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Bacterial Community Profiling of Plastic Litter in the Belgian Part of the North Sea

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Supporting Information

ABSTRACT: Bacterial colonization of marine plastic litter (MPL) is known for over four decades. Still, only a few studies on the plastic colonization process and its influencing factors are reported. In this study, seafloor MPL was sampled at different locations across the Belgian part of the North Sea to study bacterial community structure using 16S metabarcoding. These marine plastic bacterial communities were compared with those of sediment and seawater, and resin pellets sampled on the beach, to investigate the origin and uniqueness of plastic bacterial communities. Plastics display great variation of bacterial community composition, while each showed significant differences from those of sediment and seawater, indicating that plastics represent a distinct environmental niche. Various environmental factors correlate with the diversity of MPL bacterial composition across plastics. In addition, intrinsic plastic-related factors such as pigment content may contribute to the differences in bacterial colonization. Furthermore, the differential abundance of known primary and secondary colonizers across the various plastics may indicate different stages of bacterial colonization, and may confound comparisons of free-floating plastics. Our studies provide insights in the factors that shape plastic bacterial colonization and shed light on the possible role of plastic as transport vehicle for bacteria through the aquatic environment.

INTRODUCTION

Plastic debris, an inevitable consequence of living the "Plastic Age", is dominating our oceans and seas and poses a worldwide threat to aquatic wildlife.[1] Floating or drifting plastic creates environmental hazards including the risks of plastic ingestion, starvation, and entanglement of aquatic organisms.[2,3] It also provides novel aquatic vehicles for a wide range of rafting species, such as microalgae, Bryozoa, insects, and even macrobenthos, posing a threat to introduce invasive species.[4−8]

Microplastics are small ubiquitous plastic particles with a diameter <5 mm directly introduced in the environment through the use of plastic microbeads as an ingredient in cosmetics or through wastewater from domestic washing machines, or derived from the fragmentation or degradation of plastic debris.[9−14] Nowadays, laboratory trials and the evaluation of wild species in their natural habitat have shown the ingestion, accumulation, or translocation of microscopic plastic fragments for numerous species, for example, plankton, deposit and filter feeders, crustacean and fish, showing that plastics ubiquitously make their way into the food chain.[16−23] The impact of collateral effects of microplastic ingestion, such as the occurrence of mobilized chemicals in organism's tissues or transfer of potential pathogenic organisms, is still unknown.[24−26] In conclusion, major concerns remain about the ecological risks from (micro)plastics to marine ecosystems, food safety and public health.[14,15]

Not only rafting species are able to colonize plastic as a transport vehicle, but also bacteria live the "Plastic Age". The presence of microorganisms on marine plastic was first documented in 1972, when diatoms of the Sargasso Sea were identified on plastic fragments and rod shaped Gram-negative bacteria were isolated of polystyrene spherules.[27,28] Microbial colonization of these plastic particles in a marine environment

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Table 1. Plastic Properties of Samples Collected at Different Locations Across the Belgian Part of the North Sea[a]

location	number	sampling date	polymer type	litter type	color	detected pigment	depth (m)
SD	BP 1	10/03/'14	PE	beach pellet	blue	/	0.0
	BP 2	10/03/'14	PE	beach pellet	yellow	/	0.0
	BP 3	10/03/'14	PE	beach pellet	white	/	0.0
	BP 4	10/03/'14	PE	beach pellet	black	/	0.0
OO	MPL 1	05/03/'14	PE	monofilament	blue	PB15	8.5
	MPL 2*	05/03/'14	PE	sheet	blue	/	8.5
	MPL 3	05/03/'14	PE	monofilament	orange	/	8.5
	MPL 4	01/09/'14	PE	monofilament	blue	PB15	8.5
	MPL 5*	01/09/'14	PE	monofilament	blue	/	8.5
	MPL 6*	01/09/'14	/	monofilament	white	/	8.5
	MPL 7*	01/09/'14	/	monofilament	black	/	8.5
NP	MPL 8	05/03/'14	PE	monofilament	blue	PB15	6.5
	MPL 9	05/03/'14	PE	monofilament	orange	/	6.5
	MPL 10	05/03/'14	PE	sheet	transparant	/	6.5
	MPL 11	28/08/'14	PE	monofilament	blue	PB15	7.2
	MPL 12*	28/08/'14	PE	monofilament	blue	PB15	7.2
ZB	MPL 13	06/03/'14	PE	monofilament	blue	PB15	6.5
	MOL 14	29/08'14	PE	monofilament	blue	PB15	6.7
	MPL 15	29/08/'14	PE	monofilament	blue	PB15	6.7
	MPL 16	29/08/'14	PE	monofilament	orange	/	6.7
	MPL 17	29/08/'14	PE	monofilament	orange	/	6.7
	MPL 18	29/08/'14	PE	sheet	transparant	/	6.7
ZBbis	MPL 19	29/08/'14	PE	monofilament	blue	/	6.5
	MPL 20	29/08/'14	PE	monofilament	orange	/	6.5
OObis	MPL 21	01/09/'14	PP	monofilament	blue	/	31.3
	MPL 22	01/09/'14	PE	monofilament	orange	/	31.3

[a]*Samples excluded from analysis due to too low sequencing depth; "/" indicates "no information was available for this piece of plastic".

occurs relatively fast. Formation of a microbial biofilm on plastic bags in seawater was detectable after 1 week.[29] Likewise, bacterial colonization of low density polyethylene microplastics occurred after 7 days exposure in coastal marine sediments.[30] Particularly bacteria and diatoms, but also invertebrates were identified as plastic colonizers.[31−33] Study of the composition of bacterial communities on marine plastic litter showed differences from those of the surrounding seawater. Plastic marine debris is therefore suggested as a distinct microbial habitat called "The Plastisphere".[33] Similar results were obtained for microplastic particles in a freshwater environment.[34] Microbial communities on plastics could vary with substrate type, but are also influenced by the season and geographical location.[33,35] Still, the microbial community may reflect its direct environment (seawater, sediment) and research is needed to define the origin and preferences of bacterial families. Characterization of the microbial communities on plastic is essential for several reasons. First, comparison of the microbial community and its surrounding environment (seawater, sediment) is needed to define the origin of bacterial colonization. Second, the response of a bacterial community to environmental factors may help elucidate the drivers of colonization. Third, the capacity to metabolize plastic or plastic-associated chemical compounds as a nutritional source could give certain species adaptive advantages, thus selecting for specific bacterial communities.[36] Fourth, plastic may serve as a transport vehicle for bacteria, including pathogens, that become associated with the biofilm and can be transported to novel environments where they do not normally occur.[25]

Previous studies of the bacterial community on marine plastic litter focused predominantly on particles floating near the sea surface. However, the vast majority of plastic debris accumulates in the sediment, particularly in coastal areas.[30,37,38] For instance, it is estimated that the vast majority of debris entering the North Sea area will eventually sink to the seafloor (70%) while only a minor part keeps floating (15%) or is deposited on beaches (15%).[39] Moreover the major part of the sunken debris (95%) is comprised of plastic in the Belgian part of the North Sea.[40]

In this study, seafloor plastic debris was sampled at five locations across the Belgian part of the North Sea. The bacterial diversity of this marine plastic litter (MPL) was investigated using 16S rDNA sequencing and compared with those of resin pellets found on the beach (here called beach pellets) and bacterial communities of the surrounding (sediment, seawater) and broad (seawater) environment. A large diversity of bacterial community profiles across plastic samples was observed and factors influencing bacterial colonization on plastics were examined. We expected to observe a different bacterial community on plastic compared to the other environmental samples, in which location-dependent environmental factors, plastic-related properties and differences in biofilm formation stages are proposed as the main drivers for plastic bacterial composition differences in the marine environment.

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MATERIALS AND METHODS

Sample Collection. All samples were collected in 2014 at the Belgian part of the North Sea. In March, three sites (NP (51.16°N; 2.71°E), OO (51.22°N; 2.85°E), ZB (51.33°N; 3.13°E)) near the mouths of the Belgian coastal harbors were selected for plastic, sediment and seawater sampling. In August, sampling at these locations was repeated for plastic and sediment collection, and two additional locations were added for plastic collection (OObis (51.45°N; 3.23°E), ZBbis (51.45°N; 2.61°E)). Because only two sampling dates were used for sampling, seasonal variation will not be studied here, and the samples can be seen as independent samples. Additionally, seawater was sampled in June at 14 different locations to create a broader environmental context (Supporting Information (SI) Figure 1).

Plastic fragments located on top of the sediment were collected using a beam trawl equipped with a fine-meshed shrimp net with mesh size of 12 mm and a width of 3 m. Individual plastic pieces (>25 mm) were sorted with sterile forceps, individually placed in a sterile 15 mL falcon tube and immediately frozen at −20 °C.

Per location sediment samples were collected using three replicate Van Veen grabs. The upper (0 to 5 cm) layer of the Van Veen grab content was collected and 40 mL of this sediment was sampled in a sterile 50 mL falcon tube.

Water samples were taken 1 m below the water surface and on the sea floor using a carrousel of six 4 L Niskin bottles. Per replicate 1 L seawater was filtered through a 0.22 μm Millipore membrane filter (Merck Millipore, Billerica, MA; samples March), or through a sterivex filter (Merck Millipore, Billerica, MA; samples June). Per location two surface water and two seafloor water samples were collected. Collected sediment samples and membrane filters were stored at −20 °C.

Resin pellets (<5 mm) found on the beach (here called beach pellets) were collected at the Spinoladijk (SD) in Oostende (SI Figure 1). The beach pellets were picked up with sterile forceps and stored per two (based on color) in a sterile 15 mL falcon tube at −20 °C until further use. Pellets stored together were combined as one sample for further processing.

Sample Characteristics. Physico-chemical characteristics for sediment and seawater samples were recorded per location (Table 1). Plastic properties were categorized based on sampling location or date, plastic shape (monofilament, sheets or beach pellets) and color (Table 1). Raman spectra for polymer identification were recorded using a Bruker Optics "Senterra" dispersive Raman spectrometer with a BX51 microscope. Measurements were performed using a red diode laser (785 nm), an aperture of 50 μm and the 20× objective lens with a spot size of approximately 10 μm on the sample. The system uses a thermoelectrically cooled CCD detector, operating at −65 °C. The instrument is controlled by OPUS software, version 7.2. The power of the laser can be set up to 37 mW at the sample for the 785 nm laser. Number of accumulations, measuring time and laser power were set at 60 times, 30 s and 15.4 mW, respectively, to obtain good signal-to-noise ratio.

Sediment samples were categorized per sampling location and date. Sediment organic matter or total organic carbon (TOC) on the upper sediment layer (0−5 cm) was measured using the "dichromate method".[41] Carbonate content was measured on the same sediment fraction as "loss on ignition".[42] Grain size distribution was estimated using laser diffraction particle sizing and expressed as median grain size. All samples were analyzed using a Malvern Mastersizer 2000G hydro version 5.40.[43] Grain size fractions were determined as volume percentages according to the Wentworth scale.[44] Throughout this study, the clay and silt fractions have been combined as clay/silt (<63 μm) (SI Table 1).

Water samples were categorized per sampling location and date. Environmental properties were measured using the CTD SBE-19plus (SI Table 1).

DNA Extraction and 16S Amplicon Sequencing. DNA of sediment and plastic samples was extracted using the Powersoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Due to a relatively low yield (<5 ng/μL) DNA retrieved from the plastic samples was eluted in 50 μL buffer. If plastic particles were large, a piece of 15 cm (monofilament) or with a surface of 16 cm^2 (sheet) was cut off and used for DNA extraction. Otherwise the total sample was used and only a small fragment was kept for polymer type determination. 250 mg sediment was used for DNA extraction.

DNA extraction of the Millipore filters was done according to the protocol of Zettler et al.[45] The Gentra Puregene kit (QIAGEN, Germantown, MD), lytic enzyme (QIAGEN, Germantown, MD) and proteinase K (QIAGEN, Germantown, MD) were used for DNA extraction, comprising two incubation steps of 37 °C for 30 min and 65 °C for 1 h.

The taxonomic profiles of bacterial communities were determined using next-generation amplicon sequencing of the V3−V4 variable region of the 16S rRNA gene, based on the Illumina 16S metagenomic sequencing library preparation protocol.[46] Adaptor sequences were added to the gene specific primers S-D-Bact-0341-*b*-S-17 and S-D-Bact-0785-a-A-21.[47] The following PCR conditions were used: initial denaturation at 95 °C for 3 min, 25 cycles of 95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s, and a final extension at 72 °C, 5 min. Thirty PCR cycles were used instead of the standard 25 cycles for plastic samples. Dual indices and sequencing adapters were attached using the Nextera XT index kit (Illumina, San Diego, CA) and the same PCR conditions as the first PCR with only eight cycles of denaturation, annealing, and extension. Mastermixes for both PCRs were prepared using the Kapa HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA) according to the manufacturer's instructions to a total volume of 25 μL and 50 μL respectively. After each PCR step, the Highprep PCR reagent kit (MAGBIO, Gaithersburg, MD) was used for cleanup. Quality control of the final library samples was done using the Qiaxcel Advanced using the Qiaxcel DNA High Resolution kit (QIAGEN, Germantown, MD) and concentration was measured using the Quantus double-stranded DNA assay (Promega, Madison, WI). The final barcoded libraries of each sample were diluted to 10 nM and pooled in equal amounts. The resulting libraries were sequenced using Illumina MiSeq v3 technology (2 × 300bp, paired-end) by the Nucleomics Core, Leuven, Belgium.

Processing of the Sequence Reads. The data set was demultiplexed by the sequencing provider and barcodes were clipped off the reads. The raw sequence data is available in NCBI's Sequence Read Archive under the accession number SRA233339. Primers were removed using Trimmomatic v0.32.[48] Different programs of the USEARCH software v7.0.1090 were used for the following steps.[49] Forward and reverse reads were merged using a minimum overlap length of 40 bp (with a maximum of 15 bp differences) and a minimum

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resulting length of 350 bp using the "fastq_mergepairs" program. The resulting sequences were quality filtered using "fastq_filter" with a maximum expected error of 3. Next, sequences of all samples that needed to be compared to each other were merged, dereplicated ("derep_fulllength") and sorted by size ("sortbysize"). UPARSE ("cluster_otus") was used for clustering the reads into operational taxonomic units (OTUs) at 97% identity level.[50] Chimeras were removed using UCHIME ("uchime_ref") with the RDP Gold database as a reference.[51] Finally, sequences of individual samples were mapped back to the representative OTUs using the "usearch_global" algorithm (97% identity) and converted to an OTU table using "biom convert".[52] This procedure resulted in an average of 59,962 sequences per sample with an average length of 420 bp (112 samples in total).

Downstream Data Analysis and Statistics. OTU tables were analyzed with the QIIME software package (v1.8.0).[53] Representative OTU sequences were aligned to the Greengenes[54] 97% core OTU set (v13_8) with a minimum percent identity of 97% using the PyNast algorithm[55] with QIIME default parameters. Rarefaction analyses were performed using an upper rarefaction depth of 20 000 sequences and Shannon−Wiener diversity and Chao1 richness indices were calculated. Based on rarefaction analyses, only samples with a minimal sequence count of 10 000 were retained for further analyses (SI Figure 2). Based on this criterion, data of five seawater samples (location 7 and 10, SI Figure 1) and five plastic samples (MPL2, MPL5, MPL6, MPL7, MPL12, Table 1) were not used in the downstream analyses.

Throughout this study, only OTUs representing at least 0.01% of the total community in at least one sample were used.

ANOVA analyses of the Chao1 richness and Shannon−Wiener diversity indices were done and differences between sampling groups were analyzed using the Tukey HSD test. These analyses and the construction of segmented bar charts were done with the basic R program version 3.1.0.[56]

The R package vegan (version 2.0−10) was used for the multivariate analysis of the data. The betadisper function was used to study the multivariate spread of the data.[57] If multivariate homogeneity of group dispersions was fulfilled, differences between community types were analyzed using PERMANOVA analysis using 4 a priori defined groups according to the sample origin: beach pellets, MPL, seawater and sediment. These significances were further visualized by constructing non-Metric Multidimensional Scaling (nMDS) plots, using the Bray−Curtis index as dissimilarity index. The vegan package was also used to fit environmental variables to the ordination plot (function envfit). *P*-values of the environmental variables were calculated by permutation and only the variables with a significant difference ($p < 0.05$) were fitted on the plot.

To measure similarity in OTU tables between the MPL samples the Jaccard similarity index was calculated. These Jaccard overlaps in pairwise comparisons were displayed as a heatmap (Figure 2).[58]

Corbata was used to search for core sets of OTUs that are shared across a number of plastic samples.[59] To identify core members, the OTUs needed to be present in at least 95% of the samples. In parallel, QIIME was used to define a core microbiome and the OTUs present in each sample, where we varied the minimum abundance. Results of both methods were similar.

RESULTS AND DISCUSSION

Bacterial Community Structure. To investigate the diversity in microbial communities on plastic and to identify drivers of bacterial colonization, we did a random sampling of plastic (MPL and beach pellets) at the seafloor. Initially, we aimed to compare them to the bacterial communities of their surrounding environment, that is, seawater and sediment sampled at the same location and time, which could be in contact with the plastic during the period of sampling and could serve as potential sources of bacterial colonization. However, plastic is mobile and can be transported through ocean currents over longer distances, but also over smaller areas, like the North Sea.[60−62] Therefore, the surrounding environment could be limited, or even inappropriate, as reference, and we expanded the comparison of plastic and environment communities by sampling seawater at 14 different locations across the Belgian part of the North Sea.

Complexity and composition of the bacterial communities of the four different sample types (beach pellets, MPL, seawater, sediment) were analyzed. Bacterial community complexity was investigated by estimating the total number of observed species (rarefaction analysis) and estimation of the bacterial richness (Chao1 index) and diversity (Shannon−Wiener diversity index). At 10 000 sequence counts, rarefaction curves showed an average of 295, 535, 1031, and 1688 different OTUs of beach pellets, seawater, MPL, and sediment, respectively (SI Figure 2), indicating variance in the number of unique species between sample types. Significant differences in the Chao1 richness (ANOVA, *p*-value: 1.22×10^{-6}) and Shannon−Wiener diversity (ANOVA, *p*-value: 2.47×10^{-8}) indices, proved community complexity differences between the four sample types. Bacterial richness and diversity were significantly different between all sample types, with the exception of seawater communities, which showed similar diversity as those of MPL (Tukey range test, *p*-value: 0.92) and beach pellets (Tukey range test, *p*-value: 0.08) and similar richness values (*p*-value: 0.98) as sediment communities. The highest community richness and diversity was measured in the sediment, after which MPL contained the second most diverse community (SI Table 2).

To study differences in community composition between samples, taking into account the taxonomy and relative abundances of the species, we did a non-Metric Multidimensional Scaling (nMDS) analysis (Figure 1). Beach pellets, MPL, seawater and sediment bacterial communities showed a separate clustering in the nMDS plot, with no observed overlap between the sample types, indicating differences in bacterial community composition (Figure 1). Significant differences in Bray−Curtis dissimilarity indices (PERMANOVA, $p < 0.001$) confirmed the separation of community composition profiles, although these differences in dissimilarity could partly be caused by a considerable difference in multivariate spread (permutation based, $p < 0.001$).

Differences in observed number of species and the separate clustering of sample types in the nMDS plot show a clear distinction between bacterial communities of MPL, beach pellets, sediment and seawater (SI Figure 1, Figure 1). nMDS analysis further showed that the bacterial communities of seawater sampled in June, representing the "broad" environmental scan across the Belgian part of the North Sea, clustered together with seawater sampled at the time and location of plastic sampling (surrounding environment) and separately

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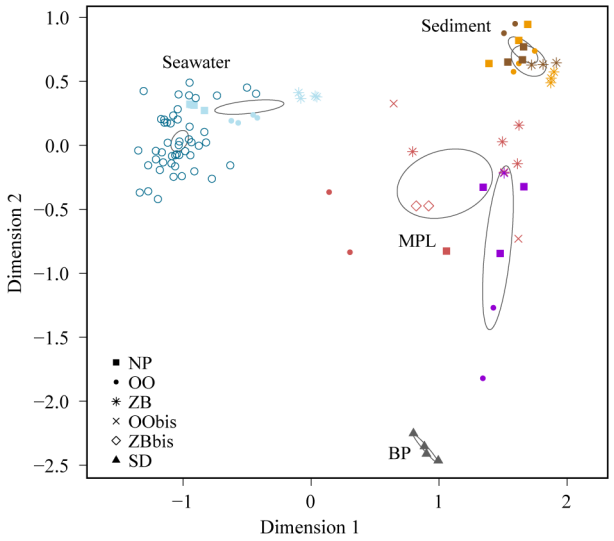

Figure 1. Non-Metric Multidimensional Scaling (nMDS) profile of pairwise community dissimilarity (Bray–Curtis) indices of 16S sequencing data of samples collected across the Belgian part of the North Sea (Dimensions: 4; Stress: 0.095). 95% confidence ellipses were constructed for each sample type. Shape represents different sampling locations. Seawater samples are indicated in light (sampled in March) and dark blue (sampled in June). Sediment samples are indicated in yellow (light: March, dark: August). MPL sampled in March and August are colored in red and purple, respectively. BP: Beach pellets.

from the other sample types. This indicates that bacterial communities colonizing plastic substrates are markedly different from the bacterial communities of seawater and sediment, both in the surrounding as "broad" environment, and irrespective of the time of sampling. The substantial variation observed within sample types may still be caused by spatiotemporal dependent factors, such as environmental parameters, as discussed further below. Taken together, our results denote MPL as a distinct microbial niche in the marine environment, called the "Plastisphere", and further confirm previous results of Zettler et al., who showed a difference in community composition between floating plastic debris and the surrounding seawater.[33]

Origin of MPL Bacterial Communities. To determine how MPL samples obtain their characteristic bacterial profiles, and to identify potential sources of bacterial colonization, we compared the taxonomic profiles on phylum and family level between the sample types (SI Figure 3 and SI Figure 4). A high variability between plastic bacterial communities of MPL and beach pellets was observed (nMDS plot), by which we decided to compare individual MPL and beach pellets taxonomic profiles (Figure 1). Community profiles of seawater and sediment samples were grouped per sampling location and date. Strikingly, most of the bacterial families found on MPL were also found in seawater and/or sediment, but with clear differences in relative abundances, causing accordingly part of the separation in sample types in the nMDS plot (SI Figure 4). This indicates the role of the marine environment serving as a bacterial source for plastic colonization. This role is further illustrated by the distinct bacterial profile found on beach pellets. The difference between beach pellets and other sample types was caused mainly by the relatively high abundance of Actinobacteria on beach pellets, whereas Proteobacteria dominated the other sample types (SI Figure 3, SI Figure 4). Contrary to MPL, beach pellets were sampled on the beach, that is, an intertidal environment with influences of both aquatic and terrestrial environments, explaining the high abundance of Actinobacteria.[63]

Besides the shared bacterial families between MPL and their surrounding environment, certain bacterial groups, for instance the Vibrionaceae or Pseudoalteromonadaceae, are commonly detected on MPL but barely observed in seawater and sediment communities (SI Figure 4). It is expected that foreign bacteria well-adapted to MPL properties could attach to the surface and travel with the plastic particle.[25] In addition, plastic can originate from different land- and sea-based sources,[60−62] each with their natural occurring bacterial communities. We expect that if the affinity for the plastic material is high enough, microorganisms could stay attached on the MPL despite changing environments. In that way not only the marine or aquatic environment (and changes thereof) can shape the community, but also the plastic's transportation history is important in the bacterial colonization process.

Because we found similar families on the different MPL samples, we tried to establish a core microbiome, hypothesizing that these species play a major role in the plastic colonization process and/or in plastic degradation. However, to obtain core members, minimum OTU abundances need to be set at a very low percentage (0.01%), resulting in thirty-six OTUs that could be defined as "core organisms". Conversely, together these only represent approximately 18% of the total number of OTUs, showing that the vast majority of the OTUs is not common. Strikingly, the high diversity in community profiles across our set of plastics precludes the identification of core microbiomes, in contrast to previous studies on fewer samples.[33] Therefore, we investigated three aspects that potentially drive bacterial colonization and may explain the observed microbial diversity on MPL. First, environmental parameters such as physicochemical properties (temperature, oxygen, salinity) may affect the plastic bacterial community. Second, bacterial communities on plastics may be in different stages of biofilm formation, as biofilm formation is a dynamic process. Third, physicochemical properties of the plastic itself may affect attachment of bacteria, either as a solid hydrophobic surface to anchor bacteria or by providing selective nutritional resources for metabolic degradation by specific species.

Evironment-Related Properties. First we established whether any spatiotemporal structure could explain the diversity in bacterial communities, and then analyzed whether this structure overlaps with variance in environmental parameters at the location and time of sampling. Relatedness between the taxonomic profiles of the 17 MPL samples was visualized in a heatmap, using the Jaccard similarity index for pairwise comparisons. Samples were ordered according to sampling location to visualize whether samples with a high fraction of shared OTUs were derived from the same location.

The highest number of OTUs and the highest number of shared OTUs were mainly detected in the samples of ZB, which indicates that location-related properties influence the bacterial composition of MPL. For each location, several environmental parameters were measured and considered as possible factors influencing the bacterial colonization of plastic (SI Table 1). We constructed an nMDS plot of the MPL samples, to which the correlation with significant (p-value <0.05) environmental data of seawater and sediment was fitted (Figure 3; SI Table 1). Differences in salinity, temperature, oxidation reduction potential, turbidity, oxygen content, and density of the

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seawater, and the total organic carbon and inorganic carbon content of sediment appear to be correlated to the diversity in MPL bacterial communities. Temperature, oxygen content and ORP seem to be correlated with sampling date, because higher temperature and ORP and a lower oxygen content were measured in August compared to March. This could explain the slightly separate clustering of the samples taken at the different time periods (Figure 3). The influx of freshwater from the Rhine/Meuse and the Scheldt Estuary by horizontal dispersion, lowers the seawater salinity in the ZB region, which could explain the observed correlation of salinity to the bacterial structure, and the discrimination between locations (Zeebrugge vs others) in the heatmap (Figure 2; Figure 3).[64] In marine

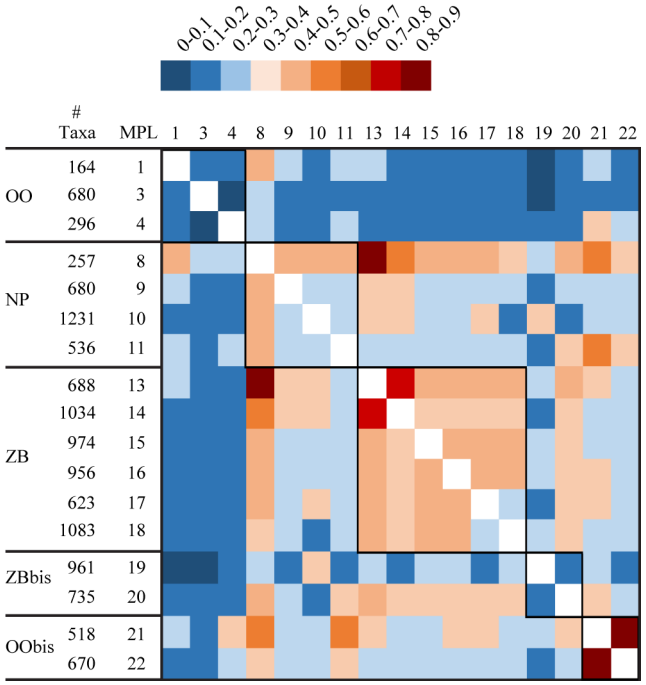

Figure 2. Heatmap construction of the MPL samples (Table 1). Jaccard similarity indices were calculated for all plastic pairs, representing the fraction of shared OTUs between MPL samples. Similarity in OTUs between samples is indicated by a color scheme (blue: low amount of shared OTUs, red: high amount of shared OTUs). Location of sampling and total number of OTUs that represents more than 0.01% of the sample are indicated next to the heatmap.

environments the median grain size of the sediment, together with other factors such as phytoplankton blooms, amount of suspended organic material and marine snow, determine the turbidity. A smaller median grain size and a high rate of dredged material deposition will lead to more cloudy water. Turbidity was therefore highest in coastal areas, especially ZB, which contains a lot of sludge and dredged material. In addition, the small median grain size and high organic content measured in ZB, makes these sediments more susceptible to environmental pollution,[18,65] which could have a complementary effect on the bacterial profile and provide an alternative explanation of the clustering of the ZB samples (Figure 2).

Biofilm Formation Stages. The hydrophobic surface of plastics promotes microbial colonization and biofilm formation. Biofilm formation is a dynamic process and the taxonomic composition changes over time.[30,33] Therefore, we investigated

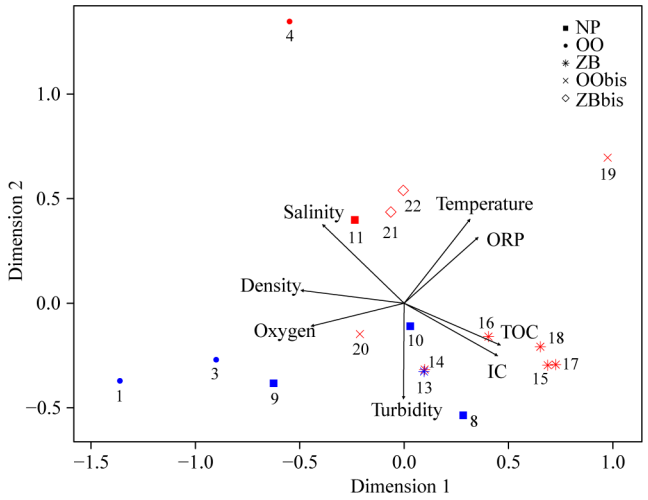

Figure 3. Correlation of environmental variables and the nMDS profile of pairwise community dissimilarity (Bray–Curtis) indices of 16S sequencing data of the MPL samples (Dimensions: 4; Stress: 0.053). Only those environmental parameters of seawater and sediment that were significantly different (p-value <0.05) between samples were fitted to the plot, where the length of the arrow is proportional to the correlation.

whether variation in biofilm formation stages may explain at least part of the variation in MPL bacterial composition.

MPL community profiles showed a dominance of Proteobacteria and Bacteroidetes (SI Figure 3). Previous studies have shown that Alpha- or Gammaproteobacteria are characteristic for primary biofilm colonization in the marine environment and Bacteroidetes act as secondary colonizers, as their abundance increases over time.[63,66,67] It is important to note that the actual age of the biofilm on our plastic samples could not be established, due to the unknown history of randomly sampled free-floating plastics. Instead, we estimated the relative abundances of Alpha- and Gammaproteobacteria and Bacteroidetes for each sample, and used these as putative signatures of biofilm formation stages (Figure 4). Three different groups could be discriminated in the sample set: MPL samples where Proteobacteria classes dominated, samples where the abundance of Proteobacteria classes and Bacteroidetes were similar and samples where Bacteroidetes dominated (Figure 4). With the exception of MPL1, the plastics with a low number of OTUs (MPL3, MPL4, MPL9) had the highest number of Proteobacteria classes (Figure 2; Figure 4), suggesting that these plastics display characteristics of early stages of biofilm formation, whereas the others could represent later stages of biofilm formation. This implies that at least part of the large variation in bacterial composition across our plastic samples could be explained by differences in stages of biofilm formation, possibly due to varying exposure times of the plastic to the marine environment. To confirm this observation, and to be able to "map" bacterial profiles onto a microbial biofilm developmental time scale, we have initiated a controlled exposure experiment using long-term time series at a fixed location to study formation and maintenance of microbial biofilms.

Chemical Composition of Plastics. We investigated whether factors inherent to the plastic, such as polymer type (e.g. polyethylene or polypropylene), plastic shape (monofilament, sheet) and presence of pigment dyes could explain part of the diversity of microbial colonization of plastic particles.

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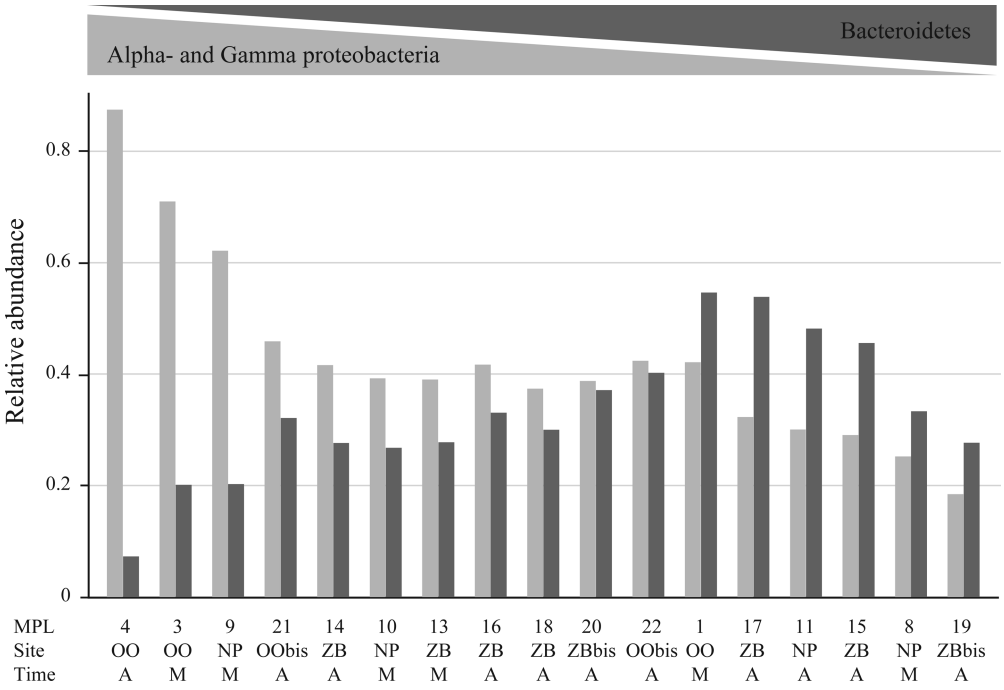

Figure 4. Representation of the primary (Alpha- and Gammaproteobacteria) and secondary (Bacteroidetes) biofilm colonizers in a marine environment. MPL were ordered according the relative abundance of the Proteobacteria and Bacteroidetes classes. (A) Dominance of Proteobacteria classes, (B) Similar amounts of Proteobacteria classes and Bacteroidetes, and (C) Dominance of Bacteroidetes.

Because 16 polyethylene samples but only one polypropylene sample were obtained, the influence of polymer type on the bacterial community profile could not be separated from the other factors (Table 1; SI Figure 5; SI Figure 6). No clear difference in bacterial profile was observed between the polypropylene sample and the polyethylene samples and even a high number of shared OTUs between the polypropylene sample (MPL21) and the most closely related polyethylene sample (MPL22) was observed (SI Figure 4; Figure 2). This is in contrast with previous observations of Zettler et al., who showed differences in microbial communities between three polyethylene and three polypropylene samples.[33] Next, the other plastic properties were fitted to the nMDS plot described above, like for the environmental factors (Table 1; Figure 3). No correlation of any of these parameters was found with the variation in MPL bacterial communities.

The capacity to metabolize polyethylene or polypropylene polymers as carbon source could give certain bacterial species an adaptive advantage. Likewise, we hypothesized that dyes incorporated in the plastic could attract specific bacterial species with the capacity to metabolize those components. One species of the Mycobacteriaceae, *Mycobacterium frederiksbergense*, caught our attention, because its high abundance (21−29%) on the yellow and blue colored beach pellets, whereas it could barely be detected on the other pellets and plastics. Notably, *M. frederiksbergense* is known for its degrading capacity of diverse polycyclic aromatic hydrocarbons like anthracene.[68,69] Anthracene is mainly used for the production of anthraquinone, a precursor for dye synthesis.[70] Several patents describe the use of anthraquinone derivatives for coloring resin pellets,[71−73] which suggests the presence of anthracene derivates on the blue and yellow beach pellets and could explain the high abundance of *M. frederiksbergense*. While black and white pellets were abundant at the time and location of sampling at the Spinoladijk, blue and yellow pellets were rather rare and the material collected was sufficient for bacterial taxonomic profiling, but not for chemical profiling. White and black pellets, however, were also used for chemical profiling, revealing very low levels of anthracene on these beach pellets, as expected for uncolored resins.[66] For now, the observation of *M. frederiksbergense* on blue and yellow beach pellets, the documented use of anthraquinone derivatives as pigments for blue and yellow resin pellets, taken together with the putative capacity of *M. frederiksbergense* to metabolize anthracene derivates as carbon source, indeed suggest that presence of dyes and adsorbed chemicals, or perhaps pollutants, could influence MPL bacterial colonization. Parallel studies in our laboratory identified more than 250 different chemical compounds on plastic debris (synthetic rope and sheets) of the Belgian part of the North Sea,[67] indicating that the relationship between chemical profile and bacterial colonization may be quite complex and requires large numbers of samples to capture both the chemical and taxonomic diversity.

Taken together, our results indicate that environmental parameters can influence the plastic bacterial community by serving as a bacterial source for plastic colonization. We expect that next to the observed influence of environmental parameters like salinity, temperature, oxygen levels and possibly pollution, and the influence of biofilm formation stages, also pigment content and adsorption of chemicals play a role in the microbial colonization process. More research however is needed to disentangle all separate influences on the microbial population of plastic and to discriminate between the relative roles of drivers of microbial colonization of plastic.

Plastic can act as vehicle for a wide range of rafting species[4−8] and our results show that also bacteria can use plastic as transport vehicle and survive in environments where they are normally not detected. This could have major ecological impacts, because pathogens or invasive species can thus travel to other environments, changing the original ecosystem.

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Knowledge of the factors shaping the plastic biofilms that determine their taxonomic constitution and metabolic properties may help to identify species that can potentially degrade the plastics and mitigate the problem of plastic pollution.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b01093.

SI Figure 1 shows the sites used for plastic, sediment and/or seawater sampling. SI Figure 2 shows the rarefaction analysis per sample type. SI Figures 3 and 4 represent taxonomic plots of the most important bacterial phyla and families, respectively of seawater, sediment and plastic sampled in March and August. SI Figure 5 and 6 represent the Raman spectra of samples MPL1 and MPL21 compared to the reference spectra for polypropylene (PP), polyethylene (PE) and a pigment of the Phthalo Blue family. SI Table 1 provides environmental data of sampled sediment and seawater. SI Table 2 provides the chao1 and Shannon−Wiener diversity indices for each sample (PDF)

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Notes

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