



A Predictive Functional Metagenomic Re-analysis of the Marine Plastisphere

Hudzaifah An-naufal Fazasmara

Study Program of Biology, Faculty of Military Mathematics and Natural Sciences, The Republic of Indonesia Defense University. Indonesia
Peace and Security Center (IPSC) Sentul, Bogor 116810, Indonesia.
Correspondence: hudzaifahannaufal@gmail.com

Abstract

The accumulation of plastic debris in the ocean has created a novel ecological niche known as the "plastisphere." While the taxonomic composition of plastic-associated biofilms has been extensively documented, the functional and metabolic capabilities of these communities remain poorly understood. To address this, our study presents a predictive functional metagenomic re-analysis of mature (week-four) plastisphere communities. We leveraged publicly available 16S rRNA amplicon sequencing data from an *in situ* colonization experiment originally conducted in the coastal waters of South Australia (Silva et al., 2023). This dataset encompassed six commercial plastic polymers, alongside a wood control and ambient seawater. Utilizing the PICRUST2 predictive pipeline and ALDEx2 for rigorous compositional data analysis, we evaluated the overarching metabolic structure and identified differentially abundant pathways. Our results revealed a profound global functional redundancy across all substrates (PERMANOVA, $p = 0.703$), indicating that the core metabolic machinery of the marine microbiome remains fundamentally stable regardless of the attachment surface. Despite this macroscopic redundancy, micro-scale metabolic specializations were evident, with 29 specific pathways exhibiting significant differential abundance (FDR-adjusted $p = 0.047$). The plastisphere exhibited a massive enrichment in anaerobic respiration (denitrification) and alternative carbon fixation (Wood-Ljungdahl pathway), suggesting the development of hypoxic micro-niches within the mature biofilm matrix. Conversely, a significant depletion in the ppGpp alarmone metabolism pathway implied alleviated nutritional stress on plastic surfaces, corroborating the plastisphere contribution in trapping and concentrating organic nutrients. These findings underscore that while the plastisphere shares a broad functional framework with the surrounding ocean, plastic surfaces actively trigger specific, highly localized metabolic adaptations critical for biofilm survival.

Keywords: 16S rRNA amplicon; Denitrification; Functional redundancy; Marine biofilms; MetaCyc; Plastisphere; Predictive metagenomics

1. Introduction

The proliferation of plastic debris in marine environments has introduced a vast array of artificial, hydrophobic substrates into the ocean, fundamentally altering microbial ecology. These anthropogenic surfaces are rapidly colonized by diverse microbial communities, forming a distinct ecological niche commonly referred to as the "plastisphere" (Zettler et al., 2013). While naturally occurring organic substrates like wood or macroalgae are transient and biodegradable, the recalcitrant nature of commercial plastic polymers allows for the prolonged persistence and long-distance transport of these biofilm-associated communities. Consequently, understanding the ecological dynamics of the plastisphere is critical, as it has the potential to significantly impact global biogeochemical cycles.

Over the past decade, extensive research has characterized the taxonomic composition of the plastisphere. For instance, recent studies, such as the *in situ* colonization experiment by Silva et al. (2023), have meticulously detailed the successional dynamics and taxonomic differentiation of microbial communities across various plastic types. However, a significant knowledge gap remains regarding the

functional potential of these communities. Taxonomic profiling via 16S rRNA gene sequencing alone often fails to comprehensively capture the actual metabolic machinery operating within the biofilm, as phylogenetic relatedness does not always directly correlate with functional capabilities (Douglas et al., 2020). Because marine ecosystems frequently exhibit high functional redundancy where diverse, unrelated taxa can perform the same biogeochemical processes relying solely on taxonomic data severely limits our understanding of how plastic surfaces actively reshape microbial metabolism (Louca et al., 2018).

Understanding the functional repertoire of the plastisphere is essential to determine whether plastics merely act as passive attachment sites for opportunistic marine bacteria or if they actively select for specific, survival-driven metabolic pathways (Oberbeckmann & Labrenz, 2020). Mature biofilms on impermeable plastic surfaces can create unique physical microenvironments, such as localized nutrient traps or sharply stratified oxygen-depleted zones (Stewart, 2003; Wright et al., 2020). These micro-niches may necessitate specialized biochemical adaptations such as anaerobic respiration that are otherwise completely unnecessary or rare for free-living planktonic communities in the well-aerated surrounding seawater.

To bridge this gap, this study aimed to investigate the predictive functional metagenomic profiles of late-stage (mature) plastisphere communities. By re-analyzing the 16S rRNA amplicon sequencing data from Silva et al. (2023) using the PICRUST2 predictive pipeline, we compared the metabolic pathways enriched on six commercial plastic polymers against an organic control (wood) and the ambient seawater. Through rigorous differential abundance testing, this research sought to determine the extent of functional redundancy within the plastisphere and to identify the specific micro-scale metabolic specializations such as alternative carbon fixation and anaerobic respiration that drive biofilm survival on marine plastic debris.

2. Materials and Methods

2.1. Data Acquisition and Sample Selection

To evaluate the functional potential of late-stage plastisphere communities, raw 16S rRNA amplicon sequencing data were retrieved from the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA893373 (Silva et al., 2023). The original in situ colonization experiment was conducted in the coastal marine environment of South Australia. To capture the metabolic profiles of mature biofilms rather than early colonizers, data selection was strictly limited to the week four sampling point. A total of 24 independent Sequence Read Archive (SRR) runs were utilized. This dataset encompassed three biological replicates for each of the eight distinct substrates: six commercial plastic polymers (high-density polyethylene [HDPE], low-density polyethylene [LDPE], polyester, polypropylene [PP], polystyrene [PS], and polyvinyl chloride [PVC]), alongside balsa wood serving as an organic control, and ambient seawater representing the surrounding environmental baseline.

2.2. Amplicon Data Pre-processing via UseGalaxy

All initial bioinformatic processing of the raw sequencing reads was executed utilizing the web-based European Galaxy Server (UseGalaxy.eu) computational platform (<https://usegalaxy.eu>) (The Galaxy Community, 2024). Paired-end read data for the 24 SRR accessions were retrieved and extracted utilizing the *Faster Download and Extract Reads in FASTQ format from NCBI SRA (Galaxy Version 3.1.1+galaxy1)* module (Leinonen et al., 2010). To streamline downstream batch processing, the extracted raw sequences were subsequently consolidated into a single dataset using the *Merge collections (Galaxy Version 1.0.0)* tool. The merged raw data were then imported into a format compatible with the QIIME 2 ecosystem (Bolyen et al., 2019) utilizing the *qiime2 tools import-fastq (Galaxy Version 2025.10.0+dist.hbfe50fe0)* function. Following standard quality filtering and taxonomic resolution to generate Amplicon Sequence Variants (ASVs) within the QIIME 2 framework with *qiime2 dada2 denoise-paired* tools (Bolyen et al., 2019; Callahan et al., 2016). The resulting feature abundance matrices were extracted using the *qiime2 tools export (Galaxy Version 2025.10.0+dist.hbfe50fe0)* utility (Bolyen et al., 2019). This step generated a BIOM2 formatted file, which was subsequently converted into a tabular format (.tsv) using *Convert between BIOM table formats (Galaxy Version 2.1.15+galaxy1)* tools to serve as the direct input for downstream functional prediction (McDonald et al., 2012).

2.3. Predictive Functional Metagenomic Profiling

The tabular ASV abundance matrix was subjected to functional prediction utilizing the PICRUST2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software pipeline (Douglas et al., 2020). By aligning the observed taxonomic profiles with established reference genomes, the genetic and functional capacities of the microbial communities were strictly inferred against the MetaCyc metabolic pathway database (Caspi et al., 2018). The primary output of this stage was an unstratified functional pathway abundance matrix, detailing the specific repertoire of MetaCyc biochemical functions present across the varying substrates.

2.4. Statistical Analysis and Differential Abundance

All subsequent statistical evaluations and data visualizations were performed within the R programming environment (R Core Team, 2021). To address the inherent compositional bias and skewness typical of microbiome sequencing datasets, differential abundance testing was conducted using the ALDEx2 (ANOVA-Like Differential Expression) package (Fernandes et al., 2013; Gloor et al., 2016). The raw functional pathway counts were first transformed via the centered log-ratio (CLR) method utilizing the `aldex.clr` function (configured with 128 Monte Carlo Dirichlet instances) to eliminate sequence-depth discrepancies among samples.

To visualize the global functional community structure, a Principal Coordinates Analysis (PCoA) was plotted based on the Euclidean distances of the CLR-transformed data. The overall variance distinguishing the three primary sample categories (plastic, wood, and seawater) was statistically evaluated through a Permutational Multivariate Analysis of Variance (PERMANOVA) running 999 permutations.

For the identification of specific metabolic pathways exhibiting significant differential abundance, a Kruskal-Wallis test (`aldex.kw`) was applied to the entire dataset. Resulting *p*-values were adjusted for the false discovery rate using the Benjamini-Hochberg procedure (`kw.eBH`), establishing an adjusted $p < 0.05$ threshold for statistical significance. To determine the biological magnitude of these differences independent of intra-group variance, standardized effect sizes (`aldex.effect`) were calculated pairwise, specifically contrasting the plastic samples against the seawater controls. Pathways demonstrating substantial median differences (`diff.btw`) and robust effect sizes were flagged as significantly enriched or depleted in the plastisphere.

2.5. Data Visualization

A total of 29 functional pathways demonstrating the most statistically robust differential abundance were isolated for targeted visualization. Raw count values for these specific pathways underwent a $\log_{10}(x+1)$ transformation to minimize the influence of extreme outliers, followed by row-wise standardization (Z-score scaling) to clearly delineate relative enrichment or depletion patterns across the samples. A clustered heatmap was constructed utilizing the *pheatmap* R package (Kolde, 2025), applying hierarchical clustering to group both the sample replicates and the functional pathways. The final graphical output was rendered and exported at a resolution of 900 DPI to meet publication-grade standards.

3. Results

3.1. Global Functional Redundancy within Biofilm Communities

To evaluate the overall functional community structure across the different substrates, the functional pathway abundance matrix was transformed using the centered log-ratio (CLR) method and visualized via Principal Coordinates Analysis (PCoA) (Figure 1). The PCoA projection revealed that the first principal coordinate (PCoA 1) accounted for a highly dominant 87% of the total variance, while the second coordinate (PCoA 2) explained an additional 9.5%. Although visual inspection suggested a potential clustering trend along the primary axis, global inferential statistics did not support a significant structural separation.

PERMANOVA confirmed the absence of statistically significant differences in the global functional profiles among the microbiomes colonizing plastic, wood, and the ambient seawater control ($R^2 = 0.040$, F

= 0.438, $p = 0.703$). The exceptionally low R^2 value, explaining merely ~4% of the total variance, strongly indicates a high degree of functional redundancy. This suggests that, at a macroscopic community level, the core metabolic machinery of these marine microbiomes remains remarkably stable and ubiquitous, regardless of the physical substrate they colonize.

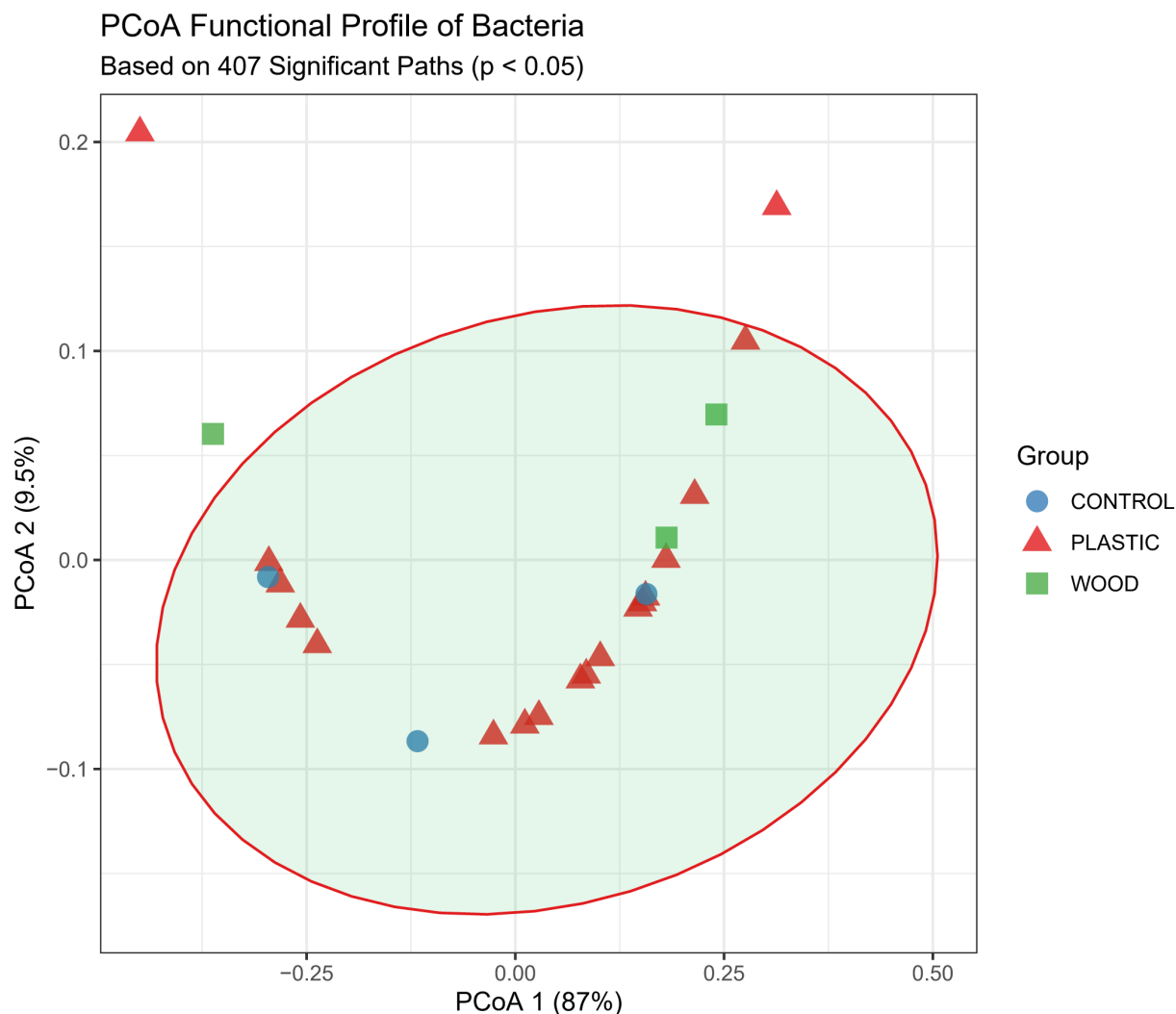


Figure 1. Principal Coordinates Analysis (PCoA) based on the Euclidean distance of centered log-ratio (CLR) transformed functional pathway abundances. The ordination illustrates the global functional community structure across the plastisphere (commercial plastic polymers), organic control (wood), and ambient seawater after a four-week colonization period. Percentages on the axes indicate the proportion of total variance explained by the first two principal coordinates. Global structural differences were not statistically significant (PERMANOVA, $p = 0.703$).

3.2. Pathway-Specific Functional Differentiation in the Plastisphere

Despite the overarching global functional redundancy, differential abundance testing using ALDEx2 (Kruskal-Wallis) successfully identified 29 specific metabolic pathways exhibiting statistically significant variations (FDR-adjusted $p = 0.047$) across the three experimental groups. Visualization via a clustered heatmap (Figure 2) demonstrated that these 29 functional pathways possessed highly distinct abundance patterns, with the plastic samples forming a separate expression cluster diverging from both wood and seawater.

To quantify the biological magnitude of these divergences and account for high intra-group dispersion, standardized effect sizes were calculated comparing the plastic substrates against the seawater control (Table 1). The majority of these 29 pathways were not merely statistically significant but also demonstrated robust biological effects (Effect Size > 11.51).

Specifically, the plastisphere exhibited a consistent enrichment in pathways associated with alternative energy metabolism, carbon fixation, and cellular stress responses. The biochemical denitrification pathway (DENITRIFICATION-PWY) emerged as one of the most profoundly impacted functions, displaying a massive effect size of -4.41, indicating a sharp enrichment within the plastic cohort. Additionally, the reductive acetyl-CoA carbon fixation pathway, also known as the Wood-Ljungdahl pathway (CODH-PWY, Effect Size = -2.11), and the isoprene biosynthesis pathway (PWY-6876, Effect Size = -4.56) were detected at substantially higher abundances on the plastic surfaces relative to the surrounding natural environment.

Conversely, several basal functions were observed to be depleted in the plastisphere yet highly abundant in the controls. Notable examples include the ppGpp alarmone metabolism (PPGPPMET-PWY, Effect Size = 3.17) and photorespiration (PWY-181, Effect Size = 4.97). The dynamic shift of these 29 functional pathways underscores a critical ecological narrative that is while the plastisphere community shares a broad, common framework of marine metabolism, exposure to plastic polymers triggers micro-scale functional specializations essential for biofilm survival on these artificial substrates.

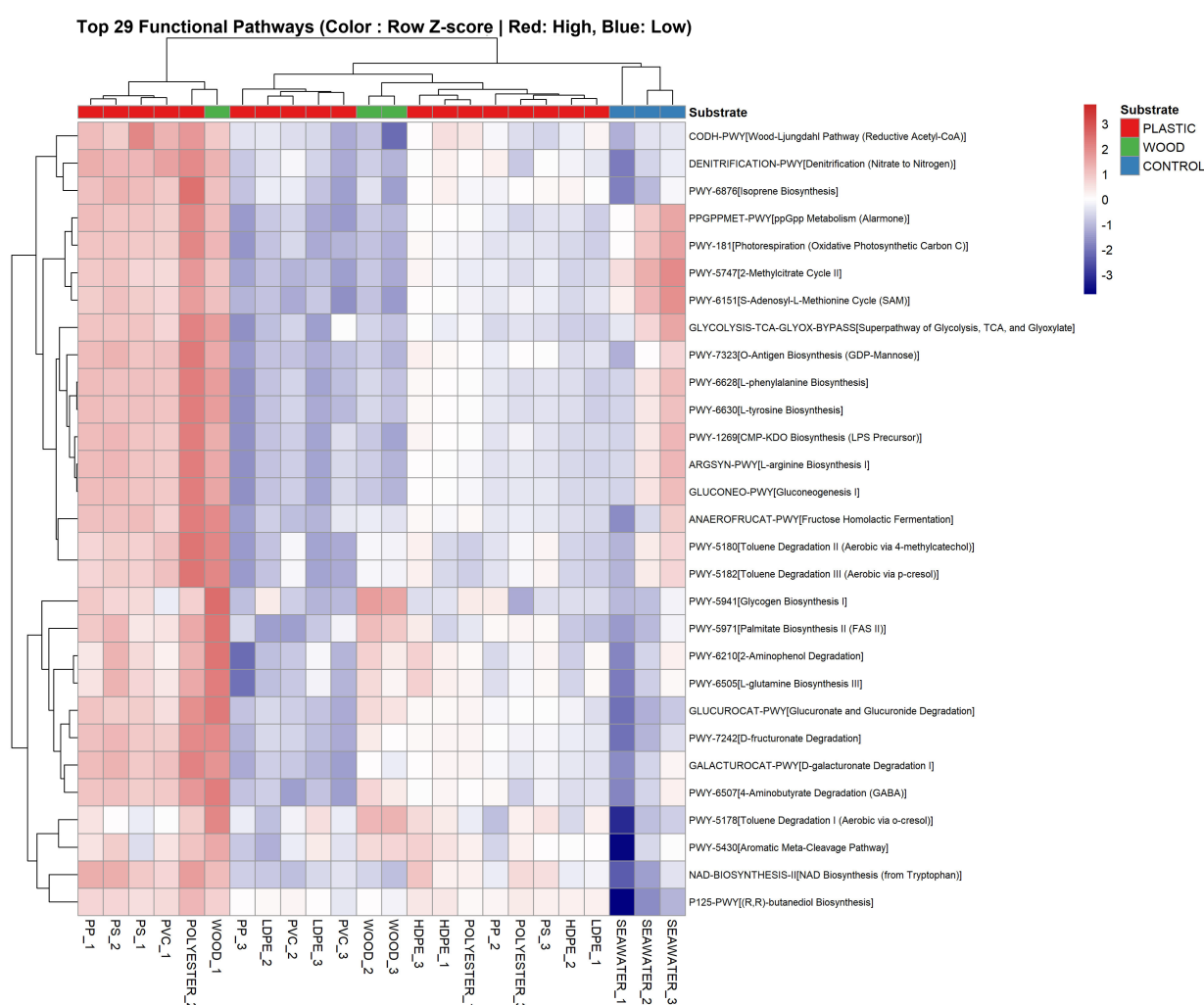


Figure 2. Hierarchically clustered heatmap displaying the relative abundance of the 29 significantly differential functional pathways across plastic, wood, and seawater samples. Raw abundance counts were $\log_{10}(x+1)$ transformed and standardized via row-wise Z-score scaling to highlight enrichment (red) and depletion (blue) patterns. Statistical significance was determined using a Kruskal-Wallis test adjusted for false discovery rate (FDR-adjusted $p = 0.047$). Both the samples (columns) and the functional pathways (rows) are clustered based on their similarity profiles.

Table 1. Top 29 significantly differential functional pathways between the plastisphere and ambient seawater. All pathways listed exhibited an identical level of statistical significance (Kruskal-Wallis FDR-adjusted $p = 0.047$). Pathways with a negative effect size indicate enrichment on plastic surfaces, whereas positive values indicate depletion relative to the seawater control.

Pathway Name	Median Difference (diff.btw)	Effect Size (effect)
CODH-PWY [Wood-Ljungdahl Pathway (Reductive Acetyl-CoA)]	-1.165	-2.116
DENITRIFICATION-PWY [Denitrification (Nitrate to Nitrogen)]	-1.407	-4.411
PWY-6876 [Isoprene Biosynthesis]	-1.459	-4.569
PPGPPMET-PWY [ppGpp Metabolism (Alarmones)]	0.450	3.178
PWY-181 [Photorespiration (Oxidative Photosynthetic Carbon C)]	0.496	4.976
PWY-5747[2-Methylcitrate Cycle II]	0.991	7.771
PWY-6151[S-Adenosyl-L-Methionine Cycle (SAM)]	0.918	4.913
GLYCOLYSIS-TCA-GLYOX-BYPASS [Superpathway of Glycolysis, TCA, and Glyoxylate]	0.336	4.373
PWY-7323 [O-Antigen Biosynthesis (GDP-Mannose)]	-0.420	-3.610
PWY-6628 [L-phenylalanine Biosynthesis]	0.016	0.240
PWY-6630 [L-tyrosine Biosynthesis]	-0.012	-0.133
PWY-1269 [CMP-KDO Biosynthesis (LPS Precursor)]	0.002	0.027
ARGSYN-PWY [L-arginine Biosynthesis I]	0.032	0.613
GLUCONEO-PWY [Gluconeogenesis I]	0.014	0.382
ANAEROFRUCAT-PWY [Fructose Homolactic Fermentation]	-0.782	-1.409
PWY-5180 [Toluene Degradation II (Aerobic via 4-methylcatechol)]	-0.245	-0.684
PWY-5182 [Toluene Degradation III (Aerobic via p-cresol)]	-0.210	-0.612
PWY-5941 [Glycogen Biosynthesis I]	-1.196	-0.927
PWY-5971 [Palmitate Biosynthesis II (FAS II)]	-1.550	-1.685
PWY-6210 [2-Aminophenol Degradation]	-1.191	-1.203
PWY-6505 [L-glutamine Biosynthesis III]	-1.348	-1.507
GLUCUROCAT-PWY [Glucuronate and Glucuronide Degradation]	-1.828	-10.261
PWY-7242 [D-fructuronate Degradation]	-1.643	-10.164
GALACTUROCAT-PWY [D-galacturonate Degradation I]	-1.019	-3.869
PWY-6507 [4-Aminobutyrate Degradation (GABA)]	-1.013	-1.638
PWY-5178 [Toluene Degradation I (Aerobic via o-cresol)]	-3.489	-1.426
PWY-5430 [Aromatic Meta-Cleavage Pathway]	-2.331	-0.828
NAD-BIOSYNTHESIS-II [NAD Biosynthesis (from Tryptophan)]	-2.049	-3.601
P125-PWY [(R,R)-butanediol Biosynthesis]	-5.422	-3.187

4. Discussion

The overarching similarity in global functional profiles across plastic, wood, and seawater as evidenced by the lack of statistical significance in the PERMANOVA test ($p = 0.703$) highlights a profound functional redundancy within the marine microbiome. Functional redundancy is a well-documented ecological phenomenon in ocean ecosystems, wherein diverse taxonomic assemblages harbor highly overlapping metabolic capabilities to ensure ecosystem stability (Louca et al., 2018). While Silva et al. (2023) demonstrated significant taxonomic successional changes on these distinct plastic polymers, our functional prediction suggests that the core metabolic machinery required to colonize these surfaces remains fundamentally uniform. This indicates that the initial colonization of artificial substrates is likely driven by opportunistic attachment rather than an immediate need for specialized metabolic pathways, corroborating the hypothesis that plastics initially act as passive attachment sites similar to natural organic matter rather than selecting for fundamentally different core metabolisms (Bryant et al., 2016).

Despite this global redundancy, targeted differential abundance analysis revealed critical micro-scale metabolic specializations within the plastisphere, most notably the massive enrichment of the denitrification pathway (DENITRIFICATION-PWY, Effect Size = -4.41). Denitrification is an anaerobic respiration process, and its pronounced upregulation on plastic surfaces strongly suggests the development of mature, thick biofilms that create localized hypoxic or anoxic microenvironments, even within well-oxygenated surface seawater (Wright et al., 2020). As the biofilm thickens on the impermeable plastic surface, oxygen diffusion is severely restricted (Stewart, 2003), forcing inner-layer bacteria to switch to nitrate as an alternative electron acceptor. This structural characteristic distinguishes plastic biofilms from free-living planktonic communities in the ambient seawater, fundamentally altering local nitrogen cycling.

A compelling ecological narrative further emerges from the significant depletion of the ppGpp alarmone metabolism pathway (PPGPPMET-PWY, Effect Size = 3.17) in the plastisphere relative to the seawater control. The alarmone ppGpp is a universal signaling molecule synthesized by bacteria in response to starvation and environmental stress, triggering the "stringent response" (Potrykus & Cashel, 2008). The significantly lower abundance of this pathway on plastic substrates implies that bacteria within the plastisphere are experiencing reduced nutritional stress compared to their free-living counterparts in the oligotrophic open ocean. This supports the mechanism similar to ZoBell effect in marine plastisphere which posits that solid surfaces like plastic debris act as nutrient trap, concentrating dissolved organic matter and limiting nutrients, thereby providing a more favorable, nutrient-rich microhabitat (Zettler et al., 2013; Zobell, 1943).

Moreover, the enrichment of the reductive acetyl-CoA (Wood-Ljungdahl) pathway (CODH-PWY, Effect Size = -2.11) on plastic surfaces points towards highly specialized carbon metabolisms. The Wood-Ljungdahl pathway is classically recognized as a strictly anaerobic, highly energy-efficient mechanism for autotrophic carbon dioxide fixation (Ragsdale & Pierce, 2008). The presence of this pathway in the plastisphere further corroborates the existence of deeply embedded anaerobic niches within the mature biofilm. It also raises the possibility of autotrophic carbon fixation occurring deep within the plastisphere matrix, potentially supporting the biofilm community where photosynthetic capabilities (such as the depleted photorespiration pathway, PWY-181, Effect Size = 4.97) are physically obstructed by biofilm depth or lack of light penetration.

5. Conclusions

This study highlights a complex ecological dichotomy within the marine plastisphere: macroscopic functional redundancy coupled with micro-scale metabolic specialization. Despite the distinct physical and chemical properties of commercial plastic polymers, the global predictive functional profiles of mature plastisphere biofilms do not significantly diverge from those colonizing natural wood or residing in the ambient seawater. This pronounced functional redundancy suggests that the core biogeochemical machinery of marine microbiomes remains highly stable, with early colonization likely driven by opportunistic attachment rather than strict metabolic selection.

However, targeted differential abundance analysis revealed that the prolonged presence of impermeable plastic debris actively shapes localized microenvironments. The significant enrichment of the denitrification and reductive acetyl-CoA pathways on plastic surfaces indicates the formation of dense, mature biofilms harboring distinct anaerobic niches capable of alternative energy metabolism and carbon fixation. Furthermore, the notable depletion of the ppGpp alarmone metabolism pathway strongly supports the nutrient trap and concentrate phenomenon on marine plastisphere, characterizing the plastisphere as a nutrient-rich refuge that alleviates the starvation stress typically experienced by free-living marine bacteria. Ultimately, while plastics may not entirely reinvent the functional wheel of the ocean microbiome, they act as active micro-ecological islands that trigger highly specific, localized metabolic adaptations. Future research integrating direct multi-omics approaches (e.g., metatranscriptomics) will be crucial to empirically validate these predicted metabolic shifts and fully comprehend their impact on global marine biogeochemical cycling.

Supplementary Materials: This journal does not provide hosting for supplementary materials on its website.

Acknowledgments: This research was conducted within the framework of the omics technology course project under the guidance of Dr. Rizky Dwi Satrio, S. Si., M. Si.. During the preparation of this manuscript/study, the author(s) used Gemini Generative AI for helps explain the technical steps for data processing on UseGalaxy.eu computational platform and data visualization on R Studio. The authors have reviewed and edited the output and accept full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

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