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Microplastics as a vector for the transport of the bacterial fish pathogen species *Aeromonas salmonicida*

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ABSTRACT

Microplastics is widespread in the marine environment where it can cause numerous negative effects. It can provide space for the growth of organisms and serves as a vector for the long distance transfer of marine microorganisms. In this study, we examined the sea surface concentrations of microplastics in the North Adriatic and characterized bacterial communities living on the microplastics. DNA from microplastics particles was isolated by three different methods, followed by PCR amplification of 16S rDNA, clone libraries preparation and phylogenetic analysis. 28 bacterial species were identified on the microplastics particles including *Aeromonas* spp. and hydrocarbon-degrading bacterial species. Based on the 16S rDNA sequences the pathogenic fish bacteria *Aeromonas salmonicida* was identified for the first time on microplastics. Because *A. salmonicida* is responsible for illnesses in fish, it is crucial to get answers if and how microplastics pollution is responsible for spreading of diseases.

1. Introduction

Marine litter is regularly observed everywhere in the oceans. According to estimations by Jambeck et al. (2015), 8 million metric tons of plastic waste makes its way into the world's oceans every year. Emissions of plastic come from point and diffuse land-based sources, as well as from fishing and from marine-based industry sources, and can travel long distances before being stranded (Galgani et al., 2015). Plastics constitute a large part, even close to 100%, of floating litter (Galgani et al., 2015). This is a cause of worry as plastics production keeps rising. Global production of plastics in 2015 reached 322 million metric tons, of which 58 million metric tons were produced in Europe alone, and this number does not even include the production of textile fibers (PET, PA, PP, polyacryl) made of effectively the same materials (PlasticsEurope, 2016). Assuming that 10% of all plastic waste ends up in the oceans (Thompson, 2006) and due its slow degradation, we can only conclude that the quantities of macrolitter and, consequently, microplastics (< 5 mm) in the marine environment will continue increasing in the future.

Over the past few years, a significant effort has been made to quantify microplastics in the seas (Lusher, 2015). From the seafloor to the water column and seacoast, microplastics measurements dominate the sea surface. Specifically, the sea surface microplastic concentrations found in the Pacific ocean are: Northern Pacific (NP) Subtropical gyre 20,000–450,000 particles/km^2(Goldstein et al., 2013); NP central gyre 85,184 particles/km^2 (Carson et al., 2013); NP central gyre 334,271 particles/km^2 (Moore et al., 2001), and in the Atlantic ocean they are: North Atlantic gyre 20,328 particles/km^2 (Law et al., 2010); Northwest Atlantic 490 particles/km^2 (Wilber, 1987); Caribbean 1414 particles/km^2 (Law et al., 2010).

Microplastics can accumulate on shorelines, on the sea surface and on the seafloor. Accumulation rates vary significantly as they are influenced by diverse factors such as the presence of large cities, shore use, maritime activities (Galgani et al., 2015), and oceanographic features such as currents and waves (Galgani, 2014; Andrady, 2011; Browne et al., 2011). It appears that accumulation rates are lower in the southern than in the northern hemisphere (Galgani et al., 2015).

In EU, the Marine Strategy Framework Directive (MSFD, 2008/56/EC) has highlighted concerns for the environmental implications of marine litter (Zarfl et al., 2011). In the Mediterranean Sea, which is one of the most polluted seas (Costello et al., 2010), the presence of microplastics in waters has already been confirmed (Galgani et al., 1996). In surface waters of the northwest Mediterranean 1,330,000 particles/km^2 (Collignon et al., 2012) were found and in the bay of Calvi on

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Corsica Island as 62,000 particles/km^2 were found. The Adriatic Sea, a small, shallow and semi enclosed basin, represents a hotspot for pollution (Halpern et al., 2008). Gajšt et al. (2016) reported an average of 406.000 particles/km^2 in the Slovenian part of the North Adriatic and indicated high locational and temporal variation attributed to currents and winds.

The negative effects of plastic marine litter and microplastics on animals, such as benthic invertebrates, birds, fish, mammals and turtles as a result of entanglement or ingestion, are well documented (Kühn et al., 2015; Lusher, 2015). Furthermore, it is generally assumed that microplastics may act as a vector for the transport of chemicals adsorbed on or contained in plastic particles, such as persistent organic pollutants (POPs) or additives (Koelmans, 2015). Since plastics have a much longer half-life than most natural floating marine substrates and a hydrophobic surface that promotes microbial colonization and biofilm formation, it serves as pelagic habitat for microorganisms and invertebrates (Reisser et al., 2014). Zettler et al. (2013) referred to the diverse microbial community composed of heterotrophs, autotrophs, predators, and symbionts that inhibit and live on plastic particles as the "plastisphere".

The formation of biofilms on the plastic's surface strongly influences plastic degradation processes (Artham et al., 2009) in two ways. The organisms can indirectly increase longevity of plastic particles (Carson et al., 2013) by protecting them from ultraviolet radiation and photocatalysis either directly via decreased buoyancy resulting in sinking (Andrady, 2011), or organisms can actively accelerate the degradation process (Balasubramanian et al., 2010; Zettler et al., 2013).

The transport of microorganisms attached to plastics is of a great concern, since communities present on plastic particles differ from those present in the surrounding marine environment (Zettler et al., 2013). The transport of species over long distances therefore presents a potential change in their natural ranges, possibly allowing them to become non-native or even invasive species (Reisser et al., 2014) or disease vectors (Goldstein et al., 2014; Maso et al., 2003). Bacteria such as human pathogen *Vibrio* spp. (Zettler et al., 2013; Kirstein et al., 2016; Foulon et al., 2016) and other Vibrionaceae (De Tender et al., 2015) have been found colonizing microplastics particles in the marine environment.

Even though it is known that all surfaces of microplastics in the marine environment are rapidly colonized by bacteria (Harrison et al., 2014), the actual taxonomic composition of biofilms on marine microplastics remains largely unexplored (Kirstein et al., 2016). There are no known data on bacterial communities colonizing microplastic particles in the North Adriatic Sea, which is a known area of high microplastic pollution and a highly populated sea. The main goal of our research was therefore to: 1) determine the abundance and chemical composition of sea surface microplastics in the North Adriatic Sea, 2) isolate bacterial DNA from the biofilms on microplastics, and to 3) construct a clone library, perform phylogenetic analysis and identify the bacterial species in biofilms on microplastics from the sampled area.

2. Methods

2.1. Microplastics sampling

Samples were collected along the Slovenian coast (from Piran Bay to Koper Bay) of the North Adriatic Sea during two periods of time. The first sampling was carried out in August 2014 (25th and 26th August 2014), when six transects were sampled in 2 days (Fig. 1, S1–S4 and R1–R2) and the second in May 2015 (11th May 2015), when 4 transects were sampled (S1–S4 on the same locations as in August 2014). The sampling transects were selected so that the majority of the Slovenian coastal sea area was included in the survey (from the south to the north border) and the areas influenced by all the main Slovenian coastal cities (Piran, Izola, Koper). Samples were collected using a manta net with 308 μm pore size, according to the methodology described in the protocol for microplastics sampling on the sea surface and sample analysis developed within the DeFishGear project (Kovač Viršek et al., 2016). The boat speed was approx. 2.5 knots and the time of sampling was 30 min. Transects were approx. 1.3 nm long.

All collected samples were analyzed in the laboratory. First, samples were cleaned of organic material and artificial objects larger than 5 mm by visual observation. After that, samples were checked for the presence of microplastic particles using a stereomicroscope. No degradation of the organic material from field samples was carried out. Microplastic particles were placed into 6 categories (fragments, filaments, films, pellets, granules and foams).

In order to avoid contamination from air transported fibers, microplastics separation was performed in a clean room.

2.2. Chemical characterization of microplastics

Chemical identification of microplastic particles sampled in 2014 was carried out using ATR FT-IR microscopy (LUMOS, Bruker, Germany), while microplastic particles sampled in 2015 were analyzed using ATR FT-IR spectroscopy (Spectrum Two, Perkin Elmer, USA). When an ATR-FT-IR microscope was used, microplastic particles were placed on a glass filter and the ATR germanium crystal was cleaned using 80% alcohol and a lint free cloth. The filter with the microplastic was placed on the automatic scanning table and the joystick was used to locate the sample and to record an optical image. The measured area was 20 × 20 μm in size. The obtained spectra were compared with spectra in the BRUKER Polymer library and ATR-FTIR Polymer library (S.T. Japan) collections to identify the composition of particles. When the ATR FT-IR spectrometer was used, microplastic particles were placed on a diamond crystal ATR holder and spectra were collected and compared with a spectra database (Hummel spectra library).

2.3. DNA isolation from microplastics

For the analysis of bacterial community one additional transect was sampled on 26th of August 2014 for 20 min on the route S2 (see Fig. 1) using a manta net. The measured sea temperature was 24.4 °C and salinity was 35.5 PSU. Material caught in the manta net was rinsed into a sterile bottle by sterile distilled water and was immediately stored in a cool box and transferred to the laboratory. On the same day, microplastic particles were separated with sterile tweezers into a sterile petri dish and frozen at − 20 °C. DNA from microplastics' samples was extracted using three different isolation methods. Two of them were direct methods, where DNA was directly isolated from microplastic particles using the PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA) (1$^{\#}$ clone library) or PowerBiofilm DNA isolation kit (MOBIO Laboratories, Carlsbad, CA) (2$^{\#}$ clone library) according to the manufacturer's instructions. In these methods, 30 to 50 mg of microplastics was used for DNA extraction. While we hypothesized that pre-culturing bacteria from a microplastic particle in a bacterial growth media (LB) could amplify the signal, also a pre-culturing method was tested. There microplastic (1 piece) was cultured in Lauria Bertani (LB) medium overnight at 37 °C and DNA was extracted from the bacteria grown overnight using a DNeasy Tissue DNA isolation kit (Qiagen) (3$^{\#}$ clone library). The yield of bacterial DNA ranged from 450 to 4450 ng. To exclude the possibility of contamination, only sterile MiliQ water and other material were used. All procedure were done in PCR box with UV disinfection. In the polymerase chain reaction MiliQ water was used as a negative control. In the case, when microplastic fragment was precultured in LB media, as a negative control only growth media was incubated overnight.

2.4. 16S RNA PCR amplification and clone library construction

16S RNA was amplified from genomic DNA samples using primers Bac27F (5′-AGAGTTTGATCMTGGCTCAG) and Univ1492R (5′-