within the same action
- 3:04 - please use a fade/cross-dissolve rather than a hard cut here to convey the passage of time within the same action
  Response: Thanks for the comments. We have inserted a cross-dissolve to make the before and after shots connected more natural.

- 3:12-3:26 feels rushed. Consider holding on the last shot of ""chapter 2"" for a moment longer before proceeding to the ch 3 title card. Then consider holding on the card until the narrator finishes reading its corresponding narration, so that the ""carefully take out the sample...."" can begin earlier, during the first shot when the sample is being removed. This should also help the following ""and place it on the center"" line up with when the sample is shown being placed.
  Response: Your suggestion is greatly appreciated. We extended the time of the last shot of Chapter 2 and the Chapter 3 title card to make sure that the narrator finish reading the narration. We also adjusted the length of the first shot of Chapter 3 so that the pace was smooth and not too rushed.

- 3:54 - right before the from the spectra to the wide shot, the color profile changes
  Response: Thanks for the comment. We recalibrated the colors of these two shots to make the whole video uniform in color.

- 3:55-3:57 - This wide shot is brief and has no accompanying narration.

Consider either removing it or drawing it out and adding narration describing what the author is doing in the shot.
Response: Thanks for your comment. This section is preparatory work before the experiment, so there is no corresponding narration. We have removed this shot in order to highlight the point more clearly.

- 4:46 - consider holding here a moment to give viewers a moment
  Response: Thank you for reminding us. We have extended the shot to provide more time to the viewers.

- 5:17 - consider holding on this image until a second after the corresponding narration concludes to give viewers time to digest the information
- 5:42 - consider holding on this image until a second after the corresponding narration concludes to give viewers time to digest the information
  Response: Thanks for your comments. Yes, we added two seconds to give the audience more time to absorb and digest the information.

Audio Comments:

- Please reduce the volume of the background music.
  Response: Thanks for your suggestion. We have reduced the volume of the background music in order to make the narration clearer.

- 1:28 - consider pushing back audio that starts here ""after shaking..."" to ~1:32 so that it falls mainly under the clip of the bottle being removed as described
  Response: Thanks for your comment. We pushed back the audio at 1:28 to 1:32 so that the narration and video content are better matched.

- 1:35 - similarly, audio here begins ""Sample preparation ..."" Since this is reading the title card, please wait until the title card appears before beginning this narration
  Response: Your suggestion is greatly appreciated. We adjusted the narration "Sample preparation…" here to make sure that the narration started when the title card appears.

- 2:04 - audio describing this step seems to continue until 2:08, but video shifts to the next step at 2:05. Consider lingering on the set-up shot for 3 more seconds before proceeding to the next step, so that the relevant footage can start at the same time as the corresponding audio (""then transfer..."")
  Response: Your suggestion is greatly appreciated. We added 3 more seconds on this shot until the corresponding audio finished. Then

started with the next shot “then transfer…” to make sure the video and audio are in sync.

- 2:05-2:14 - while related, these shots are not showing the actions described in the accompanying narration. The shots that follow them (2:15-2:30) are the ones that show the described filtering, but they currently play in silence. Consider excising the shots 2:05-2:14 and moving up the subsequent shots so that the video and audio match in content and there is less video without accompanying narration
  Response: Thank you so much for your suggestion. We removed some of the unnecessary shots and properly adjusted the video speed here to make the video and audio are in sync and to reduce the number of shots without voice-over.

- 4:21 - there's a ""beat drop"" in the music here following a build-up that is distracting from the results narration. While the music is similar in earlier chapters, it paired better with the movement of the footage. For the more static results section, please consider a reduction in volume or a less intense song.
  Response: Thanks for reminding us. We adjusted and reduced the volume of the background music so that the audience can focus on the results.

Responses to Reviewers

Reviewer #1:

Manuscript Summary:
The authors describe and show a method on how to determine microplastics (MPs) release from baby feeding bottles (BFB) made of PP during formula preparation. They use a standard protocol recommended by the WHO to sterilize and clean the bottles before use. Afterwards, they migrate the bottles with hot (70 °C) water while shaking and leave the water cool down. The migrate is filtered for Raman microscopy (RM) or dried on silicon wafers for atomic force microscopy (AFM). RM is used for MPs identification, AFM for surface characterization of some MPs.

Major Concerns:
Major Concerns 1, It is well known that plastics leach many substances, e.g. additives or slip agents, when getting in contact with water, especially when hot. Therefore, one must assume that several chemicals were dissolved in the migrate of the BFB. When cooling down or when drying (for AFM), these substances might crystallize and become particles. These particles are retained during filtration or are formed on the silicon wafer for AFM. Thus, they will be recognized as potential MPs. In theory, these crystallized particles can be distinguished from PP MPs based on their spectra. However, when having a look on the sample spectra presented in Figure 3, they do not look like PP and are of poor quality. Based on my expertise, I would identify the corresponding "particles" as slip agents, such as erucamide. These are not classified as plastics and thus are not MPs. This theory of "crystallized additives" is further supported by the fact, that the particles shown in Figure 4 b are typically sphere shaped. Further, as stated in Line 270-271: „In general, the thickness of MPs release from BFB is around tenth of the lateral size. It is also noticeable that the surface texture of MPs is rich with nano-sized bumps and valleys." Such regular shaped "particles" would typically be formed during crystallization. This mistake was already done by several researchers and was already discussed based on the publication of Hernandez et al. (https://doi.org/10.1021/acs.est.9b02540) and the corresponding comment (https://doi.org/10.1021/acs.est.0c03182). Instead of cooling the migrate or drying it, it should be filtered hot, as then dissolved chemicals would pass the filter and would not be recognized as particles/potential MPs. In sum, the "MPs" numbers detected via the shown method are highly overestimated.

Response: Many thanks for reviewer's comments, however we believe that there is no overestimation of MPs number. Let me explain. We agree that the Raman spectrum of additive erucamide might be similar to that of PP-MPs obtained in Fig 3 when you have a quick look. However, if we

carefully compare the two spectra (erucamide and MP, see following figure a-c), the differences are very obvious. For example, for erucamia, the Raman intensity ratio between 1450 and 2885 cm^{-1} is around 2 (fig a, data from ref [1], reference details see last 2 pages). In comparison, this ratio for PP-MP is less than 0.25 (fig b). This is consistent with PP rather than erucamia. In addition, the clear peak at 2956 cm^{-1} can be found for PP-MPs (fig c), which is typical PP polymer, while there this peak is not found for erucamide. Hence, the determination using Raman is practical and reliable.

Please note that the Raman spectrum provided in Fig 3 is a typical spectrum of PP microplastics with reasonable signal/noise (S/N) ratio. One may notice that the spectrum of PP-MPs is not as smooth/distinctive as the spectrum from bulk PP. It is because the spectrum of MPs may change with crystallinity and chemical property change during the chain scission process. This is well known in the MPs research area (see ref [2] for the details).

Drop casting on silicon wafer and teabag publication: please note that we fully understand the high uncertainty of the silicon wafer drop-casting method for MPs determination and we never use this method for MPs number determination. The Si wafer used in our study is just a rigid substrate to facilitate AFM imaging (the rigid substrate is necessary for AFM test obtaining 3D image of MPs) and is not used in actual counting measurements of MPs. These particles for AFM test have been verified the nature of PP microplastics by using Raman mapping.

Why not hot filter: Our method is developed based on WHO guideline for baby feeding process. To accurately mimic the daily use scenario, the water was allowed to cooldown as would be the case in formula preparation and to reflect the possible level of exposure to infants. The contents were then poured out for analysis and again, Raman can screen out for any potential contamination, including additives. On this basis our protocol provides no overestimation of MPs number due to contamination.

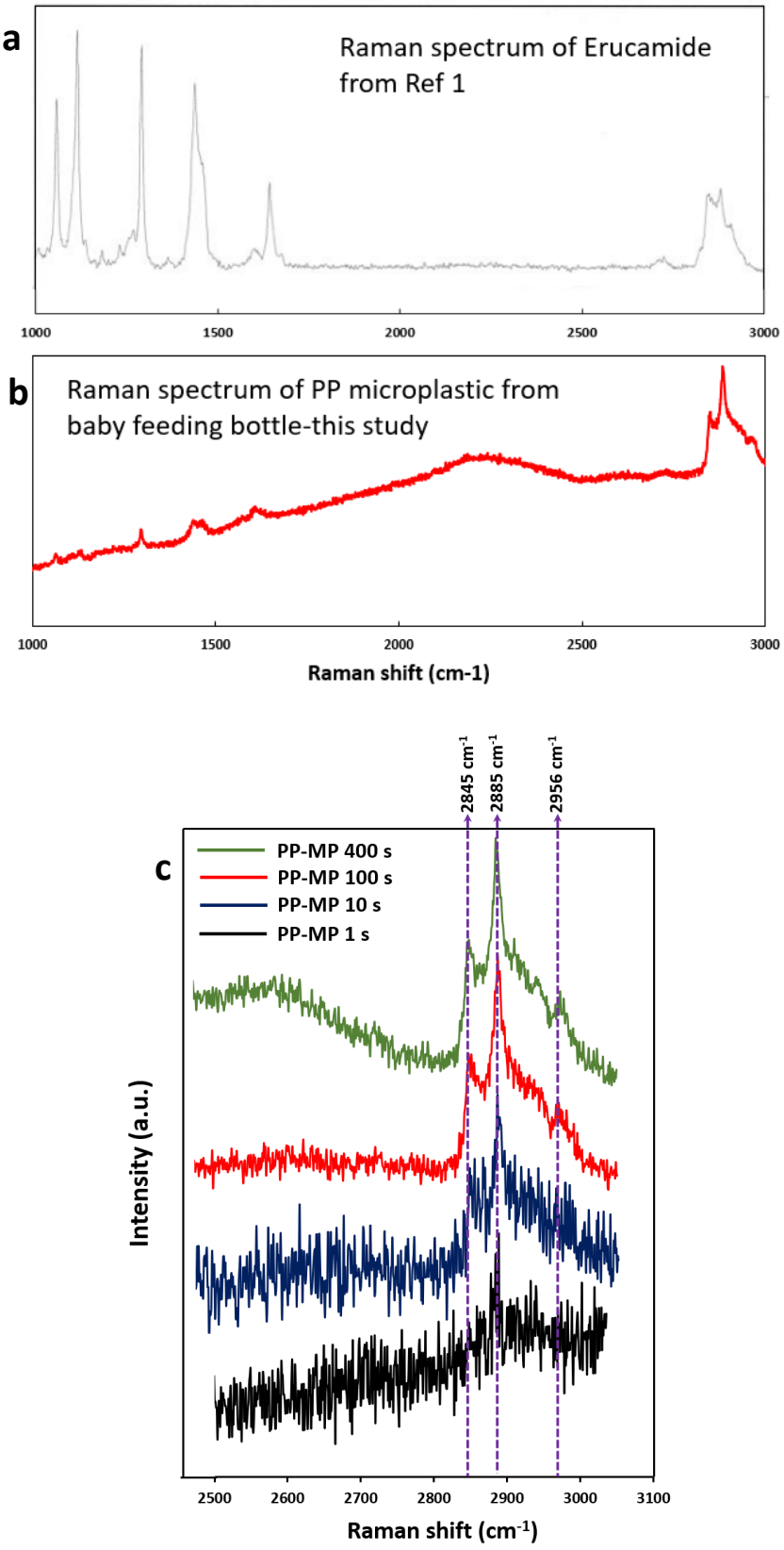

Figure Raman spectra of (a) standard erucamide; (b) PP-MP detected on membrane filter; (c) Zoomed image of PP-MP, Raman shift between 2500-3050 cm-1 with different acquisition time (1s, 10s, 100s, 400s).

Major Concerns 2, Further, there is a huge lack of measures to prevent sample contamination during sample preparation and measurement. In MP analytics, it is usual to work in a laminar flow bench to avoid sample contamination via air. In the video, it can be clearly seen that work is done in a usual lab. Researchers wear cotton lab coats, which is not described in the text. In the video, person wore protective masks, which are made from PP and can lead to sample contamination. They used a plastic tape to fix glass petri dishes to the BFB (video 1:18). Gloves are also known to cause contamination, precisely, they can leach slip agents (see above), mimicking PE MPs (https://doi.org/10.1021/acs.est.0c03742). What is even worse, the authors did not describe if or how they controlled a possible sample contamination. Studies without performing adequate contamination monitoring via the analysis of blank samples should not be published, as no one knows about sample contamination.

Response: We made huge attention to the prevention of contamination, we undertook blank sample testing and ensured that no sample contamination occurs if the user follows our protocol. As suggested, we added the blank result in the paper, please see the revision in line 266-268.

Here is how we prevent contamination.

First, we screened out the real contamination sources/steps. We found out that the clean process using sonication bath (which is very common in lab work) can significantly damage the plastic sample and substantially increase MP release. In addition, the preparation of hot water using plastic kettle or sampling using normal plastic transfer tips (extremely common in lab) can induce high number of MPs and must be avoided. We suggest that ***all items and containers that come into contact with the water samples should be made by glass rather than plastics*** to prevent any potential contamination. We have highlighted these in the protocol and discussion section (see line 119-141 and line 312-331).

Meanwhile, we confirmed that using plastic items (such as plastic gloves) during the MPs sample preparation ***is not the problem***, ***directly contacting*** the water sample with plastic items ***is the real problem,*** such as the use of plastic tips for sample transfer. The paper mentioned by the reviewer strongly supports the recommendations in our JOVE paper. See following figure from glove paper[3] cited by the reviewer (https://doi.org/10.1021/acs.est.0c03742), the author directly soaked the plastic gloves (no pre-wash) in water for 5 h, in addition to filling it with heavy sand to stretch the gloves. As a result, around 8-6000 MPs were released into the water. This is consistent to what we recommend: do not

allow the water sample to come into contact with plastic items, such as plastic transfer pipette tips. Otherwise you risk the release of MPs into the test sample and give false positive result.

Our use of plastic gloves is a very different story. During our experiments, the gloves were washed first and only touch the external surface of bottle and only in areas that are far from the bottle mouth. Using blank tests, we found no PP MP or PE MP introduced into the water samples. Actually, the use of particle-free gloves is the most common procedure applied in most of MPs studies, for example, ref [4], cited over 110 times. For the same reason, the use of cotton lab coats have been employed in most MPs study, for example ref [4] as well as the paper cited by reviewer [3] (https://doi.org/10.1021/acs.est.0c03742).

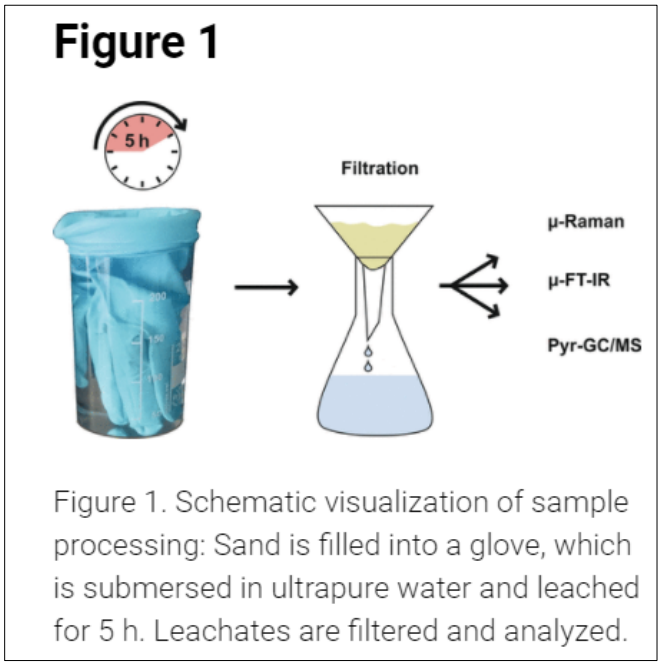

Figure Glove test procedure from the reviwer mentioned paper [3] (https://doi.org/10.1021/acs.est.0c03742).

Finally, the necessity of laminar flow bench depends on the sample types. Please note that the use of usual lab bench is very common for MPs detection, for example, ref [5] and [6] , cited over 250 and 400 times, respectively. The potential concentration of MPs in an open air lab environment are around 0.0054 MPs/L[7], which is around 9 orders of magnitude lower than that of MPs concentration in baby feeding bottle (around 10 million MPs per L). During our test, we found no PP MP or PE MP induced from lab air to the water samples. Moreover, we always insured that water samples are covered by clock glasses and all filtered samples are stored in glass containers.

In summary, our protocol was specifically developed with the aim to establish a robust method to screen out contamination sources/steps and

so as to not to cause any undue alarm about the real level of MPs that are being released from plastic products.

Major Concerns 3, The authors describe in line 217-228 that one should choose "representative spots" on the filter to analyze for MPs using RM. However, they do not mention or discuss how to do this and how large the error is, which is introduced when analyzing only a subarea of the filter. They do not state how large the actual filter area was and do not tell the reader, which factor was used to calculate back to the entire filter area or the sample. Thus, it is not possible to evaluate the uncertainty included in the results.

Response: As suggested, we clearly stated the representative spots location, area and calculation method in the manuscript. Please see the revision in protocol part Line 225-226 and Line 235-236 and Figure 3C.

In brief, we chose four representative spots. 2 spots are in the middle area while other 2 spots close to the edge of working area. For more details see the following figure. The total test area is 1.5 mm^2. The MPs concentration in water sample was obtained based on the tested area, total working area (227 mm^2) and filtered sample volume.

Please note that the test of representative area was widely used at MPs study to benefit the test efficiency, such as ref [8], cited >220 times.

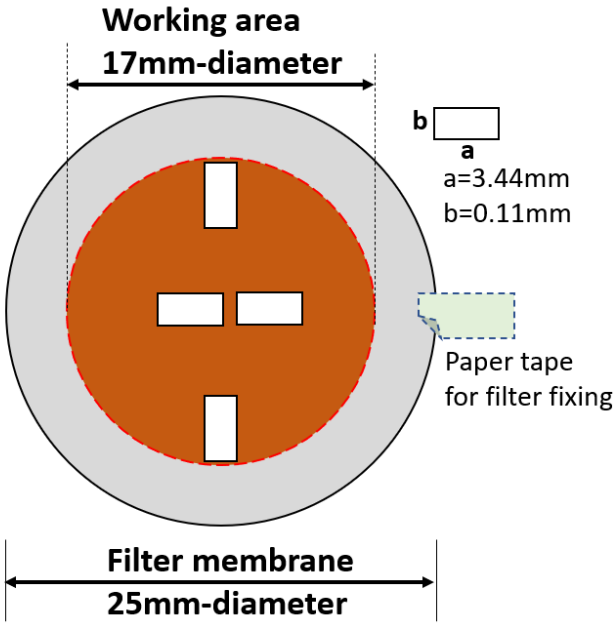

Fig representative spots tested. The total diameter of filter membrane is 25 mm of diameter with 17 mm-diameter real working area. The four white boxes indicate the full representative spots for Raman test.

Major Concerns 4, Based on these three very critical points and the following specific comments, I recommend rejection of the article. The scientific community is flooded with articles reporting about MPs amount in different matrices (https://doi.org/10.1016/j.scitotenv.2020.141426). Even if the call for valid methods producing representative results is getting louder, still lots of papers are published without using them.

Response: We agree with the reviewer's comment about the necessity for reliable MPs detection method. This in fact was our main purpose in publishing this paper as well. Based on our responses and the clarification provided to these three key questions, we believe that our protocol is robust and effective and reports reliable and accurate levels of MP release.

Further Concerns:
Further Concerns 1, Abstract:
Line 34-35: Which MPs release is "extremely high"?

Line 36: How do want to "control" a risk?

Response: we mean plastic products, such as plastic single-use cup and plastic kettle, we have revised as suggested. Please see revision in abstract Line 34.

In terms of "control" a risk, we believe establishing a reliable MPs testing protocol is the first and key step for controlling the risk of misreporting MP release levels.

Further Concerns 2, Line 41-42: How do you justify the statement that "MPs released from plastic products is much smaller than that from marine/fresh water"? Studies on marine/fresh water might have focused on larger MPs, but we do not know whether a much higher amount of smaller MPs are present. Scientists simply did not investigate this, yet.

Response: We understand the concern. However, scientists have undertaken studies about smaller MPs in marine/fresh water. For example, focusing on the micro-sized MPs, the study of freshwater ecosystem (subalpine Lake Garda, Italy[8]) found the average size of MPs is around 130 μm (the filter pore size is 2.2 μm with Raman test). In comparison, the study focusing on daily-use plastic bottle (used for bottled water[9]) found the average MPs release is around 10-20 μm (the filter pore size is 3.0 μm with Raman test as well). Based on current publications, it is evident that " MPs released from plastic products is much smaller than that from marine/fresh water".

However, to avoid any potential controverse, we deleted it as suggested. Please the revision at line 41.

Further Concerns 3, Line 44 & 48-49 & 51 & 109: In my eyes, this protocol or more general, this type of analysis is not cost efficient. One has to buy a Raman microscope (incl. AFM!). Furthermore, gold-coated PC filters are no cheap consumables. As you did not describe any automation, one has to spend lots of time during manual analysis of single particles and the subsequent data evaluation.

Lin 45-46 1): You did not describe measures, which are usually applied during microplastics analytics, such as working in a laminar flow box or wearing of cotton lab coats. In contrast, you used gloves and plastic tape (to fix the glass petri dish to the PP bottle), which can cause contamination. And you did not monitor a possible contamination via blank samples. Thus, "prevention of the potential contamination during sampling and detection" was not described appropriately.

Line 47: I my eyes, you did not perform an "accurate" collection of MPs. You collected all substances which migrated to the hot water and crystallized during cooling.

Response: we have clearly answered these questions in the first 3 key concerns. In brief, we believe that our protocol is robust and effective and reports reliable and accurate levels of MP release.

Further Concerns 4, Introduction:
Line 59-64: You cite a lot of literature, which found adverse effects of MPs on different species. However, there are also many studies, who found that there is no effect. You should mention these studies, too; and discuss this discrepancy.

Response: As suggested, we clearly stated that the risk/adverse effect study is based on animals, and the risk to human being is unknown. Please see the relevant revision in line 62-63.

Further Concerns 5, Line 81-83: "The methods required … already exist." What do you mean with this sentence, especially with "room temperature"?
Response: With regard to room temperature: During the fresh/marine MPs study, many studies directly contact the water samples with plastic equipment, such as plastic filter/sieve to separate MPs with different sizes. At room temperature, it might be OK if pre-washing the sieves repeatedly. Because at room temperature, MPs release from plastic are

usually very low. However, with high temperature (let's say 70 ^{o}C), MPs release rate significantly increases. Hence, for MP study of daily-use plastic experiencing high temperature, the room temperature marine/freshwater sampling protocol is not suitable.

Further Concerns 6, Line 99-100: PP baby bottles are surely not "one of the most commonly used plastic products in daily life".

Response: For most people, they may use the plastic baby feeding bottle on a daily basis when they are young. For example, In UK, the breastfeeding rate for 12-month-old baby is 0.5% [10]. Market data show that over 90% of baby feeding bottles are made of plastic, with several feedings each day Hence, around 90% of people in UK use the plastic BFB quite often at least during the first few years of life. A similar tendency can be found at many other countries, such as US and Australia. Hence, it is fair to say "**one** of the most commonly used plastic products in daily life".

Further Concerns 7, Protocol:
Please define "DI water". Which quality did you use? How did you check for MP contamination?
Line 118: How did you clean the glassware?
Line 120-122: Why do you benefit from less glass particles in your samples?

Response: The DI water used had a resistivity of 18.2 M Ω-cm. In addition, our blank test confirms there is no contamination. All glassware used in the sample preparation was thoroughly cleaned using 4 steps: detergent water wash (step 1, repeat 3 times), distilled water wash (step 2, repeat 3 times), sonication for 30 mins in distilled water (step 3) and finally rinsed with DI water (step 4, repeat 3 times). From our experience, replacing glass with other wares risks introducing contamination into the process.

Further Concerns 8, Line 124-127: Is it important that the microwave oven was brand-new? Are you sure that each microwave will heat the hot water to 70 °C, when heated for 2.5 minutes at "full power"? The same problem occurs for water of 95 °C in line 129-130. How did you check the temperature of the water?

Response: We used a brand-new one. We concerned a used one may have a lot of food residuals and likely contaminate the samples. However, it doesn't matter if the microwave is brand-new, as long as the the users ensure that microwave is clean.

For hot water preparation, it depends on the power of microwave. Hence, the users should conduct the pre-test to determine their own time for hot water preparation. We used a thermal sensor to check the temperature. After heating with setting time, we gently shook the glass beaker to eliminate any temperature gradients due to the potential uneven heating. Then the temperature sensor was immediately soaked in the water to record the temperature.

Further Concerns 9, Line 124 & 156: How did you determine the volume of the water? (This is missing in the video, too.)
Line 142: How did you choose the detergent? Are you sure that you do not introduce MPs via the detergent/Cleaning process?

Response: There are volume marks in the glass filter, hence you will know the filter volume during the filtration.

We are sure that detergent is not a contamination source. Blank test confirmed no contamination. Please see the relevant content at Line 266-268.

Further Concerns 10, Line 148 & 304-205: Really "one minute"? How did you prove that the surface of the bottles is damaged?

Response: Yes, the damage is very quick by sonication. After sonication, we saw the melting-like spots at the surface of BFB, that are visible to the naked eye and confirmed by optical microscopy.

Further Concerns 11, Line 157-159: How did you fix the glass petri dish to the bottle when shaking? In the video, it looks like that you used a plastic film. How did you deal with the potential contamination?

Response: No, that is not a plastic film. We fixed the petri-dish on the mouth of BFB using plastic tape. The tape is around 2 cm lower than the mouth and separated by the glass petri dish. Blank tests once again confirmed no contamination.

Further Concerns 12, Line 164-165: How long did you sonicate? Did you rinse the glass material after sonication?

Response: For glassware, the sonication time is 30 mins, followed by 3 times of DI water rinse.

Further Concerns 13, Line 174-183: Did you clean the glass pipette

before transferring the sample? How did you deal with particles sticking to the pipette? Which sample volume did you transfer? How did you ensure homogeneity of the sample? Did you analyze all filters, when you needed 3-5 membrane filters to filter the entire volume?

Response: Yes, before transferring, we cleaned the glass pipette using DI water. After the sample was filtered, we used the same pipette and sucked some DI water to wash the funnel. During this process, any particles sticking to the pipette should be washed out as well. For homogeneity, we have compared the MPs number in the middle and edge area of filter (see fig 3C), and found no significant differences. In term of 3-5 membrane filters, we analysed all of them.

Further Concerns 14, Line 192-194: When drying a sample in which substances are dissolved, they will crystallize and form particles. How did you deal with this phenomenon?

Response: Please note that we did not use this method to determine the MPs number, so it has no relation to MPs number determination.

Further Concerns 15, Line 207-211: A laser intensity of "10 %" is too unspecific, as the actual laser power varies among different instruments. You should instead state the laser power at the sample in mW. An exposure time of 10-20s with 10-40 accumulations is really high. But the spectra presented in Figure 3 are really worse. The spectral quality does not match to the Raman parameters. Further, the spectra obtained from the sample do not match the reference spectrum of PP. Instead, it looks more like PE or as recently stated by Witzig et al. (10.1021/acs.est.0c03742), like slip agents.

Response: As suggested, we added the specific laser power (around 0.18 mW). Please see the revision at Line 217.

In terms of slip agents and the poor spectra, please see the detailed response to first 3 key concerns (major concern part, 1-3). The signal from small size of PP microplastics is weak then required long time exposure/accumulation. Please note that the Raman spectra provided here is not poor but very typical for microplastics.

Further Concerns 16, Line 213-214: Really in one minute? 50 % is not a low laser power.
Line 220-223: How did you ensure that spectra were identified correctly as PP? Did you use something like a hit quality index (HQI)? How many particles did you analyze indeed? Were all particles recognized via ImageJ

identified via Raman?
Line 249-250: How did you spike a sample with exactly 4.5 Mio. PS particles?
Figure 2 & 3 are of poor quality.

Response: Yes, we confirmed that many PP-MPs can be burned by 100% laser power very quicky. However, 10-50% laser can significantly lower the burning problem. In our case, The Raman signals from PP-MPs have reasonable intensity to determine, hence we did not use HQI.

For spiking PS process, we started from standard PS powder, first made the stock solution with a higher concentration (determined using the filter method, around 100 million PS MPs per liter). Then it is easy to obtain the specific 4.5 million PS MPs per liter by diluting.

Please note that the Raman spectra provided here are not poor but very typical for PP microplastics. One may notice that the PP-MPs spectrum is not as smooth/distinctive as bulk PP. This is well studied in MPs research area, because the spectrum of MPs may change with crystallinity and chemical property change during the chain scission process. The details can be found at ref 2^2. The so-called high-quality MPs spectrum is likely obtained by testing the very large/thick MPs particles (at least 100 μm based on our experience). Given that the most particles release from baby feeding bottle are smaller than 20 μm in lateral size as well as the thickness is just one tenth of the lateral size, which make S/N ratio poorer. Here we provide a very typical spectrum of PP-MPs with a reasonable S/N ratio.

Further Concerns 17, Legend Figure 5: What do you mean with SEM? SEM is a common abbreviation for scanning electron microscopy.
Response: Here it indicates standard error of the mean value. As suggested, we revised it with full name. please see the revision in legend figure 5.

Further Concerns 18, Video:
Missing:
- Procedure of heating water & determining water volume
- Procedure for "4. Sample prepared for topography characterization"
- 3:14 Fixing of filter with paper tape
1:15: Did you use a plastic tape to fix the glass petri dish to the PP bottle?
2:23: Here, you can see bubbles in the pipette. Did you use the pipette correctly?
5:28: The statement that you showed that daily used plastic products are

a very important source for MPs is too general.
The music ends before the video.

Response to Video: There are volume marks in the glass filter, hence you will know the filter volume during the filtration. At the video, we would like to show the key steps for sampling and detection, such as contamination prevention and MPs detection. Hence, for some very normal steps, such as volume measurement, are skipped.

Yes, we fixed the filter (non-working area, details see fig 3c) using the paper tape. In addition, we fixed the petri-dish on the mouth of BFB using plastic tape. The tape is around 2 cm lower than the mouth and separated by the glass petri dish. Blank test confirmed no contamination.

We apologize for the small bubble in the glass pipette tip, which because our researcher was nervous and inexperience during filming. We fully understand how to use the tool. However, we believe our message is clear: use ***glass*** pipette tip, do ***not*** use normal ***plastic*** pipette tips, it may induce MPs contamination.

Finally, as suggested, the music has been extended to match the whole video. Please see the revision in updated video.

Reviewer #2:

Ms. Ref. No: JoVE62545
Title: Sampling, identification and characterization of microplastics release from polypropylene baby feeding bottle during daily use

Thanks for the opportunity to review the work by Li et al. (2021) for a possible publication in JoVE journal. The paper is well written, organized with advantages and limitations related to the protocols required to study microplastics in feeding bottles and other household plastic products. Despite the authors giving extensive highlight to the method, there are some other issues about the work that should also be carefully reconsidered. In my opinion, it is suggested for publication after the major revision. Therefore, the paper be published with some major improvements suggested as follows.

1, Line 70-71: it can be better phrased to express the exposure of humans to microplastics via direct ingestion when using the daily use products.

Response: Many thanks for the suggestion. As suggested, we added the exposure process of MPs when using the daily products.

Please see the revision at line 78-80.

Added content: *Given that these daily-use plastic products are designed for food and beverage preparation, the release of high quantities of MPs is likely and their consumption is a potential threat to human health.*

2, Line 80: what are the household plastic products, authors are referring to here? Provide examples.

Response: The typical household plastics products, such as plastic kettle, plastic single-use cup and plastic teabag. We clearly stated this as suggested.

Please see the revision at line 82-83.

3, Line 99: I am curious here that whether the protocol described in this study is based on the authors previous findings? That is https://www.nature.com/articles/s43016-020-00171-y. If so, would it be a good idea to mention and rewrite the principal objectives of the work.

Response: Yes, this paper detailed the protocol used in our last paper. We clearly stated this as suggested.

Please see the revision at line 102-104.

4, Line 107: Provide particle size interval measured in Raman spectroscopy. I ask the authors to present the size distribution of microplastics documented using ImageJ.

Response: The particle size interval can be found at Fig S2 of our last paper (https://www.nature.com/articles/s43016-020-00171-y.). It is not appropriate to repeatedly report it at the new paper. In addition, the main goal of this paper is to report/visualize the robust method using in MPs detection.

5, Line 121: Can authors please specify more when and at what conditions, the borosilicate 3.3 releases low quantity of glass particles.

Response: As suggested, we specified glass particles release conditions.

Please see the revision at line 124-129.

Added content: *The pre-exiting scratches or imperfection spots on glassware can release particles during heating and shaking process. We suggest the users check the glassware and avoid the use of the scratched glassware.*

6, Line 133: Please provide the reference.

Response: As suggested, we added the ref [11] .

Please see the revision at line 140.

7, Line 141-145: Please do explain why there is a need for three different pre-cleaning steps of feeding bottle involving detergent water and distilled water before microplastic extraction. do mention what detergent was implied for the said purpose?
Whether the distilled water and other associated liquids have been filtered prior to the microplastic experiments with the feeding bottles?

Response: Bottle cleaning using detergent water is suggested by WHO guideline and manufacturers. To mimic daily scenario, we cleaned the bottle using detergent water, followed by distilled water and DI water wash to remove any pre-existing particles in the bottle. After the cleaning process, we checked the bottle and found that the bottle was completely clean. Checking process: We gently poured 125 ml of DI water into the cleaned bottle, waited for 30 mins, and filtered the water using membrane filter. We found there was no MPs.

The DI water for rinsing and sample preparation was pre-filtered (0.22 μm of pore size).

8, Line 304-305: Is this proven elsewhere in literature?

Response: Yes, this damage can be found in previous reports as well. We added the reference and relevant content.

Please see revision at Line 319-321.

Added content: Similar polymer breakage due to sonication was also reported previously[12], which indicates that sonication is not a suitable cleaning method for plastic sample preparation in MP studies.

9, Figure 2: Legend can be improved.

Response: As suggested, we improved the figure legend and resolution to make it clearer.

Please see relevant revision of Fig1, Fig 2, Fig 3, Fig 5 and Fig 6.

10, Figure 5: Please expand SEM.

Response: Here it indicates standard error of the mean value. As suggested, we revised it with full name. please see the revision in legend figure 5-Line 306.

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Reviewer #3:

Manuscript Summary:
This manuscript reports a new protocol for sampling, identification and characterization of microplastics release from polypropylene baby feeding bottle during daily use. Based on this protocol, the recovery percentage using standard polystyrene microplastic was 92.4-101.2%, which also allows cost-effective chemical determination and physical topography mapping of MPs released from baby feeding bottles. The reported protocol is of interest to other scientists and there is sufficient introduction to this protocol. This work can be accepted after addressing the following issues:

Minor Concerns:
1, L166 Are these membranes made by yourself or commercial available products? What is the thickness of the gold film?

Response: Many thanks for review. These membranes are commercially available products, we purchased from APC company, Germany. The thickness of the surface gold is 40 nm. We added this content, as suggested.

Please see revision at Line 173-174.

2, L193 Why did the authors choose the temperature of 103℃? Will high temperature affect the characteristics of plastics?

Response: In general, to dry the particles as well as avoid particle damaging, the standard methods (published by American Public Health Association) suggest 103 ℃. In addition, the melting point of Polypropylene is around 160 ℃, so 103 ℃ should in any way damage the PP.

3, L208 Why did the authors choose 532nm laser instead of 785 or 633?
Response: Our system equipped with 532 nm laser, which has good performance during the MPs test. We expect that 785 or 633 laser can achieve the good performance as well.

4, L217 How are the four representative spots chosen?

How much is the smalles size one can use this protocol to detect microplastics?

Response: As suggested, we clearly stated the representative spots location, area and calculation method in the manuscript. In brief, we

chose four representative spots, 2 spots are in the middle area while other 2 spots close to the edge of working area. Details see following figure. The total test area is 1.5 mm^2.

Please see the revision in protocol part Line 225-226 and Figure 3C.

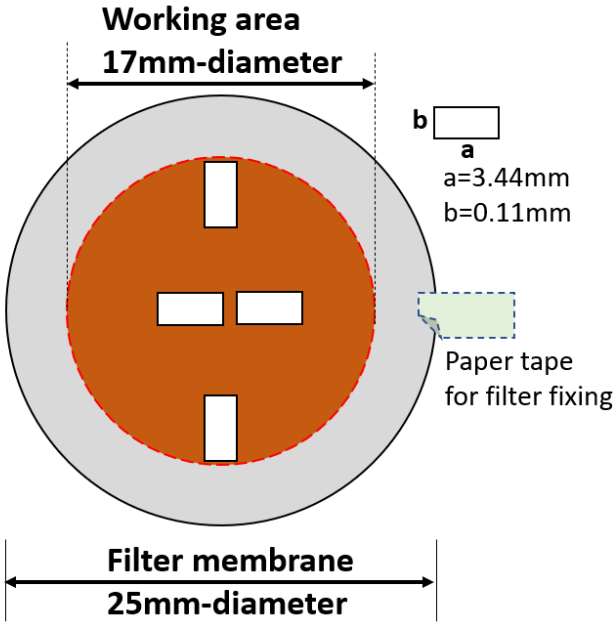

Fig representative spots tested. The total diameter of filter membrane is 25 mm of diameter with 17 mm-diameter real working area. The four white boxes indicate the full representative spots for Raman test.

In general, Raman spectroscopy can test MPs down to 1 μm. But our system can test down to around 0.5 μm. The test limit depends on the spectral spatial resolution of the experimental system given as "Spatial resolution = 0.61 λ/NA". In our measurement, the laser wavelength is 532nm and the objective NA is 0.7, therefore the test limit (spatial resolution) of our set up is 464nm.

reference

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Dear Editor,

We would like to thank the editor for giving us a chance to resubmit the video. Here we submit a new version of the video, which has been modified according to the reviewer's suggestions.

Changes to be made by the Author(s) regarding the video:
• 2:16 - I appreciate the sequence of actions and shots here. To maintain continuity, consider holding slightly longer on the shot at 2:15 to give the reaercher's arms time to assume the position they're shown at in the following shot. Or better yet, if the action occurs earlier in the wider shot, consider showing the tail end of the arm rising into that position to link the motion of the two shots.
• 2:20 - Similarly, while I appreciate the correct use of a hard cut here because these actions happen immediately in sequence, the discontinuity--between the raised and occupied arms in the first shot and a hand already grasping and shaking the bottle in the second--makes the transition disorienting. Consider a fade here, or ending or starting with a "cleared frame" so mitigate the continuity issue. By "cleared frame," I mean the reseacher is no longer visible in the frame, so it is believable that they are free to take the next action.

Response: Thank you for the valuable advice. We changed the order here to make the "take the water sample from the bottle with pipette" step more coherent to prevent confusion for the viewer. At the same time, we also modify the order of narration so that the picture and narration better correspond.

Audio Comments
• please increase volume of the narration so that it peaks between -12 and -6dB as per JoVE's ASV criteria

Response: Thanks for reminding us. We increased the volume to ensure most peaks are between -6 to -12 dB (software: Adobe Premiere Pro).