Marine Environmental Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Mature biofilm communities on synthetic polymers in seawater - Specific or general?

Inga V. Kirstein[a,*], Antje Wichels[a], Georg Krohne[b], Gunnar Gerdts[a]

[a] Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research, Biologische Anstalt Helgoland, Helgoland, Germany
[b] University of Würzburg, Biocenter, Imaging Core Facility, Würzburg, Germany

ARTICLE INFO

Keywords:
Plastics
Synthetic polymer
Substrate specificity
Plastisphere
Bacteria
Fungi
Community composition
Biofilm
Multivariate analysis

ABSTRACT

To understand the ecological impacts of the "Plastisphere", those microbes need to be identified that preferentially colonize and interact with synthetic polymer surfaces, as opposed to general surface colonizers. It was hypothesized that the microbial biofilm composition varies distinctly between different substrates. A long-term incubation experiment was conducted (15month) with nine different synthetic polymer films as substrate as well as glass using a natural seawater flow-through system. To identify colonizing microorganisms, 16S and 18SrRNA gene tag sequencing was performed. The microbial biofilms of these diverse artificial surfaces were visualized via scanning electron microscopy. Biofilm communities attached to synthetic polymers are distinct from glass associated biofilms; apparently a more general marine biofilm core community serves as shared core among all synthetic polymers rather than a specific synthetic polymer community. Nevertheless, characteristic and discriminatory taxa of significantly different biofilm communities were identified, indicating their specificity to a given substrate.

1. Introduction

During the last decade, there has been a growing concern about the ecological impact of plastics in the marine environment. The longevity of plastics in the marine environment is a matter for debate, and estimates range from hundreds to thousands of years depending on the chemical and physical properties of the synthetic polymer (Barnes et al., 2009). Indeed, plastics remain much longer in the marine environment than most natural substrates; they represent a new microbial habitat and due to floating characteristics, they could function as a vector for the dispersal of pathogenic species (Kirstein et al., 2016; Zettler et al., 2013).

Because synthetic polymers are physically and chemically distinct from naturally occurring substrates, they offer a new type of substrate to the microbial community. As any surface in the marine environment, synthetic polymers are rapidly colonized by microorganisms (Harrison et al., 2014) and subsequently by a myriad of organisms building up complex biofilms (Dobretsov et al., 2010). Using a culture-independent approach, Zettler et al. (2013) explored for the first time microbial communities on marine plastic litter. They showed that microbial communities on marine plastic debris differ consistently from the surrounding seawater communities and coined these specific biofilms "Plastisphere". Amaral-Zettler et al. (2015) reported that "Plastisphere" communities of the Atlantic and Pacific Ocean clustered to a greater extend by geography than by synthetic polymer type. Also, Oberbeckmann et al. (2014) found that the composition of biofilm communities present on synthetic polymers in marine habitats is driven by spatial and seasonal effects, but also varies with the plastic substrate type of randomly sampled plastics. However, in a short-term exposure experiment located in the North Sea they could not perceive significant differences between glass and PET associated communities (Oberbeckmann et al., 2014, 2016). Despite the increasing research effort in analysing and understanding the spatial, seasonal, habitational, or substrate parameters influencing the "Plastisphere", there is still no consistency concerning the specificity of microbial communities on different synthetic polymers and other surfaces.

Although some studies have analysed marine plastic biofilms, using a culture-independent approach (Amaral-Zettler et al., 2015; Bryant et al., 2016; De Tender et al., 2015, 2017; Debroas et al., 2017; Oberbeckmann et al., 2014, 2016; Zettler et al., 2013), little is known on the specificity of marine biofilms on chemically distinct (e.g. polyesters, polyolefines) synthetic polymers under comparable conditions. Recently, Oberbeckmann et al. (2018) investigated wood, HDPE and PS associated communities in a short term experiment (14 days)

* Corresponding author. Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research, Biologische Anstalt Helgoland, Postbox 180, 27483, Helgoland, Germany.
E-mail address: inga.kirstein@awi.de (I.V. Kirstein).

https://doi.org/10.1016/j.marenvres.2018.09.028
Received 23 April 2018; Received in revised form 28 September 2018; Accepted 30 September 2018

and found no significant differences comparing both polymers. Ogonowski et al. (2018) incubated cellulose, glass, PE, PP and PS for two weeks in pre-filtered seawater and found significant differences between plastic and non-plastic substrates, but the specificity of marine biofilms on the respective chemically distinct substrates remains unclear. Furthermore, in order to understand the ecological impacts of the "Plastisphere", those microbes that preferentially colonize and interact with synthetic polymer surfaces, as opposed to generalists that colonize other surfaces, need to be identified (Harrison et al., 2014). Recently, De Tender et al. (2017) identified a core group of 25 single OTUs, belonging to the phylum *Proteobacteria, Bacteriodetes and Verrucomicroboa,* on polyethylene (PE), but it remains unproved whether these "core organisms" are specific for an environment or whether they are also found on other types of synthetic polymers.

In the present study, it was hypothesized that the composition of marine biofilm communities varies significantly depending on the substrate type. A long-term experiment was designed in which nine different synthetic polymers as foils as well as glass slides were incubated in a natural seawater flow-through system. Previous studies focused essentially on the prokaryotic or bacterial community composition (Amaral-Zettler et al., 2015; De Tender et al., 2015; Harrison et al., 2014; Oberbeckmann et al., 2014; Zettler et al., 2013), whereas only a few studies addressed the complete eukaryotic, or fungal, communities of synthetic polymer biofilms (Bryant et al., 2016; De Tender et al., 2017; Kettner et al., 2017; Oberbeckmann et al., 2016). The composition of both prokaryotic and eukaryotic communities on the different substrate types was determined by 16S and 18S rRNA gene tag sequencing and substrate specificity assessed. Furthermore, characteristic and discriminatory genera of synthetic polymer and glass biofilms were identified, and compared those to previously described synthetic polymer associated biofilms.

2. Materials and methods

2.1. Experimental design and sample preparation

Synthetic polymers were incubated from August 2013 to November 2014 in the dark (max. light intensity 0.1033 µmol/m^2/s) in a natural seawater flow-through system (Fig. S1a) in conventional slide frames (5 × 5 cm) (Fig. S1b) located at the "Biologische Anstalt Helgoland" (North Sea, Germany, Latitude 54.18286, and Longitude 7.888838) approximately 60 km off the German coastline. North Sea water was directly pumped through the system (flow rate of approx. 5800 l/day). The experimental setup simulates sunken plastic, which is largely protected from photochemical degradation, enabling a well-defined interaction between the different synthetic polymers and the microbial community. The different exposed synthetic polymers represent the most frequent polymer types in the marine environment and were provided by various suppliers: high-density polyethylene (HDPE) (ORBITA-FILM GmbH), low-density polyethylene (LDPE) (ORBITA-FILM GmbH), polypropylene (PP) (ORBITA-FILM GmbH), polystyrene (PS) (Ergo.fol norflex GmbH), polyethylene-terephthalate (PET) (Mitsubishi Polyester Film), polylactic acid (PLA) (Folienwerk Wolfen GmbH), styrene-acrylonitryle (SAN) (Ergo.fol norflex GmbH), polyurethane prepolymer (PESTUR) (Bayer), polyvinyl chloride (PVC) (Leitz) (Table S1). As control substrate, glass slides were incubated in parallel. Glass is inert opposed to most natural surfaces and therefore enables the development of a general marine biofilm community. Using foils allowed us to 1. Separately incubate each piece without touching each other, so that even biofilms can develop. 2. It enables us of taking subsamples of the same piece of foil/biofilm for different approaches (e.g. future FISH studies). After 15 months of incubation, five replicates of each synthetic polymer with the associated microbial biofilm were taken (Fig. S1c). Environmental data including salinity (S), water temperature (T) and chlorophyll *a* (Chl *a*) were recorded in parallel as part of the Helgoland Roads time series (Wiltshire et al., 2008) (Fig. S1d). Each foil was cut into strips and glass was broken into fragments of 1 cm^2 using ethanol sterilised forceps, scalpels and scissors. To remove the unspecific loosely attached part of the biofilm, each polymer strip was washed in 1 mL 0.2 µm filtered and autoclaved sterile seawater three times for 30 s (vortex) with transferring the strip after each washing step in a new 1.5 mL tube. Synthetic polymer strips and glass fragments were stored at −20 °C for further analysis.

2.2. SEM

Strips or fragments of subsamples of two replicates (out of five) of each synthetic polymer and glass were fixed at 4 °C in sterile sea water containing 2.5% glutaraldehyde and 50 mM sodium cacodylate (pH 7.2) and stored at 4 °C (4–10 days) until processing. Before, one subsample of each replicate (n = 2) was washed to remove the unspecific loosely attached part of the biofilm as described above; the other one remained untreated to visualize the whole community. Samples were stepwise dehydrated in ethanol, critical point dried (BAL-TEC CPD 030; Balzers, Liechtenstein) and sputter coated (BAL-TEC SCD 005; Balzers, Liechtenstein) with gold-palladium before SEM analysis (JEOL JSM-7500F; Freising, Germany).

2.3. DNA extraction

DNA of microbial biofilms was extracted using a modified protocol from Sapp et al. (2006). Each replicate of each substrate (n = 5) was individually transferred into 2 mL screw cap reaction tubes containing a mixture of 100 µm Zircona/-Silica beads, 700 µL Sodium Chloride –Tris – EDTA (STE) - Buffer was added before mechanically pulped (FastPrep® FP 120, ThermoSavant, Qbiogene, United States) for 40 s on level 4.0. DNA concentrations were quantified with a PicoGreen assay (Invitrogen, Waltham, MA) using a Tecan Infinite M200 NanoQuant microplate reader (Tecan, Switzerland).

2.4. 16S & 18S rRNA gene tag sequencing of biofilm communities

16S and 18S rRNA gene tag sequencing was performed at LGC Genomics GmbH (Berlin, Germany). Community DNA samples were sent to LGC for generation of 16S V3/V4 and 18S V4 rRNA amplicon libraries for Illumina sequencing. Community DNA was amplified using amplification primers targeting the V3/V4 region of the 16S rRNA gene using 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). Eukaryotic community DNA was amplified using amplification primers targeting the V4 region of the 18S rRNA gene using Eu565F (5′-CCAGCASCYGCGGTAATTCC-3′) and Eu981R (5′-ACTTTCGTTCTTGATYRATGA-3′) (Piredda et al., 2017). The amplicons were paired-end sequenced 2 × 300 bp on an Illumina MiSeq platform. The paired-end reads were merged using BBMerge 34.48 software (http://bbmap.sourceforge.net/) and processed through the SILVAngs pipeline (Quast et al., 2013). All sequences were de-replicated at 100% identity and further clustered with 98% sequence identity to each other. Representative sequences from operational taxonomic unit clusters (OTUs) were classified up to genus level against the SILVA v123 database using BLAST as first described by Ionescu et al. (2012). Sequences having an average BLAST alignment coverage and alignment identity of less than 93% were considered as unclassified and assigned to the virtual taxonomical group "No Relative" (Quast et al., 2013). Finally, 3,517,422 (99.37%) classified sequences were obtained for bacteria and archaea, and 5,163,443 (86.49%) classified sequences were obtained for eukaryotes. For following downstream analyses, classifications on the genus-level were used to generate the final abundance matrixes. All classifications contained the sum of all sequences represented by OTUs with the equal taxonomic path. Sequence data was deposited in the European Nucleotide Archive (Toribio et al., 2017) under the accession number PRJEB22051, using the data brokerage service of the German

Federation for Biological Data (Diepenbroek et al., 2014), in compliance with the Minimal Information about any (X) Sequence (MIxS) standard (Yilmaz et al., 2011).

2.5. Statistics and downstream data analysis

All multivariate analyses were carried out with the Primer 6 software package plus the add-on package PERMANOVA + (PRIMER-E Ltd, UK). The entire prokaryotic and eukaryotic communities were analysed separately. The virtual taxonomical group "No Relative" was removed from the analysis. Subsequently, counts per classification were normalized by calculating their relative abundances to the total number of SSU rRNA gene reads per sample. For prokaryotes OTUs with a minimal mean relative abundance of 0.1% ($n = 5$) in at least one substrate type were considered for further analysis. Beta diversity analysis and related hypothesis testing of the complete eukaryotic community was carried out on the basis of presence-absence metrics. OTUs with a total abundance of 1 read were excluded from downstream analyses. To visualize patterns in community composition, principal coordinates analysis (PCO) was performed using Hellinger distance (D17 (Legendre and Legendre, 2012);) or Jaccard index for eukaryotes. Binary (presence/absence) or square root transformed relative abundances of sequence read numbers were used for distance matrix calculation. To test for statistically significant variance among the biofilm communities attached to the different substrates, PERMANOVA with fixed factors and 9999 permutations at a significance level of $p < 0.05$ was performed. Tests of significant differences in the within-group dispersion among the substrate groups were accomplished by performing tests of homogeneity of dispersions (PERMDISP) using 9999 permutations at a significance level of $p < 0.05$. Similarity percentage analysis (SIMPER) allowed us to calculate the total similarity within and dissimilarity between the different groups of substrates, and to determine characteristic and discriminatory OTUs. SIMPER analysis was performed using Bray Curtis similarity (S17) by the use of binary (presence/absence) or fourth root transformed relative abundances (Clarke, 1993).

3. Results

3.1. Prokaryotic and eukaryotic biofilm composition of nine synthetic polymers & glass

After 15 month of exposition in the natural sea water flow through system, a dense microbial biofilm colonized all provided substrates (Fig. S1 (c)). SEM was used to examine the biofilm in addition to DNA based techniques. The synthetic polymer and glass associated biofilm communities analysed by 16S and 18S rRNA gene tag sequencing contained in total 1479 prokaryotic and 692 eukaryotic different operational taxonomic units (OTUs). SEM confirmed a highly diverse biofilm community growing on all substrate types (Fig. 1A(a-k)) consisting of prokaryotic and eukaryotic microorganisms of different morphologies. Different flagellates were observed being part of the biofilm community. Exemplarily Fig. 1A (i) shows a flagellate cell having a substantial covering or pellicle. Mature loricae of *Acanthoeca spectabilis* (Leadbeater et al., 2008) belonging to the detected class *Acanthoecida* (Fig. 1C) were often observed by SEM being part of the biofilm community (Fig. 1A (d)). Fig. 1A (k) shows a striking specimen what appear to be a surface arrangement of scales and a peripheral array of long flexuous spines with obconical meshwork bases. The most closely similar specimens are attributable to the genus *Luffisphaera* spp. (VØRS, 1993). Prokaryotic biofilm communities of all substrates were dominated (mean relative abundance > 1% in at least one substrate type) by OTUs assigned to 20 classes (Fig. 1B). All biofilms consisted of a high proportion of *Proteobacteria* (42–47%) with most abundant classes of *Alpha-* (11–15%), *Delta-* (11–13%) and *Gammaproteobacteria* (13–16%). Beside the high proportion of *Proteobacteria* the taxonomic classes of *Nitrospira* (7–12%), *Planctomycetacia* (5–8%), *Caldilineae* (4–7%), *Acidimicrobiia* (4–7%), *Sphingobacteria* (3–7%) and an unclassified OTU of *Planktomycetes* OM190 (2–4%) were more abundant in all biofilm communities (Fig. 1B). Interestingly, the biofilms on glass displayed clear differences in community composition compared to all synthetic polymers. For example, an unclassified *Latescibacteria* and the unclassified *Proteobacteria AEGEAN-245* were more abundant on glass (Fig. 1B).

In contrast to the relative homogenous prokaryotic community composition among all synthetic polymers, the eukaryotic biofilm communities were highly heterogeneous (Fig. 1C). *Intramacronucleata*, belonging to the SAR clade, was one of the most abundant eukaryotic classes (4–25%) within the biofilm communities of both synthetic polymers and glass. The diverse class of crustaceans *Maxillopoda* had a mean relative abundance between 0.8 and 22%. An unclassified OTU belonging to *Gastrotricha* made up a portion of between 0.2 up to 24% of the eukaryotic biofilm community. *Demospongiae*, a highly diverse class of the phylum *Porifera*, appeared with abundances in between 3 and 21% and *Chromadorea*, belonging to the phylum *Nematoda*, appeared with abundances between 0.8 and 23% within the eukaryotic biofilm communities. Interestingly, animals like *Maxillopoda* or *Nematoda* were not observed by SEM as opposed to regularly seen *Diatomea* and *Sponges* (data not shown). Considering the proportion of Fungi within the eukaryotic community, *Chytridiomycetes* represented the highest abundances among biofilms of all substrates with 3% on PET and 1.2% on glass (Fig. 1c).

3.2. Substrate specificity of the prokaryotic biofilm communities

To determine whether microbial communities colonizing the different substrates are distinct from each other, the community structure on the genus level of biofilms attached to nine different synthetic polymers and those colonizing glass was compared. Samples of synthetic polymers and the control substrate glass clustered clearly in bisection (Fig. 2a). The 16S rRNA gene sequence comparisons showed significant differences between the glass associated biofilm communities and those associated with synthetic polymers ($p < 0.05$; pairwise PERMANOVA, Table S3). A separate test of dispersion using PERMDISP revealed that the differences among the specific synthetic polymers to glass were at least partially driven by different within-system heterogeneities in five cases (Table S4). Significant differences were also observed in 15 out of 36 possible synthetic polymer-pair combinations, between different polymer-colonizing communities (Table S3). PLA communities were significantly different from seven other synthetic polymer communities, followed by PESTUR and PVC communities that significantly differed from five and four further synthetic polymer communities. HDPE, PS, PET and SAN communities differed significantly from three, PP and LDPE communities differed significantly from one other synthetic polymer communities (Table S3).

Prokaryotic biofilm communities associated with different synthetic polymers differed between 3.9 and 5.5% from each other, and between 5.5 and 7.6% from the control substrate glass (Table S5). Considering the relative abundances of single OTUs, nine OTUs appeared with relative abundances > 3% of the total community composition including e.g. *Nitrospira* (OTU 576), the unclassified *Deltaproteobacteria SH765B-TzT-29* (OTU 1123) and an uncultured unclassified *Caldilineacea* (OTU 359) (Fig. 3).

Five OTUs were predominantly discriminating the biofilm on glass from synthetic polymer biofilm communities: the unclassified genus *Acidobacteria* AT-s3-28 (OTU 13), *Halophagae* Sva0725 of the subgroup 10 (OTU 37), the genus *Gilvibacter* (OTU 231), *Leptobacterium* (OTU 240), and the *Candidatus Entotheonella* (OTU 1058) (Fig. 3). The unclassified *Halophagae* Sva0725 and *Gilvibacter* were more characteristic for synthetic polymer communities (Table S7), with relative abundances of > 1%, respectively. The unclassified genus *Acidobacteria* AT-s3-28 contributed to the total dissimilarity between glass and all synthetic polymers, and was always more characteristic for glass biofilm communities, with relative abundances < 1% (Fig. 3, Table S7). The

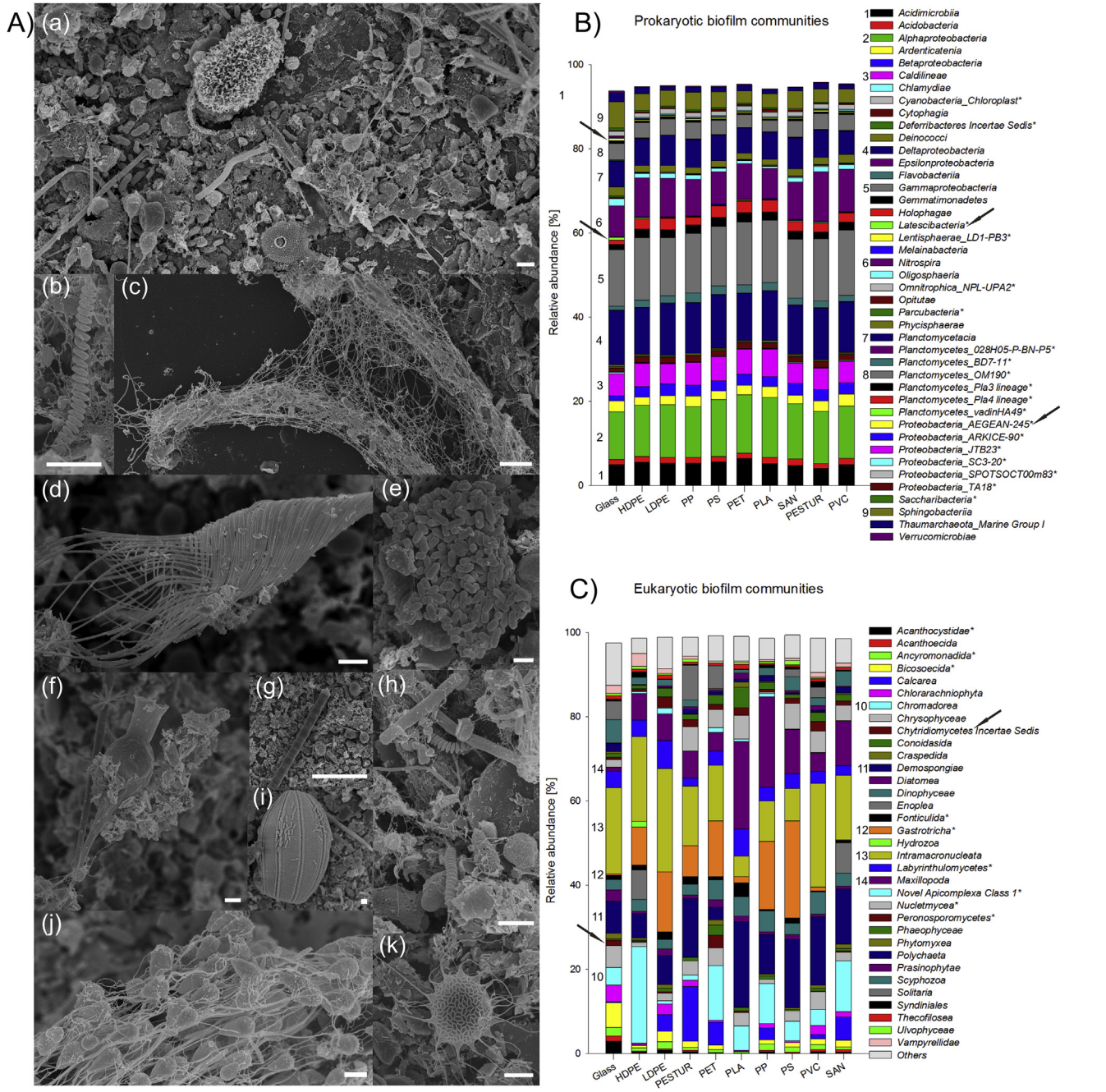

Fig. 1. Biofilm community composition on different synthetic polymers and glass. A: Scanning electron microscopy images of the biofilm community attached to synthetic polymers and glass. Scale bar = 1 µm. (a) Region of the highly diverse marine biofilm observed on PVC. (b) Spirochete embedded in EPS (HDPE). (c) Organized rod-shaped bacteria embedded in EPS (glass). (d) *Acanthoeca spectabilis* showing left-handed helical arrangement of costae in stalk and vase (PESTUR). (e) Box-shaped bacteria (LDPE). (f) Stalked *Salpingoeca* sp. (PS). (g) Belike *cyanobacteria* (PP). (h) Region of a biofilm with rod- and spiral shaped bacteria (PET) (i) Flagellate (PET). (j) Belike *fungi* spores and hyphae (HDPE). (k) *Luffisphaera* sp. (PESTUR). Images a), c), e) and i) show biofilms without, images b), d), f), g), h), j) and k) show biofilms after excessive washing. **B:** Abundance profiles of prokaryotic and **C:** eukaryotic classes on different synthetic polymers and glass. OTUs with a mean relative abundance of at least 0.1% in one substrate type (n = 5) were analysed. Displayed are prokaryotic taxonomic classes with abundances of > 0.1% and eukaryotic classes of > 1% in at least one substrate type for. The group `others' was made up of classes with abundances < 1%. A * indicates the term `unclassified class'. Numbers indicate highly abundant prokaryotic (1–9) and eukaryotic (10–14) classes. Arrows indicate differences in glass biofilms (B) and the most abundant class of fungi (C).

Candidatus Entotheonella, with relative abundances of > 3%, contributed more to total similarity of glass biofilm communities (Fig. 3, Table S7).

Beside the detected differences of glass and synthetic polymer communities, PLA associated communities showed significant differences to seven synthetic polymer community groups (Table S3). The largest dissimilarities between PLA and all other substrates was caused by an OTU belonging to the genus *Leptobacterium* (OTU 240), with overall relative abundances < 1% (Fig. 3). While the genus *Leptobacterium* was characteristic for PLA communities, the unclassified *Acidobacteria* AT-s3-28 also contributed to the total dissimilarities of PLA by being characteristic of glass communities (Table S7). Further, five OTUs contributed explicitly to the total dissimilarities between PLA and the other synthetic polymer associated biofilm communities.

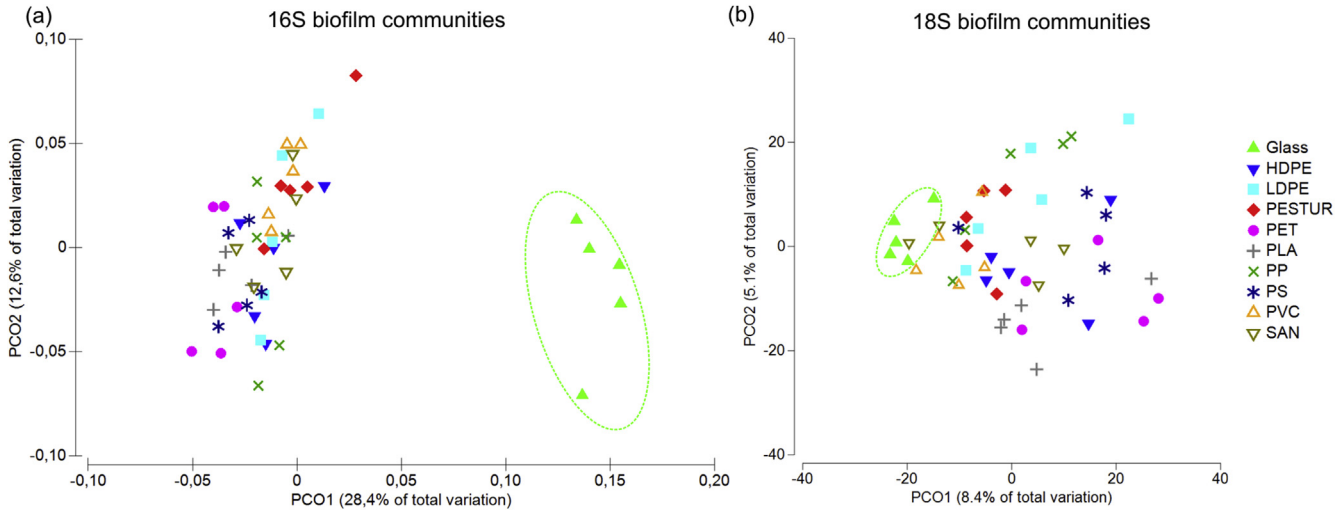

Fig. 2. Principle Coordinate Ordination (PCO) relating variation in microbial community composition between different synthetic polymers and glass biofilm communities. PCOs representing similarity of biofilm communities based on relative abundances (prokaryotes) and presence/absence (eukaryotes) of OTUs across samples. Displayed are comparisons of (a) prokaryotic and (b) eukaryotic communities of synthetic polymer attached and glass attached 15 month old biofilm communities.

Genera contributing explicitly to the total dissimilarities between PLA and the other synthetic polymers were an unclassified *Holophagae* CA002 of the Subgroup 10 (OTU 35), *Ardenticatenales* (OTU 355), an unclassified *Oligosphaeria* (565), *Nitrospira* (OTU 576) and *Nitrospina* (OTU 1059). The unclassified *Holophagae* CA002 was most characteristic for PLA (Table S7). The unclassified *Oligosphaeria* contributed least to the total similarity of PLA. *Nitrospira* clearly discriminated PLA from PESTUR communities. The unclassified genus *Ardenticatenales* contributed highly to the total dissimilarities, explained by relative abundances of 0.9% for PLA and 1.1% for PVC communities, compared to relatively low contributions of 0.2% for HDPE communities (Fig. 3).

With exception of *Nitrospira* (OTU 576) and *Candidatus Entotheonella* (OTU 1058), the OTUs contributing most to the total dissimilarity between substrates were not the most abundant ones. Instead, less abundant OTUs like the unclassified *Acidobacteria* AT-s3-28, being more characteristic for glass communities, contributed strongly to the total dissimilarity between glass and synthetic polymer biofilm communities (Fig. 3, Fig. S3, Table S7).

3.3. Substrate specificity of the eukaryotic biofilm communities

Considering the possible bias due to preferential amplification of primers resulting variation in copy numbers which might affect the relative abundance estimates of all species in the sample by over-representation of specific taxa, Beta diversity and related hypothesis testing of the general eukaryotic community was carried out on basis of presence-absence metrics. In contrast to the prokaryotic communities, for eukaryotes no clear clustering between the different synthetic polymers or the control substrate glass was observed (Fig. 2b). Eukaryotic biofilm communities differed between 44.1 and 56.3% from each other (Table S6). Furthermore, there was a significant difference between the HDPE-, LDPE-, PESTUR-, PP-, PS-, PET-, and PLA to glass associated eukaryotic communities. However, a separate test of dispersion using PERMDISP revealed that these differences among

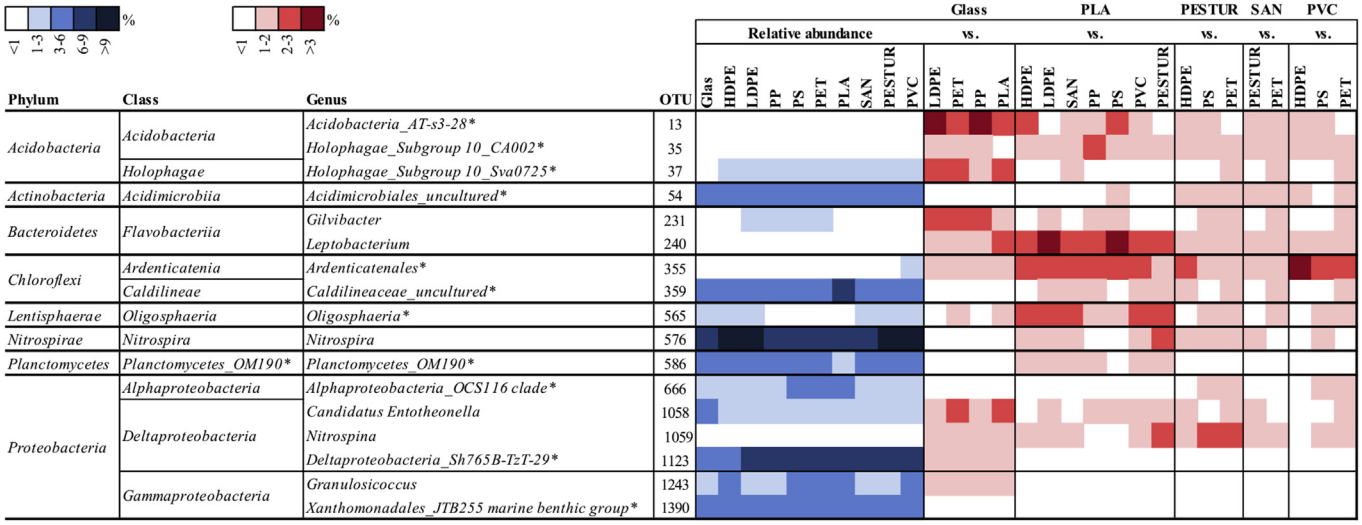

Fig. 3. Most abundant and discriminative prokaryotic OTUs of the nine different synthetic polymers and glass (n = 5). OTUs with a mean relative abundance of at least 0.1% (n = 5) in at least one substrate type were analysed. Displayed are OTUs with a mean relative abundance of at least 3% or jointly contributing, with a minimum of 2%, to the total dissimilarity between different statistically significant (PERMANOVA $p < 0.05$) glass and synthetic polymer groups. Groups showing both, PERMANOVA and PERMDISP significant p values were rejected. The amount of contribution is indicated by the colour of cells, darker colours represent higher contributions. Bold lines indicate OTUs contributing to the same phylum. A * indicates the term "unclassified".

substrates were most likely driven by different within-system heterogeneities (Table S4). Significant differences, devoid of within-system heterogeneities, were also observed in synthetic polymer-pair combinations. Eukaryotic communities colonizing PLA significantly differed to PP-, PVC and PESTUR associated communities ($p < 0.05$; pairwise PERMANOVA, Table S3). Furthermore, communities colonizing PS significantly differed to PESTUR. LDPE communities differed significantly to PET ($p < 0.05$; pairwise PERMANOVA, Table S3).

Explicitly discriminant of the PLA communities as compared to communities on PP-, PVC and PESTUR was an OTU belonging to the genus *Hatena* (*Cryptophyceae,* OTU 71) and *Gyromitus* (*Rhizaria,* OUT 499) both absent on PLA. An OTU belonging to the class of *Asteroidea* (*Metazoa,* OUT 144) contributed to the total dissimilarities between PLA, PVC and PS. Another genus discriminating PLA from PP communities was the dinoflagellate *Prorocentrum* (OTU 442). The overall variation between synthetic polymer eukaryotic communities was in total not driven by fungal OTUs (Fig. S4).

3.4. Biofilm vs. free living communities

To demonstrate the distinctness of microbial biofilm communities, commonly found marine prokaryotic microbial seawater communities of weekly collected samples of a one year time series at Helgoland Roads (March 2012–February 2013 (Lucas et al., 2015)), were compared to the pooled microbial biofilm communities (Fig. 4, Table S9) on the class level. The percentage of shared classes across the two habitats (Fig. 4, Table S9) reflects the distinctness of seawater and biofilm communities. More classes were detected in biofilm samples than in seawater samples, the former were partly consisting of single OTUs that could not be assigned to a taxonomic class (Table S9). Seven classes (14%) were exclusively detected within seawater communities including i.e. *Actinobacteria*, *Cyanobacteria*, *Deferribacteres* and *Thermoplasmata* (Table S9). Further 26 classes (52%) were exclusively detected within biofilm communities, including i.e. *Acidobacteria*, *Ardenticatenia*, *Caldilineae*, *Caldilineae*, *Deinococci*, *Holophagae*, *Melainabacteria*, *Nitrospira*, *Oligosphaeria* and *Phycisphaerae* (Table S9). Overall, 34% of the classes were common to biofilm and seawater communities and included members of *Acidimicrobiia*, *Alphaproteobacteria*, *Betaproteobacteria*, *Cytophagia*, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Flavobacteria*, *Gammaproteobacteria* and *Gemmatimonadetes*.

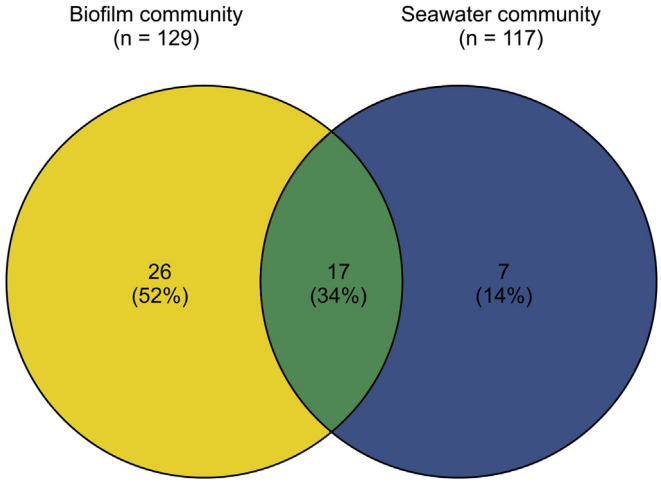

Fig. 4. Venn diagram showing prokaryotic taxonomic class overlap for pooled biofilm samples (n = 50, incubated in Helgoland seawater from August 2013–November 2014, OTUs with a mean relative abundance of at least 0.1% (n = 5) in at least one substrate type were analysed.) associated to nine different synthetic polymers and glass, and seawater samples (n = 42, collected weekly from March 2012–February 2013 OTUs with a mean relative abundance of at least 0.1% (n = 42)) at Helgoland Roads (Lucas et al., 2015); n = number of OTUs per group. Numbers inside the circles represent the number of shared or unique classes for the given environment. Images were generated using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

4. Discussion

The substrate specificity of microbial communities on synthetic polymer remains under debate as many studies conducted so far lack in systematic and statistically robust analysis of distinct synthetic polymers. Former studies focussed on the comparisons of randomly collected diverse marine synthetic polymers of unknown exposure time and origin (Amaral-Zettler et al., 2015; De Tender et al., 2015; Oberbeckmann et al., 2014; Zettler et al., 2013) which impede a proper evaluation of substrate specificity. A few studies were conducted over short time scales (Kettner et al., 2017; Oberbeckmann et al., 2016, 2018), considering that synthetic polymers remain over long time periods in natural marine environments, incubation over longer timescales allows mimicking more realistic conditions. Here, a thorough analysis of substrate specificity of prokaryotic and eukaryotic North Sea biofilms with regard to the taxonomic structure and composition of 15 month old microbial biofilms as compared on different synthetic polymer types in a natural seawater flow-through system was carried out.

Comparison of biofilm and seawater communities showed that, despite possessing classes in common, both communities are generally distinct. This finding supports several previous studies (Amaral-Zettler et al., 2015; Bryant et al., 2016; De Tender et al., 2015, 2017; Oberbeckmann et al., 2014, 2016; Zettler et al., 2013) pointing toward a consensus that free-living seawater communities are different from synthetic polymer attached ones. A possible explanation might be the much higher cell density in biofilms as compared to seawater; hence higher cell density may support the development of matrix-stabilized, synergistic micro-consortia.

Synthetic polymer associated prokaryotic biofilm communities were different from glass biofilm communities. Furthermore, significant differences between the prokaryotic and eukaryotic community composition of different synthetic polymers communities were found. In contrast to clearly distinct prokaryotic seawater communities, differences between substrates were generally low (3.9–7.6%). A few notable OTUs uniquely discriminated the biofilm communities across the diverse substrates, suggesting that physicochemical properties of the substrate shape synthetic polymer communities. Complex biofilms include a diversity of organisms with different metabolic capacities and physiologies which generates on the one hand competition but also provides on the other hand opportunities for cooperation (Flemming et al., 2016).

In contrast to the homogenous prokaryotic communities analysed here, substantial heterogeneity between eukaryotic communities on the diverse substrates was observed. Statistical analyses of eukaryotic communities revealed significant differences between diverse substrates, surprisingly mainly due to OTUs predominantly assigned to mobile organisms e.g. *Dinoflagellata* or starfish (*Asteroida*). Chesson and Kuang (2008) assumed that competition dynamics at lower trophic levels (bacteria and microflagellates) might have consequences for protists' dynamics. Thereby, the polymer characteristics may select for microorganisms and they, in turn, might attract different grazers. However, this mobile organism may not be specific for a substrate and may not be found as discriminating organisms in other studies. For clarification, the polymer strips were washed excessively in that loosely attached biofilm parts were removed. This suggests that reads assigned to mobile organisms could also originate from detritus or eggs strongly embedded in the EPS, this is also an explanation why, beside others, starfish have been identified only by molecular tools but not by SEM. Furthermore, based on the general heterogeneity of eukaryotic communities it can be assumed that this observation may be coincidental.

Analysing the eukaryotic community composition, the class of

Chytridiomycetes (*Chytridiomycota*) was found with highest abundances across all detected fungal classes. Recently, Kettner et al. (2017) investigated fungal communities attached to PE and PS from the River Warnow to the Baltic Sea but found no significant differences comparing both substrates communities. Interestingly, in the study of Kettner et al. (2017), the majority of fungal 18S rRNA reads were assigned to *Chytridiomycota*, which is consistent with our findings. Since fungi are of particular interest in their role as potential plastic degraders in the environment (Grossart and Rojas-Jimenez, 2016; Krueger et al., 2015), the repetitive detection of highest abundances of *Chytridiomycota* associated to marine plastics in both studies suggests that further investigations on their role in plastic biofilms are required.

In general, differences in the biofilm community composition are related to different factors, for example the substratum physicochemical properties e.g. hydrophobicity, roughness, vulnerability to weather but also surface chemodynamics like surface conditioning or nutrient enrichment (Dang and Lovell, 2016). Particularly primary colonizers, sensing the synthetic polymer surface, impact community formation, dynamics, and function (Dang et al., 2008). In respect of PLA, which is known to be biodegradable when composted, the degradation mechanism start with chemical hydrolysis in the presence of water at elevated temperatures (60 °C and above), followed by biological degradation (Shah et al., 2008). Since North Sea water temperatures were never above 18 °C during the 15 month of our experiment, biotic degradation is unlikely.

Beside physicochemical surface properties, it has been shown that the composition of biofilm communities associated to synthetic polymers differed distinctly with respect to different ocean basins (Amaral-Zettler et al., 2015) and underlies both seasonal and spatial effects e.g. in North Sea waters (Oberbeckmann et al., 2014). Biofilms in this study were sampled at one time point, thus seasonal and temporal changes in the taxonomic composition were not investigated. However, these biofilms were exposed to seasonal variation of several environmental factors in the North Sea such as temperature or nutrient variation within the seawater flow-through system. To delineate the effects of seasonal variation on the community composition biofilms should be monitored at close intervals best over more than one seasonal cycle. The incubation conditions applied in this setting of a natural seawater flow-through system with e.g. less shear forces and lack of light, in contrast to incubation in the open sea, may have influenced the establishment of a synthetic polymer specific community. It is known that biofilm community composition is strongly driven by the factor environment (Salta et al., 2013). Recently, in a long-term exposure experiment of PE in two different environments, harbour and offshore, De Tender et al. (2017) demonstrated a shift toward more secondary colonizers of PE biofilms at later stages, interestingly, only in the harbour environment, an environment which is less exposed to shear and current forces. To the best of our knowledge, the only other study which compared PET with glass-communities, after exposure in the open sea (i.e. high shear stress), found no distinct communities (Oberbeckmann et al., 2016). In contrast, in the present study clear differences were observed between prokaryotic communities on synthetic polymers as compared to glass after exposure in a seawater flow-through system with low shear stress. However, the time of exposure in our experiment was much longer than in the study of Oberbeckmann et al. (2016), thus the latter synthetic surfaces (i.e. glass vs. PET bottles) were colonized by a relatively “young” biofilm community after exposure of 5–6 weeks as opposed to the 15 month “old” biofilm, investigated in the present study. Hence it can be presumed that early colonizers might be more generalists than specialists and specific biofilm communities evolve over a longer period of time or/and in semi enclosed environments.

OTUs with a mean relative abundance of at least > 0.1% in one substrate type were analysed, and found that along these, even if sometimes rare (< 0.1%) all prokaryotic OTUs were detected on synthetic polymers and glass. Hence, the dissimilarities in the prokaryotic community composition observed as a function of the synthetic polymers investigated resulted from variable relative abundance profiles of dominant OTUs. Recently, De Tender et al. (2017) identified a core group of 25 single OTUs based on their abundance profiles on PE in the Belgian North Sea. Comparison with our data revealed that four of the reported genera were also present, with relative abundances > 0.1%, in the 15 month old biofilm communities analysed in the present study, belonging to *Anderseniella*, an uncultured *Rhodobacteraceae*, *Sulfurovum*, and the unclassified OTU belonging to *Proteobacteria* of the marine benthic group JTB255 (Fig. S3, Table S7). It remained unclear whether these indicator organisms are specific for the environment or whether they are commonly found more generally on different types of hard substrates. First, these organisms seem to be rather unspecific for the tested environment and may be therefore useful as indicator organisms for biofilm development in several parts of the North Sea. Second, with the exception of *Sulfurovum*, the above-mentioned genera were present on all substrate types without notably discriminating the different biofilm communities, suggesting that these organisms are common members of North Sea biofilms. Third, the overall dissimilarities between the analysed prokaryotic communities were generally low, which indicates that the shared core of the various biofilms is rather substrate unspecific. Fourth, the strongest contribution to the total dissimilarity between the diverse substrates was often given by less abundant OTUs (< 1%). Consequently, identification of a core group of indicator organisms of polymer specific biofilms based on the dominant OTUs is limited, because it illustrates a more general marine biofilm core community rather than a synthetic polymer specific one.

Significant differences between various substrates for prokaryotes and eukaryotes were detected but also substantial heterogeneity between eukaryotic biofilms. The present study, as well as other research about the composition and function of eukaryotes in marine biofilms, suffers from a gap in current taxonomic reference databases. Only 86.49% of the sequences obtained for eukaryotes were classified (coverage and alignment identity of min. 93%). This illustrates the current need to combine molecular based techniques and visual tools like SEM. *Luffisphaera* (VØRS, 1993) probably represents one of those taxa which probably counted among the unclassified sequences (13.5%). Even though the genus *Luffisphaera* has been described, and comprises several species, tag sequence data is not available yet and the phylogeny of this protist is still unresolved. Furthermore, visual inspection by SEM enables to identify species, e.g. *Acanthoeca spectabilis*, verify the presence/absence of mobile organisms, e.g. starfish (*Asteroida*), which were detected only by rRNA gene tag sequencing. Concerning the repetitive detection of highest abundances of *Chytridiomycota* associated to marine plastics, the use of fungi specific primers in upcoming studies needs to be considered, to gain detailed insights in their taxonomy. To date due to short read lengths, a conclusive identification of discriminative biofilm members on the species level is not reliable. However, synthetic polymer “specialists” might be represented by rather rare species, thus they would have been missed them since the sequencing approach was not deep enough for analyses of the rare biosphere. Since phylogenetic assignment based on rRNA gene tag sequencing is not linked to specific functions or metabolic activity, specific roles of the discriminating members related to the synthetic polymers remain theoretical. To gain insights into the function and activity of microbial biofilm communities, including the rare biosphere, attached to synthetic polymers further experiments including “omics” need to be conducted. To identify those specialised microbes that are preferentially able to colonize and interact with synthetic polymer surfaces, those organisms need to be selected and enriched from the shared core biofilm community and to test their potential degradation ability.

5. Conclusion

Our study represents a systematic and statistically robust analysis of 15 month old biofilms associated to distinct synthetic polymers, and

therefore enrich our knowledge on the substrate specificity of the "Plastisphere". First and foremost, it has been proofed that mature biofilms attached to synthetic polymers are significantly different from glass biofilms. Although differences of prokaryotic communities between synthetic polymers were generally low (3.9–5.5%), significant differences between biofilms on diverse polymers were observed. Furthermore, it was shown that a more general prokaryotic marine biofilm core community serves as shared core among all synthetic polymers rather than a specific synthetic polymer community. However, the general heterogeneity of eukaryotic communities was much higher, concluding that observations of significant differences may be coincidental. These findings indicate that the term "Plastisphere" is valid for mature prokaryotic but may not be for eukaryotic biofilm communities.

Acknowledgments

We would like to thank Dr. Barry Leadbeater, University of Birmingham, for helping us with the identification of eukaryotic organisms visualized by SEM. We thank also Maike Timmermann for her assistance in the laboratory. Furthermore, we would like to thank Dr. Marlies Reich, University of Bremen, for her support. We thank Dr. Cédric Meunier for fruitful discussions. We are grateful for kind provision of environmental data by Prof. Dr. Karen Wiltshire. This work was founded by the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Helmholtz Centre for Polar and Marine Research and supported by the German Federal Ministry of Education and Research (Project BASEMAN - Defining the baselines and standards for microplastics analyses in European waters; BMBF grant 03F0734A).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2018.09.028.

References

Amaral-Zettler, L.A., Zettler, E.R., Slikas, B., Boyd, G.D., Melvin, D.W., Morrall, C.E., Proskurowski, G., Mincer, T.J., 2015. The biogeography of the Plastisphere: implications for policy. Front. Ecol. Environ. 13, 541–546.

Barnes, D.K., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Phil. Trans. Roy. Soc. Lond. B Biol. Sci. 364, 1985–1998.

Bryant, J.A., Clemente, T.M., Viviani, D.A., Fong, A.A., Thomas, K.A., Kemp, P., Karl, D.M., White, A.E., DeLong, E.F., 2016. Diversity and activity of communities inhabiting plastic debris in the North Pacific Gyre. mSystems 1, e00024-00016.

Chesson, P., Kuang, J.J., 2008. The interaction between predation and competition. Nature 456, 235–238.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143.

Dang, H., Li, T., Chen, M., Huang, G., 2008. Cross-ocean distribution of Rhodobacterales bacteria as primary surface colonizers in temperate coastal marine waters. Appl. Environ. Microbiol. 74, 52–60.

Dang, H., Lovell, C.R., 2016. Microbial surface colonization and biofilm development in marine environments. Microbiol. Mol. Biol. Rev. 80, 91–138.

De Tender, C., Devriese, L.I., Haegeman, A., Maes, S., Vangeyte, J., Cattrijsse, A., Dawyndt, P., Ruttink, T., 2017. Temporal dynamics of bacterial and fungal colonization on plastic debris in the North sea. Environ. Sci. Technol. 51, 7350–7360.

De Tender, C.A., Devriese, L.I., Haegeman, A., Maes, S., Ruttink, T., Dawyndt, P., 2015. Bacterial community profiling of plastic litter in the Belgian part of the North sea. Environ. Sci. Technol. 49, 9629–9638.

Debroas, D., Mone, A., Ter Halle, A., 2017. Plastics in the North Atlantic garbage patch: a boat-microbe for hitchhikers and plastic degraders. Sci. Total Environ. 599–600, 1222–1232.

Diepenbroek, M., Glöckner, F.O., Grobe, P., Güntsch, A., Huber, R., König-Ries, B., Kostadinov, I., Nieschulze, J., Seeger, B., Tolksdorf, R., 2014. Towards an Integrated Biodiversity and Ecological Research Data Management and Archiving Platform: the German Federation for the Curation of Biological Data (GFBio), GI-jahrestagung. pp. 1711–1721.

Dobretsov, S., Dürr, S., Thomason, J., 2010. Marine Biofilms. Biofouling, pp. 123–136.

Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. Nat. Rev. Microbiol. 14, 563–575.

Grossart, H.-P., Rojas-Jimenez, K., 2016. Aquatic fungi: targeting the forgotten in microbial ecology. Curr. Opin. Microbiol. 31, 140–145.

Harrison, J.P., Schratzberger, M., Sapp, M., Osborn, A.M., 2014. Rapid bacterial colonization of low-density polyethylene microplastics in coastal sediment microcosms. BMC Microbiol. 14, 1–15.

Ionescu, D., Siebert, C., Polerecky, L., Munwes, Y.Y., Lott, C., Häusler, S., Bižić-Ionescu, M., Quast, C., Peplies, J., Glöckner, F.O., Ramette, A., Rödiger, T., Dittmar, T., Oren, A., Geyer, S., Stärk, H.-J., Sauter, M., Licha, T., Laronne, J.B., de Beer, D., 2012. Microbial and chemical characterization of underwater fresh water springs in the dead sea. PloS One 7, e38319.

Kettner, M.T., Rojas-Jimenez, K., Oberbeckmann, S., Labrenz, M., Grossart, H.P., 2017. Microplastics alter composition of fungal communities in aquatic ecosystems. Environ. Microbiol. 19 (11), 4447–4459.

Kirstein, I.V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., Gerdts, G., 2016. Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic particles. Mar. Environ. Res. 120, 1–8.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41 e1-e1.

Krueger, M.C., Harms, H., Schlosser, D., 2015. Prospects for microbiological solutions to environmental pollution with plastics. Appl. Microbiol. Biotechnol. 99, 8857–8874.

Leadbeater, B.S., Henouil, M., Berovic, N., 2008. Choanoflagellate lorica construction and assembly: the nudiform condition. II. Acanthoeca spectabilis Ellis. Protist 159, 495–505.

Legendre, P., Legendre, L.F., 2012. Numerical Ecology. Elsevier.

Lucas, J., Wichels, A., Teeling, H., Chafee, M., Scharfe, M., Gerdts, G., 2015. Annual dynamics of North Sea bacterioplankton: seasonal variability superimposes short-term variation. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 91 fiv099-fiv099.

Oberbeckmann, S., Kreikemeyer, B., Labrenz, M., 2018. Environmental factors support the formation of specific bacterial assemblages on microplastics. Front. Microbiol. 8.

Oberbeckmann, S., Loeder, M.G., Gerdts, G., Osborn, A.M., 2014. Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European waters. FEMS Microbiol. Ecol. 90, 478–492.

Oberbeckmann, S., Osborn, A.M., Duhaime, M.B., 2016. Microbes on a bottle: substrate, season and geography influence community composition of microbes colonizing marine plastic debris. PloS One 11, e0159289.

Ogonowski, M., Asa, M., Karolina, I., Eva, H., Zandra, G., U.K, I., Zoltan, B., Elena, G., 2018. Evidence for selective bacterial community structuring on microplastics. Environ. Microbiol. 0.

Piredda, R., Tomasino, M.P., D'Erchia, A.M., Manzari, C., Pesole, G., Montresor, M., Kooistra, W.H.C.F., Sarno, D., Zingone, A., 2017. Diversity and temporal patterns of planktonic protist assemblages at a Mediterranean long term ecological research site. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 93 fiw200-fiw200.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596.

Salta, M., Wharton, J.A., Blache, Y., Stokes, K.R., Briand, J.F., 2013. Marine biofilms on artificial surfaces: structure and dynamics. Environ. Microbiol. 15, 2879–2893.

Sapp, M., Wichels, A., Wiltshire, K.H., Gerdts, G., 2006. Bacterial community dynamics during the winter–spring transition in the North Sea. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 59, 622–637.

Shah, A.A., Hasan, F., Hameed, A., Ahmed, S., 2008. Biological degradation of plastics: a comprehensive review. Biotechnol. Adv. 26, 246–265.

Toribio, A.L., Alako, B., Amid, C., Cerdeño-Tarrága, A., Clarke, L., Cleland, I., Fairley, S., Gibson, R., Goodgame, N., ten Hoopen, P., 2017. European nucleotide archive in 2016. Nucleic Acids Res. 45, D32–D36.

VØRS, N., 1993. Marine heterotrophic amoebae, flagellates and heliozoa from Belize (Central America) and Tenerife (Canary Islands), with descriptions of new species, *Luffisphaera bulbochaete* N. Sp., *L. longihastis* N. Sp., *L. turriformis* N. Sp. and *Paulinella intermedia* N. Sp. J. Eukaryot. Microbiol. 40, 272–287.

Wiltshire, K.H., Malzahn, A.M., Wirtz, K., Greve, W., Janisch, S., Mangelsdorf, P., Manly, B.F.J., Boersma, M., 2008. Resilience of North Sea phytoplankton spring bloom dynamics: an analysis of long-term data at Helgoland Roads. Limnol. Oceanogr. 53, 1294–1302.

Yilmaz, P., Kottmann, R., Field, D., Knight, R., Cole, J.R., Amaral-Zettler, L., Gilbert, J.A., Karsch-Mizrachi, I., Johnston, A., Cochrane, G., 2011. Minimum information about a marker gene sequence (MIMARKS) and minimum information about any (x) sequence (MIxS) specifications. Nat. Biotechnol. 29, 415–420.

Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the 'Plastisphere': microbial communities on plastic marine debris. Environ. Sci. Technol. 47 (13), 7137–7146.