Hungarian University of Agriculture and Life Sciences - MATE

Doctoral School of Environmental Sciences

ANALYSIS OF PLASTIC-ASSOCIATED BACTERIA IN FRESHWATER IN HUNGARY

Ph.D. Thesis

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1. Background

Plastics have appeared in the early 20th century; they were mainly used in military applications until the second world war. 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. 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. 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. It has also been reported that plastic pollution is responsible for the transfer of invasive species between ecosystems.

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 and some of others from micro and mesofauna. 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.

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. Different methods have been used for this purpose, such as; the collection of microplastic from sea water using a manta net, collecting plastic particles from the beach or shallow water, or incubating plastic particles in seawater under controlled lab conditions.

Freshwater ecosystems are considered the main destination of various pollutants including plastics. The reason is that freshwater bodies are usually located in valleys and low-lying areas. The occurrence of plastic waste in freshwater bodies significantly affects biodiversity and presents a serious threat to freshwater ecosystems.

However, limited data is available regarding microplastics in freshwater compared to marine water, especially in terms of the associated microbial communities. Therefore, I hope that this research work will help in filling the knowledge gap by investigating microplastic associated communities in freshwater.

2. Aims and scopes

The main objectives of this PhD thesis are:

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.

For the first and the second objectives a 'plastic colonizer' was designed from simple materials; stainless steel ball-shaped tea filter were sterilized and filled with polypropylene (PP) straws (<5mm in size) and attached to fishing line. It was then submerged below water surface of a freshwater lake near to the village of Vácszentlászló, Pest county in Hungary for three-months period. The PP associated bacterial communities were investigated using molecular techniques for both the PP and compared to surrounding water.

For the third objective a possible novel bacterial strain isolated from PP straws collected from marine water was described as per the "Notes on the characterization of prokaryote strains for taxonomic purposes" by Tindal *et al.*, 2013, using molecular, physiological, and morphological tests. However, this new isolate was originated from marine litter, but helped us to perform the above mentioned plastic colonizing method for isolation and analyzation of plastic associated bacterial communities in freshwater.

3. MATERIALS AND METHODOLOGY

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 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 aerobic 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 incubated at 28ºC for 72 hours. Colonies were selected randomly and subsequently purified twice on Marine agar medium at 28°C.

3.1.2 16*S* RNA phylogeny for species identification

In order to identify the bacterial isolates, DNA was isolated followed by 16S rRNA gene sequencing. 16S rRNA similarity-based searches was done against EzBioCloud's databases.

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 new study has been developed in Hungary. The study was conducted in a freshwater lake located near to the village of Vácszentlászló, Pest county (47°33’37.0”N 19°33’09.4”E). The lake is a shallow reservoir with an area of approx. 47 hectares and a mean depth of 2 m. The water in the lake is eutrophic, and the water level fluctuation is normally up to 1m. 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.

3.3 Plastic colonizers method and first colonization test

3.3.1 Design of plastic colonizers

Self-designed plastic colonizers were prepared as follows; commercially available stainless-steel ball-shaped tea filters 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 straw pieces cut into less than 5mm small pieces (see **Figure 1**). 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 **Figure 2**.

Figure 1a **Figure 1b**

Figure 1. Plastic colonizer design **a**: open plastic colonizers made from commercial stainless-steel filter filled with 3 grams of cut plastic straws. **b**: closed plastic colonizer filled with plastic straw pieces (<5mm)

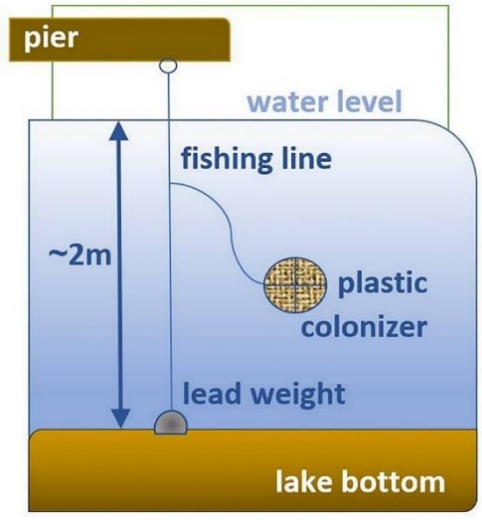

Figure 2. Installation of plastic colonizers by attachment to wooden pier and submerging 50 cm under water surface and fixed under water with lead weight (Figure by Istvan Szabó)

3.3.2 Recovery of microbial biofilm from plastic colonizers

The 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 the first month, two 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 (PP straw particles were mixed and 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 and identification of culturable bacteria on LB agar from plastic

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. The genomic DNA was then isolated and identified using 16SrRNA genes.

3.3.4 DNA Isolation of plastic-associated and lake water bacterial communities

After inoculating 1ml aliquots of the microplastic suspension onto LB agar, the remaining initial 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 ambient 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

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*.

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. (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 one empty (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 colonization test.

3.6 Statistical analysis

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

Testing the difference between microbial communities on microplastic surfaces compared to surrounding water is of high importance to measure the significance level of difference. Thus, a Paired Samples t-test using SPSS software was used to determine whether there are statistically significant differences between the two data sets.

3.6.2. Statistical analyses of bacterial communities from different materials from second colonization test

To identify similarities between microbial communities on microplastic surfaces and in the surrounding water, principal component analyses (PCA) were performed. 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) from the surface of the collected marine plastic litter in the initial study. For description of novel bacterial strains of possible novel species should be further tested and compared with the closest relatives using different phenotypic and genotypic aspects such as cell morphology, gram staining behavior, growth conditions, fatty acids analysis, polar lipids, respiratory quinones, motility, scanning electron microscope image, genome phylogeny, DNA-DNA hybridization, Average Nucleotide Identity, and gene content (Tindall et al., 2010). Besides these methods whole-cell matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was also performed for justification.

4. RESULTS AND DISCUSSIONS

4.1 Bacterial isolates from the initial study

Five bacterial strains 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). Among these isolates one was recognized as a possible new bacterial species (ZS-1/3). Accordingly, further genotypic and phenotypic analysis were conducted to describe it as novel bacterial species.

4.2 Results of the first colonization test

4.2.1 Bacterial isolates of LB agar from first colonization test

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**.

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.

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
PLA-26	*Bacillus megaterium*	February 2019	415	1
PLA-27	*Bacillus altitudinis*	February 2019	470	1
PLA-28	*Pseudomonas synxantha*	February 2019	603	1

IDs	Bacterial spp	Date	Length of the sequenced region of 16S (bp)	Risk group*
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

Table 1: Identified bacterial species isolated from microplastic surfaces from LB agar incubated aerobically at 28°C for 72h. * 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

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

According to the results of Illumina 16S rRNA gene amplicon sequencing, 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.

By the identification of OTUs, 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 3** compares the most abundant microbial phyla between microplastic surface samples and lake water samples.

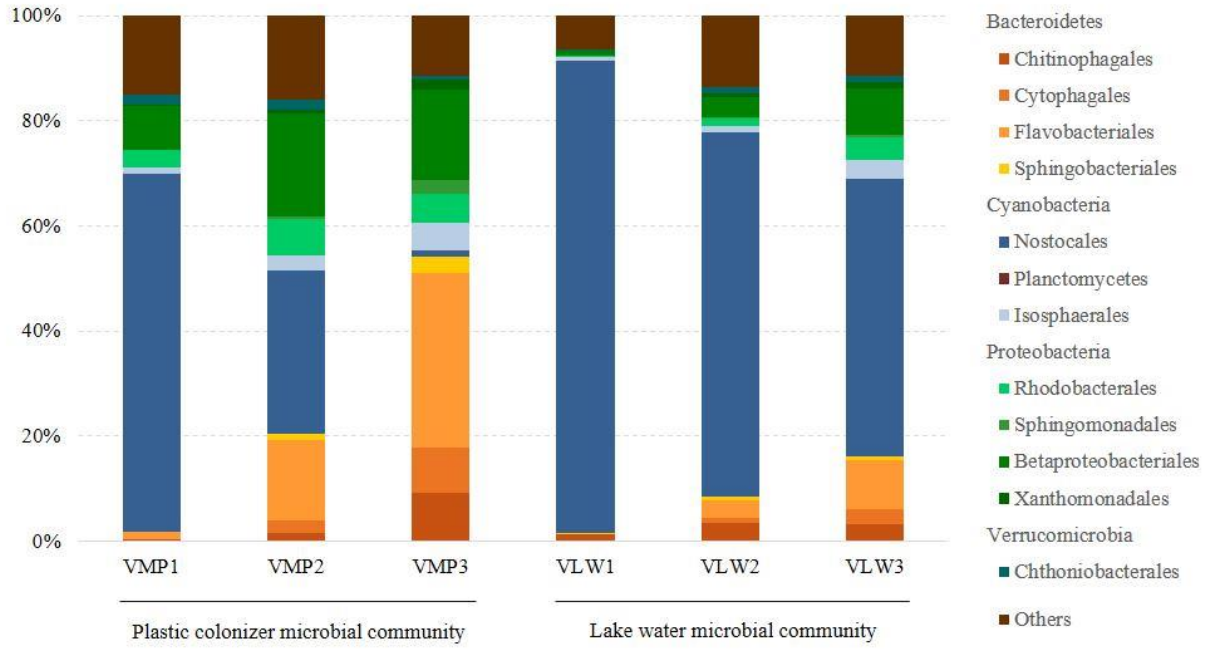

Figure 3. Dominant bacterial phyla and orders associated with microplastics and surrounding water samples.

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 (plastispheric) sample, followed by Burkholderiaceae in all microplastic originated 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 4**. 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.

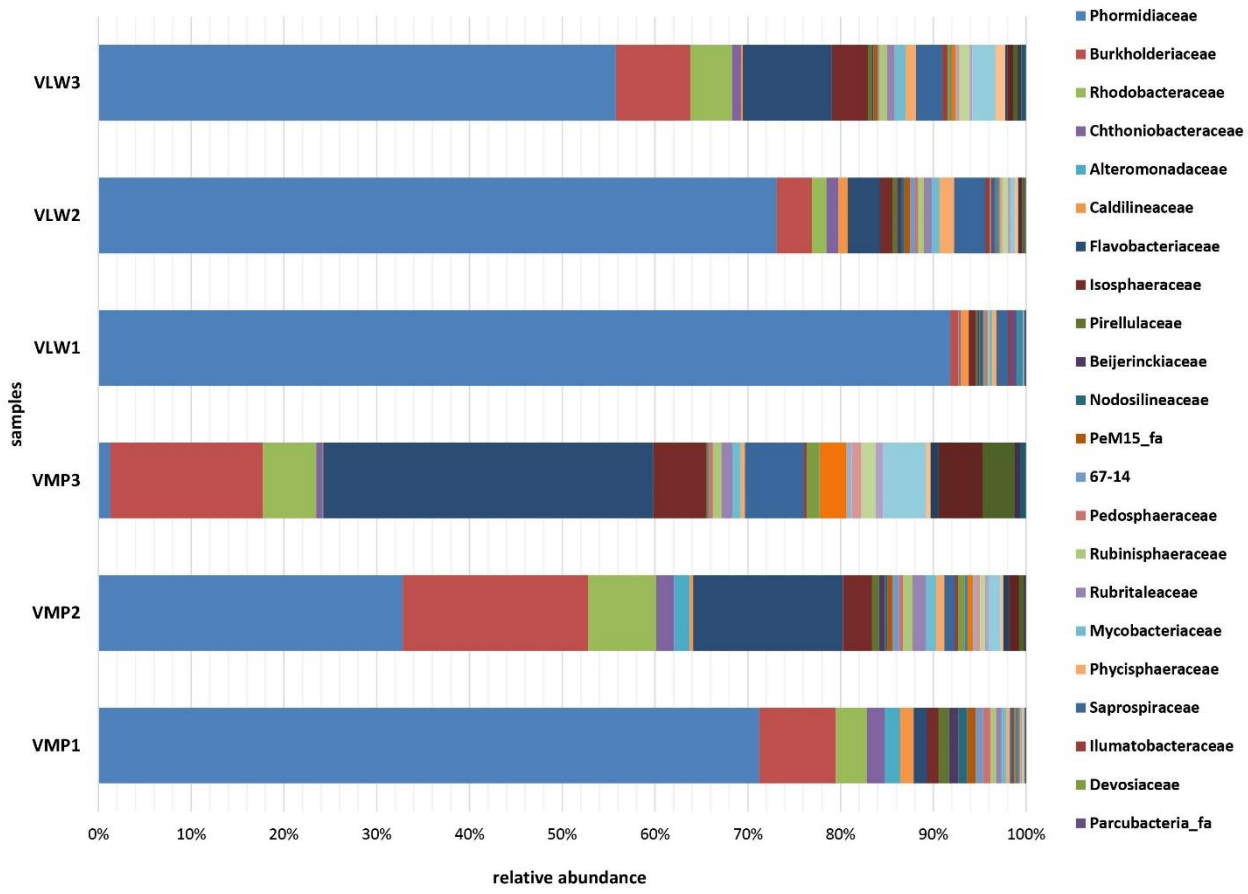

Figure 4. Dominant bacterial families associated with microplastics, and surrounding water based on relative abundance more than 1%; VMP1: microplastic from plastic colonizer, first month, VMP2: microplastic second month, VMP3: microplastic third month, VLW1: surrounding water first month, VLW2: surrounding water second month, VLW3: surrounding water third month

On the genus level, 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.

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 5**).

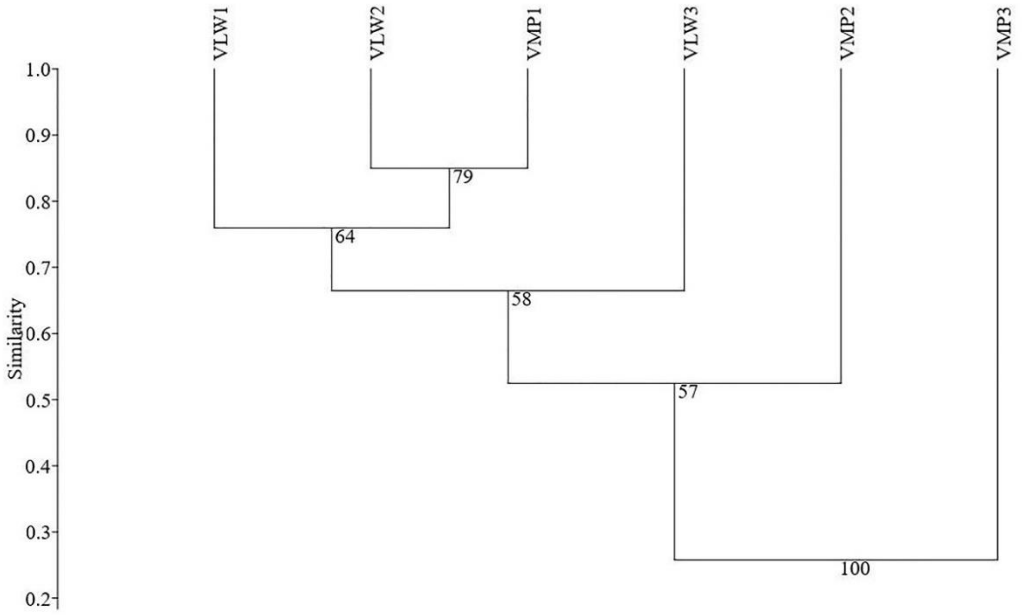

Figure 5. Clustering analysis of bacterial communities in the six samples (three microplastic and water) based on OTU abundance-based Bray-Curtis similarity coefficients; VMP1: microplastic associated community from plastic colonizers, first month, VMP2: microplastic second month, VMP3: microplastic 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 (group1) compared with surrounding water (group2) is highly significant (P-value is less than 0.05) when analyzing over the whole study period (P-Value = 0.000; see **Table 2**), So the zero hypothesis (H0): There is no (statistically) significant difference between the microbial community on microplastic surfaces compared to the surrounding water, was rejected.

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

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

Another hypothesis was tested again among the months 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 show a high significance again among December (P-Value = 0.000), January (P-Value = 0.010) and February (P-Value = 0.023) see **Table 3**. Thus the differences between these samples found also significant between the pairs from the same sampling months.

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. 2018	Equal variances assumed	42.326	.000	4.086	1322	.000	9.675	2.368	5.030	14.321
Jan. 2019	Equal variances assumed	19.670	.000	2.568	1322	.010	16.497	6.423	3.897	29.097
Feb. 2019	Equal variances assumed	17.938	.000	2.284	1322	.023	21.508	9.418	3.031	39.984

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))

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

The second colonization test was performed to probe the distinctness of bacterial communities associated to plastic surfaces from others of different materials. 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 6** below. By these results it is 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 especially in terms of relative abundance. Moreover, PP and PLA communities clustered closely to each other compared to other communities

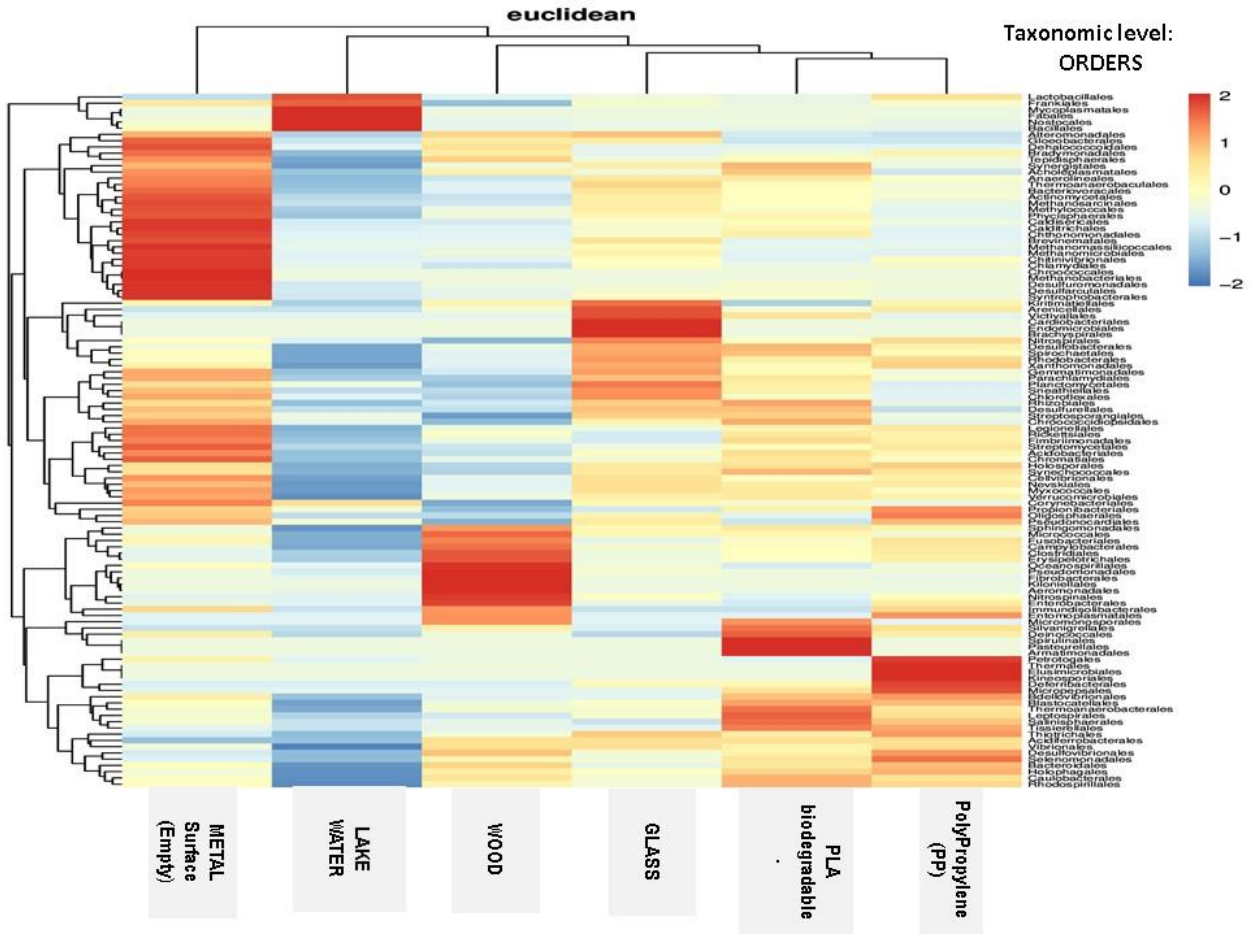

Figure 6. Heat-map of microbial community structure on the order level with relative abundance of more than 1%; Poly Propylene, degradable plastic, glass, wood, water, steel surface (empty colonizer) The color intensity in each panel shows the percentage in a sample, color key is at the right side.

4.4 Taxonomic description of the novel bacterial strain ZS-1/3^{T}

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, 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*. (see **Figures (7 and 8)** and **Tables (4 -6)** below. It was given the name *Parvularcula mediterranea* (me.di.ter.ra'ne.a. N.L. fem.adj. mediterranea pertaining to Mediterranean Sea, from where this strain was isolated). It is one of the rare species isolated from plastic surface.

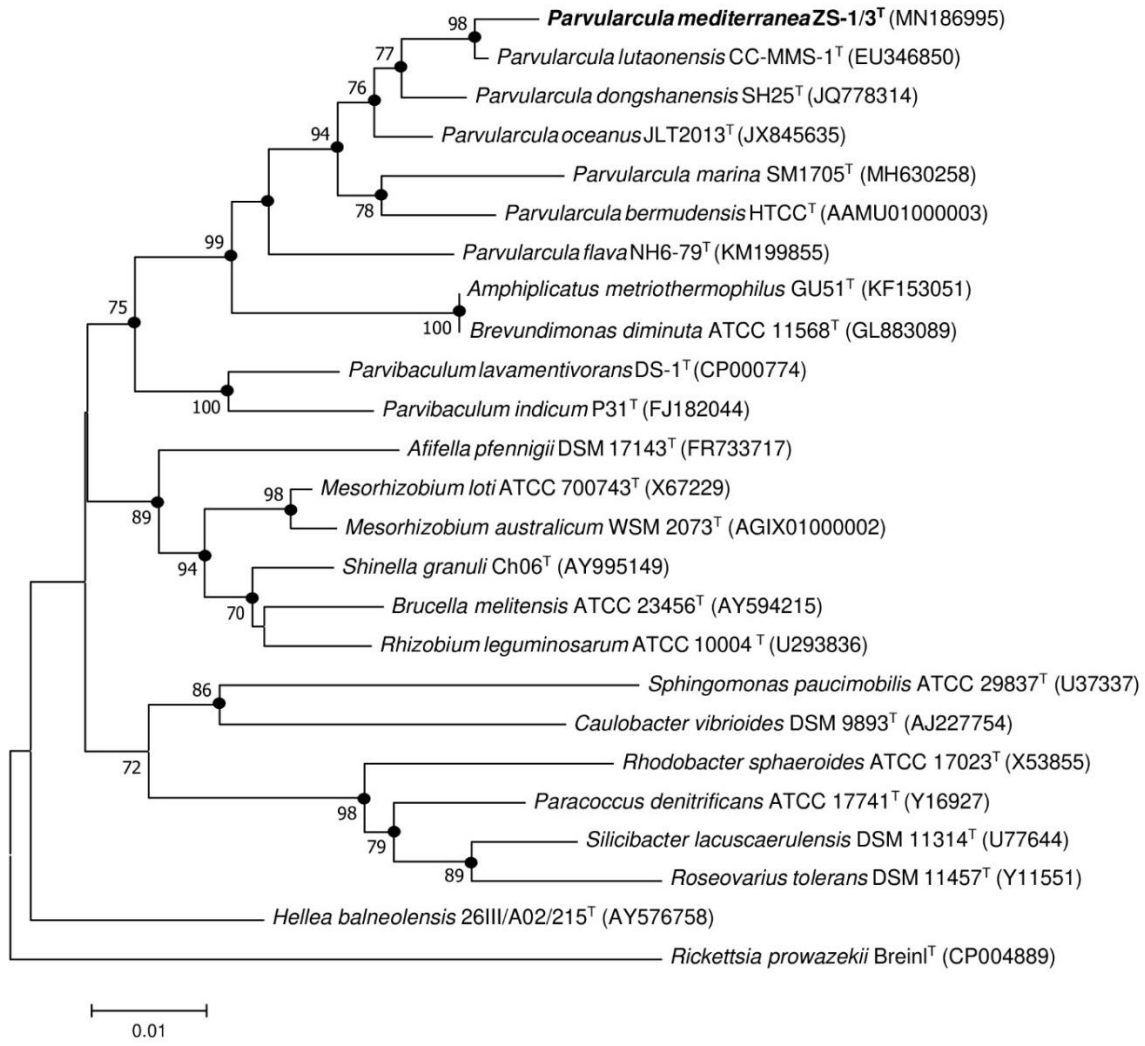

Figure 7. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic positions of strain ZS-1/3^T and related species.

Table 4 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

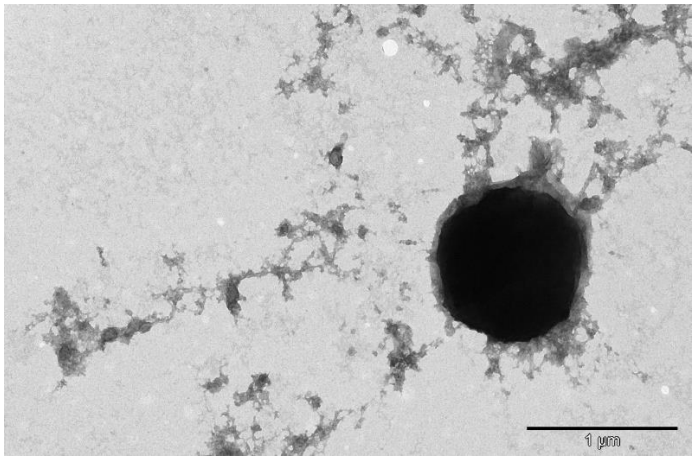

Figure 8. Shadow casting electron microscopic image of *Parvularcula mediterranea*

Table 5 Characteristics that differentiate strain ZS-1/3^T from the closest relative *P. lutaonensis* KCTC 22245^T

Characteristic	**ZS-1/3^T**	***Parvularcula lutaonensis* KCTC 22245^T**
Colony pigmentation	Orange	Orange
Growth temperature	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

Table 6 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.

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), the polar lipids of the closest relative *P. lutaonensis* consisted of four unknown phospholipids and four unknown glycolipids.

5. CONCLUSION AND RECOMMENDATIONS

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 environment. 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 reproducible and comparable method.

Our method was successfully used over the wintertime 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).

By statistical analyses of amplicon sequencing results of plastic associated microbial communities, it has been found that microplastics provide a unique ecological niche 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 waterborne communities also. However, PP associated communities were close to PLA (biodegradable) plastic.

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.

In line with that, a novel bacterial species was isolated from marine originated 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.

6. New scientific results

6.1 Bacterial strain ZS-1/3, isolated by us 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.

6.2 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.

6.3 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.

6.4 The results of 16s rDNA amplicon sequencing, by comparing the abundance of OTUs of samples from different materials, shows notable differences 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.

7. Publications

7.1 Al-Omari, J., Szabó, I., Szerdahelyi, G. S., Radó, J., Kaszab, E., Griffitts, J., Táncsics, A., & Szoboszlay, S. (2021). *Parvularcula mediterranea* sp. nov., isolated from marine plastic debris from Zakynthos Island, Greece. International Journal of Systematic and Evolutionary Microbiology, 71(1), 004608. Q1

7.2 Szabó, I., **Al-Omari**, J., Szerdahelyi, G. S., Farkas, M., Al-Omari, Y., Szabó, P. M., Sebők, R., Griffitts, J., Kriszt, B., & Szoboszlay, S. (2021). In Situ Investigation of Plastic-Associated Bacterial Communities in a Freshwater Lake of Hungary. Water, Air, & Soil Pollution, 232(12), 1-17. Q2

7.3 Szabó, I., **Al-Omari**, J., Szerdahelyi, G.S., Radó, J., Kaszab, E., Táncsics, A., Szoboszlay, S. and Kriszt, B., 2021. *Dyadobacter subterraneus* sp. nov., isolated from hydrocarbon-polluted groundwater from an oil refinery in Hungary. International Journal of Systematic and Evolutionary Microbiology, 71(7), p.004916 Q1

- Publication **7.3** does not belong to PhD research topic.

Cumulative impact factor (IF) of publications: 8.26