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■ RESEARCH ARTICLE

A Rapid One-step Field-based Detection Method for Nanoplastics in Water

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ABSTRACT: The widespread use of plastics has raised concerns about their persistence in aquatic and terrestrial environments. Tiny plastic particles, microplastics, and nanoplastics generated due to degradation lead to real environmental problems. Rapid identification of potential hotspots of nanoplastics contamination has been challenging due to a lack of field-based detection methods. This study aimed to develop a single-step portable method to detect nanoplastics in water samples. Using a custom-designed fluorometer, an optimized Nile Red-based method has been successfully extended to detect nanoplastics within 10 minutes from wastewater samples with a limit of detection of 35 µg/mL. This method can be broadly used to monitor nanoplastic load during wastewater treatment and in different surface water streams which drain into urban watersheds.

KEYWORDS: Earth and Environmental Sciences; Water Science; Nanoplastics; Detection Method; Field-technique.

■ Introduction

Plastics are widely used because of their low manufacturing cost, durability, and versatile use in many consumer goods. According to a recent study in 2020, the annual global production of plastic has increased to 360 metric tons per year.[1,2] The vast amount of plastic waste has caused environmental concern due to the contamination of soil and water bodies such as rivers and oceans. Moreover, plastics can adsorb many toxic aromatic hydrocarbons, heavy metals, and pharmaceutical and personal care products and serve as reservoirs for these toxic agents.[1,2] Microplastics and nanoplastics can pose severe health issues such as inflammation and physical damage to lung epithelial cells.[3] Quantification and tracking of plastic pollution have been further complicated due to the trapping of debris in complex matrices such as wastewater samples.[1,2] Macroplastics are easy to detect by visual inspection. Still with additional expensive laboratory detection techniques, degraded plastic products such as microplastics and nanoplastics are often easier to quantify in field samples.

Small plastic fragments are generated due to the action of chemical and environmental agents such as, soil fungi. Commonly used household products, packaging materials, clothing and bath scrubs release many plastic fragments into the daily wastewater reservoirs.[1,2] Plastic fragments > 100 nm to < 5mm are characterized as microplastics and fragments < 100 nm are called as nanoplastics. Microplastics and nanoplastics in river streams and oceans can harm aquatic life and human health.[3] Microplastics but not nanoplastics in water can be detected under the microscope, but the method could be more reliable due to differences in size, transparency, and fiber types.[4] More sophisticated techniques, such as Fourier Transform infrared or Raman Spectroscopy techniques, are useful for reliably detecting the chemical composition of the polymer types.[4] However, these detection methods are expensive and often time-consuming for routine field use.

Microplastics are hydrophobic and can be visualized by staining with lipophilic fluorescent dyes such as Nile Red.[5-8] The interaction of plastics with these fluorophores is facilitated in the presence of organic solvents such as chloroform, acetone, or methanol. The staining protocol is usually conducted with different microplastics using a filter paper method and quantifying the particles by visual or semi-automated counting techniques. The Nile Red method was proven effective in staining several plastic types, including polyethylene, polypropylene, and polystyrene.[5-8] However, these staining protocols often involve several steps of sample preparation, washing, and data capture.

Two recent studies have described a plate-based method to detect nanoplastics using polystyrene beads to generate a standard curve.[9-10] Plate-based readers are expensive and unsuitable for routine field-based testing. Moreover, the non-specific binding of nanoplastic beads to these clear plastic plates has not been ruled out. In the study by Gagne *et al.*, known amounts of 100 or 50 nm diameter nanoplastics were added to tissue extracts prepared from freshwater adult mussels (*Elliptio complanata*), which shifted the emission peak to 623 nm from the normal Nile Red peak of 660 nm.[9] The authors also tested the effects of detergents such as Triton X-100 or Tween-20 as a proxy for lipid rich-environments. The fluorescent intensity increased with increasing concentration of the detergents, consistent with the solvatochromic nature of Nile Red.[9] A recent study that used similar experimental conditions to the Gagne *et al.* paper showed that the proportion of methanol over water in the microwells was not reproducible using a different motor rotor dye, DCVJ to detect nanoplastics.[10] Methanol concentrations of 20% were optimal to detect a 620 nm emission peak with DCVJ. The study by Moraz and Breider, however, did not

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compare the performance of Nile Red dye using these optimal conditions.[10] As suggested earlier, these studies used 96-well clear plastic plates, which did not wholly rule out non-specific binding of Nile Red or DCVJ to the plates in the absence of nanoplastics.[9,10]

The goal of the present study was to develop a cost-effective, simple, portable, and preferably a one-step nanoplastic detection method suitable for field applications. A custom-designed hand-held portable fluorometer with Ex/Em of 450/620 nm was used with commercial 50 nm polystyrene nanoplastic beads to optimize various assay parameters such as linearity of signal, effects of shaking, and assay incubation times. After optimization of the assay conditions, field samples from influent and effluent wastewater samples were filtered using a 0.45 µm syringe filtered before staining with Nile Red dye in the presence of 20% methanol. This optimized protocol from this study has broader applications to detect hotspots of nanoplastics contamination in urban and rural water streams.

Methods

Instrumentation:

An easily portable hand-held single-tube fluorometer was custom-built by Amiscience Corporation (Fremont, CA). The instrument was set up to read a single wavelength with excitation/emission wavelengths of 450/620 nm. The instrument had a touch-screen LCD display and operated in either a 5V DC power adaptor or 4 AA batteries (Figure 1) Signals were read as relative fluorescent units (RFU) and converted to relevant plastics concentrations using a standard curve with 50 nm polystyrene beads.

Figure 1: Hand-held fluorometer and mini-glass tubes.

Materials

Nile Red was purchased from Cayman Chemical Company (Ann Arbor, MI). Glass tubes (6 mm outer diameter and 300 mm length) were obtained from Amiscience Corporation. Milli-Q water was obtained from a water purification system from Merck Millipore. Eppendorf tubes and 1 ml tuberculin syringes were purchased from Thermo Fisher (Waltham, MA) and BD biosciences (Franklin Lakes, NJ), respectively. Technical grade methanol and 0.45 µM syringe filters (Hydrophobic PTFE fluorophore) were obtained from Alliance Chemical (Taylore, TX) and Millipore Sigma (St. Louis, MO), respectively. Polystyrene nanoplastic beads of 50 nm size were supplied as aqueous suspension (stock concentration of 25 mg/ml) and purchased from Polysciences Inc (Warrington, PA).

Methods

A stock solution of 10 mM Nile red was prepared using methanol, and a working solution of 40 µM Nile Red in methanol was used for all experiments. Polystyrene beads were adjusted to 400 µg/mL concentration in Milli-Q water, and 1:1 dilutions of beads in water were prepared for standard curve analysis. The reaction mixture was prepared by transferring 160 µL of water sample with 40 µl of Nile red working solution (final volume 200 µL) to the glass tubes to maintain the mixture's 20% final methanol concentration. Unless otherwise specified, samples were incubated for 10 minutes at room temperature. For standard curve analysis, increasing concentrations of known bead solution were mixed with Nile Red working solution. Blank samples were prepared by mixing Milli-Q water with Nile Red Dye ratio similar to experimental samples. Glass tubes were inserted into the hand-held fluorometer, and data was read at excitation/emission wavelengths of 450/620 nm. All samples were analyzed in triplicate, and data were expressed as mean+/- SD.

Results and Discussion

Instrument Reproducibility:

Instrument reproducibility was studied by mixing 160 µL of Milli-Q water with 40 µL of either methanol or Nile Red working solution (final Nile Red concentration is 8 µM) and incubated for 10 minutes at room temperature. Results were generated from 7 independent trials. As shown in Table 1, the background signals from methanol and Nile Red solution were at 1000 and 10,000 RFUs, respectively, in all seven trials pointing to a high level of reproducibility in instrument performance.

Table 1: Comparison of assay performance from 7 independent trials using blank samples incubated with methanol or Nile Red.

Trial No.	Methanol	Nile Red
1	1000	10000
2	1000	10000
3	1000	10000
4	1000	10000
5	1000	10000
6	1000	10000
7	1000	10000

Standard Curve Generation Using the Bead Titration method:

Calibration curves were generated using serial dilutions of polystyrene beads in water at 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562 and 0 µg/mL. In addition, a mixture containing 160 µL of beads with 40 µl of 40 µM Nile Red solution was incubated for 10 minutes at room temperature. Samples were read at 450 nm. As shown in Figure 2, a linear relationship was obtained with increasing concentration of the beads with a limit of detection (LOD) of 35 µg/mL using the formula 3.3µ/S, where µ is the standard deviation of the response and S is the slope of the calibration curve. Bead concentrations above

400 µg/mL produced fluorescence signals but the signals were not linear.

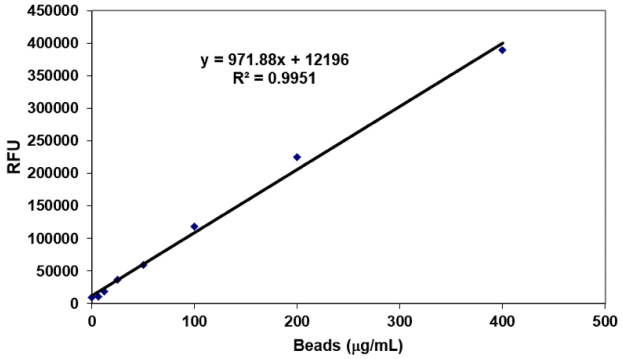


Figure 2: Calibration curve with polystyrene beads.

Effect of different incubation times on calibration curve performance:

The impact of different incubation times on the slope of the linear regressions was tested. The assay mixture with increasing bead concentrations was incubated for 10, 20, 30, or 60 min at room temperature, and RFUs were read using the hand-held fluorometer. As shown in Table 2, the slope of the linear regression obtained at 620 nm is remarkably consistent across different incubation times. Therefore, an incubation time of 10 min for subsequent experiments was chosen for assay optimization.

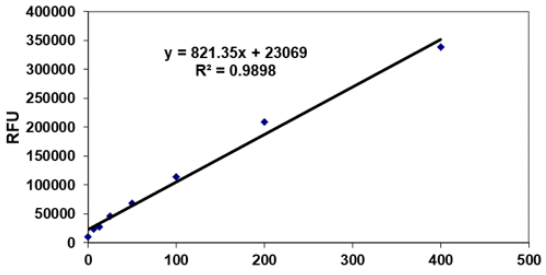
Table 2: Effect of incubation times on slope of linear regression at 620 nm.

Time (min)	Nile Red	
	Slope	R²
10	971.88	0.995
20	933.66	0.991
30	964.9	0.990
60	1003.6	0.990

Effect of shaking on calibration curve performance:

The effect of optimal mixing of the final solution without (Figure 3A) or with (Figure 3B) shaking was tested using an orbital shaker. Serial dilutions of bead samples were mixed with Nile Red dye and left on an orbital shaker at 200 rpm. Mixed samples without shaking were left on the benchtop for 10 min as control. As shown in Figure 3, the slope of linear regression obtained by both methods was very similar.

A. No Shake (10 min)



B. Shake (200 rpm, 10 min)

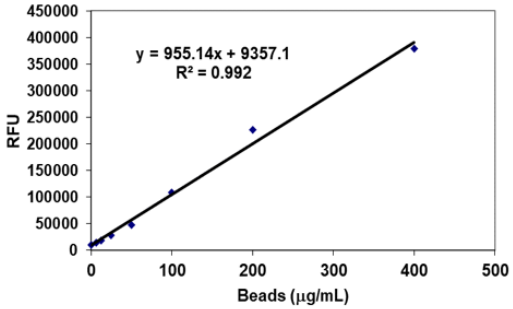


Figure 3: Effect of shaking on the calibration curve.

Based on the optimization of different parameters, the protocol utilized to test nanoplastics in influent and effluent wastewater is shown in Figure 4.

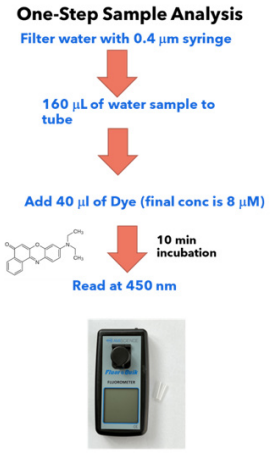


Figure 4: One-step nanoplastic detection in water samples.

Influent and Effluent parameters in a wastewater treatment facility:

Sewage water is treated daily at the TriCo Regional Sewer Facility (Zionsville, IN). The plant collects about 4 million gallons of wastewater from the residents in the western half of Carmel, IN, and sections of Indianapolis, IN. Several water parameters are collected daily from influent and effluent water samples to monitor quality when the treated water is allowed back into the Indiana White River. The facility tested influent and effluent water samples collected on five independent days for different parameters and data was provided for this study. As shown in Tables 3A and B, the effluent samples showed a significant reduction in total suspended solids, total ammonia, and phosphorus content.

Table 3: Influent and Effluent water quality

A.

	Suspended Solids - mg/L	
Day	Influent	Effluent
1	214	6.5
2	244	5.1
3	280	4.4
4	220	6.1
5	164	5.2

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B.

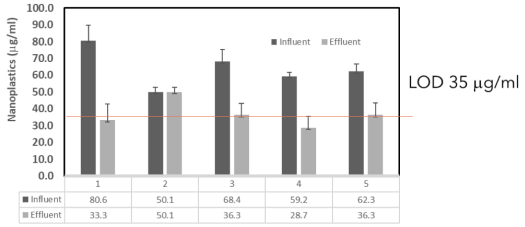
Day	pH		Phosphorus (mg/L)		Ammonia (mg/L)	
	I	E	I	E	I	E
1	7.20	7.53	4.38	0.816	25.7	0.058
2	7.58	7.55	3.94	1.385	24.8	1.085
3	7.30	7.63	4.14	0.951	24.2	0.500
4	7.32	7.64	4.15	0.633	24.6	0.046
5	7.29	7.57	6.28	0.953	23.2	0.036

Legends: I -Influent; E- Effluent

Nanoplastics levels in influent and effluent wastewater samples:

Nanoplastics levels were measured using the one-step fluorometric technique. First, wastewater samples were filtered using a 0.45 µm syringe filter to remove all suspended debris. As described in the materials and methods section, 160 µL filtered wastewater was mixed with 40 µL of Nile Red working solution and incubated for 10 minutes at room temperature before reading at 450 nm. As shown in Table 4, nanoplastics were detected from influent samples from all five collection days. On the other hand, a significant reduction of nanoplastics was observed in effluent water samples from 4 out of 5 days of collection likely due to the trapping of nanoplastics in the sewage sludge waste. In all cases, the levels were near or lower than the limit of detection of the assay. Interestingly, the levels of nanoplastics were similar in the influent and effluent water samples on day 2. These data clearly point to the utility of the one-step nanoplastic detection technique to monitor plastic load in treated water samples.

Table 4: Nanoplastics in influent and effluent wastewater samples.



A comparison of the nanoplastic content of influent samples to the number of particles revealed a strikingly high nanoplastic load in all five collection days (Table 5).

Table 5: Nanoplastics numbers in effluent wastewater samples. Standard 50 nm polystyrene particles were available at 25 mg/ml at a concentration of 3.64 x 10^{14} particles/mL. Nanoplastics concentration at µg/ml was converted to particles per mL using this information from the package insert.

Nanoplastics (µg/ml)	Nanoparticles (x 10^{14}/ml)
80.6	293.3
50.1	182.2
68.4	248.9
59.2	215.5
62.3	226.6

Conclusions

In this study, a one-step nanoplastic detection method using a custom-built portable device has been developed for routine screening of field samples. The additional step of water filtration allows the detection of nanoplastics without the interference of signals from the solid particulate materials with a limit of detection of 35 µg/mL. This technique does not require sample shaking, and results can be obtained within 10 min, making it suitable for rapidly detecting hotspots of plastic contamination in our water sources. This technique is intended to serve as the first screen for nanoplastic load in water. Further studies are required using spectroscopic methods to differentiate plastic types or shapes in these contaminated samples. Additional studies are needed to directly compare split samples with the quantification of nanoplastics using the hand-held fluorometric method to the standard mass spectroscopic methods. The utility of the method proposed in the current study is limited to detecting nanoplastic load only in water samples since nanoplastics detection, and quantification from solid sludge waste would require additional sample processing protocols.

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Author

Vidhatri Iyer is a freshman at high school and is very interested in environmental research. She has conducted prior research to monitor water quality in a wastewater treatment facility and is passionate about developing field-based techniques to detect plastic contaminants in water samples. In addition, she is interested in a medical career and would like to be a physician-scientist to pursue her passion for medicine and research.

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