universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Characterization of the seasonal variability of the microbial community composition on plastics in the Northern Adriatic Sea“

verfasst von / submitted by

Paula Irene Polania-Zenner, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2018 / Vienna 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 833
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ecology and Ecosystems
Betreut von / Supervisor:	Univ.-Prof. Dr. Gerhard J. Herndl

Acknowledgements

I would like to express my gratitude to the Microbial Oceanography Department for all the support for this project and the Ruđer Bošković Institute in Rovinj.

Thanks to the group of MSc students (Gabriela, Christian, Julie, Meriel and Teresa) and the people who have helped me out with their abilities as well as for the good time at the University.

Special thanks to my family in my country and my "Austrian" family for providing me with unconditional support and continuous encouragement throughout this study.

To my supervisor Gerhard J. Herndl for giving me the opportunity to work in his group and on this project, for the knowledge and the support provided. Without him it would have not been possible to reach this point in my studies.

My special gratitude and thanks goes to my co-supervisor Maria Pinto for sharing her knowledge and expertise, her guidance and support made it possible for me to work on this interesting topic.

Thank you!

Abstract

The amount of marine plastic debris in the ocean is increasing globally and its influence on marine organisms, such as mammals, birds and fish has received increasing attention lately. There are still much unknown aspects regarding the impact of plastics in the environment and the effects on lower trophic level marine organisms. The interaction between the microbial biofilm community associated with plastic and its role in the biogeochemistry of the ocean is largely unknown and was focus of this thesis work. Different types of marine plastics (polypropylene [PP], polyethylene [PE] and polyethylene terephthalate [PET]) and of various sizes (> 9.5 mm, 9.5 -4.75 mm; 4.75– 1.4 mm; 1.4 mm-500µm) were collected in the northern Adriatic Sea in February, August and November, to determine whether the composition of the microbial community attached to the plastics differs from that of their free-living counterpart. Most of the plastic pieces collected in this study were of small size (microplastic) and colonized by microbes. All size classes and all types of marine plastics were colonized by microbes throughout the seasonal cycle.

The microbial community composition of the plastic types differed from that of the ambient water. The community bacterial community composition of PE and PP was similar to each other. Overall, the microbial communities of the Northern Adriatic Sea did not show any preferences to specific plastic types and sizes. This study suggests that plastic provides a new spatial habitat for the microbial community in the ocean. Abundant plastic-attached taxa belong to the phyla Proteobacteria, Cyanobacteria, Bacteriodetes and Planctomycetes. We identified some member of the Alteromonadaceae, Oscillatoriaceae, Flavobacteriaceae, Saprospiraceae, Rhodobacteraceae families known to degrade hydrocarbons and show a preferential particle –associated life style. Taken together, we conclude that the plastic-associated microbial community differs from that of the free-living community and harbors bacteria groups tentatively capable of utilizing hydrocarbons. The extent to which marine plastics can be degraded by members of the plastic-associated bacterial community remains to be investigated.

Keywords: Marine plastic debris, Bacteria, Community composition, Northern Adriatic Sea.

Introduction

Plastic pollution represents an environmental threat in all the oceans and became an increasing concern over the last decade because of its possible negative impacts on the marine food web. Through physical, chemical and biological degradation, plastic is fragmented and dispersed throughout the oceans by currents and winds and concentrating it mainly in the subtropical gyres (Lebreton et al. 2012; Eriksen et al. 2014; Shah et al. 2008). Eriksen et al. (2014) suggested that a minimum of 233,400 tons of large plastic is floating in the oceans compared to 35,540 tons of microplastics. Previously, Eriksen et al. (2013) reported 26,898 particles/km² in the size range between 0.355 mm to 4.750 mm in the south Pacific subtropical gyre. Moore et al. (2001) reported also smaller plastic size categories (< 4.760mm) for the North Pacific central gyre and by multicellular fouling organisms. The abundance of 334,271 pieces/km² and the mass of 5,114 g/km² of neustonic plastic was the largest recorded for the North Pacific central gyre (Moore et al. 2001). PlasticEurope (2016) reports that in the year 2015, 322 million tons of plastics have been produced annually. For the Mediterranean Sea, Cózar et al. (2015) showed that there are major accumulation zones of plastic debris with plastic concentrations comparable to that in the five subtropical ocean gyres. Suaria et al. (2016) demonstrated that microplastics abundance in the Mediterranean Sea is among the highest in the world's ocean.

Many different types of plastics are being produced. Plastics are synthetic or semi-synthetic organic polymers that are cheap, lightweight, strong, durable and corrosion resistant (Derraik 2002). Many common classes of plastic are composed of long-chain hydrocarbons (American Chemistry Council 2015). Depending on the plastic composition, its density and its shape, it might be positively buoyant and dispersed over long distances (Hansen 1990). PE is the most commonly produced plastic worldwide (Lee et al. 1991). It is one of the most inert synthetic polymers and one of the most resistant to microbial attack (Orr et al. 2004). The distribution of plastics in the water column depends on its specific density; plastics with a specific density >1, like polystyrene (PS), polyethylene terephthalate (PET) and polyvinyl chloride (PVC) are

usually found at and in the sediments; while lower density plastics (<1), such as low-density polyethylene (LDPE), polyethylene (PE) and polypropylene (PP) are mostly found in surface waters (Moret-Ferguson et al. 2010). Plastic debris is divided into two categories, large plastics (> 5mm) known as macroplastics and small plastics, coined microplastics (< 5mm) (Derraik 2002; Moore 2008). Primary microplastics are deliberately manufactured as microplastics (Cole et al. 2011). Most primary microplastics are generated for cosmetics, clothing and industrial and domestic purposes (Moore 2008). Secondary microplastics are microscopic breakdown products of larger debris (Ryan et al. 2009). Both types are recognized to persist in the environment at high concentrations, particularly in aquatic and marine ecosystems (Browne et al. 2015).

Biofouling, which is the establishment of a biofilm on a solid surface, increases the specific density of the plastic resulting in a sinking of plastics though the water column (Andrady 2011; Moret-Ferguson et al. 2010). Within the biofilm, bacteria constitute a major fraction of biomass. Orr et al. (2004) found that *Rodococcus ruber* utilized polyethylene films as sole carbon source. In their study, bacteria formed a biofilm on the polyethylene surface and degraded up to 8% of the polyolefiln within 30 days.

Polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene tetraphthalate (PET) and polyvinyl chloride (PVC) are used for packaging; the exact amount of this plastics ending up in the ocean is unknown (Andrady 2011).

It is known from many studies that the input of plastic debris from land to the ocean increased over the years (Jambeck et al. 2015; Law et al. 2010). Land-based sources contribute about 80% of the plastic debris in the ocean (Derraik 2002). Plastic waste is transported by river systems and waste treatment systems into the marine environment (Cole et al. 2011; LI et al. 2016). Plastics in the ocean form a habitat for specific microbes, particularly for those with a preference to have a particle-attached life mode. Figure 1 illustrates the heterogeneity of a plastic-associated biofilm. The first report about the colonization of plastics by microorganisms is from Carpenter & Smith (1972). Microbial biofilms develop rapidly on submerged plastic, concomitant with

significant changes in the physicochemical properties of the plastic (Lobelle & Cunliffe 2011). Lobelle & Cunliffe (2011) reported that the biofilm was visible after one week, and they found a significant increase in microbial abundance over the course of the experiment. It is known that biofilm formation is an important mechanism for survival of marine bacteria in oligotrophic environments (Jefferson 2004). Marine biofilms are composed of complex microbial communities with a plethora of interactions among their individual members (Dang & Lovell 2000).

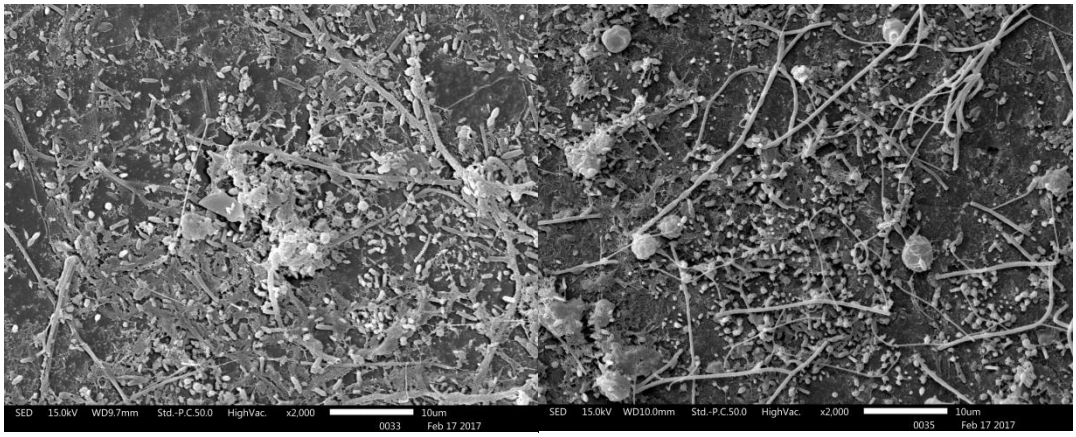

Figure 1. SEM Scanning electron micrograph of the biofilm on plastic collected in the Northern Adriatic Sea off Rovinj (Croatia). Plastic sample P.88 (left) and P. 232 (right) collected during the summer.

Hydrocarbons are organic compounds consisting entirely of hydrogen and carbon and can be naturally found in crude oil. It is known that bacteria have acquired the capacity to utilize hydrocarbons as source of carbon and energy (Oberbeckmann et al. 2016; 2014; Zettler et al. 2013). Many microbes are known to have developed metabolic pathways to degrade hydrocarbons, some to the extent that they thrive only in the present of crude oil components (Chronopoulou et al. 2015; Hazen et al. 2010; Dubinsky et al. 2013). Yakimov et al. (2007) found several bacteria that are even known to feed on hydrocarbon, and that play a significant role in the biological removal of petroleum from polluted marine waters. Floodgate (1984) listed 25 genera of hydrocarbon degrading bacteria which were isolated from marine environments.

Seasonal and spatial factors shape the microbial community composition associated with different plastic types in marine environments. Oberbeckmann et al. (2014) demonstrated that there is considerable variability in the distribution of

prokaryotic and eukaryotic communities along spatial and temporal gradients on PET fragments in the North Sea. Zettler et al. (2013) found different microbial assemblages colonizing PE and PP in the North Atlantic subtropical gyre. Bryant et al. (2016) observed that microplastics create a habitat for metabolically active and net autotrophic communities and identified fundamental differences in the functional composition of the microorganisms associated to the plastic in comparison to their free-living counterparts in the ambient water in the North Pacific subtropical gyre.

In this study I aimed at characterizing the microbial community composition of the biofilm in different types and sizes of plastics collected in three different months in the Northern Adriatic Sea. I hypothesize that the microbial community will be *(i)* different between the free-living and the plastic-attached community, *(ii)* change between different plastic types and sizes (bigger plastics have a more diverse community than smaller plastics) and *(iii)* more stable in the plastic-associated biofilm than in the ambient water over the different seasons.

Material and Methods

Collecting microplastics

Floating plastics were collected in the Northern Adriatic Sea off the coast of Rovinj (Croatia), 0.5 km to 1 km off the coast during February, July-August and November 2016.

Sampling of the plastic debris was performed using two different nets. In February, plastics were collected with a plankton net with 200 µm mesh size (2 tows for 10 min). A microplastic net with 500 µm mesh size was used in July- August and November (1 tow for 30 min). In both cases the net was towed at the surface collecting all material which was floating in the sea surface layer. The microplastic net had a 1 m wide opening and was 0.8 m long. The collected material was kept in ambient seawater until processing at the Ruđer Bošković Institute in Rovinj. In the laboratory, the samples were separated into four size classes by passing them through a series of

four sieves with mesh sizes of 9.5 mm, 4.75mm, 1.4mm and 300 µm. The samples were rinsed in the sieves with fresh 0.2 µm filtered seawater and transferred into petri dishes with filtered water. Plastic pieces were immediately sorted from the rest of the collected material and rinsed 3 times with filtered seawater. Samples for DNA extraction were stored at -80°C. Samples for scanning electron microscopy (SEM) were fixed in 2% glutaraldehyde (final conc.), and for fluorescence in situ hybridization (FISH) in 2% formaldehyde (final conc.) and also stored at -80°C.

In this study, we defined four plastic size classes according to the mesh size of the sieves: >9.5 mm; 9.5-4.75 mm; 4.75–1.4 mm; 1.4 mm-500 µm.

To compare the microbial community composition of the plastic biofilm and the ambient water, surface water samples were collected in each of the seasons as well. The water was filtered through 0.2 µm polycarbonate filters and the filter stored frozen at -80°C until further processing.

Bacterial community composition

To characterize the microbial community composition attached to the plastics, DNA was extracted with the Puregene Tissue DNA extraction Kit (Qiagen, Valencia, CA) using a modified bead-beating approach adapted from Zettler et al. (2013). Further details on the DNA extraction protocol are given in Supplement A. To identify the plastic type, the extracted plastic was kept frozen at -20°C for Raman spectroscopy.

A total of 41 plastic samples and three ambient water samples were selected for sequencing of the 16S rRNA gene (Table 1).

PCRs were performed to amplify the V4 region of the 16S rRNA gene (~460 bp fragments) using the primer pair 341F_ill (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG **CCTACG GGNGGCWGCAG**) and 802R_ill (5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAC **TA CHVGGGTATCTAATCC**) with adapters on a Mastercycler (Eppendorf). The PCR protocol was realized with an

initial denaturation of 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and the final elongation at 72°C for 30 sec.

The amplification of the gene was checked on a 1% agarose gel electrophoresis (1X TBE buffer) at 100V for 30 min and stained with SYBR Gold for 20 min. The visualization of the gel was carried out using the program Quantity One* in the Bio-Rad Chemi Doc imaging system.

After a positive amplification of the gene with the 30 PCR cycles, another PCR with 20 cycles using the same primer pair 341F_ill and 802R_ill was performed for subsequent amplicon next generation sequencing. The PCR protocol was performed with an initial denaturation at 95°C for 3 min, followed by 20 cycles at 98°C for 20 sec, 56°C for 30 sec and 72°C for 30 sec, and the final elongation at 72°C for 5 min (Mastercycler Eppendorf). DNA concentrations were quantified with the Quant-iT™ PicoGreen® Assay Molecular Probes/Invitrogen (http://probes.invitrogen.com/media/pis/mp07581.pdf).

The Agencourt AMPure XP PCR protocol was used to purify the PCR products. (www.beckmancoulter.com/customersupport/support. © 2016 Beckman Coulter, Inc.). The purified PCR products were sequenced at Microsynth AG Laboratories. Sequencing of paired-end reads was carried out on an Illumina MiSeq, v2, 2x250 bp reads. Samples with less than 5,000 reads were not included in the analysis. The transformation of the reads into OTUs was done with UPARSE OTU clustering (UPARSE-OTU algorithm) using the USEARCH tool Kit (Edgar 2013). The taxonomic affiliation was added to the OTU table with BLAST N (Basic Local Alignment Search Tool). A python script was used for this step. Libraries were constructed with the SSURef_123_ SILVA database. A quality filtering was done following the normal directives in the Fast Q file (500 length) maximum error one per 1000. The singletons for all the samples were excluded. The data set was checked for chimaeras.

The operational taxonomic units (OTUs) were constructed applying 97% sequence identity; samples with less than 5,000 reads were excluded from the analysis.

We acquired 41 plastic samples shown in Table 1 with 17 samples for July-August, 18 in November and 6 in February, with the different plastic types and sizes. For each month, a sample of ambient water was collected as well to compare the bacterial community composition associated to the plastics with that of the ambient water.

Table 1. Overview of the number of microplastics used for sequencing the 16S rRNA gene.

Month	Plastic Type	Plastic size >9.5mm-4.75 mm	Plastic size 4.75mm-500 µm	Total samples	Ambient Water
February	Polyethylene	3	3	6	1
August	Polyethylene Polypropylene	6 8	2 1	8 9	1
November	Polyethylene Polypropylene Polyethylene terephthalate	7 5	3 2 1	10 7 1	1
Total		29	12	41	3

Determining the plastic type and visualizing the plastic associated biofilm using SEM

Raman spectroscopy was used to determine the type of plastic collected using the Raman microscope at the Division of Microbial Ecology (DOME) of the University of Vienna (Table 1). The microbial biofilm was visualized by SEM from plastic fragments.

Data analyses and statistical methods

All statistical analyses were performed in RStudio. Permutational Multivariate Analysis of Variance (PERMANOVA) was used to test for significant differences between month, plastic type and size and free-living and plastic-attached communities based on a Bray-Curtis resemblance matrix and 999 permutations.

To identify possible differences in the bacterial community composition of the plastics biofilm between the three months, the plastic type and size, a NMDS (Non

Metric Multidimensional Scaling) plot was generated using Bray-Curtis distances with the Vegan R package.

The Limma R package was used to calculate Venn Diagrams.

To generate the stacked bar plot for the most abundant OTUs, phyla, families and genera with the variables (months and plastic type) we used the Package ggplot2 of the software package R.

The normalization by the coverage of the data as suggested by Chao et al. (2014) was performed to calculate microbial richness for the different months, plastic types and sizes and of the ambient water. To determine the diversity of the microbial community, the Shannon Diversity, Simpson Diversity and the Species Richness was calculated using the iNEXT package in R. Anova was used to detect significant differences between the variables. We used the TukeyHSD to determine significant differences between the variables. We excluded the PET sample because we obtained only one sample. Boxplot with means were used to visualize the results.

Because of the low sample number obtained from ambient water, we computed the diversity estimates (estimateD) for the minimum sample coverage among all samples (0.918) using the diversity and species richness data. The Anova was used to detect significant differences between the variables with the newly generated data set.

Results

The average surface water temperature in February was 9.7°C, in July-August 25.1°C and in November 6.4°C. (https://www.seatemperature.org/europe/croatia/rovinj-february.htm)

A total of 1,327 floating plastic pieces was collected in our study (Table 2). The majority of the collected plastic pieces were smaller than 5mm (microplastics) making up 92% of the total collected plastics, with 58% in the size range between 500 µm and 1.4 mm and 34% in the size range between 4.75 – 1.4 mm (Figure 2).

Table 2. Total number of collected plastic pieces and sizes for the three months.

Plastic size	February	August	November	Total plastic/size
> 9.5 mm	5	24	13	42
4.75 and 9.5 mm	1	40	30	71
1.4 and 4.75 mm	10	236	203	449
500µm and 1.44 mm	132	374	259	765
Total	148	674	505	1327

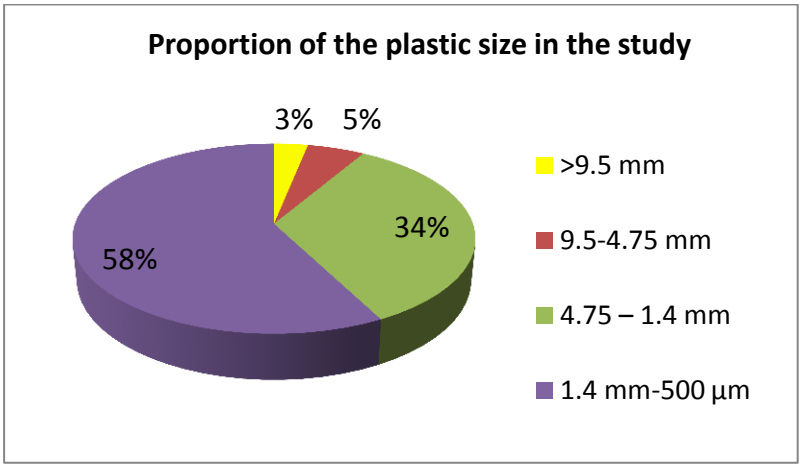

Figure 2. Proportion of the plastic sizes collected from the sea surface layer in the Northern Adriatic Sea off Rovinj (Croatia).

In February the 1.4mm - 500µm size was the most abundant plastic size class with 89%, followed by the size between 4.75 -1.4mm (7%), and the biggest ones with 4% (Figure 3).

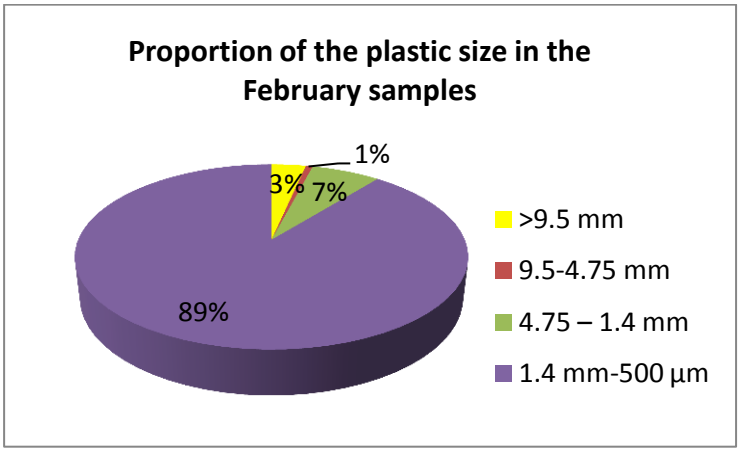

Figure 3. Proportion of the plastic sizes collected from the sea surface layer in the Northern Adriatic Sea off Rovinj (Croatia) in February.

For the plastics collected in August and November, the most abundant size class was the range 1.4mm-500µm contributing 89% to the total abundance of plastics. Generally, plastic abundance increased with decreasing size of plastic material (Figure 4a, b). In all three months, the plastic size less than 1.4mm was the most abundant.

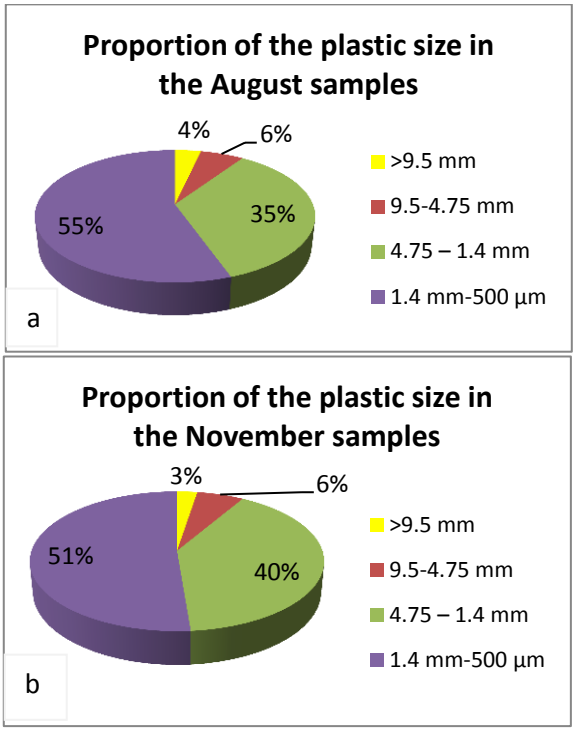

Figure 4 of different plastic size classes to the total abundance of plastics in the Northern Adriatic Sea in a) August and b) November.

Bacterial community composition

In total, bacterial libraries of 16S rDNA were comprised of 3,702 OTUs, distributed in 37 phyla, 248 families (25 uncultured bacteria, 1 unknown), 98 classes (14 uncultured bacteria, 1 unidentified), 175 orders (21 uncultured bacteria, 1 unidentified, 1 unknown), 509 genera (40 uncultured bacteria, 4 unidentified), 464 species (118 uncultured bacteria, 3 unidentified, uncultured organism).

The microbes associated with plastics were distinct from those of the ambient water (PERMANOVA, p= 0.043*). The microbial community composition shows significant difference between the three months (p=0.009*), February differs significantly. No significant difference, however, was found between the bacterial

community composition among the different plastic size classes (p=0.853) and types of plastics (p=0.709).

To determine whether there are differences in the bacterial community compositions between the plastic types, sizes and ambient water in the three months an NMDS was performed. As indicated in Figure 5A, no similarities in the community composition between the plastic samples and the ambient water, in the three different months was observed. In August and November, the ambient water community was very similar to each other. In general, the NMDS indicated a high variability in the microbial community composition. We also performed an NMDS without one of the most abundant taxa, the autotrophic Cyanobacteria. The community composition in February was distinctly different from that of August and November, which were more related to each other. These results are similar to those obtained using PERMANOVA.

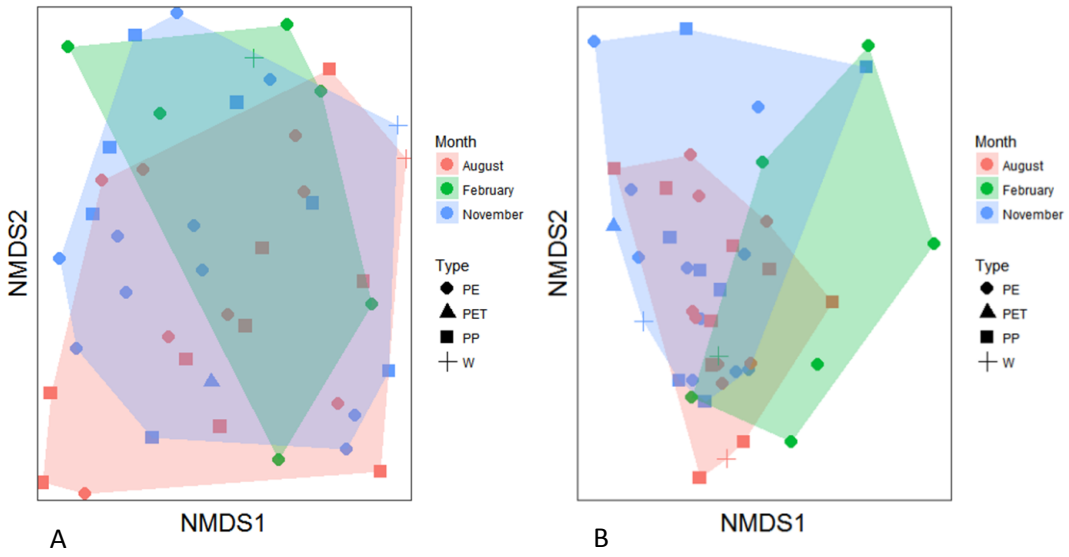

Figure 5. Non-metric multidimensional scaling indicating the changes in bacterial community composition in the ambient water and the plastic types in the three months. Values of Non-metric fit **R^2=0.962 and the linear fit R^2=0.84. PE**= Polyethylene, **PP**= Polypropylene, **PET**= Polyethylene terephthalate, **W**= Ambient water**. A)** NMDS with Cyanobacteria. **B)** NMDS without Cyanobacteria.

There were no differences detectable in the community composition of between different plastic size classes (Figure 6) regardless of whether cyanobacteria were included in the analyses or not shows no similarities in the community

composition between the four plastic sizes in the three different months (Figure 6A, B). Again, these results agree with those obtained using PERMANOVA.

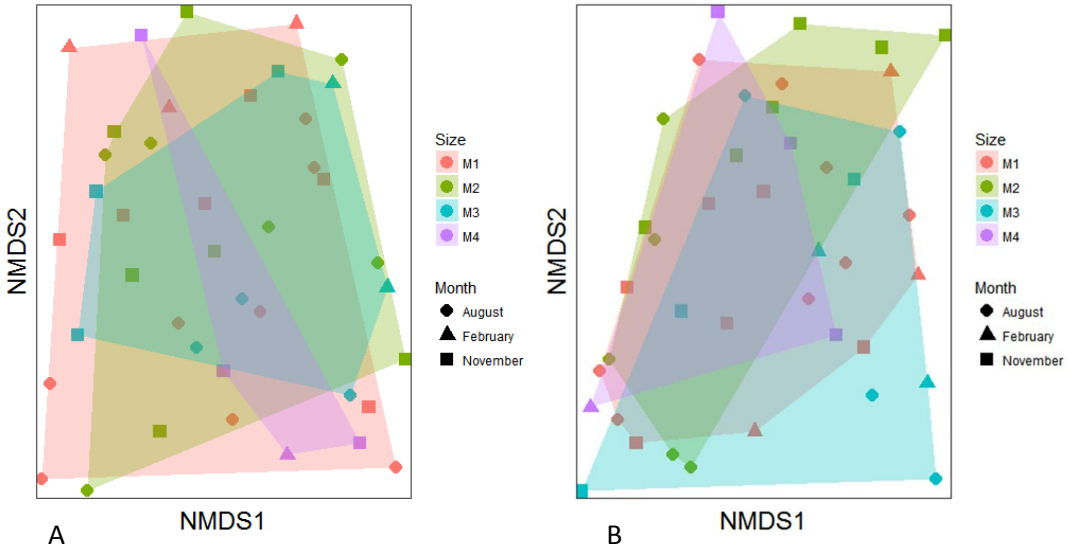

Figure 6. Non-metric multidimensional scaling indicating the changes in the community composition between the plastic size classes in the three months. Values of Non-metric fit **R^2=0.961 and the linear fit R^2=0.853. Plastic sizes. M1**: >9.5 mm **M2**: 9.5-4.75 mm; **M3**: 4.75 – 1.4 mm; **M4**: 1.4 mm-500 µm**. A)** NMDS with Cyanobacteria. **B)** NMDS without Cyanobacteria.

We also performed an NMDS analysis to test whether differences between the composition of the plastic-attached community and that of the ambient water are detectable. As indicated in Figure 7A, B with and without Cyanobacteria, respectively, significant differences were obtained in the composition between the ambient water and the plastic attached bacterial community, supporting the results obtained by PERMANOVA.

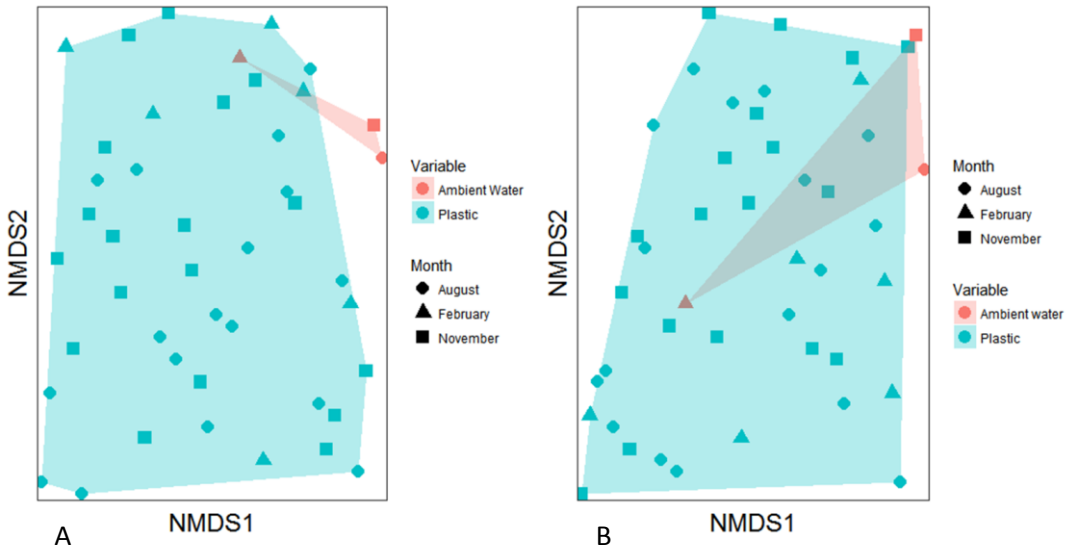

Figure 7. Non-metric multidimensional scaling relating the similarity in the community composition between the ambient water and the plastic attached over the three months. Values of Non-metric fit **R^2=0.961 and the linear fit R^2=0.853.**A) NMDS with Cyanobacteria. B) NMDS without Cyanobacteria.

Richness and diversity of the bacterial community

As indicated by the Shannon and Simpson Diversity indices, plastic size or type and its month of collection do not influence the diversity of the plastics-associated bacterial community. The OTU richness indicates a significant difference between the type variable (p value= 0.00154**) and the month variable (p value= 0.0126*) both included the ambient water (Table 3).

Table 3. ANOVA results of Shannon, Simpson Diversity and OTU Richness of the three variables.

Variable	**Shannon Diversity**		**Simpson Diversity**		**Species Richness**	
	F value	**Pr(>F)**	**F value**	**Pr(>F)**	**F value**	**Pr(>F)**
Month	0.72	0.493	0.822	0.447	0.974	0.387
Month and AW	0.666	0.651	0.813	0.548	3.386	0.0126*
Type	0.843	0.438	0.382	0.685	2.052	0.142
Plastics and AW	1.081	0.368	0.906	0.447	6.148	0.00154**
Size	0.o47	0.986	0.015	0.997	0.262	0.852

**Significant differences

The boxplot (Figure 6) with the mean illustrates that the ambient water samples had the highest mean species richness and PP the lowest. The results of the TukeyHSD (honest significant difference test) in Table 4 show that the OTU richness is significantly different between the two plastic types and the ambient water (p value= 0.0049*) for PE and (p value= 0.0008*) for PP respectively.

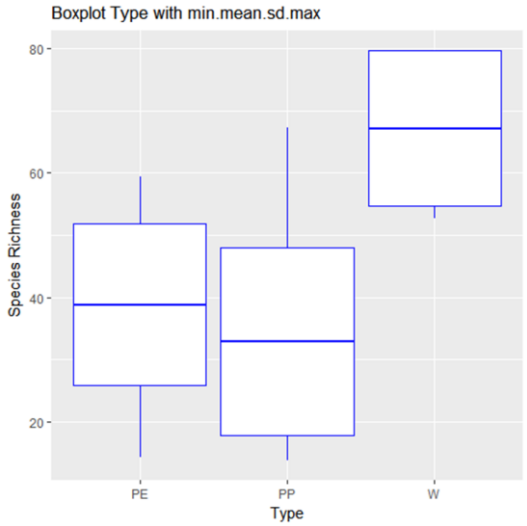

Figure 6. Species Richness of the plastic type and the ambient water (**PE**= Polyethylene, **PP**= Polypropylene, **W**= ambient water).

Table 4. TukeyHSD test results for the plastic types and the ambient water**.**

Type	diff	lwr	upr	p adj
PP-PE	-5.955.833	-16.811.760	4.900.093	0.3843508
W-PE	28.351.250	7.753.578	48.948.922	0.0049337*
W-PP	34.307.083	13.144.987	55.469.180	0.0008957*

PE= Polyethylene**, PP=** Polypropylene**, W=** ambient water. **diff**= difference in the observed means, **lwr**= lower point of the interval, **upr** = upper point, **p adj** the p-value after adjustment for the multiple comparisons.*****Significant difference

The data obtained by computing the diversity estimates for the minimum sample coverage among all samples are illustrated in Table 5, the OTU richness also indicated that there are no significant differences in OTU richness between the ambient water and the two plastic types (p value = 0.449). As indicated by the Shannon, Simpson Diversity and Species Richness indices, month of collection influence the diversity of the seasonal-associated bacterial community. The OTU diversity and richness indicates a significant difference between the sample months (August and November differs from February) with and without the ambient water.

Table 5. ANOVA results of Shannon, Simpson Diversity and OTU Richness of the three variables for the minimum sample coverage**.**

Variable	Shannon Diversity		Simpson Diversity		Species Richness	
	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Month	3.864	0.0297 *	3.454	0.0418 *	4.27	0.0212 *
Month and AW	4.168	0.0225 *	3.875	0.0288 *	4.108	0.0237 *
Type	0.077	0.926	0.218	0.805	0.132	0.877
Plastics and AW	0.791	0.506	0.76	0.523	0.902	0.449
Size	1.149	0.342	1.465	0.24	0.895	0.453

*Significant difference

The OTU diversity and richness for the size indicates no significant difference during the three sampled months. November presented significant difference (p value= 0.016*) between 4.75 – 1.4 mm (microplastic) and >9.5 mm (macroplastics) (Supplement B). The mean species richness observed was higher in 4.75 – 1.4 mm than in >9.5 mm size.

Phylogenetic affiliation of the OTUs

DNA sequence analysis indicated a different community composition on plastics and the ambient water. In the 44 samples submitted to sequencing, 3702 OTUs were identified. Figure 8 displays the relative abundance of the 30 most abundant OTUs in this study.

In August, the composition of the PE attached bacterial community was composed to 20% of the OTU_1 (Cyanobacteria), 15% by OTU_6 (uncultured Rhodobacteraceae bacterium); and by 10% of OTU_4 (uncultured member of the Pseudoalteromonadacea). The PP associated bacterial community was composed of 20% OTU_2 (uncultured *Alteromonas*), to 15% by OTU_1 and to 10% of OTU_6. Overall, PE and PP exhibited a similar pattern in the bacterial community composition (Figure 8).

In February, (Figure 8) the most abundant OTUs in the PE community were OTU_13 (Exiguobacterium, unidentified marine bacterioplankton) contributing 35%, followed by the OTU_1 contributing 12% and OTU_19 (uncultured *Oleiphilus*) with 10% of relative abundance.

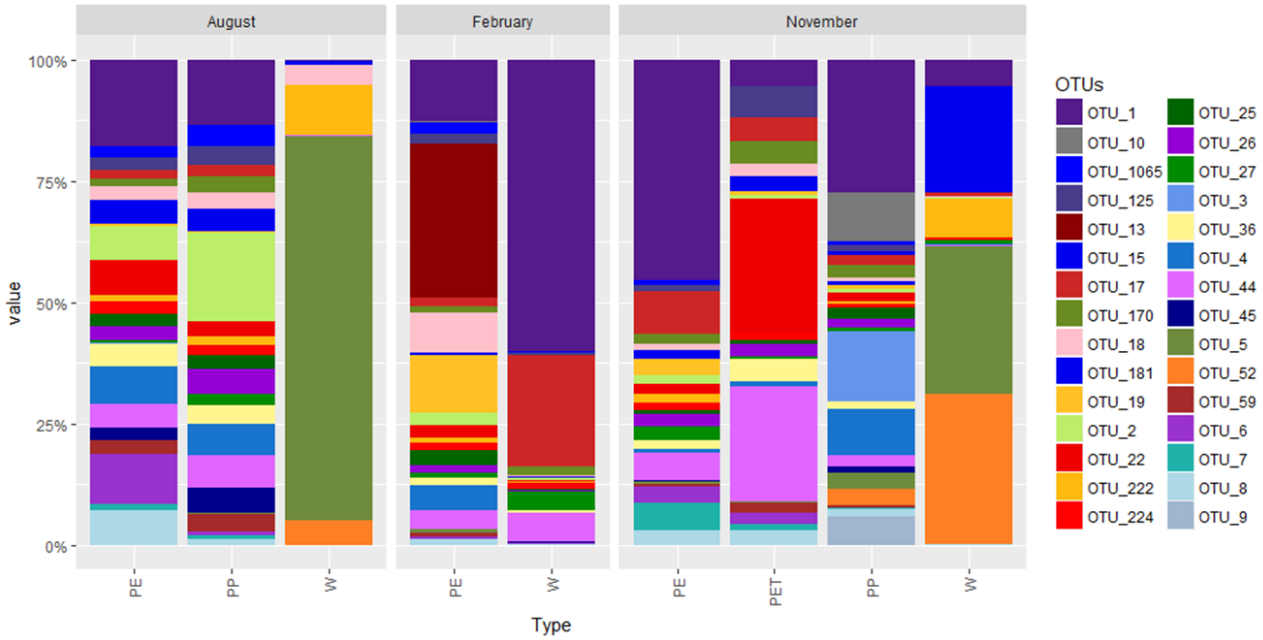

Figure 8. Relative abundance of bacterial OTUs. The most abundant (30) bacterial OTUs present in three months in the different plastic types and ambient water are shown. **PE**= Polyethylene, **PP**= Polypropylene, **PET**= Polyethylene terephthalate, **W**= Ambient water. Total number of samples =44. The most abundant OTUs are listed below.

OTU_1=Bacteria, Cyanobacteria, Chloroplast ,uncultured bacterium; **OTU_4=**Bacteria,Proteobacteria,Gammaproteobacteria,Alteromonadales, Pseudoalteromonadaceae, Pseudoalteromonas ,uncultured proteobacterium; **OTU_5**=Bacteria, Cyanobacteria, Cyanobacteria, SubsectionI, FamilyI, Synechococcus,unidentified marine bacterioplankton; **OTU_2=**Bacteria, Proteobacteria,Gammaproteobacteria, Alteromonadales,Alteromonadaceae, Alteromonas,uncultured Alteromonas sp. ; **OTU_44**=Bacteria, Proteobacteria, Alphaproteobacteria,Rhodobacterales,Rhodobacteraceae,Roseovarius ,uncultured bacterium**; OTU_3=**Bacteria,Proteo**bacteria**, Gammaproteobacteria, Thiotrichales,Thiotrichaceae,uncultured,uncultured bacterium; **OTU_17=** Bacteria ,Cyanobacteria ,Chloroplast, unculturedbacterium; **OTU_22=**Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Winogradskyella, unculturedbacterium; **OTU_52**=Bacteria, Proteobacteria, Alphaproteobacteria, SAR11, clade, Surface1, unidentified marine bacterioplankton; **OTU_6**=Bacteria, Proteobacteria,Alphaproteobacteria,Rhodobacterales,Rhodobacteraceae,Roseovarius, uncultured Rhodobacteraceae bacterium; **OTU_8**=Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Mesoflavibacter, uncultured marine microorganism; **OTU_10**=Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Maribacter, uncultured bacterium; **OTU_26**=Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, uncultured alpha proteobacterium; **OTU_36=**Bacteria, Proteobacteria,Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium; **OTU_181**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Pseudoruegeria, uncultured bacterium; **OTU_13**=Bacteria, Firmicutes, Bacilli,Bacillales,Family XII, Exiguobacterium, unidentified marine bacterioplankton; **OTU_18**=Bacteria, Proteobacteria,Alphaproteobacteria,Rhodobacterales,Rhodobacteraceae, Nautella,unidentified marine bacterioplankton; **OTU_15=**Bacteria, Actinobacteria, Acidimicrobiia,Acidimicrobiales,OM1 clade,Candidatus Actinomarina ,uncultured marine bacterium; **OTU_170**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, Ahrensia,uncultured bacterium; **OTU_25**=Bacteria, Proteobacteria,Alphaproteobacteria ,Rhodobacterales, Rhodobacteraceae, Marivita, uncultured bacterium; **OTU_19**=Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oleiphilaceae, Oleiphilus,uncultured Oleiphilus sp.; **OTU_125**=Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured bacterium; **OTU_222**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium; **OTU_1065**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Sulfitobacter,uncultured bacterium; **OTU_7**=Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillaceae,uncultured bacterium; **OTU_45=**Bacteria, Cyanobacteria, Cyanobacteria, SubsectionIII ,FamilyI, Phormidium, Plectonema sp. F3; **OTU_224**=Bacteria ,Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Octadecabacter, Octadecabacter sp. R-1-R-9; **OTU_9=**Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Maritimimonas, uncultured Bacteroidetes bacterium; **OTU_27**=Bacteria, Cyanobacteria, Chloroplast,uncultured bacterium. **OTU_59**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Sulfitobacter, uncultured bacterium.

In November, OTU_1 contributed about 50% to the relative abundance of the microbial biofilm of PE, followed by OTU_17 (Cyanobacteria, uncultured bacterium) with 10%; and OTU_7 (Oceanospirillaceae, uncultured bacterium) with 7% (Figure 8). In Polyethylene terephthalate (PET), OTU_22 was most abundant comprising 40% of the relative abundance (Winogradskyella uncultured bacterium, Flavobacteriacea) followed by OTU_44 (*Roseovarius* genus, coming from the Rhodobacteraceae) with 25% and OTU_125 (Hyphomonadaceae) with 12%. The PP attached community was

dominated by OTU_1 (30%), followed by OTU_3 (Thiotrichaceae) with 20% and OTU_10 (*Maribacter* genus) with 10% of the relative abundance (Figure 8).

The relative abundance of the community composition in the ambient water was similar in August and November and different from February. In August, the community composition in the ambient water was comprised by about 80% of the OTU_5 while the community in February was composed mainly of OTU_1 (60%). In November, 30% to the relative abundance was composed by OTU_52 (SAR11 clade) and OTU_5 (*Synechococcus*) with 30% (Figure 8).

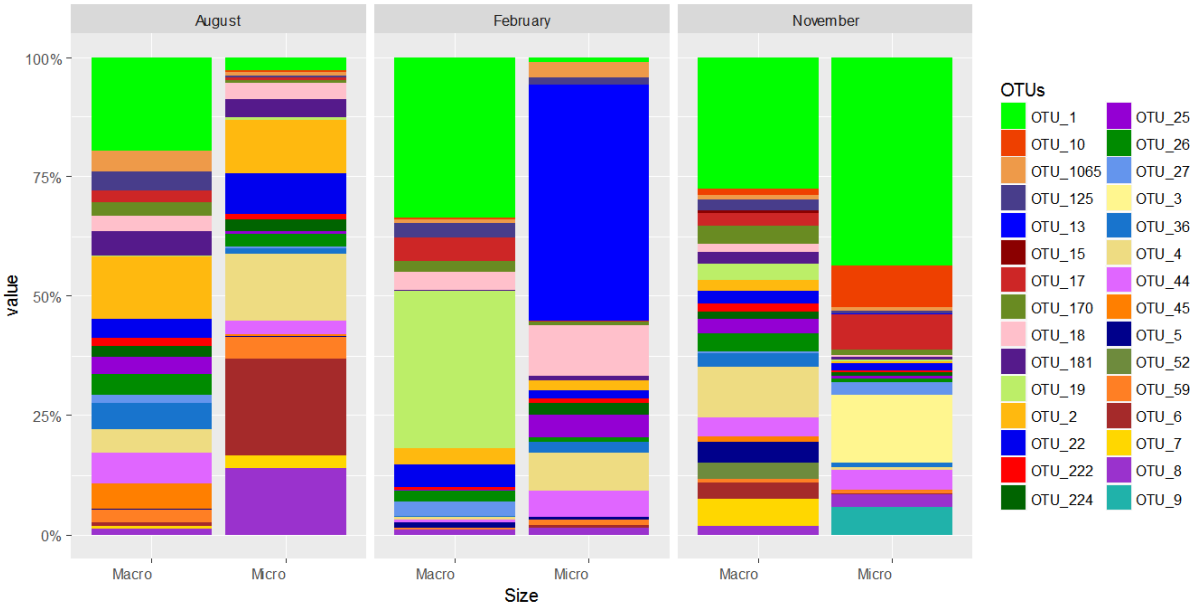

Figure 9. Relative abundance of bacterial OTUs. Most abundant (30) bacterial OTUs present in the three months in the different plastic size classes, macro- and Micro-plastics and ambient water. Total number of samples =41. The most abundant OTUs are listed below.

OTU_1=Bacteria, Cyanobacteria, Chloroplast ,uncultured bacterium; **OTU_4=**Bacteria,Proteobacteria,Gammaproteobacteria,Alteromonadales, Pseudoalteromonadaceae, Pseudoalteromonas ,uncultured proteobacterium; **OTU_5**=Bacteria, Cyanobacteria, Cyanobacteria, SubsectionI, FamilyI, Synechococcus,unidentified marine bacterioplankton; **OTU_2=**Bacteria, Proteobacteria,Gammaproteobacteria, Alteromonadales,Alteromonadaceae, Alteromonas,uncultured Alteromonas sp. ; **OTU_44**=Bacteria, Proteobacteria, Alphaproteobacteria,Rhodobacterales,Rhodobacteraceae,Roseovarius ,uncultured bacterium**; OTU_3=**Bacteria,Proteo**bacteria**, Gammaproteobacteria, Thiotrichales,Thiotrichaceae,uncultured,uncultured bacterium; **OTU_17=** Bacteria ,Cyanobacteria ,Chloroplast, unculturedbacterium; **OTU_22=**Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Winogradskyella, unculturedbacterium; **OTU_52**=Bacteria, Proteobacteria, Alphaproteobacteria, SAR11, clade, Surface1, unidentified marine bacterioplankton; **OTU_6**=Bacteria, Proteobacteria,Alphaproteobacteria,Rhodobacterales,Rhodobacteraceae,Roseovarius, uncultured Rhodobacteraceae bacterium; **OTU_8**=Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Mesoflavibacter, uncultured marine microorganism; **OTU_10**=Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Maribacter, uncultured bacterium; **OTU_26**=Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Erythrobacteraceae, Erythrobacter, uncultured alpha proteobacterium; **OTU_36=**Bacteria, Proteobacteria,Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium; **OTU_181**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Pseudoruegeria, uncultured bacterium; **OTU_13**=Bacteria, Firmicutes, Bacilli,Bacillales,Family XII, Exiguobacterium, unidentified marine bacterioplankton; **OTU_18**=Bacteria, Proteobacteria,Alphaproteobacteria,Rhodobacterales,Rhodobacteraceae, Nautella,unidentified marine bacterioplankton; **OTU_15=**Bacteria, Actinobacteria, Acidimicrobiia,Acidimicrobiales,OM1 clade,Candidatus Actinomarina ,uncultured marine bacterium; **OTU_170**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, Ahrensia,uncultured bacterium; **OTU_25**=Bacteria, Proteobacteria,Alphaproteobacteria ,Rhodobacterales, Rhodobacteraceae, Marivita, uncultured bacterium; **OTU_19**=Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oleiphilaceae, Oleiphilus,uncultured Oleiphilus sp.; **OTU_125**=Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Hyphomonadaceae, uncultured, uncultured bacterium; **OTU_222**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, uncultured, uncultured bacterium; **OTU_1065**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Sulfitobacter,uncultured bacterium; **OTU_7**=Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillaceae,uncultured bacterium; **OTU_45=**Bacteria, Cyanobacteria, Cyanobacteria, SubsectionIII ,FamilyI, Phormidium, Plectonema sp. F3; **OTU_224**=Bacteria ,Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Octadecabacter, Octadecabacter sp. R-1-R-9; **OTU_9=**Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Maritimimonas, uncultured Bacteroidetes bacterium; **OTU_27**=Bacteria, Cyanobacteria, Chloroplast,uncultured bacterium. **OTU_59**=Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, Sulfitobacter, uncultured bacterium.

Figure 9 gives a general overview of the community composition in the different plastic sizes (macro- and microplastics) over the three months. The dominant OTUs in the biofilm of the macroplastics in the three months were OTU_1 (Cyanobacteria), on the microplastics the most dominant OTU was the OTU_6 (uncultured Rhodobacteraceae) in August. In February, the OTU_13 (Exiguobacterium, unidentified marine bacterioplankton) was the most predominant and in November the OTU_1.

A total of 3367, 3049 and 898 OTUs were identified in PE, PP and ambient water, respectively (Figure 10) with 752 OTUs shared by the ambient water and both plastic types, while 2012 bacterial OTUs were shared only by the two plastic types. PE had the largest number of unique OTUs (571), followed by PP (192) whereas only 21 OTUs were unique to ambient water (Figure 10). For this analysis the PET sample was excluded.

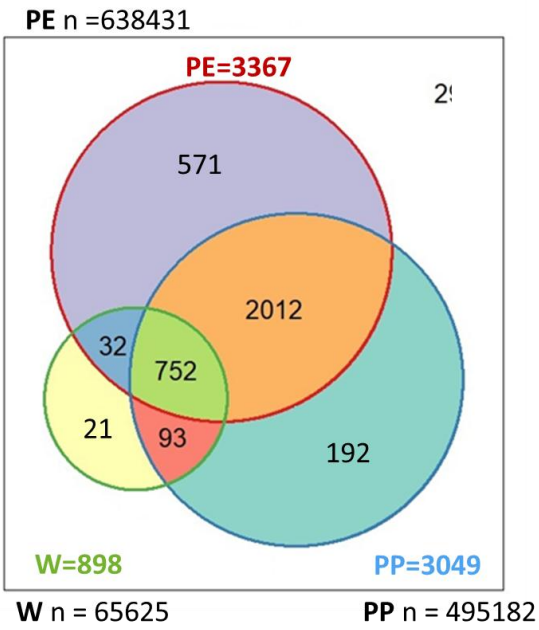

Figure 10. Distribution of the OTUs. **n**= Number of sequenced reads per group, **PE**= polyethylene, **PP**= polypropylene, **W**= ambient water. Numbers inside the circles represent the number of shared or unique OTUs.

Distribution of bacteria on the phylum level

The community composition of the 37 phyla found in the different plastic types and ambient water along the months in this study, are shown in Figure 11. Proteobacteria were the most abundant phylum (50%) in all the samples except in the ambient water in February when Cyanobacteria dominated (60%). The second most abundant phyla across the samples were Cyanobacteria (18%), followed by Bacteroidetes (15%) and Planctomycetes (10%). The relative abundance of the phyla was relatively similar between the different plastic types and in both August and November (Figure 11). The ambient water exhibited a different community composition between the three months. In August the two most dominant phyla were Proteobacteria (40%) and Cyanobacteria with 35% relative abundance. Cyanobacteria dominated (60%) the relative abundance in February and in November Proteobacteria with 40%.

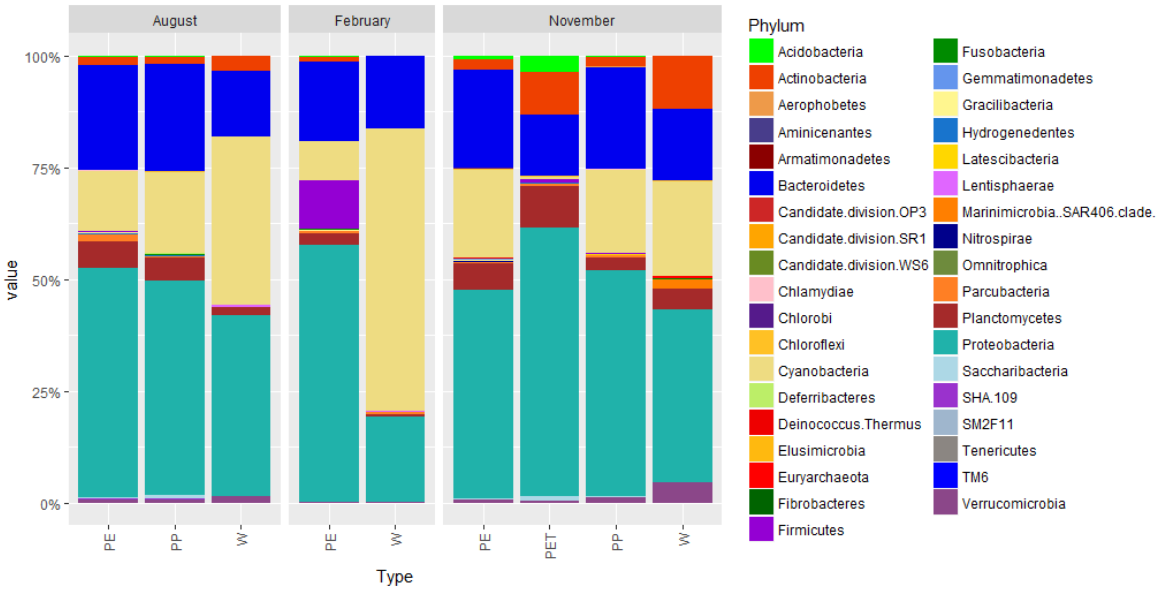

Figure 11. Distribution of the 37 bacterial phyla present in three months, at the plastic types and in the ambient water. **PE**= polyethylene, **PP**= polypropylene, **PET**= polyethylene terephthalate, **W**= ambient water. Total number of samples =44.

The PE and PP attached community in August showed the same pattern as in November. The most abundant phyla were Proteobacteria, Bacteroidetes and Cyanobacteria contributing about 50%, 25% and 20%, respectively, to the total relative abundance.

In February the composition between PE and the ambient water was most pronounced. PE colonizing taxa belonged mostly to the phyla Proteobacteria (60%), Bacteroidetes (20%) and Firmicutes (15%) in contrast to the ambient water where Cyanobacteria (70%) were the most dominant followed by Proteobacteria (20%) and 15 % Bacteroidetes (Figure 11).

In November Proteobacteria accounted for nearly 50% of all taxa in all plastic types and the ambient water. Cyanobacteria covered nearly 20% of PE and PP in all the months, in the ambient water the distribution was higher. The plastics and the ambient water almost the same community composition consisting of the phyla Proteobacteria, Cyanobacteria and Bacteroidetes over the three months (Figure 11).

A total of 35, 33 and 20 phyla were identified in PE, PP and the ambient water respectively. Armatimonadetes, Elusimicrobia and Omnitrophica were present only in PE. Candidate division WS6 and Tenericutes were present only in PP. These unique phyla were in general less abundant compared to the other phyla found in this study, present only in one or two samples (Figure 12).

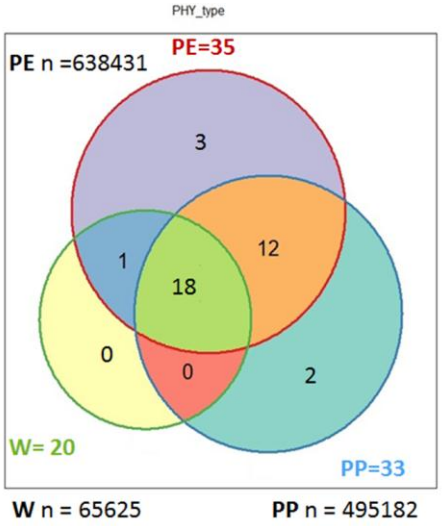

Figure 12. Distribution of phyla among PE, PP and ambient water. **n**= Number of sequenced reads per group, **PE**= polyethylene, **PP**= polypropylene, **W**= ambient water.

Figure 12 illustrates also that collectively the two plastic types shared 12 phyla that were not present in the ambient water (Aerophobetes, Candidate division OP3, Candidate division SR1, Chlorobi, Deferribacteres, Deinococcus-Thermus, Fibrobacteres, Hydrogenedentes, Latescibacteria, SHA-109, SM2F11, TM6). PE and the

ambient water shared 19 phyla, PP and the ambient water shared 18 phyla. There was no phylum present in the ambient water that was not present in plastic pieces.

Distribution of bacteria on the family level

A total of 230, 221 and 138 bacterial families were identified in the biofilm of the PE, PP and in the in the ambient water, respectively (Figure 13). Twenty families were unique to PE and 10 to PP (Table 7) while 137 families were shared by PE, PP and the ambient water and 73 families were shared between PE and PP but were not found in the ambient water.

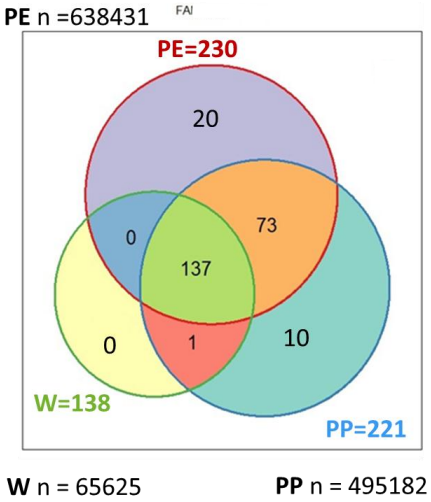

Figure 13. Distribution of bacterial families among PE, PP and the ambient water. **n**= Number of sequenced reads per group, P**E**= polyethylene, **PP**= polypropylene, **W**= ambient water.

Table 7. Unique bacterial families in the biofilm of polyethylene and polypropylene

Unique Families Polyethylene	**Unique Families Polypropylene**
X01D2Z36	ABS.19
Acetobacteraceae	Chlamydiaceae
Acidimicrobiales.Incertae.Sedis	Microbulbiferaceae
Aeromonadaceae	Mycoplasmataceae
Algiphilaceae	Porphyromonadaceae
BD2.7	Rhodocyclaceae
Beijerinckiaceae	Rubrobacteriaceae
Chthonomonadaceae	uncultured.Chromatiales.bacterium
cvE6	uncultured.Gemmatimonadetes.bacterium
DA101.soil.group	uncultured.proteobacterium
DUNssu371	
Eel.36e1D6	
Holosporaceae	
Neisseriaceae	
Nevskiaceae	
Opitutaceae	
Paenibacillaceae	
Saccharospirillaceae	
Thiotrichales.Incertae.Sedis	
Xiphinematobacteraceae	

A total of 248 families were identified in all samples. The distribution of the 30 most abundant families, over the plastic types and the ambient water over the three months is shown in Figure 14. Generally Flavobacteriaceae, Rhodobacteraceae, Fam1 (Family of OTU_1=Bacteria, Cyanobacteria, Chloroplast, uncultured bacterium), FamilyI and Saprospiraceae were the most abundant families. The relative abundance of the families appeared to be relatively similar between the different plastic types in August and November.

The bacterial family composition in August were very similar in the two plastic types (PP and PE), dominated by the taxa Rhodobacteraceae with 25%, Flavobacteriaceae 20% and FamilyI 10% of the total of the relative abundance. The ambient water was composed by 50% of FamilyI, followed by Rhodospirillaceae (12%) and Alteromonadaceae (10%), (Figure 14).

The most abundant PE-colonizing taxa in February belonged to the families Rhodobacteraceae representing 20%, Flavobacteriaceae 12% and Family.XII 10% of the relative abundance. Fam1 (Family of the OTU_1=Bacteria, Cyanobacteria, Chloroplast, uncultured bacterium) was the most abundant taxa in the ambient water representing up to 50% of the total abundance, followed by Rhodobacteraceae representing 20% of the total relative abundance (Figure 14).

In November (Figure 14), the composition of the families in PE and PP was similar, with Flavobacteriaceae, Fam1, both nearly 20% and Rhodobacteraceae with 12% of the total of the relative abundance. Planctomycetaceae was the more abundant family in PET with 20%, followed by Flavobacteriaceae 12% and 10% Rhodobacteraceae of the total of the relative abundance. The ambient water was represented by FamilyI (30%), OM1.clade (15%) and Surfrace 1 (12%) of the relative abundance. The microbial composition of the ambient water in February was different from that in August and November (Figure 8, 11,14).

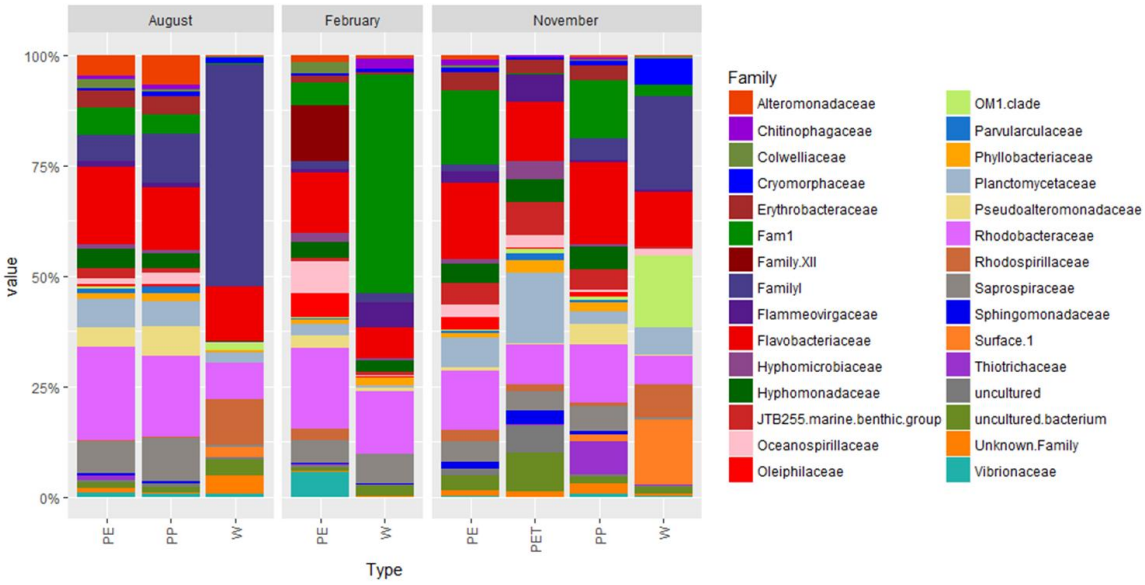

Figure 14. Relative abundance of bacterial families in the biofilm of PE, PP and ambient water. Most abundant (30) bacterial families present in the three months, in the plastic types and ambient water. **PE**= polyethylene, **PP**= polypropylene, **PET**= polyethylene terephthalate, **W**= ambient water. **Fam1**= family coming from the OTU_1=Bacteria, Cyanobacteria, Chloroplast, uncultured bacterium. Total number of samples =44.

Members of known hydrocarbon degrading families (Alteromonadaceae, Flavobacteriaceae, Hyphomonadaceae, Pseudoalteromonadaceae, Rhodobacteraceae

and Saprospiraceae) were present in all months and plastic types, but less abundant in the ambient water.

Distribution of bacteria on the genus level

The 30 most abundant genera associated with the different types of plastics and the ambient water in February, August and November indicated that, overall, the members of the genus *uncultured*, *Gen1* (from the OTU_1=Bacteria, Cyanobacteria, Chloroplast, uncultured bacterium), *uncultured bacterium* were most dominant (Figure 15). Most of these lineages represented uncultured microorganisms. We identify 509 genera in this study. The genus *Hyphomonas* was present in all the plastic types in the three months and less abundant in ambient water. Ambient water of each of the three month revealed a different pattern.

In August, the most abundant genus in the ambient water was *Synechococcus* (60%), in February *Gen 1* (60%) and in November (45%) *unidentified marine bacterioplankton.* In August, the two plastic types exhibited a similar community structure on the genus level with *uncultured* being the most abundant, followed by the *Gen 1*. The biofilm of the PP exhibited a higher relative abundance of the genus *Psychrosphaera* than on the PE while in the biofilm of the PE *Mesoflavibacter* was more abundant than on the PP. In February, the PE-associated taxa belonged to the *uncultured* group, *Exiguobacterium, Vibrio* and *Oleiphilus* genus. The *uncultured* group constituted almost 60% of the PET sample in November 20%. In November, PE and PP were colonized mostly by the *Gen 1* and the *uncultured* group; *Maribacter* was more abundant in PP than in PE (Figure 15).

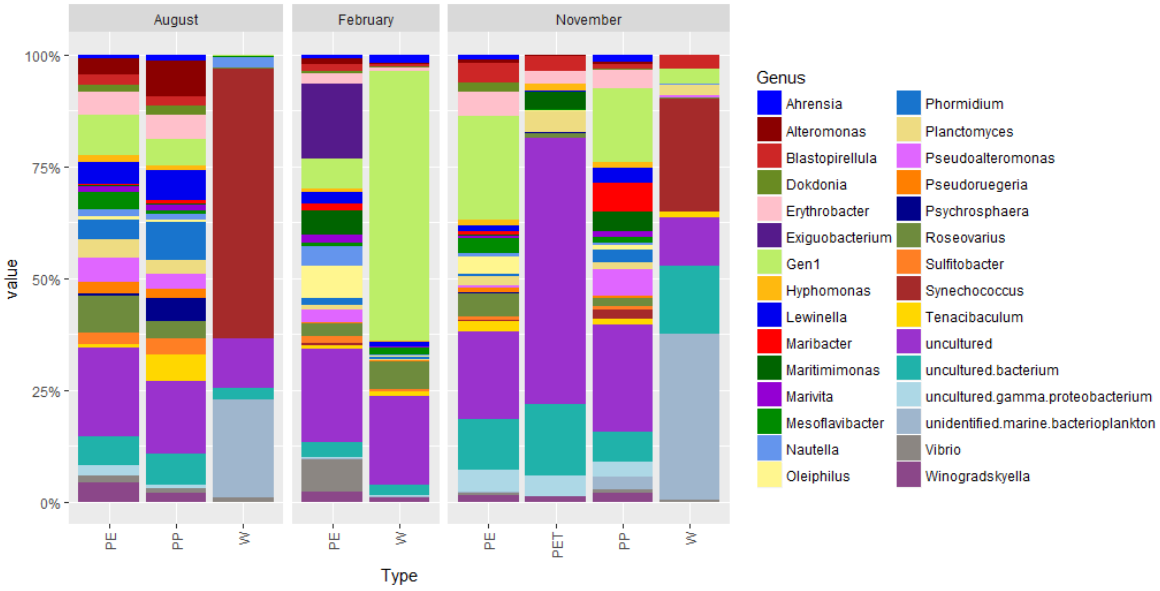

Figure 15. Relative abundance of bacterial genera. Most abundant (30) bacterial genera present in three months, on the plastic types and ambient water. **PE**= polyethylene, **PP**= polypropylene, **PET**= polyethylene terephthalate, **W**= ambient water. **Gen1=**Genus from the OTU_1=Bacteria, Cyanobacteria, Chloroplast, uncultured bacterium. Total number of samples =44.

We detected potential hydrocarbon degrading genera as listed in Table 8. These genera belong to the families Alcanivoracaceae, from which 7 genera were found, 3 genera of Hyphomicrobiaceae, 8 belonging to the Alteromonadaceae, 5 affiliated to Oceanospirillaceae and 9 genera of the Oleiphilaceae. *Oleiphilus* genus (OUT_19) were found in the 30 most abundant.

Table 8. Potential hydrocarbon-degrading genera associated to the different types of plastics and the ambient water**.** Total number of samples =44

OTU´s	Phylum	Family	Genus/species	PE	PP	PET	W	obs.
OTU´s _249			Alcanivorax uncultured bacterium	X	X			
OTU´s _284			Alcanivorax D6881	X	X			
OTU´s _538			Alcanivorax uncultured gammaproteobacteria	X	X	X		
OTU´s _526			Alcanivorax sp P2S70	X	X			
OTU´s _1131			Alcanivorax uncultured gammaproteobacteria	X	X			
OTU´s _1289			Alcanivorax alcanivoracaceae bacterium MOLA 388	X	X			
OTU´s _2070	Proteobacteria	Alcanivoracaceae	Alcanivorax uncultured bacterium	X	X			
OTU´s _969			Devosia uncultured bacterium	X	X			
OTU´s _1343			Devosia uncultured bacterium	X	X			
OTU´s _1221	Proteobacteria	Hyphomicrobiaceae	Devosia uncultured bacterium	X	X			
OTU´s _31			Marinobacter lipolyticus SM19	X	X			
OTU´s _325			Marinobacter sp. 908115	X	X			
OTU´s _391			Marinobacter uncultured Marinobacter sp.	X	X			
OTU´s _478			Marinobacter uncultured bacterium	X	X			
OTU´s _2343			Marinobacter unidentified marine bacterioplankton	X	X			
OTU´s _1963			Marinobacter uncultured bacterium	X	X			
OTU´s _1430			Marinobacter uncultured bacterium	X	X			
OTU´s _1722	Proteobacteria	Alteromonadaceae	Marinobacter uncultured Marinobacter sp.	X	X			
OTU´s _611			Thalassolituus uncultured Oleiphilus sp.	X	X		X	November
OTU´s _104			Oceaniserpentilla haliotis	X	X			
OTU´s _3095			Oceaniserpentilla uncultured Oceanospirillales bacterium	X				
OTU´s _133			Neptunomonas uncultured Neptunomonas sp	X	X		X	August
OTU´s _693	Proteobacteria	Oceanospirillaceae	Neptunomonas japonica	X	X			
OTU´s _19			Oleiphilus uncultured Oleiphilus sp.	X	X	X	X	November
OTU´s _64			Oleiphilus uncultured bacterium	X	X	X		
OTU´s _2564			Oleiphilus uncultured gamma proteobacterium	X	X	X	X	all months
OTU´s _543			Oleiphilus uncultured bacterium	X	X			
OTU´s _763			Oleiphilus uncultured bacterium	X	X			
OTU´s _340			Oleiphilus uncultured bacterium	X	X	X		
OTU´s _2082			Oleiphilus uncultured bacterium	X	X	X		
OTU´s _2591			Oleiphilus uncultured bacterium	X	X			
OTU´s _733	Bacteroidetes	Oleiphilaceae	Oleiphilus uncultured gamma proteobacterium	X				

PE= polyethylene, P**P**= polypropylene, **PET**= polyethylene terephthalate, **W**= ambient water.
Obs= month of the water sample collected where the taxa was found.

Discussion

We found plastic debris in all the net tows carried out in the Northern Adriatic Sea. Different types of plastic were identified; this study only analyzes the community composition attached to the PE, PP and PET. Zettler et al. (2013) reported that the majority of plastic sizes collected at multiple locations in the North Atlantic was less than 5 mm in size. Eriksen et al. (2014) also reported that the two size classes representing the particles between 1.00-4.75 mm (microplastic) collected for the South Pacific subtropical gyre represented 55 % of the total number of plastic particles.

In this study 92% of the plastic pieces were < 5 mm (microplastic), 58% were between 500 µm and 1.4 mm and 34% between 4.75 – 1.4 mm (Figure 2).

The plastic associated bacterial community is more similar within the three months than in the ambient water (Figure 8), according to our third hypothesis. This pattern might be caused the plastic pieces might have been for a long time in the sea and the microbial community had more time to colonize the plastics more stable physical and chemical conditions within the biofilm than in the ambient water. The plastic pieces are typically floating, driven by winds and transport into potentially over wide distances (Derriak 2002). The accumulation of floating plastic in the Mediterranean Sea is related to the semi-enclosed basin (Cozar et al. 2015).

In August and November, the bacterial communities of the ambient water were similar to each other but dissimilar to the plastic samples (Figures 8). The bacterial richness differs on the plastics and the ambient water (Figure 6). There are studies reporting a higher bacterial richness in the ambient water (Didier et al. 2017), while others report higher richness on plastics (Zettler et al. 2013; Bryant et al. 2016).

The island theory (MacArthur & Wilson 1967) suggests that the species richness increases with the size of the island and decreases with the distance to the source of the potential colonizers (Lyons et al. 2010). Hence, in our study the OTU richness should increase with the size of the plastic particle. In contrast to what is predicted by the island theory, however, species richness did not generally increase with the size of the plastic material. Only in November, a higher bacterial OTU richness in the plastic size class 4.75 – 1.4 mm than in the plastic size class >9.5 mm was detected. Generally, the bacterial richness on the plastics was lower than in the ambient water.

Differences in the composition of the free-living and attached bacterial communities were observed (Figures 8, 14). The plastic associated bacterial community was different from the free-living, ambient water community, consistent with previous studies in different systems (Zettler et al. 2013; Oberbeckmann et al. 2014; Bryant et al. 2016). However, no differences were detectable in the bacterial community composition between different plastic types and sizes.

The composition of the plastic associated bacterial community is similar in the biofilm of the PE and PP while that on the PET is different (Figure 8, 14). These differences in the colonization of different plastic types might be related to differences in the surface structure of the different plastic types or due to differences in the composition of the plastic additives.

Phylogenetic composition of the plastic associated bacterial community

Generally, more unique taxa were found on the plastic than in the ambient water (Figure 10), which is in contrast to a previous study (Zettler et al. 2013). The development of the biofilm on the plastic depends on the duration the plastics have been submerged in the water. Microbial biofilms, however, develop within days on the plastics (Lobelle & Cunliffe, 2011).

Rath et al. (1998) report Planctomycetes, the Cytophaga-Flavobacteria-Bacteriodetes lineage, the alpha, gamma, delta and epsilon subdivisions of proteobacteria on macroagreggates in the northern Adriatic Sea. Delong et al. (1993) indicated that marine snow-type macro-aggregates are colonized by bacteria belonging to the taxa Cytophaga, Planctomycetes, Gammaproteobacteria and Alphaproteobacteria. These taxa are also reported to be pioneer populations colonizing solid substrates in the marine environment (Lee et al. 2008). In this study, we also observed these plastic-attached taxa (α- γ-Proteobacteria, Planctomycetes and Bacteroidetes) also in low abundance in the ambient water. Pioneer populations are generally present in seawater with low abundance but have a rapid growth on substrata surfaces to make detectable differences in community compositions (Lee et al. 2008). Bacteroidetes were found to be an important PE-attached taxon (Zettler et al. 2013) and also PET-attached taxon (Oberbeckmann et al. 2014; 2016). We identified this phylum in to both the plastics and the ambient water (Figure 11). Didier et al. (2017) found that PET was colonized mostly by the phyla Proteobacteria and Actinobacteria. In our study, PET was also mainly colonized by Proteobacteria and

Cyanobacteria. PP and PE attached communities were dominated by Proteobacteria as also reported by Zettler et al. (2013).

The 12 phyla detected only on the plastics (Figure 12) might be opportunistic organisms that could growth in various substrata surfaces. Armatimonadetes, Omnitrophica and Elusimicrobia were found to be only present on PP. The phylum Armatimonadetes was present with one genus of the family Chthonomonadacea. Omnitrophica was present with two unidentified genera and Elusimicrobia with one unidentified genera. For the PE we identified two unique colonizing phyla, candidate division WS6 with one genus, and a representative of the family Mycoplasmataceae of the Tenericutes phylum. Member of the Verrucomicrobia were found in both the free-living and the plastics-assocaited communities in a proportion distribution, consistent with Oberbeckmann et al. (2016), who also found this phylum in both, the plastic-attached and the free-living community.

The most dominant PET-attached families (Figure 14) were Planctomycetaceae, Flavobacteriacea, Rhodobacteracea, consistent with Oberbeckmann et al. (2016) who's described in their Baltic Sea study, that the families Flavobacteriaceae, Cryomorphaceae, Saprospiraceae, Rhodobacteraceae and Alteromonadaceae, were the most dominant member of the PET-colonizing communities. At family level, Bryant et al. (2016) reported that Rodobacteraceae and Flavobacteraceae were the only abundant clades found in plastic samples as well as in the ambient water. In this study, we also found these families in all the plastic types (PE, PP and PET) and in the ambient water, significant more abundant in the plastic associated community (Figure 14). Members of the Rhodobacteraceae family are known to switch between the planktonic and attached lifestyle and are capable of rapid responses to various carbon sources (Polz et al. 2006). This might explain their high abundance in PP, PE, PET and the ambient water over the three months. We identified members of the Alcanivoracaceae family only in the three plastic types, while Oberbeckmann et al. (2016) found this family associated to PET.

Collectively, 73 families were identified in the two types of plastic (PE and PP) while absent from the ambient water (Figure 13). Similar to other studies we found bacterial clades with members considered to degrade hydrocarbons. Members of Alteromonadaceae, Flavobacteriaceae, Saprospiraceae, Rhodobacteraceae were found in this study in high relative abundance, Oscillatoriaceae was less abundant. These families are well recognized to degrade complex carbon substrates (Palinska & Marquardt 2008; López-Pérez 2012; Freitas et al. 2012). Dubinsky et al. (2013) found members of Pseudoalteromonas that degrade hydrocarbons during the deep-water Horizon oil spill. We found these taxa associated to the different plastic types and in lower abundance in the ambient water. Orr et al. (2004) isolated a strain of *Rhodococcus ruber* and identified it as polyethylene biofilm degrader. We found two genera of *Rhodococcus* associated to four polyethylene (PE) samples.

Hazen et al. (2010) found that Oceanospirallales dominates the bacterial community of deep-sea oil plumes in the Gulf of Mexico. We found this order more abundant on the plastics than in the ambient water. Members of the family Alcanivoraceae are well-known oil degraders (Schneiker et al. 2006) and in our study, were present only on the plastics.

Members of the Cryomorphaceae family contain dioxygenases and haloacid dehalogenases (Riedel et al. 2012) and have been identified as hydrocarbon degraders in soils (Ozaki et al. 2007). Also we found that members of this family are more abundant on plastics than in ambient water.

A group of marine obligate hydrocarbonoclastic bacteria (*Alcanivorax, Marinobacter, Thallassolituus, Cyclocasticus, Oleispira*) plays a significant role in the biological removal of petroleum from marine waters (Yakimov et al. 2007). We found these taxa *Alcanivorax, Marinobacter* and *Cyclocasticus* at low relative abundances only on plastics, while *Thallassolituus* was found in both plastics and ambient water and *Oleispira* were not present. For the unique families (Table 7) detected in PE (20)

and PP (10), we have no evidence that members of these families can use hydrocarbons as their sole carbon source.

Many of the plastic-attached taxa are opportunistic organisms that could grow on any surface including aquatic pathogens (Lyons et al. 2010). Generally, attached bacteria exhibit higher metabolic rates and functional diversity. Also higher concentrations of culturable *Vibrios* and fecal indicator bacteria are found in particle associated than free-living (Lyons et al. 1010). Thus floating plastics might be favorable habitats for these bacteria, specifically vibrios. We detected *Vibrio* spp. in 40 of the 41 plastic samples while in the ambient water vibrios exhibited a much lower relative abundance.

Conclusion

Most of the plastic pieces collected in this study were of small size (microplastics), showing a positive colonization, by microorganisms. From the sequencing data, it can be concluded that there are bacteria attached to plastic pieces of different sizes in the northern Adriatic Sea throughout the seasonal cycle. The community composition of PE and PP was similar between each other along August and November. The plastic community is more similar along the three month of this study than the ambient water. The bacterial community composition of the ambient water changed from February to August and remained then fairly stable until November. In this study the microbial communities of the Northern Adriatic Sea did not show any preferences for the plastic type and sizes.

References

1. American Chemistry Council (2015). Resin review. Washington, DC: American Chemistry Council.

2. Andrady AL. (2011). Microplastics in the marine environment. Marine Pollution Bulletin. 62: 1596–1605.

3. Bryant JA, et al. (2016). Diversity and activity of communities inhabiting plastic debris in the North Pacific Gyre. mSystems 1(3):e00024-16.doi:10.1128/mSystems.00024-16.

4. Browne MA, et al. (2015). Sources and Pathways of Microplastics to Habitats. Springer International Publishing. pp. 229–244. doi:10.1007/978-3-319-16510-3_9. ISBN 9783319165097

5. Carpenter EJ, Smith Jr. KL. (1972). Plastic on the Sargasso Sea surface. Science. 175: 1240-1241.

6. Chao A, et al. (2014). Rarefraction and extrapolation with Hill numbers: a framework for sampling and estimation in species diversity studies. Ecological Monographs. 84 (1) 45-67.

7. Chronopoulou PM, et al. (2015). Generalist hydrocarbon-degrading bacterial communities in the oil-polluted water column of the North Sea. Microbial Biotechnology. 8, 434–447.

8. Cole M, et al. (2011). Microplastics as contaminants in the marine environment. A review. Marine Pollution Bulletin. 62, 2588-2597.

9. Cózar A, et al. (2015). Plastic Accumulation in the Mediterranean Sea. PLoS ONE 10(4): e0121762. doi:10.1371/journal.pone.0121762.

10. Dang H, Lovell CR. (2000). Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNAg gene restriction analysis and sequence analysis of 16S rRNA genes. Applied and Environmental Microbiology. 467-475.

11. Delong EF, et al. (1993). Phylogenetic diversity of aggregate-attached vs free-living marine bacteria assemblages. Limnology and Oceanography. 38(5):924-934.

12. Derraik JGB. (2002). The pollution of the marine environment by plastic debris. A review. Marine Pollution Bulletin. 44:842-852.

13. Didier D, et al. (2017). Plastics in the North Atlantic Garbage Patch: A boat-microbe for hitchhikers and plastic degraders. Science of the Total Environment. 599-600:1222-1232.

14. Dubinsky EA, et al. (2013). Succession of Hydrocarbon-Degrading Bacteria in the Aftermath of the Deepwater Horizon Oil Spill in the Gulf of Mexico. Environmental Science & Ecology. 47:10860-10867.

15. Edgar RC. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads, Nature Methods [Pubmed: 23955772, dx.doi.org/10.1038/nmeth.2604].

16. Eriksen M, et al. (2014). Plastic pollution in the World´s Oceans: more than 5 Trillon Plastic Pieces weighing over 250.00 Tons Afloat at Sea. PloSONE9(12):e111913. doi:10.1371/journal.pone.0111913.

17. Eriksen M, et al. (2013). Plastic pollution in the South Pacific subtropical gyre. Marine Pollution Bulletin. 77:177-182.

18. Freitas S, et al. (2012). Global distribution and diversity of marine Verrucomicrobia. The ISME Journal. 6(8):1499–505.

19. Floodgate G. (1984). The fate of petroleum in marine ecosystems," in Petroleum Microbiology, R. M. Atlas, Ed., pp. 355–398, Macmillion, New York, NY, USA.

20. Hansen J. (1990). Draft deposition statement on plastic debris in marine environments. Fisheries. 15, 16-17.

21. Hazen TC, et al. (2010). Deep-Sea oil plume enriches indigenous oil-degrading bacteria. Science. 330; 204-208.

22. Jambeck JR, et al.(2015). Plastic waste inputs from land into the ocean. Science. 547 (6223):768-71.

23. Jefferson KK. (2004). What drives bacteria to produce a biofilm? FEMS Microbiology Letters. 236, 163–173.

24. Law KW, et al. (2010). Plastic accumulation in the North Atlantic Subtropical Gyre. Science. 392,1185-1188.

25. Lebreton LC, et al. (2012). Numerical modeling of floating debris in the World´s Ocean. Marine Pollution Bulletin. 64 (3):653-661.

26. Lee B, et al. (1991). Biodegradation of Degradable Plastic Polyethylene by Phanerochaete and Streptomyces Species. Applied and Environmental Microbiology. 57:678-685.

27. Lee JW, et al. (2008). Bacterial communities in the initial stage of marine biofilm formation on artificial surfaces. The Journal of Microbiology. 46(2):174-182.

28. LI WC, et al. (2016). Plastic waste in the marine environment: A review of sources, occurrence and effects. Science Total Environment. 566-567: 333-349.

29. Lobelle D & Cunfiffe M (2011). Early microbial biofilm formation on marine plastic debris. Marine Pollution Bulletin. 62:197-200.

30. López-Pérez M, et al. (2012). Genomes of surface isolates of Alt*eromonas macleodii*: The life of a widespread marine opportunistic copiotroph. Scientific Reports. 2:696. PMID: 23019517.

31. Lyons MM, et al. (2010). Theory of island biogeography on a microscopic scale: organic aggregates as islands for aquatic pathogens. Aquatic Microbial Ecology. 60:1-13.

32. MacArthur RH, Wilson EO. (1967). The Theory of Island Biogeography. Princeton University Press, Princeton, NJ.

33. Moore CJ. (2008). Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. Environmental Research. 108:131-139.

34. Moore CJ, et al. (2001). A comparison of plastic and plankton in the North Pacific Central Gyre. Marine Pollution Bulletin. 42:1297–1300.

35. Moret-Ferguson et al. (2010). The size mass and the composition of plastic debris in the western North Atlantic Ocean. Marine Pollution Bulletin. 60: 1873-1878.

36. Oberbeckmann S, et al. (2014). Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European Waters. FEMS Microbial Ecology. 90: 478–492.

37. Oberbeckmann S, et al. (2016). Microbes on a Bottle: Substrate, Season and Geography Influence Community Composition of Microbes Colonizing Marine Plastic Debris. PLoS ONE 11(8): e0159289. doi:10.1371/journal.pone.0159289.

38. Orr G, et al. (2004). Colonization, biolfilm formation and biodegradation of polyethylene by strain of *Rhodococcus Ruber.* Applied Microbial Biotechnology. 65:97-104.

39. Ozaki S, et al. (2007). Change in the predominant bacteria in a microbial consortium cultured on media containing aromatic and saturated Hydrocarbons as the sole carbon source. Microbes and Environments. 22(2):128-135.

40. Palinska KA, & Marquardt J. (2008). Genotypic and phenotypic analysis of strains assigned to the widespread cyanobacterial morphospecies *Phormidium autumnale* (Oscillatoriales). Archives of Microbiology. 189 (4):325–35. PMID: 18034229

41. PlasticEurope (2016). Plastics – The facts Brussels. http://www.plasticseurope.org/plastics-industry/market -and- economics.aspx. Accessed on October 5, 2017.

42. Polz MF, et al. (2006). Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. Philosophical Transactions of the Royal Society B. Biological Sciences. 361:2009–2021.

43. Rath J, et al. (1998). High phylogenetic diversity in a marine-snow-associated bacterial assemblage. Aquatic Microbial Ecology. Vol. 14:261-269.

44. Riedel T, et al. (2012). Genome sequence of the orange pigmented seawater bacterium *Owenweeksia hongkonensis* type strain (**UST20020801^T).** Standards in Genomic Science. 7(1):120-30.

45. Ryan PG, et al. (2009). Monitoring the abundance of plastic debris in the marine environment. Philosophical Transactions of the Royal Society. B Biology Science. 364 (1526), 1999-2012.

46. Schneiker S, et al. (2006). Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax bokumensis.* Nature biotechnology. 24(8):997-1004.

47. Shah AA, et al. (2008). Biological degradation of plastics. A comprehensive review. Biotechnology Advances. 26 (3):246-265.

48. Suaria G, et al. (2016). The Mediterranean Plastic Soup: synthetic polymers in Mediterranean surface waters. Scientific Reports. 6:37551.DOI: 10.1038/srep37551.

49. Zettler ER, Mincer TJ & Amaral-Zettler LA (2013). Life in the "Plastisphere": Microbial communities on plastic marine debris. Environmental Science & Technology. 47:7137-7146.

50. Yakimov MM, et al, (2007). Obligate oil-degrading marine bacteria, Current Opinion in Biotechnology. Vol. 18, no. 3, pp. 257–266.

51. https://www.seatemperature.org/europe/croatia/rovinj-february.htm

I Supplementary Information

A) DNA extraction protocol modified bead-beating approach

1. Produce 1000 u/ml Lysozyme
2. Add 500 µl cell lysis solution in each tube
3. Add 1 plastic piece to each tube (2)
4. Add 10 µl of lysozyme
5. Incubate at 37°C for 30 min
6. Add 5 µl proteinase K and invert 25 times
7. Add 0.325 g of beads per tube
8. Bead-beat twice for 45 sec
9. Incubate at 55°C for 30 min
10. Add 4 µl RNAse and mix by inverting 50 times
11. Incubate at 37°C for 30 min
12. Incubate on ice for 5 min
13. Add 250 µl Protein precipitation solution and vortex at high speed for 20 sec.
14. Centrifuge for 3 min at 1400 rcf (put on ice for 5 min if proteins are not tightly packed)
15. Take out the plastic piece in a tube and stored in at -4°C
16. Pipet 750 µl 100% Isopropanol into clean tube
17. Add supernatant from (14)
18. Mix by inverting 50 times
19. Centrifuge for 5 min at 1400 rcf
20. Discard supernatant and drain tube by inverting on clean piece of paper
21. Add 750 µl ethanol and invert several times
22. Centrifuge for 3 min at 1400 rcf
23. Discard supernatant and drain tube on paper
24. Air dry for 15 min, until ethanol in evaporated
25. Re-suspend in 40 µl Hydration solution
26. Incubate alt 65°C for 45 min
27. Store at -80°C

B) Table.TukeyHSD test result for the plastic sizes collected in November

$Size	diff	lwr	upr	p adj
M2-M1	20128.97	-5623.203	45881.14	0.1500071
M3-M1	38117.47	6577.628	69657.31	0.0164913*
M4-M1	24669.97	-11749.102	61089.04	0.2416345
M3-M2	17988.50	-13551.342	49528.34	0.3749019
M4-M2	4541.00	-31878.072	40960.07	0.9825040
M4-M3	-13447.50	-54165.261	27270.26	0.7687480

Plastic sizes: M1: >9.5 mm; M2: 9.5-4.75 mm; M3: 4.75 – 1.4 mm; M4: 1.4 mm-500 µm. diff= difference in the observed means, lwr= lower point of the interval, upr = upper point, p adj the p-value after adjustment for the multiple comparisons.*Significant difference.

Zusammenfassung

Die Menge an marinen Plastikabfällen im Ozean nimmt weltweit zu und ihr Einfluss auf Meeresorganismen wie Säugetiere, Vögel und Fische hat in letzter Zeit zunehmend Aufmerksamkeit erfahren. Die Auswirkungen von Kunststoffen auf die Umwelt und die Auswirkungen auf Meeresorganismen an der Basis der Nahrungsnetze sind noch weitgehend unbekannt. Die Wechselwirkung zwischen der mikrobiellen Biofilmgemeinschaft in Verbindung mit Plastik und ihrer Rolle in der Biogeochemie des Ozeans wurde im Rahmen dieser Arbeit untersucht. Verschiedene Plastiksorten (Polypropylen [PP], Polyethylen [PE] und Polyethylenterephthalat [PET]) verschiedener Größe (> 9,5 mm, 9,5-4,75 mm; 4,75-1,4 mm; 1,4 mm-500 um) wurden in der nördlichen Adria im Februar, August und November gesammelt, um festzustellen, ob sich die Zusammensetzung der mikrobiellen Gemeinschaft, die an den Kunststoffen haftet, von der freilebenden Bakteriengemeinschaft unterscheidet. Die meisten der in dieser Studie gesammelten Plastikstücke waren klein (Mikroplastik) und von Mikroben besiedelt. Alle Größenklassen und alle Arten von Kunststoffen wurden während des gesamten saisonalen Zyklus von Mikroben kolonisiert.

Die Zusammensetzung der Mikrobengemeinschaft der Kunststofftypen unterschied sich von der des umgebenden Wassers. Die Zusammensetzung der bakteriellen Gemeinschaften auf PE und PP war ähnlich. Insgesamt zeigten die mikrobiellen Gemeinschaften der Adria keine Präferenzen für bestimmte Kunststofftypen und -größen. Diese Studie legt nahe, dass Plastik einen neuen Lebensraum für die mikrobielle Gemeinschaft im Ozean bietet. Viele auf Plastik haftende Bakterien gehören zu den Phyla Proteobacteria, Cyanobacteria, Bacteroidetes und Planctomycetes. Wir identifizierten einige Mitglieder der Familien Alteromonadaceae, Oscillatoriaceae, Flavobacteriaceae, Saprospiraceae und Rhodobacteraceae, von denen bekannt ist, dass sie Kohlenwasserstoffe abbauen und einen vorzugsweise partikelassoziierten Lebensstil aufweisen. Zusammenfassend stellen wir fest, dass sich die Plastik-assoziierte mikrobielle Gemeinschaft von der freilebenden Gemeinschaft unterscheidet und Bakteriengruppen enthält, die vermutlich Kohlenwasserstoffe

nutzen können. Das Ausmaß der Abbaubarkeit von Kunststoffen im Meer durch Mitglieder der kunststoffassoziierten Bakteriengemeinschaft muss noch untersucht werden.