A Silicon Nanomembrane Analysis Pipeline (SNAP) for Multimodal Analysis of Microplastics in Drinking Water

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Abstract

The biological impact of microplastics in human food and water sources is largely unknown, and drinking water sources are not exempt from microplastic contamination. Here, we demonstrate a streamlined approach for capturing, quantifying, and identifying microplastics in drinking water. We present an analytical workflow termed Silicon Nanomembrane Analysis Pipeline (SNAP) that takes advantage of novel silicon nitride nanomembranes which enable a significant “concentration factor," consolidating suspended particles into a planarized observation area for individuated, quantifiable, and multimodal particle analysis on the same substrate. Drinking water samples sourced in the Rochester, NY region were collected and analyzed using SNAP. Particles in each sample were characterized by optical and electron microscopy, Raman spectroscopy, and various identified constituents were quantified in proportion to total captured particles.

Introduction

Microplastics are defined as synthetic polymers measuring smaller than five millimeters in size[1, 2.] Microplastic (MP) pollution is a growing human health, environmental, and ecological concern. MP contamination has been found in water sources of all types; freshwater, oceans, and even drinking water sources[3]. MPs are generated from the breakdown of primary plastic sources[4], as well as synthetic textiles[5, 6, 7]. MPs are ubiquitous and have been found in human tissues, such as human placenta, feces, and blood[8, 9, 10]. There is an increasing interest in MPs research, as evidenced by more than 4,100 and 4,600 publications in 2023 and 2024, respectively (PubMed data; keyword “microplastics”), as well as an increasing public concern regarding human exposure to MPs. Responding to a growing public concern, several government regulatory bodies such as the State of California[11] and several EU countries[12], have been (or will be) implementing regulations on MP levels in drinking water. Considering all of the above,

the U.S. Environmental Protection Agency has recently announced MPs as a new contaminant of concern. However, human risk evaluation is severely impeded by a lack of methods for the reliable and reproducible detection of MPs.

Characterization of MPs relies on analytical methods such as optical and electron microscopy[13], and spectroscopic and spectrometric techniques[14, 1, 15] to understand visual characteristics of MPs[7] and their composition, respectively. Samples containing MPs should include multiple analyses to characterize, identify, and quantify MPs, and at least require spectroscopy/spectrometry (i.e., Raman spectroscopy, infrared spectroscopy, or pyrolysis gas chromatography mass spectrometry, etc.) as per some journal minimal requirements for the measurement of plastics in environmental samples, such as Science of the Total Environment[16]. Raman spectroscopy is more versatile than infrared (IR) spectroscopy, especially with respect to material identification in biological samples. Raman spectroscopy is a light scattering technique, enabling it to analyze smaller sized particles more easily than IR, an light absorbing technique that necessitates larger sized particles and has more constraints on sample placement[17]. It has been previously suggested that substrates for Raman spectroscopy comprising of silicon or silicon oxide can yield reliable results with minimal background noise[18]. Substrates for certain analytical techniques that are usually compatible with one type of analysis (i.e., gold coated substrates required for IR spectroscopy) are not often compatible with transmission and/or reflectance light microscopy. Subsequently, subsampling is popular among investigators to produce samples appropriate for each separate analysis, but subsampling is time consuming and could exclude low abundance particulates[6, 1, 2]. Typically, manual transfers of particulates from one substrate to the next is necessary to analyze the same particle for characterization and identification by different analytical modalities, but these slow, manual transfers increase the likelihood of bias, contamination, and loss, as well as limit analysis of MPs to only the largest sized particles that can be manually manipulated[13]. These suboptimal workflows introduce numerous challenges and reliability issues, contributing to the significant variability in MP quantification reported in the literature[3, 7].

Here, we present an analytical workflow we call the Silicon Nanomembrane Analysis Pipeline (SNAP) to capture, characterize, identify, and quantify MPs, and demonstrate the utility of SNAP by analyzing drinking water samples sourced from the Rochester, NY region. SNAP is enabled by conventionally-sized 25 mm diameter filter disks from SiMPore, Inc. with integrated silicon nanomembranes, which offer a uniform substrate for concentrating and analyzing MPs from drinking water using multiple techniques[15, 19]. After sequentially concentrating and capturing MPs onto nanomembranes with decreasing cut-off sizes (20 µm microporous silicon nitride (MPSN) with cylindrical pores and 8.0 µm microslit silicon nitride (MSSN) with rectangular prism pores), SNAP

facilitates three consecutive analytical steps: 1) sample staining, optical microscopy, and MP quantification, 2) particle identification via Raman spectroscopy, followed by 3) particle characterization via scanning electron microscopy (SEM), enabling multiple analyses of the same particles captured onto one nanomembrane substrate. We demonstrate multiple SNAP pipelines by utilizing both uncoated silicon nitride (SiN) and gold-coated silicon nitride (Au-SiN) nanomembranes: Pipeline A) SNAP performed on SiN and sequentially characterizing the same MPs through multiple analytical techniques; Pipeline B) SNAP performed on Au-SiN with Raman spectroscopy; and Pipeline C) SNAP performed on Au-SiN with SEM equipped with energy dispersive X-ray spectroscopy (EDX) **(Figure 1)**. SNAPs variations are thus flexible, streamlined analytical workflows that can be used for a number of sample types and analytical techniques. We demonstrate SNAP's utility for the analysis of MPs in four drinking water samples. Samples were collected from four distinct water sources around the greater Rochester, NY area. All samples included in this study originated from different surface water sources, such as Lake Ontario, Hemlock Lake, a mix of Hemlock and Ontario, and Canandaigua Lake **(Figure 2)**. Captured particles from these drinking water sources included widespread plastics like polystyrene (PS) and polyethylene (PE), heavy metals like iron, silica, and non-synthetic particles.

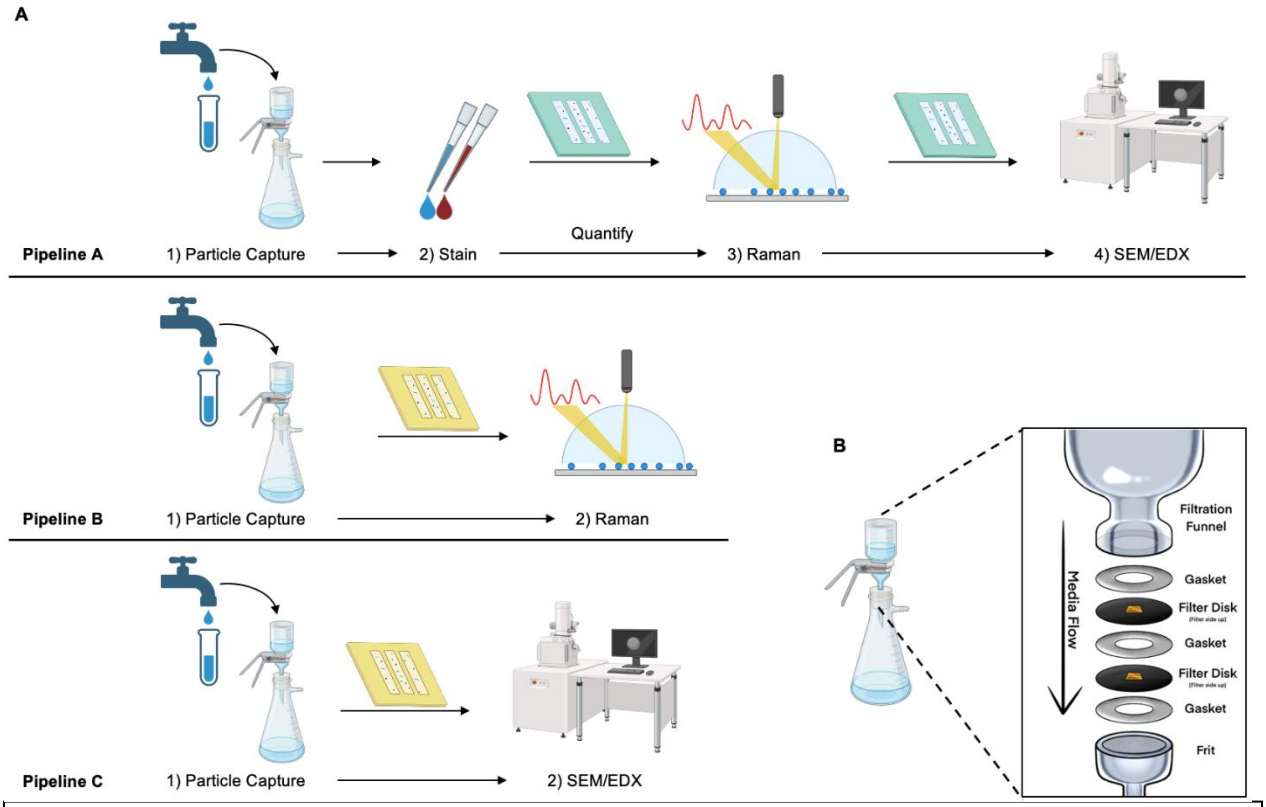

Figure 1. SNAP Pipelines. A) Schematics of SNAP pipelines, characterizing the same particles on the same nanomembrane via a series of different analytical techniques. **Pipeline A)** demonstrates **1)** Captured particles of interest from drinking water samples via vacuum filtration are **2)** stained with Nile Red and Trypan Blue for differentiation of synthetic vs. non-synthetic polymeric particles, respectively, followed by **3)** particle identification via Raman spectroscopy, and **4)** SEM/EDX to characterize particle morphology and elemental identification. **Pipeline B)** demonstrates **1)** Particle capture onto Au-SiN followed by **2)** Raman spectroscopy. **Pipeline C)** demonstrates **1)** Particle capture onto Au-SiN followed by SEM/EDX. **B)** Schematic of the filter disk and silicone gasket in the filtration setup in the vacuum apparatus, depicting the sequential filtration through a 20 µm MPSN, then 8.0 µm MSSN nanomembrane, separated by silicone gaskets.

Protocol

Section 1 - Quality Control Procedures and Solution Preparations

A *- Laminar Flow Hood and Glove Cleaning*

1. Don PPE (personal protective equipment): 100% cotton lab coat and nitrile gloves.

2. Rinse nitrile gloves by spraying them with 99% Isopropyl alcohol (IPA). Scrub hands as if washing hands with soap, then rinse with ~18 MOhm, 0.22 µm-filtered (e.g., MilliQ) water. Conduct this in triplicate to remove all release agent or box dust particulates from the gloves.
3. Fold a Kimwipe into quarters, then spray with 70% IPA.
4. Wipe the hood surface from back to front in long strokes, re-folding the Kimwipe to an unused surface every two strokes.
    a. Repeat step 4 if there is more space to clean and no unused surface of the Kimwipe.
5. Uncover the silicone roller mat, and roll across the surface of the hood to pick up any remaining particles.
    a. To clean the silicone roller, spray with 99% IPA and scrub with a gloved hand, then rinse with MilliQ water. Repeat in triplicate, then allow to air-dry in the hood.
        i. Re-rinse gloves as in Step 2.

B *- Ultrapure Water and Isopropyl Alcohol Generation*

6. Fill a 1L beaker with MilliQ water and place it in the hood.
7. Prime a 60 mL syringe and attached 0.22 µm cut-off syringe filter by filtering at least 200mL of MilliQ water through the syringe and attached filter.
    a. **NOTE** - a syringe pump will be of use to save time, but is not a requirement to conduct the described protocol.
8. Triplicate-rinse a glass screw-cap container with 0.22 µm syringe-filtered MilliQ water.. Fill with syringe-filtered MilliQ water.
    a. All syringe-filtered water from here forward are now referred to as "Ultrapure water".
9. For generating Ultrapure Isopropyl Alcohol (IPA), repeat this section with the desired % concentration of isopropyl alcohol in place of MilliQ water.
    a. Instances of "Ultrapure X% IPA" are generated using the above process.

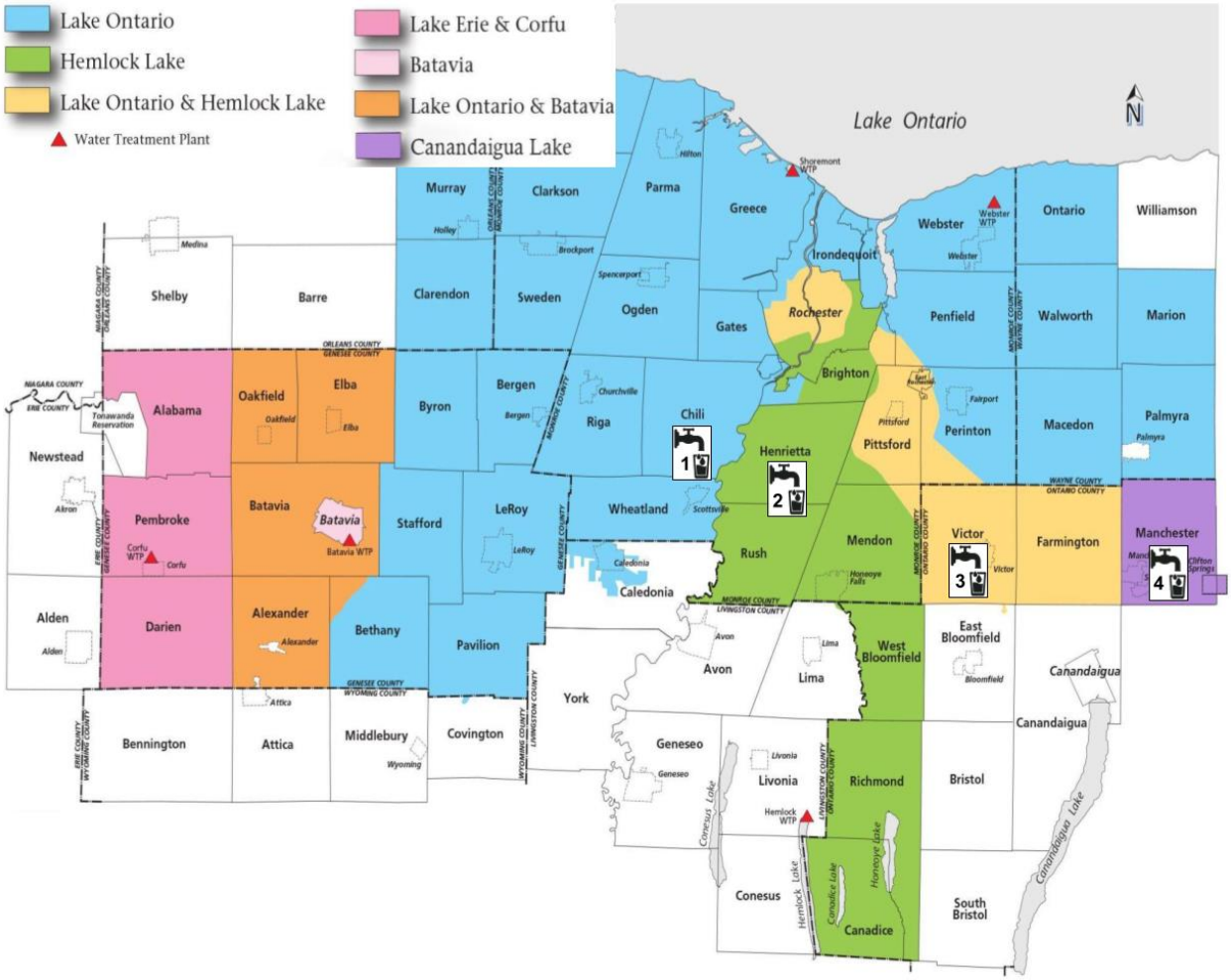

Figure 2. Drinking Water Sample Collection Sources. A regional map published by the Monroe County Water Authority depicting the locations from which each of the four drinking water samples were collected in the greater Rochester, NY, region. Samples originated from different surface water sources: Lake Ontario (blue), Hemlock Lake (green), both Hemlock Lake and Lake Ontario (yellow), and Canandaigua Lake (purple).

Section 2 - Particle Capture from Liquid Samples via Filtration

NOTE: The below protocol may be utilized with liquid samples other than water. Validated sample collection methods should be left up to the discretion of the investigator based on the needs of their study and the needs of the sample in question.

1. Don PPE.
2. Ensure gloves and hood surfaces are cleaned in accordance with Section 1A.

3. Spray a silicone gasket with Ultrapure 99% IPA, scrubbing with gloved fingers, then rinse with Ultrapure water. Repeat this in triplicate for each of the gaskets, then allow to dry in the hood.
    a. The number of gaskets to be used in a filtration equals the number of filter disks to be used, plus 1. Refer to **Figure 1B** for the visual assembly graphic.
4. Utilizing the syringe primed in Section 1B, triple-rinse the inside of the 60 mL syringe with the Ultrapure water by taking up 30mL of Ultrapure water into the syringe, then 30mL of air. Screw on a syringe filter, then shake vigorously and dispense. Repeat this in triplicate.
5. Assemble the filtration apparatus as shown in the visual assembly graphic in **Figure 1B**. Use clean tweezers to handle filter disks and gaskets during assembly and disassembly for best practice.
    a. For each additional SiN filter disk to be added in sequential filtrations, simply add one disk, then one more gasket atop the disk.
    b. Ensure that the disks are ordered with the largest cut-off on top of the stack, and the smallest cut-off on the bottom (i.e., stack a 20 µm cut-off filter disk on top of an 8 µm cut-off filter disk, separated by a gasket).
6. Turn on the vacuum to the apparatus so that there is negative flow through the filter disk stack.
7. With the rinsed syringe, dispense 50 mL of Ultrapure water slowly over the nanomembrane in the center of the top disk to ensure that all the dispensed liquid is filtered.
    a. If filtering larger sample volumes (i.e., > 50 mL), a glass filtration funnel can be utilized. Clean the glass filtration funnel with the procedure outlined in Section 1, Step 8.
        i. **NOTE**: additional labware may incur additional particle contamination risk. Maintain diligence with cleaning procedures and plan accordingly.
8. Once all the Ultrapure water has filtered, allow the vacuum to remain on for approximately a minute to allow the filter disks to dry, then turn the vacuum off.
9. Carefully remove the filter disks from the gaskets using clean tweezers and place disks into the appropriate clean and labeled container for storage, such as a glass petri dish or darkened box.
10. Repeat steps 2-9 in triplicate to for measuring background particle contamination.
    a. **NOTE**: These triplicate repeats enable an evaluation of the effectiveness of the syringe cleaning and the Ultrapure media generation, and a determination of background fparticles contributed by the system's components.
11. Image the filter disks under microscopy for optical analysis, particle counting, etc.
12. For additional liquid samples, repeat step 3 for the gaskets.
13. Obtain a new syringe and syringe filter for each unique liquid media sample (e.g., tap water, digested and conduct steps 2-6.

    a. Any sized syringe may be utilized in place of the 60 mL volume listed in the above section as is appropriate for the sample of interest.
14. Utilizing the sample of interest, take the now-cleaned syringe and uptake the desired volume of the sample, then dispense slowly over the nanomembrane in the center of the top disk to ensure all the dispensed liquid has been filtered.
15. Repeat steps 8 and 9 for each replicate to be performed of the sample of interest.
16. Repeat Step 2 of Section 1 as needed for gloves as they are changed.

Section 3 - Staining

A *- Nile Red (NR)*

NOTE - Do not conduct staining if the sample of interest is to be analyzed with a Raman laser incompatible with fluorescence signal suppression (i.e., 532 nm laser). Use a longer wavelength laser as an alternative.

NOTE - The filter disk can be stained with either NR or Trypan Blue (TB) first - it is not required to start with one or the other stain. TB counterstaining is used to differentiate non-synthetic polymers from biological sources vs. synthetic polymers stained by NR, thereby providing an indication of potential MP false positives.

1. Don PPE.
2. Clean gloves as outlined in Section 1, Step 2.
3. Complete sample filtration as outlined in Section 2.
4. Triplicate rinse two glass screw cap containers with Ultrapure water.
5. Prepare a 0.1mg / mL solution of NR in Ultrapure 99% IPA into a clean glass container.
    a. Gently invert 10 times to mix.
6. Into the second glass screw cap container, filter the prepared NR solution with a smaller cut-off filter than that of the filter onto which the samples were captured and will be stained.
    a. A filter disk with a 1 µm cut-off MSSN membrane was utilized for this process, but any porous membrane (e.g., PCTE, PTFE, or sterile bottle-top filters) with a smaller cut-off than the lowest cut-off of the utilized filter disk will suffice - as long as this chosen membrane does not shed particulates into the filtrate.
7. Place the filter disk to be stained onto the support frit of the vacuum collection flask.
8. Pipette 20 µL of the 0.1mg/mL NR solution onto the nanomembrane at the center of the filter disk.

    a. The stain should fully cover the porous surface of the nanomembrane. If 20 µL is not enough, add additional stain until all porous areas of the nanomembrane are suitably covered in stain. This additional volume should be noted for future reference.
9. Allow the stain to incubate for 5 minutes.
10. Once the stain has incubated, vacuum-filter the stain.
11. Triplicate rinse with 1 mL of Ultrapure 99% IPA. This will remove any excess NR stain.
    a. Proceed to the Trypan Blue staining section below after this step if counterstaining is to be performed.
12. Allow the filter disk to sit on the support frit with the vacuum on for 2 minutes to filter and dry any residual liquid. If not dry after 2 minutes, transfer the filter disk using a clean glass petri dish to a 70°C oven for 2-5 minutes.
13. Once dry, perform fluorescence microscopy as described in Section 4.

B *- Trypan Blue (TB)*

1. Don PPE.
2. Ensure the hood surface and gloves are clean as outlined in Section 1.
3. Complete sample filtration as outlined in Section 2, or continue counterstaining procedure after NR staining.
4. Triplicate rinse a glass screw cap container with Ultrapure water.
5. Into the clean glass screw cap container, filter the TB stain with a smaller cut-off filter than that of the filter onto which the samples were captured and will be stained.
    a. A filter disk with a 1 µm cut-off MSSN membrane was utilized for this process, but any porous membrane (e.g., PCTE, PTFE, or sterile bottle-top filters) with a smaller cut-off than the lowest cut-off of the utilized filter disk will suffice - as long as this chosen membrane does not shed particulates into the filtrate.
6. Place the filter disk to be stained onto the support frit of the vacuum collection flask.
7. Pipette 20 µL of the 0.4% TB stain onto the nanomembrane at the center of the filter disk.
    a. The stain should fully cover the porous surface of the nanomembrane. If 20 µL is not enough, add additional stain dropwise until all porous areas of the nanomembrane are suitably covered in stain. This additional volume should be noted for future reference.
8. Allow the stain to incubate for 5 minutes.
9. Once the stain has incubated, vacuum-filter the stain.
10. Triplicate rinse with 1 mL volumes of Ultrapure water.

11. Allow the filter disk to sit on the support frit with the vacuum on for 2 minutes to filter and dry any residual liquid. If not dry after 2 minutes, transfer the filter disk using a clean glass petri dish to a 70°C oven for 2-5 minutes
12. Once dry, perform fluorescence microscopy as described in Section 4.

Section 4 – Particle Quantification via Fluorescence Microscopy

Two different fluorescent microscopes were used for the same set of filter disks in this study for particle detection to demonstrate reproducibility between instruments, and no specificity towards any instrument. Fluorescent microscopy was performed per instrument instructions utilizing brightfield for grayscale imaging, TRITC channel (EX. ~543 nm; Em. ~593 nm) for NR stained particle imaging, and Cy5 channel (Ex. ~649 nm; Em. ~667 nm) for TB stained particle imaging, as applicable. To display the results of the staining, we recommend constructing an overlay of the channel images (we use FIJI/ImageJ for image processing), at a resolution of 2048 x 2040 pixels and a 16-bit depth. Whole nanomembrane images can be displayed as in **Figures 4A** and **B**, or as selected regions of interest as shown in **Figures 3A** and **B**. Other analyses from these images include measuring the longest and shortest dimensions or area of selected particles. To perform these analyses, the pores of the nanomembranes (i.e., measuring the length of an individual 8 x 50 µm pore of a MSSN nanomembrane as a known distance) can be used to set the scale bar in ImageJ, followed by using the line function to take measurements. The procedure described below is an example of step-by-step instructions for an Olympus BX61 Fluorescence Microscope but are applicable to any fluorescence enabled microscope with the proper resolution camera.

A *– Optical Microscopy Image Capture*

1. Immobilize the filter disk on a microscope slide using a piece of silicone.
2. Move the sample to be imaged to the microscope stage.
3. Image the nanomembrane using brightfield illumination so that the maximum detected counts are ~90% of the detector camera's maximum range.
    a. A 16-bit detector (65,535 white levels) should have a maximum pixel intensity of ~59,000. Adjust the illumination source or the camera exposure to reach this value.
4. Image the nanomembrane using fluorescent illumination so the maximum pixel intensities are around ~25% of the detector camera's maximum range.
    a. The lower intensity provides time savings compared to the brightfield illumination.
5. Save the image in a 16-bit composite TIFF format.

B *– Particle Quantification via Fluorescence Microscopy*

1. Using FiJi, separate the fluorescent channel(s) of the composite TIFF generated in Step 5 from the brightfield channel using the ‘Split-Channels’ function.
2. Threshold the pixel intensities using the ‘Threshold’ function to a value of 10,000.
    a. If bloom is present on the particles, the threshold is too low and should be raised.
    b. Save a separate image in PNG format.
3. Despeckle the image to remove noise pixels using the ‘Despeckle’ Function.
4. Watershed the image using the ‘Watershed’ function.
    a. **NOTE**: This function separates adjoining particles of a similar size, at the cost of breaking up large contiguous particles into smaller pieces. As there are often many more small particles than large particles present, this can be a good tradeoff, but can have unintended effects, such as breaking up long fibers into strands of small particles. Inspect image results before accepting data.
5. Set the scale of the thresholded fluorescent images by measuring the width of a pore in the brightfield image. ‘Set Scale’ in pixels/um.
    a. For rectangular pores, the smallest width will match the product label. For circular pores, the diameter will match the product label specifying the pore size (i.e., cut-off) of the nanomembrane.
6. Apply FiJi’s ‘Count Particles’ Function to the thresholded fluorescent image, evaluating the Area, min and max.
7. Process the fluorescent and brightfield images to create composites that visualize the fluorescent domains.
8. Threshold the fluorescent image using the same value as in Step 7.
9. Merge channels using FiJi.
10. Assign the fluorescent channel to the ‘Red’ channel.
11. Assign the brightfield image to the ‘Grays’ Channel.
12. Save composite as a PNG file.

Section 5 - Raman Spectroscopy

Two different Raman spectroscopy microscopes were used in this study to demonstrate the differences in lasers and reproducibility across instruments. It should be noted that shorter wavelength lasers (i.e., 532 nm) are not suitable for fluorescence signal suppression and thus the analysis of Nile Red stained particles with this wavelength laser is not ideal. However, longer wavelength lasers, such as 830 nm, are more suitable for Nile Red stained particle identification. It is important to acknowledge the size limit of detection for each Raman microscope. Perform Raman spectroscopy analysis per manufacturer’s instructions, including all necessary calibration files and control samples. Reliable identification of selected particles will yield a Pearson’s coefficient (r) greater than 70% ($r > 0.70$). MP identification results are useful in determining potential sources of the MP pollution. Most Raman spectroscopy microscopes are equipped with a camera able to take an image of the particle being analyzed. To display particle identification results, an image and spectra of the analyzed

particle can be shown as in **Figures 3C** and **4C**. The following section details instructions for particle identification using an open-source spectral library, OpenSpecy.

Section 6 - Particle identification via OpenSpecy:

NOTE - Pre-processing or calibration of data collected is not expressly necessary prior to use with OpenSpecy - there is a baseline correction tool within the site itself. Additionally, utilizing spectral processing tools or software prior to use with OpenSpecy is equally acceptable.

1. If a purchased or self-built Raman spectral database is not available, navigate to the open-source spectroscopy library at: https://www.openanalysis.org/openspecy/
    a. OpenSpecy is an open source Raman and IR spectroscopy database that includes 636 spectra from three different libraries of 276 polymers and materials applicable to identifying environmental MPs and fibers[20].
2. Save Raman spectra as individual .csv files. Column A should be labeled as "wavenum" followed by the wavenumbers of the spectra and column B should be labeled as "intensity" followed by the peak intensity values per wavenum.
3. Open the spectra in OpenSpecy.
4. Turn on "Preprocessing" and "Identification".
5. Under Preprocessing, turn on "Threshold Signal-Noise", "Min-Max Normalize", "Smoothing/Derivative", "Conform Wavenumbers", and "Baseline Correction".
    a. The specific settings therein can be adjusted depending on the individual characteristics of the spectra being analyzed.
6. Under "Identification", select "Raman" as the spectrum type, and "Derivative" as the Library Transformation. This library can be used for IR spectroscopy as well.
7. The analysis window will show a white spectrum, the uploaded spectrum for analysis, and a red spectrum, the suspected identification match.
8. OpenSpecy is not an exhaustive source - ensure that the suspected match is a visual good fit before accepting the spectral identity.
9. With any spectra that does not return a match $r > 0.70$, or reasonable peak shape correlations, utilize the top 5 suggested results as a place to begin researching potential matches as preferred.
    a. Ensure that the measured peak values are within +/-10 wavenumbers of a corresponding peak in the selected reference spectra before confirming a match for that peak.
10. Record spectral correlations, their correlation values, and the database from which the suspected match originates.
11. Using the download function, files to consider downloading include: top matches, adjusted spectra, and snapshots of the spectrums.

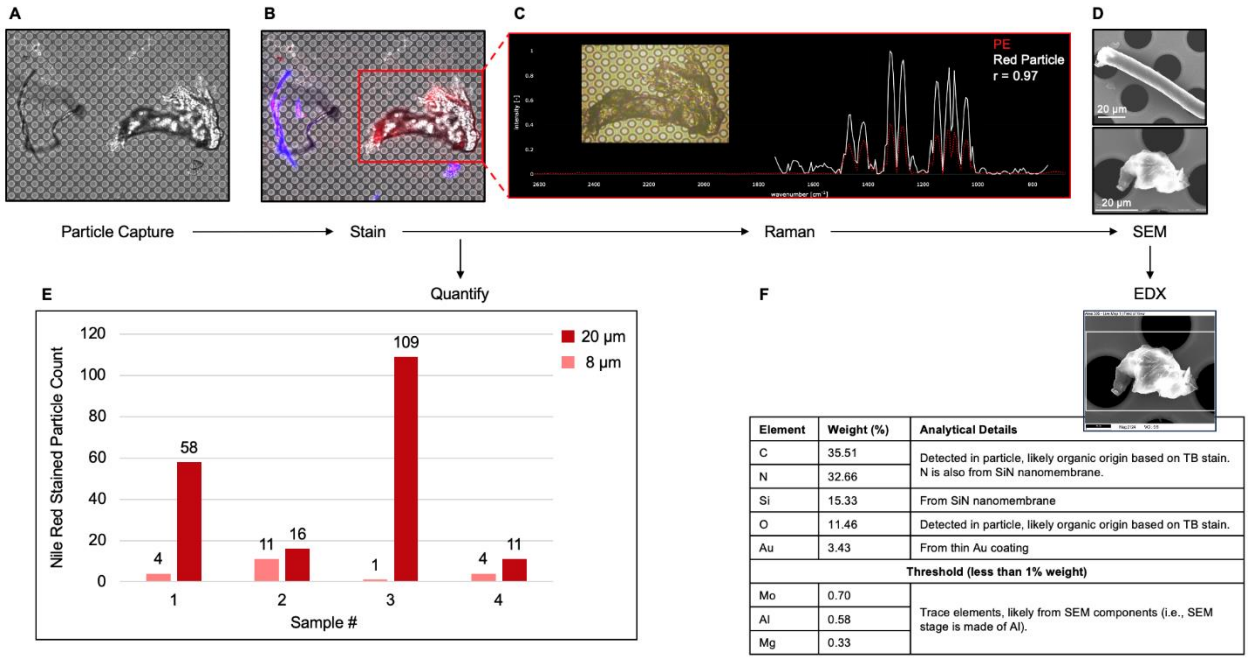

Figure 3. Summary of Analyses from SNAP Pipeline A Performed on Particles Captured from Drinking Water Samples. A) Greyscale image of captured particles from one representative drinking water sample on a 20 µm cut-off SiN nanomembrane. **B)** False colored overlay image of counterstained particles to represent Nile Red (false colored red particles) and Trypan Blue (false colored blue particles). **C)** Raman spectra identifying a Nile Red stained particle as polyethylene (PE) with Pearson's r value of 0.97, with representative image (insert) of the particle analyzed. **D)** Electron micrograph of two different particle types demonstrating particle morphology analysis of a fiber (top panel) and fragment (bottom panel), both taking up the Trypan Blue stain. **E)** Quantification of Nile Red stained particles sequentially captured onto both 20 µm and 8 µm cut-off nanomembranes. **F)** Summarized EDX elemental analysis of a randomly selected fragment and analytical details of the results with representative SEM image (inlet) of the particle analyzed.

Section 7 - Scanning Electron Microscopy

Perform SEM analysis per instrument instructions. If your instrument is equipped with EDX, then determine if gold (Au) or platinum (Pt) coating is best for your analysis. Coating samples enables higher contrasting images and higher surface resolution of particles, but can also affect EDX results. In this study, particles were coated with a thin layer of Au using a Denton Prep Sputtering System (6 nm thick, 100-150 mTorr, 15 mA, 60 s). SEM images of particles were obtained on a Zeiss Auriga SEM. Imaging was done between 200x – 3000x magnification with a 20kV beam power and focal length of 5 mm. Ideally, images of the full nanomembrane area, followed by magnified images of selected particles are acquired. Respective SEM images of selected particles are

shown in **Figure 3D**. Using the line measuring tool, size measurements of the longest and shortest dimensions of selected particles can also be performed, as well as measuring the area of the particle. After SEM image acquisition of each sample, the CCD camera of the SEM was turned off and EDX mapping data was collected for 5 minutes with the APEX software per manufacturer's instructions. Select elements of interest to be analyzed via EDX. Representative EDX results are shown in **Figure 3F**. Elements detected with a percent weight less than 1% are usually considered trace or unreliable; therefore, we set a threshold at 1%. If selected particles for EDX analysis were coated, a detectable amount of the coating will be present. If using uncoated SiN nanomembranes, Si and N signals will be observed, as shown in **Figure 3F**. Most synthetic polymer particles will contain the elements C, H, O, and unfortunately, as most non-synthetic particles from biological sources are also made of the same elements, but could also include elements such as S, as in the case of proteins. This similarity in composition is why the counterstaining method with Nile Red and Trypan Blue is useful for differentiating between synthetic vs. non-synthetic particles, but is not an exhaustive identification tool, as Nile Red is also capable of staining non-synthetic particles in certain cases as well. Additional analysis parameters as the ones suggested above should be utilized in conjunction with any staining procedures to confirm results. Additionally, EDX can be a useful tool for potentially identifying particles that are outside of the size limit of detection for Raman spectroscopy (i.e., nanoplastics measuring < 1 µm).

Representative Results

Process Blanks and Background Contamination

An acceptable blank result is one that contains either no particles, or so few particles that the background (i.e., process) contamination (if any) does not run a high likelihood of confusing or disrupting the results of the experiment. A suboptimal blank result is one that contains many particles that the particles of interest will be difficult to distinguish from the background contamination.

The acceptable level of process contamination should be at or as close to zero as possible, but if zero cannot be achieved, then consistency in the number of observed background particles is key. Run several blank samples to determine consistency of results. Diligence in quality control protocols (refer to Section 1), contamination mitigation, and proper environmental conditions during processing are paramount to maintaining low background contamination levels. Background contamination results and acceptability may also depend heavily upon the cut-off of the nanomembranes being used, and the overall cleanliness of laboratory conditions. Take into account that when analyzing for smaller sized particles with lower cut-off membranes (e.g., < 8 µm), the relative abundance of the particles of interest and similarly sized background contaminants will both increase exponentially, necessitating higher levels of stringency in quality control be applied[21]. While any level of contamination is not acceptable, as long as conditions are tightly controlled and a high degree of consistency is achieved, good analyses of environmental microplastics or analytics of interest may still be performed.

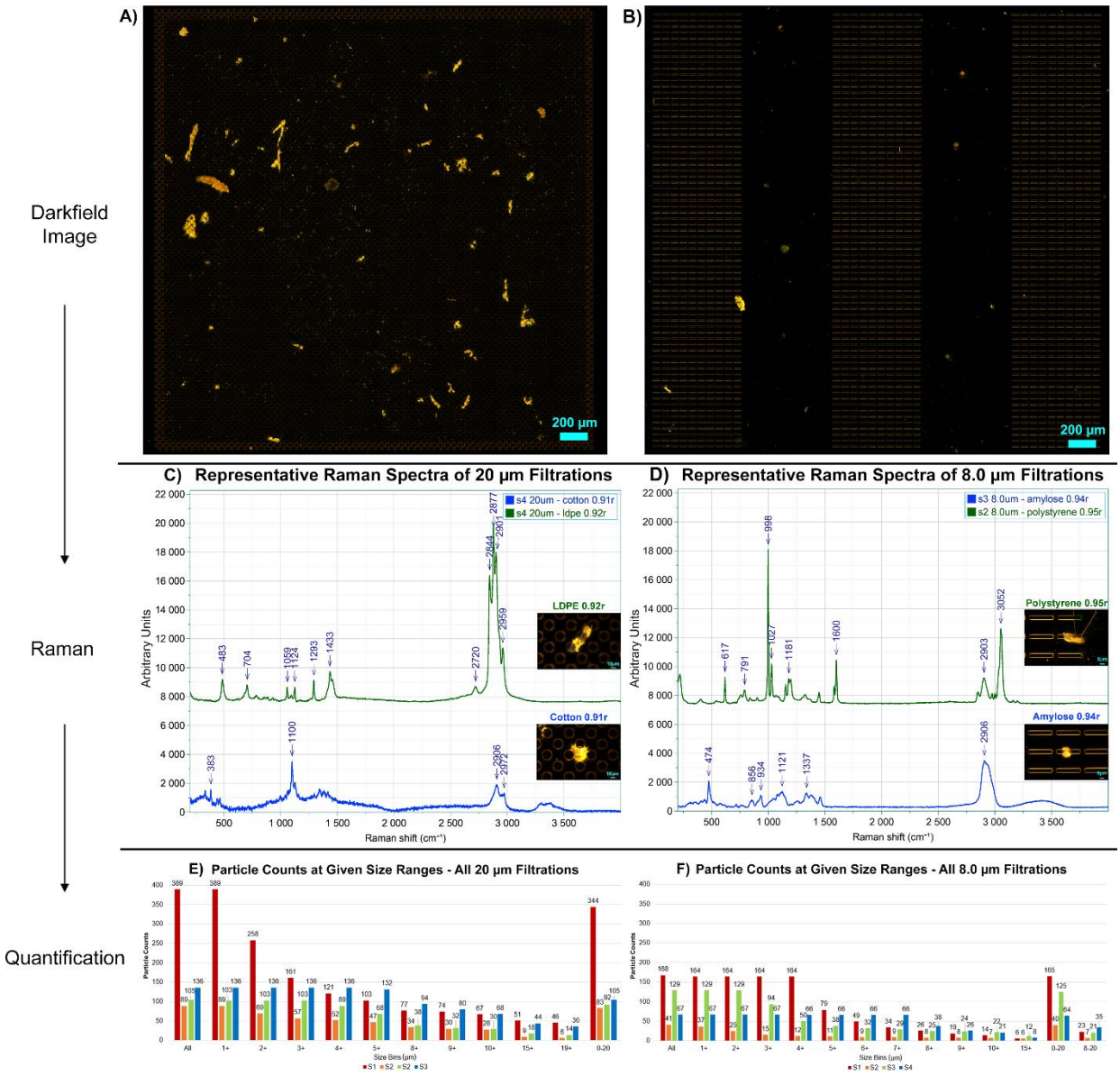

Figure 4. Summary of Analyses from SNAP Pipeline B Performed on Particles Captured from Drinking Water Samples. Raman spectral analysis of 3 randomized particles from four household drinking water samples captured on 20 µm MPSN and 8.0 µm MSSN Au-SiN nanomembranes. Representative darkfield images of the total active membrane area for the 20 µm and 8 µm Au-SiN membranes are respectively shown in **A)** and **B)**. Particles appear yellow in this imaging mode while the background remains dark, allowing for ease of automated particle counting. Representative Raman spectra of synthetic and non-synthetic particles on **C)** 20 µm and **D)** 8.0 µm Au-SiN nanomembranes. Total particle counts for **E)** 20 µm and **F)** 8.0 µm Au-SiN nanomembranes are binned by size ranges determined by the automated particle finding software.

Sample Filtration

An ideal sample filtration will result in well-dispersed particles across the nanomembrane surface (e.g., with approximately one-third membrane surface area coverage). Poorly dispersed particles, such as the case with particle aggregation (large clumps of overlapping particles), will confuse both manual and automated particle counting methods, as overlapping particles are not easily differentiated with confidence. Such data is usually excluded from analysis, which may lead to the loss of valuable information of interest. Well-dispersed particles, as shown in **Figure 4A** and **4B**, ensure that any spectroscopic or quantification analyses are not complicated by stacked or overlapping particles of varying compositions, which could return results that are more difficult to accurately interpret, identify, and quantify.

Nie Red and Trypan Blue Staining

Representative images of optimal fluorescent particle staining events are shown in **Figure 3B** on a 20 µm cut-off SiN nanomembrane. Under-rinsing of the NR or TB stain from the nanomembrane surface may create false positive results. Residual particulates that are comprised of either stain retained on the membrane's surface due to insufficient rinsing may be false interpreted as stained particles. A sign that insufficient rinsing occurred is if areas of the nanomembrane without particles or detectable residual films/residues from the filtered sample are fluorescing during observation, as shown in **Figure 5A**. After imaging, as long as the filter disk was maintained in clean conditions, a re-rinse may be conducted by placing the individual nanomembrane filter disk back onto the vacuum filtration apparatus (minus the gaskets), turning on the vacuum, and filtering another volume of Ultrapure 99% IPA (for NR) or Ultrapure water (for TB) onto the filter disk's nanomembrane. Record the total volume of additional rinsing media used. For best results, take microscopy images of the entire nanomembrane surface before conducting a re-rinse to ensure that any additional contamination from the re-rinse steps is accounted for.

Raman Spectroscopy

Raman spectroscopy performed on non-coated SiN may produce a prominent silicon peak depending on the laser, and this peak may mask low abundance signals approximately within the 520 cm^{-1} wavenumber region[22]. If Raman spectra of interest are sufficiently separated from the SiN background peak, using non-coated SiN filter disks should yield adequate Raman spectroscopy results. In this report, Pipelines B and C both used Au-SiN membrane-containing filter disks. The gold coating of Au-SiN masks the inherent Si peak, allowing low abundance spectra to be collected more reliably near or at 520 cm^{-1} wavenumbers. Additionally, Au-SiN can be used with IR spectroscopy if Raman is not available. Always start with the lowest power settings (i.e., laser intensity) on the instrument and then gradually increase, as some particles of interest can be fragile, such as oxidized particles, and may be damaged or destroyed by the laser.

When analyzing chosen particles with Raman spectroscopy, determining the viability of a returned spectra is crucial. Some particles may auto-fluoresce, and this fluorescence will add noise into the spectral measurement that may mask or complicate particle

signal interpretation. This noise will take the form of a high intensity band that spans a wide range of wavenumbers. Proper spectra, demonstrated in **Figure 3C** and **Figures 4C** and **4D**, even before baseline correction, will have clearly defined peaks across shorter spans of wavenumbers. If no peaks arise that are at least three times larger than any of the surrounding signals, then it is likely that the collected spectra are either background noise, or that different instrument settings are required. Ensure the proper laser wavelength is selected for each sample. Lower wavelength lasers, such as a 532 nm, cannot sufficiently handle the signal generated by fluorescence. A higher wavelength laser, such as a 782 nm or 830 nm laser, can sufficiently ignore enough of the fluorescence to return a spectrum. Depending on the type of sample, different lasers will be suitable for different particle types, and if possible, may be interchanged during data collection on a single filter disk. Suboptimal Raman data is shown in **Figure 5B**, where $r < 0.70$.

Scanning Electron Microscopy

Both SiN and Au-SiN membranes are compatible with SEM immediately after particle capture by filtration. If the SiN membranes are not disposed with Au or Pt prior to SEM, some particles may be more readily “charged-up” by the ionization beam of the SEM, which may cause the particle to either glow or appear very bright in images. This brightness/glowing can cause issues with visualization due to poor contrast, such as masking the morphology of the particle. To mitigate, perform optical/fluorescence microscopy and Raman spectroscopy before SEM to mark any hard-to-analyze or small particles of interest for later SEM/EDX analysis, then coat nanomembrane with Au or Pt as outlined in Section 7 and perform SEM/EDX.

EDX measurements may be performed both before and after metal coating. EDX can reveal the specific elemental composition of a given particle or area, which pairs well with any particles that are smaller than the size limit of detection for Raman analysis, or elemental compositions difficult to analyze with certain Raman laser wavelengths such as metal, alloys, water-saturated particulates, and particles that fluoresce strongly when exposed to the Raman laser beam(s) available for use.

Both SiN and Au-SiN may be coated with Au or Pt prior to SEM analysis to prevent the charge-up effect during imaging, as particles are now no longer susceptible to ionization under the disposed metal layer. This coating improves contrast and resolution, so a greater morphological understanding of a given particle can be obtained, as seen in **Figure 3D** (fiber vs fragment) or surface morphologies, such as pits and fissures on oxidized particles. Metal coating particles prior to SEM analysis will permanently prevent them from being further analyzed with spectroscopy or fluorescence imaging, rendering only EDX analysis still possible. Coating the particles and performing SEM/EDX thus

should be the final steps in any process if intended.

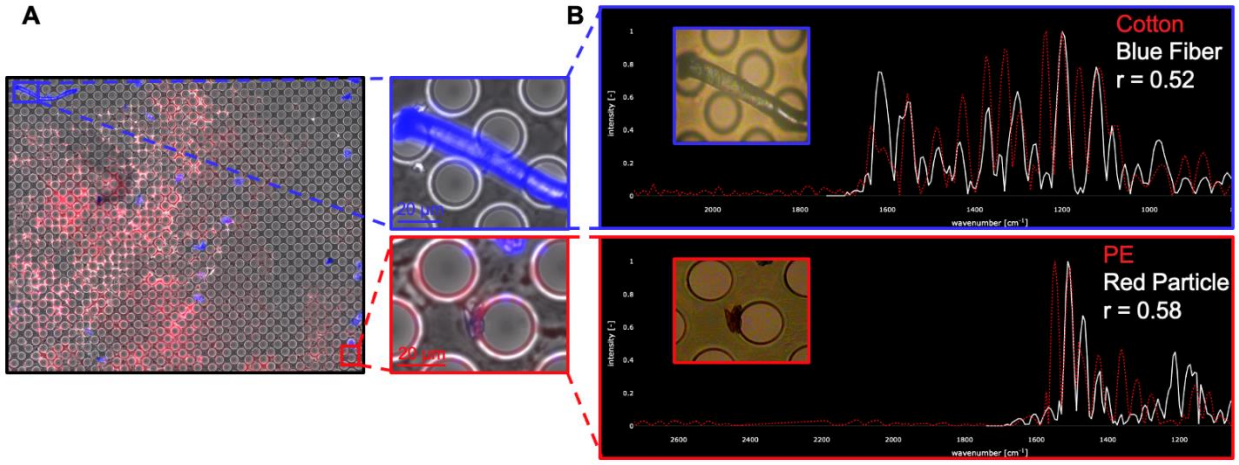

Figure 5. Suboptimal Fluorescent Imaging and Raman Spectroscopy Results. **A)** Depicts a suboptimal fluorescence image of stained particles (left panel), where the membrane retained the NR stain, likely from under-rinsing. Two particles, a TB-stained fiber and a NR stained fragment (right panel), were selected for Raman spectroscopy. **B)** Depicts suboptimal Raman spectroscopy results where the Pearson's coefficient (r) is less than 70 % (r < 0.70).

Discussion

This report describes flexible workflows based on variations of our developed SNAP methods for the analysis of MPs in liquid samples. The sample must be vacuum filtered such that the particles are captured and isolated onto a SiN nanomembrane housed inside a filter disk. Sufficient mitigation of background contamination is critical for meaningful results. Any contaminating particles on the dispensing syringe or glass vacuum funnel during filtration may contaminate the sample and be captured by the SiN membrane, so following the Section 1 quality control protocols diligently is crucial for accurate filtrations. Media-only controls are recommended to inform investigators of background contamination specific to individual processes and laboratory conditions. The protocol described herein relies on vacuum filtration of a liquid sample, as well as capturing and isolating particles from the sample on two different cut-off SiN membranes housed in filter disks. For all pipelines, particle capture and isolation must precede other methods, but all other identification methods can be done in the order most suitable for the investigator. Nile Red is commonly used to stain synthetic polymer MPs, but can stain non-synthetic polymers from biological sources due to its lipophilic and hydrophobic nature, potentially introducing false positives that distort MP quantification results[23, 24, 25]. A Trypan Blue counterstaining step as outlined in Section 3b is recommended to differentiate any false positive events. Nile Red fluorescence of particles is a concern with Raman spectroscopy analysis as it may significantly increase

background signal. In this study, however, we counterstained polymers with Nile Red and Trypan Blue prior to Raman spectroscopy with an 830 nm laser and little noise was observed, as shown in **Figure 3.** Some wavelengths of lasers suitable for Raman spectroscopy are not able to ignore fluorescence from stained particles, such as a 532 nm laser, and therefore may introduce significant difficulty in correctly estimating the composition of the particle of interest. Metal coating the particles after capture is useful for enhancing the contrast during SEM imaging, but is not suitable for any spectroscopic or fluorescent analysis afterwards. Make note of all desired measurements prior to experimental setup to ensure that the process flow and coating types are taken properly into account. The Au-SiN nanomembrane filter disks can be utilized for all the above analyses where transmission microscopy is not crucial to the workflow or when IR spectroscopy is used.

The methods shown here as well as the nanomembranes used to capture and isolate particles of interest can be modified to suit individual investigators' needs. The nanomembranes are suitable for a number of spectroscopic analyses, and the investigator may swap the media being filtered for another sample media of interest, including but not limited to digested animal tissues[26], injectable pharmaceuticals, and oil samples. The planarized capture of particles onto a uniformly flat membrane, as well as the membrane's regular array of pores, enables predictable, automated imaging. The 400 nm thickness of the SiN membranes and their silicon nitride composition endows them with excellent optical transparency, but may also return an intense silicon peak in some spectroscopic instrument parameters. Care should be taken when utilizing bare, non-metal-coated SiN membranes with Raman analysis. The Si peak may function as a calibration signal, but the intensity of the peak may also mask the signals of lower-intensity spectra in the same wavenumber region as the Si's signal. Such Si peaks were not observed when using the 830 nm laser in this study.

The ultrathin nature of the SiN (400 nm thick) and Au-SiN (520 nm thick) nanomembranes used here endowed them with their excellent filtration, optical, and spectroscopic properties. However, these membranes must be protected from physical damage, such as by Excessive differential pressure (i.e., ≥-206 kPa), direct touching by tweezers or fingers, or by improper gasket placement. The filter disc housing the membranes protects them and enables ease of handling under normal usage. When manipulating the filter disks, touch only the black outer ring, and never the active membrane area. Although not quantitatively reported here, the collection of captured particles onto a small amount of membrane surface area (3x3 mm for 20 µm and 3x0.7x3 mm for 8.0 µm cut-off membranes, respectively) potentially lowers total sample analysis time, by reducing the time needed to find and analyze particles of interest. This "concentration factor" is worthy of future exploration and may offer a unique benefit of SiN nanomembrane filter disks for investigators.

We heuristically demonstrated the utility of the SNAP variations on local tap water samples. The protocol described here focuses on MP capture from drinking water sources around Rochester, NY. Sample collection proceeded as follows: for each sample collection, at least one liter of water was run through the faucet before collection started. New, sealed polypropylene bottles were taken to the running faucet and opened directly before collection. Bottles were not rinsed with drinking water before collection proceeded. Each sample consisted of two 500mL bottles collected one directly after the other. Each water sample came from a distinct household's pipe system, and pipe material was not consistent throughout sampling. Variables like amount of recent water usage, time of collection, age of pipes, and the age of the home from which the sample came could not be controlled for in this study. This was not a validated sample collection protocol. Each investigator should develop and validate their own collection procedures. However, for the sake of complete disclosure of methods, the above description is included.

The filter disks allow repeated analysis on the same substrate for a number of analytical methods. No subsampling or multiple samples are needed outside of standard trial replicates. Eliminating the need for subsampling and multiple samples increases confidence in capturing information on low abundance targets.

Microplastic mitigation is a growing subject of concern; and the first step of mitigation is to identify the presence of contamination. A streamlined method of capture and immediate analysis is crucial to saving investigators time and preserving sensitive sample metrics. The methods above show a single substrate for optical microscopy, particle quantification, electron microscopy, and spectroscopy.

Materials

Name of Material/ Equipment	Company	Catalog Number
1 L Glass beaker	Pyrex	1000-1L
1 L Glass vacuum collection flask	Millipore Sigma	Z290459-1EA
100% cotton lab coat	Landau	LA-3172
100mL glass filtration funnel	Advantec	311000
99% isopropyl alcohol	Fisher Scientific	A416-20
APEX EDS	EDAX	
Denton Prep Sputtering System	Denton Vacuum	DESK II : 293618089
FIJI, image analysis platform	ImageJ	V 1.54F
Glass screw-cap bottle	Corning	1395-250
Kimwipes	Kimtech	06-666-11C

LabSpec6	Horiba Scientific	
Laminar flow hood	Air Science	VLF-72A
Microscope	Olympus / Nikon	BX61 / Ti2e
MPSN SiN filters	SiMPore Inc.	FD25-8.0-Au; FD25-8.0-NC FD25-20.0-Au, FD25-20.0-NC
Monoject 60 mL syringes	Coviden	8881560265
Nile Red powder	TCI	N0659
Nitrile Gloves	Ansell Microflex	19-167-17
OpenSpecy	openanalysis.org	
Oven	VWR	1310
P20 Pipette	Brandtech	705872
Pipette tip	Premium Vials	GS 151140R
Pourous support frit	Advantec	311000
Raman instrument	Horiba	Xplora PLUS
Rubber stopper	Advantec	311000
SEM instrument	Ziess	Auriga
Silicone gaskets	SiMPore	GASKET-25-R
Silicone Roller	Sanyue	(ASIN: B07XDTNPS3)
SmartSEM	Zeiss	V8
Spray bottle	Uline	S-7273
Syringe filters	Thermo Scientific	CH2225-PES
Syringe pump	New Era Pump Systems, Inc.	NE-1000
Trypan Blue - 0.4%	Millipore Sigma	T8154-20ML
Tweezers	SiMPore	K6TWZR
Vacuum pump	Welch	847-676-8800
Vacuum tubing	Grainger	ZUSA-HT-4037

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Disclosures

JR and JM are founders and stock holders of SiMPore Inc. JR is co-inventor on a patent application enabling use of Silicon Nitride filters like those depicted in this study.

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