Marine Pollution Bulletin 146 (2019) 117–124

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Paint particles are a distinct and variable substrate for marine bacteria

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ARTICLE INFO

Keywords:
Microplastic
Biofilm
16S
Sediment
Desulfobacteraceae
Desulfatitalea tepidiphilia

ABSTRACT

While paint particles are an important part of the microplastic sphere, they have, as yet, received much less research coverage, particularly regarding microplastic-microbiological interactions. This study investigated the biofilm communities of a variety of paint particles from brackish sediment using 16S rRNA gene sequencing. Paint particle biofilm communities appear to be distinct from natural (water and sediment), non-synthetic particle (cellulose) and common microplastic biofilm communities. Notably, there appears to be 1 group of sulphate-reducing bacteria from the Desulfobacteraceae family, *Desulfatitalea tepidiphilia*, that dominate certain paint biofilms. Of the 8 investigated paint-associated communities, four paints displayed this high Desulfobacteraceae presence. However, it is currently unclear from the chemical analysis performed of the paint surface chemistry (ATR FT-IR spectroscopy, Raman spectroscopy, SEM-EDX) what the drivers behind this might be. As such, this study provides important insights as the first to analyse microplastic-paint biofilm communities and paves the way for future research.

1. Introduction

Microplastics have been recognised as an omnipresent aquatic pollutant in recent years. The various attributes which they possess are cause for additional concern over large plastic pieces and have been well discussed by a multitude of authors (Besseling et al., 2013; Browne et al., 2011, 2008; Cole et al., 2013, 2011; Tagg and Labrenz, 2018; Thompson et al., 2004). However, paint particles, generally considered to be a subset within the umbrella-term “microplastics”, present further concerns. Paints are compositionally different to plastics, but share certain characteristics. Plastics are overwhelmingly composed of a single, repeated, hydrocarbon-based monomer, although some plastics, particularly plastic fabrics, can have a considerable proportion of additives. However, while paints and common plastics share a polymeric basis, paints additionally contain larger amounts of additives, much of which are inorganic elements. Some of these, often included for their anti-biofouling properties, such as zinc or copper (Yebra et al., 2004), could influence the microbiome of marine sediment, wherein paint particles are known to contaminate (Imhof et al., 2016). As such, paint particles represent both an important aquatic pollutant and microbial substrate within the microplastic sphere.

Microplastic-biofilm interactions have already been well-examined in a variety of environments (Harrison et al., 2018; Hoellein et al., 2014; Kesy et al., 2017; McCormick et al., 2014; Oberbeckmann et al., 2015) and the analysis of such can now be considered an independent research field (Ivar do Sul et al., 2018). For example, two recent studies of microplastic-microbial interactions found significantly different communities on microplastics compared to natural substrates (Ogonowski et al., 2018; Miao et al., 2019). In particular, Ogonowski et al. (2018) also studied particles exposed to Baltic aquatic conditions and observed distinctly lower diversity within all biofilm communities compared to the surrounding water, suggesting substrate was a major selective driver. However, the majority of research investigating paint-microbial interactions are concerned with the effectiveness of anti-biofouling paints. As yet, there is very little research concerning microbial interactions with paint particles in marine sediments. It is essential to understand the microbial assemblages that may persist on paint particles in the marine environment, and whether paint particle-associated communities are distinct from microplastic communities as well as compared to natural microbial niches (sediment, natural particle-attached and whole water communities). This is important because paints have different physiology (chemistry, density, shape) than common plastics, as well as different applications. They can, therefore, be expected to have differing point and diffuse sources as well as

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https://doi.org/10.1016/j.marpolbul.2019.06.013
Received 13 May 2019; Received in revised form 5 June 2019; Accepted 6 June 2019

differing final sinks compared to common microplastics. As such, areas in which paints congregate could be influenced by the biofilm present on paint particles. Paint particle ingestion by (and other interactions with) biota may well also differ from other microplastics. Additionally, given the antimicrobial, metallic compositions of some paints, the microbiota which persist on paint particles in the marine environment may be significantly different from natural communities. As such, paints may be a discrete vector for certain microorganisms, in ways microplastic may not. This concern is compounded by recent research that has shown that some antifouling paints which contain heavy metals can pre-select for antibiotic resistance in biofilm communities (Flach et al., 2017). Paint particles can be highly abundant in certain environments, particularly in sediments, and even succeed the abundance of common microplastics (Imhof et al., 2016). A better understanding of the microbial interactions with these highly abundant particles is needed given the potential effects on the aquatic environment generally, and sediment microbiome specifically.

This study is designed to provide a first glimpse at paint particle biofilms and demonstrate how paint-associated biofilms differ from biofilms on both other synthetic as well as natural marine particulate substrata.

2. Materials and methods

2.1. Sampling

Sampling was undertaken on November 17th 2017. The sampling location was an inlet of the Baltic Sea adjacent the mouth of the Warnow river in north-eastern Germany known as the Alter Strom. The area is heavily occupied with both commercial tourism and small fishing vessels. Samples were taken from the middle of the waterway by means of a small sampling boat. The approximate co-ordinates of the sampling location are 54°10′45.8″N 12°05′16.4″E.

Sediment grabs were taken using a Van-Veen grab and sealed in 3 separate 2 l glass jars (1 jar per grab; $n = 3$). Before sealing, a 2 ml tube (VWR, Germany) was filled with sediment taken from each jar to be separately analysed for the sediment community. Each jar of sediment was carefully washed through a 500 µm steel mesh using seawater taken from the same location. The material collected on the mesh was retained for particle analysis. 6 × 1 l water samples were also retrieved by submersion of 1 l glass bottles (VWR, Germany). All collected samples were stored on ice until they could be further processed in a laboratory. Temperature and pH were also measured at the time using a HQD portable meter and probes (Hach, USA). 20 ml water from the same location was syringe-filtered through a GFF filter (Whatman, UK) and stored in a 50 ml centrifuge tube (VWR, Germany) at −20 °C. Nutrients were measured colorometrically according to Grasshoff et al. (1999) by means of a Seal Analytical QuAAtro constant flow analyser (Seal Analytical, Germany). A sediment core was also taken of the top 10 cm and air-sealed. Pore water was extracted from the core using a rhizome sampler (Rhizosphere Research Products B.V., The Netherlands) and the hydrogen sulphide (S^{2-}) concentration was then measured according to Cline (1969). All environmental measurements are included in the supplementary information (Table S1).

All post-sampling sample preparation procedures were conducted within a Safe 2020 laminar flow cabinet (Thermo Fisher Scientific, USA) to avoid contamination. Each sediment jar sample (following mesh rinsing) was carefully visually examined and particles resembling plastic or paint fragments were selected with sterile tweezers. Each particle had a small portion removed using a sterile scalpel. The bulk of the particle was placed in a clean 2 ml Eppendorf tube and suspended in 1 ml sterile seawater (prepared earlier by filtering Baltic seawater through a 0.2 µm polycarbonate membrane filter and stored at 4 °C until use). The smaller piece of each particle was also placed in a clean Eppendorf tube and retained for surface chemistry analysis (Fourier Transform Infrared [FT-IR] and Raman spectroscopy; see below). A total 17 particles were isolated from across the 3 × 2 kg sediment grab jars: grab #1: $n = 6$; grab #2: $n = 4$; grab #3: $n = 7$. Water samples were separately filtered through a 3 µm polycarbonate membrane filter ($n = 3$) and a 0.2 µm membrane filter (n = 3) (Millipore Sigma, USA). Each filter was placed in an individual clean Eppendorf tube. For separate sediment samples, ~3 g of each sediment sample was placed in an individual Eppendorf tube. While working, all samples were placed on ice and once all samples had been prepared, all samples were flash-frozen and stored in a liquid nitrogen-filled storage container. Samples which could not immediately be processed for DNA extraction were transferred to a − 80 °C storage freezer.

2.2. DNA extraction, PCR and sequencing

A MoBio Powersoil DNA extraction kit (Qiagen, Germany) was used for DNA extraction. Particles were transferred from storage suspension to bead-beating tubes with sterile tweezers. ~0.25 g of each sediment sample was transferred using a small sterile spatula. For water samples, filters were cut into small (~2 mm^2) pieces using a sterile scalpel in a sterile glass petri dish before being transferred to bead-beating tubes. Procedures for extraction were followed as per the manufacturer's methodology except for elution volumes. Particle-samples were eluted in 15 µl 10 mM tris solution, water samples in 30 µl and sediment samples in 50 µl.

The V4 region of the 16S rRNA region (SSU) was targeted for investigating bacterial communities (Yarza et al., 2014) using the primers 515F (5′- GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′; Caporaso et al., 2011) with forward (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3′) and reverse (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3′) adaptors. PCRs and sequencing were performed as per the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina, n.d.) unless where hereby specified. An alternative touchdown approach was used for amplicon PCR with an initial denaturing step of 98 °C for 2 min followed by 9 cycles of denaturation at 98 °C for 15 s, annealing at 65 °C for 15 s and extension at 68 °C for 30 s with a 1 °C reduction per cycle, followed by 24 cycles of denaturation at 98 °C for 15 s, annealing at 55 °C for 15 s and extension at 68 °C for 30 s, with a final extension step at 72 °C for 5 min. Additional exceptions to the Illumina protocol were performing Bioanalyzer analysis following both amplicon and indexing PCRs. Additionally, libraries were normalized to 4 nM before being pooled, denatured and diluted, with a final concentration of 5pM. 10% PhiX control was spiked into the final pool. 4 pM of the final library pool was subjected to 1 individual sequencing run using a 500 (251 forward/251 reverse) cycle V2 chemistry kit on an Illumina MiSeq machine. During the run, roughly 800 (k/mm^2) clusters were sequenced, generating about 25 million reads passing filter specs. Over 81% of the sequencing and index reads were found with a Qscore ≥ 30. All raw data FASTQ files were recovered from the machine and used for further sequence data processing as outlined below.

2.3. Sequence data processing

Adapter, barcode, and primer sequences were clipped. Further quality filtering of reads (permitted length = 225–275 bp, max. number of ambiguous bases per sequence = 0, maximum number of homopolymers per sequence = 8, chimera filtering via vsearch), taxonomy assignment (Wang classification, reference database SILVA SSU nr_v132, required bootstrap value ≥85%, removal of chloroplasts, mitochondria, eukaryotes, unknown sequences) and picking of operational taxonomic units (OTUs, label = 0.03) were carried out using Mothur v.1.39.5 (Schloss et al., 2009). OTUs with an abundance of ≤3 across the dataset were excluded from downstream analyses as well as any OTU identified in blank samples at > 0.001 relative abundance. In order to avoid biases associated with uneven sequences numbers across samples, the whole dataset was randomly subsampled to 18,005

sequences per sample. 4 particle-communities were removed from further biofilm analysis either due to very low, below rarefaction cut-off DNA yields or prominent presence of extraction blank OTUs (due to such low DNA yields that blank OTU appear prominently). Rarefaction, as well as alpha diversity metrics, were calculated using the QIIME software package (MacQIIME 1.9.1-20150604; Caporaso et al., 2010). Analysis of similarity (ANOSIM) was performed in R (v. 3.2.1; R Core Team, 2013) using the package "vegan" (Oksanen et al., 2013).

Further beta diversity analyses were carried out with the software PRIMER 7 including the PERMANOVA + add-on package (PRIMER-e, UK). Relative abundances were square root-transformed and used to calculate Bray-Curtis similarity matrices. Based on these similarities, hierarchical cluster analyses (group average) were performed, and, to highlight the most abundant groups per sample, a shade plot was compiled, displaying solely the OTUs with a relative abundance > 0.05 in a given sample. Also based on Bray-Curtis similarity matrices (using square root relative abundance), non-metric multidimensional scaling (nMDS) was performed of certain subsets of the data, and BEST analyses (BVSTEP, Spearman rank correlation) identified the OTUs explaining best the community pattern of these subsets.

A basic tree of important Desulfobacteraceae OTUs was reconstructed in ARB (Ludwig et al., 2004) based on related public sequences of approximately 1500 nucleotides. These were reduced to unambiguously alignable positions using group-specific 50% conservation filters. The outgroup consisted of seven sequences of the Archangiaceae. An evolutionary distance dendrogram was constructed using the Jukes-Cantor correction and neighbour joining.

All raw sequence files, including sequencing controls, are available from the NCBI Short Read Archive (SRA; BioProject PRJNA497355).

2.4. Surface chemistry analysis

All FTIR measurements were done in Attenuated Total Reflection (ATR) with the spectrometer Vertex 80v (Bruker, Germany) and the Golden Gate ATR unit with a diamond crystal (Specac, UK) with 100 scans. The spectral resolution was 4 cm^{-1}. Spectral searches were performed using the Bruker OPUS software with the Hummel Polymer spectra library as well as the in-house developed FTIR spectral library at the Leibniz Institute of Polymer Research Dresden (IPF).

All Raman measurements were performed with the Qontor microscope (RENISHAW, UK) and a 20× objective. The integration time was 1 s with 100 accumulations. The laser power was dependent from the thermal stability of the microparticles and was between 0.1 and 15 mW. Spectral searches were performed using the Renishaw software WIRE with spectral databases from STJapan for polymers and inorganic substances and as well as the in-house databases for polymers, pigments and paints at the IPF.

Synthetic particles identified with prior FT-IR and Raman spectroscopy were further analysed for elemental composition using energy-dispersive X-ray (EDX) spectroscopy. Before scanning, samples were mounted on carbon tape and vacuum sputter-coated in elemental carbon to provide good electrical conductivity. Analysis was performed using a Zeiss Merlin Compact scanning electron microscope (Zeiss, Germany) and Oxford Instruments EDX using Inca feature 5:04 software (Oxford Instruments, UK). Measurements were taken using an aperture of 30 µm, a working distance of 8.5 mm and a kV of 15.00. 10 individual point-based readings per particle were selected. Additionally, an average-area selection was also included. Elemental composition-averages for each particle are included in the SI.

Surface area was measured of images obtained during SEM analysis. Where the particles were too large to be viewed in entirety, light microscopy was used. Surface area of each particle was calculated using ImageJ (version 1.52a; Schneider et al., 2012) and these calculations are included in the SI. These surface area calculations refer to the part-particles retained for surface chemistry analysis, yet the differences between part-particle surface area are proportionate to those subjected to microbial analysis, if not the true size of individual particles subjected to DNA extraction.

3. Results and discussion

3.1. Surface chemistry analysis

Polymer analysis revealed a range of different particles. In total, 17 suitable particles were isolated from the 3 sediment samples. Of these, 12 paints were identified, 10 of which were polyester (predominantly alkyd-based) resins, while 2 paints were epoxy resins. Further pigment distinctions were identified by FT-IR and Raman analysis and a comprehensive results table is included in the SI. Three particles were identified as common plastics (1 polypropylene [PP], 1 polyamide [PA] and 1 polyvinyl chloride [PVC]) and 2 particles were identified as non-synthetic polymers (1 cellulose fibre and 1 metallic fibre). The particles removed due to low DNA yields or presence of blank OTUs were 1 polyamide, 2 polyester resins and 1 epoxy resin (indicated in Table S2). Henceforth these material distinctions will be summarized to paints (A-H), common plastics and non-synthetics throughout this article. EDX results are also presented in the SI (Fig. S1). EDX results show the epoxy paint (H) is distinguished from the polyesters by having the highest amount of sulphur and a lower elemental diversity on average, although it is clear that paint elemental composition is highly variable even among the polyester paints (A - G).

3.2. Biofilm analysis

Biofilm community analysis based on Bray-Curtis similarity demonstrated that paints formed two different groups (Fig. 1). Thus, paint particles appear to present a distinct surface or substrate for bacterial communities in the brackish sediment environment. The whole water samples show high (> 80%) similarity within replicates and are the most distinct (< 5% similarity) from all other samples, most likely due to these samples being the only to comprise free-living microbial communities. Interestingly, the 2 common plastic particles appear highly dissimilar, with the PVC sample indicating a community-based similarity with natural particle-attached water communities and sediment, while PP demonstrates an apparently independent community, dissimilar to all other samples with < 10% shared similarity with other samples. It is unfortunate that there are no further common plastic particles to better explain this apparent dissimilarity of common plastic biofilm communities, however it is certainly interesting that paints, by comparison, share reasonable similarity in biofilm community composition across multiple ($n = 8$) samples. The differentiation of the 2 distinct paint-biofilm communities is further explained by the shadeplot (Fig. 1, bottom).

It appears particle surface area has little to do with microbial community composition in this study (see Table S3). This can be observed by comparing the two common microplastic particles. These two particles had the largest surface areas of all particles (PP = 51.315 and PVC = 29.077 compared to average particle size of 9.743 and median of 3.729) yet, can be observed from Fig. 1, these particles support completely different microbial communities being, in fact, the most dissimilar of all particles analysed in this study.

A high abundance of Desulfobacteraceae/*Desulfatitalea* OTUs (OTUs 5, 6 and 7) within paints A-D (Group 1 paints) and complete absence of these OTUs in paints *E*-H (Group 2 paints) was observed (Fig. 1). The stark appearance and absence of these OTUs may well explain the distinction of these 2 paint groups. A non-parametric multidimensional scaling (nMDS) plot of the Bray-Curtis similarity matrix (Fig. 2A) showthat non-synthetic particles appear to be well distinguished from other samples, as well as PP, which is well-distinguished from all other samples in Fig. 1. The presence of the most influential OTUs based on BEST analysis demonstrate that, in confirmation with that shown in Fig. 1, both Chloroflexi and Aegiribacteria (OTUs 22 and 47) are highly

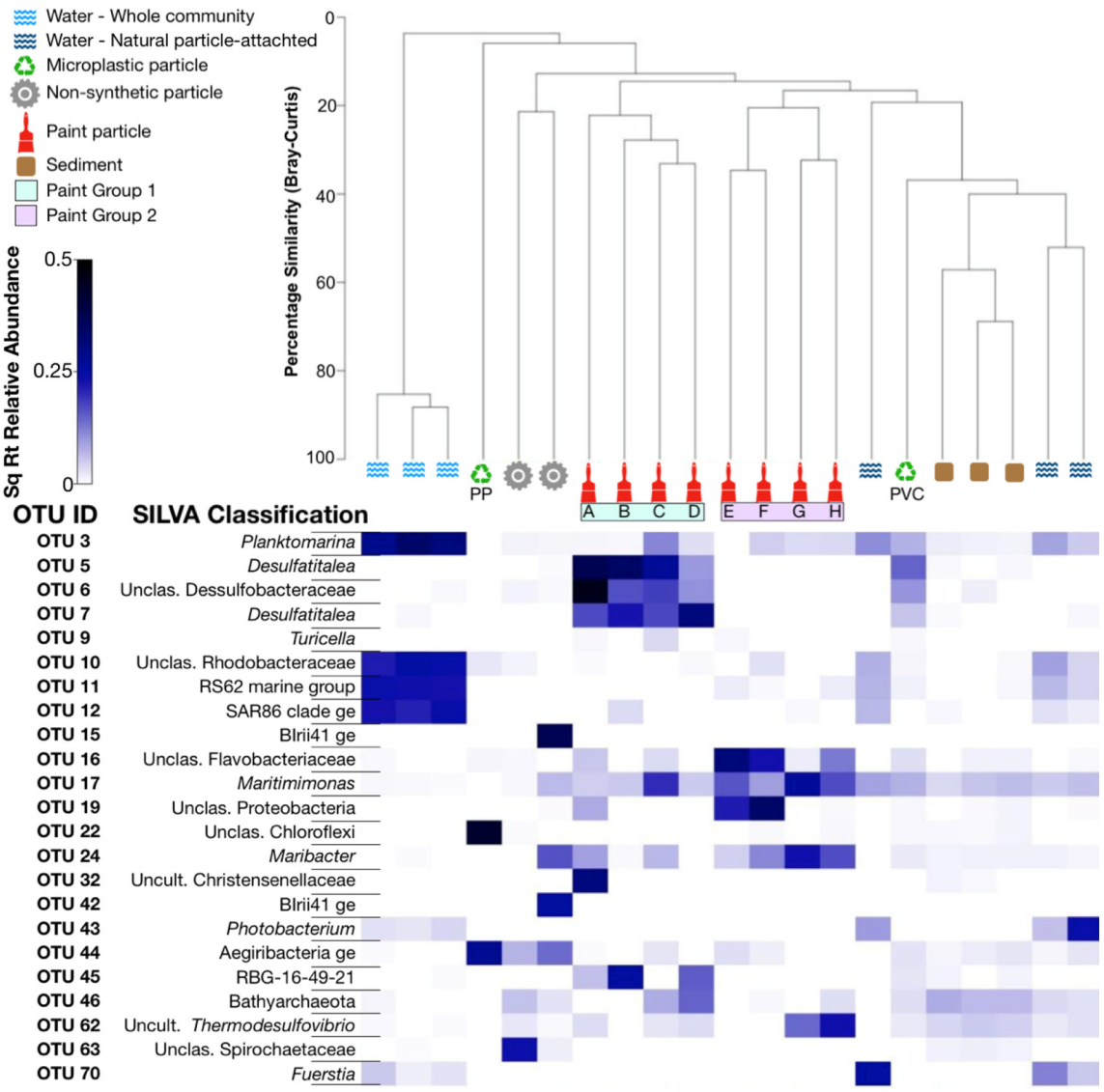

Fig. 1. Top: Sample similarity tree distanced by Bray-Curtis dissimilarity matrix. Bottom: Shade plot based on square root-transformed relative abundance (sqrt-RA) including any OTU that has at least a 0.05% sqrt-RA within the entire dataset. In some cases, SILVA classification adjusted to match NCBI accepted spelling where inconsistences in the nomenclature exist. For further classification details via BLAST, see Table S3. Unclas. = unclassified; Uncult. = uncultured.

important in the distinction of PP compared to the other particle-associated biofilms. Fig. 1A also indicates that the PVC-associated biofilm was well-distinguish from paint samples while Fig. 2A indicates that this biofilm community appears to bridge the similarity between the 2 distinct paint groups. Indeed, the PVC-associated biofilm appears to show some similarities to Group 1 paints, since PVC also appears to have high abundance of the prior-mentioned *Desulfatitalea* OTUs (OTUs 5, 6 and 7). ANOSIM analysis comparing the 2 paint group communities confirm that paint groups 1 and 2 are significantly different ($P = 0.028$; $R = 0.8333$). However, when PVC is included with Group 1 paints, the differences are more stark ($P = 0.006$; $R = 0.7188$). This indicates that the PVC biofilm does indeed bridge the similarities between the 2 paint groups, with higher similarities to Group 1 paints, likely due to the significant presence of *Desulfatitalea* OTUs. This indicates how important these *Desulfatitalea* OTUs are within Group 1 paint biofilm communities, since these paints show greater similarity to PVC than other paint communities.

While it is clear *Desulfatitalea* (OTU 5) is the most influential OTU of the Group 1 paints, the consistent appearance of the archaeal genus "*Candidatus* Lokiarchaeum" of the newly-described phylum Lokiarchaeota (OTU 30) alongside OTU 5 is interesting (Fig. S1). This phylum was first described in 2015 where it was obtained during the sampling of communities dependent on hydrothermal vent activity (Spang et al., 2015), where the genus was presented as a bridge between prokaryotes and eukaryotes. The consistent co-occurrence of this archaea along with *Desulfatitalea* could be indicative of some kind of interactive relationship. Particularly given the sulphate-metabolising attributes of *Desulfatitalea* (Higashioka et al., 2013) and the discovery of Lokiarchaeota within hydrothermal vent microbial communities, it is possible there is some shared metabolism mechanisms between these 2 taxa that explain the observed co-occurrence. This could be an important finding, as given the prominence of *Desulfatitalea* at determining a significantly different group of paints, the future study of this taxon in relation to paint-particle biofilms is important, and it may be that investigative research into this taxon should also consider Lokiarchaeota presence.

When only the paints are examined using the same analysis as performed and exhibited in Fig. 2A it can be seen that the previously key *Desulfatitalea* OTU (OTU 5) is absent, with BEST analysis identifying different Desulfobacteraceae OTUs as important (Fig. 2B). Fig. 3 presents a phylogenetic tree of key Desulfobacteraceae OTUs (characterized by analyses performed in Figs. 1 and 2) where OTUs 5, 6 and 7 all

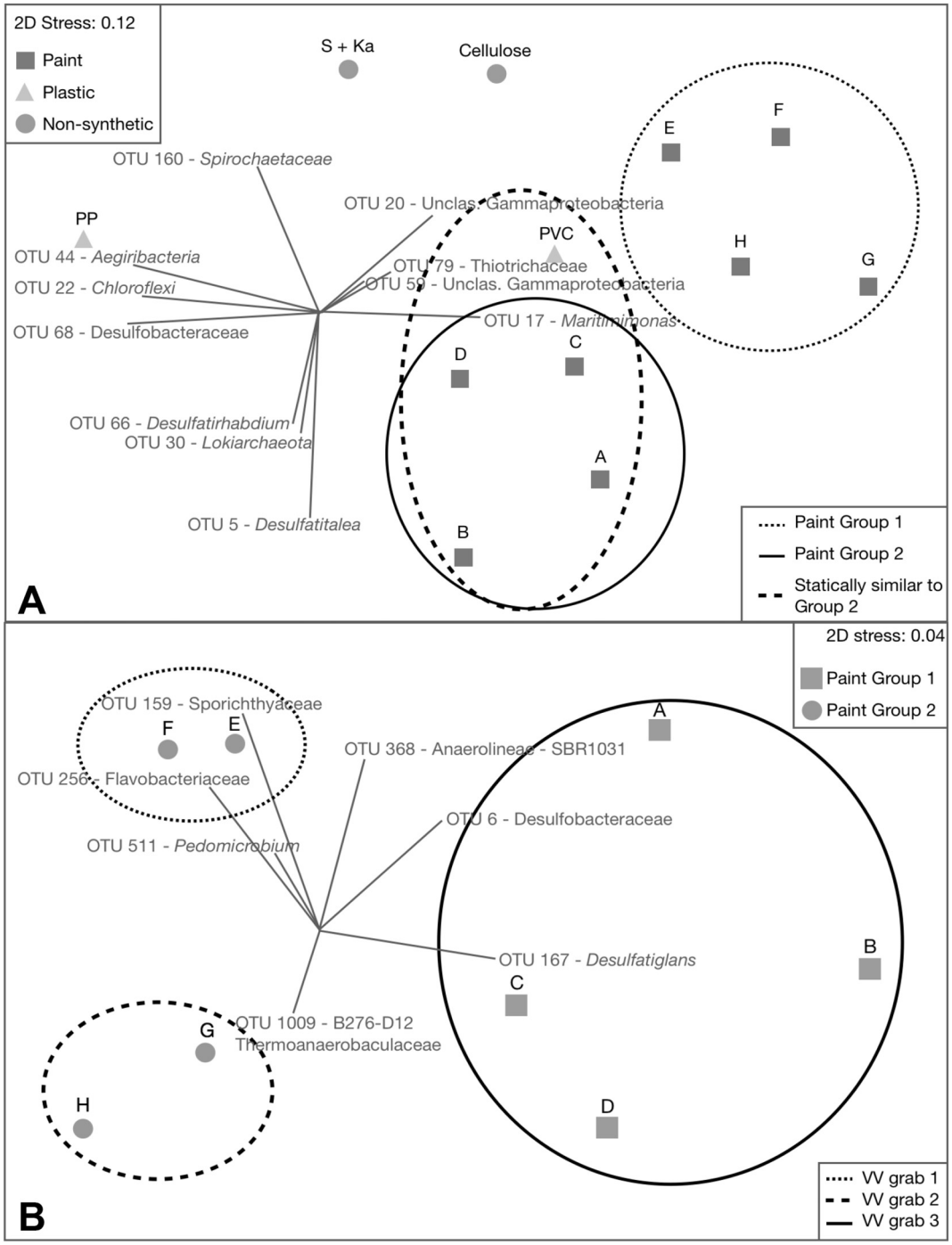

Fig. 2. Non-metric multi-dimensional scale plot of anthropogenic little particle-associated biofilm communities distances by Bray-Curtis dissimilarity matrix. Key OTUs in determining the dissimilarity of samples are included, calculated by BEST analysis. A: Showing the development of 2 distinct paint groups and the associated OTUs influencing this distinction, as well as how the PVC-associated community is associated to Paint Group 2; B: Demonstrating how the different Van-Veen grabs may be associated with the differentiation of paint-associated communities.

appear to as part of the same cluster with *Desulfatitalea tepidiphilia*. This species was identified in 2013, with the type strain (S28bF^{T}) being isolated from tidal sediment in Japan (Higashioka et al., 2013). However, this Japanese study also isolated 2 other strains from a crude oil enrichment culture, and it is to 1 of these strains, S28OL2, that OTUs 5, 6, and 7 are clustered in Fig. 3. Given that these OTUs were very prominent in Group 1 paint biofilms, it may be that these presumed *Desulfatitalea tepidiphilia* strains are able to use some aspects in the paint particle substrate as electron donors. However, while this strain was identified within a crude oil enrichment culture, it is unlikely that hydrocarbons are acting directly as electron donors in this case as the authors reported that no growth was observed for strain S28OL2 within toluene or n-hexane growth media (Higashioka et al., 2013).

OTU 68 is the only prominent Desulfobacteraceae OTU that is not associated to paint particles, instead a key member of the PP biofilm (Fig. 2A). Furthermore, while this is closely related to OTUs 5, 6 and 7, it appears to be an evolutionarily distinct cluster. As such, and given that PP, unlike paints, is purely hydrocarbon-based, it may be that the bacteria represented by OTU 68 is able to utilise some part of the PP substrate, given that sulphate-reducing bacteria have the potential to

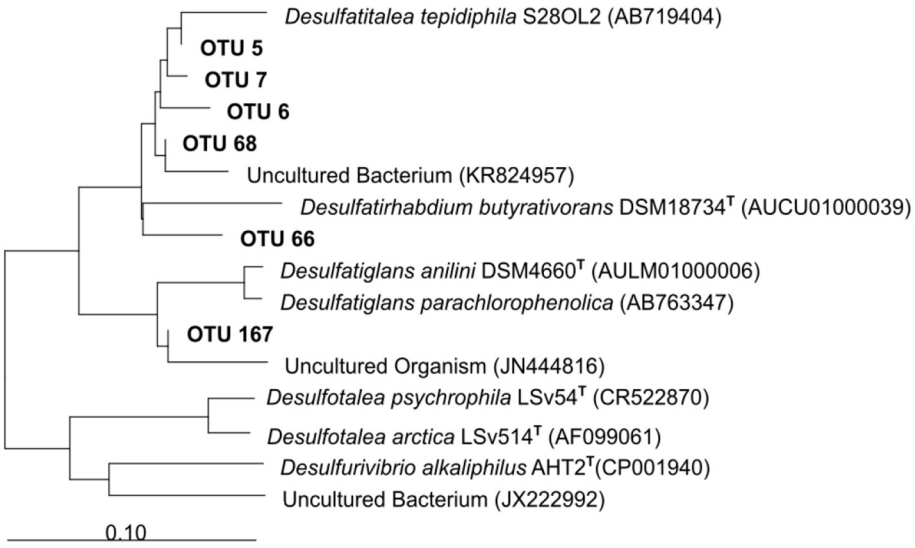

Fig. 3. A re-construction of a phylogenetic tree using neighbour joining and the Jukes-Cantor correction demonstrates the phylogenetic position of key OTU within the Desulfobacteraceae family. These OTUs are any Desulfobacteraceae that appear in prior figures, either through BEST analysis (Fig. 2) or be appearing with > 0.05% sqrt-RA within the entire dataset (Fig. 1).

utilise hydrocarbons (Kleikemper et al., 2002). However, due to the low bioavailability of microplastics, it would be more probable that hydrophobic organic substances/pollutants adsorbed to PP are potentially degraded by OTU 68 (Klaeger et al., 2019). Unfortunately, since this OTU is part of an uncultured cluster, more research is needed to better understand whether and to what extent this might be a true association.

Even when culture-based information is available and can be assigned to key OTUs driving the observed distinction of the two paint groups, it can be difficult to acutely determine what the interactions might be, due to the complexity of paint particle physiology when compared to common plastic substrates. This challenge is exhibited when examining the surface chemistry of the paints. FT-IR and Raman spectroscopy reveal 7 of the 8 paints (A – G) to be polyester-based resins; 1 being epoxy-based (H). However, unlike plastic, the polymer basis is only part of the overall chemistry. Most paints contain a variety of other chemicals, chief of which are the colorants, which are often metallic in basis (see Fig. S1). Yet the pigmentation information appears to be unimportant in distinguishing the 2 paint groups. 2 paints of Group 1 (A and D) exhibit the pigment blue 15 (phthalocyanine blue), while this exact pigment chemistry is demonstrated in 1 paint (F) from Group 1. 1 paint from each of the 2 distinguished groups also exhibit the same red pigmentation (red 3 (((4-methyl-2-nitrophenyl)azo)-2-naphthol)). Given the difficulties in explaining the 2 paint groups using pigmentation spectroscopy matches, EDX-enabled SEM analysis was also undertaken. This surface chemistry analysis also proved inconclusive at determining the dynamic behind the division of the 2 paint groups, with no apparent differences between the elemental composition of these groups according to cluster analysis and resemblance matrices (Euclidean distance) of EDX elemental data. These results show that the distinction between the two groups is likely not determined by the chemistry of the particles. However, an important factor to consider, particularly when paint particle-associated biofilms are being compared to other types of anthropogenic litter, is the stark variability of the surface composition. This is particularly well illustrated in Fig. 4, which demonstrates scanning electron microscopy images of 3 paint particles (see figure legend for paint composition details) and 1 common plastic (PP). The variability in substrate composition of the paint particle surfaces is quite stark in comparison to the highly uniform plastic substrate. This is also demonstrated in the EDX analysis (Fig. S1) where the paints show a plethora of different elemental composition while PVC is composed of only chlorine (since hydrogen and carbon cannot be measured) and PP has no measurable chemistry (being 100% hydrocarbon-based). The high variability in the elemental composition of paint particles can make it very difficult to explain whether chemistry is a determining factor in biofilm composition. Particle surface area was also found to be not significant between paint groups A and B (Students *t*-test; $P = 0.226$). While the 16S rRNA gene analysis shows that a distinction between two paint groups certainly exists, this distinction cannot be explained by the chemistry or size of the paint particles.

For this reason, comparing the results found in this study with those from other similar studies investigating microplastic biofilm communities can be challenging. This is the first study to analyse and compare the biofilm communities of microplastic-paint particles, and since paint can be a vastly different surface chemically and morphologically compared to traditional plastics, comparisons may be limited. However, some studies can offer useful, if limited, comparisons. When examining core members of PE plastics from the seafloor of a North Sea harbour, *Sulfitobacter* was described as being a core member of the biofilm community in the late-stages of environmental exposure (after 31 days). Although PE is quite dissimilar to the paint particles within this study, there are similarities, such as the habitats where the particles resided being saline, coastal marine environments influenced by high human maritime activity. Additionally, while it is not possible to state how long the particles were within the sediment, the time is likely to be considerable, with biofilms also representing late stages of community development. Therefore, it is interesting to see the emergence of 2 bacteria likely involved in the sulphur cycle (Higashioka et al., 2013; Sorokin, 1995) within the 2 studies. Another study which investigated plastics incubated in marine sediment found *Arcobacter* as a core biofilm member (Harrison et al., 2014), species of which have been shown to be potential sulphide oxidisers (Voordouw et al., 1996). It is not clear yet what effects microplastics, and particularly microplastic-paint particles may have on the marine sulphur cycle, but the results of this study, combined with similarities identified within other studies, suggest that there may be a notable interaction. However, comparisons with other studies do not immediately suggest why only half of the paints demonstrate dominance of sulphate-reducing taxa.

It is possible that spatial influences could explain why two paint groups appear to form in the data. Three independent grabs were made from the same geographic location (see Experimental Section) and it could be that the microbial conditions in the sediment may be notably different within the range of a Van-Veen grab deployed at the same surface point (assumedly around a 0–2 m range). Most microplastic particle biofilms studies that examine location as a variable have consistently found that this is the most potent variable at determining biofilm composition (De Tender et al., 2017; Hoellein et al., 2014; McCormick et al., 2014; Oberbeckmann et al., 2014). While these

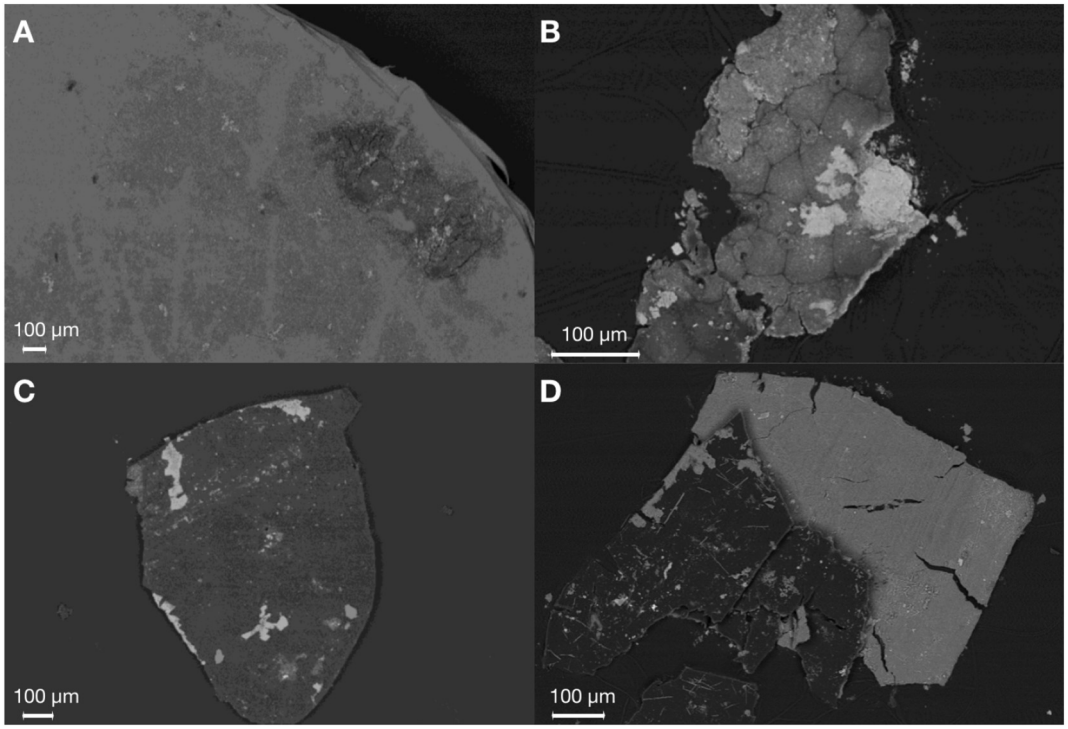

Fig. 4. Comparison of paint and plastic particles using scanning electron microscopy. A: PVC; B: Polyester resin + pigment blue 15 (phthalocyanine blue) + pigment white 6 (titanium oxide); C: Polyester resin + pigment red 3 (((4-methyl-2-nitrophenyl)azo)-2-naphthol); D: epoxide resin + pigment white 6 (titanium oxide) + pigment red 48:2 (2-Naphthalenecarboxylic acid).

studies examined spatial variability at a far greater scale than a few meters (i.e. completely alternate geographic locations), it is possible that even at such a fine scale (by comparison) location-based effects may still dominate biofilm community composition. This can be seen in a distance matrix (Fig. 2B) where grab number appears to partially explain paint-biofilm differences. While this spatial influence appears to be the most profound influence (given the lack of influence demonstrated by surface chemistry analysis), it does not completely explain the paint biofilm variability. This is because the independent sediment community samples were taken from each grab individually, yet the sediment samples do not exhibit the variability demonstrated by the paint particles. Notably, the sediment sample from grab 3 shows no greater abundance of the key Desulfobacteraceae that are important in the distinction of the 2 paint groups. A second complication is the similarity of the PVC biofilm to the biofilms of Group 1 paints. This PVC particle was obtained from the grab 1, yet it shows high affinity (and high *Desulfatitalea tepidiphilia* OTUs) to the paints obtained from grab 3. The earlier ANOSIM analysis comparing the two paint groups demonstrated a greater significant distinction when PVC was included as part of the Group 1 paint communities. Therefore, while fine-scale (0–2 m) spatial variability appears to be an influential variable in distinguishing the biofilms of paint particles, some, as yet unidentifiable, factor appears to also be influencing paint biofilms in marine sediment.

4. Conclusion

Paint particles appear to support a distinct microbial community in marine sediments. These communities appear to be more consistent than the biofilms of other common microplastics, which appear to be highly variable. However, when examined closely, there are significant differences in the biofilms of paint particles. While some of this variability appears to be attributed to the spatial variability within the meter range, other factors appear to influence the observed distinctions, although paint surface chemistry (both functional-group and elemental) appears to be an insignificant factor. It appears that, for certain paint particles in marine sediments, taxa from the Desulfobacteraceae family are abundant in the biofilm communities, particularly *Desulfatitalea tepidiphilia*, which may have a co-existent relationship with the archaea Lokiarchaeota, originally isolated from hydrothermal vents. Given the importance that these taxa may have in the sulphur metabolism cycle, further research into how paint particles may influence marine sediments is essential.

Acknowledgements

We would like to thank Jana Bull at the University of Rostock for performing the PCRs and sequencing. We would also like to thank Andrea Käppler and Franziska Fischer from the Leibniz Institute for Polymer Research in Dresden for assisting in the FT-IR and Raman spectroscopy. Thanks also goes to the following people at the Leibniz Institute for Baltic Sea Research Warnemünde: Sascha Plewe for assisting in the SEM and EDX analysis; Christian Burmeister for performing water nutrient analysis; Heide Schulz-Vogt, Iris Schmiedinger and Jan Henkel for assisting in the measuring of sulphides; Katharina Kesy for providing DNA extraction materials and Robin Lenz for assisting in the collecting of samples. Purchase of the Illumina MiSeq was kindly supported by the EU-ERDF (European Funds for Regional Development) program and funded by the University of Medicine Rostock awarded to BK. This study resulted from funding provided by the German Leibniz Association (MikrOMIK SAW-2014-IOW-2), the BONUS MICROPOLL project, supported by BONUS (Art 185), funded jointly by the EU and BMBF (03F0775A), and by BMBF project MicroCatch_Balt, grant number 03F0788A.

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2019.06.013.

References

Besseling, E., Wegner, A., Foekema, E.M., van den Heuvel-Greve, M.J., Koelmans, A.A., 2013. Effects of microplastic on fitness and PCB bioaccumulation by the lugworm Arenicola marina (L.). Environ. Sci. Technol. 47, 593–600. https://doi.org/10.1021/es302763x.

Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C., 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, Mytilus edulis (L). Environ. Sci. Technol. 42, 5026–5031.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines worldwide: sources and sinks. Environ. Sci. Technol. 45, 9175–9179. https://doi.org/10.1021/es201811s.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522. https://doi.org/10.1073/pnas.1000080107.

Cline, J.D., 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14, 454–458. https://doi.org/10.4319/lo.1969.14.3.0454.

Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. Mar. Pollut. Bull. 62, 2588–2597. https://doi.org/10.1016/j.marpolbul.2011.09.025.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646–6655. https://doi.org/10.1021/es400663f.

De Tender, C., Devriese, L.I., Haegeman, A., Maes, S., Rgen Vangeyte, J., Andrécattrijsse, 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. https://doi.org/10.1021/acs.est.7b00697.

Flach, C.-F., Pal, C., Svensson, C.J., Kristiansson, E., Östman, M., Bengtsson-Palme, J., Tysklind, M., Larsson, D.G.J., 2017. Does antifouling paint select for antibiotic resistance? Sci. Total Environ. 590–591, 461–468. https://doi.org/10.1016/J.SCITOTENV.2017.01.213.

Grasshoff, K., Kremling, K., Ehrhardt, M., 1999. Methods of Seawater Analysis, 3. Wiley-VCH, Weinheim, Germany.

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, 232. https://doi.org/10.1186/s12866-014-0232-4.

Harrison, J.P., Hoellein, T.J., Sapp, M., Tagg, A.S., Ju-Nam, Y., Ojeda, J.J., 2018. Microplastic-associated biofilms: a comparison of freshwater and marine environments, Handbook of Environmental Chemistry. https://doi.org/10.1007/978-3-319-61615-5_9.

Higashioka, Y., Kojima, H., Watanabe, M., Fukui, M., 2013. Desulfatitalea tepidiphila gen. nov., sp. nov., a sulfate-reducing bacterium isolated from tidal flat sediment. Int. J. Syst. Evol. Microbiol. 63, 761–765. https://doi.org/10.1099/ijs.0.043356-0.

Hoellein, T., Rojas, M., Pink, A., Gasior, J., Kelly, J., 2014. Anthropogenic litter in urban freshwater ecosystems: distribution and microbial interactions. PLoS One 9, e98485. https://doi.org/10.1371/journal.pone.0098485.

Illumina, n.d. 16S metagenomic sequencing library preparation [WWW Document]. Part # 15044223 Rev. B. URL https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf (accessed 10.29.18).

Imhof, H.K., Laforsch, C., Wiesheu, A.C., Schmid, J., Anger, P.M., Niessner, R., Ivleva, N.P., 2016. Pigments and plastic in limnetic ecosystems: a qualitative and quantitative study on microparticles of different size classes. Water Res. 98, 64–74. https://doi.org/10.1016/j.watres.2016.03.015.

Ivar do Sul, J.A., Tagg, A.S., Labrenz, M., 2018. Exploring the common denominator between microplastics and microbiology: a scientometric approach. Scientometrics 117, 2145–2157. https://doi.org/10.1007/s11192-018-2936-y.

Kesy, K., Hentzsch, A., Klaeger, F., Oberbeckmann, S., Mothes, S., Labrenz, M., 2017. Fate and stability of polyamide-associated bacterial assemblages after their passage through the digestive tract of the blue mussel Mytilus edulis. Mar. Pollut. Bull. 125, 132–138. https://doi.org/10.1016/j.marpolbul.2017.08.016.

Klaeger, F., Tagg, A.S., Otto, S., Bienmüller, M., Sartorius, I., Labrenz, M., 2019. Residual monomer content affects the interpretation of plastic degradation. Sci. Rep. 9, 2120. https://doi.org/10.1038/s41598-019-38685-6.

Kleikemper, J., Schroth, M.H., Sigler, W.V., Schmucki, M., Bernasconi, S.M., Zeyer, J., 2002. Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. Appl. Environ. Microbiol. 68, 1516. https://doi.org/10.1128/AEM.68.4.1516-1523.2002.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüssmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.-H., 2004. ARB: a software environment for sequence data. Nucleic Acids Res.. 32, 1363–1371. doi:https://doi.org/10.1093/nar/gkh293.

McCormick, A., Hoellein, T.J., Mason, S.A., Schluep, J., Kelly, J.J., 2014. Microplastic is an abundant and distinct microbial habitat in an urban river. Environ. Sci. Technol. 48, 11863–11871. https://doi.org/10.1021/es503610r.

Miao, L., Wang, P., Hou, J., Yao, Y., Liu, Z., Liu, S., Li, T., 2019. Distinct community structure and microbial functions of biofilms colonizing microplastics. Sci. Total Environ. 650, 2395–2402. https://doi.org/10.1016/J.SCITOTENV.2018.09.378.

Oberbeckmann, S., Loeder, M.G.J., 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. https://doi.org/10.1111/1574-6941.12409.

Oberbeckmann, S., Löder, M.G.J., Labrenz, M., 2015. Marine microplastic-associated biofilms - a review. Environ. Chem. 12, 551–562. https://doi.org/10.1071/EN15069.

Ogonowski, M., Motiei, A., Ininbergs, K., Hell, E., Gerdes, Z., Udekwu, K.I., Bacsik, Z., Gorokhova, E., 2018. Evidence for Selective Bacterial Community Structuring on Microplastics. https://doi.org/10.1111/1462-2920.14120.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Wagner, H., 2013. Vegan: Community Ecology Package. R Package Version 2.0-10.

R Core Team, 2013. R: A Language and Environment for Statistical Computing. Vienna, Austria.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541. https://doi.org/10.1128/AEM.01541-09.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Sorokin, D.Y., 1995. Sulfitobacter pontiacus gen. nov., sp. nov. – a new heterotrophic bacterium from the Black Sea, specialized on sulfite oxidation. Mikrobiologiya 64, 354–365.

Spang, A., Saw, J.H., Jørgensen, S.L., Zaremba-Niedzwiedzka, K., Martijn, J., Lind, A.E., Van Eijk, R., Schleper, C., Guy, L., Ettema, T.J.G., 2015. Complex archaea that bridge the gap between prokaryotes and eukaryotes. Nature 521, 173–179. https://doi.org/10.1038/nature14447.

Tagg, A.S., Labrenz, M., 2018. Closing microplastic pathways before they open: a model approach. Environ. Sci. Technol. 52, 3340–3341. https://doi.org/10.1021/acs.est.8b00961.

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., McGonigle, D., Russell, A.E., 2004. Lost at sea: where is all the plastic? Science 80 (304), 838. https://doi.org/10.1126/science.1094559.

Voordouw, G., Armstrong, S.M., Reimer, M.F., Fouts, B., Telang, A.J., Shen, Y., Gevertz, D., 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. Appl. Environ. Microbiol. 62, 1623–1629 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC167936/pdf/621623.pdf.

Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K.-H., Whitman, W.B., Euzéby, J., Amann, R., Rosselló-Móra, R., 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat. Rev. Microbiol. 12, 635–645. https://doi.org/10.1038/nrmicro3330.

Yebra, D.M., Kiil, S., Dam-Johansen, K., 2004. Antifouling technology—past, present and future steps towards efficient and environmentally friendly antifouling coatings. Prog. Org. Coat. 50, 75–104. https://doi.org/10.1016/j.porgcoat.2003.06.001.