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Nile Red Microplastic Validation: Enhancing the Study of Microplastics in Oregon's River Water

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Enhancing the Study of Microplastics in Oregon's River Water

By

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Abstract
Microplastics are ubiquitous in our environment and can be found in rivers, streams, oceans, and even tap water. In Oregon, eight sites along four freshwater rivers were evaluated for the presence of microplastics. Nile Red dye was tested as a method to improve the accuracy of microplastic counts in plankton tow samples from each of the sites along the Columbia, Willamette, Deschutes, and Rogue Rivers. Samples were stratified to separate microplastics from organic debris using a hypersaline solution. All samples were analyzed using traditional light microscopy for initial microplastic counts, then Nile Red dye was applied to each filter and re-analyzed using light microscopy for a before/after comparison. The difference in microplastic counts between before and after dye application was quantified using a Wilcox Test. The Wilcox Test comparing fibers produced a p-value of 0.0675, and the test comparing particles produced a p-value of 0.0007. The count difference is significant for particles before and after dye application. Overall the trend indicates that Nile Red aided in the identification of microplastics, specifically particles more so than fibers.

Introduction

Synthetic polymers derived from petroleum, otherwise known as plastics, have become a part of daily life in the products that we rely on. Global plastic production was estimated at about 250 million tons per year in 2011, and that number is projected to increase annually by about ten percent (Claessens et al. 2011). Plastic contamination is now a widespread concern in the aquatic environment. The durability of plastic makes it both appealing as a product and challenging to dispose of properly. Even with recycling, a majority of plastic products still end up in landfills and experience degradation over time. In fact, land-based sources of waste contribute roughly 80 percent of the plastic litter in the marine environment (Sharma and Chatterjee 2017). The marine environment has been studied extensively with respect to plastic pollution, but information gaps remain for freshwater systems (Horton et al. 2017).

Microplastics are particles smaller than five millimeters in diameter, and can be introduced into the environment in many different ways. Primary microplastics are manufactured as these small particles, while secondary microplastics are the result of fragmentation of larger plastic debris (Barboza et al. 2018). Microplastics are generally categorized into six types, which includes pellets, fragments, fibers, granules, plastic films, and Styrofoam (Van Cauwenberghe et al. 2015). The groups “fiber” and “particle” will be used for the purpose of this study.

There is great potential for microplastics to cause harm to both marine life and the humans that ingest those aquatic organisms. Once microplastics are ingested by aquatic species, studies have revealed that several physiological consequences can result, such as physical damage to internal mechanisms during digestion and absorption of chemicals added to the plastic during manufacturing (Cole et al. 2011). These ingested particles can then accumulate up the food chain as organisms are preyed upon, ultimately bioaccumulating up to marine mammals and humans (Rochman et al. 2015).

Plankton tows can be used to establish the presence of microplastics in an aquatic environment, even at the lowest levels of the food chain. For example, an Australian study from 2014 (Hall et al.) used subsurface plankton tows to establish the presence of microplastics in reef water. The specific types of plastics from the Hall study included polyurethane, polystyrene, and polyester, which are commonly associated with anthropogenic activities like shipping and fishing. Most plastics found were less than one millimeter and fibrous, which suggested that the microplastics were of the secondary variety resulting from fragmentation (Hall et al. 2014).

Cole's 2016 study also supported the notion that fibers are one of the most prevalent microplastic types. Synthetic fibers are generally manufactured as nylon, polyester, or polypropylene, which are commonly used in the production of textiles and fishing gear. Sources of introduction include washing machines, the degradation of cigarette butts, and the fragmentation of nautical equipment like fishing nets (Cole 2016).

This project began as a collaboration with Oregon Public Broadcasting (OPB), to quantify microplastic pollution in Oregon's rivers for an Oregon Field Guide episode (Plastic Rafts 2019). A total of 24 samples plus a field control for each of the eight sites was collected. The sites were selected by OPB and included the Columbia, Willamette, Rogue, and Deschutes Rivers (Figure 1).

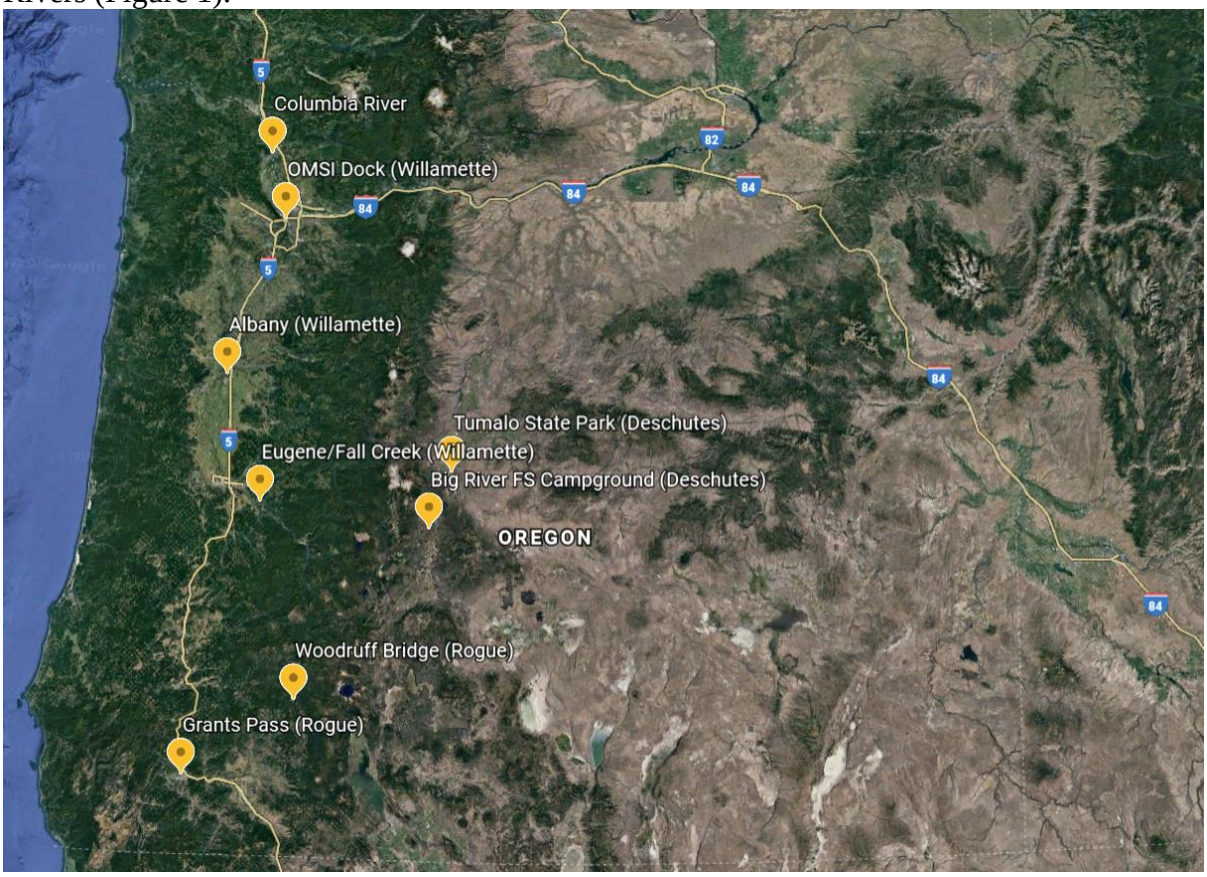

Figure 1: A Google Earth plotting of the eight sampling locations and their respective names.

All sample collection took place between September 7th and 14th, 2018. Initial microscope analysis was conducted between October 17th and 31st, 2018. This initial analysis differentiated the microplastics by color, which included blue, black, red, white, clear, green, and purple fibers and particles. Field controls were factored in, and average microplastic count per site was calculated.

The objective of this thesis was to validate the initial results using Nile Red. Nile Red is a lipophilic fluorescent dye with common applications in biology for the study of cell walls, bacteria, yeast, and microalgae (Maes et al. 2017). Because of its ability to highlight lipid materials, Nile Red has been gaining popularity for the study of microplastics since plastics are petroleum-derived products. Traditional microscopy presents several problems when conducting microplastic research. Data reliability can be low especially when particles are exceedingly small

or clear/white in coloration. Additionally, organic matter such as natural debris can be mistaken as plastic. This is why validation is required to confirm that the materials being counted are in fact plastic. Other methods for validation exist, including infrared or Raman spectroscopy, but these methods require expensive equipment that is often inaccessible and requires technical expertise to operate (Shim et al. 2016). Nile Red is a cost-effective alternative that is inexpensive to replicate. Nile Red causes plastics to fluoresce under LED light conditions by binding to lipids during the staining process. This allows for more accurate results while saving time and expense.

This thesis sought to answer the question, *does the application of Nile Red dye significantly change recorded microplastic presence in plankton samples*? The resulting hypothesis is that *significantly more microplastics will be counted after applying Nile Red because of the uncertainty surrounding traditional microscopy*. The samples were analyzed using traditional microscopy, then the dye was applied and the samples were analyzed a second time in order to create a before/after comparison.

Methods

Sample Collection

A total of 24 samples were collected: three per site, plus a field control for each of the eight sites. The sampling method was 15-minute stationary plankton tows held in the water column. A fine mesh plankton net with a caught-end (jar) was submerged in the river to collect the organisms and sediment floating or being pulled downstream by the current. The caught-end was attached at the end of the cone-shaped net to collect plankton as water flowed through the mechanism. The excess water exited through the mesh netting and plankton was trapped in the jar at the end (Vinzant 2016). The plankton tows for this study were horizontal, held by a person on shore or in a boat rather than dragged through the surface water. The net was submerged one to four feet deep in the water. This was repeated three times per site.

Tissue Dissolution

The samples contained a significant amount of biological material that made it difficult to visually inspect microplastics under a microscope. In order to avoid misidentification, a potassium hydroxide (KOH) digestion was performed to remove naturally-occurring biological material in the samples. A 10% KOH solution was prepared by transferring 40 grams of KOH to 400 milliliters of deionized (DI) water. The KOH solution was added to each sample and samples were placed on a hot plate with a stir bar for 24 hours to allow digestion of organic material. Some of the samples also contained larger biological debris like leaves that were not removed before digestion. As a result, some samples were digested multiple times with little success. Given the still-muddy sample conditions, density separation was explored as an alternative method to more effectively isolate the plastics from the biological material.

Density Separation

Quality Control: All glassware and lids were rinsed twice with DI water to avoid microplastic contamination. Glassware was inverted or covered if not in use, and contamination was recorded at each processing step. Proper lab attire included nitrile gloves, a 100% cotton t-shirt, and a 100% cotton lab coat to further avoid contamination.

The samples were originally collected in the Fall of 2018 and had since dried out in their glass petri dishes. DI water was added to the dishes to coat each sample to rehydrate them. While the samples rehydrated, the hypersaline solution was prepared using a ratio of 320 grams of salt

(sodium chloride, NaCl) to one gallon of water. The solution was mixed in a two-liter mason jar, so the ratio was used as a conversion factor, resulting in 168.4 grams of salt being added to the two liters of DI water.

Once the hypersaline solution was mixed thoroughly, one-quart mason jars were prepared by transferring 400 milliliters of the hypersaline solution into the quart jar. The sample in the corresponding petri dish was scraped with a shucking tool to loosen the sample from the bottom of the dish. The plankton/DI solution was then poured into the quart jar. The jar was sealed and shaken vigorously for 60 seconds. The jar was then put back on the lab bench for the contents to separate and stratify. The hypersaline solution caused the heavier sediment particles to sink to the bottom of the jar, while the lighter plastic particles floated to the top. This procedure was repeated for each sample (Thompson et al. 2004).

Once the solution had stratified, the liquid was vacuumed out using the apparatus in Figure 2. The vacuum apparatus consisted of a large glass Erlenmeyer flask connected to the sink faucet by a rubber tube. One glass filter (Whatman 1820-047 Glass Microfiber Binder Free Filter, 1.6 Micron, 4.3 s/100mL Flow Rate, Grade GF/A, 4.7cm Diameter, Amazon) was placed atop the Erlenmeyer flask, and the beaker component was stacked on top of the filter paper. The two glassware pieces were secured together using a metal clamp. The vacuum suction began when the sink was turned on. The quart sample jar was then opened and the top layer was poured out in order to ensure that the plastics were filtered but no sediment was included from the bottom. The water in the beaker was sucked into the Erlenmeyer flask, and the plastic particles were left on the filter paper between the beaker and the flask. The filter paper was lifted and transferred to a new petri dish using Excelta 5-SA stainless steel precision tweezers. The petri dish lid was secured with two rubber bands, and the filter papers were stored in a cardboard box for microscope analysis.

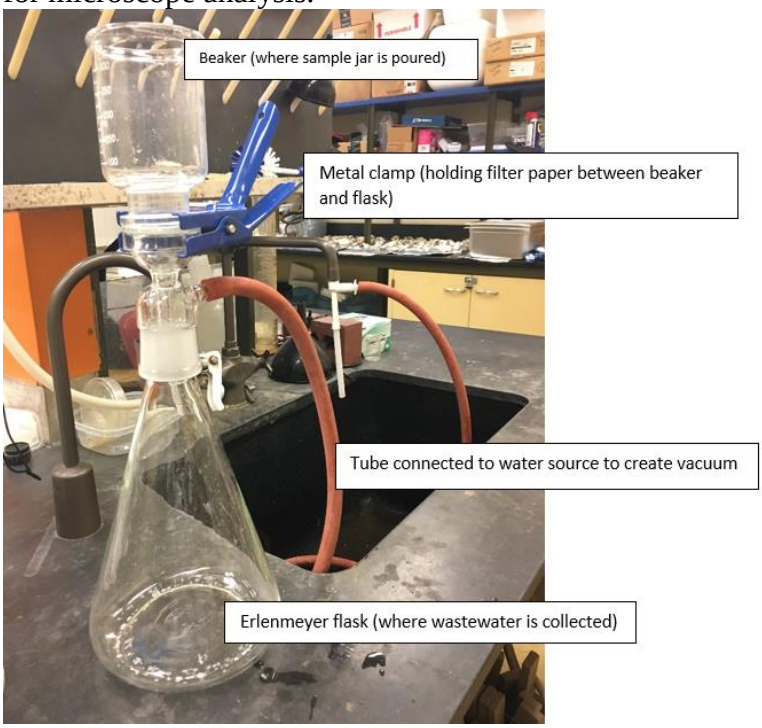

Figure 2: The vacuum apparatus setup, including the Erlenmeyer flask, the connected tube, the beaker component, and the metal clamp.

Microscope Analysis

Microscope methodology was adopted from the Marine & Environmental Research Institute ("Guide to Microplastic Identification" nd). Each filter was viewed on a Leica MZ6 light microscope using 40x magnification. The general method involved starting analysis at the top right of the filter and moving left in a straight line across the width of the paper. Once one row was complete, the next row down would be read from left to right and so forth until the bottom of the filter was reached. At each supposed microplastic, the physical characteristics were assessed using precision tweezers to test malleability. Parameters like thickness, homogenous color, and any cellular structures were assessed visually to differentiate plastic from potential natural materials. Each microplastic was photographed and recorded on a data sheet, along with its type (fiber versus particle) and color (blue, red, clear, white, black, green, or purple). While each filter was being viewed, a petri dish with DI water sat at the back of the microscope to collect any potential contamination from the microscope lab room. This control dish was also analyzed under the scope after each filter paper, and contamination was recorded separately. This procedure was repeated for each of the filter papers during April and May of 2019.

Dye Application

The dye application procedure was adopted from Wiggin & Holland (2019). In this procedure, 1 mg Nile Red (Santa Cruz Biotechnology, SC-203747C) was mixed with 1 mL acetone to create a stock solution, which was then diluted with 100 mL of hexane to create a working solution of 10 µg Nile Red/mL. After thorough mixing with a stir bar over several hours, the working solution was transferred into an amber dropper bottle, and the solution was applied to each filter paper until coated (about nine drops) and allowed to dry on a 12-hour, 30°Celsius cycle in a drying oven. Microscope analysis was repeated for each of the filter papers. To create the proper light conditions for fluorescence, the lab room was completely dark and orange safety goggles were taped under the microscope lens to create an orange viewing environment. A 455nm LED flashlight (Arrowhead Forensics PART NO: A-6994FK) was used to illuminate the samples, causing fluorescence (Figure 3).

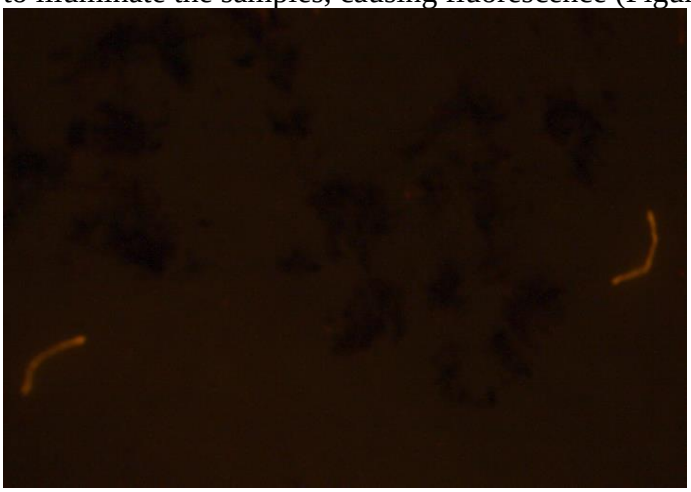

Figure 3: An example of the Nile Red fluorescence from the Eugene 3 sample.

Data Analysis

All statistical analyses were performed in RStudio version 1.1.453. To test for significant differences, nonparametric t-tests were run between the number of fibers and particles before and after dye application. Shapiro Tests revealed data were abnormal, thus the use of a nonparametric analysis. A Wilcox Test was used to compare microplastic counts before and after dye application.

Results

The highest fiber counts were found at the OMSI sampling location, both before and after the application of Nile Red (Figure 4). As the large error bars on the histogram suggest, the data did not meet normality assumptions. The nonparametric data were put through a Wilcox Test, which produced a p-value of 0.0675 when comparing fibers before and after Nile Red application (Table 1).

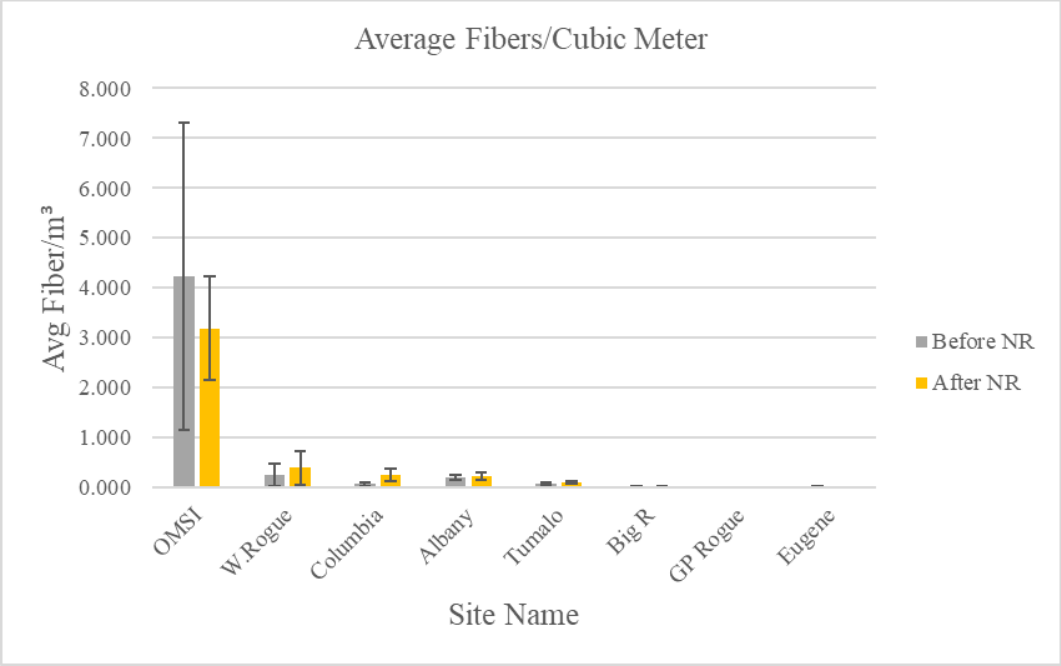

Figure 4: Number of fibers per cubic meter of water at each site. Sites are arranged in descending order from "after" values, and before/after is differentiated by bar color. The lines on each bar represent standard error.

The highest particle averages were initially found at W. Rogue, but particle values changed noticeably after the application of Nile Red (Figure 5). The Albany site became the location with the highest particle averages, and changes were also seen in values for W. Rogue, GP Rogue, OMSI, and Tumalo. Again the data did not meet normality assumptions. This nonparametric data also underwent a Wilcox Test, which produced a p-value of 0.0007 when particle counts were compared before and after the application of Nile Red (Table 1).

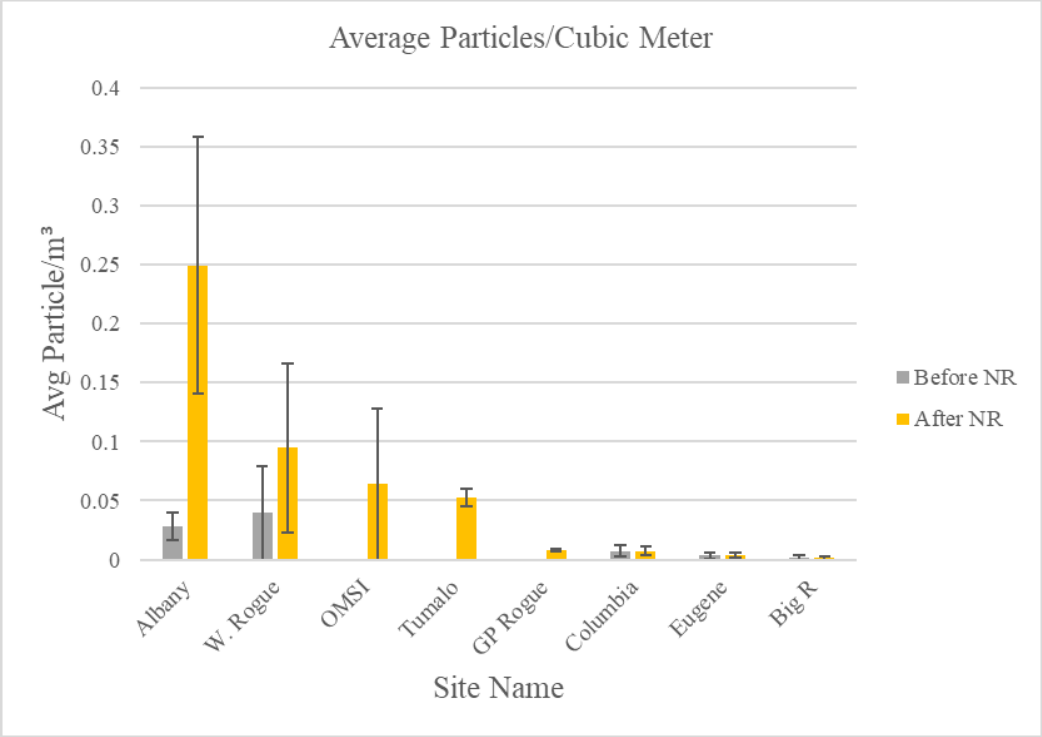

Figure 5: The average particles per cubic meter of water at each site. Sites are arranged in descending order from "after" values, and before/after is differentiated by bar color. The lines on each bar represent standard error.

Table 1: A summary of the Wilcox results for non-normal data.

Test	**W**	**P-value**
Comparison of Fibers before and after Nile Red dye	181	0.0675
Comparison of Particles before and after Nile Red dye	118.5	0.0007

Table 2: A representation of the average microplastic contamination recorded at each site, standardized by volume. Contamination includes plastics that could have been introduced in the field, in the lab, and in the microscope room. Contamination is reported separately in alignment with Rochman et al. 2019.

Contamination		
Site	Average Contamination / Cubic Meter Before NR	Average Contamination / Cubic Meter After NR
Albany	0.1433827	0.0394366
Big R	0.1029361	0.0066076
Columbia	0.0634588	0.0128183
Eugene	0.0127818	0.0000000
GP Rogue	0.0265957	0.0028625
OMSI	1.2259482	0.6202643
W. Rogue	1.2690119	0.1103489
Tumalo	0.1255762	0.0059736

Discussion

Based on Wilcox Tests, fiber differences were not significant but particle counts did differ significantly before and after dye application (Table 1). Figure 4 illustrates that the fiber values did not change noticeably, possibly indicating that "before" counts were fairly accurate given that fibers are more easily distinguished as plastic than particles. More changes were noted with respect to particles as seen in Figure 5. This reveals that Nile Red bolsters particle results since particles tend to be more difficult to identify given their non-uniform characteristics. This study indicates that Nile Red helps researchers visualize plastic better than without the dye.

With respect to saving time, Nile Red did seem to help by making it easier to isolate plastics. Any sediment that was crowding the filter essentially disappeared when the lights were turned out (Figure 3), which eliminated a lot of background material that originally took significant time to separate out. Although Nile Red may be an improvement in current practice, it is not a perfect method. Organic debris on the filter paper creates the potential for co-staining biological material. Thus it is important to employ knowledge of plastic behavior and visual qualities to identify each potential plastic. Methods like chemical digestion and density separation are essential for samples to be as "clean" as possible before being dyed.

However, several sources of error also exist in the study. Some samples had a lot of remaining organic debris on them, even after the hypersaline solution, which made it difficult to estimate accurate "before" counts. Additionally, flow was not recorded for one sample, so Eugene averages were based on two samples instead of three, potentially leading to inaccuracies in average microplastic counts for the Eugene site. Microplastic contamination from field or lab exposure (Table 2) could also be contributing to microplastic counts.

Overall, the study supports the existing literature that Nile Red aids in microplastic research, especially given the uncertainty of particle identification. Potential areas for future work include validating Nile Red results with some of the more costly analyses like infrared or Raman spectroscopy, to further test the reliability of this new method in future research.

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References

Barboza, L. G. A., Vethaak, A. D., Lavorante, B. R., Lundebye, A. K., & Guilhermino, L. Marine microplastic debris: An emerging issue for food security, food safety and human health. *Marine Pollution Bulletin*, 133, 336-348 (2018).

Claessens, M., De Meester, S., Van Landuyt, L., De Clerck, K., & Janssen, C. R. Occurrence and distribution of microplastics in marine sediments along the Belgian coast. *Marine Pollution Bulletin*, 62(10), 2199-2204 (2011).

Cole, M. A novel method for preparing microplastic fibers. *Scientific Reports*, 6, 34519 (2016).

Cole, M., Lindeque, P., Halsband, C., & Galloway, T. S. Microplastics as contaminants in the marine environment: a review. *Marine Pollution Bulletin*, 62(12), 2588-2597 (2011).

Guide to Microplastic Identification. *Marine & Environmental Research Institute*, retrieved from https://sfyl.ifas.ufl.edu/media/sfylifasufledu/flagler/sea-grant/pdf-files/microplastics/MERI_Guide-to-Microplastic-Identification.pdf.

Hall, N. M., Berry, K. L. E., Rintoul, L., & Hoogenboom, M. O. Microplastic ingestion by scleractinian corals. *Marine Biology*, 162(3), 725-732 (2015).

Horton, A.A., Svendsen, C., Williams, R.J., Spurgeon, D.J. and Lahive, E. Large microplastic particles in sediments of tributaries of the River Thames, UK–Abundance, sources and methods for effective quantification. *Marine Pollution Bulletin*, 114(1), 218-226 (2017).

Maes, T., Jessop, R., Wellner, N., Haupt, K., & Mayes, A. G. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. *Scientific Reports*, *7*, 44501 (2017).

Plastic Rafts; Plastics in Rivers; Oysters With A Side of Plastic. *Oregon Public Broadcasting*, retrieved from https://www.opb.org/television/programs/ofg/episodes/3008/ (2019).

Rochman, C.M., Brookson, C., Bikker, J., Djuric, N., Earn, A., Bucci, K., Athey, S., Huntington, A., McIlwraith, H., Munno, K. and De Frond, H. Rethinking microplastics as a diverse contaminant suite. *Environmental Toxicology and Chemistry*, 38(4), 703-711 (2019).

Rochman, C.M., Tahir, A., Williams, S.L., Baxa, D.V., Lam, R., Miller, J.T., Teh, F.C., Werorilangi, S. and Teh, S.J. Anthropogenic debris in seafood: Plastic debris and fibers from textiles in fish and bivalves sold for human consumption. *Scientific Reports*, *5*, 14340 (2015).

Sharma, S., & Chatterjee, S. Microplastic pollution, a threat to marine ecosystem and human health: a short review. *Environmental Science and Pollution Research*, 24(27), 21530-21547 (2017).

Shim, W. J., Song, Y. K., Hong, S. H., & Jang, M. Identification and quantification of microplastics using Nile Red staining. *Marine Pollution Bulletin*, 113(1-2), 469-476 (2016).

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W., McGonigle, D. and Russell, A.E. Lost at sea: where is all the plastic? *Science*, 304(5672), 838-838 (2004).

Van Cauwenberghe, L., Devriese, L., Galgani, F., Robbens, J., & Janssen, C. R.. Microplastics in sediments: a review of techniques, occurrence and effects. *Marine Environmental Research*, 111, 5-17 (2015).

Vinzant, Alisa. Plankton Tow. *Catalina Island Marine Institute*, retrieved from https://cimioutdoored.org/plankton-tow/ (2016).

Wiggin, K. J., & Holland, E. B. Validation and application of cost and time effective methods for the detection of 3–500 μm sized microplastics in the urban marine and estuarine environments surrounding Long Beach, California. *Marine Pollution Bulletin*, 143, 152-162 (2019).