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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 CHROMagar™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.

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 algal 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

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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, rod-shaped chemoorganotrophic 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. cholera* (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, GUILDLINE, 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