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Ocean acidification alters bacterial communities on marine plastic debris

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Abstract

Rising quantities of plastic waste in the ocean means is providing a growing and more widespread novel habitat for microbes. Plastics have taxonomically distinct microbial communities (termed the 'Plastisphere') and can raft these unique communities over great distances. In order to understand Plastisphere properly it will be important to work out how major ocean changes (such as warming, acidification and deoxygenation) are shaping microbial communities on waste plastics in marine environments. Here, we show that common plastic drinking bottles provides rapidly become colonized by novel biofilm-forming bacterial communities, and that ocean acidification greatly influences the composition of plastic biofilm assemblages. We highlight the potential implications of this community shift in a coastal community exposed to enriched CO_2 conditions.

Introduction

Plastic pollution has become an emerging environmental issue in recent years, with increasing quantities of this synthetic material being introduced into the marine environment (Bergmann et al., 2017; Ryan & Moloney, 1993). It is estimated that around 360 million metric tons (Mt) of plastic were produced globally in 2018 (PlasticsEurope, 2019), with an estimated 4.8-12.7 Mt entering the oceans as macroscopic litter and microplastic particles each year (Geyer et al., 2017; Worm et al., 2017). Plastics have a number of impacts, such as through ingestion and entanglement (Boerger et al., 2010; Codina-García et al., 2013; Schuyler et al., 2014), which often has fatal consequences (Thompson et al., 2009; Worm et al., 2017). Fragmentation of plastic debris results in micro- and nano-plastics, with growing concern that they can be transferred through food webs along with any chemicals or pathogens they contain (Galloway & Lewis, 2016). As this breakdown is slow, macro plastic debris are accumulating in the ocean and pose a pervasive threat.

Due to their very slow degradation, plastics provide novel and durable substrata for rapid microbial colonisation, which can be transported across and between ocean basins via currents or after extreme events (e.g. the Great East Japan Earthquake and tsunami in 2011 washed away an estimated 5 million tons of debris which dispersed across the Pacific ocean basin; Murray et al., 2018). This rafting of biota is a cause for concern (Jokiel, 1990), since it could aid the global dispersion of invasive species (Barnes, 2002; Rochman et al., 2016), pathogens (Zettler et al., 2013), harmful bloom-forming algae (Masó et al., 2003) and persistent organic pollutants (Hirai et al., 2011). Plastic associated microbial communities - the 'Plastisphere' (Zettler et al., 2013) – have a different taxonomic composition to that of the surrounding water (McCormick et al., 2014; Zettler et al., 2013). Uncertainty remains as to whether recruitment of microbial communities onto plastics enrich some taxa (a 'core Plastisphere community') (Amaral-Zettler et al., 2020; Zettler et al., 2013), or are simply being driven by conventional

marine biofilm processes (i.e. plastic provides an available surface for colonisation; Oberbeckmann et al., 2016).

When surfaces are submerged in seawater they are rapidly colonised by microorganisms (Salta et al., 2013), eventually forming a biofilm matrix which provides resources, protection and improved opportunities for physiological homeostasis of the bacteria (Dang & Lovell, 2016). As early colonisers of marine biofilms, bacteria can determine the structure, dynamics, and function of mature biofilm communities (Dang et al., 2008). The formation of marine biofilm communities is strongly shaped by environmental conditions (e.g. temperature, salinity, pH; Toyofuku et al., 2016), and this is no different for plastic-associated microbial communities (Oberbeckmann et al., 2018) which will also be strongly influenced by biogeography, local conditions, season and human impacts (Amaral-Zettler et al., 2015, 2020; Oberbeckmann et al., 2014). Investigating the role of environmental factors in shaping the community composition of plastic-associated microbial communities will help understand the potential effects these communities will have (e.g., rafting of non-natives, and pathogens). However, the longevity and pervasive nature of plastics means that future projected environmental conditions need to be considered, and there remains a lack of information concerning how the Plastisphere will be influenced by other major anthropogenic changes to the ocean.

Ocean acidification, the alteration of seawater carbonate chemistry due to rising atmospheric CO_2, can play a strong role in determining the community composition and dynamics of marine ecosystems (Agostini et al., 2018; Gaylord et al., 2015; Harvey et al., 2019). Our understanding of processes driving responses to increasing levels of CO_2 in seawater has been greatly advanced by laboratory experiments (Dupont & Pörtner, 2013; Riebesell & Gattuso, 2015) but it is difficult to scale-up from these studies to assess the effects of these changes on ocean systems (Hall-Spencer & Harvey, 2019). Natural analogues, such as CO_2 seeps, offer

opportunities to investigate the long-term ecological consequences of increasing CO_2, while retaining natural pH variability and ecological interactions (Agostini et al., 2018; Hall-Spencer et al., 2008). The effects of rising CO_2 on plastic-associated microbial communities has not previously been investigated. Previous studies using natural CO_2 seeps have, however, shown that ocean acidification can influence the community composition and diversity of biofilm communities (Kerfahi et al., 2014; Lidbury et al., 2012; Taylor et al., 2014). Plastics provide a novel ecological habitat and we assert that ocean acidification will likely play a role in shaping the community composition and functioning of the Plastisphere.

Here, we studied the bacterial composition of biofilms grown on plastic drinking bottles (which are a conspicuous marine litter problem worldwide) along a natural gradient in seawater carbonate chemistry at carbon dioxide gas seeps off Japan. We sequenced bacterial 16S rRNA to investigate the effects of ocean acidification on plastic bottle biofilms and compared them to the free-living and particle-associated bacteria from the same locations. Our hypotheses were that: 1) plastic surfaces would have a different bacterial community to that found on suspended particles or free-living in the plankton, as is the case on plastic debris in the Baltic Sea (Oberbeckmann et al., 2018); and 2) that a gradient in seawater pCO_2 would cause significant changes in bacterial biofilm communities - similar to those observed in biofilms grown on microscope slides (Lidbury et al., 2012) and sediment (Kerfahi et al., 2014) in the Mediterranean Sea.

Materials and methods

Study locations

We assessed the response of biofilm-forming bacterial communities along a natural gradient in pCO_2 at a volcanic seep off Shikine Island, Japan (34°19′9″ N, 139°12′18″ E). We used four locations along the pCO_2 gradient, and named them with reference to their equivalent Intergovernmental Panel on Climate Change (IPCC) Representative Concentration Pathway (RCP) scenarios (IPCC, 2013). These sites were: 'Reference' (mean pCO_2: 410 ± 73 µatm), which was outside the influence of the CO_2 seep; 'RCP 2.6′ (mean pCO_2: 493 ± 158 µatm); 'RCP 6.0′ (mean pCO_2: 971 ± 258 µatm); and '>RCP 8.5′ (mean pCO_2: 1803 ± 1287 µatm, Table 1). The locations used in this study as well as the carbonate chemistry was previously reported in Harvey *et al*. (2019); the methods used are reported in the supporting online material. We used the >RCP 8.5 location to assess the response of bacterial communities to ocean acidification beyond average sea surface predicted levels due to human CO_2 emissions. The locations used in this study had the same temperature, dissolved oxygen, total alkalinity, and were all at 5 m depth (Agostini et al., 2015, 2018).

Table 1. Carbonate chemistry of the Reference, RCP 2.6, RCP 6.0, and >RCP 8.5 locations at Shikine Island, Japan (Previously reported in Harvey et al., 2019). pH_T, temperature, salinity (n = 336), and total alkalinity (A_T, n = 4) are measured values. Seawater pCO_2, dissolved inorganic carbon (DIC), bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), saturation states for calcite (Ωcalcite), and aragonite (Ωaragonite) are values calculated using the carbonate chemistry system analysis program CO2SYS (Pierrot et al., 2006). Values are presented as mean ± S.D. RCP refers to the representative concentration pathways used in IPCC projections.

Station	pH_T	Temp (°C)	Salinity (psu)	A_T (µmol kg^{-1})	pCO_2 (µatm)	DIC (µmol kg^{-1})	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	Ωcalcite	Ωaragonite
Reference	8.041	23.086	34.129	2281.9	409.965	2007.341	1798.117	196.978	4.76	3.115
	0.067	0.603	0.741	6.80	73.383	38.944	61.612	24.859	0.596	0.392
RCP 2.6	7.983	21.437	35.056	2282.93	493.011	2044.255	1855.972	173.103	4.144	2.703
	0.119	1.273	0.125	6.57	158.004	53	81.439	32.771	0.781	0.501
RCP 6.0	7.719	22.896	34.91	2271.84	970.706	2144.537	2008.7	106.928	2.568	1.681
	0.095	0.937	0.211	3.03	257.68	33.169	43.845	17.716	0.423	0.274
>RCP 8.5	7.529	22.072	34.723	2277.62	1803.047	2218.975	2088.23	75.92	1.823	1.19
	0.234	1.212	0.742	20.50	1287.448	82.982	82.43	33.368	0.799	0.519

Field Deployment and Collection

Clear polyethylene terephthalate (PET) 500 ml bottles were deployed in each location by a SCUBA diver (six replicates per location, 24 replicates in total). Each PET bottle was deployed approximately 20 cm above the seabed, and attached using anchor bolts and 5 mm thick rope, with a small float attached to the top in order to maintain positive buoyancy (Figure 1). Each PET bottle was washed with mild bleach then distilled water before deployment. The PET bottles were deployed for three-weeks (26th June 2018 until 17th July 2018) to capture the early-stage biofilm colonisation. Upon sample collection, a small cutting of the bottles (approximately 2 × 5 cm per piece) was collected underwater using sterilised scissors and immediately placed into a ziplock bag. Immediately following collection, the plastic samples were rinsed with sterile water to remove residual debris, and transferred into a new ziplock bag, and stored at -20 °C until DNA extraction. At each location, seawater samples (six replicates per location) were also collected. One litre of seawater was filtered through a 3 µm filter for particle-associated communities (three replicates per location), and 0.5 litre of seawater was filtered through 0.22 µm filters for free-living communities (three replicates per location). Filters were also stored at -20 °C until DNA extraction.

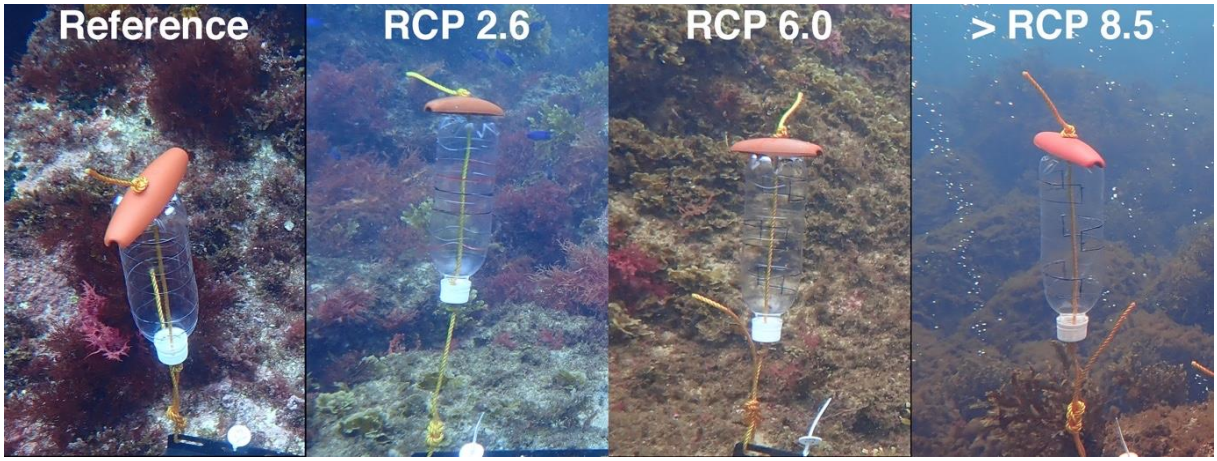

Figure 1. Example deployment of polyethylene terephthalate (PET) drinking bottles in each of the locations (Reference, RCP 2.6, RCP 6.0, and >RCP 8.5) at Shikine Island, Japan. NOTE: the presence of CO_2 bubbles in the > RCP 8.5 location.

DNA extraction and PCR amplification

DNA was extracted from the plastic bottles (for study of biofilm on plastic) and the filters (for the comparison of bacteria in the surrounding seawater). Identical cut plastic sections of 2 cm × 2 cm were taken from the side of each bottle, and further cut in small pieces. The filters were each cut into several pieces and transferred for processing with the DNA extraction kits (FastDNA SPIN Kit for Soil; MP Biomedicals, LLC, USA) according to the manufacturer's instructions. The DNA isolated from each sample was amplified using the universal primers, 515 F (5′-barcode- GTGCCAGCMGCCGCGGTAA -3′) and 907 R (5′-barcode- CCGYCAATTCMTTTRAGTTT -3′), targeting the V4–V5 regions of the prokaryotic 16S rRNA gene. The barcode is an eight-base sequence unique to each sample. PCR experiments were performed under the following conditions: 95 ºC for 5 min, 5 cycles of 57 ºC for 30 s and 72 ºC for 30 s, and 25 cycles of 95 ºC for 30 s and 72 ºC for 30 s. PCRs were performed in duplicate using 24 μL reaction volumes, consisting of 20 μL of Emerald AMP GT PCR 1× Master Mix (Takara Bio, Shiga, Japan), 0.5 μL (10 μM) of each barcoded PCR primer pair and 3 μL of DNA template (10–50 ng of DNA). PCR products were purified using an AMPure XP bead purification kit (Beckman Coulter, Brea, CA, USA) and were pooled in equal concentrations. An Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA, USA), was used to confirm the correct concentration needed for sequencing.

Sequence analysis

The sequenced data generated from Miseq sequencing was processed using QIIME platform version 1.9.1 (Caporaso et al., 2010) and quality-filtered with Trimmomatic (Bolger et al., 2014), using the following criteria: LEADING:3 TRAILING:3 SLIDINGWINDOW:50:20 MINLEN:240. After chimera removal, all sequences were clustered into OTUs at 97 % identity and classified against SSURef_132_SILVA database using the VSEARCH pipeline. The

sequences used in this study have been deposited in the NCBI Sequence Read Archive under SRA accession number PRJNA600662.

Statistical analysis and methods

Analyses was performed using R (v 3.6.0) (Team & R Development Core Team, 2019), with the R markdown file of the analysis presented in the supporting online material. The QIIME v.1.9.1 BIOM file was read into R using the 'Biomformat' (McMurdie & Paulson, 2016) and 'Taxa' (Foster et al., 2018) packages. To correct for differences in number of reads, all samples were rarified at an even sequencing depth of 14,158 reads per sample using the 'Metacoder' package (Foster et al., 2017). Rarefaction curves were calculated using the 'rarefy' function in the 'Vegan' package (Oksanen et al., 2019), and are presented in Figure S1.

Alpha diversity of the samples was calculated with 'Inverse Simpson's diversity' metric (calculated with the 'Vegan' package, Oksanen et al. 2019). One-way analysis of variance (ANOVA) and *post-hoc* Tukey HSD were used to assess for differences in alpha diversity. For the substrata (Plastic, particle-associated, and free-living), data conformed to normality (QQ) but needed to be square-root transformed in order to meet the assumptions of homogeneity of variance (Levene). For the differences between locations (Reference, RCP 2.6, RCP 6.0, and >RCP 8.5), data conformed to both normality (QQ) and homogeneity of variance (Levene). Beta diversity of the samples between the substrata (Plastic, particle-associated, and free-living) and locations (Reference, RCP 2.6, RCP 6.0, and >RCP 8.5) was calculated using PERMANOVA with a Bray-Curtis dissimilarity matrix in the 'Vegan' package (Oksanen et al., 2019). For beta diversity, all data conformed to the test for multivariate homogeneity of group dispersion, assessed used 'betadisper' in the 'Vegan' package (Oksanen et al., 2019). All of the alpha and beta diversity plots, and the taxonomic compositions (used to show the

relative abundance of the different substrata and locations at the Phylum and Class order) were produced using the 'ggplot2' (Wickham, 2016) and 'ggpubr' (Kassambara, 2019) packages.

The linear discriminant analysis effect size (LEfSe) method (Segata et al., 2011) was performed to highlight which taxonomic groups are responsible for the differences in the biofilm-forming bacterial communities when assessing between the different substrata and locations. This method uses Kruskal-Wallis to identify features with significant differences between groups, and then performs the linear discriminant analysis to evaluate the effect size of each feature. The LefSe approach is Python-based, and so was performed in R using a wrapper (see supporting online material for more detail), with the default parameter settings and an 'all-against-all' comparison strategy applied. The differences between groups were also visualized using a differential heat tree. The differential heat trees were produced using the 'Metacoder' package (Foster et al., 2017), and used a Wilcoxon Rank Sum test to compare the $\log_2$ of ratio of median abundances of the different taxa between the two groups being compared. We used the differential heat trees to compare between 1) the community composition of the plastic-associated bacteria, and the seawater-associated bacteria (combination of particle-associated and free-living bacteria); and 2) the community composition of the different locations (Reference, RCP 2.6, RCP 6.0, and >RCP 8.5) for the plastic-associated bacteria.

Results

We obtained 2,238,565 quality sequences in total, which were classified into 2063 operational taxonomic units (OTUs) at 97 % similarity level. OTUs not belonging to the Bacteria Kingdom were removed, as well as any OTUs associated with chloroplasts or mitochondria, resulting in a final 2042 OTUs. Samples were then rarefied to 14,158 sequences for subsequent analysis.

Alpha and beta-diversity of the plastic, particle-associated and free-living substrata

Plastic bottles had a more diverse bacterial community compared to either the particle-associated (1.5-fold increase) or free-living (2-fold increase) bacterial samples (Alpha diversity, diversity - ANOVA: $F_{2, 45} = 10.34$, $p < 0.001$; Figure 2A), with greater evenness (Alpha diversity, evenness - Kruskal-Wallis: $\chi^2 = 18.45$, $p < 0.001$; Figure 2B), as well as demonstrating a significantly different community composition (Beta diversity, PERMANOVA: $F_{2,47} = 9.54$, $p < 0.001$; Figure 2C). The alpha diversity of the plastic surfaces was mostly similar between the pCO_2 locations, with only RCP 2.6 showing reduced diversity (Alpha diversity, diversity - ANOVA: $F_{3, 20} = 9.46$, $p < 0.001$; Figure 2D) and evenness (Alpha diversity, evenness - Kruskal-Wallis: $\chi^2 = 11.24$, $p < 0.05$; Figure 2E) compared to all other locations. In terms of community composition, however, the beta diversity of the bacterial communities on the plastic was significantly altered by pCO_2 (Beta diversity, PERMANOVA: $F_{3,23} = 4.44$, $p < 0.001$; Figure 2F) with only the reference and RCP 2.6 (least acidified) location being similar (i.e. non-significant difference). The richness (Alpha diversity, diversity - ANOVA: $F_{3,20} = 1.57$, $p = 0.23$; Figure 2G) and evenness (Alpha diversity, evenness - ANOVA: $F_{3,20} = 2.95$, $p = 0.06$; Figure 2H) of the seawater samples did not significantly differ between locations. Although beta diversity showed a significant main effect (Beta diversity, PERMANOVA: $F_{3,23} = 2.55$, $p = 0.014$; Figure 2I), the Bonferroni-corrected pairwise comparisons between locations were all non-significant ($p > 0.05$). Overall, this demonstrates

that the richness and composition of the surface-associated bacterial communities on the plastic differed from that of the surrounding seawater, and were altered by the changes in carbonate chemistry; whereas the seawater bacterial communities were relatively homogenous among the changing carbonate chemistry (Figure 2).

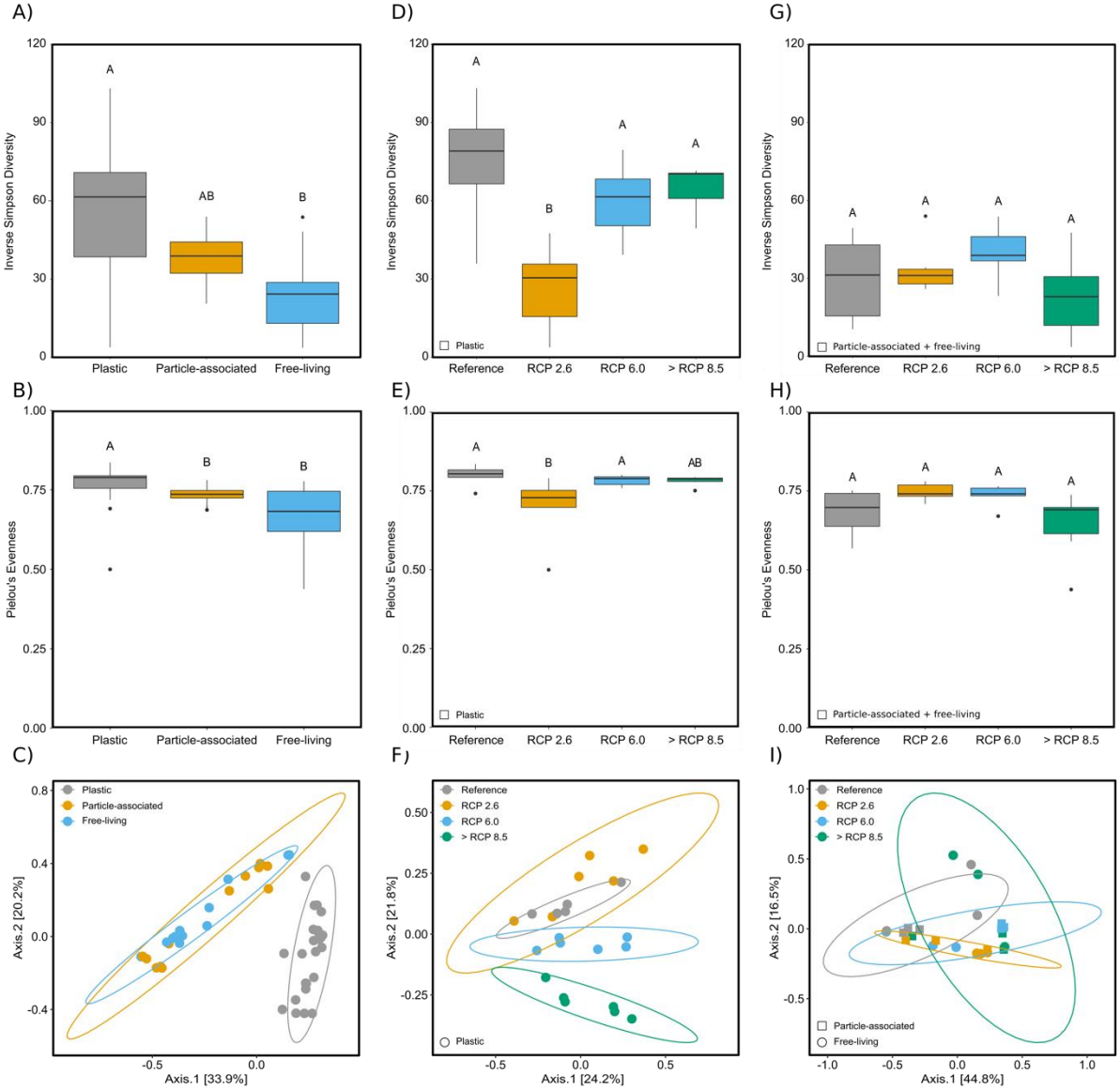

Figure 2 – (**A-C**) Alpha diversity richness (**A**) and evenness (**B**), and beta diversity (**C**) of the different substrata (Plastic, particle-associated, free-living). (**D-F**) Alpha diversity richness (**D**) and evenness (**E**), and beta diversity (**F**) of the plastic-associated biofilm-forming bacteria among the different pCO_2 locations (Reference, RCP 2.6, RCP 6.0, > RCP 8.5). (**G-I**) Alpha diversity richness (**G**) and evenness (**H**), and beta diversity (**I**) of the seawater bacterial communities among the different pCO_2 locations (Reference, RCP 2.6, RCP 6.0, > RCP 8.5).

Differences in community composition between plastic and seawater substrata

The dominant bacteria our plastic and seawater samples were Bacteroidetes representing an average of 36 % of total reads, followed by Proteobacteria (30 %), Firmicutes (15 %) and Cyanobacteria (12 %). The most abundant remaining phyla detected were Actinobacteria, Verrucomicrobia, Planctomycetes, Chloroflexi, and Tenericutes, which each had a relative abundance of less than 2 % of the total reads. The phyla Acidobacteria, Patescibacteria and Spirochaete were only detected in our plastic samples, but at very low abundances. When assessing which taxonomic groups were primarily responsible for the differences between the surface-associated bacterial communities on the plastic and the particle-associated and free-living species in the seawater (Figure 2B), we found that a large number of taxonomic groups

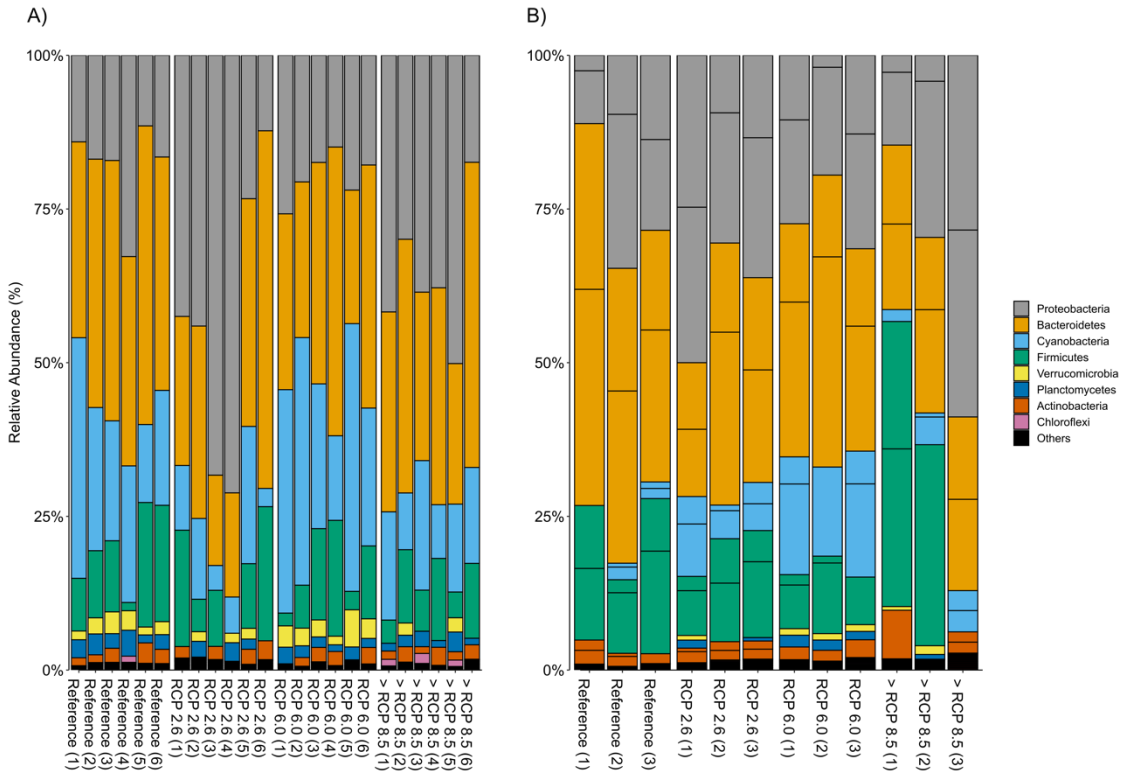

Figure 3 – Community composition at the Phyla level for the plastic-associated communities (**A**) and the seawater communities (**B**; combination of the free-living and particle-associated) across the different pCO_2 locations (Reference, RCP 2.6, RCP 6.0, and > RCP 8.5). Phyla with less than 1 % relative abundance were grouped into 'Others'.

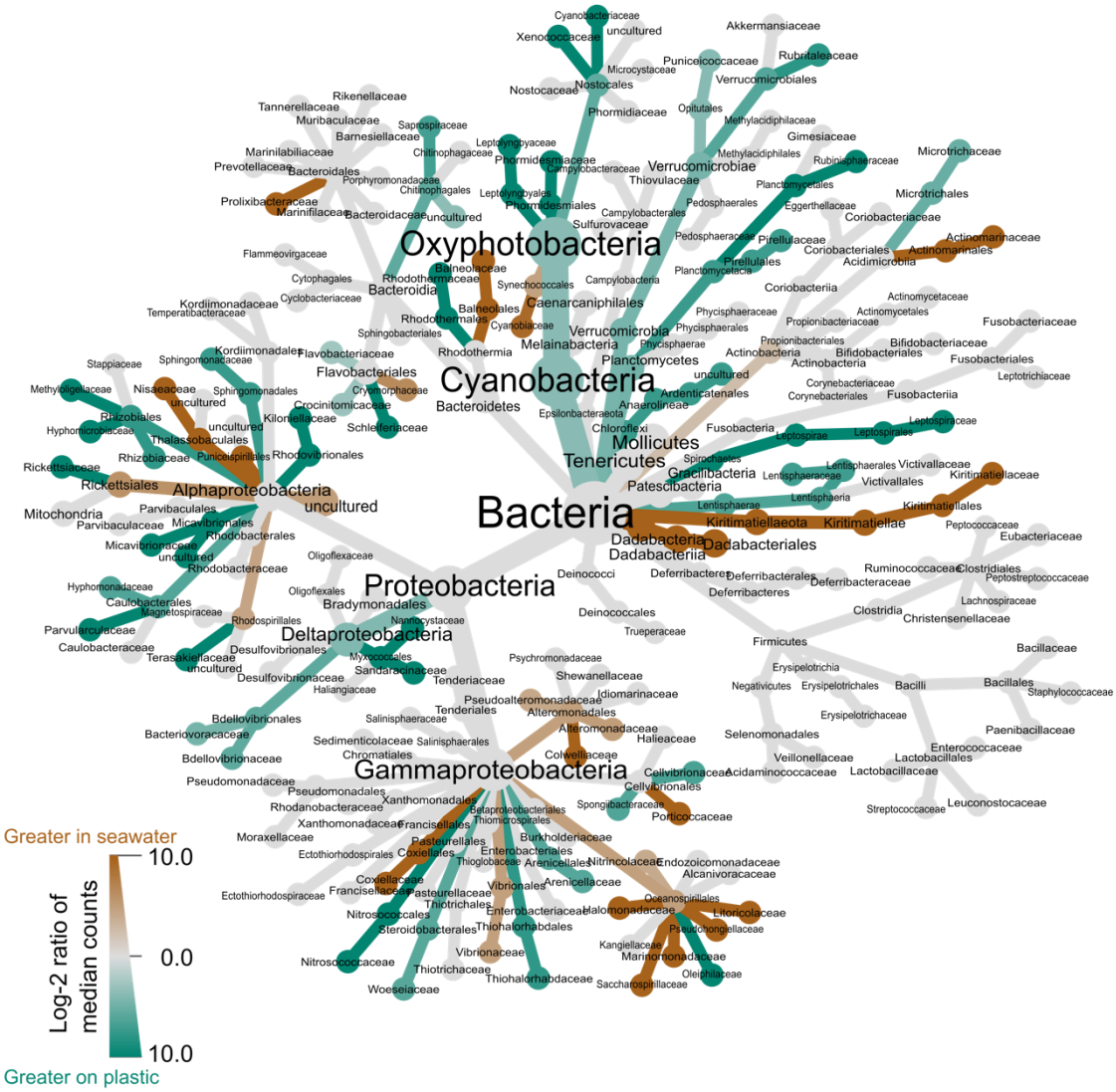

Figure 4 - Differences in the relative abundance between plastic (Green) and seawater (Brown, particle-associated and free-living combined). Aggregated at family-level. Grey indicates where differences were non-significant.

were significantly enriched on their respective substrata (Figures 3 and 4; Figure S2). We found that OTUs belonging to the Phyla Cyanobacteria, Chloroflexi, Planctomycetes and Verrucomicrobia (Figure 3A), along with the (Bacteroidia) Chitinophagales and *Flavobacteriaceae*, (Gammaproteobacteria) Alteromonadales and Thiohalorhabdales, (Alphaproteobacteria) Caulobacterales, Sphingomonadales, Rhizobiales and *Rhodobacteraceae*, and Deltaproteobacteria taxonomic groups, were all significantly enriched in the plastic samples (LDA, $p < 0.05$; see Figure S2 for full list) compared to the

seawater samples (Figure 4). In contrast, the following groups were significantly enriched in the seawater samples relative to the plastic substratum (LDA, $p < 0.05$; see Figure S2 for full list): the phyla Actinobacteria, Dadabacteria and Kiritimatiellaeota (Figure 3B), as well as (Alphaproteobacteria) SAR11 clade, Puniceispirillales and Rhodospiralles, (Bacteroidia) *Cryomorphaceae* and NS9 marine group, (Gammaproteobacteria) SAR86 clade, Oceanospirillales and Vibrionales, and the (Oxyphotobacteria) Synechococcales (Figure 4). In total, 263 OTUs were shared between plastic, free-living and particle-associated samples. Among them, 348 OTUs were unique on plastic, 158 OTUs in particle-associated and 101 unique OTUs in free-living water samples (Figure S3).

Differences in community composition with altered carbonate chemistry

When considering only the communities on the plastic bottles, the specific differences in the community composition (Figure 2D) can be explained by the characteristic OTUs found at each of the different levels of pCO_2. For the Reference pCO_2 location, these characteristic OTUs were (Cyanobacteria) Phormidesmiales, the (Bacteroidia) Chitinophagales, Cytophagales and some assorted *Flavobacteriaceae* and *Crocinitomicaceae* species, (Planctomycetes) OM190 group and Phycisphaerales, (Verrucomicrobia) Verrucomicrobiales, (Gammaproteobacteria) Vibrionales, OM182 clade and SZB50, and (Deltaproteobacteria) *Nannocystaceae* (Figure S4). For the RCP 2.6 location, the characteristic OTUs were the (Gammaproteobacteria) Alteromonadales, Arenicellales, Enterobacteriales and Oceanospirillales, (Alphaproteobacteria) Kordimonadales, (Spirochaetes) Leptospirae, and the (Verrucomicrobia) Methyacidiphilales (Figure S5). For the RCP 6.0 location, the characteristic OTUs were (Cyanobacteria) Oxyphotobacteria, (Verrucomicrobia) Verrucomicrobiae, (Gammaproteobacteria) Thiohalorhabdales, and

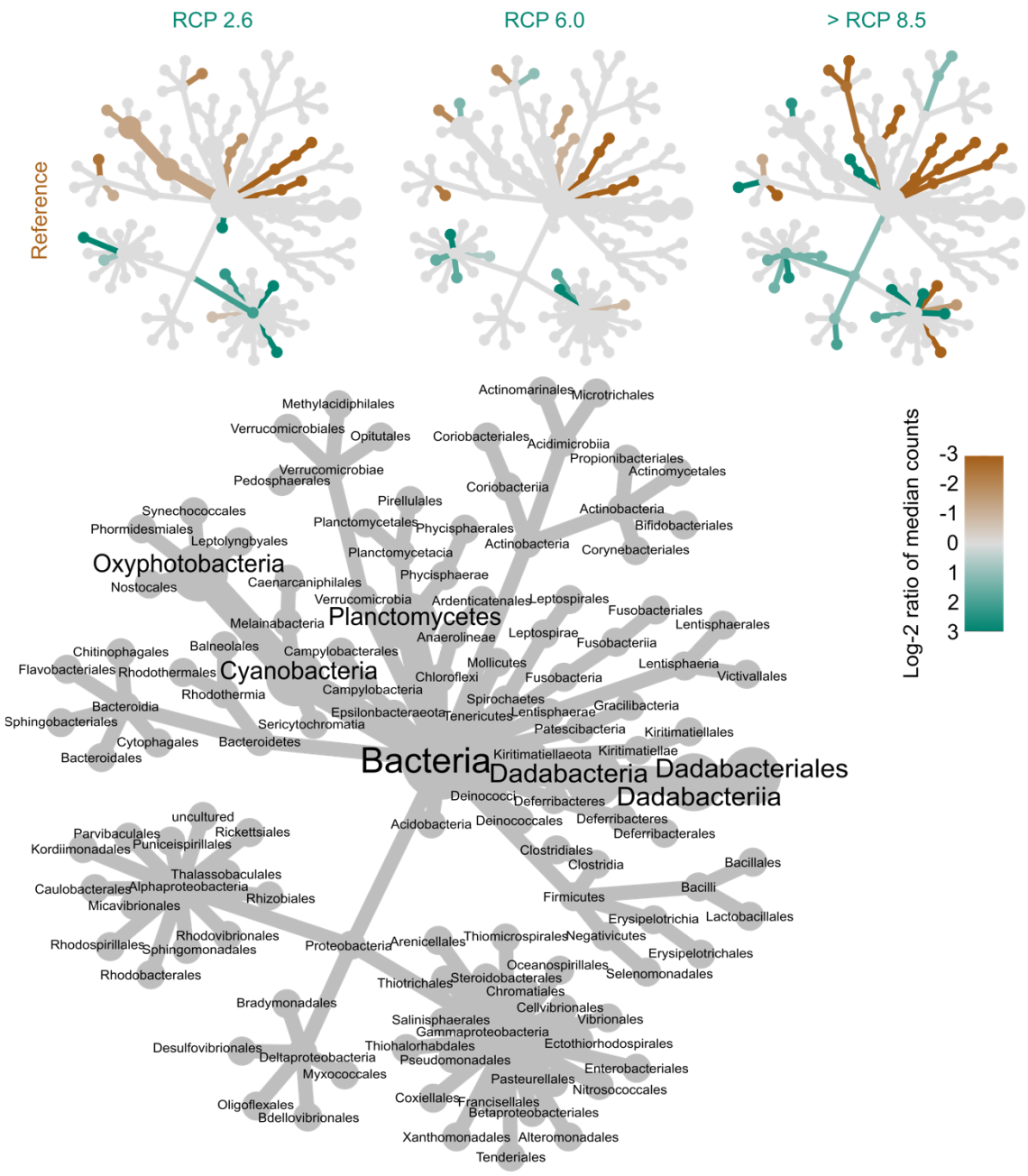

Figure 5 – (**Upper**) Differences in the relative abundance of plastic surfaces between the Reference location (Brown) vs. (from left to right) RCP 2.6, RCP 6.0 and > RCP 8.5 (Green). Grey indicates where differences were non-significant. (**Lower**) Indicates the taxonomic information for the highlighted differences in the upper panel. Aggregated at Order level.

(Lentispharae) Lentisphaeria (Figure S6). Finally, for the '> RCP 8.5' location, the characteristic OTUs were the Chloroflexi and Epsilonbacteraeota phyla, (Alphaproteobacteria) Rhodobacterales, Sphingomonadales, Rhizobiales and Micavibrionales, (Gammaproteobacteria) Thiotrichales, and B2M28 and K189A clade, (Cyanobacteria)

Synechococcales, (Deltaproteobacteria) Bdellovibrionales, and the (Planctomycetes) Pla3_lineage (Figure S7).

The Phyla Fusobacteria, Spirochaetes and Verrucomicrobia, and taxonomic groups of (Bacteriodia) Cytophagales, and (Gammaproteobacteria) Alteromonadales, Oceanospirillales and Vibrionales were all negatively impacted by pCO_2 compared to the Reference pCO_2 location (Figure 5). In contrast, the (Alphaproteobacteria) Koriimonadales, Rhodobacterales, Sphingomonadales and Rhizobiales, and (Gammaproteobacteria) Thiotrichales, Chromatiales, and Arenicellales were enriched in the elevated pCO_2 locations compared to the reference pCO_2 location (Figure 5). Bacterial communities colonising the plastic bottles shared 352 bacterial OTUs across the four locations, with the reference pCO_2 location possessing 129 unique OTUs; while the RCP 2.6, RCP 6.0 and > RCP 8.5 locations had 86, 65 and 111 unique OTUs, respectively (Figure S8).

Discussion

About 5 to 13 million tons of plastics end up in the ocean annually with the trend increasing ten-fold during decade 2015-2025 (Jambeck et al. 2015). The increasing quantity of plastic waste will continue to provide a novel ecological habitat for a wide range of marine microbial communities for millenia (Barnes, 2002; Masó et al., 2003; Zettler et al., 2013), and because many plastics are buoyant this Plastisphere will spread between continents (Wright et al., 2020). Ocean acidification is expected to shape the composition and function of the Plastisphere since increasing levels of seawater CO_2 change sediment (Kerfahi et al., 2014) and epilithic bacterial community composition (Kerfahi et al., 2020; Lidbury et al., 2012; Taylor et al., 2014). Here, we show that bacterial communities that quickly grew on a widely used type of plastic drinking bottle differed from those in the water column, and that elevated CO_2 altered these plastic surface bacterial communities. We suggest that distinct ecological processes will underpin the assembly of these bacterial communities, and highlight the potential implications of this community shift in a coastal community exposed to enriched CO_2 conditions.

Differences in bacterial community composition between plastic and seawater

Microbial communities on plastics consistently differ from those found in the surrounding seawater (Amaral-Zettler et al., 2015; Kirstein et al., 2019; Oberbeckmann et al., 2014, 2016) and, because there are fundamental differences in surface-associated and free-living lifestyles of marine microorganisms (Dang & Lovell, 2016), it is not surprising that our plastic-associated and water-borne communities differed in taxonomic composition. The structure of the plastic bottle communities showed that their formation was not simply a product of adsorption of cells or particles from the water column. We also found that the plastic community differed from the particle-associated bacterial communities (as also reported by Dussud et al., 2018; Kirstein et al., 2019), highlighting that plastic substrata promote novel bacterial communities. Generally, microbial communities found on plastic include many natural biofilm-forming species that usually live attached (rather than free-living) (Kirstein et al., 2019). Some of the taxa that characterised our Plastisphere communities included the often surface-associated Cyanobacteria, *Rhodobacteraceae*, Sphingomonadales and Caulobacterales (Alphaproteobacteria group), Alteromonadales (Gammaproteobacteria group), *Flavobacteriaceae* (Bacteroidia group), Deltaproteobacteria group, and Planctomycetes (Salta et al., 2013). Many of these bacteria are highly adapted to surface living, including the extracellular degradation of organic matter (e.g. Flavobacteria, Williams et al., 2013), surface adherence using a polar holdfast structure (e.g. Caulobacters, Stahl et al., 1992) and show close associations with the phycosphere (e.g. between diatoms and Bacteroidetes, *Rhodobacteraceae* and Gammaproteobacteria, Schlundt et al., 2019). *Rhodobacteraceae*, in particular, are ubiquitous and abundant bacteria associated with primary surface colonisation (Dang et al., 2008; Elifantz et al., 2013) with the Bacteroidetes (e.g. *Flavobacteriaceae*) thought to dominate the second wave of succession (Dang & Lovell, 2016; Russell et al., 2014). The prevalence of

commonly attached and surface-associated bacteria on the plastics may support the previous notion that plastic surfaces are not necessarily selecting for specific bacteria, and are instead being driven by conventional biofilm formation processes by providing additional available substrata (Oberbeckmann et al., 2016).

The following taxa are characteristic of the Plastisphere, and were found on the plastic bottles used in this study; Alphaproteobacteria (*Sphingomonadaceae* and *Rhodobacteraceae* families), Gammaproteobacteria (*Alteromonadaceae* family) and Bacteroidia (*Saprospiraceae* and *Flavobacteriaceae* families) (Amaral-Zettler et al., 2015; Bryant et al., 2016; De Tender et al., 2015, 2017; Debroas et al., 2017; Jiang et al., 2018; Oberbeckmann et al., 2014, 2016, 2018; Ogonowski et al., 2018; Schlundt et al., 2019; Zettler et al., 2013). The proliferation of these taxa on plastic indicates that they might use it as a carbon source (McCormick et al., 2014; Oberbeckmann et al., 2014; Zettler et al., 2013) and play a role in plastic biodegradation (Ogonowski et al., 2018; Zettler et al., 2013). In contrast to previous studies (Kirstein et al., 2016; Zettler et al., 2013), but in agreement with others (Bryant et al., 2016; Oberbeckmann et al., 2018), we found that the potentially pathogenic Vibrionaceae was not significantly enriched on the plastic surfaces and was in fact more common in the seawater samples. Such considerations help determine the behaviour of the plastic debris (Lobelle & Cunliffe, 2011), and potential for rafting opportunities (Jokiel, 1990). In agreement with most studies, we found that species evenness was greater on plastics compared to the surrounding seawater; in contrast to most previous studies(reviewed in Amaral-Zettler et al., 2020), however, we found higher species richness on plastics. Taken together, with the same groups often demonstrating enrichment on plastic, it has been suggested that these species may represent some of the core species of the Plastisphere (De Tender et al., 2017; Oberbeckmann et al., 2014).

Differences in community composition with altered carbonate chemistry

Mesocosm studies have shown that the structure of free-living bacterioplankton communities are altered by elevated pCO_2 (Allgaier et al., 2008). Here, particle-associated and free-living communities did not significantly differ between the pCO_2 locations. It is likely that this is due to hydrodynamics, since the seeps are open systems with water exchange between locations (González-Delgado & Hernández, 2018). The initial assembly of biofilm communities is considered to be stochastic (Jackson et al., 2001) and therefore influenced by the immigration rate of propagule cells sourced from nearby macrophyte, coral and microbial communities. The rocky reef marine biological communities in each pCO_2 location were distinct (see Figure 1) due to differences in carbonate chemistry (described in Agostini et al., 2018). Our reference pCO_2 location had a diverse mix of hard scleractinian corals, crustose coralline algae and canopy-forming macroalgae (Agostini et al., 2018), with a shift towards lower ecosystem diversity, species richness and spatial heterogeneity as pCO_2 levels increased (Hall-Spencer & Harvey, 2019); eventually being dominated by turf algae and benthic diatoms in the areas with highest levels of seawater pCO_2 (Harvey et al., 2019). Diatoms, for example, interact with Alphaproteobacteria and Bacteriodetes (Schäfer et al., 2002) so the source populations of Plastiphere colonization will likely differ with future ocean acidification, and as with the effects of biogeography (Oberbeckmann et al., 2018), will be a major driver of biofilm diversity.

In contrast to the seawater-based communities, which were mixed by the turbulence caused by currents and waves, the bacterial communities on plastic bottles were significantly altered by the changes in pCO_2. These PET bottles were attached to bedrock and constantly held at their respective treatments of carbonate chemistry (i.e. Reference, RCP 2.6, RCP 6.0 and > RCP 8.5), while still retaining natural pCO_2 variability (Agostini et al., 2018). This suggests that physiological responses of specific bacteria and/or changes in ecological interactions drive

responses to elevated pCO_2. Different families of heterotrophic bacteria have distinct pH-homeostasis mechanisms for responding to elevated pCO_2, each mechanism with particular bioenergetic requirements (Bunse et al., 2016). Such distinctions between the bacterial families will influence the sensitivities of marine bacteria to elevated CO_2, and affect the metabolism and growth of the dominant bacterial groups (del Giorgio et al., 2011). Microbial communities have ecological interactions between the bacteria which shape their composition (Rendueles & Ghigo, 2012). For example, marine predatory bacteria (including some members of Proteobacteria, Actinobacteria, Bacteroidetes, and Chloroflexi) affect the abundance and composition of communities (Richards et al., 2012). Any changes to the ecological interactions or bioenergetics of marine predatory bacteria (such as due to ocean acidification) could also influence their heterotrophy (Rendueles & Ghigo, 2012). This hypothesis has yet to be tested but highlights the importance of moving beyond monocultures and studying interspecies interactions within mixed communities.

We found that the abundance of Fusobacteria, Spirochaetes and Verrucomicrobia, as well as the Cytophagales (Bacteriodia group), and the Alteromonadales, Oceanospirillales and Vibrionales (Gammaproteobacteria) were all negatively impacted by pCO_2 compared to the Reference pCO_2 location. Many members of these groups play important roles within bacterial communities (Dang & Lovell, 2016). For example, *Alteromonadaceae*, a widespread and common species has an exceptional carbon cycling capacity, with the ability to degrade and use a wide range of organic substrates (Allers et al., 2007; Dang & Lovell, 2002; Nelson & Wear, 2014). *Vibrioaceae*, which include several potential pathogens, are typically a subdominant and opportunistic bacteria (Eilers et al., 2000), and so their reduced abundance under elevated CO_2 may highlight the competitive dominance of the primary colonisers. Such changes might influence their future potential for spreading pathogens, however, other non-

pathogenic marine Vibrios species in biofilms can also contribute positively, such as inducing the settlement of invertebrates (Huggett et al., 2008). Whereas some plastic-associated taxonomic groups were negatively affected by elevated pCO_2, others were enriched by it. These included Alphaproteobacteria (Koriimonadales, Rhodobacterales, Sphingomonadales and Rhizobiales) and Gammaproteobacteria (Thiotrichales, Chromatiales, Arenicellales). Using plastics from the North Atlantic garbage patch, Debroas et al. (2017) found that both the Rhodobacterales and Rhizobiales (along with Cyanobacteria and Streptomycetales) are considered keystone bacterial groups. Keystone bacteria are those that have a disproportionate influence on the structure and help define the community, despite often only being of moderate abundance. With the enrichment and declines of different taxonomic groups due to altered carbonate chemistry, it is likely that the core-species of the plastic microbiome (De Tender et al., 2017; Oberbeckmann et al., 2014) will be altered by future ocean acidification. Given the numerous roles of these bacterial groups, this implies that the functioning of biofilm-forming bacterial communities will also be influenced by increased pCO_2.

Wider implications and future research considerations

Given the ubiquitous persistence of plastics, there is increasing interest in the microbial ecology and evolution of the Plastisphere; including the need to understand to what extent the 'core' bacterial community is unique to plastics (Amaral-Zettler et al., 2020). The bacterial communities that formed biofilms on plastic bottles were distinct from those found in the surrounding seawater. However, without a suitable contrasting substratum for comparison (e.g. glass) we are unable to disentangle whether certain microbes preferentially colonised the plastic (although the assemblage did differ between the plastic and particle-associated bacteria). Such differences between glass and plastic have been demonstrated in some studies (Kirstein et al., 2018) with no difference found in others (Oberbeckmann et al., 2016).

Environmental conditions and biogeography clearly influence microbial communities of the plastisphere, and we demonstrate that future conditions may further influence their composition. However, since previous studies have been carried out at a range of geographic scales, it makes the synthesis of the results problematic (Amaral-Zettler et al., 2020). Taken together, this highlights the need for more studies on the plastisphere covering a range of environmental conditions and plastic materials to better understand how plastics biodegrade and how they transport potentially pathogenic bacteria across ocean basins.

Conclusions

As the Plastisphere is the interface between plastic and its marine environment it drives every interaction that the debris has with its surrounding milieu, from food web interactions, ecotoxicology, and the ultimate fate of the material (Wright et al., 2020). We showed that plastic drinking bottles quickly developed bacterial biofilms that were very different to those assemblages that were found on particles and free-living in the surrounding seawater, with the plastic communities showing a greater taxonomic richness. The composition of the plastic communities was strongly influenced by changes in seawater carbonate chemistry caused by increasing levels of CO_2. The increasing presence of plastic marine debris is providing a novel habitat for bacteria and ocean acidification will greatly influence the composition of these biofilm assemblages. Our results highlight that environmental conditions and local ecological processes will play an important role in determining the composition and functioning of the Plastisphere.

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Competing interests

The authors declare that they possess no competing interests.

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