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Environmental factors control microbial colonization of plastics in the North Sea

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ABSTRACT

Large quantities of plastic enter the oceans each year providing extensive attachment surfaces for marine microbes yet understanding their interactions and colonization of plastic debris remains limited. We investigated microbial colonization of various plastic types (polyethylene, polystyrene, polyethylene-terephthalate, and nylon) in ex-situ incubation experiments. Plastic films, both UV-pretreated and untreated, were exposed to seawater from a coastal and an offshore location in the North Sea. 16S rRNA amplicon sequencing was employed to assess microbial community structures after 5, 10, 30, and 45 days of incubation. Our findings show the significant influence of time, seawater origin and plastic type on microbial community succession. We also identified several genera associated with hydrocarbon or plastic degradation potential as well as genera selecting for specific plastics such as *Ketobacter* and *Microbacterium*. Our results highlight potential role of microorganisms in plastic biodegradation and support the idea that microbial colonizers on marine plastics debris seemingly select distinct substrate types.

1. Introduction

Industrial scale production of plastic polymers started in the 1950s, and since then, global production figures increased exponentially (Geyer et al., 2017). Currently, >390 million metric tons of plastic are produced worldwide per year (Plastics Europe, 2022). This increase has also led to a rise in mismanaged plastic waste, resulting in substantial amounts of disposed plastic ending up in terrestrial and aquatic environments; It is estimated that about 0.1 % (Cózar et al., 2015) up to 4.1 % (Jambeck et al., 2015) of the annual global plastic produced ends up in the ocean. The ingress into marine environment occurs via multiple pathways (Li, 2018), including atmospheric deposition (Zhang et al., 2020; Allen et al., 2022), transportation via rivers (Lebreton et al., 2017; Meijer et al., 2021), or littering at sea (Lebreton et al., 2022). The annual amount of plastic litter entering the ocean is estimated to amount to ~0.5 Mt. (Kaandorp et al., 2023) to up to 12.7 Mt. (Jambeck et al., 2015). These estimates are often based on investigations of plastic in surface waters or are based on modelling studies (Wayman and Niemann, 2021). However, plastic marine debris (PMD) has been found in all marine compartments across horizontal gradients from shorelines to coastal- and open waters, and vertical gradients from the sea surface to (deep sea) sediments (Erni-Cassola et al., 2019; Morales-Caselles et al., 2021; Zhao et al., 2023).

The fate of PMD that accumulates in surface waters remains uncertain. Once in the ocean, floating plastics are subjected to a wide range of physical (solar UV radiation, wave action, etc.) and biological processes (biofouling), that may contribute to plastic degradation (Wayman and Niemann, 2021). For biodegradation to occur, the first step is the attachment of marine microbes to plastic and the formation of biofilms. The occurrence of PMD, as any immersed particle/object, provides artificial surfaces for microbial attachment, and hence the opportunity for a sessile lifestyle (Zettler et al., 2013; Vaksmaa et al., 2021a; Zhao et al., 2023; Goudriaan et al., 2024). Although marine pelagic environments are characteristically dominated by free-living microbes, living within a biofilm offers various advantages comprising protection from UV radiation, predation, and environmental fluctuations (Vaksmaa et al., 2021a). Furthermore, plastics feature a wide range of molecular diversity and contain substantial amounts of chemical energy. Plastic polymers can be alkane-like, such as polyethylene (PE) or can have a more complex molecular structure, e.g. with aromatic rings, like polystyrene (PS). Some plastics, like polyethylene terephthalate (PET) and nylon also include other non-carbon heteroatoms such as oxygen (O)

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and nitrogen (N).

A few marine microbes have been shown to degrade plastic polymers in laboratory settings with plastic as the sole carbon source. The most up-to-date database compiling information on microorganisms with the potential for plastic degradation is Plastic DB (Gambarini et al., 2022). It needs to be noted that the 'potential for plastic degradation' is relatively loosely defined in Plastic DB. It includes solid experimental proof where degradation products were detected (as has e.g. been demonstrated for the PET degrader *Ideonella sakaiensis* (Yoshida et al., 2021); or where labelled carbon was traced from the plastic matrix into degradation products and microbial biomass (Zumstein et al., 2018; Goudriaan et al., 2023). In addition, plastic DB also lists organisms for which (only) indications of plastic degradation have been documented such as the formation of clearance zones on plastic films (Brunner et al., 2018) or polymer weight loss upon exposure to these microorganisms (Raghavendra et al., 2016). Plastic DB also list organisms that (only) have genes that encode for polymer degrading enzymes (Zampolli et al., 2022), which is no ultimate proof for plastic biodegradation ability (Vaksmaa et al., 2021a). These microbes are thus considered potential plastic degraders. Furthermore, several bacteria that are reported in plastic DB are hydrocarbonoclastic bacteria (HCB), for which the ability to degrade the somewhat chemically similar but more complex plastic polymer, has typically not been demonstrated. Additionally, biodegradation can be either polymer specific, as found for *Alcanivorax borkumensis*, which can degrade PE and other alkanes (Yakimov et al., 1998; Delacuvellerie et al., 2019). Some plastic degraders may also break down multiple polymers: For example, *Rhodococcus ruber* was found to degrade PE, PS, and hydrocarbons (Orr et al., 2004; Zhukov et al., 2007; Mor and Sivan, 2008; Goudriaan et al., 2023).

As for the degradation of other particulate organic matter, the plastic degrading microbes likely need to be in close vicinity to the plastic particle for an efficient use of their degrading enzymes and to scavenge degradation products. It is assumed that plastic degrading microbes thus attach to plastic particles similar to what has been found for other degraders of particulate organic matter (Vaksmaa et al., 2021a). The mechanisms involved in polymer colonization, and whether specific polymers host distinct microbial communities including plastic degrading microbes or rather opportunistic organisms, are rationales of ongoing research. Studies investigating these topics relied on incubation setups (De Tender et al., 2015; Oberbeckmann et al., 2016; Xu et al., 2019; Zhang et al., 2022) or sampling of 'wild plastics', i.e. PMD that has been collected from the ocean (Frère et al., 2018; Delacuvellerie et al., 2019; Lacerda et al., 2020; Vaksmaa et al., 2022). Nevertheless, comparing results between incubation studies remains difficult due to the disparity in factors and experimental conditions. For example, the polymers used typically differ, comprising virgin or weathered variants. Also, the types of control surfaces, location, and the incubation time usually differ. Finally, the origin and the history of a 'wild plastics', its geographical trajectory, the amount of UV radiation it had received and the succession of microbes that have colonized the particle remain unconstrained.

As a biogeographical region, the North Sea can be regarded as a shelf sea model system, which is influenced by plastic pollution (Lorenz et al., 2019; Materić et al., 2022) and undergoes a major transition. It is one of the regions with the highest shipping activity on the globe and features an abundance in hydrocarbon extraction infrastructures and other offshore installations such as wind and solar power parks are planned. The coast is densely populated, and several major river systems shed water, including pollutants, into the coastal North Sea.

In this study, we incubated four plastic polymers PE, PS, PET, and nylon, both virgin and UV weathered in North Sea coastal and open waters. Our aims were (i) to analyze the microbial communities developing on these polymers through time, (ii) to unravel the factors (location, polymer, and weathering state) influencing the microbial assemblages on plastic, and (iii) to identify potentially relevant taxa for plastic degradation.

2. Material & methods

2.1. Sampling and experimental setup

North Sea (NS) surface water was collected with Niskin bottles from 2 stations at 1 m water depth during a research cruise with R/V Pelagia (PE462, October 2019). One station was located close to the coast (52.07°N, 4.16°E) and the other station in the open Dutch North Sea (54.8°N, 4.11°E) (Figure 1)

Plastic film sheets of 1 mm thickness of virgin PE, PS, PET, and nylon (Goodfellow, UK: order Nrs. ET313010, ST313120, and ES303010, respectively) were cut into strips of 20 cm × 1 cm prior to incubation. Half of the strips were UV weathered in a custom-built UV chamber with a UV lamp (Supratec HTC 400241; Osram, Germany) emitting a UV spectrum similar to solar UV-A/B light (Delre et al., 2023). Irradiance was 250 W m^{-2} at the height of the samples and the strips were exposed in the chamber for 3 days.

For the incubation setup, 8 times 5 l GL-45 borosilicate bottles were used (prior to use, these were washed with 10 % HCl and thereafter MilliQ water). These were rinsed with the corresponding water and filled with 4 l of either coastal NS water (12 °C at the time of sampling) or open NS water (10 °C at the time of sampling). Into each bottle, 3 strips of the same virgin polymer and 3 strips of UV weathered polymer and one natural polymer (beech wood strip), serving as a control were immersed hanging by a string made of hemp into the water. These bottles were kept in the dark at room temperature (20 °C).

To monitor microbial community succession, we sampled the immersed strips by successively cutting off 1 cm in a flow cabinet after 5, 10, 30 and 45 days of incubation. Samples were then stored at −20 °C in sterile cryovials prefilled with 1.5 ml of RNAlater (Sigma Aldrich) for molecular analysis, and in cryovials prefilled with an SEM grade 1.5 % glutaraldehyde solution for microscopy analysis. In total 280 samples were collected for each downstream analysis.

2.2. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to visualize microbial biofilms on the incubated polymers. For this, the samples stored in 1.5 % glutaraldehyde solution (taken after 5 and 45 days of incubation) were

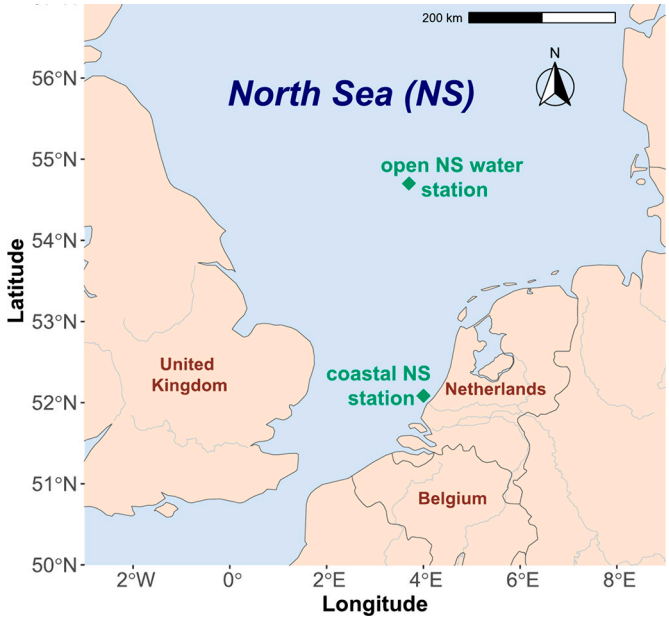

Fig. 1. Map of the southern North Sea (NS) displaying the two stations: coastal NS station and open NS water station that were sampled for the incubation setup.

washed with ethanol solutions of 50 %, 70 % and 90 % ($EtOH/H_2O$, *v*/v) and then air dried in a fume hood. The plastic films were mounted on carbon tape and a ~ 3 nm thin gold coating was applied (JEOL JFC-2300HR sputter coater, using a JEOL FC-TM20 thickness controller). Thereafter, the samples were imaged with a scanning electron microscope (JEOL Neoscope II JCM-6000, Japan). Measurements were conducted at 0.1–0.3 mbar vacuum and with a high accelerating voltage (10 or 15 kV) and by using a backscatter electron detector.

2.3. DNA extraction and PCR amplification

DNA from individual samples was extracted using the ZymoBIOMICS™ DNA Miniprep Kit (D4300) (ZR, Zymo Research, Irvine, CA, USA). The instructions provided by the manufacturer were followed except for adding a bead beating step to break the cells. This was done using the OMNI Bead Ruptor Elite (SKU 19-040E) set to 5 cycles of 75 s at 5 m/s and 5 min of dwell time in between cycles.

The extracted DNA was subjected to PCR amplification by using the universal primers 515F—Y (5′-GTGYCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3′) (Parada et al., 2016). The forward and reverse primers were both barcoded with unique Golay 12 nucleotide barcode combinations. The total volume of the PCR reaction mix was 25 μl. It contained 12.75 μl of PCR grade water, 5 μl of 5× Phusion HF buffer, 2 μl dNTPs (2.5 mM), 2 μl BSA (20 mg/ml), 0.25 μl (2 U/μl) Phusion polymerase, and 1.5 μl of each forward and reverse primer (10 μM) and 1 μl of DNA. The thermocycler program was as follows: 98 °C for 2 min followed by 25 cycles of 98 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s, and with a final elongation of 72 °C for 7 min. PCRs were carried out in three technical replicates for each sample. Amplification products were kept at 4 °C. All three technical PCR-replicated reactions were quantified via gel quantification, then pooled in equimolar amounts and subjected to PCR purification by AMPure XP (Beckman Coulter, Inc). Illumina MiSeq 2 × 300 nt sequencing was carried out using the MiSeq reagent kit v3 at Macrogen (Seoul, South Korea).

2.4. Data processing

The raw sequencing data were processed using the CASCABEL pipeline V4.6.2 (Abdala Asbun et al., 2020) with configuration parameters as described in detail in the supplementary material (Supplementary document). Further data analysis was carried out using the R Statistical Software v4.2.0 (R Core Team, 2022). Alpha diversity indexes were calculated using the package Phyloseq (McMurdie and Holmes, 2013) and multivariate statistical analysis was carried out using the package Vegan (Oksanen et al., 2022). Genera reported in plastic DB (Gambarini et al., 2022), were compiled in a list regardless of polymer type or degradation evidence. We also built a curated database of 65 HCB (see Data availability) and used the Plastic DB database to extract genera which could be involved in plastic breakdown and hydrocarbon degradation. The metabolic capabilities of the organisms listed as HCB or plastic degraders (PD) in both these databases concern specific strains. Since our analysis is based on the genus level, the extracted genera are then considered as potential HCB and potential PD.

3. Results

3.1. Microscopy of early and late biofilm developmental stages

SEM images were prepared from biofilms on plastic polymer strips sampled after 5 and 45 days of incubation (Supplementary Figs. 1–4). The plastic strips exposed to UV light had cracks on their surfaces while the non-UV weathered samples featured smooth surfaces. Colonization by microbes was evident on plastic strips already after 5 days of incubation. For both the coastal and open water station, biofilms after 45 days of incubation time were visually denser, indicating that biofilm coverage on the plastic strips increased over time during the incubation. Microbial cells (likely bacteria), hyphae (likely fungi) and diatoms were visible on multiple polymer types. Additionally, we observed that non-UV weathered nylon and non-UV weathered PE incubated in open NS water featured less dense biofilms when compared to their UV weathered counterparts and polymers PS and PET. The films exposed to coastal NS water had a more diverse microbial coverage. Furthermore, biofilms on UV weathered plastics generally expanded into the cracks, irrespective of polymer type and station.

3.2. Microbial diversity in biofilms across taxonomic ranks

3.2.1. Kingdoms

On the kingdom level (Supplementary Fig. 5), we detected a clear dominance of Bacteria with relative abundance values of 97 ± 3 % across all samples. The reads assigned to Archaea accounted for 0.3 ± 2 % and reads assigned to Eukaryotes accounted for 3 ± 2 %. With the subsequent taxonomic composition analysis, we will be focus on the kingdom Bacteria to uncover changes and trends in microbial community composition.

3.2.2. Phyla

In total, 33 bacterial phyla were detected in our samples. The phyla with highest relative abundances were Actinobacteria, Bacteroidota, Campylobacterota, Firmicutes, Myxococcota, Patescibacteria, Planctomycetota, Proteobacteria and Verrucomicrobiota (Figure 2). For the coastal NS station, we found that the phyla Proteobacteria and Campylobacterota dominated on day 5 with relative abundances of 61 ± 16 % and 31 ± 17 %, respectively. On day 10, the proportion of Campylobacterota decreased (except for PE), while the proportion of Bacteroidota increased to up to 12 ± 6 %. In the open water station, we found that the phylum Proteobacteria dominated on day 5 with a relative abundance averaging 87 ± 3 %. On day 10, this had decreased to 75 ± 5 %, while the Bacteroidota increased in all samples to 17 ± 5 %.

On day 30, Planctomycetota were detected with relative abundance averaging 16 % ± 5 % in the coastal NS water and 18 % ± 11 % in the open NS water. While the proportion of Campylobacterota decreased to <1 % on the polymers irrespective of the sampling station or treatment. Furthermore, the relative abundance of Actinobacteria on nylon (irrespective of UV weathering and station) was 23 ± 4 %, which was not the case for any of the other polymers, where Actinobacteria were detected in relative abundance <5 %. This pattern prevailed for Actinobacteria on day 45 reaching 52 ± 17 % for nylon in coastal NS water and 24 ± 8 % for nylon in open NS water. On all other polymers except nylon, Proteobacteria, Planctomycetota and Bacteroidota were the most abundant phyla (irrespective of UV weathering and station).

3.2.3. Order

On the order level, 268 unique orders were detected. In general, the community dynamics at the order and phylum (see above) levels were similar. The orders Campylobacterales (phylum Campylobacterota) and Enterobacterales (phylum Proteobacteria) dominated on day 5 with relative abundances 31 ± 17 % and 42 ± 13 %, respectively at the coastal NS station (Fig. 3). The proportion of Campylobacterota decreased over time from 15 ± 24 % on day 10 to 0.1 ± 0.2 % on day 45. Like the Campylobacterales, the relative abundance of Enterobacterales decreased over time as well from an average of 26 ± 14 % after 10 days of incubation, to an average of 3 ± 2 % and 2 ± 2 % respectively, after 30 and 45 days of incubation.

In contrast to the coastal station, Campylobacterales was not detected as a dominant order at any time at the open NS water station; there the community was dominated on day 5 by Enterobacterales, Rhodobacterales and Pseudomonadales. Over the course of the incubations, similar to the open NS station, the relative abundance of Enterobacterales decreased from 57 ± 6 % on day 5 to 6 ± 6 % on day 45. Overall, the proportion of Pseudomonadales (phylum Proteobacteria)

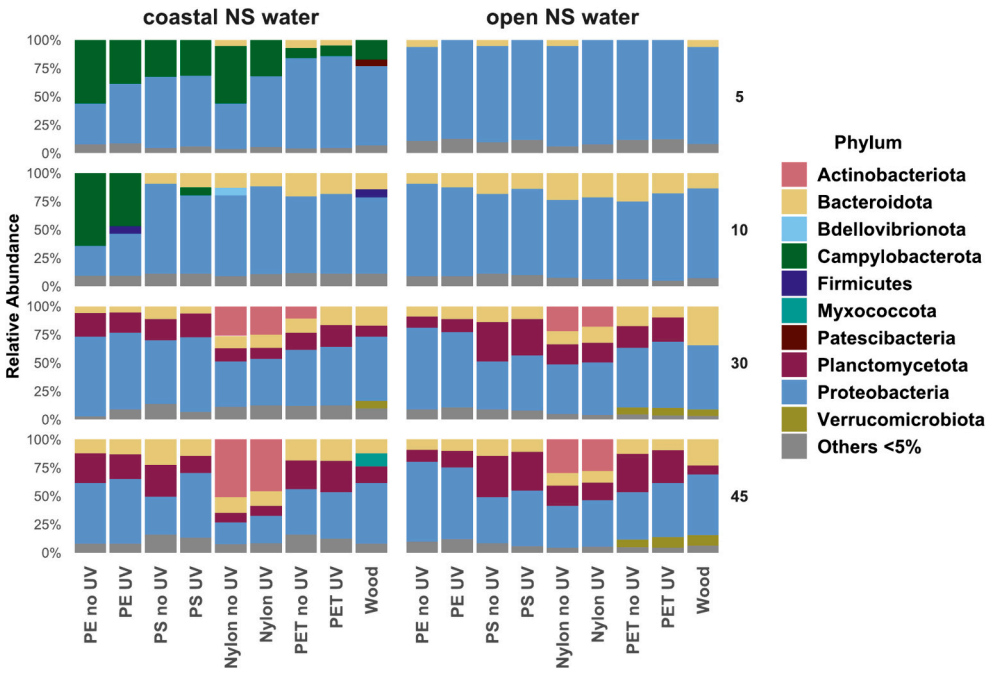

Fig. 2. Average relative abundances expressed in percentages of detected phyla according to stations, time of incubation and polymer type and treatment. The phyla for which the relative abundance was less than 5 % were labeled as "Others <5%".

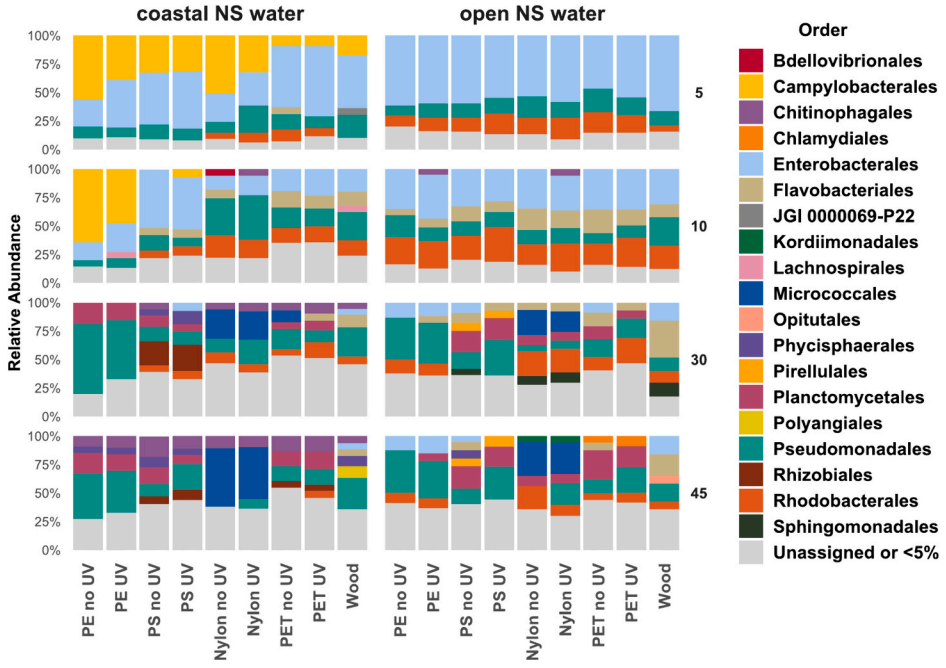

Fig. 3. Average relative abundances expressed in percentages of detected orders according to stations, time of incubation and polymer type and treatment. The orders which could not be assigned and/or for which the relative abundance was less than 5 % were labeled as "Unassigned or <5%".

remained stable ranging from 14 % ± 4 % on day 5 to 20 % ± 11 % on day 45. The order Micrococcales belonging to the phylum Actinobacteria reflected the same increase as observed for the whole phylum, from being undetectable on day 5 to 39 ± 12 % on day 45 on nylon.

3.3. *Multivariate statistical analysis of microbial community compositions*

Alpha diversity indexes were calculated prior to discarding low abundance ASVs. The Gini-Simpson diversity index was relatively high for all samples (Supplementary Fig. 6). However, there was no significant difference between the Gini-Simpson diversity index values of different types of samples within stations.

Principal Components Analysis (PCA) showed a discrimination between time points on the x-axis, which explained 18 % of variance and a discrimination between stations on the y-axis, which explained 14.7 % of the total variance (Fig. 4). However, the polymer type did not show clear distribution patterns in the PCA.

A first PERMANOVA including our full dataset determined that time, locations and material type (wood, PE, PET, PS and nylon) were significantly impacting the structure of the microbial community. To

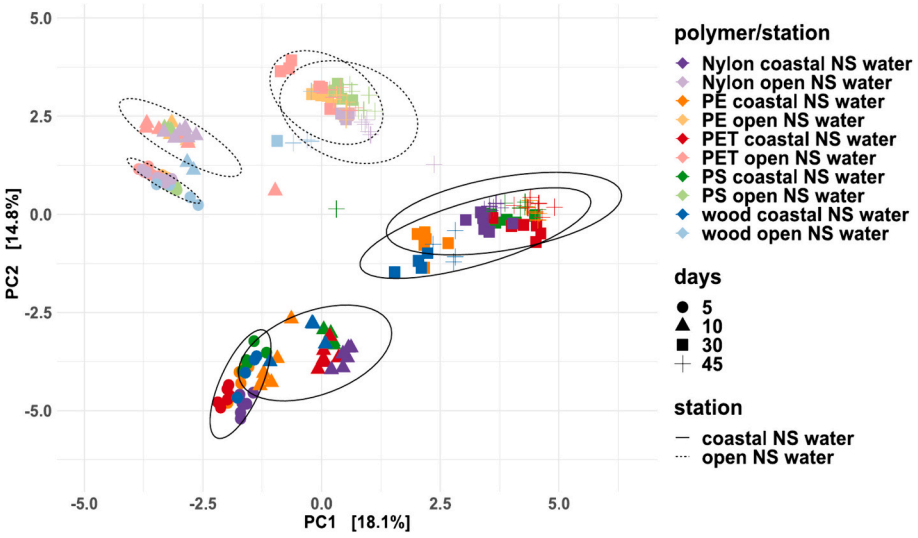

Fig. 4. Principal Components Analysis (PCA) ordination of the Centered Log Ratio (CLR) transformed data. The percentages of variance explained is displayed for each axis. The ellipses are drawn around 95 % of the days-station combination data.

focus on the effect of the plastic polymer type and UV weathering, we applied further statistical analysis. For this, we did not consider the control samples (wood) for the subsequent analysis as these were not replicated and did not feature a UV weathered subset. The PERMANOVA results indicated that the factors time, location, and polymer type were significant. To confirm this, the assumption of group variances' homogeneity was tested. This showed significant differences in groups dispersions. We therefore applied an analysis of similarities (ANOSIM) to all variables. ANOSIM showed that time, location, and polymer type were indeed significantly affecting the microbial communities' distribution (p value = 0.001) while UV weathering was not found to be significant (p value >0.05) (Supplementary table 1).

Fig. 5. Evolution development of the relative abundance of the top 3 genera per time point/location/polymer/weathering combination. Genera in blue are potential hydrocarbon degraders. Genera in green are those reported in plastic DB as polymer degraders. Genera in purple are those that are both potential hydrocarbon degraders and reported in plastic DB as polymer degraders.

3.4. Community succession and potential plastic and hydrocarbon degraders

The top 3 genera in the seawater inoculums and in each combination of the variables time, location, polymer type and treatment were extracted. This resulted in a total of 44 genera (Fig. 5). We detected relative abundances >50 % for some of the genera such as *Ketobacter* (on PE incubated in coastal NS water) and *Microbacterium* (on nylon incubated in coastal NS water). Other genera were comparably low in relative abundance, e.g. *Aliiglaciecola, Pelagicoccus* and *Thalassolitus*. Amongst the top 3 intersection genera, we generally found some HCB as well as some genera previously defined as potential PD in the plastic DB database (Gambarini et al., 2022). Some of these (e.g., *Alteromonas, Pseudoalteromonas* and *Sulfitobacter*) were present on all plastic polymers, while others (e.g., *Microbacterium, Ketobacter*) were only present on specific ones. Mostly, UV and non-UV weathered plastic samples showed similar patterns. Typically, these genera were early colonizers (i.e., they appeared at the beginning of the incubation and decreased in abundance over time, (e.g., *Colwellia, Oleibacter, Oleispira* and *Pseudarcobacter*), whereas some also increased in abundance towards the end of the experiment (e.g., *Gimesia*, *Oleiphilus*, *Ketobacter* and *Microbacterium*).

Alteromonas, *Colwellia, Marinomonas, Pseudarcobacter* (only on polymers incubated in coastal NS water) as well as *Pseudoalteromonas*, *Sulfitobacter* and *Vibrio* were detected with highest relative abundances during the early stages of colonization. Among those "early colonizers", *Pseudoalteromonas* is a genus that is both a potential HCB and PD. *Colwellia, Marinomonas* and *Sulfitobacter* are potential HCBs. *Pseudoalteromonas* and *Vibrio* are reported as potential PD. Amongst the late colonizers, we detected *Fuerstia, Gimesia, Ketobacter, Lewinella, Microbacterium* and *Saccharospirillum*. *Ketobacter and, Lewinella* are potential HCBs, whereas *Microbacterium* has been reported as a PD (Zhang et al., 2024). Additionally, some genera appeared only on specific polymers. In incubations with coastal water, for example, *Ketobacte*r was highly abundant on PE (61 ± 16 % on non-UV PE and 30 ± 6 % on UV weathered PE on day 30 and respectively 27 ± 4 % and 26 ± 8 % on day 45). Although at lower relative abundance, *Ketobacter* was also detected on PE samples incubated in open NS waters, but we could not detect this on other polymers. We observed a similar trend for *Microbacterium,* which was a late colonizer showing highest relative abundance on nylon in both stations reaching an average of 48 ± 7 %, while it was low in relative abundance on the other polymers.

4. Discussion

Increasing mass production and waste mismanagement have made plastic waste a severe environmental problem with large quantities of plastic debris ending up in the ocean. Any exposed surface in the marine realm is rapidly colonized by microbes such as bacteria, archaea, fungi, and algae. Plastics in this regard have become a new artificial habitat, offering a surface for microbial colonization and biofilm formation (Zettler et al., 2013; Vaksmaa et al., 2021a; Zhao et al., 2023; Goudriaan et al., 2024). These biofilms are composed of a complex community of microorganisms and the formation of the biofilm is influenced by a variety of physicochemical and biological factors (Vaksmaa et al., 2021b; Wayman and Niemann, 2021). Polymer type, plastic surface texture, location and habitat, nutrient availability, presence of additives and contaminants, and environmental conditions have been described to influence which microbes colonize the plastics (Amaral-Zettler et al., 2020; Wright et al., 2020, 2021b; de Vogel et al., 2024; Goudriaan et al., 2024). Understanding the impacts of these factors is crucial for comprehending the dynamics of biofilm formation on plastic polymers and for identification of members of the microbial community which are potentially involved in plastic degradation. Here we investigated microbial colonization of plastics in North Sea waters.

4.1. Plastic attached microbial communities

In this study we investigated the influence of polymer type, UV weathering, incubation time, and habitat (coastal NS versus open NS) on the composition of microbial biofilms forming on plastic polymers. High microbial diversities and richness indexes for microbes on plastic have been reported for plastics sampled at multiple time points (Frère et al., 2018), or on incubated polymers compared to surrounding seawater (Zhang et al., 2022). In contrast, a lower alpha diversity was found on epi-plastic communities than on natural substrates (cobblestone and wood) in freshwater laboratory incubations setup (Miao et al., 2019).

In our setup we considered day 5 and day 10 to be "early colonization stages" and day 30 and day 45 to be "late colonization stages". Comparison of alpha diversity indexes did not reveal any significant change when comparing the different parameters of our experiment (Supplementary Fig. 5). Findings from Erni-Cassola et al. (2020) showed an increase in evenness and diversity from day 2 to day 9 in incubation experiments using thermo-oxidized and non-weathered PE in Mediterranean Sea. However, contrasting with our findings, their study found that oxidized polyethylene had lower diversity and evenness than non-weathered polyethylene. This distinction between weathered and non-weathered plastic polymers was not observed in our case. Similar to our findings, Dussud et al. (2018) found that the developmental stages of biofilm formation on plastics in Mediterranean mesocosms did not affected alpha diversity indexes. Jacquin et al. (2021) made a similar observation and found that the Pielou evenness index was stable for 40 days during incubations of polypropylene (PP) as well as other biobased plastics. However, we caution not to consider only alpha diversity indexes as indicators for the dynamic of epi-plastic communities. It is important to note that these indexes were developed for plant and animal ecology where specimen of a given species can be enumerated for a precise area. While for the dynamics of plastic attached microbial communities, it is important to note that the vast majority of these indexes do not account for the compositional nature of microbiome sequencing data (Willis, 2019).

Although time, location and polymer type did not influence the diversity and richness indexes, we found that these factors (based on ANOSIM) were the main drivers for the differentiation of microbial communities. The distinction according to time and biogeography was evident in the PCA as well (Fig. 4). Residence time in water was identified as a major driver for change in the composition of plastic attached microbial communities in other studies (Jacquin et al., 2021) including incubation experiments comparable to our experimental setup (De Tender et al., 2017; Pinto et al., 2019). The high influence of residence time indicates that the composition of microbial communities on plastics follows traditional marine biofilm development processes such as the initial attachment, species recruitment and maturation. Indeed, early biofilms are usually created by opportunistic microbes from surrounding waters (Lee et al., 2024). In addition, the communities settle when an ecological balance is found leading to a mature microbial biofilm. This could explain the smaller variance in the later stages of colonization in our experiment (between day 30 and day 45) (Fig. 4). Finally, abiotic environmental factors such as salinity, temperature and light availability were found to affect free-living as well as surface attached microbial communities (Lee et al., 2024).

We found distinct microbial communities on plastic exposed to open or coastal NS water. Moreover, considering that these habitats differ in multiple abiotic factors, this finding is consistent. Although free living pelagic microbes are distinct from surface attached ones (Zettler et al., 2013), microbial communities which colonize plastic surfaces are recruited from surrounding waters. The properties of the initial seawater inoculum are hence important in structuring the epi-plastic community on a regional scale. Coastal and open ocean habitats differ in the importance of several abiotic factors like the availability of nutrients (Giraud et al., 2008), and concentration of pollutants, which is typically higher in coastal areas (Doney, 2010). Location has been shown on a

local scale (de Vogel et al., 2024) and on a more global scale to be a determining factor, too (Amaral-Zettler et al., 2020; Wright et al., 2020). We argue that this is determined by the inoculum because the microbial communities in the epi-plastic biofilm ultimately originate from the surrounding water. The combination of abiotic (e.g. polymer type, temperature, salinity) and biotic factors (e.g. physiological functioning, competition) select and determine the composition of the epi-plastic biofilm. Finally, stochastic effects were also discussed to influence the composition of epi-plastic biofilms, specifically during the early stage of development (Goudriaan et al., 2024).

Studies investigating epi-plastic biofilms often focused on 'wild plastic', i.e., fragments that have been in aquatic environments for an undetermined duration (Bryant Jessica A. et al., 2016; Di Pippo et al., 2020; Cappello et al., 2021; Vaksmaa et al., 2021b, 2022). The stochastic elements related to sample collection, particle history and diversity of polymer types and additives make it difficult to adequately assess substrate specificity. Our setup aimed to expose different plastic polymers to the same abiotic conditions without exposure to uncontrollable disturbance. In our incubation setup, where only polymer type and UV pre-treatment were variable factors, we could statistically show that polymer types were shaping the epi-plastic community. Previous studies also reported the plastic type affects the composition of epi-plastic communities (Kirstein et al., 2018, 2019; Miao et al., 2019; Hansen et al., 2021). However, this was not the case in other studies, where no effect of the polymer was detected (Oberbeckmann and Labrenz, 2020; de Vogel et al., 2024). This indicates that the polymer effect is relatively weak and might not always be apparent or is quickly overprinted even by minor variations of abiotic factors (Goudriaan et al., 2024). While a community specific for distinct plastic types may be difficult to detect, we suggest that future studies investigate if a core microbial community exists that favors plastics in general. Graphically, our PCA shows the dissipation of the effects time and station and thus indicates that microbial communities converge and become more similar with time. This has been observed for PE and PP incubated in the Adriatic Sea when bacterial community composition differences dissipated with time (Pinto et al., 2019). At first, this might indicate that plastic surfaces act primarily as a platform for attachment, rather than favoring the recruitment of microbes specifically adapted to plastic colonization.

To confirm if specific plastic polymers or polymers overall harbor distinct microbial communities, we also exposed a control surface -wood- to the same incubation conditions. The PCA and the ANOSIM showed that the microbial community which developed on wood was distinct in comparison to the plastic polymers, which could suggest the existence of microbial community selecting for plastic surfaces (at least when compared to wood as a surface). Previous studies have used either surrounding seawater or other hard surfaces as controls to identify if polymers select for a specific microbial community. A clear differentiation of microbial communities was previously observed when comparing plastics and surrounding seawater (Zettler et al., 2013; Vaksmaa et al., 2022; Zhang et al., 2022), comparing glass and plastics (Kirstein et al., 2018; Pinto et al., 2019) or even comparing plastics with cobblestone and, as done here, wood (Miao et al., 2019).

4.2. Microbial community succession through time

Overall, our results show a dominance of bacteria compared to archaea and eukaryotes in the epi-plastic biofilms on all samples. Only considering bacterial phyla, the location and time played a key role in community shaping. Some taxa where rather early colonizers with highest relative abundance detected on day 5 and 10 of the incubation, other taxa showed the opposite trend, with later stage maxima in relative abundance. One of the most abundant phyla in the early stage and in the late stage of the incubation was Proteobacteria in both coastal and open NS waters. This phylum comprises remarkably diverse microbes that are involved in the carbon and nitrogen cycles, and exhibit both anaerobic and aerobic metabolisms. Proteobacteria are dominant in many marine biofilms, as has been observed for high-density PE, stainless steel, and titanium in a coastal site in the Laccadive Sea (Sushmitha et al., 2021), on PE in the Belgian North Sea (De Tender et al., 2015), on PE, PP, PS, PET and glass in the German sector of the North Sea (Kirstein et al., 2018), on PE, PET, PP, PS and nylon in Caribbean coastal waters (Goudriaan et al., 2024) and on collected plastic particles in the Pacific ocean (Vaksmaa et al., 2022).

Additionally, we detected a high relative abundance of Campylobacterota in the coastal station at the beginning of the incubation. This phylum is the re-classification of the class Epsilonproteobacteria (Waite et al., 2017, 2018), which was previously considered as a part of the Proteobacteria. The relatively recent reclassification makes it challenging to trace Campylobacterota in earlier studies. The main driver for the high abundance of Campylobacterota in our incubations with coastal water was linked to a single genus, *Pseudarcobacter* belonging to the family Arcobacteraceae, which are ubiquitous in seawater but also in wastewater treatment plant effluents (Venâncio et al., 2022). These organisms might hence originate from a terrestrial/lacustrine source in our study, possibly shed with the effluents of the rivers Rhine and/or Scheldt into the North Sea (the coastal station was close to the estuaries of these rivers (Fig. 1). On the genus level, and in all incubations, the genera *Alteromonas*, *Pseudoalteromonas*, *Marinomonas, Colwellia* and *Vibrio* appeared to be early colonizers. These genera contain species that might play a role in hydrocarbon and or plastic degradation (Fig. 5).

For later stage colonizers in our study (day 30 and 45), there was a shift in microbial community composition. Proteobacteria and Campylobacterota's proportions decreased gradually suggesting that these taxa might comprise more opportunistic settlers that got outcompeted by more specialized microbes; Actinobacteria, Bacteroidota, Planctomycetota for example increased in incubations with waters from both stations. Dussud et al. (2018) also observed an increase of the phylum Planctomycetota on PE. The main late colonizers genera were *Fuerstia, Gimesia, Lewinella, Saccharospirillum* and *Pseudomonas*. The latter genus was also observed on PS incubated in artificial sea water (Ye et al., 2021).

Our study combines an incubation design with different plastics in fully controlled marine microcosms that were sampled at multiple time points for investigating microbial community compositions; such studies are scarce (Wright et al., 2020), which makes comparison to literature difficult. It has been argued that early microbial colonization of plastics is often supported by easily accessible and metabolizable short chain molecules for example polymeric oligomers produced through plastic degradation (Gewert et al., 2018; Romera-Castillo et al., 2018; Erni-Cassola et al., 2020; Goudriaan et al., 2023). Nevertheless, these initial energy sources are believed to be quickly exhausted within a few days (Romera-Castillo et al., 2022). Hence, a consistent energy supply becomes more beneficial. This may derive from organic matter (OM) other than plastic, for example seawater DOM or from photosynthetic products produced by pioneering phototrophic organisms in the epi-plastic biofilm. In our experimental setup, there was no supply of new OM or photosynthetic products (we conducted incubations in the dark). Still, in both stages of colonization, we found genera that comprise species with a potential role in plastic degradation. The biofilm dynamics found here contrast with other hypotheses which attribute a higher importance of early colonizers to plastic biodegradation in marine environments.

4.3. Potential metabolic capabilities of plastic attached microbial communities

Due to their chemical resemblance to naturally occurring polymers like petroleum, plastic polymers could serve as potential substrates for microorganisms (Vaksmaa et al., 2021a). Several previous studies found microbes in plastic associated biofilms that might be metabolically linked to plastic biodegradation (Vaksmaa et al., 2021b; Wright et al., 2021a; Goudriaan et al., 2024). In our analysis, we used "plastic DB"

(Gambarini et al., 2022) as a database; this lists genera/species which could play a role in plastic degradation. It must be noted, however, that the studies on which the database relies do not always provide unambiguous proof of degradation (see the introduction section). However, it also contains species for which unambiguous proof of plastic degradation was found. In addition to plastic degraders, plastic DB also contains organisms that were shown to degrade (longer chain) hydrocarbons - hydrocarbonoclastic bacteria that are also referred to as hydrocarbon degrading bacteria (HCB). HCB are ubiquitous in the marine environments and known to be involved in oil and pollutant degradation (see also Supplementary document 2 for a list of HCB). These bacteria are often enriched on plastic surfaces, but it is not clear if they indeed use plastics as substrates as carbon source or for energy gain or shorter chain leachates (Erni-Cassola et al., 2020; Vaksmaa et al., 2021b; Goudriaan et al., 2024).

Genera listed as PDs and HCBs comprised *Ketobacter* and *Microbacterium* that made up to 70 % of relative abundance in some of our samples (Fig. 5). *Colwellia, Erythrobacter, Marinomonas, Oleibacter, Oleiphilus* and *Sulfitobacter* were present in high relative abundances as well. *Oleiphilus* and *Oleibacter* are common in different marine habitats but are known to increase in abundance in oil contaminated sites (Gutierrez, 2019). *Marinomonas* and *Colwellia* are ubiquitous in marine environments. *Colwellia* species are secondary producers, detected in most cold marine habitats as well as temperate marine habitats, and involved in decomposing organic material like hydrocarbons, lipids, proteins and polysaccharides (Bowman, 2014). Similarly, *Sulfitobacter* members are often found in environmental marine samples and algal microbiomes (Beiralas et al., 2023). Some of these were detected as part of biofilms on collected floating plastic particles in the Mediterranean Sea (Vaksmaa et al., 2021b), the subtropical gyres of the North Atlantic and the North Pacific (Debroas et al., 2017; Vaksmaa et al., 2022) as well as on plastics incubated in the coastal Caribbean Sea (Goudriaan et al., 2024).

From the early colonizers *Aliiglaciecola, Alteromonas, Marinobacter, Pseudoalteromonas and Vibrio*, *Pseudoalteromonas* (Wright et al., 2021a, 2021b), *Alteromonas* and *Vibrio* (Amaral-Zettler et al., 2020) are furthermore considered to be part of a hypothetical global plastisphere and have been listed as potential plastic degraders (Gambarini et al., 2022).

Furthermore, we detected some HCBs only on specific polymers, suggesting a substrate specificity of these genera. Most prominently, we found *Ketobacter* in high relative abundance on PE, with no difference between UV weathered and non-weathered polymer. Similarly, a high proportion of this genus on PE was previously reported in a 2 year long incubation experiment (Pinto Maria et al., 2022). The species identified as responsible for the high proportion of *Ketobacter* is *K. alcanivorans,* an n-alkanes degrader (Kim et al., 2018). Due to the molecular resemblance between PE and alkanes, it was hypothesized that the metabolic processes responsible for PE biodegradation bear similarities to those involved in breaking down hydrocarbons, particularly alkanes (Restrepo-Flórez et al., 2014). Furthermore, we found that *Microbacterium* only colonized nylon. *Microbacterium* is a genus comprising many species some of which are known human pathogens; in the marine environment they thrive in oil contaminated sites and several strains were described as HCBs or methanotrophs (Schippers et al., 2005; Smit et al., 2021; van Spanning et al., 2022) and a hydrocarbon monooxygenase from *Microbacterium* has been described (Coleman et al., 2012). In plasticDB, *Microbacterium* is not described as a nylon degrader. Instead, it was reported in relation to PE and polyhydroxybutyrate (PHB) degradation (neither of which share large similarities in chemical structure amongst each other or compared with nylon).

Our and previous observations of distinct genera present at elevated abundance on distinct types of polymers indicate substrate specificity and that these genera might potentially be involved in plastic degradation. To analyze more accurately the capability of plastic colonizing microbes and their roles in plastic degradation, we suggest combining metagenomics and metatranscriptomics. This provides a useful overview of the community's global gene pool and gene expression.

5. Summary and conclusion

Plastic pollution creates a novel habitat for microbial colonization in the ocean. This study aimed at unraveling microbial succession from initial colonization to a later stage epi-plastic biofilm on the most important types of plastics in the ocean and the significance of the abiotic factors (UV weathering, biogeography, plastic type and time). Our results reveal a segregation of early and late stage colonizers; the temporal shift in community composition indicates a maturation towards a potential plastic attached core microbial community. Furthermore, location played a significant role in structuring the plastic attached microbial community. We suggest that this is related to abiotic factors such as salinity, temperature, mixing or presence of contaminants differing between the coastal or open water NS station. Though to a lesser degree, the plastic type was also significant in shaping the epiplastic community implying that some taxa select preferentially for a distinct polymer. Together, our results show that the composition of epiplastic biofilms is determined by a combination of several factors which may mask each other thus complicating attempts to determine a (global) plastic core microbiome. Plastics create a new ecological niche colonized by the surrounding sea water microorganisms, whose biome differs across geographical scales. The open niche may thus drive selection of colonizers based on their ecological function, rather than taxonomic identity. High abundances of genera with a metabolic potential for plastic and/or hydrocarbon degradation are a common feature of epiplastic biofilms. Hence, substrate availability (either in the form of the complex plastic polymer or as smaller daughter products) for energy gain and growth may also constitute an important microbial selection parameter.

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CRediT authorship contribution statement

Emna Zeghal: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Annika Vaksmaa:** Writing – review & editing, Validation, Methodology. **Judith van Bleijswijk:** Writing – review & editing, Validation. **Helge Niemann:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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

Raw sequencing data is available under accession number PRJNA1098063. The hydrocarbonoclastic bacteria (HCB) database is accessible via https://doi.org/10.25850/nioz/7b.b.bh. The ASVs and taxonomy data with calculated relative abundances is available at https://dataportal.nioz.nl/doi/10.25850/nioz/7b.b.mh. Scripts to generate treated data and figures are available via the github repository PE462_seq_analysis.

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Appendix A. Supplementary data

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