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RESEARCH ARTICLE

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Plastics select for distinct early colonizing microbial populations with reproducible traits across environmental gradients

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Abstract

Little is known about early plastic biofilm assemblage dynamics and successional changes over time. By incubating virgin microplastics along oceanic transects and comparing adhered microbial communities with those of naturally occurring plastic litter at the same locations, we constructed gene catalogues to contrast the metabolic differences between early and mature biofilm communities. Early colonization incubations were reproducibly dominated by Alteromonadaceae and harboured significantly higher proportions of genes associated with adhesion, biofilm formation, chemotaxis, hydrocarbon degradation and motility. Comparative genomic analyses among the Alteromonadaceae metagenome assembled genomes (MAGs) highlighted the importance of the mannose-sensitive hemagglutinin (MSHA) operon, recognized as a key factor for intestinal colonization, for early colonization of hydrophobic plastic surfaces. Synteny alignments of MSHA also demonstrated positive selection for *mshA* alleles across all MAGs, suggesting that *mshA* provides a competitive advantage for surface colonization and nutrient acquisition. Large-scale genomic characteristics of early colonizers varied little, despite environmental variability. Mature plastic biofilms were composed of predominantly Rhodobacteraceae and displayed significantly higher proportions of carbohydrate hydrolysis enzymes and genes for photosynthesis and secondary metabolism. Our metagenomic analyses provide insight into early biofilm formation on plastics in the ocean and how early colonizers self-assemble, compared to mature, phylogenetically and metabolically diverse biofilms.

INTRODUCTION

Plastic marine debris is now recognized as an urgent threat to global ecosystems and has become ubiquitous in the ocean. Approximately 5.25 trillion plastic fragments weighing over 250,000 tons are afloat on the ocean's surface (Eriksen et al., 2014), with microplastics (<5 mm) accounting for nearly 93% of the so-called 'global particle count'. Since 2010, estimates indicate that between 4.8 and 12.7 million metric tons, or

approximately 5% of annual plastic production is input to the global ocean each year (Jambeck et al., 2015) but could be as high as 11% of annual production, or 23 million metric tons (Borrelle et al., 2020). By the year 2050, the projected global plastic production is expected to surge to >1300 metric tons, nearly a four-fold increase from the current standard today (Bergmann et al., 2015), with limited infrastructure and policies in place to manage plastic waste.

Approximately 40%–80% of archaeal and bacterial cells on Earth reside in biofilms, and these substrate-associated biofilms provide the foundation for many biogeochemical processes (Flemming & Wuertz, 2019). Microplastic pollution presents a unique ecological niche in seawater, with the microbial community colonizing the surface of plastic, termed the 'plastisphere' (Zettler et al., 2013). Conservative estimates suggest that between 2.1×10^{21} and 3.4×10^{21} cells, or approximately between 1% and 5% of cells in the neuston layer alone colonize plastic globally and in biogeochemical terms, between 1496 and 11,416 tons of carbon biomass reside on marine plastics (Zhao et al., 2021). Many plastisphere studies have amplified hypervariable regions of the 16S rRNA gene for archaeal/bacterial diversity, fewer studies have profiled exclusively eukaryotic or fungal diversity using the 18S rRNA gene locus or the ITS2 marker, and only five studies have used shotgun metagenomics from plastic to determine the functional composition of epiplastic communities (Table S1). No metagenomic data are available for early colonization of oceanic plastic (<7 days), despite this being shown to be a critical time window that shapes the trajectory of communities on chitin particles (Datta et al., 2016). Given the pervasiveness of microplastics in the marine environment, more functional metabolic data are necessary to gain a deeper understanding of the ecological implications of plastic substrates in the ocean. At the same time, plastic pollution stands as a global and evolutionarily recent perturbation that offers the prospect to study microbial ecological and evolutionary adaptations in near real time.

Regardless of sequencing methodologies, almost all previous plastic incubation studies have moored plastic particles to fixed locations and exposed these particles to local environmental conditions. However, the true nature of the epiplastic communities is dynamic, spanning many environmental gradients, and therefore plastic represents a unique opportunity to study how metabolism and community membership varies on the same plastic particle across environmental gradients. Previous community relative abundance data (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2014, 2016, 2018) suggest that seasonal and biogeographical drivers facilitate the structuring of epiplastic communities, whereas other studies (Kesy et al., 2019; Oberbeckmann et al., 2018; Ogonowski et al., 2018; Song et al., 2022; Xu et al., 2019) suggest salinity and temperature as primary influences on microbial community structure. The physicochemical properties (e.g., chemical structure, hydrophobicity, surface roughness) of surfaces have also been shown to initially influence microbial colonization (Kettner et al., 2017; Oberbeckmann et al., 2018; Rummel et al., 2017), with adhesion generally occurring faster with increasing hydrophobicity (Bendinger et al., 1993; Bruinsma et al., 2001) and biofilm foundational properties structuring mature biofilms rather than substrate-specific properties (Datta et al., 2016; Oberbeckmann et al., 2014). While the possibility of a core set of functional traits and taxonomy being present in epiplastic communities has been discussed, many studies have used mature, equilibrated biofilms and none have studied early colonizing communities from a functional metabolic perspective. Therefore, the metabolic constraints for initial plastic colonization, as well as how these early colonizers impact secondary settlement on plastic remain an open-ended question.

Prior metagenomic studies of mature biofilms have hinted that there are advantageous gene sets for a plastic-associated lifestyle (Bhagwat et al., 2021; Bryant et al., 2016; Oberbeckmann et al., 2021), although few data exist on the beneficial gene sets used for colonizing the surface that plastic initially provides. While other studies have sampled DNA during the first week of colonization and from multiple time points to study community succession (De Tender et al., 2017; Dudek et al., 2020; Erni-Cassola et al., 2020; Kesy et al., 2019; Latva et al., 2022; Lee et al., 2008; Ogonowski et al., 2018; Pinto et al., 2019; Pollet et al., 2018), only small-subunit ribosomal RNA gene sequencing was used to provide community profiling information. Consequently, the functional metabolic potential (i.e., attachment mechanisms, chemotaxis, plastic hydrolysis genes) of early colonization of plastic remains poorly understood, although this information is critical for understanding general biofilm formation on hydrophobic surfaces. Though in different settings, the key themes of early colonization and biofilm formation on marine plastic surfaces may be applicable on plastics and other inanimate materials used in agriculture, biomedicine and food processing facilities, which have been shown to harbour human pathogens (Wißmann et al., 2021).

In this study, we address the questions: (1) What genomic traits do early colonizing microorganisms possess compared to mature biofilms? and (2) How do the genomic traits of early colonizing microorganisms vary across environmental gradients and substrate? To that end, we incubated virgin plastic particles along two latitudinal transects that were harvested incrementally underway to mimic an epiplastic community as it drifted through surface marine waters up the Eastern Seaboard (Figure 1). These plastic incubations were

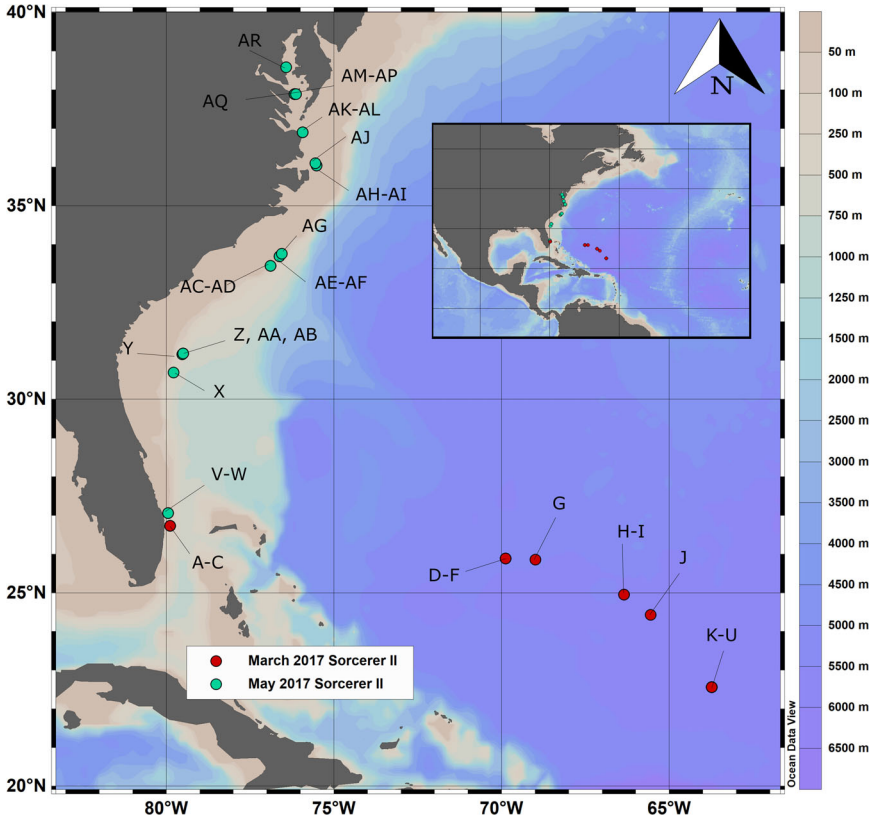

FIGURE 1 Map of sampling stations during the March 2017 (red shaded circles) and May 2017 (teal shaded circles) cruises aboard the *Sorcerer II*. Refer to Table S2 for corresponding sample labels.

carried out alongside collections of free-drifting plastic particles (and cardboard and wood [also referred to as lignin]), that served as proxies for mature biofilms from the same sampling locations. Using whole-genome-shotgun sequencing and a suite of bioinformatic tools, we established taxonomic and functional gene inventories for early and mature epiplastic biofilms to compare overarching traits between biofilm phases. We further performed comparative genomic analyses with metagenome assembled genomes (MAGs) of the dominant early plastic colonizers to compare their genomic characteristics and describe the potentially advantageous gene sets for initial colonization and lifestyle on plastic.

EXPERIMENTAL PROCEDURES

Sample collection

The Drifter experiment was developed to simulate drifting plastic particles in surface marine waters and sample the associated epiplastic microbial community along a marine transect at different time points. Aquarium seawater and plastic samples were collected during two cruises aboard the R/V *Sorcerer II* from West Palm Beach, FL to Marigot on the island of St. Martin (19–26 March 2017) and West Palm Beach, FL to Annapolis, MD (14–18 May 2017; Figure 1). Samples were collected in two different ways: (1) shipboard incubations (2–7 days), which were incubated underway and harvested incrementally across the cruise track; and (2) Manta trawls (333 μm) that were deployed alongside locations where the short-term incubation samples were harvested. The short-term incubations represented the early colonization component of this study, whereas the particles collected via Manta trawl represented a proxy for mature biofilms collected at the same sample locations.

The short-term shipboard incubations consisted of incubating polyethylene (PE) 'Infant Water' jug punches from Walmart, pre-industrial PE pellets (Dow), polyhydroxyalkanoate (PHA) pellets (Meridian Holdings) and polystyrene (PS) pellets (source unknown) in a 4 L pre-cleaned aquarium aboard the ship, and regularly replenishing the aquarium with surface seawater along the transect for one week. Plastic pellets ranged in size from 3 to 5 mm. More detailed information on the physicochemical properties of the incubated polymer types can be found at https://www.polymerdatabase.com/. Chemical structures of the polymers incubated in this study can be viewed in Amaral-Zettler et al. (2020). The March and May Drifter experiment set ups included ~200 circular 'punches' from the high-density PE jug, ~200 PE pellets, ~100

PHA pellets and ~100 PS pellets. All plastic particles and the aquarium were pre-cleaned in 10% bleach then rinsed in distilled water for 30 min, followed by three rinses with laboratory grade deionized water. A 20 L plastic bucket was also pre-cleaned and used as a reservoir for the experiment. For the March cruise, plastic particles were loose within the aquarium, and seawater in the 20-L bucket was pumped continually through the aquarium and replaced with surface seawater approximately every 2 h during the day. For the May cruise, a slow flow-through seawater system was developed to continually add surface seawater to the 20 L bucket. The aquarium was covered with 1-mm fibreglass netting to retain the plastic in case of rough seas, and to provide some shading. The aquarium was maintained on the ship deck and exposed to natural sunlight, with nearly all collections taking place just before solar noon. Starting at two days, approximately 10 pellets of PE, PHA and PS punches and/or pellets were harvested using sterile forceps and preserved for DNA extraction and imaging (see later). Using a peristaltic pump, 2 L of aquarium seawater from the 20-gallon incubator bucket was filtered through 0.22 μm Sterivex filters (Merck, Germany) periodically during the short-term incubations to compare the epiplastic community on the plastic pellets with cells in the surrounding aquarium seawater or that might be sloughing off plastic surfaces in the bucket.

Samples were further categorized based on the source (aquarium seawater, cardboard, plastic and wood), as well as the colonization phase of microorganisms (free-living; early and mature biofilms). The measured carbonyl index of each plastic along with the appearance of each plastic's surface (e.g., thick biofilm) were used to differentiate early from mature epiplastic biofilms. More detail is provided in Supporting Information. Environmental data, including phosphate, nitrate, nitrite, silica, oxygen, salinity and temperature were collected in conjunction with plastic sampling (Table S2).

Sample preservation

At each time point, incubated microplastics and plastics collected with Manta trawls were preserved for both molecular analysis and imaging. Multiple punches and pellets were pooled for each purpose to increase the amount of biofilm available, while net samples were cut into two pieces for molecular analyses and imaging. In each case, the samples for molecular analysis were immediately transferred to 1 mL Puregene cell lysis solution (Qiagen, Valencia, CA) and frozen at −20°C for downstream whole-genome-shotgun sequencing. Samples for imaging were fixed in 4% paraformaldehyde (EMS, Hatfield, PA) then transferred to a 1:1 phosphate buffer/ethanol mix and stored at −20°C until prepared for SEM imaging. SEM sample preparation and imaging followed the approach described in Zettler et al. (2013), except a Leica EM CPD300 was used for critical point drying. Sterivex filters were flooded with Puregene cell lysis solution, sealed and frozen at −20°C.

Bioinformatics pipeline

Total genomic DNA was extracted using the Gentra Puregene Tissue DNA Isolation kit (Qiagen, Valencia, CA). DNA extractions were performed using a modified bead beating approach described in Amaral-Zettler et al. (2015). Library preparation was done using the Swift2S (Swift Biosciences INC., Ann Arbor, MI) library preparation kit following the manufacturer's instructions, and DNA was sequenced using whole-genome-shotgun sequencing on a 150 base pair paired-end Illumina NextSeq at the J. Craig Venter Institute, La Jolla, CA. Raw reads were pre-processed to remove ligated adapters, regions of low complexity and low-quality sequences using fastp (v0.12.5), with default parameters (Chen et al., 2018). PolyG tail trimming (--poly_g_min_len 50) and a quality threshold (--qualified_quality_phred 12) were used, and the quality of pre-processing steps was assessed using FastQC (Andrews, 2010). The freely available software and data platform KBase (KBase, http://kbase.us/) was leveraged to assemble our quality-controlled, paired-end sequences using metaSPAdes (Nurk et al., 2017), with kmers [21, 33, 55] and a minimum contig length of 500 nucleotides.

A Unix PowerShell script was created to take all metagenomic assembly files (.fa) in a working directory and enumerate gene counts for all genes in each assembly to construct a composite gene catalogue. This script is available at https://github.com/Echiostoma. Each metagenomic assembly was iteratively converted into an Anvi'o (v7.0 dev, Eren et al., 2021) compatible contig database using the anvi-gen-contig-database command. Open reading frames were predicted using Prodigal (v2.6.3, Hyatt et al., 2010) and putative genes were annotated using COG20 Categories and Functions, KOfams and Pfams with an e-value threshold of $1e^{-4}$ (filtered with awk). Annotated genes for each assembly were iteratively exported using the anvi-export-functions command. The exported gene annotations were then processed by iteratively identifying gene calls in each file, sorting them alphabetically, and enumerating the number of appearances of each gene name and displaying those counts. At the same time, a comprehensive list of all gene calls for all assemblies was tabulated and sorted while only retaining unique appearances of each gene. The list of all unique genes identified in the previous step was then used to query each list of assembly annotations using the grep function. In this case, the

grep function exactly matched gene names between files and pulled the number of counts of that gene observed in each assembly or wrote a '0' to indicate gene absence to a temporary file that was progressively built over time. Ultimately, a gene catalogue containing a list of all unique genes and counts for those genes for each assembly was retained.

Functional gene inventories between early and mature biofilms were statistically compared for differential abundance using the DESeq2 algorithm (Love et al., 2014). The DESeq2 algorithm (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) is traditionally used for RNA-seq read count analyses but can also be used to study differential abundance of genes based on a negative binomial distribution followed by *p*-value adjustment using the false discovery rate/Benjamini–Hochberg correction. DESeq2 internally normalizes count data by calculating the geometric mean for each gene in every sample and dividing it by that mean. This normalization also corrects for library size and gene composition bias.

Metagenomic reads were taxonomically identified using Metagenomic Intra-species Diversity Analysis System (MIDAS; Nayfach et al., 2016) for strain-level differentiation of all communities. The MIDAS pipeline quantifies bacterial species abundance and strain-level genomic variation using single-nucleotide polymorphisms from shotgun metagenomes by leveraging a database of more than 31,000 prokaryotic genomes. However, the MIDAS database is geared towards studying the human microbiome, and consequently does not contain a representative database of marine microorganisms. Therefore, Kaiju (v1.8, Menzel et al., 2016) was used to complement MIDAS taxonomic annotations. Kaiju assigns each sequence in a read library to a taxon in the NCBI database with protein-level sequence homology against selectable sequence repositories. Here, we used the parameters (-x -m 9 -a greedy -e 5 -s 70 -z 8 -v) on a random subsample of reads from each read library to make a BLAST search against the non-redundant database containing bacterial and eukaryotic reads.

MAGs were binned from early colonizer metagenomes using Metabat2 (Kang et al., 2019), with inclusion of only contigs greater than or equal to 2500 base pairs in length. Each MAG was processed and functionally annotated like each metagenomic assembly as described earlier. The quality of MAGs based on completion and contamination were assessed using CheckM (Parks et al., 2015), with a minimum percent completion of 70% and contamination less than 2%. In addition to functional metabolic annotations, taxonomic affiliation of each MAG was determined using a combination of approaches, which included taxonomic estimation with anvi-run-scg-taxonomy, anvi-run-hmms, CheckM and GTDB-Tk (Dataset S5; Chaumeil et al., 2020).

Statistical analyses

Functional and taxonomic matrices generated from aquarium seawater, cardboard, plastic and wood communities were converted into a Bray–Curtis dissimilarity matrix and clustered using non-metric multidimensional scaling. These relative abundance data were statistically compared using a Permutational Analysis of Variance (PERMANOVA), with post-hoc multiple comparisons where applicable. Analyses of similarity (ANOSIM) were used to compare the statistical resemblance between and within levels of the factors: colonization phase, polymer type and substrate. The relative abundance of COG categories for aquarium seawater communities and epiplastic communities were statistically compared using ANOVA or Kruskal–Wallis tests, with post-hoc multiple comparisons. The DESeq2 algorithm was used to statistically compare the differential abundance of KOfams between early colonization and mature epiplastic biofilm communities. Carbonyl indices were statistically compared between early colonization and mature epiplastic biofilms with Mann–Whitney–Wilcoxon tests. All statistical analyses were conducted in the freely available software R (R Core Team, 2013), with packages EnhancedVolcano, ggplot2, ggpubr, pheatmap and vegan.

RESULTS

Given the 1-week sampling duration of this cruise, the short-term plastic drifter incubations of known age established the early colonization component of this study, whereas collections of plastic debris collected by Manta nets alongside the drifter incubations represented a proxy for a timepoint of each mature epiplastic biofilm (Figure 1). Comparisons between the carbonyl index (oxidative state) of plastics used in early colonization incubations and mature epiplastic biofilms (Figure S1), as well as the presence of thick biofilms on free-drifting plastics substantiated that older biofilm communities were present on these aged plastics. SEM imaging of the plastics harvested from the drifter incubations and the marine environment further underscored the differences between incubated and Manta-trawl collected plastics. Free-drifting plastic surfaces displayed clear signs of mechanical weathering such as altered surface topology and degradation including cracks and extensive micron sized honeycomb-like pits, whereas our short-term incubation samples exhibited none of these topological features, except for PHA day 7 that functionally and taxonomically resembled a mature community (Figure 2; see discussion later).

Whole-genome-shotgun sequencing followed by pre-processing yielded ~19,000,000 high-quality, paired-end reads per read library. Assembly information for all metagenomes can be viewed in Tables S2

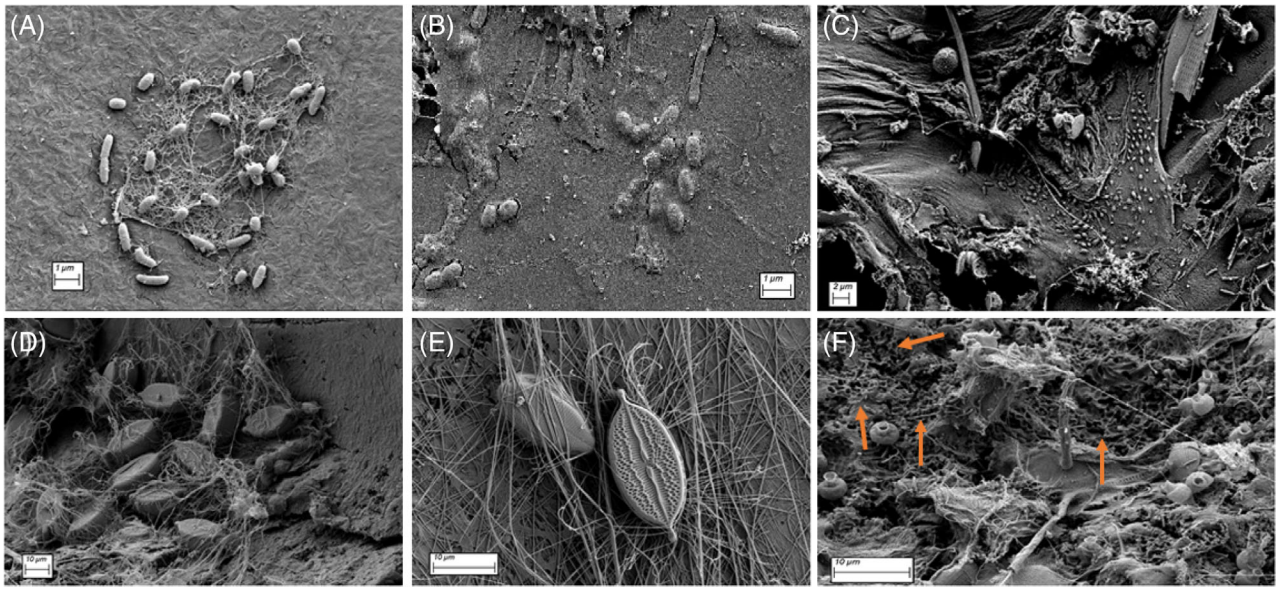

FIGURE 2 SEM images of early (A–C) and mature epiplastic biofilms (D–F): (A) Microcolony of bacterial cells observed on polyethylene. Lateral flagella and secreted pili are present, indicating irreversible attachment; (B) various bacterial morphotypes embedded in the exopolysaccharide matrix on polystyrene; (C) early diverse biofilm on polyhydroxyalkanoate consisting of different morphotypes of bacteria and eukaryotes; (D,E) a cluster of diatoms entangled in secreted chitin 'fingers' that are used for attachment; (F) the surface of highly weathered plastic collected via Manta trawl. Orange arrows denote areas where honeycomb-like, presumptive degradative pits are present.

and S3. The supplement includes the heatmap containing the top 50 bacterial species (Figure S2) and taxonomic breakdown at the family level generated via MIDAS (Figure S3) for aquarium seawater, cardboard, plastic and wood communities. Figure 3 displays the Kaiju annotated relative abundances of bacterial and eukaryotic families observed for each respective sequence library from aquarium seawater, cardboard, plastic and wood. Aquarium seawater communities were replete with bacteria from the families Pelagibacteraceae, Prochloraceae and Synechococcaceae, but also included members from Flavobacteriaceae and Rhodobacteraceae (Figure 3). Early epiplastic communities were dominated by Alteromonadaceae, which was composed of *Alteromonas*, *Marinobacter* and *Marisediminitalea*, and in most of these read libraries, this family constituted >50% relative abundance on PE, PHA and PS. Given that Kaiju and MIDAS employ fundamentally different approaches (taxonomy based on sequence homology vs. curated phylogenomic trees), they provided a complementary and corroborative confirmation that Alteromonadaceae were the dominant early colonizers of plastic in this study. Flavobacteriaceae and Oceanospirillaceae were also present in most early colonization incubations. Thirty-seven high-quality MAGs, including 14 *Alteromonas*, 4 *Marinobacter* and 8 *Marisediminitalea* MAGs were recovered from early colonization incubations, and have been annotated and described in Dataset S5. Multiple strains of *Alteromonas macleodii* (AD006, AD037, Balearic Sea AD45, Black Sea 11, English Channel 673) were detected on PE, PHA and PS during the drifter experiment, yet *A. macleodii* strain Black Sea 11, which was highly abundant on PHA, was rarely observed on PE and PS (Figure S2).

Alteromonadaceae, which dominated early colonization (comprising a relative abundance of 50%–60%), was not observed in aquarium seawater (0%) and rarely detected in mature epiplastic biofilm communities (<1% relative abundance). By contrast, Flavobacteriaceae and Rhodobacteraceae constituted low relative abundances in early biofilm communities, yet were present in all mature epiplastic biofilms, with Rhodobacteraceae being dominant in mature biofilms. Erythrobacteraceae and Hyphomonadaceae were also abundant in these mature biofilms, despite their low relative abundances in early colonization and aquarium seawater (Figure 3). There was a significant difference (PERMANOVA, $p = 0.001$) between the taxonomic composition of aquarium seawater and early and mature epiplastic biofilm communities (Figure S4), with all levels being statistically different from one another (post-hoc multiple comparisons, p-adj $= 0.003$, aquarium seawater vs. early colonization, 0.006, aquarium seawater vs. mature epiplastic biofilm, 0.003 early colonization vs. mature epiplastic biofilm). ANOSIM was used to discern the taxonomic resemblance between aquarium seawater and early and mature epiplastic biofilm communities and within each group, and these communities were significantly different from one another, but varied little within each respective group (ANOSIM, $R = 0.929$, $p = 0.001$). Cardboard and wood

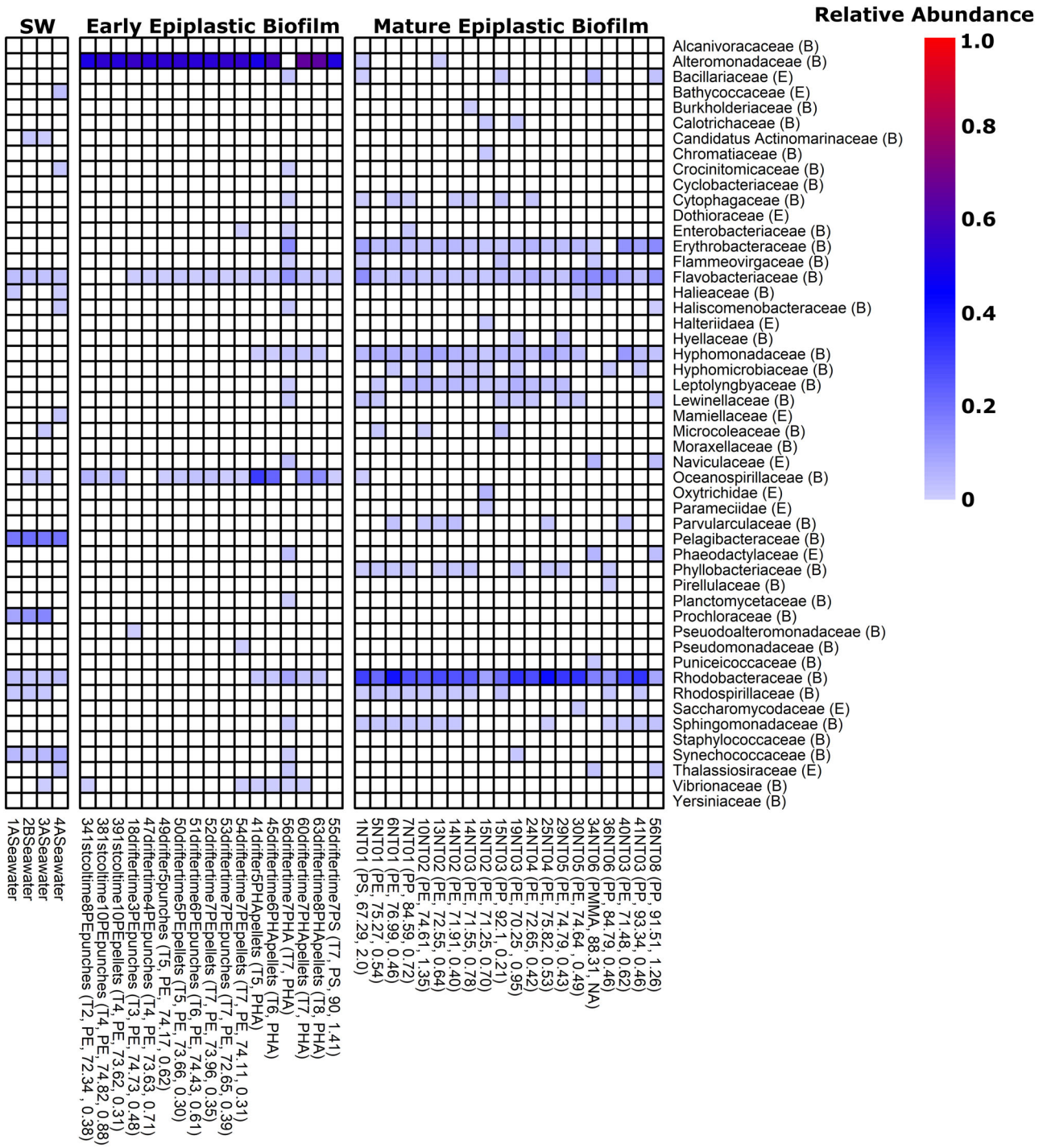

FIGURE 3 Taxonomic heatmap of bacteria (B) and eukaryotic (E) families annotated from aquarium seawater, cardboard, early and mature epiplastic and wood communities using Kaiju annotations. SW indicates aquarium seawater metagenomic read libraries. Each column represents a unique read library. Relative abundances range from 0 to 1. PE, PHA, PS, PMMA and PP indicate polyethylene, polyhydroxyalkanoate, polystyrene, poly(methyl methacrylate) and polypropylene, respectively. Read library names are displayed on the bottom portion of this figure followed by polymer type, percent match similarity with reference spectra, and carbonyl index.

samples were used for ordination but excluded from multivariate statistics because of the low sample size.

When comparing functional metabolic potential between early and mature epiplastic biofilms and free-living communities there was a significant difference, with all communities being functionally distinct from one another (Figure S5A, PERMANOVA, $p = 0.001$, p-adj $= 0.002$ aquarium seawater vs. early colonization, 0.004 aquarium seawater vs. mature epiplastic biofilm, 0.002 early colonization vs. mature epiplastic biofilm).

ANOSIM was used to discern the functional metabolic resemblance between aquarium seawater and early and mature epiplastic biofilm communities and within each group; these communities varied little within each respective group (ANOSIM, $R = 0.778$, $p = 0.001$). When exploring functional potential observed in early and mature epiplastic biofilms, a total of 8314 unique KOfams were annotated, with 1452 and 1029 KOfams being enriched in early colonization and mature epiplastic biofilm communities, respectively (Figure S5B; Datasets S6 and S7). When comparing large-scale genomic characteristics (all KOfams) among the dominant early colonizer MAGs across our sample locations, ~80% of annotated KOfams were identical between phylogenetically distinct MAGs, despite the presence of salinity (7.2–36.8 PSU) and temperature changes along our latitudinal gradients (18.3–28.3°C; Figure 4; Table S2). When compared with mature epiplastic biofilms, early colonizers reproducibly possessed enriched metabolic and regulatory characteristics involved with adhesion, biofilm formation, chemotaxis, environmental stress, iron utilization and motility (Figure 5A). In mature epiplastic biofilm communities, there was a significantly higher proportion of genes for transport and catabolism of the organic acids fumaric, malic and succinic acids, as well as enrichment of carbohydrate active enzymes (Dataset S7). Additional enriched gene sets participating in

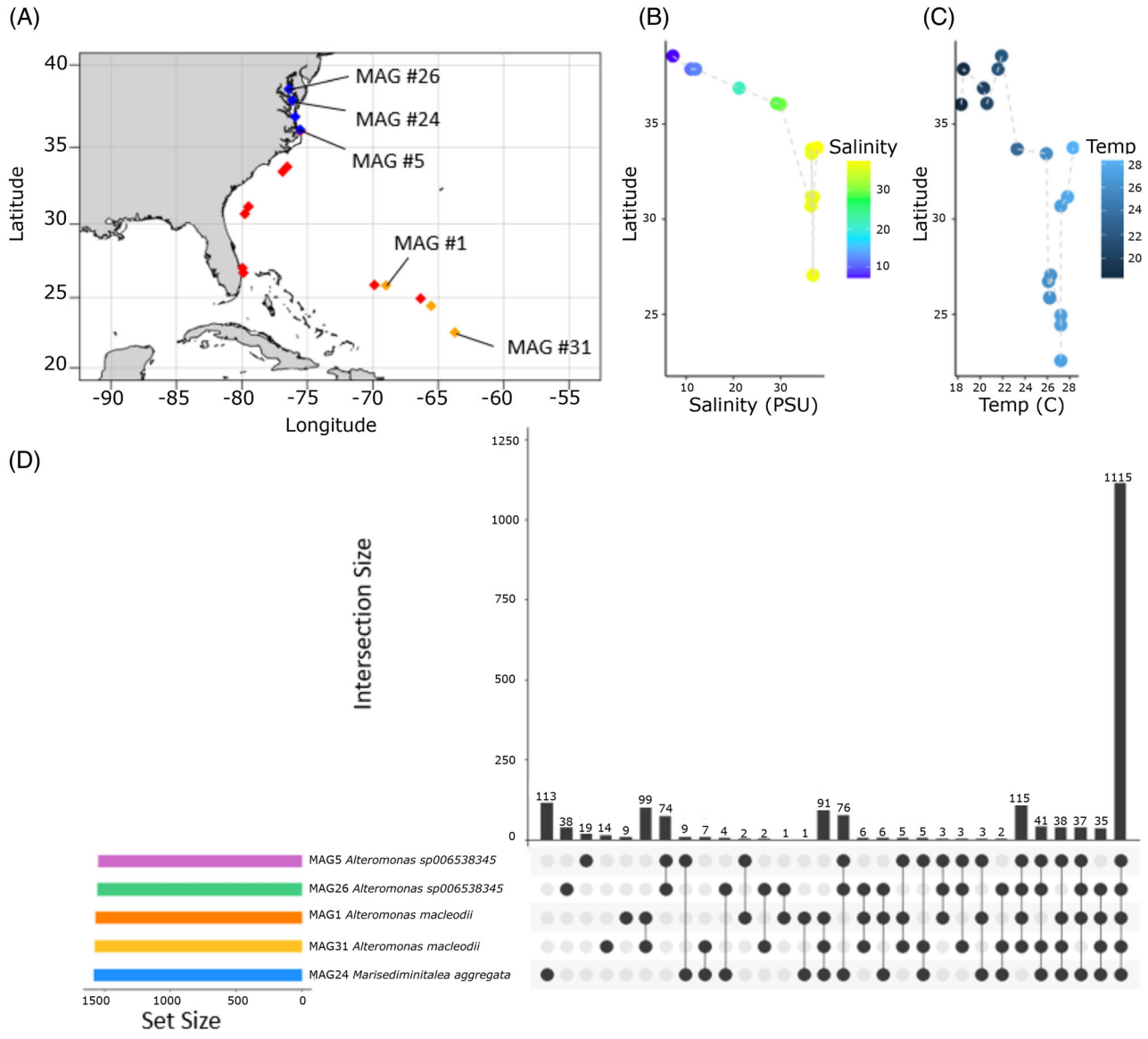

FIGURE 4 (A) Dominant metagenome assembled genomes (MAGs) sampled along our drifter incubation transects. Blue diamonds indicate where polyhydroxyalkanoate (PHA) substrates were used, whereas orange and red diamonds represent polyethylene (PE) and mature plastic biofilm samples, respectively. (B) Salinity and (C) temperature data collected across the latitudinal sampling gradient. (D) Upset plot showing the shared annotated KOfams between the dominant early colonizer MAGs sampled across our cruise transects. Vertical bars represent shared KOfams between each MAG.

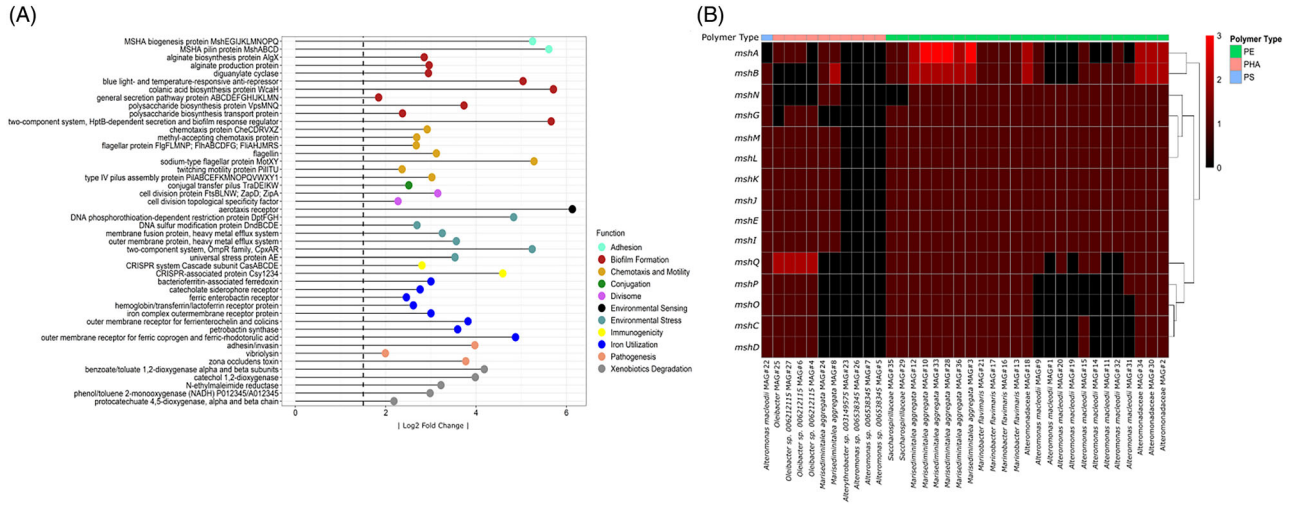

FIGURE 5 (A) The mean Log_2 fold changes of enriched KOfams with adjusted *p*-values (<0.05) and Log_2 fold changes greater than the absolute value of 1.5 for early colonizers of plastic (when compared with mature plastic biofilms). (B) Copy number (0–3) of genes from the MSHA operon present in metagenome assembled genomes (MAGs) binned from polyethylene (PE), polyhydroxyalkanoate (PHA) and polystyrene (PS) during early colonization (<7 days). See Dataset S5 for functional and taxonomic annotations corresponding with each MAG.

oxidative phosphorylation, phosphonate metabolism, photosynthesis, secondary metabolism and type IV secretion system pathways were observed in mature biofilm communities (Figure 6). Further functional metabolic comparisons between substrates with use of COG categories can be viewed in Figure S6.

Using gene catalogues and read mapping, comparative genomic analyses with metagenomes and MAGs revealed that putative operons for the type IV mannose-sensitive hemagglutinin (MSHA) pilus were significantly enriched in early colonization and positively correlated with substrate hydrophobicity (Figure 5B; Datasets S5, S6 and S8). The same was true for *mshA*, except this gene was highly positively correlated with substrate hydrophobicity (Spearman's correlation, $\rho = 0.757$). Our *A. macleodii* MAGs recovered from PHA, which annotated as *Alteromonas* Kul 49, did not possess any components of the MSHA operon, whereas the *Alteromonas, Marinobacter* and *Marisediminitalea* strains from PE and PS possessed full or fragmented portions of the operon, with some MAGs possessing multiple copies of *mshA*, each copy with a unique amino acid sequence (Datasets S5 and S8). *A. macleodii*, *Marinobacter flavimaris* and *Marisediminitalea aggregata* synteny alignments of the MSHA operon and neighbouring genes, that displayed high synteny and apparent positive selection for *mshA*, can be viewed in Figures S7–S9.

DISCUSSION

The metagenomic data from plastics collected via Manta trawl and week-long shipboard PE, PHA and PS incubations present a stark contrast between early and mature epiplastic biofilm communities (Figures 2–5) and further our understanding of bacterial successional dynamics. In the present study, the genus *Alteromonas* reproducibly dominated (50%–60% of mapped reads) early colonization incubations across all time points but was rarely detected in any of the mature biofilms on net captured particles, or in aquarium seawater samples (Figure 3). These observations suggest that virgin plastic substrates select for distinct bacterial populations in seawater when compared with mature epiplastic and seawater communities. Experimentally determining that *Alteromonas* was reproducibly the major early colonizer across environmental gradients, with high agreement between Kaiju (taxonomy based on sequence homology) and MIDAS (taxonomy based on curated phylogenomic trees) is notable. The high reproducibility and fidelity of large-scale genomic characteristics, such as the presence or absence of the MSHA operon, observed in phylogenetically distinct early colonizers across environmental gradients is of greater interest for understanding functional turnover and traits at different stages of a biofilm (Pascual-García et al., 2022). Based on high consensus between our metabolic annotations, *Alteromonas* has an efficient genome chassis for early particle colonization, with subtle functional differences between polymer types and water parcels, that reproducibly contains enriched characteristics such as accelerated adhesion (see later), biofilm formation, chemotaxis, hydrocarbon degradation (see later) and motility (Datasets S5 and S8). The gene sets mentioned earlier, which decrease in relative abundance in mature biofilm communities, may provide a competitive advantage for niche occupation and enable bacteria to overcome the barriers required for locating, swimming towards and colonizing plastic surfaces. Critically, our

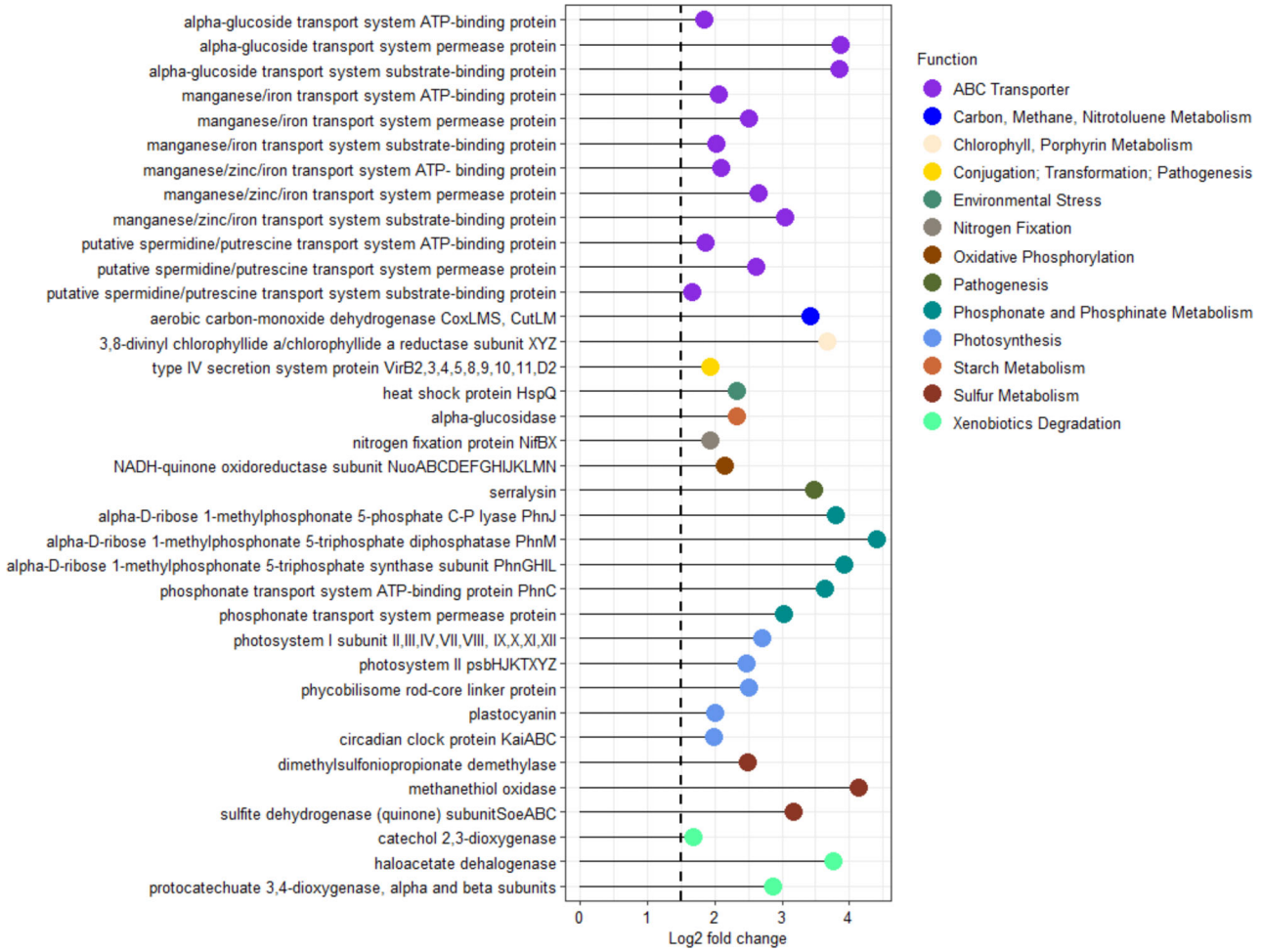

FIGURE 6 The mean Log_2 fold changes of enriched KOfams with adjusted *p*-values (<0.05) and Log_2 fold changes greater than the absolute value of 1.5 for mature epiplastic biofilms (when compared with early plastic biofilms). Note difference in scales with Figure 5.

dominant *Alteromonas* spp. MAGs shared ~80% of annotated KOfams, despite being sampled across salinity (7.2–36.8 PSU) and temperature (18.3–28.3°C) gradients (Figure 4; Table S2; see also Figure S11 for further nutrient correlation between functional metabolism and biofilm phases). As such clear reproducibility is rarely observed in even controlled laboratory settings, these in situ observations are not idiosyncratic of our sampling locations, but rather suggest one of two possibilities: (1) the presence of a core functional metabolic and taxonomic microbiome in early plastic communities; or (2) *Alteromonas* represents an early core of substrate attached communities, rather than specificity to plastic, as *Alteromonas* has been shown to be a metabolic generalist (Dang & Lovell, 2016). What remains to be studied is whether these genome characteristics are hallmarks of initial microbial settlement in the marine environment or specific for plastic colonization.

A novel observation is presence of the MSHA operon, a collection of adhesive genes best characterized in *Vibrio* pathogenesis (Floyd et al., 2020), in nearly all phylogenetically distinct early colonizers of petroleum-based plastic surfaces. A notable exception were *Alteromonas* spp. MAGs binned from hydrolysable PHA (see discussion later; Figure 5B; Dataset S5). The MSHA pilus machinery, crucial for virulence, is reported to be acquired as a mobile genetic element (Marsh & Taylor, 1999). The genes directly flanking the MSHA operon are the rod-shape determining proteins *mreBCD* on one end and a single-stranded DNA binding protein on the opposite side, observable in some of our MAGs (Dataset S5). By leveraging synteny alignments of the MSHA operon between each clade's closest reference genomes and our *A. macleodii*, *M. flavimaris* and *M. aggregata* MAGs, all MAGs that were binned from plastic metagenomes appear to display high synteny, as well as apparent positive selection for alleles of *mshA* across all species that possessed the operon for reliable comparisons (Figures S7–S9). Therefore, acquisition of this operon may be an evolutionary adaptation to the novel surface that plastic provides, allowing microorganisms to overcome the physical barriers of colonizing a highly

hydrophobic surface. Specifically, acquisition of the MSHA pilus may be a result of adaptive radiation, whereby this horizontally transferred operon may facilitate plastic colonization between primary colonizers and secondary consumers (discussed later)—a result previously observed in alginate degradation (Hehemann et al., 2016).

The observation that *A. macleodii* strain Black Sea 11 was rarely present on PE and PS samples but highly abundant on PHA, further underscores the importance of MSHA for early colonization of highly hydrophobic surfaces. Strain Black Sea 11 does not possess MSHA, and the same is true for the *A. macleodii* MAGs binned from PHA, which annotated as *Alteromonas* Kul 49, a species that also does not possess the MSHA operon (Figure S2; Datasets S5 and S8). Importantly, a MAG does not represent clonal individuals, but rather a phylogenetically defined group of organisms that co-occur in space and time, in this case *Alteromonas* spp., providing insight into the metabolism of a particular genus. The observation that MSHA was not observed in any *Alteromonas* MAGs binned from PHA is striking. These data suggest that possession of MSHA and multiple copies of *mshA* may provide a competitive advantage for early colonization of highly hydrophobic surfaces. Our data support this idea, as the MSHA operon was positively correlated with substrate hydrophobicity, or water contact angle (PE water contact angle, $\theta = 101.7^{\circ}$, De Geyter et al., 2008), PS ($\theta = 87^{\circ}$, Li et al., 2007) and PHA ($\theta = 75^{\circ}$; Kang et al., 2001). Other properties like surface roughness, surface free energy or electrostatic interactions could also partly explain the observed trend (Rummel et al., 2017), however this is less likely since all polymers were virgin and of similar production finish. Nevertheless, gene-expression and genetic manipulation studies with model systems will be necessary for understanding MSHA's role for early plastic colonization and will provide important implications for how microbial communities self-assemble on plastic.

Primary adhesion, more specifically, the traits of early bacterial colonizers are important for community assembly because the drivers of initial particle attachment ability are complex (e.g., adherence mechanisms, biofilm production, chemotaxis and motility). Based on our data, secondary/tertiary colonizers are not metabolically equipped to fulfil the primary colonizer niche (biofilm establishment) but can colonize surface-modified plastics. The questions that remain are: why would bacteria selectively colonize plastic substrates over naturally occurring aggregates that are presumably easier to metabolize? Did the early colonizers observed here obtain MSHA through horizontal gene transfer to access plastic materials more easily? Does the hydrophobic surface or leachates that plastic provides 'trick' bacteria into colonizing, much like a seabird may mistakenly target plastic as a prey item, due to olfactory cues or are these microorganisms adapted specifically to locating and metabolizing plastic substrates? Plastics are known to off-gas dissolved organic matter (Romera-Castillo et al., 2018; Royer et al., 2018) from photochemical dissolution when irradiated with ultraviolet light (Zhu et al., 2020), and low-molecular weight compounds such as ethylene (C_2H_4), butylene (C_4H_8), propylene (C_3H_6) and short-chain fatty acids (CH_2O) may serve as a lure for hydrocarbonoclastic bacteria. When paired with the observation of enriched chemotaxis gene sets and hydrocarbonoclastic functions present in early colonizers such as acetate kinase ($C_2H_3O_2$), catechol and protocatechuate dioxygenases (Buchan et al., 2001), general short-chain fatty acid transporters, formate transporters, as well as genes involved in polyethylene glycol degradation $(CH_2OH)_2$, these observations suggest that bacteria may be keying in on and metabolizing low-molecular weight hydrocarbons (see for example honeycomb-like pits formed from biodegradation or photochemical dissolution; Figure 2F). However, targeted microfluidic assays with plastic dissolved organic matter are still required to verify whether leached additives and hydrocarbons lure microorganisms, and our metagenomic, as well as spectral observations (Table S4; Dataset S9) warrant further annotations on attraction/predilection of chemotaxis genes.

Metagenomic and metaproteomic studies to date suggest it unlikely that microorganisms are holistically biodegrading plastic in the ocean due to the presence of labile compounds in epiplastic biofilms that could be prioritized over recalcitrant plastic carbon (Oberbeckmann et al., 2021). While we do not have exometabolomic or transcriptomic data, our metagenomic data suggest that the apparent wholesale functional and taxonomic shift between early and mature epiplastic biofilms may change from particle-degrading specialists (Alteromonadaceae, Oceanospirillaceae) to secondary consumers (Flavobacteriaceae, Rhodobacteraceae). Namely, community succession may be driven by the initial metabolic responses to leaching plastic materials (in early colonization) to the types of recalcitrant plastic materials that have become hydrolysable over time, or by-product cross-feeding from the metabolic products of the early colonizers themselves. For example, *Alteromonas, Marinobacter, Marisediminitalea* and *Oleibacter* MAGs possessed several putative hydrocarbon degradation genes such as alkane 1-monooxygenase, benzoate and toluate monooxygenases, catechol and protocatechuate dioxygenases and multiple alkane degradation pathway regulators that suggest plastic hydrolysis can take place (Dataset S5). The total degradation of plastic produces CO_2, CH_4, H_2O and organic acids (Alexander, 1978, Narayan, 1993), so it could be expected that secondary colonizers, that cannot hydrolyse long chain plastic oligomers could persist in epiplastic communities by

utilizing metabolic by-products from the primary colonizers. Our data support this idea, as there is a significantly higher proportion of genes for transport and catabolism of the organic acids fumaric, malic and succinic acids, as well as enrichment of carbohydrate hydrolysis enzymes in mature epiplastic biofilms. Pairing gene annotations from the present study with those from the plastic hydrolysis database (Gambarini et al., 2022) also suggest that polyethylene glycol may be an important energy source emanating from plastic, as D-lactate alcohol and aldehyde dehydrogenases were abundant in early plastic biofilms.

Community succession may be simultaneously driven through by-product cross-feeding from the metabolic products of the early colonizers themselves. The significantly higher abundance of iron receptors and iron utilization genes in early colonization is of particular interest because some *Alteromonas* and *Marinobacter* produce siderophores (Manck et al., 2020) that can be utilized by all members of the community that possess the compatible transporters such as periplasmic protein TonB. The *A. macleodii* MAGs binned from PE and PS possessed the gene petrobactin synthase (Barbeau et al., 2002; Manck et al., 2022), whereas the *A. macleodii* MAGs that were binned from PHA and found in low abundance on PE and PS did not have this gene present, and neither did their closest related type of strain. All of the PE, PHA and PS colonizing *A. macleodii* strains, as well as *Marisediminitalea* MAGs possessed putative catecholate siderophore and TonB receptors (Dataset S5), irrespective of siderophore producing ability or lack thereof. These observations, paired with significantly higher abundance of iron utilization genes involved in siderophore production and utilization during our drifter experiment suggest that early colonizers may incentivize future colonization of plastic by autotrophic organisms, as well as aid in overcoming iron limitation, and therefore may be harbingers of the succeeding biofilm community.

Plastic remains in the environment for long periods of time and tertiary colonizers are exposed to completely different selective pressures than the primary colonizers. Varied conditions notwithstanding, there appears to be a highly reproducible shift to heterotrophic reliance on oxygenic photosynthesis, and colonization by autotrophs may highlight a key turning point in community metabolism, whereby any substantial amount of plastic hydrolysis is supplanted by metabolism of carbon sources from primary producers. Either this functional convergence is predictable based on the traits of early colonizers, or eventually mature communities share the same fate, irrespective of the environment they are pulled from. Future study is warranted to address this question. Our metagenomic data support the observation that there is more available carbon produced by primary producers in mature epiplastic biofilms (Bryant et al., 2016). In our mature communities, there was no significant difference in the functional KOfam matrix between polymer types and substrates (ANOSIM, $p = 0.002$, $R = 0.4493$; PERMANOVA, $p = 0.132$, $R^2 = 0.32041$), which suggests that mature epiplastic communities may also be functionally predictable (Figure S10). The metabolic and taxonomic data from our PHA incubations support this notion, as the appearance of Bacillariaceae and Leptolyngbyaceae are marked by the absence of Alteromonadaceae and Oceanospirillaceae, concomitant with a significant increase in circadian clock proteins, photosystem I and photosystem II operons, phycobillisomes and plastocyanins, as well as carbohydrate degradation enzymes as communities become mature (Figure 5). Secondary colonizing, autotrophic organisms like Bacillariaceae, Phaeodactylaceae and Leptolyngbyaceae, which were newly present in mature epiplastic biofilms, would benefit from siderophore production (via early colonizers), as iron is often a limiting nutrient in the ocean that is required for photosynthesis. Concurrently, a positive feedback loop could be created, whereby vitamins secreted by bacteria could fuel autotroph metabolism, and in return, autotrophs could provide labile, fixed carbon for bacteria. For example, vitamin B12, a cobalt-containing complex, cannot be created by diatoms, and must be provided by bacteria through growth or lysis (Croft et al., 2005; Tang et al., 2010; Warren et al., 2002). The significant increase in cobalt and zinc transporters, as well as methionine and tetrahydrofolate metabolism and methylmalonyl-CoA mutase may provide evidence for acquisition of vital nutrients by diatoms (Bertrand & Allen, 2012). Colonization by the former diatom families also highlights important algal–bacterial interactions that can take place in plastic communities and can directly influence bacterial diversity.

Previous particle studies have investigated the dynamics of early and mature biofilms using chitin, a known biodegradable compound that has been around for millions of years, as a model substrate in controlled laboratory settings (Datta et al., 2016; Enke et al., 2018; Pascual-García et al., 2022; Pollak et al., 2021; Pontrelli et al., 2022). The time-series generated using plastic substrates from this study is therefore distinct and expands our understanding of foundational biofilm formation and functional dynamics of geologically recent plastic inputs, both biodegradable and recalcitrant polymers. Our experimental design, which analyses early and mature adhered biofilms across the ocean environment, as opposed to controlled laboratory settings, reveals clear reproducibility across natural environmental gradients. This consistency of large-scale genomic characteristics of early biofilms suggests that the interplay between environments, microbes and plastic surfaces abide by chemical and physical constraints and allow prediction of emergent properties of biofilm stages. Of particular interest, MSHA may facilitate colonization by

secondary colonizers. Whether MSHA can provide an avenue to address plastic pollution or waste valorization of plastic products via coordination between primary and secondary colonizers remains to be determined, however inclusion of MSHA in biotechnological applications warrants consideration. More metagenomic data taken at early-stage colonization are required to better comprehend the fate of plastic marine debris, and these data may be useful for better understanding biofouling and biofilm inhibition not just on plastic but other substrates in aquatic environments as well.

AUTHOR CONTRIBUTIONS

Ryan P. Bos: Data curation (lead); formal analysis (lead); validation (lead); writing – original draft (lead); writing – review and editing (equal). **Drishti Kaul:** Data curation (supporting); formal analysis (supporting); writing – review and editing (supporting). **Erik R. Zettler:** Conceptualization (equal); funding acquisition (equal); investigation (equal); writing – review and editing (supporting). **Jeffrey M. Hoffman:** Investigation (equal); writing – review and editing (supporting). **Christopher L. Dupont:** Conceptualization (equal); data curation (supporting); formal analysis (supporting); funding acquisition (supporting); writing – review and editing (supporting). **Linda A. Amaral-Zettler:** Conceptualization (equal); data curation (supporting); formal analysis (supporting); funding acquisition (equal); project administration (equal); supervision (supporting); validation (supporting); writing – review and editing (supporting). **Tracy J. Mincer:** Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); project administration (equal); supervision (lead); validation (supporting); writing – review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Raw sequencing data generated and analysed in the present study have been deposited at the NCBI Sequence Read Archive under BioProject ID: PRJNA777294 and BioSample accession numbers: SAMN24661204–SAMN24661247. Code used to create gene catalogues can be found at https://github.com/Echiostoma/.

ETHICS STATEMENT

Our manuscript has been approved by all authors, and all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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