



Dangerous hitchhikers? Evidence for potentially pathogenic *Vibrio* spp. on microplastic particles

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ABSTRACT

The taxonomic composition of biofilms on marine microplastics is widely unknown. Recent sequencing results indicate that potentially pathogenic *Vibrio* spp. might be present on floating microplastics. Hence, these particles might function as vectors for the dispersal of pathogens. Microplastics and water samples collected in the North and Baltic Sea were subjected to selective enrichment for pathogenic *Vibrio* species. Bacterial colonies were isolated from CHROMagarTM*Vibrio* and assigned to *Vibrio* spp. on the species level by MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionisation – Time of Flight Mass Spectrometry). Respective polymers were identified by ATR FT-IR (Attenuated Total Reflectance Fourier Transform – Infrared Spectroscopy). We discovered potentially pathogenic *Vibrio parahaemolyticus* on a number of microplastic particles, e.g. polyethylene, polypropylene and polystyrene from North/Baltic Sea. This study confirms the indicated occurrence of potentially pathogenic bacteria on marine microplastics and highlights the urgent need for detailed biogeographical analyses of marine microplastics.

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1. Introduction

The production of synthetic polymers started over 100 years ago and meanwhile the worldwide production reached up to 311 million tons per year (PlasticsEurope, 2015). As a consequence of improper disposal synthetic polymers represent the most rapidly growing form of anthropogenic debris entering and accumulating in the oceans (Andrady, 2011; Thiel and Gutow, 2005).

Due to their durability most synthetic polymers are poorly degradable in the marine environment but become brittle and subsequently break down in small particles, so called microplastics (Andrady, 2011; Corcoran et al., 2009). Several size categorizations of plastics have been suggested by various researchers (Gregory and Andrady, 2003; Moore, 2008) while plastic fragments smaller than 5 mm are categorized as microplastics by Barnes et al. (2009). Once floating on seawater, plastic debris can be transported over long distances by wind, currents and wave action (Barnes et al., 2009).

As all surfaces in the marine environment microplastic is rapidly colonized by bacteria (Harrison et al., 2014) and subsequently by a plethora of organisms building up complex biofilms (Dobretsov, 2009). Harrison et al. (2014) detected bacterial colonization of low density polyethylene microplastics already after 7 days exposure in marine sediments. Also Lobelle and Cunliffe (2011) proved biofilm formation on plastics after 1 week of incubation in seawater via quantitative biofilm assays. Prior studies evidenced that even harmful al-

gal species were detected in biofilms on plastic debris (Masó et al., 2003). Being highly heterogeneous environments, biofilms offer important ecological advantages such as the accumulation of nutrients, as protective barrier, for mechanical stability (Flemming, 2002) or the formation of micro-consortia of different species that orchestrate the degradation of complex substrates (Wimpenny, 2000).

Zettler et al. (2013) showed that microbial communities on marine plastic debris differ consistently from the surrounding seawater communities and coined the term “Plastisphere” for this habitat. Furthermore, Amaral-Zettler et al. (2015) reported that “Plastisphere” communities are genetically unique from the free marine water communities that envelop them and possess dominant taxa that are highly variable and diverse. Moreover, the composition of biofilm communities on plastic in marine habitats varies with season, geographical location and plastic substrate type (Oberbeckmann et al., 2014).

Zettler et al. (2013) have suggested that plastic particles may serve as vectors for the dispersal of human pathogens (*Vibrio* spp.). Using a culture-independent approach, the author's detected sequences affiliated to *Vibrio* spp. on marine plastic debris (Zettler et al., 2013), i.e. on plastic particles in the North Atlantic by using molecular tools (Amplicon Pyrotag Sequencing). Furthermore, De Tender et al. (2015) recently detected Vibrionaceae on marine plastics from the Belgian North Sea, by using next-generation amplicon sequencing. However, due to short read lengths, a conclusive identification on the species level was not provided so far (De Tender et al., 2015; Zettler et al., 2013).

Species of the genus *Vibrio* belong to the class *Gammaproteobacteria* and are highly abundant in sediments, estuaries and marine coastal waters (Barbieri et al., 1999). *Vibrios* are gram-negative,

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rod-shaped chemorganotrophic and facultatively anaerobic organisms. Besides occurring free-living in aquatic environments, *Vibrio* spp. are known to colonize a variety of marine organisms, utilizing released nutrients on these living surfaces (Huq et al., 1983; Visick, 2009) or living in symbiosis (McFall-Ngai and Ruby, 1991; McFall-Ngai, 2002; McFall-Ngai and Ruby, 1998).

Some *Vibrio* species are known as animal pathogens invading coral species and causing coral bleaching (Ben-Haim et al., 2003) and others are classified as human pathogens causing serious infections (Morris, 2003). Especially *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* are known as water-related human pathogens which cause wound infections associated with recreational bathing, septicemia or diarrhea after ingestion of contaminated foods (Thompson et al., 2004a).

Although *Vibrio* infections are common in tropical areas, the last decade showed a significant increase in documented cases also in European regions, such as in the Mediterranean Sea (Gras-Rouzet et al., 1996; Martinez-Urtaza et al., 2005) or in the more temperate Northern waters (Eiler et al., 2006). Prior studies reported that the number of *Vibrio* infections correspond closely with the sea surface temperature pointing to a possible link to climate change related phenomena (e.g. global warming, heat waves) (Baker-Austin et al., 2010, 2012).

Böer et al. (2013) reported that *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* occurred in water and sediments in the central Wadden Sea and in the estuaries of the rivers Ems and Weser. The most prevalent species were *V. alginolyticus* followed by *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (Böer et al., 2013), reflecting earlier findings on the composition of *Vibrio* communities in other parts of the North Sea (Bauer et al., 2006; Collin and Rehnstam-Holm, 2011; Hervio-Heath et al., 2002; Schets et al., 2011). While *V. vulnificus* and *V. cholerae* were detected mainly in the Baltic Sea, *V. parahaemolyticus* occurred as the main potential pathogenic *Vibrio* spp. in the North Sea (Böer et al., 2013; Oberbeckmann et al., 2011b; Ruppert et al., 2004; Schets et al., 2010).

As already mentioned most synthetic polymers are poorly degradable and are rapidly colonized by microorganisms. Microplastics could be transported over long distances in marine environments, as compared to naturally occurring polymers, and therefore function as a vector for the dispersal of harmful or even human pathogenic species. To verify or falsify the occurrence of potentially pathogenic *Vibrio* spp. on marine plastics, we analysed plastics and corresponding water samples of the North and Baltic Sea with respect to potentially human pathogenic *Vibrio* spp. by using cultivation-dependent methods (alkaline peptone water (APW), CHROMagar™*Vibrio*), followed by state of the art identification of bacteria on the species level by MALDI-TOF MS (Erler et al., 2015). The main focus of the study was on detecting the main potentially human pathogenic species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Polymers were identified by ATR FT-IR (Attenuated Total Reflectance Fourier Transform - Infrared Spectroscopy).

2. Materials and methods

2.1. Sampling

To detect *Vibrio* spp. attached to microplastics, neustonic particles were collected during two research cruises in 2013 and 2014 at 62 sampling stations in the North and Baltic Sea (see Table A1). Neuston samples were taken with a Neuston Catamaran equipped with a 300 µm net. The Catamaran was towed alongside the vessel for about 30–45 min per station. The volume passing the Neuston net was recorded by use of a mechanical flowmeter (Table A2). Further

samples were taken at the drift line of the south port beach at the island Helgoland at low tide in August 2013 (station 63). Particles recovered in the cod end of the Neuston net or sampled at the drift line of Helgoland were sorted by stereo microscopy and using a Bogoroff chamber and finally transferred to Petri dishes containing sterile seawater. Single particles identified visually according to the definition by Barnes et al. (2009) in a size range of 0.5–5 mm and to colour and texture as being synthetic polymers were picked with sterile forceps and washed three times with 10 ml of sterile seawater, to remove loosely attached organisms.

For comparison of microplastic-attached and waterborne *Vibrio* spp., additional surface seawater samples were taken on both research cruises with a thoroughly flushed bucket or rosette sampler (SBE 911 plus, Sea-Bird Electronics, US) and a maximal volume of 1 l was filtered onto 0.45 µm sterile membrane filters (Sartorius stedim biotech, US). Environmental parameters (temperature, salinity) were recorded by a ship-based thermosalinograph (SBE 21SeaCAT, Sea-Bird Electronics, US) or by the sensors of the rosette sampler. The temperature of Helgoland was measured manually with a thermometer and the salinity was recorded with a salinometer (Autosal, GUILDLIN, Canada) (Table A3).

2.2. Enrichment & isolation of *Vibrio* spp.

All particles and membrane filters (seawater samples) were immediately transferred individually into sterile glass tubes with alkaline peptone water (15 ml APW) and incubated in a rotating incubator at 37 °C for 48 h in the dark for the growth of a broad spectrum of mesophilic and potentially pathogenic *Vibrio* spp., enabling their selective enrichment.

After APW incubation the tubes were visually checked for growth and turbid samples were plated by using an inoculation loop or Spiral-plater (easySpiral® Dilute; Interscience, France) on selective CHROMagar™*Vibrio* (MAST Diagnostica GmbH, Germany) (Di Pinto et al., 2011). All inoculated CHROMagar™*Vibrio* were incubated at 37 °C for 24 h in the dark. The appearing colonies were checked with respect to distinct colony colorations typical for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* according to the manufacturers' instruction. Representative colonies for each coloration were picked and differentially streaked out on marine broth agar (Oppenheimer and ZoBell, 1952) with reduced salinity (MB-50% = 16PSU). Incubation was performed at 37 °C for 24 h in the dark.

Even though CHROMagar™*Vibrio* is a selective medium for the isolation of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, other species have the ability to grow on these media appearing with the same colony colorations. For instance, *Vibrio fluvialis* occurred in mauve coloured colonies distinct from *V. parahaemolyticus* and *Vibrio mimicus* in turquoise coloured colonies distinct for *V. vulnificus* and *V. cholerae*. Hence for a conclusive identification all presumptive *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* strains were further analysed by MALDI-TOF MS.

2.3. MALDI-TOF MS

For MALDI-TOF analysis, all isolates were grown overnight on MB-50% agar plates as described above. To create high quality mass spectra, proteins of the strains isolated during the cruise in 2013 were extracted using a previously described formic acid/acetonitrile extraction method (Mellmann et al., 2008). For fast identification, all other strains (cruise 2014 and Helgoland samples 2013) were analysed via the direct transfer procedure according to manufacturers' recommendations (Bruker Daltonics Inc., Germany, Bremen). This involved

picking colonies after 24 h of cultivation with sterile toothpicks and directly transferring onto the MALDI-TOF MS target plate (MSP 96 target polished steel) as thin layer. Each sample spot was first overlaid with 1 μ l formic acid (70% v/v) followed by an overlay with 1 μ l matrix solution (saturated solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and directly screened. All spectra were acquired using the microflex LT/SH system (Bruker Daltonics Inc., Germany, Bremen). Species identification was done by using the Biotyper™ software (version 3.1) according to the manufacturer's instructions, where 70 most prominent mass peaks were compared to the mass spectra of the Bruker library as well as the "VibrioBase" library (Erler et al., 2015).

In order to check the reliability of the species assignment via MALDI-TOF MS all *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* were verified by PCR amplification of species-specific genes and additionally screened for virulence-associated genes (section 2.4).

2.4. PCR of regulatory and virulence-related genes

As described previously (Oberbeckmann et al., 2011a), DNA extraction of *Vibrio* strains identified by MALDI-TOF MS was carried out using lysozyme/SDS lysis and phenol/chloroform extraction, followed by isopropanol precipitation. Prior to PCR experiments, DNA quantity and quality was determined photometrically (TECAN infinite M200, Switzerland). Species-specific PCR for toxR genes was performed with all *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* strains respectively using the universal forward primer *UtoxR* together with the species specific primers *VptoxR*, *VvtoxR* and *VctoxR*, respectively (Bauer and Rorvik, 2007; Di Pinto et al., 2005). Specific PCRs targeting thermostable direct haemolysin (*tdh*) (Nishibuchi and Kaper, 1985) and the *tdh* related haemolysin (*trh*) (Honda et al., 1991; Honda and Iida, 1993) genes were performed with the primer sets *tdhD3F/tdhDIR* and *trhFR2/trhRR6* to strains assigned to *V. parahaemolyticus* (Bauer and Rorvik, 2007; Tada et al., 1992). To test *V. cholerae* strains for the presence of a unique chromosomal region indicating the serotypes O139 (Albert et al., 1997) and O1 (Hoshino et al., 1998) and the cholera toxin gene *ctxA* (Singh et al., 2002) a multiplex PCR was performed with the primer sets *O139F/O139R*, *O1F/O1R* and *ctxA1/ctxA2* (Bauer and Rorvik, 2007; Mantri et al., 2006; Nandi et al., 2000). All reactions were performed in duplicate. In case of discordant results, a third PCR was carried out. The PCRs were performed as described by Böer et al. (2013) with the exception that 20 ng of template DNA was used. The following reference strains were used as positive controls: *V. vulnificus* ATCC 27562 (*VvtoxR*) (The Federal Institute for Risk Assessment, BfR), *V. parahaemolyticus* RIMD 2210633 (*VptoxR*; *tdh*) (German Collection of Microorganisms and Cell Cultures, DSMZ), *V. parahaemolyticus* CM12 (*tdh*; *trh*), *V. parahaemolyticus* CM24 (*trh*) (provided by Carsten Matz, HZI), *V. cholerae* CH 111 (*VctoxR*; *O1*), *V. cholerae* CH 187 (*VctoxR*; *O139*; *ctxA*) and *V. cholerae* CH 258 (*VctoxR*; *ctxA*; *O1*) (BfR). *Vibrio harveyi* ATCC 25919 (DSMZ) was used as negative control in each PCR. PCR products were confirmed to be of the expected size by a MultiNA Microchip electrophoresis system (MCE-202 MultiNA, Shimadzu Biotech).

2.5. FT-IR analyses of particles

After incubation in APW, all particles were rinsed using deionized water and dried at 60 °C overnight. Prior to analysis, particles were rinsed with ethanol (70% v/v) and the surface was scraped with a scalpel to avoid organic contamination interfering with FT-IR analysis. The FT-IR spectra of particles were recorded by the attenuated to-

tal reflectance (ATR) technique using a Tensor 27 spectrometer with a Platinum ATR unit (Bruker, Germany). For each analysis 16 scans in the range 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and 6 mm aperture were performed and averaged. The obtained IR spectra were compared to reference-spectra of an in-house database covering 143 spectra of different synthetic polymers and the IR Library from Bruker Optics containing 350 entries. Spectra processing and database comparisons were performed by using OPUS 7.2. (Bruker, Germany).

3. Results

3.1. Occurrence and characterization of microplastics

Particles were collected from 39 stations in the North Sea and 5 stations in the Baltic Sea. In total, 170 particles were collected in the North Sea and 15 particles in the Baltic Sea, mostly abundant at stations 17, 56, 58 and 61, with ≥ 10 particles from each station, respectively (Table A4). Almost all particles showed signs of weathering, including cracks and pitting. Most particles were covered at least partially with dense biofilms on their surface, indicating colonization by various biota. Polymer identification of presumptive synthetic polymer particles, (ATR FT-IR (Table A3)) confirmed 141 as synthetic polymers, 14 particles were non-plastics such as chitin or keratin, and 30 could not be further identified. All of the 15 presumptive microplastics of Helgoland drift line were identified as synthetic polymers. The most abundant synthetic polymer throughout all sampling sites was polyethylene, comprising over 40% of the collected particles at all sites. Polypropylene and polystyrene were also frequently found at all sites, representing 14–20% and 5–7% of all particles, respectively (Fig. 1).

3.2. Identification and geographic distribution of *Vibrio* spp. in water samples

Water samples were taken from all stations in the North and Baltic Sea with the exception of Helgoland drift line (station 63) resulting in 326 APW enrichment cultures. Out of these, 323 displayed growth and were subjected to further isolation of bacteria on selective CHROMagar™ *Vibrio* agar plates, with respect to *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*.

From all water samples, 151 pure cultures of representative mauve and turquoise blue colonies were grown on marine broth agar and subjected to MALDI-TOF MS. Out of these, 104 were identified as *Vibrio* spp. by MALDI-TOF MS.

With the exception of three isolates, all *Vibrio* water strains could be identified by MALDI-TOF MS on a conclusive species level. We identified 38% out of all *Vibrio* water isolates (104) as *V. parahaemolyticus*, 16% as *V. vulnificus* and 11% as *V. cholerae*. Further on, 21% of the strains were classified as *V. fluvialis*, 7% as *V. mimicus*, 5% as *Vibrio diazotrophicus*, 1% as *Vibrio metschnikovii* (Table A6).

A single *V. parahaemolyticus* strain (VN-4212) isolated from water (station 3) carried the virulence-associated gene *tdh*, while *trh* was not detected in any strain (Table A6). No *V. cholerae* strain belonged to the *O1/O139* type or carried the *ctxA* gene.

In general, *V. parahaemolyticus* was detected only in North Sea waters (Fig. 2) in a temperature range of 14.9–21.1 °C and at salinities between 16.9 and 32.4 PSU (Table A3). The potentially pathogenic species *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* occurred mainly in coastal and estuarine regions of the North Sea. *Vib-*

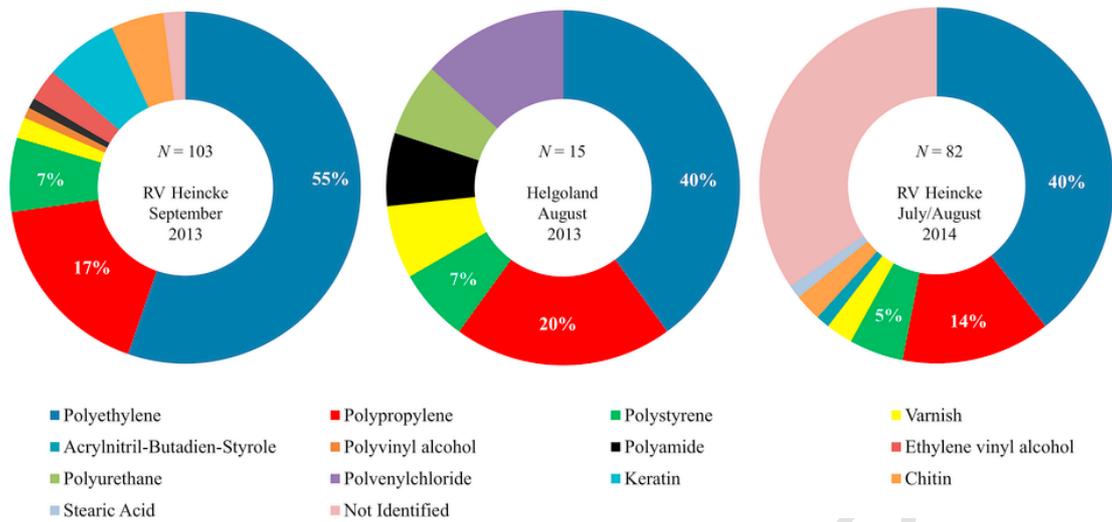


Fig. 1. Proportions of synthetic polymers and other particles collected during research cruises in the North and Baltic Sea and the drift line of Helgoland. Sampling took place in September 2013 (left), July/August 2014 (middle) and July 2013 (right). Particles were characterized using ATR FT-IR spectroscopy. Also given are numbers of total particles (*N*) and percentages of polyethylene, polypropylene and polystyrene particles.

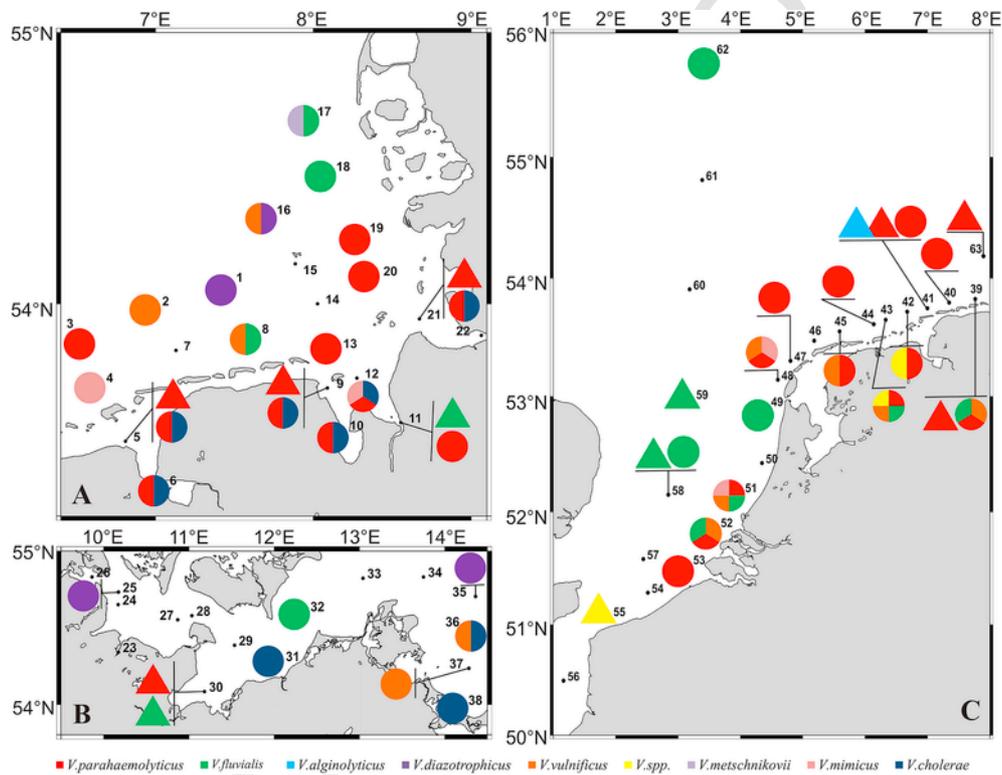


Fig. 2. Geographical occurrence of *Vibrio* spp. On microplastics and surface water of a) the North Sea from research cruise HE409 on RV Heinke in September 2013 b) the Baltic Sea from research cruise HE409 on RV Heinke in September 2013 and c) North Sea from research cruise HE430 on RV Heinke in July/August 2014 and the drift line of Helgoland (station 63). (○) species detected from surrounding seawater (△) species detected on microplastic particles.

rio fluvialis was the only species that was detected in open waters in the North Sea (Fig. 2 a, c).

In the Baltic Sea both species, *V. vulnificus* and *V. cholerae* appeared close to the Polish border at 14.5–14.9 °C and 5.7–7.3 PSU (station 36, 37, 38). *V. cholerae* occurred also nearby to Rostock at 14.1 °C and 11.7 PSU (station 31) (Fig. 2 b; Table A3). *Vibrio fluvialis* was detected once in Baltic surface water inside Germany and Denmark (station 32).

3.3. Identification and geographic distribution of *Vibrio* spp. on microplastics

All collected particles of North Sea, Baltic Sea and Helgoland drift line were subjected to selective APW enrichment resulting in 200 APW cultures. Out of these 161 displayed growth and were processed as described previously. From 15 microplastic particles from the North and Baltic Sea, in total 37 putative (according to the

colony colorations) *V. cholerae*, *V. vulnificus* or *V. parahaemolyticus* strains were isolated. At the drift line of Helgoland 4 putative *V. parahaemolyticus* strains from 4 different microplastic particles were isolated. Of these 41 strains, 22 were identified as *Vibrio* spp. by MALDI-TOF MS. Thirteen strains were identified as *V. parahaemolyticus* (59%), six as *V. fluvialis* (27%) and one as *V. alginolyticus* (5%) (Table A5). Even though we isolated representative coloured colonies neither *V. vulnificus* nor *V. cholerae* could be detected on microplastic particles.

V. parahaemolyticus was isolated from three polyethylene fibres and four polyethylene fragments during the cruises in the North Sea at temperatures between 14.8 and 21.1 °C and salinities between 12.6 and 32.4 PSU (Table A3). These were collected in the Ems estuary (station 5), near the uninhabited island Mellum (station 9), the Elbe estuary (station 21), and close to the Frisian islands (stations 39 and 41) (Fig. 2a, c). Additionally *V. parahaemolyticus* was isolated from two polyethylene films and two polypropylene fragments of Helgoland drift line at a water temperature of 16.6 °C and a salinity of 30.2 PSU (station 63) (Fig. 2c). *V. fluvialis* was detected on four non-identified particles collected between the UK and the Netherlands (stations 58, 59) and on a polyethylene fragment of the Weser estuary (Germany, station 11). *V. alginolyticus* was detected on one polyethylene fragment close to the Frisian island Juist (station 41). In the English Channel (station 55) an unspecified *Vibrio* spp. was detected on a polyethylene fragment (Fig. 2c).

One polypropylene film (station 30; Fig. 2b) collected close to the coastal regions of Wismar in the Baltic Sea at 14.8 °C and 12.6 PSU (Table A3) was colonized by both species, *V. parahaemolyticus* and *V. fluvialis*. *Vibrio parahaemolyticus* was detected only once on this single microplastic particle in the Baltic Sea (Fig. 2b).

4. Discussion

Although the microbial colonization of marine plastic particles was reported already in the 1970s, (Carpenter et al., 1972; Carpenter and Smith Jr., 1972) this issue received increasing attention in the last years due to the discovery of the large oceanic garbage patches (Kaiser, 2010; Ryan, 2014) and the general perception of microplastics being an emerging environmental topic of concern. In this context, it was also hypothesized, that microplastics may function as a vector for dispersion of invasive species including toxic algae but also pathogenic organisms (Masó et al., 2003; Zettler et al., 2013).

Recently the microbial community on marine plastics was targeted in several studies, highlighting the composition and diversity of plastic-attached microorganisms (Carson et al., 2013; Zettler et al., 2013; Oberbeckmann et al., 2014; Reisser et al., 2014; Amaral-Zettler et al., 2015; De Tender et al., 2015). Within the microbial community on the “Plastisphere” (Zettler et al., 2013) sequences related to the genus *Vibrio*, a group of bacteria also containing serious pathogens, were found (De Tender et al., 2015; Zettler et al., 2013). However, in both studies a conclusive identification on the species level could not be provided so far due to the usage of next-generation amplicon sequencing and the short read lengths inherent to the methodology.

In our study we were able to prove the presence of potentially pathogenic *V. parahaemolyticus* on twelve floating microplastics for the first time by a selective cultivation approach and identification on species level by MALDI-TOF MS.

4.1. Microplastics in the North and Baltic Sea

In the present study, we observed more microplastic particles in North Sea waters compared to the Baltic Sea. Up to now, information on the abundance of microplastics in coastal waters of the North and Baltic Sea is scarce, and a comparison of the findings is problematic due to missing standard operational procedures (SOP) for sampling, extraction and analysis of microplastics (Löder and Gerdt, 2015).

During both cruises in 2013 and 2014, 77% of all collected and identified microplastics as well as all collected microplastics at the drift line of Helgoland, occurred as fragments with rough and uneven edges clearly indicating a breakdown of larger plastics (Thompson et al., 2004). Brittleness of particles including cracks and pitting could be detected on collected microplastics which might be the result of degradation processes or wind and wave actions (Andrady and Neal, 2009). Thus it could be suggested that most of the collected microplastics were exposed long enough to the marine environment to get brittle and be transported over long distances. Thiel et al. (2011) reported hotspots of accumulating microplastics in the North Sea and a rapid transport through the German Bight due to strong westerly winds. In contrast, based on the relationship between litter accumulation on Helgoland beaches and southerly winds, Vauk and Schrey (1987) suggested that these winds might push anthropogenic debris from source regions which results in accumulation on local beaches. Galgani et al. (2000) proposed that the predominant northward currents in the eastern part of the German Bight transport floating debris and accumulate it in an area to the west of Denmark. However due to the focus of our study (*Vibrio* spp.), these findings should be interpreted with care since we were not aiming at monitoring microplastics explicitly and in a systematic way.

By far the majority of microplastics from the North and Baltic Sea as well as from the Helgoland drift line was identified as polyethylene, followed by polypropylene and polystyrene (Fig. 1). Prior studies already reported high portions of these three polymers in the course of various samplings in marine and coastal environments which mirrors our results (Browne et al., 2010; Moret-Ferguson et al., 2010; Oberbeckmann et al., 2014) and furthermore reflect the usage of these polymers in the worldwide economy. In the United States polyethylene, polystyrene, polypropylene and polyethylene terephthalate are the most widely produced and disposed synthetic polymers (Barnes et al., 2009). In Europe polyethylene and polypropylene are the synthetic polymers with the highest demand in various application segments, especially in packaging (PlasticsEurope, 2015).

4.2. *Vibrio hitchhikers*

Biofilm communities on environmental plastic samples were recently characterized in several studies applying molecular tools. The diverse microbial communities on marine plastic debris differed clearly from the surrounding seawater (Zettler et al., 2013; Oberbeckmann et al., 2014; Amaral-Zettler et al., 2015; De Tender et al., 2015).

The herein described presence of potentially human pathogenic *Vibrio* spp. on microplastics has to be discussed in the light of these latter studies. The first indication of the presence of *Vibrio* spp. on marine microplastics was published by Zettler et al. (2013), who reported the dominance of this genus that constituted nearly 24% of the whole biofilm community on a single polypropylene particle collected from the North Atlantic. In 2015, De Tender et al. (2015) reported the detection of members of the family Vibrionaceae on marine plastics from the Belgian North Sea. Recently a review of

Keswani et al. (2016) highlights the lack of knowledge about the persistence of potentially pathogenic *Vibrio* spp. on plastic debris. Our study clearly confirmed the presence of cultivable *Vibrio* spp. on 13% of all marine collected microplastic particles. Amongst others, potentially pathogenic *V. parahaemolyticus* strains were detected on 12 microplastic particles. Only collected polyethylene, polypropylene and polystyrene fragments were colonized by *Vibrio* spp.

In general *Vibrio* spp. tends to colonize marine biotic surfaces like corals or zooplankton/phytoplankton surfaces. *V. cholerae* strains, both *O1* and non-*O1* serovars, as well as *V. parahaemolyticus* strains were found to be attached to the surfaces of copepods in natural waters (Huq et al., 1983). In comparison to naturally occurring polymers like chitin, synthetic polymers are poorly degradable and could therefore function as a mechanism for the transport and persistence of *Vibrio* species. Pruzzo et al. (2008) reviewed substrate-specificity of *V. cholerae* on the naturally occurring polymer chitin. They reported close interactions between *V. cholerae* and chitin surfaces in the environment including cell metabolic and physiological responses e.g. chemotaxis, cell multiplication, biofilm formation, and pathogenicity. With respect to plastic microbial communities, Oberbeckmann et al. (2014) found that the structure and taxonomic composition of these plastic associated communities vary with plastic type, but also with geographical location and season. Moreover, Amaral-Zettler et al. (2015) found that "Plastisphere" communities of the Atlantic and Pacific Ocean clustered more by geography than by polymer type, with exception of polystyrene that showed significant differences to polyethylene and polypropylene.

The substrate specificity of *Vibrio* spp. on synthetic polymers is still not investigated. However, since polyethylene, polypropylene, polystyrene and polyethylene terephthalate are the most widely disposed synthetic polymers globally (Barnes et al., 2009), it can be supposed that our results are biased due to the high accumulation of these specific synthetic polymers in our oceans.

Potentially pathogenic *V. parahaemolyticus* as well as *V. fluvialis* occurred in water as well as on microplastic particles. Recent studies report that *V. parahaemolyticus* and *V. alginolyticus* are prevailing inhabitants of North Sea waters (Böer et al., 2013; Oberbeckmann et al., 2011b). In contrast, *V. vulnificus* and *V. cholerae* are more abundant in the Baltic Sea (Böer et al., 2012), which is also reflected by our findings. As already shown elsewhere, free-living bacterial communities in general differ significantly from plastic-attached ones (Amaral-Zettler et al., 2015; De Tender et al., 2015; Oberbeckmann et al., 2014; Zettler et al., 2013), which holds also for microplastics investigated here. With respect to potentially pathogenic *Vibrio* spp., the species *V. vulnificus* and *V. cholerae* were only isolated from seawater samples but not identified on microplastics in the framework of our study. In contrast, *V. parahaemolyticus* was detected in both, water and on microplastic particles (Fig. 2). Additionally, *V. parahaemolyticus* was detected once in the Baltic Sea and only on a microplastic particle throughout the entire cruise.

Plastic is a persistent material and may serve as a reservoir and vector for potentially pathogenic microorganisms. The drift of potentially harmful algae species, barnacles and bryozoans on plastic debris (Barnes, 2002; Masó et al., 2003) is already well documented. Our results fuel the evidence for potentially pathogenic bacteria being dispersed on microplastic particles by wind or currents. However, although we identified *V. parahaemolyticus* on microplastics to species level, due to the high intra-species diversity information on the geographical origin of these hitchhikers or the microplastics is not possible, since the assignment of *Vibrio* species down to specific ecotypes was not successful.

Vibrio spp. on microplastics were detected mainly close to the coast and only occasionally offshore. However, microplastics and

seawater samples carrying *V. parahaemolyticus* were located exclusively in estuarine and coastal areas of the North and Baltic Sea. *V. parahaemolyticus* occurrences in seawater were already addressed in several studies in Northern European waters (Bauer et al., 2006; Böer et al., 2013; Collin and Rehnstam-Holm, 2011; Ellingsen et al., 2008; Lhafi and Kühne, 2007; Oberbeckmann et al., 2011b; Schets et al., 2011; Schets et al., 2010). Environmental parameters, such as temperature, salinity or plankton abundance have an effect on *Vibrio* spp. communities and abundances (Blackwell and Oliver, 2008; Caburlotto et al., 2010; Drake et al., 2007; Martinez-Urtaza et al., 2008; Thompson et al., 2004b; Turner et al., 2009; Vezzulli et al., 2009). Vezzulli et al. (2010) and Schets et al. (2010) identified seawater temperature as a key factor influencing the presence of *Vibrio* spp., for instance it is well documented that *V. parahaemolyticus* favours warmer water temperatures (Sobrinho et al., 2010). Recently, pathogenic *V. parahaemolyticus* was detected even in temperate European waters (Baker-Austin et al., 2010; Martinez-Urtaza et al., 2005). Martinez-Urtaza et al. (2008) observed higher occurrence of this taxon during periods of lower salinity and in general this taxon was primarily detected in areas of reduced salinity close to freshwater discharge runoff, which is also in agreement with our findings.

In our study *V. parahaemolyticus* occurred also on microplastics collected from the drift line at Helgoland. Oberbeckmann et al. (2011b) detected *V. parahaemolyticus* during summer months and reported that the abundance of *Vibrio* spp. was influenced by specific environmental conditions like the decrease in salinity due to an inflow of coastal water at Helgoland Roads (North Sea, Germany). Each *Vibrio* group was influenced by different combinations of environmental parameters but no single environmental parameter could explain the whole community structure of *V. alginolyticus* and *V. parahaemolyticus* populations in the German Bight (Oberbeckmann et al., 2011b). The authors also reported that free-living and plankton-attached *Vibrio* spp. abundances were mainly driven by the same environmental parameters (Oberbeckmann et al., 2011b). This suggests that the potentially pathogenic *V. parahaemolyticus* detected both on North Sea microplastics and in seawater samples of one station were influenced equally by environmental conditions.

5. Conclusion

This study successfully evidences the occurrence of potentially pathogenic *Vibrio* spp. on the species level on marine microplastics by use of MALDI-TOF MS for the first time. In most of the cases, these species co-occurred also in surrounding seawater, suggesting that seawater serves as a possible source for *Vibrio* colonization on microplastics. The fact that we for the first time detected *V. parahaemolyticus* exclusively on polyethylene, polypropylene and polystyrene particles, points to the urgent need to further address the biogeography and persistence of these hitchhikers on marine microplastics. Studies on the co-occurrence of specific *V. parahaemolyticus* genotypes on microplastic and surface water from the North Sea are particularly important specifically with reference to the potential health impacts of microplastic-colonizing microbial assemblages.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.marenvres.2016.07.004>.

Uncited reference

Webb et al., 2009.

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