Analytical Methods

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A review of microscopy and comparative molecular-based methods to characterize "Plastisphere" communities†

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Plastic is currently the most abundant form of debris in the ocean. Since the early 70's, investigators have recognized the presence of life such as pennate diatoms, bryozoans and bacteria on plastic debris, sometimes referred to as the "Plastisphere". This review provides an overview of molecular and visualization techniques used to characterize life in the Plastisphere, presents a new data portal located on the Visual Analysis of Microbial Population Structures (VAMPS) website to illustrate how one can compare plastic debris datasets collected using different high-throughput sequencing strategies, and makes recommendations on standardized operating procedures that will facilitate future comparative studies.

Introduction

Since its first mass production in the 50's, the use of plastics has been growing steadily. Currently, more than 300 million tons of plastic are produced worldwide each year.[1] Plastic's durability has led to its persistence in the environment and its role as a common environmental pollutant. By the early 1970s, plastic began appearing alongside plankton in oceanographic sampling nets.[2,3] Now plastic is the most abundant form of debris in the ocean, reaching an estimated number of 5.25 trillion particles scattered over the oceans and seas.[4] A few years after Carpenter and Smith[2] reported diatoms, bacteria, and hydroids on the surfaces of plastic debris (PD) collected in the Sargasso Sea, Sieburth[5] noted the ubiquity of microbial colonization on man-made surfaces including high-density polyethylene (HDPE) plastic like bleach bottles in his book "Microbial Seascapes" (Fig. 1A and B). These Scanning Electron Microscopy (SEM) images of pennate diatoms, filamentous cyanobacteria, coccoid bacteria, and bryozoans were perhaps the first published glimpses of what has been referred to as the "Plastisphere"[6] – the diverse assemblage of taxa that inhabit the

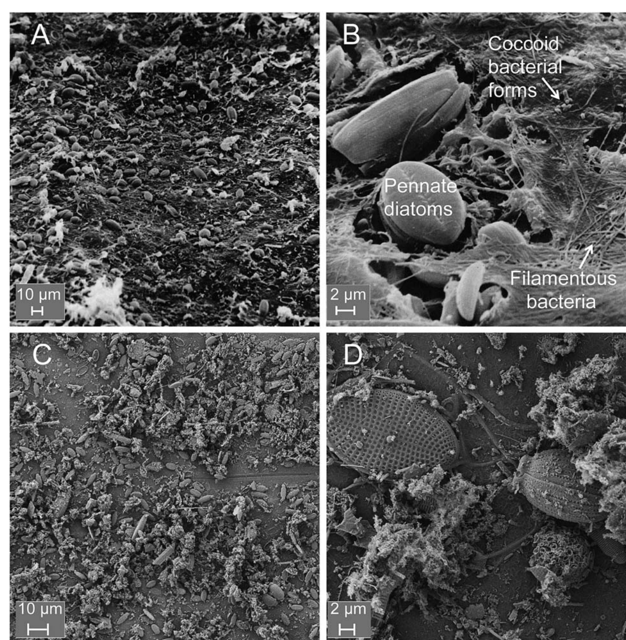

Fig. 1 Images A&B: SEM images of microbial communities on plastic surfaces first recorded by Sieburth.[5] Panel A shows diatoms colonizing the surface of a bleach bottle in Narragansett Bay, Rhode Island, USA. Panel B shows a higher resolution view of the same microbial biofilm. Images C&D: SEM images of similar communities on polyethylene samples in waters off Grenada in 2014.

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thin layer of life on the outer surface of PD.[6] In terrestrial, lacustrine, and marine settings, PD is widespread and carries with it a collection of diverse life worthy of study at multiple scales and varying approaches.

The hard surface of plastics provides a suitable environment for microbial colonization, but several marine physicochemical factors influence microbial succession. Dang and Lovell[7] were among the first to explore the dynamics of early-stage bacterial colonization on plastic plates top-coated with different polymers of varying degrees of hydrophobicity in the marine environment (polyethylene glycol (PEG-S and PEG-L), polyvinyl alcohol (PVA), and bis(2-hydroxyethyl ether) of tetrabromobisphenol (BR)). Within the Rhodobacterales, the *Roseobacter* clade appeared as a primary surface colonizer in temperate coastal waters.[7,8] Several studies that followed, employing a diversity of plastic substrates, revealed that diverse microbial biofilms colonize PD after initial colonization.[9–11] The amount of biomass increased with exposure time of the plastic to seawater or marine sediment in both fossil fuel-based and biodegradable/compostable plastic.[12] Lab experiments showed that the microbial cell densities were five to eight times higher on biodegradable plastic bags than on polyethylene (PE) bags when both were buried in sediments.[13] In addition to hydrophobic surface properties,[14] surface roughness (rugosity) of the plastic material also impacts colonization.[15–17] Geographical location, season, and polymer type also influence the formation of biofilms on plastic substrates in aquatic environments.[12,18–22] In freshwater ecosystems, location is the major factor influencing biofilm composition, with polymer type being a secondary factor,[20] a pattern also detected at global scales in the marine environment.[22] Given the impact of geography and season on the colonization of plastic substrates, it is challenging to compare studies across time and space.

To study the structure and composition of biofilms formed on PD, exclusive use of SEM is not sufficient. Therefore, researchers have used a variety of molecular methods from genetic fingerprinting techniques to high-throughput sequencing, as well as light microscopy coupled with Fluorescence *In Situ* Hybridization (FISH) to visualize and study the living community on PD. In this review, we present an overview of different microscopy and molecular methods and their suitability to visualise and study PD microbial communities in aquatic environments. Based on the validation of the different methods, we propose a standardized method to investigate microbes on PD to facilitate comparability of different studies. Furthermore, we present a new data portal located on the Visual Analysis of Microbial Population Structures (VAMPS: http://vamps.mbl.edu [23]) website called the Plastisphere Portal to illustrate how one might compare datasets collected using different high-throughput sequencing strategies.[6,22,24] This portal will aid in comparative studies of microbial communities specifically on PD, from a variety of environments both aquatic and terrestrial.

Visualising the Plastisphere through microscopy

To visualize the composition and the spatial structure of microbial communities on plastic, SEM still offers a useful first-hand look at bacteria, diatoms and other protists, and small invertebrates on plastic surfaces.[6,11,12,17,21,25] For example, SEM observations enabled the detection of microbes sitting in pits and grooves, suggesting degradation of the plastic surface.[6,12,25] However, while SEM offers a detailed look at life on the surface of plastic litter, it is limited in terms of the taxonomic resolution recovered from this approach. Apart from morphologically distinct protists such as diatoms and select filamentous cyanobacteria, the ability to discern different groups of microbes to the species level is extremely difficult if not impossible using SEM alone. In addition to being costly and labor-intensive, SEM suffers from a present lack of automation and challenges associated with data interpretation and quantification.

To investigate microbial communities on PD with greater taxonomic detail, epifluorescence microscopy coupled with the application of phylogenetic probes *via* FISH[26] has the potential to facilitate our understanding of how microbes interact with each other on PD. An example of FISH applied to a microbial community residing on the surface of low-density polyethylene (LDPE) pieces incubated for 1 month in July 2014 at the Marine Biological Laboratory dock in Woods Hole, Massachusetts, USA, is shown in Fig. 2. Alexa-488 labelled universal bacterial oligonucleotide probe EUB-338 (http://www.microbial-ecology.net/probebase) fluorescing green and chlorophyll fluorescing red with all cells counterstained with 4′,6-diamidino-2-phenylindole (DAPI binding to any double stranded DNA) reveals a spatial structure of filamentous and individual bacteria, as well as diatoms showing a dense biofilm with heterogeneous distribution of bacterial and eukaryotic cells. FISH has several advantages: (1) it provides taxonomic information about the community composition on the plastic surface; (2) shows the spatial structure of the community on PD; and (3) has the ability to provide actual abundance data *versus* relative abundance information for the cells being targeted. The investigation of the spatial distribution of microbes can elucidate the physiology and interactions of microbial communities because the function of biological systems is often associated with its structure.[27]

However, a potential challenge with FISH methods is that phylogenetic probes target and hybridize with ribosomal RNA (rRNA) in the cell. For communities that are not growing actively, the fluorescent signal may be weak, leading to overlooked taxa. Thus, different techniques are required to enhance the fluorescent signal such as catalysed reporter deposition FISH (CARD-FISH),[28] next-generation *in situ* hybridization chain reaction (HCR)[29] or a nested FISH approach using multiple probes. However, applying signal enhancement techniques can increase the complexity of a method. Harrison *et al.*[11] successfully performed CARD-FISH to investigate the bacterial community on LDPE exposed to different types of coastal marine sediments. They localized the genera *Arcobacter* and *Colwellia* on their PD samples using genus-specific probes. Additionally, they compared the spatial structure of these genera with the distribution pattern of all bacteria on their PD samples by staining all cells with DAPI. They showed that *Colwellia* spp. populations were site-specific on their samples, although the microbial biofilm was equally distributed on PD.

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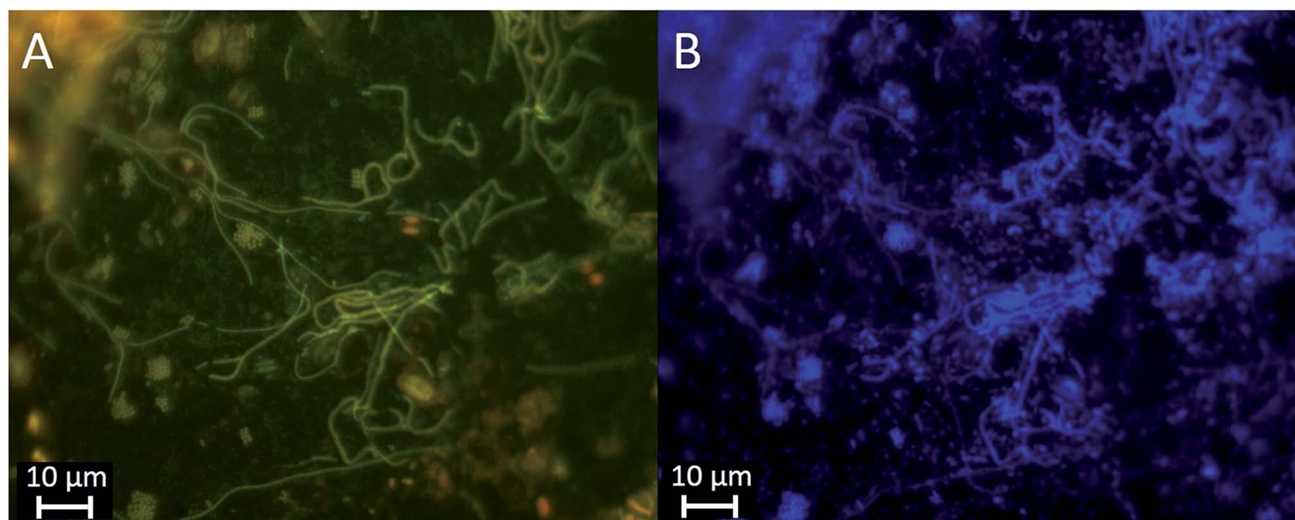

Fig. 2 Application of FISH to Plastisphere communities colonizing a piece of LDPE plastic incubated *in situ* off Woods Hole, MA for 1 month in July 2014. Image A shows results of FISH probing using probe Eub-338 hybridizing to filamentous and individual bacteria on the surface (some red fluorescence from chlorophyll in eukaryotic diatoms is also visible). Image B is the same field of view showing DAPI staining of DNA.

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An additional limitation of FISH techniques is that they require an investment in fluorescently-labelled probes that presupposes molecular knowledge of the communities to be interrogated with these techniques. For instance, Harrison *et al.*[11] used clone libraries in combination with Sanger Sequencing and Terminal Restriction Fragment Length Polymorphisms (T-RFLP) to identify the taxonomic affiliations of the microbes before they applied CARD-FISH. Until recently, FISH also suffered from a limitation in the number of different fluorophores (up to 3) that could be used at one time. This limits the number of taxa that can be identified in each sample. However, a new FISH technique called the Combinatorial Labelling and Spectral Imaging FISH (CLASI-FISH) method might overcome this problem[27] by using many different fluorophores (6 or more) in parallel. A given taxonomic group can be labelled with a combination of different fluorophores (2–3) simultaneously, and these combinations of fluorophores expand the number of taxa that can be labelled in a single sample. With this approach the number of distinguishable microbes can be greatly expanded, theoretically up to hundreds of different microbes in a single FISH experiment, depending on the number of fluorophores used. The fluorophores can be distinguished by using a combination of spectroscopy with microscopy and statistical analysis of digital fluorescence microscopy images. This method was successfully applied to human oral microbiomes[30] and is being tested and applied to PD, which presents an exciting new avenue for future work.

Comparative molecular methods to study PD microbial community composition

Within ecological studies, two central questions are typically asked regarding microbes: (1) What kind and how many microorganisms are present?; and, (2) How do these microorganisms interact and how do their activities relate to ecosystem functions?[31] The study of the microbial community on PD is relatively young and underexplored. Therefore, research is still focused on the first question related to diversity: Which microorganisms are present on PD, what influences this colonization and how stable is the community?

Cultivating microorganisms in the laboratory enables identification using Sanger sequencing of rRNA genes and other molecular markers, physiological characterization and ecological experimentation. However, greater than 99% of the microorganisms in any environment are not cultivable by standard cultivation techniques,[32] leaving the bulk of the microbial community unexplored. With the introduction of culture-independent methods, it became possible to explore the uncultivable microbial majority in the environment.

Molecular methods that have been previously used to characterize microbial communities on PD in aquatic environments include: clone libraries, Denaturing Gradient Gel Electrophoresis (DGGE), T-RFLP and high-throughput sequencing. These techniques are based on the polymerase chain reaction (PCR) and therefore, subject to PCR-bias, and thus unable to provide information on absolute species abundances. The advantages and disadvantages of these different molecular methods are summarized in Table 1. Table 2 summarizes the results of PD studies using these different molecular methods and/or visualization tools.

Based on published research, SEM is the most commonly used method to visualize microbial communities on PD. While no one molecular technique has dominated attempts to characterize the individual species on the plastic particles (Table 2), high-throughput methods are gaining momentum as the method of choice for profiling Plastisphere communities.

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Table 1 Advantages, disadvantages, and characteristics of the different molecular methods used to characterize microbial communities on PD

Method	Advantages	Disadvantages	Taxonomic level	Yield (% of community screened)	16S rRNA gene region fragment length	Number of samples simultaneously screened
Clone libraries	Good taxonomic/phylogenetic assignment (complete 16S rRNA gene)	Labor intensive	Species	<5%	±1500 bp	1
	Combination with ARDRA yields information on bacterial community structure	Time consuming Sample size limited, only minor part of bacterial diversity characterized				
DGGE	Fast	Minor part of bacterial diversity identified	Order/family/genus	1–2%	<500 bp	10–16
	Multiple samples analyzed simultaneously	Overestimation of diversity also possible as one bacterium can contain multiple copies				
	Comparative analysis Possible	Limited phylogenetic information One band can represent multiple species, due to similar melting temperature PCR biases				
T-RFLP	Fast	No assignment to a certain taxonomy	None	?	<500 bp	>10
	Multiple samples analyzed simultaneously	Underestimation of species diversity				
	Comparative analysis possible Robust community diversity index	PCR biases				
High-throughput sequencing: Amplicon sequencing	Low labor intensity and cost per base pair Multiple samples analyzed simultaneously	Limited sequence information for taxonomic assignment PCR biases	Order/family/genus	Up to 100%	<600 bp (Illumina)	100 or more
	Fast	High computational and analysis demands Requires expensive instrumentation				
High-throughput sequencing: Shotgun metagenomics	Multiple samples analyzed simultaneously Functional information	Need for high storage and computational capacity Requires expensive instrumentation and advanced bioinformatics skills	Order/family/genus	Less than 100%	<500 bp (Illumina)	10–12

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Table 2 Different methods used to study microbial composition of plastics in aquatic habitats ([a] Polyethylene, [b] Polypropylene, [c] Polystyrene, [d] Polyethylene terephthalate, [e] Polyvinyl alcohol, [f] Polyvinyl chloride)

Method	Plastic size	Geographic location	Habitat/laboratory	Plastic types	References
SEM	Microplastic	North Atlantic Ocean	Marine offshore	PE[a], PP[b]	Zettler *et al.*[6]
		North Pacific Gyre	Marine offshore	PE, PP, PS[c]	Carson *et al.*[17]
		Lab test	Microcosm experiment	PE	Harrison *et al.*[11]
		Australia-wide coastal and ocean region	Marine coastal waters and offshore	Unidentified	Reisser *et al.*[25]
		Urbanized river in Chicago, USA	River water	Unidentified	McCormick *et al.*[58]
		North Pacific and North Atlantic Ocean	Marine offshore	PE, PP, PS, PET[d], other	Amaral-Zettler *et al.*[22]
	Macroplastic	Mediterranean Sea	Marine offshore	PE, biodegradable plastic	Eich *et al.*[12]
		North Sea, UK	Marine coastal waters and offshore	PET	Oberbeckmann *et al.*[21]
CARD-FISH	Microplastic	Lab test	Microcosm experiment	PE	Harrison *et al.*[11]
Clone libraries	Microplastic	Lab test	Microcosm experiment	PE	Harrison *et al.*[11]
	Macroplastic	West Pacific Ocean, Qingdao	Marine coastal waters	PVA[e], PVC[f], unknown	Dang & Lovell;[7] Dang *et al.*[8]
DGGE	Micro- and macroplastic	North Sea, UK	Marine coastal waters and offshore	PET, PS, PE, PP	Oberbeckmann *et al.*[21]
T-RFLP	Microplastic	Lab test	Microcosm experiment	PE	Harrison *et al.*[11]
Amplicon sequencing	Microplastic	North Atlantic Ocean	Marine offshore	PE, PP	Zettler *et al.*[6]
		North Pacific Ocean, North Atlantic Ocean	Marine offshore and coastal	PE, PP	Amaral-Zettler *et al.*[22]
		Coast, BE	Beach	PE	De Tender *et al.*[24]
		Urbanized river in Chicago, USA	River water	Unidentified	McCormick *et al.*[58]
	Macroplastic	North Sea, BE	Marine coastal waters and offshore	PE, PP	De Tender *et al.*[24]
		North Sea, UK	Marine offshore	PET	Oberbeckmann *et al.*[57]
Shotgun metagenomics	Micro- and macroplastic	North Pacific Subtropical Gyre	Marine offshore	Unidentified	Bryant *et al.*[59]

Clone library construction and Sanger sequencing

The 16S rRNA gene is by far the most commonly used marker gene for taxonomic classification of environmental DNA samples. The use of this gene has several benefits: (1) it is present in all domains of life; (2) it follows the evolutionary trajectory of a species; and, (3) has the largest known reference database of sequence data for comparative purposes.[33] These characteristics are among the reasons Woese[33] referred to rRNA as the "ultimate molecular chronometer". With the construction of clone libraries, the 16S rRNA gene can be sequenced completely or partially through Sanger sequencing. Complete 16S rRNA gene sequence data provide for superior taxonomic/phylogenetic assignments and in most cases, bacteria can be classified to the species level. However, making clone-libraries is labor-intensive and time-consuming and therefore most studies sequence less than 1000 clones in a given library.[31] Consequently, only a minor part of the complete community richness is screened, typically less than 5%.[34] Dang and Lovell[7] were the first to examine bacterial communities on plastic plates using clone libraries. In general, individual gene fragments are cloned, sequenced and compared to a known sequence database, *e.g.* GenBank, Ribosomal Database Project (RDP) or Greengenes[35] and used to infer phylogenetic placement of unknown environmental sequences.

The construction of clone libraries can be used in combination with amplified rRNA gene restriction analysis (ARDRA) to screen the clones and measure bacterial community structure. The PCR-amplified 16S rRNA fragments are digested and cut at specific sites with restriction enzymes. The resulting digest is then separated by gel electrophoresis. The combination with ARDRA is often done to study microbial diversity because it is simple, rapid, and cost-effective.[36] Dang and Lovell identified 26 and 121 clones in an initial and subsequent study, respectively, most of which fell into the Proteobacterial phylum.[7,8]

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Genetic fingerprinting techniques

DGGE and T-RFLP are genetic fingerprinting techniques (Table 1) that make use of PCR products amplified from environmental DNA.[37] Fingerprinting techniques have the advantage that they are fast and target multiple samples at the same time, which makes comparative analyses between samples possible. However, both DGGE and T-RFLP are limited in that they only capture 1–2% of the total microbial population in an environment (Table 1).[38]

Oberbeckmann *et al.*[21] first introduced DGGE to study the bacterial community on polyethylene terephthalate (PET) bottles, revealing that microbial communities on PD vary both with season, geographical location and substrate type. In DGGE, sequences are separated according to their melting properties and provide a unique band profile.[39,40] To determine the phylogenetic identities of PD colonizers, selected bands can be excised from the gel, re-amplified and sequenced. These sequences can then be compared to the sequences from nucleotide databases.[41,42] The sequence lengths recovered from DGGE are typically limited to 500 bp. Taxonomic assignments are not quantitative, nor always accurate, as one band on the gel can represent several species, because some DNA fragments can have the same melting points. In addition, different 16S rRNA gene copies can result in multiple bands on the gel but still represent one species. In this study 34 sequences from the DGGE gel included members of the Proteobacteria, Bacteroidetes and Cyanobacteria.[21]

Harrison *et al.*[11] used T-RFLP to compare bacterial communities of LDPE fragments with the bacterial communities from surrounding sediments during a 14 day microcosm exposure in coastal marine sediment from Spurn Point, Humber Estuary, UK. They demonstrated that bacteria from coastal sediments can rapidly colonize PD, but that the composition and structure of the Plastisphere is different from the microbial assemblage of sediment. In contrast to DGGE, complete 16S rRNA gene fragments are separated in T-RFLP based on length, resulting in a specific pattern for each sample.[43] A major limitation of T-RFLP compared to other molecular methods is that no taxonomic information can be retrieved for the different sequences. Nonetheless the technique is interesting as it gives a robust index of community diversity and is well correlated with the results from clone libraries.[44]

High-throughput sequencing: amplicon sequencing

The arrival of high-throughput sequencing has increased read depth for 16S rRNA marker genes, overcoming many of the limitations of first generation sequencing approaches (Table 1).[45,46] High-throughput sequencing has been commercially available since 2005, when Roche introduced the 454 sequencer platform.[47] Roche no longer supports this platform and therefore the number of published 454 studies is declining in the literature. The most widely used high-throughput sequencing method is now the Solexa method, which was acquired by Illumina in 2007.[48]

In the amplicon sequencing approach, genetic markers such as the 16S rRNA gene are amplified and sequenced for multiple environmental samples simultaneously, resulting in massive parallelization of the sequencing reactions.[46,47] This technique has two advantages. First, through the enhanced parallelization of the sequencing reactions, the cost per sequence is reduced extensively.[49] Second, separation of the templates is done without the need to transform fragments into a bacterial host, reducing labor costs considerably. In addition, considering the high numbers of sequences generated, the technique is rather fast.[50,51] Thus far amplicon sequencing is limited to marker gene regions of ±500 bp in length, limiting the ability to perform phylogenetic inference as a means of classifying unknown reads.[52]

Several high-throughput sequencing approaches are used to study microbial communities on substrates such as PD. Currently, amplicon sequencing is the most widely employed technique to survey environmental microbial communities.[53–56] Zettler, Mincer and Amaral-Zettler[6] first applied this amplicon sequencing approach to PD communities from the North Atlantic subtropical gyre in 2013. They detected that Plastisphere communities were distinct from, displayed greater evenness (less dominance), and exhibited overall higher beta diversity than surrounding seawater communities. They detected more than 1000 Operational Taxonomic Units (OTUs) on PE and polypropylene (PP) microplastic fragments recovered from the North Atlantic. Subsequent work using this approach confirmed that Plastisphere communities were distinct from the surrounding marine environment (seawater and sediment),[24] but that the biofilm related bacteria of PD resembled those of other hard substrates in the North Sea.[57] This study also recovered more than 1000 OTUs from pieces of microplastic. Two other similar studies were carried out on plastic fragments originating from fresh water.[20,58] The bacterial community of PD in freshwater contained more OTUs compared to PD of the marine environment, ranging from 1500 to more than 3000, though the surface area of plastic extracted was not standardized. For both freshwater studies, Proteobacteria, Bacteroidetes and Firmicutes were the primary colonizers of the plastic fragments examined.

High-throughput sequencing: shotgun metagenomics

Existing molecular approaches described in this review are based on the sequencing of all or part of the 16S rRNA gene to identify bacterial species on PD. These techniques are limited to providing only taxonomic identification of taxa. To obtain the taxonomic composition of all organisms present on PD and extract metabolic diversity as well, shotgun metagenomics can be applied as Bryant *et al.* performed on micro- and macroplastics samples from the North Pacific Subtropical Gyre.[59] This molecular strategy involves two goals: first, annotated genomic DNA fragments can be analyzed independently; and second, fragments can be assembled into genomic bins and analyzed from a comparative genomic context.[60] This technique offers the most comprehensive approach for extracting both phylogenetic and functional diversity in an environment and enables discovery of new metabolic functions.[60] However, the cost of sample preparation required and the data complexity limit the total number of samples run compared to amplicon sequencing. In addition, the data are complex, which requires

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access to computational infrastructure with high storage and analysis capacities.[60]

The 16S rRNA genes extracted from the Bryant *et al.* shotgun data showed a high abundance of Cyanobacteria and Alphaproteobacteria on 12 plastic fragments, and to a lesser extent Flavobacteria, Cytophaga, Sphingobacteria, Gamma- and Deltaproteobacteria. In addition, they recovered eukaryotic 18S rRNA genes of animal taxa including Bryozoa, Hydrozoa, Maxillopoda and Aphragmophora.[59]

The major advantage of shotgun metagenomics is that it also provides information on functional genes. Bryant *et al.* compared the functional genes in the plastic metagenomes to those of seawater of the North Pacific Subtropical Gyre and found several genes in higher abundance on plastic including: *che* genes, secretion system genes and *nifH* genes, suggesting enrichment for chemotaxis, cell-to-cell interaction and nitrogen fixation.[59] Because this is so far the only study describing the functional potential of the plastic debris metagenome in the marine environment, further studies in other environments and under different sampling conditions should be undertaken to confirm and complement these results.

Comparative analysis using the Plastisphere portal for Visualization and Analysis of Microbial Population Structures (VAMPS) platform

Strides in amplicon sequencing have contributed large volumes of microbial diversity data to nucleotide repositories. However, this explosion of available sequence data necessitates appropriate computational tools and skillsets to process and analyze. VAMPS addresses these challenges by providing an intuitive and interactive platform for studying microbial communities and relationships between communities. Over 16914 open-access datasets are already available on VAMPS, including sequences retrieved from marine microplastic debris summarized in the Plastisphere portal. Users can upload their marker gene sequences and associated metadata for analyzing their sequences and compare it with existing datasets.

Using VAMPS, microbial communities can be analyzed at single or multiple taxonomic and abundance levels. For each selected taxon, VAMPS also returns the underlying sequence data. VAMPS provides a variety of visualization tools for data analysis and presentation such as heatmaps, dendrograms, bar and pie charts, and taxonomy. Compared to other tools to analyze amplicon sequencing data, such as QIIME, MG-RAST and Megan6,[61–63] the VAMPS portal offers three benefits: (1) it is constructed specifically for amplicon sequencing data, where others are intended primarily for shotgun metagenomics data (MG-RAST, Megan6); (2) no bioinformatics skills or software are necessary because the sequences only need to be uploaded and the program is web-based; and (3) the portal can serve as a database for "Plastisphere" sequences from several research institutes, making comparison between datasets possible. In addition, other environmental community sequences can be uploaded. For more details about VAMPS please see the publication by Huse *et al.*[64]

For demonstration purposes in this review, we compared datasets from the De Tender and the Amaral-Zettler groups

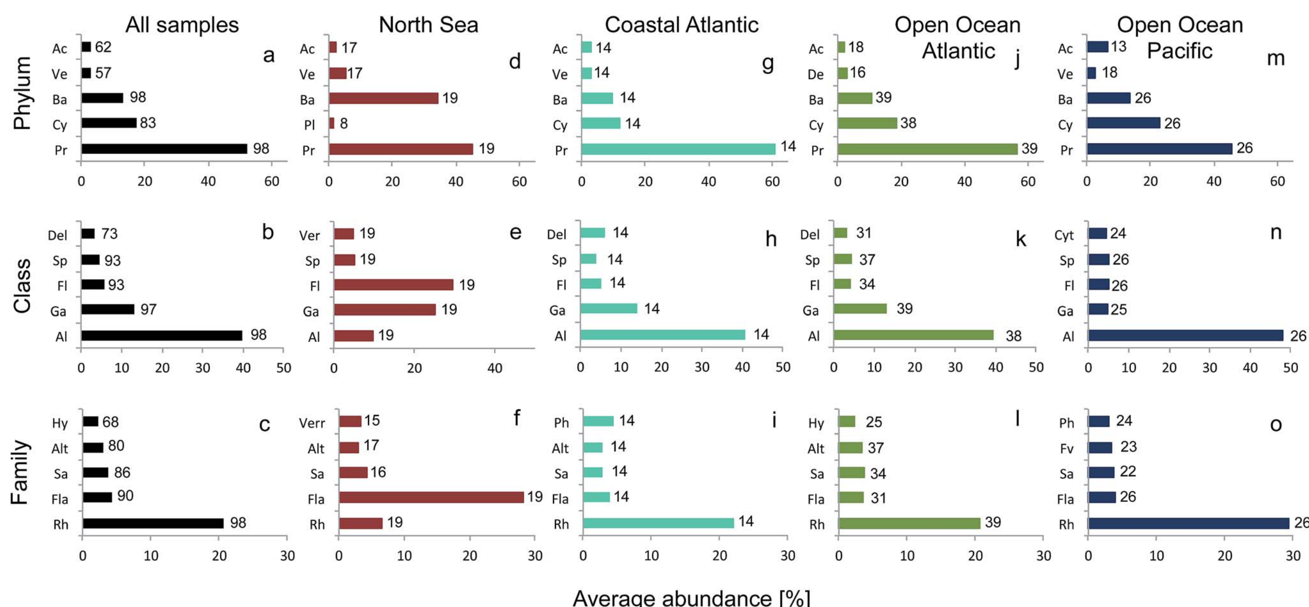

Fig. 3 An overview of the five most abundant taxa on PE samples in the different marine regions at different taxonomic levels. Numbers adjacent to the bars represent the numbers of samples of the 98 examined that included these taxa. Of the 98, 19 were from the North Sea, 14 from the coastal Atlantic, 39 from the open ocean Atlantic and 26 from the open ocean Pacific. The following abbreviations apply: Phylum: Ac Actinobacteria; Ve Verrucomicrobia; Ba Bacteroidetes; Cy Cyanobacteria; Pr Proteobacteria; Pl Planctomycetes; De Deferribacteres. Class: Del Deltaproteobacteria; Sp Sphingobacteria; Fl Flavobacteriia; Ga Gammaproteobacteria; Al Alphaproteobacteria; Ver Verrucomicrobiae; Cyt Cytophagia. Family: Hy Hyphomonadaceae; Alt Alteromonadaceae; Sa Saprospiraceae; Fla Flavobacteriaceae; Rh Rhodobacteraceae; Verr Verrucomicrobiaceae; Ph Phylobacteriaceae; Fv Flammeovirgaceae.

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available through the Plastisphere portal. We selected 98 bacterial community samples from these projects derived from PE plastic marine debris. This PD was sampled either in the Belgian part of the North Sea,[24] the North Atlantic[6,22] or Pacific Ocean.[22]

These data were differentially sequenced targeting either V3–V4 [24] or the V6 [6,22] hypervariable regions of the 16S rRNA gene and either high-throughput Illumina[22,24] or amplicon pyrotag sequencing[6] on the Roche 454 platform. Since the projects differed in many methodological respects (*i.e.* location, sampling period, 16S rRNA gene hypervariable target, and high-throughput sequencing method), we restricted our comparison between bacterial communities to taxonomy-based comparisons and community membership alone (see Rubin *et al.*[65] for a discussion on bias in amplicon sequencing). An extended overview of the metadata including sample locations corresponding to the project datasets is provided in ESI Table 1 and Fig. S1.†

Taxonomic groups in each sample were normalized and expressed as relative abundances between 1 and 100% of the total community abundance in that sample. Proteobacteria, Bacteroidetes and Cyanobacteria were the dominant phyla colonizing PD in every sample analyzed. Proteobacteria constituted the most common phylum and usually dominated, making up between 23 to 88% of samples (with an average of 52%) (Fig. 3 and 4). Bacteroidetes were less abundant (2 to 58%, average 13%) but also occurred in every sample. Cyanobacteria were observed on 85% of the analyzed PD and constituted 1 to 60% (18% on average) of the community. These same three phyla dominated PD samples collected in the Northern European waters by Oberbeckmann *et al.*[21,57]

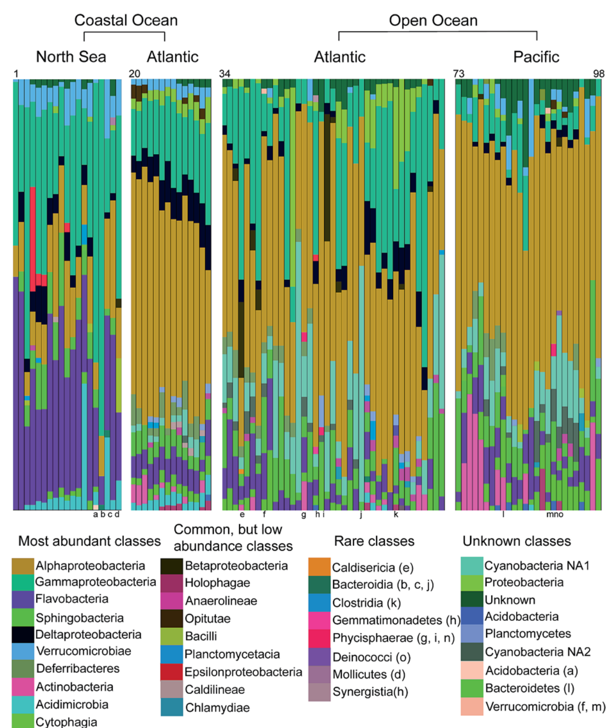

Fig. 5 Overview of the relative abundance and taxonomic breakdown of bacterial classes on PE plastic debris sampled in different marine regions. Only classes that contributed ≥1% relative abundance are shown. Letters at bottom of bar graphs indicate samples where rare taxa occurred. The sample order in the figure is the same as provided in Table S1,† numbers above bar charts are provided for orientation.

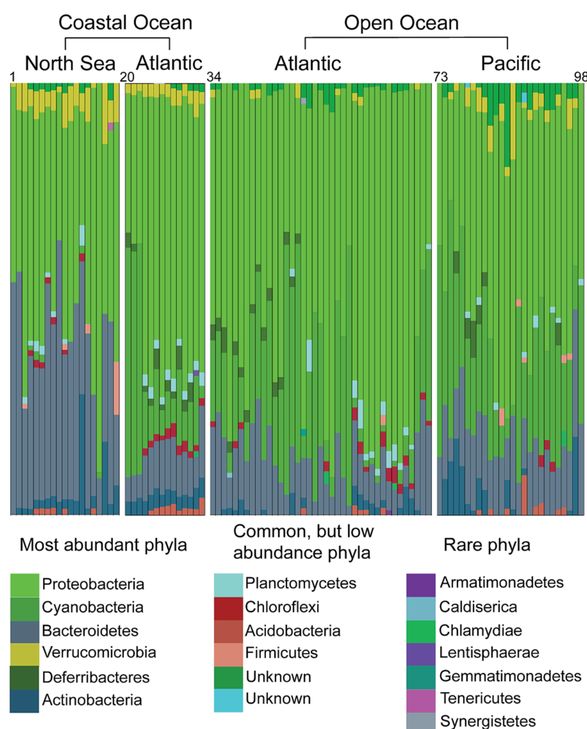

Fig. 4 Overview of the relative abundance and taxonomic breakdown of bacterial phyla on PE plastic debris sampled in different marine regions. Only phyla that contributed ≥1% relative abundance are shown. The sample order in the figure is the same as provided in Table S1,† numbers above bar charts are provided for orientation.

The most common class in our datasets was the Alphaproteobacteria representing 5 to 67% (40% on average) of the relative abundance of each sample, followed by Gammaproteobacteria (range 1.6 to 80%; on average 13%) and Flavobacteriia (range 1 to 52%; on average 6%) (Fig. 3 and 5). Dang and Lovell[7] and Dang *et al.*[8] also observed these classes in initial colonization communities in a salt marsh. Additionally, Oberbeckmann *et al.*[21] observed Flavobacteriia *via* DGGE but not Alphaproteobacteria on PET after a six-week incubation.

In our VAMPS meta-analysis, the most common bacterial family was the Rhodobacteraceae occurring at 20% relative abundance on average in each sample (Fig. 6). In temperate coastal waters, members of the Rhodobacteraceae, *e.g. Roseobacter* clade, were often the major primary colonizers of surfaces such as PD.[8] Rhodobacteraceae were also identified as the most dominant bacteria in biofilms on substrates of the Eastern Mediterranean Coastal waters.[66] This is in contrast to other studies where Flavobacteriaceae were the most dominant family on PET bottles exposed to the North Sea[57] followed by Cryomorphaceaea, Saprospiraceae and Rhodobacteraceae. Within our datasets, Flavobacteriaceae and Saprospiraceae were the second most abundant families occurring

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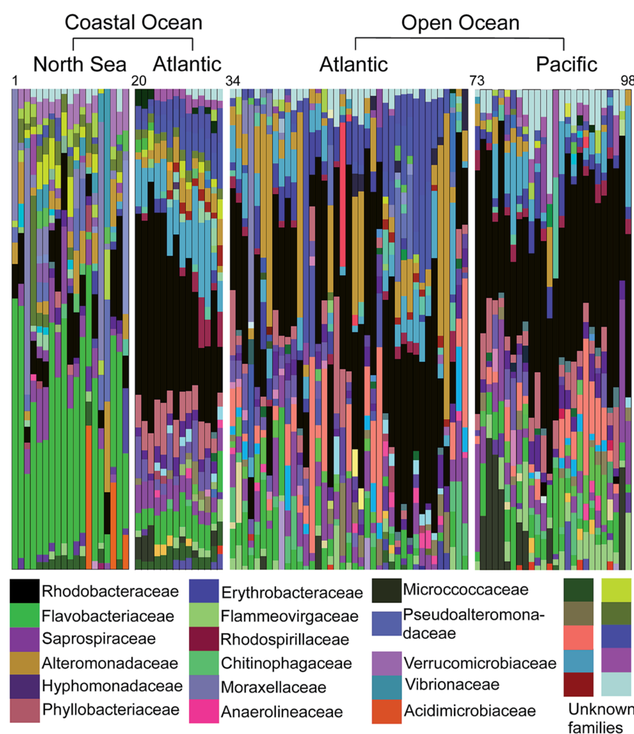

Fig. 6 Overview of the relative abundance and taxonomic breakdown of bacterial families on PE plastic debris sampled in different marine regions. Only families that contributed ≥1% relative abundance are shown. The sample order in the figure is the same as provided in Table S1,† numbers above bar charts are provided for orientation.

on 90 and 86 samples, respectively, but were low in relative abundance (both around 4% on average) on PE.

To identify possible "core" Plastisphere microbiome members for different marine regimes, we compared the five most abundant and common taxa of four different marine regions (Fig. 3) against each other: offshore *versus* coastal regions and Atlantic Ocean *versus* Pacific Ocean *versus* North Sea. Clear differences could be observed between the North Sea and all other regions. For the Atlantic and Pacific Oceans, all datasets exhibited similar dominance structures with Proteobacteria, Cyanobacteria, and Bacteroidetes as the most abundant phyla (representing >80% of the relative abundance), without showing differences between the coastal *versus* open ocean. In comparison to these regions, the North Sea samples varied in composition. Cyanobacteria were observed in only one quarter of the samples, and next to Proteobacteria and Bacteroidetes, the Verrucomicrobia were the third most dominant fraction.

At the class level, Alphaproteobacteria was the most abundant class in the Atlantic and Pacific Oceans, whereas Flavobacteriia and Gammaproteobacteria were the most abundant classes in the North Sea (Fig. 6). The same trend was also observed at the family level, where Rhodobacteraceae were most abundant in all regions except for the North Sea where Flavobacteriaceae dominated on average. Since this pattern was also observed in a different set of samples and a separate study from the North Sea,[53] it suggests that despite differences in sampling region and sequencing techniques, there may be core taxa that characterize this region (Fig. 4) and that: (i) plastic marine debris favors the attachment of particular microbial taxa in a given region; and, (ii) different methods used to assess microbial community composition may produce comparable results at some level of taxonomic resolution.

Conclusions and perspectives

To date the number of studies examining community structures of the Plastisphere is limited, but the introduction of high-throughput sequencing allows for in-depth comparative characterizations. *Via* the VAMPS platform, we have compared datasets of three projects characterizing PD bacterial communities using amplicon sequencing albeit from different geographical locations, targeting a different hypervariable region of 16S rRNA gene, and using various generations and modes of sequencing techniques. Despite the diversity of approaches used in generating these datasets, striking patterns do emerge in the data that suggest there may in fact be a Plastisphere "core microbiome" within given marine regions. We advocate that a more powerful approach would employ a standardized protocol, used by all research groups studying Plastisphere communities.

To this end, agreeing upon the following aspects of a standard operating procedure would facilitate comparisons of Plastisphere communities:

(1) Standardized sample preservation. We recommend storing plastic samples in lysis buffer at −20 °C to preserve DNA. We tested several options for storing plastics (*i.e.* in seawater, in seawater at −20 °C, at −80 °C, at −20 °C, and at −20 °C in lysis buffer) in the laboratory. We determined that storage at −20 °C in lysis buffer yielded the highest DNA concentration after extraction (De Tender *et al.*, unpublished results).

(2) Standardized DNA extractions. A recent study of Debeljak *et al.*[67] tested different DNA extraction methods used in the literature for Plastisphere communities. The Qiagen Puregene Tissue kit yielded the highest DNA concentrations for most sizes and amounts of plastics at relatively low cost and short processing time.

(3) Standardized 16S rRNA gene hypervariable target region for amplification. Since most studies have employed part or all of the V6 hypervariable region, continuing to include this region would provide the most comparative potential.

(4) Deposition of amplicon data from PD studies into the VAMPS Plastisphere portal.

One could also recommend using the same sequencing platform across studies, however since sequencing technology evolves so rapidly, this may not be a feasible recommendation.

Studying the Plastisphere using amplicon sequencing[6,22,24,57] provides taxonomic information but only limited information on metabolic functions associated with microbial taxa.[68] The next step in studying the Plastisphere communities is the use of comparative "omics" studies, such as metagenomics and metatranscriptomics, which provide information on the biological functions encoded in the metagenome and the microbial activity, respectively. Unlike other microbiome studies in the

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marine environment,[69–72] it is not possible for Plastisphere communities to be size fractionated to reduce data complexity. This makes it potentially difficult to assemble whole genomes because of the disproportionate contributions of eukaryotes such as dinoflagellates that have large genome sizes. However, recently developed methods make it possible to study metagenomes without the use of genome assembly and allow for the recovery of information from short reads.[73,74] Another challenge of the whole genome studies, however is that library construction is quite difficult due to the low biomass that often occurs on PD. This is especially problematic in metatranscriptomics studies, but can be overcome with genomic amplification methods, which, however, introduce inherent amplification biases. Nevertheless, more comparative "omics" studies are in progress and will not only provide glimpses of the metabolic potential of the Plastisphere members, but also expand our knowledge of diversity in these communities. So far, few studies have emphasized taxa beyond bacterial communities using high-throughput sequencing.[6,59] SEM images, however, proved the presence of several eukaryotes, *e.g.* Bryozoa.[2,3] Making use of shotgun metagenomics will thus give us a more thorough understanding of the complete microbial community including eukaryotes present on marine plastic debris. We believe that a thorough understanding of the complete microbial community is necessary for two main reasons. First, plastic has the ability to serve as a vector for harmful microorganisms.[75] It has already been shown that pathogenic *Vibrio* and harmful algal blooming species can be present on PD.[76,77] In terms of transport to other regions, knowledge regarding the microbial community is therefore important. Second, in terms of degradation of PD, it is important to study other community members beyond bacteria, such as fungi. In terrestrial environments, both bacterial and fungal micro-organisms can degrade plastics.[78–82] Expanding the PD microbial community studies to fungal organisms will therefore likely provide novel insights into the ecology and functional diversity of the Plastisphere.

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