QUANTIFICATION OF COMMON MICROPLASTIC PARTICLES IN FRESHWATER SEDIMENTS USING NILE RED

by

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ABSTRACT

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Under the Supervision of Professor John A Berges

Identifying and quantifying microplastics (i.e., plastic particle that is smaller than 5 mm) in the environment is critical to our understanding of them because of potential harm to humans and wildlife. Traditional identification tools such as observing and sorting under microscopes are tedious and error-prone, especially in sediments. The lipid stain Nile Red can also stain plastics and has been used previously with some field samples. In the present study I applied it to some of the smaller size categories of particles than have previously been considered, in freshwater beach sediments. My objectives were: 1) to develop a Nile Red staining method and 2) to demonstrate its feasibility by sampling beach sediments on part of the coast of Lake Michigan. Beach sediment samples were collected from multiple sites along the Lake Michigan coast in March, June, and August, year. Plastics were separated out using floatation (with 10 M $ZnCl_2$) and digestion (with 30% H_2O_2 and 7.2 mM $Fe(II)SO_4$) protocols, followed by Nile Red (1 µg/mL in 55% DMSO) staining. Measuring the fluorescence of stained particles (epifluorescence microscopy at 40 x magnification, with excitation at 480 nm and emission at 535 nm) allowed for several plastic polymers to be easily and quickly identified, and quantification was automated using image processing software (Fiji package of ImageJ) and a machine learning algorithm. Particle numbers per gram of sediment ranged from 450 to 1211 and particle size from 5.1 µm (the resolution limit of reliable detection of particles at this magnification) to 458 µm. It was estimated that plastics averaged approximately 2.85 µg per kg of sediment on these beaches. With one exception (Lakeshore Park on June 2nd, 2021) there were no significant ($p > 0.05$) changes in particle density throughout the seasons sampled, nor among sample sites. The present study detected far more microplastic particles (a total of 50,276 in present study), and far smaller particles (15.1 – 30.0 µm mean diameter) than previous studies. I conclude that the

coupling of Nile Red epifluorescence staining with automated analysis may be invaluable in correctly accounting for the microplastics currently in waterways, sediments, and wildlife.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

DDW	Distilled deionized water
MP	Microplastics is an umbrella term for any piece synthetic resin that is smaller than 5 mm.
NR	Nile Red is a lipid stain which binds to many nonpolar materials, including plastics.
PMP	Primary microplastics are manufactured to a desired scale and shape.
SMP	Secondary microplastics are fragments and filaments resulting from fragmentation of larger primary plastics.

ACKNOWLEDGEMENTS

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INTRODUCTION

Microplastics (plastic particles < 5 mm (Barnes et al. 2009)) have been identified in every aquatic ecosystem on Earth (Li et al. 2016, Coppock et al. 2017). Furthermore, microplastics have been found to become incorporated in organisms and biomagnify through trophic levels (Provencher et al 2017). The rate of production, use, and waste of plastic products has increased the sources of microplastic in the environment (Cole et al. 2011, Okoffo et al. 2021). Total global plastic production has increased from 1.5 million tonnes in 1950 to 367 million tonnes in 2020 (Plastics Europe 2022). Since 1972 and the first identification of microplastics as a potential ecological hazard, global plastic production and waste have steadily increased (Carpenter and Smith 1972, Okoffo et al. 2021, Prata 2018). While we may not be able to accurately determine the historical rates of microplastic deposition, we can conclude that the more plastic humans produce, the more potential microplastic arrive in ecosystems.

Microplastic waste in the environment can be separated into two categories: Primary Microplastics (PMPs) and Secondary Microplastics (SMPs) (Cole et al. 2011, Eerkes-Medrano et al. 2015). Primary microplastics are manufactured to a specification. This often will be in the form of beads that offer a variety of industrial purposes from desiccation to product uniformity. Primary plastics are often the proxy used in most scientific research related to microplastic pollution (Ogonowski et al. 2016, Jaikumar et al. 2019). However, the vast majority of microplastics in ecosystems is Secondary Microplastic – microplastic pieces resulting from the fragmentation of larger plastics particles exposed to the elements (Wang et al. 2021). Any piece of macroplastic (> 5 mm) waste can shed microplastics. Abrasion, radiation, and erosion all will produce uneven remnants of the larger parent piece (Song et al. 2017). The irregular

nature of secondary microplastics is often overlooked in studies using uniform primary microplastics as the primary material of study (Jaikumar et al. 2019). There is some conflicting data as to whether using PMPs or SMPs in the laboratory will lead to potential overestimates of the biological impacts experienced by organisms in nature (Ogonowski et al. 2016, Jaikumar et al. 2019). While there are examples of primary microplastics being deposited in environments and the resulting damage: secondary microplastics are understudied in natural environments (Waldman and Rillig 2020, Alnajar et al. 2021).

Microplastics in aquatic environments

SMPs have a wide distribution throughout the aquatic environment (Sighicelli et al. 2018, Lenaker et al. 2019, Townsend et al. 2019). While freshwater microplastics will eventually fall to the sediment or wash up on beaches, the regular refresh rate via pollution ensures SMPs will always be present in every level of the freshwater ecosystem (Driegder et al. 2015). Because of this, microplastics research (and pollution studies in general) has had to be relatively flexible in sampling methods to quantify material found throughout the water column, in the sediment, and potentially in the biota of the ecosystem.

Microplastics can enter freshwater systems through several pathways including wastewater (Browne et al. 2010, Turner 2019), runoff from land (Andrady 2003, Browne et al. 2011), dumping (Browne et al. 2010) and plastic degradation in water (Song et al. 2017). However, microplastics arrive in freshwater basins, once they arrive, they are subject to the same hydrogeologic forces as detritus or sediment (Sadri and Thompson 2014). The majority of microplastics are less dense than water (i.e., buoyant) when they are first introduced to the environment (Chubarenko et al. 2016). Overtime biofouling over the surface of the particle

leads to a slow sinking process which keeps microplastics present at every level of the water column (Kaiser et al. 2017). This also makes microplastic exposure an unavoidable fact of human water usage in addition to the impact on aquatic and semi-aquatic species (Browne et al. 2011).

Freshwater lakes are a large portion of the sinks for microplastic pollution in inland environments (Erikson et al. 2013, Hoellein et al. 2014). Eventually, this material will be transported to the oceans, but the rate of plastic production renders lakes into 'temporary waste holding points' for other environments (Wetzel 2001). Without many of the geostrophic forces experienced in the ocean (e.g. gyres), plastics in freshwaters have no major factor drawing them to concentrate in specific central points (Law et al. 2010, Erikson et al. 2014). This leads to freshwater microplastic pollution being more commonly found as flotsam at waters edges or washed up on shores and beaches (Browne et al. 2010, Hoellein et al. 2014). This is not the universal fate for all microplastics however, as some particles will become biofouled and embed into benthic lake sediments (Kaiser et al. 2017, Kooi et al. 2017, He et al. 2020).

The impact of microplastics on organisms varies. Unlike many human-sourced pollutants, microplastics do not always provide an acute or chronic toxicological effect on their own (Anbumani and Kakkar 2018). When ingested by freshwater species, plastics have been found to cause false satiation (Ryan 1988, Besseling et al. 2013, Welden and Cowie 2016), gut damage (Lei et al. 2018), lowered reproduction (Lee et al. 2013, Ogonowski et al. 2016, Lei et al. 2018), decreased survivorship (Mazurais et al. 2015, Renzi et al. 2019), and (with respect to compounds that adsorb to them) bioaccumulation (Ma et al. 2016, Alnajar et al. 2021), and

biomagnification (Setala et Fleming-Lehtinen 2014, Batel et al. 2016, Costa et al. 2020). It should be noted that these experiments have primarily used PMPs which are present in some but not all freshwater systems (Erikson et al 2013). There are additional examples of studies where no major impacts from microplastic exposure on organisms were found (Penderson et al. 2020, Yardi and Callaghan 2021). The variation of MPs being a potential harm or harmless to wildlife leads MPs being in an odd category of pollutants which cannot always be identified as a source of harm to every ecosystem they are found in. However, MPs themselves may not be harmful to all member of a freshwater ecosystem, they may act as vectors in order to concentrate other dilute forever chemicals such as PFAS or PAHs as organisms collect microplastics (Mato et al. 2001, Zarfl and Matthies 2010).

Plastic samples are expected to mainly be a result of pollution of the Greater Milwaukee area flowing down the Milwaukee River, rather than localized beach litter. The present study assumes that summer is the largest period of plastic pollution for the watershed due to the large abundance of festivals, activities, and consumption occurring near the Milwaukee River and Lake Michigan. Over the course of the summer months, it might be expected that levels of MP pollution at the sample sites could vary with river discharge, which might carry plastic pollution to beaches. According to USGS data river discharge varied from 28.3 m3/s in June 2021 to 11.4 in August 2021 and 11.3 in March 2022 (WaterData - USGS). Thus, there is no clear correlation with plastic particles found, however, we do not know over what time scales the particles collect and therefore what period of time the plastics we observe integrate.

Our hypotheses assumes that plastics particle find their way from a river source to beaches based simply on distance from the river. Plastic particles, however, may not evenly

radiate into the Milwaukee Harbor as they are discharged from the Milwaukee River. Much like bacterial species discharged into Harbor, local currents, winds, and recent precipitation may limit how far particles can travel (Scopel et al. 2006, Newton et al. 2013, Silva et al. 2014). Initial expectations were that microplastics would behave more similarly to pollution such as pharmaceuticals or personal care products rather than bacteria (Blair et al. 2013). Conditions may lead to where currents do not distribute MPs evenly throughout the harbor system but instead create a large glut of particles cannot be distributed from where they were discharged into the river – leading to significantly higher pollution on nearby beaches.

Methods of MP analysis

Sampling and quantification of MPs have traditionally provided novel challenges for research teams. The human eye is relatively adept at identifying a plastic fragment compared to a grain of sand or cluster of algae (Prata et al. 2018). Additionally, the limited number of synthetic compounds manufacturers have produced, restricts the number of MPs compounds we can expect to find in the environment (PlasticsEurope Plastics – the Facts 2021). While several techniques have been developed and published for MP quantification, no scientific standard has yet been agreed upon.

For the quantification of MPs, most research groups have hand counted particle density with the aid of microscopy (e.g., Cole 2016, Erni-Cassola et al. 2017, Maes et al. 2017, Prata et al. 2021).

MPs can thus be easily identified chemically using Raman (analysis of the change in energy of photons scattering off an object; Cole et al. 2013, Käppler et al. 2016) or Fourier-

transform infrared spectroscopy (FTIR, analysis of selective absorption of IR by molecular bonds; Nor and Obbard 2014). The difficulty in assessing MP contamination come less from problems in identification and more from difficulties in quantification. Samples have historically been manually counted for the number of particles with the aid of microscopy (Erikson et al. 2013, Sighicelli et al. 2018, Abel et al. 2021). However, many research teams have noted this is not only tedious but time consuming, turning even small sediment samples into hours of sorting (Erni-Cassola et al. 2017, Prata et al. 2019).

Our goal in the present study would be a methodology that can effectively identify common plastics quickly and accurately in aquatic environments as well as sediments. Our priority is the identification of common consumer plastics as these are the most common MPs found in most ecosystems (Erikson et al. 2013, Hoellein et al. 2014, Driedger et al. 2015, Prata et al. 2018, Townsend et al. 2019, Lenaker et al. 2019, Okoffo et al. 2021). We chose Nile Red – a fluorescent lipid stain – to identifying MP's. Nile Red is a benzophenoxazone dye with strong fluorescence in many nonpolar solvents (Greenspan et al. 1985). This stain has been found to be effective by many teams due to its high affinity with nonpolar materials – including plastics. (Cole 2016, Erni-Cassola et al. 2017, Maes et al. 2017, Konde et al. 2020, Prata et al. 2021). While thus far the primary use of Nile Red stain has been as a marker to assist with hand counting particles (Cole 2016, Prata et al. 2021); we believe this process could be automated to improve the efficiency of large-scale sampling techniques (Erni-Cassola et al. 2017, Prata et al. 2019).

The primary hurdle with using Nile Red is the potential for false positives due to its high affinity to all nonpolar materials (Stanton et al. 2019). Before effective staining can occur, the

samples must go through rigorous sorting and processing to ensure all organic material is removed. For example, while entire stained exoskeletons of crustaceans could be clearly identified by the observer and removed from analyses, amorphous fragments of organic material would be much more difficult to differentiate from fragments of plastic. To prevent this, samples need to be processed to isolate plastics, based on their key characteristics, such as low density and resistance to degradation.

The overall aim of this research was to develop a rapid and simple method that allowed accurate counts of commonly found microplastic particles in freshwater ecosystem samples. This method would provide an alternative to manual counting of microplastic particles under a microscope (although such counts will always be useful for verification).

Specifically, I tried to:

- develop and refine published methods using Nile red staining of microplastics
- demonstrate the feasibility of the method for a limited set of in water and sediment samples collected from coastal Lake Michigan

The central hypotheses of this present study were that: 1. Because the methods developed in the present study aim to stain smaller plastic particles that would be difficult to identify by other means, smaller particles would be found than have typically been identified in previously published work. 2. If the Milwaukee River were a major source of plastic particles to Lake Michigan beaches, then particles would be more abundant in beach sites closer to the river and less abundant at beaches further away. Additionally, over the course of the summer

months, if human outdoor activities increase, then particle pollution should rise during summer months (i.e. June and August) over spring activity (March).

MATERIALS AND METHODS

Plastics Studied

The plastics examined in the present study were a mixture of PMPs (Primary Microplastics: manufactured PS beads), artificial SMPs (Secondary microplastics: post-consumer plastics that had been machined into MPs), and SMPs found at sample sites in the environment. PMP samples were all polystyrene [resin code 6] microspheres (#PPS-6K Spherotech Inc Lake Forest, Il). Artificial SMP samples (selected due to their largescale consumer use) included polyethylene terephthalate [resin code 1], high density polyethylene [resin code 2], low density polyethylene [resin code 4], and polypropylene [resin code 5] samples (ASTM International 2020). Polystyrene was not used as an artificial SMP because it was difficult to grind such light material into workable samples smaller than 50 µm, while butadiene was not used because previous work showed that it was poorly stained with Nile Red (Wang et al. 2021).

Field Sampling

Four beaches along the coast of Southeastern Wisconsin selected based on proximity to the Milwaukee River, the primary source for microplastics) (Lenaker et al. 2019) were sampled for plastics in the water and beach sediment: Atwater Beach, Bradford Beach, Lake Shore Park Pond Beach, and South Shore Beach (Fig 1).

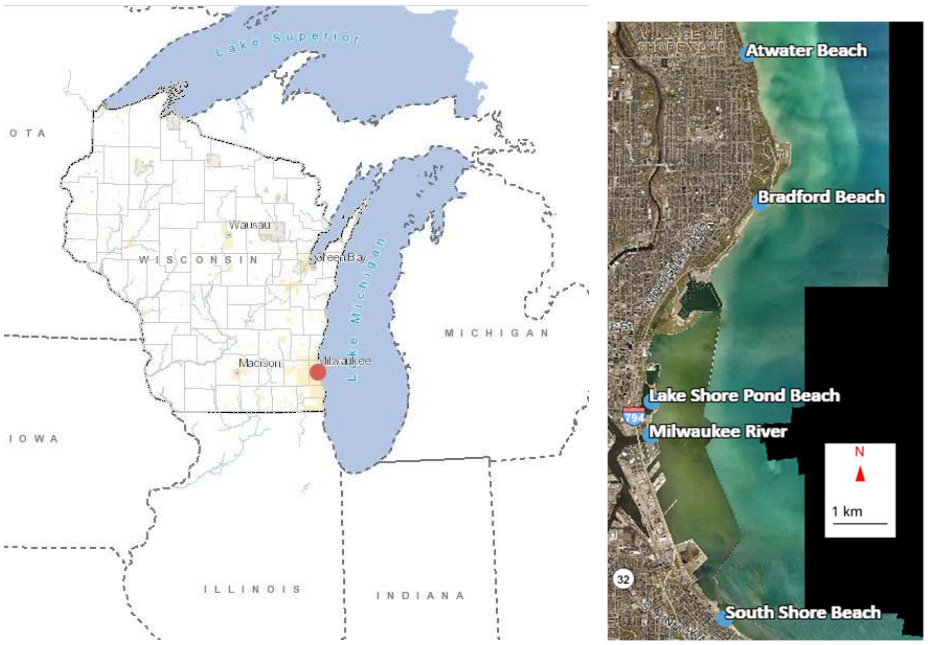

Figure 1: Regional map of Midwestern USA and sampling location in Southeastern Wisconsin [43°*0389' N, 87°9065' W] (source Wisconsin Dept of Natural Resources & US Dept of Agriculture). Combined aerial photography image of Milwaukee County coastline (source Milwaukee Co GIS & Land Information). Beaches sampled included (distance from Milwaukee River delta) Atwater Beach (7.32 km), Bradford Beach (4.72 km), Lake Shore State Park Pond Beach (0.64 km), & South Shore Beach (3.64 km).*

Samples were collected on June 2nd, 2021, August 2nd 2021, and March 2nd 2022. Sediment samples were collected using modified 140 mL polypropylene syringes (8881114030 Covidien Monoject™) which had the end of the barrels cut off, creating a coring tool. 10 cm-deep cores were extracted using the syringes. The upper half (5 cm) was removed from each core and stored in darkness [an opaque plastic bag] at 16 °C in glass vials until needed.

Sample Floatation

Samples were collected and dry mass was measured for SMPs. In the case of PMPs, number of particles per sample - were counted using a dissecting microscope (Olympus SZ51, 40x).

Samples were placed in a 30 mL syringe (301033 B-D) fitted with a luer-lock valve, allowing the syringe to be used functionally as a separatory funnel. Samples were flushed with 2 mL of 0.1% Tween 20 in distilled deionized water (DDW) to prevent MPs from adhering to the surfaces of equipment. To isolated plastics, 5 mL of 10.05 M $ZnCl_2$ was then added to the syringe and allowed to sit for 30 min which resulted in plastics floating, but denser particles (e.g., inorganic sediments) sinking. Syringes were drained of all but the last 1 mL of liquid (containing plastics) which was then recovered on a 0.043 mm mesh stainless steel filter (# 3AJV2, Grainger) using vacuum filtration, along with material backwashed from the syringe with an additional of 10 mL of DDW.

For beach sediment samples, an initial floatation was done in conical centrifuge tubes (339651, Fisher Scientific, Waltham) and for 30 min, after which the top 2 mL of supernatant was pipetted out into syringes. 3 mL of DDW was added to the centrifuge tube and 2 mL was also pipetted out to ensure complete collection. A second floatation performed as described above.

Samples were vacuum filtered onto 43 µm stainless steel mesh (# 3AJV2, Grainger) and rinsed with DDW water. The filters were transferred to glass test tubes (10 mL, 14-962-10C, Fisher Scientific, Waltham) and pressed to the bottom.

Organic Contaminant Removal

Organic matter in samples was removed by oxidation using a 2:1 mixture of 30% hydrogen peroxide and Iron (II) Sulfate. The H_2O_2 oxidizes the Iron (II) into Iron (III) forming a hydroxyl radical and a hydroxide ion, thus creating a more reactive solution than the H_2O_2 alone (Fenton's reaction, Fenton 1894). Four mL of 30% (v/v) H_2O_2, and 2 mL of 7.2 mM $Fe(II)SO_4$

were added to the test tube containing the stainless-steel filter with sample. Samples were acidified by adding 2 mL of 2.5% HCl solution, incubated for 10 min at 20 *℃ in a* water bath. The samples were retrieved from the Fenton's reaction using vacuum filtration on to 25 mm glass fiber filters (61630, Gelman A/E Pall Co, New York). The test tube and stainless-steel mesh filter were backwashed twice with 15 mL of DDW. This backwash captured any remaining plastic particles and removed any remaining superoxides before being vacuum filtered onto the glass fiber filter.

Glass filters were place in aluminum weight boats (08-732-101, Fisher Scientific, Waltham) and stained with Nile Red (No 659, TCI, Tokyo) by pipetting 400 µL of a 1 µg/mL solution in 55% DMSO (D128-500, Fischer Scientific, Waltham) on top of the filter and incubating in the dark for 10 min. We found that this concentration of Nile Red, though lower than that used in previous work, was optimal, balancing sufficiently strong staining of plastics with minimum background staining of filters, against which particles were visualized. Additionally, the relatively low concentration of DMSO solvent in the stock solution was important to prevent the possibility that the solvent (DMSO) might actually dissolve MP particles; we found that 100% DMSO would dissolve polyamide membrane filters (and potentially MPs in sample) (unpublished data). Excess stain was removed from filter using gentle vacuum filtration.

Particle Visualization

Glass fiber filters were transferred onto microscope slides and observed under epifluorescence microscopy. Samples were observed and photographed at 40x with an Olympus BX41 epifluorescence microscope with FITC filters and digital camera (Olympus DP70). Images were

focused and captured using dedicated software (DPController version 1.2.1.108 The rest of this is probably unnecessary-: Dell OptiPlex 9020: Windows 7 Professional). [Exposure was changed depending on the amount of iron oxide that remained on the glass filter, which would also fluoresce -to a lesser extent- at the same excitation and emission range]. Up to five images were collected at random coordinates on the filter.

Particle data was collected from the images using the Fiji package of ImageJ (version 2.9.0/1.53t, (Schindelin et al. 2012, Erni-Cassola et al 2017). All images were calibrated for the conversion of 930 pixels per millimeter (confirmed by previous work). Color images were split into red, green, and blue channels, and the red and blue channels discarded. Uniquely shaped particles were differentiated using the plugin machine learning algorithm Trainable Weka Segmentation (Arganda-Carreras et al. 2017, Li et al. 2021, Yang et al. 2022). Prior to sample analysis, a classifier was produced using Trainable Weka Segmentation based on Nile Red-stained microspheres: this classifier was the baseline for all image trainings. Particles were grouped into class 1 (Objects) and all other materials (filter and nonfluorescent material) into class 2 (Background). Approximately four examples of each were added to each class and the classifier was then trained for the current image. Once successful a probability image was collected and converted to an 8-bit image. Minor thresholding changes were made by only

focusing on pixels with a greyscale index [brightness] between 160 and the maximum of 255, and particle data was then collected.

Data Analysis

Samples from all sites were compared based on the total number of particles found at the site, the estimated mean diameter and volume of an average particle, and the mass of plastics per gram of sediment at the site. Diameter and volume estimates were taken from based on the two-dimensional area provided by Fiji and converted to radii assuming all particles in the sample were spherical beads. Objects with an area smaller than 19.6 μm^2 [calculated radius of 2.5 µm] could not be confidently confirmed to be plastics and were discarded as background. Mass of plastics was further extrapolated based on the calculated volume and assuming all particles in the sample were the same material and density. The present study assumed all particles were lightest possible polyethylene ([LDPE] with an approximate density of 0.92 g cm^{-3}) based on the high volume of polyethylene found in the sediment of the Milwaukee River and estuary by other researchers (Batra 2014, Lenaker et al. 2019).

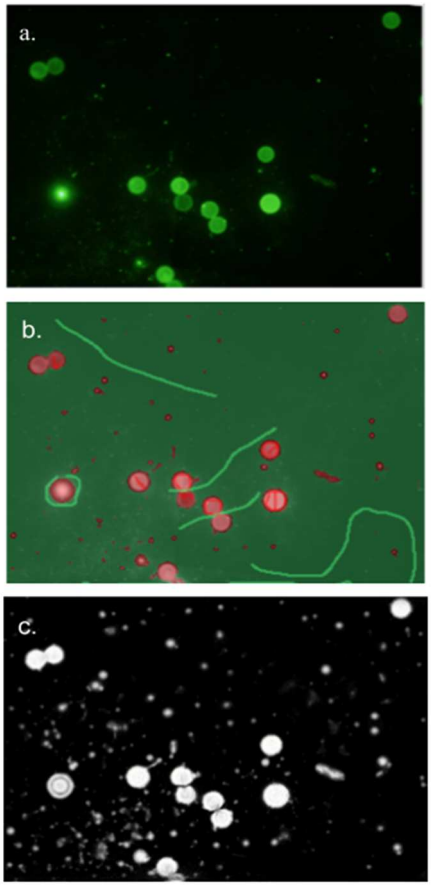

Figure 2: Process for training the Trainable Weka Segmentation model to recognize stained microplastics. (a) Original epifluorescence image of 200 µm microspheres taken at 40x. (b) Post segmentation image with category 1 [objects] highlighted red and category 2 [background] highlighted green. (c) Probability map of items belonging to category 1 [objects] – this image was one of several used to train the base model for particle recognition.

Statistical analysis was performed using SigmaPlot (version 12.5, Systat Software Inc.). Data were tested for normality using Shapiro-Wilk tests prior to analyses. A two-way ANOVA was performed on beach sediment samples, testing particle abundance and particle diameter among sampling dates and sampling location, and also testing for interactions between the two factors. Where differences were found, they were analyzed using a Tukey Pairwise Multiple Comparison. In all cases, significance was judges at the 95% confidence level (i.e., 5% probability of a Type I error).

RESULTS

The present method incorporated several recent improvements in different stages of microplastic separation and analysis, and, in combination, they simplified MP analysis in sediment samples. Preliminary experiments established optimal methods, e.g. using 10.05 M $ZnCl_2$ for floatation was better than 30% saline solution] (Cole 2016, Maes et al. 2017, Coppock et al. 2017, Abel et al. 2021, Townsend et al. 2022), digestion was more complete with Fenton's reaction for 10 min versus 50% H_2O_2 for 24 h to] (Fischer et al. 2015, Erni-Cassola et al 2017, Tagg et al. 2017, Pfeiffer et Fisher 2020, Abel et al. 2021, Tirkey et Upadhyay 2021) and staining was more even and showed less background with NR stock solution 1 mg/L in DMSO & water (55% v/v) versus NR stock solution 0.5 mg/mL in acetone (Cole 2016, Erni-Cassola et al. 2017, Maes et al. 2017, Kaile et al 2020, Wang et al. 2021). Alterations to standard techniques were made one at a time, and we verified each improvement against standard samples before proceeding.

Sequential application of the floatation, digestion, and then staining protocols did not produce any adverse or confounding chemical interactions during transfers from one technique to the next.

We assessed the potential loss microplastics during analysis by running standards (500 μm fluorescent green PS microspheres) with known numbers of particles. Over six trials, recovery of standard microspheres averaged 93.5% (*± 3.3)*.

Samples were compared through time and special variation among beaches along the Lake Michigan coast. In total among 12 samples, a total of 50,276 MP particles were identified. Our search window was for particles between 5 mm to 43 μm (based on the mesh size used to transfer particles between the floatation and digestion steps), despite the fact that the samples were filtered onto 42 μm steel mesh after separation by floatation and before digestion, large numbers of smaller particles were enumerated. Such particles likely were liberated from larger aggregates (both plastic and organic) during digestion.

Initial expectations were that would be fluctuations in the number of plastics found at each site over the course of the summer months both because the increase in human activity at each of the beaches during the summer and the proximity to the Milwaukee River – a major sink for microplastics in the local basin.

Current data is unable to support the present study's second hypothesis for three of the four sampled locations (Fig. 3 a). Plastic particles concentration averaged 500 ± 46 particles per gram of sediment and was not significant (Two way ANOVA, $p > 0.05$) change in the concentration microplastics found before summer (March 2nd), in midsummer (June 2nd), and late summer (August 2nd) in Atwater beach, Bradford beach, or Southshore beach.

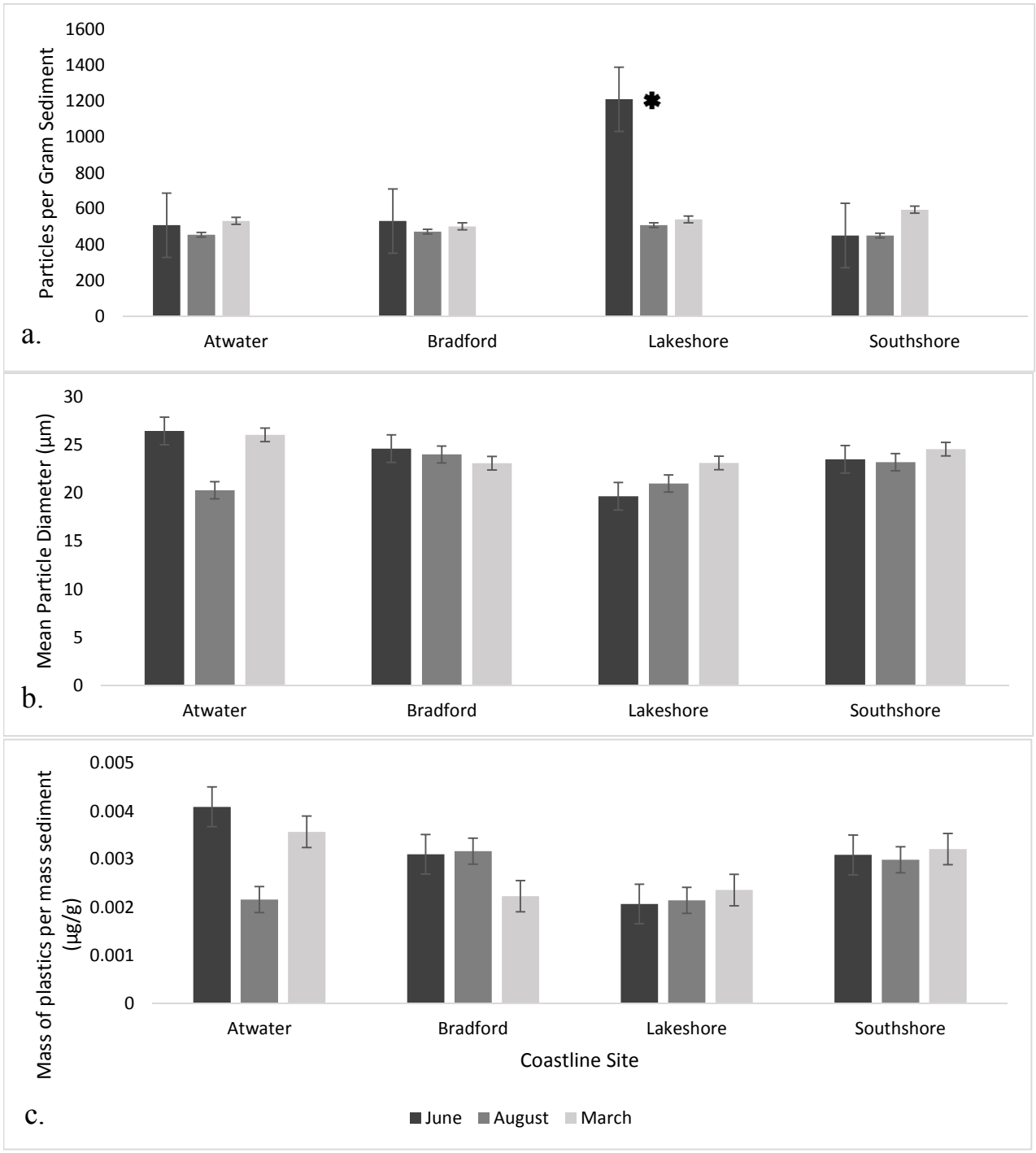

Figure 3: Abundance (±standard error) of microplastic particles at each Lake Michigan coastal sites: Atwater beach, Bradford beach, Lakeshore State Park pond beach, and Southshore beach over the sampling periods June 2nd 2021 (black bars), August 2nd 2021 (dark grey bars), and March 2nd 2022 (light grey bars). (a) Particles found at each site per gram of beach sediment. (b) Calculated mean diameter of particles µm at each site. (c) Calculated microplastic mass (assumed density of 0.92 g cm^{-3}) per gram of beach sediment.

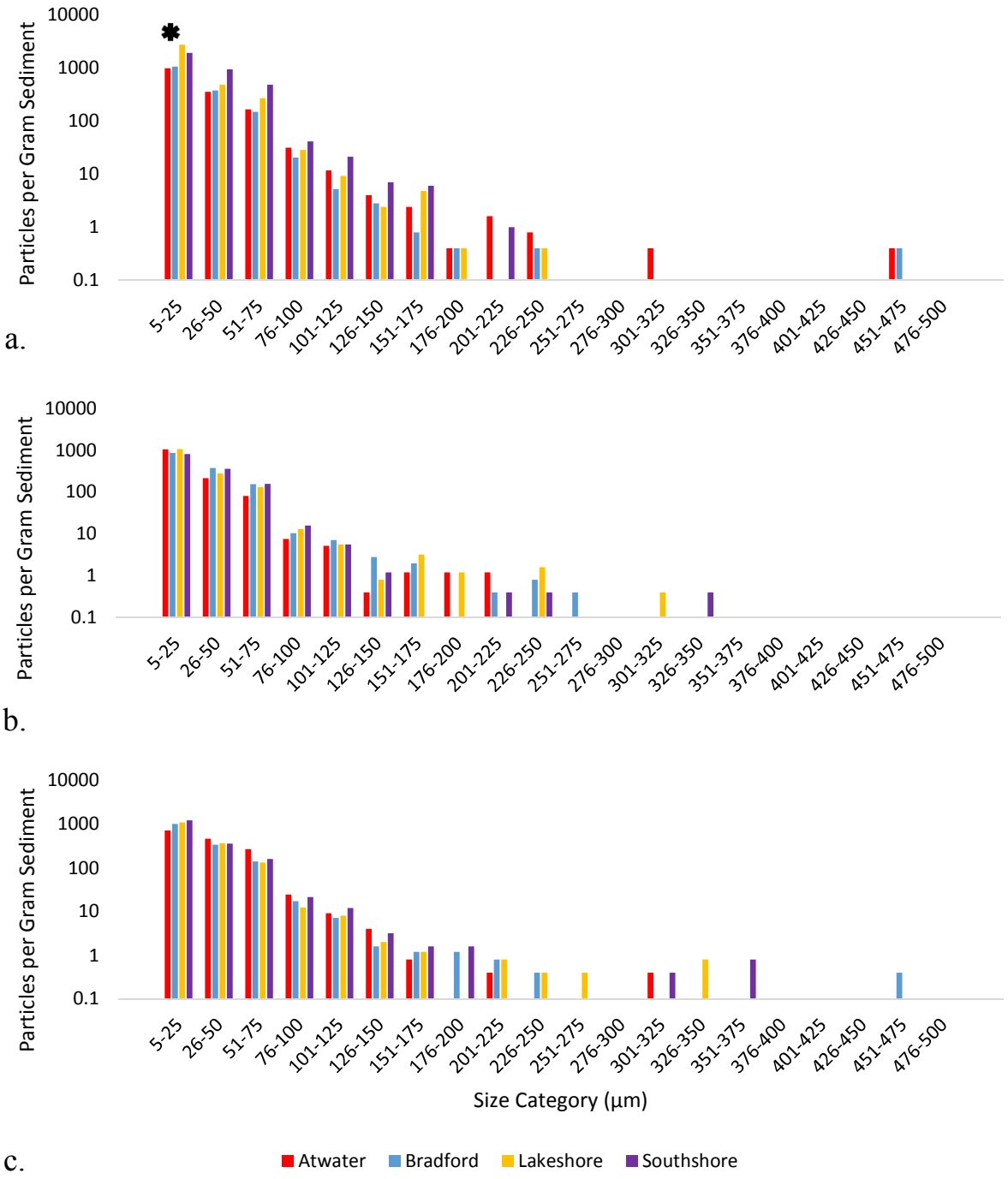

Figure 4: Array of particles per gram of sediment found at all sites (Atwater beach, Bradford beach, Lakeshore State Park Pond beach, and Southshore beach) on (a) June 2nd 2021, (b) August 2nd 2021, and (c) March 2nd 2022. The majority (65%) of particles were smaller than 25 µm. Note objects smaller than 5 µm could not be reliably classified and were not counted.

There was however variation in the number of particles present at a given time at pebble beach in the inlet at Lakeshore State Park. June samples from Lakeshore held significantly more (Pairwise Multiple Comparison, $p < 0.001$) particles than other beaches during the same day (Fig 3 a). Although this is not true later in the season as neither the August nor March samples are significantly greater than any other samples site.

We can conclude rather than the level of MPs changing on each beach with seasonality or proximity to the Milwaukee River: Atwater, Bradford, and Southshore beaches all had similar MP levels throughout the sampled season.

As to the first hypothesis, the majority of particles found in sampling were between 5 – 25 µm. This was true for all locations at all times sampled (Fig. 4).

Particles did display differing brightness during epifluorescence microscopy. Especially smaller-sized particles showed a much larger range of brightness while larger particles tend to

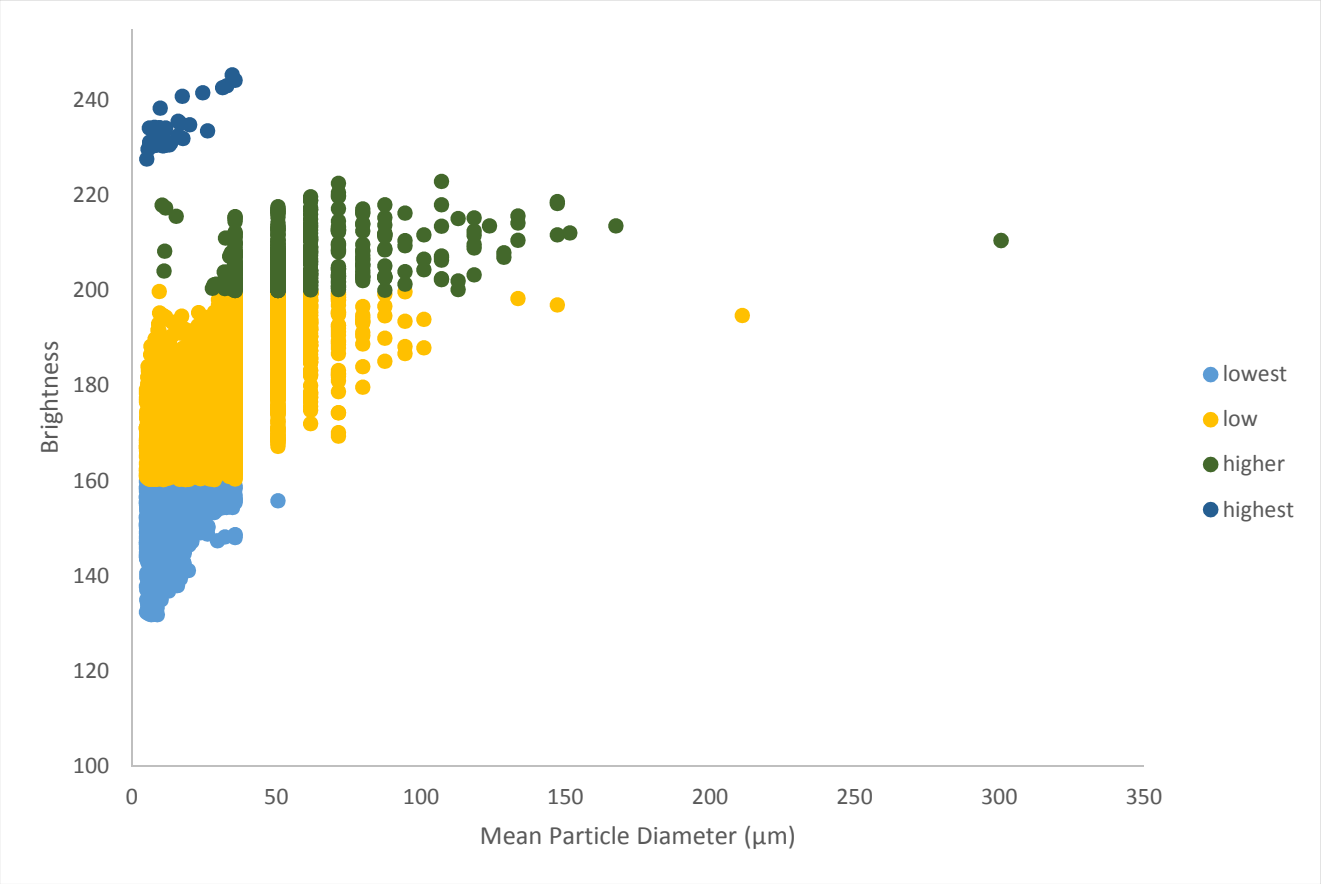

Figure 5: Differential image brightness among particles compared to the mean diameter of particles at Atwater beach on March 2nd 2022. ImageJ defined image brightness on a scale of 0 to 255 (entirely black pixels to entirely white pixels). Categories included lowest [x < 160.29], low [160.29 – 200], higher [200 – 225], and highest [x > 225] brightness.

in the higher categories of brightness (Fig 5). In some cases, discrete clusters particles were observed, e.g., a group of very small (5- 50 µm), very bright (230-250 units) particles.

DISCUSSION

Application of the method

The primary objective of this study was to adopt and verify an effective and efficient means of identifying microplastics in a variety of freshwater ecosystems. The present method was

designed to be effective in freshwater sampling in addition to sediment sampling. While no tool can be universal in MP analysis, this has been effective in recovering assorted plastics from beach sediment and has also functioned with sampled and spike freshwater (unpublished data).

Nile Red has been a widely used solution to the difficulty of identifying the wide variety of microplastics (Cole 2016, Erni-Cassola et al. 2017, Maes et al. 2017, Konde et al. 2020, Prata et al. 2021). Such a popular stain has led to a wide variety of stain stock solutions including: 500 μg mL^{-1} NR in acetone (Cole 2016), 1 mg mL^{-1} NR in acetone (Maes et al. 2017, Prata et al. 2019, Wang et al. 2021), 1 μg mL^{-1} NR in methanol (Erni-Cassola et al. 2017), 20 μg mL^{-1} NR in acetone & ethanol (50% v/v) (Konde et al. 2020), and 10 μg mL^{-1} NR in DMSO (Kaile et al. 2020). A regular issue with the more concentrated (> 10 μg mL^{-1} NR) stock solutions is the tendency for Nile Red particles to self-adhere and quench (Ray et al. 2019, Rumin et al. 2015). NR will tend to aggregate in polar solutions, and while this may not be an issue in stock solution, this must be considered when these stains will be vacuum filtered or rinsed: the greater the concentration of NR the more particles will self-adhere (and be useless). Additionally, a greater concentration of NR can result in a loss of overall fluorescence as the particles interact with the electrons in an excited state – this phenomenon is known as quenching (Rumin et al. 2015). Once again, the traditional solution to this has reduced the concentration of NR stock solution. The reduction in concentration used by the present study has had the added benefit of further preventing the staining of organic particles (although digestion is still the primary preventative measure to remove organic particles). Thus 1 μg mL^{-1} NR was selected as the optimal concentration and DMSO was found as a more environmentally friendly alternative to

methanol (note 55% DMSO was used because 100% DMSO would make both glass fiber and membrane filters unusable (unpublished data).

Field observations

The original hypothesis of the present study was that levels of microplastics found in beach sediments would fluctuate throughout the summer months. Additionally, this study expected beaches closer to the Milwaukee River (a known source of pollutants) would show higher levels of microplastic contamination than urban beaches further from the river.

Several previous studies have measured the MP content in coastal beaches, estuaries, and river sediments (Cannas et al. 2017, Lenaker et al. 2019, Dodson et al. 2020 Townsend et al. 2022). The majority of these studies analyzed kilograms of sample sediments and after separation techniques, plastics were identified by observers with the assistance of microscopy. Recently the critical assumption that representative samples can correctly represent the sediment even a meter away (Abel et al. 2021). Thus, a smaller scale (kg to g) approach was deemed more appropriate for analyzing samples. Another standard practice in most studies is to hand count the number of microplastic particles found in a given sample. This may be in line with current standards of many regulatory institutions (US EPA 2020); however, it is also time consuming and costly. Automated identification has been repeatedly suggested as a solution to these issues (Erni-Cassola et al. 2017, Prata et al. 2019). The primary hurdle for the mass adoption of automated identification tools in the study of microplastics has been the problem of misidentification: background or organics material can be incorrectly identified as plastic, while lightly stained plastic particles can be missed (Stanton et al. 2019). Two solutions to this

problem have been employed: minimize application of Nile Red to reduce staining on low affinity materials, and to filter the particle identification process through a machine learning algorithm to better identify MP particles.

The particles recovered and identified by the present study have been generally different than those of other studies. The majority of studies that have employed observer hand sorting as their primary means of particle identification and categorization found at most a few thousand total particles (Table 1). This is in stark contrast to the 50276 particles found over the course of 12 samples in the present study. While this may initially suggest high error, what is also remarkable is the difference in mean diameter of particles. Previous studies have found much larger particles (up to 5 mm in length): this may be a result of sampling bias by what objects previous teams were able to locate. The majority of the particles recovered in the average sample fell between 5 – 25 μm total diameter (Fig 4). It is important to note, this is smaller than the material that should be identified by the present study. After the floatation step, samples were collected on 43 μm stainless steel mesh to allow for all particles collected to be digested together (which should remove all the particles in the largest observed size category). There are a few potential explanations for this incongruity. The mesh likely clogged as a total of 5 mL of $ZnCl_2$ (2 mL of supernatant + 3 mL of backwash) was vacuum filtered across the mesh. This would allow for partial collection of MPs smaller than 42 μm. Additionally, the objects captured by the mesh may not be singular objects, but conglomerations of smaller particles. After digestion, clusters of particles may break apart into their individual subunits which were then observed in ImageJ analysis. While the counts provided in the present study may be closer to the number of very small microplastics (with a diameter of 5 – 25 μm): further

study is needed on the behavior of very small microplastics. Any material found smaller than 5 µm was not counted because it could not be accurately resolved at a magnification of 40X. This exponential increase may suggest even greater presence of very small particles including nanoplastics that currently cannot be accounted for.

Table 1. Comparison of various estimates of plastic particles in various aquatic sediments

Area Sampled	Method of Identification	Total particles found	Mean particles per g sediment	Mean Diameter of particles (µm)	Source
Lake Michigan	Epifluorescence microscopy	50276	450.779 – 1211.826	15.1 – 30.0	Present study
Lake Michigan	Hand Sorting	4218	0.0329 – 6.229	125 – 355	Lenaker et al. 2019
Chesapeake Bay Virginia	Hand Sorting	3564	0.596 – 2.224	500 – 5000	Dodson et al. 2020
Melbourne, Australia	Hand Sorting	913	0.002 – 0.147		Townsend et al. 2022
Tuscany, Italy	Hand Sorting	1027 ± 782	0.042 – 1.069		Cannas et al. 2017
Kuril-Kamchatka Trench	FlowCam	630	0.014 – 0.209		Abel et al. 2021

The number of particles automatic identified here does potentially bring into question if previous estimates of microplastic pollution have been too conservative. This is not to deride the rigorous effort of previous teams, but reliance on human observation (even improved with a dissecting microscope) may be able to more accurately count the consumer plastics found in ecosystems.

Caveats

The majority of microplastics found in sediments were between 5 – 25 µm; our use of a 43 µm to move particles between floatation (in $ZnCl_2$) to digestion (in H_2O_2 and $Fe(II)SO_4$) was thought to be satisfactory in capturing the majority of particles that would be recovered. While clogging of the mesh did save some portion of the particles smaller than 43 µm, we do no know how many similar small particles would have been collected on a similar mesh with a pore size of 5 µm. The counts observed in the present study show an improvement in the identification of microplastics but should be considered the minima of microplastics in Lake Michigan beaches.

Human observers are generally effective in the identification of microplastic particles. However, there is a large constraint in the time samples take to sort based on observer selection (Erni-Cassola et al. 2017). The ability to automate human pattern recognition has been functionally replicated in the use of machine-learning algorithms in particle analysis (Li et al. 2021, Yang et al. 2022). Fluorescence images alone would often provide a poor representation of a fiber or large fragment if the object experienced differential staining across the surface. Image analysis of such particles could lead to the misidentification of a single 4 µm fiber as a cluster of four 1 µm fragments. By using the ImageJ add-on Trainable Weka

Segmentation prior to particle analysis, objects can be correctly sized and classified (Fig 2). The best technique found for avoiding any confusion within the machine-learning algorithm was to create a baseline identifier model using images of microspheres and once sufficiently powerful (77 attributes): reload and retrain on each image. This was less efficient than simply running the saved model and data but training the algorithm on each image made for far misidentifications.

Different plastics did display a high variance in fluorescence emission both in data from the sample sites and artificial seeded samples (unpublished data). Whether this is a result of a larger surface area having a greater power to emit light or a potential tool to identify different plastics based on affinity to Nile Red stain remains to be seen. Furthermore, while it would at first appear to be a simple question of the polarity of what polymers are being stained, this does not always appear to be the case. We found that polyvinyl chloride (PVC) would stain using the present method, despite vinyl chlorides making up the structure being polar. Nile Red staining of PVC and other hydrophilic polymers has been observed previously although it is typically weaker than hydrophobic plastics (Erni-Cassola et al. 2017, Wang et al. 2021). This means that Nile Red staining may not only be a result of nonpolar – nonpolar interactions but also from small scale interactions by particles collecting nearby chemicals (such as stain) (Hüffer et Hofmann 2016).

Current limitations to the present method would include the inability to identify plastics that are commonly found in freshwater ecosystems. Butadiene from automotive tires has been found in Lake Michigan and the Milwaukee River in high concentrations (Lenaker et al. 2019). This plastic does not stain with Nile Red (Erni-Cassola et al. 2017, Wang et al. 2021). While the

intention was to create an assay for the most common consumer plastics found in the ecosystem, the inability to account for butadiene could mean a significant amount more plastics is unaccounted for in this method.

Additionally, the current floatation protocol requires 10 mL of 10.05 M $ZnCl_2$ per 2.5-gram sample of sediment or 2 mL of sample water. This is a high demand of zinc, which produces a large environmental hazard when disposed (Zyadah et Abdel-Baky 2000). It would be preferable to find a more ecologically friendly floatation solution with similar properties to $ZnCl_2$.

Future work

There may yet be even further untapped potential in the use of Nile Red staining in identification of microplastics. The unique polymer structures of different plastics do appear to have varying affinity to Nile Red stain (Wang et al. 2021). While the present study has been more concerned with what plastics stain well with Nile Red and avoiding those that stain not at all. A spectrum of effective staining may be not only useful for determining ideal stain concentrations, but also for a rough identification method for objects on masse. Objects that only partially stained typically showed a lower average brightness – and this could provide clues to the composition of particles with the assistance of FTIR or Raman spectroscopy (Fig 5). The reason for clusters of plastics with different fluorescence must still be determined before affinity identification can be analyzed. The difference in staining could be the result of an unforeseen variable such as particle damage.

Should stain affinity identification be viable it would be best ground-truthed using known mixtures of primary microplastics. For example, a series of artificial sediment samples could be seeded with known concentrations of multiple plastics that stain but at different levels (i.e., polypropylene and polystyrene). Should differential staining be a viable index for particle composition, particles should form categories of brightness which match the ratio of the known plastic mixture (i.e., 50 – 50 w/w to 50% 'bright' and 'dim' particles).

A major component in microplastics not included is the precise chemical identification of samples using FTIR or Raman spectroscopy (Coleman et al. 1988, Nor et Obbard 2014, Lares et al. 2018, Araujo et al. 2018). The benefit of the present method is that Nile Red staining does not have an impact on Raman spectroscopy (Erni-Cassola et al. 2017, Prata et al. 2021, unpublished data). Samples can be taken directly from epifluorescence imaging to subsequent Raman analysis. For example, seeded artificial sediment samples could be created with a single known polymer (such as polystyrene) and both stained and unstained samples could be compared under Raman spectroscopy to see if identification, and particle counts are similar. A chemical confirmation should be the standard for any further work using this method.

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APPENDICES

Appendix A: Comparisons of particles detected by eye and using image analysis

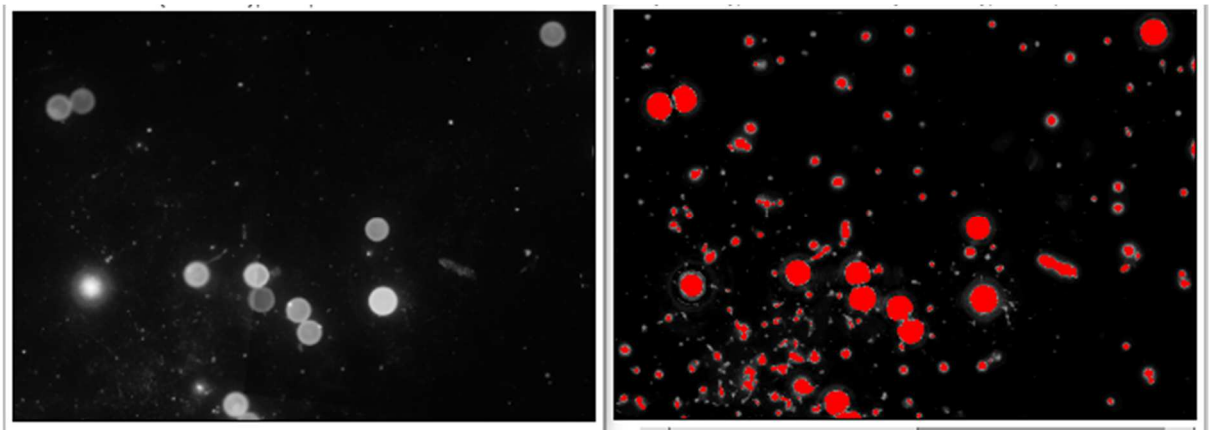

Figure 6: Side by side comparison of the same control image counted by a human observer (left) and the software ImageJ after trainable segmentation (right). In the right image the area in highlighted in red is the area that has been selected for using the Threshold tool. The human observer identified a total of 100 objects in the image and ImageJ identified 129.

Appendix B: Two-way analysis of variance

Table 2: Two-Way Analysis of Variance of microplastics abundance data. Location represents four sampling location Atwater beach, Bradford beach, Lakeshore State Park Pond beach and Southshore beach, Date represents sampling dates June 2nd 2021 August 2nd 2021 and March 2nd 2022. See Materials and Methods for details.

Source of Variation	DF	SS	MS	F	P
Location	3	2500343	833447	5.800	0.004
Date	2	1521891	760945	5.296	0.012
Location x Date	6	4380450	730075	5.081	0.002
Residual	24	3448656	143694		
Total	35	11851342	338609		

Table 3: Pairwise Multiple Comparison Procedures (Holm-Sidak) of microplastics abundance data: dates within locations. Location represents four sampling location Atwater beach, Bradford beach, Lakeshore State Park Pond beach and Southshore beach, Date represents sampling dates June 2nd 2021 August 2nd 2021 and March 2nd 2022.

Comparison: Date at Atwater	Difference of Means	t	P
March vs August	197.000	0.636	0.896
June vs August	134.667	0.435	0.889
March vs June	62.333	0.201	0.842
Comparison: Date at Bradford	Difference of Means	t	P
March vs August	139.333	0.450	0.960
June vs August	75.000	0.242	0.964
March vs June	64.333	0.208	0.837
Comparison: Date at Lakeshore	Difference of Means	t	P
March vs August	1707.000	5.515	<0.001
June vs August	1626.667	5.256	<0.001
March vs June	80.333	0.260	0.797
Comparison: Date at Southshore	Difference of Means	t	P
March vs August	353.667	1.143	0.602
June vs August	347.667	1.123	0.471
March vs June	6.000	0.0194	0.985

Table 4: Pairwise Multiple Comparison Procedures (Holm-Sidak) of microplastics abundance data: locations within dates. Location represents four sampling location Atwater beach, Bradford beach, Lakeshore State Park Pond beach and Southshore beach, Date represents sampling dates June 2nd 2021 August 2nd 2021 and March 2nd 2022.

Comparison: Location in June	Difference of Means	t	P
Lakeshore vs Southshore	1836.000	5.932	<0.001
Lakeshore vs Atwater	1697.667	5.485	<0.001
Lakeshore vs Bradford	1647.667	5.323	<0.001
Bradford vs Southshore	188.333	0.608	0.908
Atwater vs Southshore	138.333	0.447	0.884
Bradford vs Atwater	50.000	0.162	0.873
Comparison: Location in August	Difference of Means	t	P
Lakeshore vs Southshore	135.000	0.436	0.999
Lakeshore vs Atwater	125.333	0.405	0.997
Lakeshore vs Bradford	80.000	0.258	0.998
Bradford vs Southshore	55.000	0.178	0.997
Atwater vs Southshore	9.667	0.146	0.987
Bradford vs Atwater	45.333	0.146	0.987
Comparison: Location in March	Difference of Means	t	P
Lakeshore vs Southshore	138.333	0.447	0.986
Lakeshore vs Atwater	8.667	0.028	0.978
Lakeshore vs Bradford	85.333	0.276	0.990
Bradford vs Southshore	223.667	0.723	0.980
Atwater vs Southshore	147.000	0.475	0.994
Bradford vs Atwater	76.667	0.248	0.963

Table 5: Two-Way Analysis of Variance of mean diameter of microplastics data. Location represents four sampling location Atwater beach, Bradford beach, Lakeshore State Park Pond beach and Southshore beach, Date represents sampling dates June 2nd 2021 August 2nd 2021 and March 2nd 2022.

Source of Variation	DF	SS	MS	F	P
Location	3	51.148	17.049	1.589	0.218
Date	2	27.301	13.650	1.272	0.298
Location x Date	6	69.185	11.531	1.075	0.405
Residual	24	257.539	10.731		
Total	35	405.173	11.576		

Table 6: Least square means of mean diameter of microplastics data. Location represents four sampling location Atwater beach, Bradford beach, Lakeshore State Park Pond beach and Southshore beach, Date represents sampling dates June 2nd 2021 August 2nd 2021 and March 2nd 2022.

Least square mean for location	Mean
Atwater	24.279
Bradford	23.922
Lakeshore	21.272
Southshore	23.774
Standard Error	1.092
Least square mean for date	Mean
June	23.575
August	22.138
March	24.222
Standard Error	0.946
Least square mean for location x date	Mean
Atwater x June	26.479
Atwater x August	20.303
Atwater x March	26.056
Bradford x June	24.636
Bradford x August	24.027
Bradford x March	23.105
Lakeshore x June	19.671
Lakeshore x August	21.001
Lakeshore x March	23.143
Southshore x June	23.515
Southshore x August	23.223
Southshore x March	24.585
Standard Error	1.891