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ANALYSIS OF PLASTIC-ASSOCIATED BACTERIA IN FRESHWATER IN HUNGARY

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LIST OF ABBREVIATIONS

PE	Polyethylene
PVC	Poly (vinyl chloride)
PS	Polystyrene
PET	Poly (ethylene terephthalate)
PP	Polypropylene
PU	Polyurethane
WHO	World health organization
PCBs	Polychlorinated biphenyls
MPs	Microplastics
SEM	Scanning electron microscope
T-RFLP	Restriction fragment length polymorphism
LDPE	Low-density polyethylene
AFM	Atomic force microscopy
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
HCB	Hydrocarbonoclastic bacteria
MPL	Marine plastic litter
CS	Cobblestone
WWTP	Wastewater treatment plant
OTU	Operational taxonomic unit
PE-OXO	Polyethylene additivated with a prodegradant catalyst to make it biodegradable

ARGs	Antibiotic resistance genes
PAH	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PCL	Polylcaprolactone
PLA	Polylactic acid
PCA	Principle component analysis
dDDH	Digital DNA–DNA hybridization values
GGDC	Genome-to-genome distance calculator
OrthoANI	Orthologous average nucleotide identity
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
DSMZ	German Collection of Microorganisms and Cell Cultures
MCs	Microcystins
PCP	Pentachlorophenol
BC	Bacterial cellulose
GL	Glycolipids
PL	Phospholipid
PG	Phosphatidylglycerol

1 INTRODUCTION

Although plastics appeared in the early 20th century, they were mainly used in military applications until the second world war(Geyer *et al.*, 2017). After that, plastic production has increased exponentially and entered a wide range of industries; in 1950, plastic production reached 1.5 million tons and in 1989 100 million tons were produced. In 2019, the number jumped to 368 million tons (Statista, 2020). Plastics are used extensively in many applications in human life, and this is due to the unique characteristics that plastics have, such as versatility, durability, cost-effectiveness, limited maintenance, resistance to corrosion, lightweight, flexibility, and many others. The uses of plastics include; construction, electrical and electronic applications, packaging, transport industry (cost-effective, economic fuel consumption) (British Plastic Federation, 2020). Due to the huge increase in plastic production worldwide, more and more plastics are being released and accumulating in the environment.

Plastic pollution has serious threats on most ecosystems; it threatens marine wildlife due to the ingestion and entanglement of hundreds of marine animals. Large numbers of marine animals die due to starvation as their stomachs become full of plastic, in addition plastics limit their ability to swim and increase the occurrence of internal injuries (Pinto *et al.*, 2020). It has also been reported that plastic pollution is responsible for the transfer of invasive species between ecosystems. A wide range of species associated with plastic materials are invasive macrofauna in addition to toxic microorganisms (García-Gómez *et al.*, 2021).

Like other surfaces, plastic surfaces, including both macroplastic (>5mm) and microplastic (<5mm) in size, have been found to be colonized by organisms including bacteria, viruses, algae, fungi. Heterotrophic, autotrophic, symbiotic, and even pathogenic microbes have been detected in the plastic associated biofilm. The attachment of microbes to plastic surfaces helps in the dispersion to other locations and various environments, including rivers, lakes, oceans, ground water as well as wastewater (Wright *et al.*, 2020).

Microbial communities associated with microplastic have been frequently studied in marine water. Many studies have revealed that associated microbial communities are different from the surrounding water or other natural surfaces. (Miao *et al.*, 2019; Yang *et al.*, 2020; Zettler *et al.*, 2013). Various environmental, chemical, and physical factors were analyzed to study their impact on microbial colonization (Oberbeckmann *et al.*, 2018). Early community pioneers colonizing microplastic surfaces were also described (Oberbeckmann, Löder and Labrenz, 2015). Different methods have

been used for this purpose, such as; the collection of microplastic from sea water using a manta net (Viršek *et al.*, 2017), collecting plastic particles from the beach or shallow water (Guo *et al.*, 2020), or incubating plastic particles in seawater under controlled lab conditions (Wu *et al.*, 2019).

Freshwater ecosystems are considered the main destination of various pollutants released into the watershed. The reason for that is that freshwater bodies are usually located in valleys and low-lying areas. Incorrect disposal of plastic lead to the transfer of plastic waste into freshwater ecosystems. Moreover, direct disposal of waste including plastic into rivers is an old tradition in many urban areas. The occurrence of plastic waste in freshwater bodies significantly affects biodiversity and presents a serious threat to freshwater ecosystems. Recently, a growing concern has been noticed because microplastics have been detected in various freshwater bodies such as rivers and lakes (Koelmans *et al.*, 2019), even detected in drinking water (WHO, 2019; Li *et al*., 2018). However, limited data is available regarding microplastics in freshwater compared to marine water, especially in terms of the associated microbial communities, but we hope that our research work will help in filling the knowledge gap by investigating microplastic associated communities in freshwater.

1.1 Research Objectives

1. Development of an easy-to-use method to study the microbial colonization of microplastics in freshwater that can be used *in situ*.
2. Investigation of microplastic associated bacteria in a freshwater lake in Hungary.
3. Description of possible novel bacterial species associated with plastics.

2 LITERATURE REVIEW

2.1 Synthetic polymers

Polymers are made synthetically in the laboratory mainly from petroleum origin. Synthetic polymers, or so-called plastics, were discovered in the nineteenth century. PVC was the first to be polymerized in the period between (1838-1872). In 1907 the first real synthetic plastic (Bakelite) was created and produced in mass quantities by Leo Baekeland, a Belgian-American chemist (PlasticEurope, 2020).

Today synthetic polymers include polyethylene (PE), poly (vinyl chloride) (PVC), polystyrene (PS), polyamides (nylon), synthetic rubber, epoxy, teflon, and many others. Their backbone is made from monomers of carbon-carbon bonds (Figure 1) (Gewert *et al.*, 2015). In the presence of heat, pressure, and catalysts, these monomers hold together in stable structures (Shrivastava, 2018).

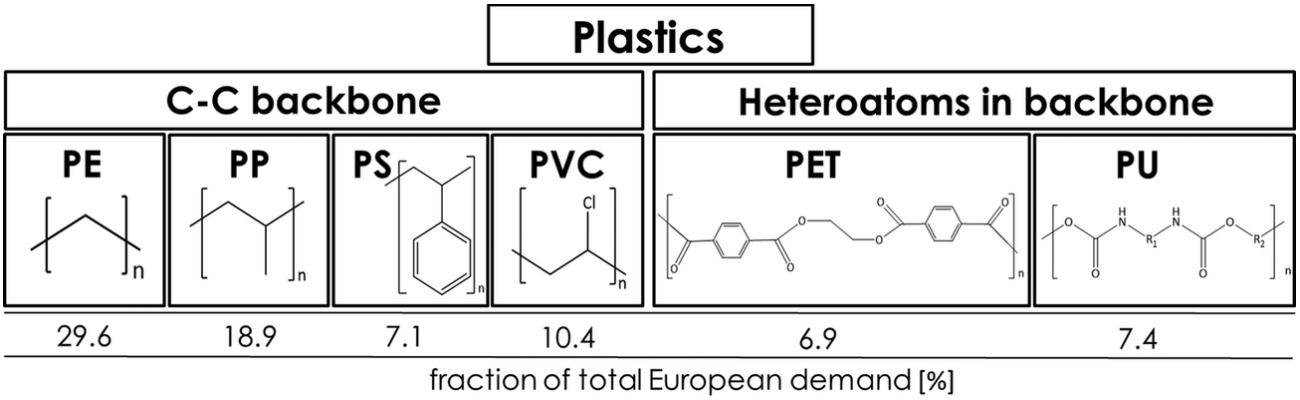

Figure1 Polymer types with structures and their fraction of the total European demand (PE – polyethylene, PP – polypropylene, PS – polystyrene, PVC – poly(vinyl chloride), PET – poly(ethylene terephthalate), PU – polyurethane)

Polymer degradation can be defined as the alteration in polymer properties because of physical, chemical, or biological reactions resulting in bond scissions and subsequent chemical transformation. Degradation changes the optical, mechanical, or electrical properties of materials such as cracking, discolouration, and erosion. (Singh and Sharma, 2008). Polymer degradation can be classified according to the nature of causative factors into two categories; abiotic and biotic degradation. Microbes are responsible for biotic degradation, while abiotic degradation occurs due to other physical and chemical factors (Gewert *et al.*, 2015).

Photooxidation is the most common abiotic factor that precedes the microbial attack that will start the biodegradation of plastic polymers (Rummel *et al.*, 2017). This kind of degradation is of high importance in the initiation of degradation. Visible light and UV mostly start the first step of synthetic

polymer primary degradation, normally photo-irradiation start generating ester, propyl, formate and aldehyde groups in the soft part of the polymer, then degradation starts there (Singh and Sharma, 2008). Photodegradation results in the alteration of physical and chemical properties of the polymers resulting in visual appearance changes, molecular weight change, mechanical integrity (Nagai *et al.*, 1999).

2.2 Plastics in the environment

Marine litter or debris is composed of items made or used by people, and they are discarded into the water bodies accidentally or intentionally. This debris causes serious economic, environmental, and health threats and may lead to the degradation of ecosystems. Plastics have been recognized as the major content of marine debris; it is estimated that 50-90% of the total marine debris is plastic (P *et al.*, 2019). Plastic debris is a mixture of polymers and associated chemicals; diverse plastic items have been found in plastic debris such as cigarette butts, bottles, lids, bags, fishing gear, food packaging, etc. Compared to other organic and inorganic marine debris, plastic debris is persistent in the environment, and the degradation rate is very slow (hundreds of years) (Gallo *et al.*, 2018). It is estimated that two-thirds of the plastic debris ends up on the seabed, half of the remaining third washes up on beaches, and the other half is the floating amount observed in the oceans; therefore, considering only the floating amount underestimates the plastic problem in the oceans (Andrady, 2011).

The first reported plastic debris occurrence in coastal water was in 1970 (Carpenter and Smith, 1972). More concern has been given to the plastic pollution problem when a new term "microplastic" (plastic pieces of size <5mm) was proposed (Thompson *et al.*, 2004). Microplastics (MPs) can be classified into two categories based on their origin; primary microplastics (microbeads), including those generated to be used in facial cleansers (Zitko and Hanlon, 1991), medicine (Patel *et al.*, 2009), and cosmetics (Guerranti *et al.*, 2019). The second category of microplastics is the secondary microplastics produced from the breakdown of macroplastics (plastic particles with a diameter ≥ 5 mm) (Thompson *et al.*, 2004). Plastics can be degraded in the environment by several means; chemical (corrosion, heat, photodegradation), mechanical (abrasion, wave action, scarification on rocks), or biodegradation via the activity of bacteria and fungi (Zettler *et al.*, 2013; Andrady, 2011).

Their durability, weight, and buoyancy are unique features that enable microplastics to travel and spread in the environment. Land-based sources are the main source of microplastics (80%), while 20% are usually generated by sea-based sources (Barboza *et al.*, 2019). Microplastics were detected in beaches (Stolte *et al.*, 2015), sediments of the deep sea (Woodall *et al.*, 2014), effluent wastewater

(Carr *et al.*, 2016; Edo *et al.*, 2020), freshwater bodies (Wagner *et al.*, 2014; WHO, 2019; Blettler *et al.*, 2017), surface water (Zhao *et al.*, 2014; McCormick *et al.*, 2016; Egessa *et al.*, 2020), and even in the arctic ocean (Kanhai *et al.*, 2020).

Further degradation of microplastics generate smaller particles called nanoplastics (<100nm). Nanoplastics are considered a recent emerging environmental pollutant. Due to their high surface area, nanoplastics could play an essential role in the bioaccumulation of other pollutants such as polychlorinated biphenyls (PCBs), pesticides, and heavy metals. Additionally, they have the capability to cross biological membranes and consequently to affect cell function (Pinto *et al.*, 2016).

2.3 Threats to the aquatic environment

Microplastics can have severe effects on the aquatic environment; because of their small size, they might be ingested by variable organisms, such as planktonic and higher organisms like mammals, fish, or birds (Kühn *et al.*, 2020; Reynolds and Ryan, 2018; Nelms *et al.*, 2019) (Figure 2). The exact toxicity mechanism is poorly understood, but it is believed that toxicity potentially occurs due to three reasons: 1) ingestion stress such as egestion expended energy, reduced feeding efficiency, 2) associated contaminants like heavy metals, organic pollutants or biological agents 3) exposure to plastic additives (Anderson *et al.*, 2016). The severity of microplastic toxicity effects depends on the organism egestion capability, the level of accumulation in tissues, and trophic transfer potential (Wright *et al.*, 2013).

Figure 2 An Albatross feeding his baby pieces of plastic (Chris Jordan, 2021)

On the low trophic level, plastic debris provides a new surface for the colonization of some animals (e.g. molluscs) and different microbes. Due to its long durability compared to most natural surfaces, microbial communities associated with plastics were found to consist of heterotrophs, predators, autotrophs, and symbionts; such communities were given the name 'Plastisphere' (Zettler *et al.*, 2013). Several studies have revealed that the bacterial communities associated with microplastics in aquatic environments are distinct from surrounding water (McCormick *et al.*, 2014; Frère *et al.*, 2018; Miao *et al.*, 2019). Plastispheric bacterial communities may contain opportunistic human or animal pathogens such as *Vibrio*; therefore, when microplastics are ingested by birds or fish, they might introduce a risk of transferring infectious diseases (Zettler *et al.*, 2013; Thompson *et al.*, 2004).

Several studies have been conducted to analyze the structures of bacterial communities associated with microplastics. However, the majority of these studies were conducted in marine water (Debroas *et al.*, 2017; Dudek *et al.*, 2020; Ogonowski *et al.*, 2018; Oberbeckmann *et al.*, 2018).

2.4 Colonization of plastic by microorganisms

Any surface in the aquatic environment can be colonized by microorganisms as well as macroorganisms. The formation of biofilm develops via four stages: organic and inorganic molecules adsorption, bacterial attachment, followed by attachment of unicellular eukaryotes and finally spores

and larvae attachment (Dobretsov, 2010). The adsorption of organic and inorganic molecules occurs quickly (within seconds) after the contact of the virgin surface with the surrounding water (Loeb and Neihof, 1975). Microorganisms get into contact with surfaces due to the interactions of repulsion and attraction among the surface, the cell wall, and the surrounding medium (water) (Rummel *et al.*, 2017). The process of bacterial attachment is highly regulated and controlled. Following the attachment stage, bacteria start to divide and multiplicate and produce an extracellular polymer layer leading to irreversible attachment (Costerton *et al.*, 1995). Bacterial attachment on any surface is affected by the surface roughness, charge, free energy, electrostatic interactions, and hydrophobicity (Rummel *et al.*, 2017).

Colonization of plastics by microorganisms is also a rapid process, usually happening within hours (Harrison *et al.*, 2014). Evidence for marine plastic colonization by microorganisms was first presented in the 1970s as diatoms, and other microbes were detected on plastic surfaces samples in the Sargasso Sea and North Atlantic (Carpenter and Smith, 1972**;** Colton *et al.*, 1974). Colonization of plastics has been vigorously studied afterwards in different environments; marine water, wastewater, freshwater, and soil (Lobelle and Cunliffe, 2011; Zettler *et al.*, 2013; McCormick *et al.*, 2014; Oberbeckmann *et al.*, 2018).

Due to their high molecular weight and unavailability of environmental analogues to plastic, the chemical reactivity of plastic is limited. The degradation rate is very slow; it can persist in the environment for hundreds to thousands of years. In the deep sea, it may take a longer time (Worm *et al.*, 2017; Barnes *et al.*, 2009). Thus, it can provide a habitat for the growth and colonization of microbial communities.

Plastic is one of these surfaces that are rapidly colonized by microbes. Certain factors can help to initiate the process of microbial colonization. The surface structure and chemistry can be altered by UV radiation and hydrolytic degradation reaction; they can induce crack formation, reduce molecular weight and enhance surface oxidation which can contribute to biofilm development. It is believed that for polymers with a carbon backbone, abiotic degradation usually comes before biodegradation (Gewert *et al.*, 2015).

The structure of the plastisphere can be influenced by the particle age; for example, plastic particles in rivers and streams have shorter residence time compared to lakes and oceans where it can stay for decades; therefore their exposure to UV radiation will be higher (Harrison *et al.*, 2018). Other ambient conditions like salinity, pressure, temperature, oxygen and light availability have effects on microbial

biofilms associated with plastic. For example, in the deep sea where no light is available, the temperature is low (<5C), the pressure is high, the oxygen concentration is limited. Under these conditions, the structure of the plastisphere is expected to be greatly different compared to the shallow water plastisphere (Oberbeckmann *et al.*, 2015; Harrison *et al.*, 2018; Amaral-Zettler *et al.*, 2015). Furthermore, open ocean water is considered relatively poor in nutrients compared to inland and coastal waters, which are rich in nutrients received from the surrounding sources (Battin *et al.*, 2016).

Biofilm formation was found to significantly affect the physiochemical characteristics of plastic surfaces in water, such as hydrophobicity, which increases notably after the biofilm development. Plastic buoyancy was also found to be influenced by biofilm formation; most plastics are positively buoyant but become neutrally buoyant upon biofilm formation (Lobelle and Cunliffe, 2011). Changes in the hydrophobicity and buoyancy of plastic due to biofilm formation will affect the vertical transfer in the water column and consequently the sorption and release of contaminants (A. Glaser, 2020).

2.5 Bacterial colonization of microplastics in marine water

Colonization of microplastics in marine water has been studied extensively; in a study conducted by Zettler *et al*., marine plastic debris was collected by neuston net with 333 μm from different locations in the north Atlantic subtropical gyre. Microbial communities were investigated and described on PP and PE pieces and compared with communities in the surrounding marine water. Phenotypic tools using scanning electron microscope (SEM) and genotypic molecular sequencing tools (amplicon sequencing) were used to analyze the microbial communities. SEM images showed that PP and PE samples were rich in bacterial communities and eukaryotes (Zettler *et al.*, 2013). On the other hand, the DNA sequencing results revealed that microbial communities associated with plastic pieces were notably different compared with communities of the surrounding water. For instance, the cyanobacterial members *Phormidium* and *Rivularia* were detected in the plastic community but not in surrounding seawater which was dominated by the phototroph *Prochlorococcus*. Regarding heterotrophic bacteria, *Pelagibacter* and other free-living picoplanktonic bacteria were dominant in seawater samples but were much different in plastic communities in terms of relative abundance. Moreover, *Vibrio* genus was dominant in the plastic communities, especially in PP samples, especially strains of *V. natriegens*. Using alpha diversity analysis (average species diversity in a sample or local habitat), there were two main differences in bacterial communities between plastic and the surrounding seawater; 1) the observed species richness (number of species in a community) was much higher in surrounding seawater communities. 2) greater species evenness (relative abundances of

species in a community) was observed in plastic associated communities (Zettler *et al.*, 2013; Moore, 2013).

In a study that compared the early-stage bacterial communities on different surfaces, three kinds of surfaces were used; acryl, glass and steel coupons. They were submerged under 2m depth in Sacheon harbor (Republic of Korea), for a period of thirty-six hours. Bacterial communities for the three surfaces and the surrounding water were analyzed and compared to each other using terminal restriction fragment length polymorphism (T-RFLP). The results revealed that there were no significant differences between different surfaces, but a notable change of the bacterial communities was observed on all surfaces between 9-24 hours. Furthermore, members of Gammaproteobacteria (P*seudomonas*, *Alteromonas*, *Acinetobacter*) were the dominant community in the early period of 0-9 hours, suggesting that Gammaproteobacteria might be a pioneering population in marine biofilm (Lee *et al.*, 2008).

In 2018 Dussud *et al.* investigated the colonization of both biodegradable and degradable plastic by marine microorganisms. In their study, four polymer types were used; PE, OXO (PE additivated with D2W OXO based on manganese and iron), AA-OXO, which was thermally treated for 180 days to change its physiochemical characteristics, and the fourth polymer was poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). Five identical aquariums (filled with natural seawater) were used to incubate pieces of each type of plastic polymers; one aquarium had only seawater as control. Atomic force microscopy (AFM) was used to study the surface state of polymers, amplicon sequencing by Illumina MiSeq was used to investigate the incubated (13°C) microbial communities associated with polymers and seawater. Samples were taken and analyzed after 7, 15, 22, 30, and 45 days. The results have shown that the biodegradable polymers AA-OXO and PHBV have had higher colonization of bacteria compared with the non-biodegradable plastics PE and OXO. Furthermore, members of hydrocarbonoclastic bacteria (HCB) such as *Alcanivorax* sp., *Aestuariicella hydrocarbonica*, *Marinobacter sp.*, *Lutibacterium anuloederans*, and *Neptuniibacter sp*. have dominated the plastisphere of all polymer types (Dussud *et al.*, 2018).

In 2015 a study was conducted in the Belgian part of the North Sea, where marine plastic litter (MPL) was investigated for the associated bacterial communities. Three kinds of samples were collected; plastic (MPL and beach pellet), sediment, and seawater. The sample collection occurred over a period of years; additionally, physiochemical characteristics were measured for water and sediments samples (De Tender *et al.*, 2015). To investigate the associated bacterial communities, DNA was isolated, then

16S amplicon sequencing was used. Rarefaction analysis, species richness estimation, and diversity (Shannon−Wiener diversity index) were used to investigate the complexity of the bacterial community.Plastic samples showed high variation in terms of bacterial community composition. Bacterial communities associated with plastic was significantly different from communities associated with seawater and sediments. Most of the bacterial taxa found in the plastisphere were also associated with sediments and/or seawater, but with notable differences in terms of relative abundances, suggesting that the marine environment is the bacterial source of plastic colonization; this was proved by the finding that beach pellet communities were notably distinct from marine plastic debris community. Regarding the identified bacterial taxa, Actinobacteria was dominant in the beach pellet community, whereas Proteobacteria dominated all other samples. Furthermore, in spite of many shared taxa, specific taxa such as Vibrionaceae and Pseudoalteromonadaceae were commonly detected on MPL but scarcely was found in sediment and seawater communities. The study also revealed that environmental factors such as salinity, temperature, oxygen concentration, as well as some intrinsic plastic-related factors like pigment content, might influence the bacterial community structure associated with plastic (De Tender *et al.*, 2015).

A similar study has been conducted with sediments samples in Humber Estuary in the United Kingdom. Sediments from the surface top centimetre were collected from three locations, sediments samples were then spiked with Low-density polyethylene (LDPE) fragments and stored at 4°C in sterile artificial sea water for up to two weeks. Fragments of LDPE were taken at different periods (immediately, 6 hours, 1d, 2d, 4d, 7, and 14d), and T-RFLP and SEM were used to investigate the microbial communities' changes over time in addition to comparison with the surrounding control sediments. Results have shown that bacterial communities rapidly colonized the LDPE as shown by SEM. Furthermore, the bacterial community structure on LDPE was significantly different from the surrounding sediments community. With regards to time effect, it was found that the bacterial community structure had greatly changed over time, bacterial genera *Arcobacter* and *Colwellia* were found to be dominant (84%-93%) after 14 days at LDPE, their results demonstrated the rapid selection of LDPE-associated bacterial assemblages (Harrison *et al.*, 2014).

2.6 Bacterial colonization of microplastics in intertidal zones and freshwater

Because of the ecological importance of the plastisphere, it is important to study microbial colonization in different environments in addition to marine water, such as the intertidal zone, which is defined as the area between the high tide and low tide mark. The intertidal zone is quite important

since it serves as a hydrographic link between human activities in upland and the neighbouring marine environment. Bacterial communities associated with microplastics samples collected from the Yangtze estuary intertidal zone in China were studied, samples were collected from three different sites in the estuary. Next-generation amplicon sequencing was used; Illumina MiSeq v3 technology, for data treatment, MOTHUR v.1.33.3 software was used. Plastic polymer types were found to be PE, PP, and PS, and there was no significant difference between polymer types in terms of microbial communities. However, diverse microbial communities were found in microplastic samples from all sites; Proteobacteria, Cyanobacteria, Bacteroidetes, and Actinobacteria dominated microplastic communities from the three sites. Bacterial genera *Phormidium*, *Vibrio*, and *Pseudomonas*, were detected in the plastisphere. Generally, the bacterial assemblages on microplastic from all sites originated from sedimentary areas as well as from aquatic areas (Jiang *et al.*, 2018).

In a study conducted by Miao *et al.*, 2019, the composition of microbial communities associated with two plastic substrates (PE and PP) were compared with some natural substances (wood and cobblestone (CS), all substrates were incubated under controlled conditions for 21 days with the microbial community which was retrieved from freshwater. Illumine sequencing was then used to analyze the microbial communities associated with each substrate type, bacterial diversity was investigated by calculating species richness, evenness, and Shannon diversity index. The species richness, evenness and Shannon index were also variable between all substrates, indicating that the community complexity is different between the four types of substrates, the highest values observed in wood communities, whereas the lowest was observed in PE communities. Proteobacteria followed by Bacteriodetes were the most dominant phyla in all substrate communities. In natural substrate communities, Cyanobacteria, Acidobacteria, Chloroflexi, and Actinobacteria were significantly more dominant compared with PE and PP. Betaproteobacteria and Deltaproteobacteria relative abundances were higher on natural substrates in comparison with microplastic, whereas Gammaproteobacteria was the most dominant class on microplastic. These results suggest that the microplastic communities are notably different from natural substrates (Miao *et al.*, 2019).

McCormick *et al.* 2016, investigated the bacterial assemblage on microplastic collected from the water column of nine rivers in Illinois, United States, and compared them to bacterial communities associated with the surrounding water and the suspended organic matter. The nine rivers receive treated water from the effluent of a wastewater treatment plant (WWTP), microplastics and organic matters were collected from rivers downstream and upstream of the WWTP. Amplicon sequencing results have shown that associated microplastic communities have the lowest taxon richness and

community diversity. In contrast, their results showed the downstream organic matter had the highest richness and diversity, the bacterial assemblage composition of all substrates was significantly different in terms of observed operational taxonomic units (OTU) (McCormick *et al.*, 2016). In terms of relative abundance of bacterial taxa, there was a clear difference among substrates; Bacteriodetes relative abundance decreased from upstream water to downstream water, organic matter, and microplastic, respectively. In comparison, the relative abundance of Proteobacteria was increasing in the same direction, and Actinobacteria was most abundant in water samples compared to organic matter and microplastic, Firmicutes dominated the microplastics. Furthermore, bacterial taxa Pseudomonadaceae was the most dominant in the microplastic community. The study also showed that there are differences in relative abundance of bacterial taxa among different streams (McCormick *et al.*, 2016).

In another study which was conducted in a man-made lake in Turkey, the structure of bacterial biofilm associated with microplastic was compared with the surrounding lake water. The method used to study microbial composition was the culture-based method using solid culture media; R2A agar, faecal coliform (mFC), eosin-methylene-blue (EMB) agar, UTIC, Cetrimide agar). A diverse profile of bacterial communities was found on microplastic surfaces; members of phyla Proteobacteria, Firmicutes, and Bacteroidetes were detected, including pathogenic species such as *staphylococcus*, *Acinetobacter* sp., and *Pseudomonas* sp., however, the bacterial richness in water samples was higher than microplastic surfaces (Tavşanoğlu *et al.*, 2020).

2.7 Bacterial colonization of microplastics in wastewater

Wastewater treatment plants(WWTP) eliminate between 83 and 95% of the plastic particles, but the remaining amount of plastics is still high (Dris *et al.*, 2015). Therefore, several studies have been investigating whether wastewater effluents contain specific microbial communities associated with microplastics. In a study conducted by Eckert *et al.*, the inflow of wastewater effluent into a freshwater lake was simulated by a continuous culture setup with a microplastic concentration gradient. Continuous cultures in chemostats were used where freshwater collected from lake Maggiore, and wastewater effluent from Verbania (Italy) was used as concomitant water, large particles and grazers were removed by 126 μm and 10 μm plankton nets. They monitored the occurrence of Integrase 1; a proxy gene that is linked to antimicrobial resistance and anthropogenic pollution; microbial community structure was also studied. It was found that the microbial community in the plastisphere is more similar to wastewater effluent than the surrounding water community. Additionally, the

Integrase 1 gene increased in the plastisphere as microplastic concentration increased (Eckert *et al.*, 2018).

Kelly *et al*. investigated the role of WWTPs in modifying the structure of the plastisphere community. They analyzed the microplastic associated biofilm in raw sewage, effluent, and treated sludge. Sludge plastisphere showed higher species richness than influent plastisphere, and effluent plastisphere had a lower abundance of potentially pathogenic bacteria than influent plastisphere such as members of *Campylobacteraceae*. Several bacterial taxa which were linked to plastic biodegradation, such as *Pseudomonas*. *Klebsiella* were more abundant in the effluent plastisphere compared to the influent microplastic bacterial community—suggesting that WWTPs have a significant role in the alteration of microplastic bacterial assemblage (Kelly *et al.*, 2021).

To study the early biofilm formation on microplastics deployed with WWTPs effluents, Martinez-Campos *et al*. used sterilized metallic cages filled with seven types of polymers PLA, PHB, PCL, PET, POM, PS, and LDPE. All metallic cages were incubated for 48h at a depth of 20cm at the exit of WWTP. All of the microplastic pellets were then collected along with one litre of the surrounding water was collected and filtered with a 0.22 μm membrane Millipore filter. DNA was then extracted from all collected samples, followed by amplicon sequencing. Higher diversity of bacteria was seen in microplastic communities compared to the surrounding water community. Furthermore, specific core microbiomes were observed for each type of polymer suggesting that each polymer type might select its early colonizers (Martínez-Campos *et al.*, 2020)

2.8 Microplastics as vectors for pathogens and antibiotic-resistant bacteria

Microplastics can be a global vector for the transmission of animal and human pathogens because it can travel long distances. Animal pathogens transmitted via microplastics can result in high economic loss in farmed fish, shrimp and mollusks. *Aeromonas salmonicida* was identified for the first time on a plastic surface collected from the North Adriatic sea. Strains of *Aeromonas salmonicida* are a known pathogen for salmonids, cyprinids and marine flatfish (Viršek *et al.*, 2017).

In a study conducted by Radisic *et. al*, 37 bacterial isolates from plastic particles were collected from the west coast of Norway using Mueller–Hinton and MacConkey agars. Bacterial isolates were identified, using the Illumina MiSeq platform, as potential fish pathogens such as *Aeromonas salmonicida.* Additionally, two opportunistic human pathogens; *Acinetobacter beijerinckii*, and *Morganella morganii* were isolated. Moreover, several antibiotic resistance genes (ARGs) were

detected in variants of *Acinetobacter beijerinckii*, and *Morganella morganii* such as β-lactamases and chloramphenicol acetyltransferase (Radisic *et al.*, 2020).

Plastic surfaces as a vector for harmful microorganisms was first reported by Masó *et al.* In their study, potential harmful microalgae (dinoflagellates) species were observed on the surface of plastic debris such as *Ostreopsis, Coolia*, *Alexandrium* species. Plastic debris was collected from the Catalan coast (northwestern Mediterranean) (Masó *et al.*, 2003). *Ostreopsis* sp was connected to respiratory and skin irritation problems in humans in addition to the production of palytoxin (PTX)-like toxins (Tichadou *et al.*, 2010). *Coolia* sp. was associated with the production of ciguatoxin responsible for Ciguatera Fish Poisoning (CFP) (Tibiriçá *et al.*, 2020), and *Alexandrium taylori* involved in the production of saxitoxin which causes paralytic shellfish poisoning (PSP) in humans (Emura *et al.*, 2004).

Several studies reported the detection of potentially human and/or fish pathogens associated with plastic surfaces. For instance, Forty-four pathogenic *E.coli* strains and 18 pathogenic strains of *Vibrio* species (*Vibrio cholerae* , *Vibrio vulnificus*, and *Vibrio mimicus*) were isolated from the surface of plastic debris collected from Guanabara Bay, RJ, Brazil (Silva *et al.*, 2019)

Vibrio species was first detected on marine plastic surfaces by Zettler *et al.*, who reported that genus *Vibrio* was dominant in the plastic associated community by 24% (Zettler *et al.*, 2013). Potentially pathogenic *Vibrio* species, such as *V. cholerae* and *V. vulnificus*, were also isolated by culturable method (chromogenic agar) from microplastics collected from the North and Baltic Seas. Additionally, potentially pathogenic *Vibrio parahaemolyticus* was found on the surface of PE, PP, and PS. The presence of virulence genes was confirmed by multiplex PCR (Kirstein *et al.*, 2016).

As was mentioned above, microplastics not only transfer pathogenic bacteria but antibiotic-resistant bacteria (Keswani *et al.*, 2016). The occurrence of ARGs on microplastic surfaces has been investigated by several studies. Yang *et al.* studied the abundance of ARGs on microplastics and macroplastics collected from North Pacific Gyre. Based on the Shannon-Wiener indices and richness, they found that the abundance of ARGs was notably greater than the surrounding sea water. It was also found that there was no significant difference between microplastic and macroplastic in the occurrence of ARGs, suggesting that particle size has no effect (Yang *et al.*, 2019). Plastic associated biofilm can be a reservoir of ARGs in various aquatic environments such as: marine environment (Y. Liu *et al.*, 2021). Guo *et al.* found that the abundance of ARGs in PE and PP biofilm in the Yangtze Estuary, China was higher than in the surrounding water and the sediments (Guo *et al.*, 2020). In the

freshwater environment, Wu *et al.* found that microplastic biofilm developed on PVC incubated in freshwater river contains ARGs hosted by two human pathogens (*Pseudomonas monteilii*, *Pseudomonas mendocina*) (Wu *et al.*, 2019).

2.9 Microplastic bacterial communities; effects of polymer type

The effect of the different types of plastic polymers on the structure of the microbial biofilm has been investigated by many studies. Frère *et al*. have found that microbial community structure was significantly affected by the polymer family, PS associated community had a distinct structure compared to PP and PE, a great heterogeneity in dispersion was observed for the PE communities compared to PS and PP, which displayed more homogeneous clustered groups (Frère *et al.*, 2018).

Kirstein *et al.* have compared microbial communities associated with nine polymer types; HDPE, LDPE, PP, PS, PET, PLA, styrene-acrylonitryle (SAN), polyurethane prepolymer (PESTUR), PVC in addition to glass as a control surface. Their results showed that the glass community was significantly different from the nine analyzed polymer's associated ones. PLA community was significantly different from the other polymers communities, however significant differences between various polymers communities were also observed (Kirstein *et al.*, 2018).

Rosato *et al.* have investigated the bacterial colonization of different microplastic pellets in anaerobic laboratory conditions using microcosms of marine sediment. Microplastic pellets types were PE, PET, PS, PP, and PVC. They found that microbial colonization developed rapidly on all microplastic polymer types, and they also noted that the biofilm structure significantly differed between the five tested types of plastic (Rosato *et al.*, 2020). On the other hand, Wu *et al.* found that the chemical structure and the plastic type had no significant effect on the microbial community structure associated with plastic (Wu *et al.*, 2020).

2.10 Microplastic bacterial communities: seasonal changes

Although microbial colonization in freshwater and marine water have been investigated by many studies, most of these studies have been conducted over short periods. Few studies have investigated long-term plastic microbial colonization. Oberbeckmann *et al.* investigated the structures of microbial communities associated with PET bottles in marine water for a period of six weeks over three seasons, winter, spring, and summer. Shannon diversity indices revealed that the highest diversity of the plastisphere community was in summer and the lowest was in winter, suggesting that plastisphere communities might differ according to season (Oberbeckmann *et al.*, 2014). Frere *et al.* have found

similar results regarding marine water; the plastisphere community in samples taken in October was different from communities in December samples(Frère *et al.*, 2018).

In an *in situ* experiment, Zhang *et al.* have investigated the microbial communities associated with different polymers incubated in the Yellow Sea for six weeks over three seasons, they found that the biomass increased with time in summer samples in most polymers, in winter the biomass rate of increase was slower, and the biofilm density was less than summer biofilm. They suggest that the differences in microbial communities were due to the differences in environmental samples like temperature (the main factor), dissolved oxygen (DO), salinity, and nutrient availability. The autumn communities were similar to summer communities with a slight decrease in biofilm development after the end of the fourth month. Moreover there was no significant differences between microbial communities associated with different polymers (Zhang *et al.*, 2021).

2.11 Biodegradation of plastic

Biodegradation of plastic occurs via several steps, polymers are turned into oligomers and monomers by enzymatic actions (hydrolytic division), followed by the further breakdown of oligomers and monomers into H_2O and CO_2 by microorganisms (mostly bacteria and fungi) via different enzymatic and metabolic mechanisms (Ahmed *et al.*, 2018). Different polymers can be degraded depending on the nature of the catalytic activity and specificity of the enzymes. For instance, *Bacillus* species produce proteases that contribute to different polymers degradation, whereas Fungi can degrade lignin due to the production of laccases that is needed for oxidation of aromatic and non-aromatic compound (Sivan, 2011; Mayer and Staples, 2002).

Various plastic types were found to be biodegradable, such as polyesters which consist of monomers linked together by an ester bond that is easily hydrolyzed. Esterase enzymes involved in ester linkage degradation are abundant in microorganisms. Polyhydroxyalkanoates (PHA) are biodegradable polyesters produced by various microorganisms (Shimao, 2001). Polyhydroxybutyrate (PHB) is a polyester that accumulates in specific bacterial cells as a source of energy and carbon, such as *Pseudomonas stutzeri*, *Alcaligenes faecalis*, and *Bacillus subtilis*. PHA and PHB are used frequently by the plastic manufacturer to produce bioplastic (Shimao, 2001; Bioplastics, 2021).

Polylcaprolactone (PCL) is another kind of biodegradable polymer that is degraded by lipases and esterases. Bacteria that can degrade PCL are ubiquitous in the environment; evidence was provided that the fungal pathogen Fusarium can degrade polycaprolactone(Murphy *et al.*, 1996). Polylactic acid

(PLA) is a biodegradable polymer made from renewable sources like corn starch. Several types of microorganisms are capable of PLA degradation, such as PLA-degrading *Amycolatopsis sp* strain and a thermophilic bacterium *Bacillus brevis* (Pranamuda *et al.*, 1997; Tomita *et al.*, 1999). Polyvinyl alcohol is a water-soluble polymer that can be degraded by pathogenic fungi *Fusarium lini* and different bacterial species such as *Pseudomonas*, *Bacillus* and *Alcaligenes* (Chiellini *et al.*, 2003).

Many plastic-degrading microorganisms and enzymes have been isolated and identified, including different polymer types like PE, PS, PVC, PP, PUR, and PET. They were isolated from various environments such as soil of plastic dumping sites, seawater, sewage, crude oil contaminated soil, the digestive tract of plastic-eating animals, and landfill. Various bacterial taxa have been involved in plastic degradation, among these are; members of Cyanobacteria, *Enterobacter*, *Bacillus*, *Actinomycetes*, *Rhodococcus*, *Exiguobacterium*, *Pseudomonas*, and others. *Ideonella sakaiensis* (Yoshida *et al.*, 2016) is a PET degrading bacteria that can degrade PET fibers into monomers, which can then be used as a carbon source. Additionally, a number of Fungi taxa have been documented to be plastic degraders, such as *Aspergillus flavus*, *Penicillium janthinellum*, *Aspergillus niger*, *Phanerochaete chrysosporium*, *Aureobasidium pullulans*, and others (Ru *et al.*, 2020).

As we have seen in the literature, the majority of studies investigated microplastic associated bacteria was in marine water. Additionally, the methods used were mostly depends on collection of microplastic particles from the environment, either by filtering the water using nets and meshs or by direct collection of microplastic particles from shallow water, or incubating microplastic particles under environmental conditions. Therefore, we believe that a standardized, reproducible method is needed, which can be used to compare plastic associated microbes between different freshwater bodies and ecosystems. We hope that the method used in this study will facilitate the investigation of microplastic associated microbial communities in the natural environment. Furthermore, we hope that the results of our study will be present a valuable data regarding microplastic associated bacteria in freshwater environment.

3 MATERIALS AND METHODS

In September 2018, ten floating polypropylene straw samples were randomly collected from shallow seawater of the Mediterranean Sea near the public beach of Laganas in Zakynthos Island by my supervisor. In this initial study plastic samples were kept in seawater in a sterile container and transferred to the laboratory and bacterial strains were isolated and identified. Based on the results about the plastic litter collection and bacterial isolation from marine environment a new study has been developed in Hungary. Self-designed plastic colonizers were prepared and two colonization test methods were performed to describe the plastic associated bacterial community and find possible novel bacterial species. These methods are summarized in supplementary table 1 and a flow chart (Figure 3) below:

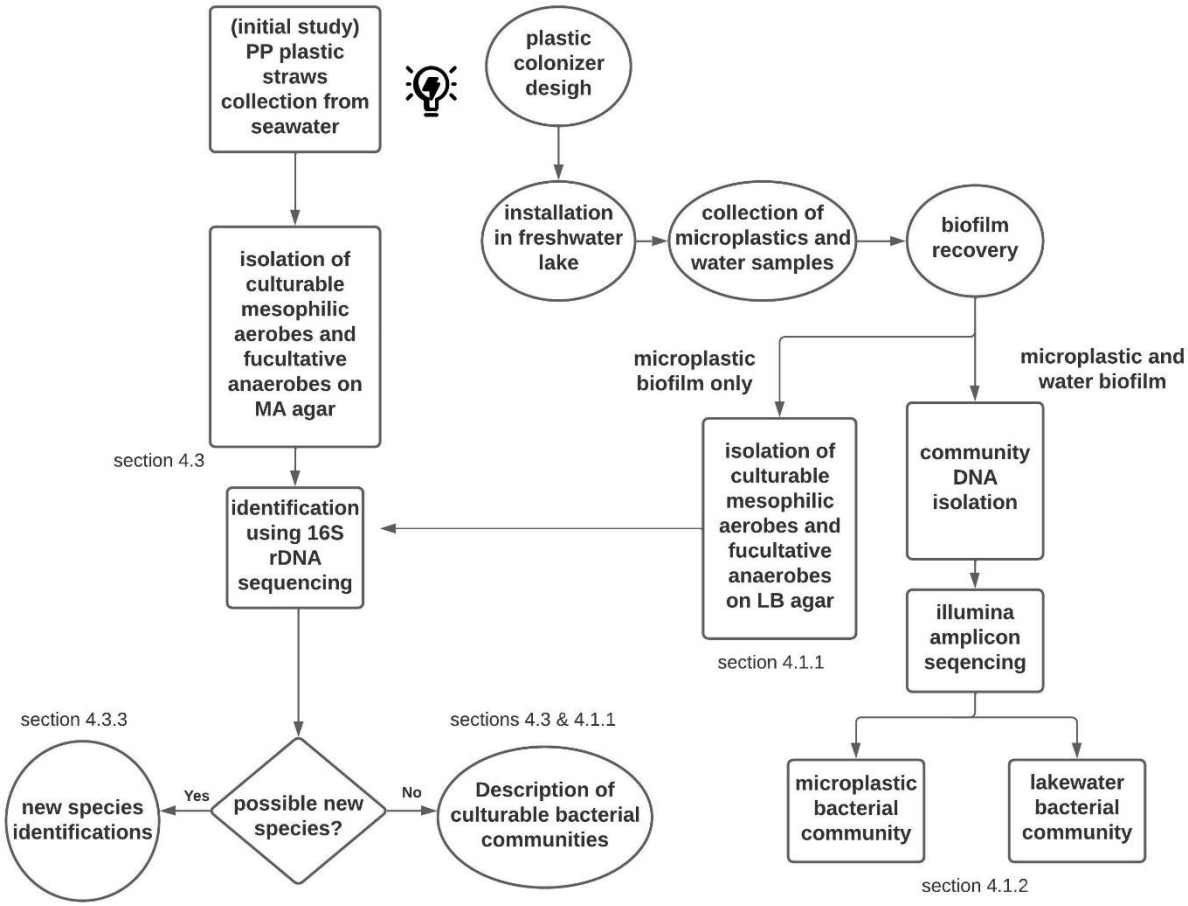

Figure 3 A flow chart about all applied methods in my study

3.1 Methods used in the initial study

In September 2018, ten floating polypropylene straw samples were randomly collected from shallow seawater of the Mediterranean Sea near the public beach of Laganas in Zakynthos Island, Greece (37° 43' 9" N 20° 51' 42" E) by my supervisor. Samples were kept in seawater in a sterile container and transferred to the laboratory, and stored at 4°C until analysis (see below).

3.1.1 *Isolation of bacterial species from the plastic surface in the initial study*

Mesophilic aerobic and facultative anaerobic bacteria were isolated from the plastic straw samples by rinsing in 90 ml normal saline (NaCl at 0.9 % w/v) with glass beads and stirred in a shaking incubator at room temperature for one hour. The samples and the serial dilution were then plated using pour plate method in marine agar (MA) prepared from marine broth (from Carlroth, Germany, Art. No. CP73.1) composed of Peptone 5g/L, Yeast extract 1g/L, Ferric Citrate 0.1g/L, Sodium Chloride 19.45g/L, Magnesium Chloride 5.9g/L, Magnesium Sulfate 3.24g/L, Calcium Chloride 1.8g/L, Potassium Chloride 0.55g/L, Sodium Bicarbonate 0.16g/L, Potassium Bromide 0.08g/L,) and 18 g agar agar l-1 (from Carlroth, Germany, Art. No. 2266.3) in pH 7.0 distilled water and incubated at 28ºC for 72 hours. Colonies were selected randomly and subsequently purified twice on Marine agar medium at 28°C.

3.1.2 *16S RNA phylogeny for species identification*

In order to identify the bacterial isolates, 16S rDNA gene sequencing has been done as a widely used method for the identification of bacterial species. As the first step, genomic DNA was extracted using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, USA). Subsequently, the 16S rRNA gene was amplified using 27F and 1492R primers (Lane, 1991). Amplification was performed by using an Eppendorf Mastercycler (Eppendorf, Germany). PCR products were purified with NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel GmbH, Germany). The almost complete 16S rRNA gene sequence of the strain was determined by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Sequencing products were separated on a Model 3130 Genetic Analyzer (Applied Biosystems, USA).

3.2 The study area of first and second colonization tests

Based on the results about the plastic litter collection and bacterial isolation from marine environment a brand new study has been developed in Hungary. The study was conducted in a freshwater lake located near to the village of Vácszentlászló, Pest county in Hungary (47°33'37.0"N 19°33'09.4"E)

approximately 20 km from our microbial laboratory where samples were analyzed (see Figure 4 & 5). The lake is a shallow reservoir with an area of approx. 47 hectares and a mean depth of 2 m. The dam on the Hajta-stream was constructed in the 1960s. Formerly the main purpose of the reservoir was duck hunting and irrigation water supply for the surrounding agricultural areas. Nowadays, recreational fishery (catch and take angling) has increasing importance. The water in the lake is eutrophic, and the water level fluctuation is normally up to 1m. For water quality of the sampling lake (see supplementary table 2); (classified as class1) based on ECE standard statistical classification of surface freshwater quality for the maintenance of the aquatic life (Helmer and Hespanhol, 1998). Fish fauna is dominated by omnivore *Cyprinidae* species. In this lake our self-designed plastic colonizing methods were used first to collect information about plastic associated (plastispheric) bacterial communities by classical and molecular methods.

Figure 4. Location of the Vácszentlászló lake where the microplastic colonizers were installed

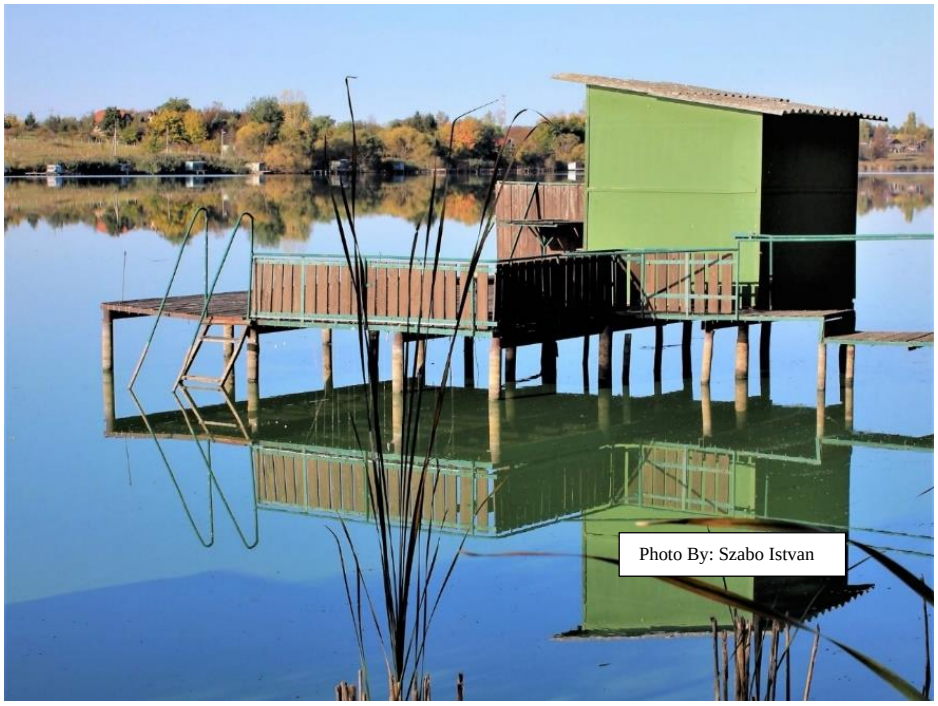

Figure 5. Sampling pont in Vácszentlászló lake, the pier where the plastic colonizers were hanged

3.3 Plastic colonizers method

3.3.1 *Design of plastic colonizers*

Self-designed plastic colonizers were prepared as follows; commercially available stainless-steel ball-shaped tea filters (Easy Filter 6.5×6cm. No 1082, Mingwei, China) were wrapped in aluminium foil and pre-sterilized in an autoclave at 121 °C, under 1,2 Atm pressure for 15 minimum minutes. Filters were then filled with 3 grams (for each) of commercially available polypropylene plastic straws (produced by Perfect home no. 72226) cut into less than 5mm small pieces (see Figure 6). Six of these plastic colonizers were hung on a wooden pier next to each other with a strong fishing line and submerged under the water surface (around 50cm depth). Their positions were fixed with fishing lead weights to keep the colonizers under the water's surface (see Figure 7).

Figure 6a **Figure 6b**

Figure 6. Plastic colonizer design **a**: open plastic colonizers made from commercial stainless-steel filter filled with 3 grams of cut plastic straw. **b**: closed plastic colonizer filled with plastic straw pieces (<5mm)

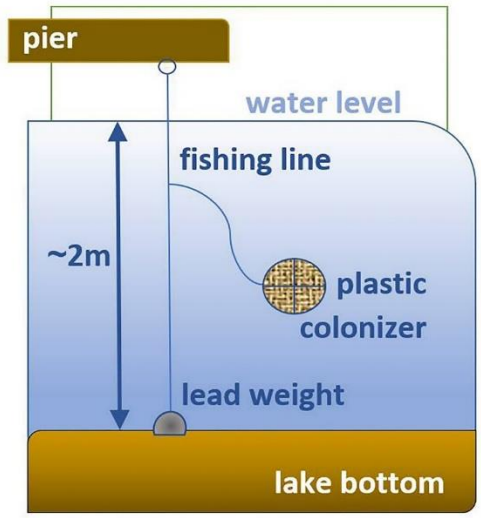

Figure 7. Installation of plastic colonizers by attachment to wooden pier and submerging 50 cm under water surface and fixed under water with lead weight (Figure by Istvan Szabó)

3.3.2 *Recovery of microbial biofilm from plastic colonizers*

The submerged plastic colonizers were collected and transferred to the laboratory directly within one hour in a pre-sterilized container filled with ambient lake water. In the laboratory, two plastic colonizers were opened (treated as one sample), and plastic particles were transferred to a pre-sterilized stainless-steel mesh using a sterilized spatula, then washed through with sterilized normal saline to remove the stuck debris but keep the plastic associated biofilm. Then the water-washed

plastics were transferred to an Erlenmeyer flask containing sterilized 90 ml distilled water, 30g of glass beads and 13.5 µl of TWEEN 80. It was incubated at room temperature in a shaker with 170 rpm speed for one hour to recover the microbial biofilm attached to plastic surfaces.

3.3.3 *Isolation of culturable bacteria on LB agar from plastic*

Plastic surfaces can be a unique ecological niche for bacterial communities; thus, it could be possible to find undescribed species here. In our study, LB agar was used to isolate fast-growing cultivable species from the plastic surface to get information about their pathogenicity or find novel ones. From the one hour shaking suspension of plastic particles, the initial 1 ml was serially diluted up from 10^0 to 10^{-6} using 9 ml of sterile normal saline. 1 ml from each dilution steps was plated on LB agar and incubated at 28 °C for three days. From every sample maximum of eight colonies with different morphology were transferred and purified twice on LB agar.

3.3.4 *16S rRNA gene sequencing for identification of bacterial isolates*

For the identification of bacterial strains isolated in 3.3.3, genomic DNA was isolated and 16S rRNA genes were amplified as it was performed in section 3.1.2 above.

3.3.5 *DNA Isolation of plastic-associated and lake water bacterial communities*

After inoculating 1ml aliquots of the microplastic suspension onto LB agar (see below), the remaining suspension (~90 ml) was centrifuged at 4˚C and 4000 rpm speed for 25 minutes, the supernatant was discarded, and the pellet was stored at -80°C for subsequent DNA isolation. For isolation of community DNA from lake water, 150 ml were centrifuged under the same conditions to have a sufficient pellet. Community DNA was then isolated by using DNeasy UltraClean Microbial Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions

3.4 Bacterial community assessment - first colonization test

The first colonization test was conducted over a period of three months, from December 2018 (the first month) until the end of February 2019 (the third month). By the end of all three months, two plastic colonizers were harvested, as mentioned above. To assess the composition of bacterial communities in plastic colonizers Illumina 16S rDNA amplicon sequencing was used (in the laboratory of SeqOmics Biotechnology Ltd., Morahalom, Hungary). Samples were identified as *VMP1* (first month, 2018 December); *VMP2* (second month, 2019 January); *VMP3* (third month, 2019 February), and the connected lake water samples from the same time as *VLW1, VLW2* and *VLW3*.

To assess the composition of bacterial communities in plastic colonizers (first month (December); VMP1, second month (January); VMP2, and third month (February); VMP 3) and in lake water (first month; VLW1, second month; VLW 2 and third month; VLW 3) Illumina 16S rDNA amplicon sequencing was used (in the laboratory of SeqOmics Biotechnology Ltd., Morahalom, Hungary).
For paired-end 16S rDNA amplicon sequencing, the variable V3 and V4 regions of the 16S rRNA gene were amplified by using 16S amplicon PCR forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTA CGGGNGGCWGCAG-3′) and reverse (5′-GTCT CGTGGGCT CGGAGATGTGTATAAGAGAC AGGACTACHVGGGTATCTAATCC-3′) primers, with Illumina adapter overhanging nucleotide sequences (Klindworth *et al.*, 2013). PCR reaction mixture volume was 25 µl with 12.5 ng of DNA, 0.2 µM of each Illumina 16S primers and 12.5 µl of 2X KAPA HiFi HotStart Ready Mix (KAPABiosystems, London, United Kingdom). The temperature profile was as follows; initial denaturation (3 min at 25°C), 25 denaturation cycles for 0.5 min at 95°C, the annealing temperature was 25°C for 0.5 min, and 0.5 min elongation at 72 °C. ProFlex PCR System (Applied Biosystems by Life Technologies, USA) was used for all amplification steps. Analysis of amplicon was performed under UV after electrophoresis in 1% (w/v) agarose gel stained with EtBr. Paired-end fragment reads were generated on an Illumina MiSeq sequencer using MiSeq Reagent Kit v3 (600-cycle). Primary data analysis (base-calling) was carried out with Bcl2fastq software (v2.17.1.14, Illumina). Sequences were processed using mothur v1.41.1 (Schloss *et al.*, 2009) as recommended by the MiSeq SOP page (http://www.mothur.org/wiki/MiSeq_SOP) (Kozich *et al.*, 2013). Sequence lengths were screened by setting minimum length to 400 base pairs then were assorted based on the alignment using SILVA 132 SSURef NR99 database (Quast *et al.*, 2013). Chimera detection was performed with mothur's uchime command (Edgar *et al.*, 2011), and 'split.abund' command was also used to remove singleton reads according to (Kunin *et al.*, 2010). Taxonomic assignments were made against SILVA release 132 applying a minimum bootstrap confidence score of 80%. Operational taxonomic units (OTUs) were assigned at 97% similarity threshold level for prokaryotic species delineation (Tindall *et al.*, 2010).

3.5 Microbial communities associated with different materials - second colonization test

In order to assess if the microbial community structures are plastic-specific, four colonizers were filled with three grams of polypropylene plastic, biodegradable (polylactic acid) plastic, wood, glass, in addition to anempty one (from stainless steel surface). They were all installed as mentioned above,

but for two months period (from September to November 2019). At the end of that, all colonizers filled with different materials plus a water sample were collected and genomic DNA was isolated, and amplicon sequencing was again conducted as above. Bacterial diversity was determined as it was described above in the bacterial community assessment at the first colonisation test.

3.6 Statistical analysis

3.6.1 *Statistical analyses of the results between plastic and water communities from the first colonization test*

Testing the difference between microbial communities on microplastic surfaces (Group1) compared to surrounding water (Group2) is of high importance to measure the significance level of difference. A t-test is used to compare between the means of two data sets, assuming that the data sets are normally distributed, in large samples (> 30 or 40), the sampling distribution tends to be normal, regardless of the shape of the data (Ghasemi and Zahediasl, 2012), and the two groups of data are also assumed to be independent of one another. Thus, a Paired Samples t-test using SPSS software was used to answer the research question and determine whether there are statistically significant differences between the two data sets.

H0: There is no (statistically) significant difference between the microbial community on microplastic surfaces compared to the surrounding water.

H1: There is a (statistically) significant difference between the microbial community on microplastic surfaces compared to the surrounding water.

3.6.2 *Statistical analyses of bacterial communities from different materials (second colonization test)*

To identify similarities between microbial communities on microplastic surfaces and in the surrounding water, we performed principal component analysis (PCA). Z-score calculated from the number of OTUs to have unit variance before the PCA analysis. All PCA-related data analysis was performed with R 4.0.2 for Linux using the stats (version 3.6.2) and ggfortify (version 0.4.10) packages.

3.7 Methods for novel species description

Based on the results of 16S rDNA phylogeny, one possible novel species was isolated (ZS-1/3) in the initial study. Description of novel bacterial strains has great importance in prokaryotes systematics. Various methodologies have been developed and used over the past 100 years. Both the traditional methods and the newly developed methods are considered as key elements to decide whether a strain

is novel or not. Nowdays, after the 16S rDNA sequencing results, strains of possible novel species should be further tested and compared with the close relatives using different phenotypic and genotypic aspects such as cell morphology, gram staining behaviour, growth conditions, fatty acids analysis, polar lipids, respiratory quinones, motility, MALDI-TOF MS, scanning electron microscope image, genome phylogeny, DNA-DNA hybridization, Average Nucleotide Identity, and gene content (Tindall *et al.*, 2010).

3.7.1 *Molecular methods*

The 16S rDNA gene sequence of strain ZS-1/3 was compared to the type strains of closest relatives from the members of the genus *Parvularcula* obtained from GenBank (Kim *et al.*, 2012) (Kim *et al.*, 2012). Multiple alignments of 16S rDNA gene sequences were made with CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou and Nei, 1987) methods with Kimura's two-parameter calculation model and the maximum-parsimony algorithm (Kimura, 1980) using MEGA X 10.0.5 (Kumar *et al.*, 2018). Tree topologies and distances were evaluated by bootstrap analysis based on 1000 replicates. Whole-genome sequencing, including G+C determination, was carried out in SeqOmics Biotechnology, Morahalom, Hungary. The whole-genome sequencing of ZS-1/3 was conducted based on the procedure described by (Borsodi *et al.*, 2019), mate-paired libraries were generated using Nextera Mate Pair Sample Preparation Kit (Illumina, USA) according to manufacturer protocol of gelplus version after minor modifications. to produce a robust smear within the 7-11 kbp region 13 µl of Mate-Paired Tagment Enzyme was used. Zymoclean Large Fragment DNA Recovery kit (Zymo Research, USA) was used to excise the 7-11 kbp DNA fraction from the gel, then the circularized DNA was sheared using Covaris S2. The quality measurements were conducted using TapeStation 2200 instrument (Agilent, USA). Qubit (ThermoFisher, USA) was used to quantify the final libraries, which were sequenced on an Illumina MiSeq instrument using MiSeq Reagent Kit v2 (500 cycles) sequencing chemistry. De novo assembly and scaffolding were performed with CLC Genomics Workbench Tool v11 (Qiagen, Germany).

Automatic annotation of the genome was performed by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP) v4.5 (Tatusova *et al.*, 2016). Digital DNA–DNA hybridization values (dDDH) among strain ZS-1/3^T and related species were determined using the Genome-to-Genome Distance Calculator (GGDC, https://ggdc.dsmz.de/) version 2.1. (Meier-Kolthoff *et al.*, 2013). For the calculation of orthologous average nucleotide identity (OrthoANI) values between strain ZS-1/3^T and

its closest relatives, the OAT software was used (Lee *et al.*, 2016). Reference genomes for comparison purposes were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

3.7.2 *Physiology and Chemotaxonomy*

Samples were taken for electron-microscopic morphology from 48-hours old cultures grown in Marine Broth (Art. No. CP73.1, Carlroth) at 28°C. The cell morphology and flagellation type of strain ZS-1/3 were investigated during the exponential growth phase using transmission electron microscopy (H-7100; Hitachi) by applying the shadow-casting technique described by Ohad *et al.*(Ohad and Danon, 1963). Carbon-source utilization and enzyme activities were tested by using API 20, API 20NE, and API ZYM test kits (bioMérieux, France) according to the manufacturer's instructions. All API tests were carried out in parallel with strains ZS-1/3 and *Parvularcula lutaonensis* KCTC 22245^{T}. Examination of test, catalase activity were fulfilled by the methods from (Barrow, J.I & Feltham, 1993) verifying the API tests. Gram-reaction was performed by using the nonstaining method, as described by (Buck, 1982). Growth at different temperatures (5, 10, 15, 20, 28, 37, and 42 ºC) and pH (pH 4.0–11.0, in increments of 1 pH units at 28 °C) was assessed after ten days incubation in marine broth. After autoclaving marine broth pH was controlled (S220 SevenCompact, Mettler Toledo) and adjusted by adding sterile solutions of HCl or NaOH (1 M each), the following buffers were used depending on the tested pH; MES, MOPS, Tris, CHES, and CAPS buffer. Salt tolerance was tested after ten days incubation in marine broth supplemented with 0.0–10 % (w/v) NaCl (at 28 ºC). Growth on nutrient agar (prepared from basic ingredients), trypticase soy agar (TSA, from VWR Cat No.470015-844) , and R2A agar (from Carlroth Art.-Nr.CL1.1) was all evaluated at 28ºC for 72h.

Chemotaxonomic analyses (quinoine and fatty acid methyl ester analysis and polar lipid) were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunsweig, Germany. *Parvularcula lutaonensis* has been grown under the same conditions before the fatty acid compositions were made. Analysis of cellular fatty acids was made by conversion into fatty acid methyl esters by saponification, methylation and extraction according to the method described by Miller (1982) and Kuykendall *et al.*, (1988) with minor modification (Miller, 1982; Kuykendall *et al.*, 1988). The fatty acid methyl esters mixtures are separated by gas chromatography and detected by a flame ionisation detector using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.). Peaks are automatically integrated, and fatty acid names and percentages are calculated by the MIS Standard Software (Microbial ID), followed by identification by TSBA40 and TSBA6 methods (Miller, 1982; Kuykendall *et al.*, 1988). Respiratory

quinones and polar lipids were extracted from 200 mg of freeze-dried cell material using the two-stage method described by (Tindall, 1990a; Tindall, 1990b; Tindall *et al.,* 2007).

3.7.3 *MALDI-TOF MS:*

To compare the strain ZS-1/3 with the closest relatives, whole-cell matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling was performed using a Bruker Biotyper instrument (Bruker Daltonics, Leipzig, Germany). Bacterial samples were prepared in six replicates according to the instructions of Bruker Daltonics. The standard extraction protocol is based on the extraction with acetonitrile/formic acid. Target was overlayed with 1 µl HCCA (α-cyano-4-hydroxycinnamic acid) used as a matrix as it was recommended (Krizova *et al.*, 2014). Samples were measured automatically by the MALDI Biotyper 3.0, spectra were transformed to .mzML files (m/Z intensity lists) using flexAnalysis 3.3. software (Bruker Daltonics, Leipzig, Germany). The .mzML files were processed and analyzed by the free statistical software Mass-Up (López-Fernández *et al.*, 2015) following the instructions and settings described previously (Fernández-Álvarez *et al.,* 2018).

Based on the results obtained by the initial study, the idea then came up to design a standardized method that uses PP straws in a simple and controlled design that can be used *in situ* to study the bacterial colonization of microplastics in freshwater. The study area had to be chosen to be a freshwater body that is accessible, close to our laboratory to facilitate the collection of the samples and to maintain the integrity of samples.

4 RESULTS

4.1 Results of the first colonization test

4.1.1 *Bacterial isolates of LB agar from first colonization test*

Based on the idea of the initial study a plastic colonizer was designed and used in Vácszentlászló Lake, Hungary (as it was mentioned in sections 3.3., 3.4., 3.5.). Variable bacterial species were isolated from microplastic surfaces on LB agar. A full list of isolated bacterial species that were identified based on 16S rRNA gene sequencing (> 99% similarity) are mentioned in **Table 1,** taking into consideration that only eight strains were isolated from each plastic sample, some species were found more than once. Risk groups of these species were described according to the data from DSMZ (German Collection of Microorganisms and Cell Cultures) in which risk group 1 includes bacteria of a low individual or community risk, and unlikely to cause disease. Risk group 2 includes bacteria of moderate risk, meaning exposure might cause disease, but the risk is not significant to lab workers or the environment.

The bacterial isolates in the table below were dominated by *Bacillus* and *Pseudomonas* species, three isolates were classified as risk group 2 (*Shewanella putrefaciens PLA-12*, *Brevundimonas vesicularis PLA-6*, *Aeromonas sobria PLA-21*) which means that they can cause human illness for patients with no properly working immune system. *Aeromonas bestiarum* (*PLA-13*) which is a well-known fish pathogen was also isolated.

Table 1: Identified bacterial species (based on 16S rDNA sequence similarity), isolated from microplastic surfaces (first colonization test) on LB agar incubated aerobically at 28°C for 72h.

IDs	Bacterial spp	Date	Length of the sequenced region of 16S (bp)	Risk group*
PLA-8	*Bacillus simplex*	December 2018	544	1
PLA-9	*Shewanella hafniensis*	December 2018	398	n/d
PLA-10	*Pseudomonas antarctica*	December 2018	524	1
PLA-12	*Shewanella putrefaciens*	December 2018	582	2
PLA-13	*Aeromonas bestiarum*	December 2018	507	1
PLA-15	*Streptomyces pratensis*	December 2018	507	n/d
PLA-6	*Brevundimonas vesicularis*	January 2019	553	2
PLA-16	*Exiguobacterium undae*	January 2019	662	1
PLA-17	*Jeotgalibacillus campisalis*	January 2019	637	1
PLA-18	*Bacillus zhangzhouensis*	January 2019	600	n/d
PLA-21	*Aeromonas sobria*	January 2019	535	2
PLA-22	*Pseudomonas helmanticensis*	January 2019	590	1
PLA-25	*Bacillus tequilensis*	January 2019	549	n/d

IDs	Bacterial spp	Date	Length of the sequenced region of 16S (bp)	Risk group*
PLA-26	*Bacillus megaterium*	February 2019	415	1
PLA-27	*Bacillus altitudinis*	February 2019	470	1
PLA-28	*Pseudomonas synxantha*	February 2019	603	1
PLA-29	*Rhizobium ipomoeae*	February 2019	578	n/d
PLA-30	*Pseudomonas peli*	February 2019	604	1
PLA-31	*Cellulomonas oligotrophica*	February 2019	606	1

* classified according to the German Collection of Microorganisms Cell Cultures (DSMZ.de); **Risk group 1**: bacteria of a low individual or community risk, unlike to cause disease. **Risk group 2**: bacteria of moderate risk, exposure might cause disease, but the risk is not significant to lab workers or environment. **n/d:** not defined

4.1.2 *Microbial diversity based on Illumina 16S rRNA gene amplicon sequencing (first colonization test)*

The Illumina 16S rRNA gene amplicon sequencing provided 29718, 28837, 28453, 30742, 29366, 29386 reads for VMP1, VMP2, VMP3, VLW1, VLW2, VLW3 (VMP1: microplastic surface sample from first month (2018 December);VMP2: same, second month (2019 January); VMP 3: third month (2019 February); and in lake water samples VLW1 from first month; VLW 2 second month, and VLW3 third month;), respectively The rarefaction curves of the samples indicated that the data contained enough sequence depth to ascertain the full bacterial diversity. High sequencing coverage was reached in all samples, see Figure 8.

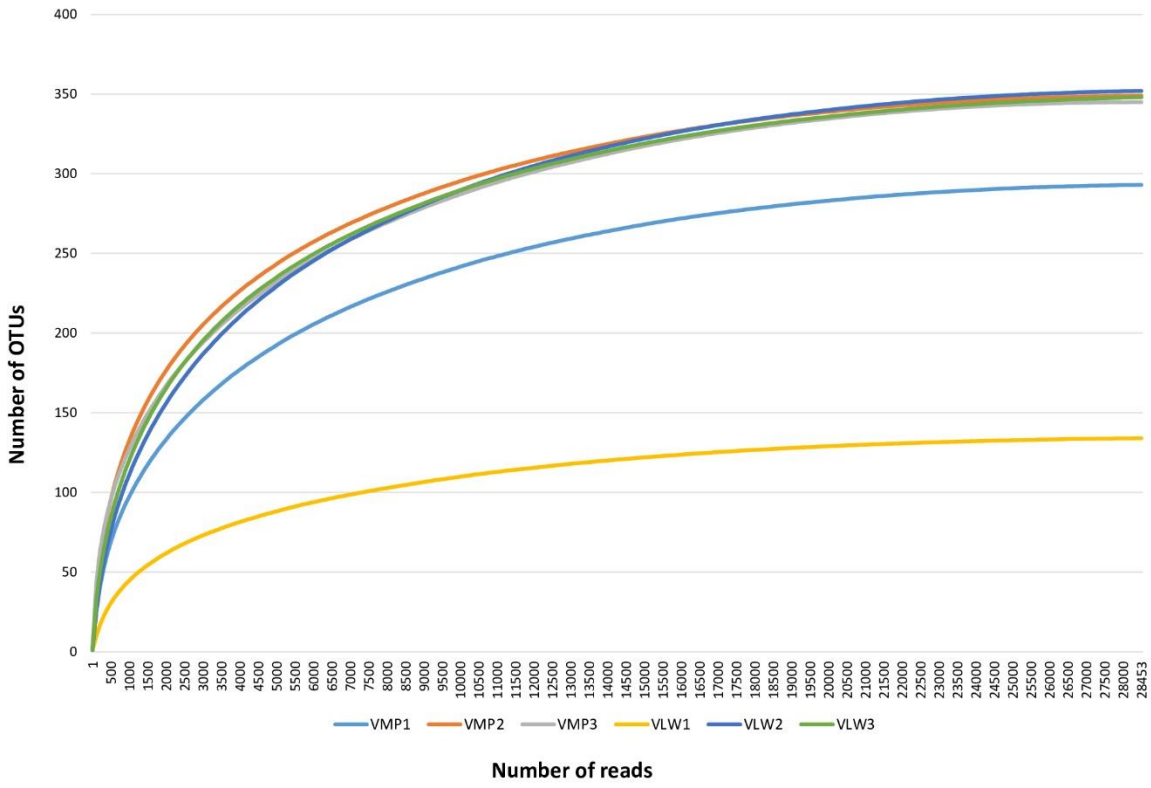

Figure 8. Rarefaction curves of the three-month samples from the plastic surface (VMP1, VMP2, VMP3) and ambient water (VLW1, VLW2, and VLW3). As the number of reads increase, the number of operational taxonomic unit increase.

Diverse bacterial communities were found on microplastic surfaces and in lake water as well. The amount of different OTUs observed on microplastics surfaces after the first (VMP1), second (VMP2), and third month (VMP3) were 293, 394, 345, respectively. Over the same period, the amount of different OTUs observed in lake water samples were 134 (VLW1) in the first, 352 (VLW2) in the second, and 348 (VLW3) in the third month (See supplementary figure 1-5.)

In the lake water, the notable microbial assemblages after the same first month (December 2018, sample ID: VLW1) were: Cyanobacteria (90.6%), Proteobacteria (2.3%), Planctomycetes (1.7%), Bacteroidetes (1.6%), Verrucomicrobia (1.2%), Actinobacteria (1.0%). In the second (January 2019, sample ID: VLW2) Cyanobacteria (70.0%), Proteobacteria (9.5%), Bacteroidetes (8.5%), Planctomycetes (4.1%), Verrucomicrobia (3.0%), Actinobacteria (2.8%) were the most abundant. After the third month (February 2019, sample ID: VLW3) the following phyla dominated the community: Cyanobacteria (53.0%), Proteobacteria (19.2%), Bacteroidetes (16.0%), Planctomycetes

(6.0%), Actinobacteria (2.4%), Verrucomicrobia (2.4%). Thus, it was found that Cyanobacteria and Proteobacteria dominated in all lake samples over all months.

In the first month (December 2018, sample ID: VMP1) the notable (>1% in abundance) microplastics surface-associated microbial assemblages on the phylum level were: Cyanobacteria (69.3%), Proteobacteria (16.6%), Verrucomicrobia (3.6%), Planctomycetes (3.6%), Actinobacteria (2.7%), Bacteroidetes (2.0%) and Chloroflexi (1.4%). In the second month (January 2019, sample ID: VMP2), Proteobacteria became the most abundant (34.0%), followed by Cyanobacteria (31.3%), Bacteroidetes (20.6%), Planctomycetes (5.9%), Verrucomicrobia (4.1%), Actinobacteria (2.8%). In the third month (February 2019, sample ID: VMP3) the most dominant phyla were: Bacteroidetes (54.2%), Proteobacteria (32.9%), Planctomycetes (6.8%), Verrucomicrobia (2.2%), Actinobacteria (1.5%), Cyanobacteria (1.2%). Figure 9 compares the most abundant microbial phyla between microplastic surface samples and lake water samples.

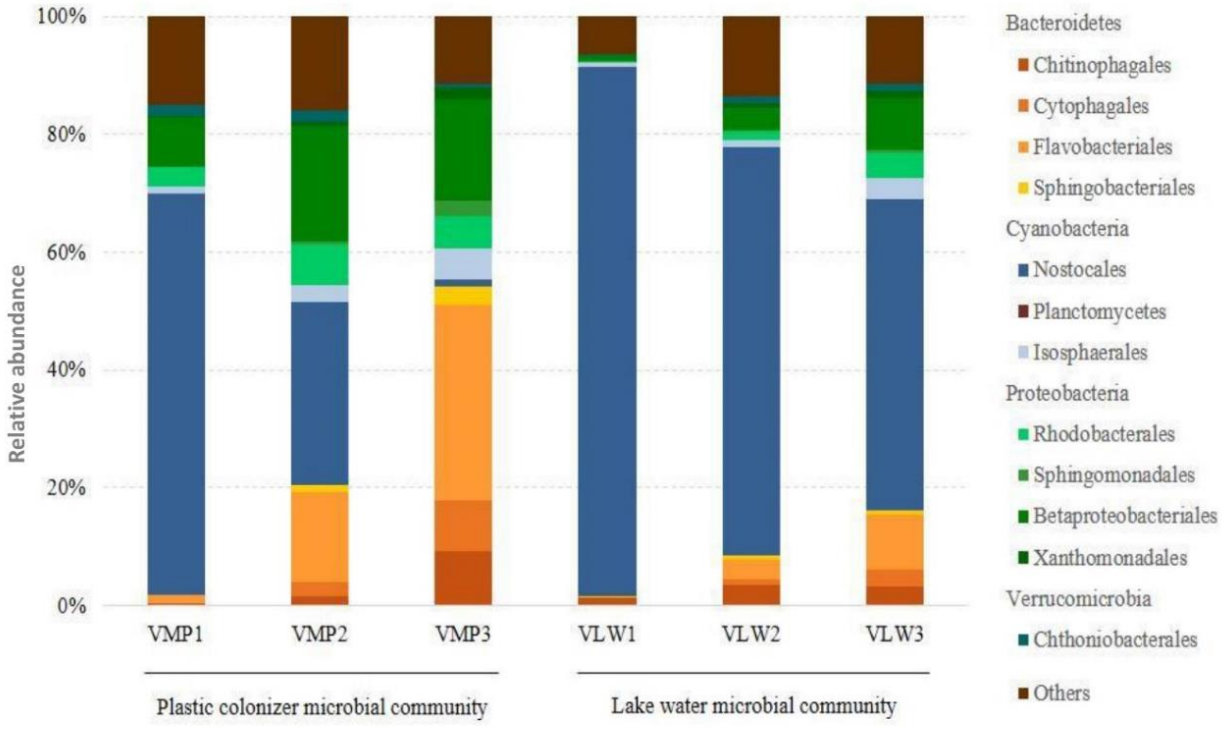

Figure 9. Dominant bacterial phyla and orders associated with microplastics and surrounding water samples. based on relative abundance more than 1%; VMP1: microplastic from plastic colonizer, first month, VMP2: microplastic second month, VMP3: microplastic third month, VLW1: surrounding water first month, VLW2: surrounding water second month, VLW3: surrounding water third month

Order Nostocales which belongs to phylum Cyanobacteria, was the most dominant in microplastic samples at the order level in the first and second month, while Flavobacteriales, a member of order Bacteroidetes, was dominant in the third month in the microplastic associated bacterial community, followed by order Betaproteobacteriales in all microplastic samples. All lake water samples were dominated by Nostocales, followed by Chitinophagales order that belongs to Bacteroidetes phylum in the first month, Betaproteobacteriales in the second month, and Flavobacteriales in the third month see Figure 10.

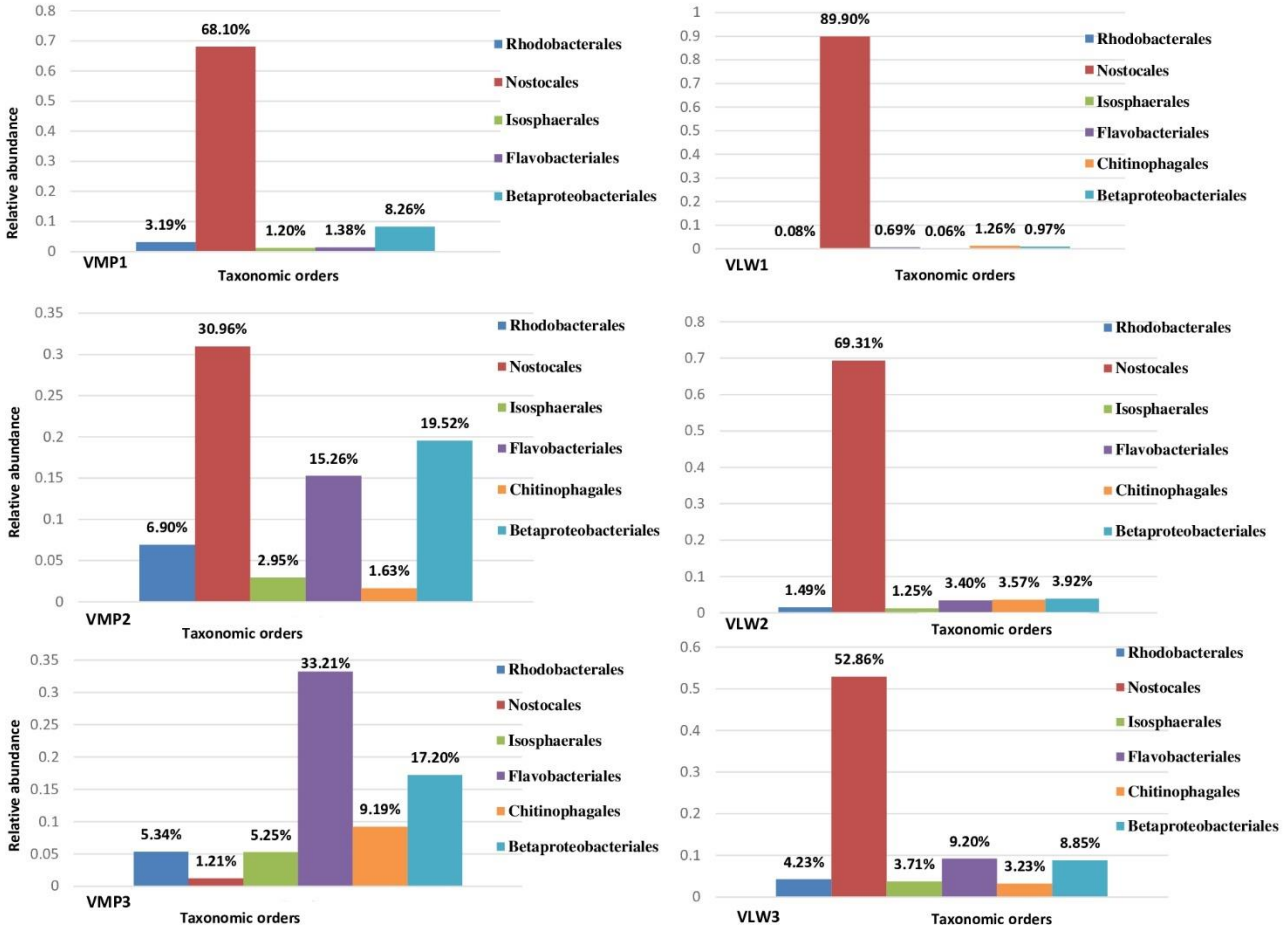

Figure 10. Dominant taxonomic orders associated with microplastics and surrounding water based on relative abundance more than 1%; VMP1: microplastic from plastic colonizer, first month, VMP2: microplastic second month, VMP3: microplastic third month, VLW1: surrounding water first month, VLW2: surrounding water second month, VLW3: surrounding water third month

The Phormidiaceae family was the most dominant in microplastic originated samples in the first and second month, while Flavobacteriaceae was dominant in the third month in the microplastic associated sample, followed by Burkholderiaceae in all microplastic samples. Phormidiaceae was also prominent in all water samples, followed by Saprospiraceae, Burkholderiaceae, Flavobacteriaceae in the first, second, and the third month respectively, see Figure 11. At the genus level, Planktothrix was the most

dominant in microplastic samples in the first and second month, while Flavobacterium was dominant in the third month in microplastics, followed by the unclassified genus of Burkholderiaceae in the first month, Flavobacterium in the second month, and Rhodoferax in the third month. Genus Planktothrix was prominent in all water samples, followed by the unclassified genus of Burkholderiaceae in the first month and Flavobacterium in the second and third months.

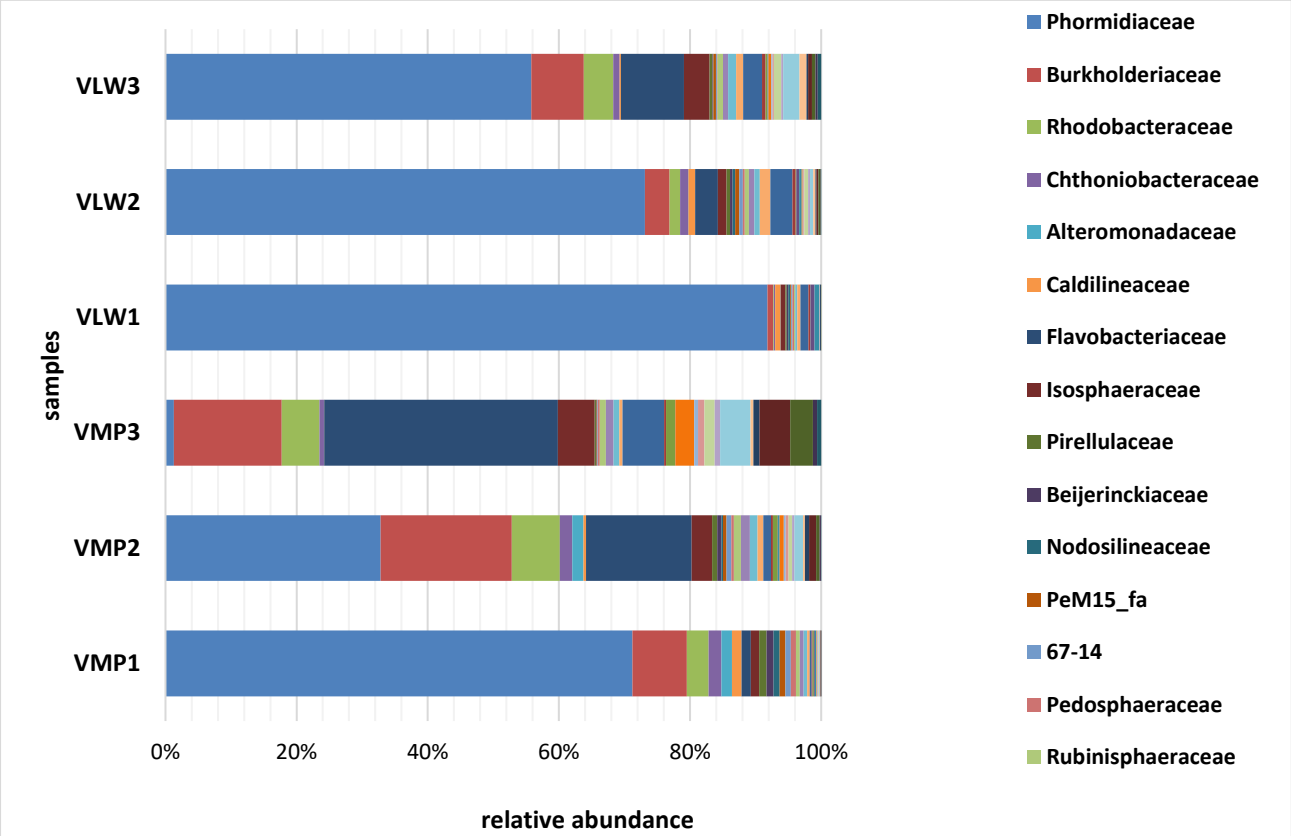

Figure 11. Dominant bacterial families associated with microplastics and surrounding water based on relative abundance more than 1%; VMP1: microplastic from plastic colonizer, first month, VMP2: microplastic second month, VMP3: microplastic third month, VLW1: surrounding water first month, VLW2: surrounding water second month, VLW3: surrounding water third month

In order to visualize differences between bacterial community structures, a heat map analysis was performed at the genus level (Figure 12). For some genera, the tendencies of abundance changing were likely the same during the three-month period in both microplastic and surrounding lake water communities, e.g. Albidiferax, Algoriphagus. But in most cases, distinct differences in dominant genera between microplastic surface samples compared to the surrounding lake water were observed. For example, Planktothrix genus, which belongs to Cyanobacteria, is frequently present in all lake water samples across all months, whereas in the microplastic samples, it decreased in abundance from the first month to the third month. With regards to Flavobacterium it was less frequent in the first

month in both microplastic and lake water associated samples but became more abundant in the second and third months on microplastics compared to lake water samples. Overall, Sphaerotilus was more abundant in microplastic samples as compared to lake water samples.

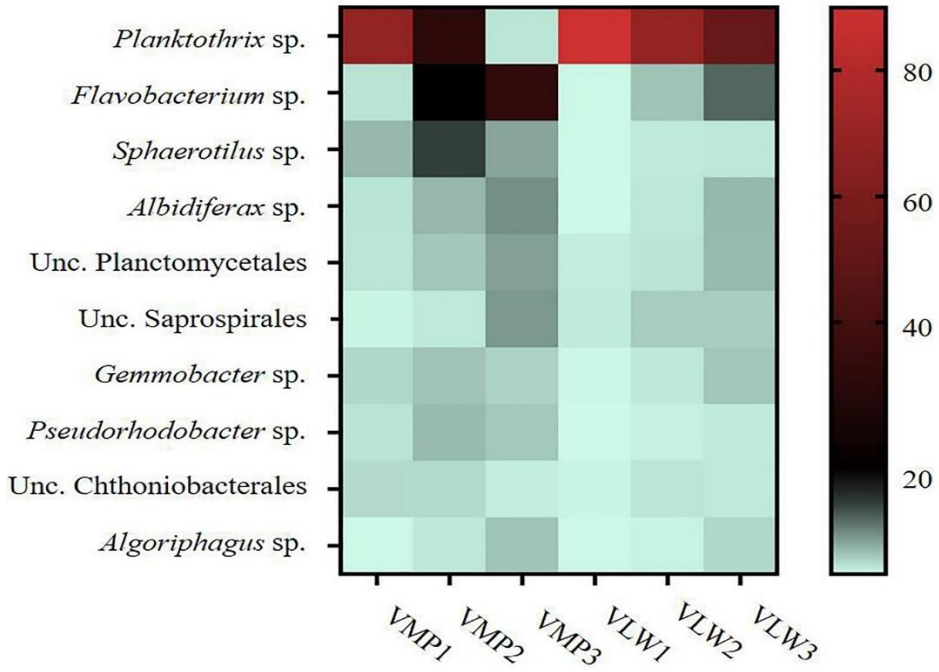

Figure 12. Heat-map of microbial community structure on the genus level with relative abundance of more than 1%; VMP1: microplastic first month, VMP2: microplastic second month, VMP3: microplastic third month, VLW1: surrounding water first month, VLW2: surrounding water second month, VLW3: surrounding water third month. The color intensity in each panel shows the percentage in a sample, color key is at the right side.

At the species level, *Planktothrix rubescens* was mostly dominant in microplastic samples in the first and second month, followed by *Sphaerotilus montanus*, while *Flavobacterium lacus* was dominant in the third month in microplastics followed by *Flavobacterium chungnamense*. *Planktothrix rubescens* was dominant in all water samples, followed by *Leptothrix cholodnii*, *Lewinella nigricans*, *Tundrisphaera lichenicola* in the first, second, and third month respectively see in (supplementary table 3).

Cluster analysis of OTUs with higher abundance of 1%, based on Bray-Curtis similarity, demonstrated that the dissimilarities between microplastic surfaces (VMP samples) and the surrounding water (VLW samples) increased with time. The bacterial community of the first month (VMP1) on microplastic surfaces belongs to a close cluster with the samples of the surrounding water (>60%). From the second month, the microplastic associated bacterial community differentiated from the surrounding water (around 50% similarity). The highest differences between the microplastic surface

and the surrounding water were observed in the third month, where the similarity decreased to less than 30% (Figure 13).

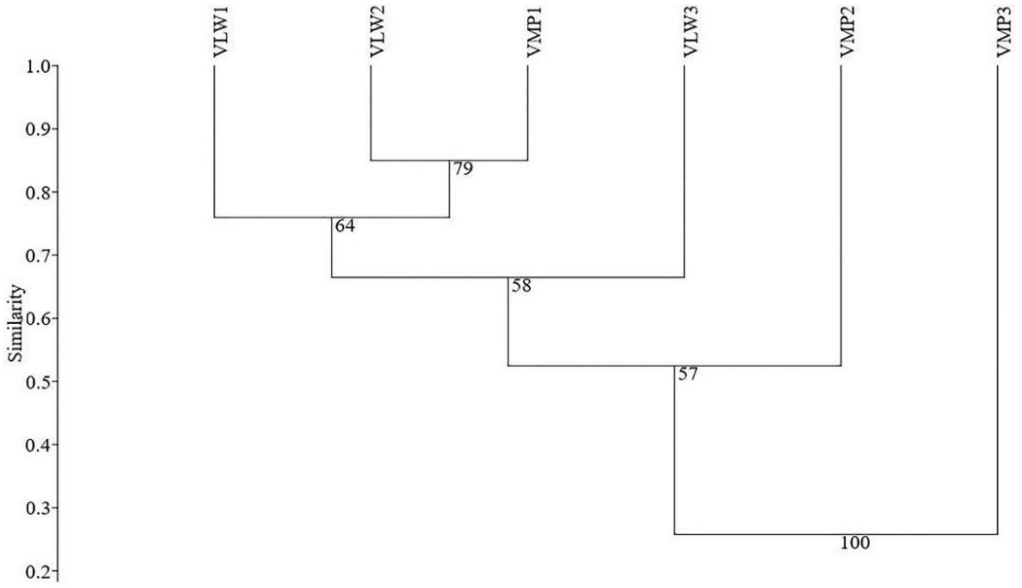

Figure 13. Clustering analysis of bacterial communities in the six samples (three microplastic and water) based on OTU abundance-based Bray-Curtis similarity coefficients; VMP1: microplastic associated community from plastic colonizers, first month, VMP2: microplastic second month, VMP3: microplastic third month, VLW1: surrounding lakewater first month, VLW2: surrounding water second month, VLW3: surrounding water third month.

Based on the statistical analysis above, it was found that the difference in microbial community compositions of microplastics compared with surrounding water is highly significant (significant if P-value is less than 0.05) when analyzing over the whole study period (P-Value = 0.000; see **Table 2**), So the H0: There is no (statistically) significant difference between the microbial community on microplastic surfaces compared to the surrounding water, hypothesis was rejected.

Table 2 Independent Samples Test table (testing differences between the microplastic associated bacterial communities [group 1] and surrounding water [group 2] over the study period), Note: Otu0001 were removed from the analysis for the three months due to extreme size

Size	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% CI of the Difference	
								Lower	Upper
Equal variances assumed	49.247	.000	4.085	3970	.000	15.893	3.891	8.266	23.521
Equal variances not assumed			4.085	2465.578	.000	15.893	3.891	8.264	23.522

This hypothesis was also tested again among the months; below are the results for testing the differences between the microplastic associated bacterial communities (group 1) and surrounding water (group 2) on monthly basis (December, January, February). The results of the t-test and Levene's Test show a highly significance among December (P-Value = 0.000), January (P-Value = 0.010) and February (P-Value = 0.023) see **Table 3**.

Table 3 Independent Samples Test table (testing differences between the microplastic associated bacterial communities [group 1] and surrounding water (group 2) on monthly bases (December, January, February))

Month		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% CI of the Difference	
									Lower	Upper
Dec.	Equal variances assumed	42.326	.000	4.086	1322	.000	9.675	2.368	5.030	14.321
	Equal variances not assumed			4.086	828.653	.000	9.675	2.368	5.027	14.323
Jan.	Equal variances assumed	19.670	.000	2.568	1322	.010	16.497	6.423	3.897	29.097
	Equal variances not assumed			2.568	768.770	.010	16.497	6.423	3.888	29.106
Feb.	Equal variances assumed	17.938	.000	2.284	1322	.023	21.508	9.418	3.031	39.984
	Equal variances not assumed			2.284	843.606	.023	21.508	9.418	3.022	39.993

4.2 Bacterial amplicon sequencing results for different materials (second colonization test)

The second colonization test was performed to probe the distinctness of bacterial communities from plastic surfaces from others of different materials. By the results of amplicon sequencing, the most dominant bacterial phyla in all materials and on microplastic were Proteobacteria, Bacteroidetes, Verrucomicrobia, Cyanobacteria, Actinobacteria, and Firmicutes. The differences were with the relative abundance of these phyla between microplastic and the different materials. On the other hand, Cyanobacteria dominates the water associated communities, followed by Firmicutes and Proteobacteria.

On the order level, orders Bacteroidales (16.3%), Verrucomicrobiales (12.9%), Clostridiales (11.9%), Rhodobacterales (11%), Xanthomonadales (8%), and Desulfovibrionales (6.7%) were the most abundant (>5%) in polypropylene plastic associated communities. In degradable plastic communities, orders; Verrucomicrobiales (13.9%), Bacteroidales (13.4%), Nostocales (11.3%) , Rhodobacterales (9.5%), Clostridiales (8.3%), Xanthomonadales (5.8%), Desulfobacterales (5.7%) were the most dominant. Rhodobacterales (14.6%), Verrucomicrobiales (13.9%), Xanthomonadales (9.6%), Nostocales (9.6%), Bacteroidales (7.8%), Desulfobacterales (6.3%), and Clostridiales (5.8%) were mostly dominant in glass-associated community. The wood-associated bacterial community was dominated by Clostridiales (21.5%), Bacteroidales (15.1%), Pseudomonadales (11.6%), Verrucomicrobiales (9%), Desulfovibrionales (5.8%), and Erysipelotrichales (5.5%), whereas water-associated bacterial communities were dominated by the following taxonomic orders: Nostocales (92.2%), Bacillales (4.1%), Planctomycetales (0.6%), and Rhizobiales (0.4%).

To represent similarities between microbial communities and samples we drew clustered heatmaps where we performed hierarchical clustering on both the samples and microbial communities using Euclidean method as distance measure (Figure 14).

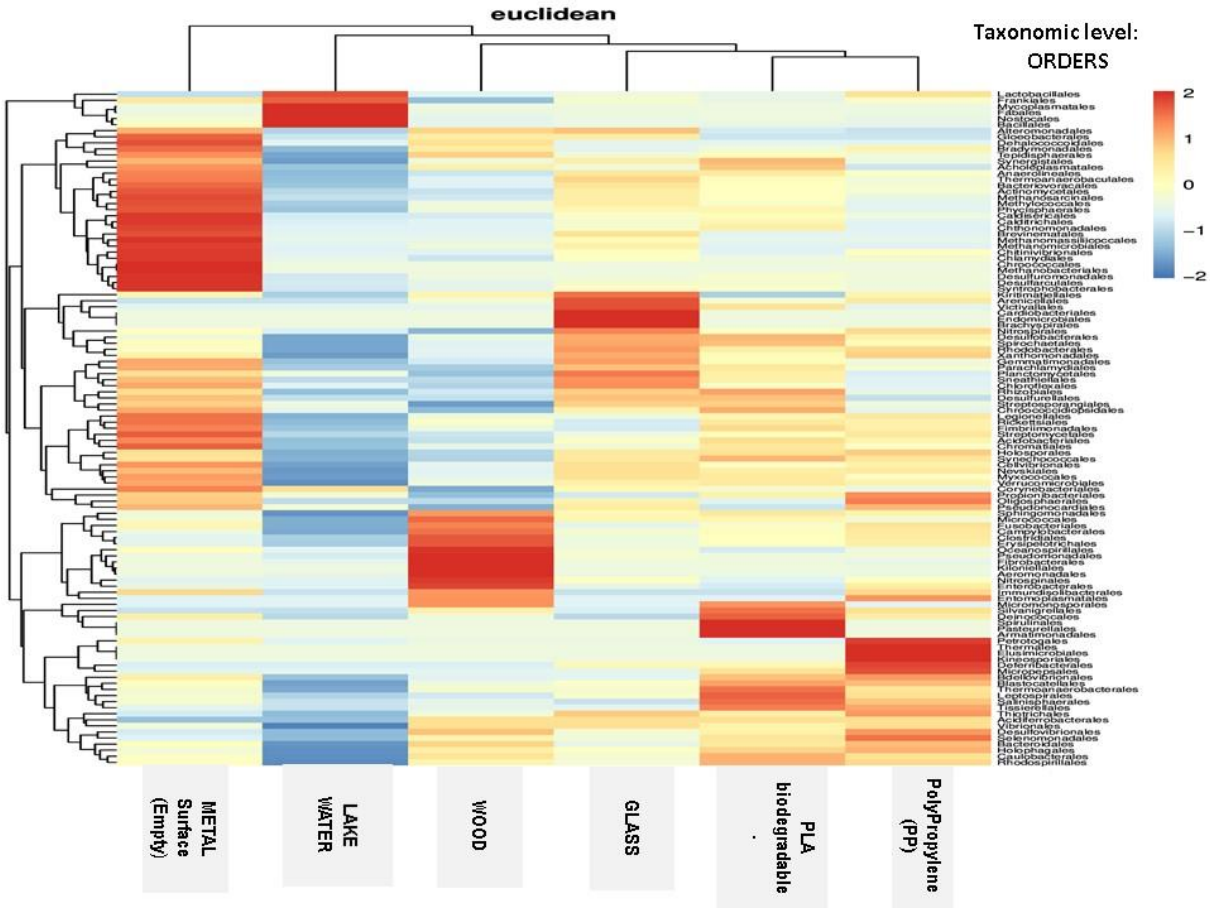

Figure 14. Heat-map of microbial community structure on the order level with relative abundance of more than 1%; Poly Propylene, degradable plastic, glass, wood, water, steel surface (empty colonizer) The color intensity in each panel shows the percentage in a sample, color key is at the right side. *This figure can be reached in bigger size in the Supplementary Materials at the end of the dissertation.*

On the family level, Rubritaleaceae and Rhodobacteraceae were the most dominant in the stainless steel, glass, microplastic and degradable plastic communities, with notable differences in relative abundances of these families among them. The wood community, was dominated by Pseudomonadaceae followed by Clostridiales, whereas the water community was highly dominated by Bacillaceae.

These results of ours were published in Water, Air, and Soil Pollution (Q2, IF: 2,49) in 2021 (Szabó *et al.*, 2021).

4.3 Description of novel bacterial species from marine waste

Variable bacterial species were isolated in the initial study on marine agar from PP straws collected from seawater. A list of isolated bacterial species that were identified based on around 500 base pairs (bp) long 16S rDNA gene sequencing (> 99% similarity) are presented in **Table 4**. A strain of *Aeromonas ichthiosmia*, which is a synonym of *Aeromonas veronii* a known fish pathogen, was

isolated (Hickman-Brenner *et al.*, 1987; Rahman *et al.*, 2002), two strains of *Halobacillus locisalis*, (found in solar salterns first) (Yoon *et al.*, 2004) were also isolated from PP straws surface. Another bacterial isolate (Zs-1/5) was also detected. It has shown 99.77% similarity with *Idiomarina aestuarii* which was first isolated from the south sea in the Republic of Korea (Park *et al.*, 2010).

Table 4 Identified bacterial species isolated from marine plastic waste (PP straws) on MA agar incubated aerobically at 28°C for 72h.

Isolated strain	Close relative	16S rDNA Similarity	Isolation date	Risk group*
ZS-1/1	*Aeromonas ichthiosmia*	98.46%	September 2018	1
ZS-1/2	*Halobacillus locisalis*	99.77%	September 2018	2
ZS-1/3	*Parvularcula lutaonensis*	98.09%	September 2018	1
ZS-1/4	*Halobacillus locisalis*	99.81%	September 2018	2
ZS-1/5	*Idiomarina aestuarii*	99.77%	September 2018	n/d

* classified according to the German Collection of Microorganisms Cell Cultures (DSMZ.de); **Risk group 1**: bacteria of low individual or community risk, unlike to cause disease. **Risk group 2**: bacteria of moderate risk, exposure might cause disease, but risk is not significant to lab workers or environment. **n/d**: not defined

Bacterial isolate ZS-1/3 was recognized as possible new bacterial species, because 16s rDNA sequence similarity was not genetically high with the closest relative. Therefore, for novel species description, further genotypic and phenotypic analysis were conducted as explained in section 3.1.3 in the Materials and methods section. The results of these tests of ZS-1/3 bacterial isolate will be discussed in the following sections.

4.3.1 *Genomic characteristics of the novel species*

The complete 16S rDNA gene sequence of strain ZS-1/3T determined in this study was a continuous stretch of 1371 bp (positions 50–1458 with respect to the *Escherichia coli* numbering system). Sequence similarity calculations using the EzTaxon server (http://www.eztaxon.org/) verified that by the closest relatives of strain ZS-1/3T were *Parvularcula lutaonensis* (98.09% sequence similarity) and *Parvularcula oceanus* (95.89%). Moreover, on the basis of the 16S rRNA gene sequence analysis, the phylogenetic position of strain ZS-1/3T among the other members of the genus *Parvularcula* is unique and distinct (Figure 15). The overall topology of the maximum-likelihood tree was similar to that of the neighbour-joining and maximum parsimony trees.

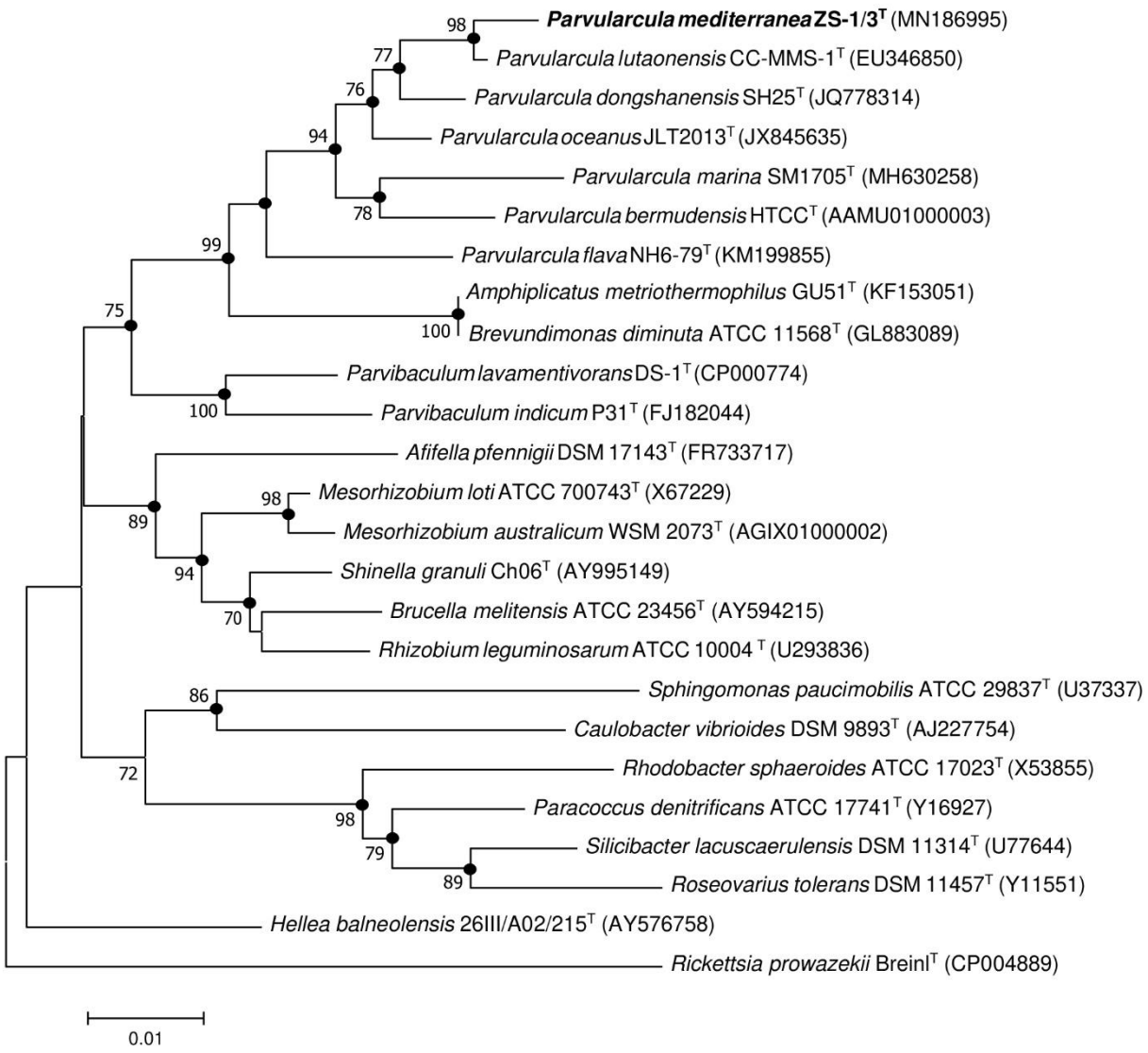

Figure 15. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic positions of strain ZS-1/3^T and related species.

The final draft genome of ZS-1/3 comprised 3 scaffolds (N50= 1726715 bp) and 5 contigs, with a total genome size of 3214726 bp, the total number of genes was 3096 and 3031 coding genes, one complete rRNA operon (5S-16S-23S) and 41 tRNA were found in the genome, the total number of RNA genes was 48. Furthermore, the sequence coverage was 205.24-fold, the DNA G+C content of strain ZS-1/3T was 62.5.0 %. The OrthoANI, dDDH values between strain ZS-1/3T and the closest *Parvularcula* relative species, *P. lutaonensis*, were 74.9, 19.1 respectively. The OrthoANI, dDDH values for other *Parvularcula* relatives are shown in **Table 5**. The ANI and dDDH values were much lower than the threshold values of ANI (95~96%) and DDH (70%) to discriminate bacterial species.

Table 5 Average nucleotide Identity (ANI) and digital DNA–DNA hybridisation (dDDH) values between strain ZS-1/3^T and closest *Parvularcula* relatives.

Strain	ZS-1/3^T	
	ANI (%)	dDDH (%)
P. lutaonensis CC-MMS-1^T	74.9	19.1
P. bermudensis HTCC2503^T	67.9	19.9
P. flava NH6-79^T	68.8	20.1
P. oceani JLT2013^T	70.7	19.1
P. dongshanensis SH25^T	70.7	19.9

Based on the evaluation of intra-strain and inter-strain biomarker peaks detected in the bacterial spectra by MALDI TOF MS, hierarchical clustering analysis verified that the novel strain and the closest relative *Parvularcula lutaonensis* can be clearly distinguished and are different enough to be separate species (see supplementary Figure 7 A., B. and C).

4.3.2 *Physiology and Chemotaxonomy of the novel species*

The coccoid-shaped cells are about 1 μm long and 1 μm in diameter. The surface of the cells is totally smooth with no flagella. Electron-microscopic morphology of strain ZS-1/3^T is seen in Figure 16. Cell morphology was also observed at 1000x magnification with a light microscope (Leica) using cells grown for 72 hours at 28ºC on marine agar plates. Colony morphology on marine agar was small (approximately 0.5 mm), dark orange pigmented, circular, convex, smooth and shiny surface. On R2A, nutrient agar, and tryptic soya agar, no growth was observed after 72h.

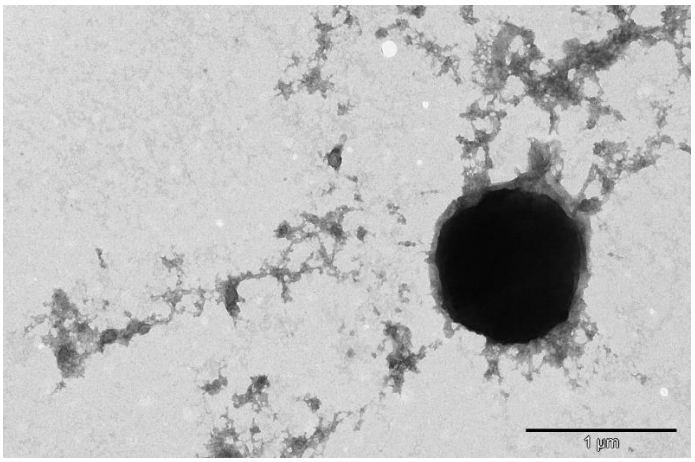

Figure 16. Shadow casting electron microscopic image of *Parvularcula mediterranea*

All other phenotypic and genotypic characteristics are summarized in **Table 6**.

Table 6 Morphological, biochemical, and physiological characteristics of the novel bacterial strain ZS-1/3^T and the closest relative *P. lutaonensis* KCTC 22245^T

Characteristic	**ZS-1/3^T**	***Parvularcula lutaonensis* KCTC 22245^T**
Colony pigmentation	Orange	Orange
Growth temperature range	20-38	25-50
Growth in NaCl%	2-7	1-6
pH range	5-10	6-8
β galactosidase	-	+
Gelatin	+	-
Nitrate reduction	-	-
Indole	-	-
glucose	-	-
Urease	-	-
Aesculin	+	+
ONPG	+	+
D mannitol	-	-
α glucosidase	-	+
Lipase (C14)	-	-
L arabinose	-	-
Alkaline phosphatase	+	+
Esterase	+	+
DNA G+C content (%)	62.5	59.0

The isolated ZS-1/3^T grew well only on marine agar. Other physiological characteristics of strain ZS-1/3^T are summarized in the species description. The only respiratory quinone was Q-10 (100 %). Although similar fatty acid profiles were observed for strains of species belonging to the genus *Parvularcula*, in which $C_{18:1}$ ω 7*c* and $C_{16:0}$ are the major fatty acids (Zhang *et al.*, 2016), (Arun *et al.*, 2019), the profile differs from the closest relative in the proportions of these latter fatty acids. The fatty acid cyclo-$C_{19:0}$ ω8*c* was not detected in strain ZS-1/3^T but was present in *P. lutaonensis* (see **Table 7**). This finding also confirms that strain ZS-1/3^T differs at the species level from other members of the genus *Parvularcula*.

Table 7 cellular fatty acids compositions (%) by TSBA 40 method of strain ZS-1/3^T and the closest relative *P. lutaonensis* KCTC 22245^T

Fatty acid	**ZS-1/3^T**	***P. lutaonensis* KCTC22245^T**
$C_{18:1\ \omega7c}$	**56.8**	**63.9**
$C_{16:0}$	**27.5**	**22.6**
$C_{18:0}$	2.2	4.9
$C_{12:0}$	1.4	0.7
$C_{14:0}$	0.9	0.1
cyclo-$C_{19:0}$ $\omega8c$	-	0.3
Unknown 11.799	**8.3**	**5.1**

Major fatty acids (>5 %) in each strain are shown in bold.
-, not detected/reported.

On the basis of the 16S rRNA gene sequence similarities between strain ZS-1/3^T and its closest relative within the genus *Parvularcula*, and considering 98.65% as the threshold for differentiating two species (Kim *et al.*, 2014), in addition to the results of the genomic, chemotaxonomic, biochemical and physiological analysis, strain ZS-1/3^T is considered to represent a novel species within the genus *Parvularcula* for which the name *Parvularcula mediterranea* sp. nov. was proposed.

4.3.3 *Description of Parvularcula mediterranea sp. nov.*

Parvularcula mediterranea (me.di.ter.ra'ne.a. N.L. fem.adj. *mediterranea* pertaining to Mediterranean Sea, from where this strain was isolated).

Cells are Gram-negative, obligate aerobe, non-spore-forming, motile with single flagella, 1μm in size. Colonies grown on Marine agar (MA) plates for 72h were small (approximately 0.5mm), dark orange pigmented, circular, convex, smooth and shiny surface. On R2A, nutrient agar, and tryptic soy agar no growth was observed after three days. Growth was observed at temperatures between 20-38 ºC and pH 5-10. The optimal growth temperature and pH are 28ºC and pH 7.0. No Growth occured in the absence of NaCl; growth was observed at a concentration of NaCl from (2.0-7.0% (w/v), with optimum growth at (3.0%). It was oxidase positive and catalase-negative. API tests showed it was positive for gelatin, alkaline phosphatase, α-Chymotrypsin, acid phosphatase, trypsin, Leucine arylamidase, *β*-glucosidase, estrerase (C4), esterase lipase (C8) *β*-galactosidase, *β*-glucosidase, and esculine ferric citrate and negative reactions were observed for nitrate reduction, glucose fermentation, arabinose, mannitol, sorbitol, indol, urease, *β*-glucuronidase, for the complete results of API ZYM, 20E, and 20NE see supplementary table 4.

Major fatty acids were $C_{18:1\ \omega7c}$, $C_{16:0,}$ $C_{18:0}$, and $C_{12:0}$. Quinone 10 (Q-10) is the predominant (100 %) respiratory quinine. The polar lipids of strain ZS-1/3^T consisted of eight unknown glycolipids (GL), and one unknown phospholipid (PL), and one unknown phosphatidylglycerol (PG) (Supplementary figure.6), the polar lipids of the closest relative *P. lutaonensis* consisted of four unknown phospholipids and four unknown glycolipids(Arun *et al.*, 2019). The DNA G+C content of the type strain is 62.5 %. Strain, ZS-1/3^T (= NCAIM B 02654^T = CCM 9032^T) was isolated from a floating plastic straw sample from the Mediterranian Sea near the public beach of Laganas in Zakynthos Island, Greece.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JABFCX000000000. The version described in this paper is version JABFCX000000000.1, the accession number for the 16S rRNA gene sequence is MN186995.

This result of ours were published in International Journal of Systematic and Evolutionary Microbiology (Q1, IF: 2,51) in 2020 (Al-Omari et al, 2020)

5 DISCUSSION

5.1 The 'plastic colonizer' method

Comparing the self developed plastic colonizer method with the majority of methods used to study the microbial colonization of microplastic in water, it can be stated that some other methods are mainly focused on the collection of microplastic particles through a mesh or net or the collection of plastic litter (Zettler *et al.,* 2013; Frère *et al.,* 2018; McCormick *et al.,* 2016; Viršek *et al.,* 2017; Jiang *et al.,* 2018). Other methods were used in which plastic particles were incubated in situ, but mostly for short incubation periods (Oberbeckmann *et al.*, 2018; Dudek *et al.*, 2020; Oberbeckmann *et al.,* 2014). Other studies incubate the plastic particles in a controlled environment *in vitro* (Dussud *et al.,* 2018; Kirstein *et al.,* 2019; Wang *et al.,* 2020). In contrast, the method of plastic colonizers used in this study can be used in situ in different environments like freshwater lakes and rivers. Periodic changes in microbial communities and the possible alteration of microplastic surfaces might also be monitored since it is expected to stand for changeable environmental conditions. Thus it can be used to monitor the changes over the seasons. It might also be used in marine environments to study microbial communities that associate with microplastics. Future studies based on this same easy-to-use colonizing method can be compared with each other .

Furthermore, based on our results, limitations of the plastic colonizers should be tested in saline water and for long-term periods (minimum one year) to verify the stability of the complex structure. By the results of our tests, it can be stated that the self-designed plastic colonizer is an appropriate method to describe plastic associated communities. It can be used to collect comparable data about plastispheric bacterial communities from different water bodies. Martínez-Campos *et al.* also affirmed that as they used a similar method for the description of plastic associated communities in a waste water treatment system (Martínez-Campos *et al.*, 2020).

In line with previous studies (Zettler *et al.*, 2013; Miao *et al.*, 2019; Oberbeckmann *et al.*, 2014), the microbial community structures of microplastics in our study were also different from those in the surrounding water. The community structures were changing over the period of three months. The dominance of microbial phyla was changing in terms of relative abundance over the three-month period of the first colonization test. For example, Cyanobacteria was the most dominant phylum in the lake water samples over the studied time, but with decreasing relative abundance over the first,

second, and third month respectively (91%, 70%, 53%). On microplastic surfaces, the relative abundance of Cyanobacteria decreased from 69%, to 31%, and 1% over the three months, but Bacteroidetes became more and more dominant (2%, 21%, 54% respectively). This result is consistent with Jiang *et al.* (2018) where they also found that Cyanobacteria and Proteobacteria are suggested to be early microplastic colonizers in freshwater, followed by Bacteroidetes as secondary microplastic colonizers (Jiang *et al.*, 2018), which is also in line with Lee *et al.* (2008) regarding the early and secondary colonizers in marine water (Lee *et al.*, 2008). Furthermore, the occurrence of Cyanobacteria, which is an oxygen producer, is probably due to its importance for polymer oxidation. Planktothrix species dominated the investigated samples. The high abundance of bloom-forming *Planktothrix agardhii* and *P. rubescens* are regularly observed in European waters (Nõges and Ott, 2003; Willame *et al.*, 2005; Churro *et al.*, 2017). While they cause strong red-colored water-bloom, these species can produce microcystins (MCs), which inhibit eukaryotic protein phosphatases. Bioactive peptides are also produced by this species, which presumably enhance the colonization potential and possible dominance in habitats (Kurmayer *et al.*, 2016). The cyanotoxins as microcystins can cause human and animal poisoning. MCs might be responsible for tumor promotion (Bogialli *et al.*, 2013). This finding supports the hypothesis that microplastics may serve as a vector for pathogenic bacteria (Zettler *et al.*, 2013). It is also verified with the bacterial isolates of the initial study and the first colonization test of ours, because some bacterial strains from microplastic surfaces have been identified as species belonging to fish and facultative human pathogens such as *Aeromonas bestiarum, Shewanella putrefaciens, Brevundimonas vesicularis,* and *Aeromonas sobria.*

During the three months, the Bacteriodetes became more and more dominant on microplastic surface samples. The most abundant Flavobacteriaceae family was also observed in the second and third months of lake water but was absent in the first month. Flavobacteriaceae composed around one-third of the identified plastic associated communities (Oberbeckmann *et al.*, 2016). Flavobacteria were described as a major colonizer of Diatoms detritus (Abell and Bowman, 2005). Therefore, the increased relative abundance of Flavobacteriaceae on microplastic surfaces is probably because of the noted algae colonizing of the surface of the plastic particles inside the colonizers. However, we cannot prove this hypothesis since we have no data about eukaryotic (eg. Alveolata) members of the community. On microplastic surfaces, *Flavobacterium lacus* was the dominant species in the third month, followed by and *Flavobacterium chungnamense*. However other members of Flavobacterium were also present. Members of this genus are chemo-organotroph aerobes and can be found in various aquatic habitats such as freshwater, wastewater, seawater, and can adapt to very cold environments

like Antarctic lakes. Flavobacterium spp. was isolated from the surface of low density polyethelene microplastics and considered as a serious fish pathogen (Li *et al.*, 2014; Gong *et al.*, 2019). Some Flavobacterium species are also known to degrade complex polymers such as Pentachlorophenol PCP (Saber and Crawford, 1985), diesel (Chaudhary *et al.*, 2019), and nitriles (Egelkamp *et al.*, 2017). Nylon oligomer has been degraded by Flavobacterium sp. KI 725 when provided as the sole source of carbon and nitrogen (Negoro, 2000), therefore the increased abundance of Flavobacteria on microplastic surfaces with time might be due to a potential role in biodegradation.

Lacihabitans were also present on microplastic surfaces; this genus, by the time of writing contains only one valid species *Lacihabitans soyangensis*, it belongs to the Cytophagaceae family, which is widely distributed in environments like freshwater, marine water, soil. Members of this family are known to have the capability to degrade several organic compounds such as starch, chitin, and cellulose (Joung *et al.*, 2014). Bacterial cellulose (BC) is usually abundant in biofilm due to its role in intra- and inter-domain interactions. BC is known to be produced by proteobacteria which was abundant on microplastic surfaces in this study. Therefore this might be the reason for the occurrence of *Lacihabitans* spp. on microplastics.

Algoriphagus marisflavi was first isolated and identified in estuarian water in the Yellow Sea (Korea), it can grow at low temperatures 4°C and at 0–2.0 % (w/v) NaCl (Park *et al.*, 2017). Members of genus *Algoriphagus* were isolated from different habitats; seawater, freshwater, marine sediments, and algae (Nedashkovskaya *et al.*, 2004; Liu *et al.*, 2009). We suggest that its presence in the plastisphere in our study might also be due to the notable abundance of algae on the microplastic surfaces.

Burkholderiaceae was identified on microplastic surfaces over the three months period as the second most dominant with a relative abundance of 7.9%, 18.8%, 15.3%, respectively. Members of Burkholderiaceae were also identified in bacterial communities associated with plastic surfaces in different environments such as wastewater treatment systems (Pal *et al.*, 2012) and drinking water facilities (Kalmbach *et al.*, 2000). Therefore, the occurrence of these family members might be connected to the plastic surface rather than the availability of nutrients.

Members of genus Gemmobacter have been isolated from diverse environments; freshwater spring, snow samples, birds, planktonic seaweeds, and marine environments (Chen *et al.*, 2013; Liu *et al.*, 2014; Yoo *et al.*, 2019), Gemmobacter belongs to the family Rhodobacteraceae which was frequently

identified on plastics (Zettler *et al.*, 2013; Bryant *et al.*, 2016), and was described as a primary biofilm colonizer in seawater (Elifantz *et al.*, 2013). Additionally, Rhodobacteraceae was suggested as one of the candidate bacterial families for plastic degradation (Roager Line and Sonnenschein Eva, 2019).

The PVC superphylum member uncultured Planctomycetales bacteria's (closest relative Tundrisphaera lichenicola 89,7% 16S rRNA similarity) abundance showed an increasing trend within the plastic colonizer community. Planctomycetes usually prefer a surface-attached lifestyle. DeLong *et al.* (1993) found a greater abundance on the surface of marine aggregates than in the bacterioplankton (DeLong *et al.*, 1993). Analyzing four lakes in Germany, Allgaier and Grossart (2006) found a complete lack of Planctomycetes among the free-living microorganisms, while the group appeared abundant among the surface colonizers (Allgaier *et al.*, 2006).

The distribution of another PVC group member uncultured Chthoniobacterales bacteria (closest relative *Terrimicrobium sacchariphilum* 91,42% 16S rRNA similarity) was not so specific, since it was present in all samples.

Members of the Mycobacterium genus were also identified on microplastic surfaces in our study. This genus contains many pathogenic species associated with pulmonary diseases. Additionally, Mycobacterium has been reported as a potential degrader of polyethylene (Sudhakar *et al.*, 2008; Fusco Da Costa *et al.*, 2015).

Based on the former publications about the identified species, it can be verified that potential fish and human pathogenic strains can be isolated from microplastic surfaces. This result of ours is in line with studies that mention the potential occurrence of pathogens on microplastic surfaces (Keswani *et al.*, 2016; Zettler *et al*., 2013; Wu *et al.*, 2019). For example, the identified isolates, *Aeromonas bestiarum* species was described as a fish pathogen in carp and trout (Vet *et al.*, 2010). *Aeromonas sobria* strains have also been described as a pathogen of silver carp as well as a rare human pathogen (Dar *et al.*, 2016). Plastic colonizing *Shewanella putrefaciens* is a potential opportunistic human pathogen. Some strains of this species were reported as a causative agent of osteitis, erysipelas, abscess, and rare cases of bacteremia and soft tissues infections (Hochedez *et al.*, 2013). Strains of Bacillus simplex were reported as a suspected cause of human brain abscesses (Pesce *et al*., 2016).

The amplicon sequencing results of the different materials (second colonization test) have verified that bacterial communities associated with different materials (plastic, degradable plastic, glass, metal, and wood) are clearly different from the surrounding water on both phylum and order levels. Plastic associated communities were also different from other materials colonizing communities in line with the results of (Oberbeckmann *et al.*, 2018; Oberbeckmann *et al.*, 2021). According to the comparison of results, the differences between bacterial communities associated with polypropylene plastic and degradable plastic surface were smaller than in the case of wood metal and glass and surrounding water; mainly, these differences were in the relative abundance of different taxa. The clustering of our samples shows that community structures of polypropylene plastic and degradable plastic were the closest to each other, followed by glass, wood, water and finally, metal surface.

5.2 Parvularcula mediterranea

As far as we know there are few numbers of novel bacterial species isolated from plastic surfaces. *Croceimicrobium hydrocarbonivorans* was isolated from a microbial consortium that is used to degrade PET. The consortium was enriched from a deep-sea sediment sample (Liu *et al.*, 2021). Another three novel *Rubripirellula* species were isolated from PS and PET particles submerged in Baltic sea and the river Warnow in Germany (Kallscheuer *et al.*, 2019). *Ideonella sakaiensis* which was discovered in plastic bottle recycling factory in Japan has the capability to degrade PET, the resulting compounds are environmentally-friendly products (Palm *et al.*, 2019; Yoshida *et al.*, 2016). In line with that we hope that our novel *Parvularcula mediterranea* isolated from PP straws will be an important member of the plastisphere who might have a role in plastic biodegradation in the future.

6 CONCLUSIONS

The investigation of microplastic associated communities in freshwater has been conducted using different methods, such as manta net, collection of plastic particles from beaches, shallow water, or incubation under lab conditions in a controlled environmrents. Some of these methods are not easy to be used in different locations, but the plastic colonizer method invented in this study can be applied to study microplastic associated microorganisms among different locations. Therefore, it will hopefully enable the researcher to accurately investigate microplastic colonization in freshwater bodies in different geographical areas, countries, or even continents, by using a reproducable and comparable method. Our research aimed to provide an easy to use method for the investigation of microplastic associated microorganisms in freshwater. The self-invented plastic colonizer method described in this study can be used in various freshwater bodies. This method was successfully used over the winter time under hard conditions when the lake was frozen. By this method, not only cut PP straws were used but also different materials like glass, biodegradable plastic, and wood. Therefore, it can be concluded that this method can be used to study the plastispheric microbial communities associated with different polymers, and this will be helpful in determining the polymer selectivity to microbial communities. Moreover, the plastic colonizer method can be used for longer periods to study the seasonal changes in microbial communities, since it resisted the hard winter weather conditions. *This method was published in Water, Air, and Soil Pollution (Q2, IF: 2,49) in 2021 (Szabó et al, 2021).*

Various bacterial species were isolated from these plastic colonizers by culturing method using LB agar. Among these isolates *Aeromonas bestiarum* was found, which is a known fish pathogen that can cause chronic skin ulcer in carps. Moreover, facultative human pathogens were also isolated, such as *Shewanella putrefaciens, Brevundimonas vesicularis,* and *Aeromonas sobria.* The role of microplastics in the transfer of harmful bacteria as "hitchhikers" was also supported by the results of our work. The occurrence of harmful bacteria on microplastic surfaces in freshwater should be further studied and investigated.

By statistical analyses of amplicon sequencing results of plastic associated microbial communities, it has been found that microplastics provide a unique ecological nich for microbial colonization. As revealed by the amplicon sequencing results of the first colonization test, the microbial community structure associated with PP microplastics is distinct from the surrounding lake water. This finding was also supported by the results of our second colonization test: the amplicon sequencing of the

second colonization test, the associated microbial communities among PP, PLA, wood, glass, and stainless steel were different from each other and from waterborned communities also. However, PP associated communities were close to PLA (biodegradable) plastic.

In line with that, a novel bacterial species was isolated from PP straws in our initial study. The isolated bacterial strain described as novel species which belongs to the genus *Parvularcula* and it was named as *Parvularcula mediterranea*. Species description was *published in International Journal of Systematic and Evolutionary Microbiology (Q1, IF: 2,51) in 2020 (Al-Omari et al, 2020)*. These results support and comply with the studies which revealed that microplastic surfaces provide a novel ecological niche for the colonization of selective bacterial communities.

7 NEW SCIENTIFIC RESULTS

7.1 Bacterial strain ZS-1/3, isolated from marine plastic waste, had been identified as a strain of an unknown novel bacterial species. It was verified by all recommended analyses and named as *Parvularcula mediterranea,* as a refer of its origin.

7.2 A repeatable easy-to-use method, named as ‘plastic colonizer’, has been designed to study microplastic associated microbes in freshwater environments, and it was tested to use *in vivo* in freshwater lake of Hungary.

7.3 According to the results of 16s rDNA amplicon sequencing of samples from a freshwater lake in Hungary (Vácszentlászló), plastic associated bacterial community, isolated from plastic colonizers, differs from the surrounding water. This result is in accordance with the theory that plastic associated bacterial community is different from the surrounding water’s.

7.4 The results of 16s rDNA amplicon sequencing, by comparing the abundance of OTUs of samples from different materials, shows notable differencies between the microbial communities associated with wood, glass, stainless steel, poly lactic acid (PLA) and polypropylene microplastics, as well as surrounding water according to our two months long plastic colonizing test, made in a freshwater lake of Hungary. PLA associated communities are clustered closer to polypropylene originated ones than others.

8 SUMMARY

Plastic pollution has become one of the most serious environmental threats. The huge production of disposable plastic products and the bad practices of disposal have contributed to the occurrence of plastic wastes almost everywhere in the environment. Plastic debris is classified generally based on size; macroplastic with a size greater than 25mm, mesodebris between 25 and 5mm, and microplastic which is less than 5mm. Like any other surface, microbes colonize plastic surfaces and form a biofilm, Huge diversity of (even pathogenic and antibiotic resistant) microbes were detected on plastic surfaces in different environments such as marine water, freshwater, wastewater and soil.

The initial idea of this study came after the collection of plastic straws from Zakynthos island in Greece. From these marine litter various bacterial species have been isolated by cultural methods using marine agar. Also a novel bacterial species was isolated and described by us from the surface of the straw. Based on that, an easy-to-use plastic colonizer method has been designed; where PP straws were cut into small pieces (less than 5mm) and put into commercial stainless steel tea/spice filters. These, as we call it plastic colonizers, were installed 50 cm under the water surface of a freshwater lake in Hungary. After a period of three months, the bacterial communities associated with these microplastics were investigated and found to be different from the surrounding water bacterial communities. The study was repeated with different materials such as glass, wood, stainless steel (empty filters) and biodegradable plastic; the results have shown that the bacterial communities were different among these materials as well.

By these results, we have confirmed that the structure of bacterial communities associated with microplastics is different from the surrounding freshwater. Additionally, the microplastic surface could be a source of novel bacterial species that haven’t been described before; hopefully, these isolates could play a role in plastic biodegradation. The designed plastic colonizer method introduced in this study can be easily and successfully used to study the microplastic associated microbes in freshwater environments. It is recommended to use this method to investigate the microplastic colonization in different freshwater environments in other surface waters since the results can be compared.

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Supplementary table (1) summarized Methods used in the initial study, the first and the second colonization test, and the novel species description indicating the responsibility.

Initial study	
Methodology	**Conducted by**
Culturable mesophilic aerobes and facultative anaerobes on marine agar	Supervisor and the research group
16S rRNA gene sequencing for identification of bacterial isolates	Supervisor and the research group
First colonization test	
Recovery of microbial biofilm from plastic colonizers	PhD candidate
Isolation of culturable bacteria on LB agar from plastic	PhD candidate
16S rRNA gene sequencing for identification of bacterial isolates	PhD candidate
DNA Isolation of plastic-associated and lake water bacterial communities	PhD candidate
Illumina 16S rDNA amplicon sequencing	Seqomics Biotechnology Ltd
Statistical analyses of the results between plastic and water communities from (first)test	Publication's coauthor
Second colonization test	
Community DNA Isolation of different materials, plastic, and lake water	PhD candidate
Illumina 16S rDNA amplicon sequencing	Seqomics Biotechnology Ltd
Statistical analyses of bacterial communities from different materials (second colonization test)	Publication's coauthor
Novel species description	
Whole-genome sequencing G+C content	Seqomics Biotechnology Ltd
Gram stain Cell and colony morphology Temperature, pH, and NaCl growth ranges Genome phylogeny Ortho ANI dDDH API 20, API 20NE, and API ZYM Growth on NA, TSA, R2A agars	PhD candidate
Scanning electron microscope image	Eötvös Loránd University (ELTE)
MALDI-TOF MS:	WESSLING Hungary Kft
Fatty acid profile Polar lipid respiratory quinones	German Collection of Microorganisms and Cell Cultures GmbH (DSMZ)

Supplementary Table (2) water quality data for sampling lake (Vácszentlászló)

Parameters	Measurement Unit	Value
pH		8.38
Electrical conductivity	µS/cm	1080
Nitrate	mg/dm^3	<5
Nitrite	mg/dm^3	0.14
Ammonium	mg/dm^3	0.71
Carbonate	mg/dm^3	12
Bicarbonate	mg/dm^3	519
Orthophosphate	mg/dm^3	0.61
Total hardness	$mgCaO/dm^3$	298
Total suspended solids	mg/dm^3	11
Alkalinity	$mmol/dm^3$	8.9

Supplementary Table (3) Community composition and bacterial diversity of the investigated samples based on the Illumina 16S rRNA gene amplicon sequencing dataset

	VMP 1	VMP 2	VMP 3	VLW 1	VLW 2	VLW 2
Initial sequence read numbers	29718	28837	28453	30742	29366	29386
Sequence read numbers after streaming						
Coverage	0.999865	0.999861	0.999930	0.999902	0.999830	0.999660
Shannon diversity_*H*	2.027082	3.449380	3.934962	0.727749	2.049799	2.752970
A Taxonomic composition at the CLASS level (relative abundance, %)						
Acidimicrobiia	0.21%	0.31%	0.28%	0.24%	0.47%	0.55%
Actinobacteria	1.60%	1.81%	1.10%	0.53%	1.81%	1.69%
Bacteroidia	1.97%	20.62%	54.25%	1.58%	8.51%	16.15%
Anaerolineae	1.43%	0.36%	0.05%	0.82%	0.97%	0.21%
Oxyphotobacteria	69.14%	31.30%	1.21%	90.22%	69.67%	52.91%
Planctomycetacia	3.18%	5.04%	6.38%	1.22%	2.63%	5.04%
Alphaproteobacteria	5.44%	9.93%	11.36%	0.72%	2.95%	6.70%
Gammaproteobacteria	10.85%	23.08%	20.53%	1.40%	5.46%	11.07%
Verrucomicrobiae	3.64%	4.15%	2.28%	1.19%	3.04%	2.41%
A Taxonomic composition at the order level (relative abundance, %)						
Microtrichales	0.20%	0.31%	0.28%	0.24%	0.47%	0.55%
Flavobacteriales	1.38%	15.26%	33.21%	0.06%	3.40%	9.20%
Caldilineales	1.40%	0.35%	0.03%	0.82%	0.97%	0.20%
Nostocales	68.10%	30.96%	1.21%	89.90%	69.31%	52.86%
Isosphaerales	1.20%	2.95%	5.25%	0.69%	1.25%	3.71%
Pirellulales	1.06%	0.71%	0.17%	0.21%	0.49%	0.43%
Rhizobiales	1.65%	1.66%	1.45%	0.27%	0.70%	0.45%

Rhodobacterales	3.19%	6.90%	5.34%	0.08%	1.49%	4.23%
Alteromonadales	1.57%	1.60%	0.05%	0.00%	0.01%	0.01%
Betaproteobacteriales	8.26%	19.52%	17.20%	0.97%	3.92%	8.85%
Chthoniobacterales	2.07%	1.89%	0.76%	0.22%	1.26%	1.10%
A Taxonomic composition at the family level (relative abundance, %)						
Ilumatobacteraceae	0.17%	0.27%	0.26%	0.24%	0.44%	0.53%
Flavobacteriaceae	1.35%	15.16%	33.10%	0.04%	3.30%	9.06%
Caldilineaceae	1.40%	0.35%	0.03%	0.82%	0.97%	0.20%
Phormidiaceae	68.00%	30.84%	1.18%	89.90%	69.26%	52.77%
Isosphaeraceae	1.20%	2.95%	5.25%	0.69%	1.25%	3.71%
Pirellulaceae	1.06%	0.71%	0.17%	0.21%	0.49%	0.43%
Beijerinckiaceae	1.02%	0.61%	0.08%	0.20%	0.41%	0.11%
Rhodobacteraceae	3.19%	6.90%	5.34%	0.08%	1.49%	4.23%
Alteromonadaceae	1.57%	1.60%	0.05%	0.00%	0.01%	0.00%
Burkholderiaceae	7.91%	18.81%	15.32%	0.86%	3.58%	7.61%
Chthoniobacteraceae	1.83%	1.80%	0.63%	0.20%	1.19%	0.93%
A Taxonomic composition at the Genus level (relative abundance, %)						
CL500-29_marine_group	0.13%	0.27%	0.26%	0.24%	0.41%	0.52%
Flavobacterium	1.32%	14.98%	32.72%	0.04%	3.19%	8.85%
uncultured	1.40%	0.35%	0.03%	0.82%	0.97%	0.20%
Planktothrix_NIVA-CYA_15	68.00%	30.84%	1.18%	89.90%	69.26%	52.77%
uncultured	1.20%	2.95%	5.25%	0.69%	1.25%	3.71%
Pseudorhodobacter	1.23%	3.64%	2.90%	0.01%	0.45%	0.89%
Rhodobacteraceae_unclassified	1.93%	3.15%	2.29%	0.08%	0.99%	3.05%
Rheinheimera	1.54%	1.60%	0.05%	0.00%	0.01%	0.00%
Burkholderiaceae_unclassified	3.83%	11.19%	4.95%	0.07%	0.88%	1.06%
Leptothrix	1.32%	1.27%	0.79%	0.23%	0.60%	0.50%
Rhodoferax	1.27%	3.90%	6.28%	0.01%	1.06%	3.87%
LD29	1.82%	1.80%	0.63%	0.20%	1.19%	0.93%
A Taxonomic composition at the species level (relative abundance, %)						
Planktothrix rubescens v agardhii	68.1%	30.8%	1.2%	90.0%	69.2%	52.8%
Flavobacterium lacus	0.3%	3.9%	13.5%	0.0%	1.2%	1.7%
Flavobacterium chungnamense v koreense	0.4%	6.5%	9.6%	0.0%	1.2%	2.6%
Sphaerotilus montanus	3.2%	9.7%	3.1%	0.0%	0.6%	0.4%
Tundrisphaera lichenicola	1.0%	2.8%	5.2%	0.7%	1.2%	3.7%
Albidiferax ferrireducens	0.7%	2.8%	4.9%	0.0%	0.7%	2.9%
Gemmobacter tilapiae v. Tabrizicola sediminis v. alkalilacus	1.2%	3.6%	2.9%	0.0%	0.5%	0.9%
Terrimicrobium sacchariphilum	1.8%	1.8%	0.6%	0.2%	1.2%	0.9%
Flavobacterium buctense	0.1%	1.9%	4.0%	0.0%	0.3%	0.4%
Algoriphagus marisflavi	0.0%	1.0%	3.1%	0.0%	0.3%	1.9%
Lacihabitans soyangensis	0.0%	0.8%	4.0%	0.0%	0.3%	0.5%
Flavobacterium psychrolimnae	0.2%	0.7%	1.2%	0.0%	0.3%	2.6%
Lewinella xylanilytica v maritima	0.0%	0.3%	3.6%	0.0%	0.3%	0.7%

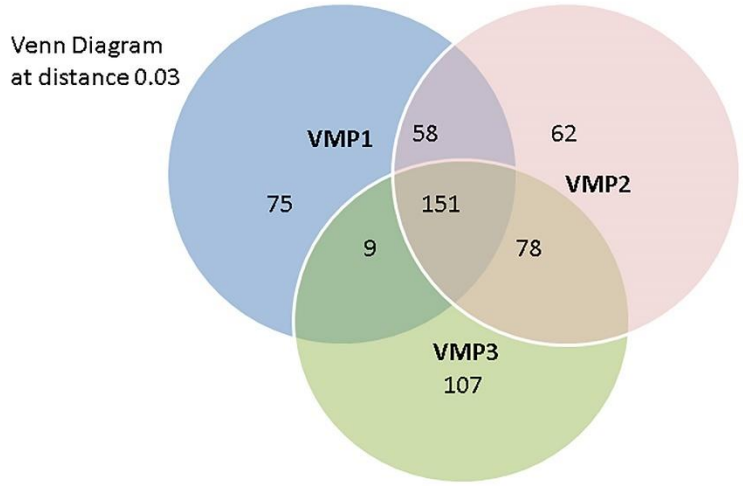
Mycobacterium interjectum v paraense	0.5%	1.1%	0.8%	0.3%	0.8%	1.1%
Algisphaera agarilytica	0.3%	0.8%	0.4%	0.4%	1.5%	1.0%
Rhodobacter thermarum	0.6%	1.7%	0.6%	0.0%	0.4%	1.1%
Tabrizicola aquatica	0.6%	0.6%	0.8%	0.1%	0.5%	1.6%
Lewinella nigricans	0.1%	0.2%	0.2%	0.6%	1.7%	1.0%
Fuerstia marisgermanicae	0.6%	1.0%	0.9%	0.2%	0.6%	0.8%
Luteolibacter algae	0.5%	1.2%	0.9%	0.0%	0.7%	0.5%
Hydrogenophaga taeniospiralis	0.5%	0.82%	1.60%	0.26%	0.21%	0.32%
Flavobacterium aquatile	0.1%	0.76%	2.44%	0.00%	0.07%	0.20%
Leptothrix cholodnii	1.1%	0.84%	0.30%	0.23%	0.50%	0.15%
Rhodoferax fermentans	0.3%	0.58%	1.06%	0.01%	0.22%	0.58%
Sphingorhabdus rigui	0.1%	0.23%	1.67%	0.00%	0.07%	0.20%
Nannocystis exedens	0.1%	0.27%	0.45%	0.00%	0.35%	1.03%

Supplementary table (4) Results of API ZYM, API 20E, and API 20NE for the novel species *Parvularcula mediterranea* and the closest relative *Parvularcula lutaonensis*

API ZYM	**Parvularcula mediterranea ZS-1/4**	***Parvularcula lutaonensis***
alkaline phosphatase	+ (5)	+ (5)
esterase c4	+ (3)	+ (3)
Esterase lipase	+ (3)	+ (3)
Lipase	- (2)	- (0)
Leucine arylamidase	+ (5)	+ (5)
Valine arylamidas	- (2)	+ (3)
cystien arylamidase	- (2)	+ (3)
trypsin	+ (5)	+ (5)
α-Chymotrypsin	+ (5)	+ (5)
acid phosphatase	+ (3)	+ (4)
Naphthol-AS-BI-phosphohydrolase	+ (4)	+ (5)
alfa galactosidase	- (0)	- (0)
B galactosidase	- (0)	+ (5)
B glucoronidase	- (0)	- (0)
Alfa-glucosidase	- (0)	+ (5)
B glucosidase	+ (5)	+ (3)
n-Acetyl-b-glucosaminidase	- (0)	- (0)
α-Mannosidase	- (0)	- (0)
α -Fucosidase	- (0)	- (0)
API 20 E		
ONPG	+	+
ADH	-	-
LDC	-	-
ODC	-	-
CIT	-	-
H2S	-	-
URE	-	-
TDA	-	-
IND	-	-
VP	-	-

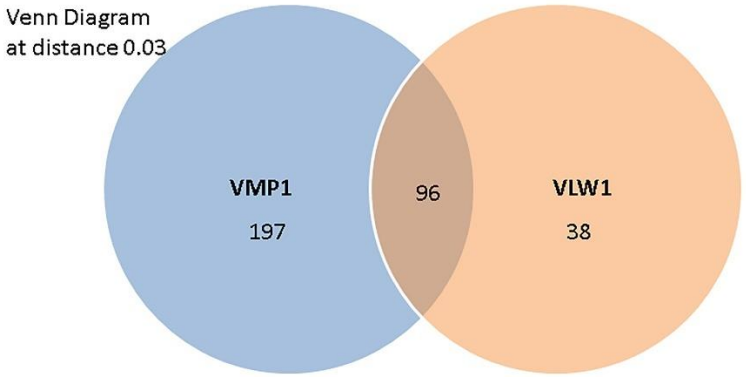
GEL	+	-
GLU	-	-
MAN	-	-
INO	-	-
SOR	-	-
RHA	-	-
SAC	-	-
MEL	-	-
AMY	-	-
ARA	-	-
API 20 NE		
NO3	-	-
TRP	-	-
GLU	-	-
ADH	-	-
URE	-	-
ESC	+	+
GEL	-	+
PNG	+	+
GLU	-	-
ARA	-	-
MNE	-	-
MAN	-	-
NAG	-	-
MAL	-	-
GNT	-	-
CAP	-	-
ADI	-	-
MLT	-	-
CIT	-	-
PAC	-	-
OX	+	+

Supplementary figure 1



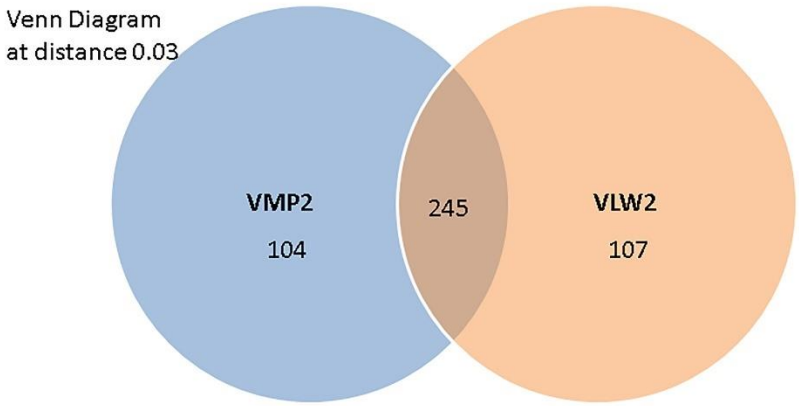
The number of species in group VMP1 is 293, and the number of sequences is 28453
The number of species in group VMP2 is 349, and the number of sequences is 28453
The number of species in group VMP3 is 345, and the number of sequences is 28453
The total richness of all the groups is 540
The number of species shared between groups VMP1 and VMP2 is 209, and the number of sequences is 55689
The number of species shared between groups VMP1 and VMP3 is 160, and the number of sequences is 51670
The number of species shared between groups VMP2 and VMP3 is 229, and the number of sequences is 55680
The total shared richness is 151, and the number of sequences is 78517

Supplementary figure 2



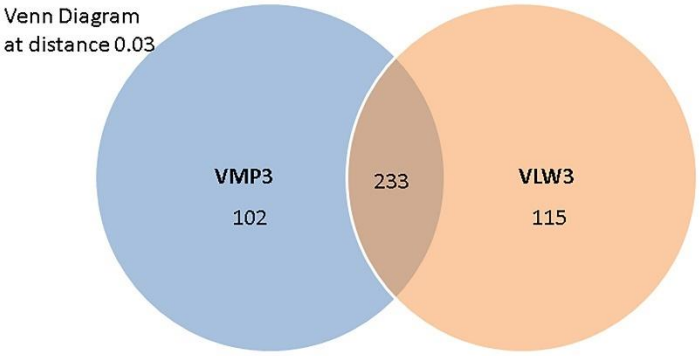
The number of species in group VMP1 is 293, and the number of squences is 28453; 2515 sequences are not shared
The number of species in group VLW1 is 134, and the number of sequences is 28453; 189 sequences are not shared
The number of species shared between groups VMP1 and VLW1 is 96, and the number of sequences is 54202; 95.2483% of these sequences are shared.
Percentage of species that are shared in groups VMP1 and VLW1 is 29.003
The total richness for all groups is 331

Supplementary figure 3



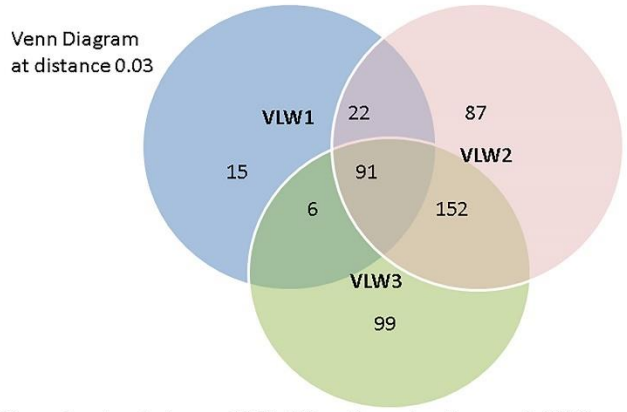
The number of species in group VMP2 is 349, and the number of squences is 28453; 1010 sequences are not shared
The number of species in group VLW2 is 352, and the number of squences is 28453; 402 sequences are not shared
The number of species shared between groups VMP2 and VLW2 is 245, and the number of squences is 55494; 97.5187% of these sequences are shared
Percentage of species that are shared in groups VMP2 and VLW2 is 53.7281
The total richness for all groups is 456

Supplementary figure 4



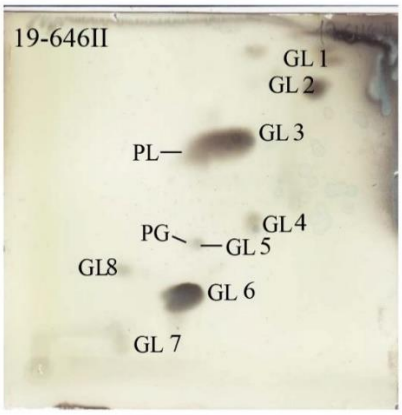
The number of species in group VMP3 is 345, and the number of squences is 28453; 494 sequences are not shared
The number of species in group VLW3 is 348, and the number of squences is 28453; 638 sequences are not shared
The number of species shared between groups VMP3 and VLW3 is 233, and the number of squences is 55774; 98.0108% of these sequences are shared
Percentage of species that are shared in groups VMP3 and VLW3 is 50.6522
The total richness for all groups is 460

Supplementary figure 5



The number of species in group VLW1 is 134, and the number of squences is 28453
The number of species in group VLW2 is 352, and the number of squences is 28453
The number of species in group VLW3 is 348, and the number of squences is 28453
The total richness of all the groups is 472
The number of species shared between groups VLW1 and VLW2 is 113, and the number of squences is 53908
The number of species shared between groups VLW1 and VLW3 is 97, and the number of squences is 49347
The number of species shared between groups VLW 2and VLW3 is 243, and the number of squences is 56008
The total shared richness is 91, and the number of squences is 74678

Supplementary figure 6: Two-dimensional TLC polar lipid images of strain ZS-1/3^T

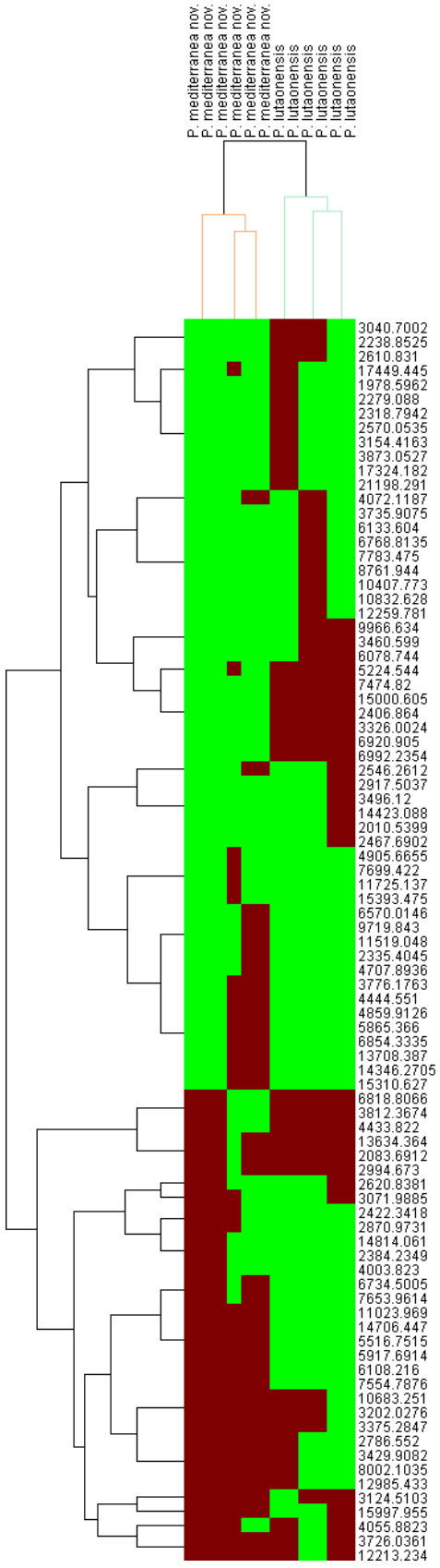


GL = Glycolipid

PL = Phospholipid

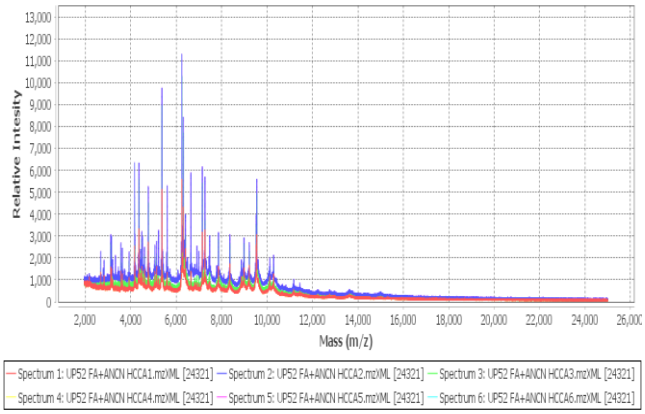
PG = Phosphatidylglycerol

Supplementary figure 7. A Dendrogram generated from hierarchical cluster analysis of MALDI-TOF mass spectra of *P. lutaonensis* and *P. mediterranea* nov. (6 replicates)

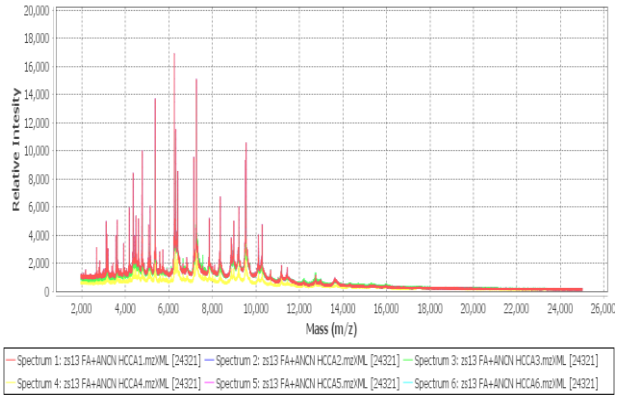


Supplementary figure 7. B *P. mediterranea* nov. and *P. lutaonensis* MALDI-TOF MS spectra (6 replicates)

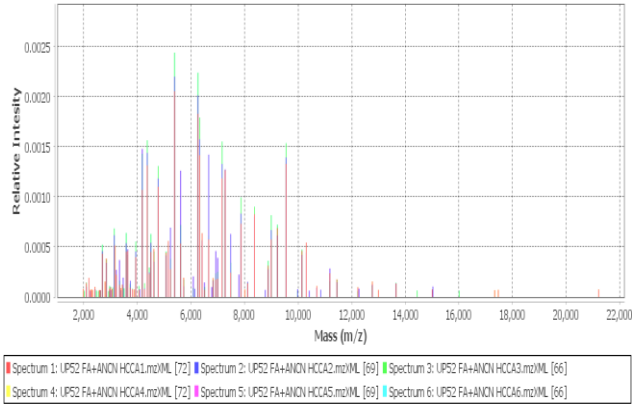
***P. lutaonensis* MALDI-TOF MS spectra (6 replicates)**



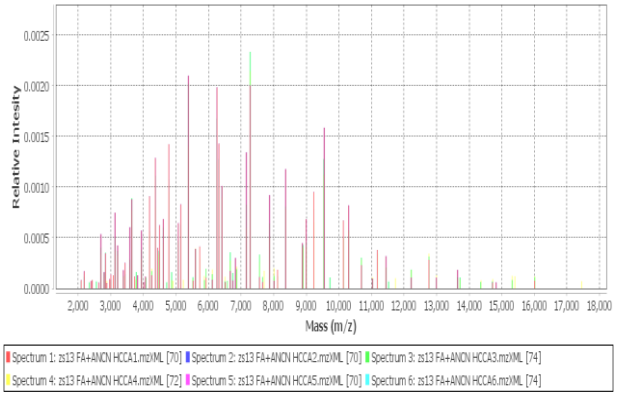
***P. mediterranea* nov. MALDI-TOF MS spectra (6 replicates)**



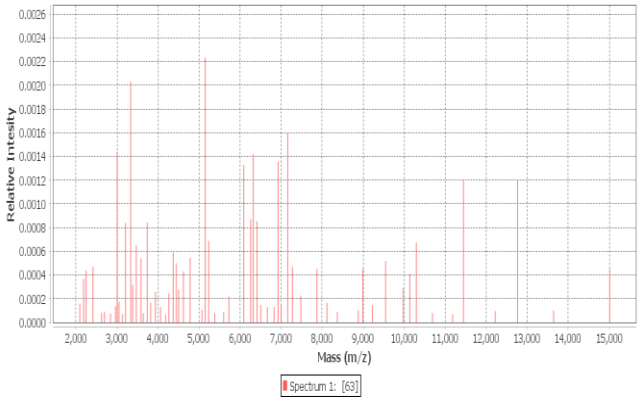
***P. lutaonensis* labeled peak list (6 replicates)**



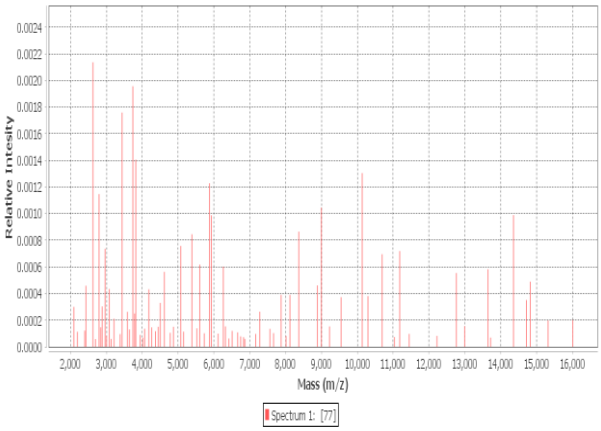
***P. mediterranea* nov. labeled peak list (6 replicates)**



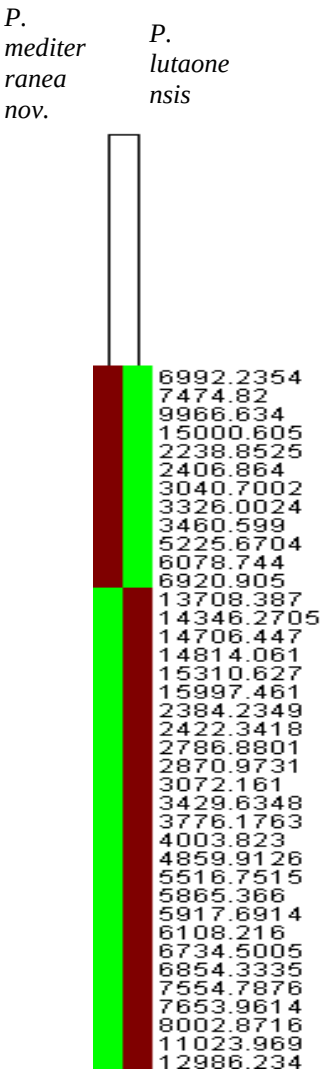
***P. lutaonensis* matched peak list set**



***P. mediterranea* nov. matched peak list set**



Supplementary figure 7.C Hierarchical clustering analysis of *P. lutaonensis* and *P. mediterranea* nov.



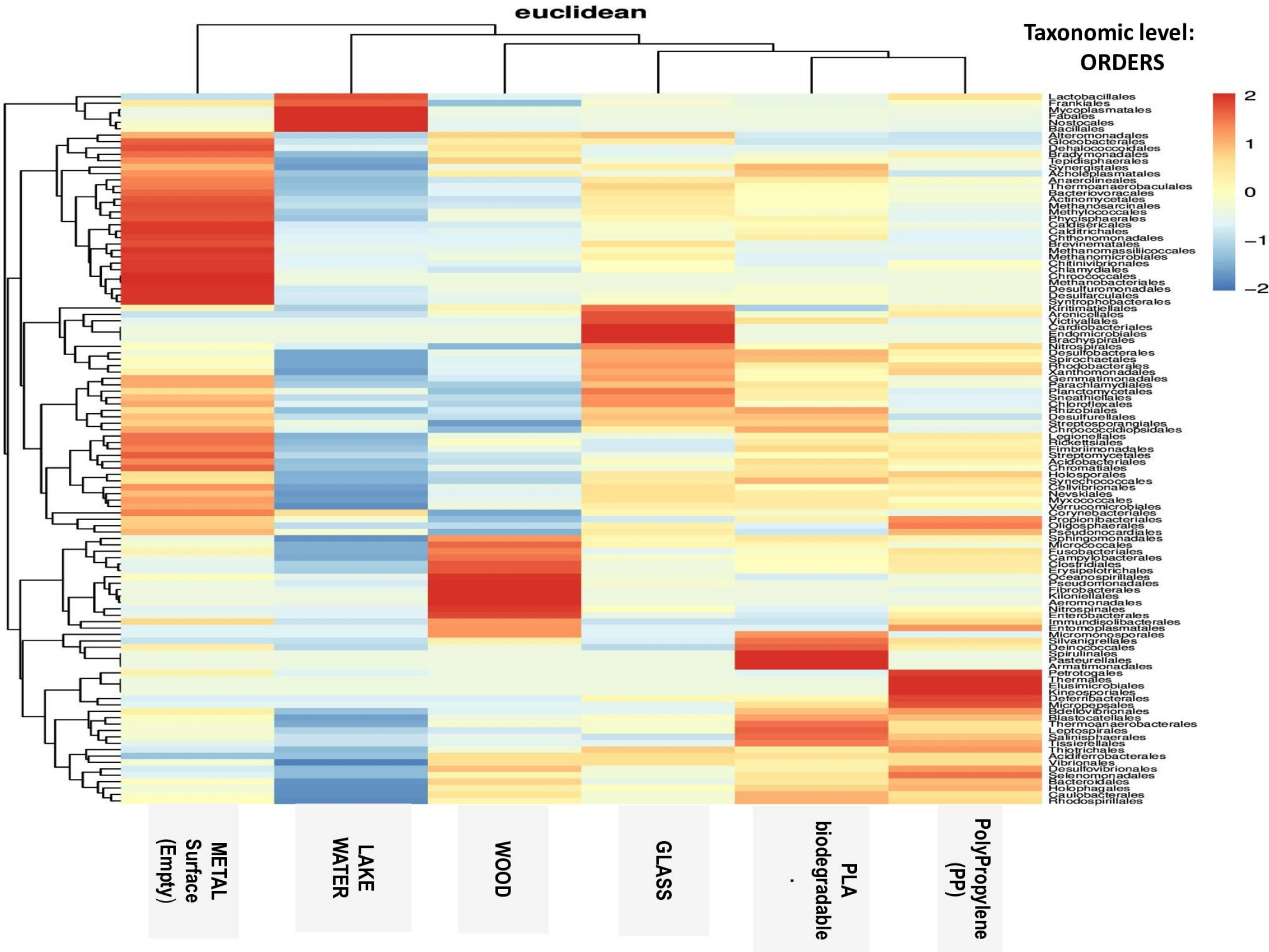


Figure 14 Heat-map of microbial community structure on the order level with relative abundance of more than 1%; Poly Propylene, degradable plastic, glass, wood, water, steel surface (empty colonizer) The color intensity in each panel shows the percentage in a sample, color key is at the right side.