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Biodegradable plastics in Mediterranean coastal environments feature contrasting microbial succession

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HIGHLIGHTS

- The fate of biodegradable plastics in different marine environments is understudied.
- Marine microbes of all three domains of life colonize biodegradable plastics.
- Habitat more than polymer type drives community selection in diverse environments.
- Putative biodegrading bacterial and fungal taxa occurred in all habitats.
- Disintegration patterns did not match relative abundance of putative biodegraders.

GRAPHICAL ABSTRACT
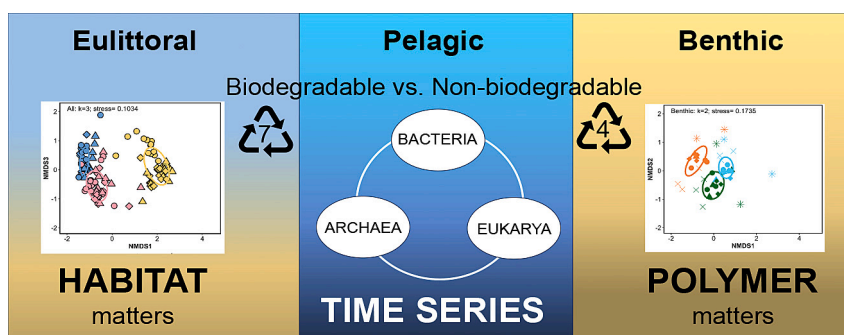
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ABSTRACT

Plastic pollution of the ocean is a top environmental concern. Biodegradable plastics present a potential "solution" in combating the accumulation of plastic pollution, and their production is currently increasing. While these polymers will contribute to the future plastic marine debris budget, very little is known still about the behavior of biodegradable plastics in different natural environments. In this study, we molecularly profiled entire microbial communities on laboratory confirmed biodegradable polybutylene sebacate-co-terephthalate (PBSeT) and polyhydroxybutyrate (PHB) films, and non-biodegradable conventional low-density polyethylene (LDPE) films that were incubated in situ in three different coastal environments in the Mediterranean Sea. Samples from a pelagic, benthic, and eulittoral habitat were taken at five timepoints during an incubation period of 22 months. We assessed the presence of potential biodegrading bacterial and fungal taxa and contrasted them against previously published in situ disintegration data of these polymers. Scanning electron microscopy imaging complemented our molecular data. Putative plastic degraders occurred in all environments, but there was no obvious

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"core" of shared plastic-specific microbes. While communities varied between polymers, the habitat predominantly selected for the underlying communities. Observed disintegration patterns did not necessarily match community patterns of putative plastic degraders.

1. Introduction

Plastic pollution in the World's Ocean is a top environmental concern linked to and rivaling climate change (Ford et al., 2022; United Nations Environmental Program, 2021). Like climate change, marine litter does not respect territorial waters or exclusive economic zones and thus represents a global environmental crisis (Arp et al., 2021; Jahnke et al., 2017; MacLeod et al., 2021). Biodegradable plastics are often mentioned as a potential "solution" in combating the accumulation of plastic pollution, and there are applications in which using biodegradable plastics is a logical choice (Bauchmüller et al., 2021; Dilkes-Hoffman et al., 2019; Flury and Narayan, 2021; United Nations Environmental Program, 2023). Currently their use only represents <1 % of the current global plastic production, though production is increasing rapidly and expected to almost triple in the next five years (European Bioplastics, 2022).

Biodegradable plastics not only disintegrate into smaller fragments, but are per definition, also fully available for microbial utilization and mineralization. This conversion results in only CO_2, CH_4 (in anaerobic conditions), H_2O and biomass. Complete biodegradation, however, is usually tied to specific technical environments (e.g. industrial composting facilities), and very little is known about the behavior of biodegradable plastics in natural environments (Zumstein et al., 2019). Like conventional plastics, biodegradable plastics will contribute to the future plastic marine debris (PMD) budget, because appropriate disposal and waste management will never capture all that is produced worldwide (Borrelle et al., 2020; Dilkes-Hoffman et al., 2019; Jambeck et al., 2015). Consequently, knowledge of the interactions of microbes with biodegradable synthetic polymers in the ocean is important, to constrain this budget. Biodegradation experiments are typically performed in the laboratory using standard test methods. Little is known about the microbial communities that actually interact with and break down these new biodegradable plastics in different marine settings in situ. Also, the effects these interactions have on local ecosystems pose a gap in our understanding of environmental microbiology (Arp et al., 2021; Harrison et al., 2011; Jahnke et al., 2017; MacLeod et al., 2021).

In the aquatic environment, plastic waste experiences abiotic influences such as ultraviolet radiation and mechanical stress, including wave action and abrasion with sediment. This in turn leads to photo-oxidation and disintegration (Andrady et al., 2022). However, plastic substrates are also colonized by a wide variety of bacterial and eukaryotic microbes in a matter of hours (Harrison et al., 2014; Kesy et al., 2020; Latva et al., 2022; Lee et al., 2008). This colonizing community, often referred to as the plastisphere (Zettler et al., 2013), consists of heterotrophs, phototrophs, mixotrophs, parasites, predators, and symbionts. Many residents of this community profit from the advantages of living in a biofilm instead of a free-living state (reviewed in: Amaral-Zettler et al., 2020; De Carvalho, 2018; Wright et al., 2020). The plastisphere is influenced by factors including geographical location, temperature, salinity, contact with sediment, season, incubation time and polymer type (Oberbeckmann and Labrenz, 2020; Wright et al., 2021). However, rarely has the interplay between different factors in situ been investigated in one integrated experiment over a long period of time, and few studies contrast conventional, non-biodegradable plastics and (biobased) biodegradable polymers (Dussud et al., 2018a; Odobel et al., 2021; Philippe et al., 2023). Several studies have identified the presence of potential plastic biodegraders in the plastisphere (e.g. Dussud et al., 2018b; Erni-Cassola et al., 2020). These microbes, by means of their enzymes, can either attack the virgin polymer matrix or utilize the partially photodegraded polymer (Andrady et al., 2022; Gewert et al.,

2015; Vaksmaa et al., 2021a). This drives the biodegradation of plastics into their basic molecular building blocks, but the extent to which this happens is not well characterized (Amaral-Zettler et al., 2020; Zeghal et al., 2021).

In this study, we used a three-domain rRNA marker gene sequencing approach, as well as scanning electron microscopy (SEM) analysis on plastic samples from the EU 7th framework Open-Bio study. Polybutylene sebacate-co-terephthalate (PBSeT) and polyhydroxybutyrate (PHB) films, which were both confirmed to biodegrade in laboratory tests (Briassoulis et al., 2020; Lott et al., 2021), were incubated for two years together with conventional low-density polyethylene (LDPE). This was done for three scenarios in coastal environments in the Mediterranean Sea: (1) eulittoral, where plastic arrives on an intertidal sandy beach and is eventually buried; (2) pelagic, where plastic is neutrally floating in the open water; and (3) benthic, where plastic has sunken to the seafloor and is in contact with both seawater and the sediment (Lott et al., 2020). While disintegration was observed in situ (Lott et al., 2021), microbes present and involved in biodegradation at these field sites remained unknown. Our research was motivated by the following questions: Is there a connection between the microbial community and the observed disintegration of the polymers? Are known biodegrading genera present in all environments? Do the same polymers attract the same biodegrading microbes in different environments? We hypothesized that the two biodegradable substrates would select for plastisphere members distinct from those on conventional non-biodegradable plastics and exhibit high relative abundances (RA) of biodegrading microbes.

2. Materials and methods

2.1. Polymer materials

Field incubations were carried out with two biodegradable plastic films, PBSeT (Novamont, Novara, Italy) and PHB (MIREL™ P5001, Metabolix, Cambridge, USA), and one conventional plastic, low-density polyethylene (LDPE, LUPOLEN 2420 K, LyondellBasell, USA). The polymers were in the form of films of 25 μm (PBSeT), 85 μm (PHB), or 30 μm (LDPE) thickness.

2.2. Field incubations

Field incubations were conducted as detailed in Lott et al. (2021) in the Mediterranean Sea in coastal waters off the Islands of Elba and Pianosa (Supplementary Fig. A.1). Incubations included a eulittoral (beach), pelagic (open water) and benthic (sublittoral seafloor) scenario. The polymer films were mounted in "HYDRA® test frame" holders (260 mm × 200 mm external and 200 mm × 160 mm internal dimensions) leaving a surface of 320 cm^2 of material directly exposed. In these frames, films were held between a mesh (PET) and plastic frames (PE) that were assembled with plastic nuts and bolts (Nylon 6.6), to minimize mechanical impact on the sample and prevent loss of small fragments. During the experiments, environmental parameters and physical, chemical and biogeochemical properties of the water column and sediments were measured (Lott et al., 2021).

2.2.1. Eulittoral incubations

Polymer films were buried in sediment retrieved from Fetovaia Beach (Elba, Italy, 42°44′00.1″N 010°09′15.3″E) and placed inside 60 L plastic bins in a former salina basin on the Island of Elba, Terme di San Giovanni, Portoferraio (42°48′12.1″N 010°19′01.0″E). The polymers

experienced flooding during high tides and falling dry during low tides. Incubations ran from April 2014 to March 2016.

2.2.2. Benthic and pelagic incubations

The benthic and pelagic incubations were performed in the marine protected area of the National Park Tuscan Archipelago off the island of Pianosa (42°34′41.4″N 010°06′30.6″E). For the pelagic incubations, the HYDRA® test frames were attached to a mooring and kept suspended at a water depth of ~20 m. For the benthic incubations, the test frames were placed on the seafloor at ~40 m depth and fixed to the sediment surface with iron U-bars, close to the mooring of the pelagic samples. These incubations ran from July 2014 to June 2016.

2.3. Plastic sampling, preservation, and imaging

Samples/films were retrieved at five time-intervals: 2.5 months, 5 months, 7.5 months, 10 months and 22 months (Supplementary Table A.1) and processed in the lab, as described earlier (Lott et al., 2021). Samples were collected in triplicate, except for timepoint five, where benthic and pelagic set-ups were only sampled in duplicate, and PHB in the eulittoral scenario only retained enough material for a duplicate sample due to deterioration. Hence forward, the combination of biological replicates is defined as treatments. Subsamples of the plastic films were preserved for DNA extraction in RNAlater (25 mM Sodium Citrate, 10 mM EDTA, 700 g/L ammonium sulfate, pH 5.2) (Lader, 1998) at −20 °C, and subsamples for SEM imaging were preserved with Trumps fixative (Electron Microscopy Sciences, Hatfield, PA, USA) at 4 °C. The samples for SEM were prepared and imaged as described in Theirlynck et al. (2023).

2.4. DNA extractions and SSU rRNA V4-V5 hypervariable region amplicon sequencing

DNA extractions were performed as described in Vaksmaa et al. (2021b). In short, polymer samples were removed from RNAlater solution and extracted with the Powersoil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), with the following modifications to the manufacturer's recommendations. After gentle shaking to remove RNA later, film subsamples of 0.5 × 0.5 cm were directly added to the PowerBead tubes. We added a bead beating step (4.00 m/s for 30 s, 3 times, 30 s dwell time) to the protocol, replacing the original cell lysis step. The final elution volume was carried out with 30 μL of elution buffer.

The V4-V5 region of SSU rRNA genes was amplified in triplicate using the universal primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3′) (Parada et al., 2016) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3′) (Quince et al., 2011), that amplify all three domains of life. Each amplification contained 12.5 μM of both the forward and reverse primer, 5 mM dNTPs (Genecraft, Cologne, Germany), 5 μL 5× SuperFi buffer and 0.25 μL (2 U/μL) Platinum SuperFi DNA polymerase v1 (Invitrogen, Waltham, MA, USA), and 0.15 to 3 μL of extracted DNA. The reaction volume was adjusted to 25 μL using PCR grade water. Negative control amplifications were carried out in each batch of PCR amplifications by replacing the DNA template with PCR grade water. The forward and reverse primers contained a unique 12-nucleotide Golay barcode for each sample (Caporaso et al., 2012). Amplification was performed in a thermocycler with the following program: 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 50 °C for 15 s, 72 °C for 30 s, with final elongation at 72 °C for 5 min.

After amplification of individual reactions was confirmed by gel electrophoresis, the three technical replicates were pooled, and gel electrophoresis with a 2 % agarose gel containing DNA quantification standards was performed. The 16S and 18S rRNA gene amplicons were quantified individually using a G:Box F3LFB gel imager (Syngene, Cambridge, UK). Then, both the 16S rRNA gene fragment and 18S rRNA amplified product (if observed) were pooled separately in equimolar

amounts. Concentration of the pooled samples was done with the QIAquick PCR purification kit (Qiagen, USA), after which they were run on a 1 % agarose gel. The equimolar pooled 16S and 18S rRNA gene amplicons were then excised from the gel and purified with the QIAquick gel extraction kit (QIAGEN, Venlo, Netherlands). After quantification with a Qubit (Invitrogen, Waltham, MA, USA), the 16S and 18S rRNA gene product pools were pooled together in an equimolar ratio. Illumina MiSeq 2 × 300 bp sequencing (Illumina, San Diego, CA, USA) was then carried out at the USEQ (Utrecht Sequencing Facility) in Utrecht, Netherlands. Raw sequence data have been deposited in the European Nucleotide Archive (ENA) available through the European Bioinformatics Institute under BioProject PRJEB71695. Associated metadata is compiled in MIMARKS table format, which can be found in Supplementary file B.

2.5. Sequencing data analysis

We analyzed our sequencing data with the amplicon sequence data analysis pipeline Cascabel v4.5.2 (Abdala Asbun et al., 2020), set to the Amplicon Sequence Variants (ASV) workflow. The differences in sequence length between the (archaeal and bacterial) 16S and (eukaryotic) 18S rRNA hypervariable regions allows for the simultaneous analysis of all three domains of life (Needham and Fuhrman, 2016). Hence, we used Cascabel with the regular paired-end workflow for the shorter 16S rRNA gene amplicon fragments, and the paired-end unpaired workflow for the longer 18S rRNA gene fragments, of which the read pairs did not have a minimum overlap of 10 nucleotides. The Cascabel pipeline settings for both workflows and the corresponding report files can be found in Supplementary file C. Archaeal and bacterial taxonomy was assigned using the ARB Silva database v138.1, ref.nr 99 (Quast et al., 2012) and eukaryotic taxonomy was assigned using PR2 v5.0.0 (Guillou et al., 2012). This resulted in separate ASV count tables and representative sequence taxonomic assignments for both the archaeal/bacterial (16S) and eukaryotic (18S) datasets. Single singletons (sequences with only one read count across the entire dataset) were removed, and the resulting ASV matrices were used for further analysis in R-studio version 4.2.1 (R Core Team, 2022). Unless otherwise specified, all plots were generated with the R-package ggplot2 v3.3.6 (Wickham, 2016). Used scripts are available upon request.

MicroDecon v1.0.2 (McKnight et al., 2019) was used to remove contaminating reads present in negative PCR controls from all samples in their respective PCR amplification batches, using the *remove.cont* and *remove.thresh* functions. A subsequent filtering step retained ASVs classified as Archaea or Bacteria and removed chloroplast and mitochondrial reads from the 16S rRNA gene ASV matrix, while only ASVs taxonomically classified as Eukaryota were retained in the 18S rRNA gene ASV matrix. ASVs that were unassigned on the domain level were removed from both matrices.

2.6. Microbial community analysis and multivariate statistics

We assessed our depth of sequencing using rarefaction via the R package Vegan v2.6–2 (Oksanen et al., 2022). Venn diagrams, to identify the number of overlapping ASVs between habitats and polymers, were created using ggVennDiagram v1.2.0 (Gao et al., 2021). Phyloseq v1.40.0 (McMurdie and Holmes, 2013) was used for data curation, and calculating alpha diversity indices. For the 16S rRNA gene dataset, the ASV RA was calculated. For bubble plot/heatmap visualizations, we determined where overlap of the top taxa per sample occurred. Due to the low recovery of eukaryotic reads, the 18S data frame was transformed into a presence/absence matrix before all assigned taxa on the chosen taxonomic level were plotted.

Community beta diversity analyses were performed with Vegan on square root transformed RA data. We performed NMDS ordinations based on Bray-Curtis distances on the ASV level and used the function *veganCovEllipse* to calculate the mean and the standard deviation of the

NMDS scores of the samples per grouping factor. Permutational multivariate analysis of variance (PERMANOVA) was performed on the Bray-Curtis distance matrix used for NMDS using Vegan and pairwiseAdonis v0.4 (Martinez Arbizu, 2020), to test for significant differences between sample sets. We also performed PERMANOVA on the habitat data subsets, with polymer and timepoint as factors. For every model, 9999 permutations were run, and the resulting *p*-values were adjusted by the Benjamini-Hochberg procedure to obtain q-values and reduce false discovery rates. Permutational Multivariate Analysis of Dispersion (PERMDISP) with 9999 permutations was performed with Vegan after PERMANOVA, to test for homogeneity of dispersion between sample sets.

Similarity Percentage (SIMPER) analysis was performed (on the Bray-Curtis distance matrix) to determine key genera responsible for community differences between and within habitats, polymers, timepoints, and interactions of these factors (with 9999 permutations). The ten genera with the highest influences, ordered by cumulative contribution (*cusum*), were selected as key genera for each pairwise test.

Differential abundance of genera was determined with the ALDEx2 package 1.32.0 (Fernandes et al., 2013; Fernandes et al., 2014; Gloor et al., 2016). Taxa that were not present on at least 5 % of the samples and at least 2 replicates with at least 5 reads per sample were excluded from the test. We selected taxa with a Benjamini-Hochberg corrected *p*-value of Wilcoxon test (wi.eBH <0.05) as significantly differentially abundant taxa and presented only significant results. From these results, we filtered taxa that were found influential in SIMPER and/or in the top-4 intersection of the most abundant genera per sample and plotted the effect size of these taxa.

2.7. Identifying plastic biodegrading microbes

Obtained ASV taxonomic assignments were compared against PlasticDB (version of 19-April-2023) (Gambarini et al., 2022), to identify potential plastic degrading genera. Polymers in PlasticDB were categorized into 4 main groups, in relation to our tested polymers: PBSeT, PHA, PE, and other (Supplementary Table A.2). The PBSeT-group contained aliphatic–aromatic *co*-polyesters, PBS and terephthalate containing compounds. The PHA-group contained the general formulized polyhydroxyalkanoates, as well as specific forms with a 3-carbon through 9-carbon backbone, and with various side groups. The PE-group contained polymers consisting of multimeric PE units. The "Other" group contained all other polymers that degrading organisms were described for.

3. Results

3.1. 16S rRNA amplicon sequence analysis of archaeal and bacterial communities

We obtained DNA sequence data for a total of 123 out of 128 samples collected (Supplementary Table A.1). A total of 177,853 archaeal sequences were found to belong to 523 unique ASVs. Bacterial reads were far more abundant, comprising a total of 7,054,424 sequences, representing 28,324 unique ASVs. On average, samples contained 340 bacterial ASVs, and 9 archaeal ASVs. Rarefaction curves for the bacterial and archaeal ASVs (Supplementary Fig. A.2) demonstrated that sequencing depth was sufficient to reach diversity detection saturation in the eulittoral, and for most samples in the benthic habitat, but less so in the pelagic datasets. In the benthic and eulittoral habitats, community alpha diversity peaked on LDPE and was relatively similar for both biodegradable polymers (Supplementary Fig. A.3).

Archaea were present at all sampled locations, though not on all biological replicates. Archaeal RA on samples from the benthic and eulittoral incubations was 3–22 %, while on pelagic samples it was <2 % (Fig. 1A). In total, we detected 10 archaeal and 56 bacterial phyla. Proteobacteria, Bacteroidota, Cyanobacteria, Planctomycetota,

Actinobacteria, Desulfobacterota and Nanoarchaeota, together made up between 75 % - 95 % of the 16S rRNA amplicon reads on all samples. At a finer phylogenetic level, we detected a total of 12 archaeal and 301 bacterial orders. The ten orders with the highest RA were: Pseudomonadales, Rhodobacterales, Caulobacterales, Flavobacteriales, Cythophagales, Rhizobiales, Cyanobacteriales, Desulfobacterales, Desulfobulbales, and Woesearchaeales (Fig. 1B), accounting for 20 % - 85 % of the reads on all samples. We detected a total of eight archaeal and 432 bacterial families. Of these families 98 had an RA >1 %, and 30 unique families belonged to the top 8 of all samples (Fig. 1B). The ten families with the highest RA were Microtrichaceae, Saprospiraceae, Desulfocapsaceae, Desulfobacteraceae, Flavobacteriaceae, Rhodobacteraceae, Cyclobacteriaceae, Cellvibrionaceae, Hyphomonadaceae and Desulfosarcinaceae, comprising 15 % - 75 % of all reads on all samples. Of the ASVs, 11,385 out of 28,847 could be classified to the genus level. This resulted in 968 unique bacterial genera, of which 218 had a RA >0.5 %, but no archaeal genera. The ten genera with the highest abundance were: *Desulfatitalea, Desulfobacula, Muricauda, Desulforhopalus, Desulfobacter, Hirschia, Blastopirellula, Ketobacter, Desulfoconvexum* and *Agarilytica*, representing up to 90 % of all reads per sample (Supplementary Fig. A.4).

The highest number of ASVs was detected in the benthic samples (14,784), followed by the pelagic (13,079) and eulittoral (8604) samples. When we compared the habitats, we found that the pelagic and benthic habitat had the most bacterial and archaeal ASVs (19 %) in common, and that 3 % (980) of the ASVs were shared between all three habitats (Supplementary Fig. A.5). When comparing the polymers, 22 % (6235) of the bacterial and archaeal ASVs were shared. The highest number of unique ASVs were found on LDPE (7695), followed by PHB (5315) and PBSeT (5124) (Supplementary Fig. A.5). Within the same habitat, the fraction of shared ASVs between polymers was highest in the pelagic (28 %), compared to the benthic (13 %) and eulittoral habitat (12 %). Considering the polymers, 2 % of the ASVs were generally shared per specific polymer among all three habitats. Only 17 ASVs overlapped between all polymers-habitat combinations, 14 of which were classified in the phylum of Proteobacteria (data not shown).

3.2. 18S rRNA amplicon sequence variant analysis of the eukaryotic community

We detected eukaryotic reads in all 42 pelagic samples, while benthic samples only had reads in 40 out of 41 samples and eulittoral samples in only 32 out of 40 samples (Supplementary Table A.1). On average, we recovered 5910 eukaryotic reads on pelagic samples, 965 on benthic samples, and 420 on eulittoral samples. Similarly, pelagic incubations yielded the largest number of ASVs (1295), followed by the benthic (616), and eulittoral (177) samples.
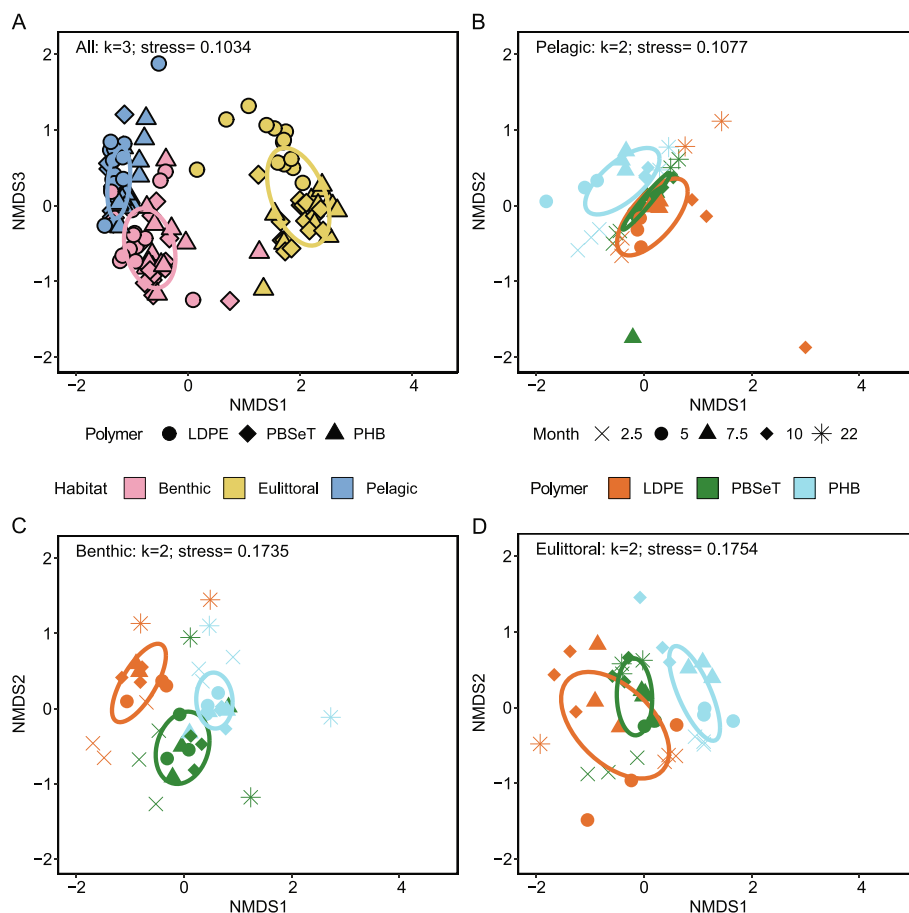
Due to the low sequencing depth of eukaryotes, rarefaction curves not plateauing in most samples (Supplementary Fig. A.2), and issues with large rRNA gene copy number variation in Eukarya, we performed presence/absence analyses for this domain (Fig. 2). The eukaryotes present on most treatments included: annelids, arthropods, bryozoans, cnidaria, diatoms (Bacillariophyceae and Mediophyceae), dinoflagellates (Dinophyceae), and spirotrich ciliates.

All habitats had a total of 39 ASVs in common, while 251 ASVs were shared among polymers, regardless of habitat (Supplementary Fig. A.5). Hydroids were the most commonly detected among these shared ASVs, followed by other metazoans including those from annelid, nematode, molluscan, and crustacean ASVs. Fungal ASVs included the Basidiomycote *Malassezia*, while red algal ASVs included members of the order Ceramiales. Members of the Opisthokonta, (e.g. Ascomycota and Basidiomycota) were found on all pelagic samples and to a lesser extent the two other habitats. Even though the light intensity at the pelagic test system (20 m depth) was measured to be 13 % and at the benthic test system (40 m depth) 3 % of the surface intensity (during sunny middays) and ultraviolet light was considered to be negligible at the tested depths
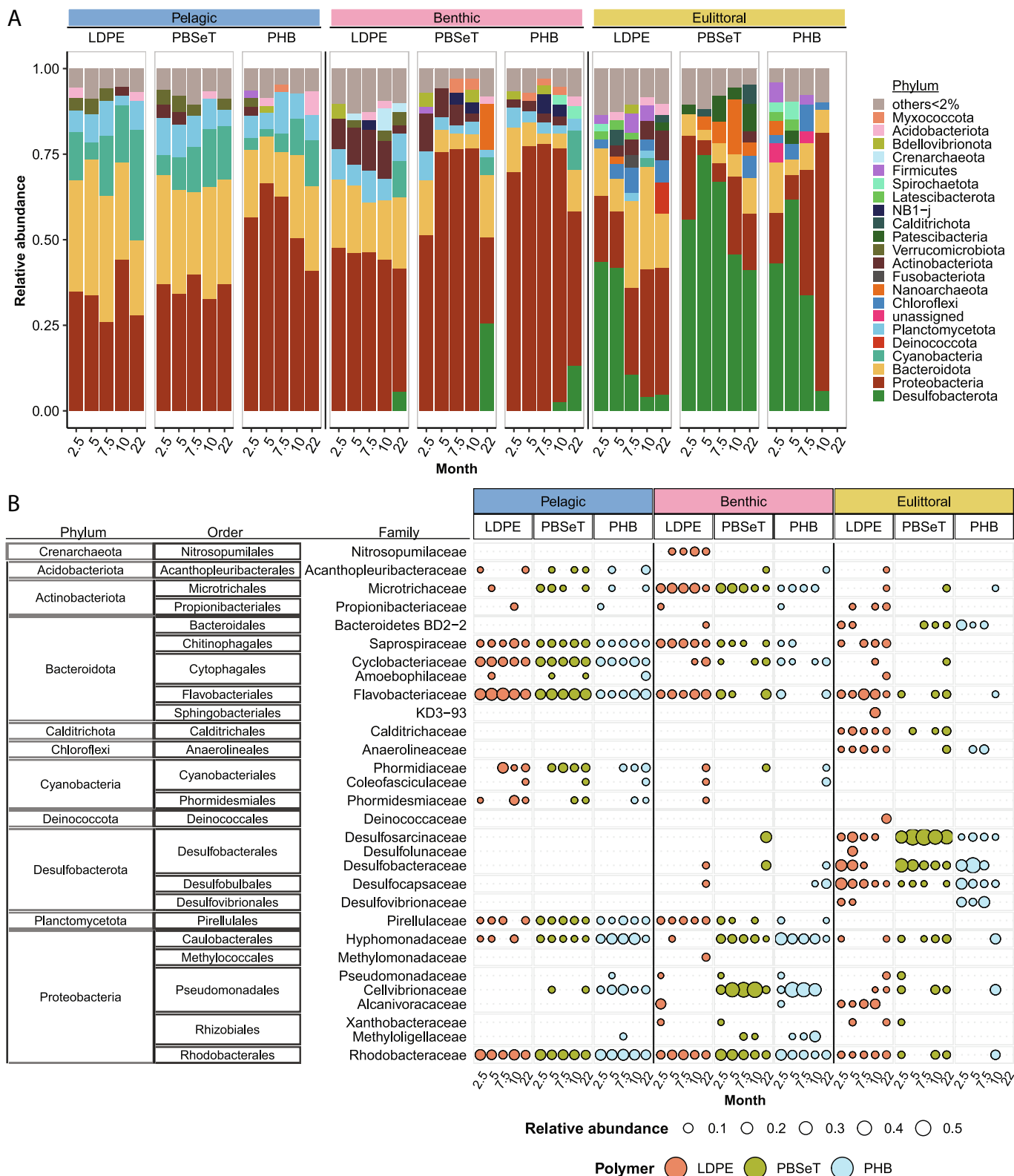
Fig. 1. The most abundant bacterial and archaeal phyla (A) and families (B) on the plastic films at the 5 sampling points (indicated by the time in months on the x-axis). The panels are subdivided into the three different habitats: pelagic (blue), benthic (pink), and eulittoral (yellow), showing the three incubated polymers LDPE (red), PBSeT (green) and PHB (light blue). The bubble plot includes only the most abundant families using the top 8 most abundant families per treatment having a RA of >1 %.

Fig. 2. Presence/absence matrix of eukaryotic taxa, detected on the samples at the 5 sample points (indicated on the x-axis). The three panels represent the 3 different habitats, pelagic, benthic and eulittoral, and the colors the three polymers LDPE, PBSeT, and PHB. The matrix shows a presence when taxa were present on at least 1 replicate per treatment.

(Lott et al., 2020), phototrophs including centric and pennate diatom ASVs occurred in all three habitats (e.g. *Navicula, Lampriscus, Pleurosigma* and *Triceratium*). Phaeophyceae and Pelagophyceae phototrophs were also found across habitats. Rare but detectable ASVs from potential harmful algal blooms or pathogenic species included the dinoflagellates *Alexandrium andersonii, Alexandrium insuetum* and a mesomycetozoean from the family Rhinosporidae.

3.3. Beta diversity bacterial/archaeal community

3.3.1. NMDS

We explored compositional dissimilarity of the combined bacterial and archaeal communities using NMDS. The three different habitats formed distinct clusters, with the eulittoral habitat being most distinct from the pelagic and benthic (Fig. 3A). Within the habitats, samples of

Fig. 3. Nonmetric multidimensional scaling (NMDS) plots of square root-transformed Bray-Curtis dissimilarity indices. (A): All samples from all investigated habitats plotted in 2 dimensions after a 3-dimensional NMDS was performed showing a distinction between habitats (benthic in pink, eulittoral in yellow, and pelagic in blue), and polymers (LDPE (circles), PBSeT (diamonds), and PHB (triangles)). In the other panels, samples are plotted per habitat, after a 2-dimensional NMDS was performed: (B) pelagic; (C) benthic and (D) eulittoral, with a separation visible between the polymers LDPE (orange), PBSeT (green) and PHB (light blue). Timepoints represented in these plots are 2.5 months (cross), 5 months (circle), 7.5 months (triangle), 10 months (diamond) and 22 months (star). The centroid and radius of the ellipses in all panels represent the mean and the standard deviation of the ordination of the samples in the indicated groups.

the different polymers clustered together (Fig. 3B-D). In the eulittoral habitat, PHB samples separated clearly from the two other polymers and in the pelagic habitat the PHB was slightly separated from the other polymers. In the benthic habitat, LDPE separated from the biodegradable polymers. We also observed a clear time gradient as the communities changed in response to seasonal changes. This effect was more pronounced in the pelagic and eulittoral habitats. Pelagic samples formed clusters that migrated through the NMDS space with each successive sampling (Supplementary Fig. A.6), and also clustered into the three different seasons in which they were sampled, namely autumn, winter, and two subsequent springs (Supplementary Fig. A.7). In the eulittoral habitat there was a separation between samples from the first two timepoints, and the three later ones, which corresponded to summer and autumn samples separating from winter samples. In the benthic habitat the pattern was less clear, with only clustering of samples by first- and second-year sampling.

3.3.2. Statistical analyses: PERMANOVA and PERMDISP

Statistical analysis using PERMANOVA on the complete dataset showed significant effects of all factors (habitat, time, season, and polymer) and most interactions thereof (q-values <0.0004, Supplementary Table A.3). Inter-habitat pairwise PERMANOVA (on the complete dataset) testing polymers and habitats, and the combination habitat × polymer as factors (Supplementary Table A.4 and A.5 respectively), resulted in significant differences between all three habitats (q = 0.0001), and all three polymers (q < 0.0007) and showed that

communities on a given polymer significantly differed between habitats (q = 0.0011). Testing time as a factor (Supplementary Table A.6) showed low significant differences in 2.5 versus 5 months and 10 versus 22 months (q = 0.05), and no significant differences between the other consecutive timepoints (q > 0.1), which aligns with the temporal trends observed by NMDS. We uncovered that the observed differences during the overall test were in most cases a reflection of the individual habitats, by performing intra-habitat pairwise PERMANOVA to further investigate the differences between polymers and timepoints within habitats (Supplementary Table A.7). All polymers differed significantly in both the eulittoral and benthic habitat (q = 0.001), while in the pelagic habitat significant differences were found between PHB and both PBSeT and LDPE (both q = 0.001), but not for other polymer combinations. In all habitats, significant differences were detected between samples taken after 2.5 and 5 months (q < 0.03). Furthermore, samples taken after 10 and 22 months differed significantly in the benthic and pelagic (q < 0.01), and additionally the time gradient in the pelagic was confirmed by weakly significant differences between timepoints 5 versus 7.5 and 7.5 versus 10.

Test for homogeneity of dispersion (PERMDISP) on the complete dataset (Supplementary Table A.8) showed differences in dispersion for habitats (q = 0.0007), which also influenced interactions with habitat (data not shown), indicating that besides differences in cluster location dispersion might contribute to the observed differences. For the other factors and interactions (time, polymer, and time × polymer) differences in dispersion were not significant (q > 0.16). We tested whether the

overall observed dispersion of clusters was caused by inter-habitat differences or if they might be habitat-specific, with intra-habitat pairwise PERMDISP tests. These showed no significant differences in dispersion between timepoints in all three habitats (Supplementary Table A.8). Furthermore, cluster dispersion affected most of the intra-polymer differences in the eulittoral, with the exception of PHB versus LDPE, but had no effect on pairwise polymer-polymer differences in the two other habitats (Supplementary Table A.8).

3.4. SIMPER analysis

Pairwise SIMPER analysis was conducted to identify the key genera contributing to dissimilarities between habitats and polymers. From a total of 15 tests performed, we selected the top 10 influential genera based on cumulative differences. On the genus level, ungrouped analysis revealed a 72.9 % overall dissimilarity, with the top 10 genera responsible for 10 % (Fig. 4A). For the specific habitats, overall dissimilarities were ~70 % (eulittoral), ~65 % (benthic) and ~50 % (pelagic). Again, the top 10 genera accounted for a similar range (~10 %) in cumulative dissimilarity. In the eulittoral, however, this was 20 % and higher (Fig. 4B-D). Generally, between-habitat dissimilarity for single polymers surpassed intra-habitat polymer dissimilarity. This was most obvious between the eulittoral and benthic habitat. However, the contribution of single genera showed the highest dissimilarities when comparing polymers within the eulittoral habitat, specifically PBSeT against PHB (top 10 cumulative difference of 31.2 %). For the pelagic zone, we found the lowest dissimilarities for all of the single tests. On a temporal scale, the highest dissimilarity for single genera was found when comparing the sequential timepoints 10 versus 22 months, irrespective of habitat.

However, since this timeframe spanned a year between sampling, we compared the first timepoint (2.5 months) with 10 months as well, and that showed dissimilarity caused by single genera was generally higher between those timepoints.

Single genera typically accounted for <1 % dissimilarity. Genera surpassing 1 %, and also the average contribution of ungrouped tests, were deemed influential in driving the inter-group variations identified by NMDS and PERMANOVA. Most notably, *Hirschia* affected differences between benthic and eulittoral habitats, and distinguished PBSeT and PHB in the eulittoral compared to the other habitats. The latter was also the case for *Cognatishimia*, but to a lesser extent. The third most influential genus, *Arenicella*, mostly differentiated PHB in the benthic versus eulittoral habitat. In the pelagic sample subset, *Roseofilum* AO1-A contributed not only to the differences observed between polymers, but also between sampling points. *Desulfatitalea*, *Alcanivorax* and *Agarilytica* contributed greatly to the dissimilarity between PBSeT and PHB in the eulittoral subset. In the subset of benthic samples, only *Agarilytica* showed a similar impact. From the top 10 most influential genera, *Alcanivorax* and *Desulfovibrio* (eulittoral subset) and *Pseudomonas* (pelagic subset) are reported in PlasticDB.
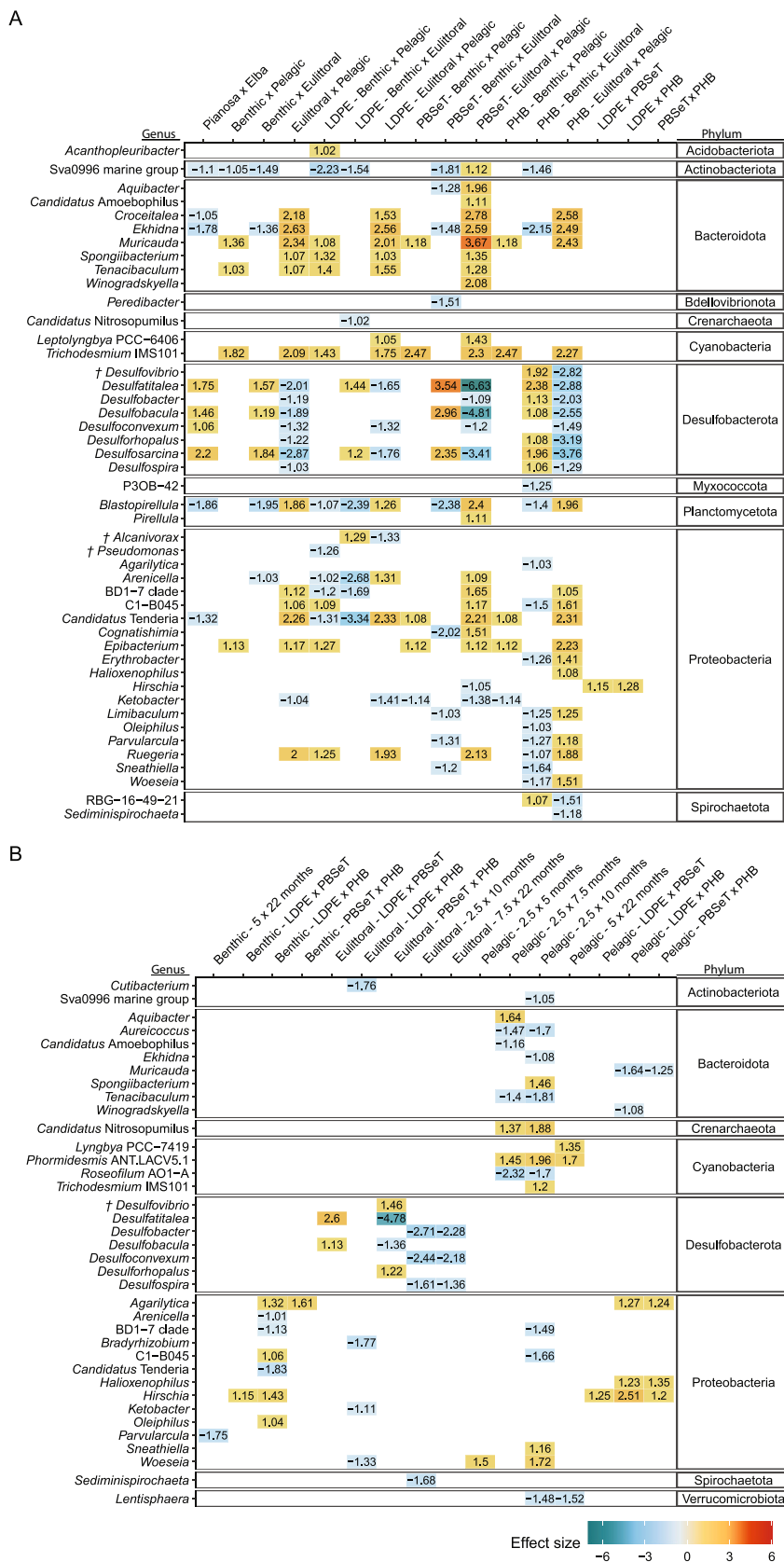
3.5. Differential abundance analyses

In total 62 pairwise differential abundance tests were performed at the genus level, of which 32 yielded significant outcomes, resulting in 261 unique assigned differentially abundant genera in all tests combined. Of these genera, 77 (Fig. 5) were also influential in the SIMPER analysis (Fig. 4) and/or found in the top five intersection (Supplementary Fig. A.4).
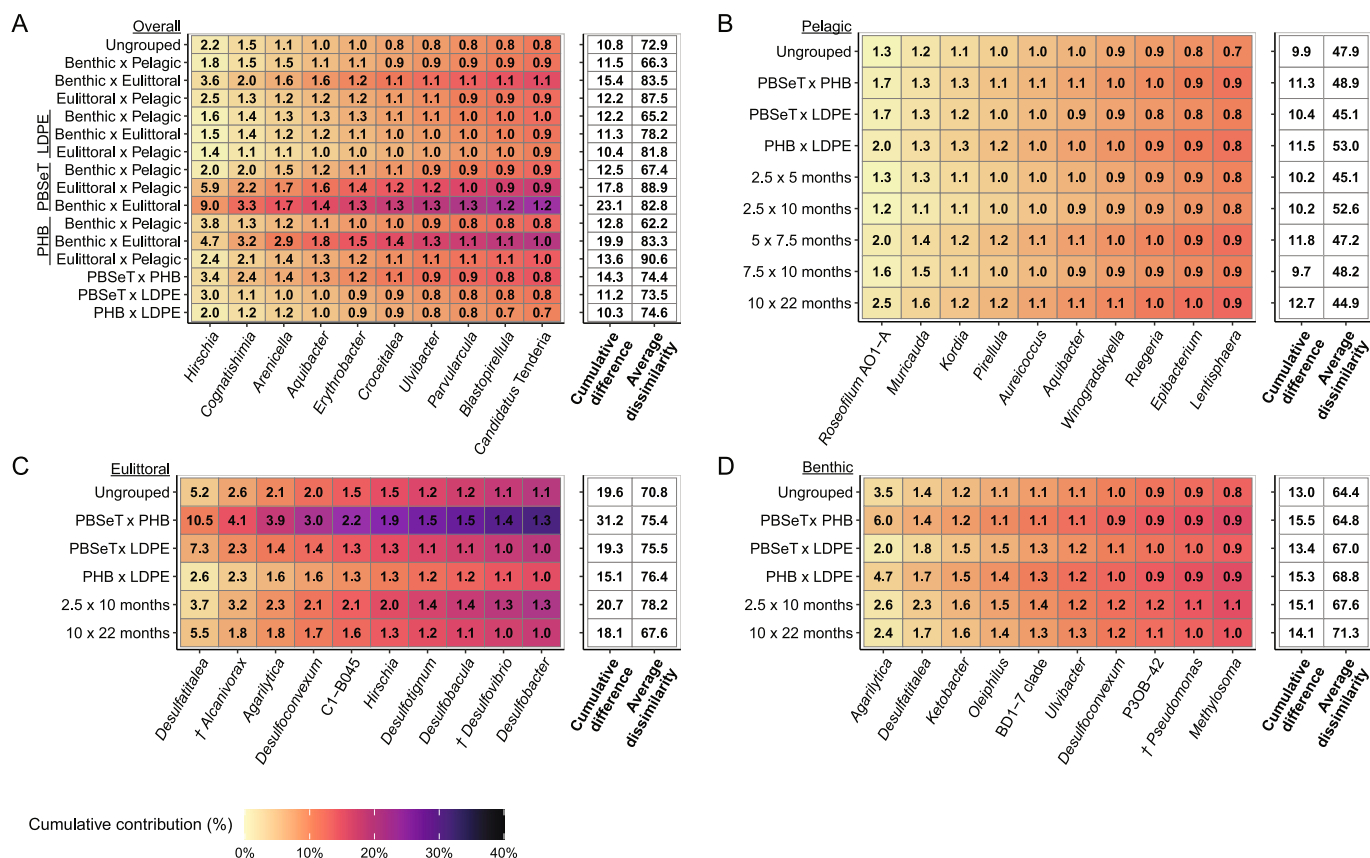
Fig. 4. Outcome of the overall SIMPER tests (A), and tests performed with samples exclusively from pelagic (B), eulittoral (C) and benthic (D) habitats. The ungrouped analysis highlights the top ten most influential genera (x-axis), with pairwise analysis providing insight into the specific impact of these genera. The numbers indicate the percentage of contribution to Bray-Curtis distances by these genera. The cumulative difference of these ten influential genera and average dissimilarity (the average difference between groups based on Bray-Curtis dissimilarity), are shown on the right side in each panel. Only sample groups that were significantly different according to PERMANOVA were compared. Colors indicate cumulative difference, and genera found in PlasticDB are indicated with †.

Fig. 5. Differentially abundant genera between tested conditions (wi.eBH <0.05), with an absolute effect size >1, also found to be influential in SIMPER analysis and/or found in an intersection of the top-5 genera per sample (Supplementary Fig. A.4). Panel A shows tested comparisons between habitats, while panel B shows tested comparisons within habitats. Effect size gives change in RA between two conditions. Negative means decrease and positive means increase between two listed habitats/polymers. Genera found in PlasticDB are indicated with †.

Between the Pianosa and Elba sites, 165 distinct genera exhibited differential abundance. Our pairwise tests yielded the following results: 101 genera exhibited differential abundance between the eulittoral and benthic habitats, 204 between the eulittoral and pelagic habitats, and 130 between the pelagic and benthic habitats. At the phylum level, Desulfobacterota exhibited a higher RA in the eulittoral scenario (Elba site) compared to the benthic scenario (Pianosa site), and a higher RA in the benthic scenario compared to the pelagic scenario (Pianosa site). Conversely, Bacteroidota showed higher RA in the pelagic habitat compared to both the benthic and eulittoral habitats and Proteobacteria displayed higher RA in the benthic habitat compared to the pelagic habitat and higher RA in the pelagic habitat compared to the eulittoral habitat.

Comparison of the three different polymers revealed three genera with differential abundance between LDPE and PBSeT, 24 between LDPE and PHB, and 8 between PBSeT and PHB. In terms of overall differences between the various polymers, we observed a higher RA of *Hirschia* on both biodegradable polymers compared to LDPE. In the overall comparison of the biodegradable polymers (i.e. without considering habitat), the genera *Agarilytica, Halioxenophilus,* and *Salinimonas* had a slightly higher RA (effect size >1) on PHB than PBSeT.

To pinpoint genera driving the differences on biodegradable polymers, we analyzed sample subsets of individual polymers. For biodegradable polymers, Proteobacteria and Desulfobacterota significantly distinguished the eulittoral habitat (Elba site) from the other two (Pianosa site), and several Desulfobacterota genera favored the eulittoral habitat over the benthic, and the benthic over the pelagic. Proteobacteria, particularly the orders of Caulobacterales, Enterobacterales, Pseudomonadales, and Rhodobacterales, were identified as key contributors to biodegradable polymer distinctions. Specific differentially abundant genera are discussed in more detail in Section 4.3. Both biodegradabe polymers had a higher RA of *Epibacterium, Muricauda* and *Trichodesmium* IMS101 (Cyanobacteria) in the pelagic habitat, while *Ketobacter* (Gammaproteobacteria) dominated in the benthic habitat. On both biodegradable polymers, *Blastopirellula, Ekhidna, Granulosicoccus, Parvularcula, Sneathiella*, and the Sva0996 marine group demonstrated higher RA in the benthic over the eulittoral. Both biodegradable polymers displayed a higher RA of BD1–7 clade*, Blastopirellula, Muricauda, Croceitalea, Ekihdna* and *Trichodesmium* IMS101 in the pelagic. Additionally, *Bradyrhizobium, Hirschia,* and *Ketobacter* displayed higher RA in the pelagic on PBSeT. The numbers of different genera found per test suggested that the differences in communities on LDPE were mainly driven by sediment versus water column, while on both PBSeT and PHB geographical location seemed to be the main driver.

The analysis of habitat-specific differences among the three polymers revealed significant variations in RA for multiple genera. Notably, the distinctions between LDPE and the biodegradable polymers were more pronounced and numerous compared to the disparities between the two biodegradable polymers. In the eulittoral habitat, PBSeT and PHB showed significant RA differences in 14 genera, while benthic and pelagic habitats exhibited differences in 2 and 15 genera respectively (data not shown). In the eulittoral habitat, PHB displayed higher RA of *Desulfovibrio* and *Desulfoconvexum*, while PBSeT showed higher RA of *Arcticiflavibacter, Desulfobacula*, and *Desulfatitalea*. In the benthic habitat, *Hirschia* had higher RA on the biodegradable polymers than LDPE, and *Desulfobacula* along with *Desulfatitalea* had higher RA on PBSeT. In the pelagic habitat, PBSeT showed higher RA of *Winogradskyella* and *Muricauda*, whereas PHB exhibited higher RA of *Pseudooceanicola, Halioxenophilus*, and *Agarilytica*. The RA of *Hirschia* in the pelagic habitat, was highest on PHB, followed by PBSeT, and lowest on LDPE. Four genera were found in plasticDB: *Staphylococcus, Pseudomonas, Desulfovibrio*, and *Alcanivorax*.

3.6. SEM imaging of plastics and their plastisphere members

Disintegration of the polymers in situ was observed only for the PHB and PBSeT films in the benthic and eulittoral habitats (Lott et al., 2021). Our SEM imaging of these samples corresponded to the disintegration data, showing a clear increase in the size and number of cracks in the surface of the PBSeT and PHB films in the eulittoral and benthic samples over time (Fig. 6). This crack formation was not observed in the pelagic habitat (Supplementary Fig. A.8 and Fig. A.9), and the LDPE films did not show any obvious signs of surface deterioration in any habitat (Supplementary Fig. A.10). The disintegration half-life of PHB was shorter in the eulittoral than the benthic habitat, and the half-life of PBSeT was generally longer, but comparable in the eulittoral and benthic habitats. PHB films in the eulittoral habitat indeed had a similar disintegration pattern at 10 months as in the benthic habitat at 22 months. However, in our SEM images, bigger cracks were observed in the PBSeT film in the eulittoral habitat compared to the benthic habitat at 2.5 and 22 months, as well as for PHB films at 2.5 months.

SEM imaging also showed biofilm development on all films, irrespective of habitat. Both the area covered by microbes, and a thicker biofilm developed over time. This was especially the case with the substrates in the pelagic habitat. We also found a high abundance of diatom cells on pelagic samples, with fewer on benthic samples. Apart from an occasional intact diatom, mostly fragments of diatom frustules were observed in the biofilms of eulittoral samples. Various structures resembling fungal hyphae were also identified on different polymer samples (Supplementary Fig. A.11).

3.7. Plastic degrading bacterial genera

Several of the bacterial and fungal genera in our dataset are reported in the PlasticDB as having plastic degrading potential. In total 64 bacterial genera with plastic degrading potential were identified (Supplementary Fig. A.12), 33 of which had a RA >0.1 % on at least one replicate (Fig. 7). These genera accounted for 3.7 ± 8.7 % (mean ± standard deviation) of the total read abundance on average per sample, with an abundance of 2.3 ± 1.6 %, 2.6 ± 2.5 % and 6.3 ± 8.2 % in the pelagic, benthic and eulittoral habitats, respectively. On the polymers, the average abundance of genera from PlasticDB was 1.7 ± 1.4 %, 3.6 ± 8.0 %, and 5.8 ± 3.2 % on PBSeT, PHB and LDPE, respectively. Of all treatments, the highest number of plastic degrading genera was present on LDPE in the eulittoral habitat, on PHB in the pelagic habitat, and PBSeT in the benthic habitat. The RA of potential plastic degraders generally decreased over time on PBSeT and PHB. The exception was with PHB at 7.5 months, which had high RA of *Desulfovibrio*. Plastic degraders found on the biodegradable polymers but not on LDPE included *Schlegelella, Bacteroides, Rheinheimera,* and *Shewanella*. The genera *Marinobacter, Variovorax, Paracoccus, Schlegelella, Bacteroides, Micrococcus, Microbacterium* and *Oleispira* were detected on PBSeT but not on PHB. In contrast, *Pseudoxanthomonas, Rheinheimera* and *Shewanella* were found on PHB but not PBSeT. Furthermore, certain plastic degraders were found on LDPE but not the biodegradable polymers, e.g. *Alcanivorax, Brevundimonas, Sphingobacterium, Bacillus, Pantoea, Duganella, Nocardioides, Mycobacterium, Ralstonia, Flavobacterium,* and *Kocuria*.

The top 9 plastic degrading genera on the two biodegradable polymers, over all habitats and polymers were *Desulfovibrio, Stenotrophomonas, Aestuariibacter, Aliiglaciecola, Alteromonas, Corynebacterium, Staphylococcus, Bdellovibrio*, and *Pseudomonas* (Fig. 7). *Desulfovibrio* had an especially high RA on PHB and LDPE in the eulittoral habitat. *Stenotrophomonas* was detected on all three polymers and had the highest abundance in the eulittoral habitat on PHB and LDPE. *Aestuariibacter* was mainly detected in the pelagic and benthic habitats, according to differential abundance analysis. PHA degradation has been reported for *Aestuariibacter*, but it showed no significant preference for PHB in our study. *Aliiglaciecola* was detected on both PBSeT and PHB, and it had the highest RA in the pelagic, followed by the benthic. *Alteromonas* was predominantly detected in the pelagic on PHB. *Corynebacterium* was present on all three polymers, and had the highest
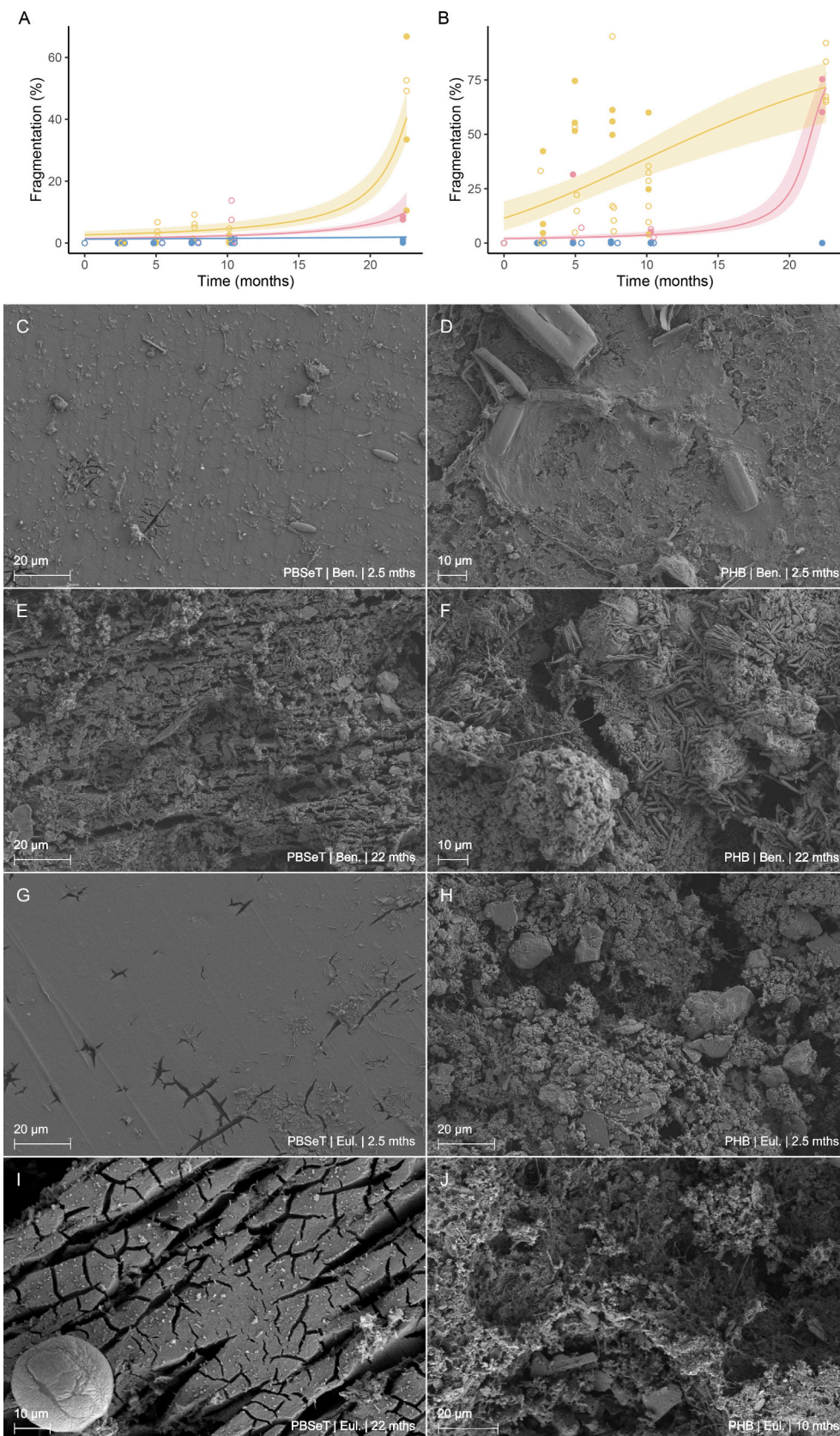
Fig. 6. The observed disintegration data of the PBSeT (A) and PHB (B) films (adapted from (Lott et al., 2021)) in the different habitats: pelagic (blue), benthic (pink) and eulittoral (yellow). Filled points indicate samples that were sequenced and imaged for this study. Scanning Electron Microscopy (SEM) images were taken of representative fields of view from benthic incubated samples of PBSeT (C) and PHB (D) retrieved habitat after 2.5 months, and PBSeT (E) and PHB (F) retrieved after 22 months. Also imaged were the eulittoral incubated samples of PBSeT (G) and PHB (H) retrieved after 2.5 months, and PBSeT retrieved after 22 months (I) and PHB after 10 months (J).
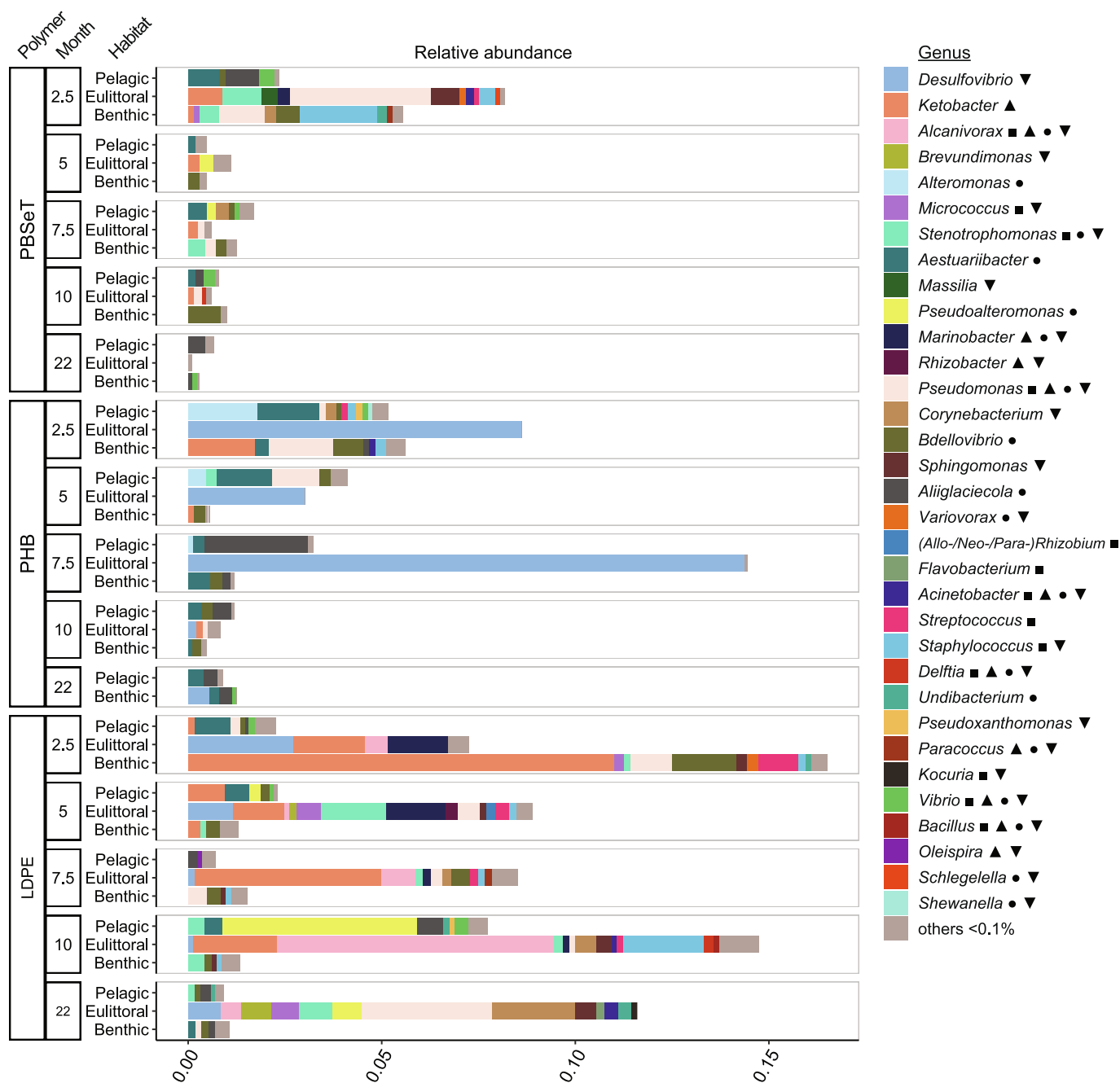
Fig. 7. Relative abundance of bacterial genera in our study with known plastic degrading representative species. Symbols after genus names indicate polymers for which degradation was reported for a given genus in the PlasticDB database (Gambarini et al., 2022) (▲ = PBSeT, ■ = PE, ● = PHA, ▼ = Other).

abundance in the eulittoral, followed by the pelagic. *Staphylococcus* was mainly detected on LDPE and PBSeT, but is not reported for degrading these polymers. It had the highest RA in the benthic, followed by the eulittoral. *Bdellovibrio* was widespread on all polymers in all habitats, and, it had the highest abundance in the benthic, and on LDPE and PHB. According to differential abundance analysis, *Pseudomonas* had the highest abundance in the benthic, followed by the eulittoral, and it was mainly found on LDPE and PBSeT. In total, 18 genera from PlasticDB were differentially abundant (Supplementary Fig. A.13), of which *Alcanivorax*, *Desulfovibrio* and *Pseudomonas* were also influential according to SIMPER analysis (Fig. 4).

3.8. The presence of fungi

In total, we detected 30 fungal genera, belonging to phyla Aphelidiomycota, Ascomycota, Basidiomycota and Chytridiomycota (Fig. 8). Eulittoral LDPE harbored the greatest number of fungal genera overall, followed by benthic LDPE, and pelagic LDPE. Presence of fungal genera on PBSeT followed the order of benthic > eulittoral > pelagic. On our PHB incubations, we found the most fungal genera in the benthic habitat, with only one fungal type associated with PHB incubations in the pelagic (*Malassezia*), and eulittoral (*Ascosphaera*) samples respectively. *Malassezia* was the most cosmopolitan fungal type we detected across habitats, timepoints, and polymer types.

Six of the fungal genera we detected on our plastics were included in PlasticDB. *Acremonium* sp. and *Debaryomyces* sp. were reported to
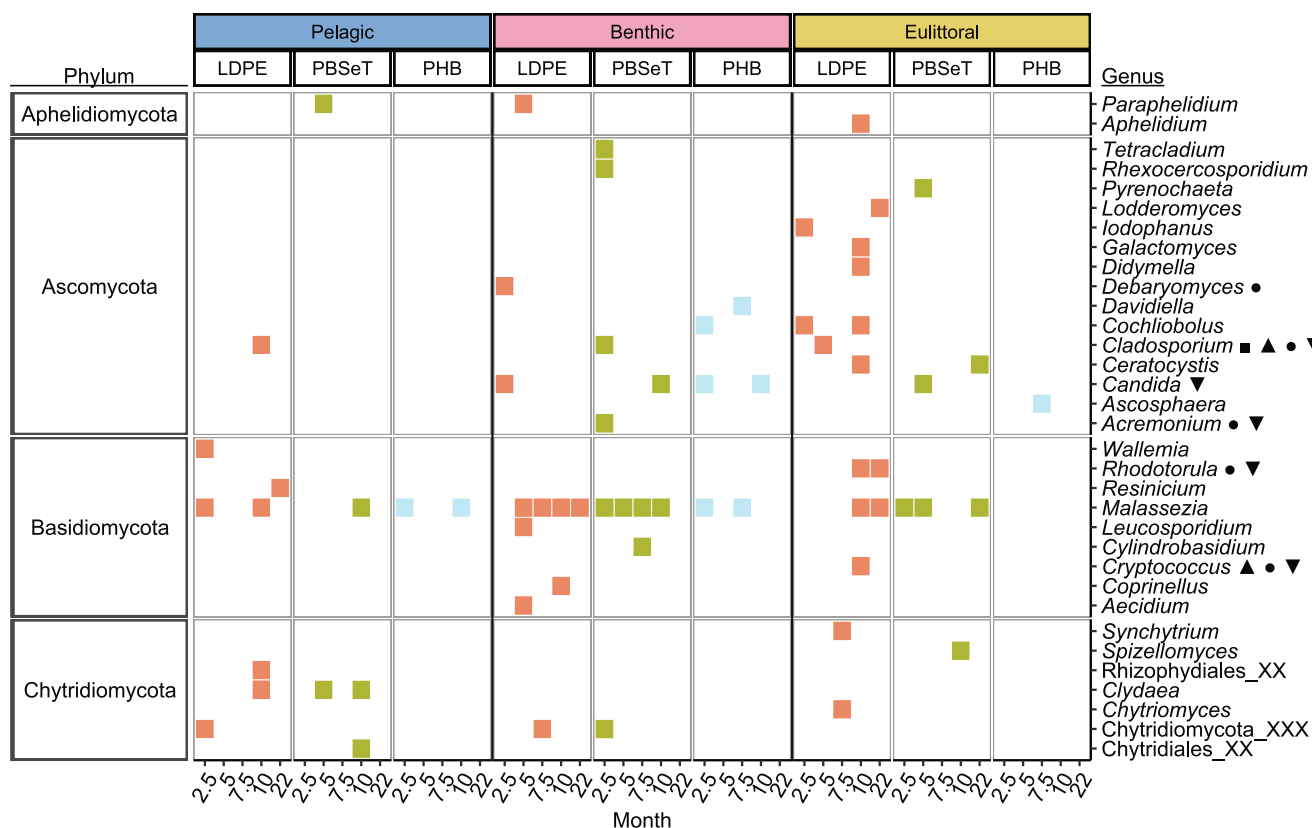
Fig. 8. Presence/absence of fungal genera on our sampled polymers. Symbols behind genus names indicate the polymers for which biodegradation was discovered, per genus, as included in the PlasticDB database (Gambarini et al., 2022). (■ = PE, ▲ = PBSeT, ● = PHA, ▼ = Other).

biodegrade PHB and other PHAs, but we did not detect them in our PHB incubations. *Candida* sp. was found on the three different polymers in the benthic habitat and on PBSeT in the eulittoral habitat but was not reported to degrade any of our substrates, only polyurethane. *Clado-sporium* entries in PlasticDB agreed partially with the polymers we found it on, as we detected it on PE and PBSeT, however not on PHB. Both *Rhodotorula* sp. and *Cryptococcus* sp. occurred only on LDPE in the eulittoral samples and not on either of our biodegradable polymers despite being reported to biodegrade PHB in the case of *Rhodotorula* and PHA, PHB and PBS in the case of *Cryptococcus*.

4. Discussion

The development of biofilms on biodegradable plastics is still poorly understood, since our understanding of microbial succession during colonization of plastics under field settings is largely shaped by incubation studies of conventional (petroleum-based), non-biodegradable plastics. Overall, abiotic environmental factors including season and location are considered the major drivers in shaping the microbial community (Amaral-Zettler et al., 2020; Wright et al., 2021). However, we do not know if this also holds true for biodegradable polymers, which are fundamentally different from 'inert' surfaces since they can be degraded and used as a carbon or energy source. This means the surface changes over time and interacts with the community, which means the community might experience stronger influences from the substrate. In the present study, we investigated long-term in situ incubations in three different coastal habitats, comparing biodegradable PBSeT and PHB with non-biodegradable LDPE. This allowed us to assess the interplay between various factors influencing biofilm development and gain a better understanding of microbial succession on these polymers under realistic field conditions.

4.1. Bacterial communities show significant differences between polymers and habitats

An ongoing question in the microbial ecology of the plastisphere is whether communities differ between polymer types and if these differ from natural hard substrates (e.g. shell, glass or wood). Our overarching hypothesis was that biodegradable polymers would attract a distinct microbial community capable of utilizing these substrates as an energy source, with a focus on observing differentiation between different polymers rather than between timepoints or seasons.

Sample clustering based on polymer type was visible in our NMDS plots, and PERMANOVA and SIMPER analysis confirmed there were significant differences and substantial dissimilarities between the polymer groups. The degree of dissimilarity observed with these tests, however, was habitat specific. In the eulittoral and benthic habitats, all polymers were significantly different, while in the pelagic these differences were less pronounced (Figs. 3 and 4). Contrary to our expectations, LDPE was not always more dissimilar from the biodegradable polymers than the biodegradable polymers from each other. Note that here we are combining results from biofilm samples that varied in age from 2.5 to 22 months, and it has been suggested that different plastics initially select for distinct colonizing communities when microbes are interacting with the plastic surface chemistry and physics, but that communities converge over time, as the biofilm develops and there is less influence of the plastic surface on a colonizing microbe (Datta et al., 2016; Pinto et al., 2019; Wright et al., 2019).

Past incubation studies observed significantly different communities on different conventional plastic polymers (e.g. Kirstein et al., 2018; Oberbeckmann et al., 2017). However, most of these studies were conducted on polymers from the water column at one geographical location, for relatively short times. Nonetheless, ocean surface plastic samples, show clear differences in plastisphere communities between global

ocean basins (Amaral-Zettler et al., 2015). Additionally, a recent meta-analysis (specifically targeting conventional plastic matrices) demonstrated that across wider scales, environmental and biogeochemical variables, rather than polymer type, appeared as key factors affecting the bacterial and archaeal composition of the plastisphere (Oberbeckmann and Labrenz, 2020; Wright et al., 2021). The question of whether these large-scale biogeographic differences extend to smaller scales is still an open question, as comparative incubation studies performed in different habitats in one geographic area are limited (Latva et al., 2022), especially over longer times (Lemonnier et al., 2022).

Corroborating our PERMANOVA and NMDS analyses, SIMPER and differential abundance analysis identified both geographical location and habitat as the main drivers of community differences, more so than the polymer effect. Samples from the eulittoral differed the most from the two other habitats. Substrates here were buried 10 cm deep in beach sand, at a different geographical location that is more coastal and has more anthropogenic influences. They were also impacted by the daily tidal cycle, which was also reflected in the measured environmental factors. These fluctuated severely during the incubation experiment, i.e. pH from 7.6 to 8.2, salinity from 36 to 42 ppt, oxygen availability of 20–80 % air saturation, and daily temperatures of 11–30 °C (Lott et al., 2020), and this would put considerable stress, as well as the need to adapt on the microbial community. Especially the oxygen availability reflects that at least some oxygen limitation would have occurred. Our eulittoral samples also contained more (anoxic) sediment-associated microorganisms (e.g. sulphate reducers). The salina basin in which the bins with the beach sediment were set up was also filled with very fine anoxic sediment. The water that flushes the beach sediment bins would have therefore been influenced by that anoxic community or contain this DNA. Our results support the conclusion that habitat was a prime element in shaping microbial communities associated with biodegradable plastics in our study.

Given the long sampling intervals during our study, we anticipated pronounced differences in community composition between time points due to seasonality, but also since disintegration rates of the biodegradable polymers varied between timepoints. The pelagic and the eulittoral incubations experienced more seasonal environmental fluctuations over the course of the incubations, while conditions in the benthic remained relatively constant (Lott et al., 2021). On the biodegradable polymers, degradation was also observed by SEM, after 2.5 and 22 months of incubation in the eulittoral and benthic habitats (Fig. 6). The changes over time/season, however, exerted a stronger influence on the observed microbial communities (Fig. 3, Table A.3), but the differences were again habitat dependent. Despite the observed time gradients, however, differentiation between polymers did not progress with time in the eulittoral and benthic habitats according to our NMDS and PERMANOVA analysis, while polymer types converged over time in the pelagic.

4.2. The core plastisphere

A re-occurring question regarding plastisphere membership is whether there is a habitat-independent "core-community" of common microbial members in every plastisphere (Coons et al., 2021; De Tender et al., 2017; Debroas et al., 2017; Roager and Sonnenschein, 2019). More specific to our study, is the question: Are general biodegraders present in all the habitats and on all plastics, in addition to the general colonizers one would find on any surface? Based on the observed disintegration patterns, we expected to see differences in the microbial communities among different habitats, particularly between the eulittoral habitat and the other two. We also expected that biodegradable substrates would select for plastisphere members distinct from those on conventional plastics regardless of season and location.

Biodegradable polymers require specific enzymes capable of breaking the polymer bonds and utilizing the released products, and this can result in differentiated microbial communities between biodegradable and conventional plastics. Prior research noted polymer-dependent

(functional) diversity and richness within the plastisphere (Miao et al., 2019), and identified significant diversity differences between biodegradable and non-biodegradable polymers (Bos et al., 2023; Dussud et al., 2018a). Other studies observed no differences in diversity between different polymers, or polymers and natural substrates during exposure to the marine environment (Kirstein et al., 2019; Oberbeckmann et al., 2016). Performing a meta-analysis of multiple plastic colonization studies, there seems to be no core community when comparing one polymer from multiple sites (Wright et al., 2021).

Our data indeed showed that communities on different polymers were significantly different from each other and shared relatively few ASVs across habitats (Supplementary Fig. A.5). Shared taxa on one polymer across different habitats were almost always found on all polymers, and similarly the dominant taxa within a habitat were generally detected on all polymers. Hence, it seems like the core microbiome on different polymer types is mostly habitat dependent, rather than just polymer specific. Dominant families and phyla on a given polymer were relatively stable and mostly persisted, similar to trends reported before with longer timeseries incubations (De Tender et al., 2017). We did not observe significant differences between timepoints for most polymer/habitat combinations. This could mean there is a tightly attached core microbiome (Kirstein et al., 2019) that remains present over time, or that the biofilm is already too mature after three months to see any major shifts in community composition between later sample points (De Tender et al., 2017).

4.3. Microbial members that differentiated between polymers and habitats

We identified several genera that were differentially abundant, and thus suggest they were influenced by habitat, polymer, timepoint, or a combination thereof. The genus *Hirschia* played a significant role in differences between LDPE and the biodegradable polymers, according to both SIMPER and ALDEx2 analysis, since it occurred almost exclusively on PBSeT and PHB. However, while SIMPER analysis identified it as a key genus for the eulittoral habitat (Fig. 4C), ALDEx2 analysis and community profiling indicated that the genus preferred the geographical location of Pianosa island (Fig. 5A-B; Supplementary Fig. A.4), where it seemed to prefer the benthic over the pelagic. *Hirschia* is a facultatively anaerobic oligotrophic organism that has been identified in biofilms on plastics before (Kirstein et al., 2019; Nguyen et al., 2021). Within the same order (Caulobacterales), the genus *Parvularcula* was found to be overall influential according to SIMPER analysis (Fig. 4A). Similar to *Hirschia*, its presence was highest near Pianosa Island, especially in the benthic habitat, where it appeared to prefer PHB over the other polymers. This aerobic genus is often isolated from coastal and surface waters. It is known to accumulate intracellular PHB granules. Furthermore, it has been detected in biofilms associated with (bio)plastics in the past (Kirstein et al., 2019; Nguyen et al., 2021; Zettler et al., 2013) and identified as a hydrocarbonoclastic genus (Ramírez et al., 2020).

Some genera were specific to certain habitats according to both SIMPER and ALDEx2 analysis. Genera that were notably specific for the eulittoral habitat, according to both ALDEx2 and SIMPER, included genera belonging to the phylum of Desulfobacterota, namely *Desulfatitalea, Desolfoconvexum, Desulfobacula, Desulfovibrio, Desulfotignum*, and *Desulfobacter*. Most of these genera also have a high RA in the two sediment habitats (Supplementary Fig. A.4). This phylotype occurred more commonly on PBSeT among the polymers, since it exhibited a higher effect size and RA on this polymer compared to the other two (Fig. 5A-B; Supplementary Fig. A.4). Orders in this phylum show a preference for anaerobic environments, like coastal and marine sediments, and hydrocarbon contaminated environments, such as the Elba site that is located close to a harbor.

Both *Agarilytica* and *Alcanivorax*, from the order Pseudomonadales were found to be sediment-associated according to SIMPER analysis, with *Agarilytica* being influential in both the benthic and eulittoral, and *Alcanivorax* in the eulittoral only. *Alcanivorax* was found to be specific to

LDPE in the eulittoral habitat according to both SIMPER and ALDEx2 analysis. This halophilic marine genus, found before in biofilms on plastics in the upper water layers of the ocean (Coons et al., 2021; Delacuvellerie et al., 2019; Vaksmaa et al., 2021b), is described as obligate hydrocarbonoclastic (Yakimov et al., 2007). These species have a broad spectrum of different enzymes for oil degradation and often become the predominant microbes in oil-contaminated marine environments. *Alcanivorax* species were also mentioned in PlasticDB for their ability to degrade LDPE, PHB, and PHA, among other polymers. According to ALDEx2 analysis, *Agarylitica* had a higher RA at the Pianosa sites (especially the benthic habitat) than at the Elba site, and preferred the biodegradable polymers over LDPE, especially PHB (Fig. 5; Supplementary Fig. A.4). An *Agarilytica* strain was recently found on PHBV and contained 13 genes encoding for extracellular PHA depolymerases expressed at high levels (Suzuki et al., 2022).

Genera from the order Flavobacteriales showed a preference for the pelagic habitat. In both ALDEx2 and SIMPER analyses, *Winogradskyella* was found to be a major taxon. Its presence seemed to be dependent on geographical location with a preference for PBSeT, and to a lesser extent LDPE. *Winogradskyella* is an aerobic bacterial genus, often isolated from biofilms in coastal marine zones, and it has been found to be enriched on PMD in the Mediterranean (Vaksmaa et al., 2021b), and on polymers incubated in sediments in the laboratory (Delacuvellerie et al., 2019). Similarly, *Muricauda* and *Kordia* appeared to be mainly present at Pianosa, in the pelagic habitat. These genera appeared to prefer LDPE, followed by PBSeT for *Muricauda*. Both these genera have been enriched in incubations with crude oil (Chernikova et al., 2020), and *Muricauda* has been found on PMD in the Pacific (Bryant et al., 2016).

The genera *Ruegeria* and *Epibacterium* from the order Rhodobacterales were found to be influential in the pelagic habitat according to SIMPER analysis (Fig. 4B) and exhibited a higher RA at Pianosa compared to the Elba site (Fig. 5A). *Ruegeria* was found in association with marine plastics before (Pinto et al., 2019), and both *Ruegeria* and *Epibacterium* have been reported as members of hydrocarbonoclastic consortia (Dell'Anno et al., 2020; Kumar et al., 2019).

From the order *Pirellulales*, *Pirellula* and *Blastopirellula* were found to have a higher RA at the Pianosa site than at Elba, with a preference for the pelagic over the benthic (Fig. 5A). *Pirellula* was also identified as influential in the pelagic with SIMPER analysis (Fig. 4B), while *Blastopirellula* had a high RA at Pianosa (Supplementary Fig. A.4). These two genera have been found to be enriched on LDPE bags deployed in river waters (Martínez-Campos et al., 2023), and in our results showed higher RA on LDPE and PBseT than on PHB.

4.4. Polymer disintegration and abundance of potential biodegraders

In the field study from where our samples originated (Lott et al., 2021), disintegration of the polymers was used as a proxy for calculating biodegradation, after it was confirmed that these polymers truly biodegraded in laboratory experiments (Briassoulis et al., 2020; Lott et al., 2021). The half-lives for PHB and PBSeT in field incubations were shorter in the eulittoral than the benthic habitat, and essentially no disintegration was reported in the pelagic habitat. This was supported by our SEM images showing the disintegration patterns on the plastic surface (Supplementary Fig. A.8-A.10).

Disintegration rates might be higher in the presence of sediment, due to sediment-associated microbes contributing to higher biodegradation rates. Their biofilms can have a higher substrate specificity, and there is a higher biomass availability in general compared to the water column (Oberbeckmann et al., 2017; Wartell et al., 2021). Furthermore, biodegradation could be hampered in the pelagic field incubations due to the availability of alternative carbon sources produced by phototrophs or a deficit of nutrients owing to the ultra-oligotrophic open waters of the Mediterranean. For instance, the cyanobacterium *Roseofilum* AO1-A appeared as significant element in our pelagic habitat incubations, with significant temporal changes of the RA on the plastics.

Hence, we expected substantial variations in the abundance of plastic biodegraders not only for the different polymers, but also in the different habitats. We anticipated that the RA of potential plastic degraders would be highest in the eulittoral, and lowest in the pelagic environment, with the highest abundance of plastic degraders on PHB, followed by PBSeT and then LDPE. However, we did not observe this in our bacterial dataset.

Potential plastic biodegrading bacteria identified with PlasticDB did not dominate our biodegradable plastic samples, but they were always present, representing 1–16 % of the bacterial/archaeal community (Fig. 7). Within the three habitats, the RA of plastic degrading bacteria was generally highest in the eulittoral habitat, and the lowest in the benthic habitat. Surprisingly, LDPE in many cases showed a higher RA of plastic-degraders compared to PHB and especially PBSeT, even though we observed no cracks in our SEM images of LDPE samples and no disintegration was reported in the original field and lab incubations (Lott et al., 2021). The abundance of plastic-degraders, but an absence of degradation patterns on the polymers, can be partially explained by the fact that most microbes capable of plastic biodegradation can also utilize other carbon sources including more easily metabolized dissolved organic matter in the water and biofilms on the plastic surfaces. Several studies have speculated that eukaryotes play an important role in the degradation of PMD, either by performing enzymatic biodegradation, or enabling colonization of biodegraders (Amaral-Zettler et al., 2021; Dudek et al., 2020). We did not take into account the RA of possible biodegrading eukaryotes like fungi, neither did we employ quantitative measurements of cell counts with our SEM imaging in general. There was however a clear absence of Ascomycota in the pelagic habitat, compared to the benthic and eulittoral habitat (Fig. 8).

We expected the highest RA of plastic-biodegrading microbes to match the observed disintegration rates of the polymers. This would mean, a high RA at 2.5 months on PHB in the eulittoral habitat, and an increasing RA with time for PHB in the benthic habitat and PBSeT in both the eulittoral and benthic habitats, particularly at 10 and 22 months. With the exception of the PHB films at 2.5 months in the eulittoral habitat, we did not observe this trend. Total disintegration and disintegration rates were the highest at the final two sampling points compared to earlier timepoints. Despite this, however, the RA of identified plastic-biodegraders was generally lowest on all polymers and comparable in all habitats at 10 and 22 months. We assume that biodegradation was ongoing at the later timepoints but may have slowed down before complete biodegradation was achieved. This phenomenon was observed with our incubated polymers in the laboratory (Briassoulis et al., 2020; Lott et al., 2021). Our study combined with Lott et al. (2021), shows that the mere presence of certain microbes is no proof for biodegradation activity and that the number of ASVs detected does not reflect the biodegradation or disintegration rate of plastic per se. More research including metabolomics, metatranscriptomics, metagenomics, and the use of stable- or radio isotopes is needed to allow for specific conclusions regarding biodegradation taking place and identifying the major (bio)degraders.

The mechanisms underlying preferential colonization by microbes in the plastisphere are still not fully understood (Pinto et al., 2019; Yu et al., 2023). The chemical composition of different plastics can influence bacterial attachment, especially when they are also composed of a food source for microbes. In prior studies, it has been observed that while biodegradable substrates are initially populated by predominantly substrate biodegrading microbes, subsequent colonization is accompanied by the emergence of cross-feeding consumers, for instance on chitin (Datta et al., 2016; Wright et al., 2019) and other particulate organic matter (Enke et al., 2019). Community assembly followed rapid successional dynamics that were independent of the substrate, while substrate biodegraders were suppressed by these secondary consumers (Enke et al., 2019). Additionally, oxygen limitation at the polymer surface might play a role in loss of certain (biodegrading) microbes, as was suggested by Lott et al. (2020), and observed by Briassoulis et al.

(2020). The final stages of disintegration might also have been caused by deterioration of the biofilm and its extracellular polymeric substances (EPS) layer holding the remaining polymer together, signs of which are visible in our SEM pictures (Fig. 6 and Supplementary Figs. A.8-A.10), and/or by physical forces after enzymatic activity degraded the polymer, even though the experimental set-up was designed to minimize this (Lott et al., 2020).

4.5. Potential bacterial biodegraders, archaeal and microbial eukaryotic members

4.5.1. Bacteria

Given that biodegradability of both PBH and PBSeT was demonstrated in laboratory tests, we expected to identify one or several most likely candidates involved in the biodegradation of these polymers in our field incubations, and to see a difference between PBSeT and PHB hydrocarbon-degrading communities. Structurally, PBSeT contains an aromatic group in its carbon backbone, located adjacent to ester groups, compared to PHB which does not have an aromatic group. Furthermore, there are different enzymes described in the literature related to depolymerization of the respective polymers. PHAs are hydrolyzed by PHA depolymerases, and lipases, while terephthalate and succinate containing polyesters are hydrolyzed by PETases, cutinases and lipases (reviewed in Gricajeva et al., 2022).

In the original field studies, PHB disintegration occurred relatively quickly, with the highest rate in the eulittoral habitat (Lott et al., 2021). We detected *Desulfovibrio* in all PHB eulittoral samples, but only at the later timepoints in the benthic habitat, which matches the disintegration curves (Fig. 6). This genus had a very high RA in eulittoral habitat on PHB at the first three timepoints, it was also detected on LDPE, but rarely on PBSeT. Sulfur-reducing organisms have been found to be enriched on PHAs before (Pinnell and Turner, 2019), and PHA in sediment can serve as a carbon source and electron source in sulfite reduction (Urmeneta et al., 1995).

Members of the genus *Stenotrophomonas* can play a role in the degradation of a wide variety of xenobiotic hydrocarbons (Ryan et al., 2009), and they were detected on all three of our polymers. They had the highest RA in the eulittoral habitat on PHB and LDPE, but also reached >1 % RA on PBSeT in both the benthic and eulittoral habitat after 2.5 months. It was no longer detected at the last sampling point on both of the biodegradable polymers. Members from this genus are known to degrade various PHAs, including PHB (Boyandin et al., 2012; Gangoiti et al., 2012; Kim et al., 2007; Mergaert and Swings, 1996; Ramsay et al., 1994; Wani et al., 2016). In addition, several terrestrial strains have been described for degrading PE (Dey et al., 2020; Jeon and Kim, 2016; Nadeem et al., 2021; Oluwole et al., 2022; Peixoto et al., 2017). Furthermore, recent publications report degradation of PET (Din et al., 2023; Huang et al., 2022) and PBAT by this genus (Jia et al., 2021). Surface deterioration of PBS by *Stenotrophomas* sp. was reported by Ghaffarian et al. (2017), and actual biodegradation (measured by CO_2 evolution) of PBS was enhanced when the fungus *Fusarium solani* was co-cultured with *Stenotrophomas melophilia* (both were isolated from a degraded PBS film that was buried in soil) (Abe et al., 2010).

4.5.2. Archaea

Studies on the plastisphere mainly focus on bacteria, and our knowledge of archaeal communities and diversity in the plastisphere remains limited (Yu et al., 2023). Up until now, there seems to be no archaeal analogy to the bacterial core community (Vaksmaa et al., 2021b), and little is known about whether archaea preferentially colonize certain polymers. In this study, we took advantage of primers capturing all three domains of life to examine microbial succession on plastic by domains in addition to bacteria.

While bacteria dominated the microbial communities in the biofilms on our samples, archaeal reads constituted up to 22 % of the total RA on several samples. Furthermore, we found a higher RA of archaea on PBSeT and LDPE compared to PHB (Fig. 1A). Moreover, the two archaeal phyla Halobacteriota and Thermoplastmatota, have been reported to contain extracellular PHA depolymerases (Viljakainen and Hug, 2021), and both phyla were also detected on our polymers (data not shown).

Biofilms on plastic surfaces typically have a low abundance of archaea (Dussud et al., 2018a; Oberbeckmann et al., 2016; Vaksmaa et al., 2022), but this observation may be biased due to most studies focusing on pelagic environments. We detected the most archaeal signatures on polymers in the benthic and eulittoral habitats, and since all current biodegradable plastic polymers are denser than seawater, more research into archaea in relation to sediments and biodegradable polymers is needed.

4.5.3. Eukaryotes

Eukaryotes play an important role in structuring the plastisphere ecosystem and can colonize along with their own bacterial communities (Amaral-Zettler et al., 2021). As with bacteria, colonization can be impacted by incubation site and type of plastic (Philippe et al., 2023). However, only a limited number of plastisphere studies have considered eukaryotic diversity (Wright et al., 2020), so our results address an important gap in understanding of potential eukaryotic plastic degraders.

Our pelagic samples contained a very large portion of eukaryotic phototrophs at all timepoints, as expected from previous studies (Lott et al., 2020). In addition, we consistently detected molecular signatures of diatoms (Bacillariophyceae) on our polymers in all habitats, and these microalgae have been reported as common colonizers on marine plastics (Amaral-Zettler et al., 2021; Dudek et al., 2020; Eich et al., 2015; Masó et al., 2016). We even detected their molecular signature on the buried eulittoral films, though SEM imaging showed few intact diatom cells on these films. This could be a result of the mixing of the sediment during the incubation set-up preparation (Lott et al., 2020), but diatoms are also known to migrate vertically down into the sediment in response to tides and other stimuli (Saburova and Polikarpov, 2003; Serôdio et al., 2023).

Metazoans were the most represented ASVs among the eukaryotes in our samples and may contribute to deterioration of polymer surfaces due to attachment of fast-holds, but fungi are often cited as the most likely eukaryotes contributing to hydrocarbon biodegradation in the environment (Harms et al., 2011; Zeghal et al., 2021). Fungal communities are an integrated part of the plastisphere (Philippe et al., 2023), however there is an overall lack of published studies reporting fungal diversity during biodegradation, so our knowledge on their role in the plastisphere remains limited (Yu et al., 2023).

In general, we detected more fungal groups on LDPE than our biodegradable polymers. Ascomycota and Basidiomycota were the most encountered fungal phyla. Philippe et al. (2023) reported that Basidiomycota were more frequently associated and with higher abundance on biodegradable plastics, and Ascomycota were encountered more and with higher abundance on conventional plastics. In our study, Ascomycota were detected more on biodegradable plastics in the benthic habitat, but more on LDPE in the eulittoral habitat. They were rarely detected in the pelagic habitat. Aphelidiomycota and Chytridiomycoata, although encountered less, were detected only on LDPE and PBSeT.

We observed the genus *Malassezia* frequently across all three polymer types in all three habitats, this genus has not been reported as a plastic degrader but seems to be omnipresent in the marine environment (Amend et al., 2019). Several of the fungal genera that we detected on our plastics were included in PlasticDB as plastic degraders (Gambarini et al., 2022).

Cladosporium spp. were present in all three environments on some of the PBSeT and LDPE samples. Members of this genus have been reported for degradation of PBSA in soil (Yamamoto-Tamura et al., 2020) and a synthetic polymer containing terephthalic acid and ethylene glycol from a museum specimen of an Apollo space suit (Breuker et al., 2003), as well as for surface deterioration of oxo-degradable PE (Bonhomme et al.,

2003), and LDPE and HDPE that contained pro-oxidant additives (Koutny et al., 2006). We did not detect *Cladosporium* spp. on our PHB films, and while terrestrial *Cladosporium* spp. have been reported for degrading PHB, PHB-PLA blends (Jeszeová et al., 2018) and PHBV, several marine species did not show the capacity to degrade PHB or PHBV (Matavuly and Molitoris, 2016).

Acremonium sp. (Boyandin et al., 2013; Mergaert and Swings, 1996) and *Debaryomyces hansenii* (Gonda et al., 2000; Matavuly and Molitoris, 2016) have been reported to degrade PHB and PHBV. However, we detected *Acremonium* sp. and *Debaryomyces* sp. only in the benthic environment on PBSeT, and LDPE respectively. *Candida* sp. was found on the three different polymers in the benthic habitat and on PBSeT in the eulittoral habitat but has not been reported to degrade any of our substrates and is only mentioned in relation to polyurethane in PlasticDB.

Lastly *Rhodotorula* sp. occurred only on LDPE in the eulittoral samples, and not on either of our biodegradable polymers despite being reported to biodegrade PHB (Gonda et al., 2000). While not included in PlasticDB at the time of analysis, *Rhodotorula mucilaginosa* has been shown recently to be able to accumulate carbon derived from UV-irradiated PE (Vaksmaa et al., 2023). The presence of *Rhodotorula* on LDPE in our incubations and not the biodegradable plastics substrates suggests that it is indeed degrading the leachates of LDPE and not actively engaging in biodegrading the biodegradable plastic substrates. Similarly, we detected the genus *Cryptococcus* only on LDPE in the eulittoral habitat, but it is not known to (bio)degrade this polymer. Instead, a purified cutinase-like enzyme containing a signal peptide from *Cryptococcus* sp. was identified being able to degrade PBS, PCL, PHB and PLA (Masaki et al., 2005), as well as a lipase was identified for PBS and PBSA degradation (Thirunavukarasu et al., 2016).

In general, we found low correspondence between fungal groups reported as capable of biodegrading a certain plastic and their presence in our datasets. This could potentially be due to the relatively low read abundance of eukaryotic amplicons we recovered as we did not employ fungal specific primers.

5. Conclusions

As biodegradable plastic use increases, our understanding of its behavior in the marine environment becomes more important. Our study provided among the first insights into long-term (22 months) colonization of biodegradable plastics by the three domains of life, in three contrasting coastal habitats. We found potential plastic degraders in all environments, but there was no obvious "core" of shared plastic specific microbes. Communities varied between polymers, but the habitat was highly influential in selecting for specific communities. The time courses of disintegration did not match community patterns of potential plastic degraders. This could be due to a lag between when active biodegradation was occurring and when the plastic began to disintegrate, or the presence of unknown biodegraders. Archaea and eukaryotes were consistently present as part of the plastic communities we analyzed, sometimes at relative read abundances of over 20 %, but the functionality of these domains in plastic degradation in marine systems remains understudied. Future experiments should combine field disintegration rates and laboratory biodegradation studies with samples of microbial biofilms earlier in the biodegradation process. This, combined with molecular analyses including metagenomic, metatranscriptomic and metaproteomic approaches will provide additional insights into the metabolic potential and activity of plastisphere communities on biodegradable plastics. These data are needed to understand the fate of biodegradable plastics and to inform policy makers to avoid their accumulation in marine environments.

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CRediT authorship contribution statement

Fons A. de Vogel: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maaike Goudriaan:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Erik R. Zettler:** Writing – review & editing, Visualization, Investigation. **Helge Niemann:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization, Funding acquisition. **Andreas Eich:** Writing – review & editing, Resources, Data curation, Visualization. **Miriam Weber:** Writing – review & editing, Resources, Funding acquisition. **Christian Lott:** Writing – review & editing, Resources, Funding acquisition. **Linda A. Amaral-Zettler:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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