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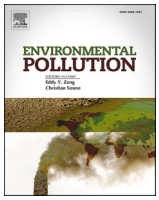

A closer look at plastic colonisation: Prokaryotic dynamics in established versus newly synthesised marine plastispheres and their planktonic state☆

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ABSTRACT

The taxonomy of marine plastisphere communities has been extensively studied, demonstrating the ubiquity of hydrocarbonoclastic bacteria of potential biotechnological significance. However, prokaryotic functioning on plastic surfaces has received limited attention, and the question of whether these microorganisms are active and expressing specific molecular mechanisms underpinning plastisphere colonisation remains to be addressed. The aim of this study was to investigate the plastic colonisation process, to identify the active taxa involved in biofilm formation and the mechanisms used to initiate colonisation. To achieve this, a marine plastisphere characterised by active hydrocarbonoclastic genera was used as the inoculum for a short-term microcosm experiment using virgin low-density polyethylene as the sole carbon source. Following incubation for 1 and 2 weeks (representing early and late colonisation, respectively), a taxonomic and comparative metaproteomic approach revealed a significant shift in plastisphere diversity and composition, yet highlighted stability in the predominance of active Proteobacteria spanning 16 genera, including *Marinomonas*, *Pseudomonas*, and *Pseudoalteromonas*. Relative quantification of 1762 proteins shared between the initial plastisphere inoculum, the microcosm plastisphere and the planktonic cells in the surrounding artificial seawater, provided insights into the differential regulation of proteins associated with plastisphere formation. This included the upregulation of proteins mediating cellular attachment in the plastisphere, for example flagellin expressed by *Marinomonas*, *Cobetia*, *Pseudoalteromonas*, and *Pseudomonas*, and curli expressed by *Cobetia*. In addition to the differential regulation of energy metabolism in *Marinomonas*, *Psychrobacter*, *Pseudomonas* and *Cobetia* within the plastisphere relative to the surrounding seawater. Further, we identified the upregulation of amino acid metabolism and transport, including glutamine hydrolysis to glutamate in *Marinomonas* and unclassified Halomonadaceae, potentially coupled to ammonia availability and oxidative stress experienced within the plastisphere. Our study provides novel insights into the dynamics of plastisphere formation and function, highlighting potential targets for regulating plastisphere growth to enhance plastic bioremediation processes.

1. Introduction

Plastic pollution has reached critical levels in the marine environment, with present estimates of the mass of plastic debris reaching thousands of kilotonnes, derived from trillions of individual pieces distributed throughout all major ocean basins (Eriksen et al., 2023; Kaandorp et al., 2023). In the marine environment, plastics interact with a range of abiotic and biotic components of the surrounding ecosystem, such as dissolved nutrients, chemical pollutants, ultraviolet radiation, and macro- and microorganisms (Bhagwat et al., 2021a; Camacho et al., 2019; Pauli et al., 2017; Reisser et al., 2014; Wang et al., 2020). These interactions contribute to changes in the surface properties of plastics, creating a nutrient-rich conditioning film, increasing rugosity, and exposing recalcitrant polymers and additives (Fernández-González et al., 2021). As these processes facilitate biotic interactions, organisms rapidly colonise the plastic surface (Latva et al., 2022), creating the ecological niche now widely referred to as the plastisphere (Zettler et al., 2013).

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The phylogenetic diversity of the plastisphere has been well documented over the last decade. Through taxonomic marker gene surveys and microscopy studies, the plastisphere is now known to encompass Eukaryotes, Bacteria, and Archaea, which interact to form a complex and distinct microbial community (Amaral-Zettler et al., 2020; Erni-Cassola et al., 2020; Schlundt et al., 2020). Specifically, many copiotrophic bacterial classes such as Gamma- and Alphaproteobacteria, which are often also hydrocarbonoclastic, and members of Bacteroidota (Lee et al., 2023; Oberbeckmann and Labrenz, 2020; Scales et al., 2021) represent prominent members of the plastisphere community, in addition to photosynthetic organisms, including diatoms, dinoflagellates, and multicellular algae which appear to be active within warmer climates (Dudek et al., 2020; Latva et al., 2022; Messer et al., 2024; Wright et al., 2021b). Indeed, Oceanospirillales, Alteromonadales (Wright et al., 2021b), Rhodobacteraceae and Flammeovirgaceae (Scales et al., 2021) appear to be widespread colonisers of marine plastic pollution, suggesting that specific adaptations of these lineages allow the colonisation of plastic surfaces.

Microorganisms are known to utilise a range of attachment strategies to initiate surface colonisation (Dang and Lovell, 2015), and recent metagenomic analyses suggest that marine microorganisms can use pili, fimbriae, flagella, quorum sensing, and chemotaxis to establish a biofilm on the surface of plastics (Bhagwat et al., 2021b; Bos et al., 2023; Lee et al., 2023). This is indeed consistent with the known morphology and phenotypic behaviours of many of the prokaryotic lineages associated with marine plastisphere communities (e.g. Oceanospirillales, Alteromonadales, Rhodobacteraceae and Flammeovirgaceae). However, the specific molecular mechanisms underpinning the observed trends in plastisphere taxonomy remain poorly characterised yet could provide important insights into plastic bioremediation processes and inspire new avenues of biotechnological discovery (Howard and McCarthy, 2023).

The functional gene content of established plastisphere communities provides some insights into the dynamics of the marine plastisphere and the ecological roles of specific microorganisms. Indeed, microorganisms inhabiting this niche possess the ability to photosynthesise, or metabolise organic carbon, and/or use inorganic and organic nitrogen and phosphorous to acquire energy and nutrients (Bryant et al., 2016; Mincer et al., 2016). Furthermore, the plastisphere encodes mechanisms to withstand heavy metal toxicity, exposure to antibiotics, and oxidative stress (Bhagwat et al., 2021b), reflecting the diversity of co-pollutants accumulated at the plastic surface (Lee et al., 2023) and the potential adaptive strategies of colonising microorganisms. Some microorganisms isolated from the plastisphere can degrade plastic and their associated additives through the expression of hydrolases, esterases, and depolymerases (Gambarini et al., 2022; Wright et al., 2021a, 2020), while the potential to degrade aromatic compounds has also been detected within environmental plastisphere metagenomes (Bryant et al., 2016; Messer et al., 2024). Taken together, it is evident that the collective activity of this diverse community will impact the indirect and direct breakdown, or indeed persistence, of marine plastic pollution, with broad ranging implications for ecosystem and human health. However, as gene content only reflects the potential function of a community, and the activity of cultured isolates is often distinct to the activity of microorganisms in the environment, a detailed understanding of the expressed proteins underpinning plastisphere establishment and metabolism is required to fully resolve the fate of plastic pollution in the environment.

Comparative metaproteomic approaches, in which microbial genotype-phenotype relationships are determined through the identification and annotation of expressed proteins, provide insights into complex community interactions, community function, and the diversity of active taxa (Géron et al., 2021). To date, metaproteomic analyses of the marine plastisphere have revealed the importance of active photosynthetic microorganisms including diatoms and cyanobacteria, and to a lesser extent Rhodobacteraceae, Flavobacteriaceae, and Erythrobacteraceae, with the expression of an assortment of transporters suggesting the active uptake of nutrients, amino acids, and sugars (Delacuvellerie et al., 2022; Oberbeckmann et al., 2021). More recently, the activity of hydrocarbonoclastic genera within the plastisphere has been observed, along with the expression of proteins associated with adhesion and biofilm formation, such as flagellin and elongation factor Tu, and proteins potentially mediating inter-community interactions, such as acyl-homoserine lactone synthase (Messer et al., 2024). Whether these functions enabled colonisation of the plastic surface is unclear, but comparative metaproteomics provides a unique opportunity to determine the specific adaptations and molecular mechanisms enabling microorganisms to exploit the plastic pollution niche, when applied to the study of plastisphere formation.

Here, our aim was to determine the functioning of the plastisphere during plastic colonisation, to identify, i) the taxonomy of the present and active taxa contributing to biofilm formation, ii) the phenotypes and behaviours used to initiate plastic colonisation, and iii) the regulation of proteins within the plastisphere relative to planktonic counterparts. To achieve this, a well-characterised marine plastisphere (Messer et al., 2024) was used as an inoculum for a microcosm experiment employing virgin low-density polyethylene (LDPE) as a sole carbon source within oligotrophic minimal media. We tested the hypothesis that active microorganisms closely associated with the plastic surface would be enriched when exposed to a new plastic substrate under oligotrophic conditions, whereas loosely associated microorganisms would be outcompeted during the incubation due to nutrient limitation. However, we also posited that some microorganisms may be indirectly supported by the metabolic activity ('cross-feeding') of the plastisphere itself through growth in the oligotrophic planktonic phase. Using 16S rRNA gene sequencing to determine the taxonomy of the present microorganisms, and comparative metaproteomics to link the active taxa to their expressed functions, we provide novel insights into the formation of the plastisphere and identify specific adaptations to the plastic niche, which represent possible targets for biotechnological manipulation of the plastisphere in the future.

2. Materials and methods

2.1. Sample collection and plastisphere recovery

Marine plastic debris (transparent and coloured films) were collected along a 100 m transect following the high tide mark of the westward expanse of Gullane Beach on the North Sea (Scotland, United Kingdom), on April 18th, 2022 during boreal spring (Messer et al., 2024). Environmental metadata were collected from the adjacent seawater using a handheld probe meter to measure the temperature and dissolved oxygen, and salinity was measured using a refractometer. Plastic debris were placed into sterile sampling bags, and upon return to the laboratory, samples were rinsed with complete artificial seawater (ASW) (Eguchi et al., 1996) to remove loosely attached organisms and sand. Transparent and coloured films, representing plastic with similar properties to the LDPE used in the microcosm, were sorted from the collected mixed debris based on their visual properties. Samples were briefly dried at room temperature under laminar flow prior to plastisphere detachment. Triplicate samples consisting of 6.43g, 5.26g, and 4.57g of the transparent and coloured films were submerged in 15 ml ASW and subjected to three rounds of 10 min bead-beating with 1g of 1 mm glass beads at maximum speed (Vortex Genie 2). The supernatants were recovered, briefly mixed by vortex and 2 ml aliquots were taken from each sample for the incubation inoculum (referred to as, T0-P), with the remaining cells used for DNA and protein co-extraction (Messer et al., 2024) (Fig. 1). All cells were pelleted by centrifugation at 12,000×*g* for 15 min at 4 °C and ASW was removed prior to downstream processing.

2.2. LDPE-microcosm incubations

Pristine, virgin LDPE film (0.1 mm) was cut into 7 × 7 cm pieces using a scalpel and sterilised under laminar flow in 70% ethanol,

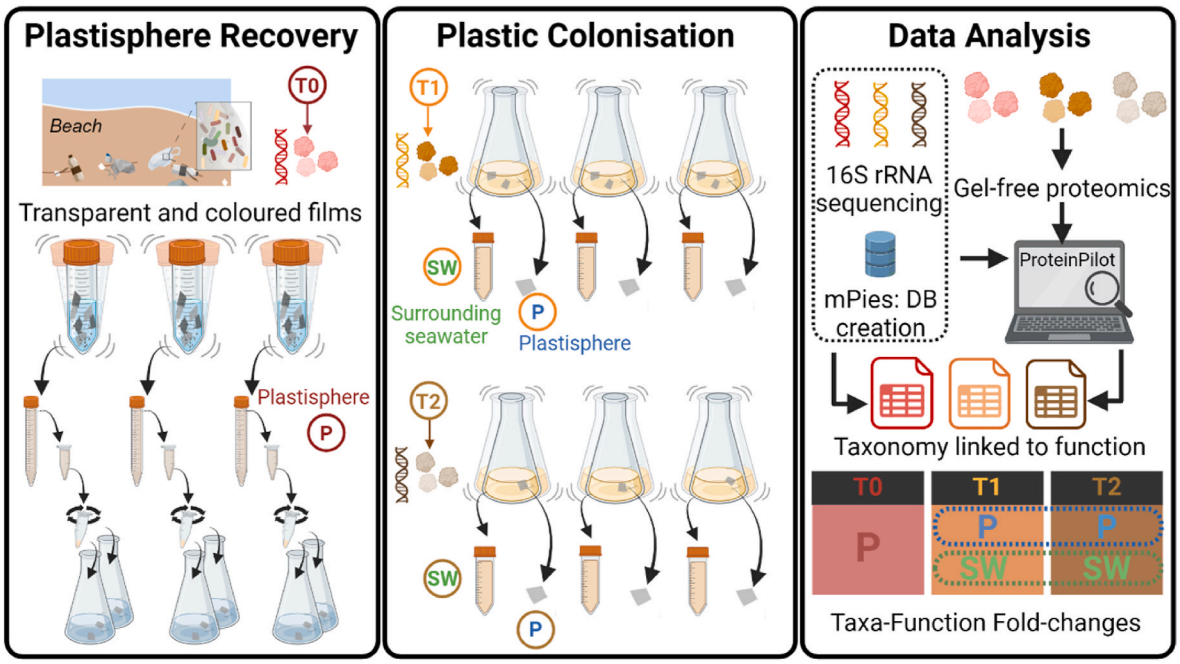

Fig. 1. Schematic representation of the experimental design used in this study, including the recovery of the beach plastisphere inoculum, microcosm incubation, and data analysis strategy. The initial plastisphere (T0-P) was recovered from transparent and coloured films through bead-beating in artificial seawater, DNA and proteins were co-extracted and an aliquot of plastisphere cells was used in microcosms to study plastic colonisation. Low-density polyethylene was used as the sole carbon source in minimal oligotrophic artificial seawater in microcosm incubations with recovered plastisphere cells. After 1 week (early colonisation; T1) and 2 weeks (late colonisation; T2), cells from the surrounding seawater (SW) and plastisphere (P) were collected and DNA and proteins co-extracted. Microbial community composition was determined using 16S rRNA gene sequencing and the identified taxa used to create a custom protein search database for identification of proteins following gel-free proteomics. Examination of significant differences in the present and active taxa and their functions between T0-P and T-P (T1 and T2 plastisphere), and T-P and T-SW (T1 and T2 seawater), revealed specific adaptations to the plastic niche. Created with BioRender.com.

ultrapure water, and finally UV-treatment for 20 min (each side). Sterile Bushnell Hass Broth was prepared according to the manufacturer's instructions and supplemented with 3% NaCl prior to autoclaving, followed by the addition of 1 ml filter-sterilised vitamin solution (Eguchi et al., 1996) under laminar flow, creating an artificial oligotrophic seawater. The cell pellets from T0-P (Section 2.1) were resuspended in 500 μl artificial seawater and divided equally between two Erlenmeyer flasks (technical replicates) containing two 7 cm^2 sterile LDPE pieces and 200 ml media. All flasks were incubated at 15 °C with 80 rpm shaking, with 1 piece of LDPE and 50 ml of the planktonic cells in the surrounding artificial seawater recovered after 1 (T1) and 2 weeks (T2) of incubation for DNA and protein co-extraction (Fig. 1). Triplicate no-inoculum controls were included, in which sterile LDPE was incubated in artificial seawater for two weeks under the same conditions.

2.3. Co-extraction of microbial community DNA and proteins

DNA and proteins were co-extracted from the initial plastisphere T0-P (Section 2.1), the LDPE-plastisphere (T1-P and T2-P) and planktonic cells in the surrounding seawater (T1-SW and T2-SW) (Section 2.2), following a previously optimised protocol (Messer et al., 2024). Briefly, technical replicates from the same starting inoculum were combined, and cell pellets were lysed in 2 ml SDS-based buffer [2 % SDS, 50 mM Tris-HCl pH 8, 10 mM DTT] with repeated probe sonication (1s gap, 1s pulse, 40% amplification, 2 × 30s) on ice, and cell debris was removed by centrifugation at 8000×*g*, for 15 min at 4 °C. The recovered cell lysate was concentrated to ~300 μl via ultra-filtration at 7000 rpm maintained at 4 °C (3K centrifugal filter unit, Pall Corporation), and a 50 μl aliquot was retained for DNA precipitation. Proteins were precipitated from the remaining sample using ice-cold acetone (1:4 v/v) incubated overnight at −80 °C. Proteins were recovered via centrifugation at 16,000×*g*, for 15 min at 4 °C, and pellets were washed twice with 100 μl ice-cold acetone to limit residual cell lysis buffer contamination (Zhang et al., 2018). Protein pellets were briefly air-dried under laminar flow and resuspended in 30 μl Urea-Ammonium bicarbonate buffer [8M Urea, 50 mM ammonium bicarbonate]. Sonication (10s; 1s gap, 1s pulse, 40% amplification) followed by centrifugation (13,000 rpm, 15 min, 4 °C) (Géron et al., 2019) were used to remove any insoluble material. Protein quantification was performed on diluted protein isolates (to ensure [Urea] < 2M) using the Bradford Assay with bovine-gamma-globulin as a protein standard. Gel electrophoresis was used to confirm protein yields by loading 20 μg of protein per sample into a NuPage Gel (4–12% Bis-Tris, Invitrogen) prepared in accordance with the manufacturer's instructions, and proteins were visualised following SDS-PAGE using silver staining (Pierce).

DNA was recovered from the concentrated cell lysate as previously described (Messer et al., 2024). Briefly, protein was removed using Protein Precipitation Solution (1:0.5 v/v; Promega) with a 5 min incubation on ice followed by centrifugation at 16,000×*g* for 15 min at 4 °C. Sodium acetate (3M, pH 5.2; 1:10 v/v) and fresh ≥95% ethanol (3:1 v/v) were added to the aspirated supernatant and DNA was precipitated overnight at −20 °C. Precipitated DNA was recovered by centrifugation at 16,000×*g* for 15 min at 4 °C, and pellets were washed twice with fresh 70% ethanol to reduce any residual contaminants. Following centrifugation, pellets were air-dried under laminar flow to remove residual ethanol, and DNA was resuspended in 20 μl Ultra-Pure water (Oksanen et al., 2019). To facilitate dissolution, resuspended DNA samples were incubated at 55 °C for 10 min followed by 5 min on ice, prior to quantification on a NanoDrop 2000 spectrophotometer. Following co-extraction of the no-inoculum controls, no quantifiable proteins or DNA were detected, and therefore these samples were not analysed further.

2.4. 16S rRNA amplicon sequencing and bioinformatics

Prokaryotic community composition was determined by 16S rRNA

gene sequencing using primers targeting the V4–V5 region of the 16S rRNA gene (515F–Y 5′-GTGYCAGCMGCCGCGGTAA-3′, 909R 5′-CCCCGYCAATTCMTTTRAGT-3′) (Parada et al., 2016; Tuan et al., 2014; Walters et al., 2016; Wang et al., 2018). Illumina MiSeq 2 x 300 nt paired-end sequencing was performed with V3 chemistry (StarSEQ® GmbH, Germany), including negative and positive controls. The 16S rRNA gene sequences were analysed using QIIME2 (v2022.2) (Bolyen et al., 2019), including demultiplexing, adaptor trimming with cutadapt (Martin, 2011), paired end read merging using vsearch (Rognes et al., 2016), and quality control (including read correction, chimera and low-quality reads removal) and ASV generation using Deblur (Amir et al., 2017). Taxonomy was assigned using a naïve-Bayes approach of the scikit learn Python package (Bokulich et al., 2018) with the SILVA 138 nonredundant 99% reference dataset (Quast et al., 2013). Alpha diversity metrics were generated for rarefied 16S rRNA ASVs (8949 sequences per sample) using the QIIME2 q2-diversity core-metrics-phylogenetic plugin. Whereby representative sequences were aligned using MAFFT (Katoh and Standley, 2013) for phylogenetic tree construction using FastTree (Price et al., 2010), in order to compute Faith's Phylogenetic Diversity.

2.5. Gel-free metaproteomics: liquid chromatography tandem mass spectrometry, protein identification, and annotation

Isolated proteins (20 μg) were reduced, alkylated and precipitated with acetone prior to overnight trypsin digestion (1:25 enzyme/substrate ratio) at 37 °C, as previously described (Géron et al., 2019). The trypsin digestions were terminated with 0.1 % formic acid (v/v, final concentration), and analysed on an ultra-high-performance liquid chromatography–high-resolution tandem mass spectrometer (UHPLC-HRMS/MS) system, including a Eksigent NanoLC 400 and AB SCIEX TripleTOF 6600. MS/MS spectra were acquired with the instrument operating in data-dependent acquisition (DDA), using 2 μg of tryptic peptides, with micro injection (75 min LC separation) modes.

The generation of a non-redundant protein search database utilised the 16S rRNA genus-level taxonomic assignments as input (16S-TaxDB = 7.3 Gb) using the mPies database_creation workflow (v1.0) (Werner et al., 2019). Briefly, mPies retrieves all available proteomes from the Uniprot database based on the NCBI taxon ID of the identified taxa and removes redundancies (100% sequence similarity) from the final protein search database. 16STax-DB was used within ProteinPilot (v5.March 0, 1029, 9521aa4603a; Paragon Algorithm: 5.March 0, 1029, 1029; AB SCIEX), levering the AB SCIEX OneOmics software package, to identify protein groups with the parameters: Sample Type: Identification, Cysteine Alkylation: Iodoacetamide, Digestion: Trypsin, ID Focus: Biological Modifications and Amino acid substitutions, Search effort: Thorough ID, Detected Protein Threshold [Unused ProtScore (Conf)]>: 0.05 (10.0%), including 1% False Discovery Rate analysis at the protein level. The identified proteins were annotated using mPies through DIAMOND BLAST (Buchfink et al., 2015) alignments against the non-redundant UniProt (Swiss-Prot) and NCBI (nr) databases to assign function, and through implementation of the Last Common Ancestor approach of MEGAN (Bağcı et al., 2021) (cut-off, 80%) to assign taxonomy, following (Werner et al., 2019).

2.6. Statistical analyses

Significance tests on prokaryotic community alpha diversity metrics were conducted in QIIME2 using pairwise Kruskal Wallis tests as implemented in the q2-diversity plugin. ASV by sample tables were exported from QIIME2 and converted to relative abundances, prior to Hellinger transformation and Bray-Curtis distance matrix generation for input into PERMANOVA using the vegan R package (v2.6-2) (Oksanen et al., 2019). Significant differences in the mean relative abundances of ASVs were determined using a T-test and examination of fold change in R (v4.3.0). For the comparative metaproteomics analyses, ProteinPilot. group files were imported into Skyline to perform spectra integration, normalisation, and relative quantification of expressed proteins (Pino et al., 2020). Significant up- and down-regulation of proteins between treatment groups (T0-P, n = 3 samples; T-P, n = 5 samples; T-SW, n = 6 samples) was determined using the MSstats package (Choi et al., 2014). A P-value of <0.05, adjusted to control for the False Discovery Rate (P-adj.), and fold change ≥ 2 was considered significant.

3. Results

3.1. Establishing LDPE microcosms to explore plastic colonisation

To determine the extent of taxonomic stability in the marine plastisphere during the colonisation of a representative new plastic surface, an environmental community recovered from transparent and coloured plastic films was used as inoculum for an LDPE-microcosm experiment. The diversity of the initial plastisphere community from beach plastics (T0-P), and from the LDPE-plastisphere (T1-P, T2-P) and planktonic cells within the surrounding artificial seawater (T1-SW, T2-SW) was assessed by 16S rRNA gene sequencing, revealing the dominance of the phyla Bacteroidota and Proteobacteria across all samples (Fig. 2A). The T0-P community used as inoculum was dominated by the orders (mean relative abundance ± s.d.; n = 3) Flavobacteriales (48.7 ± 4.6%), Alteromonadales (34.5 ± 6.5%), Rhodobacterales (5.0 ± 0.8%), and Pseudomonadales (4.0 ± 0.9%; Fig. 2B). A significant reduction in alpha diversity was observed between T0-P and, T1-P and T2-P, and, T1-SW and T2-SW, such that Shannon's Entropy and Faith's Phylogenetic Diversity were significantly different between T0-P and all microcosm treatments and timepoints (Kruskal-Wallis, $P \leq 0.05$, $q < 0.1$; Supplementary Table 1). While Pielou's measure of community evenness was only significantly distinct between T0-P and T2-P, and T0-P and T2-SW (Kruskal-Wallis, $P \leq 0.05$, $q < 0.2$; Supplementary Table 1).

The composition of the prokaryotic communities diverged over time between T0-P and T1-P and T2-P, and, T1-SW and T2-SW, underpinned by shifts in the relative abundances of lineages within the Bacteroidota and Proteobacteria (Fig. 2), and the loss of 10 Phyla present at T0-P compared to the microcosm plastisphere and artificial seawater communities (Supplementary Table 2). Specifically, ASVs assigned to the Bacteroidota reduced from a mean of 49.6 ± 4.9% at T0-P, to 5.6 ± 4% at T1-P and 16.6 ± 5.9% at T1-SW, before increasing slightly to 16.6 ± 6.7% at T2-P and 26.5 ± 4.0% at T2-SW. Conversely, Proteobacteria ASVs increased from a mean relative abundance of 47.5 ± 6.2% at T0-P, to 93.8 ± 3.9% at T1-P and 82.2 ± 5.2% at T1-SW, and 82.9 ± 6.5% T2-P and 73.2 ± 3.9% at T2-SW (Fig. 2A). Correspondingly, the structure and composition of the prokaryotic communities differed significantly between T0-P and early colonisation at T1 (Pairwise PERMANOVA, F = 51.3, P-adj. < 0.05), and T0-P and late colonisation at T2 (Pairwise PERMANOVA, F = 52.7, P-adj. < 0.05). However, no significant differences were observed in community structure and composition between T1-P and T2-P (Pairwise PERMANOVA, F = 3.6, P-adj. = 0.1), or between T1-SW and T2-SW (Pairwise PERMANOVA, F = 2.2, P-adj. = 0.1). This is likely owing to consistency in the relative abundances of ASVs within the orders Flavobacteriales, Oceanospiralles, Pseudomonadales, and Rhodobacterales within the microcosms (Fig. 2B). Specific differences in the present and active taxa at lower taxonomic ranks were therefore explored by grouping the plastisphere (T1-P and T2-P = T-P), and seawater (T1-SW and T2-SW = T-SW) samples in subsequent comparisons.

3.2. Community streamlining towards specific plastisphere taxa

When comparing the structure and composition of genus-level ASVs, significant dissimilarity was observed between the initial community T0-P (n = 3) and the combined plastisphere samples T-P (n = 6; PERMANOVA, F = 72.5, P-adj. = 0.016), and between T0-P and the combined surrounding artificial seawater samples T-SW (n = 6;

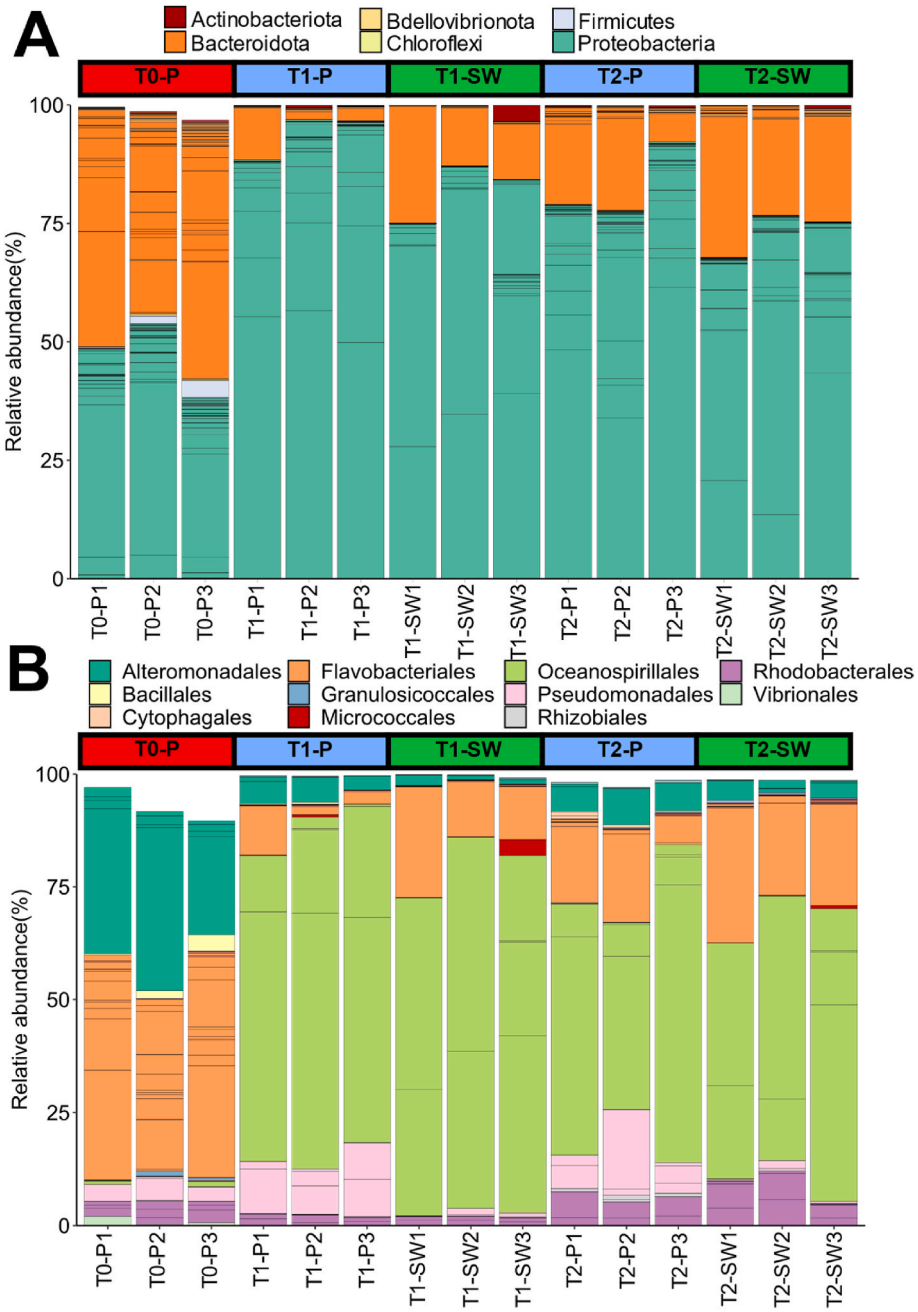

Fig. 2. Microbial community composition of the plastisphere and planktonic cells in the surrounding artificial seawater explored using 16S rRNA gene sequencing. Individual amplicon sequence variants (ASV's; ≥0.1% relative abundance) are separated by black lines and grouped by colour in A) according to phylum, and in B) by order-level taxonomic ranks. Biological replicates corresponding to the initial plastisphere community are denoted by "T0-P1-3", the microcosm plastisphere community by "T1-P1-3 and T2-P1-3", and the cells in the surrounding artificial seawater by "T1-SW1-3 and T2-SW1-3", where "T1" = 1 week and "T2" = 2 weeks of incubation in oligotrophic artificial seawater at 15 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PERMANOVA, F = 64.8, P-adj. = 0.016). Examination of significant fold change in genus-level ASV relative abundances revealed that the dissimilarity in the prokaryotic communities was driven by changes in a small subset of genera. For instance, *Pseudoalteromonas* and *Gillisia* were significantly more abundant in T0-P compared with both T-P and T-SW (T-test, $P < 0.05$, fold change >2; Fig. 3A and B). While *Marinomonas*, *Cobetia*, *Albirhodobacter*, and *Brumimicrobium* displayed significantly greater relative abundances within the microcosm relative to T0-P, for both T-P and T-SW (T-test, $P < 0.05$, fold change >2; Fig. 3A and B).

Further restructuring of the prokaryotic community was observed between T-P and T-SW within the microcosms. Overall, no significant differences were observed in the alpha diversity of ASVs between these two niches (Kruskal-Wallis, $P > 0.05$), however the composition of the prokaryotic communities were distinct (PERMANOVA, F = 6.3, P-adj. < 0.05; n = 6 for both). Comparison of ASV fold changes revealed that T-P contained significantly greater relative abundances of *Pseudomonas*, *Psychrobacter*, and *Pseudoalteromonas* compared to T-SW, while the latter encompassed significantly greater relative abundances of *Marinomonas* and *Marinobacterium* (T-test, $P < 0.05$, fold change >2; Fig. 3C).

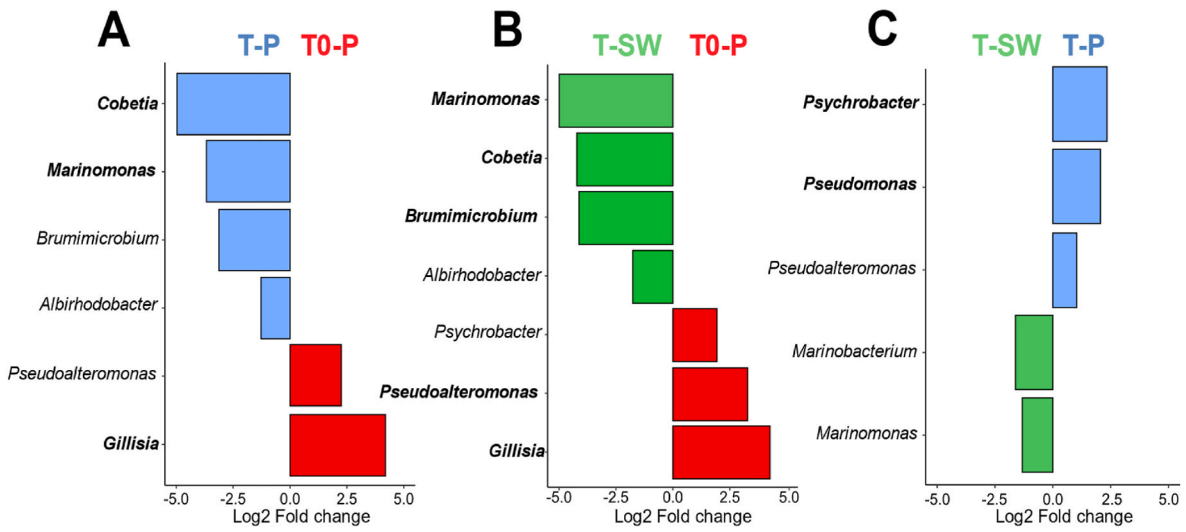

Fig. 3. Community re-assembly during plastisphere colonisation is driven by specific genera. Significant enrichment (T-test, $P < 0.05$, Fold change >2) of genus-level ASV's between A) the microcosm plastisphere T-P (n = 6) and the initial plastisphere T0-P (n = 3), B) the free-living cells T-SW (n = 6) and the initial plastisphere T0-P (n = 3), and C) the free-living cells T-SW (n = 6) and the microcosm plastisphere T-P (n = 6). Genera shown in bold represent those displaying the greatest fold changes in the pairwise comparisons.

3.3. Present and active Proteobacteria dominate the plastisphere

To determine the active taxa within T0-P, the newly formed plastisphere T-P, and the free-living T-SW, the identification of protein groups and peptides was obtained using metaproteomics employing a custom protein search database derived from the 16S rRNA taxonomic assignments (16S-TaxDB). This resulted in a minimum of 34 to a maximum of 2328 protein groups (≥2 peptides), between 52 and 8021 distinct peptides, and peptide spectra coverages between 2.3 and 34.3%, across individual samples (Supplementary Table 3). One plastisphere metaproteome was poorly characterised (T1-P2, n = 34 proteins, n = 52 peptides, spectra coverage = 2.3%) and this was removed from the downstream analyses. Qualitative comparisons of the taxonomic annotations of identified proteins grouped by treatments, revealed that Proteobacteria represented 90% of the active taxa within T0-P (355 annotated proteins; n = 3 samples), primarily of the orders Alteromonadales (45%), Pseudomonadales (18%), Rhodobacterales (17%), and Flavobacteriales (5%). These lineages were all shared between the

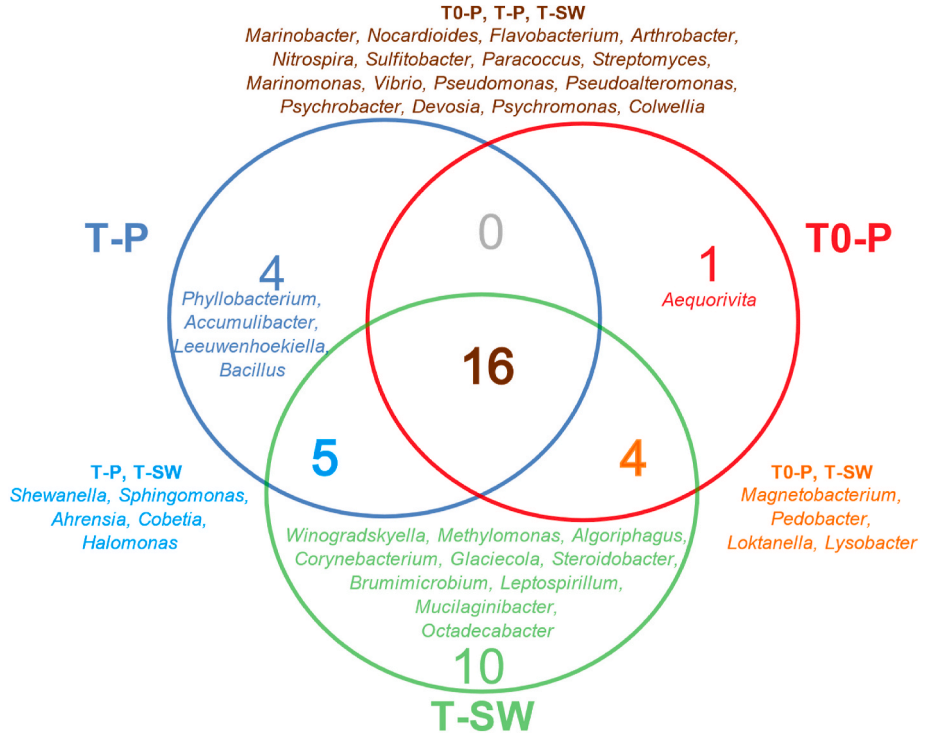

Fig. 4. Qualitative identification of the active taxa using metaproteomics. Proteins were identified using ProteinPilot by combining the biological replicates from each individual treatment group in a 'multi-file search' with the custom database 16S-TaxDB. Identified protein groups were then annotated with consensus taxonomic and functional annotations using the bioinformatic tool mPies. Genus-level taxonomic annotations are shown for the initial plastisphere community T0-P (n = 3), the microcosm plastisphere T-P (n = 5), and the planktonic T-SW (n = 6).

taxonomic annotations of the microcosm plastisphere T-P (985 annotated proteins; n = 5 samples) and free-living T-SW (2260 annotated proteins; n = 6 samples). Thus, the active taxa within the microcosms were similarly Proteobacteria-dominated (98% and 97% for T-P and T-SW, respectively). However, within the microcosms the Oceanospirillales were the most active, representing 59% of T-P and 72% of T-SW annotated proteins respectively, followed by Pseudomonadales (T-P, 22%), and Alteromonadales (T-SW, 7%). Although less proteins were annotated to the lower taxonomic rank of genus (T0-P = 81%, T-P = 66%, and T-SW = 78%, of annotated proteins), genus-level taxonomic annotations revealed that 16 of the active genera at T0-P, including the dominant taxa identified using 16S rRNA gene sequencing (*Pseudomonas Pseudoalteromonas*, *Marinomonas*, *Psychrobacter*), were also active within T-P and T-SW (Fig. 4). Only 1 genus, *Aequorivita*, was uniquely active in T0-P, while 5 genera were active within T-P and T-SW but not T0-P, including *Shewanella*, *Sphingomonas*, *Ahrensia*, *Cobetia*, and *Halomonas* (Fig. 4). Interestingly, 4 genera, including *Phyllobacterium, Accumulibacter*, *Leeuwenhoekiella*, and *Bacillus*, were active only within T-P, while a further 10 genera were identified as being uniquely active within T-SW, suggesting several inactive taxa from the initial plastisphere community proliferated when presented with a new plastic surface within the oligotrophic artificial seawater.

3.4. Connecting structure to function during plastisphere formation

Qualitative assessment of the functional annotations of proteins identified within the plastisphere samples T0-P and T-P revealed that 66 non-redundant protein functions were shared between the two communities, 22 were unique to the T0-P, and 207 were specific to T-P (Supplementary Table 4). The shared proteins included those associated with translation, ribosomal structure and biogenesis, energy production and conversion, and posttranslational modification, protein turnover, and chaperones, in addition to specific proteins previously associated with inter-community and microbe-plastic interactions, such as phenyloxazoline synthase, flagellin, OmpA-like domain-containing protein, alkyl hydroperoxide reductase, and superoxide dismutase (Messer et al., 2024). Interestingly, other protein functions associated with inter-community interactions were unique to T0-P, implicating them in the functioning of mature plastisphere communities, including acyl-homoserine-lactone synthase, LuxP autoinducer 2-binding periplasmic protein, beta-lactamase domain-containing protein, Type II secretion system protein K, Big_12 domain-containing protein, and a phage tail component protein. In comparison, unique functions within T-P reflected active substrate uptake within the newly formed plastisphere, such as a secretin and TonB N terminus-domain containing protein, an ABC-type glycine betaine transport system (OpuAC), sodium/proline symporter, and membrane fusion family protein, in addition to the regulation of cellular morphology/growth (and protein synthesis) and membrane integrity, including cell division and shape (CpoB, FtsZA, ZapB, and MreB), lipopolysaccharide assembly (LptD, AsmA), curli production (CsgG), and small- and large-conductance mechanosensitive channels, as well as pilus- and Von Willebrand factor-mediated adherence.

To determine significant differential protein regulation during plastic colonisation, relative quantification was performed on 1762 proteins shared across T0-P, T-P, and T-SW which were identified using a combined multi-file search in ProteinPilot software (n = 14 samples). These proteins were taxonomically and functionally annotated, and the examination of significant fold changes revealed that 120 proteins representing 100 unique protein groups were significantly differentially expressed between T0-P and T-P (T-test, Fold change >2; P-adj. <0.05; Supplementary Table 5; Fig. 5). Of these, 86 proteins representing 73 unique protein groups were upregulated within T0-P, compared to 34 proteins and 27 unique groups upregulated in T-P. The T0-P upregulated proteins largely belonged to the families Oceanospirillaceae (n = 38 proteins), Halomonadaceae (n = 11), and Vibrionaceae (n = 8), and the genera *Marinomonas* (n = 26), *Cobetia* (n = 7), and *Vibrio* (n = 8), respectively. While the upregulated proteins within T-P primarily belonged to the families Pseudomonadaceae (n = 11), Oceanospirillaceae (n = 10), unclassified Halomonadaceae (n = 4), and Alteromonadaceae (n = 3), and the specific genera, *Pseudomonas* (n = 11), *Marinomonas* (n = 10), and *Marinobacter* (n = 3), respectively. In terms of function, upregulated proteins in T0-P belonged to the categories, energy production and conversion (n = 24), amino acid metabolism (n = 9), posttranslational modifications, protein turnover, and chaperones (n = 9), nucleotide metabolism (n = 9), and inorganic ion transport and metabolism (n = 9), among others (Fig. 5). In T-P, the upregulated proteins were also primarily associated with energy production and conversion (n = 15), followed by translation, ribosomal structure and biogenesis (n = 7), and amino acid metabolism (n = 3; Fig. 5).

The differential regulation of proteins associated with energy

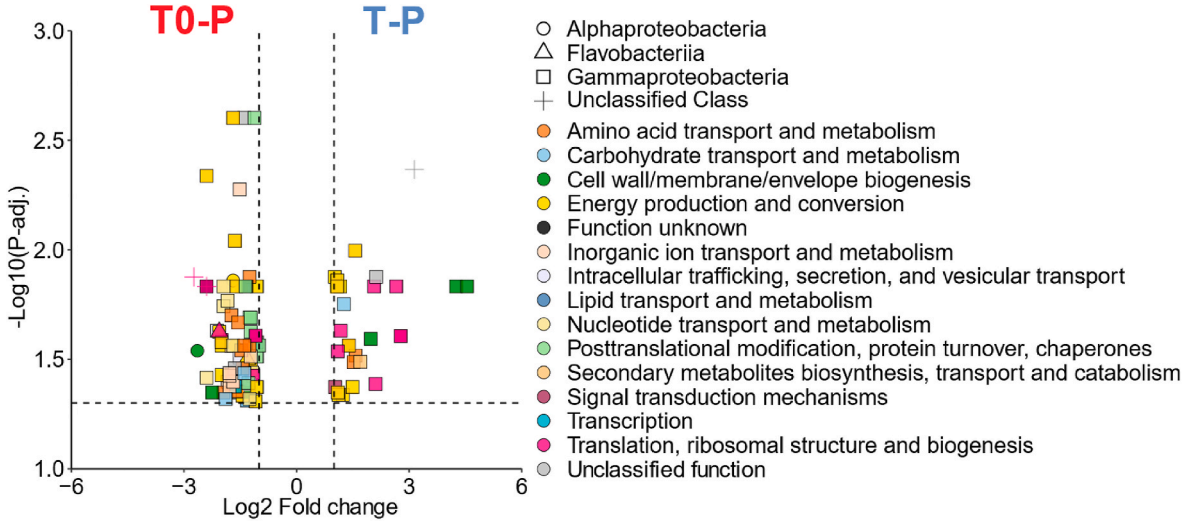

Fig. 5. Differential protein regulation between the established and newly synthesised plastisphere. Points represent individual proteins identified as being significantly differentially expressed (T-test, P-adj. <0.05, Fold change >2) between the initial plastisphere community T0-P (n = 3) and the microcosm plastisphere T-P (n = 5). Class-level taxonomic assignments are identified by shape, and functional categories by colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

metabolism, translation, ribosomal structure and biogenesis, amino acid metabolism, and proteins associated with stress responses, reflected the shift in the growth and metabolic state of key taxa within the plastisphere. Specifically, upregulated proteins associated with energy metabolism in T0-P relative to T-P, included those mediating oxidative phosphorylation and the citrate cycle in several genera, such as inorganic pyrophosphatase expressed by *Marinomonas*, ATP synthase expressed by *Psychrobacter*, *Sphingomonas*, *Pseudoalteromonas* and unclassified *Halomonadaceae* and *Rhodobacteraceae*, NADP-dependent isocitrate dehydrogenase expressed by *Winogradskyella* and *Pseudoalteromonas*, succinate–coA ligase by *Marinomonas*, *Vibrio,* and unclassified Gammaproteobacteria, and fumarate hydratase expressed by *Marinomonas*. In contrast, upregulated proteins mediating energy metabolism in T-P relative to T0-P, reflected a shift in the taxa mediating key steps in the citrate cycle, such as succinate dehydrogenase flavoprotein expressed by *Marinobacter* and *Marinomonas*, and succinate–coA ligase and NADP-dependent isocitrate dehydrogenase expressed by *Pseudomonas*. Upregulated proteins associated with amino acid metabolism in T0-P relative to T-P were primarily expressed by *Marinomonas,* and these were related to the biosynthesis of leucine, threonine, and aromatic amino acids, including 3-isopropylmalate dehydrogenase, 2-isopropylmalate synthase, threonine synthase, and chorismate mutase. On the other hand, upregulated proteins associated with amino acid metabolism in T-P relative to T0-P were associated with amino acid transport, including solute-binding protein family 3, expressed by *Pseudomonas* and *Vibrio*. The regulation of translation, ribosomal structure and biogenesis in T0-P relative to T-P, was characterised by the expression of lysine and aspartate–tRNA ligases by *Marinomonas*, and proteins involved in protein folding such as DnaK, peptidyl-prolyl cis-trans isomerase, and GroEL, expressed by *Shewanella*, *Marinomonas*, and *Pseudomonas*, respectively. In contrast, upregulated proteins mediating translation, ribosomal structure and biogenesis in T-P relative to T0-P included 30S and 50S ribosomal proteins (S5, S7, L1, L2) and an isoleucine–tRNA ligase, expressed by *Pseudomonas*, *Marinobacter*, unclassified Halomonadaceae, and *Marinomonas,* respectively. Interestingly, proteins mediating defence against oxidative stress were also significantly upregulated in T0-P relative to T-P, including glutathione-dependent peroxiredoxin, catalase-peroxidase, and catalase, expressed by *Marinomonas* and *Cobetia*.

3.5. Physiological shifts between plastic-associated and planktonic states

Within the microcosms, qualitative assessment of functional annotations for the plastisphere samples T-P, and planktonic cells within surrounding seawater samples T-SW, revealed that 251 non-redundant protein functions were shared between the two communities, with 22 unique to T-P and 108 specific to T-SW (Supplementary Table 4). Notably, several proteins associated with stress responses and redox homeostasis were unique to T-SW, including glutathione peroxidase, ferredoxin, thioredoxin peroxidase, and catalase peroxidase. In addition to proteins mediating the metabolism of a diversity of amino acids and those associated with nitrogen availability, including glutamine synthetase, an ammonium transporter, and nitrogen regulatory protein, perhaps reflecting the survival and adaptation of taxa within the oligotrophic minimal seawater media. In order to identify the specific molecular mechanisms associated with the plastisphere niche, relative quantification was performed (as above) and significant fold changes were examined between T-P and T-SW. In total, 147 proteins representing 123 unique protein groups were identified as being significantly differentially expressed (T-test, P-adj. <0.05, Fold change >2; Supplementary Table 6), with 82 proteins spanning 78 protein groups and 17 unique functional categories upregulated in T-P, compared to 55 proteins encompassing 45 protein groups and 6 functional classes upregulated T-SW. The taxonomic assignments of the upregulated proteins reflected differences in protein expression by *Marinomonas, Pseudomonas, Psychrobacter, Cobetia*, and several other genera present in both T-P and T-SW (Fig. 6). Functional annotation of the differentially expressed proteins revealed that the majority were associated with energy metabolism (n = 38), translation, ribosomal structure and biogenesis (n = 20), amino acid metabolism (n = 17), cell wall/membrane biogenesis (n = 12), posttranslational modification, protein turnover, chaperones (n = 11), and cell motility (n = 10), among several other categories.

At the protein level, the differential expression of key pathways and specific functions in taxa identified as dominant in both the plastic-associated and planktonic communities revealed the physiological and morphological differences between these specific niches. For instance, upregulated proteins associated with energy metabolism in T-SW (n = 26 proteins) relative to T-P (n = 12), highlighted the production of ATP and oxidative decarboxylation by *Marinomonas*, *Shewanella*, and *Vibrio*, along with unclassified Alteromonadales and Gammaproteobacteria, through the activity of ATP synthase, NADP-dependent isocitrate dehydrogenase, and succinate–coA ligase (Fig. 6). Conversely, the upregulated proteins associated with energy metabolism in T-P relative to T-SW, primarily mediated orthophosphate production through inorganic pyrophosphatase in *Marinomonas* and *Psychrobacter*, and oxidative decarboxylation through NADP-dependent isocitrate dehydrogenase in *Pseudomonas* and oxoglutarate dehydrogenase in *Cobetia* (Fig. 6). Furthermore, differences in the metabolism of amino acids between T-P (n = 7) and T-SW (n = 10) included the upregulation of glutamine hydrolysis by *Marinomonas* (CarB) and unclassified Halomonadaceae (CarA) in T-P, whereas glutamine biosynthesis (GlnA) mediated by *Marinomonas* and unclassified Proteobacteria was upregulated within T-SW (Fig. 6). Moreover, dapD associated with the diaminopimelate pathway of lysine biosynthesis expressed by *Marinomonas,* was upregulated in T-SW relative to T-P, while O-succinylhomoserine sulfhydrylase (metZ), expressed by *Marinomonas* for the conversion of homocysteine, was upregulated in T-P (Fig. 6). While the differential regulation of protein synthesis between T-SW (n = 7) and T-P (n = 13), was evident through the up-regulation of several ribosomal proteins and tRNA ligases expressed by *Halomonas*, *Pseudomonas*, *Pseudoalteromonas*, and *Marinomonas* in T-P, relative to the upregulation of elongation factor G by unclassified Gammaproteobacteria, *Pseudomonas* and *Marinomonas* in T-SW (Fig. 6). Notably, the upregulation in proteins associated with cell membrane biogenesis (n = 12) and motility (n = 10) was observed in T-P, including flagellin expressed by *Marinomonas*, *Cobetia*, *Pseudoalteromonas*, *Pseudomonas*, and unclassified Gammaproteobacteria, curli expressed by *Cobetia*, and an OmpA-like domain containing protein expressed by *Psychrobacter, Pseudomonas,* and unclassified Gammaproteobacteria (Fig. 6).

4. Discussion

In this study, a combined taxonomic and comparative metaproteomic approach was used to explore the active taxa, their functions, and the molecular mechanisms giving rise to the formation of the marine plastisphere under controlled oligotrophic conditions. Interestingly, significant restructuring of the initial active plastisphere community was observed, with the selection of specific taxa during the re-development of the plastisphere and proliferation of several genera within the surrounding artificial seawater, likely co-dependent on the plastisphere due to the oligotrophic nature of the microcosm. The use of complementary molecular approaches revealed differences between the dominant present and active taxa, highlighting nuances of the plastisphere overlooked when relying upon taxonomic observations alone. However, we also identify several lineages present within the plastisphere whose functional roles remain unknown due to poor genomic and proteomic representation, providing an important avenue of future research. Together, the taxonomic and functional annotations of differentially upregulated proteins provided insights into the specialised functions expressed by keystone taxa associated with the plastisphere, supporting the notion that marine plastic pollution is colonised by microorganisms

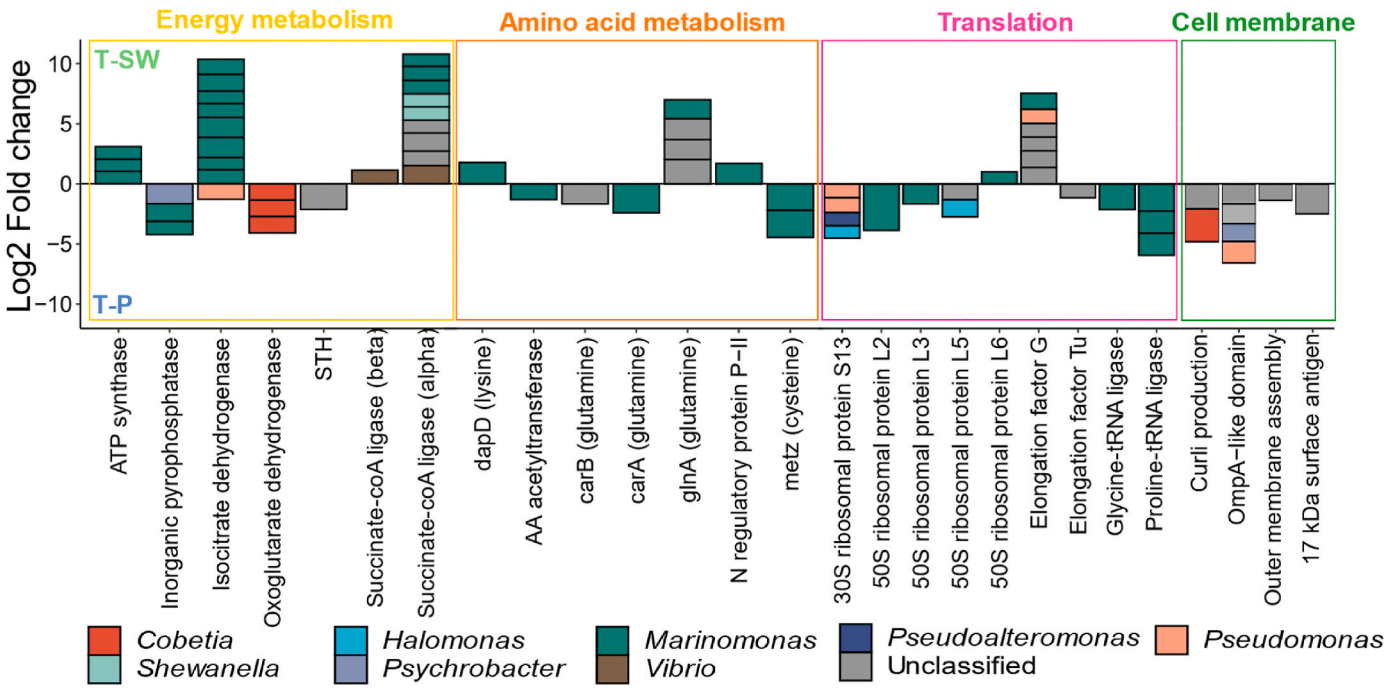

Fig. 6. Functional and physiological differences between the plastisphere and planktonic state. Significant differentially expressed proteins (T-test, P-adj. <0.05, Fold change >2) identified between the microcosm plastisphere (T–P) and free-living cells within the surrounding seawater (T-SW). Differentially expressed proteins of the functional categories, energy metabolism, amino acid metabolism, translation, ribosomal structure, and biogenesis, and cell wall/membrane biogenesis, are coloured by their genus-level taxonomic assignments. STH = soluble pyridine nucleotide transhydrogenase.

with the specific ability to exploit this niche.

4.1. Community re-assembly within the plastisphere

In-depth characterisation of the beach plastisphere used as the inoculum supports that it was highly diverse, consisting of over 21 bacterial families. The level of plastisphere complexity suggests this was a mature biofilm composed of both specific and generalist taxa, including key hydrocarbonoclastic lineages such as *Pseudoalteromonas*, *Pseudomonas*, and *Colwellia*. The presence and active state of these taxa was likely influenced by local environmental structuring factors, such as temperature, nutrients, and coastal processes, driving selection within the initial microbial community (Lee et al., 2023; Messer et al., 2024). Moreover, previous research has demonstrated a proclivity for hydrocarbonoclastic bacteria in marine waters North of Scotland, likely due to proximity to natural reserves of oil and gas which may have contributed to pre-selection in the plastisphere community (Angelova et al., 2021). It also contained a Eukaryotic community composed of primary producers, secondary consumers, and saprotrophs (Kettner et al., 2019; Kirstein et al., 2019; Messer et al., 2024; Wallbank et al., 2022), which although not investigated, may have influenced the patterns observed in the prokaryotic community in the present study. The composition of the plastisphere community within the experimental microcosm diverged over time from the initial diverse inoculum to a community dominated by Oceanospirillaceae, Halomonadaceae, and Crocinitomicaceae. A reduction in plastisphere diversity is commonly observed in microcosm incubations (Delacuvellerie et al., 2019; Huang et al., 2022; Wright et al., 2021a) regardless of whether the source inoculum is derived from a plastisphere biofilm (Delacuvellerie et al., 2019; Huang et al., 2022; Wright et al., 2021a), or free-living communities (Ogonowski et al., 2018), suggesting that specific interactions between microorganisms and the plastic surface do contribute to the restructuring of the community (Kirstein et al., 2019). Indeed, the dominance of gammaproteobacterial families within the plastisphere is consistent with previous literature (Lee et al., 2023; Wright et al., 2021b), but the taxonomic succession of the plastisphere in the environment typically includes the primary colonisers such as diatoms, cyanobacteria, and Alteromonadaceae (Bos et al., 2023; Schlundt et al., 2020), and the subsequent proliferation of additional heterotrophic bacteria.

Interestingly, our observations of a reduction in Bacteroidota and enrichment of Proteobacteria during early colonisation reflects the findings of a global meta-analysis of plastisphere composition during colonisation (Wright et al., 2021b). Members of the Bacteroidota are typically representative of mature plastisphere communities and likely represent secondary colonisers (De Tender et al., 2015; Wright et al., 2021b). Indeed, the increase in Bacteroidota by the late colonisation stage (2 weeks of incubation herein) may represent the re-establishment of the Bacteroidota community, suggesting consistent community structuring factors even within the microcosm setting. However, phylum level changes provide only coarse resolution of the dynamics of plastic colonisation and biofilm succession, and specific patterns may be masked depending on several features of the experimental design (Erni-Cassola et al., 2020; Latva et al., 2022). In the present study, specific enrichment of plastisphere taxa within the microcosm was explored at the level of ASVs and their genus level annotations, and reflected proliferation of *Marinomonas*, *Cobetia*, *Brumimicrobium,* and *Albirhodobacter*, which were present in relatively low abundances in the initial inoculum. Further exploration of the specificity of the microcosm plastisphere community relative to the free-living cells in the surrounding artificial seawater, revealed enrichment of *Pseudomonas*, *Psychrobacter*, and *Pseudoalteromonas* within the biofilm – consistent with the initial plastisphere community. Collectively, the enriched taxa represented several hydrocarbonoclastic genera, implying the presence of molecular mechanisms or metabolic functions supporting their ability to colonise the plastic niche (Erni-Cassola et al., 2020). In contrast, the enrichment in the relative abundances of *Marinomonas* and *Marinobacterium* within the surrounding seawater relative to the plastisphere, suggested an indirect reliance of these taxa on the plastisphere for carbon and other essential nutrients not supplied within the oligotrophic minimal media (Wright et al., 2021a; Yu et al., 2009).

4.2. Functioning of the plastisphere during colonisation

Previously, we conducted a detailed characterisation of the functioning of the plastisphere used as inoculum in the present study (Messer et al., 2024). This analysis demonstrated that the dominant genera (e.g. *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*) were active within the plastisphere, expressing proteins associated with oxidative phosphorylation, the citrate cycle, glutamine and proline biosynthesis, aromatic compound degradation, and fatty acid beta-oxidation, in addition to mechanisms associated with biofilm formation, such as motility, chemotaxis, and adhesion, and inter-community interactions, including quorum sensing and virulence factors (Messer et al., 2024). The identification of relatively few (<10%) differentially expressed proteins between the inoculum and microcosm plastisphere, and the microcosm plastisphere and planktonic cells, suggested a degree of functional stability, consistent with the notion of a specialised plastisphere community (Delacuvellerie et al., 2019). This functional stability was maintained over time within the microcosm, with no significant differences in protein expression detected between early and late colonisation for both the plastisphere and cells in the planktonic state.

The taxonomic and functional annotations of the proteins that were differentially expressed provided novel insights into the functioning and metabolism of the active taxa. For instance, the overrepresentation of proteins associated with translation, ribosomal structure and biogenesis, suggested upregulated growth and metabolic state of *Pseudomonas*, *Marinobacter*, unclassified Halomonadaceae, and *Marinomonas* within the microcosm, relative to the initial plastisphere. Intriguingly, the initial plastisphere metaproteomes were characterised by the overrepresentation of proteins associated with oxidative stress in *Marinomonas* and *Cobetia*, relative to the microcosm plastisphere. The presence of co-pollutants, additives, or free radicals produced through photooxidation (Zhu et al., 2019), within the plastisphere may result in exposure of microorganisms to reactive oxygen species, resulting in an increased requirement to produce antioxidants to maintain cellular homeostasis (He et al., 2017). This in turn may result in the limitation of growth in some lineages within the initial plastisphere sampled from the environment (Lee et al., 2023), perhaps leading to the results observed herein.

4.3. Differential protein regulation between plastic-associated and planktonic states

Our analyses provided insights into the potential regulation of the metabolism of plastisphere taxa between their different niche states. For example, over-representation of proteins associated with energy metabolism was a prominent finding of the comparative metaproteomic analyses. Changes in energy production and conversion pathways are known to elicit specific responses in microorganisms, resulting in either biofilm proliferation or cellular dispersal into the plankton (Martín--Rodríguez, 2023). The most efficient mechanism of ATP generation employs membrane-associated ATP synthases, which were overrepresented in the initial plastisphere in *Pseudoalteromonas*, *Psychrobacter*, *Sphingomonas*, and unclassified Halomonadaceae and Rhodobacteraceae, relative to the microcosm samples. Perhaps reflecting the fact that biofilm formation is an energy-intensive process (Scribani Rossi et al., 2022). Our results highlighted changes in the production of ATP and the mediation of the citrate cycle by *Marinomonas*, *Marinobacter*, and *Pseudomonas* as a discriminating feature of the microcosm communities, relative to the initial plastisphere. However, upregulation of ATP production in *Shewanella* and *Vibrio* within the cells in the surrounding seawater, and overrepresentation of orthophosphate production by *Marinomonas*, differentiated energy metabolism between the planktonic cells in the surrounding seawater and plastisphere community within the microcosm, respectively. Although the availability of key electron acceptors likely underpins the nature of the regulation of energy metabolism and the individual responses of specific taxa (Martín-Rodríguez, 2023), these findings suggest differences in the efficient production of ATP impact the colonisation of the new plastic substrate, which has implications for the persistence, dispersal, and survival of microorganisms, including potential pathogens, within the plastisphere.

Besides energy metabolism and nutrient availability, microbial cell wall composition and morphology in addition to behaviours such as chemotaxis and motility, facilitate the colonisation of surfaces for biofilm formation. In-line with this, very few proteins associated with cell wall, membrane and envelope biogenesis and motility displayed differential regulation between the initial plastisphere community and the microcosm plastisphere. Whereas >20 proteins associated with these functions were collectively over-represented in *Marinomonas*, *Cobetia*, *Pseudomonas*, *Pseudoalteromonas*, and *Psychrobacter*, within the microcosm plastisphere relative to cells within the surrounding seawater. These proteins included flagellin, curli, an OmpA-like transmembrane domain, and a cell surface antigen involved in host-cell recognition, virulence, and biofilm formation (Smith et al., 2007; Viale and Evans, 2020; Vila-Farrés et al., 2017), and reflect phenotypic differences between plastisphere taxa when growing in either a biofilm-associated or planktonic state. Moreover, identification of the specific mechanisms enabling the reestablishment of the plastisphere provides insights into potential targets for biotechnological advancement of plastics bio-recycling. For example, bioremediation could be enhanced through the optimisation of cellular attachment and interaction between biodegraders and the plastic surface (Howard and McCarthy, 2023). This has previously been achieved for heterocyclic hydrocarbons using curli-overproducing mutants of *Cobetia* sp. to promote biofilm formation (Dinamarca et al., 2018), and for polyethylene terephthalate biodegradation through the modulation of Cyclic-di-GMP (Howard and McCarthy, 2023). Similar examples have also been used in biofilm-mediated heavy metal remediation (Tay et al., 2017), and represent exciting avenues of further research for the environmental plastisphere.

Amino acids are also important determinations of microbial biofilm formation and represent important potential targets for the regulation of biofilm growth (Scribani Rossi et al., 2022). Indeed, significant changes in the biosynthesis, hydrolysis, and transport of amino acids were observed between the initial plastisphere community, the microcosm plastisphere, and free-living cells. This was represented by a significant downregulation in the diversity of amino acids produced by *Marinomonas,* and the upregulation of amino acid transport in *Pseudomonas* and *Vibrio*, within the microcosm relative to the initial plastisphere. These findings suggest different amino acid requirements of plastisphere-associated microorganisms, depending on their growth within the plastisphere or as free-living planktonic cells. Interestingly, the specific metabolism of the amino acid glutamine distinguished plastisphere-associated and planktonic *Marinomonas* and unclassified Halomonadaceae. Glutamine plays a key role in microbial physiology, not only as an important amino acid for protein synthesis, but also as signalling molecule, a mediator of bioavailable nitrogen compounds *in vivo,* and in the oxidative stress response as a precursor of the antioxidant glutathione (Forchhammer, 2007). Furthermore, the availability of glutamine has been shown to play a critical role in biofilm proliferation and is closely regulated within established biofilms (Hassanov et al., 2018; Pisithkul et al., 2019; Prindle et al., 2015). Consistent with this, our results suggest that the regulation of glutamine may be associated with differential ammonium availability and oxidative stress within our low-nutrient microcosm. For instance, the potential regeneration of ammonia through glutamine hydrolysis to glutamate was overrepresented within the microcosm plastisphere, while the potential assimilation of ammonia during glutamine biosynthesis mediated by glutamine synthetase, was upregulated in the surrounding seawater community. This was supported by the overexpression of P-II by *Marinomonas* in the surrounding seawater, which functions as a nitrogen regulatory protein of glutamine synthetase. Moreover, the qualitative identification of proteins associated with antioxidant responses unique

to cells in the surrounding seawater relative to the microcosm plastisphere, such as glutathione peroxidase, highlights the potential link between nutrient availability and oxidative stress leading to opportunistic biofilm formation within oligotrophic environments. These dynamics would have implications for microbial colonisation and growth within the plastisphere in oligotrophic marine waters (Mincer et al., 2016), and further suggest that plastic surfaces may contribute to nitrogen regeneration within marine ecosystems.

5. Conclusions

This study explored the taxonomic and functional re-assembly of an environmental plastisphere and revealed the molecular mechanisms and phenotypic differences underlying plastisphere formation and function. Although microcosm experiments are less representative of the natural environment, the increase in total identified proteins (>2000), numbers of proteins available for relative quantification (1,762), and relatively high peptide spectral coverage (range 8–34%) reported herein, facilitated improved representation of the active taxa and their functions during plastic colonisation. This enabled identification of the plastisphere taxa with the ability to exploit the plastic niche directly through expressed attachment mechanisms, and indirectly through growth in the surrounding seawater in which plastic was the sole carbon source. Our findings demonstrate significant filtering of the original environmental plastisphere community during colonisation, with convergence in the active enrichment of hydrocarbonoclastic lineages. Our results also revealed the differential regulation of key pathways, such as energy and glutamine metabolism, within specific taxa inhabiting both plastic-associated and planktonic niches, identifying possible mechanisms of nutrient limitation and remineralisation and recognising potential biotechnological targets for plastisphere manipulation. However, the colonisation of plastic by eukaryotes and their subsequent interactions with prokaryotes, may influence the patterns observed herein, therefore determining the mechanisms used by the eukaryotic community is an important avenue of future research. Overall, this study has contributed to an improved mechanistic understanding of the development and function of the marine plastisphere, providing novel insights into this evolutionarily novel yet ubiquitous niche in marine ecosystems.

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CRediT authorship contribution statement

Lauren F. Messer: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ruddy Wattiez:** Resources, Methodology, Funding acquisition. **Sabine Matallana-Surget:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition.

Declaration of competing interest

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

Data availability

The sequence data generated in this study have been submitted to the NCBI Sequence Read Archive database and are available at BioProject accession PRJNA1123065. The metaproteomic data have been submitted to ProteomeXchange and are available under the accession PXD053111.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.124479.

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