Characterizing the Diatom Communities of Freshwater Polyethylene Terephthalate and Polypropylene Plastispheres

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The introduction of the first synthetic plastics in the early 20th century launched not only a new industry of versatile and cheap material but a new era of environmental pollution: that of aquatic plastic waste. Nowadays, Du et al. (2022) estimates that without intervention, the rate of plastic pollution in 2021 could double by 2030. While large-scale impacts of plastic pollution are publicized and commonly studied, the microbial impact of plastics is not.

Upon entering a body of water, microorganisms immediately colonize plastic particles to form biofilm communities, a phenomenon dubbed the 'plastisphere'. Diatoms are a major component of plastispheres and are crucial ecosystem health indicators, so understanding diatom responses to plastic pollution will reveal how plastic affects the entirety of an aquatic ecosystem (B-Béres et al., 2022).

There is a general lack of scholarly research on the plastisphere and diatoms, especially in freshwater settings. Additionally, plastispheres are thought to differ based on geographic location. Therefore, the goal of this study is to morphologically characterize and compare the composition of diatom communities in polyethylene terephthalate (PET) and polypropylene (PP) plastispheres in a Pacific Northwest freshwater environment over a period of 5 weeks. A quasi-experimental method, where treatment groups are compared without fully controlling independent variables, was used to collect both quantitative and qualitative data in a five-part method: experiment installation, sampling, biofilm quantification, diatom characterization, and analysis. Data was analyzed in terms of biofilm richness and diatom diversity (Amaral-Zettler et al., 2015). This quasi-experimental work may serve as a pilot-study for future, more rigorous projects.

Literature Review

Plastic Pollution

Plastics are an ideal material for a variety of applications due to their versatility, light-weightiness, strength, potential transparency, oxygen and moisture barrier properties, and bio-inertness. As a result, the large-scale production of plastic polymers beginning in 1950 has reached over 300 million tons annually today. Annual global demand for plastics consistently increases, with a compound annual growth rate of 8.4% for plastic production from the years 1950 to 2015. Polyethylene (PE), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polypropylene (PP), polyvinyl-chloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR), polystyrene (PS), and polyester represent 92% of all plastic ever made. About 42% of all non-fiber plastics produced, made up mostly of PE, PP, and PET, are used as consumer packaging material. Packaging material, which reaches its end-of-life the same year it is produced, comprised 54% of the non-fiber plastic waste leaving use in 2015. Most plastic monomers are derived from fossil hydrocarbons, none of which are biodegradable on a human time scale. Consequently, as plastic production has increased over the past 6 decades (Fig. 1a), so has global plastic waste generation (Fig. 1b) (Andrady, 2011; Geyer et al., 2017; Azevedo-Santos et al., 2021).

Fig. 1a

Global Primary Plastics Production (in million metric tons) According to Polymer Type from 1950 to 2015 (Geyer et al., 2017)

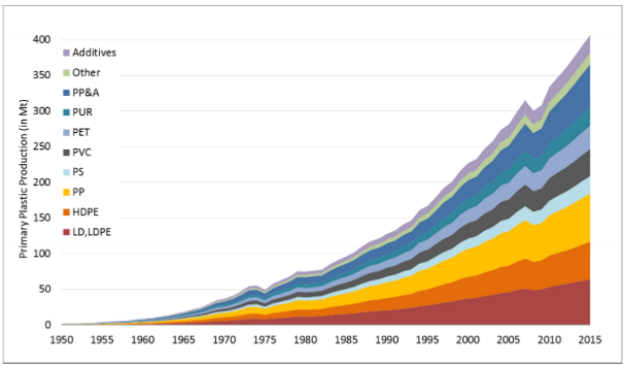
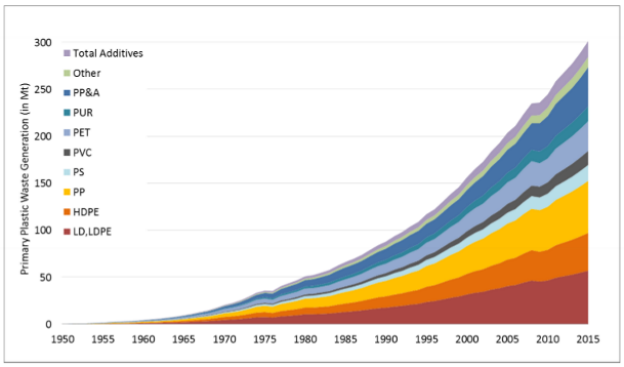


Fig. 1b

Global Primary Plastics Waste Generation (in million metric tons) According to Polymer Type from 1950 to 2015 (Geyer et al., 2017)



The first reports of plastic litter in the oceans date back to the early 1970s. Nowadays, it is estimated that over 51 trillion fragments (236,000 tons) of plastic are in the marine environment. 60% of all plastic ever produced is currently accumulating in landfills and the natural environment. With plastic debris having been transported throughout all five world subtropical gyres and in freshwater ecosystems, "near permanent contamination of the natural environment with plastic waste is a growing concern" (Geyer et al., 2017). As anthropogenic climate change worsens, extreme tsunamis and storms carry large pulses of plastic into marine ecosystems from coastal landfills and urban areas (Andrady, 2011; Dey et al., 2022; Zettler et al., 2013).

Two methods of plastic degradation are of concern in this study. Photo-oxidative degradation results from exposure to UV-B radiation and is often the most effective first step to breaking down plastics. Biodegradation is when living organisms convert the carbon in plastic polymers into carbon dioxide to incorporate into biomass. With the help of photo-oxidative degradation, all plastics biodegrade extremely slowly in the marine environment. However,

biofouling of plastic pieces may increase their density enough to sink them out of the sunlit pelagic zone, negating the chance of photo-oxidative degradation. In addition, microbial species that biodegrade polymers are rare (Andrady, 2011).

When examining plastic debris' effect on aquatic ecosystems, most studies focus on the entanglement of large organisms and the ingestion of plastic particles. For example, at least 44% of marine bird species are recorded to have ingested plastics. Ingestion of microplastics, synthetic polymers ranging from 1 micrometer to 5 millimeters in size, by microbiota can have a significant toxic effect from the presence of persistent organic pollutants (POPs), which concentrate twice as much on plastics compared to the surrounding seawater. Once adsorbed by microplastics, these highly concentrated POPs become bioavailable and move through food webs via ingestion. Similarly, floating microplastics are a vector to spread infectious diseases and invasive species across large aquatic distances. Microbial infections via plastic consumption in fish, mollusks, and crustaceans are known to damage the aquaculture industry, affecting economic prosperity, especially along the coasts (Andrady, 2011; Dey et al., 2022; Du et al., 2022; Zettler et al., 2013).

The Plastisphere

Plastics also function as habitats for microorganisms. The 'plastisphere' refers to the distinct and diverse microbial community that attaches to plastic surfaces, often in the form of a biofilm. Biofilms are organized structures of microorganisms with specialized functions. The physical properties of plastics, including roughness and hydrophobicity, as well as the concentration of nutrients on plastics makes them an ideal habitat for bacterial communities. These physical properties and environmental factors select for bacterial colonizers and affect the evolution of a plastisphere community. Plastispheres start forming when pioneer bacteria rapidly

cover the surface area of a plastic. These pioneers excrete extracellular polymeric substances that allow secondary microorganisms to colonize, and over time, as species form niches and are outcompeted, community succession leads to specialized, plastic-specific microbes living in mature biofilms. These take several months to form (Kirstein et al., 2019; Dey et al., 2022; Bamford et al., 2023; Du et al., 2022).

Understanding how plastisphere biofilms affect the plastics they attach to and their surrounding environments is a critical area to research (Bamford et al., 2023). Studies by Dey et al. (2022), Eich et al. (2015), and Du et al. (2022) found that diatoms are pioneer colonizers that dominate the plastic surface and are important for biogeochemical activity.

Diatoms

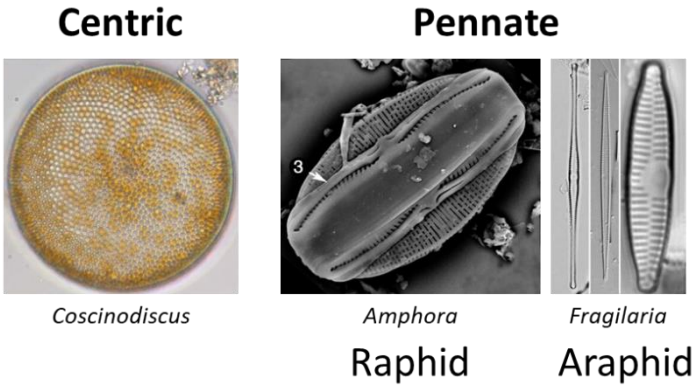
Diatoms are unicellular, photosynthetic eukaryotes and the most diverse group of phytoplankton. They exist as single cells or chains of connected cells in both planktonic and benthic habitats and range from a size of a few micrometers to a few millimeters (Fig. 2a).

Fig. 2a

Diatoms at 400x Magnification

Fig. 2b

Centric, Penate, Rapid, and Araphid Diatoms (Spaulding et al., 2021)



Diatoms are classified into two overarching morphological groups: Centric, or circular with radial symmetry, and Pennate, or elongated with primarily bilateral symmetry (Fig. 2b).

Pennate diatoms are further divided into Raphid and Araphid diatoms, categories that indicate the presence or absence of a raphe. A raphe is a slit in the frustule responsible for motion and attachment to substrates. The frustule, which is a diatom's key identifying feature, is a cell wall made of hydrated silica that forms species-specific patterns. The two valves of a frustule are joined by silica bands called a girdle, and the walls of a frustule may be perforated by pores called areolae (Fu et al., 2022; Virginia, 2009; B-Béres et al., 2022).

Ecosystem Services of Diatoms

Supporting Services

Diatoms were essential to the oxygenation of the Earth and today are responsible for up to 40% of global primary production, serving as the base of aquatic food webs. 40% of total oceanic carbon sequestration is due to the sedimentation of diatoms, making them essential for transporting nutrients from pelagic to benthic zones and for the biological carbon pump. Diatoms, especially freshwater species, also control the biogenic cycling of silica. In addition to providing nutrients to zooplankton, diatoms are used as food additives in the aquaculture industry and serve as habitats for other organisms like nitrogen-fixing cyanobacteria. According to Dudek et al. (2020), diatoms may be an important habitat and recruiter for hydrocarbon-degrading bacteria in plastispheres (B-Béres et al., 2022; Fu et al., 2022; Virginia, 2009).

Regulating Services

The sequestration of carbon by diatoms is crucial for climate regulation. By storing nitrogen and removing heavy metals in wastewater, diatoms help prevent potentially toxic cyanobacteria blooms in freshwaters overburdened with surplus nutrients from human activities. In addition, biofilms that diatoms form protect other organisms and 'glue' together sediment particles, reducing erosion (B-Béres et al., 2022).

Provisioning Services

Diatoms produce several immunostimulants like polyphenols and carotenoids that are used for antibiotic applications and disease treatment. Their silica cell wall is also a model for nanotechnology and drug-delivery vehicles, and diatoms are heavily involved in genetic engineering studies. In the Southern Ocean, proliferation of marine diatoms corresponds with 10% of the world's supply of oil and gas, indicating sites of economic potential. Diatoms' lipid content is ideal for biofuel production, and diatomaceous earth had an estimated processed value of $260 million in 2020 (B-Béres et al., 2022).

Cultural Services

According to B-Béres et al. (2022), the "photo documentation of diatoms revolutionized the connection of science and art." Because of these organisms' aesthetic value, exhibitions are an appealing way to educate the public about diatoms and related topics. Diatoms are also ideal "indicator" organisms for ecosystem health, as they respond sensitively to environmental changes. Currently, diatom-based reconstruction of climate impacts is one of the few ways to understand past climate events and predict future ones.

Negative Effects

Diatoms can also impact ecosystems negatively. Massive algae blooms and the transport of invasive species can lead to decreases in biodiversity at the entire ecosystem level. Diatoms' rapid proliferation may exclude native species and threaten aquaculture, fisheries, tourist activities, municipal water systems, and human health. Importantly, "many of the above-mentioned negative impacts of diatoms are the consequences of anthropogenic activities," underlining the importance of utilizing diatoms as ecosystem indicators in an era of environmental pollution and anthropogenic climate change (B-Béres et al., 2022).

The impacts of human activity on diatom biodiversity, which is essential to ecosystem services and human well-being, are severely underrepresented. One of these influential activities is the presence of plastic pollution in aquatic environments. B-Béres et al. (2022) notes that for "diatoms, protection of individual species is impossible: only habitat protection may preserve them." Therefore, it is essential to understand how aquatic plastic pollution affects diatom populations and habitats. The supporting, regulating, provisioning, and cultural services provided by diatoms can then be used as links between scientists, policymakers, and the public to improve the natural environment.

Knowledge Gaps

Previous studies used SEM, 16S and 18S rRNA profiling, and microfluidics to study the composition of diatoms in plastispheres (Dey et al., 2022). Dudek et al. (2020) found no significant difference in eukaryotic communities among different plastic types but observed that Raphid and Araphid diatoms were most abundant in initial biofilms and decreased over time on all plastic types. Some diatoms were found on all plastics, but some exhibited polymer preference. Eich et al. (2015) and Lobelle et al. (2011) reported a significant increase in biofilm amount on all plastic samples over time, and Kirstein et al. (2019) found that microbial communities on marine plastic debris differ from the surrounding seawater. Kirstein et al. (2019) also observed no significant difference in short-term biofilms among plastic types but saw a significant difference in mature 15-month biofilms.

The first knowledge gap this study addresses is the general lack of microbial plastisphere research. Previous studies note that "the composition of the biofilm community and its activity … remains to be investigated …" (Eich et al., 2015), that "very little is known about the communities of microbes that develop on [plastic marine debris] …" (Amaral-Zettler et al.,

2015), and that “studies of plastic-associated microbial communities are lacking” (Zettler et al., 2013).

Secondly, although plastic pollution increasingly threatens freshwater ecosystems, “the consequences of plastics in freshwaters remain poorly known” (Azevedo-Santos et al., 2021). The literature search for this study found no previous freshwater plastisphere studies to draw upon. Freshwaters are often the main destination for pollutants released in a watershed and frequently transport plastics from inland to marine environments: lotic ecosystems are responsible for inputting over one million tons of plastic into marine ecosystems, and over 150 freshwater fish species have ingested plastics. Therefore, the freshwater knowledge gap is an important one to fill.

Lastly, Amaral-Zettler et al. (2015) found that plastispheres tend to reflect their local surroundings more than their origins. Eich et al. (2015) and Dudek et al. (2020) agree that the structure of the plastisphere is heavily dependent on geography. While it is still controversial whether plastispheres vary by geographic location – Du et al. (2022) found that the stress tolerance of microplastics may counteract geographical variation – it is important to consider locational influence on the plastisphere. To the author’s knowledge, this is the first plastisphere study conducted in the Pacific Northwest of the United States.

Methods

This study was modeled after Eich et al.’s 2015 paper *Biofilm and Diatom Succession on Polyethylene (PE) and Bioedgradable Plastic Bags in Two Marine Habitats: Early Signs of Degradation in the Pelagic and Benthic Zone?*, a similar study investigating the effect of water depth on plastisphere formation on polyethylene and biodegradable plastic in the Mediterranean Sea.

Experiment Installation

PET and PP are the third and second most wasted plastic types, respectively, and these two packaging plastics are commonly found in freshwaters (Geyer et al., 2017). Thus, PET and PP were selected as test subjects. PET was sourced from clear Ziploc bags and PP from clear binder pockets. After cutting into 1.5 inch squares (Fig. 3a), 20 PET squares were stapled into each of six wire-reinforced mesh polyester bags and 20 PP squares were stapled into another six identical bags.

Fig. 3a

PET and PP Squares

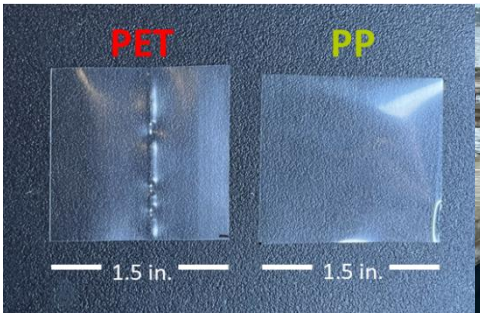


Fig. 3b

Installed Experiment at Settler's Landing

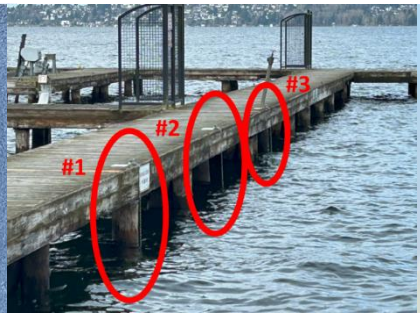


Fig. 3c

Location of Settler's Landing (City of Kirkland Parks Department, 2018)

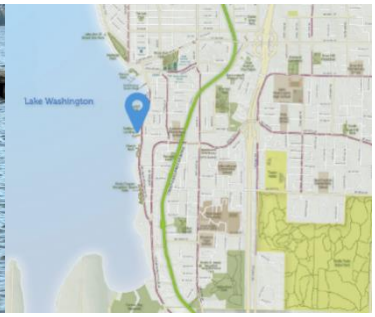


Fig. 3d

A Sample Group of Two PET and Two PP Bags Chained to a Dock Cleat

Fig. 3e

Sample Group Submerged at a 1 Meter Water Depth

Bags were randomly organized into three sample groups, with each group containing two PET bags and two PP bags for a total of 40 squares of PET and 40 squares of PP per sample group (Fig. 3d). After washing all bags with 91% isopropyl alcohol, each group was secured to a chain using a carabiner and duct tape (Fig. 3b).

With the appropriate permissions from various city, county, and state departments, this experiment was installed at Settler's Landing, a small City of Kirkland dock on Lake Washington in the Puget Sound (Fig. 3c). Settler's Landing was the ideal location due to its low popularity, water depth, and the presence of dock cleats.

Since diatoms reach maximum biomass in the spring, starting in March 2024, each sample group was locked to a dock cleat and submerged at a 1 meter water depth, simulating plastic debris floating in the water column (Fig. 3e). A 1 meter depth was chosen to ensure the plastics remained in the sunlit pelagic zone, which diatoms tend to inhabit. The average water temperature during exposure time was 10.187 ± 0.34671 °Celsius. Additionally, 1.8 liters of freshwater were sterilized using a vacuum filter with a pore size of 0.22 μm for use in lab protocols. The cells filtered out of this water were used to characterize diatoms in the water column (B-Béres et al., 2022; Dey et al., 2022).

Sampling

According to Dey et al. (2022), a stable plastisphere may be achieved in a few weeks, so sample groups were exposed for 3, 4, and 5 weeks. After retrieval, sample bags were submerged in unfiltered freshwater for transportation. All lab analysis was conducted within 72 hours of retrieval. For each plastic type, three sample squares were selected from both bags using a simple random sampling (SRS) method and submerged gently in sterile freshwater to remove loose biofilm. From those six selected plastics, another SRS was used to assign three squares to a

biofilm quantification assay and the other three to a diatom characterization assay. At this point, a modification to the original method was added: three 1.5 inch squares were also sampled from the polyester bags (PLYS) for diatom characterization.

Fig. 4a

CV PET and PP Samples Stained with Crystal Violet

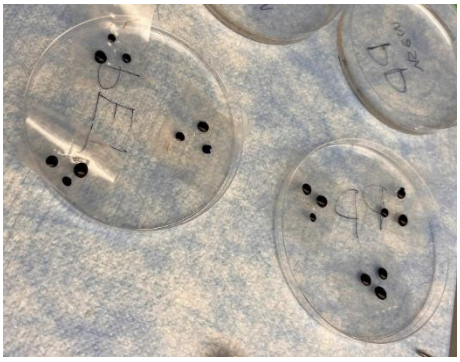
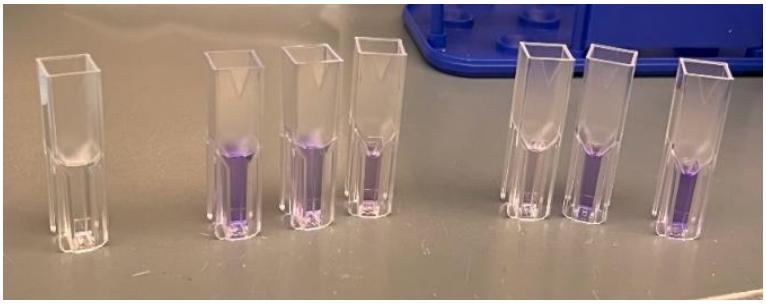


Fig. 4b

Stained Ethanol in Cuvettes



Biofilm Quantification: Crystal Violet Assay (CV)

The total amount of biofilm growth (richness) per plastic sample was quantified using a crystal violet assay modelled after Lobelle and Cunliffe (2011). Plastics were air-dried for 45 minutes at room temperature and then stained with three drops of crystal violet (1% w/v) using a Pasteur pipette (Fig. 4a). After air-drying again for 45 minutes, stained samples were washed with 5 mL of sterile freshwater (SFW) and air-dried for another 45 minutes. Each plastic square was then inserted carefully into a 15 mL Falcon tube with 1 mL ethanol (95% v/v), taking care not to crumple the plastic, and vortexed to destain the plastic. The 1 mL of ethanol was then transferred to a cuvette (Fig. 4b). After blanking with ethanol, optical density was measured at 595 nm.

Diatom Characterization (DC)

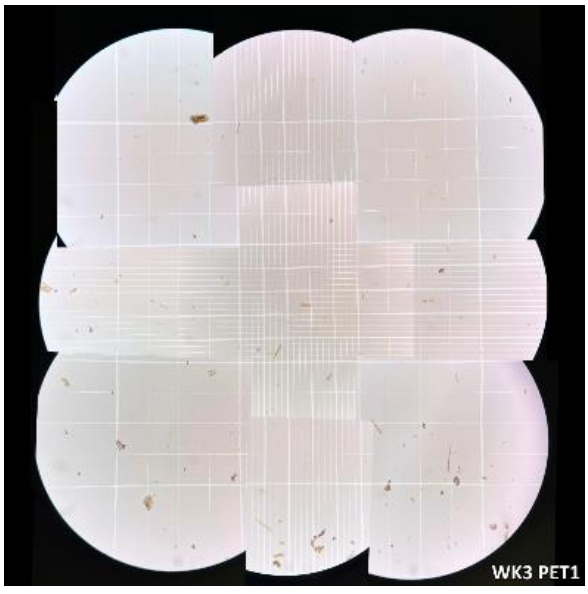
Since, according to Fu et al. (2022), traditional classification of diatoms is based exclusively on morphological characteristics of the frustule, a light microscopy assay was used to characterize diatoms. Although Davidov et al. (2020) advocates for genetic sequencing of the 16S and 18S rRNA genes as a more precise, accurate, and widely used method, light microscopy was selected to abide by financial and temporal limitations. Each plastic square was inserted into a 50 mL Falcon tube with 30 mL of SFW and vortexed for 30 seconds to dislodge plastic biofilms (Fig. 5a). Samples were centrifuged at 4,000 rpm for 15 minutes and decanted. A

Fig. 5a

PLYS in 30 mL of SFW

Fig. 5b

10 µl of Sample WK3 PET1 Loaded into a Hemocytometer at 100x Magnification



milliliter of SFW was then added, the biofilm pellet was resuspended, and then transferred to a microcentrifuge tube. This was centrifuged at 10,000 rpm for 5 minutes. 800 µl were decanted before mixing the sample and storing at 4 °Celsius until ready for microscopical analysis. When

ready, 10 µl were loaded into a hemocytometer and viewed at 100x and 400x magnification (Fig. 5b). New diatom morphologies, when observed, were photographed. Analysis was performed for the entire slide.

Analysis

Diatoms were classified into 42 distinct morphological groups, with G42 representing unidentifiable but recurring morphologies (Fig. 6). Photographs for each plastic sample were reviewed and the presence of morphological groups was recorded. Using the Diatoms of North America database, the 41 identifiable morphological groups were sorted into nine morphological categories (Table 1). Potential genera were then identified for each group. Microsoft Excel was used to construct heat maps, graphs, and data charts, and the coding language R was used to conduct crystal violet t-tests. DC data was used to analyze morphological group overlap and diversity. Diversity was defined as the percentage of morphological groups found on all triplicates of a sample out of all morphological groups or out of morphological groups per category.

Fig. 6

Visual Key of 42 Identifiable Morphological Groups

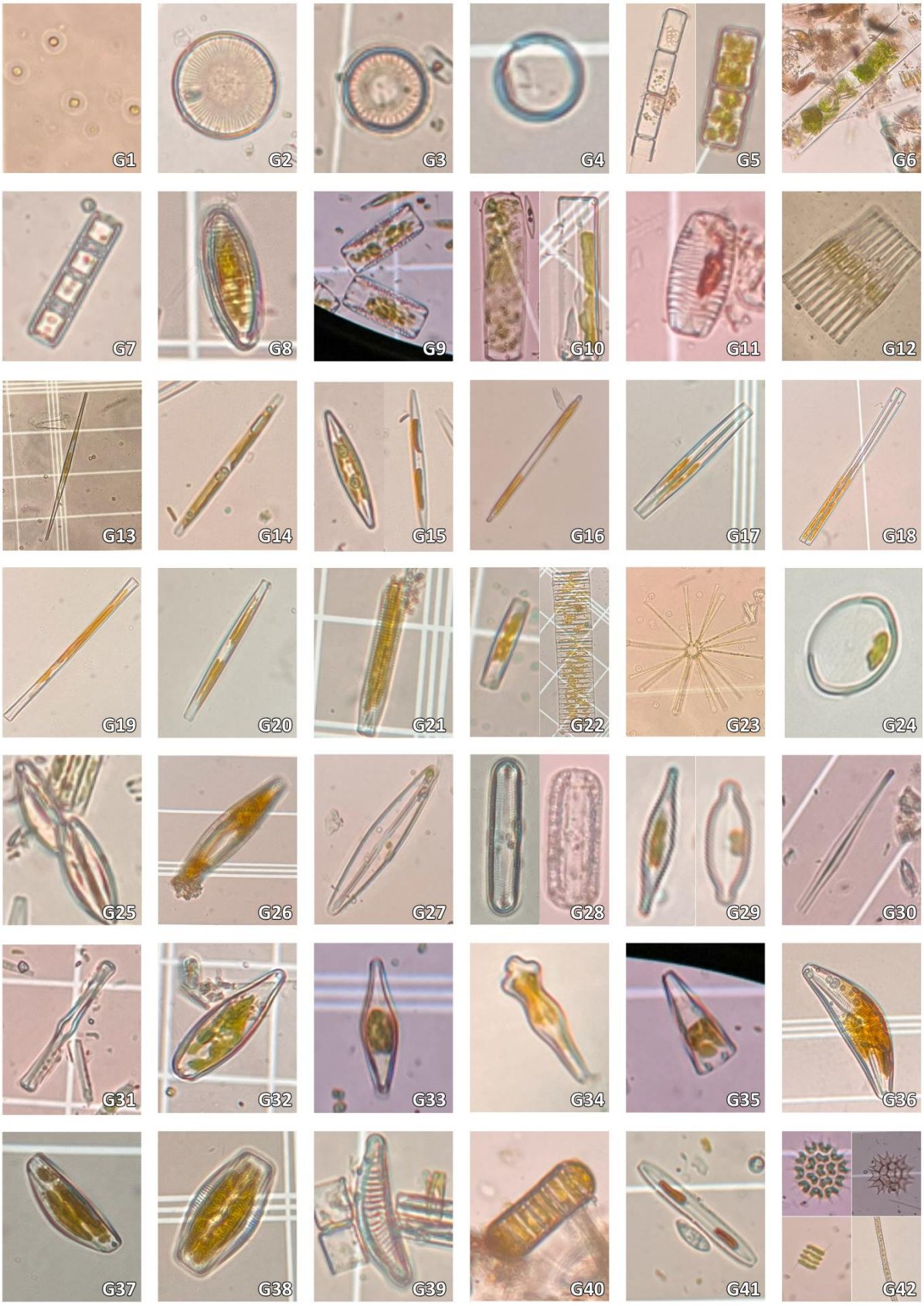


Table 1

Diatom Groups Based on Morphological Features

Diatom Groups Based on Morphological Features			
Group	**Category**	**Morphology**	**Genus**
G1	Centric	Small, circular/rectangular frustule.	
G2	Centric	Circular frustule with radial striae organized into bundles. Spines on valve margin.	*Stephanodiscus* spp.?
G3	Centric	Circular frustule with radial striae. May have aerolae in center.	*Stephanocyclus* spp.? *Lindavia* spp.? *Cyclotella* spp.?
G4	Centric	Circular frustule. No visible striae.	*Stephanocyclus* spp.? *Lindavia* spp.? *Cyclotella* spp.?
G5	Centric	Rectangular frustules. May grow in long colonies linked at valve face.	*Melosira* spp.?
G6	Centric	Rectangular frustule, diagonal symmetry. Mantle edge is recurve. Long external tubes at mantle edge.	*Trieres* spp.? *Guinardia* spp.?
G7	Centric	Rectangular frustule. May grow in colonies.	*Aulacoseira* spp.?
G8	Araphid	Ovoid frustule with transapical striae. May grow in chain colonies.	*Pseudostaurosira* spp.?
G9	Araphid	Rectangular frustule. Ribs with along both longitudinal sides.	*Diatoma* spp.?
G10	Araphid	Rectangular frustule.	*Diatoma* spp.?
G11	Araphid	Rectangular frustule that swells in center. Transapical striae.	*Diatoma* spp.?
G12	Araphid	Frustule narrows to slender point. Cells form band-shaped colonies.	*Fragilaria* spp.? *Tabellaria* spp.? *Microtabella* spp.?
G13	Araphid	Frustule gradually tapers to a slender point. Fusiform.	*Fragilaria* spp.? *Ulnaria* spp.?
G14	Araphid	Slender, rectangular frustule with transapical chloroplasts.	*Nitzschia* spp.? *Synedra* spp.?
G15	Araphid	Pointed ellipsoidal frustule. Transapical or lateral chloroplasts.	*Synedra* spp.?
G16	Araphid	Ellipsoidal frustule. Fusiform.	*Synedra* spp.?
G17	Araphid	Rectangular frustule that swells in center. Longitudinal channel. Fusiform.	*Synedra* spp.? *Thalassionema* spp?
G18	Araphid	Rectangular frustule. Longitudinal channel. Fusiform.	*Synedra* spp.? *Thalassionema* spp?
G19	Araphid	Rectangular frustule. Fusiform.	*Synedra* spp.? *Thalassionema* spp?
G20	Araphid	Rectangular frustule. Fusiform.	*Synedra* spp.? *Thalassionema* spp?
G21	Araphid	Rectangular frustule with striae extending across central canal.	*Synedra* spp.?
G22	Araphid	Rectangular frustule. May grow in zig-zag chains.	*Synedra* spp.?
G23	Araphid	Forms stellate colonies, attached by mucilage pads at end. Symmetrical to apical axis.	*Asterionella* spp.?
G24	Symmetric Biraphid	Ovoid frustule with raphe, interrupted in center.	*Amicula* spp.?
G25	Symmetric Biraphid	Ovoid frustule with raphe.	*Caponea* spp.?
G26	Symmetric Biraphid	Ellipsoidal frustule, striae reach towards longitudinal canal (do not continue across entire valve).	*Biremis* spp.? *Pinnularia* spp.?
G27	Symmetric Biraphid	Ellipsoidal frustule, longitudinal ribs appear to have slits with a small central area. Forms a point at valve ends.	*Craticula* spp.? *Frustulia* spp.?
G28	Symmetric Biraphid	Oblong frustule with transapical striae. Longitudinal canals around raphe.	*Muelleria* spp.? *Neidiopsis* spp.? *Pinnularia* spp.?
G29	Symmetric Biraphid	Ellipsoidal frustule coming to an abrupt point.	*Navicula* spp.? *Kobayasiella* spp.? *Mastogloia* spp?
G30	Symmetric Biraphid	Frustule comes to a slender point. Fusiform.	
G31	Symmetric Biraphid	Pennate diatom. Swells in center.	
G32	Asymmetric Biraphid	Rounded frustule tapering to a point on one end.	*Gomphonema* spp.?
G33	Asymmetric Biraphid	Rounded frustule tapering gradually to a point on both ends.	*Gomphonema* spp.?
G34	Asymmetric Biraphid	Pennate diatom, frustule swells at center, headpole is broad and ends abruptly in a point.	*Gomphonema* spp.?
G35	Asymmetric Biraphid	Triangular frustule.	*Rhoicosphenia* spp.?
G36	Asymmetric Biraphid	Frustule is wedge shaped, striae extend to raphe.	*Amphora* spp.? *Cymbella* spp.?
G37	Asymmetric Biraphid	Frustule is wedge shaped, striae extend to central canal(s).	*Amphora* spp.? *Cymbella* spp.?
G38	Asymmetric Biraphid	Ellipsoidal frustule with interrupting central canal.	*Amphora* spp.? *Cymbella* spp.?
G39	Asymmetric Biraphid	Crescent frustule with striae.	
G40	Nitzschioid	Oblong frustule with transapical striae.	*Denticula* spp.?
G41	Nitzschioid	Pennate diatom, frustule narrows towards center. Has a raphe, transapical chloroplasts.	*Tryblionella* spp.?
G42	Other	These organisms appeared multiple times among samples but were unidentifiable.	

Results

Biofilm Quantification

The crystal violet assay returned extremely variable results with outliers for control values, PET, and PP (Table 2). Because of this variability, comparisons between the two plastic types and conclusions about total biofilm richness per sample cannot be drawn. T-tests comparing biofilm amounts for each plastic over time produced p-values much greater than the tested alpha value ($\alpha = 0.05$) (Fig. 7a, Fig. 7b). Therefore, there is no convincing statistical evidence from this method that the biofilm amounts on PET and PP changed significantly over time.

Table 2

All CV Values Recorded for PET and PP

Crystal Violet Biofilm Quantification: Optical Density at 595 nm												
Week	**Date**	**Days**	**PET1**	**PET2**	**PET3**	**PET Mean**	**PET SD**	**PP1**	**PP2**	**PP3**	**PP Mean**	**PP SD**
0	3/20/2024	0 (Control)	0.8	0.41	N/A	N/A	N/A	0.25	0.24	N/A	N/A	N/A
3	3/25/2024	21	0.29	0.12	6.6	2.3366667	3.693133	0.12	0.16	0.31	0.196667	0.100167
4	4/1/2024	28	0.25	2.2	0.3	0.9166667	1.11168	0.08	0.37	0.17	0.206667	0.148436
5	4/8/2024	35	4.8	3.4	0.33	2.8433333	2.286402	0.18	0.51	0.67	0.453333	0.249867

Fig. 7a

P-values for PET CV Data

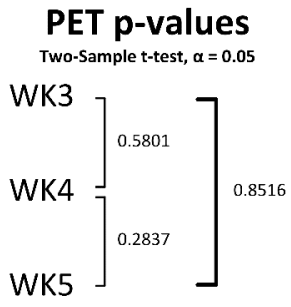


Fig. 7b

P-values for PP CV Data

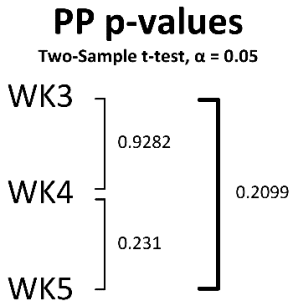
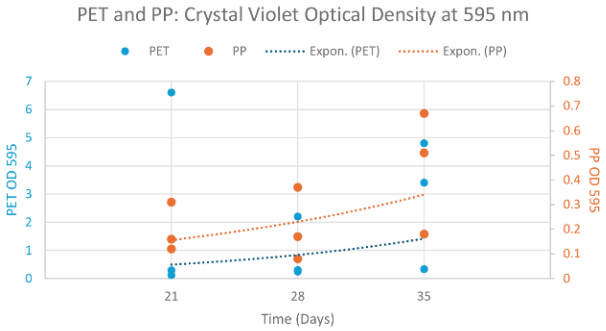


Fig. 8

PET and PP: Crystal Violet Optical Density at 595 nm



However, when graphed, both plastic types show increases in growth despite the variability of sample values (Fig. 8). Additionally, anecdotal visual evidence of plastic squares (Fig. 9a), as well as the PLYS bags (Fig. 9b), shows an increase in biofouling over the weeks. Thus, we assume biofilm amount increased over time on all three plastic types.

Fig. 9a

Biofouling of PET, PP, and PLYS Across all 3 Weeks

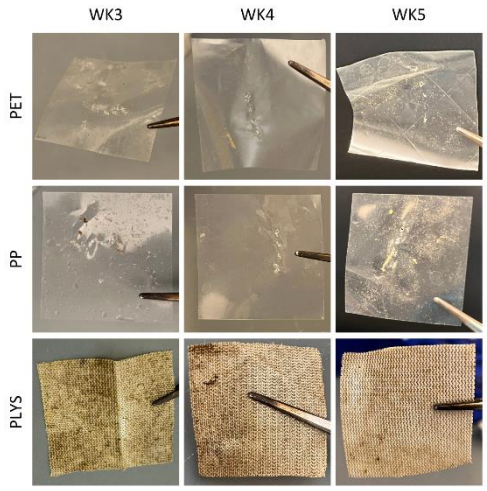


Fig. 9b

Biofouling of Sample Bag Groups

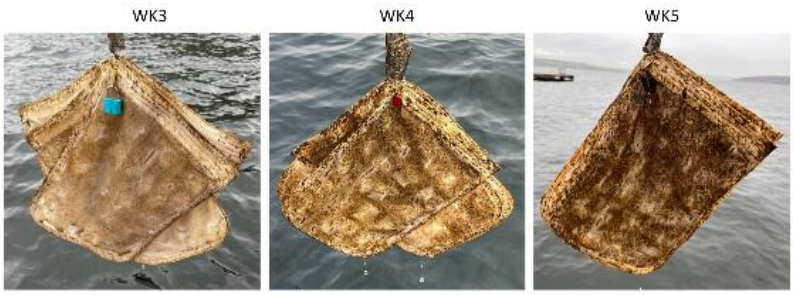


Fig. 10a

Triplicate Frequency of Diatom Morphologies by Time

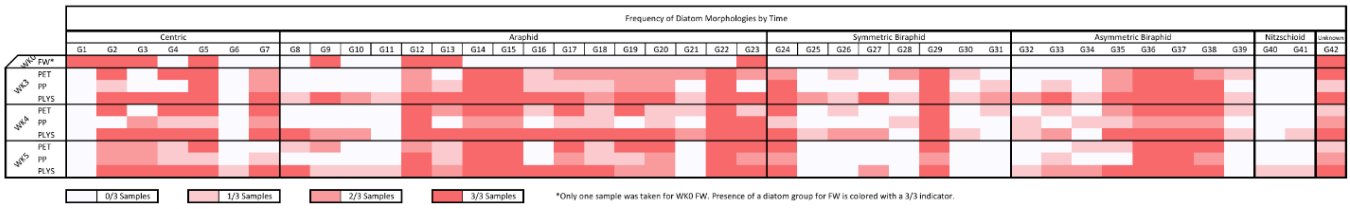


Fig. 10b

Triplicate Frequency of Diatom Morphologies by Plastic Type

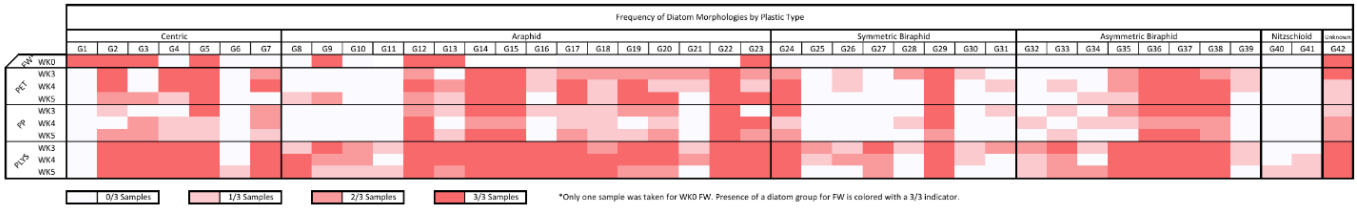


Table 3a

Diatom Diversity by Time

		Diatom Diversity by Time																		
		Centric			Araphid			Symmetric Biraphid			Asymmetric Biraphid			Nitzschioid			Total		
		2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %
WK0	FW*	N/A	4	57.14%	N/A	4	25.00%	N/A	0	0.00%	N/A	0	0.00%	N/A	0	0.00%	N/A	8	19.51%
WK3	PET	1	3	42.86%	7	3	25.00%	2	1	12.50%	2	2	25.00%	0	0	0.00%	12	9	21.95%
	PP	1	1	14.29%	3	3	18.75%	0	2	25.00%	1	3	37.50%	0	0	0.00%	5	9	21.95%
	PLYS	0	5	71.43%	2	11	68.75%	2	3	37.50%	1	5	62.50%	0	0	0.00%	5	24	58.54%
WK4	PET	4	0	0.00%	1	6	37.50%	0	2	25.00%	1	3	37.50%	0	0	0.00%	6	11	26.83%
	PP	2	0	0.00%	2	3	18.75%	0	1	12.50%	3	0	0.00%	0	0	0.00%	7	4	9.76%
	PLYS	0	5	71.43%	3	12	75.00%	2	2	25.00%	1	4	50.00%	0	0	0.00%	6	23	56.10%
WK5	PET	2	1	14.29%	2	7	43.75%	0	2	25.00%	0	3	37.50%	0	0	0.00%	4	13	31.71%
	PP	2	0	0.00%	3	4	25.00%	2	0	0.00%	1	2	25.00%	0	0	0.00%	8	6	14.63%
	PLYS	0	5	71.43%	3	9	56.25%	1	2	25.00%	2	4	50.00%	0	0	0.00%	6	20	48.78%

*Only one sample was taken for WK0 FW. Presence of a diatom group for FW is included in the 3/3 column.

Table 3b

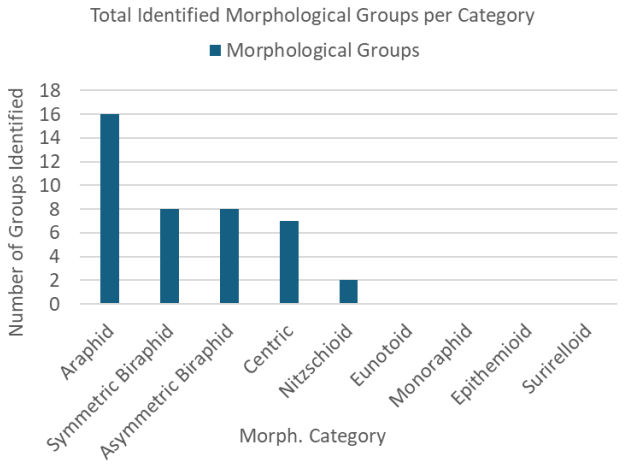
Diatom Diversity by Plastic Type

		Diatom Diversity by Plastic Type																		
		Centric			Araphid			Symmetric Biraphid			Asymmetric Biraphid			Nitzschioid			Total		
		2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %	2/3	3/3	(3/3)/(Total) %
FW	WK0	N/A	4	57.14%	N/A	4	25.00%	N/A	0	0.00%	N/A	0	0.00%	N/A	0	0.00%	N/A	8	19.51%
PET	WK3	1	3	42.86%	7	3	25.00%	2	1	12.50%	2	2	25.00%	0	0	0.00%	12	9	21.95%
	WK4	4	0	0.00%	1	6	37.50%	0	2	25.00%	1	3	37.50%	0	0	0.00%	6	11	26.83%
	WK5	2	1	14.29%	2	7	43.75%	0	2	25.00%	0	3	37.50%	0	0	0.00%	4	13	31.71%
PP	WK3	1	1	14.29%	3	3	18.75%	0	2	25.00%	1	3	37.50%	0	0	0.00%	5	9	21.95%
	WK4	2	0	0.00%	2	3	18.75%	0	1	12.50%	3	0	0.00%	0	0	0.00%	7	4	9.76%
	WK5	2	0	0.00%	3	4	25.00%	2	0	0.00%	1	2	25.00%	0	0	0.00%	8	6	14.63%
PLYS	WK3	0	5	71.43%	2	11	68.75%	2	3	37.50%	1	5	62.50%	0	0	0.00%	5	24	58.54%
	WK4	0	5	71.43%	3	12	75.00%	2	2	25.00%	1	4	50.00%	0	0	0.00%	6	23	56.10%
	WK5	0	5	71.43%	3	9	56.25%	1	2	25.00%	2	4	50.00%	0	0	0.00%	6	20	48.78%

*Only one sample was taken for WK0 FW. Presence of a diatom group for FW is included in the 3/3 column.

Fig. 11

Total Identified Morphological Groups per Category



Diatom Characterization

In total, 1,849 microscope images were analyzed. Five out of the nine morphological categories recognized by the Diatoms of North America database were identified in this study. Of those five categories, Araphid (with 16 groups), Symmetric Biraphid (with eight groups), and Asymmetric Biraphid (with eight groups) were the most diverse categories (Fig. 11).

The freshwater sample (FW) contained only eight of the 41 identifiable morphological groups, making it the least diverse sample in this study. Four morphological groups were

recorded each for the Centric and Araphid categories, making Centric the most diverse FW category (57.14%). Symmetric Biraphid, Asymmetric Biraphid, and Nitzschioid diatoms, the remaining categories from the five identified in this study, were not present. Notably, G1 is the only group that appeared exclusively on FW (Fig. 10a, Fig. 10b).

Regarding total diatom diversity, PLYS, PET, and PP consistently had the greatest to least measures across all three sample times: PLYS always had the greatest percentage of diatoms recorded out of the 41 identified groups and PP always had the least percentage of diatoms recorded (Table 3a). Every morphological group except for G1 was recorded on PLYS at some point over the course of the study. For PP and PLYS, total diatom diversity generally decreased over time, meaning that WK4 and WK5 diversity was always less than or equal to WK3 diversity (Table 3b). On the contrary, PET diversity increased over time.

Like total diatom diversity, PLYS, PET, and PP consistently had the greatest to least diatom diversity per morphological category (Table 3a). Araphid was the most diverse category and the category with the most morphological groups identified for PET and PP (Table 3b). While Centric was the most diverse category identified for PLYS, Araphid also had the largest number of morphological groups identified for this plastic type. There was no clear increasing or decreasing trend of diversity within morphological categories across time for any of the plastics. PLYS recorded significantly more 3/3 attachments than 2/3 attachments compared to PET and PP. Additionally, G11, G25, G27, and Nitzschioid (G40, G41) diatoms only appeared on PLYS and rarely in a 3/3 capacity.

Venn diagrams were constructed using 3/3 data points to compare the distribution of morphological groups across FW, PET, PP, and PLYS samples for each morphological category. Diatom groups that only appeared in a 0/3, 1/3, or 2/3 capacity are included outside the Venn

Fig. 12a

Overlapping Centric Morphological Groups

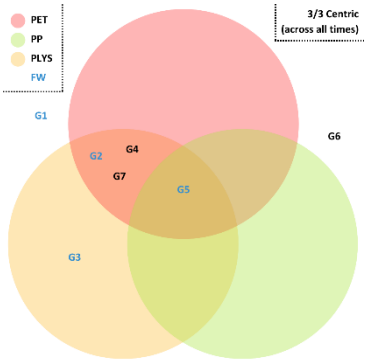


Fig. 12b

Overlapping Araphid Morphological Groups

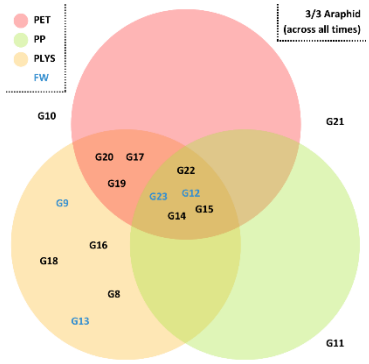


Fig. 12c

Overlapping Symmetric Biraphid Morphological Groups

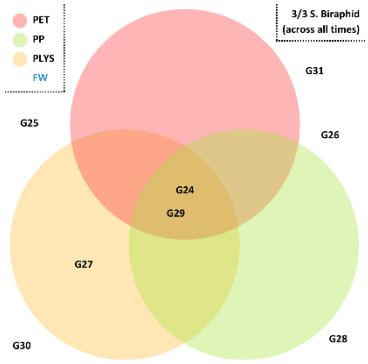


Fig. 12d

Overlapping Asymmetric Biraphid Morphological Groups

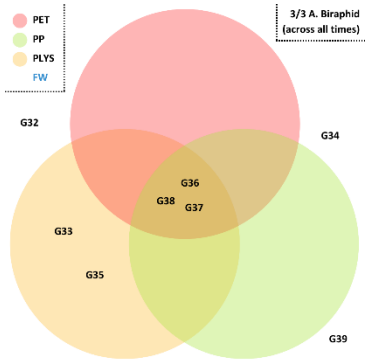


Fig. 12e

Overlapping Nitzschioid Morphological Groups

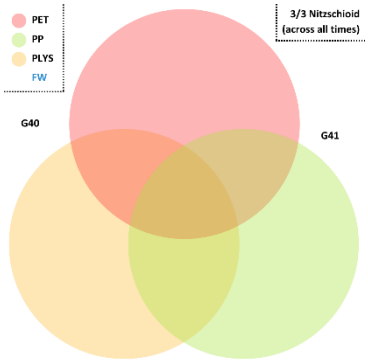


diagram. In every morphological category, no groups were found solely on PET or PP, and no groups overlapped on PET and PP or PP and PLYS. All morphological groups belonged to one of the following areas: solely on PLYS, overlapping on PET and PLYS, or overlapping on PET, PP, and PLYS. Diatoms found in the FW sample were distributed across these three areas.

The following diatom groups appeared on all plastic types at all time points in either a 2/3 or 3/3 capacity:

Araphid

- G12: *Fragilaria* spp.? *Tabellaria* spp.? *Microtabella* spp.?;
- G14: *Nitzschia* spp.? *Synedra* spp.?;
- G15: *Synedra* spp.?;
- G22: *Synedra* spp.?

Symmetric Biraphid

- G29: *Navicula* spp.? *Kobayasiella* spp.? *Mastogloia* spp?

Asymmetric Biraphid

- G36: *Amphora* spp.? *Cymbella* spp.?;
- G37: *Amphora* spp.? *Cymbella* spp.?;
- G38: *Amphora* spp.? *Cymbella* spp.?

Analysis

Biofilm Quantification

The crystal violet assay proved too imprecise to develop claims related to biofilm richness on each plastic. Variability in this assay may be decreased by testing a larger number of samples. The increase of biofouling over time for all three plastic types implies that plastic particles may sink out of the sunlit pelagic zone into benthic sediments over time, since biofouling increases plastic density. Eich et al. (2015) corroborates this, reporting that about 70% of all plastic particles in the sea sink to the floor because of biofouling. Additionally, biofouling can lead to the fragmentation of larger plastic items into microplastics. Plastic particles buried in benthic sediments are more difficult to study and, undisturbed, develop mature biofilm that may have different environmental impacts compared to plastics in the pelagic zone (Du et al., 2022).

Diatom Characterization

The absence of Eunotioid, Monoraphid, Epithemioid, and Surirelloid diatoms, and the lack of 3/3 data points in the Nitzschioid category, suggests that these morphological categories may not attach to initial plastisphere communities. It is possible they only appear in mature biofilms or do not colonize the plastisphere at all. Araphid and Biraphid categories containing the most morphological groups directly supports Dudek et al. (2020), which reports these two groups are the most found plastisphere diatoms. This finding is an example of a similarity between freshwater and marine plastispheres.

Despite being identified on the three plastic types, Symmetric Biraphid, Asymmetric Biraphid, and Nitzschioid diatoms were absent from the FW sample, indicating that they may only be benthic colonizers. G1 may be a purely planktonic diatom, as it was only found in FW. Centric and Araphid diatoms are observed to grow in both the FW and plastic samples, establishing evidence for planktonic taxa in freshwater plastispheres (Zettler et al., 2013).

The order of PLYS, PET, and PP for greatest to least total diversity reflects the order of most to least textured plastics used in this study. This indicates that texture, a variable not considered in the initial hypothesis, may play an important role in total diatom diversity. Eich et al. (2015) supports this, finding that “a kind of specialization [seems] to have taken place probably due to the small-scale differences in the plastic surface structure.” Total diatom diversity decreasing over time for PP and PLYS indicates that time may be a selecting variable for diatom diversity, leading to a more specific community. This shift to lower community diversity demonstrates that general organisms are gradually outcompeted through high nutrient demand and grazing pressure, leaving plastic-specific microbes behind. In support, Dudek et al. (2020) reports that the clustering of eukaryotic plastispheres apart from the water column

occurred because of time more than of plastic type. PET diversity increased over time. One possible explanation for this may be a longer initial biofilm-forming stage specific to PET (Kirstein et al., 2019; Zettler et al., 2013).

While PLYS varied from PET and PP in that Centric was the most diverse category (instead of Araphid), there does not appear to be a significant difference in which morphological category attaches to which plastic category, since Araphid dominated all three plastics as the category with the most morphological groups identified. The greater proportion of 3/3 data for PLYS indicates there is a stronger relationship between PLYS and the organisms that attach. However, the greater diversity of Centric diatoms and this stronger attachment on PLYS may be related more to the confounding variable of texture rather than the plastic type. Similarly, the presence of morphological groups only on PLYS in a 1/3 or 2/3 capacity may be due to the mesh texture of the plastic catching diatoms floating in the water column.

The presence of diatoms appearing on all plastics at all time points may be evidence for a core group of diatoms attracted to all plastic substrates. Kirstein et al. (2019) found that such a biofilm core is substrate unspecific. This indicates that plastic "specific" microorganisms are represented by rarer species. Further justifying this claim, a previous study by Zettler et al. (2013) found that PE and PP samples shared 30-40% of their original taxonomic units. In addition, four of the 10 potential genera identified (*Navicula*, *Nitzschia*, *Mastogloia*, *Amphora*) are known to be widely found in the plastisphere (Du et al., 2022).

The differences in diatoms among the FW and plastic samples, as well as the evidence for a core group of substrate-unspecific diatoms suggests that plastic waste entering freshwater ecosystems provides a new ecological niche for benthic, core-biofilm diatoms. Adding to this, the CV results indicate that biofilm richness increases over time and the DC results show the

community composition, in terms of diversity, changing over time. Considering plastics last in aquatic environments for hundreds to thousands of years, these findings imply that the effect of the plastisphere on aquatic ecosystems evolves over time (Kirstein et al., 2019).

Conclusion

Implications

While ecological implications of the plastisphere are poorly understood, ecosystem balances, plastic degradation, biogeochemical cycles, and climate change are important areas of impact. As evidenced by the differences in planktonic and benthic diatoms, plastics provide new, unexpected opportunities for certain species to proliferate and spread. Biofilms are also known hotspots for horizontal gene transfer, leading to rapid acquisition of new traits that may enable species to dominate. These highly variable populations may introduce invasive species to ecosystems, decimating biodiversity. Potential pathogens like *Vibrio* bacteria may concentrate in diatom plastisphere communities and spread (Zettler et al., 2013; Amaral-Zettler et al., 2015; Bamford et al., 2023).

The biofouling and resulting sinking of plastics in freshwater ecosystems removes plastics from areas where photo-oxidative degradation is possible, leading to lower rates of biodegradation. Considering the impracticality of removing all plastics from aquatic environments, microbial biodegradation is an important mitigation for plastic pollution. However, mature biofilms may provide habitats for microbes specialized in plastic biodegradation. The beginnings of this specialized habitat were seen with the observed decrease in total diatom diversity (Andrady, 2011; Du et al., 2022).

As plastisphere diatoms are transported on floating plastics, their metabolic processes may change in different environments. Shifts in diatom carbon, nitrogen, and silicon composition

may affect important biogeochemical cycles like the global carbon pump, which is essential for regulating anthropogenic climate change. These reductions in carbon dioxide sequestration will exacerbate climate change, which in turn will favor smaller phytoplankton and harm diatom populations (Fu et al., 2022; Virginia, 2009; B-Béres et al., 2022).

Limitations

As a quasi-experiment, a general lack of control over variables like water temperature, salinity, pH, plastic texture, and surface area, which are known to affect microbial adhesion, prevented this study from drawing causal conclusions. The short nature of this study also prevented study of more mature biofilms, which are reported to differ in microbial composition starting after 6 weeks (Dey et al., 2022; Kirstein et al., 2019).

The variability of the CV assay may be due to using an imprecise Pasteur pipette to measure crystal violet drops and a standard 5 mL of water to wash the samples. The varied texture of the PET plastic may have led the crystal violet to stain some samples more than others.

For the DC method, recording cell counts of each morphological group was originally intended. However, the DC method was too imprecise to standardize across samples for accurate cell counts. Kirstein et al. (2019) finds that plastic-specific microbes attach strongly to substrates. The vortexing used in this method may not have separated plastic-specific microbes, and therefore they may be missing from the data collected. Lastly, the low-resolution quality of microscope images and the author's inexperience with identification methods may have introduced bias when categorizing cells.

Next Steps

Important next steps include developing a more thorough biofilm quantification method and pressure-wash method to isolate plastic-specific microbes. Machine learning algorithms,

which are shown to classify diatom species with an accuracy rate of 95%+, or genetic sequencing technology may be used to provide a more comprehensive picture of the diatom plastisphere. Scanning-electron-microscopy is another precise method for morphological identification (Kirstein et al., 2019; Fu et al., 2022; Davidov et al., 2020).

To isolate the variable of plastic type, plastics may be incubated in freshwater samples in controlled lab setups to study microorganismal activity, including that of biodegradation. Extending plastisphere studies over a period of 12-15 months will provide an opportunity to compare the composition of young to mature biofilms and understand how the plastisphere's environmental impact evolves over time. This research should be conducted in diverse, real-world environments (Dey et al., 2022).

Considering that diatoms are an important link between science and art, spreading education about microbial ecology with diatoms as a representative of the plastisphere is another important step.

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