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Decoding Microbial Plastic Colonisation: Multi-Omic Insights into the Fast-Evolving Dynamics of Early-Stage Biofilms

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Abstract

Marine plastispheres represent dynamic microhabitats where microorganisms colonise plastic debris and interact. Metaproteomics has provided novel insights into the metabolic processes within these communities, however the early metabolic interactions driving the plastisphere formation remain unclear. This study utilised metaproteomic and metagenomic approaches to explore early plastisphere formation on low-density polyethylene (LDPE) over three (D3) and seven (D7) days, focusing on microbial diversity, metabolic activity, and biofilm development. In total, 2,948 proteins were analysed, revealing dominant proteomes from Pseudomonas and Marinomonas, with near-complete metagenome-assembled genomes. Pseudomonas dominated at D3, while at D7, Marinomonas, along with Acinetobacter, Vibrio, and other genera became more prevalent. Pseudomonas and Marinomonas showed high expression of reactive oxygen species (ROS) suppression proteins, associated with oxidative stress regulation, while granule formation, and alternative carbon utilisation enzymes, also indicated nutrient limitations. Interestingly, 13 alkane and other xenobiotic degradation enzymes were expressed by five genera. The expression of toxins, several type VI secretion system (TVISS) proteins, and biofilm formation proteins by Pseudomonas indicated their competitive advantage against other taxa. Upregulated metabolic pathways, including those relating to substrate transport also suggested enhanced nutrient cross-feeding within the biofilm. These insights enhance our understanding of plastisphere ecology and its potential for biotechnological applications.

Decoding Microbial Plastic Colonisation:

Multi-Omic Insights into the Fast-Evolving Dynamics of Early-Stage Biofilms

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Abbreviations

AD Alcohol dehydrogenase

Ahp Alkyl hydroperoxide reductase

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ALDH Aldehyde dehydrogenase

ArnA Polymyxin resistance protein

ASW+G Artificial seawater + vitamins, trace metals, and glucose

BHB+v Bushnell Haas broth + vitamins, and trace metals

CopA Copper resistance protein A

CSD Cold shock domain-containing protein

D3/D7 Day 3/7

FC Fold change

Fhp Flavohemoprotein

HGT Horizontal gene transfer

Kat Catalase peroxidase

LAD Large adhesive protein

LDPE Low-density polyethylene

MAG Metagenome-assembled genome

Omp Outer membrane protein

PPS Protein precipitation solution

ROS Reactive oxygen species

TelA Toxic anion resistance protein

TerD Tellurium resistance protein

TIISS/TVISS Type- II/VI secretion system

Keywords

Plastisphere; Metaproteome; Marine biofilms; Biofilm formation; Plastic biodegradation

Word count : 6757

Abstract

Marine plastispheres represent dynamic microhabitats where microorganisms colonise plastic debris and interact. Metaproteomics has provided novel insights into the metabolic processes within these communities, however the early metabolic interactions driving the plastisphere formation remain unclear. This study utilised metaproteomic and metagenomic approaches to explore early plastisphere formation on low-density polyethylene (LDPE) over three (D3) and seven (D7) days, focusing on microbial diversity, metabolic activity, and biofilm development. In total, 2,948 proteins were analysed, revealing dominant proteomes from *Pseudomonas* and *Marinomonas,* with near-complete metagenome-assembled genomes. *Pseudomonas* dominated at D3, while at D7, *Marinomonas,* along with *Acinetobacter, Vibrio, and other genera became more prevalent* . *Pseudomonas* and*Marinomonas* showed high expression of reactive oxygen species (ROS) suppression proteins, associated with oxidative stress regulation, while granule formation, and alternative carbon utilisation enzymes, also indicated nutrient limitations. Interestingly, 13 alkane and other xenobiotic degradation enzymes were expressed by five genera. The expression of toxins, several type VI secretion system (TVISS) proteins, and biofilm formation proteins by *Pseudomonas* indicated their competitive advantage against other taxa. Upregulated metabolic pathways, including those relating to substrate transport also

suggested enhanced nutrient cross-feeding within the biofilm. These insights enhance our understanding of plastisphere ecology and its potential for biotechnological applications.

Significance of the study

Given the increasing plastic pollution in marine environments, understanding early plastisphere assembly is essential from both ecological and biotechnological perspectives. This study advances knowledge by identifying 13 pollutant-degrading enzymes in addition to those found through prior metaproteomic research, shedding light on the bioremediative potential of multi-species biofilms. Their expression by constitutive genera, particularly *Marinomonas* , also underscores the recognised association of these genera with environmental bioremediation. Additionally, interspecies competition and oxidative stress responses, shaped by resource limitations, were found to govern biofilm dynamics. The selection for generalist species and potential pathogens is concerning due to plastic's ability to travel through marine ecosystems. However, the cooperative behaviour among plastisphere members supports prior research demonstrating biofilms as resilient microbial solutions. This research not only deepens our understanding of microbial colonisation and interaction but also highlights the utility of metaproteomics in studying complex environmental communities. Insights from this study also contribute to the broader field of plastisphere ecology, offering pathways for future research into managing plastic pollution and developing biotechnological strategies for marine ecosystem resilience.

Introduction

Over 400.3 million metric tonnes of plastic are manufactured, utilised, and discarded annually [1]. Despite diverse waste management strategies implemented by national governments and public initiatives aimed at controlling plastic waste, an estimated 19 to 23 million tonnes of plastic pollution enter the marine environment each year [2, 3]. Marine plastic debris, along with the resulting microplastics, is now anticipated to enter nearly all marine ecosystems, threatening the health of our oceans [4]. Direct studies of marine plastic debris reveal a complex relationship between plastics and the ocean's most abundant biogeochemical cyclers and producers: microorganisms [5, 6]. As natural biofilm-forming organisms, and secondary surface colonisers, these microorganisms directly interact with marine plastic debris through the formation of the 'plastisphere' [7].

Marine plastispheres are complex, multi-species biofilms that consist of both eukaryotic and prokaryotic producers, hydrocarbonoclastic organisms, pathogens, and other biogeochemical cyclers [6-10]. The composition of these communities is influenced by several factors, including the type of plastic, its chemical additives, environmental location, seasonal changes, and the time available for colonisation [6]. Among these factors, the duration of plastisphere development (i.e., the stage at which the plastisphere is collected) appears to exert the most consistent influence across studies. Microbial abundance and diversity are often low in the initial hours following colonisation, peak within 1-2 weeks, and subsequently decline [10-13]. Metaproteomic studies focusing on biofilm formation during these early stages have uncovered both competition and cooperation between species, interactions that are likely to be prevalent within the first days of plastisphere development [14, 15]. This early plastisphere is particularly significant, as it may be the only stage of biofilm development where direct microbe-plastic interactions occur. As biofilms continue to form, new layers often cover older ones, shielding the upper biofilm layers from direct contact with the plastic surface [11].

Prior metaproteomic research on multi-species biofilm formation [14, 15] and established marine plastispheres [8, 16, 17] has offered insights into how microorganisms interact with plastic substrates and with each other. A recent study by our research group highlighted the importance of comparing new versus established plastispheres, demonstrating a significant shift in microbial diversity and composition while maintaining a core assemblage of active *Proteobacteria* , including *Marinomonas* , *Pseudomonas* , and*Pseudoalteromonas* [9]. Additionally, this study highlighted the differential regulation of proteins involved in cellular attachment and energy metabolism during the first 2 weeks of colonisation, offering important context for understanding the evolution of biofilm dynamic. However, the molecular mechanisms driving the functioning of marine plastisphere communities in the earliest stages of development remain poorly characterised at the proteome

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level.

In the present study, we employ metaproteomics to dissect the molecular basis of plastisphere formation within the first 7 days of colonisation, with a particular focus on interspecies interactions and microbe-plastic dynamics. Our aim was to build on this previous research by providing a deeper understanding of how microorganisms interact with plastic in a marine environment and how these interactions influence community structure and function in the young plastisphere.

As addressed in our prior work [18], metaproteomics poses several challenges, which influenced the experimental design of this study. We collected samples at three (D3) and seven (D7) days post-inoculation to track the development of the nascent plastisphere [11-13]. While limiting the time available for biomass accumulation on the plastic restricts the amount of material for analysis, it is crucial for capturing the earliest stages of plastisphere formation [10]. To increase sample biomass, we enriched the plastisphere communities collected from the environment, prior to plastic inoculation. Low-density polyethylene (LDPE), one of the most widely produced plastics globally (45.7 Mt annually) [1], was selected as the test substrate to ensure relevance to real-world environmental conditions.

1. Materials and Methods
2. Recovery of plastisphere stock communities Beached plastics were collected from Oban Bay (56° 24' 50.4' ' N, 5deg 28' 19.2' ' W; Scotland) on August 6th, 2022 and washed in artificial seawater [19] to remove loosely attached organisms, prior to preservation at -20 until use. To increase plastisphere biomass, transparent plastics were placed into glass containers with 300ml artificial seawater supplemented with vitamins, trace metals, and glucose (ASW+G), and incubated with shaking (65 rpm) at 15 for three days, equalling Oban Bay's highest seawater temperature. Excess ASW+G was carefully removed, and plastics were vortexed in the remaining media and rinsed with additional ASW+G to ensure the biofilm was fully detached. The plastics were then removed and the detached plastisphere cells were pelleted by centrifugation (14, 5000 g, 7mins), resuspended in 15ml ASW+G, and preserved with 30% glycerol at -80 until further use.
3. Plastisphere growth

Five pieces of low-density polyethylene (LDPE; 2x10 cm folded in/to 8ths; ET31-FM-000101; Goodfellow, England) per replicate were added to 150ml of Bushnell Haas Broth supplemented with vitamins and trace metals (BHB+v) [20], and inoculated with 50µl of plastisphere stock community. This was then incubated (65 rpm, 15°C) in 250ml glass Erlenmeyer flasks for 3 or 7 days.

All experimental conditions were performed in quadruplicate.

[Figure 2]

Key functional categories driving early plastisphere development

The proteins expressed on D3 and D7 were classified into functional categories, with 'translation, ribosomal structure and biogenesis,' 'energy production and conversion,' and 'amino acid transport and metabolism' being the most abundant across most genera (Figure 3a). These categories were then followed by functions associated with 'cell wall/membrane/envelope biogenesis', 'cell motility', and biofilm formation.

Within 'energy production and conversion,' proteins linked to the TCA cycle were prominent and differentially expressed between time points. These include succinate dehydrogenase, expressed by *Pseudomonas* (>D3; FC 2.83), *Marinomonas* (>D7; FC 4.53), *Paracoccus* (>D7; FC 2.72),*Acinetobacter* (>D7; FC 4.27), and *Vibrio* on both days, as well as by *Rhodobacter* and *Shewanella* on D7. Additionally, citrate synthase was expressed by *Pseudomonas* (D3; D7), *Marinomonas* (D7), *Acinetobacter* (D7), and*Psychromonas* (D7), while malate dehydrogenase was expressed by*Pseudomonas* , *Marinomonas* (>D7; FC 3.6),*Paracoccus* (>D7; FC 2.92), and*Pseudoalteromonas* (>D7; FC 4.13) on both days. Isocitrate dehydrogenase was expressed by *Pseudomonas* (>D7; FC 3.01 ± 0.79), *Marinomonas* (>D7; FC 3.21 ±0.52), *Psychromonas* (>D7; FC 3.85), and*Flavobacterium* (>D7; FC 4.07) (Supplementary File S3).

Interestingly, proteins associated with 'amino acid transport and metabolism' were differentially regulated across nine genera (Figure 3a), accounting for 11.5% (± 1.5) of the total differentially regulated proteins. These include enzymes involved in glutamate cycling (e.g. glutamate methylesterase, >D7 *Marinomonas* , FC 3.03; glutamine synthetase, >D7 *Marinomonas* , FC 2.7; >D7 *Paracoccus* , FC 2.1), arginine cycling (e.g. acetylornithine aminotransferase, >D7 *Marinomonas* , FC 3.79), and the regulation and transport of branched-chain amino acids (e.g. acetohydroxy-acid isomeroreductase, >D7*Flavobacterium* , FC 4.95).

Within the categories of 'cell wall/membrane/envelope biogenesis', and 'cell motility',

several membrane proteins facilitating substrate binding and biofilm formation were expressed. Key adhesive structures include lipoprotein (expressed by *Pseudomonas* on D3 and *Marinomonas* on D7), large adhesive protein (LAD; expressed by *Pseudomonas* on D3), and outer membrane protein A (OmpA; expressed by *Thioclava* ,*Shewanella* , *Pseudomonas* , *Rheinheimera* on D7), with*Acinetobacter* showing the highest expression on D7 (FC 4.4 ± 0.14; Figure 3). Additionally, Type V secretory adhesin AidA (FC 2.92 ± 0.76) and elements of the Type II secretion system (TIISS) were expressed by *Pseudomonas* on D3 (Figure 4). Pili were also expressed by *Pseudomonas* on both D3 and D7, with pili assembly proteins PilZ (*Pseudomonas* , D3) and FimV (*Marinomonas* , D7) similarly expressed. In parallel, the curli assembly/transport component CsgG was identified in *Marinomonas* on D7 (Figure 4), indicating potential involvement in biofilm formation. (Figure 4) Additionally, flagellin, which may facilitate substrate adhesion, exhibited higher expression on D7 compared to D3. This increase was particularly noted in*Marinomonas* (FC 4.86 ± 0.79), *Pseudoalteromonas* (FC 4.5 ± 0.13), *Rheinheimera* (FC 4.02), and *Pseudomonas* (FC 2.96 ± 0.32). However, certain *Pseudomonas* species demonstrated elevated flagellin expression on D3 (FC 3.35 ± 0.31), suggesting variation in flagellar activity between strains across different time points.

Proteins associated with biofilm arrangement including components of calcium-gated (EF-hand domain-containing protein, dCache_2 domain-containing protein, *Pseudomonas*), and potassium channels (potassium uptake protein TrkA, *Marinomonas*) were expressed on D3 and D7, respectively. One instance of quorum sensing, via the expression of the autoinducer 2-binding periplasmic protein LuxP, was found on D7, expressed by an uncharacterised species of *Vibrio* . Further examples of cell-cell interactions were identified through the expression of competence protein ComEA (*Pseudomonas* , D3), involved in horizontal gene transfer (HGT), the OAA-family lectin-sugar binding domain-containing protein, (>D3 *Pseudomonas* , FC 4.72), and TIVSS protein (*Marinomonas* , D7). Proteins involved in purine nucleotide biosynthesis (e.g. bifunctional purine biosynthesis protein PurH), which are also thought to play a role in early biofilm formation, were expressed on both days (Figure 3; Supplementary file S3).

[Figure 3]

3.3 Stress-driven resilience and competition in a nascent marine plastisphere

Proteins associated with the regulation of oxidative stress, nutrient limitation, and interspecies competition were abundantly expressed, reflecting the plastisphere's resilience to environmental stress and the intense competitive dynamics within these biofilms (Figure 4). Evidence of nutrient starvation was observed through the expression of cobalamin biosynthesis protein, CobW (*Marinomonas*), and stringent starvation protein (*Marinomonas, Psychromonas*) on D7. Additionally, poly(3-hydroxyalkanoate) granule-associated proteins PhaF, Phal and poly(R)-hydroxyalkanoic acid (Pha) synthase expressed by*Pseudomonas* on D3 (Figure 4), provided evidence for starvation responses due to nutrient limitation. Moreover, evidence for alternative carbon source utilisation was observed, including three enzymes associated with the alkane degradation pathway. Including, catalase-peroxidase (Kat) expressed by *Marinomonas* (>D7; FC 2.96), *Psychromonas* (D7), and*Pseudomonas* (D3), aldehyde dehydrogenase (ALDH) expressed by*Pseudomonas* (D3; D7), *Thioclava* (D7), *Rhodobacter* (D7), and *Marinomonas* (D7), and alcohol dehydrogenase (AD) expressed by *Marinomonas* (D7; Figure 4). Protein identification using PlasticDB - a database containing proteins specifically mediating plastic biodegradation – identified the polyethylene-degrading enzyme laccase expressed by *Psychromonas* , on D7 (Figure 2; Supplementary file S2).

Proteins associated with carbon metabolism, under the functional classification 'carbohydrate transport and

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metabolism' (Figure 3), were consistently upregulated by *Pseudomonas* on D3, and by*Marinomonas* on D7. CreA family proteins and response regulatory domain-containing proteins were also expressed by *Pseudomonas* on D3. Tricarboxylic transporters were expressed by *Pseudomonas* on D3, and by *Pseudomonas, Pseudorhodobacter, Rhodobacter* and*Marinomonas* on D7 (Supplementary file S3). Interestingly, enzymes involved in aromatic hydrocarbon biodegradation were characterised, including dienelactone hydrolase, mostly expressed by *Marinomonas* on D7 (FC 2.03; Figure 3), and by *Pseudomonas* on D3. Nine other enzymes associated with this process were discovered on both days, including 3-oxoadipate-CoA transferase, 3-hydroxyacyl-CoA dehydrogenase, acetyl-CoA carboxylase, acetyl-CoA acetyltransferase, acetyl-CoA dehydrogenase, pyruvate aldolase, p-cresol methylhydroxylase (PCMH)-type protein, thiolase I, and thiopurine s-methyltransferase. Of these, acetyl-CoA acetyltransferase and acetyl-CoA dehydrogenase were most expressed on D7 (FC 4.51 and 2.48, respectively). Evidence for nitrogen cycling was found through the constitutive expression of nitrate reductase (*Pseudomonas* , D3; *Pseudomonas, Marinomonas,* D7), nitrous-oxide reductase (*Pseudomonas,* D7), and nitrogen regulatory protein PII (*Pseudomonas* , D3; *Pseudomonas, Acinetobacter* , D7) on D3 and D7. Nitrogen and urea transporters were also expressed by *Pseudomonas, Marinomonas,* and *Paracoccus* on both days.

In response to intracellular ROS, the plastisphere communities expressed a variety of stress-response proteins, including alkyl hydroperoxide reductase (Ahp), catalase (CAT), catalase peroxidase (Kat), glutathione peroxidase (Gpx), thiol peroxidase (TP), and thioredoxin peroxidase (TPx) in response to hydrogen peroxide (H_2O_2), flavohemoprotein (Fhp) to respond to nitric oxide (NO), and superoxide dismutase (SOD) to neutralise superoxide radicals. The most frequently expressed stress-response proteins were Ahp (*Pseudomonas* , D3;*Pseudomonas* , *Paracoccus* , D7; >D7*Acinetobacter* , FC 5.83; >D7 *Marinomonas* , FC 4.32), Fhp (*Pseudomonas* , D3; *Acinetobacter* , D7; >D7 *Marinomonas* , FC 3.5), Kat (*Pseudomonas,* D3; *Psychromonas* , D7; >D7 *Marinomonas* , FC 2.96), and SOD (*Paracoccus* , D3; *Acinetobacter* ,*Psychromonas* , *Rhodobacter* , D7; >D3*Pseudomonas* , FC 3.92; >D7 *Marinomonas* , FC 2.74) due to their consistent expression across multiple genera (Figure 4; Supplementary file S3). In response to metals and anions,*Marinomonas* (D7), and *Paracoccus* (D7) expressed copper resistance protein A (CopA), and toxic anion resistance protein (TelA), respectively, and *Pseudomonas* (D3) expressed tellurium resistance protein (TerD) (Figure 4). Osmotic stress proteins OsmC (*Pseudomonas, Marinomonas,* D7), and glucans biosynthesis protein C (*Pseudomonas* , D3) were also found. The downstream effects of stress were apparent in the expression of membrane repair proteins ATP-dependent zinc metalloprotease (FtsH), α-2 macroglobulin (α2m), and phage shock protein (Psp). Additionally, transcriptional response proteins such as HU, RecA, RdgC, cold shock-domain (CSD) proteins, and DNA repair proteins such as GrpE, Lon protease, and methionine sulfoxide reductase were also adundant. Further stress mitigation was supported by thioredoxin/thioredoxin reductase systems, along with a suite of chaperones and chaperonins (e.g., ClpA, ClpB, DnaJ, DnaK, GroEL, GroES, HtpG, SurA), and the redox enzyme glucose-6-phosphate dehydrogenase (Figure 4). Among these, the cold shock-domain protein was the most abundantly expressed protein, found in the proteomes of*Pseudomonas* (>D3 FC 3.8), *Marinomonas* (D7),*Acinetobacter* (D7), *Paracoccus* (D7), and *Shewanella* (D7). Further protective mechanisms were also identified, such as dipicolinate synthase expression by *Pseudomonas* on D3 and capsular biosynthesis protein on D7. Interestingly, proteins linked to reactive oxygen species (ROS) generation, including sarcosine oxidase (*Pseudomonas* , D3) and Na(+)-translocating NADH-quinone reductase (*Marinomonas* ; > D7 FC 7.01), were also expressed.

Competition for resources was evidenced by the expression of proteins associated with competitive stress. This included the type VI secretion system (TVISS) proteins, such as TssM, contractile sheath large subunit, and secretion proteins Evp, IcmF, and VasK, expressed by*Pseudomonas* (D3; FC 1.74 ± 0.12), *Pseudoalteromonas* (D7; FC 3.54), and Acinetobacter (D7) (Figure 4; Supplementary file S3). Competitive advantage was also indicated by the expression of hemolysin-related proteins and the actin cross-linking toxin VgrG2 by*Pseudomonas* on D3. In potential response to antibiotic production, resistance mechanisms were expressed, such as polymyxin resistance protein ArnA and OprM by *Pseudomonas* on D3, and β-lactamase by *Pseudomonas* and colicin-I receptor proteins by*Shewanella* on D7.

Interestingly, the potential pathogens *Pseudomonas syringae* and *Pseudomonas aeruginosa* , expressed two

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TVISS proteins on D3, while *P. syringae* also expressed tol-pal system proteins and a FeADH domain-containing protein. These results indicate a complex competition network within the biofilm, where both resource limitation and interspecies antagonism shape the microbial community structure and resilience.

[Figure 4]

Discussion

In this study, we analysed 2,948 non-redundant proteins from the LDPE plastisphere, providing near-complete proteomes for the two dominant genera *Pseudomonas* and *Marinomonas* within the first days of colonisation. This extensive dataset offered valuable insights into the metabolic activity of these key taxa, as well as the broader functioning of the young plastisphere community. In particular, we revealed the intense competition between taxa within the rapidly developing community and identified the molecular mechanisms underpinning plastisphere resilience to nutrient limitation and oxidative stress. Our results also revealed molecular interactions between the early plastic colonisers and the plastic itself, with important implications for the modulation of biofilm formation and the exploration of plastic biodegradation. These results provide new insights into the dynamics of the nascent plastisphere and their biotechnological potential.

Dominance of key taxa in the young marine plastisphere

The activity of the young marine plastisphere was dominated by heterotrophic bacteria, particularly Gammaproteobacteria, consistent with our previous studies examining plastisphere formation and function [6, 9, 16], and with studies investigating the taxonomic diversity of the plastisphere [7, 28, 29]. Herein and in our previous work, plastisphere samples were collected from the Scottish coast, and biogeographic location appears to play a significant role in shaping plastisphere communities, as noted in several studies including our own [6, 9, 16]. Community development in the present study mirrored previous observations demonstrating that plastisphere diversity increases over time [10-13]. Indeed, we observed a plastisphere community whose activity was dominated by *Pseudomonas* after three days of colonisation, shifting to an active community comprised of Bacteroidetes after seven days. *Pseudomonas* , the pioneering genus in this study and one of two high-quality MAGs recovered from the marine plastisphere, is known for its metabolic versatility and biotechnological potential. Its species span biogeochemical cyclers, symbionts, decomposers, denitrifying bacteria, and pathogens [30]. This genus is particularly known for its biofilm-forming capabilities, facilitated by the secretion of specialised membrane proteins and secretion systems which likely facilitate interactions with plastic (Section 4.3) [31]. We have previously observed *Pseudomonas* within the marine plastisphere, but at $< 25\%$ of the active community [9, 16], indicating that the functional role of *Pseudomonas* is most significant within the initial stages of plastisphere development. As such, the expressed proteome of this organism provides important insights into microbe-plastic interactions at the time of most significance, where colonisers specifically attracted to the plastic surface may utilise it as a substrate [32]. Nevertheless, the most abundant and active genera found on D7, *Marinomonas* , was the second high-quality MAG recovered and is also a marine-adapted genus typically associated with pollutant bioremediation [33, 34]. Its enrichment on the LDPE substrate suggests that beached plastic provided an ideal niche for colonisation, but likely as a secondary coloniser. Consistent with this, in our previous work, *Marinomonas* was also the dominant and active taxa following 1-2 weeks LDPE colonisation, but it was less important within the mature marine plastisphere. Additionally, further bioremediative genera such as *Thioclava* [28] were active on D7 (relative abundance 1.5%), alongside the presence of potential pathogens *P. aeruginosa,* and *P. syringae* which represented 4.6% and 17.5% of all annotated species, equalling 285 non-redundant proteins [35, 36]. Indeed, *P. syringae* was the most annotated species in the D7 plastisphere, indicating a concerning enrichment of potential pathogens within the portion of the plastisphere (19%) that could be annotated to this level. **The observed** diversity within this early-stage plastisphere highlights the biofilm as a dynamic environment, initially dominated by one or two taxa, likely shaped by significant competitive interactions.

Biofilm formation

Biofilm formation is a key survival strategy for microbial proliferation in challenging, nutrient-poor marine

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environments [37, 38]. Several proteins involved in surface adhesion were found to be repetitively identified across different taxa known for their propensity to form biofilms. In this way, biofilm formation might have been promoted in *Pseudomonas* , *Marinomonas* , *Acinetobacter* , and *Vibrio* through the expression of secretion systems I, II, and IV, as well as flagellin, adhesin, curli, Omps, lipoprotein, and LAD [39-41]. These surface adhesion mechanisms were predominantly expressed by *Pseudomonas* , potentially aiding their dominance and rapid colonisation of plastic at D3. The succession of *Marinomonas* after the initial colonisers further supports their biofilm-forming ability, which is well-documented in marine environments [34]. The detection of quorum sensing molecules expressed by *Vibrio* on D7 could indicate that this genus was involved in processes potentially related to surface adhesion, such as the regulation of membrane sorting proteins, which are often associated with coordinated biofilm development [42].

Biofilm formation may have also been supported by the upregulation of purine biosynthesis, the TCA cycle, arginine, and glutamate cycling proteins, which are crucial for energy and resource allocation within the biofilm community [37, 38, 43]. Moreover, our previous findings highlighted the critical role of glutamine in biofilm proliferation [16]. Its regulation was found to be closely linked with ammonium availability and oxidative stress within low-nutrient environments, with *Marinomonas* showing distinct glutamine metabolism within the plastisphere [9]. This suggested a differential nutrient strategy between biofilm-associated and planktonic communities, further supporting the notion of a resilient and metabolically adaptive plastisphere biofilm [16]. The observed increase in microbial diversity over time implies that the resources produced by pioneering species (e.g., carbon, amino acids) may have facilitated the growth of subsequent colonisers, creating a more complex and resilient biofilm [15, 44].

Importantly, plastisphere biofilms offer distinct advantages to microorganisms in these environments. For instance, the expression of TIVSS and a competence protein suggests the potential for HGT within the plastisphere [39], allowing microbes to share adaptive traits. Moreover, the expression of calcium-gated channels (e.g., EF-hand and dCache_2 domain-containing proteins) and potassium uptake proteins (e.g., TrkA) points to a potential cooperative exchange of nutrients between neighbouring cells, further enhancing the biofilm's capacity to thrive under nutrient-limited conditions [45]. Moreover, nitrogen sufficiency is suggested by the expression of nitrogen cycling proteins like nitrate reductase, nitrogen regulatory protein PII, and glutamine. Carbohydrates and amino acids were metabolised and transported within the community, indicating possible resource sharing through symbiosis or cross-feeding, contributing to the overall resilience and stability of the biofilm [15, 44].

However, it is important to note that biofilm communities are not only cooperative but also competitive, as microorganisms compete for dominance and resources within these complex ecosystems.

Resilience and competition

Proteins associated with the regulation of metabolic stressors were among the most abundant expressed non-housekeeping proteins found in our datasets. Enzymes responsible for mitigating oxidative stress were frequently expressed by multiple genera (e.g., *Pseudomonas* ,*Marinomonas* , *Acinetobacter* , *Paracoccus* ,*Psychromonas* , and *Rhodobacter*), with nearly the entire process of reactive ROS suppression (antioxidant production - DNA regulation - DNA repair) being characterised [46]. Oxidative stress in multi-species biofilms may be triggered by spatial constraints, forced oxygen gradients, competition, nutrient limitation, and the accumulation of abiotic stressors such as metals (e.g., CopA, TelA, TerD) [6, 47]. The plastic itself could also contribute to oxidative stress through the accumulation of ROS under UV-light exposure [48, 49]. The multifunctionality of proteins identified here, such as the frequently expressed CSD protein (*Pseudomonas* ,*Marinomonas* , *Acinetobacter* , *Paracoccus* ,*Shewanella*), suggests that they may have been induced by a combination of these stressors. Indeed, in addition to their role in cold adaptation, cold shock proteins are crucial for maintaining protein stability and proper folding under stress conditions, extending their protective function against oxidative and metabolic stress [50].

Granule storage, competition, and the expression of nutrient-limitation-related proteins highlight nutrient stress during both sampling days. Interestingly, granule formation is often triggered by the scarcity of other

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essential nutrients outside carbon sources [51]. Additionally, we found evidence for the utilisation of diverse potential carbon sources through the expression of enzymes associated with alkene and consequentially polyethylene degradation (AD, ALDH, Kat, laccase), also as noted by Delacuvellerie et al. (2022).*Pseudomonas* enriched at D3 are also known to be associated with the biodegradation of polyethylene [52-54]. Enzymes related to aromatic hydrocarbon metabolism, such as dienelactone hydrolase [55, 56], were also detected, though no definitive evidence of plastic or aromatic hydrocarbon biodegradation was found. The synthesis of granules and the presence of carbohydrate metabolism proteins suggest an available carbon source, though potentially through cross-feeding.

Nutrient limitation is known to cause dysregulation of core metabolic processes, leading to the accumulation of ROS, as well as an increased competition for resources [57, 58]. In support of this, the expression of hemolysin and VgrG2 toxins by *Pseudomonas* may suggest competitive interactions within the plastisphere biofilm. The production of these toxins, alongside other virulence factors, could potentially induce membrane damage in neighbouring microorganisms through the action of the TVISS [59, 60]. Interestingly, we identified a near-complete annotation of the TVISS machinery (i.e. TVISS proteins IcmF, VasK, VipB, membrane subunit TssM, contractile sheath small subunit, and large subunit, Hcp1 family TVISS effector, EvpB family TVISS protein) expressed by active members of the marine plastisphere [8, 16, 17]. These findings suggest that interspecies competition within the biofilm could be a significant factor in shaping plastisphere community structure and dynamics.

Using the TVISS as an indication of virulence [61],*Pseudomonas* were most competitive on D3 within a predominantly single genera biofilm. Previously, *Pseudomonas* , and*Pseudoalteromonas* have also been found to outcompete other microbial species through the secretion of toxins [62-64], perhaps actively antagonising other members of the biofilm. The expression of proteins used to convey antibiotic resistance (i.e. b-lactamase, efflux RND transporters, capsule protein) [39, 64, 65] by*Pseudomonas* and *Marinomonas* on D7, highlight the intense competition within the diversified plastisphere community. These mechanisms are important for maintaining cell integrity in a stressful environment, but their expression puts great demand on the cell's intracellular resources [39, 57, 61, 62], including those which may be used in key metabolic processes. The deregulation of these processes can further precipitate oxidative stress – similar to nutrient deprivation [57, 58] – and limit the growth of impacted bacteria [46]. Interspecies competition may have therefore contributed to this plastisphere's function and composition, confirming the observations made by Herschend *et al.* (2017) and Guillonneau*et al.* (2018) in model biofilm communities [14, 15].

Metabolic versatility for plastic degradation

The proteins expressed here which relate to the utilisation of alternative carbon sources are of interest within the context of plastic biodegradation. As stated in *Section 4.3* , we do not find this to be conclusive evidence of pollutant biodegradation within these plastispheres. However, it is interesting to note that the two enzymes expressed here which align with Delacuvellerie's (2022) depiction of the alkene degradation pathway (AD, ALDH) are expected to be involved in the initial depolymerisation process of polyethylene degradation by Tao (2023) [8, 66]. Catalase peroxidase, also found here, but not in the alkane degradation pathway, is expected to play a role in the first steps of polyethylene biodegradation. Our recent paper on the late-stage plastisphere also revealed the expression of acyl-CoA dehydrogenase [16], another element of the alkene degradation pathway, which was also expressed in Delacuvellerie's (2022) study alongside ferredoxin and ferredoxin reductase [8]. In combination, five enzymes associated with the alkene degradation pathway have now been annotated from within the marine plastisphere, plus an additional enzyme which may directly depolymerise polyethylene.

In alignment with our previous investigation, 10 proteins involved in fatty acid beta-oxidation (i.e. acetyl-CoA carboxylase, acetyltransferase, and dehydrogenase) were found in these plastispheres [16]. This process, like the alkene degradation pathway, has been linked to both plastic biodegradation and the biodegradation of other xenobiotic hydrocarbons due to the need to dismantle their similarly structured hydrocarbon base [67]. Metabolites from xenobiotic degradation are also likely funnelled directly into the fatty acid beta-oxidation pathway once initially depolymerised [66]. Dienelactone hydrolase is not linked to this pathway

but is associated with the final stages of aromatic hydrocarbon (chlorophenol) degradation [55, 56]. The discovery of such enzymes is interesting because no such xenobiotics were present in these samples, and the plastics used were purportedly free of additives. Another trend of note is the upregulation of these proteins by *Marinomonas* . Even if these organisms did not actively biodegrade aromatic hydrocarbons in these samples, this may reveal a little of their adaptability to other xenobiotics such as crude oil in the marine environment [33].

The combined expression of enzymes which may be used by bacteria to biodegrade plastics, and other noted xenobiotics here may not indicate that plastic biodegradation is occurring in this scenario. However, it may suggest that organisms found here and in other marine plastispheres have the capacity to perform this biodegradation. Interestingly, the incomplete annotation of these processes can rarely be attributed to a singular genus. Instead, several genera express these enzymes in tandem. This may have significant implications for the field of plastic bioremediation, encouraging the exploration of complex microbial communities rather than relying on single species for more effective plastic degradation.

Conclusion

To date, the molecular mechanisms underpinning the initial days of microbial colonisation of marine plastic debris have remained uncharacterised. To resolve the interactions between the earliest colonisers and plastic surfaces, we utilised multi-omics to investigate the activity of the marine plastisphere community grown on LDPE for 3 and 7 days. Our findings reveal a complex relationship between pioneering and secondary colonisers, shaped by stressors such as reactive oxygen species (ROS) and nutrient limitation, while also highlighting the interplay of cooperation and competition among microorganisms. Consistent with previous research, the majority of these nascent plastisphere microorganisms were primarily composed of heterotrophic bacteria, indicative of marine plastisphere communities found in colder environments. The near-complete assembly of metagenome-assembled genomes (MAGs) for *Pseudomonas* and*Marinomonas* significantly enhanced our ability to characterise the associated proteomes and elucidate their precise functions within the dynamic young plastisphere. In this study, *Pseudomonas,* the pioneering genera, was likely aided through its known ability to form biofilms, while the genera detected in the later plastisphere diversified over time, potentially fuelled by metabolites produced within these biofilms. Proteins related to the biodegradation of plastic and other xenobiotics were expressed by *Pseudomonas* ,*Marinomonas* , and three additional genera, highlighting the potential for these microorganisms to contribute to plastic biodegradation processes. Further studies exploring the functioning of the nascent plastisphere are required, to determine their role in those processes. Overall, this study significantly enhances our understanding of the early formation of the plastisphere and its interactions with plastic, providing valuable insights essential for addressing the growing environmental challenge of plastic pollution.

Associated data

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org/) via the MassIVE partner repository [68, 69] with the dataset identifier PXD056358 (for reviewers only: ftp://MSV000095986@massive.ucsd.edu).

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Figure legends

Figure 1 . Three- and seven-days growth of a marine microbial community on LDPE, and analysis of biofilm structure and functioning. ASW+G, artificial seawater supplemented with vitamins, trace metals, and glucose; BHB+v, Bushnell Haas Broth supplemented with vitamins and trace metals.

Figure 2. Relative abundance of microbial species found within the metaproteome at each time point. Calculated using the number of proteins associated with each genus relative to all the proteins found in that condition. Values used for doughnut plot shown in table below, as well as corresponding metagenomic data.

Figure 3. Total differentially regulated proteins (FC>1.5, P-adj. <0.05) according to function and taxonomic classification. Positive fold change (purple) in a indicates greater abundance of proteins on D7 (purple), and negative fold change (orange) indicates greater expression on D3.

Figure 4. Graphic representation of biologically relevant processes found within this study's most abundant bacterial genera from relative quantification and qualitative analysis of the metaproteomic dataset. Purple = more expressed on day 7, orange = more expressed on day 3, black = qualitative results, * = qualitative results with 1 entry, grey = not found, but can be assumed. Colour code indicates all basal processes active within the genus at that time (>1 protein each). All basal processes active in the largest, annotated microorganisms. Acronyms: **Figure elements** : H2O2, hydrogen peroxide; HGT, horizontal gene transfer; NO, nitric oxide; O2, superoxide; PE, polyethylene; PHA, polyhydroxyalkanoate granule; Xn, xenobiotic. **Stress** (osmotic, oxidative, heat/cold, interspecies interaction, xenobiotic): Ahp, alkyl hydroperoxide reductase; ArnA, polymyxin resistance protein; α2M, alpha-2-macroglobulin; β-L, β-lacatamse; Cap, capsular

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biosynthesis protein; CAT, catalase; CopA, copper resistance protein A; CSD, cold shock domain containing protein; Fhp, flavohemoprotein; FtsH, ATP-dependent zinc metalloprotease; G6PD, glucose-6-phosphate dehydrogenase; Gpx, glutathione peroxidase; GrpE, DnaK nucleotide exchange factor; GSH, glutathione; HU, transcriptional regulator HU; Kat, catalase peroxidase (also PE degradation) ; LonP, lon protease; MsrP, methionine sulfoxide reductase; OP, 5-oxoprolinase; OprM, outer membrane efflux system protein; Osm, osmotically inducible protein; Psp, phage shock protein; RdgC, recombination-associated protein; RecA, SOS response protein ; SOD, superoxide dismutase; OpuAC, substrate binding domain of ABC-type glycine betaine transport system; TerD, tellurium resistance protein; TP, thiol peroxidase; TPx, thioredoxin peroxidase; Trx, thioredoxin; TrxR, thioredoxin reductase; Chaperones- ClpA, ClpB, DnaJ, DnaK, HtpG, SurA; Chaperonins- GroEL, GroES. **Virulence and biofilm formation** : 1B, hemolysin co-regulated protein; BepC, outer membrane efflux protein; Ccda, control of cell death protein; ComEA, competence protein; EL-Tu, elongation factor Tu; hcp, hemolysin-coregulated protein; Hly, hemolysin activation/ secretion protein; Omp, outer membrane protein; RND, efflux resistance-nodulation-division (RND) transporter; SecYEG, translocon; TISS, type-I secretion system; TIISS, type-II secretion system; TIVSS, type-IV secretion system; TVISS, type-VI secretion system; VgrG2b, actin cross-linking spike protein; Translocons- IcmF, Tld/PmbA, Vask, VipB.**Starvation** : CobW, cobalamin biosynthesis protein; PhaF, polyhydroxyalkanoate (Pha) granule-associated protein F; Phal, Pha granule-associated protein L; PHAs, PHA synthase; SSP, stringent starvation protein. **Adhesion** : AidA, adhesin; CGC, calcium-gated channel; CsgG, curli assembly protein G; LAD, large adhesive protein; LP, lipoprotein; OAA, OAA-family lectin sugar binding domain containing protein; TrkA, Potassium uptake protein. **Aromatic hydrocarbon degradation** : 3-OCoAT, 3-oxoadipate-CoA transferase; 3-HA-CoAd, 3-hydroxyacyl-CoA dehydrogenase; ACC, acetyl-CoA carboxylase; A-CoAAT, acetyl-CoA acetyltransferase; A-CoAD, acetyl-CoA dehydrogenase; CMBL, dienelactone hydrolase; PA, pyruvate adolase; PCMH-p, p-crestol methylhydroxylase (PCMH) type protein; TI, thiolase I; TMPT, thiopurine s-methyltransferase. **PE degradation** : AD, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

Supplementary files

Supplementary File_S1 Overview of the total metaproteome identified at D3 and D7 showing the taxonomic annotation (LCA score > 80%) obtained from mPies using our Combined-Metagenome Database.

Supplementary File_S2 Protein identification obtained from the publicly available databases for antimicrobial resistance (CARD), plastic metabolism (PlasticsDB), and virulence factor (VFDBs).

Supplementary File_S3 Fold-change analysis summary using Skyline when comparing protein abundance ratio at D7 and D3.

Supplementary File_S4 Peptide Summary Exports generated by Protein Pilot, including peptide sequences, and associated metadata for each condition and replicate.

Supplementary File_S5 Protein Summary Exports generated by Protein Pilot, and associated metadata for each condition and replicate.

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Figure.4.pptx available at https://authorea.com/users/840919/articles/1231089-decoding-microbial-plastic-colonisation-multi-omic-insights-into-the-fast-evolving-dynamics-of-early-stage-biofilms

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