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Microbial pioneers of plastic colonisation in coastal seawaters

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ABSTRACT

Plastics, when entering the environment, are immediately colonised by microorganisms. This modifies their physico-chemical properties as well as their transport and fate in natural ecosystems, but whom pioneers this colonisation in marine ecosystems? Previous studies have focused on microbial communities that develop on plastics after relatively long incubation periods (i.e., days to months), but very little data is available regarding the earliest stages of colonisation on buoyant plastics in marine waters (i.e., minutes or hours). We conducted a preliminary study where the earliest hours of microbial colonisation on buoyant plastics in marine coastal waters were investigated by field incubations and amplicon sequencing of the prokaryotic and eukaryotic communities. Our results show that members of the Bacteroidetes group pioneer microbial attachment to plastics but, over time, their presence is masked by other groups – Gammaproteobacteria at first and later by Alphaproteobacteria. Interestingly, the eukaryotic community on plastics exposed to sunlight became dominated by phototrophic organisms from the phylum Ochrophyta, diatoms at the start and brown algae towards the end of the three-day incubations. This study defines the pioneering microbial community that colonises plastics immediately when entering coastal marine environments and that may set the seeding Plastisphere of plastics in the oceans.

1. Introduction

Plastic pollution is a global problem, now found in every corner of our planet (Hale et al., 2020; Stubbins et al., 2021). Plastics are both durable and light-weight, and due to their high transportability, much of the mismanaged plastics accumulate in the marine environment (Hale et al., 2020; Jambeck et al., 2015; Lebreton et al., 2017) with largely unknown consequences. Plastic, as any surface in the marine environment, gets instantaneously coated by a film of organic matter – eco-corona – followed by rapid attachment and colonisation by marine micro-organisms (Galloway et al., 2017; Harrison et al., 2014; Caruso, 2020). Micro-organisms colonizing marine plastic debris – coined 'the Plastisphere' (Zettler et al., 2013) – largely differ from their free-living planktonic counterparts (Oberbeckmann et al., 2018a; Erni-Cassola et al., 2019a; Bryant et al., 2016) and, in some cases, from biofilms on other materials (Oberbeckmann et al., 2018a; Kirstein et al., 2018; Muthukrishnan et al., 2018). Since the first comprehensive (i.e., conducted using Next-Generation Sequencing, NGS) characterization of microbial communities on marine plastic debris was published in 2013 (Zettler et al., 2013), numerous studies of a similar nature have followed, often attempting to detect and describe a plastic-specific 'core microbiome' (De Tender et al., 2017a; Roager and Sonnenschein, 2019; Debroas et al., 2017). The presence of possible pathogens like *Vibrio* spp. (Zettler et al., 2013; Kirstein et al., 2016; Laverty et al., 2020) as well as taxa with the potential to degrade complex or recalcitrant carbon compounds, including members of the obligate hydrocarbonoclastic bacterial (OHCB) group (Zettler et al., 2013; Erni-Cassola et al., 2019a; Wright et al., 2020a), recurs in these kinds of studies, prompting speculations of the potential role of the latter in the biodegradation of marine plastics. The drivers and roles of their presence in these biofilms are, however, unclear (Bryant et al., 2016; Dudek et al., 2020). It seems likely that, in order to degrade plastic, these hydrocarbonoclastic taxa

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would require direct access to the polymer surface, thereby acting as primary colonisers before a thick mature biofilm develops (Wright et al., 2020b). To date, however, very little is known about these very early steps of Plastisphere formation.

The early stages of marine surface colonisation are highly dynamic (Dang and Lovell, 2000; Pollet et al., 2018; Kesy et al., 2021), and marine biofilm communities have been reported to transition from the first stage of colonisation to the second, following just 9 h' immersion in seawater (Lee et al., 2008). There is, however, very little information available on the community succession on marine biofilms in general, especially at the very early stages of succession (Pollet et al., 2018; Abed et al., 2019; Rampadarath et al., 2017). This lack of data also applies to community analyses of the Plastisphere despite the recent boom of publications (Wright et al., 2020a; Wright et al., 2020b). These studies have looked into microbial communities either on ‘aged’ plastics collected from the marine environment with unknown life-histories and exposure times, or after relatively long incubation periods on virgin plastics incubated either in situ or with natural marine communities in laboratory-settings; nevertheless, very little data seems to be available regarding the earliest stages (< 1 day) of colonisation on buoyant plastic types – such as polypropylene (PP) – in marine surface waters. The early stages of colonisation may, however, play an important role in the fate of marine plastic debris, as indicated by previous studies conducted on the natural polymer chitin. These studies have shown that chitin particles exhibit positive selection towards substrate-degrading taxa during the early successional stages, before being overtaken by secondary colonisers that are not able to use the substrate as a carbon source and, as a consequence, chitinase activity is drastically reduced (Datta et al., 2016; Wright et al., 2019). Recent evidence suggests a similar process might also apply during early stages of plastic colonisation when plastic-associated weathering products are available for primary colonisers (Erni-Cassola et al., 2019a).

Among the multitude of Plastisphere community investigations as reviewed in (Wright et al., 2020b), only six field studies conducted in the natural marine environment included a time series for community succession analysis using NGS methods (Erni-Cassola et al., 2019a; De Tender et al., 2017a; Pollet et al., 2018; Pinto et al., 2019; Dang et al., 2008; Xu et al., 2019). The earliest timepoint in any of these studies was one day (Pollet et al., 2018; Dang et al., 2008) and, in most cases, the incubation period in these studies ranged from weeks to months. Such timescales appear lengthy, considering (1) that marine biofilm communities have been shown to exhibit distinct successional stages with selective enrichment of substrate-degrading organisms and hydrolytic activity occurring within the first one or two days (Erni-Cassola et al., 2019a; Datta et al., 2016; Wright et al., 2019) and (2) that initially distinct marine Plastisphere communities are known to converge as early as within the first 9 days after immersion in seawater (Erni-Cassola et al., 2019a). Some field studies on the very early stages on marine biofilm formation do, however, exist. Lee et al. (Lee et al., 2008), for example, investigated bacterial biofilm succession on acryl, glass and steel surfaces at timepoints of 3, 9, 24, and 28 h in a marine harbour in Korea. This study, though, used Terminal Restriction Fragment Length Polymorphisms (T-RFLP) method instead of implementing NGS analyses for prokaryotic and/or eukaryotic communities, and used acryl as a substrate. Acryl is a high-density polymer which sinks to the seafloor in natural conditions and, as such, may not be the most environmentally relevant material to use in colonisation studies in the upper layers of the water column. Harrison et al. (Harrison et al., 2014) investigated colonisation on polyethylene (PE) with timepoints as early as 2 min and 6 h, followed by consecutive timepoints at 1, 2, 4, 7 and 14 days. Misic and Covazzi Harriague (Misic and Covazzi Harriague, 2019) studied biofilm development on polyethylene terephthalate (PET) bottles with the first timepoint at 10 h after the start of incubations. Finally, Ramsperger et al. (Ramsperger et al., 2020) recently investigated structural diversity of very early (0, 0.5, 1, 2, 5, 7, 11 and 14 days) biofilms on PET, polyvinyl chloride (PVC) and polyamide (PA). All of these studies were, however, laboratory-based and not incubated under natural settings, as well as also lacking a comprehensive microbial community identification by NGS. It has now been shown that microbial Plastisphere communities obtained from laboratory-based incubations are significantly different to those obtained from in situ field studies (Wright et al., 2020a). More recently, Kesy et al. (Kesy et al., 2021) investigated the very early (1 h, 5 h and 10 h) colonisation of PE and polystyrene (PS) pellets in coastal and estuarine waters of the Baltic Sea, but their NGS-based analysis focused only on members of the bacterial genus *Vibrio*.

Compared with community analyses focused on prokaryotes, eukaryotic communities within the Plastisphere have been heavily understudied (Wright et al., 2020b; Amaral-Zettler et al., 2020). To our knowledge, only three field-based Plastisphere analyses have characterized a eukaryotic community fraction over a time series using NGS (18S rRNA gene (Dudek et al., 2020); fungal ITS gene (De Tender et al., 2017a); v4–5 region of small subunit rRNA (Amaral-Zettler et al., 2021a)), all having the first timepoint at or beyond one week. Other, early colonisation metabarcoding studies on marine eukaryotes have mainly focused on detection and monitoring of non-indigenous or pest species (e.g., (Zaiko et al., 2016): days 1, 5 and 15 on PVC; (Pochon et al., 2015): 1 month on acryl) or identifying factors controlling biofilm community development (days 7, 14, 19, 28, 42 and 56 on acryl (Tobias-Hünefeldt et al., 2020)).

In the present study we fill an important research gap by investigating the very early colonisation and succession stages of both prokaryotic and eukaryotic communities on PP incubated in natural marine settings. PP together with PE are globally the two most abundantly-produced plastics and, due to their low density, they are by far the most abundant plastics found floating on sea surfaces (Erni-Cassola et al., 2019b).

2. Materials and methods

2.1. Experimental site

Field incubations were conducted in the coastal waters of the Mediterranean Sea in South-West Majorca (Spain; coordinates: 39.494269N, 2.740099E) over 6–9th April 2018. The site, in the vicinity of a quiet residential area, consisted of a rocky shore environment with predominant currents coming from open oligotrophic waters (Fig. 1a). Sea water temperature was 15 °C and solar radiation was 5.86 kWh m^{-2} day^{-1} spread over 13 h of daylight (BalearsMeteo.com).

2.2. Material preparation, incubation and collection

Plastic strips (0.5 cm × 3 cm in size) were cut out of commercial, clear, disposable polypropylene drinking cups. Three plastic strips were tied onto a nylon fishing line with enough space between them to prevent the strips from overlapping each other. The lines with tied strips were kept in absolute ethanol until submerged in seawater and installed as shown in Fig. 1b, *i.e.,* in 2 m-deep coastal waters keeping the strips over 1 m from the sea floor and 0.5 m from the sea surface. The tidal range in the Mediterranean Sea is negligible and, therefore, the incubation depth remained constant throughout the experiments. Five fishing lines in total were placed at an open coastal location with no shading (hereafter referred to as ‘OpenCoast’), and another five lines were placed inside an open coastal cave with low-light conditions (‘Cave’; Fig. 1a). Predominant currents made open waters flow through the cave, although the turnover was not determined. OpenCoast (= high-light) and Cave treatments (= low-light) were chosen to investigate how light availability affected microbial colonisation and succession on plastics.

Three plastic strips were collected at each timepoint – 15 min, 4 h, 6 h, 28 h and 76 h – by collecting one line from each treatment (i.e., OpenCoast and Cave). Unfortunately, the last line from the Cave incubation was lost and, hence, there is no 76-h timepoint for this treatment.

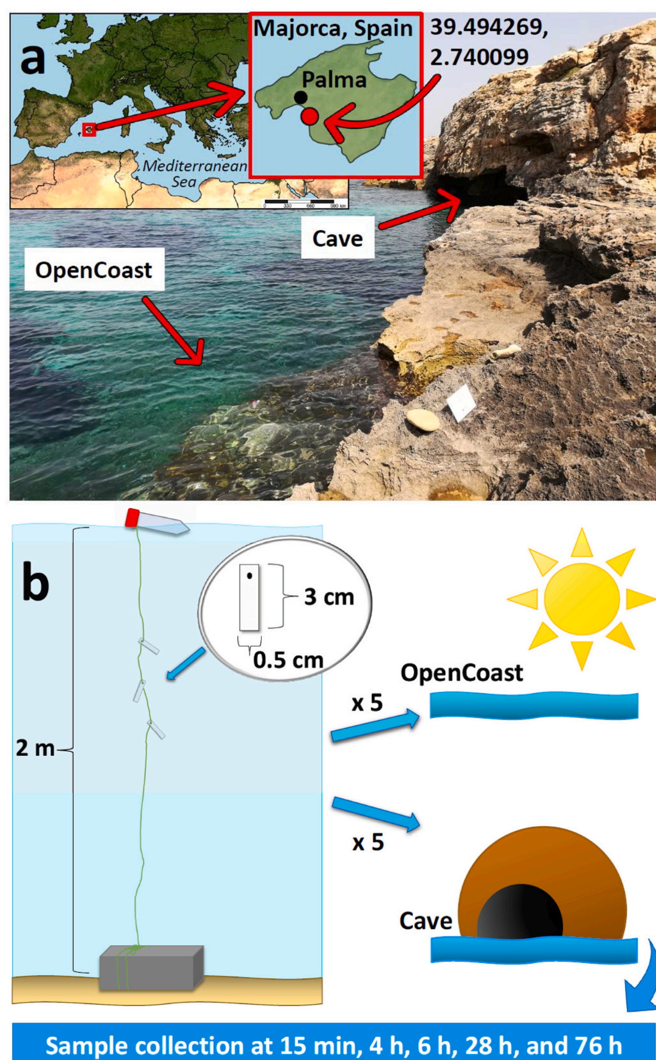

Fig. 1. Location (a) and experimental design (b) of the in situ incubations using polypropylene plastic strips.

Plastic strips were immediately cut from the line directly into 2-mL Eppendorf tubes containing 1 mL of lysis buffer (Buffer AL, Qiagen, Netherlands). Samples in lysis buffer were stored at −20 °C until further processing in the laboratory.

2.3. DNA extraction

DNA was extracted from plastic particles using the DNeasy PowerBiofilm Kit (Qiagen, Netherlands) following the manufacturer's instructions. The initial step of bead beating was conducted for two 30 s cycles at 30 Hz (Qiagen Tissue Lyser), and samples were allowed to rest for 30 s between the cycles. DNA was eluted in 30 μL of sterile milliQ water. Non-submerged plastic strip controls ($n = 3$; 'plastic controls') as well as procedural controls (i.e. lysis buffer without plastic samples; $n = 3$; 'kit controls') were processed in parallel.

2.4. Amplicon sequencing

Amplification of the v4–5 regions from the 16S rRNA gene was performed using primers 515F-Y and 926R (Parada et al., 2016), while the v8–9 regions of the 18S rRNA gene were amplified with primers V8F and 1510R (Bradley et al., 2016). After amplicon purification and PCR indexing, all PCR products were visualized on 1% agarose gels stained with ethidium bromide to confirm the presence of a PCR product. Due to the large number of 18S rRNA gene samples for which no PCR product was visible, PCR was repeated for these samples using a nested PCR method, as previously done by Oberbeckmann et al. (Oberbeckmann et al., 2018b). Briefly, the extracted DNA was first amplified using the full-length 18S rRNA gene primers 63F and 1818R (Lepere et al., 2011) and then these PCR products were amplified again using the V8-9 18S rRNA primers V8F and 1510R. These amplicons were then purified and used for PCR indexing. Library preparation for 300 bp paired-end amplicon sequencing (including all controls) was carried out as previously described (Wright et al., 2021). Briefly, libraries were pooled and quantified using the NEBNext Library Quant Kit for Illumina (New England Biolabs, USA) and diluted to 4 nM. Libraries were denatured using 0.2 N NaOH and MiSeq amplicon sequencing was carried out using the MiSeq Reagent Kit v3 (600 cycles; Illumina, USA), following the manufacturer's instructions for a 14 pM library with 2% phiX as an internal reference. Illumina BaseSpace was used to demultiplex reads.

2.5. Data processing

Raw sequencing data was processed in R (version 3.5.1; (R Core Team, 2020)) using the DADA2 pipeline (Callahan et al., 2016a; Callahan et al., 2016b). Briefly, forward and reverse primer sequences were trimmed and, after sequence quality inspection, amplicon sequences were truncated to lengths of 250 bp (forward) and 200 bp (reverse) for both the 16S rRNA gene and the 18S rRNA gene, in order to remove low-quality nucleotides. Taxonomy was assigned using the naïve Bayesian classifier method (Wang et al., 2007) using the Silva reference database v132 (Quast et al., 2013). Species level taxonomy assignment by exact matching was performed only on the 16S rRNA gene dataset as this option was not available for other marker genes, such as the 18S rRNA gene, at the time. It is, however, important to note that species level information cannot be reliably inferred from short amplicon sequences (Johnson et al., 2019) and should therefore be interpreted with caution.

Further filtering as well as all downstream analyses were performed in RStudio (versions 1.3.1073 and 4.0.2 for RStudio and R, respectively; (RStudio Team, 2020)) using R package Phyloseq (McMurdie and Holmes, 2013). At kingdom level, all unassigned sequences were removed, whilst also removing all eukaryotic sequences from the 16S rRNA gene dataset, and all bacterial and archaeal sequences from the 18S rRNA gene dataset. Further filtering steps for both datasets included removal of all unassigned or ambiguously annotated sequences at the phylum level, as well as removal of sequences assigned to Chloroplasts, Mitochondria, and Mammalia. All sequences with less than five occurrences in each respective dataset, as well as samples belonging to an unrelated treatment, respectively, were likewise removed.

After exploring the datasets and confirming that procedural controls differed from the actual samples (Fig. S1), controls were removed from the datasets for downstream analyses. Samples with less than 788 (16S rRNA gene) or 1613 (18S rRNA gene) reads were likewise removed from further analyses (i.e., 16S rRNA gene samples 'OpenCoast_28h_Rep3' and 'OpenCoast_76h_Rep3', and 18S rRNA gene sample 'Cave_28h_Rep3'), leaving at least two biological replicates for all conditions. For the final datasets, the average number of reads per sample (as summarised by the 'summarize_phyloseq' function from the R package Microbiome (Lahti and Shetty, 2019)) was approximately 12,277 for the 16S rRNA gene dataset and 10,422 for the 18S rRNA gene dataset. Sequencing depth of samples in both (16S and 18S rRNA gene) datasets was visually inspected via rarefaction curves (function 'ggrare' from package Ranacapa (Kandlikar, 2020)).

2.6. Statistical analyses and data visualisation

Alpha diversity measures (observed richness, Shannon diversity, and inverse Simpson's diversity) were used to compare within-sample diversities between different sample groups by boxplots generated using the Phyloseq function 'plot_richness' (McMurdie and Holmes, 2013).

Significant differences in alpha diversity, and the effect of treatment, timepoint and their interaction, were further investigated by two-way analyses of variance (ANOVAs) and post-hoc Tukey's HSD tests using the R package Stats (R Core Team, 2020) for Shannon diversity ('estimate_richness', R package Phyloseq (McMurdie and Holmes, 2013)). Shannon diversity was chosen for statistical testing because it is more robust towards differences in library sizes compared with the other alpha diversity metrics (Erni-Cassola et al., 2019a; Knight et al., 2018). To test whether the assumptions for ANOVA were met, normality of residuals was evaluated by histograms and Q-Q plots (R packages Car (Fox and Weisberg, 2019), Graphics, and Stats (R Core Team, 2020)) as well as Shapiro-Wilk normality tests (R package Stats (R Core Team, 2020)), while homogeneity of variances was confirmed via Levene's tests (R package Car (Fox and Weisberg, 2019)).

Beta diversity, i.e., between-samples diversity, was investigated by Principal Coordinates Analysis (PCoA) plots using Bray-Curtis and binary Jaccard distance measures in order to find out if microbial community compositions differed between different sample groups, and whether these differences were driven by species abundance (i.e., by a few dominant taxa) or species richness (i.e., by rare taxa), respectively. Permutational Multivariate Analyses of Variance (PERMANOVAs) were subsequently performed with 999 permutations and both distance measures using the 'adonis' function in the R package Vegan (Oksanen et al., 2019) to assess whether any observed differences were statistically significant and, in order to detect which groups differed significantly from each other, pairwise-PERMANOVA (function 'pairwise.adonis'; (Martinez Arbizu, 2020)) tests were employed. The pairwise tests were run separately on each variable because the function (pairwise.adonis2) that accepts interactions was still under development. Multivariate homogeneity of group dispersions, an assumption for PERMANOVA, was permutationally tested using functions 'betadisper' and 'permutest' from the Vegan package. Main patterns and features of microbial community composition and succession were further interpreted at different taxonomic rank levels by exploring abundance tables and visualising relative abundances of top-10 most abundant taxa within a chosen rank with bar graphs using function 'plot_composition' from the R package Phyloseq.extended (Mariadassou, 2020). Functions 'fantaxtic_bar' (package Fantaxtic (Teunisse, 2018)) and 'bubble_plot' (package MetagMisc (Mikryukov, 2017)), respectively, were applied to further visualise the succession of the dominant classes within the most abundant phyla as well as the top-20 most abundant Amplicon Sequence Variants (ASVs) in both datasets. The amount of shared and unique ASVs between treatments over the first 28 h of incubation was investigated by generating Euler diagrams using R packages MicEco (Russel, 2020) and Venneuler (Wilkinson, 2011). All ASVs that were observed at least once in a respective group were included in the Euler diagrams.

Data were normalised by transformation to relative abundances in order to account for uneven library sizes in all analyses except for rarefaction curves, Euler diagrams, top-10 abundance bar graphs, and alpha diversity estimations, for which raw counts were used as input data. In order to avoid confounding effects and problems with unbalanced designs caused by the uneven amount of timepoints with three replicates available for the two different treatments, statistical tests were run with data from only the first three timepoints (15 min, 4 h, and 6 h) where triplicate samples were available for both treatments. Additional R packages, such as ggplot2 (Wickham, 2016) and RColorBrewer (Neuwirth, 2014), were used for data visualisation.

2.7. Data availability

All raw data is available in the NCBI repository under the BioProject accession number PRJNA758446.

3. Results

3.1. Plastics are colonised within minutes of submersion in coastal seawater

Plastic strips made of PP were rapidly colonised by a characteristic marine Plastisphere as demonstrated by the strong distinction of all plastics incubated in coastal seawater – either in well-lit coastal waters or in a low-light cave – compared to the non-submerged plastic controls (Fig. S1). Plastics incubated as little as 15 min already began to group with all other in situ-incubated plastics whereas the non-submerged plastic strips (i.e., 'PlasticControl') grouped with the procedural controls ('KitControl'; Fig. S1).

Despite the scarce DNA extracted, PCR amplification of the 16S (prokaryotic community) and 18S (eukaryotic community) rRNA genes, and subsequent amplicon sequencing provided sufficient reads from most samples to perform the community analysis. Only three replicates (i.e., 16S rRNA gene: OpenCoast_28h_rep3 and OpenCoast_76h_rep3; 18S rRNA gene: Cave_28h_rep3) were below the threshold and discarded from further downstream analyses (Fig. S2). All samples from the last timepoint (76 h) of Cave treatment were likewise excluded, as these were lost in the field. The average number of reads per sample was 12,277 for the 16S rRNA gene dataset and 10,422 for the 18S rRNA gene dataset. The prokaryotic and eukaryotic datasets included 6210 and 1229 taxa, respectively.

3.2. Community variation between treatments and over time (beta diversity)

Differences in community composition between sample groups from prokaryotic and eukaryotic communities were evaluated by applying Bray-Curtis (i.e., where taxa abundance is considered) and binary (i.e., presence-absence) Jaccard distance measures on data transformed to relative abundances in order to investigate whether any differences observed were due to dominant (i.e., high-abundance) or rare (i.e., low-abundance) taxa, respectively. Statistical tests were run with a dataset containing only the first three timepoints (15 min, 4 h, 6 h) for which triplicate samples were available in both treatments.

3.2.1. Prokaryotic community beta diversity

Treatment and timepoint, as well as their interaction, had a significant effect on prokaryotic community composition (PERMANOVA, $p = 0.001$; Table S1). Comparison of R^2 values as well as inspection of PCoA plots indicated that differences between communities were mainly driven by dominant rather than rare taxa, and that timepoint had a stronger effect on the very early community composition than treatment (i.e., light exposure; Table S1, Fig. 2). Permutation tests for homogeneity of variance of multivariate dispersions, however, showed that group dispersions were heterogeneous for timepoint when using Bray-Curtis distance ($p = 0.046$, Table S2), meaning that the differences detected by PERMANOVA may have been caused by within-group rather than between-group variances when using Bray-Curtis distance. To find out which groups differed significantly from each other, pairwise PERMANOVA tests were performed with p values adjusted for multiple comparisons by the Bonferroni method. Prokaryotic communities were confirmed to be significantly different between the two treatments (i.e., OpenCoast vs. Cave; p.adjusted <0.005; Table S3). Comparing timepoints across the first 6 h, the results indicated that overall communities were significantly different only between timepoints 15 min and 4 h (p.adjusted <0.05; Table S3), as well as between 15 min and 6 h (p.adjusted <0.01; Table S3). Communities did not differ significantly from each other with either distance measure between the 4-h and 6-h timepoints (p.adjusted >0.05; Table S3). Likewise, comparisons between 'treatment at timepoint' sample groups (e.g., 'OpenCoast_15min' vs. 'Cave_15min') did not result in any significant differences between communities (p.adjusted >0.05; Table S4).

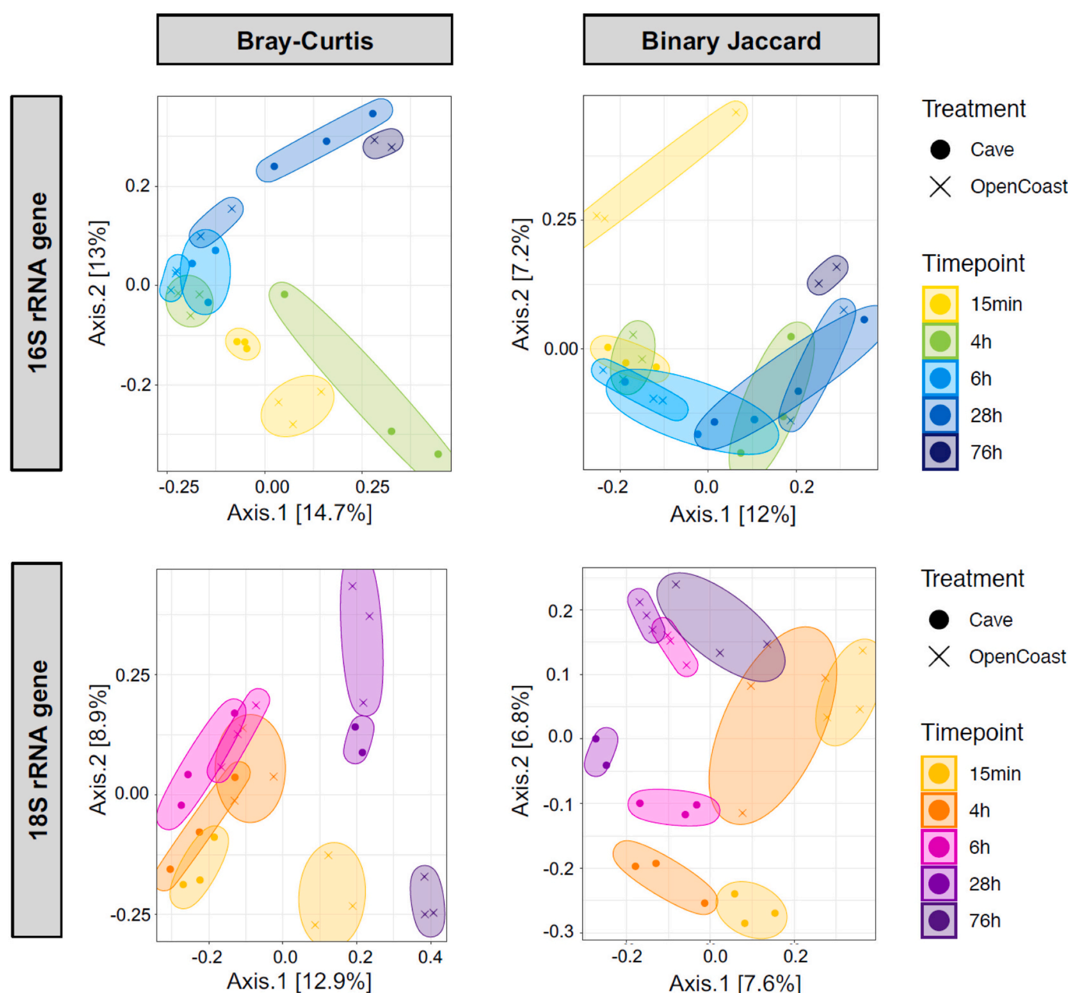

Fig. 2. PCoA plot showing beta diversity of the prokaryotic and eukaryotic Plastisphere communities (i.e., 16S and 18S rRNA genes, respectively) that pioneered the colonisation of the polypropylene strips in the two different marine locations (i.e., treatments 'Cave' and 'OpenCoast'). Beta diversity was assessed by both Bray-Curtis dissimilarity (accounting for taxon abundances) and binary Jaccard distance (accounting only for presence/absence).

3.2.2. *Eukaryotic community beta diversity*

Similarly to prokaryotes, the very early eukaryotic communities were significantly affected by treatment ($p = 0.001$), timepoint ($p < 0.01$), and their interaction ($p < 0.01$; Table S5). Again, the higher R^2 values obtained with Bray-Curtis, as opposed to binary Jaccard, distance indicated that dominant taxa had more impact on the community differences than rare taxa, and that timepoint had a greater effect on community composition than treatment (Table S5), although PCoA plots showed that the rare communities in particular also clustered by treatment (Fig. 2). Permutation tests for homogeneity of multivariate dispersions showed that the dispersions were homogeneous for all variables (treatment, timepoint, sample group; $p > 0.05$; Table S6) with both distance measures, thereby complying with the assumptions of PERMANOVA. Pairwise PERMANOVA comparisons confirmed that communities were significantly different between the two treatments (p.adjusted <0.01; Table S7). When the first three timepoints were compared, significant differences between overall communities were detected between timepoints 15 min and 4 h as well as 15 min and 6 h using Bray-Curtis distance that emphasises high-abundance taxa (p.adjusted <0.05), while the 'rare' communities (i.e., binary Jaccard distance data) differed only between timepoints 15 min and 6 h (p.adjusted <0.01; Table S7). Again, no significant differences were detected between the overall communities between timepoints 4 h and 6 h with either distance measure (p.adjusted >0.05). Likewise, no significant differences were observed in pairwise comparisons between different 'treatment at timepoint' sample groups (p.adjusted >0.05; Table S8).

3.3. *Community diversity (alpha diversity)*

Alpha diversity was generally higher among the prokaryotic communities, while the different metrics also displayed overall higher median values for richness and diversity in the OpenCoast compared with the Cave treatment in both prokaryotic and eukaryotic communities (Fig. 3). Interestingly, a decreasing trend in median richness and diversity over time was observed in both community fractions (i.e., prokaryotic and eukaryotic communities) and both treatments (i.e., OpenCoast and Cave).

3.3.1. *Prokaryotic community alpha diversity*

ANOVA results suggested that, over the first 6 h, both treatment ($p < 0.05$) and timepoint ($p < 0.01$), as well as their interaction ($p < 0.01$), had a significant effect on Shannon diversity (Table S9), while post hoc Tukey's test further confirmed that Shannon diversity was significantly higher in the OpenCoast treatment compared with the Cave (Table S10; $p < 0.05$). Among timepoints, significant differences in Shannon diversity were only observed between timepoints 15 min and 4 h, the latter having a lower diversity than the former ($p < 0.01$), but pairwise comparisons between 'treatment at timepoint' sample groups revealed that this observation may have been caused in part by the anomalous

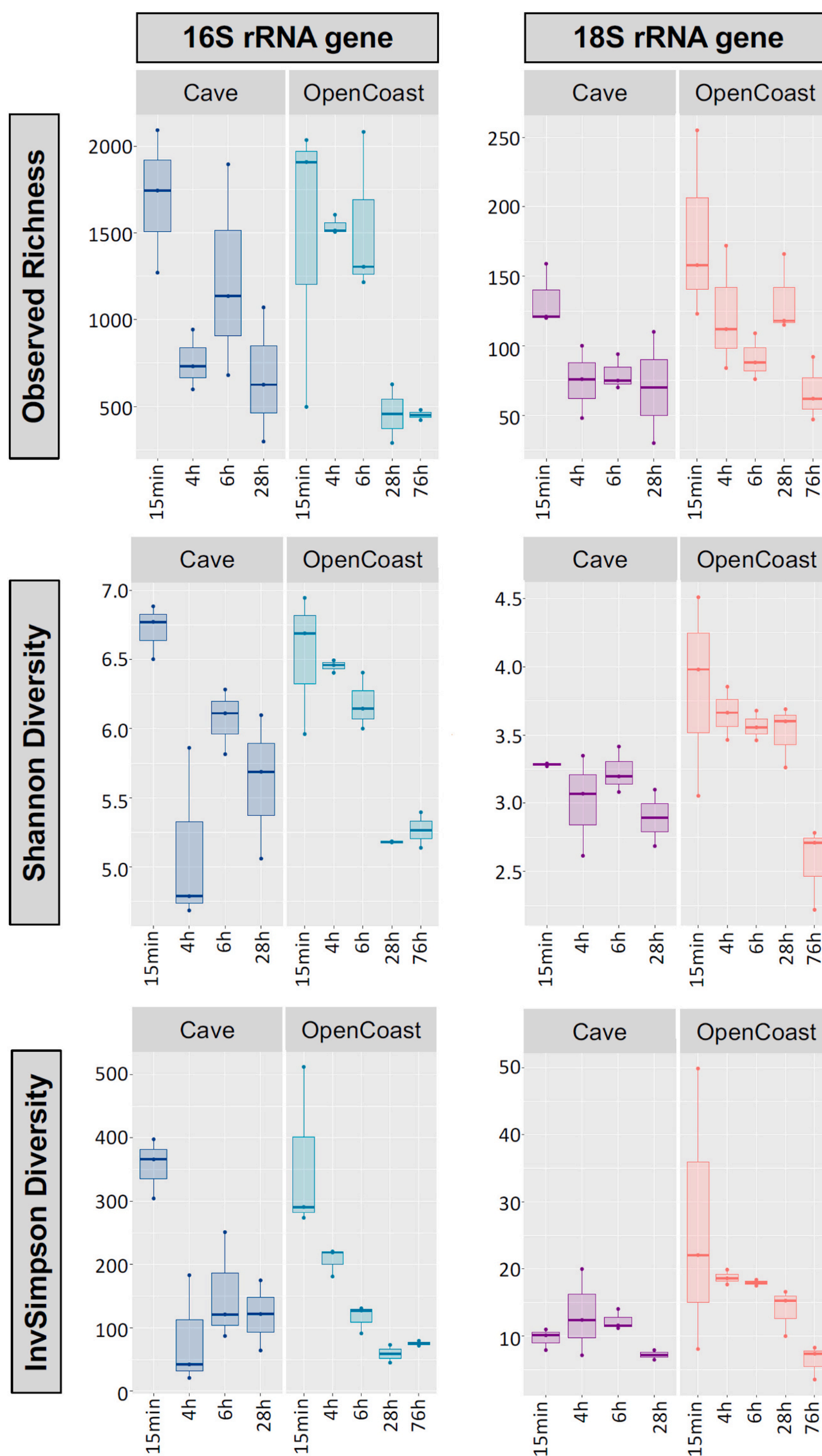

Fig. 3. Alpha diversity of the Plastisphere pioneering the colonisation of polypropylene strips incubated in situ in coastal marine waters (i.e., 'Cave' and 'OpenCoast') over time. Note the different scales on the y-axes.

Cave_4h community that had a significantly lower Shannon diversity than any other sample group (p < 0.05; Table S10, Fig. 3). A Shapiro-Wilk test ($p > 0.05$; Table S9), along with inspection of histograms and Q-Q plots, indicated that the residuals of the Shannon diversity data were normally distributed, while Levene's test confirmed that all variables had equal variances ($p > 0.05$; Table S9). Assumptions for ANOVA were thereby met.

3.3.2. *Eukaryotic community alpha diversity*

As with prokaryotes, the median richness and diversity values generally decreased over time among the eukaryotic communities (Fig. 3). Interestingly though, despite the median richness and diversity values being the highest at timepoint 15 min in all other instances, in the Cave the median inverted Simpson's diversity was slightly lower at 15 min compared with the two consecutive timepoints (Fig. 3), potentially indicating lower evenness in that sample group (Erni-Cassola et al., 2019a). Statistical analyses revealed that only treatment had a

significant effect on Shannon diversity of eukaryotic communities over the first three timepoints (ANOVA, $p < 0.01$; Table S11) and that Shannon diversity was significantly higher in the OpenCoast than the Cave communities (Tukey's test, $p < 0.01$; Table S12). Pairwise comparisons did not reveal significant differences in Shannon diversity between any other groups (Tukey's test, $p > 0.05$; Table S12). The assumptions of equal variances as well as normal distribution of residuals, as required for ANOVA, were again met (Table S11).

Rarefaction curves, which depict whether the sequencing depth was sufficient to capture the whole diversity and species richness in the community, did not level off for most of the prokaryotic community samples whereas the curves did reach saturation in most of the eukaryotic communities (Fig. S3). This, added to the information above on the number of taxa and ASVs detected for both communities and the overall alpha diversity values (Fig. 3), highlights the expected higher diversity extant within the prokaryotic community when compared with the eukaryotic one.

3.4. Community composition

The overall prokaryotic and eukaryotic community compositions over time in OpenCoast and Cave samples can be found in Fig. 4. It is interesting to note the much higher stability of the prokaryotic community at the phyla level in all samples (Fig. 4a) as compared with the more stochastic colonisation of eukaryotes (Fig. 4b). This stochasticity at such early stages of colonisation is particularly apparent in the Cave

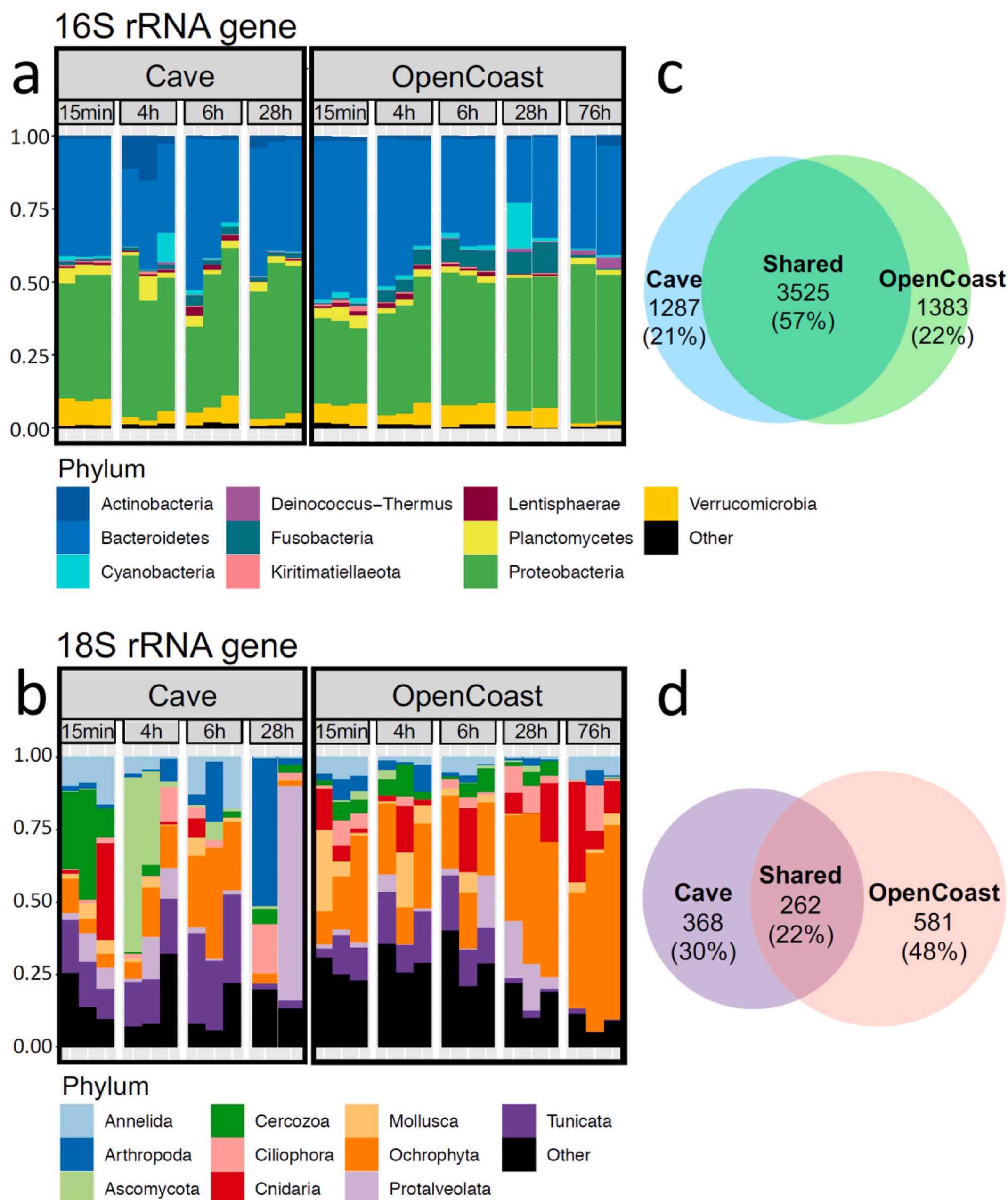

Fig. 4. Phylum-level taxonomic analysis of the prokaryotic (16S rRNA gene; a) and eukaryotic (18S rRNA gene; b) communities that pioneered plastics colonisation in coastal marine waters. *Euler diagrams* show the number of ASVs from the prokaryotic (c) and eukaryotic (d) communities that were exclusive to each one of the two locations (i.e., 'Cave' or 'OpenCoast') as well as the number of ASVs that were shared between the locations. The Euler diagrams include all ASVs that were detected at least once in any respective group. Furthermore, only samples from the first four timepoints (i.e., up to 28 h) are included in the Euler diagrams as these timepoints were available for both treatments.

samples, whereas Plastisphere communities exposed to light in the OpenCoast samples seem to stabilise after 28 h, although longer incubation timepoints would be required in order to confirm this. Light exposure clearly showed a much stronger influence in the selection of the eukaryotic community than on the prokaryotic one as demonstrated by the number of shared ASVs between samples of the Cave and OpenCoast treatments: 57% of the prokaryotic ASVs were present in both conditions (Fig. 4c), whereas only 22% of the eukaryotic ASVs were shared between treatments (Fig. 4d).

3.4.1. Prokaryotic community composition

Proteobacteria and Bacteroidetes were by far the most abundant phyla within the prokaryotic Plastisphere fraction (42.6% and 39.1%, respectively, of total relative abundance of all taxa). Nevertheless, over the first 28 h for which data from both treatments was available, Bacteroidetes were slightly more abundant within the prokaryotic OpenCoast communities (41.3% vs. Proteobacteria 38.3%), while Proteobacteria dominated in the Cave environment (45.0% vs. Bacteroidetes 37.4%). Whilst the relative abundances of Proteobacteria and Bacteroidetes remained relatively equal over time in the Cave, the OpenCoast Plastisphere experienced a transition from a pioneering colonisation by Bacteroidetes (i.e., 53.3% Bacteroidetes vs. 28.2% Proteobacteria) at the 15-min timepoint, to a clear dominance of Proteobacteria when the Plastisphere matured at the 76-h timepoint (i.e., 37.7% Bacteroidetes vs. 52.3% Proteobacteria; Fig. 5). Bacteroidia was by far the most abundant class among the phylum Bacteroidetes (97.8%), whereas Proteobacteria was dominated by the classes Gammaproteobacteria (61.7% of Proteobacteria) and Alphaproteobacteria (34.5%), the latter becoming more abundant in more established Plastisphere communities (i.e., 76 h) in OpenCoast samples (Fig. 5).

Apart from Bacteroidetes and Proteobacteria, other phyla were also found within the Plastisphere communities although in much lower abundance (e.g., Verrucomicrobia 5.3%, Fusobacteria 2.9%; Fig. 4a). Cyanobacteria was the sixth most abundant phylum in the overall dataset (2.0% of total relative abundance), being more abundant in the OpenCoast samples (2.7%) compared with the Cave samples (1.6%) over the first 28 h. Archaea contributed to the overall prokaryotic community with a total relative abundance of only 0.15%, which remained similar across treatments (0.15% vs. 0.18% in Cave and OpenCoast communities, respectively, over the first 28 h).

3.4.2. Eukaryotic community composition

Among eukaryotes, Ochrophyta (25.3% in total; 13.7% in Cave vs. 28.2% in OpenCoast over the first 28 h for which data from both treatments was available), Tunicata (11.9%; 16.8% vs. 10.2%), Protalveolata (7.6%; 11.3% vs. 6.1%) and Cnidaria (7.1%; 3.8% vs. 7.6%) were the most abundant phyla in the overall dataset, while metazoans made up over 35% of the eukaryotic taxa (Fig. 4b).

As expected, the phylum Ochrophyta, mainly composed of photosynthetic primary producing organisms, was most abundant in the light-exposed treatment dominating the OpenCoast communities throughout the sampling period, particularly in the later incubation timepoints 28 h and 76 h (45.1% and 56.3%, respectively; Figs. 4b and 6). When investigating the taxonomic diversity within the phylum Ochrophyta in OpenCoast samples we observed a rapid increase of diatoms over time reaching a maximum abundance after 28 h of submersion (38.3% of total relative abundance in the OpenCoast_28h group), but this group was replaced by brown algae of the class Phaeophyceae after 76 h (i.e., 11.9% of Diatomea vs. 44.5% of Phaeophyceae at OpenCoast timepoint 76 h; Fig. 6). Interestingly, the distribution of diatom and brown algal classes within each Plastisphere was fairly stochastic between samples (Fig. 6).

In the Cave treatment, where light was scarce and the development of photosynthetic organisms to feed the Plastisphere was compromised, we observed a much higher abundance of organisms involved in acquiring organic carbon from the surrounding waters as the main source of carbon and energy, e.g., the filter feeding sea squirts from the phylum Tunicata (16.8% in Cave vs. 10.2% in OpenCoast over the first 28 h), as well as members of Protalveolata (11.3% vs. 6.1%), Arthropoda (8.4% vs. 3.1%), Cercozoa (8.2% vs. 4.8%), Annelida (6.8% vs. 3.0%), and Basidiomycota fungi (2% vs. 0.4%).

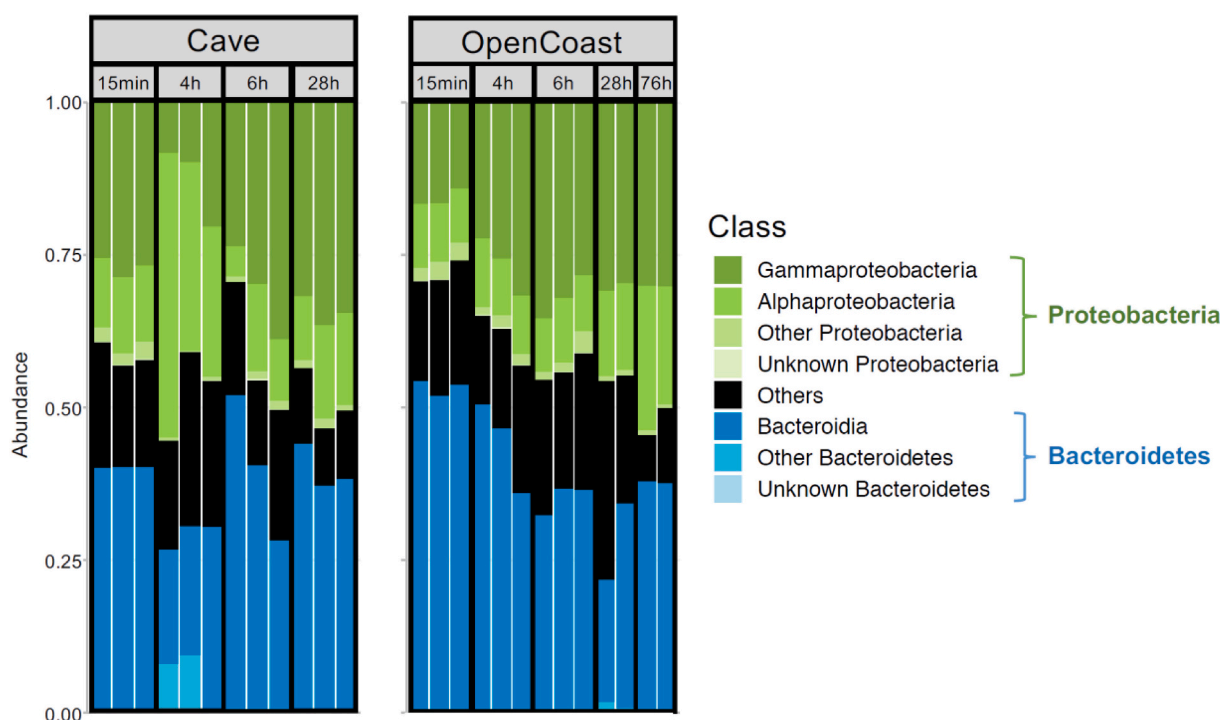

Fig. 5. Evolution over time of the two major prokaryotic phyla involved in early colonisation of plastics in coastal seawaters, i.e., Proteobacteria and Bacteroidetes. The most representative classes within each phylum are indicated.

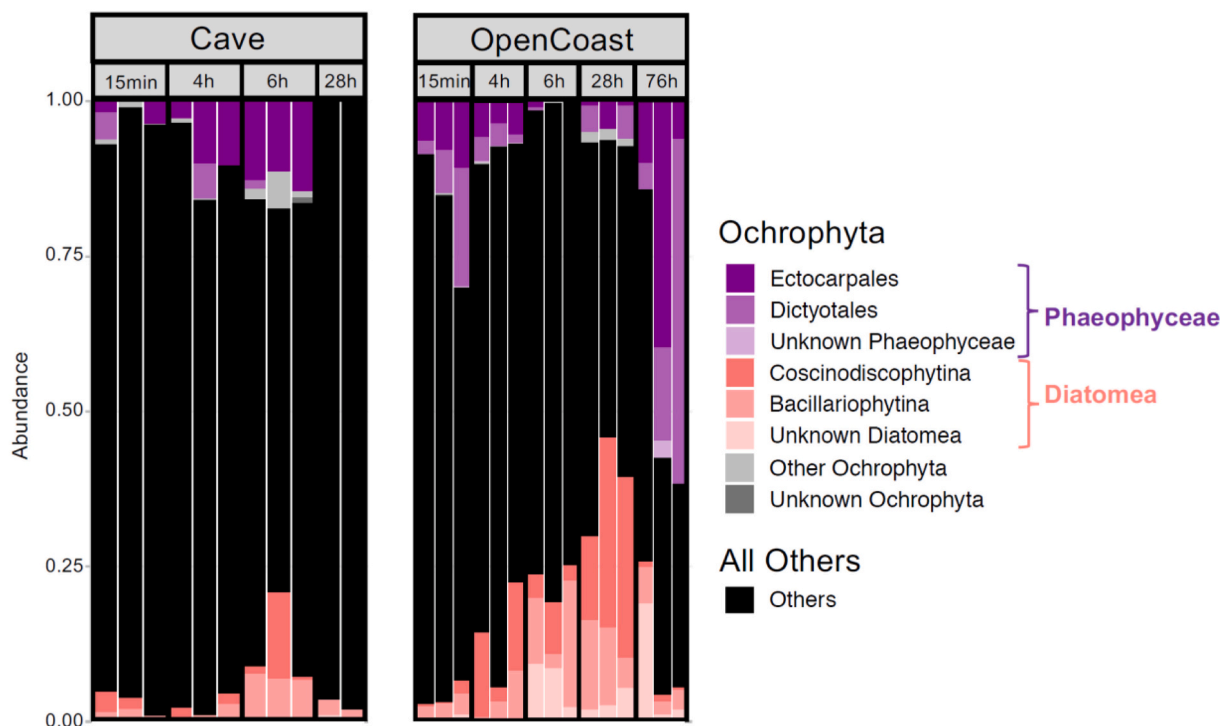

Fig. 6. Evolution over time of the major photosynthetic phylum pioneering colonisation in the Plastisphere communities on PP strips incubated in coastal seawaters, i.e., the eukaryotic phylum Ochrophyta. The most representative orders within the classes Phaeophyceae (brown algae) and Diatomea (diatoms) are indicated.

3.5. Relevant and most abundant species (ASVs) pioneering plastic colonisation

Prokaryotic ASVs pioneering the colonisation of plastic were fairly reproducible between samples and consistent over time, while eukaryotic ASVs were much more stochastic between samples (Fig. 7). As expected, most of the abundant prokaryotic ASVs belonged to the phyla Bacteroidetes (e.g., ASV5, *Polaribacter dokdonensis*-like, 0.9% of the total relative abundance of prokaryotic ASVs; and ASV7, Flavicella, 0.7%) and Proteobacteria (e.g., ASV3, *Photobacterium*, 1.5%; ASV4, *Vibrio*, 1.7%; interestingly, both from the family *Vibrionaceae*), although ASV2, a *Propionigenium*-like marine anaerobe belonging to the phylum Fusobacteria was curiously the most abundant ASV (2.5%), particularly in the OpenCoast samples (3.8% of the total abundance of prokaryotic ASVs in OpenCoast vs. 1.2% in Cave samples; Fig. 7a). ASV36 and ASV94 (1.0% and 0.9%, respectively), both assigned to *Granulosicoccus*, a genus associated with macroalgae (Bengtsson et al., 2012; James et al., 2020), were particularly abundant only in the more mature Cave and OpenCoast Plastisphere communities (ASV36, 4.9% and 3.1% of the total abundance of prokaryotic ASVs in sample groups Cave_28h and OpenCoast_76h, respectively; ASV94, 4.1% and 3.8% of the sample groups Cave_28h and OpenCoast_76h, respectively), suggesting that the eukaryotic community on plastics may be a strong driver of the prokaryotic Plastisphere.

Eukaryotic ASVs from the phylum Ochrophyta dominated the OpenCoast samples, as expected, from the groups of diatoms (e.g., ASV3, Family *Fragilariales*, 5.1% of the total abundance of eukaryotic ASVs in OpenCoast samples; ASV21, Diatomea-like, 2.8%) and brown algae (e.g., ASV25, genus *Spatoglossum*, 5.4% of the total 18S rRNA dataset and up to 51.9% in one of the replicates of timepoint 76 h; ASV36, order Ectocarpales, 2.5% of all OpenCoast 18S rRNA reads; ASV20, also Ectocarpales, was more abundant in the Cave, comprising 2.1% of the total abundance of eukaryotic ASVs in Cave samples; Fig. 7b). The Tunicata ASV2, from the Stolidobranchia order, was the most abundant eukaryotic ASV in our dataset (7.8%), being particularly abundant during the very early stages of colonisation (i.e., the first 6 h, averaging 11.3% in the Cave and OpenCoast samples) but then disappeared at later stages. Also remarkable is the presence of the amoebal Cercozoa genus *Rhogostoma* (ASV4, 20.5% of Cave_15min samples), as well as ASV7 from the detritivorous Annelida order Terebellida, mainly in the first 6 h of incubation in the Cave samples (4.6% of the total abundance of eukaryotic reads in Cave samples).

Two 4-h Cave replicates appeared with anomalously abundant prokaryotic and eukaryotic ASVs that were barely observed in other sample groups, e.g., *Erythrobacter* (ASV8, 19.0% and 10.1% of reads in Cave_4h replicates 1 and 2, respectively; 0% in all other samples), and members of two fungal (Ascomycota) genera, i.e., *Pleospora* (ASV17 25.4% and 13.1%, and ASV22 16.2% and 15.7%; max 0.1% in any other sample) and *Knufia* (ASV28, 18.6% and 3.5%; max 0.4% in any other sample). This could have occurred due to a punctual and temporal attachment of floating aggregates.

3.6. Colonisation by pathogen-like taxa

Despite the challenges and additional determinants needed to correctly assess 'pathogenic' microbes or harmful taxa (Wright et al., 2020b), we estimated the abundance of pathogen-like genera during the early colonisation of plastics in coastal marine waters. Most notably, *Vibrio* spp. (which includes members that can cause illness in humans (Baker-Austin et al., 2018)) and *Tenacibaculum* spp. (known to include fish-pathogens (Bridel et al., 2018)) were the third and the fifth most abundant genera observed in our study (i.e., at least 2.59% and 2.06%, respectively, of total relative abundance of prokaryotic reads; Fig. S4c). Both genera were among the most abundant genera at most timepoints in both treatments, and particularly dominated the community in the Cave_6h sample group (*Tenacibaculum* 5.45% and *Vibrio* 4.72%; Fig. S4c). The *Vibrio*-like ASV4 was also the second most abundant ASV in our prokaryotic dataset (1.7% of total prokaryotic abundance; Fig. 7a). Both of these genera have been frequently and abundantly observed on marine plastics (Roager and Sonnenschein, 2019; Wright et al., 2020a) and, interestingly, a number of studies have indicated that *Vibrio* might prefer polypropylene as a substrate for colonisation (Zettler

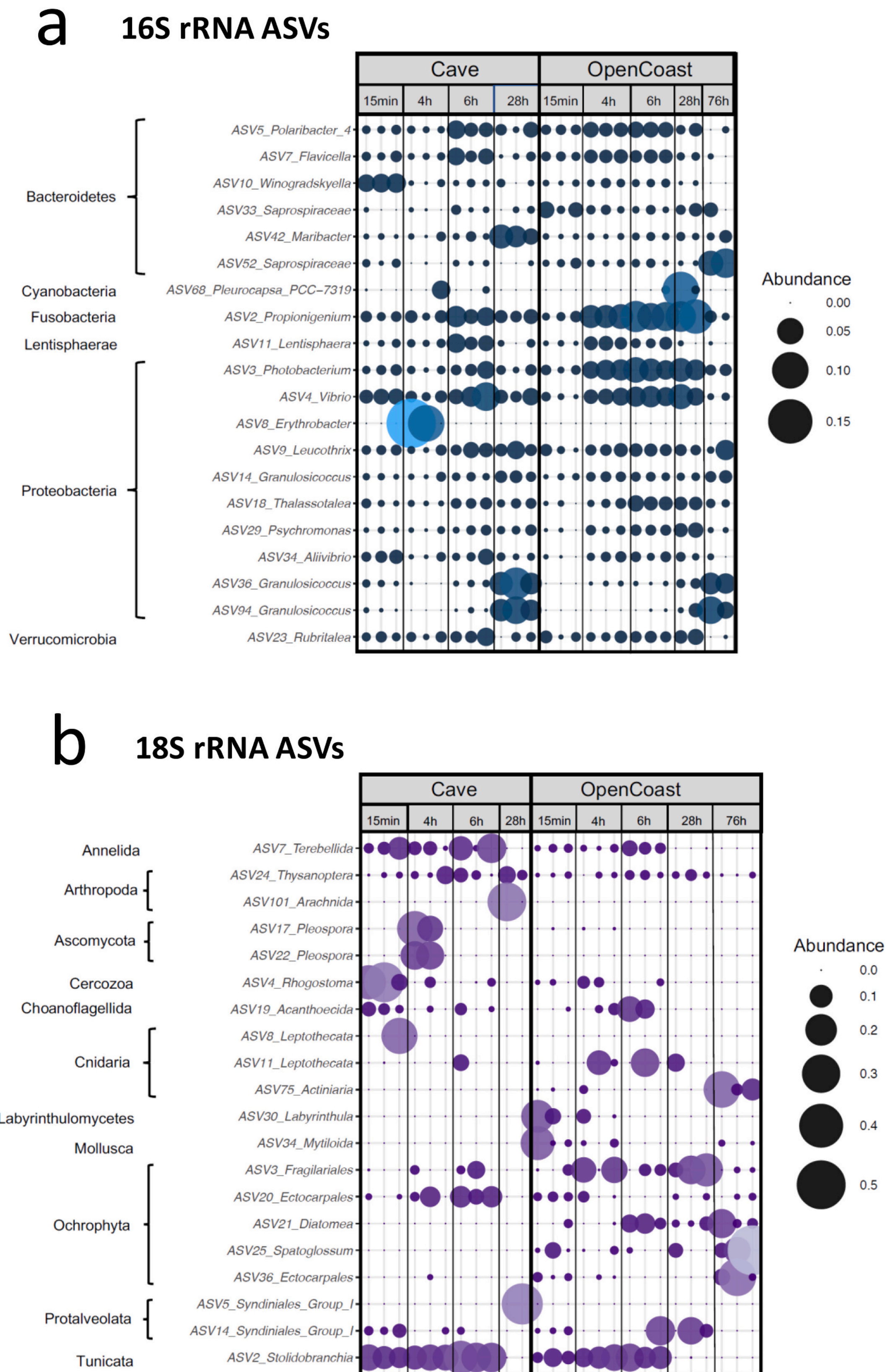

Fig. 7. Bubble plot showing the twenty most abundant prokaryotic (a) and eukaryotic (b) ASVs. The relative abundance (scaled to 1) of the ASVs in each sample and the closest related taxon is shown.

et al., 2013; Laverty et al., 2020; Hansen et al., 2021).

Although only a relatively small proportion of eukaryotic taxa were identified at genus level (i.e., 30% of relative abundance of total eukaryotic reads; Fig. S4f), some potentially harmful dinoflagellate genera were detected in the dataset, including *Gymnodinium* (e.g., ASV26: 13.8% of OpenCoast_28h replicate 1), *Scrippsiella* (e.g., ASV45, ASV131 and ASV135: 6.5%, 2.0% and 2.0% of OpenCoast_28h replicate 3), and *Gyrodinium* (e.g., ASV189 and ASV249: 7.5% and 5.3%, respectively, of Cave_15min replicate 1), all of which include members classified as Harmful Algal Bloom (HAB) forming species (Ignatiades and Gotsis-Skretas, 2010; Moestrup et al., n.d.). Contrarily, the parasitic dinoflagellates from the order Syndiniales (11.3% and 6.0% of total relative abundance in Cave and OpenCoast samples, respectively, over the first 28 h; members of order Syndiniales were not detected in OpenCoast_76h samples) may potentially control dinoflagellate 'red tide' blooms (Park et al., 2004; Guillou et al., 2008). Another interesting eukaryote in our dataset, the marine protist *Labyrinthula* (the most abundant genus in OpenCoast_15min samples, i.e., 12.1%, and the fifth most abundant in OpenCoast_4h samples, i.e., 1.3%, and e.g., ASV30 comprised 21.7% of OpenCoast_15min replicate 1), includes members responsible for seagrass disease outbreaks, which can lead to serious disruptions to critical ecosystem services thereby causing not only ecological, but also economical losses (Bockelmann et al., 2012; Sullivan et al., 2013). Similar to our findings, members of *Labyrinthulaceae* were detected as early (i.e., 1 week) colonisers of plastics (PS and PETE) incubated in the Caribbean Sea (Dudek et al., 2020), and they have also been reported from field-collected marine plastic debris (Debroas et al., 2017; Amaral-Zettler et al., 2021b).

3.7. Colonisation by biodegrader-like taxa

Taxa belonging to the 'obligate hydrocarbonoclastic bacteria (OHCB) group' and usually associated with plastic biodegradation (Roager and Sonnenschein, 2019), including the genera *Alcanivorax, Oleispira, Neptunomonas, Thalassospira, Thalassolituus, Cycloclasticus, Marinobacter* and *Oleiphilus* (Erni-Cassola et al., 2019a; Gutierrez, 2018; Radwan et al., 2019), were mostly present only in negligible amounts (combined OHCB abundance 0.4% of prokaryotes). The total relative abundance of the combined OHCB genera increased over time in both treatments (from 0.44% at 15 min to 0.92% at 28 h in Cave samples and from 0.13% at 15 min to 0.73% at 76 h in OpenCoast samples), which was mainly due to the increase in the relative abundance of *Oleiphilus* spp., predominantly in the OpenCoast treatment (Table S13). Interestingly, *Oleiphilus* spp. were found to be enriched on weathered and non-weathered PE, but not on glass, after 2 days of incubation in a previous study conducted in the same location but later in the same year (i.e., April vs. August) (Erni-Cassola et al., 2019a). Furthermore, members of some of the most abundant genera in our dataset, for example *Tenacibaculum, Polaribacter, Leucothrix, Shewanella* and *Vibrio* (Fig. 7 and Fig. S4c) have been shown to degrade hydrocarbons, such as crude oil or phenanthrene (Prince et al., 2019). Nevertheless, inferring plastic degradation from the presence of these taxa may be an overstretch as members of many hydrocarbonoclastic genera have also been associated with e.g., microalgae (such as diatoms) and other marine particles (Dudek et al., 2020; Abell and Bowman, 2005; Oberbeckmann et al., 2016), having a role in biodegrading organic matter derived from photosynthetic microbes as discussed in Wright et al. (Wright et al., 2020b).

4. Discussion

4.1. The very early colonisers – who's first?

Here we show that Bacteroidetes were the dominant prokaryotic group during the earliest (i.e., 15 min and 4 h) stages of colonisation, particularly in the OpenCoast environment, implying that members of this phylum might play a role as pioneer taxa on marine biofilms, before being masked or outcompeted by other groups. This observation contrasts with the previous understanding of Proteobacteria, namely that Gammaproteobacteria (Pollet et al., 2018; Lee et al., 2008), soon followed by Alphaproteobacteria, have previously been reported as the initial colonisers on marine surfaces (Wright et al., 2020a; Dang and Lovell, 2016). This discrepancy is most likely caused by the scarcity of very early (<1 day) timepoint data, but also by the fact that previous studies on early colonisation have either used (1) primers that underestimate the abundance of Bacteroidetes (Pollet et al., 2018) or (2) low-throughput techniques that capture only a small proportion of the overall community (De Tender et al., 2017b). Indeed, our OpenCoast results show a rapid decline in the relative abundance of Bacteroidetes during the earliest hours while the abundance of Proteobacteria, especially Gammaproteobacteria and Alphaproteobacteria, increases and eventually exceeds that of Bacteroidetes at the 6 h - timepoint (Fig. 5). This community shift, however, was not observed in the Cave environment. It is therefore highly probable that geographical location and other spatio-temporal or environmental factors (e.g., sunlight irradiation) have a role in determining the pioneer communities. Acryl plates incubated in coastal waters of Antarctica revealed that Bacteroidetes was the most abundant phylum on day 1, then subsequently decreased in abundance as Gammaproteobacteria grew more abundant, yet began increasing again after day 4 and regained the position as the most abundant phylum on day 7 (Lee et al., 2016). There is also evidence that the dominating Bacteroidetes members might be different between the early (day 1) and later (day 7) stages of colonisation (Lee et al., 2016). Our short timeline here, however, was not long enough to confirm a possible re-establishment of a secondary Bacteroidetes community.

Members of the phylum Bacteroidetes have been associated with a preference for a particle-attached lifestyle as well as being specialised in the degradation of high-molecular weight compounds such as polysaccharides (e.g., cellulose, chitin) and proteins (Fernandez-Gomez et al., 2013; Gómez-Pereira et al., 2012; Kirchman, 2002). Plastics are known to be primarily coated by organic macromolecules, i.e., the ecorona, immediately after entering natural waterbodies (Galloway et al., 2017). This adhered organic matter may facilitate and condition the first microbial colonisers of the plastic surface, which could potentially explain the high abundance of Bacteroidetes in very early biofilms in seawater (Dang and Lovell, 2016; Petrova and Sauer, 2012). Interestingly, proteins are often found as the major component of marine ecocorona during the first hours of immersion in seawater (Garg et al., 2009; Satheesh and Wesley, 2010). Thus, it could be hypothesised that these macromolecules provide a food source for Bacteroidetes, initiating the first stages of biofilm formation (Siboni et al., 2007). The following community shift may be linked to the increasing attachment by microalgae that provide labile cell exudates favoured by members of the Proteobacteria (Dang and Lovell, 2016). The re-establishment of a Bacteroidetes-dominated community during the late-stage colonisation could be explained by the ability of Bacteroidetes to utilize decaying algal matter (e.g., their polysaccharidic cell walls (Gómez-Pereira et al., 2012)) and other detritus accumulating on the mature biofilm (Dang and Lovell, 2016). Hence, while Bacteroidetes are generally associated with secondary surface colonisation in marine environments (Dang and Lovell, 2016), including Plastisphere communities (Wright et al., 2020c) where it has even been suggested that the phylum could be used as a putative indicator for longer environmental exposure times for plastic debris collected from marine waters (De Tender et al., 2015), our findings highlight the need to re-evaluate or refine this claim by defining indicators at a finer taxonomic resolution.

Diatoms and brown algae dominated the eukaryotic community fraction in our study, particularly in the well-lit OpenCoast, which is not surprising considering members of the phylum Ochrophyta are mainly photoautotrophic. Diatoms have consistently been reported as abundant and early colonisers on marine plastics (Dudek et al., 2020; Amaral-Zettler et al., 2020; Reisser et al., 2014), while brown algae have received less attention despite having been documented as abundant

Plastisphere members in a number of studies (e.g., (Zettler et al., 2013; Dudek et al., 2020; Oberbeckmann et al., 2016)). The abundance of the latter in our study may have come as a consequence of plastics being incubated in a shallow coastal environment, the time of the year, or the fact marine brown algae are mostly multicellular organisms (Song et al., 2015) which may bias 18S rRNA gene read counts towards higher abundances (Gong and Marchetti, 2019). Our eukaryotic dataset also revealed a high abundance of organisms belonging to the kingdom Animalia, e.g., annelids, arthropods, cnidarians, molluscs and tunicates. High abundances of metazoans have also been reported colonizing plastics in previous studies (e.g., (Bryant et al., 2016; Zaiko et al., 2016; Kettner et al., 2019)). Considering the small size of our mesoplastic strips, we agree with the discussion by Kettner et al. (Kettner et al., 2019) that the high relative abundances of these organisms can probably be explained by (1) the attachment of eggs, larvae and juvenile forms, (2) the presence of environmental DNA or fragments of the organisms themselves, and/or (3) the high 18S rRNA gene copy numbers in these multicellular organisms (Gong and Marchetti, 2019).

4.2. Community differences over time

Time had a significant effect on both the overall prokaryotic and eukaryotic communities, although no significant difference was observed in either dataset when comparing 4 h and 6 h incubation times indicating that sampling at 2-h intervals is probably not necessary. Time, measured at a variety of temporal resolutions, has been shown to have a significant effect on marine microbial community composition on plastics (Wright et al., 2020a; Pinto et al., 2019; Erni-Cassola et al., 2019b). Among the few very early-stage studies, bacterial communities sampled across a 36-h period were found to cluster into two distinct stages according to sampling time: stage 1 included the establishment of pioneer communities between the first 3–9 h of biofilm formation, followed by stage 2 where a successional transition occurred around 24–36 h (Lee et al., 2008). Similarly, a laboratory study found that community specialisation in the early successional stages rapidly shifted from particle attachment (8–20 h) to substrate degradation (20–44 h) and secondary colonisation (44–140 h) (Datta et al., 2016). Our study supports these findings as, even though we were not able to include all timepoints in statistical tests, our PCoA plots show that communities in both prokaryotic and eukaryotic datasets generally clustered separately between early (15 min), mid (4 h and 6 h) and late (28 h, 76 h) timepoints.

Time also had an effect on alpha diversity: the median richness, diversity and evenness values in both prokaryotic and eukaryotic sample groups generally decreased over time. Little information is available regarding changes in alpha diversity for marine microbial biofilm communities during very early (< 1 day) successional stages. Some field studies have, however, reported a decreasing trend for early-stage biofilms from day 1 onwards (Abed et al., 2019; Lee et al., 2016), while others have recorded the lowest diversity at day 1 (Pollet et al., 2018). Our observations in this study can probably be explained by high initial species richness due to rapid founding colonisation and (potentially reversible) attachment, which subsequently declines due to rapid growth of the fittest taxa and competition caused by scarcity of niches in the young biofilm (Jackson, 2003). Long-term maturation of the biofilms will cause an increase in diversity due to the enhanced complexity of the biofilm structure and thereby increase in available niches, before the diversity eventually stabilises (Jackson, 2003).

4.3. Differences between OpenCoast (high-light) and cave (low-light) environments

Our results indicated that location, likely driven by light availability, had an effect on both prokaryotic and eukaryotic community compositions, and this effect appeared to be statistically significant at least over the first 6 h. Over this time, Shannon diversity was also significantly higher in both community fractions incubated in the OpenCoast compared with the Cave, with a remarkable increase in abundance of phototrophic eukaryotes. Previous studies have found that location and other environmental factors often have a greater impact on microbial communities than, for example, the substrate material itself (Wright et al., 2020a; Oberbeckmann and Labrenz, 2020). Light regime was previously found to have a significant impact on both the overall community as well as just the heterotrophic fraction (i.e., Cyanobacteria excluded), and the differences were more pronounced at the early (1 week) than later (1–2 months) stages of biofilm formation (Pinto et al., 2019). The authors concluded that differences between communities exposed to different light conditions were most likely caused by a combination of solar radiation (such as UV-radiation) and indirect effects of sunlight-plastic interactions (e.g., increased leaching of plastic-associated compounds) and the quality and availability of organic carbon (e.g., access to photosynthate) (Pinto et al., 2019). This previous study focused only on the 16S rRNA gene community but was combined with an analysis of the eukaryotic community members using Scanning Electron Microscopy (SEM), finding that photoautotrophs such as Cyanobacteria, diatoms and algae were more abundant in the ambient compared with the dim light treatment (Pinto et al., 2019), which agrees with our dataset. These observations were further supported by a laboratory study showing that microbial communities growing on PET bottles in seawater were characterized by chlorophyll *a* production under natural light, and by hydrolytic enzymatic activities and high prokaryote abundance when grown in dark (Misic and Covazzi Harriague, 2019).

4.4. Potentially harmful rafters and hydrocarbon biodegraders

One of the main concerns regarding plastic pollution in the marine environment has been the potential for plastics to act as vectors for pathogens (Zettler et al., 2013; Kirstein et al., 2016; Laverty et al., 2020) and other harmful organisms, such as HAB-forming taxa (Masó et al., 2003; Masó et al., 2016; Casabianca et al., 2019), possibly enabling their enrichment and transport across long distances (Kiessling et al., 2015). Our dataset contained several potentially pathogenic and otherwise harmful taxa such as some species of *Gymnodinium* that can produce toxins that can accumulate in filter feeding organisms and subsequently cause paralytic shellfish poisoning in humans if consumed (Anderson et al., 1989; Margarida Rodrigues et al., 2012), or *Scrippsiella* blooms that are generally considered non-toxic but can be lethal to oyster and clam larvae (Tang and Gobbler, 2012) or lead to anoxic conditions resulting in fish kills (Hallegraeff, 1992). Nevertheless, inferring pathogenicity per se requires the determination of additional pathogenic factors that cannot be assessed via short read amplicon analysis (Wright et al., 2020b). Hence, while it is not surprising to find a high abundance of members of the genus *Vibrio* in our study (2.6% of total relative abundance of the overall dataset), it is difficult to assess the 'harm' they may cause in the environment. Whichever the case, our findings hereby highlight the potential for plastic debris to accumulate and transport potentially harmful micro-organisms within minutes of being discarded in the marine environment.

The same applies to the identification of potential plastic-biodegrading organisms where, again, further work is required to ascertain their estimated role in degrading the plastic surfaces (Wright et al., 2020b). Moreover, to the best of our knowledge there is currently no known mechanism described for the molecular hydrolysis and assimilation of polypropylene. Hence, taxa associated with the 'obligate hydrocarbonoclastic bacteria (OHCB) group' (Gutierrez, 2018; Radwan et al., 2019) require further investigation, i.e., isolation and mechanistic characterization in biodegrading processes. Interestingly, we observed a higher abundance of these microbes in the shaded Cave treatment suggesting that a reduced supply of photosynthate within the Plastisphere may encourage the proliferation of potential biodegrading bacteria.

5. Conclusions

Our preliminary study is the first one to investigate the very early microbial community composition and succession on low-density plastics in situ in coastal waters using amplicon sequencing of 16S and 18S rRNA genes. We found that polypropylene harbours a diverse and characteristic community of both prokaryotic and eukaryotic microorganisms during the very first moments after submersion in seawater (only 15 min after submersion) and that, over time, the communities show distinct yet dynamic successional developments that differ between environmental conditions. Members of Bacteroidetes were discovered as dominant pioneer colonisers at the initial stages of biofilm formation, before being masked or outcompeted by Proteobacteria in the well-lit OpenCoast environment and, hence, we may need to re-evaluate the role of Bacteroidetes in early stages of Plastisphere formation. Our analysis also supports the idea that the microbial community that develops on plastics is mainly powered by phototrophs, in this study by the eukaryotic phylum Ochrophyta (i.e., diatoms and brown algae), whereas in plastics incubated under dark conditions communities rely on available organic matter from the surrounding waters. While we observed a relatively high abundance of potentially pathogenic and harmful taxa from the very early moments after submersion, the number of potential hydrocarbon-degraders was low, possibly requiring pre-treatments or pre-weathering to enhance these taxa as done in other studies. Future early colonisation experiments of plastic in marine environments should include additional control materials (e.g., glass and/or wood, and water samples), different plastic types and treated plastics, as well as a longer time series with further timepoints.

CRediT authorship contribution statement

Conceptualisation: M.L. and J.C.-O.; field work: J.C.-O., C.J.D., and M.L.; DNA extraction: M.L.; library preparation and sequencing: R.J.W. and C.J.D.; methodology: J.C.-O., R.J.W. and M.L.; formal data analysis: M.L. (with training by R.J.W.); writing—original draft preparation: M. L.; writing—review and editing: J.C.-O.; visualisation: M.L.; supervision: J.C.-O. and M.P.; project administration: J.C.-O.; funding acquisition: M. L., J.C.-O. and M.P. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

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