Bacterial biofilms on microplastics in the Baltic Sea – Composition, influences, and interactions with their environment

kumulative
Dissertation
zur
Erlangung des akademischen Grades
Doctor rerum naturalium (Dr. rer. nat.)
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität Rostock

vorgelegt von
Katharina Kesy, geb. am 06.11.1985 in Berlin
aus Rostock
Rostock, 17.09.2019

https://doi.org/10.18453/rosdok_id00002636

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Jahr der Einreichung: 2019

Jahr der Verteidigung: 2020

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Summary

An estimated five trillion pieces of microscopic plastic particles (≤5 mm) are currently afloat at sea. Due to their great dispersal potential, microplastics have become ubiquitous in aquatic environments. These microplastic particles provide a new habitat for surface-associated bacteria but there is no final understanding on which factors are primarily driving biofilm composition on various surfaces. Surface-properties and environmental factors have both been proposed as major drivers of biofilm formation on microplastics. These microplastic-associated biofilms can have a great impact on aquatic ecosystems by adding new functional traits, enhancing bacterial activity, or as a dissemination vector for potential harmful microorganism. Bacterial genera found on microplastics include, for instance, potentially human pathogenic *Vibrio* sp.. Many *Vibrio* species are halo- to mesohalophilic, and the brackish Baltic Sea is thus a suitable habitat for them. Because vibrios are also known to form biofilms as a survival strategy, it is likely to find them in microplastic-associated biofilms. However, reported abundances of *Vibrio* on microplastics vary greatly from 24% to *Vibrio* not being detectable. This study therefore investigated if microplastics *per se* favour the enrichment of potentially pathogenic *Vibrio*, or if biofilms become enriched via a specific inoculation event. Because microplastics are so small, filter- and deposit feeders of lower trophic levels are likely to ingest them. Many aquatic invertebrates are known to host potential pathogenic bacteria within their guts, so microplastics traversing the intestinal system might acquire a potential pathogenic gut community via this route. Further, the general bacterial assemblages on microplastics can also influence the colonisation success of other bacteria. Therefore, factors driving microplastic-associated bacterial diversity, and potentially *Vibrio* abundances, were also investigated.

Feeding-experiments using the lugworm *Arenicola marina* and the blue mussel *Mytilus edulis* were conducted to assess the influence of gut passage on polystyrene (PS)- and polyamide (PA)- associated bacterial assemblages, with glass and chitin serving as control surfaces, respectively. The stability of these egested biofilms was investigated by incubating the egested particles in sea water for 24 h and 7 days. Bacterial assemblages were analysed using 16S rRNA-gene fingerprinting and the phylogenetic assignment of prominent bands via sequencing. Factors influencing biofilm development on different surfaces were investigated in incubation experiments during a Baltic Sea summer cruise,

using polyethylene (PE), polystyrene (PS), and wood as a natural control surface. The bacterial diversity was analysed using 16S rRNA-gene amplicon Illumina sequencing.

Gut-passage did not result in an enrichment of potentially pathogenic bacteria and egested biofilms were not stable. *Vibrio* was detected on microplastics after incubation in Baltic Sea water, with maximum relative abundances on PE of 0.4 ± 0.2% and on PS of 1.2 ± 0.3%. However, the highest abundances were found on wood (2.3 ± 0.5%). *Vibrio* numbers were also positively correlated to salinity. A co-occurrence network showed that *Vibrio* was not well connected to other biofilm members, only to a few saccharolytic OTUs of diverse bacterial lineages.

The surface type was generally of lower importance, although microplastic-associated assemblages were distinct to those on natural seston and free-living ones. Salinity was also the main driver in structuring bacterial assemblages on PE, PS, and wood. However, several OTUs were found exclusively, or significantly more, abundant on the plastics.

This study provides one of the first mechanistic investigations on *Vibrio* abundance and early biofilm assemblages on microplastics within the Baltic Sea. Given that *Vibrio* abundances on microplastics sampled *in situ* are often far below the abundances found in this study here, it is assumed that *Vibrio* is an early coloniser of surfaces in general and not restricted to microplastics. This assumption is also in accordance with *Vibrio* ecology of using a 'feast-or-famine'-strategy and has also been reported by other studies. Further, it corroborates that microplastics can be regarded as a novel habitat for biofilm-forming bacteria in aquatic systems. The diversity and composition of the microplastic-biofilm in general, and *Vibrio* in particular will however greatly depend on temporal, spatial, and environmental dynamics, which need to be considered when assessing the impact these biofilms will have on aquatic ecosystems and coastal societies.

Zusammenfassung

Mikroskopisch kleine Kunststoffpartikel, so genanntes Mikroplastik (≤5 mm), werden heutzutage ubiquitär in aquatischen Systemen gefunden. Mikroplastik besitzt ein großes Ausbreitungspotenzial und Schätzungen gehen davon aus, dass derzeit etwa 5 Billionen Partikel in den Ozeanen schwimmen. Diese Partikel bieten einen neuen Lebensraum für oberflächenassoziierte Bakterien. Derzeit ist noch unklar, welche Faktoren die Biofilmbildung- und Zusammensetzung auf verschiedenen Oberflächen primär beeinflussen. Sowohl Oberflächeneigenschaften als auch Umweltfaktoren werden als wesentliche Einflussfaktoren angesehen. Diese mikroplastik-assoziierten Biofilme können einen großen Einfluss auf aquatische Ökosysteme haben, indem sie neue funktionelle Merkmale hinzufügen, die bakterielle Aktivität erhöhen oder auch als Verbeitungsvektor für potenziell schädliche Mikroorganismen dienen. Zu den Gattungen, die bereits auf Mikroplastik gefunden wurden, gehört zum Beispiel *Vibrio*, welche auch potentielle Humanpathogene beinhaltet. Viele *Vibrio*-Arten sind halo- bis mesohalophil und die Ostsee, als größtes Brackwassermeer der Erde, daher ein geeigneter Lebensraum. Da Vibrionen außerdem dafür bekannt sind, Biofilme als Überlebensstrategie zu bilden, ist es wahrscheinlich, sie auf Mikroplastik zu finden. Die bisher gefundenen *Vibrio*-Abundanzen auf Mikroplastik variieren jedoch stark von 24% bis hin zu keiner Detektierbarkeit. In dieser Studie wurde daher untersucht, ob Mikroplastik an sich die Anreicherung von potenziell pathogenen Vibrionen begünstigen oder ob Biofilme durch ein bestimmtes Impfereignis angereichert werden. Durch seine geringe Größe ist es besonders wahrscheinlich, dass Mikroplastik von Suspensions- und Depositfresser der unteren trophischen Ebenen aufgenommen wird. Viele marine Wirbellose sind oft mit potenziell pathogenen Bakterien assoziiert, insbesondere im Magen-Darm-Trakt. Ob Mikroplastik durch die Aufnahme und Passage des Magen-Darm-Trakts höherer Organismen speziell mit potenziell pathogenen Keimen angeimpft werden kann und dadurch ihre Verbreitung begünstigt, wurde in dieser Dissertation als ein Hauptthema untersucht. Ob sich *Vibrio* unabhängig von einem speziellen Impfereignis auf Mirkroplastik anreichert und welche Rolle dabei auch die gesamte bakterielle Gemeinschaft des Biofilms spielt, wurde als zweiter großer thematischer Block bearbeitet. So wurde auch der Einfluss verschiedener Umweltfaktoren auf die sich entwickelnden Biofilme, insbesondere auf *Vibrio*-Abundanzen, analysiert.

Um den Einfluss der Darmpassage auf Polystyrol- und Polyamide- assoziierten bakterielle Gemeinschaften zu beurteilen, wurden Fraßexperimente mit dem Wattwurm *Arenicola marina* und der Miesmuschel *Mytilus edulis* durchgeführt, wobei Glas und Chitin als Kontrollpartikel dienten. Die Stabilität der Biofilme auf den ausgeschiedenen Partikeln wurde außerdem untersucht, indem diese für 24 Stunden, sowie 7 Tage in Ostseewasser inkubiert wurden. Die bakteriellen Gemeinschaften wurden anschließend mittels 16S rRNA-Gen fingerprinting analysiert und prominente Banden in den molekularen Fingerabdrücken mittels Sequenzierung phylogenetisch zugeordnet. Der Einfluss von Umweltfaktoren sowie verschiedener Oberflächen auf die mikrobielle Besiedlung wurde während einer Ostseeausfahrt im Sommer untersucht. Dafür wurden Polyethylen (PE) und Polystyrol (PS) als Substrat verwendet, sowie Holz als natürliche Kontrolloberfläche. Die bakterielle Gemeinschaft und Diversität wurde mittels rRNA-Gen-Amplikon Illumina-Sequenzierung analysiert.

Die Darmpassage führte nicht zu einer Anreicherung potenziell pathogener Bakterien und die Biofilme auf den ausgeschiedenen Partikeln waren nicht stabil. Allerdings konnte *Vibrio* auf Mikroplastik nach Inkubation in Ostseewasser nachgewiesen werden, mit maximalen relativen Häufigkeiten von 0,4 ± 0,2% auf PE und 1,2 ± 0,3% auf PS. Die höchste Abundanz wurde jedoch auf Holz gefunden (2,3 ± 0,5%). *Vibrio*-Zahlen waren außerdem positiv mit dem Salzgehalt korreliert. Eine Netzwerkanalyse zeigte, dass *Vibrio* nur mit wenigen, saccharolytischen Bakterien (OTUs) verschiedener phylogenetischen Linien assoziiert war.

Die Oberfläche war im Allgemeinen von geringerer Bedeutung für die Unterscheidung der bakteriellen Gemeinschaften auf Plastik und den Kontrolloberflächen. Allerdings unterschieden sich diese signifikant von denen auf natürlichem Seston und den freilebenden Gemeinschaften. Der Salzgehalt war auch der Hauptfaktor in der Strukturierung der bakteriellen Gemeinschaften auf PE, PS und Holz. Allerdings konnten mehrere OTUs entweder ausschließlich oder deutlich häufiger auf Mikroplastik identifiziert werden.

Diese Studie stellt eine der ersten mechanistischen Untersuchungen zur Häufigkeit von *Vibrio* und frühen Biofilmgemeinschaften auf Mikroplastik in der Ostsee dar. *Vibrio*-Abundanzen auf Mikroplastik, welches *in situ* beprobt wurde, sind häufig sehr viel geringer, als die hier gefundenen. Da sich diese Partikel mit hoher Wahrscheinlichkeit sehr viel länger im Wasser befunden haben, kann davon ausgegangen werden, dass *Vibrio* ein

früher Besiedler von Oberflächen im Allgemeinen ist. Diese Annahme wird durch die Ökologie von *Vibrio* als r-Strategen (wenige Zellen bei geringen Nährstoffkonzentrationen und extrem schnelles Wachstum bei Nährstoffzufuhr) bestärkt und wurde auch in anderen Studien berichtet.

Darüber hinaus kann bestätigt werden, dass Mikroplastik als neuer Lebensraum für biofilmbildende Bakterien in aquatischen Systemen angesehen werden kann. Die Diversität und Zusammensetzung der Biofilme auf Mikroplastik im Allgemeinen und von *Vibrio* im Besonderen wird jedoch stark von der zeitlichen und räumlichen Dynamik der Partikel, als auch der Umweltfaktoren abhängen. Diese müssen von daher bei der Beurteilung der Auswirkungen mikroplastik-assoziierter Biofilme auf aquatische Ökosysteme und Küstengesellschaften berücksichtigt werden.

General introduction

Biofilms, their formation, and influential factors

Biofilms are complex communities of primarily microorganisms that form on interfaces, which can be solids-aqueous, gas-aqueous, aqueous-aqueous, and also aggregates of cells. Biofilms are found in almost every environment, ranging from the deep sea to humans (Flemming and Wuertz, 2019), where they can be of clinical importance (Hall-Stoodley et al., 2004). The organisms within biofilms gain protection from environmental stressors, such as UV-radiation, osmotic stress, antibiotics and pollutants, but also from predation and offer enhanced nutrient availability (Dang and Lovell, 2016; Davey and O'Toole, 2000). Chances of acquiring new functional traits are increased due to an intensified horizontal gene transfer, providing microorganisms (especially *Bacteria*) with the opportunity of exploiting new niches (Davey and O'Toole, 2000). Biofilm formation on diverse materials has been studied extensively, but there is still no generalisable pattern that can predict biofilm assemblages on a given surface at a given time and space. However, the initialisation of formation and temporal development of a biofilm are quite well understood. After the conditioning of the surface by various biomolecules, biofilm formation starts with reversal attachment of single cells to the substratum (Stoodley et al., 2002). A multitude of mechanisms for bacterial attachment has been identified, involving sensing of the surface by a flagellum, Type IV pili, or fimbriae, resulting in a signalling cascade initialising irreversible adherence of the cells and the production of extracellular polymeric substances (EPS) (O'Toole and Wong, 2016). EPS secreted by the cells have different functions, but foremost they make up the biofilm matrix and provide structural stability. Also, the resistance of biofilm to environmental stressors and enhanced nutrient availability has been attributed to different components of the biofilm matrix (Flemming and Wingender, 2010).

According to this general pattern, different influential factors have been identified that may act at separate stages of biofilm formation and the subsequent development: Firstly, physico-chemical properties of both the given surface and the bacterial cell-surface play a role, such as the surface free energy (hydrophobic vs. hydrophilic) and electric charge, surface- roughness and hardness, and surface functional groups (Renner and Weibel, 2011; Zhang et al., 2015). Many studies have already investigated the influence of surface free energy (i.e. hydrophobic vs. hydrophilic) on bacterial attachment, but mostly using single

strains in laboratory settings. These studies usually assumed that bacteria adhere more readily to hydrophobic surfaces, since most bacteria have a net negative charged cell wall. Since these forces need to be overcome by attaching bacteria, it seems reasonable to assume that this might be one of the major factors shaping initial biofilm composition. In natural environments, however, bacteria will hardly encounter a barren surface, since a conditioning film will always form on any surface. Nonetheless, the physico-chemical surface properties can influence which and how free molecules will adsorb to it and thus alter or translate these properties through the conditioning film (Busscher and van der Mei, 2000; Schneider, 1996). However, from the vast amount of literature, it is still not clear exactly how important these factors are. One can assume that surface-properties would be especially important during the initial attachment, when cells are in direct contact with the surface's conditioning film. However, environmental factors can alter these surface properties, such as ionic strength and the pH of the liquid medium. The pH and salinity can mask the surface electric charge of both the substrate and the bacterial cell (Renner and Weibel, 2011). Also hydrodynamic forces, especially shear stress, have been shown to play a role in biofilm formation (Catão et al., 2019; Niederdorfer et al., 2016). Finally, nutrient availability can act at a physiological level, but also the growth phase is of importance, as both can influence cell wall composition- and structure. Further, nutrient availability can promote or inhibit biofilm formation in bacteria. Yet, the response to nutrients and other environmental cues is not consistent across bacterial species. In some species, starvation might induce biofilm formation, while in others it might inhibit it (Allan et al., 2002; Karatan and Watnick, 2009). As the biofilm matures, competition, predation, and viral infections may become more important in these later stages of biofilm development and are probably less linked to physico-chemical factors. During the maturation phase, a three dimensional biofilm architecture develops, which consists of microcolonies interspersed with water channels providing the deeper embedded cells with nutrients and oxygen (Stoodley et al., 2002).

Finally, in the last stage of biofilm development, cells start to disperse from the biofilm to colonise new habitats, thus starting the biofilm formation anew and completing the "biofilm life-cycle" (Stoodley et al., 2002).

The ecological importance of biofilms in aquatic systems

Estimates suggest that roughly 40–75% of all microbial cells on earth live within biofilms. In aquatic systems, biofilms can be found in sediments, on natural aggregated of organic and inorganic material, on living organisms, and on the sea surface microlayer (Flemming and Wuertz, 2019). They consist of rich assemblages of microorganisms, such as *Bacteria*, *Archaea*, viruses, and also eukaryotic organisms like protists, algae, and small metazoans (Simon et al., 2002). Microorganisms are the major catalysts for almost all biogeochemical cycles (Falkowski et al., 2008). Existing in biofilms with a close juxtaposition to other biofilm members can provide microorganisms with versatile metabolic interactions, such as the coupled nitrification process by ammonia-oxidising *Nitrosomonas* spp. and nitrite-oxidising *Nitrobacter* spp., that has been shown to occur in aggregates (Mobarry et al., 1996). Biofilms on aggregates are considered hotspots of microbial activity and contribute greatly to the carbon flux by releasing dissolved organic matter (DOM) from the sinking particles that becomes available to planktonic bacteria (Cho and Azam, 1988; Simon et al., 2002), while the remineralisation of inorganic nutrients like phosphate, nitrate, ammonium, and silicate are also highly increased (Simon et al., 2002). Aggregate-attached bacteria are of special importance in estuarine systems and coastal seas, where their productivity can make up to 90% of the total bacterial carbon production (Crump et al., 1998).

Not only are organic aggregates colonised by biofilms. Biofilms form on every surface that is submerged in water, starting by the adhesion of dissolved proteins, glycoproteins, polysaccharides and various biomolecules, forming the so-called conditioning film (Flemming and Wingender, 2010). Anthropogenic structures, such as dams, breakwaters, and ship hulls, and diverse garbage items are carriers of biofilms. So far, it is known that *Gammaproteobateria* are common inhabitants of aquatic biofilms, but *Alphaproteobacteria* dominate the early colonisation process, especially members of the marine *Roseobacter* clade and members of the *Sphingomonadaceae* (Dang and Lovell, 2016). Members of the *Bacteroidetes*, especially *Flavobacteriaceae*, are also frequently found, but are assumed to be later colonisers of inorganic material (De Tender et al., 2017; Oberbeckmann et al., 2015). *Cyanobacteria* are often abundant as well, mainly according to season or geographical location (Bryant et al., 2016; Oberbeckmann et al., 2015). Further, a diverse assembly of protists, micro- and macroalgae and metazoans are

inhabitants of biofilms, depending on the available surface area (Kaiser et al., 2017; Reisser et al., 2014).

In natural systems, biofilms can also become reservoirs for potential pathogens (Lyons et al., 2010). Coliforms like *Escherichia coli* can survive in biofilms (Shikuma and Hadfield, 2010), as well as the putative pathogens *Pseudomonas aeruginosa* and *Aeromonas hydrophilia* (Lyons et al., 2007). Most importantly, however, may be the persistence of several human pathogenic *Vibrio* species, like *V. cholerae*, *V. vulnificus*, and *V. parahaemoliticus*. These species are halo- to mesohalophilic Gram-negative bacteria, that have optimal growth at temperatures >15°C (Gomez-Gil et al., 2014). The ability of *V. cholerae*, the causative agent of the Cholera-disease, to form biofilms significantly enhances the survival of the cells in the aquatic environment and thus contributes to their transmissibility (Alam et al., 2007; Faruque et al., 2006). Also, *V. vulnificus* and *V. parahaemolyticus*, which can cause severe wound infections and gastroenteritis, respectively, have repeatedly been found in biofilms (Baker-Austin et al., 2010; Froelich et al., 2013; Yildiz and Visick, 2009). Vibrios are found on natural aggregates, but are more often associated with the chitinous carapaces of copepods (Huq et al., 1983). Biofilms are thus not only important for nutrient cycling and organic matter regeneration in aquatic systems, but are also of clinical relevance for coastal societies.

Microplastics in aquatic environments: a newly available habitat for surface associated microorganisms and possible vector for potential pathogens

The topic of the ongoing pollution of aquatic systems by anthropogenic waste, mainly plastics, has been of concern for many years. It was first described by Edward Carpenter and Kenneth Smith, after they discovered various plastic pieces in the Sargasso Sea in 1972 (Carpenter and Smith, 1972). Research into this topic firstly focused on macro-sized items, such as derelict fishing gear and single-user items from recreational activities. The impact of this macro-litter was assessed mainly on its effect on higher marine organisms, which would mistake these items for food or get entangled in it, but also as means of dispersal for biofilms and sessile aquatic organisms (Kiessling et al., 2015). This ultimately resulted in the Annex V to the International Convention for the Prevention of Pollution from Ships (MARPOL, International Maritime Organization, 1983) in 1988, to ban all deposition of plastic waste from ships in the global oceans (International Maritime

Organization, 1988). Only in the last decade has the focus shifted to plastic waste in the microscopic size, as had been first described by Carpenter and Smith 1972 (Thompson R. C. et al., 2004). These microplastics (here defined as <5 mm; Arthur et al., 2009) result from primary sources, such as scrubbing material in cosmetics, air-blasting techniques, or virgin resin pellets, that enter aquatic systems either by cargo mishandling or through the effluent of waste water treatment plants (Wilber, 1987; Zitko and Hanlon, 1991). However, the gross of microplastic pollution of aquatic systems is thought to originate from the fragmentation of larger plastic waste through photodegradation and physical forces like winds and waves (Andrady, 2011; Cooper and Corcoran, 2010; Ryan, 2015).

Again, the focus was first laid on the effect these microscopic particles might have on higher aquatic organisms. Because of their small size, microplastics are susceptible to ingestion by a variety of organisms at lower trophic levels, such as suspension- and deposit feeders (Wright et al., 2013a). Further on, many artificial polymers adsorb and accumulate persistent organic pollutants due to their hydrophobic surface characteristics (Mato et al., 2001) and the highly mobile microplastic fraction could therefore act as a vector for these to remote areas or to aquatic organisms (Koelmans, 2015; Teuten et al., 2007; Zarfl and Matthies, 2010). Ingestion-related effects include, for instance, inflammatory responses in the tissue of the blue mussel *Mytilus edulis* (Browne et al., 2008), reduced carbon uptake by the copepod *Calanus helgolandicus* (Cole et al., 2015), and reduced energy reserves in the polychaete *Arenicola marina* (Wright et al., 2013b). However, almost 10 years after the problem of microplastic pollution came to prominence, researchers started to investigate the biofilms present on these particles (Ivar do Sul et al., 2018; Zettler et al., 2013). Estimates suggest that 5 trillion pieces of plastic are currently afloat at sea, constituting a vast surface area for colonisation (Eriksen et al., 2014). Biofilms on microplastics might differ in their community composition from those on natural aggregates, and thus represent a newly available niche for microorganisms in aquatic systems (Debroas et al., 2017; Dussud et al., 2018a; Kettner et al., 2017; Oberbeckmann et al., 2016, 2018). There are different implications for the ecological assessment that arise from the formation of biofilms in this new habitat. So far, biofilm function on microplastics has mostly been inferred indirectly by comparing the phylogenetic information obtained from comparing the sequence of the 16S rRNA gene to the closest relative with a complete sequenced genome (e.g. via the PICRUSt-method; Langille et al., 2013). These results suggest an overrepresentation of genes involved in different metabolisms and xenobiotics degradation

on microplastics, compared to free-living or natural aggregate-associated communities (Debroas et al., 2017; Dussud et al., 2018a; Jiang et al., 2018). This is in accordance with metagenome studies on plastic-associated biofilms from the Pacific and Indian Ocean (Bryant et al., 2016; Rampadarath et al., 2017)

Microplastics are often colonised by photoautotrophic bacteria and microalgae (Oberbeckmann et al., 2015). Due to the accumulation of nutrients within the conditioning film, this could lead to enhanced photosynthetic activity, which would then have direct influences on the carbon flux, especially in oligotrophic systems. These systems include the Northern- and Southern Subtropical Gyres, where a high load of plastic waste is located (Law et al., 2010). The presence of a biofilm can also alter the density of the particles and thus has an effect on the sinking behaviour and the distribution of the particles. Particles can become more dense by the presence of a biofilm and thus sink faster (Ye and Andrady, 1991), while photoautotrophic organisms might prevent sinking, as these organisms often possess gas vacuoles, which would lead to more positive buoyancy (Kaiser et al., 2017). As microorganisms possess high metabolic versatility, it is also possible that members of the biofilm could degrade the sorbed contaminants – or that the biofilm could prevent the sorption of contaminants altogether (Rummel et al., 2017). The potentially high relative abundances of genes involved in the degradation of diverse polycyclic aromatic hydrocarbons might hint towards the former (Bryant et al., 2016; Debroas et al., 2017). Another hypothesis is that some bacteria could degrade the polymers themselves, which would have further implication for the oceanic carbon flux and carbon budget (Dussud et al., 2018b; Ogonowski et al., 2018; Romera-Castillo et al., 2018; Zettler et al., 2013). Finally, the formation of an organic biofilm on artificial polymers may make these particles more attractive to aquatic organisms as a food source, thus increasing the risk of accidental ingestion and its potential negative impacts (Carson, 2013; Ward and Kach, 2009). Therefore it is important to study biofilm formation on microplastics to disentangle the complex interaction of these microplastic-associated biofilms with aquatic systems in general. Biofilms are the communities that are in immediate contact with the particles, probably alter its properties, add new functions, and therefore play a major role in what impact microplastics will have on aquatic ecosystems (Fig. A).

Plastics are a common descriptor for a variety of different hydrocarbon-based polymers, which differ in their surface properties and thus may also harbour distinct bacterial assemblages. Surface physico-chemical properties, such as functional groups, surface

roughness, and the surface free energy (hydrophobic vs. hydrophilic) are believed to play a role in the attachment behaviour of cells to surfaces, but also the environment of the cells and cell-properties are of importance (Berne et al., 2018). So far, most studies on the regulation of biofilm formation have focused on specific bacterial strains in laboratory experiments, which might misrepresent the complex interaction that could influence biofilm formation in natural systems. Based on *in situ* sampling of microplastics and mechanistic studies *in-* or *ex situ*, some investigators reported differences in bacterial biofilm assemblages based on polymer type (Oberbeckmann et al., 2018; Ogonowski et al., 2018; Zettler et al., 2013), while others did not (Bryant et al., 2016; Dussud et al., 2018b; Kirstein et al., 2018). Seasonal and spatial factors also influence community composition (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2014, 2016; Oberbeckmann and Labrenz, 2020), but again there is no consistency as to how important these factor are for biofilm formation on different polymers (Bryant et al., 2016; Dussud et al., 2018b). The Baltic Sea is a one of the largest brackish water systems in the world, with a stable salinity gradient from west to north-east, which leads to almost marine conditions in the west and almost freshwater conditions in the north-east. This salinity gradient gives rise to distinct seston-attached bacterial assemblages, which are dominated by *Flavobacteria* in the marine part, by *Cyanobacteria* in the mesohaline part, and *Planctomycetes* in oligohaline parts during summer. In the winter months, *Alpha-* and *Gammaproteobacteria* became more abundant on seston in the marine part, while *Gammaproteobacteria* dominate the seston in mesohaline waters. In the olighaline part, relative abundances are more evenly distributed, with *Planctomycetes*, *Gammaproteobacteria*, and *Cyanobacteria* constituting the most abundant classes (Rieck et al., 2015). The Baltic Sea is therefore a suitable ecosystem to investigate the influence of different environmental factors on developing microplastic-associated bacterial assemblages. The brackish nature of the Baltic Sea also makes it a suitable habitat for several *Vibrio* species, foremost the putative human pathogens *V. cholerae* non-O1/non-O139 (which causes gastroenteritis but not Cholera), *V. vulnificus*, and *V. parahaemolyticus,* the latter which can also be a potential animal pathogens (Austin, 2010). Cases of *Vibrio* infections have been reported repeatedly from the Baltic Sea (Baker-Austin et al., 2013). The Baltic is also relatively shallow, regularly reaching water temperatures >15°C in the summer months (Snoeijs-Leijonmalm and Andrén, 2017). Data about *Vibrio* abundance and, to a limited extent, about *Vibrio* diversity only exists from the Swedish and the German coasts, but little is known about the

Vibrio diversity in the Baltic Sea in general, as well as the potential reservoirs for these putative pathogens. So far, sediments are thought to be a reservoir for these bacteria, but information on the complete eastern coast is still missing (Huehn et al., 2014).

Futhermore, what data does exist on *Vibrio* spp. in the Baltic Sea was mostly obtained using cultivation-based methods. Vibrios are known to enter a so-called viable but non-culturable (VBNC) state when environmental conditions become unfavourable, and therefore, these cells would be missed in cultivation-dependent surveys (Colwell et al., 1985). Although it is long known that *V. cholerae* is strongly associated with zooplankton (Huq et al., 1983), other interactions with bacteria or eukaryotic organisms, especially within biofilms, remain largely unknown. This preference for a sessile lifestyle make vibrios candidates to become enriched on microplastics, and one of the first study on bacterial assemblages on microplastics indeed reported very high abundances (24% of *Vibrio* sp.) on one polypropylene particle sampled in the Sargasso Sea (Zettler et al., 2013). The Baltic Sea's catchment area includes several highly industrialised states, that have roughly 85 million in total population, and intense agricultural practices. It is therefore one of the most anthropogenically influenced water bodies (HELCOM, 2010) and an enhanced dissemination of *Vibrio* via microplastics would have a great impact on its coastal communities.

However, there is still an ongoing debate about whether microplastics can serve as vectors for potential pathogenic organisms. While some studies have reported high relative abundances (18.6–24%) of *Vibrio* sp. (Frère et al., 2018; Zettler et al., 2013), other studies could not identify a selective enrichment of *Vibrio* on microplastics (Dussud et al., 2018b; Oberbeckmann et al., 2018; Schmidt et al., 2014). As many pathogens are known biofilm-formers, it is an emerging question if microplastics *per se* favour an enrichment of potential pathogens acting as a substrate-analogue, or if high relative abundances could be mediated by a distinct inoculation event. Many aquatic organisms harbour potential pathogens within their digestive system (Harris, 1993), and because microplastics are so small, they are susceptible to become ingested by a variety of aquatic organisms. Especially those that filter large volumes of water or sediment, such as filter- or deposit-feeders are of interest, since it is most likely that they will ingest the highest amount of microplastics (Li et al., 2016; Van Cauwenberghe et al., 2015; Wright et al., 2013b). Therefore a reasonable hypothesis is that microplastics become ingested; pass the gut of the organism to subsequently be egested again, along with the acquired gut community,

including potential pathogenic bacteria. So far, the only studies investigating *Vibrio* occurrences in the Baltic Sea on microplastics are those of Kirstein et al. (2016) and Oberbeckmann et al. (2018). Kirstein and colleagues investigated *in situ* presence of *Vibrio* spp. on microplastics sampled from the North and Baltic Sea, using a cultivation-based approach. They were able to identify *Vibrio* up to the species level using MALDI-TOF MS and isolated one *V. parahaemolyticus* and one *V. fluvialis* strain from one polypropylene particle collected in the Baltic Sea. However, they found that the diversity and abundance of *Vibrio* in the waters along the German Baltic Sea coast was much greater than on the microplastics. Oberbeckmann et al. (2018) showed that *Vibrio* spp. were enriched on incubated PE- and PS particles compared to the surrounding water, but were most abundant on wood, which was used as a control surface. These incubations were conducted within the Warnow estuary. Because brackish waters in general and especially the salinity range ≤10 PSU are preferred habitats for several potentially pathogenic *Vibrio*, especially in the summer months (Le Roux et al., 2015), it is of importance to extend the knowledge of *Vibrio* abundances on microplastics in the Baltic Sea beyond the German border and into the eastern Baltic coastlines to gain a more holistic knowledge on the possibility of microplastics as vector for *Vibrio*.

Description of research aims

Because coastal communities around the Baltic Sea would be directly affected by an increased dissemination of *Vibrio* spp. by microplastics, either due to the infections of humans or the infections of aquaculture stock, the question whether microplastics could act as a dispersal vector for potential pathogenic vibrios is of importance. However, the biofilm community in general is important due to the influence it can have on the whole aquatic system, and because virulence of *Vibrio* spp. can also depend on the non-pathogenic members of the whole community (Le Roux et al., 2015; Smith, 2000).

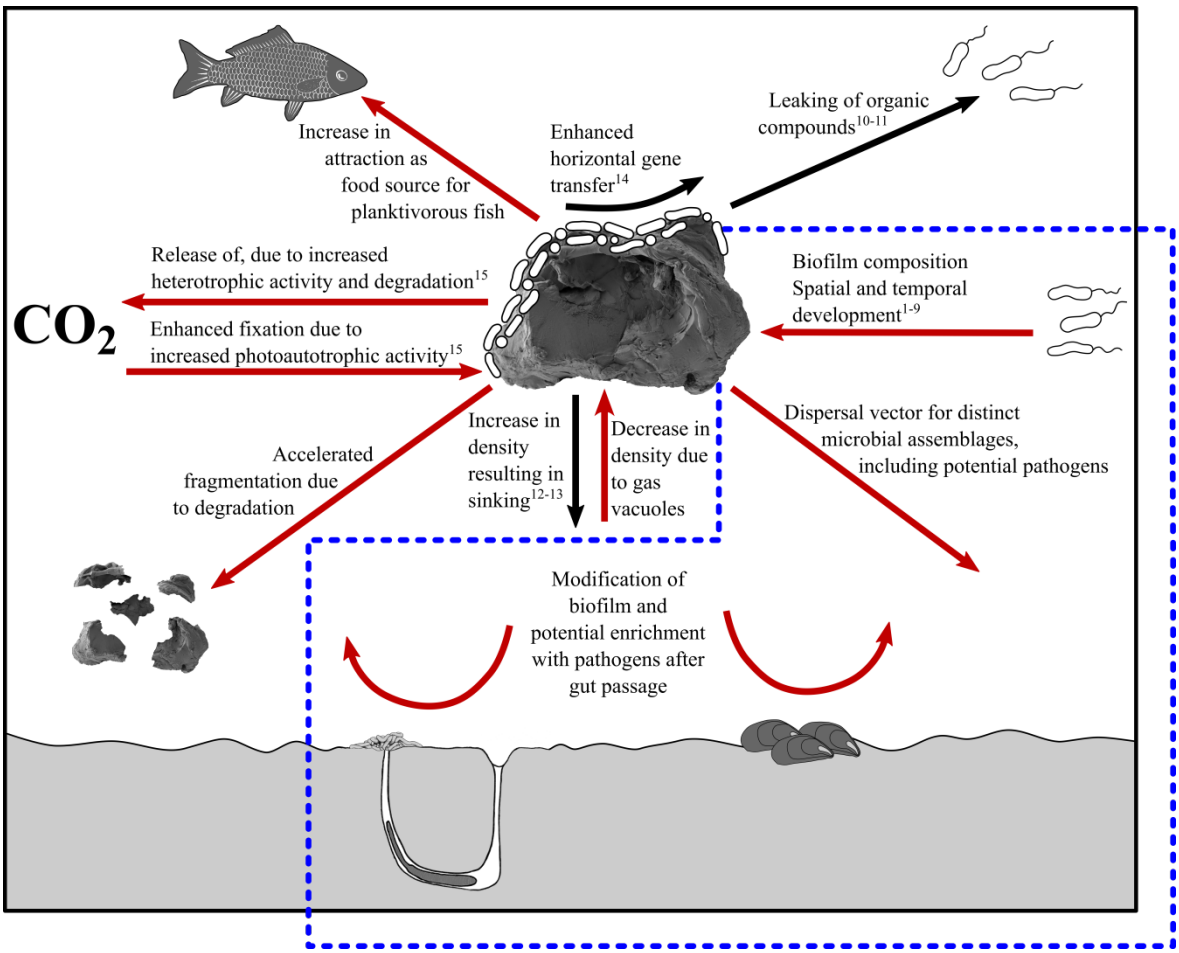

Figure A. Conceptual framework of the potential interactions of microplastic (MP)-associated assemblages in aquatic ecosystems. The various influences of microplastic-associated biofilms on the particles themselves, the environment, and higher organisms are depicted with arrows. Some interactions have already been investigated (black arrows), others are still purely hypothetical or only limited research exists (red arrows). The questions addressed in this study are encapsulated in

[1]Zettler et al. 2013; [2]Amaral-Zettler et al. 2015; [3]Oberbeckmann et al. 2016; [4,5]Dussud et al. 2018a,b; [6]Kettner et al. 2017; [7]Ogonowski et al. 2018; [8]Kirstein et al 2018; [9]Kettner et al. 2019; [10]Romera-Castillo et al. 2018; [11]Klaeger et al. 2019; [12]Ye & Andrady 1991; [13]Kaiser et al. 2017; [14]Arias-Andres et al. 2018; [15]Bryant et al. 2016

the blue box. Though data already exist on bacterial assemblages on microplastics from several environments, systematic investigations on drivers of biofilm development within the Baltic Sea ecosystem are lacking.

Due to its ecological features, the Baltic Sea possesses many prerequisites for the investigation of biofilm formation on microplastics in general and their risk potential.
In consequence, this study set out to investigate the potential impact of microplastic-associated bacterial biofilms on the Baltic Sea ecosystem, according to the key issues identified in Figure A. Because the question of microplastics as a dispersal vector for potential pathogens is of crucial importance to coastal communities, it aimed at providing information on different scenarios if and how microplastic-associated biofilms could become enriched in potential pathogenic bacteria like *Vibrio*: either mediated through passage through the gut of two important aquatic invertebrates, or if this enrichment could have happened independently because of favourable environmental conditions (brackish water and high water temperatures) within the Baltic Sea. This thesis encompasses three published experimental studies investigating biofilms on different microplastics. The first two chapters focus on the impact of the passage through the gut of two important aquatic invertebrates on microplastic-associated bacterial biofilms to assess the potential enrichment of opportunistic pathogens. The deposit-feeding marine polycheate *Arenicola marina* was used as a representative of a sediment dwelling, deposit feeding invertebrate that is highly likely to encounter microplastics with higher density reaching the bottom sediment. Worms were fed with either PS or glass as a control surface, and egested biofilms were analysed directly, but also the stability of these egested biofilms was investigated to assess the potential of microplastics as a dispersal vector for the potential faecal biofilms. Passage through the gut of this organism did not result in an enrichment of biofilms with potential pathogens on PS or glass. Rather, the gut passage resulted in more similar bacterial assemblages on the PS, the glass, and the faeces altogether, but biofilms were not stable over 24 h, resulting in a low vector potential of microplastics for gut-acquired bacteria. However, the oceanospirillum *Amphritea atlantica* was enriched in the investigated biofilms, faeces, and water only in the presence of PS. Thus, especially in areas of high PS pollution, this polymer may impact the bacterial composition of different habitats, with as yet unknown consequences for the respective ecosystems. This study is

described in chapter I: "*Polystyrene influences bacterial assemblages in Arenicola marina-populated aquatic environments in vitro*", published in *Environmental Pollution*.

In the study described in chapter II: "*Fate and stability of polyamide-associated bacterial assemblages after their passage through the digestive tract of the blue mussel Mytilus edulis*", published in *Marine Pollution Bulletin*, the bivalve *Mytilus edulis* was used as an exemplary organism for a highly active suspension-feeder. Although *M. edulis* is naturally located at the bottom above the sediment, it still has the capacity to filter the entire water column and thus ingests particles that are neutrally buoyant and present in the water column. Further, *M. edulis* becomes more and more important in the Baltic Sea in aquacultures (SUBMARINER Network for Blue Growth EEIG, 2017), in which they are usually cultivated on long ropes within the water column, making it both more likely to encounter particles throughout the whole water column, and from the long lines itself (Mathalon and Hill, 2014). In these experiments, polyamide (PA) and the biopolymer chitin were used to feed the mussels and biofilms were analysed thereafter. The egested particles were also incubated in sea water for 24 hours and 7 days to assess the stability of these biofilms. Although *M. edulis* has been shown associated with several potentially pathogenic *Vibrio* spp. (Lhafi and Kühne, 2007), no potential pathogens were detected exclusively on polyamide after gut passage. Biofilms were also not stable in these experiments, and after 7 days of incubation of the biofilms in sea water, the species richness of the polyamide assemblage was lower than that of the chitin assemblage with yet unknown impacts on the functioning of the biofilm community.

These two investigations were embedded in a broader characterisation of microplastic-associated biofilms developing in the specific Baltic Sea environmental gradients. Firstly, a general knowledge on the bacterial diversity within these biofilms is needed to assess their ecological importance for the Baltic Sea, and secondly to account for the interactions and interdependencies of single members of the assemblages, like vibrios, and the whole community. To investigate the importance of environmental parameters on the developing biofilms on different polymers, incubation experiments were conducted for seven days along the southern Baltic Sea coast along a ~2000 km transect covering a salinity gradient of 4.5–9 PSU. Two artificial polymers, polyethylene (PE) and polystyrene (PS), and wood as a natural polymer surface were used in these experiments. The developing biofilms were compared to those on natural seston and to planktonic bacterial assemblages within the incubation tanks. The results showed that habitat was the most important factor structuring

bacterial assemblages overall, significantly differentiating the biofilms on PE, PS, and wood from those on natural seston and the assemblages of the free-living fraction. However, surface properties were less significant in differentiating attached biofilms on PE, PS, and wood than environmental factors, of which mainly salinity was the most important. Nonetheless, a potential role for inorganic-nutrient limitations in surface-specific attachment was also identified. *Vibrio* was more abundant on the PE and PS biofilms than on seston, but its abundances were highest on wood and correlated positively with salinity. These results corroborate earlier findings that microplastics constitute a habitat for biofilm-forming microorganisms distinct from seston, but less from other natural polymer surfaces, such as wood. This study is described in chapter III: "*Spatial environmental heterogeneity determines young biofilm assemblages on microplastics in Baltic Sea mesocosms*", published in *Frontiers in Microbiology*. To investigate potential interaction of *Vibrio* with other biofilm members, a co-occurrence network was constructed from the more abundant OTUs (≥0.1% relative abundance) on PE, PS, and wood, which showed that *Vibrio* was not well connected with other biofilm members. This additional network analysis described in the General discussion has not been published yet.

Summary of published papers

No enrichment of *Vibrio* was found specifically on microplastics after gut passage of two aquatic invertebrates, or after incubation in seawater from different coastal stations along the Baltic Sea. Although *Vibrio* abundances were higher on PE and PS than on seston and in the free-living fraction, they were highest on wood and were significantly higher at salinities of 7.5–9 PSU. Using the widespread lugworm *A. marina* and the filter-feeder *M. edulis* as a model organism, it was found that the passage through the gut of this organism did not result in an enrichment of biofilms with potential pathogens on PS or glass, nor that a distinct faecal signal in the biofilms could be detected in general. Rather, the gut passage resulted in more similar bacterial assemblages on the PS, the glass, and the faeces altogether. However, recolonisation processes from the sediment or the water seemed to be the main driver of the biofilm assemblage processes and biofilms on the egested particles (PS, PA, glass, and chitin) changed rapidly; indicating that, though the gut passage had an effect on the biofilm assemblages, this effect could be neglected. In general, environmental parameters were the main factors influencing developing assemblages on microplastics

(PE, PS, and PA) in the Baltic Sea, with salinity probably being the most influential one. Substrate type (plastics vs. controls) was playing a secondary role. However, biofilm assemblages on microplastic- and control surfaces differed strongly from those on natural seston and the free-living ones.

Some substrate-specific colonisation could be observed: a close relative of the oceanospirillum *Amphritea atlantica* was found exclusively enriched on the PS-particles, and in other samples of the PS-treatment. Members of the putative hydrocarbonoclastic *Sphingomonadaceae*, *Devosiaceae*, and *Rhodobacteraceae*, as well as members of the *Alteromonadaceae* and the genus *Pseudomonas* were discriminant for the PE and PS-associated assemblages. No PA-specific OTU could be found, but the oceanospirillum *Neptunomonas* sp. was very abundant on PA after 7 days of incubation in sea water. This organism was also found on chitin, but at lower abundances than on the plastic particles.

General discussion

The vector potential of microplastics for putative pathogenic bacteria

Neither PS, nor PA showed an enrichment of potential pathogens after passage through the digestive tract of *A. marina* or *M. edulis*. Also, no other potential pathogen was found enriched on PS and PA in comparison to glass, chitin, sediment, or water. The vector potential of microplastics after gut passage of invertebrates living in the Baltic Sea is therefore considerably low. A colonisation of PS and PE by *Vibrio* spp. rather stems directly from the water, as is shown in the incubation experiments along the 2000 km coastal transect of the Baltic Sea. Here, a particular *Vibrio* OTU was more abundant on the PE, PS, but especially on wood, compared to the seston and the free-living fraction, but only at stations within the salinity range of 7.5–9 PSU.

Oberbeckmann et al. (2018) found *Vibrio* spp. on PE and PS in exposure experiments conducted in the Warnow estuary after 2 weeks of incubation, but in lower relative abundance (max. 0.6% on PS) than was found by Kesy et al. (2019) (max. 1.2% on PS). Interestingly, the relative abundance of *Vibrio* in the study by Oberbeckmann et al. (2018) was even higher on wood after 2 weeks (max. 13.6%) than after 1 week in the study along the Baltic Sea coastline (Kesy et al., 2019) (max. 2.3%). A different extraction method and PCR protocol were used in both studies, so this could account to some extend for the

differences in relative abundances found on PE and PS between the two studies. However, the differences in the ranges between the *Vibrio* abundances on PE, PS, and wood are comparable. Therefore, it seems that *Vibrio* is more persistent on wood than on PE or PS, and that microplastics appear to have a higher vector potential in the early stage of their time at sea. Further, we could observe differences in *Vibrio* abundances based on substrate similar to the pattern observed for the whole community (see chapter III). PE was always the substrate with lowest *Vibrio* abundance, than PS and wood, which also hints at factors such as surface complexity as an important factor for *Vibrio* colonisation. This could also be the reason for the persistence of *Vibrio* on wood. Only two studies found very high *Vibrio* abundances on microplastics samples *in situ* (24 and 18%; Frère et al., 2018; Zettler et al., 2013), all other studies investigating biofilms on microplastics did not detect an enrichment (Debroas et al., 2017; Dussud et al., 2018b; Jiang et al., 2018; Schmidt et al., 2014). *Vibrio* was also not detected on particles sampled from the Baltic Sea along the same cruise on which the incubation experiments were conducted (Sadowski, 2018). Given that particles found in the system probably had longer residence time in the system than 7 days, we could show that *Vibrio* is most likely a member of young biofilm assemblages. This assumption is strengthened by results from Datta et al. (2016), who investigated early biofilm succession on chitin particles and showed a *Vibrio* OTU being amongst the very first colonisers. Also, in an *in situ* incubation experiment using fibreglass around the island of Mauritius, Rampadarath and co-workers found *Vibrio* abundances of 5.3% after the first 24 h of immersion (Rampadarath et al., 2017).

The potential dependencies of vibrios with other biofilm members was investigated by constructing a co-occurrence network of all OTUs with relative abundance >0.1% in the PE, PS and wood biofilms after 7 d of incubation in water from different Baltic Sea stations. To prevent confounding effects of salinity or sample type on the co-occurrence inference, only the PE, PS, and wood samples were used from the incubations within the salinity range of 7–9 PSU (TF0046, MP3, MP5, MP9, and MP11). Therefore it was ascertained that stations with higher and lower *Vibrio* abundance were included, but that salinity or the sample type (e.g. seston, which had an overall low abundance of *Vibrio*) would not hamper the interpretation (Berry and Widder, 2014). From 472 OTUs with >0.1% relative abundance, 394 OTUs were connected by positive or negative edges, which represent correlations in their abundances (Fig. B.A). To investigate the degree of connection of the *Vibrio* OTU 137 within the overall network, the latter was analysed using

the OH-PIN algorithm from the CytoCluster application (Li et al., 2017) to detect sub-complexes, after which the one including the *Vibrio* OTU was extracted (highlighted in yellow in Fig. B.A). The *Vibrio* OTU was part of a smaller complex within the whole network (rank 7, Fig. B.B). Compared to the average number of neighbours of each OTU in the network (33.1), *Vibrio* sp. was only directly connected to 7 other OTUs, of which 4 were positive and 3 negatively correlated (Fig. C). A similar pattern for *Vibrio* was observed in a study investigating biofilm assemblages on floating plastics in the Mediterranean Sea, where *Vibrio* was found to co-occur mostly with a few other *Vibrio* OTUs (Delacuvellerie et al., 2019). In the incubation experiment along the Baltic Sea coast, *Vibrio* significantly co-occured with 4 other OTUs that stemmed from very diverse bacterial lineages. One OTU, OTU 471, belonged to the genus *Pusilimonas* of the family *Burkholderiaceae* and another OTU 366, was related to an unclassified *Saccharospirillaceae*, both within the class *Gammaproteobacteria*. A third, OTU 57, was assigned to the genus *Flavobacterium* within the *Bacteroidia* class. Finally, OTU 90 was

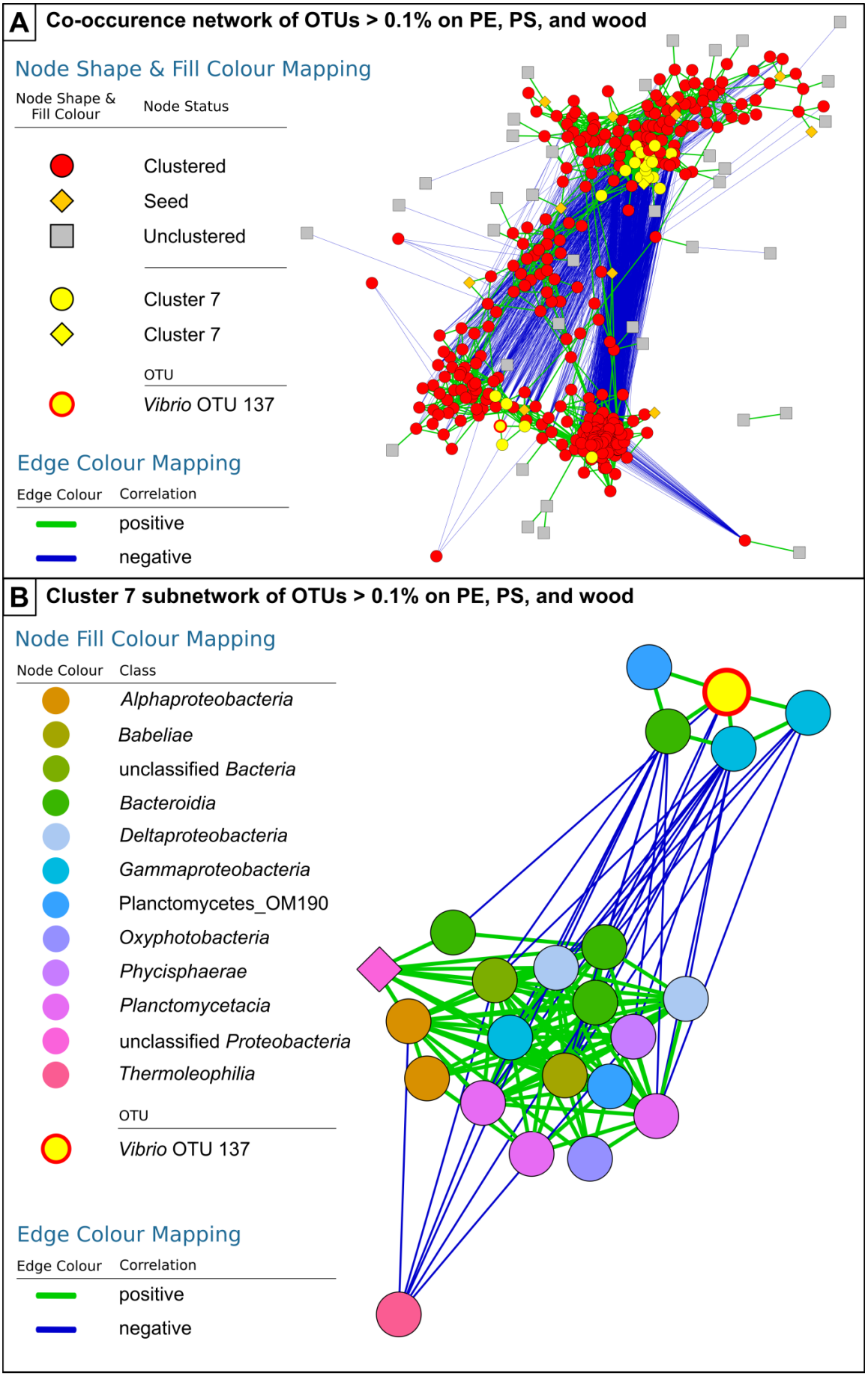
A
Co-occurence network of OTUs > 0.1% on PE, PS, and wood
Node Shape & Fill Colour Mapping
Node Shape & Fill Colour
Node Status
Clustered
Seed
Unclustered
Cluster 7
Cluster 7
OTU
Vibrio OTU 137
Edge Colour Mapping
Edge Colour
Correlation
positive
negative
B
Cluster 7 subnetwork of OTUs > 0.1% on PE, PS, and wood
Node Fill Colour Mapping
Node Colour
Class
Alphaproteobacteria
Babeliae
unclassified Bacteria
Bacteroidia
Deltaproteobacteria
Gammaproteobacteria
Planctomycetes_OM190
Oxyphotobacteria
Phycisphaerae
Planctomycetacia
unclassified Proteobacteria
Thermoleophilia
OTU
Vibrio OTU 137
Edge Colour Mapping
Edge Colour
Correlation
positive
negative

Figure B. (previous page) Co-occurrence network of OTUs with relative abundances >0.1 % on the PE, PS, and wood from the incubation experiment in the Baltic Sea. **(A)** Only stations within the salinity range of 7-9 PSU (TF0046, MP3, MP5, MP9, and MP11) were used in the construction. The network was analysed for sub-complexes using the 'Overlapping and Hierarchical modules in Protein Interaction Networks' algorithm (OH-PIN). The sub-complex including the OTU 137 classified as *Vibrio* sp. (yellow circle with red border) and identified by the OH-PIN algorithm is represented by the yellow colour of the nodes. **(B)** The sub-complex identified by the OH-PIN algorithm only depicts OTUs that are associated with the most abundant *Vibrio* OTU, either directly linked or indirectly via their neighbours.
Each OTU is represented by a node along with its status in the network based on the node's shape: Seed OTU (diamond &orange), clustered OTU (circle & red or yellow), unclustered OTU (rectangle & gray) in **A**, or based on its phylogenetic association (colour) in **B**. Nodes are connected through edges (lines) representing their interaction: Green edges between nodes represent positive correlations; blue edges between nodes represent negative correlations.

related to the uncultured planctomycetean group OM190. Although these 5 OTUs (*Vibrio* sp. included) were positively correlated, it does not necessarily imply a direct interaction or mutualistic relationship. It may well just mean that these OTUs occupy similar ecological niches and have similar habitat requirements and are also early colonisers (Faust and Raes, 2012). Indeed, planctomycetes are generally known to easily form biofilm using a holdfast structure (Youssef and Elshahed, 2014), and *Flavobacteriaceae* are also repeatedly found in biofilms (McBride, 2014). These findings corroborate our assumption that *Vibrio* is an early coloniser of biofilms in the Baltic Sea, which is not dependant on resources or secondary metabolites provided by other biofilm bacteria. Interestingly, all of the positively correlated OTUs are to some extend related to organisms that degrade a variety of sugars derived from different sources, such as algae. *Planctomycetes* are often found in biofilms on diverse macroalgae and are supposed to be able to utilise polysaccharides excreted by these organisms (Lage and Bondoso, 2014). *Flavobacteria* have repeatedly been shown to be amongst the first to respond to algal derived sugars (Buchan et al., 2014; Teeling et al., 2012). As the name says, *Saccharospirillaceae* are able to utilise a diverse range of sugars (Labrenz et al., 2003), and also *Vibrio* species are able to utilise a variety of short sugar molecules (Gomez-Gil et al., 2014). The presence of these organisms may be indicative of the utilisation of polysaccharide molecules that constitute the conditioning film. It has also been suggested that diatoms are early eukaryotic colonisers of surfaces in aquatic systems and a tight coupling of bacteria to eukaryotic communities on PE and PS

biofilms has recently been demonstrated in 2-weeks old biofilms (Kettner et al., 2019). The network results could therefore also indicate that these co-occurring bacteria OTUs share similar utilisation patterns of phytoplankton derived material in biofilms. Although eukaryotic communities were not investigated in this incubation experiment, it is known that the annual diatom-bloom in the Baltic Sea occurs in spring and ceases in the summer due to nutrient depletion (Andersson et al., 2017; Schneider et al., 2017), so diatoms probably do not play a major role in microplastic-associated biofilms formed during summer months in the Baltic. In which way microplastics could sustain diatom-growth in biofilms during summer month due to the accumulation of nutrients is out of the scope of this discussion, but an interesting emergent question.

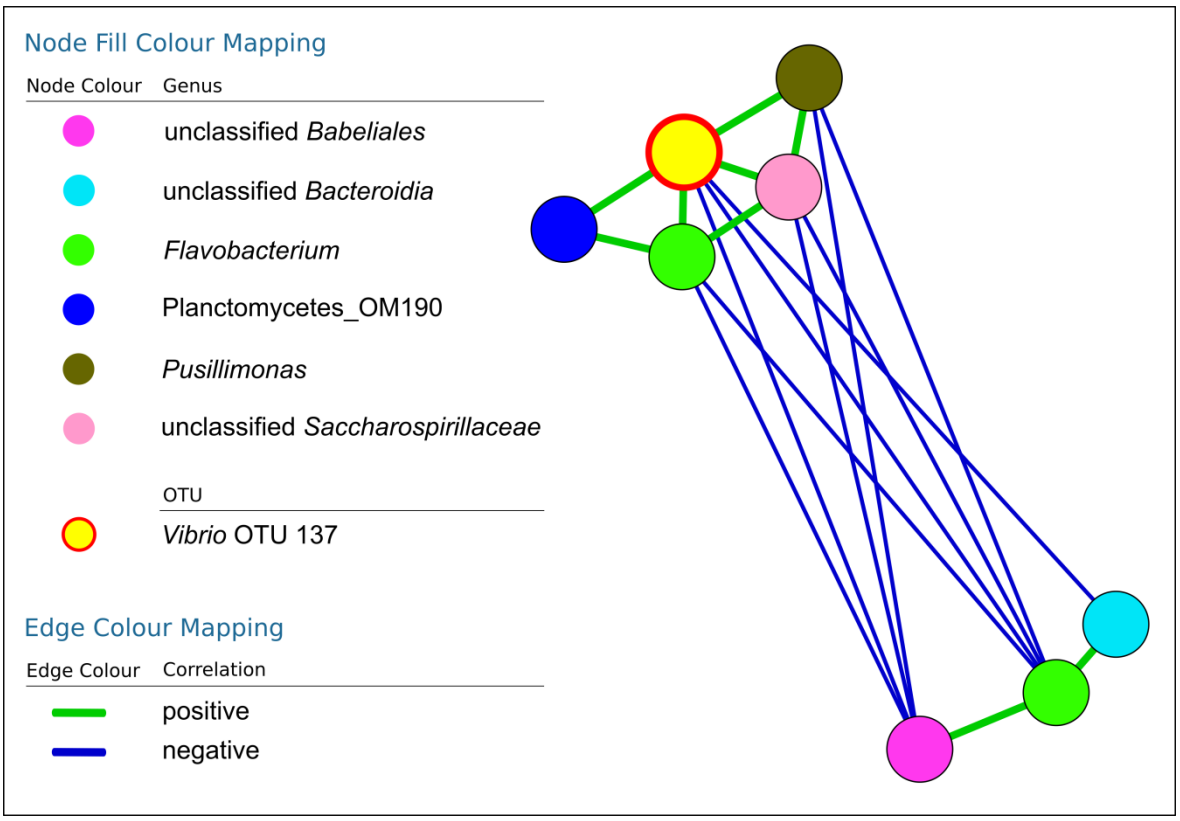

Figure C. Co-occurrence network of OTUs with relative abundances >0.1 % on the PE, PS, and wood directly associated with *Vibrio* sp. from the incubation experiment in the Baltic Sea. Each OTU is represented by a node (circle) and its phylogenetic association (colour). Nodes are connected through edges (lines) representing their interaction: Green edges between nodes represent positive correlations; blue edges between nodes represent negative correlations. This sub-complex only depicts OTUs that are directly associated with the OTU 137 (yellow node with red border), the most abundant OTU within those classified as *Vibrio* spp.

However, *Vibrio* was not found during the *A. marina* and *M. edulis* experiments, where the incubation times were in a similar range. One reason for this could be the lower water temperatures, which were below the optimal growth temperature for relevant *Vibrio* spp.. Since warmer water temperatures in summer usually give rise to growing *Vibrio* numbers (Takemura et al., 2014), it was an aim to investigate the enhanced dispersal potential via gut passage also for the mean Baltic Sea temperature, which is especially important for aquaculture organisms. Although *Vibrio* cells might be VBNC state during colder temperature, it was already shown that *V. vulnificus* and *V. cholera* are able to resurrect from this state when they are within an organism (Alam et al., 2007; Colwell et al., 1985), which would make the question of inoculation via ingestion all the more important, because this would add a potential reservoir and dispersal route even in colder temperatures in the Baltic Sea. *Vibrio* cells, even when in VBNC state are still detectable by molecular methods, which is why it is important to include these methods in surveys, or at least when investigating potential reservoirs. Nonetheless, *Vibrio* spp. were not detected after gut passage on PS or PA. However, these surfaces were not entirely new, because they had been incubated in water beforehand, then had passed the gut of the respective animals, therefore leading to an existing biofilm that was not enriched in *Vibrio*. This priority effect might have also let to *Vibrio* spp. not being able to take advantage of newly available habitat and the potential accompanying nutrient input.

Other genera that contain potential pathogens that have been detected on microplastics include *Arcobacter*, *Tenacibaculum*, and also members of *Pseudomonas* (Oberbeckmann et al., 2015). *Arcobacter butzleri* for example is a species that has been isolated from various sources, including faeces and biofilms on drinking water pipes and is associated with gastroenteritis and bacteraemia (Collado and Figueras, 2011; Lastovica et al., 2014). The genus *Tenacibaculum* includes several fish pathogens, which are often the cause of high mortality in cultured marine fish (Avendaño-Herrera et al., 2006). *Pseudomonas* species are generally considered to be metabolically very versatile and are found in almost every environment and are also known to react quickly in incubations (Madigan et al., 2012), so that the high abundance of *Pseudomonas* OTUs is not exceptional. In fact, the most abundant OTU in the whole dataset was a member of this genus. However, when the representative sequence was compared against the NCBI-database using the online BLAST-tool (uncultured species excluded), the highest identity was obtained for *Pseudomonas pelagia* (% identity 100%; % query cover 100%, Accession number

MK224745.1), which is an organism that had first been isolated from a culture of an Arctic unicellular green algae (Hwang et al., 2009). Also, *Tenacibaculum* was not detected on any samples from the Baltic Sea or during the incubation experiment with *A. marina*. It was found only in faeces of *M. edulis* with about 1.6% relative abundance (Fig. 2.3), thus, *Tenacibaculum* does not appear to be a member of young biofilms and is less relevant in the Baltic Sea. *Arcobacter* spp. were found in the experiment using *A. marina*, *M. edulis*, and during the incubations with Baltic Sea water from different coastal stations. However, it was also shown that biofilms on microplastics did not specifically enrich *Arcobacter* spp., but that the control surfaces (glass, chitin, and wood) consistently harboured higher relative abundances of *Arcobacter* (Fig. 1.6, Fig. 2.3, and Fig. D). From the literature it is noteworthy that in most cases *Arcobacter* became abundant during incubation experiments, also in the incubation waters (Fig. D) (Curren and Leong, 2019; Harrison et al., 2014; Kesy et al., 2016, 2017, 2019).

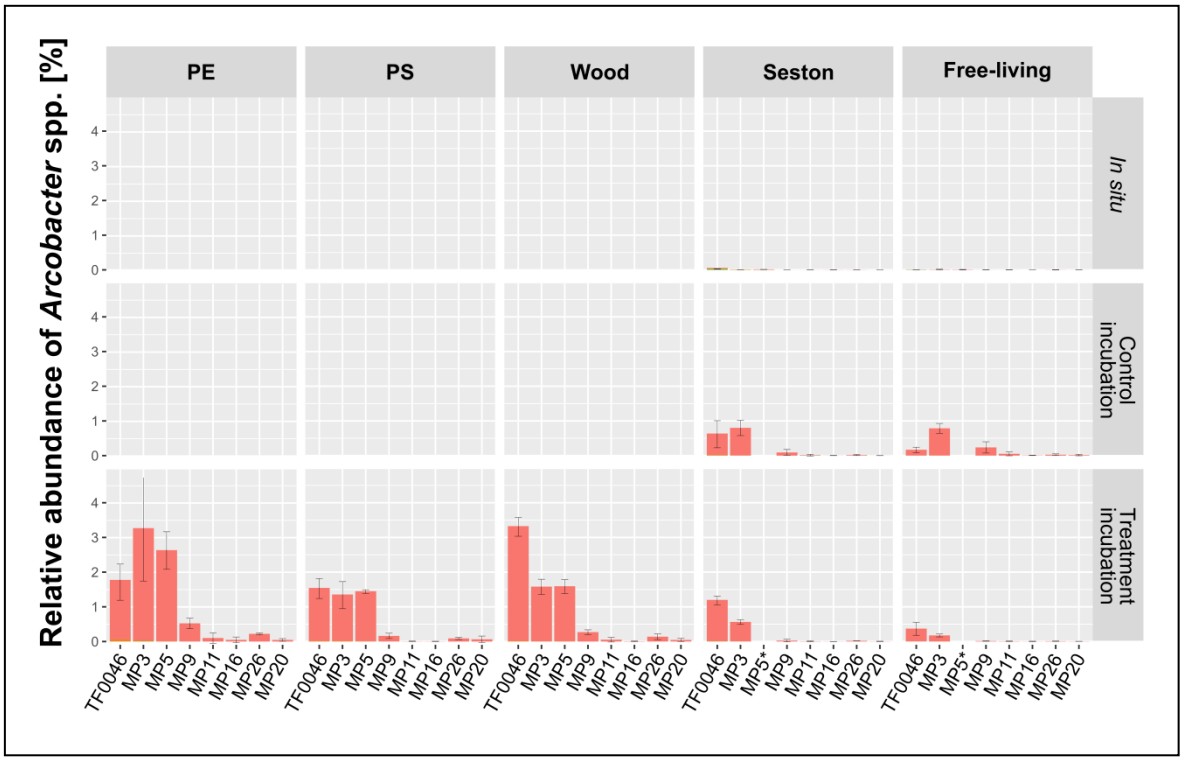

Figure D. Mean relative abundances of each *Arcobacter* OTU on seston (≥3 µm) and in the free-living fraction (3–0.22µm) at the different stations at t_0 (*in situ*) and after 7 days of incubation on PE, PS, wood and seston and in the free-living fraction. Data for both the treatment and the control incubations are shown. Bars indicate the standard deviation of the most abundant *Arcobacter* OTU. *For station MP5, incubation water samples were not available.

The increase of *Arcobacter* spp. might therefore be in part due to the incubation conditions themselves. An increase in the number of *Arcobacter* spp. was also observed in the control incubations from the Baltic Sea without the addition of any particles, unlike the case for *Vibrio* spp.. However, *Arcobacter* was still more abundant on the PE, PS, and wood than on the natural seston, showing that it preferentially colonised inert surfaces. It was also more abundant at the western stations within the salinity range of 7–9 PSU (Fig. D).

One can therefore conclude that microplastics do not comprise a dispersal vector for potential pathogens that is distinct from other natural or inert surfaces; rather it is the particle/surface itself that promotes enrichment with potential pathogenic taxa due to their preferred biofilm lifestyle. This is also, however, dependant on the succession stage of the biofilm, as shown for *Vibrio* spp. The potential for microplastics and plastic waste in general as means for pathogen dissemination therefore strongly depends on the amount of items/particles in the ecosystem and on the temporal- as well as environmental dynamics of the system. So far, experiments have been conducted with large amounts of microplastics and in case of the proof-of-the-principle approach this is also legitimate. As has been shown in the incubation study along the Baltic Sea coast, *Vibrio* abundances were also higher in the free-living fraction of the incubation water and it would be of utmost importance to be able to transfer these results to the natural Baltic Sea system. Reported microplastic concentrations in Baltic Sea waters range between 0.0068 particles (>100 µm) and 9.4 particles (>20 µm) per litre in the Gulf of Finland (Setälä et al., 2016; Talvitie et al., 2015) to 0.0077 particles (>335 µm) and 7.5 particles (>90 µm) per litre in the Stockholm area (Gewert et al., 2017; Gorokhova, 2015). These data already show that microplastic distribution can be very variable. However, for risk assessment, the highest amount should be considered, which so far has been 9.5 particles (>20 µm) per litre. These numbers are hard to put into an ecological perspective, simply because comparable data is lacking. To the best of my knowledge, there is no information available on numbers of drift wood or other inanimate particles present in the Baltic Sea, such as amber or pollen. One possibility is therefore to compare these numbers with copepod abundances, since they are also known to be associated with *Vibrio* (Huq et al., 1983). Although the dispersal behaviour from copepod-associated bacteria may be different, this at least provides a first rough estimate on the order of magnitude of available substrate. Mean copepod abundances in the Baltic Sea reach ~40 individuals per litre during spring and summer (Wasmund et al., 2018). Though the highest microplastic concentration so far reported is

9.5 particles per litre, these are not orders of magnitude different to copepod numbers and microplastics could thus represent a significant contribution to the available surface area. This may become especially important during the winter month, when copepod abundances cease to about 3 individuals per litre (Wasmund et al., 2018) but microplastics persist. In conclusion, to be able to extrapolate this observed dispersal effect of *Vibrio* into the surrounding water into the natural system; it would be of importance to use ecologically relevant microplastic concentrations to confirm whether this effect is of relevance in the Baltic Sea (Fig. E).

Microplastics provide a habitat for opportunistic biofilm-forming bacteria

Plastics comprise a newly available surface made from organic building blocks, so that it could also act as a substrate-analogue. However, the data so far suggests that nonspecific attachment is of higher importance in microplastic-associated biofilms in non-nutrient limited systems (Kesy et al., 2019; Lorite et al., 2011; Oberbeckmann et al., 2018). Further, the line between surface specificity cannot be drawn between artificial versus natural organic surfaces (such as wood or chitin). The most abundant OTUs were found both on the plastics employed in the different experiments and the control surfaces, and have been found in a variety of biofilms, such as members of the *Lentispheara* (Cho et al., 2004), *Planctomycetes*, *Flavobacteriaceae*, *Rhodobacteraceae*, *Caulobacteraceae*, *Hyphomonas*, *Pseudomonadaceae*, and *Alteromonadadales* (and within this order especially the genera *Colwellia*, *Shewanella*, *Thalassomonas*, and *Alteromonas*) (Dang and Lovell, 2016; López-Pérez and Rodriguez-Valera, 2014; McBride, 2014; Oberbeckmann et al., 2015; Youssef and Elshahed, 2014), indicating that taxa colonising plastics are rather usual biofilm forming species. It seems reasonable to assume that bacteria that have the ability to form biofilms will do so on various surfaces and a single cell can possess a battery of mechanisms to form biofilms on diverse substrates (Guilbaud et al., 2017; Marshall, 2006; Mueller et al., 2007). Therefore it is highly likely that environmental cues are important to trigger biofilm formation. On the other hand, substrate-specific attachment mechanisms are more likely to play a role if this substrate is also a nutritional source, like the attachment of *V. cholerae* to chitin, or the colonisation of natural seston by heterotrophic bacteria (Meibom et al., 2004; Stocker et al., 2008). When assessing biofilm formation on PE, PS, and wood along a spatial gradient in the Baltic Sea, it became apparent that environmental

factors were the dominant drivers of biofilm differentiation regardless of surface type. Obviously, the source community was important, but salinity seemed to be the main driver in structuring the different communities, apart from just shaping the source community. Surface properties were less important. This shows that within the already complex processes that govern biofilm formation and composition, a hierarchy of factors exists that will greatly depend on the underlying environmental gradients of these factors and the pressure they exhibit. Salinity has been shown, together with temperature, to be one of the main factors structuring the geographical distribution of almost all organisms on earth (Del Giorgio and Gasol, 2008; Hahnke et al., 2013; Lozupone and Knight, 2007; Schattenhofer et al., 2009). The cellular mechanisms of dealing either with a hyperosmotic or hypoosmotic surrounding are not easily changed, or if, only under great energetic costs (Oren, 2006). Bacteria can adapt quickly to new environmental conditions, and as a particle transitions through different aquatic environments, changes in salinity will most likely be the major selective pressure on these biofilm communities. Here again, a smaller particle will travel faster than a bigger item (Isobe et al., 2014), subduing their associated assemblages to the changing conditions more rapidly. To which extend biofilm assemblages are able to resist change when met with new conditions would be of great interest as it would help to answer just how much microplastics can be a vehicle for invasive assemblages. Although, our results from the *A. marina* and the *M. edulis* experiments suggest that biofilms are not stable when experiencing a new environment (e.g. gut passage vs. water) (Kesy et al., 2016, 2017).

However, some investigators reported differences between plastic surfaces versus glass or other inorganic surfaces (Jiang et al., 2018; Kirstein et al., 2018), providing at least some support for the hypothesis that plastics could act as substrate-analogues. The artificial polymers in these studies described were polyethylene (PE), polystyrene (PS) and polyamide (PA). All three are thermoplastics, which can be moulded, solidified and remoulded over several cycles. This is because their individual polymer chains are not cross-linked but associated by intermolecular forces to form the bulk polymer (Zheng et al., 2005). PE and PS are pure hydrocarbons that have a low free energy and are hydrophobic. High density linear polyethylene (HDPE) was used in for the incubation experiments with water from different Baltic Sea stations. HDPE is a long chain of single-linked C-C bonds and hydrogen atoms. Linear PE has very little branching side chains and thus has an even smoother surface at a molecular level than polystyrene, which is formed

by a hydrocarbon backbone with attached phenyl-groups. C-C-bonds are very stable and are thought to be rather resistant to cleavage via hydrolysis (Zheng et al., 2005). In comparison, PA is a polymer that also consists of heteroatoms, namely an amide group (-CO-NH-) that connects the hydrocarbon chain via an amide bond, making it technically a polypeptide. Because the amide bonds result in –H and –O moieties at the carbon backbone, PA is rather polar and more hydrophilic. Further it is assumed that the amide bond is more readily degradable as it is more susceptible to hydrolysis (Krueger et al., 2015; Negoro, 2000). As natural control surfaces, chitin, glass and wood were used. Chitin is, next to cellulose and lignin, the most common biopolymer on earth. It consists of chains of the sugar monomer *N*-acetylglucosamine, a derivative of glucose and is found in the call walls of insects, arthropods and fungi (Gooday, 1990). Wood consists majorly of the polysaccharides cellulose and hemicellulose, and of the heterogeneous hydrocarbon polymer lignin, which is rich in aromatic rings and responsible for the rather hydrophobic character of wood (Pettersen, 1984). The high abundance of a *Neptunomonas*-related OTU on both chitin and PA could hint an analogue colonisation, since both also offer nitrogen. Many chitinovorous bacteria are positive chemotactic towards chitin oligosaccharides that result from chitin degradation and diffuse away (Meibom et al., 2004). A study recently found that a decrease in the $^{14}C/^{12}C$ isotope ratios in the DIC content of experimental mesocosms (which could be attributed to microbial mineralisation) was indeed due to diffusion from residual mono- and oligomers out of the polymer as a result of incomplete polymerisation (Klaeger et al., 2019). These leaking mono- and oligomers could act as chemical cues for the specific colonisation, as some degree of similarity exists between the *N*-acetylglucosamine- and caprolactam-oligomers: the oligomer of one of the most abundant PA variants, Nylon 6 (Andrady, 2011).

Yet again, these results are not consistent, as other studies did not find the differentiation organic vs. inorganic substrate to be the main driver of biofilm dissimilarity, and thus underline the importance of additional factors (Hoellein et al., 2014; Miao et al., 2019; Ogonowski et al., 2018). One probable factors has been identified as nutrient limitation or the nutrient ratios, as this seemed to play a role in surface-specific dissimilarities of PE, PS, and wood associated biofilms (Kesy et al., 2019; Oberbeckmann et al., 2018). One possibility could be that the available nutrients influence the composition and concentration of the conditioning film, which is also depending on the surface properties (Schneider, 1996). On the other hand, the polymer itself could become a nutritional source

during nutrient limitation. However, PE and PS are both pure hydrocarbons, it is unlikely that they might become a carbon source when there is no nitrogen or phosphorous available for the generation of biomass, although these nutrients may be enriched in the conditioning film. In spite of this, all station water used in the incubation experiment along the Baltic Sea coast had an initial DOC concentration of >300 µM, which is an easier available carbon- and energy source, and in this concentrations excessively available (the lowest DOC concentration thought to support heterotrophic growth is ~30 µM) (Arrieta et al., 2015). Therefore it is unlikely that PE or PS were used as an actual substrate. More probable is that other physico-chemical surface properties became more important during nutrient limitation or different salinities, as these two factors may significantly influence attachment capacities of different bacteria as outlined in chapter III of this thesis.

Interestingly, in the context of Baltic Sea biofilms on plastics, a trend can be observed in which surface roughness or chemical complexity influence assemblages in a way that biofilms on PE are more similar to PS, PS more similar to wood, and wood more similar to natural seston (PE – PS – wood – seston, Fig. 3.6). That chemical complexity and material heterogeneity can influence bacterial attachment has been found by other studies as well, although the direct mechanisms in which this might influence bacteria is still in the dark (Alexander and Williams, 2017).

Rough surfaces provide a greater and more heterogeneous surface area and thus more available microniches. The consistently lower species richness of biofilms formed on smoother microplastics compared to the control surfaces, seston, and surrounding water in the studies presented here may indicate such an effect. However, there is an inconsistency in reported alpha-diversity on microplastics from various studies. Lower richness has been reported by Kettner et al. (2017); McCormick et al. (2014); Ogonowski et al. (2018); and Zettler et al. (2013), but other investigators have reported higher α-diversity on microplastics compared to those of seston or the free-living assemblages (De Tender et al., 2015; Debroas et al., 2017; Dussud et al., 2018a; Frère et al., 2018). These differences probably reflect the diversity of particles found: bigger particles will harbour greater diversity; weathered particles may also provide a greater surface heterogeneity and thus, more microniches. Finally, differences in the succession stages of the accordant biofilms will lead to differences in α-diversity. Species richness is generally attributed to greater functional diversity- and resilience (Bello et al., 2018; Spehn et al., 2005). The role microplastics play in either increasing or decreasing species richness will depend on the

particles history and properties (i.e. size, surface roughness) and cannot be extrapolated at the moment. What can definitely be confirmed is that microplastics provide a habitat for biofilm-forming bacteria that will add new functional traits to aquatic systems that are usually dominated by pelagic assemblages.

Microplastic-specific colonisation is limited to bacteria specialised in the attachment to, and potential degradation of, organic surfaces

As the majority of taxa was found on all surfaces tested (plastics and controls), biofilm formation on microplastics seems to be unspecific overall. Nonetheless, in all three experiments some discriminant taxa for plastics existed, such as *Amphritea atlantica* on PS, and members of the *Sphingomonadacea* and *Pseudomonas* on PE and PS. It is noteworthy that repeatedly members of families and genera were detected on the plastics that had been described as putative hydrocarbon degraders. *Amphritea* and also *Neptunomonas*, which became abundant on PS and PA, are both members of *Nitrincolaceae* (former *Oceanospirillaceae*) within the order *Oceanospirillales*. This order contains many members that are hydrocarbon degraders (Satomi and Fujii, 2014). The same holds true for many sphingomonads (Stolz, 2009) and members of the genus *Pseudomonas* (Onaca et al., 2007). Although there has been an ongoing debate whether or not bacteria would eventually degrade plastic waste under environmental conditions (Oberbeckmann and Labrenz, 2020), it is not negligible that these putative hydrocarbonoclastic bacteria are consistently found on diverse plastics from various habitats, such as the Mediterranean Sea, the Atlantic Ocean, and the North and Baltic Sea. Many of these bacteria are also found on natural surfaces, such as algae (Burke et al., 2011; Lachnit et al., 2011), wood (Kesy et al., 2019) and chitin (Kesy et al., 2017), or became abundant during oil spills (Hazen et al., 2010). Accordingly, the degradation of crude oil by bacteria is facilitated by biofilm formation at the oil-water interface (Sivadon and Grimaud, 2018). However, these observations do not necessarily imply a degradation of plastics by these bacteria. Even if this is not the case, it seems that the plastic surface provides cues for the colonisation by these putative hydrocarbonoclastic bacteria. Indeed, a characteristic of *Sphingomonadaceae* is the replacement of lipopolysaccharides in their cell wall with sphingoglycolipids (Glaeser and Kämpfer, 2014), which are more hydrophobic, and it has been hypothesised that these sphingolipids are an advantage for sphingomonads

in colonising hydrophobic substrates, such as the oil-water interface (Haas et al., 2015; Stolz, 2009). In other words, hydrophobicity would be an environmental cue for *Sphingomnadaceae* that they have encountered a hydrocarbon surface and thus, initialise biofilm formation. However, other studies have not detected a mechanistic relationship between the attachment of single sphingomonad strains to hydrophobic or hydrophilic surfaces in soils – substantiating again the complexity of biofilm formation and its drivers in natural systems (Cunliffe and Kertesz, 2006; Johnsen and Karlson, 2004).

It was recently calculated from experimental data that between 260 and 23,600 MT of DOC per year could leach from the plastics found in the oceans, which significantly enhances heterotrophic activity (Romera-Castillo et al., 2018). However, these data did not account for enhanced bacterial activity directly within the biofilms. Hydrocarbonoclastic biofilm-bacteria could add to heterotrophic activity by the degradation of hydrocarbons: be it of the polymer backbone, of leaching monomers or additives, or of adsorbed organic pollutants. Since the detection of putative hydrocarbonoclastic bacteria has consistently been reported from various environments and various substrates, the functional role these bacteria play in plastic-associated biofilms, or if the hydrophobic surface simply triggers biofilm formation, should be investigated to elucidate if and how they impact the carbon cycle in the global oceans.

Microplastics have the potential to alter pelagic bacterial communities

Interestingly, it was found in two out of the three experiments that the addition of particles could lead to an increase of bacteria found associated to the particles (as *Vibrio* spp., *Amphritea atlantica*, and to some extent, *Neptunomonas* sp.) in the respective incubation waters. This effect might be overrepresented in closed incubation systems; however, it shows that the addition of available surface area can influence planktonic communities. Many bacteria do not exhibit just one type of lifestyle (i.e. planktonic vs. sessile), but transition between them (Sauer et al., 2002; Stoodley et al., 2002). Dispersal from biofilms is usually described as the final maturation step in biofilm development, when cells become motile again and detach from the biofilm to colonise new habitats (Stoodley et al., 2002). Different cues trigger the detachment of cells from the biofilms. Such cue could include a depletion of nutrients or other environmental cues, signals from neighbouring cells, if cell density becomes too high (Guilhen et al., 2017), or physical forces detaching

whole microcolonies (Stoodley et al., 2002). Although it is seen as the final maturation step in biofilm development, natural biofilm assemblages are likely to undergo successional changes. Moreover, different species might respond to different cues during biofilm development and thus different time points for biofilm dispersal are likely to exist for different biofilm members. The marine *Vibrio* strain DW1 for example released daughter swarming cells 45 min after cell-adhesion to a given surface (Kjelleberg et al., 1982). Therefore, an enrichment of distinct bacterial species within the biofilm may also cause enrichment in the surrounding seawater. This could induce local shifts in the whole bacterioplankton assemblage, which could ultimately alter the functional capacities of these assemblages with unknown consequences for these ecosystems. This however, still has to be verified in natural settings.

Conclusion & outlook

A very strong influence of the surrounding environment including, its abiotic parameters, was identified to be the main drivers of biofilm assembly processes. As observed for both aquatic invertebrates – although belonging to different feeding guilds – the influence of the gut passage had very little lasting effect on the microplastic-associated assemblages for PS and PA. Rather, the colonisation of distinct bacteria, including *Vibrio*, occurred independently and was mediated by the presence of an available surface for colonisation and the right abiotic settings. Most probably, *Vibrio* acts as a general and early coloniser in biofilms in the Baltic Sea that is not dependant on other biofilm bacteria for colonisation success (Fig. E). The importance of microplastics as a dispersal vector for potential pathogenic vibrios should therefore be further investigated with regard to the temporal dynamics. This is why investigating microplastic-asscoiated biofilms is still of importance, since they have a very different distribution behaviour than macro-sized plastic debris. However, regarding the influence of this newly available habitat for aquatic ecosystems on the functional level, these investigations should also incorporate macro-sized plastic items, since a bigger surface area holds the potential to harbour a much greater richness of bacterial and eukaryotic species, as well as higher cell numbers of both, and thus also greater activity. Consequently, macroplastics can have a much bigger potential to influence or even alter ecosystem functions. This is especially important in oligotrophic systems,

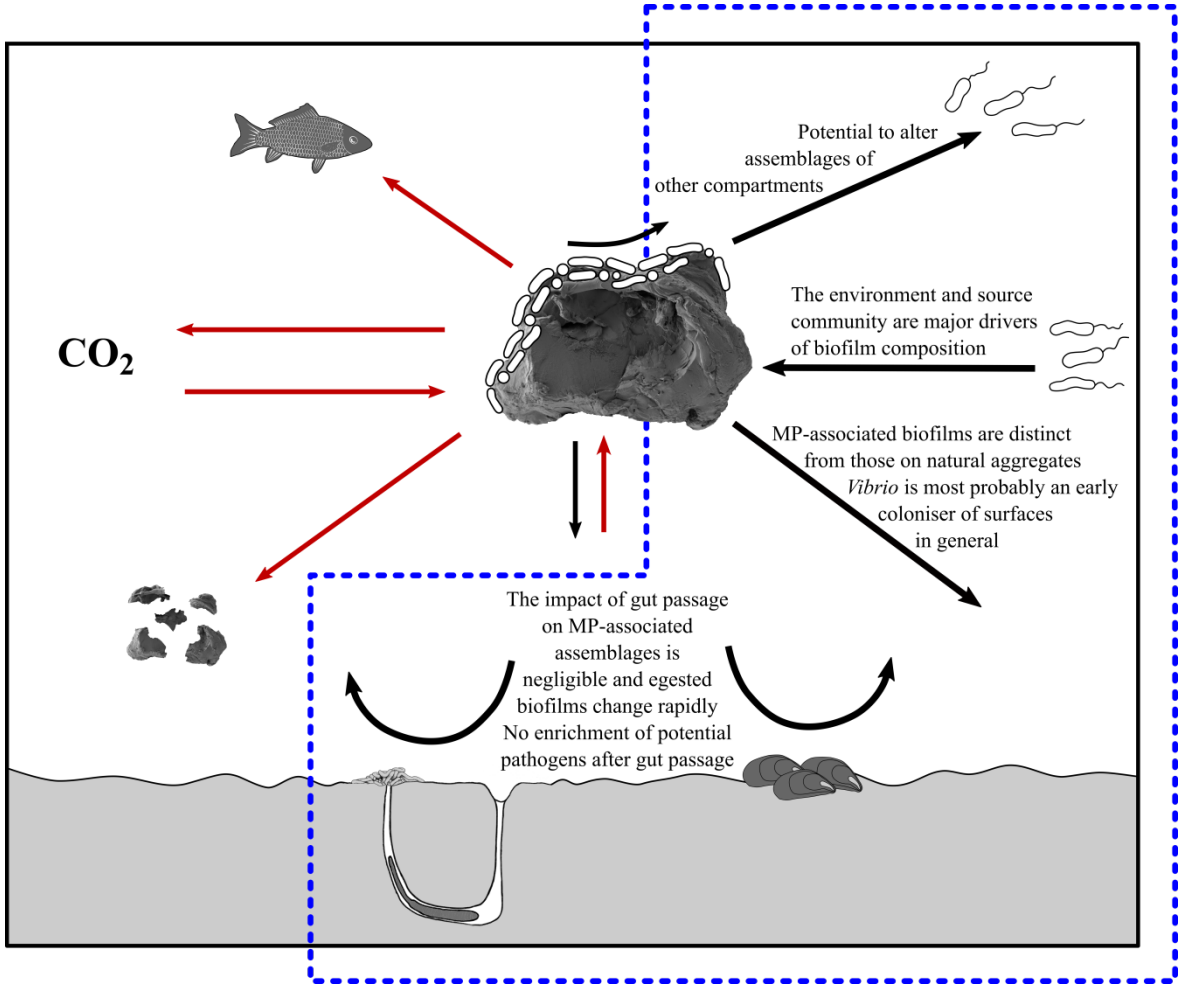

Figure E. Conceptual framework of the potential interactions of microplastic (MP)-associated assemblages in aquatic ecosystems. The various (black) and often hypothetical (red) influences of microplastic-associated biofilms on the particles themselves, the environment, and higher organisms are depicted with arrows. Results from this work are encapsulated in the broken blue box.

where nutrients are generally low and the addition of nutrient-scavenging surfaces might have profound effects on nutrient- and carbon turnover. A key aspect in assessing whether plastic-associated biofilms are truly able to significantly alter aquatic communities and ecosystem functioning is the amount of available surface area. Although reported data suggests a significant effect on pelagic heterotrophic activity, this is only a glimpse into the ramifications of the plastic-microbiome-ecosystem interactions. It is therefore of critical importance to obtain a comprehensive dataset on available surface area and its geographical, as well as vertical distribution. If not possible through direct observations, a reliable estimation of the available surface area calculated from existing data based on a

conversion factor should be attempted. This has to be accompanied by thorough functional analysis of the plastic-associated biofilms in different aquatic ecosystems, since a description based solely on 16S data is not sufficient. Likewise, production and turnover rates need to be determined, which can be included into modelling studies to fully assess the impact these biofilms may have on nutrient cycles in aquatic ecosystems. As is shown in the studies of this thesis, spatial and temporal dynamics, as well as disturbance events will play a role in the diversity of plastic-associated biofilms. While a lot of plastic waste is thought to be generated directly at sea by the global fishing and cargo fleet, these items, and also those resulting from land based sources, will travel within the aquatic ecosystem. Additionally to investigating succession and temporal dynamics of biofilms, it is necessary to include these investigations into a framework that takes into account the possible priority effects of initial colonisation by bacteria on subsequent colonisers. Although effects may be investigated thoroughly for a single colonisation event, the pressure of the changing environment has to be taken into account for plastic particles travelling down a river into the open sea. Together with the notion that biofilms provide shelter from environmental stressors, this is an interesting emerging research topic that also provides insights into more fundamental ecological questions of community assembly mechanisms.

Plastics as a possible nutritional source needs to be further investigated. Due to the great variety of plastic polymers, some materials may be more susceptible to degradation, because they have a greater structural similarity to abundant natural polymers, as PA to chitin. Thus, they could provide not only energy via bond-cleavage, but in addition be a source for nitrogen. This again would be especially important in oligotrophic environments. Obviously, these investigations need to include whether sorbed contaminants or leaking material are simply metabolised. Finally, the role of the conditioning film versus nutrient-limitation in surface specific attachment on diverse polymers needs to be addressed in specifically designed experiments. This would provide valuable information for modelling studies to be able to incorporate the environmental settings into possible predictions of the biofilm assemblage at a given situation.

Chapter I

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

The following chapter was published in the journal *Environmental Pollution* as

Katharina Kesy, Sonja Oberbeckmann, Felix Müller, and Matthias Labrenz (2016). Polystyrene influences bacterial assemblages in *Arenicola marina*-populated aquatic environments *in vitro*. *Environ. Pollut.* 219, 219–227. doi:10.1016/j.envpol.2016.10.032.

Declaration of author contributions:

Katharina Kesy conducted the experiment with the help of Sonja Oberbeckmann.

Katharina Kesy and Felix Müller performed laboratory work.

Katharina Kesy analysed the data.

Katharina Kesy, Sonja Oberbeckmann and Matthias Labrenz discussed the data.

Katharina Kesy drafted the manuscript, Matthias Labrenz critically commented on the manuscript and redrafted parts of it, Sonja Oberbeckmann critically commented on the manuscript.

The contribution of Katharina Kesy to the written manuscript was ~85%

Abstract

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 (PS) 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 PS 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 PS particles and reference glass beads became more similar, harbouring common sediment bacteria. By contrast, only in the presence of PS the potential symbiont *Amphritea atlantica* was enriched in the investigated biofilms, faeces, and water. Thus, especially in areas of high PS contamination, this polymer may impact the bacterial composition of different habitats, with as yet unknown consequences for the respective ecosystems.

1.1. Introduction

1.1.1. 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 R. C. 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 (Cooper and Corcoran, 2010; Andrady, 2011) 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 Cauwenberghe 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 R. C. 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.

1.1.2. 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. parahaemolyticus* in mussels (Lhafi 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. parahaemolyticus* on a PS 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/m^2 (Reise, 1985) and a potential sediment turnover rate of up to 80 cm^3 sediment/day have been reported (Cadeé, 1976). As the test polymer, polystyrene (PS) was chosen because it makes up ~7% of the European plastic demand (PlasticsEurope, 2015) and is widely processed into single-usage items. It is frequently detected in the marine environment (Carpenter et al., 1972; Claessens et al., 2011; Kirstein et al., 2016), due to its higher density (~1.05 g/cm^3) than seawater usually at the seafloor. Furthermore, the ingestion of PS by *A. marina* has been documented both experimentally (Besseling et al., 2013; Van Cauwenberghe et al., 2015) and in the field (Van Cauwenberghe et al., 2015).

1.2. Material & methods

1.2.1. 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.

1.2.2. 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. 1.1) 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.

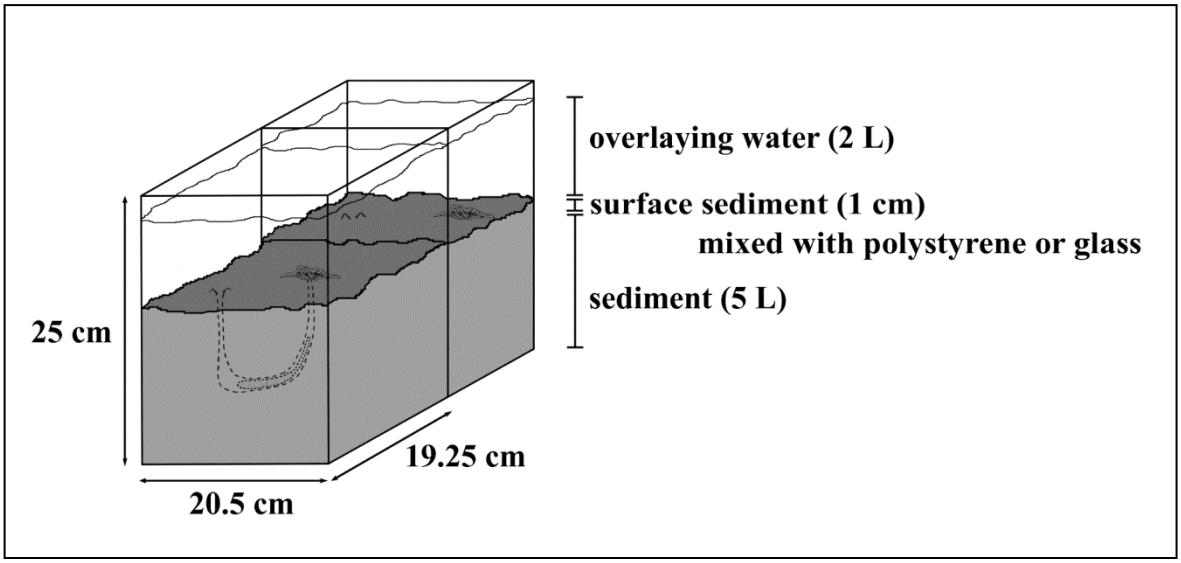

Figure 1.1. Scheme of the experimental set-up. Six independent experimental units were created by dividing aquaria with an acrylic glass insert into two distinct halves. Each unit was spiked with one *Arenicola marina* specimen. Polystyrene particles (250–400 µm) or glass beads (250–400 µm) were added to the first cm of sediment in three replicates each.

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, PS particles 250–400 µm in size (Goodfellow, UK) were added to three aquaria (PS treatment) and similarly sized glass beads (Oberflaechentechnik Seelmann, Germany) to the other three aquaria (reference treatment).

The major mineral content of the glass beads was SiO_2 ~72%, Na_2O ~14%, and CaO ~8%. The PS particles had a rough surface with many edges (Fig. S1.1a), whereas the glass beads were smooth (Fig. S1.1b). Both, the PS 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 PS 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 ± 36 g WW PS particles or 600 g WW glass beads were mixed into the first cm of sediment. The particle concentration was ~1 g WW PS and ~1.5 g WW glass per cm^3 of surface sediment, ensuring the same surface area in the two treatments. Fresh 30-µm-filtered seawater was then 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. 1.2): A 45-mL water sample from each setup 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 cm^3 of sediment and ~1 cm^3 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 PS particles or glass beads from the sediment or faeces. The filtered faeces were sub-sampled 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. The sediments, faeces and corresponding PS particles or glass beads frozen immediately after sampling represented the t_0 samples (Fig. 1.2). The remaining faeces and the corresponding PS 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 PS particles or glass beads were wrapped within a 30-µm gauze piece (faeces) or 200-µm gauze piece (PS or glass) and incubated in the respective aquaria (Fig. 1.2). These gauze pieces were retrieved after 24 h, rinsed with sterile seawater and stored at −80°C (t_{24} samples). Additional water samples were collected as described above.

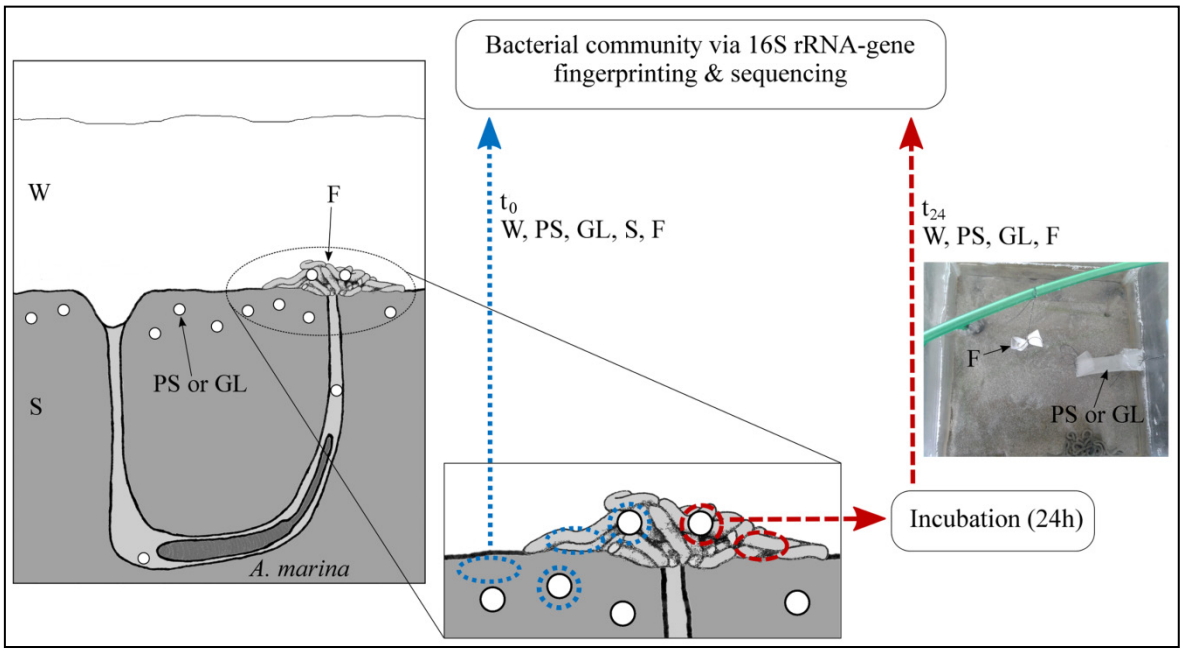

Figure 1.2. Schematic overview of the experimental procedure. Sediment (S), the corresponding polystyrene (PS) particles or glass (GL) beads and faeces (F) plus the corresponding polystyrene particles or glass beads after egestion (t_0, dotted lines) were sampled 4 days after the addition of the particles/beads to the aquaria. Faecal material and some of the particles/beads extracted from the faeces were then further incubated in seawater for 24 h (t_{24}, dashed lines). Additional water samples (W) were also collected.

1.2.3. Molecular analysis of bacterial assemblages

1.2.3.1. 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' CAGCAGCCGCGGTAATAC 3', and to nucleotides 907–925, with the sequence 5' CCGTCAATCCTTTGAGTTT 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 t_{24} samples and for the pre-incubated (before their addition to the aquaria) PS or glass samples and in duplicates for the t_0 and water samples.

1.2.3.2. 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 (Häne et al., 1993; Röling et al., 2001). This matrix was then used for non-metric multidimensional 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 S1.1 and S1.2). Statistical analyses were carried out using Primer6 and the add-on package PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK).

1.2.3.3. 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 ggplot2 package (Wickham, 2009) for R (R Core Team, 2015). The relative abundance plot of the t_{24} samples included only those OTUs exclusive to or enriched in those samples, with a relative abundance ≥5 × higher than in t_0 samples.

1.2.3.4. Quantitative PCR

To verify the relative abundance of the *Amphritea atlantica* OTU, quantitative PCR (qPCR) was conducted with the t_0 PS particles extracted from faeces (n = 3), the t_{24} PS samples (n = 2) and the t_{24} faeces samples (n = 5). *Amphritea*-specific primers (*E. coli* position 444–462, with the sequence 5' GTGAGGAAAGGTTGTAGC 3', and position 823–841, with the sequence 5' GTGTCCCAACGGCTAGTA 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).

1.3. Results

1.3.1. 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.

1.3.2. Substrate-specific bacterial assemblages in sediment and faeces at t_0

Before the ingestion by *A. marina* of PS particles and glass beads from the sediment, their respective bacterial assemblages differed significantly from those of the sediment itself (Table S1.1, Fig. 1.3). However, the most abundant 16S rRNA gene OTUs (relative abundances of 30–60%) obtained from the sediment and from the PS particles or glass

beads extracted from it were identical (Fig. 1.4). After gut passage, the differences in the bacterial16S rRNA gene fingerprint patterns between the faeces and the faecal PS particles and glass beads were no longer significant (Table S1.1). 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 S1.1). However, Pearson correlation of all the sediment and faecal fingerprint patterns still showed similarities >40% (Fig. 1.3).

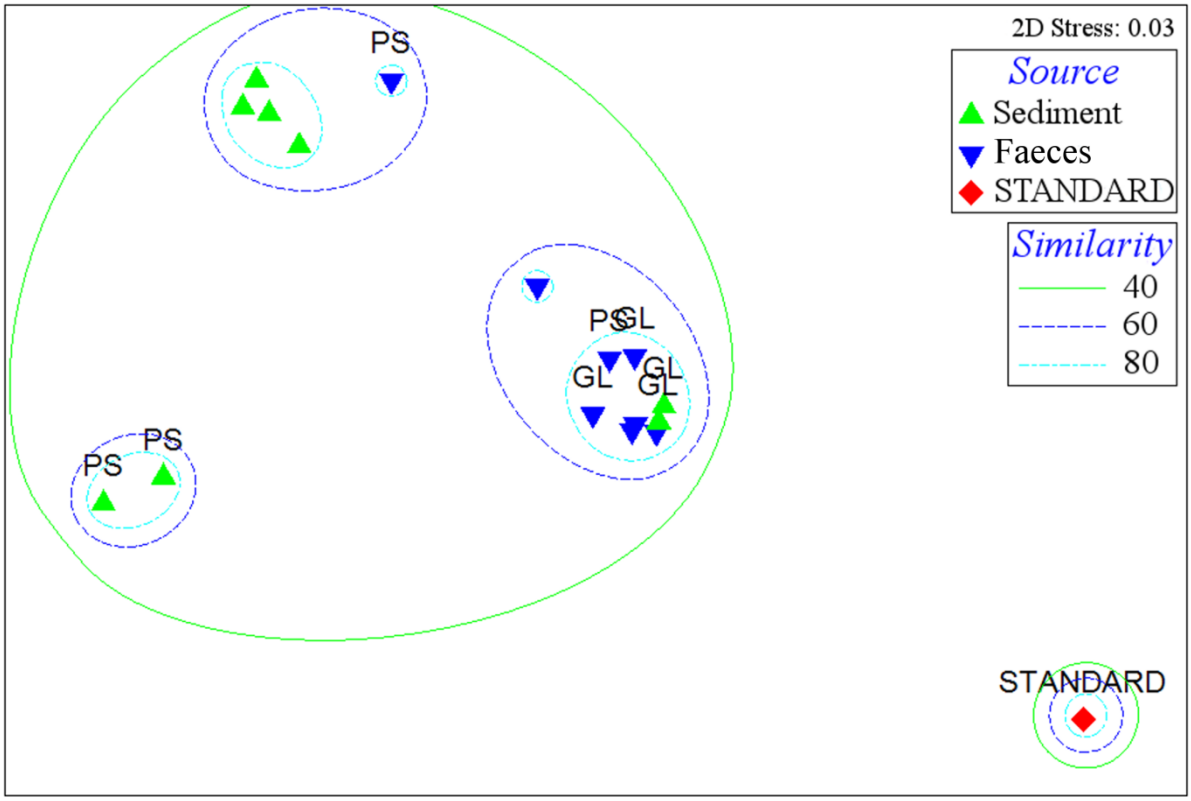

Figure 1.3. Non-metric multi-dimensional scaling plot based on Pearson correlation of the 16S rRNA gene SSCP fingerprint pattern generated from the analysed sources and substrates at time t_0. Sediment, faeces and the corresponding particles/beads were sampled 4 days after the addition of the latter to the aquaria. Symbols indicate 16S rRNA gene fingerprints of the sediment (green triangles) and faeces (blue inverted triangles) samples. PS and GL indicate the 16S rRNA gene fingerprints generated from the biofilms of the corresponding polystyrene particles and glass beads, respectively. The red trapezoid indicates the behaviour of the SSCP standards. Similarity values were derived from an accompanying cluster analysis (UPGMA).

All the sample types also shared most of the abundant bacterial OTUs (Fig. 1.4). 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 *Desulfatibacillum 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 PS particles from the sediment (Fig. 1.4). An OTU phylogenetically related to *Amphritea atlantica* (95% 16S rRNA gene similarity, NCBI BLAST) appeared solely on the PS particles from the sediment, on the t_0 PS particles from the faeces, and in the water samples of the PS treatment (Fig. 1.4). Overall, the OTUs of the water samples were clearly distinct from those of both the sediment and the faecal samples (Fig. 1.4).

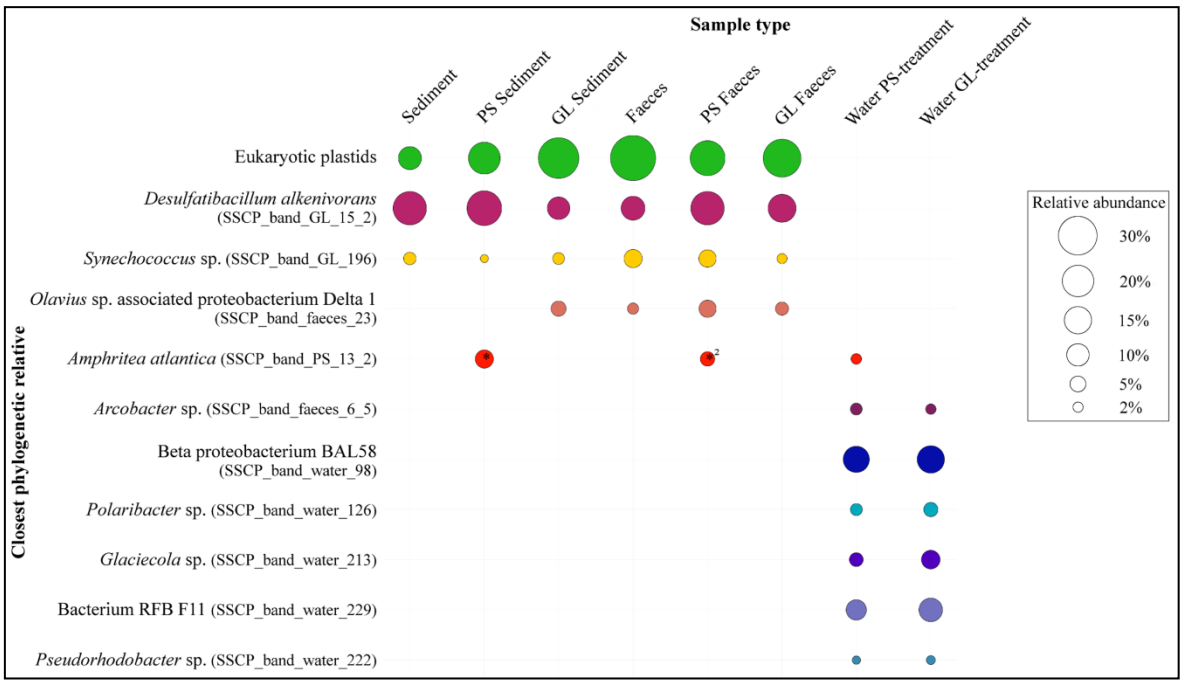

Figure 1.4. Relative abundance (%) data derived from 16S rRNA gene SSCP fingerprints for the most abundant OTUs (BLAST results) from sediment, faeces and biofilms of the corresponding polystyrene particles (PS) and glass beads (GL) sampled 4 days after their addition to the aquaria. The phylogenetic affiliations of the prokaryotic OTUs are given in Figure S1.2. *Obtained sequences with 3.5% ambiguities. *[2]qPCR was negative for the t_0 polystyrene biofilm samples from the faeces.

1.3.3. Substrate-specific bacterial assemblages at t_{24}

The composition of the biofilms on the PS particles and glass beads extracted from the faeces changed significantly within 24 h (Table S1.2), whereas the bacterial assemblage in the t_{24} faeces samples remained 60% similar to those of the t_0 samples (Fig. 1.5). As found in the t_0 samples, an OTU enriched exclusively in the PS 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 PS particles, in all independent triplicates. These relative abundances were confirmed by qPCR with 18 ± 3% (Fig. 1.6). *A. atlantica* was also detected in the t_{24} faecal samples of the PS treatment, with relative abundances of 2.8 ± 0.3%

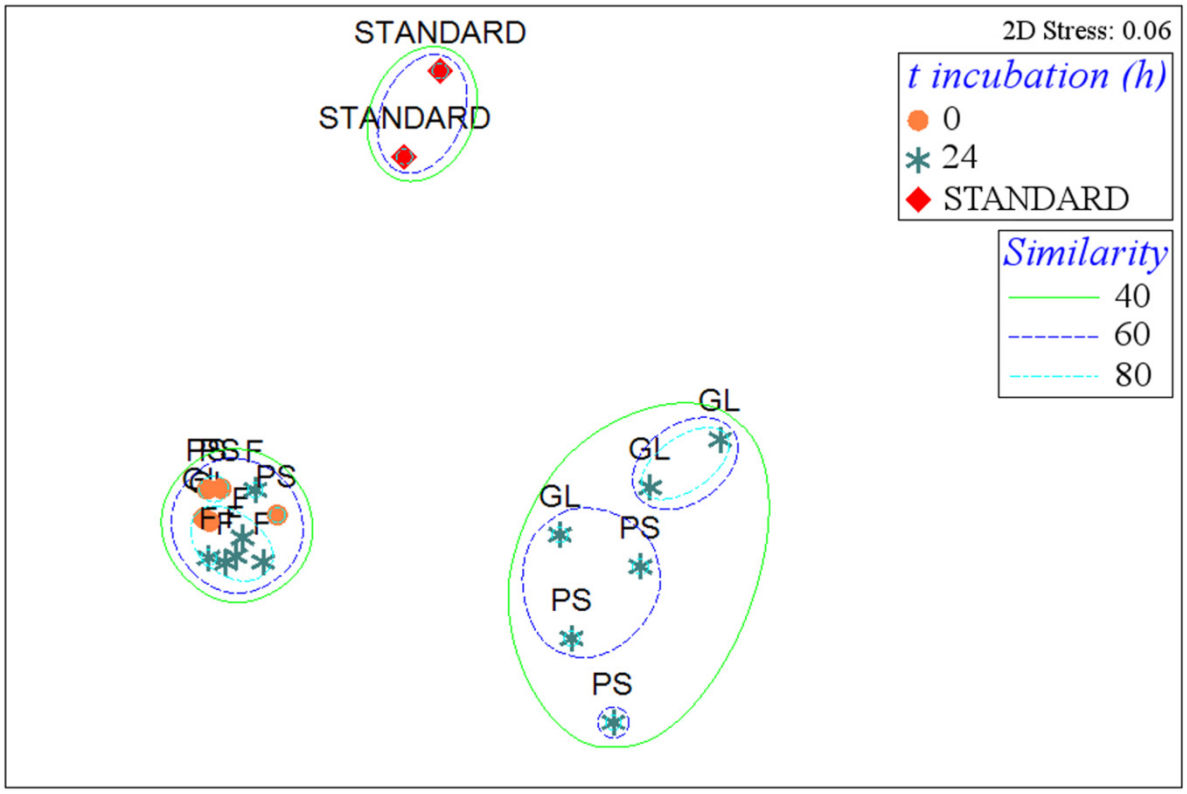

Figure 1.5. Non-metric multi-dimensional scaling plot based on Pearson correlation of the 16S rRNA gene SSCP fingerprint patterns generated from faeces and the substrates extracted from faeces before (t_0, orange dots) and after (t_{24}, dark green asterisk) a 24-h incubation. Analysed material: Faeces (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).

and 5.4 ± 2.5% as determined by SSCP and qPCR, respectively, and was enriched in the water samples from the PS treatment (19 ± 3% according to SSCP, Fig. 1.6). Other OTUs affiliated with symbiotic bacteria were also identified but they were not exclusive to the PS samples (Fig. 1.6). An OTU related to an unassigned *Arcobacter* sp. was present in all of the t_{24} samples, except in the t_{24} faeces of the glass treatment (Fig. 1.6). An OTU affiliated with *Lentisphaera marina* was enriched in the t_{24} PS 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.

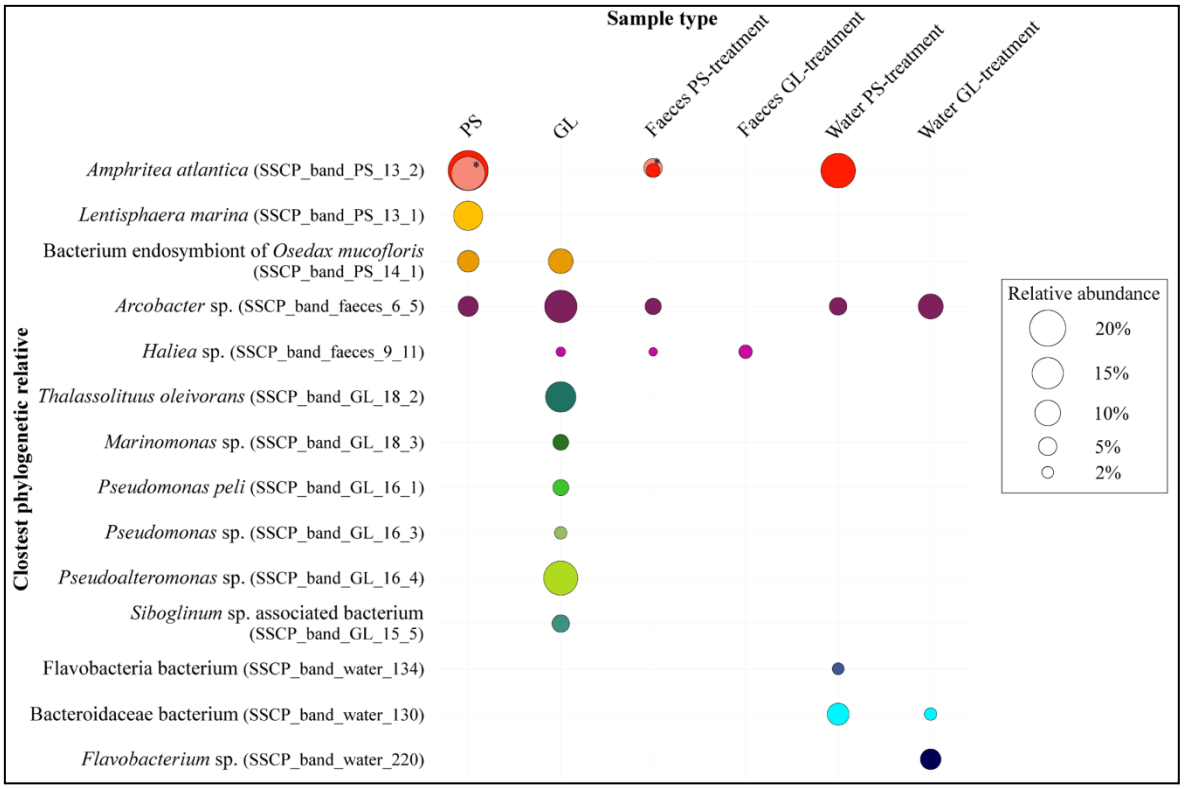

Figure 1.6. Relative abundance data (%) derived from the 16S rRNA gene SSCP fingerprints of OTUs (BLAST results) found exclusively or enriched by at least 5-fold in the 24-h incubated polystyrene particles (PS) and glass beads (GL) extracted from faeces, the corresponding 24-h incubated faecal samples and the incubation water. Phylogenetic affiliations are given in Figure S1.2. *qPCR results.

1.4. 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 PS particles and glass beads became more similar. However, the identification of a PS-specific OTU suggested that PS 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 PS treatments.

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

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

The biofilm assemblages on the PS 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 PS, glass and faeces were more similar (Fig. 1.3). 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. 1.4). Thus, the differences between faeces, PS and glass extracted from the faeces, the sediment, as well as PS 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 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 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 PS, glass or the corresponding faeces.

1.4.1.2. Low vector potential of PS 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 PS particles would become enriched in gut microflora, including potential human pathogens, we could not identify any pathogen specifically enriched on PS. An OTU affiliated with the genus *Arcobacter* was not restricted to the PS samples but was also found on the t_{24} glass particles, in the t_{24} faeces and in the water samples (Fig. 1.6). 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 PS 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 (Goñi-Urriza et al., 1999; Volkenborn et al., 2007). However, in our study *A. marina* feeding on PS particles did not result in the significant enrichment of uncommon sediment bacteria or of pathogens in microplastic-associated biofilms.

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

The most abundant OTUs in the sediment were also those on the PS particles and glass beads from the sediment. However, the fingerprint patterns of the whole biofilm assemblages on PS and glass from the sediment and those of the bulk sediment differed significantly (Fig. 1.3). 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 PS extracted from the sediment after 4 days (Fig. 1.3). An OTU affiliated with *A. atlantica* occurred only on the PS biofilm samples (Fig. 1.4), which suggested a role for substrate specificity in the development of bacterial biofilms on PS. Yet, the most abundant OTUs found in all sediment samples, all faeces samples and on the corresponding PS and glass biofilms were the same (Fig. 1.4). 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.

1.4.2. Substrate-specific enrichment of potentially symbiotic bacteria on PS

1.4.2.1. *Amphritea* sp. enrichment on PS occurs independently of *A. marina*

After passage through the digestive tract of *A. marina*, the worm's faeces and the PS 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 PS 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} PS and glass biofilm samples were still 40%, but the phylogenetic affiliations of their most abundant OTUs differed (Fig. 1.6). Thus, after 24 h the glass biofilm was dominated by members of the genera *Pseudomonas*, *Pseudoalteromonas* and *Thalassolituus*, whereas on PS an OTU closely affiliated with *A. atlantica* became highly enriched (Fig. 1.6).This gammaproteobacterium was first isolated in association with a *Bathymodiolus* clam inhabiting a hydrothermal vent system (Gärtner 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 PS incubated in seawater before its addition to the aquaria containing *A. marina* (Fig. S1.2). We also found an OTU affiliated with *A. atlantica* on PS extracted from sediment, before passage of the particles through the worm's digestive tract. Thus, *A. marina* probably did not inoculate the PS 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 PS potentially represents a substrate analogue for *Amphritea* sp. Uncultured bacteria isolated from the bone-eating worm *Osedax* 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 PS 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 PS 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 PS 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.

1.4.2.2. Potential impact of PS on microbial assemblages in pelagic systems

Amphritea sp. was exclusively enriched on PS and in the PS treatments, most noteworthy, it was also enriched in the t_{24} water samples of the PS treatment (Fig. 1.6). While particle incorporation during water sampling cannot be ruled out completely, the presence of a few PS particles would not explain the high relative abundance (~20%) of *Amphritea* sp. detected in the water samples. Besides demonstrating the direct impact of PS 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/m^3 has been determined (Song et al., 2014) – to clarify the influence of plastic-litter on pelagic assemblages and therefore on the pelagic food web (Fuhrman and Steele, 2008). Very recent experiments using European perch (*Perca fluviatilis*) larvae demonstrated that their performance and development are reduced significantly at a PS concentration of 10,000 particles/m^3 (Lönnstedt and Eklöv, 2016). Although this observation was attributed to the physico-chemical impact of PS 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.

Chapter II

Fate and stability of polyamide-associated bacterial assemblages after their passage through the digestive tract of the blue mussel *Mytilus edulis*

The following chapter was published in the journal *Marine Pollution Bulletin* as

Katharina Kesy, Alexander Hentzsch, Franziska Klaeger, Sonja Oberbeckmann, Stephanie Mothes, and Matthias Labrenz (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. doi:10.1016/j.marpolbul.2017.08.016.

Declaration of author contributions:

Alexander Hentzsch and Sonja Oberbeckmann conducted the experiment.

Franziska Klaeger and Stephanie Mothes performed laboratory work.

Katharina Kesy analysed the data.

Katharina Kesy, Sonja Oberbeckmann, and Matthias Labrenz discussed the data.

Katharina Kesy drafted the manuscript, Matthias Labrenz critically commented on the manuscript and redrafted parts of it, Sonja Oberbeckmann critically commented on the manuscript.

The contribution of Katharina Kesy to the written manuscript was ~80%.

Abstract

This study examined whether bacterial assemblages inhabiting the synthetic polymer polyamide are selectively modified during their passage through the gut of the blue mussel *Mytilus edulis* in comparison to the biopolymer chitin with focus on potential pathogens. Specifically, we asked whether bacterial biofilms remained stable over a prolonged period of time and whether polyamide could thus serve as a vector for potential pathogenic bacteria. Bacterial diversity and identity were analysed by 16S rRNA gene fingerprints and the sequencing of abundant bands. The experiments revealed that egested particles were rapidly colonised by bacteria from the environment, but the taxonomic composition of the biofilms on polyamide and chitin did not differ. No potential pathogens could be detected exclusively on polyamide. However, after 7 days of incubation of the biofilms in seawater, the species richness of the polyamide assemblage was lower than that of the chitin assemblage, with yet unknown impacts on the functioning of the biofilm community.

2.1. Introduction

Plastic pollution of the global oceans has been of concern to scientists and environmentalists for over 50 years. Since the first recognition of the presence of small plastic particles in the Sargasso Sea (Carpenter and Smith, 1972), reports on so-called microplastics have increased, especially in the last decade (Law and Thompson, 2014). Mostly, microplastics refer to particles <5 mm in diameter (Arthur et al., 2009). Their small size makes them available to lower trophic levels, with the potential for bioaccumulation (Setälä et al., 2014; Watts et al., 2014) and representing a multitude of threats to the marine environment (Wright et al., 2013a). For instance, the ingestion of microplastics was shown to reduce the fitness of the marine polychaete *Arenicola marina* (Wright et al., 2013b) and to induce an inflammatory response in the tissue of the blue mussel *Mytilus edulis* (Browne et al., 2008). Moreover, microplastics can absorb persistent organic pollutants (Mato et al., 2001), which may then be transferred to marine organisms, following particle ingestion (Browne et al., 2013).

Although the direct effects of plastics and microplastics ingestion on marine organisms have been extensively studied, the ability of plastics to provide a persistent habitat for surface-attached aquatic microorganisms has come into focus only recently. Microorganisms play key roles in all biogeochemical cycles and in ecosystem functioning (Azam et al., 1983), including the degradation of natural particles (Smith et al., 1992). While microplastics usually resist microbial degradation, they can serve as substrates for biofilm formation. Biofilms provide microbes with the advantages of versatile metabolic cooperation, enhanced horizontal gene transfer and protection from environmental stressors such as antibiotics (Davey and O'Toole, 2000). Bacterial assemblages on plastics sampled from marine waters comprise a large variety of colonising organisms. Among the bacteria that have been identified on the biofilms that form on plastics are those belonging to the phyla *Bacteriodetes*, *Proteobacteria*, *Cyanobacteria*, and *Verrucomicrobia* but also eukaryotic organisms belonging to the *Bacillariophyceae* and *Phaeophyceae* have been identified (Zettler et al., 2013; Oberbeckmann et al., 2014; Oberbeckmann et al., 2016). Moreover, members of the *Chryomorphaceae*, *Alcanivoraceae* and *Oceanospirillaceae* were significantly more abundant on plastic than on glass surfaces and in some cases were exclusively found on the plastics (Kesy et al., 2016; Oberbeckmann et al., 2016).

2.1.1. Impact of *M. edulis* on biofilm formation on microplastics

Many opportunistic pathogens are able to form biofilms, which then become reservoirs of potential pathogenic bacteria (Lyons et al., 2010). Several studies have described the attachment of *Vibrio* spp. onto microplastics in the water and in sediments (Zettler et al., 2013; De Tender et al., 2015; Foulon et al., 2016; Kirstein et al., 2016). Zettler et al. (2013) reported an abundance of ~25% of 16S rRNA gene reads of an unassigned *Vibrio* sp. on one polypropylene particle sampled from the Sargasso Sea. Kirstein et al. (2016) identified *V. parahaemolyticus* on microplastics sampled in the North and Baltic Sea, which is a potential human pathogen that causes gastroenteritis but also wound infections (Drake et al., 2007). Why microplastics could become enriched with potential pathogens and thus may serve as vectors for these is unclear. In the case of *Vibrio* spp., many are naturally widespread in marine or brackish environments, such as the Baltic Sea (Gomez-Gil et al., 2014), and they often occur in association with aquatic invertebrates, especially bivalves (Prieur et al., 1990); thus, one possibility would be that these invertebrates influence or even stimulate the development of potentially pathogenic biofilms. Although up to 98% of the bacteria ingested by bivalves may be digested (Cabello et al., 2005) not all bacteria are equally susceptible to lysis by gut lysozyme (McHenery and Birkbeck, 1982) and a smaller fraction is able to survive the gut passage of marine bivalves (Rowse and Fleet, 1982; Barillé and Cognie, 2000; Cabello et al., 2005). It has been shown that a *Vibrio* strain could even form viable microcolonies inside the digestive tract of *M. edulis* (Prieur, 1981) and that *V. vulnificus* could accumulate within the digestive tract compared to other bivalve tissue (Tamplin and Capers, 1992). This leads to the hypothesis that gut passage may alter the bacterial assemblage on the particles by favouring a selective survival (Birkbeck and McHenery, 1982) of biofilm members towards potentially pathogenic taxa, e.g. *Vibrio*.

In this context the widespread benthic filter feeder *M. edulis* could play a vital role. *M. edulis* is of high socio-economic importance, with a global *M. edulis* aquaculture production of ~185,000 tonnes in 2014 (FAO, 2017). As a resident of marine coastal ecosystems, it regularly encounters microplastics, as recently demonstrated for 22 sites along the 12,400 miles of coastline in China (Li et al., 2016). In aquaculture, the cultivation of mussels on plastic ropes could lead to their enhanced intake of microplastics (Mathalon and Hill, 2014). The effects of microplastics ingestion by *M. edulis* have been extensively studied and the ability of the mussel to ingest microplastics present in the water

column has been demonstrated in the field and in laboratory experiments (Browne et al., 2008; Van Cauwenberghe et al., 2015). *M. edulis* plays an important role in the cycling of nutrients and particulate matter (Kautsky and Evans, 1987), but has also been shown to be associated with potentially pathogenic bacteria, especially *Vibrio* spp. (Lhafi and Kühne, 2007).

In this study, we examined whether the passage of microplastics through the digestive tract of *M. edulis* could lead to a distinct and permanent bacterial biofilm assemblage and could catalyse the establishment of potentially pathogenic biofilms on microplastics. Via the dispersal of the contaminated microplastics into the environment these particles would pose a threat to aquaculture and potentially to human health because microplastics have the potential to persist much longer in the environment. These potential consequences could be amplified, as with ongoing plans for expanding mussel farming numbers of blue mussels will increase. To test our hypothesis, *M. edulis* was fed with polyamide, a polymer typically found in garments and fishing gear and a common pollutant of the marine environment (Andrady, 2011; Browne et al., 2011). Following egestion of the polyamide particles, the bacterial assemblages of the associated biofilms were investigated and compared to those of biofilms on naturally occurring particles and the biopolymer chitin.

2.2. Material & methods

2.2.1. Experimental set-up and sampling procedure

The *M. edulis* specimens used in the experiment were collected at the Marine Science Centre Rostock (Germany), cleaned, and kept at 10°C until their use in the experiments. Water for the experiments had a salinity of 11 PSU and was collected at the west mole of Warnemuende, Germany.

The optimal particle-size fraction and incubation time were determined in preliminary experiments (Hentzsch, 2013). The largest amount of particles in the faeces was obtained from mussels fed particles 30–75 µm in diameter. Particle abundance was significantly lower in the faeces than in the pseudofaeces during the first hours after feeding (repeated measures MANOVA, $p = 0.018$ for the factor "where"; amount of particles for the size fraction ≤75 µm found at t_0 in faeces and pseudofaeces were 0 ± 0 and 111.8 ± 92.9, respectively, at t_{2h} 0 ± 0 and 66.7 ± 70.2, and at t_{24h} 4.3 ± 5.2 and 4 ± 6.2, n = 6).

The experimental set-up consisted of test beakers filled with 150 mL of 30-µm filtered seawater and containing 5 mussels each. The feeding trials included a polyamide treatment, a chitin treatment as a reference and an additional control treatment in which the mussels were fed only with 30-µm filtered seawater. The polyamide and chitin particles used in the feeding trials were pre-incubated in 30-µm filtered, aerated seawater for 7 days. Each treatment was carried out in triplicate. Each mussel in the test beakers was fed separately, by introducing 3 mL of a seawater-particle suspension (0.5 g/L) directly above the mussel's siphon via a syringe. The faeces of the mussels were collected with sterile tweezers 12 h and 24 h later to account for possible effects of gut residence time on the biofilm community. All faeces from one beaker were pooled and homogenised in sterile seawater by pipetting the suspension up and down several times. This suspension was then split and the two resulting subsamples were filtered through an autoclaved 30-µm gauze piece to retain the polyamide and chitin particles. The low-level contamination of the gauze with other organic material was accounted for in the control treatment which contained the natural occurring particles. The filtrate, containing the faecal matter, was centrifuged for 20 min at ~17,400 rcf (Kryachko et al., 2012). The overlaying water was discarded and the pelleted faeces were frozen at −80°C for later molecular analysis. For each pair of subsamples, one of the gauze pieces was directly frozen at −80°C for later analysis and the other was used to investigate the stability of the polyamide- and chitin-associated biofilms over a period of time to mimic dispersion of the particles. For this, the filtered polyamide and chitin particles were resuspended from the gauze piece in 150 mL of 30-µm filtered seawater and incubated at 10°C and constant aeration for 1 day (t_1) or 7 days (t_7) (Fig. 2.1). For the 1-day incubation, the 12-h samples, and for the 7-day incubation the 24-h samples served as the corresponding t_0 source material. Because of limited egested material, one incubation was done from one deposition time. At the end of the incubation, the incubation water was filtered over a 30-µm gauze piece to retain the polyamide and chitin particles. A 45-mL water sample was collected from the filtered incubation water and handled as described for the faeces. The polyamide or chitin particles retained on the gauze were rinsed with sterile seawater and the gauze piece was frozen at −80°C. The rinse water was also collected and subjected to molecular analysis, but its bacterial assemblage did not differ from that of the incubation water. The rinse-water samples were therefore omitted from further analyses. In addition, the crude polyamide and

chitin particles were also analysed and organisms found on the crude material were excluded in the later analyses.

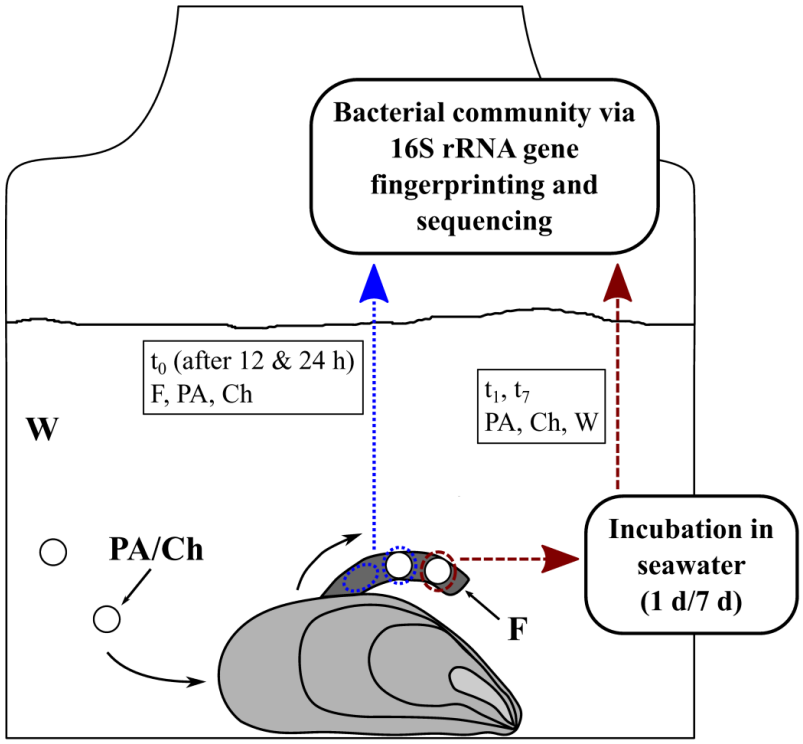

Figure 2.1. Experimental set-up and sampling procedure. Mussels were fed with a suspension of polyamide (PA) or chitin (Ch) and seawater or only with 30-µm filtered seawater (control, not shown). The particles together with the faeces (F), were collected 12 h and 24 h after feeding (t_0). Particles were isolated from the faeces via filtration.The bacterial communities of the polyamide and chitin particles and the faeces were analysed via 16S rRNA gene SSCP fingerprinting. Particles obtained from the faeces 12 h after feeding were incubated in seawater (W) for 1 day (t_1) while those obtained from faeces collected 24 h after feeding were incubated for 7 days (t_7) in seawater. The respective bacterial communities were analysed. Additional samples of the incubation water were also analysed.

2.2.2. Molecular analysis

2.2.2.1. DNA extraction and polymerase chain reaction (PCR)

DNA was extracted using a neutral phenol-chloroform extraction method (Weinbauer et al., 2002), with modifications as described in Kesy et al. (2016 and the supplement). DNA from the particle samples was extracted together with the sterile gauze because the particles were too small to separate them from the material. Bacterial DNA was amplified

using modified bacterial com-primers (Schwieger and Tebbe, 1998).The forward primer had the sequence 5' CAGCAGCCGCGGTAATAC 3' and the hybridisation position 519–536 on the *Escherichia coli* 16S rRNA gene. The sequence and hybridisation position of the reverse primer were 5' CCGTCAATCCTTTGAGTTT 3' and 907–925, respectively. Five ng of DNA was used as the template for amplification. Thermocycling (FlexCycler, analytik jena) consisted of an initial denaturation step at 94°C (1 min), followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and elongation (1.5 min at 72°C) plus a final elongation step at 72°C for 4 min. Bacterial DNA of the pre-incubated polyamide particles was difficult to amplify and no PCR product could be obtained via standard thermocycling.

2.2.2.2. 16S rRNA gene fingerprinting and diversity estimates

Single strand conformation polymorphism (SSCP) gel-electrophoresis was carried out after Dohrmann and Tebbe (2004), with modifications as described in Kesy et al. (2016). At least duplicate samples were analysed. This fingerprint method results in a specific band pattern for each sample, in which each band is considered to represent a distinct bacterial operational taxonomic unit (OTU). The SSCP-gels were digitalised and analysed using the GelCompar II software (Applied Maths), as described in Stolle et al. (2011). A similarity matrix was calculated using the Pearson correlation coefficient, which compares the densitometric profiles of the samples (Häne et al., 1993). This similarity matrix was further applied to non-metric multidimensional scaling (nMDS) using the vegan-package (Oksanen et al., 2016) in the R program (R Core Team, 2015). To test whether the samples from the different conditions differed significantly from each other with respect to their bacterial assemblages, the data were subjected to a pairwise PERMANOVA (Anderson, 2001) and an additional PERMDISP (Anderson, 2006) using the Primer 6 software with the add-on package PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK). PERMANOVAs were run only with samples from the same SSCP-gel. All p-values were based on Monte Carlo permutations. The results of the PERMDISP-tests were not significant and were therefore omitted from further consideration. OTU richness was defined as the number of bands occurring in a sample. To calculate the richness of each sample type, the band positions were obtained using the GelCompar software and identical bands between samples were assigned manually. OTU richness based on the number of bands was then calculated with the Chao2 estimator (Chao, 1987) using the iNEXT-function in the

correspondent R-package (Hsieh et al., 2016). To determine whether the estimated OTU richness differed significantly in the specific sample types collected at 12 h and at 24 h, two-sample t-tests with a Welsh correction were performed using the 't.sum.test' function of the BSDA package in R (Arnholt, 2012).

2.2.2.3. Phylogenetic 16S rRNA gene analysis and relative abundances

To identify abundant OTUs as well as OTUs exclusive to a given sample type, the corresponding bands were excised and reamplified following the protocol of Dohrmann and Tebbe (2004), with modifications as described in Kesy et al. (2016). The PCR products were sent for Sanger-sequencing to LGC Genomics (Berlin, Germany). The obtained sequences were assembled and quality checked using the Seqman program (DNAStar) and screened for chimeras using the DECIPHER Find chimeras web tool (Wright et al., 2012). For all further analyses, only sequences with <2% ambiguous base calls were considered (Quast et al., 2013). Those sequences were identified to the closest phylogenetic relative by comparing them with sequences in the GenBank database (National Center for Biotechnology Information, USA) using the basic local alignment search tool (BLAST) and the MEGABLAST algorithm (Zhang et al., 2000). Database entries of uncultured organisms were excluded from the search. Band identities were assigned manually and the relative abundances of these bands determined from the digitalised gels using GelCompar. If the BLAST results gave the same bacterial taxa but different isolated strains for bands occurring at the same position in the gel, the sequence similarities from the bands were compared using the ARB program (Ludwig et al., 2004). Bands with sequence similarities >98% were considered to belong to the same OTU. These OTUs were renamed after the closest phylogenetic affiliation based on the BLAST results and the alphanumeric first band number. To plot the relative abundances, averages from the replicate samples were calculated and visualised using the ggplot2 package for R (Wickham, 2009). Only those OTUs that occurred in at least two replicates were plotted. Sequences obtained in this study were deposited at GenBank under the accession numbers KY624507–KY624570.

2.3. Results

2.3.1. Diversity and composition of the bacterial assemblages after passage through the digestive tract

The 16S rRNA gene fingerprint patterns of the biofilms on the egested particles were compared to determine whether gut passage affected the polyamide biofilms differently than chitin or natural particle biofilms. No significant difference among biofilms on the polyamide, chitin and natural particles were identified either in the 12-h or the 24-h samples (p = 0.46–0.08). There was also no significant change in the number of bacterial taxa in each particle-associated biofilm (p = 0.41–0.9), but there was a decreasing trend in OTU richness for the polyamide and chitin biofilms (Fig. 2.2).

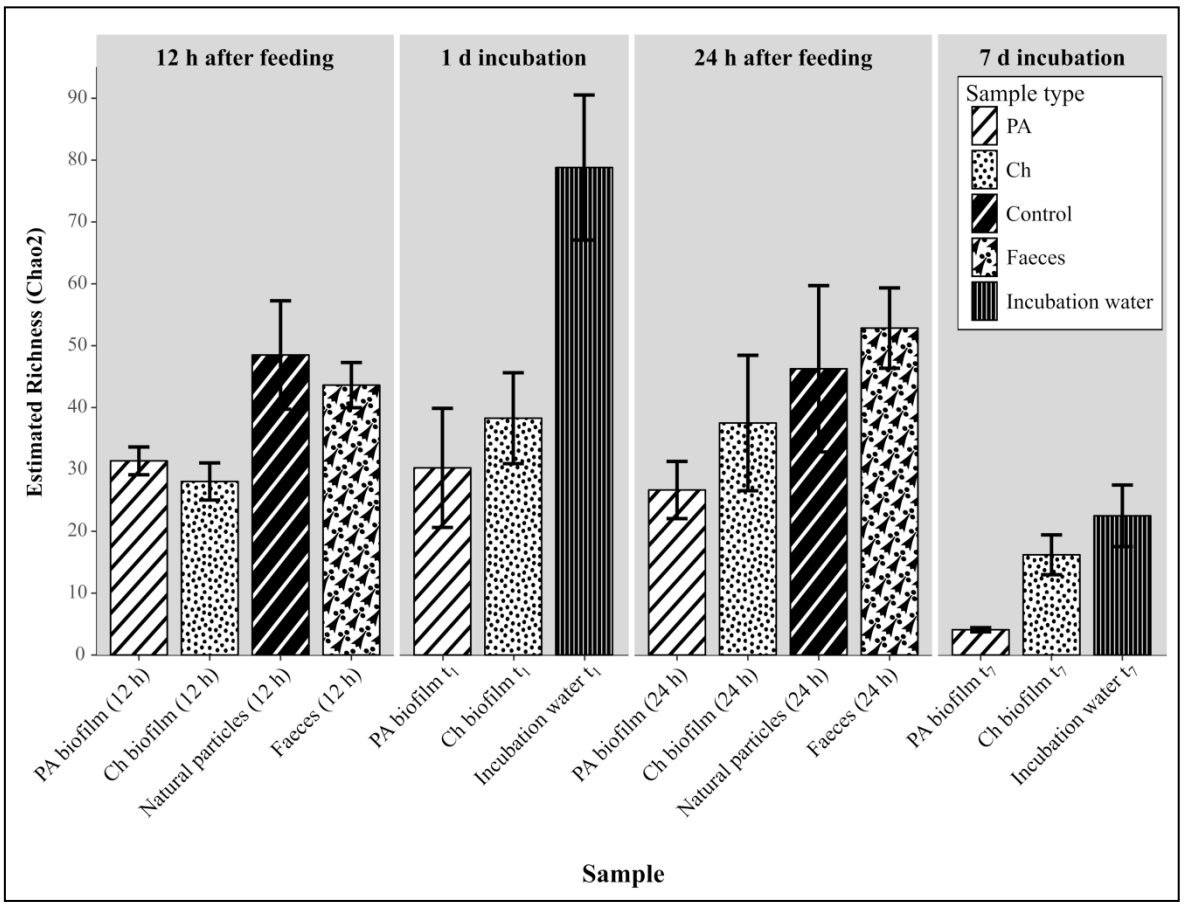

Figure 2.2. Bar chart of the estimated OTU richness (Chao2 index) and estimated standard error based on the number of bands in the 16S fingerprint of the different obtained samples. Polyamide (PA), chitin (Ch) and the natural particles from the control treatment were isolated from faecal material (Faeces) 12 and 24 h after the mussels were fed. The polyamide and chitin samples were then further incubated for 1 and 7 days (t_1 and t_7) and their OTU richness then estimated. Richness was also estimated for the incubation water of the incubated samples.

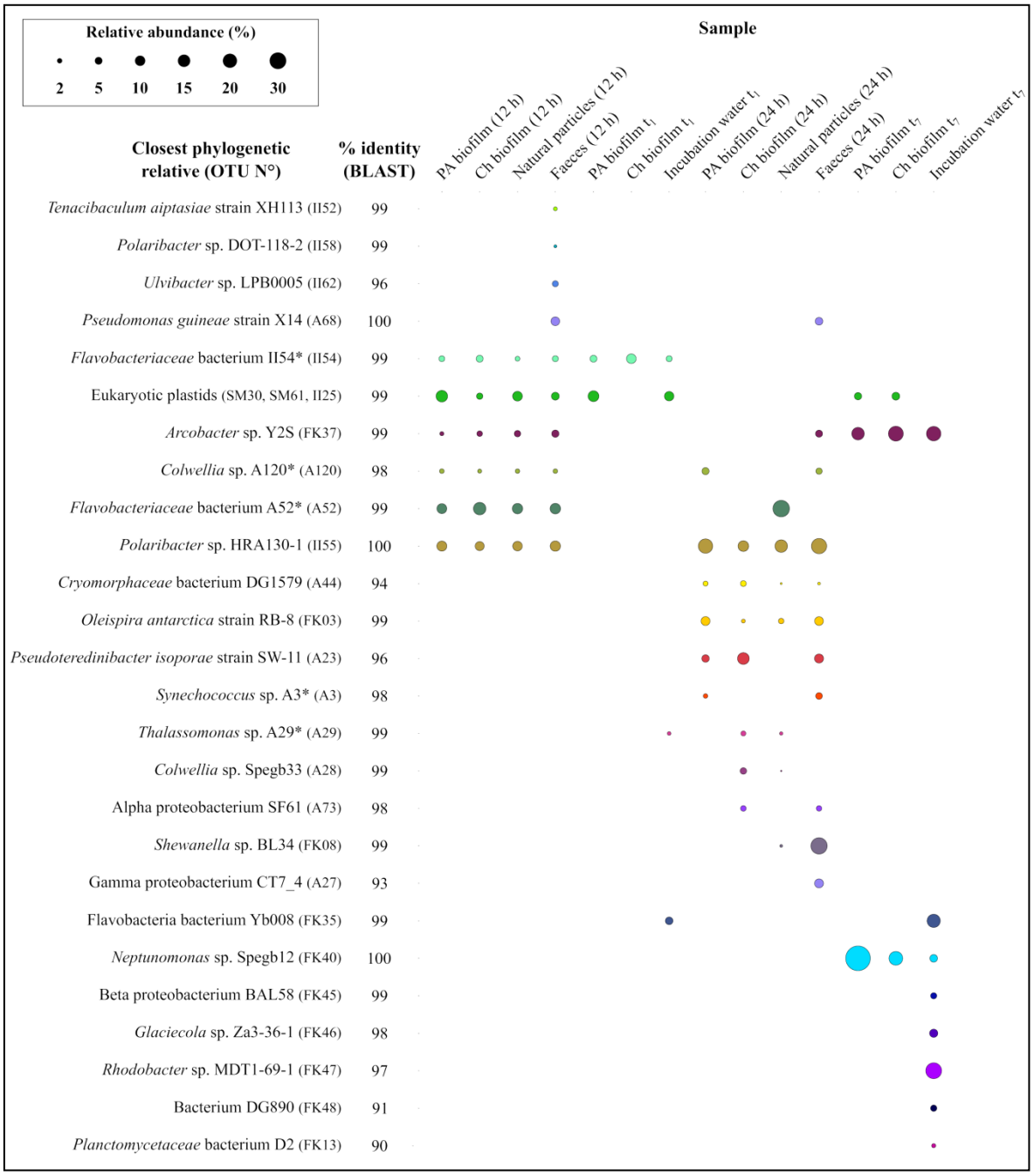

Figure 2.3. Relative abundance (%) data derived from 16S rRNA gene SSCP fingerprints for the most abundant OTUs (BLAST results) in the polyamide biofilm (PA biofilm), the chitin biofilm (Ch biofilm), on the natural particles from the control treatment (isolated from the faeces), on faeces collected 12 and 24 h after feeding and on polyamide and chitin particles subsequently incubated for 1 (t_1) and 7 (t_7) days in seawater. The relative abundances (%) of OTUs detected in the corresponding incubation water are also shown. *OTUs combined and renamed based on >98% sequence similarity (see material & methods).

Among the most abundant OTUs in our samples, there was a shift from *Flavobacteria* in the 12-h samples towards *Gammaproteobacteria* in the 24-h samples (Fig. 2.3). Within the 12-h samples, the bacterial OTUs in the polyamide, chitin and natural-particle biofilms were identical and still highly similar to those in the faeces. In addition to OTUs A52, II52, II54, II55, II58 and II62, assigned to *Flavobacteria*, plastidial DNA related to eukaryotes was also abundant. A close relative of *Arcobacter* sp. was also detected in all the 12-h samples, a genus which contains potential pathogens (Fig. 2.3). Comparably to 12 h, the most abundant OTUs in the 24-h samples were usually present in all the 24-h sample types. Besides close relatives of *Gammaproteobacteria*, OTUs affiliated with *Alphaproteobacteria*, *Bacteroidetes* and *Cyanobacteria* were detected, but these occurred in no more than two sample types. In general, the highest bacterial diversity tended to be in the faeces (Figs. 2.2 & 2.3).

2.3.2. Stability of the microbial assemblages after particle egestion and subsequent incubation in seawater

After the egestion and subsequent incubation of the polyamide and chitin particles in seawater, the bacterial assemblages changed over time (Figs. 2.3 & 2.4), with a decreasing trend in OTU richness after 7 days of incubation. This was most pronounced in the polyamide treatment, in which OTU richness decreased from 27 OTUs at t_0 to 4 OTUs at t_7 (Fig. 2.2). OTU richness on the particles did not decrease after 1 day of incubation in seawater, but only two OTUs, shared by the t_0 and t_1 particles, were detected (Figs. 2.2 & 2.3).

The 16S rRNA gene fingerprint patterns of the polyamide and chitin biofilms after 1 day of incubation in seawater did not differ significantly from each other ($p = 0.63$). This was also the case in biofilms incubated for 7 days in seawater ($p = 0.27$), but these were clearly distinct from the corresponding t_0 samples ($p = 0.002$, Fig. 2.4). The t_1 and t_7 particles shared some abundant OTUs with their incubation water (Fig. 2.3), but the bacterial assemblages comprising the biofilms of the incubated particles were clearly distinct from the bacterial assemblage of the incubation water ($p = 0.002$ and $p = 0.001$, respectively; Fig. 2.4).

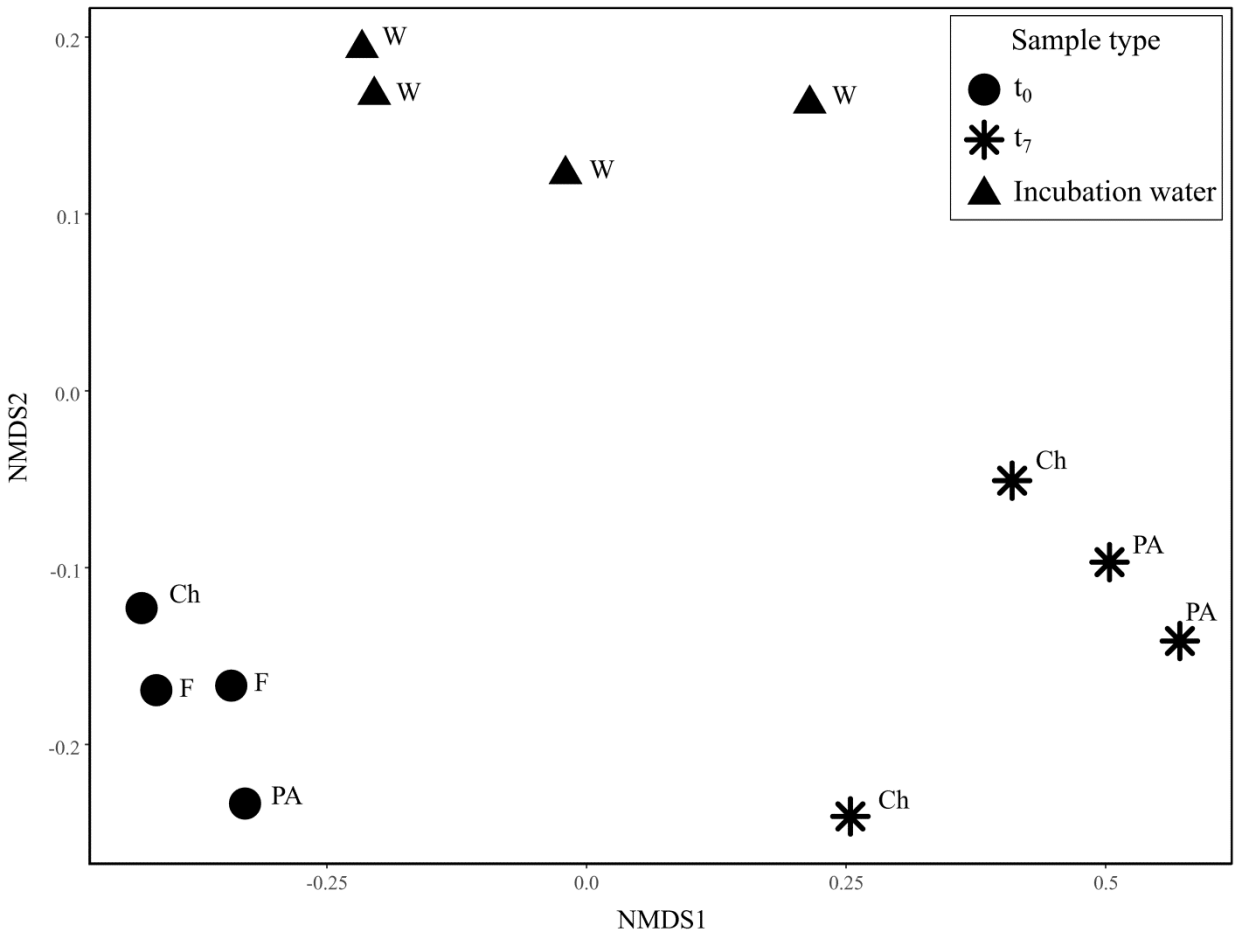

Figure 2.4. Non-metric multidimensional scaling plot based on Pearson correlation of the 16S rRNA gene SSCP fingerprint pattern generated from the polyamide biofilm (PA) or chitin biofilm (Ch) extracted from the faeces (F), and the faeces 24 h after feeding (t_0, dots) and the corresponding polyamide and chitin particles incubated for 7 days in seawater (t_7, asterisk). W, incubation water in which the particles had been incubated for 7 days (triangles). Stress = 0.044.

2.4. Discussion

This study investigated whether bacterial biofilms on artificial and natural particles differ after their passage through the gut of the blue mussel *M. edulis*. The 16S rRNA fingerprint patterns obtained from polyamide and naturally occurring chitin particles did not differ significantly. There was, however, a shift in the dominant bacterial OTUs, with *Flavobacteria* being more abundant in the faeces and on the particles of the 12-h samples and *Gammaproteobacteria* predominating in the faeces and on the particles of the 24-h

samples. This shift was consistently observed in the polyamide, chitin and control treatments.

We also tested the stability of the bacterial biofilms on polyamide vs. chitin after egestion of the particles and their subsequent incubation in seawater. The biofilm assemblages were not stable over a 24 h time period and differed significantly after 7 days. Again, this pattern was independent of the polymer type present. No potential pathogens were found exclusively in the polyamide treatment; instead, after 7 days of incubation in seawater, there was a trend towards lower OTU richness in these biofilms.

2.4.1. Highly similar bacterial assemblages on egested polyamide and chitin particles

Early cultivation-based studies of the gut microflora of aquatic bivalves repeatedly detected members of the genera *Achromobacter*, *Pseudomonas*, *Flavobacterium* and *Vibrio* as abundant gut inhabitants (Prieur et al., 1990). A cultivation study focusing on the faecal bacteria of *Mytilus* also reported members of the family *Vibrionaceae* (Bouvy and Delille, 1987). In our study, the egested polyamide and chitin biofilms, the natural particles of the control treatment, and the faeces of the mussels were dominated by *Flavobacteria* when sampled 12 h after feeding (Fig. 2.3), but none of the detected OTUs had previously been isolated from gut samples. Other known enteric bacteria or bacteria commonly associated with mussels were not detected in any of the 12-h samples. In the 24-h samples, a dominance of *Gammaproteobacteria* was consistently detected (Fig. 2.3). A previous study (Teeling et al., 2012) showed that in a diatom spring bloom in the North Sea *Flavobacteria* were the primary colonisers of algal material and were specialised in the breakdown of algal-derived macromolecules. Members of the *Gammaproteobacteria* became abundant in the later stage of the bloom and were better adapted to the uptake of smaller molecules. A study investigating free-living vs. attached bacterial assemblages along the salinity gradient of the Baltic Sea also detected a dominance of *Gammaproteobacteria* on the particle-attached fraction under mesohaline conditions in winter (Rieck et al., 2015). Whether the shift from *Flavobacteria* to *Gammaproteobacteria* in our experiment was related to a difference in the molecular composition of the egested faecal matter, perhaps due to a longer gut residence time of the 24-h samples, could not be determined. However, the similarity between the assemblage patterns in this study and

those of natural aggregate-attached assemblages suggested that during the first hours after its egestion faecal material is rapidly colonised by bacteria from the water. In previous studies, the survival of some bacteria and diatoms during passage through the gut of suspension-feeding bivalves was demonstrated (Prieur, 1981; Barillé and Cognie, 2000); thus, the regrowth of viable cells on the egested particles and in the faeces cannot be ruled out.

In a previous study of a benthic environment, the passage of polystyrene particles through the digestive tract of the deposit-feeding lugworm *A. marina* did not lead to an enrichment of potential pathogens. Instead, the environment was also shown to be an important factor influencing the bacterial assemblages on polystyrene particles in the sediment (Kesy et al., 2016). In the present work, we did not find a substrate-specific assemblage on polyamide nor was there an enrichment of potentially pathogenic bacteria on the polyamide particles after their gut passage. Thus, it seems unlikely that microplastics, at least polyamide, acquire potential pathogens through contact with wild or farmed mussels and the risk of either disease transmission via this route or adverse socio-economic impacts in temperate regions is low. However, as water temperatures rise in response to climate change, the pressure of potential pathogens, including *Vibrio*, on aquatic ecosystems is expected to increase (Martinez-Urtaza et al., 2010). Under these conditions, microplastics could act as an additional stressor on marine wildlife and thus enhance the susceptibility of mussels towards potential pathogens (Harvell et al., 2002). This could influence aquacultures through possible loss of livestock. Furthermore, whether microplastics can be vectors of potential pathogenic bacteria in tropical regions should be investigated with regard to the respective ecosystem.

2.4.2. Limited vector function of polyamide for bacterial biofilms

The taxonomic composition of abundant OTUs on polyamide and chitin shifted between the 12-h and t_1 samples. At the later time point, the only shared OTU was that of a close relative of a *Flavobacteriaceae* bacterium and DNA related to eukaryotic plastids (Fig. 2.3). After 7 days, the polyamide and chitin biofilms had changed significantly from their corresponding t_0 samples (Fig. 2.4) and the number of OTUs showed a decreasing trend (Fig. 2.2). This suggests a limited potential of polyamide as a bacterial vector. A close relative of the potential chitin-degrading gammaproteobacterium *Neptunomonas* sp. was

detected on the t_7 polyamide and t_7 chitin samples (Fig. 2.3). This observation was consistent with that of another study in which standardised chitin particles were examined for colonisation patterns on marine particles. The authors isolated two strains of *Oceanospirillaceae*, one of which was identified as *Neptunomonas* sp., which became abundant on the particles after 48–96 h of colonisation and remained an abundant component of the particle-attached assemblage even after 6 days (Datta et al., 2016). As in our study, this pattern was consistent in all the replicates and strongly suggested that the process was a naturally occurring one. Similarly, none of the organisms identified in our experiment were exclusive to the polyamide biofilms; rather the colonisation patterns of polyamide and chitin were comparable. Furthermore, the OTUs derived from the incubated particles were also detected in the incubation water, indicating colonisation of the particles by bacteria from the water (Fig. 2.3).

However, there was a decreasing trend in bacterial richness on the polyamide biofilm after 7 days of incubation in seawater. Although this decrease was observed in all the t_7 sample types, this trend was most pronounced on polyamide (Fig. 2.2). Species richness is commonly linked to the functioning and resilience of a community (Spehn et al., 2005), such that the decrease on polyamide may indicate functional deficiencies of the respective ecosystem. This possibility needs to be further investigated, especially in waters with high concentrations of polyamide. Broader confirmation of this decline in bacterial richness on polyamide would imply that this polymer offers a habitat for specialised organisms, perhaps with substrate-degrading capabilities. OTU FK40, detected both on polyamide and chitin, was affiliated with the oceanospirillum *Neptunomonas* sp., which is a potential degrader of chitin and polycyclic aromatic hydrocarbons (Hedlund et al., 1999; Datta et al., 2016). This OTU had its peak on the t_7 polyamide samples, reaching a relative abundance as high as ~69%. This high abundance might indicate that *Neptunomonas* sp. is also capable of degrading polyamide or its monomers. In a previous study we identified an OTU affiliated with the oceanospirillum *Amphritea* sp. that became highly enriched on polystyrene incubated for 24 h in seawater. Members of the *Oceanospirillacea* have been found in hydrocarbon-rich environments, such as oil-contaminated sites (Yakimov et al., 2005; Teramoto et al., 2009), and are known degraders of petroleum-related hydrocarbons (Hedlund et al., 1999; Yakimov et al., 2003; Yakimov et al., 2004). Whether members of the family *Oceanospirillaceae* can also degrade artificial polymers should be investigated in future studies.

2.5. Conclusions

In a proof of principle approach, we showed that the passage of polyamide particles through the digestive tract of *M. edulis* does not lead to an altered bacterial assemblage regarding the abundant OTUs compared to particles of the natural polymer chitin. There was also no enrichment of potential pathogens on the egested particles. Thus, despite the prominent role of *M. edulis* in coastal marine cycling of organic matter and particle turnover, there is negligible alteration of bacterial assemblages on polyamide particles that have passed through the gut of this mussel. To determine whether polyamide influences bacteria of the rare biosphere and whether that may have implication for the functioning and resilience of particle-attached biofilms, further studies are needed under the various conditions of the marine environment.

Chapter III

Spatial environmental heterogeneity determines young biofilm assemblages on microplastics in Baltic Sea mesocosms

The following chapter was published in the journal *Frontiers in Microbiology* as

Katharina Kesy, Sonja Oberbeckmann, Bernd Kreikemeyer, and Matthias Labrenz (2019). Spatial environmental heterogeneity determines young biofilm assemblages on microplastics in Baltic Sea mesocosms. *Front. Microbiol.* 10:1665. doi: 10.3389/fmicb.2019.01665

Declaration of author contributions:

Katharina Kesy conducted the experiment.

Katharina Kesy performed laboratory work.

Katharina Kesy analysed the data.

Katharina Kesy and Matthias Labrenz discussed the data.

Katharina Kesy wrote the manuscript, Matthias Labrenz, Sonja Oberbeckmann and Bernd Kreikemeyer critically commented on the manuscript.

The contribution of Katharina Kesy to the written manuscript was ~95%.

Abstract

Microplastics in aquatic environments provide novel habitats for surface-colonising microorganisms. Among the bacterial species found in microplastic-associated biofilms are potentially pathogenic *Vibrio* spp. Due to their persistence and great dispersal potential, microplastics could act as vectors for these potential pathogens and for biofilm assemblages in general. Given the continuing debate on whether substrate-specific properties or environmental factors prevail in shaping biofilm assemblages on microplastics, we examined the influence of substrate vs. spatial factors in the development of bacterial assemblages on polyethylene (PE), polystyrene (PS), wood and seston and in the free-living fraction. Further, the selective colonisation of microplastics by potential pathogens was investigated. Incubation experiments with these substrates were conducted for 7 days during a summer cruise along the eastern Baltic Sea coastline in waters covering a salinity gradient of 4.5–9 PSU. Bacterial assemblages were analysed using 16S rRNA-gene amplicon sequencing, distance-based redundancy analyses, and the linear discriminant analysis effect size method to identify taxa that were significantly more abundant on the plastics.

The results showed that the sample type was the most important factor structuring bacterial assemblages overall. Surface properties were less significant in differentiating attached biofilms on PE, PS and wood; instead, environmental factors, mainly salinity, prevailed. A potential role for inorganic-nutrient limitations in surface-specific attachment was identified as well. *Alphaproteobacteria* (*Sphingomonadaceae*, *Devosiaceae* and *Rhodobacteraceae*) and *Gammaproteobacteria* (*Alteromonadaceae* and *Pseudomonas*) were distinctive for the PE- and PS-associated biofilms. *Vibrio* was more abundant on the PE and PS biofilms than on seston, but its abundances were highest on wood and positively correlated with salinity. These results corroborate earlier findings, that microplastics constitute a habitat for biofilm-forming microorganisms distinct from seston, but less from wood. In contrast to earlier reports of low *Vibrio* numbers on microplastics, these results also suggest that vibrios are early colonisers of surfaces in general. Spatial as well as temporal dynamics should therefore be considered when assessing the potential of microplastics to serve as vectors for bacterial assemblages and putative pathogens, as these parameters are major drivers of biofilm diversity.

3.1. Introduction

Microplastics, usually defined as plastic particles ≤5 mm in size (Arthur et al., 2009), are now widely recognized as new, significant pollutants of aquatic systems (GESAMP, 2015). Although the first records of microplastics in aquatic systems date back to the 1970s (Carpenter and Smith, 1972), most research into the global pollution of aquatic systems with microplastics has been conducted only within the last 15 years (Thompson R. C. et al., 2004). The majority of these investigations have focused on the potential harm to aquatic organisms resulting from the ingestion of microplastics. Among the effects identified thus far are inflammatory responses in the tissue of the blue mussel *Mytilus edulis* (Browne et al., 2008), reproductive disruption in the Pacific oyster *Crassostrea gigas* (Sussarellu et al., 2016), a reduction in carbon uptake by the copepod *Calanus helgolandicus* (Cole et al., 2015) and reduced growth rates of the cold-water coral *Lophelia pertusa* (Chapron et al., 2018). However, the role of microplastics as a habitat for biofilm-forming microorganisms has only recently been investigated, although interest in this topic is growing (Ivar do Sul et al., 2018).

In aqueous systems, biofilms inevitably form on every submerged surface. Initially, a so-called conditioning film develops in which polysaccharides, amino acids and proteins immediately adsorb onto the surface and promote subsequent colonisation by microorganisms (ZoBell, 1943). Microorganisms are key drivers of all biochemical cycles (Falkowski et al., 2008) and the biofilms that form on surfaces have been shown to host distinct microbial communities with distinct functional traits (Dang and Lovell, 2016). In addition to enhancing microbial activity (van Loosdrecht et al., 1990), biofilms protect microorganisms from environmental stressors, such as UV-radiation, osmotic stress and antibiotics. Moreover, they provide opportunities for new niches, through versatile metabolic cooperation and horizontal gene transfer (Davey and O'Toole, 2000).

It has been estimated that >5 trillion plastic pieces are afloat at sea, accumulating in ocean convergence zones such as the northern and southern subtropical gyres (Eriksen et al., 2014). The impacts of this vast addition of newly available surfaces colonisable by biofilm-forming microorganisms on aquatic microbial communities and ecosystem functioning have yet to be fully determined. Studies from different regions of the world's oceans have shown that microbial assemblages on microplastics usually differ from their free-living counterpart and from assemblages on natural seston (Dussud et al., 2018b;

Oberbeckmann et al., 2018; Zettler et al., 2013). However, whether biofilm communities are predominantly shaped by environmental factors or surface properties is unclear and the environmental factors exerting the strongest selective pressure have yet to be identified. Oberbeckmann et al. found that the microbial assemblages on polyethylene terephthalate (PET) bottles and glass slides incubated in the North Sea for 6 weeks were shaped mainly by seasonal and geographic factors rather than by surface properties (Oberbeckmann et al., 2014, 2016). Ogonowski et al. (2018) identified a strong separation between the composition of the bacterial communities on artificial and hydrophobic polymers on the one hand and hydrophilic glass and cellulose substrates on the other after 14 days of colonisation. Amaral-Zettler et al. (2015) reported that microplastic-associated assemblages sampled from the Pacific and Atlantic oceans exhibited biogeographic patterns but only a weak relationship with the polymer type. De Tender et al. (2015) assumed that salinity, temperature and oxygen levels played a role in shaping the microplastic-associated assemblages obtained from sediments. Further, it could be shown that surface properties are more important under low nutrient conditions (Oberbeckmann et al., 2018). However, Dussud et al. (2018b) could not detect an effect of geographic location, environmental factors or different polymers on the microbial assemblages that had formed on plastics sampled in the western Mediterranean basin. There was also no effect of polymer type or sampling location on the biofilms of microplastic samples obtained from the northern Pacific Ocean (Bryant et al., 2016).

Biofilms can also serve as reservoirs for potentially pathogenic bacteria (Lyons et al., 2010). Shikuma and Hadfield (2010) found that *Vibrio*, a genus which includes potential human pathogens, was enriched in the biofilms on ship hulls compared to the surrounding water in different ports of Hawai'i, U.S.A.. Islam et al. (2007) detected *Vibrio cholerae* in biofilms on acrylic glass submerged in a canal in Bangladesh. *Vibrio* spp. were found at high relative abundance (24%) on a polypropylene particle sampled from the North Atlantic Gyre (Zettler et al., 2013), on samples from the Bay of Brest, France (1.5–18.6%) (Frère et al., 2018) and the potential pathogen *V. parahaemolyticus* was identified on microplastic particles sampled from the North Sea and Baltic Sea (Kirstein et al., 2016). However, other studies of microplastic-associated microbial assemblages found little or no enrichment of potential pathogens sampled *in situ* (Dussud et al., 2018b; Schmidt et al., 2014), or after passage through the gut of marine invertebrates (Kesy et al., 2016, 2017). Thus, whether microplastics *per se* selectively favour the colonisation of potential

pathogens such as *Vibrio* or even become enriched and thus able to serve as vectors for potentially pathogenic bacteria (Oberbeckmann et al., 2018) remains to be determined. Because of the large volumes of plastic pollutants <5 mm in size (Cozar et al., 2014; Moret-Ferguson et al., 2010) and their persistence in aquatic systems, microplastics could provide a significant route of pathogen dispersal (Pham et al., 2012). Although sediments in the Baltic Sea have been shown to act as reservoirs of *Vibrio* spp. (Huehn et al., 2014), floating microplastics, and thus their attached microbial assemblages, are more susceptible to distribution by winds and currents (Chubarenko et al., 2016) and may therefore be rapidly transported over long distances (Isobe et al., 2014). Furthermore, the microplastics sampled *in situ* are of unknown age and the attached bacterial assemblages have been shown to change over time (De Tender et al., 2017; Dussud et al., 2018b). Studies of biofilm formation must therefore be conducted under controlled conditions in addition to *in situ* investigations, to augment the knowledge on drivers of biofilm diversity and interactions with potential pathogens within the different aquatic habitats.

The Baltic Sea is a semi-enclosed sea in Northern Europe that is under strong anthropogenic pressure (HELCOM, 2010). It has a stable salinity gradient, with nearly marine conditions in its most western regions and nearly freshwater conditions in the northeast. Brackish waters are a suitable habitat for several *Vibrio* species, including the potential human pathogens *V. vulnificus*, *V. cholerae* non-O1 and *V. parahaemolyticus*, which can cause severe wound infections and gastroenteritis (Baker-Austin et al., 2010). Because *Vibrio* infections have been repeatedly reported from the Baltic Sea (Baker-Austin et al., 2013), it is a suitable ecosystem to investigate the influence of different environmental factors on biofilm formation on microplastics, including the colonisation of those biofilms by potentially pathogenic *Vibrio*. In this study, we investigated the influence of geographic location vs. habitat type on bacterial assemblages, with a focus on developing biofilm assemblages on two different polymers, and whether potential pathogens are selectively enriched on microplastics. Thus, incubation experiments using polyethylene (PE) and polystyrene (PS) microplastics were conducted. Wood particles served as a biotic control, because their properties are similar to those of floating plastics in terms of elemental structure and floating behavior. The incubations were conducted for 7 days during a cruise along ~2000 km of the southeastern coastline of the Baltic Sea, covering a salinity gradient of 4.5–9 PSU. The biofilms that developed during those 7 days can still be considered as young, which has been shown in several studies (De Tender et

al., 2017; Dussud et al., 2018b; Fischer et al., 2012). Bacterial assemblages were analysed using 16S rRNA-gene amplicon sequencing, multiple regressions and linear discriminant effect size to distinguish the effects of sampling station vs. sample type. The colonisation and potential enrichment of the particles by putative pathogenic *Vibrio* spp. were assessed by comparing the relative abundances of *Vibrio* spp. on the different sample types.

3.2. Material and methods

3.2.1. Sampling campaign and incubation experiments

Incubation experiments were carried out similar to those described by Ogonowski et al. (2018) during a cruise in August/September 2015 along the coast of the Baltic Sea with the *R/V Poseidon* (cruise POS488), covering roughly 2000 km of coastline along the eastern mesohalinic part of the Baltic Sea, from Rostock, Germany to Helsinki, Finland.
Surface water from within the first 5 m depth was collected at eight stations (Fig. 3.1 and Table S3.1) with 5-L free-flow bottles mounted on a rosette equipped with a conductivity-temperature-depth-probe (Sea-Bird SBE 9). Water from five to six bottles was mixed and then sequentially filtered in technical triplicates (500 mL each) over cellulose nitrate filters (GE Whatman) of 3-µm (seston-attached bacteria) and 0.22-µm (free-living fraction) pore-size. The bacteria on these filters represented the *in situ* samples (t_0). The filters were snap-frozen in liquid nitrogen and stored at −80°C. The remaining water was then filtered through 30-µm gauze to exclude large grazers and 1.5-L distributed into plastic tanks (SAVIC, 19.5 × 13 × 11.5 cm). The plastic and wood as control substrates used were the same as described in Oberbeckmann et al. (2018). For the treatment incubation, 80-resin polyethylene pellets (PE, HDPE HTA108, ExxonMobil, density 0.961 $g.cm^{-3}$), 80-resin polystyrene pellets (PS, polystyrene 143 E, BASF, density 1.04 $g.cm^{-3}$; both ø 3 mm, respectively) and 2-g wood pellets (1Heiz®, Germany) were introduced together into the treatment tanks (treatment incubation, n = 3, Fig. S3.1). Tanks containing only water, without plastic or wood particles, served as the control (control incubation, n = 3, Fig. S3.1). The treatment and control incubations were run for 7 days at the ambient temperature (18–20°C) and were aerated using common aquaria diffuser stones (Dohse Aquaristik, Germany). Light/dark cycles varied between 19/5 h and 18/6 h. Prior to the experiment, all materials used in the study were incubated in Milli Q water (Merck

Millipore) for at least 24 h, to allow the leaching out of any additives from the material, and then dried at 30°C. Temperature, O_2, salinity and the pH of the incubation water were monitored during the course of the experiment using a HachLange field meter and ready-to-use pH-indicator strips (Merck, Germany).

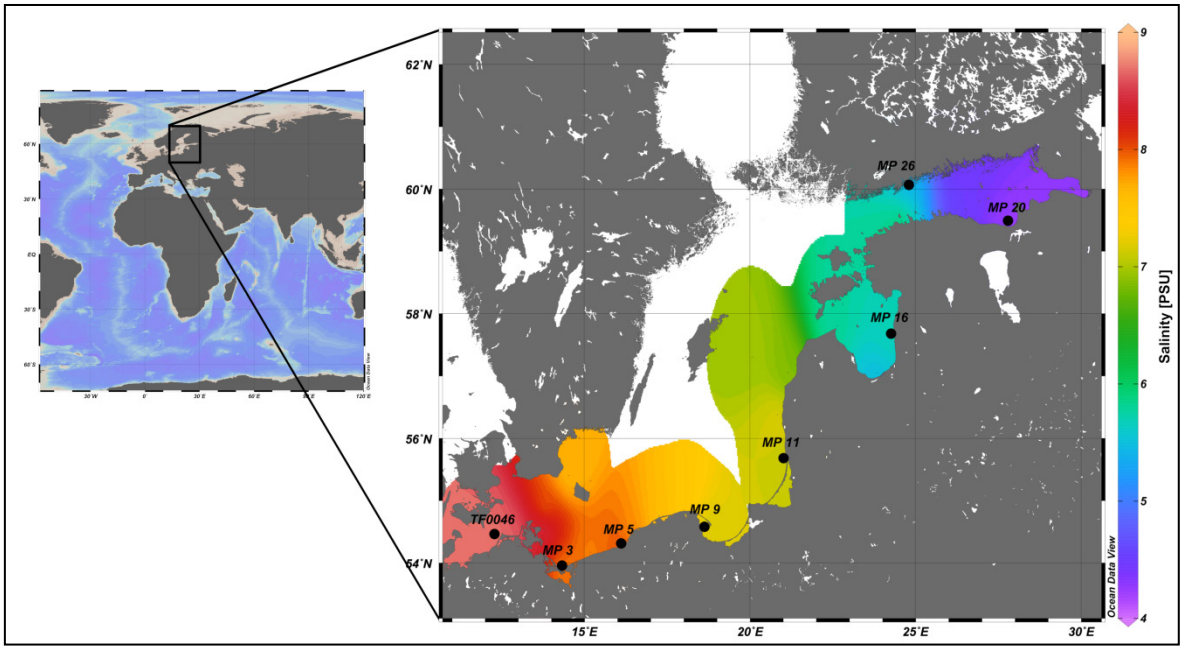

Figure 3.1. Map of the study location of the Baltic Sea and of the sampling stations included in the incubation experiments along the Baltic Sea coast (enlarged). Salinities in the surface water are those measured during cruise POS488 (some stations are not depicted) and subsequently extrapolated. The map was created using Ocean Data View v. 5.0 (Schlitzer, 2018).

After 7 days, the PE, PS and wood particles were collected using sterile tweezers, rinsed twice with sterile-filtered seawater and quickly centrifuged to remove loosely attached cells. The remaining water was removed and the particles were snap-frozen. To assess the bacterial assemblages on seston and in the free-living fraction of the incubations at t_7, water (500 mL) from all incubations was pre-filtered over a 100-µm gauze. This step was necessary to exclude smaller wood particles. The pre-filtered water was then processed as described for the *in situ* samples. All samples were stored at −80°C until further analysis. Additionally, 40 mL of water from each tank was collected, filtered through an Acrodisc 0.2 µm HT Tuffryn membrane syringe filter (PALL Life Science) to remove any particles and stored at −20°C for later nutrient analysis. Nutrient analysis for the *in situ* samples was performed on board, using standard colorimetric methods (Grasshoff et al., 1999), and for

the t_7 samples, after the cruise, using an autoanalyser (Seal Analytical). Because ammonia concentrations cannot be measured reliably after freezing and subsequent thawing of samples, they were omitted from the t_7 dataset.

3.2.2. DNA extraction and 16S rRNA-gene amplicon sequencing

DNA was extracted from all sample types using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instruction, except that DNA was eluted twice from the spin column, using the same 50 µL of PCR-grade water, to enhance the DNA yield. Twelve PE and PS pellets and 45 mg of wood were used for each DNA extraction. Blank extractions were carried out after each extraction kit package had been used, to account for possible contamination during the extraction process (Salter et al., 2014). The DNA was PCR-amplified using primers covering the V4 region of the 16S rRNA-gene (position 515F to 806R), with the forward sequence 5' GTGCCAGCMGCCGCGGTAA 3' and the reverse sequence 5' GGACTACHVGGGTWTCTAAT 3' (Caporaso et al., 2011). The PCR was preceded by a short linear amplification step to increase the DNA yield. Thermal cycling started with an initial denaturation at 98°C for 2 min, followed by an additional denaturation step at 98°C for 15 s, annealing at 65°C for 15 s and elongation for 30 s at 68°C. The last three steps were repeated nine times, with the elongation temperature reduced by 1°C per cycle (linear amplification), followed by a denaturation step at 98°C for 15 s, annealing at 55°C for 15 s and an elongation step at 68°C for 30 s (24 cycles). Thermocycling ended with a final elongation step at 70°C for 5 min (Takahashi et al., 2014). Library preparation and sequencing on an Illumina MiSeq machine were carried out according to the "Illumina 16S Metagenomic Sequencing Library Preparation Guide". DNA from a known *V. vulnificus* strain (DSM No. 10143^T) and PCR-grade water were included in each run to serve as a positive and negative control, respectively.

3.2.3. Sequence processing

Raw sequence reads were processed using the mothur pipeline v. 1.39.5 (Schloss et al., 2009) following the mothur MiSeq SOP guidelines (Kozich et al., 2013; MiSeq SOP - mothur). Quality filtered sequences were classified using the k-Nearest Neighbour algorithm and the SILVA SSURef release 132 as the reference database (Yilmaz et al.,

2014), with a required bootstrap of ≥85%. The taxonomy used in the 132 release and throughout this study incorporated several rearrangements of bacterial phyla, as proposed by Parks et al. (2018). Operational taxonomic units (OTUs) were clustered based on 97% sequence similarity and those with sequence reads ≤3 in the whole dataset were excluded. Sequences classified as Mitochondria, *Archaea*, Chloroplasts, and *Eukaryota* were also excluded.

The dataset was further filtered so that OTUs with mean read counts of 2.5 in the blank extraction or in the negative controls were discarded. The maximum library size of the PE, PS and wood pellets incubated in Milli Q water was 203 reads after filtering; these samples were therefore omitted from the dataset.

The raw sequences obtained in this study were deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA506548.

3.2.4. Chao1 richness and species turnover

For the α- and β-diversity analyses, the filtered dataset was subsampled to the smallest library size (13,926 sequences) using 100 iterations, and the mean reads per sample and per OTU were calculated (Zha et al., 2016) together with the mean OTU richness based on the Chao1 estimator and Pielou's evenness. The Kruskal-Wallis test was used to determine whether the Chao1 richness and Pielou's evenness were significantly different between sample types and between stations. If the results of the Kruskal-Wallis test were statistically significant, post hoc pair-wise comparisons were performed using the Conover-Iman test for multiple comparisons within the conover.test package v. 1.1.5 in R (Dinno, 2017). A Benjamini-Hochberg correction was applied to *p*-values for multiple testing. The results were considered significant at an α-level of 0.05. A Venn diagram was computed using the package VennDiagram in R (Chen, 2018) to assess the number of unique OTUs within each sample type. All t_0 and t_7 samples from the seston and from the free-living fraction were combined, respectively prior to computing of the Venn diagram to account for OTUs truly unique to the plastics.

3.2.5. Relative abundances of the most abundant bacterial classes

Relative abundances were calculated within mothur using the 'get.relabund' command and transformed to a percentage in the R program (R Core Team, 2017). Relative abundances at the class level were visualised for classes with a mean relative abundance of ≥1% in at least one sample, using the ggplot2 package (Wickham, 2016).

3.2.6. Plastic-specific bacteria

To evaluate OTUs that discriminated between sample types, the linear discriminant analysis effect size method (LEfSe; Segata et al., 2011) was applied to the relative-abundance-based OTU table of the filtered dataset. Default parameter settings were used and an all-against-all comparison strategy was applied. First, the PE, PS and wood samples were combined into a single group to determine whether a core community was present on the introduced particles. In a second LEfSe run, only the PE and PS samples were combined, yielding a plastics group, to evaluate OTUs that were significantly more abundant on plastics than on wood or seston or in the free-living fraction. The core OTUs of the combined PE, PS and wood samples as well as the discriminant OTUs for the wood and plastics samples alone were visualised at the family level in a phylogenetic tree constructed from all OTUs with a mean relative abundance of ≥0.1% in at least one sample type. The relaxed neighbour-joining method contained in the clearcut program within mothur (Evans et al., 2006) was used and the tree was visualised using the interactive Tree Of Life online tool (iTOL, v. 4.2.3; Letunic and Bork, 2016).

3.2.7. *Vibrio* spp. relative abundances

To evaluate the proportion of *Vibrio* spp. within the total bacterial assemblages, the mean relative abundances of each *Vibrio* OTU and the standard deviation per triplicates were calculated in R and visualised using the ggplot2 package. The Kruskal-Wallis test and Conover-Iman test for pair-wise comparisons were used to identify significant differences in the relative abundances on seston and in the free-living fraction between the treatment incubations, control incubations and the *in situ* samples. The same tests were applied to determine differences between all sample types within the treatment and control incubations. Because only two replicates were available for the seston samples from the

treatment incubations of station MP9, these comparisons were excluded, when applicable. A Spearman rank correlation (ρ) was used to correlate *Vibrio* spp. read counts to environmental parameters.

3.2.8. Multiple regression analysis of factors influencing bacterial assemblages

Data in the read-based, subsampled OTU table of the t_7 samples, as described for the Chao1 richness, were further square-root-transformed and used for all multiple regression analyses and multivariate statistics. To test whether the bacterial assemblages differed significantly from each other, global and pair-wise permutational multivariate analyses of variance (PERMANOVAs; Anderson, 2001) were calculated on the Bray-Curtis similarity matrix for a two-factorial design (sample type and station). Pair-wise comparisons were calculated for the factor "sample type" within each station to exclude possible effects between stations, using Monte Carlo random draws from the asymptotic permutation distribution (Anderson and Robinson, 2003). To account for possible dispersal effects between samples, the homogeneity of the dispersions was tested using the PERMDISP routine (Anderson, 2006). To determine whether substrate type or geographic location was the main driver of the bacterial assemblages, a distance-based redundancy analysis (dbRDA; Legendre and Anderson, 1999) was performed based on the Bray-Curtis dissimilarity matrix, using the sample types (PE, PS, wood, seston, free-living) and the different stations as constraining factors. The dbRDA was conducted in R using the 'dbrda' function from the vegan package (Oksanen et al., 2018), with Lingoes correction for negative eigenvalues (Lingoes, 1971). Significance tests of the dbRDA models and marginal tests for the factors were performed using permutation tests with the 'anova.cca' function of the vegan package (999 permutations). All regression coefficients (R^2) were adjusted for multiple testing. The contributions of constraining factors to the first two axes of the dbRDA model were assessed with Spearman rank correlations (ρ) using the basic 'cor' function in R. Because of missing water samples for the station MP5 incubations, data from this station were excluded from the dbRDA and the PERMANOVA during comparisons of all sample types. The 'ordisurf' function from the vegan package was used to fit the response surfaces of salinity, temperature, NO_2^-, NO_3^- and PO_4^{3-} (means between t_0 and t_7) onto the dbRDA plots (Bennion et al., 2012).

All tests were performed in the R program for Statistical Computation v. 3.4.3 (R Core Team, 2017) using the packages vegan v. 2.4-6 (Oksanen et al., 2018), reshape2 v. 1.4.3 for data handling (Wickham, 2007) and ggplot2 v. 3.0.0 for visualisation (Wickham, 2016). Graphs were further processed with Inkscape v. 92.0. PERMANOVA and PERMDISP tests were performed using the PRIMER7 program and its add-on package PERMANOVA+ (PRIMER-e, Quest Research Limited, Auckland, New Zealand).

3.3. Results

3.3.1. Physico-chemical parameters of the stations and inorganic nutrient concentrations over the course of the experiment

The *in situ* salinity of the experimental stations ranged from 8.7 PSU at the most western station (TF0046) to 4.4 PSU at the most eastern station (MP20) (Fig. 3.1). The temperature of the surface waters was consistently between 18.5 and 20.6°C, except at stations TF0046 (15.3°C) and MP5 (10.0°C).

Inorganic nitrogen (NO_2^-, NO_3^- and NH_4^+) was depleted at all stations (<0.5 µmol.L^{-1}), except at station MP16 (2.1 µmol.L^{-1}). Phosphate concentrations ranged between 0.05 µmol.L^{-1} at station MP20 and 0.66 µmol.L^{-1} at station MP5. Most stations were therefore extremely nitrogen-limited, with DIN/DIP ratios <2.2, except stations MP20 (DIN/DIP 17.8) and MP16 (DIN/DIP 29.8), which were rather phosphate-limited (Table S3.1).

At the end of the incubation experiments, salinity had increased slightly, by between 0.3 and 0.8 PSU, due to evaporation, and the temperature was the same in all incubation tanks, between 18°C and 20°C (Table S3.1). Dissolved inorganic nitrogen (DIN) concentrations (without NH_4^+) were still very low (<0.3–0.6 µmol.L^{-1}), except in the incubation tanks of station MP11 (1.7 ± 1.5 µmol.L^{-1}), while phosphate was nearly depleted in the incubation tanks of all stations, with concentrations <0.1 µmol.L^{-1} (Table S3.3.1). Due to the low DIN concentrations, the incubations continued to be nitrogen-limited (DIN/DIP 2.5–6.1), except at station MP11 (DIN/DIP 18.6, Table S3.1).

3.3.2. Sequence yield and quality

The four Illumina MiSeq runs generated 38,024,360 paired-end reads. Assembly of the forward and reverse reads yielded 33,166,861 sequences. The final sequence count after filtering was 14,199,783. Based on a 97% similarity, these sequences could be clustered into 12,572 OTUs. After the removal of potential contaminating OTUs, which were also found in the negative controls and blank extractions, 12,509 OTUs remained in the whole dataset.

3.3.4. Bacterial richness on different sample types and across stations

Chao1 richness across all stations on PE and the PS was 329 ± 108 and 366 ± 130, respectively. In the treatment and control incubations, the mean Chao1 richness across all stations on wood and seston and in the free-living fraction was relatively similar, ranging from 667 ± 148 on seston from the treatment incubations to 579 ± 154 on wood and 554 ± 56 in the free-living fraction of the control incubations, although some significant differences were detected (Fig. 3.2 and Table S3.2A–B). Mean Chao1 richness was significantly higher in the *in situ* samples of seston and the free-living fraction (1142 ± 534 and 733 ± 94; $p < 0.001$ and $p \leq 0.007$, respectively; Table S3.2C–D). In addition, for all sample types, except those of the free-living fraction of the treatment and control incubations, the differences in Chao1 richness between stations were significant (p = 0.003–0.05, Table S3.2A–B). Mean Chao1 richness across all sample types was generally highest at station MP3: 894 ± 496, except in the cases of PE and the free-living fraction of the treatment and control incubations (Fig. 3.2).

Pielou's evenness was relatively uniform between sample types across all the stations, ranging from 0.62 ± 0.05 on the seston samples of the control incubation to 0.74 ± 0.01 in the free-living fraction *in situ* (Fig. S3.2). Although both factors, "sample type" and "station," had a significant effect on evenness ($p < 0.001$ and 0.013, respectively), there was no obvious pattern between sample types and stations (Fig. S3.2). However, evenness was lowest on the PE and PS samples at the western stations TF0046, MP3 and MP5, ranging from 0.6 ± 0.02 for the PE samples at station TF0046 to 0.68 ± 0.01 for the PS samples at station MP3 (Fig. S3.2).

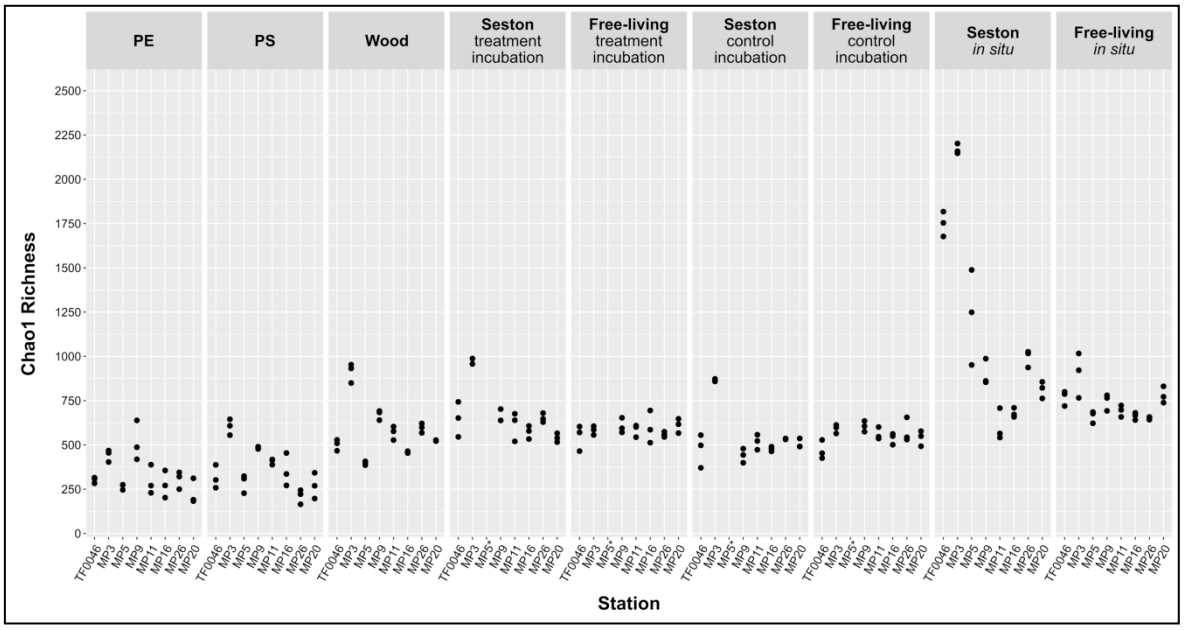

Figure 3.2. Chao1 estimator of bacterial OTU richness on seston (≥ 3 µm) and in the free-living fraction (3–0.22 µm) at different stations at t_0 (*in situ*) and after 7 days of incubation on PE, PS, wood and seston and in the free-living fraction. Data for both the treatment and control incubations are shown. *For station MP5, incubation water samples were not available.

3.3.5. β-diversity

The lowest number of unique OTUs was associated with PE, PS, and wood (50, 93, and 137 OTUs, respectively). These three sample types had 20 OTUs in common and 100 OTUs that were shared with seston. The latter had the highest number of unique OTUs (3184), followed by the free-living fraction (1772 OTUs). Among all sample types there were 1098 shared OTUs. There was also a pronounced overlap of OTUs shared by seston and wood (603 OTUs), by seston, wood and the free-living fraction (670 OTUs) and by seston and the free-living fraction (1969 OTUs) (Fig. S3.3).

3.3.6. General community composition on class level

Gammaproteobacteria, *Alphaproteobacteria* and *Bacteroidia* were the most abundant classes overall. Twenty classes of 12 phyla occurred in abundances of ≥1% in at least one sample. Some classes were found in larger quantities on PE, PS, and wood and some also differed in their occurrences depending on the station (Fig. 3.3).

Gammaproteobacteria was the dominant class in samples from the treatment and control incubations, with greater mean abundances across all stations on PE (52.1 ± 13.5%), PS (43.9 ± 12.4%) and wood (42.6 ± 7.5%) than on seston (24.9 ± 11.3% in the treatment and 20.0 ± 14.6% in the control incubations) or in the free-living fraction (23.5 ± 6.3% and 30.7 ± 6.8%, respectively) of the incubation waters. The mean abundance of *Gammaproteobacteria* was less on the seston *in situ* samples and in the *in situ* samples of the free-living fraction (7.9 ± 3.7% and 13.7 ± 3.4%, respectively). In addition, the relative abundance of *Gammaproteobacteria* differed depending on the location and was greater at the western stations TF0046, MP3 and MP5 (maximum abundance of 68.8 ± 0.6% on PE and 63.9 ± 2.4% on PS at station MP5). The lowest percentage on PE occurred at station MP9 (35.4 ± 1.4%) and on PS at station MP16 (32.4 ± 4.0%) (Fig. 3.3).

The second most abundant class was *Alphaproteobacteria*, which was also generally found in higher mean numbers in the incubations than in the *in situ* samples across all stations. *Alphaproteobacteria* were also slightly more abundant on the plastics (31.6 ± 11.5% on PE and 32.1 ± 9.4% on PS) than on wood (26.0 ± 5.7%) or seston (20.5 ± 5.4% in the treatment and 24.7 ± 12.3% in the control incubations) or in the free-living fraction (24.3 ± 3.0% in the treatment and 21.1 ± 6.7% in the control incubations). Low abundances of *Alphaproteobacteria* also characterized the *in situ* samples: 7.7 ± 1.6% on seston and 11.4 ± 1.5% in the free-living fraction. In the treatment and control incubations, the relative abundances of *Alphaproteobacteria* showed a general trend towards higher percentages at the more eastern stations (MP9–MP26). The maximum abundances on PE and PS were measured at station MP16 (44.7 ± 3.5% and 44.6 ± 2.7%, respectively), while the lowest abundance occurred at station MP5 (16.2 ± 2.0% and 18.0 ± 1.0%, respectively). This trend was not observed in the *in situ* samples (Fig. 3.3).

The occurrence of uncultured planctomycetes class OM190 was highest on particles (2.3 ± 1.9%, 3.5 ± 2.0% and 3.6 ± 2.1% on PE, PS and wood, respectively). The relative abundance of this group in the seston samples of the treatment and control incubations was in the same range (3.3 ± 2.7% and 3.0 ± 3.5%, respectively). Abundance was highest on the seston *in situ* samples (5.1 ± 2.7%). In the free-living fraction, the highest abundance was measured in the control incubations (0.7 ± 0.4%). Among the stations, the abundance of class OM190 was highest at station MP16 (6.4 ± 1.3% on PE and 7.0 ± 2.3% on PS) and lowest at station TF0046 (0.3 ± 0.1% on PE and 1.3 ± 0.3% on PS) (Fig. 3.3).

Additional classes with relative abundances of 5–45 % were either less frequent on PE, PS and wood or showed no differences in abundance between sample types. The former included *Verrucomicrobiae*, *Planctomycetacia*, *Oxyphotobacteria*, *Bacteroidia* and *Actinobacteria* and the latter *Phycisphaerae* and *Deltaproteobacteria* (Fig. 3.3). Other classes were also present but their contribution to the bacterial assemblages was minor (<3%). Within this group were representatives of obligate anaerobes (such as *Kiritimatiellae*, *Anaerolineae* and *Clostridia*), which were mainly found in the biofilms on seston (Fig. 3.3).

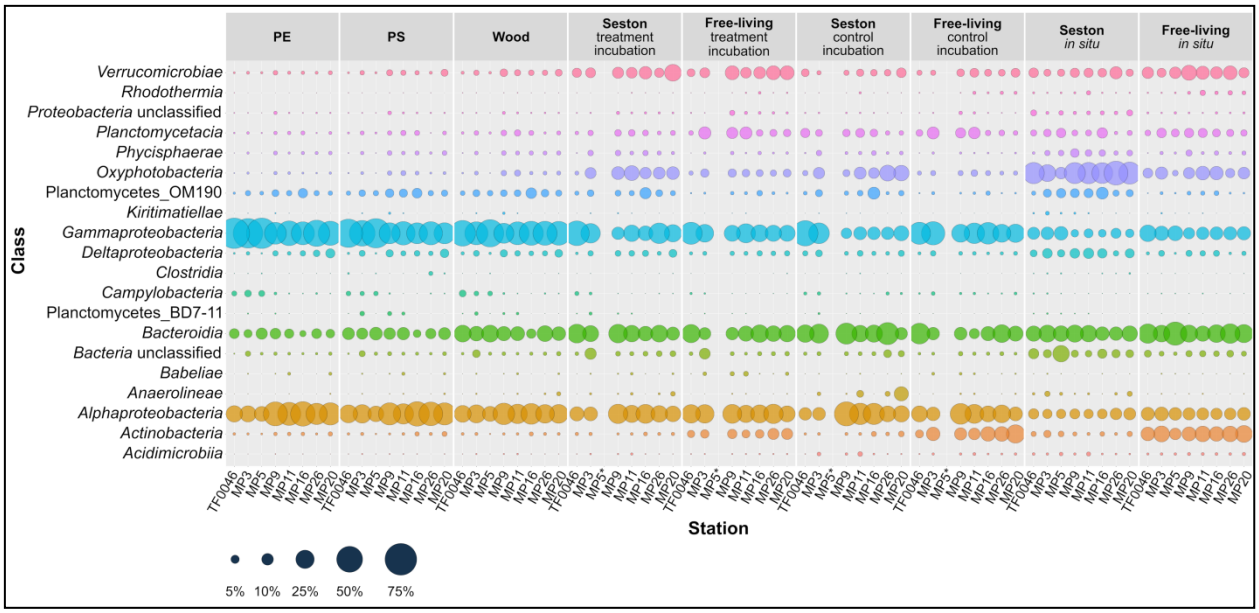

Figure 3.3. Mean relative abundances of the bacterial classes (>1% relative abundance in at least one sample) present on seston (≥3 µm) and in the free-living fraction (3–0.22 µm) at different stations at t_0 (*in situ*) and in the samples incubated for 7 days on PE, PS, wood and seston and in the free-living fraction. Data for both the treatment and the control incubation are shown. *For station MP5, incubation water samples were not available.

3.3.7. Biofilm core OTUs and discriminant OTUs for plastics

Proteobacteria were significantly more abundant on the plastics but were exclusively represented by *Alpha-* and *Gammaproteobacteria*. Within these two classes, the families *Devosiaceae* and *Sphingomonadaceae* were significantly more abundant [linear discriminant analysis (LDA) scores 3.8 and 4.6, respectively, $p < 0.001$]. The *Devosiaceae* were represented by OTUs of the genera *Devosia* (2 OTUs) and *Pelagibacterium* (1 OTU).

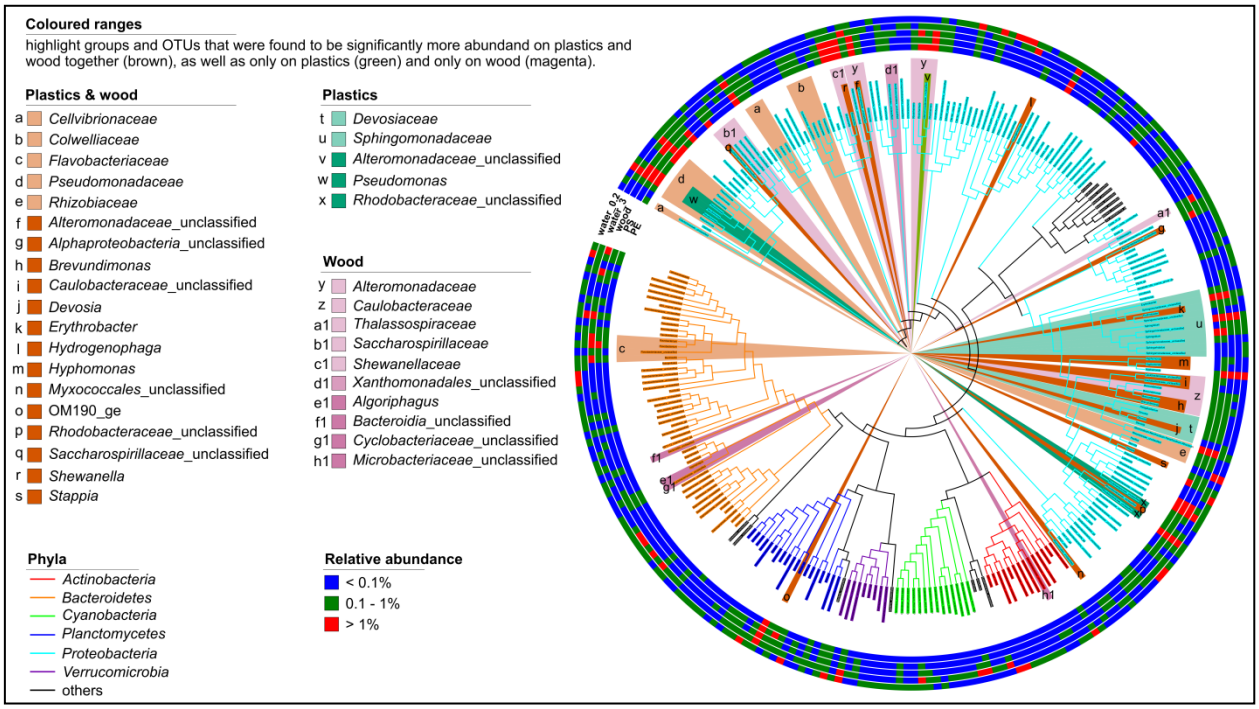

Figure 3.4. Phylogenetic tree of all bacterial OTUs with a relative abundance >0.1% in at least one sample type after 7 days of incubation. Outer rings show the relative abundances of OTUs in the PE, PS and wood biofilms, and on seston (≥3 µm, water_3) and in the free-living fraction (3–0.22 µm, water_0.2) for both the treatment and the control incubations combined. The branch color depicts the phylogenetic affiliation of the OTUs; the background color-ranges highlight the phylogenetic groups or OTUs that differentiated the assemblages on PE, PS and wood (brown) vs. those on seston and in the free-living fraction, and those on plastics (green) and wood (magenta) alone. *Proteobacteria* were discriminant for plastics but, for clarity, are not highlighted.

Among the *Sphingomonadaceae*, 5 OTUs could not be further classified, but 2 OTUs belonged to the genus *Sphingobium*, and 1 OTU each to the genera *Erythrobacter* and *Sphingorhabdus*. Three OTUs from the genus *Pseudomonas* (LDA score 4.2 and 3.2, $p <$ 0.001), one unclassified OTU representing *Alteromonadaceae* (LDA score 4.0) and another representing *Rhodobacteraceae* (LDA score 4.2 and 3.2, $p < 0.001$) were also discriminant for the plastics (Fig. 3.4). The presence of some of the discriminant groups on the plastics correlated with environmental parameters. Thus, the relative abundance of *Sphingomonadaceae* correlated negatively and that of *Pseudomonas* positively with salinity ($\rho = -0.83$ and $\rho = 0.85$, respectively). Members of the *Devosiaceae* correlated negatively with PO_4^{3-} concentrations ($\rho = -0.79$). Wood and plastics shared a core assemblage of 19 phylogenetic groups compared to the 5 differential phylogenetic groups unique to the plastic-associated assemblages (Fig. 3.4). *Alpha-* and *Gammaproteobacteria*

were the phylogenetic groups that contributed most to the differential features of the core assemblage of the combined plastics and wood samples (9 and 7 members, respectively) whereas *Deltaproteobacteria*, the uncultured planctomycetes class OM190 and *Bacteroidia* contributed one member each (Fig. 3.4).

3.3.8. *Vibrio* spp. relative abundances *in situ* and after 7 days of incubation

The relative abundances of *Vibrio* spp. were higher on PE, PS, and wood than in the *in situ* samples, but differences were also detected depending on the geographic location. In all samples, the *Vibrio* population was consistently dominated by one OTU. From the 13 OTUs classified as *Vibrio*, one OTU (OTU 137) comprised 99.6% of all *Vibrio* spp. reads. This OTU was not identical to the *V. vulnificus* OTU used as the sequencing positive control.

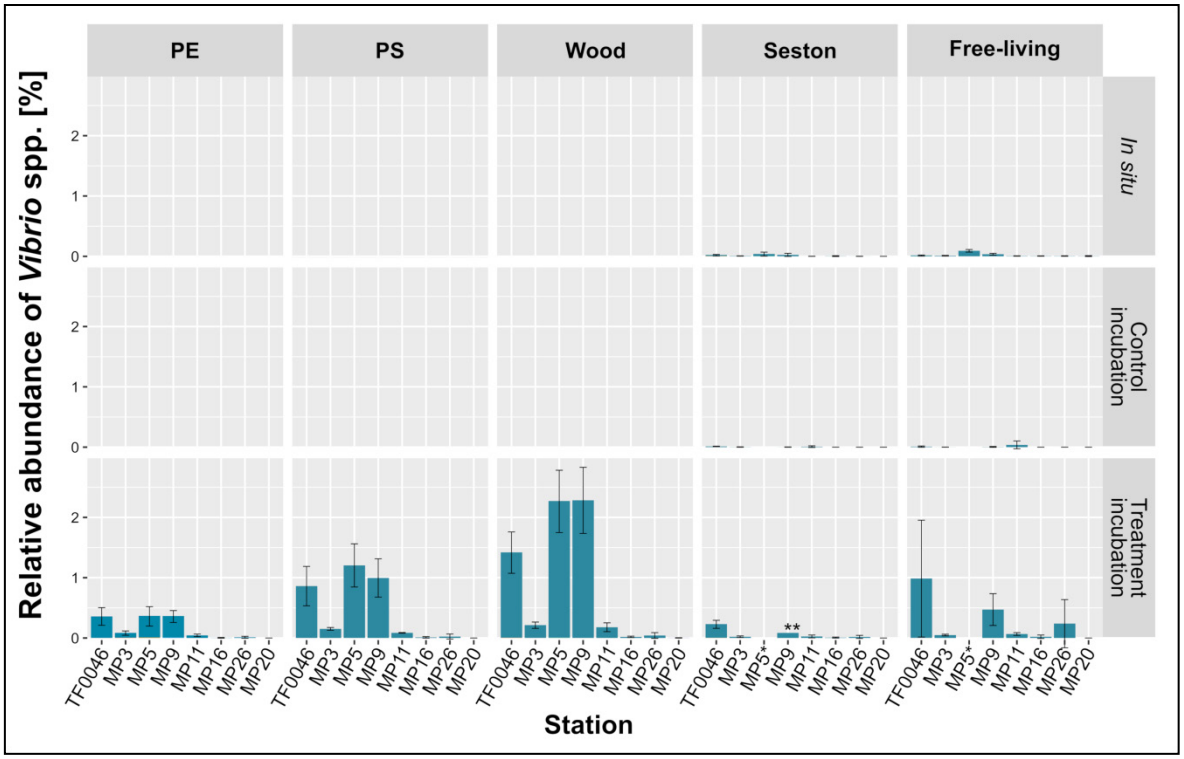

Figure 3.5. Mean relative abundances of each *Vibrio* OTU on seston (≥3 µm) and in the free-living fraction (3–0.22µm) at the different stations at t_0 (*in situ*) and after 7 days of incubation on PE, PS, wood and seston and in the free-living fraction. Data for both the treatment and the control incubations are shown. Bars indicate the standard deviation of the most abundant *Vibrio* OTU. *For station MP5, incubation water samples were not available; **only two replicates

In general, the relative abundances of *Vibrio* spp. were significantly higher on samples from the treatment than from the control incubations or compared to the *in situ* samples ($p < 0.001$, Fig. 3.5). Within the samples of the treatment incubations, relative abundances were higher on PE (0.2 ± 0.2%) and PS (0.4 ± 0.5%) than on seston (0.1 ± 0.1%), but were twice as high on wood (0.8 ± 1.0%). The concentrations of *Vibrio* spp. in the free-living fraction of the treatment incubations were in the range of those of the PE and PS samples (0.3 ± 0.5%) but were significantly higher than in either the free-living fraction of the control incubations or the *in situ* free-living fraction ($p < 0.001$ and $p = 0.02$, respectively, Fig. 3.5, Table S3.3E–H).

There was a clear difference in the relative abundance of *Vibrio* spp. between the different stations (79 out of 120 pair-wise comparisons were significant at $p < 0.001$–0.048; Table S3.3C–D). Thus, significantly higher abundances were determined at stations MP5 (0.4 ± 0.2% on PE, 1.2 ± 0.4% on PS, and 2.3 ± 0.5% on wood), MP9 (0.4 ± 0.1%, 1.0 ± 0.3%, and 2.3 ± 0.5%, respectively) and TF0046 (0.4 ± 0.1%, 0.9 ± 0.3%, and 1.4 ± 0.3%, respectively; $p < 0.001$–0.043; Table S3.3C–D). At the other stations, the mean relative abundances across all sample types were ≤0.1% (Fig. 3.5). To distinguish between the effects of sample type and stations on the relative abundances, Kruskal-Wallis tests and Conover-Iman pair-wise comparisons were conducted between sample types at each station. The relative abundances of *Vibrio* spp. differed significantly between sample types at stations TF0046, MP3, MP5, MP9, and MP11 ($p = 0.01$–0.04; Table S3.3A–B). In the Spearman correlation based on environmental parameters, only the wood samples were chosen, since they had the highest numbers of *Vibrio* reads. In these samples, the only positive correlation of *Vibrio* spp. was with salinity ($\rho = 0.76$).

3.3.9. Factors influencing the bacterial assemblages

The sample type was the most important factor driving bacterial assemblage differentiation, with a clear distinction between assemblages on PE, PS and wood versus on seston and in the free-living fraction. There was also a trend separating the PE and wood assemblages; however, when the artificially introduced substrates were investigated alone, spatial factors were dominant in shaping the biofilm assemblages. Overall, more of the variation in the complete dataset after 7 days of incubation was explained by the sample type than by the spatial factor (Fig. 3.6A). In the dbRDA plot, the different sample

types formed three clusters distributed along the first axis. Centroids of the factor "sample type" strongly correlated with the first dbRDA axis (ρ = 0.86). The assemblages on PE, PS and wood were always significantly different from those on seston and in the free-living fractions. This was the case across all stations, independent of whether the seston and free-living samples were those of the treatment or control incubations (p = 0.011–0.043, Table S3.4A–B).

However, the bacterial assemblages also differed significantly between stations (p = 0.001) and were clearly distributed along the second dbRDA axis according to station, forming two major clusters that separated stations TF0046, MP3, MP9 and MP11 from stations MP16, MP20 and MP26 (Fig. 3.6A). Centroids of the spatial factor "station" strongly correlated with the second dbRDA axis (ρ = 0.83). Together, the first two axes explained 52.5% of the fitted variation while the full dbRDA model explained 63.1% of the variation in the bacterial assemblages between the PE, PS, wood, seston and free-living fraction samples. Both were significant contributors to explaining the variation (p = 0.001) while the variation was significantly partitioned by the dbRDA axis (p = 0.001).

Tests of the homogeneity of the multivariate dispersions within groups did not yield significant results for the factor "station" (p = 0.54), whereas in some cases significant results were obtained in the global test of the factor "sample type" (p = 0.001). However, when the sample types were tested within the subset of each station, none of the pair-wise comparison produced a significant result (p = 0.22–1, Table S3.4A & C).

When only the assemblages on PE, PS and wood were compared, more of the variation between the bacterial assemblages on PE, PS and wood after 7 days of incubation was explained by the spatial factor than by sample type. "Station" was a significant factor (p = 0.001, Fig. 3.6B) and its centroids strongly correlated with the first dbRDA axis (ρ = 0.91), which explained 30.8% of the fitted variation. The samples formed four clusters along this axis: stations TF0046 and MP5 clustered together, as did stations MP9 and MP11; station MP3 formed a separate cluster between those two clusters and stations MP16, MP20, and MP26 formed a fourth, distinct cluster (Fig. 3.6B). These clusters were significantly different, as shown in pair-wise PERMANOVAs (p = 0.001). The factor "sample type" was still a significant contributor to explaining the variation (p = 0.001) but it was not one of the main factors and its centroids did not correlate with the first or second dbRDA axis (ρ = 0.05 and 0.21, respectively). The first two axes explained 51.6% of the fitted variation and the full model 71.1% of the total variation. Both the full model and the axes

significantly captured the variation within the bacterial assemblages on PE, PS and wood after 7 days of incubation (p = 0.001, respectively). The assemblages on PE differed significantly from those on wood at all stations (p = 0.02–0.042, Table S3.4B), except at stations MP20 (p = 0.075) and MP16 (p = 0.054). The assemblages on PS differed significantly from those on wood only at station MP26 (p = 0.039) whereas those on PE and PS differed significantly only at station MP3 (p = 0.035, Fig. 3.6B). The fitted response surfaces for the environmental parameters were also significant (p < 0.001), but salinity explained most of the variation (96.7%) and was also gradually arranged along the first dbRDA axis. It was therefore chosen for display in the dbRDA plot (Fig. 3.6B and Fig. S3.4).

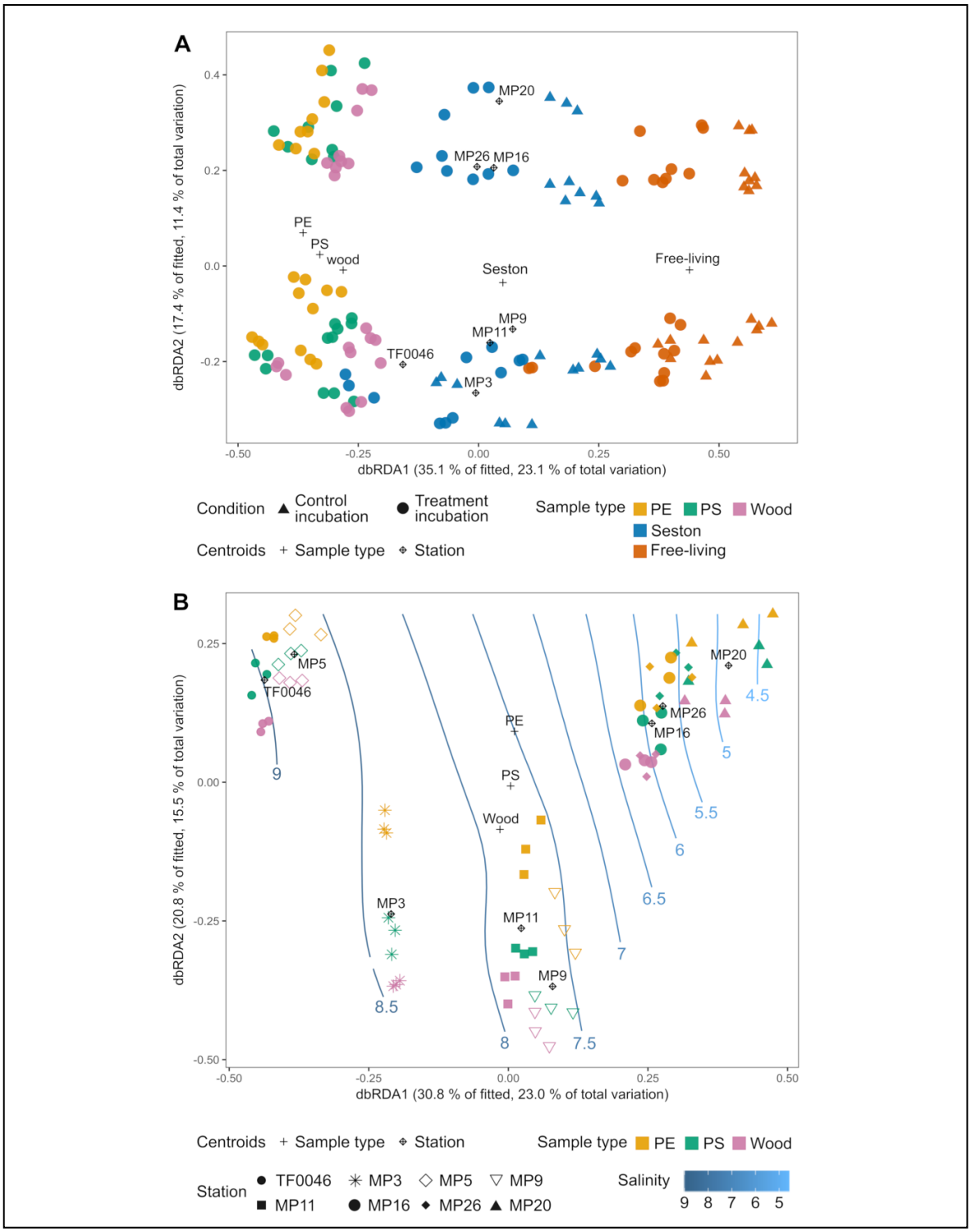

Figure 3.6. Distance-based redundancy analysis (dbRDA) ordination plots (type I scaling) based on the Bray-Curtis dissimilarities of the square-root transformed bacterial OTU read counts of (**A**) the incubated test particles (PE, PS, wood), seston (≥ 3 µm) and in the free-living fraction (3–0.22 µm) for both the treatment (filled circles) and control (filled triangles) incubations after 7 days and (**B**) the incubated test particles (PE, PS, wood) after 7 days. The plus sign and rhombus-shaped symbols depict the centroids of the constraining factors (sample type and station) used in the dbRDA model. Smooth response surfaces for salinity were fitted using penalised splines with the function 'ordisurf' from the vegan package.
For station MP5, no water samples are available and the data were therefore omitted from part A.

3.4. Discussion

In this study, young biofilms on PE and PS as well as on wood, as a natural polymer, were investigated with respect to the influence of environmental factors and different surfaces. The results were then compared to those from bacteria inhabiting seston and in the free-living water fraction. Most of the variation in bacterial assemblages could be explained by the sample type but environmental factors were dominant in the structuring of biofilm assemblages on PE, PS and wood. The relative abundances of *Vibrio* spp. were compared, on the different materials, both in the different incubations and vs. seston-attached and free-living bacteria *in situ*. The results showed that while *Vibrio* numbers were elevated on PE and PS, they were highest on wood.

3.4.1. Microplastics comprise a newly available habitat for biofilm-forming bacteria in aquatic ecosystems

Our results confirm that microplastics comprise a novel habitat in the Baltic Sea for surface-attached bacteria, as already shown in the Mediterranean Sea and in previous studies of the Baltic Sea (Dussud et al., 2018a; Oberbeckmann et al., 2018; Ogonowski et al., 2018). Despite a coastline of ~2000 km and waters of different salinities and anthropogenic inputs, in our study of the Baltic Sea the sample type was still the major factor explaining the differences between the bacterial assemblages on PE, PS and wood vs. those on seston and in the free-living fraction. However, it should be noted that the biofilms on PE, PS and wood were only 7 days old, whereas neither the age of the seston nor its colonisation history could be determined. Chao1 richness and the number of unique OTUs were lowest in the PE and PS samples, which suggests differences in the succession stages of the introduced particles vs. of seston at t_7. However, the Chao1 richness determined for wood did not differ significantly from that determined for seston. It is therefore unlikely that the dissimilarity between the bacterial assemblages on PE, PS and wood vs. either on seston or in the free-living fraction can solely be attributed to differences in succession stages. Instead, an effect of substrate type on the developing assemblages is more likely, as also shown in other *in situ* studies (Dussud et al., 2018a; Oberbeckmann et al., 2018). Differences in the assemblages present on inert surfaces and in bacteria colonising natural aggregates of biogenic origin have also been reported for

stream ecosystems (Niederdorfer et al., 2016). Although in this study the generally lower Chao1 richness within the incubation tanks might have resulted from a bottle effect arising from the incubation, comparisons among the incubations were still valid. There were also clear differences in the relative abundances of specific phylogenetic groups. *Gammaproteobacteria* was the most dominant group after the 7 days of incubation and was more abundant on PE, PS and wood than on the t_7 seston samples and in the t_7 free-living fraction. Both copiotrophic species and species identified in batch cultures as primary responders are found within the *Gammaproteobacteria* class (Eilers et al., 2000). There was a general increase of *Gammaproteobacteria* from the *in situ* samples to samples obtained from the incubation tanks at day 7, which can in part be attributed to the incubation conditions. However, other studies of aquatic biofilms also showed that *Gammaproteobacteria* are usually amongst the early colonisers of inert surfaces (Dang et al., 2008; De Tender et al., 2017; Dussud et al., 2018b; Lawes et al., 2016; Lee et al., 2008; Li et al., 2014), which according to this study includes those present in the Baltic Sea. As copiotrophs, *Gammaproteobacteria* may be able to quickly respond to the enhanced availability of the organic substances, such as proteins and polysaccharides, that adsorb to immersed surfaces.

The *Alphaproteobacteria* comprised the second most abundant group on PE, PS and wood after 7 days of incubation. Members of the *Alphaproteobacteria* are also consistently identified as primary colonisers of surfaces in aquatic systems, especially the marine *Roseobacter* clade within the family *Rhodobacteraceae* (Dang et al., 2008). Unclassified members of the *Rhodobacteraceae* were abundant on PE and PS, with ~60% of the sequences affiliated with genera within the *Roseobacter* clade (Fig. S3.5), indicating that taxa usually found in marine biofilms contribute to the young biofilm assemblages. Studies on the initial colonisation of surfaces immersed in marine waters have shown that during the first 24 h of biofilm formation *Gammaproteobacteria* were the first group to colonise glass, acrylic glass, steel and polyvinylchloride; thereafter, the surfaces were rapidly dominated by *Alphaproteobacteria* (Dang et al., 2008; Lee et al., 2008). However, studies explicitly investigating biofilm formation on artificial polymers (PE and acrylic glass) found that *Gammaproteobacteria* can dominate the assemblages during the first 7 days of incubation (De Tender et al., 2017; Dussud et al., 2018b; Lawes et al., 2016; Li et al., 2014), which may hint towards a general trend of preferential biofilm formation by *Gammaproteobacteria* on artificial polymers. *Bacteroidia*, as the third most abundant class

on PE, PS and wood, are also well known biofilm-forming bacteria occurring within marine and brackish systems (DeLong et al., 1993; Elifantz et al., 2013). Whereas they are usually the first to respond to particulate organic matter inputs such as those deriving from phytoplankton blooms (Teeling et al., 2012), on inert surfaces *Bacteroidia* seem to be late colonisers (De Tender et al., 2015, 2017), a strategy that may allow them to take advantage of the release of organic compounds by primary colonising organisms. This would explain the relatively low abundance of *Bacteroidia* in the young biofilms on PE and PS vs. on seston after only 7 days of incubation.

These results confirm that biofilms on PE, PS and wood form a habitat distinct from that of seston. However, differences between the bacterial assemblages on these three substrates were difficult to determine, despite the significantly higher Chao1 richness of the assemblages on wood. The latter observation can be attributed to the greater surface heterogeneity of wood, with its pits and cracks providing a larger number of possible microhabitats than available on the more homogeneous surface of plastics (Horner-Devine et al., 2004). Also, a pronounced phylogenetic overlap was determined between taxa discriminant for plastics only vs. plastics and wood combined. The family *Devosiaceae* was a discriminant group for plastics, but the genus *Devosia*, within the *Devosiaceae*, was also a discriminant group for plastics and wood combined. Likewise, the genus *Pseudomonas* was a discriminant taxon for plastics alone, but the family *Pseudomonadaceae* was a discriminant group for plastics and wood. Very few features were discriminant for plastics only compared to plastics and wood. Thus, in this study, the majority of the colonising organisms in the young biofilms that formed on PE and PS were general biofilm-forming taxa rather than surface-specific specialists.

Nonetheless, the families *Sphingomonadaceae* and *Devosiaceae*, the genus *Pseudomonas* and unclassified *Rhodobacteraceae* and *Alteromonadaceae* were significantly more abundant on the plastics at t_7. Many members of these groups are able to form biofilms (Dang and Lovell, 2002; López-Pérez and Rodriguez-Valera, 2014; Masák et al., 2014; Stolz, 2009). For example, *Sphingomonadaceae*, such as *Erythrobacter*, *Sphingopyxis* and *Sphingomonas*, have consistently been found in biofilms on microplastics, thus demonstrating that our results adequately reflect *in situ* conditions (Hoellein et al., 2014; Jiang et al., 2018; Oberbeckmann et al., 2018; Ogonowski et al., 2018; Zettler et al., 2013). Moreover, these organisms may represent core species of the plastic-associated microbiome. Many *Sphingomonadaceae*, including members of the genera *Erythrobacter*

and *Sphingobium*, which in this study were significantly more abundant on plastics, as well as members of the genera *Pseudomonas* and *Devosia* have been described as putative hydrocarbon degraders and have repeatedly been isolated from environments contaminated with petroleum-derived hydrocarbons (Kumar et al., 2008; Onaca et al., 2007; Stolz, 2009). They are also abundant in the biofilms that form on other organic surfaces in aquatic systems, such as brown and green algae (Burke et al., 2011; Lachnit et al., 2011; Staufenberger et al., 2008). The consistent detection of these bacteria on natural and petroleum-derived polymers has been linked to the potential degradation of marine microplastics by the respective species (Dussud et al., 2018b; Ogonowski et al., 2018; Zettler et al., 2013). Our results show that these organisms are also members of the young biofilms that develop on microplastics in the Baltic Sea. However, nothing is known whether these organisms are able to degrade the carbon-backbone of the polymers. A first metagenome study of the microplastic-associated assemblages revealed an overrepresentation of genes involved in xenobiotic degradation processes (Bryant et al., 2016), but it may also be the case that the bacteria take advantage of the volatile compounds released from the plastics even after 2 weeks, such as monomers and additives (Klaeger et al., 2019; Romera-Castillo et al., 2018), or make use of the organic pollutants that sorb to the surface of the polymers (Mato et al., 2001). These scenarios warrant further investigation.

Despite the relatively small differences between the assemblages on PE and PS vs. on wood, our study shows that plastics, as newly introduced hard substrates, are colonised by biofilm consortia that differ from those found on natural seston. Given the current quantity of plastic debris in the ocean and the predicted increase thereof (Thompson R. C. et al., 2004), the difference between plastic and natural surfaces might be negligible, with the large quantity of hard substrates newly introduced into a system that is otherwise devoid of such habitats being of much greater ecological relevance. The impact this development can have on aquatic ecosystems and its functioning needs to be acknowledged and should be carefully investigated.

3.4.2. The vector potential of microplastics for *Vibrio* depends on the life history of the particle

The relative abundance of *Vibrio* spp. was determined to facilitate comparisons across both sampling types measured in different units and different studies. Accordingly, the relative abundances of *Vibrio* spp. on PE and PS in this study were lower than those reported by Zettler et al. (2013) (24% on one sample) and Frère et al. (2018); (up to 19%). However, they were higher than the *in situ* amounts of *Vibrio* spp. on seston and in the free-living fraction collected during the study cruise. The abundances on PE, PS and wood were also higher than those reported for free-living *Vibrio* occurring in the vicinity of the Stockholm Archipelago, where the maximum was 0.002% (calculated from data in Eiler et al., 2006; and Eiler and Bertilsson, 2006). Thus, the *in situ* abundances in the free-living fraction (max. 0.09%) measured in our study were comparable to those of earlier studies and consistent with the increased abundances found on PE and PS. However, relative abundances were highest on wood (max. 2.3%), which indicated that the detected *Vibrio* OTU represented a biofilm generalist, a conclusion well in line with the findings of Oberbeckmann et al. (2018).

The relative abundances of *Vibrio* spp. on PE (max. 0.4%) and PS (max. 1.2%) in this study were higher than in most of the reported occurrences described in other studies investigating floating plastic debris in the ocean. In those studies, *Vibrio* spp. abundances ranged between 0.0032% and 0.6% (Debroas et al., 2017; Dussud et al., 2018a; Jiang et al., 2018; Oberbeckmann et al., 2018; Schmidt et al., 2014). The use of PCR-amplified amplicon sequencing in this study may have introduced a PCR-related bias (Polz and Cavanaugh, 1998). However, the *Vibrio* numbers detected are comparable to those previously obtained in a similar experimental set-up in which abundances were determined using a combination of amplicon sequencing and quantitative PCR (Oberbeckmann et al., 2018) and to the *Vibrio* abundances measured in the Baltic Sea using a quantitative competitive PCR approach (Eiler and Bertilsson, 2006), such that a severe over- or underestimation of *Vibrio* quantities in this study was unlikely. Also, the *Vibrio* abundances in the treatment incubation were significantly higher than in the control incubation after 7 days, which clearly showed that the increase in *Vibrio* spp. was not an incubation artefact.

The genus *Vibrio* is considered an r-strategist. While it is usually found in low numbers (< 0.1%) throughout the world (Eilers et al., 2000; Thompson J. R. et al., 2004), it quickly

responds to nutrient inputs (Eilers et al., 2000; Takemura et al., 2014) to reach high abundances, a reaction attributed to high growth rates and high rRNA copy numbers (Gilbert et al., 2012; Heidelberg et al., 2000; Westrich et al., 2016). This "feast or famine" strategy might explain the elevated relative abundances detected on the PE, PS and wood particles in this study after only 7 days of incubation. Thus, the identified *Vibrio* OTU may have been among the organisms able to take early advantage of the new habitats as well as the nutrients in the conditioning film. Indeed, a study investigating early succession on chitin particles showed that vibrios were amongst the very early colonisers (Datta et al., 2016).

Vibrio numbers were elevated only in the incubations with water from stations TF0046, MP5, and MP9, i.e., from Mecklenburg Bay to the Bay of Gdansk, where the salinity range is 7.7–9 PSU. The lower *Vibrio* abundances in the incubations with water from the other stations indicated that the detected *Vibrio* OTU was present along the southeastern Baltic Sea coast, but that its optimal growth occurred at salinities >7 PSU. Hood and Winter (1997) found that the attachment of different *Vibrio cholerae* strains to surfaces occurred primarily at NaCl concentrations of 1%. The attachment of *V. cholerae* and other *Vibrio* species was also shown to be impaired in the presence of low Ca^{2+} concentrations (Kierek and Watnick, 2003a, 2003b), characteristic of freshwater and waters of lower salinity (Schubert et al., 2017). The significantly lower abundances of *Vibrio* spp. on PE, PS and wood from the station MP3 incubations, in which the mean salinity was 8.2, suggested that additional factors play a role in the contribution of *Vibrio* spp. to biofilm formation. Moreover, station MP3 had the highest Chao1 richness, such that other primary colonisers may have prevented the growth of *Vibrio* sp. by outcompeting these bacteria (Rendueles and Ghigo, 2015). Our findings could account for the sporadically high abundances of *Vibrio* on the microplastics sampled *in situ* but are otherwise inconsistently detected on them. Firstly, *Vibrio* can be regarded as member of young biofilms and a putative primary coloniser of solid surfaces and would likely be absent from older particles. Secondly, environmental conditions, e.g., nutrient availability or the lack of specific salts, may have been suboptimal for *Vibrio* biofilm formation in general. Thus, the detection of elevated *Vibrio* abundances may be indicative of relatively newly colonised particles and therefore of their possible sources. Investigations of the succession of biofilm assemblages on microplastics are needed to fully assess the temporal dynamics of *Vibrio* spp. as an early

coloniser. Such studies must also take into account the "life history" of the microplastic particles to obtain a holistic risk assessment.

Chubarenko and Stepanova (2017) proposed a scheme for microplastic transport in the Baltic Sea and hypothesised that particles undergo several beaching and immersion events, which could lead to repeated cycles of colonisation, before the particles sink or are otherwise removed. This scenario suggests the importance of investigating not only the spatial scale but also the temporal dynamics of biofilm formation (De Tender et al., 2017). Of note, after 7 days the relative abundances of *Vibrio* spp. were higher in the free-living fraction in water from the treatment incubation than in the control incubation. An ability of particles to affect other compartments of the aquatic system was previously demonstrated in a study showing that a close relative of the gammaproteobacterium *Amphritea atlantica* was enriched on PS and in the respective incubation water (Kesy et al., 2016). Although this effect might be overestimated in a closed system such as an incubation tank, it still shows the potential of microplastics, including their potential leachates, to alter the assemblages in their surroundings. Accordingly, not only the changes that plastic particles and their biofilms bring to aquatic ecosystems usually void of hard substrates, but also the effect of these newly introduced substrates on the free-living bacterial assemblage must be taken into account. This is of particular importance in areas with high microplastic concentrations, such as in East Asian seas (Isobe et al., 2015).

Microplastics might not be the sole vectors for potential pathogens, as higher abundance of *Vibrio* was detected on wood. Nonetheless, with the increasing burden of microplastics in the ocean, the microplastic load may become an important dispersal vector.

3.4.3. Biofilm differentiation on microplastics differs according to the sampling location, but nutrient limitation may select for surface specificity

Oberbeckmann et al. found that location and season were prominent drivers of the biofilms that developed on PET after 6 weeks of incubation at different stations in the North Sea (Oberbeckmann et al., 2014, 2016). Amaral-Zettler et al. (2015) demonstrated that plastic-associated biofilms sampled in the Atlantic and Pacific oceans showed biogeographic patterns that separated the assemblages found in these systems. However, other studies found no differences in the plastic-associated biofilms from different geographic locations. Dussud et al. (2018a) sampled microplastics in the western Mediterranean Basin and were

unable to differentiate among the bacterial assemblages based on the sampling site. Likewise, Bryant et al. (2016) found no evidence of spatial differences along a ~2000-km transect across the Pacific Ocean. By contrast, in our study the sampling location was the most important factor structuring the plastic- and wood-associated bacterial assemblages. This was evident from the dbRDA based on the OTU level as well as the relative abundances of higher-order phylogenetic groups. The prominent geographic influence observed in this study but not in contrast to the findings of Dussud et al. (2018a) and Bryant et al. (2016) was most likely due to the environmental heterogeneity of the Baltic Sea. Thus, *Gammaproteobacteria* were more abundant on PE, PS and wood particles exposed to the higher-salinity western stations TF0046–MP5 and less abundant at the lower-salinity eastern stations MP9–MP26. The relative abundances of *Alphaproteobacteria* increased from the western towards the eastern stations (MP9–MP26) after 7 days of incubation.

We argue that salinity, and not other nutrients, was the main driver of the differentiation of the PE-, PS-, and wood-associated assemblages because salinity is the major factor differentiating bacterioplankton assemblages globally (Lozupone and Knight, 2007), including in the Baltic Sea, where bacterial assemblages were previously shown to be influenced by salinity rather than by inorganic nutrient concentrations (Herlemann et al., 2011, 2016; Rieck et al., 2015). Additionally, the three distinct clusters of the assemblages apparent from the dbRDA did not accord with the clustering of the stations when environmental parameters were considered, assuming the equal importance of each one (Fig. S3.6). However, a role for other factors in bacterial assemblage differentiation was suggested by the bacterial assemblages from station MP3 (mean salinity 8.2 PSU), which clustered between the tightly clustered samples from the higher saline stations TF0046 and MP5 (8.5–9PSU) and the cluster derived from the intermediate saline stations MP9 and MP11 (7.7 PSU).

Robust conclusions regarding the factors influencing biofilm formation require investigations performed under controlled conditions (Ogonowski et al., 2018). Thus, despite the challenges posed by extrapolating the results obtained in incubation experiments to natural systems, our interpretation can be considered as valid, since all incubations were subjected to the same environmental pressure. Although *in situ* incubations are closer to natural systems, those performed along a 2000-km transect do not

allow the exclusion of factors such as differences in hydrodynamics, solar radiation or temperature, which would make any interpretation of the results even more challenging.

Of note, we were able to differentiate early biofilms even along a relatively moderate salinity gradient (4.5–9 PSU) almost exclusively within the mesohaline range (Anonymous, 1958). Differences in biofilm assemblages likely reflect already-existing differences in the respective source community, indicating the importance of the inoculum on the resulting biofilm assemblage (Crump et al., 2012; Ruiz et al., 2014). However, even when the detected phylogenetic groups were present in equal abundances in the source community at t_0, differences in their relative abundances on PE, PS and wood emerged after 7 days of incubation (e.g. *Alphaproteobacteria*), which suggests the contribution of additional factors to the community composition of young biofilms. Studies on the attachment behaviour of bacterial isolates have shown that ionic composition and concentrations influence substrate adhesion, such that the degree of attachment of the same bacterial species on PE and PS may have been determined by the different salinities (Bakker et al., 2004; Karatan and Watnick, 2009). Adhesive and biofilm polymers of *Pseudomonas* spp. isolated from freshwaters and marine waters were previously shown to differ in their responses to electrolyte addition, resulting in reduced biofilm thickness in the freshwater isolate, but not in the marine strain (Fletcher et al., 1991).

Oberbeckmann et al. (2018) found that substrate type was more important at low nutrient concentrations and higher salinity than at high nutrient conditions and lower salinity. Inorganic nutrients are generally depleted in summer in the Baltic Sea, following the spring diatom bloom, with nitrogen being the most limiting nutrient (Schneider et al., 2017). This was also the case during the study period, in August and September 2015, except at stations MP16 and MP20 in the Gulf of Finland, which were not initially nitrogen-limited. Those stations also had the highest initial concentration of dissolved organic carbon (DOC). Notably, the biofilm assemblages on PE and wood did not differ significantly at these two stations, unlike at all other stations. This could have been due to a difference in the condition films, with the higher DOC concentrations at MP16 and MP20 masking the surface properties of the materials (Lorite et al., 2011). Alternatively, differences in surface specificity may depend on the adhesion capacity of the bacteria themselves. Previous studies have shown that the capacity to induce biofilm formation can depend on the nutritional status of the bacterial cells and that bacteria under nutrient-limitation differ in their surface attachment behaviours (Allan et al., 2002; Karatan and Watnick, 2009). In

general, these findings corroborate the results of Oberbeckmann et al. (2018) and further suggest that, even at overall low inorganic nutrient concentrations, nutrient ratios could play a role in determining surface specificity. Further research on the role of the conditioning film in surface specificity vs. whether and how limitations in inorganic nutrients serve as a driver of surface-specific bacterial attachment on diverse microplastics is needed.

To date, we still do not know much about the dynamics and successional changes in microplastic-associated assemblages that occur as the particles are subjected to different environments characterised by different local communities, such as during transport by currents and winds. Studies thus far have shown that microplastic-associated biofilms are unstable after a disturbance and that the local environment acts as a selective force (Kesy et al., 2016, 2017).

3.5. Conclusion

Even along a moderately distinct environmental gradient, the assemblages on PE, PS, and wood differed in terms of their Chao1 richness and composition from assemblages on seston and in the free-living fraction. This observation demonstrated the importance of location in determining the assemblages on these three substrates. Our study also showed that the formation of surface-specific biofilms may depend on inorganic nutrient availability and that the relative abundances of the dominant *Vibrio* OTU in the young biofilms that formed on PE, PS, and wood were linked to geographic location and correlated positively with salinity. Thus, while microplastics comprise a novel habitat for biofilm-forming bacteria, environmental factors, especially salinity, greatly influence the composition of biofilm assemblages. In contrast to other studies, we detected a higher abundance of *Vibrio* spp. on microplastics but also on wood, consistent with a role for *Vibrio* in young biofilms. Taken together, our results highlight the need to take into account spatial factors, the temporal dynamics of biofilm formation and the “life history” of the particles to assess the full importance of microplastics as a new habitat and potential vector for surface-associated bacteria in aquatic systems.

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List of figures

List of abbreviations

µL	Microlitre
µm	Micrometre
µM	Micromol/litre
16S rRNA	16S ribosomal ribonucleic acid
BLAST	Basic Local Alignment Search Tool
d	Days
dbRDA	Distance-based Redundancy Analysis
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EPS	Extracellular polymeric substances
Fig.	Figure
h	Hours
HDPE	High density polyethylene
km	Kilometres
L	Litre
LDA	Linear discriminant analysis
m	Metres
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
max.	Maximum
min	Minutes
mL	Millilitre
MP	Microplastics
MT	Megatonnes
NCBI	National Center for Biotechnology Information
nMDS	Non-metric Multidimensional Scaling
nt	Nucleotides
OH-PIN	Overlapping and Hierarchical modules in Protein Interaction Networks
OTU	Operational taxonomic unit
PA	Polyamide
PCR	Polymerase chain reaction
PE	Polyethylene
PERMANOVA	Permutational multivariate analysis of variance
PERMDISP	Distance-Based Tests for Homogeneity of Multivariate Dispersions
PET	Polyethylene terephthalate
PS	Polystyrene
PSU	Practical salinity units
rcf	Relative centrifugal force
S	Svedberg

sp.	Species
spp.	Species pluralis
SSCP	Single-strand conformation polymorphism
UPGMA	Unweight pair group method with arithmetic mean
VBNC	Viable but non-culturable
WW	Wet weight

Supplementary material

Chapter I

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

The supplementary material includes a brief description of the preliminary feeding experiments conducted and a detailed description of the molecular methods used. A scanning electron microscopy image of the polystyrene particles and glass beads used in the final feeding experiment is provided (Fig. S1.1) and a phylogenetic tree that gives the phylogenetic affiliations of sequenced SSCP bands (Fig. S1.2).
Two tables with the p-values obtained by PERMANOVA and PERMDISP are provided (Table S1.1 & S1.2)

Supplementary material and methods

Pre-experiment for assessment of optimal experimental procedure

For determination of optimal experimental settings, pre-experiments were conducted to determine optimal particle size and optimal time point for sampling of faeces to ensure particle egestion. One litre test beakers filled with 700 mL natural sediment and equipped with one specimen of *A. marina* each were tested for particle size ranging from 212 to 630 µm and particles with a determined size of 1 mm, respectively. Particles were mixed into the upper sediment layer and faeces were checked daily for occurrence of particles for 8 days. For testing optimal control particles, lignin (Sigma-Aldrich) and chitin powder (Biolog Heppe; both with an average particle size of 30 µm) were tested as described above.

DNA extraction and polymerase chain reaction (PCR)

For nucleic acid extraction, the polystyrene and glass particles were transferred with a sterilized scalpel from the gauze pieces into 2 mL reaction tubes (Eppendorf). Pelleted faeces and sediment (250–500 µL each) were suspended with SDS solution (stock solution: 4.8 mL 20% sodium dodecyl sulphate, 1.2 mL 0.5 M sodium acetate and 33.2 mL

water, PCR grade) in Falcon tubes and then transferred to 2 mL reaction tubes. Pelleted cells from the water samples were suspended with the water residue within the Falcon tubes and filtered over autoclaved 30-µm gauze to remove any possible remaining particles. Cell lysis as well as DNA extraction and purification followed a modified phenol-chloroform protocol adapted from Weinbauer et al. (2002). Six siliconised zirconium beads (3 × 2 mm and 3 × 3 mm) were added to the samples in 2mL reaction tubes. Equal volumes (500 µL) of phenol-chloroform (pH 7.5) and SDS were added and the cells were broken by bead beating for 2+1 min. The tubes were centrifuged for 2 min at 4°C and ~20,800 rcf. The aqueous phase was transferred to a new tube and the extraction step repeated. The two aqueous phases were combined, shaken, and centrifuged for 5 min at 4°C and ~20,800 rcf. The aqueous phase was purified by the addition of an equal volume of phenol (pH 7.5), followed by two consecutive washing steps with equal volumes of chloroform (5 min at 4°C and ~ 20,800 rcf). DNA was precipitated overnight at −20°C with 2.6 volumes of a mixture of ice-cold ethanol (abs.) and 3 M sodium acetate (25:1) and 1.3 µL of glycogen. The DNA was pelleted by centrifugation (50 min at 4°C and ~ 20,800 rcf), washed twice with 70% ice cold ethanol (15 min at 4°C and ~ 20,800 rcf), and then treated as described in Eichler et al. (2004). For the working solution, the DNA was resuspended in H_2O and kept at −20°C. The DNA content was measured photometrically using NanoDrop (NanoDrop Technologies). DNA was amplified using bacterial primers modified from Schwieger and Tebbe (1998), with hybridisation positions on the 16S rRNA gene in *E. coli* 519–536, with the sequence 5' CAGCAGCCGCGGTAATAC 3' (Com1f), and positions 907–925, with the sequence 5' CCGTCAATCCTTTGAGTTT 3' (Com2r). About five ng of DNA served as the template. Thermocycling (FlexCycler, analytik jena) started with an initial denaturation step at 94°C for 1 min, followed by 30 cycles (1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C) and a final elongation step at 72°C for 4 min. The amplification of bacterial DNA from the 7 d pre-incubated polystyrene particles (before their addition to the aquaria) was difficult because no PCR product could be obtained under the conditions tested. A nested PCR was therefore performed as described in Labrenz et al. (2004; and references therein).

16S rRNA gene-fingerprinting

Single-strand conformation polymorphism (SSCP) gel electrophoresis was carried out as described in Dohrmann and Tebbe (2004) with slight modifications: The PCR products were digested directly with lambda exonuclease (Thermo Scientific) for 2.5 h at 37°C and purified with the NucleoSpin kit (Macherey-Nagel) according to the manufacturer's instructions. The single-stranded DNA was concentrated in a SpeedVac (Eppendorf). The gels were silver stained according to the protocol by Lee et al. (1996) but their development was stopped using disodium-ethylenediaminetetraacetic acid (0. 5 + 2% glycine); they were then impregnated with 10% glycerol.

Phylogenetic analysis

For the phylogenetic analysis of major contributing OTUs and those OTUs occurring only in certain samples, the respective gel bands were excised and re-amplified following the protocol of Dohrmann & Tebbe (2004) except that the excised bands were eluted at 4°C overnight and up to 3 µL of the eluate served as the template for the subsequent PCR. The amplification conditions were as described above but thermocycling was reduced to 25 cycles and final elongation lasted for 10 min. The PCR products were sequenced at LGC Genomics (Berlin, Germany); the forward and reverse sequences were assembled and quality checked using the Seqman software (DNAStar). Only assembled sequences with <2% ambiguities (Quast et al., 2013) were analysed further. The obtained sequences were aligned using the SINA aligner (Pruesse et al., 2012) and a phylogenetic tree of bacterial OTUs was constructed in ARB based on the SILVA SSU ref. NR99 release 123. A neighbour-joining tree was constructed as described in Labrenz et al. (2007).

Quantitative PCR

To verify the relative abundance of the *Amphritea atlantica* OTU, quantitative PCR (qPCR) was conducted with *Amphritea*-specific primers designed within ARB using the implemented probe design tool (Ludwig et al., 2004). The primer sequences were tested *in silico* within ARB; candidates with the highest specificity were picked and synthesized at Eurofins Genomics (Ebersberg, Germany). The primers were named according to the type strain abbreviation in the SILVA tree (Pruesse et al., 2007) and the base position in *E. coli.* The forward primer AmrAtlan 444f had the sequence 5' GTGAGGAAAGGTTGTAGC 3'

(*E. coli* position 444–462) and the reverse primer AmrAtlan823r the sequence 5' GTGTCCCAACGGCTAGTA 3' (*E. coli* position 823–841). The primers were checked for specificity and optimal annealing temperature *in vitro* using the primer pair within a gradient PCR containing DNA of *A. atlantica* and *Haliea salexigens* (Urios et al., 2008) as the positive and negative controls, respectively. DNA was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). One OTU of the genus *Haliea* was found in the faecal samples. *In silico* testing of the chosen primer pair showed that *Haliea* sequences were also susceptible to amplification when one mismatch was allowed, thus it was chosen as negative control. After successful evaluation of the specificity of the primer pair towards *A. atlantica*, qPCR was run under the following conditions. The PCR mix consisted of 7.5 µL of 2 × KAPA SYBR FAST qPCR Master Mix, 0.3 µL of 10 µM of the forward and of the reverse primers, 5.9 µL of DEPC water, and 1 µL of template DNA. Both primer pairs (AmrAtlan 444f/AmrAtlan 823r and Com1f/Com2r) were used in the same run to first determine the abundance of *A. atlantica* and then the overall bacterial abundance in the sample (Labrenz et al., 2004). Serial dilutions of the samples (no dilution, 1:2, 1:5, 1:7, 1:10) were run in triplicate using an iCycler (Bio Rad) with the following protocol: Initial denaturation for 4 min at 95°C followed by 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 57°C), an elongation step (45 s at 72°C) and fluorescence detection (10 s at 83°C). Final elongation consisted of 7 min at 72°C, 30 s at 95°C, and 30 s at 50°C, followed by melting curve analysis (50°C + 0.5°C/10 s until 92°C). To determine a standard curve and calculate PCR efficiency, a dilution series (no dilution, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of an *in-vitro*-expressed amplicon (~1400 nt) of the 16S rRNA gene of *A. antlantica* was included in each qPCR run. The relative abundance of *A. atlantica* was then calculated according to Labrenz et al. (2004).

Supplementary figures

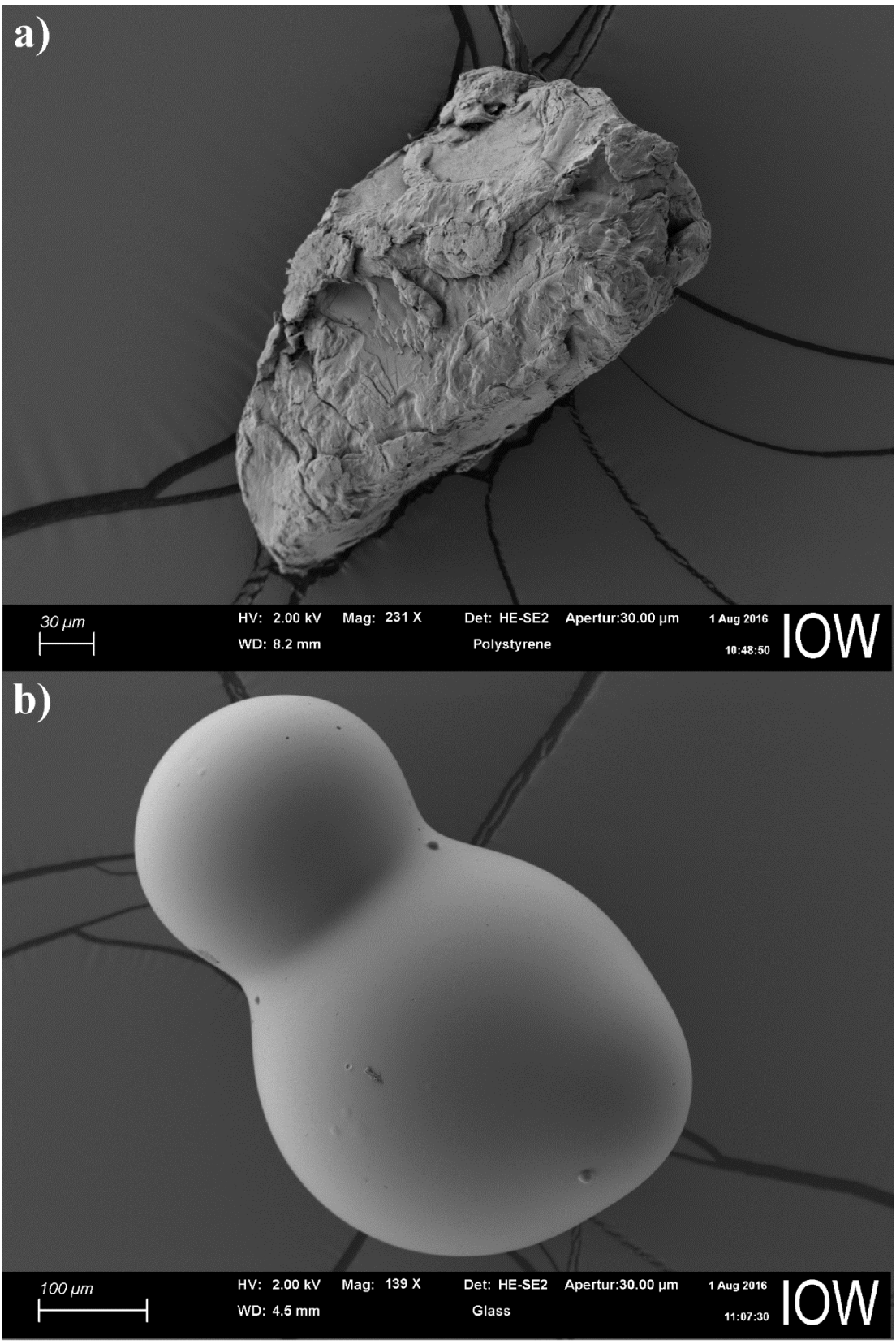


Figure S1.1. Scanning electron micrograph of the polystyrene particles (a) and glass beads (b) used in the feeding experiment.

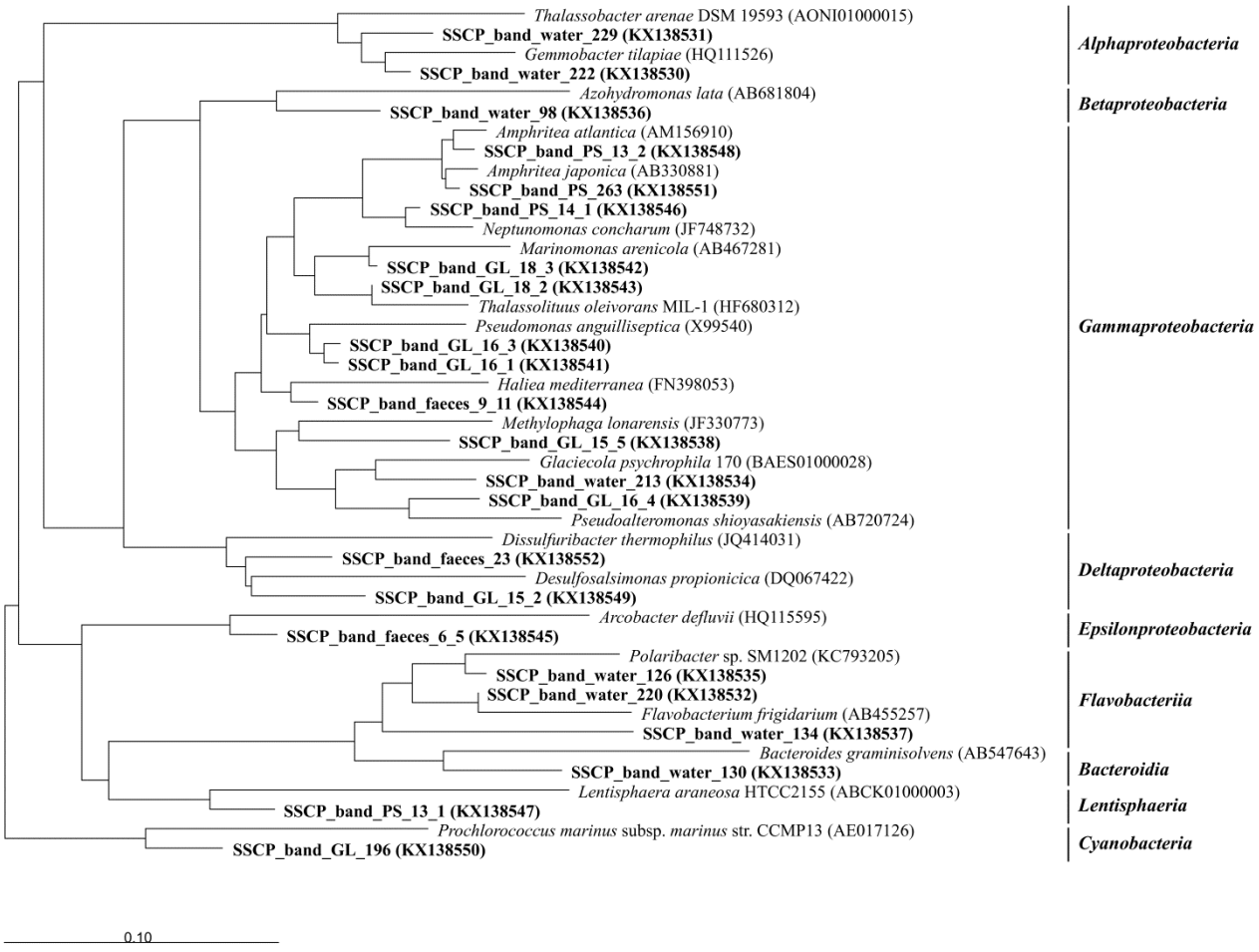


Figure S1.2. Neighbour-joining tree based on the Silva SSU ref. NR99 release 123, showing the phylogenetic affiliations of the identified SSCP bands (in bold) from faeces, water, polystyrene (PS), and glass (GL) samples. Only bacterial sequences were included. The tree was rooted with members of the family *Clostridia* as outgroup. Bar: 10 substitutions per 100 nucleotides.

Supplementary tables

Table S1.1. Pairwise comparison of sediment and faecal samples and associated particles (polystyrene or glass).

	PERMANOVA		PERMDISP	
Groups	t	P(MC)	t	P(perm)
S, PS_F	29.271	**0.028**	4.51	0.056
S, PS_S	5.322	**0.004**	0.437	0.653
S, F	73.001	**0.001**	0.119	0.927
S, GL_S	96.209	**0.002**	3.688	0.062
S, GL_F	61.739	**0.004**	0.075	0.93
PS_F, PS_S	31.513	**0.048**	No test	
PS_F, F	2.582	0.073	1.191	0.531
PS_F, GL_S	27.001	0.101	No test	
PS_F, GL_F	15.214	0.251	No test	
PS_S, F	6.325	**0.006**	0.041	1
PS_S, GL_S	85.511	**0.01**	No test	
PS_S, GL_F	59.338	**0.018**	No test	
F, GL_S	10.023	0.388	1.124	0.66
F, GL_F	10.713	0.35	0.061	1
GL_S, GL_F	15.727	0.185	No test	

PERMANOVA (pairwise comparison) based on Monte Carlo permutations [P(MC)] and PERMDISP results based on random permutations [P(perm)] between groups of particle/bead samples (PS = polystyrene, GL = glass) that were extracted from sediment (S) or faeces (F) respectively and bulk sediment (S) and bulk faeces (F) samples 4 d after addition of particles and beads to the set-ups. Significant differences obtained with PERMANOVA are highlighted in bold ($p < 0.05$).

Table S1.2. Pairwise comparison of freshly egested particle samples and egested samples, which had been incubated for 24 h in seawater.

	PERMANOVA		PERMDISP	
Groups	t	P(MC)	t	P(perm)
PS_24h, GL_24h	20.811	0.077	0.4992	0.78
PS_24h, F_24h	6.012	**0.001**	19.559	0.131
PS_24h, GL_0h	43.853	**0.01**	28.726	0.309
PS_24h, PS_0h	43.212	**0.007**	10.594	0.607
GL_24h, F_24h	65.842	**0.001**	0.871	0.574
GL_24h, GL_0h	47.663	**0.017**	15.502	0.393
GL_24h, PS_0h	46.241	**0.004**	0.2817	0.886
F_24h, GL_0h	17.406	0.076	20.706	0.221
F_24h, PS_0h	22.944	**0.011**	0.6605	0.581
GL_0h, PS_0h	10.861	0.356	25.014	0.386

PERMANOVA (pairwise comparison) based on Monte Carlo Permutation [P(MC)] and PERMDISP results based on random permutations [P(perm)] between groups of samples that were extracted from faecal material (0 h) and those samples, which had been incubated subsequently in seawater for 24 h. PS_0h = polystyrene particles isolated from faeces and analysed directly; GL_0h = glass particles that had been isolated from faeces and analysed directly; F_24h = faeces that had been incubated in seawater for 24 h; PS_24h = polystyrene particles that had been isolated from faeces and subsequently incubated in seawater; GL_24h = glass particles that had been isolated from faeces and subsequently incubated in seawater for 24 h. Significant differences obtained with PERMANOVA are highlighted in bold ($p < 0.05$).

Chapter III

Spatial environmental heterogeneity determines young biofilm assemblages on microplastics in Baltic Sea mesocosms

Supplementary material and methods

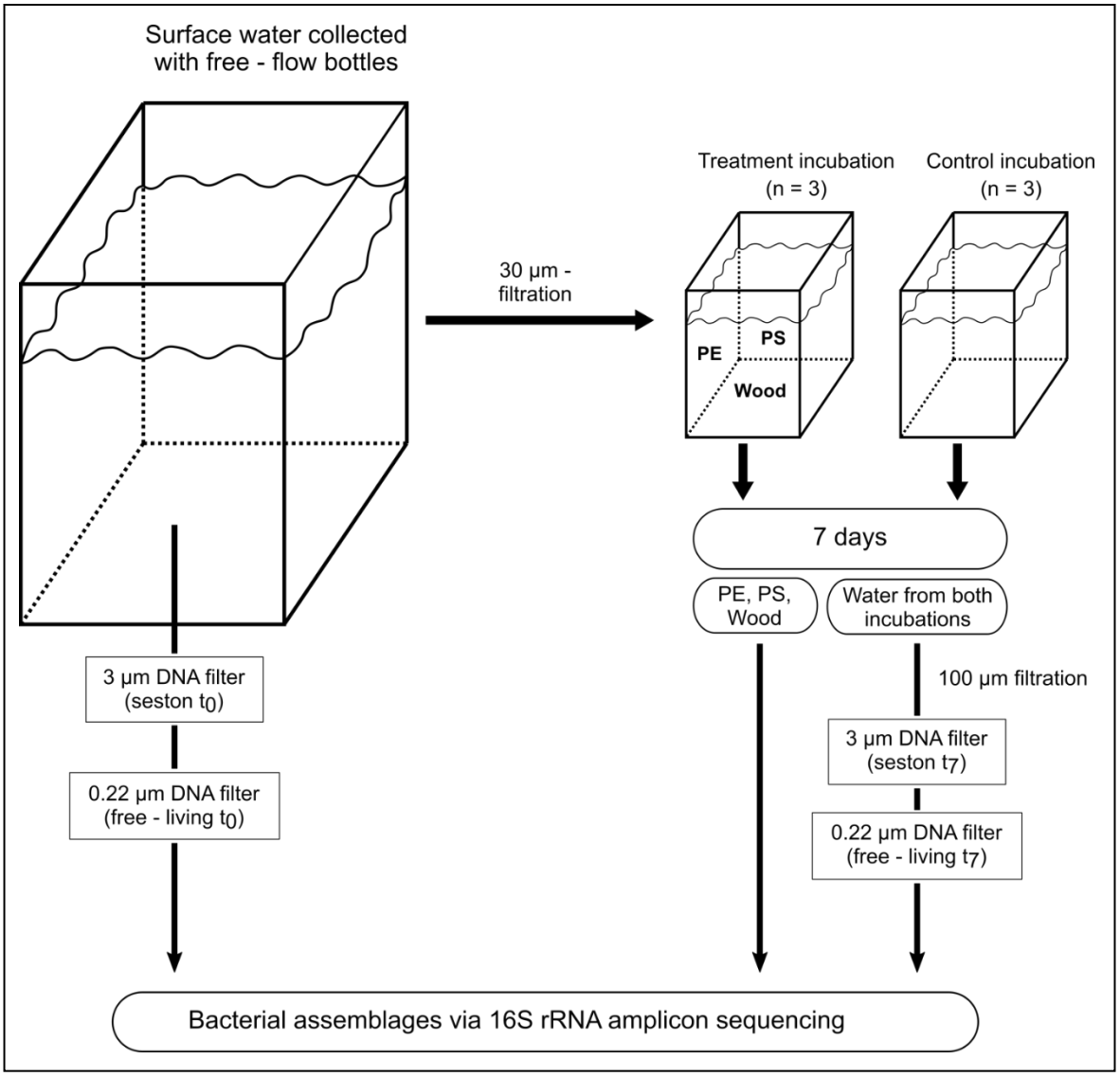
Hierarchical clustering of stations according to environmental parameters

For comparing physico-chemical parameters of the stations, hierarchical clustering based on Euclidian distance of the z-transformed data was conducted using the Ward method. The means between the t_0 and t_7 data were used as input. All calculations were done in the R program for Statistical Computation (R Core Team, 2017) using the function 'vegdist' for Euclidean distances (Oksanen et al., 2018) and 'base' functions for transformations ('scale') and hierarchical clustering ('hclust').

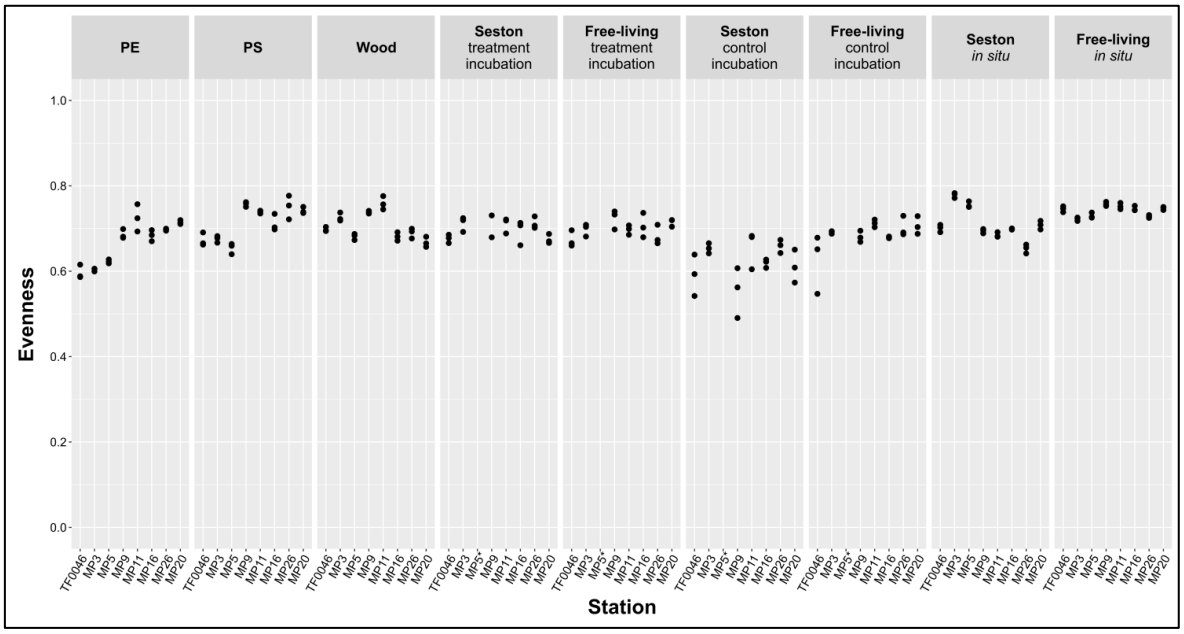
Unclassified *Rhodobacteraceae* tree

To gain insight into the phylogenetic affiliations of the unclassified *Rhodobacteraceae*-OTUs, the representative sequences for these OTUs only found on the PE and the PS were retrieved using the mother commands 'get.oturep' and 'get.lineage'. The OTUs present only on the PE and the PS were then picked manually, resulting in 116 representative sequences. These were aligned using the SINA online tool (Pruesse et al., 2012). Aligned sequences were in loaded into the complete bacterial SSU tree Ref. Nr. 99 Release 132 in ARB (Ludwig et al., 2004) using ARB parsimony. Neighbouring sequences as well as close type strains were then chosen and a baseline tree was constructed only with these sequences using the Neighbour Joining method with bootstrapping (1000) and Jukes-Cantor correction. The genus *Acidimicrobium* (*Actinobacteria*) was used as outgroup. The unclassified *Rhodobacteraceae* sequences were then added into this baseline tree using ARB parsimony. The tree was visualised using the iTOL online tool (Letunic and Bork, 2016).

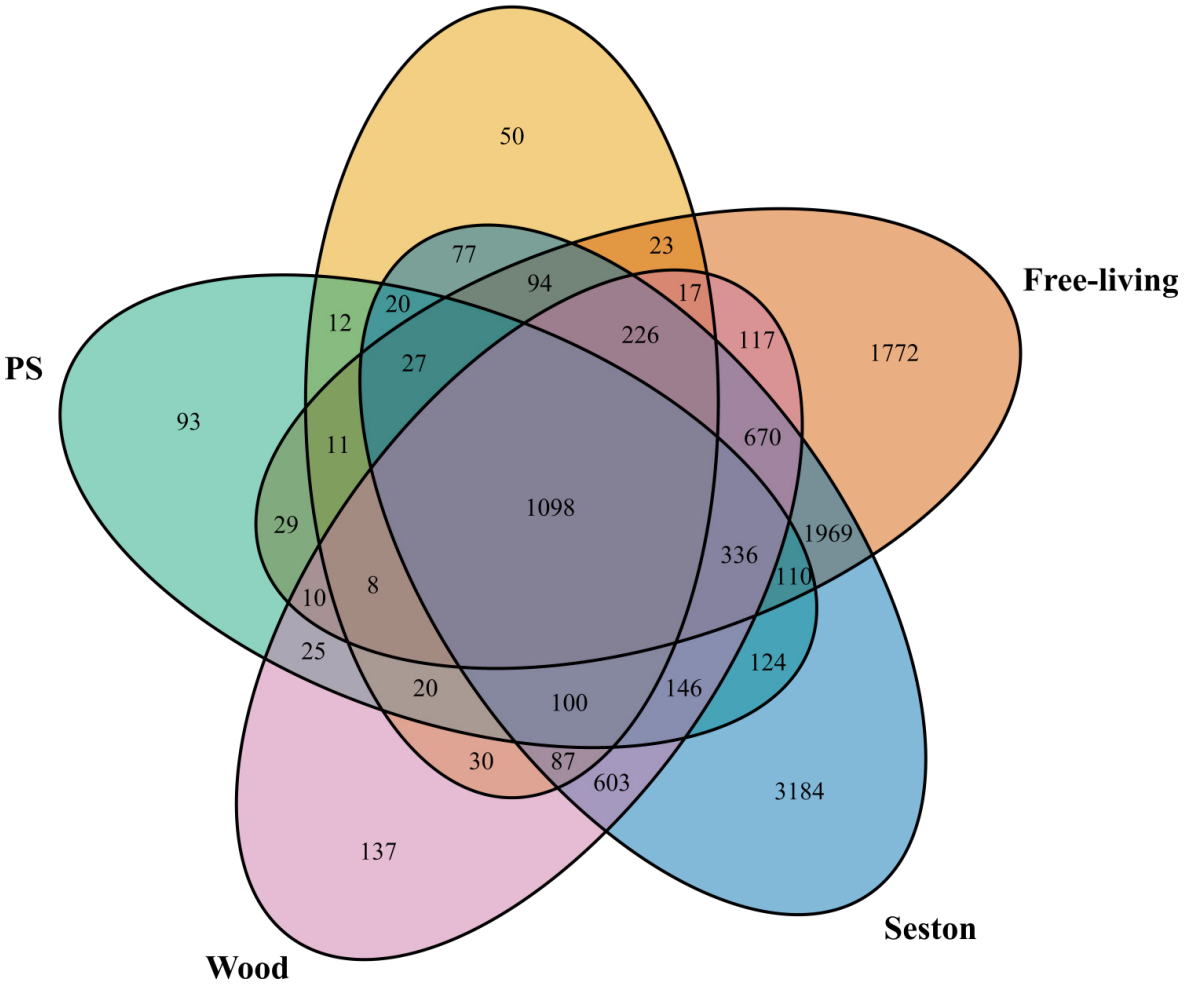
Supplementary figures



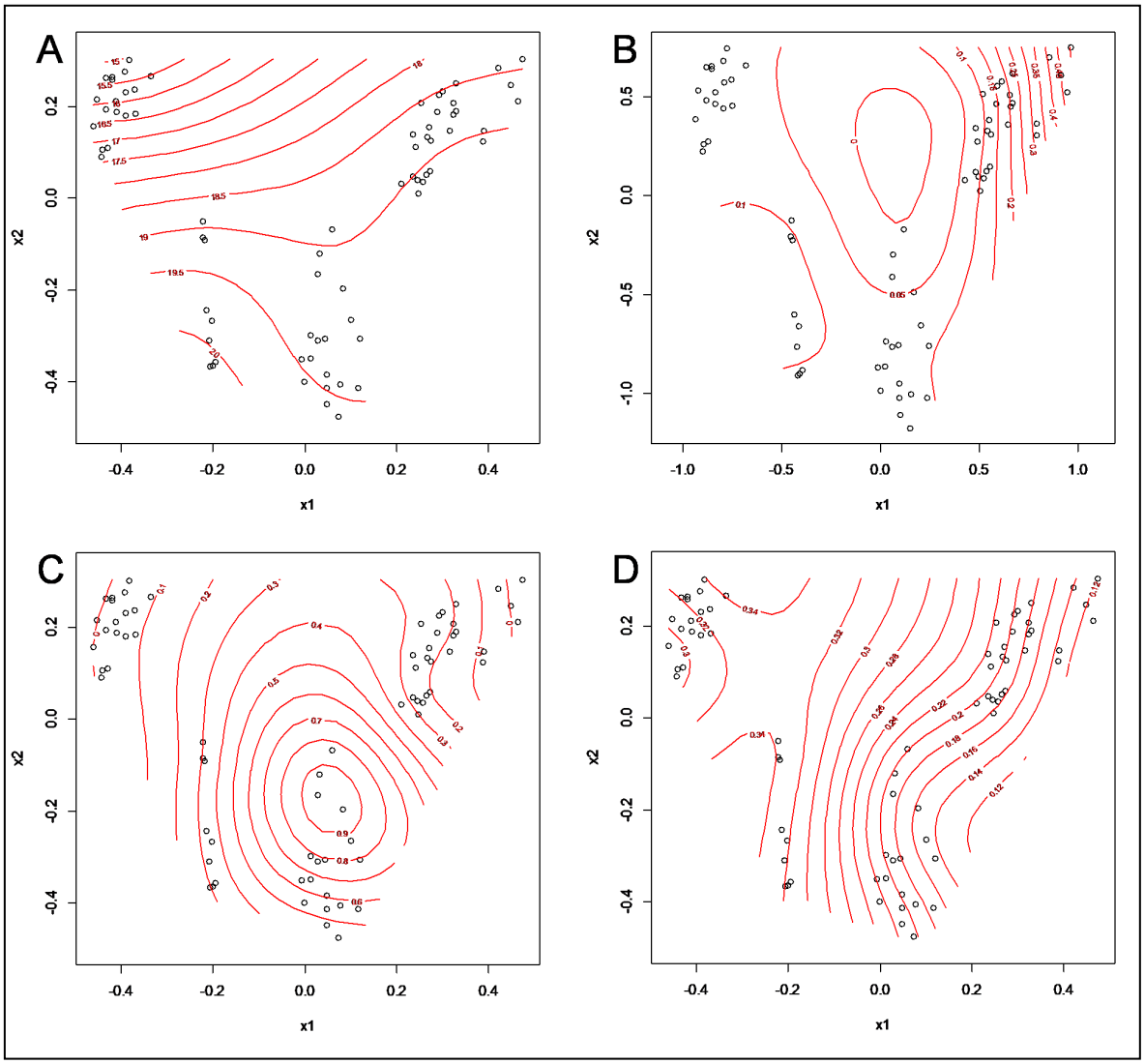
Supplementary Figure 3.1. Schematic overview over the experimental set-up and sampling procedure. Surface water from within the first 5 m was collected using a CTD-rosette equipped with free-flow bottles. Water from the bottles was mixed to create a homogenous starting community (represented as the bigger tank). Seston-attached bacteria of the t_0 community (*in situ*) were collected on 3 µm pore-size filter, and the free-living bacterial fraction on 0.22 µm pore-size filters (3 technical replicates). The water was then 30 µm filtered to exclude bigger grazers and distributed into incubation tanks (1.5 L). Polyethylene (PE), polystyrene (PS) and wood pellets were introduced into the treatment incubation, a control was run without the introduction of pellets (control incubation).
Incubations were run for 7 days at ambient temperature (20°C) and a light/dark rhythm between 19/5 h and 18/6 h. Incubations were aerated with aquarium diffuser stones.



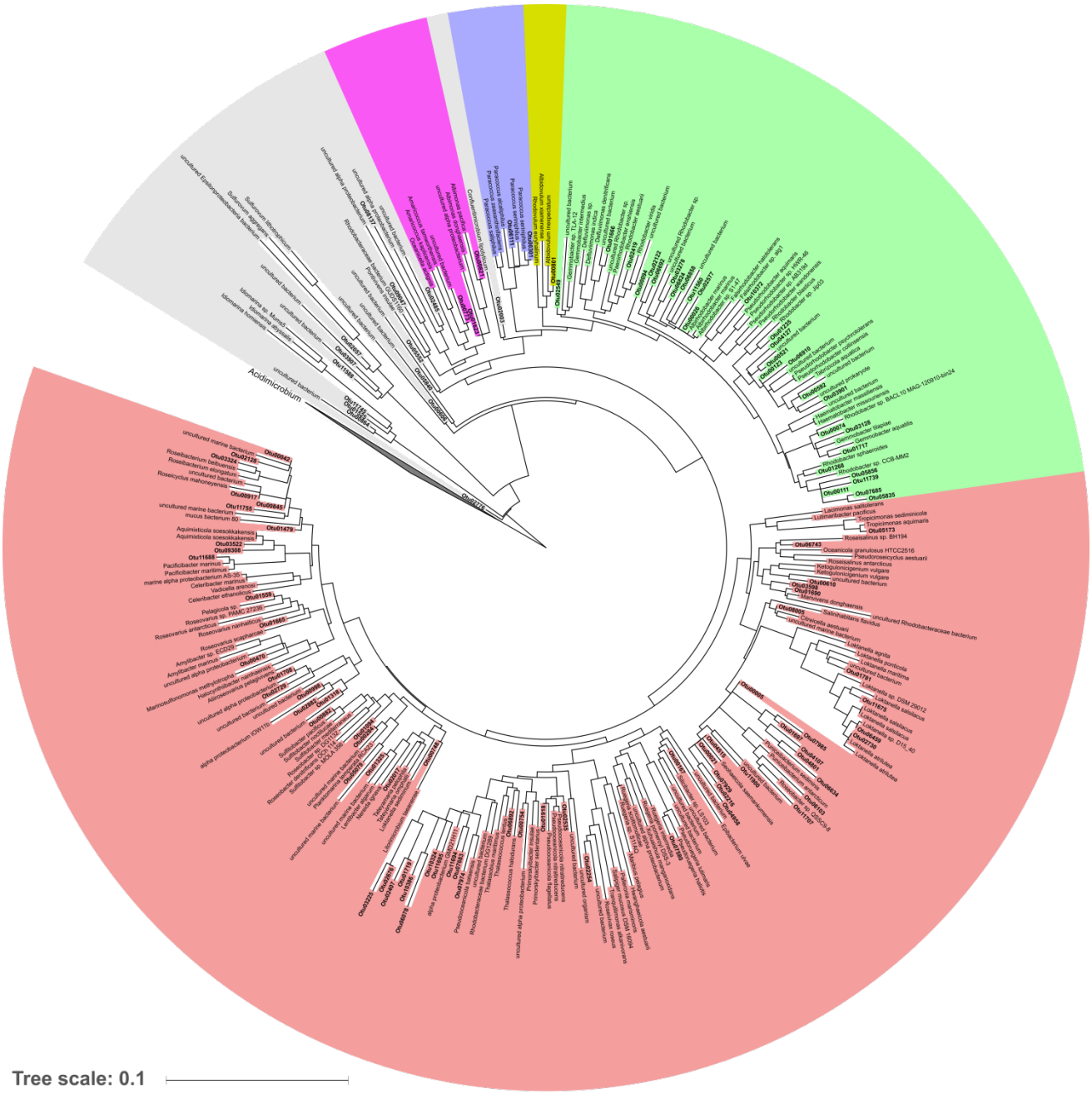
Supplementary Figure 3.2. Pielou's evenness based on bacterial OTUs on seston (≥3 µm) and in the free-living fraction (3 – 0.22 µm) of the water at different stations at t_0 (*in situ*) and after 7 days of incubation on the PE, PS and wood, and on seston and in the free-living fraction for both the treatment and control incubations. *For station MP5, incubation water samples were not available.



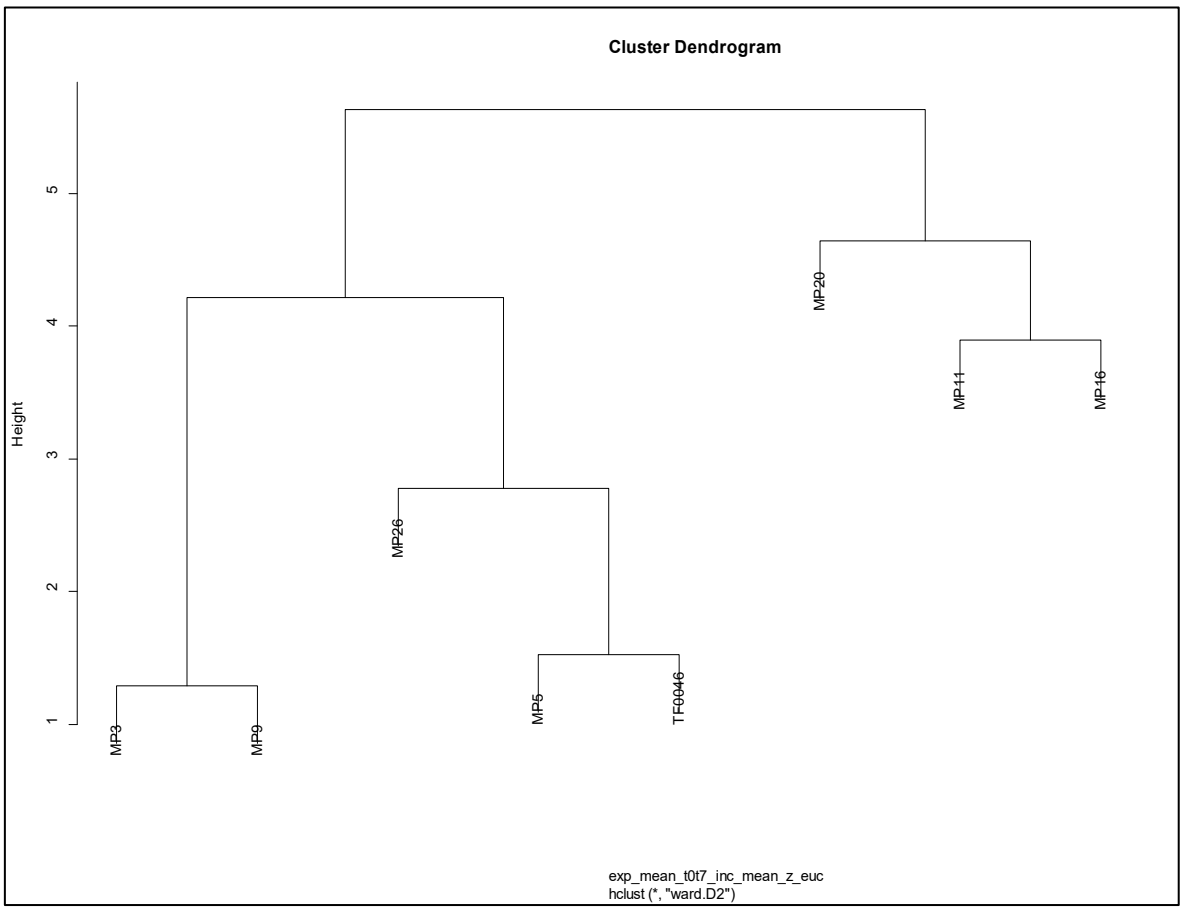
Supplementary Figure 3.3. Venn diagram depicting unique and shared fraction of bacterial OTUs on the PE, PS and wood after 7 days of incubation in seawater and assemblages on seston (≥3 µm) and in the free-living fraction (3 – 0.22 µm) of the incubation water of the treatment- and the control incubations at t_0 and t_7 combined.



Supplementary Figure 3.4. Distance-based redundancy analysis (dbRDA) ordination plots (type I scaling) based on the Bray-Curtis dissimilarities of the square-root transformed bacterial OTU read counts of the incubated PE-, PS- and wood particles after 7 days. Smooth response surfaces for (**A**) temperature, (**B**) NO_2^-, (**C**) NO_3^-, and (**D**) PO_4^{3-} were fitted using penalized splines with the function 'ordisurf' from the vegan package.



Supplementary Figure 3.5. Phylogenetic affiliations of unclassified *Rhodobacteraceae*-OTUs present only on the incubated PE and PS. Coloured ranges highlight affiliation of sequences with distinct clades within the family *Rhodobacteraceae*. Entries highlighted in bold represent OTUs found in this study. Collapsed nodes represent the outgroup.



Supplementary Figure 3.6. Dendrogram based on hierarchical clustering of physico-chemical surface water properties, averaged from t_0 and t_7 parameters of the incubation water. Data was z-transformed and the Ward-method was used for clustering based on Euclidian distance.

Supplementary tables

Table S3.1. Physico-chemical parameters of the sampling stations included in the incubation experiments along the Baltic Sea coast *in situ* (t_0) and in the incubation tanks after 7 days of incubation (t_7). For t_7, mean (± sd) are provided, where applicable.

Station	Longitute [degrees_east]	Latitude [degrees_north]	Area	Timepoint	Depth [m]	Salinity [PSU]	Temperature [°C]	PO_4^{3-} [µmlo/l]	SiO_2 [µmlo/l]	NO_2^- [µmlo/l]	NO_3^- [µmlo/l]	NH_4^+ [µmlo/l]	DIN [µmol/l]	DIN/DIP
TF0046	12.2797	54.4637	Mecklenburg Bay	t0	5.3	8.7	15.3	0.2	11.3	0.1	0.0	0.1	0.2	0.8
MP3	14.3227	53.9596	Pomeranian Bay	t0	2.9	8.1	20.1	0.6	21.3	0.2	0.0	0.2	0.3	0.5
MP5	16.1171	54.3152	Polish coast	t0	1.8	8.2	10	0.7	14.4	0.1	0.0	0.0	0.1	0.1
MP9	18.6288	54.5823	Bay of Gdansk	t0	2.1	7.4	20.6	0.4	18.8	0.1	0.0	0.2	0.3	0.9
MP11	21.0114	55.6843	Klaipeda	t0	1.4	7.3	19.1	0.1	8.8	0.1	0.0	0.2	0.3	2.2
MP16	24.2636	57.6819	Gulf of Riga	t0	1.6	5.8	20.3	0.1	12.0	0.1	0.0	2.0	2.1	29.8
MP20	27.7959	59.4951	Narva mouth	t0	2.4	4.4	18.8	0.0	5.6	0.8	0.0	0.0	0.8	17.8
MP26	24.8039	60.0652	Helsinki	t0	1.8	5.6	18.5	0.7	9.0	0.1	0.0	0.0	0.1	0.2
TF0046	12.2797	54.4637	Mecklenburg Bay	t7	5.3	9.0 (±0.09)	17.3 (±0.07)	0.1 (±0.00)	10.6 (±2.8)	0.1 (±0.00)	0.2 (±0.00)	-	0.3	2.5
MP3	14.3227	53.9596	Pomeranian Bay	t7	2.9	8.4 (±0.03)	20.0 (±0.04)	0.1 (±0.00)	23.4 (±0.4)	0.1 (±0.00)	0.2 (±0.00)	-	0.3	2.5
MP5	16.1171	54.3152	Polish coast	t7	1.8	8.6 (±0.10)	14.4 (±0.22)	0.1 (±0.02)	14.7 (±1.4)	0.1 (±0.00)	0.2 (±0.00)	-	0.3	2.2
MP9	18.6288	54.5823	Bay of Gdansk	t7	2.1	7.7 (±0.05)	19.8 (±0.07)	0.1 (±0.00)	19.9 (±0.5)	0.1 (±0.00)	0.2 (±0.02)	-	0.2	2.4
MP11	21.0114	55.6843	Klaipeda	t7	1.4	7.7 (±0.05)	18.8 (±0.06)	0.1 (±0.00)	9.2 (±0.2)	0.1 (±0.09)	1.7 (±1.58)	-	1.9	18.6
MP16	24.2636	57.6819	Gulf of Riga	t7	1.6	6.2 (±0.08)	19.6 (±0.06)	0.1 (±0.00)	14 (±0.3)	0.1 (±0.05)	0.2 (±0.09)	-	0.3	3.0
MP20	27.7959	59.4951	Narva mouth	t7	2.4	4.5 (±0.03)	18.7 (±0.06)	0.1 (±0.00)	6.5 (±0.2)	0.1 (±0.05)	0.2 (±0.02)	-	0.3	3.0
MP26	24.8039	60.0652	Helsinki	t7	1.8	5.8 (±0.02)	18.3 (±0.06)	0.1 (±0.00)	10.7 (±0.5)	0.1 (±0.00)	0.6 (±0.57)	-	0.6	6.1

Table S3.2A

Kruskal-Wallis rank sum test of Chao1 richness for factor "station" across all sample types

Kruskal-Wallis chi-squared	df	*p*-value
25.317	7	**0.0006665**

Table S3.2B

Kruskal-Wallis rank sum test for Chao1 richness for factor "station" within the subset of each sample type separated by treatment-, control and *in situ* samples.

Sample type	Kruskal-Wallis chi-squared	df	*p*-value
PE	15.187	7	0.034
PS	19.267	7	0.007
wood	21.973	7	0.003
seston_treat	11.433	5	0.043
seston_control	12.987	6	0.043
seston_in situ	21.853	7	0.002
free-living_treat	3.9654	6	0.681
free-living_control	11.048	6	0.086
free-living_in situ	18.373	7	0.01

Table S3.2C

Pair-wise comparisons (Conover-Iman-Tests with Benjamini-Hochberg correction) of Chao1 richness between stations across all sample types from the treatmet-, and control incubations and *in situ* samples together.

Comparisons	*p*-values (BH)
MP11, MP16	0.84
MP11, MP20	0.973
MP11, MP26	0.827
MP11, MP3	**0.002**
MP11, MP5	0.875
MP11, MP9	0.243
MP11, TF0046	0.963
MP16, MP20	0.801
MP16, MP26	0.576
MP16, MP3	**0.001**
MP16, MP5	0.952
MP16, MP9	0.117
MP16, TF0046	0.77
MP20, MP26	0.89
MP20, MP3	**0.003**
MP20, MP5	0.797
MP20, MP9	0.207
MP20, TF0046	0.94
MP26, MP3	**0.01**

MP26, MP5	0.553
MP26, MP9	0.551
MP26, TF0046	0.868
MP3, MP5	**0.002**
MP3, MP9	0.163
MP3, TF0046	**0.003**
MP5, MP9	0.162
MP5, TF0046	0.815
MP9, TF0046	0.221

Table S3.2D

Kruskal-Wallis rank sum test of Chai1 richness for factor sample type across all stations

Kruskal-Wallis chi-squared	df	*p*-value
136.26	8	**< 2.2e-16**

Table S3.2E

Pair-wise comparisons (Conover-Iman-Tests with Benjamini-Hochberg correction) of Chao1 richness between sample types across all stations.

Comparisons	*p* (BH)
PE, PS	0.33
PE, free-living_control	**0**
PE, free-living_in situ	**0**
PE, free-living_treat	**0**
PE, seston_control	**0**
PE, seston_in situ	**0**
PE, seston_treat	**0**
PE, wood	**0**
PS, free-living_control	**0**
PS, free-living_in situ	**0**
PS, free-living_treat	**0**
PS, seston_control	**0**
PS, seston_in situ	**0**
PS, seston_treat	**0**
PS, wood	**0**
free-living_control, free-living_in situ	**0**
free-living_control, free-living_treat	0.176
free-living_control, seston_control	0.224
free-living_control, seston_in situ	**0**
free-living_control, seston_treat	**0.002**
free-living_control, wood	0.893
free-living_in situ, free-living_treat	**0**
free-living_in situ, seston_control	**0**

free-living_in situ, seston_in situ	0.12
free-living_in situ, seston_treat	**0.007**
free-living_in situ, wood	**0**
free-living_treat, seston_control	**0.01**
free-living_treat, seston_in situ	**0**
free-living_treat, seston_treat	0.07
free-living_treat, wood	0.201
seston_control, seston_in situ	**0**
seston_control, seston_treat	**0**
seston_control, wood	0.179
seston_in situ, seston_treat	**0**
seston_in situ, wood	**0**
seston_treat, wood	**0.002**

Table S3.3A
Kruskal-Wallis rank sum test for *Vibrio* spp. relative abundances for factor "sample type" within the subset of each station from the treatment incubations.

Station	Kruskal-Wallis chi-squared	df	*p*-value
TF0046	9.9667	4	**0.04**
MP3	13.233	4	**0.01**
MP5	7.2	2	**0.03**
MP9	8.7436	3	**0.03**
MP11	11.467	4	**0.02**
MP16	3.2327	4	0.52
MP20	8.5714	4	0.07
MP26	2.283	4	0.68

Table S3.3B
Pair-wise comparisons (Conover-Iman-Tests with Benjamini-Hochberg correction) of *Vibrio* spp. relative abundances between sample types for each station from the treatment incubations.

Station	Comparison	*p*-values (BH adjusted)
TF0046	PE - PS	0.089
TF0046	PE - water_0.2	0.152
TF0046	PS - water_0.2	0.676
TF0046	PE - water_3	0.423
TF0046	PS - water_3	**0.040**
TF0046	water_0.2 - water_3	**0.041**
TF0046	PE - wood	**0.026**
TF0046	PS - wood	0.376
TF0046	water_0.2 - wood	0.259

TF0046	water_3 - wood	**0.015**
MP3	PE - PS	**0.010**
MP3	PE - water_0.2	**0.044**
MP3	PS - water_0.2	**0.000**
MP3	PE - water_3	**0.001**
MP3	PS - water_3	**0.000**
MP3	water_0.2 - water_3	**0.012**
MP3	PE - wood	**0.000**
MP3	PS - wood	**0.016**
MP3	water_0.2 - wood	**0.000**
MP3	water_3 - wood	**0.000**
MP5	PE - PS	**0.010**
MP5	PE - wood	**0.001**
MP5	PS - wood	**0.016**
MP9	PE - PS	**0.034**
MP9	PE - water_0.2	0.664
MP9	PS - water_0.2	0.051
MP9	PE - wood	**0.003**
MP9	PS - wood	0.057
MP9	water_0.2 - wood	**0.003**
MP11	PE - PS	**0.017**
MP11	PE - water_0.2	0.197
MP11	PS - water_0.2	0.125
MP11	PE - water_3	0.485
MP11	PS - water_3	**0.009**
MP11	water_0.2 - water_3	0.091
MP11	PE - wood	**0.002**
MP11	PS - wood	0.143
MP11	water_0.2 - wood	**0.012**
MP11	water_3 - wood	**0.002**

Table S3.3C
Kruskal-Wallis rank sum test for *Vibrio* spp. relative abundances for factor "station" within the subset of each sample type from the treatment incubations.

Sample type	Kruskal-Wallis chi-squared	df	*p*-value
PE	20.834	7	**0.004**
PS	21.551	7	**0.003**
wood	21.56	7	**0.003**
seston	12.231	5	**0.03**
free-living	14.948	6	**0.02**

Table S3.3D
Pair-wise comparisons (Conover-Iman-Tests with Benjamini-Hochberg correction) of *Vibrio* spp. relative abundances between stations for each sample type from the treatment incubations.

Sample type	Comparison	*p*-value (BH adjusted)
PE	MP11 - MP16	**0.016**
PE	MP11 - MP20	**0.005**
PE	MP16 - MP20	0.546
PE	MP11 - MP26	0.088
PE	MP16 - MP26	0.464
PE	MP20 - MP26	0.170
PE	MP11 - MP3	0.273
PE	MP16 - MP3	**0.002**
PE	MP20 - MP3	**0.001**
PE	MP26 - MP3	**0.009**
PE	MP11 - MP5	**0.001**
PE	MP16 - MP5	**0.000**
PE	MP20 - MP5	**0.000**
PE	MP26 - MP5	**0.000**
PE	MP3 - MP5	**0.013**
PE	MP11 - MP9	**0.001**
PE	MP16 - MP9	**0.000**
PE	MP20 - MP9	**0.000**
PE	MP26 - MP9	**0.000**
PE	MP3 - MP9	**0.010**
PE	MP5 - MP9	0.876
PE	MP11 - TF0046	**0.002**
PE	MP16 - TF0046	**0.000**
PE	MP20 - TF0046	**0.000**
PE	MP26 - TF0046	**0.000**
PE	MP3 - TF0046	**0.017**
PE	MP5 - TF0046	0.909

PE	MP9 - TF0046	0.814
PS	MP11 - MP16	**0.018**
PS	MP11 - MP20	**0.001**
PS	MP16 - MP20	0.165
PS	MP11 - MP26	**0.007**
PS	MP16 - MP26	0.635
PS	MP20 - MP26	0.315
PS	MP11 - MP3	0.123
PS	MP16 - MP3	**0.001**
PS	MP20 - MP3	**0.000**
PS	MP26 - MP3	**0.000**
PS	MP11 - MP5	**0.000**
PS	MP16 - MP5	**0.000**
PS	MP20 - MP5	**0.000**
PS	MP26 - MP5	**0.000**
PS	MP3 - MP5	**0.001**
PS	MP11 - MP9	**0.000**
PS	MP16 - MP9	**0.000**
PS	MP20 - MP9	**0.000**
PS	MP26 - MP9	**0.000**
PS	MP3 - MP9	**0.005**
PS	MP5 - MP9	0.283
PS	MP11 - TF0046	**0.002**
PS	MP16 - TF0046	**0.000**
PS	MP20 - TF0046	**0.000**
PS	MP26 - TF0046	**0.000**
PS	MP3 - TF0046	**0.043**
PS	MP5 - TF0046	**0.045**
PS	MP9 - TF0046	0.295
wood	MP11 - MP16	**0.004**
wood	MP11 - MP20	**0.000**
wood	MP16 - MP20	0.056
wood	MP11 - MP26	**0.007**
wood	MP16 - MP26	0.732
wood	MP20 - MP26	**0.029**
wood	MP11 - MP3	0.850
wood	MP16 - MP3	**0.003**
wood	MP20 - MP3	**0.000**
wood	MP26 - MP3	**0.005**
wood	MP11 - MP5	**0.000**
wood	MP16 - MP5	**0.000**
wood	MP20 - MP5	**0.000**
wood	MP26 - MP5	**0.000**

wood	MP3 - MP5	**0.000**
wood	MP11 - MP9	**0.000**
wood	MP16 - MP9	**0.000**
wood	MP20 - MP9	**0.000**
wood	MP26 - MP9	**0.000**
wood	MP3 - MP9	**0.000**
wood	MP5 - MP9	0.760
wood	MP11 - TF0046	**0.015**
wood	MP16 - TF0046	**0.000**
wood	MP20 - TF0046	**0.000**
wood	MP26 - TF0046	**0.000**
wood	MP3 - TF0046	**0.021**
wood	MP5 - TF0046	**0.030**
wood	MP9 - TF0046	0.059
Seston	MP11 - MP16	0.236
Seston	MP11 - MP20	**0.028**
Seston	MP16 - MP20	0.149
Seston	MP11 - MP26	0.483
Seston	MP16 - MP26	0.607
Seston	MP20 - MP26	0.068
Seston	MP11 - MP3	0.901
Seston	MP16 - MP3	0.271
Seston	MP20 - MP3	**0.022**
Seston	MP26 - MP3	0.509
Seston	MP11 - TF0046	**0.022**
Seston	MP16 - TF0046	**0.004**
Seston	MP20 - TF0046	**0.001**
Seston	MP26 - TF0046	**0.009**
Seston	MP3 - TF0046	**0.022**
free-living	MP11 - MP16	0.206
free-living	MP11 - MP20	**0.023**
free-living	MP16 - MP20	0.152
free-living	MP11 - MP26	0.576
free-living	MP16 - MP26	0.488
free-living	MP20 - MP26	**0.046**
free-living	MP11 - MP3	0.608
free-living	MP16 - MP3	0.519
free-living	MP20 - MP3	0.052
free-living	MP26 - MP3	1.000
free-living	MP11 - MP9	0.163
free-living	MP16 - MP9	**0.028**
free-living	MP20 - MP9	**0.002**
free-living	MP26 - MP9	0.058

free-living	MP3 - MP9	0.063
free-living	MP11 - TF0046	0.098
free-living	MP16 - TF0046	**0.020**
free-living	MP20 - TF0046	**0.002**
free-living	MP26 - TF0046	**0.048**
free-living	MP3 - TF0046	0.056
free-living	MP9 - TF0046	0.784

Table S3.3E
Kruskal-Wallis rank sum test of *Vibrio spp.* relative abundances between treatment- and control incubations and *in situ* samples.

Kruskal-Wallis chi-squared	df	*p*-value
36.663	2	1.09E-08

Table S3.3F
Pair-wise comparisons (Conover-Iman-Tests with Benjamini-Hochberg correction) of *Vibrio* spp. relative abundances between treatment- and control incubations and *in situ* samples.

Comparison	*p*-value (BH adjusted)	Remark
control - in situ	0.067	no MP5, no MP9
control - treatment	**0.000**	no MP5, no MP9
in situ - treatment	**0.000**	no MP5, no MP9

Table S3.3G
Kruskal-Wallis rank sum test for *Vibrio* spp. relative abundance in the free-living fraction between treatment- and control incubations and *in situ* samples.

Kruskal-Wallis chi-squared	df	*p*-value
21.786	2	1.86E-05

Table S3.3H

Pair-wise comparisons (Conover-Iman-Test with Benjamini-Hochberg correction) for *Vibrio* spp. relative abundance in the free-living fraction between treatment- and control incubations and *in situ* samples.

Comparison	*p*-value (BH adjusted)	Remark
in situ - control	**0.002**	no MP5
control - treatment	**0.000**	no MP5
in situ - treatment	**0.020**	no MP5

Table S3.4A

Global PERMANOVA & PERMDISP for factors "sample type" & "stations" of the samples from the treatment- and control incubations

	PERMANOVA		PERMDISP
Factor	**Unique permutations**	***p* perm**	***p* perm**
sample type	997	**0.001**	**0.001**
station	998	**0.001**	0.536
sample type x station	993	**0.001**	-

*Permutation method: Permutation of residuals under a reduced model

Table S3.4B

Pair-wise PERMANOVA for the factor "sample type" within the subset of each station for samples from the treatment- and control incubations. Comparisons are based on unrestricted permutations and Monte Carlo (MC) random draws from the asymptotic permutation distribution.

Station	Comparisons	*p*MC (BH)
TF0046	free-living_treat,wood	**0.018**
TF0046	free-living_treat,seston_treat	**0.019**
TF0046	free-living_treat,seston_control	**0.013**
TF0046	free-living_treat,free-living_control	**0.019**
TF0046	free-living_treat,PS	**0.013**
TF0046	free-living_treat,PE	**0.013**
TF0046	free-living_control,wood	**0.011**
TF0046	free-living_control,seston_treat	**0.015**
TF0046	free-living_control,seston_control	**0.019**
TF0046	free-living_control,PS	**0.011**
TF0046	free-living_control,PE	**0.011**
TF0046	seston_treat,wood	**0.042**
TF0046	seston_treat,seston_control	**0.019**
TF0046	seston_treat,PS	**0.030**
TF0046	seston_treat,PE	**0.019**

TF0046	seston_control,wood	**0.013**
TF0046	seston_control,PS	**0.015**
TF0046	seston_control,PE	**0.011**
TF0046	PE,wood	**0.022**
TF0046	PS,wood	0.111
TF0046	PS,PE	0.223
MP3	free-living_treat,wood	**0.011**
MP3	free-living_treat,seston_treat	**0.011**
MP3	free-living_treat,seston_control	**0.011**
MP3	free-living_treat,free-living_control	**0.014**
MP3	free-living_treat,PS	**0.011**
MP3	free-living_treat,PE	**0.011**
MP3	free-living_control,wood	**0.011**
MP3	free-living_control,seston_treat	**0.011**
MP3	free-living_control,seston_control	**0.013**
MP3	free-living_control,PS	**0.011**
MP3	free-living_control,PE	**0.011**
MP3	seston_treat,wood	**0.022**
MP3	seston_treat,seston_control	**0.018**
MP3	seston_treat,PS	**0.019**
MP3	seston_treat,PE	**0.011**
MP3	seston_control,wood	**0.019**
MP3	seston_control,PS	**0.013**
MP3	seston_control,PE	**0.011**
MP3	PE,wood	**0.020**
MP3	PS,wood	0.075
MP3	PS,PE	**0.035**
MP5	PE,wood	**0.042**
MP5	PS,wood	0.138
MP5	PS,PE	0.254
MP9	free-living_treat,wood	**0.011**
MP9	free-living_treat,seston_treat	**0.049**
MP9	free-living_treat,seston_control	**0.013**
MP9	free-living_treat,free-living_control	**0.019**
MP9	free-living_treat,PS	**0.011**
MP9	free-living_treat,PE	**0.014**
MP9	free-living_control,wood	**0.013**
MP9	free-living_control,seston_treat	**0.019**
MP9	free-living_control,seston_control	**0.016**
MP9	free-living_control,PS	**0.011**
MP9	free-living_control,PE	**0.011**
MP9	seston_treat,wood	**0.040**
MP9	seston_treat,seston_control	**0.047**
MP9	seston_treat,PS	**0.043**
MP9	seston_treat,PE	**0.036**
MP9	seston_control,wood	**0.011**
MP9	seston_control,PS	**0.013**
MP9	seston_control,PE	**0.011**
MP9	PE,wood	**0.041**

MP9	PS,wood	0.124
MP9	PS,PE	0.124
MP11	free-living_treat,wood	**0.011**
MP11	free-living_treat,seston_treat	**0.020**
MP11	free-living_treat,seston_control	**0.011**
MP11	free-living_treat,free-living_control	**0.022**
MP11	free-living_treat,PS	**0.011**
MP11	free-living_treat,PE	**0.011**
MP11	free-living_control,wood	**0.011**
MP11	free-living_control,seston_treat	**0.017**
MP11	free-living_control,seston_control	**0.013**
MP11	free-living_control,PS	**0.011**
MP11	free-living_control,PE	**0.011**
MP11	seston_treat,wood	**0.019**
MP11	seston_treat,seston_control	**0.041**
MP11	seston_treat,PS	**0.019**
MP11	seston_treat,PE	**0.019**
MP11	seston_control,wood	**0.015**
MP11	seston_control,PS	**0.011**
MP11	seston_control,PE	**0.011**
MP11	PE,wood	**0.021**
MP11	PS,wood	0.055
MP11	PS,PE	0.051
MP16	free-living_treat,wood	**0.013**
MP16	free-living_treat,seston_treat	**0.011**
MP16	free-living_treat,seston_control	**0.013**
MP16	free-living_treat,free-living_control	**0.018**
MP16	free-living_treat,PS	**0.011**
MP16	free-living_treat,PE	**0.011**
MP16	free-living_control,wood	**0.011**
MP16	free-living_control,seston_treat	**0.011**
MP16	free-living_control,seston_control	**0.019**
MP16	free-living_control,PS	**0.011**
MP16	free-living_control,PE	**0.011**
MP16	seston_treat,wood	**0.017**
MP16	seston_treat,seston_control	**0.022**
MP16	seston_treat,PS	**0.017**
MP16	seston_treat,PE	**0.015**
MP16	seston_control,wood	**0.015**
MP16	seston_control,PS	**0.017**
MP16	seston_control,PE	**0.013**
MP16	PE,wood	0.054
MP16	PS,wood	0.117
MP16	PS,PE	0.396
MP20	free-living_treat,wood	**0.013**
MP20	free-living_treat,seston_treat	**0.013**
MP20	free-living_treat,seston_control	**0.011**
MP20	free-living_treat,free-living_control	**0.018**
MP20	free-living_treat,PS	**0.014**

MP20	free-living_treat,PE	**0.014**
MP20	free-living_control,wood	**0.011**
MP20	free-living_control,seston_treat	**0.011**
MP20	free-living_control,seston_control	**0.018**
MP20	free-living_control,PS	**0.011**
MP20	free-living_control,PE	**0.011**
MP20	seston_treat,wood	**0.030**
MP20	seston_treat,seston_control	**0.019**
MP20	seston_treat,PS	**0.026**
MP20	seston_treat,PE	**0.023**
MP20	seston_control,wood	**0.013**
MP20	seston_control,PS	**0.019**
MP20	seston_control,PE	**0.015**
MP20	PE,wood	0.076
MP20	PS,wood	0.131
MP20	PS,PE	0.393
MP26	free-living_treat,wood	**0.011**
MP26	free-living_treat,seston_treat	**0.011**
MP26	free-living_treat,seston_control	**0.011**
MP26	free-living_treat,free-living_control	**0.016**
MP26	free-living_treat,PS	**0.014**
MP26	free-living_treat,PE	**0.011**
MP26	free-living_control,wood	**0.011**
MP26	free-living_control,seston_treat	**0.013**
MP26	free-living_control,seston_control	**0.011**
MP26	free-living_control,PS	**0.011**
MP26	free-living_control,PE	**0.011**
MP26	seston_treat,wood	**0.040**
MP26	seston_treat,seston_control	**0.015**
MP26	seston_treat,PS	**0.020**
MP26	seston_treat,PE	**0.018**
MP26	seston_control,wood	**0.011**
MP26	seston_control,PS	**0.011**
MP26	seston_control,PE	**0.011**
MP26	PE,wood	**0.024**
MP26	PS,wood	**0.040**
MP26	PS,PE	0.222

Table S3.4C
Pair-wise PERMDISP for the factor "sample type" within the subset of each station for samples fro
incubations. Comparisons are based on 999 permutations.

Station	Comparisons	*p* perm (BH)
TF0046	free-living_treat,wood	0.227
TF0046	free-living_treat,seston_treat	0.363
TF0046	free-living_treat,seston_control	0.927
TF0046	free-living_treat,free-living_control	0.806
TF0046	free-living_treat,PS	0.227
TF0046	free-living_treat,PE	0.227
TF0046	free-living_control,wood	0.227
TF0046	free-living_control,seston_treat	0.544
TF0046	free-living_control,seston_control	0.877
TF0046	free-living_control,PS	0.491
TF0046	free-living_control,PE	0.227
TF0046	seston_treat,wood	0.227
TF0046	seston_treat,seston_control	0.634
TF0046	seston_treat,PS	0.877
TF0046	seston_treat,PE	0.227
TF0046	seston_control,wood	0.227
TF0046	seston_control,PS	0.632
TF0046	seston_control,PE	0.227
TF0046	PE,wood	0.806
TF0046	PS,wood	0.227
TF0046	PS,PE	0.227
MP3	free-living_treat,wood	0.698
MP3	free-living_treat,seston_treat	0.227
MP3	free-living_treat,seston_control	0.227
MP3	free-living_treat,free-living_control	0.227
MP3	free-living_treat,PS	0.227
MP3	free-living_treat,PE	0.749
MP3	free-living_control,wood	0.227
MP3	free-living_control,seston_treat	0.698
MP3	free-living_control,seston_control	0.227
MP3	free-living_control,PS	0.632
MP3	free-living_control,PE	0.227
MP3	seston_treat,wood	0.227
MP3	seston_treat,seston_control	0.227
MP3	seston_treat,PS	0.749
MP3	seston_treat,PE	0.227
MP3	seston_control,wood	0.227
MP3	seston_control,PS	0.227
MP3	seston_control,PE	0.227
MP3	PE,wood	0.227
MP3	PS,wood	0.227
MP3	PS,PE	0.227
MP5	PE,wood	0.749
MP5	PS,wood	0.927

MP5	PS,PE	0.713
MP9	free-living_treat,wood	0.698
MP9	free-living_treat,seston_treat	0.927
MP9	free-living_treat,seston_control	0.806
MP9	free-living_treat,free-living_control	0.806
MP9	free-living_treat,PS	0.698
MP9	free-living_treat,PE	0.698
MP9	free-living_control,wood	0.651
MP9	free-living_control,seston_treat	0.806
MP9	free-living_control,seston_control	0.877
MP9	free-living_control,PS	1.000
MP9	free-living_control,PE	0.632
MP9	seston_treat,wood	0.877
MP9	seston_treat,seston_control	0.877
MP9	seston_treat,PS	0.806
MP9	seston_treat,PE	0.927
MP9	seston_control,wood	0.713
MP9	seston_control,PS	0.877
MP9	seston_control,PE	0.749
MP9	PE,wood	0.749
MP9	PS,wood	0.698
MP9	PS,PE	0.698
MP11	free-living_treat,wood	0.632
MP11	free-living_treat,seston_treat	0.227
MP11	free-living_treat,seston_control	0.227
MP11	free-living_treat,free-living_control	0.227
MP11	free-living_treat,PS	0.227
MP11	free-living_treat,PE	0.227
MP11	free-living_control,wood	0.227
MP11	free-living_control,seston_treat	0.230
MP11	free-living_control,seston_control	0.927
MP11	free-living_control,PS	0.227
MP11	free-living_control,PE	0.927
MP11	seston_treat,wood	0.227
MP11	seston_treat,seston_control	0.651
MP11	seston_treat,PS	0.227
MP11	seston_treat,PE	0.227
MP11	seston_control,wood	0.227
MP11	seston_control,PS	0.227
MP11	seston_control,PE	0.698
MP11	PE,wood	0.227
MP11	PS,wood	0.698
MP11	PS,PE	0.227
MP16	free-living_treat,wood	0.504
MP16	free-living_treat,seston_treat	0.749
MP16	free-living_treat,seston_control	0.227
MP16	free-living_treat,free-living_control	0.227
MP16	free-living_treat,PS	0.227
MP16	free-living_treat,PE	0.227

MP16	free-living_control,wood	0.227
MP16	free-living_control,seston_treat	0.227
MP16	free-living_control,seston_control	0.227
MP16	free-living_control,PS	0.227
MP16	free-living_control,PE	0.749
MP16	seston_treat,wood	0.504
MP16	seston_treat,seston_control	0.227
MP16	seston_treat,PS	0.227
MP16	seston_treat,PE	0.227
MP16	seston_control,wood	0.227
MP16	seston_control,PS	0.505
MP16	seston_control,PE	0.749
MP16	PE,wood	0.227
MP16	PS,wood	0.227
MP16	PS,PE	0.877
MP20	free-living_treat,wood	0.927
MP20	free-living_treat,seston_treat	0.877
MP20	free-living_treat,seston_control	0.504
MP20	free-living_treat,free-living_control	0.806
MP20	free-living_treat,PS	0.227
MP20	free-living_treat,PE	0.379
MP20	free-living_control,wood	0.749
MP20	free-living_control,seston_treat	0.877
MP20	free-living_control,seston_control	0.227
MP20	free-living_control,PS	0.227
MP20	free-living_control,PE	0.227
MP20	seston_treat,wood	0.749
MP20	seston_treat,seston_control	0.504
MP20	seston_treat,PS	0.227
MP20	seston_treat,PE	0.227
MP20	seston_control,wood	0.227
MP20	seston_control,PS	0.227
MP20	seston_control,PE	0.806
MP20	PE,wood	0.227
MP20	PS,wood	0.227
MP20	PS,PE	0.698
MP26	free-living_treat,wood	0.227
MP26	free-living_treat,seston_treat	0.227
MP26	free-living_treat,seston_control	1.000
MP26	free-living_treat,free-living_control	0.362
MP26	free-living_treat,PS	0.227
MP26	free-living_treat,PE	0.698
MP26	free-living_control,wood	0.227
MP26	free-living_control,seston_treat	0.749
MP26	free-living_control,seston_control	0.363
MP26	free-living_control,PS	0.227
MP26	free-living_control,PE	0.749
MP26	seston_treat,wood	0.227
MP26	seston_treat,seston_control	0.227

MP26	seston_treat,PS	0.227
MP26	seston_treat,PE	0.363
MP26	seston_control,wood	0.227
MP26	seston_control,PS	0.227
MP26	seston_control,PE	0.698
MP26	PE,wood	0.227
MP26	PS,wood	0.227
MP26	PS,PE	0.227

Table S3.4D
Global PERMANOVA & PERMDISP for the grouped station (clusters observed in the dbRDA-ordination) based on the PE, PS, and wood samples.

		PERMANOVA*	PERMDISP
Factor	**P(perm)**	**Unique permutations**	**P(perm)**
station_grouping	**0.001**	998	**0.001**

*Permutation method: Permutation of residuals under a reduced model

Table S3.4E
Pair-wise PERMANOVA & PERMDISP for the grouped station (clusters observed in the dbRDA-ordination) based on the PE, PS, and wood samples.

		PERMANOVA*	PERMDISP
Comparisons	**P(perm)**	**Unique permutations**	**P(perm)**
TF0046_MP5, MP3	**0.001**	999	**0.025**
TF0046_MP5, MP9_MP11	**0.001**	998	**0.004**
TF0046_MP5, MP16_MP20_MP26	**0.001**	999	**0.001**
MP3, MP9_MP11	**0.001**	998	**0.001**
MP3, MP16_MP20_MP26	**0.001**	999	**0.001**
MP9_MP11, MP16_MP20_MP26	**0.001**	999	**0.001**

*Permutation method: Permutation of residuals under a reduced model

Digital appendix

The accompanying DVD to this dissertation includes:

- SSCP-gel pictures obtained for chapter I & II
- Sequence data for SSCP-bands
- Relative abundance data for sequenced SSCP-bands
- The GelCompar databases for the analysis of the SSCP-fingerprint patterns
- Similarity matrices and statistics of the SSCP-fingerprint patterns
- qPCR-results from chapter I
- The mothur-script used for downstream-analysis of the amplicon sequence data obtained in chapter III
- The sub-samples OTU-table obtained in chapter III
- Result of the linear discriminant effect size analysis (LEfSe) obtained in chapter III

Acknowledgement

I am very grateful to Matthias Labrenz for providing me with this opportunity and sharing his extensive knowledge and experience – but most of all for beeing there when I needed him and keeping me motivated.
I want to thank Sonja Oberbeckmann for her great support, both work-related and personally, and Brittan Scales for proof-reading this on such short notice.

I am thankful to:

- The Leibniz-Association for funding my position in the MikrOMIK project.
- The captain and crew of the R/V Poseidon cruise POS488 and those involved in sampling, especially Andreas Müller, Stephanie Mothes and Christina Hensler.
- Present and past members of my working group – Franziska Klaeger, Sophie Charvet, Janine Wäge, Heike Benterbusch, Brittan Scales, Christin Benncke, Juliana Ivar do Sul, Alexander Tagg, Robin Lenz, and Kristina Enders, thank you all for being such a great crowd to work with.
- René Janßen and Lars Möller for being the best office-mates there will ever be! You guys so often brightened my days.
- So many other people from the institute that help with, and support, the daily routine, both by providing technical assistance and by just being fun to work with – Christian Stolle, Christian Meeske, Mercè Berga, Christian Burmeister, Christin Laudan, Jan Henkel, Philipp Braun, Falk Eigemann, Daniel Herlemann, Dandan Shen, Sarah Weber, Sarah Piehl, Ines Bartl, Rainer Bahlo, Iris Liskow, and Sascha Plewe.

I also want to acknowledge the Open-Source community for creating such great tools as R and its packages, and the StackOverflow community for being such a valuable resource, and both for sharing the knowledge.

I want to thank my family and friends, for putting things back into perspective ever so often. And especially my parents, for providing me with wings to pursue my own path, but with enough roots to have the confidence to do so.

Last but not least, and although its odd, I want to thank my past-self for being so stubborn – there were days when I would not know how to keep going, but you just did.

I want to stress, that all the above mentioned acknowledgements are not explicitly attributed to be ‘fruitful’, ‘excellent’, ‘superbe’, ‘very’, ‘extreme’, ‘tremendous’, ‘inspiring’, ‘unconditional’, ‘brilliant’, ‘perfect’, etc., in order to avoid hierarchic weighting and interpretation. All you people were needed to finish this work. (I stole this sentence from Christian Stolle, because I really really liked it.)

THANK YOU VERY, VERY MUCH!!!

Declaration of Authenticity

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den 17.09.2019