Polystyrene influences bacterial assemblages in *Arenicola marina*-populated aquatic environments *in vitro* *

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**A B S T R A C T**

Plastic is ubiquitous in global oceans and constitutes a newly available habitat for surface-associated bacterial assemblages. Microplastics (plastic particles < 5 mm) are especially susceptible to ingestion by marine organisms, as the size of these particles makes them available also to lower trophic levels. Because many marine invertebrates harbour potential pathogens in their guts, we investigated whether bacterial assemblages on polystyrene are selectively modified during their passage through the gut of the lugworm *Arenicola marina* and are subsequently able to develop pathogenic biofilms. We also examined whether polystyrene acts as a vector for gut biofilm assemblages after subsequent incubation of the egested particles in seawater. Our results showed that after passage through the digestive tract of *A. marina*, the bacterial assemblages on polystyrene particles and reference glass beads became more similar, harbouring common sediment bacteria. By contrast, only in the presence of polystyrene the potential symbiont *Amphritea atlantica* was enriched in the investigated biofilms, faeces, and water. Thus, especially in areas of high polystyrene contamination, this polymer may impact the bacterial composition of different habitats, with as yet unknown consequences for the respective ecosystems.

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1. **Introduction**

1.1. **Marine plastic pollution and its environmental implications**

Plastics are a major component of the worldwide marine litter load (Barnes et al., 2009) and have been recognised as an environmental concern for nearly 50 years (Carpenter and Smith, 1972). Although measures have been implemented to mitigate plastic pollution (MARPOL, Annex V), the ongoing accumulation of plastic litter within global oceans poses a multitude of environmental problems (Smith, 2014). Of particular concern are the so-called microplastics (Thompson et al., 2004), usually defined as being < 5 mm in size (Arthur et al., 2009; GESAMP, 2015). Microplastics in the marine environment may derive from the fragmentation of larger plastic items (Andrady, 2011; Cooper and Corcoran, 2010) or they can enter the marine environment through effluents and river runoff, as documented for fibres originating from the laundry of synthetic garments (Browne et al., 2011). While little is known about the transport mechanisms and ultimate sinks for microplastics in the ocean (Kaiser, 2010), these particles have become globally distributed and are found even in deep-sea sediments (Van Cauwenbergh et al., 2013) and in ice cores from the Arctic (Obbard et al., 2014). The small size of microplastics makes them available to lower trophic levels (Wright et al., 2013a) and the ingestion of microplastics has been reported for a variety of organisms from different habitats and with different feeding types, including zooplankton (Cole et al., 2013), bivalves (Browne et al., 2008), polychaetes (Thompson et al., 2004), fish (Carpenter et al., 1972), seabirds (Spear et al., 1995), and mammals (Eriksson and Burton, 2003). Studies on the effects of microplastic ingestion have shown that they can transport persistent organic pollutants to marine organisms (Besseling et al., 2013). Moreover, ingestion of the particles can lead to a reduction in organismal fitness or induce an inflammatory response (von Moos et al., 2012; Wright et al., 2013b). In their study of *A. marina*, Wright et al. (2013b) estimated that a 1% contamination by weight of the worm’s food source with microplastics could reduce its energy reserves by ~30%. However, investigations of microplastic ingestion by marine organisms have focused on the toxicological effects of the ingested particles, but largely ignoring the influence of gut passage on the microplastic-associated microorganisms.

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1.2. Microplastics as a substrate for marine microbial assemblages

Surfaces exposed to seawater inevitably become colonised by bacteria (ZoBell and Allen, 1935). It has been shown that spatial and seasonal factors influence the microbial assemblages on marine plastic litter (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2014), and that microbial assemblages on microplastics differ from the corresponding water and sediment assemblages (De Tender et al., 2015; Zettler et al., 2013). Gut passage following the ingestion of microplastics by marine organisms might also influence the associated microbial assemblages, by the selective removal and/or enrichment of certain bacterial taxa, thus giving rise to a distinct gut biofilm assemblage on the particles. Plante et al. (2008) analysed gut surfactants in marine polychaetes and found the selective survival of gut passage by members of the genus Vibrio. This genus contains several potentially pathogenic organisms that are often found in association with higher organisms (Harris, 1993), such as V. alginolyticus and V. para-haemolyticus in mussels (Lhai and Kühne, 2007) and crustaceans (Ashiru et al., 2012). Zettler et al. (2013) detected the enrichment of a yet unassigned Vibrio sp. (~24% of 16S rRNA gene reads) on one polypropylene particle sampled in the Sargasso Sea. The occurrence of the potential pathogen V. para-haemolyticus on a polystyrene particle sampled in the Baltic Sea was recently described (Kirstein et al., 2016). The enrichment of high-density polyethylene with Arcobacter sp., a genus that also includes potential pathogens (Collado and Figueras, 2011), was demonstrated by Harrison et al. (2014). These studies show that microplastics may carry distinct assemblages, including high abundances of potentially pathogenic bacteria. However, whether passage through the gut of marine organisms influences biofilm formation on microplastics in general or leads to the enrichment of potential pathogens is unknown. It is also unclear whether the biofilms on persistent microplastics remain sufficiently stable to allow their dispersal by ocean currents, in which case microplastics would serve as vectors of microorganisms. In densely populated coastal areas, demonstration of this route of disease transmission would have important implications for human health and socio-economic activities.

In this study, we addressed the question if the passage through the gut of a marine invertebrate could significantly alter the microplastic-associated bacterial assembly and could serve as a source for potential pathogenic bacteria on microplastics. We also determined the stability of the particle-associated biofilms after egestion. As a model organism the lugworm Arenicola marina L. (1758) was used, which is a common inhabitant of the intertidal sediments within northern Europe (Riisgård and Banta, 1998). Abundances of A. marina as high as 40 individuals/m² (Reise, 1985) and a potential sediment turnover rate of up to 80 cm³ sediment/day have been reported (Cadee, 1976). As the test polymer, polystyrene was chosen because it makes up ~7% of the European plastic demand (PlasticsEurope, 2015) and is widely processed into single-use items. It is frequently detected in the marine environment (Carpenter et al., 1972; Gaessens et al., 2011; Kirstein et al., 2016), due to its higher density (~1.05 g/cm³) than seawater usually at the seafloor. Furthermore, the ingestion of polystyrene by A. marina has been documented both experimentally (Besseling et al., 2013; Van Cauwenbergh et al., 2015) and in the field (Van Cauwenbergh et al., 2015).

2. Material & methods

2.1. Collection of A. marina and natural sediment

A. marina specimens were collected from a natural population at a small sheltered basin between Poel Island and the Isle of Langenwerder, Wismar Bay, southern Baltic Sea, Germany. The local salinity ranges between 11 and 14 PSU. Sediment was collected at the same location and sieved through a 200-μm mesh.

2.2. Experimental set-up and sample collection

Preliminary experiments were conducted to determine optimal particle size and reference particle material, faeces sampling point, and sediment volume, as described in Kesy (2013). A brief description of these experiments is provided in the Supplementary Material. The final experimental set-up consisted of six independent aquaria (Fig. 1) filled with ~5 L of 200-μm-sieved sediment (dry weight: 1.3 g/ml) and 2 L of 30-μm-filtered local seawater (salinity 12 ± 0.8 PSU). Each aquarium contained one A. marina specimen with a mean size of 9.8 ± 2.5 cm and a mean wet weight (WW) of 6.1 ± 3.1 g. The worms were allowed to acclimate for 18 days at 10 °C and a light/dark cycle of 10/14 h. The aquaria were aerated constantly throughout the experiment. For the feeding experiment, polystyrene particles 250–400 μm in size (Goodfellow, UK) were added to three aquaria (polystyrene treatment) and similarly sized glass beads (Oberflächentechnik Seelmann, Germany) to the other three aquaria (reference treatment). The major mineral content of the glass beads was SiO₂ –72%, Na₂O –14%, and CaO –8%. The polystyrene particles had a rough surface with many edges (Fig. S1a), whereas the glass beads were smooth (Fig. S1b). Both, the polystyrene particles and the glass beads, were pre-incubated in 30-μm-filtered and aerated seawater for 7 days in the absence of A. marina. The major reason for this pre-incubation was, in the case of the polystyrene particles, that plasticisers were allowed to leach out of the polymer. To account for any biofilm development during this pre-incubation time, triplicate subsamples of the beads and particles were collected prior to their addition to the aquaria and stored at ~80 °C for later molecular analysis. For particle or bead addition, the water was released from the aquaria after which 345 ml of filtered local seawater was added. Samples of water, sediment and faeces were collected 4 days after the addition of the particles or beads to the aquaria using the following procedure (Fig. 2): A 45–ml water sample from each set-up was transferred to a sterile 50-ml centrifugation tube (Falcon) and then centrifuged for 20 min at ~17,400 rcf (Kryachko et al., 2012). The supernatant was discarded and the tubes containing the pellets were stored at ~80 °C. Sediment and faeces were sampled by draining the water from the aquaria, after which ~3 cm³ of sediment and ~1 cm³ of faecal material were collected using a
sterile spoon. These samples were suspended in 10 mL of sterile seawater and vacuum-filtered through 200-μm gauze to separate the previously added polystyrene particles or glass beads from the sediment or faeces. The filtered faeces were subsampled to investigate the stability of their bacterial assemblage. The remaining filtered sediment and faecal samples were centrifuged and handled as described for the water samples. The gauze pieces containing the extracted particles or beads were rinsed with sterile seawater and then split, with one half stored at −80 °C and the other half of the faeces and the corresponding polystyrene particles or glass beads frozen immediately after sampling represented the t₀ samples (Fig. 2). The remaining faeces and the corresponding polystyrene particles or glass beads were used to further investigate the stabilities of both the particle-attached biofilms and the bacterial assemblages of the faeces. Thus, fresh 30-μm-filtered seawater was carefully added to the aquaria and the other half of the faeces and the corresponding polystyrene particles or glass beads were wrapped within a 30-μm gauze piece (faeces) or 200-μm gauze piece (polystyrene or glass) and incubated in the respective aquaria (Fig. 2). These gauze pieces were retrieved after 24 h, rinsed with sterile seawater and stored at −80 °C (t₂₄ samples). Additional water samples were collected as described above.

2.3. Molecular analysis of bacterial assemblages

2.3.1. DNA extraction, polymerase chain reaction (PCR) and 16S rRNA gene-fingerprinting

A full description of the steps used in the molecular analysis is provided in the Supplementary Material. DNA was extracted and subsequently amplified using bacterial primers modified from Schwieger and Tebbe (1998), with hybridisation positions corresponding to nucleotides 519–536 on the 16S rRNA gene of Escherichia coli and with the sequence 5’-CAGCAGCCCGCGTAATAC-3’, and to nucleotides 907–925, with the sequence 5’-CCGTCATCTTTGAGTTT-3’; for a description of the coverage, see Klindworth et al. (2013). Single-strand conformation polymorphism (SSCP) gel electrophoresis was carried out in triplicate for the t₂₄ samples and for the pre-incubated (before their addition to the aquaria) polystyrene or glass samples and in duplicates for the t₀ and water samples.

2.4. Digital processing of fingerprints and statistical analysis

The dried SSCP gels were digitalised and processed using GelCompar II (Applied Math) as described by Stolle et al. (2011). A similarity matrix was calculated for each gel using Pearson correlation, based on the densitometric profiles of the lanes (Hane et al., 1993; Roling et al., 2001). This matrix was then used for non-metric multi-dimensional scaling (nMDS). Additionally, a cluster analysis was computed using the unweight pair group method with arithmetic mean (UPGMA). To test for significant differences between groups of samples, a PERMANOVA (Anderson, 2001) was run using Monte Carlo permutations with an additional PERMDISP test (Anderson, 2006). Since the PERMDISP tests were not significant, their results are omitted in the Results section (but see Tables S1 and S2). Statistical analyses were carried out using Primer6 and the add-on package PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK).

2.5. Phylogenetic analysis

The phylogeny of the major contributing bacterial operational taxonomic units (OTUs) and of those OTUs that occurred only in certain samples was determined by excising and re-amplifying the gel bands and then sequencing the resulting PCR products (LGC Genomics; Berlin, Germany). The sequences were assembled and quality checked using Seqman (DNAStar). Only sequences with <2%
ambiguities were analysed further (Quast et al., 2013). These sequences were identified using the MEGABLAST algorithm (Zhang et al., 2000), which is implemented in the Web-based basic local alignment search tool (BLAST, Altschul et al., 1990) of the U.S. National Center for Biotechnology Information (NCBI) and deposited at GenBank under the accession numbers KX138530–KX138555. Band identities based on BLAST results were assigned manually. The relative intensities of the identified bands in each lane were determined using GelCompar. Only bands occurring in at least two of the replicates were analysed further. The relative abundances of the identified OTUs, derived from the relative intensities of the assigned bands, were visualised using the ggpplot2 package (Wickham, 2009) for R (R Core Team, 2015). The relative abundance plot of the t24 samples included only those OTUs exclusive to or enriched in those samples, with a relative abundance ≥5 × higher than in t0 samples.

2.6. Quantitative PCR

To verify the relative abundance of the Amphritea atlantica OTU, quantitative PCR (qPCR) was conducted with the t0 polystyrene particles extracted from faeces (n = 3), the t24 polystyrene samples (n = 2) and the t24 faeces samples (n = 5). Amphritea-specific primers (E. coli position 444–462, with the sequence 5’-GTGAG-GAAGGTGTAGC-3’, and position 823–841, with the sequence 5’-GTGTCACCGCTAGTA-3’) were designed within the ARB program using the implemented probe design tool (Ludwig et al., 2004) and synthesised at Eurofins Genomics (Ebersberg, Germany). The relative abundance of A. atlantica was then calculated according to Labrenz et al. (2004).

3. Results

3.1. Experimental settings

Under the experimental conditions, A. marina was able to ingest particles with a size <1 mm. The experiments were conducted with 5 L of sediment. For this set-up, the assemblage similarity between replicates was 73.8 ± 18%. Particles were first detected in the faeces of A. marina on day 3 of the incubation.

3.2. Substrate-specific bacterial assemblages in sediment and faeces at t0

Before the ingestion by A. marina of polystyrene particles and glass beads from the sediment, their respective bacterial assemblages differed significantly from those of the sediment itself (Table S1, Fig. 3). However, the most abundant 16S rRNA gene OTUs (relative abundances of 30–60%) obtained from the sediment and from the polystyrene particles or glass beads extracted from it were identical (Fig. 4). After gut passage, the differences in the bacterial 16S rRNA gene fingerprint patterns between the faeces and the faecal polystyrene particles and glass beads were no longer significant (Table S1). There were though, significant differences between the samples which had passed the gut (faecal samples) and the samples which remained in the sediment instead of being ingested (sediment samples), except for the glass beads extracted from the sediment, which did not differ significantly from the faecal samples (Table S1). However, Pearson correlation of all the sediment and faecal fingerprint patterns still showed similarities >40% (Fig. 3). All the sample types also shared most of the abundant bacterial OTUs (Fig. 4). In addition, although bacterial primers were used and bacteria were the main target, eukaryotic plastids were also amplified and detected. The OTUs of plastids isolated from the eukaryotic Bacillariophyta were amongst the most abundant (11–42% relative abundance) OTUs, followed by a bacterial OTU closely related to the deltaproteobacterium Desulfatibacillus alkenivorans (10–25% relative abundance). Further OTUs were affiliated with the cyanobacterium Synechococcus sp. and with a deltaproteobacterium associated with a tubificid worm of the genus Olavius sp. This OTU was detected in all samples except those from the sediment and the polystyrene particles from the sediment (Fig. 4). An OTU phylogenetically related to Amphritea atlantica (95% 16S rRNA gene similarity, NCBI BLAST) appeared solely on the
polystyrene particles from the sediment, on the polystyrene particles from the faeces, and in the water samples of the polystyrene treatment (Fig. 4). Overall, the OTUs of the water samples were clearly distinct from those of both the sediment and the faecal samples (Fig. 4).

3.3. Substrate-specific bacterial assemblages at t24

The composition of the biofilms on the polystyrene particles and glass beads extracted from the faeces changed significantly within 24 h (Table S2), whereas the bacterial assemblage in the t24 faeces samples remained 60% similar to those of the t0 samples (Fig. 5). As found in the t0 samples, an OTU enriched exclusively in the polystyrene treatments was closely related to *A. atlantica*. This OTU accounted for up to 25 ± 8% of the relative 16S rRNA gene abundances determined after the 24-h incubation of the polystyrene particles, in all independent triplicates. These relative abundances were confirmed by qPCR with 18 ± 3% (Fig. 6). *A. atlantica* was also detected in the t24 faecal samples of the polystyrene treatment, with relative abundances of 2.8 ± 0.3% and 5.4 ± 2.5% as determined by SSCP and qPCR, respectively, and was enriched in the water samples from the polystyrene treatment (19 ± 3% according to SSCP, Fig. 6). Other OTUs affiliated with symbiotic bacteria were also identified but they were not exclusive to the polystyrene samples (Fig. 6). An OTU related to an unassigned Arcobacter sp. was present in all of the t24 samples, except in the t24 faeces of the glass treatment (Fig. 6). An OTU affiliated with *Lentisphaera marina* was enriched in the t24 polystyrene samples but it was also present in the other samples, including those from sediment and faeces, albeit irregularly. It was therefore not included in all of the relative abundance plots.

4. Discussion

In our study, the passage of microplastics through the digestive tract of *A. marina* did not enrich atypical sediment bacteria or pathogenic bacteria; rather, the bacterial assemblages on the polystyrene particles and glass beads became more similar. However, the identification of a polystyrene-specific OTU suggested that polystyrene influences not only biofilm development but also pelagic microbial assemblages, as evidenced by the preferential enrichment of a relative of the potential endosymbiont *A. atlantica* in the polystyrene treatments.

4.1. Microbial assemblages in sediment and faecal samples and the impact of *A. marina*

4.1.1. High similarity of bacterial assemblages after passage through the *A. marina* gut

The biofilm assemblages on the polystyrene and glass extracted from the sediment differed significantly from the bacterial assemblage of the bulk sediment. After gut passage, however, the microbial assemblages on the polystyrene and glass and faeces were more similar (Fig. 3). Although the sediment and faecal samples differed from each other, based on Pearson correlation, the most abundant OTUs could be found in all sediment and faecal samples, even with similar relative abundances (Fig. 4). Thus, the differences between faeces, polystyrene and glass extracted from the faeces, the sediment, as well as polystyrene and glass extracted from the sediment seemed to result from OTUs that were less abundant. *Plante and Mayer (1994)* demonstrated that the lytic activity of *A. marina* gut fluid can cause the removal of 95% of bacterial cells; which may account for their removal in the faecal samples in our study. The
Fig. 5. Non-metric multi-dimensional scaling plot based on Pearson correlation of the 16S rRNA gene SSCP fingerprint patterns generated from faeces and the substrates extracted from faeces before (t₀, orange dots) and after (t₂₄, dark green asterisk) a 24-h incubation. Analysed material: Faeces (F), polystyrene biofilm (PS), glass biofilm (GL). The red trapezoid shows the behaviour of the SSCP standards. Similarity values were derived from an accompanying cluster analysis (UPGMA).

Fig. 6. Relative abundance data (%) derived from the 16S rRNA gene SSCP fingerprints of OTUs (BLAST results) found exclusively or enriched by at least 5-fold in the 24-h incubated polystyrene particles (PS) and glass beads (GL) extracted from faeces, the corresponding 24-h incubated faecal samples and the incubation water. Phylogenetic affiliations are given in Fig. S2. *qPCR results.
rapid recolonisation of the *A. marina* faeces by sediment bacteria (Plante and Stinson, 2003; Plante and Wilde, 2001) might explain the strong similarities of most of the abundant OTUs in all of the sample types. An *in situ* study conducted at the coast of Maine, USA, in which denaturing gradient gel electrophoresis (DGGE) 16S rRNA gene fingerprints were used, identified differences in sediment vs. faeces (Plante, 2010); but, in accordance with our findings, the majority of the DGGE bands were present in both habitats. One dominant member in all of our samples, *D. alkenivorans*, belonged to the sulphate-reducing *Deltaproteobacteria*, whose members are frequently found in coastal anoxic sediments and can comprise >20% of the overall sediment microbial assemblage (Purdy et al., 2002). The other major contributors to the relative abundances in the sediment and faecal samples in our study were phototrophic bacteria (*Synechococcus*) and microalgae (*Bacillariophyta*). Benthic microalgae are abundant inhabitants of the upper sediment layer (Consalvey et al., 2004). Detecting these taxa, which are commonly found in sediments, in all of our samples provides further support that recolonisation processes exceed the impact of ingestion by *A. marina* on the bacterial assemblages, whether on polystyrene, glass or the corresponding faeces.

4.2. Low vector potential of polystyrene for pathogens after gut passage

Members of the genera *Vibrio*, *Aeromonas*, *Pseudomonas* and *Bacillus* have been identified in the gut microflora of marine annelids (Harris, 1993), and an OTU affiliated with the *Legionella*/*Coxiella* clade was identified from *A. marina* casts (Ashforth et al., 2011). The potential of marine microplastics per se to enrich associated biofilms towards possible human pathogens, such as members of the genera *Vibrio* (Zettler et al., 2013) or *Arcobacter* (Harrison et al., 2014), has also been described. However, in contrast to our expectation that biofilm assemblages on faecal polystyrene particles would become enriched in gut microflora, including potential human pathogens, we could not identify any pathogen specifically enriched on polystyrene. An OTU affiliated with the genus *Arcobacter* was not restricted to the polystyrene samples but was also found in the *t*24 glass particles, in the *t*2 faeces and in the water samples (Fig. 6). SSCP fingerprinting detects only those organisms with relative abundances >1% and is therefore less sensitive than clone libraries or ribosomal amplicon sequencing. Nonetheless, if the passage of polystyrene particles through the gut of *A. marina* had resulted in an enrichment even slightly comparable to that detected by Zettler et al. (2013), who reported a 25% enrichment of *Vibrio* sp., this would have been apparent on the SSCP fingerprints (van Dorst et al., 2014).

Surface sediment passes through the gut of *A. marina* about 30 times a year (Reise, 1985), such that the impact of the worm on the marine benthic ecosystem is significant (Goni-Urriza et al., 1999; Volkenborn et al., 2007). However, in our study *A. marina* feeding on polystyrene particles did not result in the significant enrichment of uncommon sediment bacteria or of pathogens in microplastic-associated biofilms.

4.3. Substrate-related differences in assembly patterns occur only within sediment samples

The most abundant OTUs in the sediment were also those on the polystyrene particles and glass beads from the sediment. However, the fingerprint patterns of the whole biofilm assemblages on polystyrene and glass from the sediment and those of the bulk sediment differed significantly (Fig. 3). Biofilm assemblages on plastic pieces sampled from the sediment in shallow coastal waters along the Belgian coast differed in their composition from adjacent sediment assemblages (De Tender et al., 2015). Harrison et al. (2014) similarly found that high-density polyethylene was rapidly colonised by bacteria in a sediment microcosm and that these assemblages diverged from those of the sediment after 7 days. In our experiments, fingerprint patterns differed between sediment and polystyrene extracted from the sediment after 4 days (Fig. 3). An OTU affiliated with *A. atlantica* occurred only on the polystyrene biofilm samples (Fig. 4), which suggested a role for substrate specificity in the development of bacterial biofilms on polystyrene. Yet, the most abundant OTUs found in all sediment samples, all faeces samples and on the corresponding polystyrene and glass biofilms were the same (Fig. 4). Thus, in comparison to substrate specificity, the environment and the bacterial meta-population, as a source of colonisation, also play an important role in shaping sediment-associated bacterial assemblages.

4.4. Substrate-specific enrichment of potentially symbiotic bacteria on polystyrene

4.4.1. *Amphritea* sp. enrichment on polystyrene occurs independently of *A. marina*

After passage through the digestive tract of *A. marina*, the worm’s faeces and the polystyrene and glass extracted from them were incubated in seawater for 24 h to test the stability of the associated bacterial assemblages. Though highly similar after egestion by *A. marina*, the polystyrene and glass assemblages diverged and became very distinct after 24 h of incubation in seawater. Similarities based on Pearson correlation between the fingerprint patterns of the *t*24 polystyrene and glass biofilm samples were still 40%, but the phylogenetic affiliations of their most abundant OTUs differed (Fig. 6). Thus, after 24 h the glass biofilm was dominated by members of the genera *Pseudomonas*, *Pseudalteromonas* and *Thalassolitohus*, whereas on polystyrene an OTU closely affiliated with *A. atlantica* became highly enriched (Fig. 6). This gammaproteobacterium was first isolated in association with a *Bathymodiolus* clam inhabiting a hydrothermal vent system (Gärnert et al., 2008). Four additional species within the genus *Amphritea* have been described so far, all of them isolated in association with higher organisms (Jang et al., 2015; Kim et al., 2014; Miyazaki et al., 2008). We identified a close relative of *A. japonica* on polystyrene incubated in seawater before its addition to the aquaria containing *A. marina* (Fig. 52). We also found an OTU affiliated with *A. atlantica* on polystyrene extracted from sediment, before passage of the particles through the worm’s digestive tract. Thus, *A. marina* probably did not inoculate the polystyrene particles with *Amphritea* sp.; rather, the particles themselves seemed to be the main driver of the specific enrichment of *Amphritea* sp. in the respective treatments. The >99% similarity of the OTU sequences isolated in this study with the sequences of both *A. atlantica* or *A. japonica* prevented their assignment to one or the other species, but we assume that they represented the same species. The reason for the enrichment is unclear, but polystyrene potentially represents a substrate analogue for *Amphritea* sp. Uncultured bacteria isolated from the bone-eating worm *O峡alax* sp. are also grouped within the genus *Amphritea* (Satomi and Fujii, 2014), such that members of this genus may be capable of at least partially hydrolyse complex polymers, including plastic and collagen. This must still be confirmed in further experiments. The different surface properties of the polystyrene particles versus the glass beads regarding their surface rugosity could also play a role. But if it the enrichment of *Amphritea* sp. would only be due to the higher degree of rugosity of the polystyrene particles, we would also expect to find *Amphritea* sp. in the sediment of both treatments, as sand grains also have cracks and crevices. Thus, although we were not able to show that polystyrene serves as a vector for pathogens
based on the SSCP fingerprints, our results confirm that microplastics can serve as a substrate-specific habitat for organisms, as shown for Amphritea sp.

4.5. Potential impact of polystyrene on microbial assemblages in pelagic systems

Amphritea sp. was exclusively enriched on polystyrene and in the polystyrene treatments, most notably, it was also enriched in the T; water samples of the polystyrene treatment (Fig. 6). While particle incorporation during water sampling cannot be ruled out completely, the presence of a few polystyrene particles would not explain the high relative abundance (~20%) of Amphritea sp. detected in the water samples. Besides demonstrating the direct impact of polystyrene on biofilm formation, our results show that it alters pelagic bacterial assemblages, although this remains to be verified in situ. So far, field studies have only shown that pelagic bacterial assemblages differ from those found on microplastics (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2014; Zettler et al., 2013). The need for additional field studies is crucial in areas with a very high plastic-litter load—for example, in the area off the southern coast of South Korea, where a concentration of ~16,000 particles/m³ has been determined (Song et al., 2014). The hypothesis of plastic-litter on pelagic assemblages and therefore on the pelagic food web (Fuhrman and Steele, 2008) is still open. Recent experiments using European perch (Perca fluviatilis) larvae demonstrated that their performance and development are significantly reduced at a polystyrene concentration of 10,000 particles/m³ (Lönnstedt and Eklöv, 2016). Although this observation was attributed to the physicochemical impact of polystyrene on the larvae, also unusual biofilms could have influenced the larval development. However, further research is needed to clarify the ecological role of microplastic-associated biofilms in the environment.

Notes

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.10.032.

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