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Microbial communities of polyhydroxyalkanoate (PHA)-based biodegradable composites plastisphere and of surrounding environmental matrix: a comparison between marine (seabed) and coastal sediments (dune sand) over a long-time scale

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Abstract.

Most researches on the plastisphere in coastal environments deal with plastics floating in seawater. Comparatively smaller attention has been devoted to the plastisphere of plastics buried in marine sediments, and very little is known on that of plastics on coastal sand dunes. Yet, limited information is available on the impact of plastics, especially biodegradable plastics, on microbial organisms in their surroundings. Nevertheless, a large amount of plastics sink on the seabed or is deposited on beach-dune systems.

We investigated the succession of plastisphere microbial community on two biodegradable composites based on poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) and seagrass fibres (PHBV/PO), buried in seabed and dune sediments over a 27 months period in mesocosm. PHBV is

regarding as a valuable alternative to conventional plastics and PHBV/PO has recently been designed for applications in coastal habitat restoration. We also examined the degradation rate and impact of these plastics on the microbial communities of surrounding sediments.

Microbial communities of the surface of PHBV and PHBV/PO in seabed and dune sand differ from those of surrounding sediments, displaying a lower richness. Plastics colonization occurs largely from bacteria present in surrounding sediments, although the contribution from the water column bacterial pool could be not negligible for plastics in the seabed. No significant differences were detected between the communities of the two plastics and no significant impact of plastics on microbial community of the surrounding sediments was detected. The exceptional long duration of this study allowed to gain information on the succession of a plastisphere community over a previously unexplored time scale. Succession appears highly dynamic in dune sand even after two years, while the community structure in seabed seems to reach stability after one year. These findings highlight the importance of performing long-term studies when trying to characterize composition and dynamics of plastisphere bacterial communities.

1. Introduction.

Plastic release in natural terrestrial and aquatic environments has reached such a high level on earth, that it can now be considered a marker of the Anthropocene (Duis and Coors, 2016; Zalasiewicz et al., 2016). Despite the efforts made by many countries to organize and encourage a correct disposal of plastic wastes, a huge amount of plastics still ends up in the marine environment (Jambeck et al., 2015) and the deposition of these materials on coastal organisms and ecosystems will not decrease in a short time (World Economic Forum, Ellen MacArthur Foundation, McKinsey & Company, 2016). Therefore, mitigating this impact to preserve the quality of coastal matrices will remain an absolute priority, at least in the next future. In this frame, increasing research efforts are focusing on the development of viable alternative to conventional plastics, such as biodegradable plastics (Helanto et al., 2019; Lambert and Wagner, 2017). These plastics include those produced from natural materials such as cellulose, starch and more generally polyesters, such as

polyhydroxyalkanoates (PHAs). As the global market for biodegradable plastics is expected to rise in the future (European bioplastics, 2018), a rigorous and deep evaluation of the behavior of these materials in nature, their interaction with living organisms and, in general, of their possible impact on the health of the environmental matrices they could come in contact with, is needed.

Colonization of conventional plastics by microbial communities has been largely studied and assessed, and plastic debris are now regarded as an additional habitat for microbes, defined as the so-called "Plastisphere" (Zettler et al., 2013). Besides the identification of bacterial components involved in plastic degradation (Danso et al., 2019; Jacquin et al., 2019; Krueger et al., 2015; Restrepo-Florez et al., 2014), environmental microbiologists are focusing on one side on a deeper understanding of factors driving the composition of communities colonizing plastics and, on the other side, on the possible impact of plastics on microbial communities living in their surroundings (Bryant et al., 2016; Dussud et al., 2018; Ogonowski et al., 2018). However, most of the available information on the interactions between microbes and plastics comes from studies conducted in standardized laboratory conditions over a limited period of time (Amaral-Zettler et al., 2020; Jacquin et al., 2019). Further researches need to be performed *in situ* or in conditions resembling as much as possible the natural ones and on an appropriate timescale in order to gain a clear picture of the processes really undergoing in nature. Moreover, the majority of the studies related to the plastisphere have dealt with organisms living in the surface of floating plastics and in the water column, and little attention has been devoted to those growing in plastics buried in sediments (Amaral-Zettler et al., 2020; Jacquin et al., 2019). Nevertheless, sediments are ecologically important compartments accounting for the highest fraction in the marine environment, and a considerable amount of plastic wastes entering this environment sinks to the seabed or is stranded on the shore (Browne et al., 2007; Hidalgo-Ruz et al., 2012). Lastly, there is a paucity of information on the microbial colonization process of plastics deposited on sand dune systems, despite a relevant fraction of plastic litter ends up on beaches and sand dunes (Andriolo et al., 2020; Ceccarini et al., 2018; De Francesco et al., 2019; Rangel-Buitrago et al., 2018; Šilc et al., 2018).

Microbial communities living in marine and dune sediments are well-known to play a critical role in global biochemical cycles and energy transfer (see for example Orsi, 2018 and Whitman et al., 2014). The presence of biodegradable plastics in these sediments could influence these communities in different ways, for example acting as a physical barrier, modifying microclimate conditions and adding carbon, organic and inorganic products of their degradation. Thus, the acquisition of knowledge on potential interactions between microbial communities of these environmental comparts and biodegradable plastics is of particular ecological interest.

In this paper, we focused on the colonization of plastics made with poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), a bio-based polymer belonging to the class of PHA, and surrounding sediments by microbial communities living in seabed and dune sand. A seagrass fibre reinforced PHBV composite, hereinafter referred to as PHBV/PO (Seggiani et al., 2017; Seggiani et al., 2018), was also investigated. PHAs are a family of biodegradable polyesters naturally produced by bacteria for intracellular storage of carbon and energy (Anjum et al., 1999; Rutkowska et al., 2008; Tsuji and Suzuyoshi, 2002; Volova et al., 2010; Volova et al., 2017). The good performance of PHAs, in terms of mechanical, rheological, thermal and morphological properties and their biodegradability both in terrestrial and marine environments (Corre et al., 2012; Dussud et al., 2018; Elain et al., 2015, 2016), make these polymers a promising alternative to traditional plastics as well as potential candidates for applications in marine and coastal habitats, including restoration interventions (Balestri et al., 2019; Meereboer et al., 2020; Seggiani et al., 2018). Previous studies have shown that various bacteria are able to degrade PHAs in different conditions (Ammala et al., 2011; Boyandin et al., 2012; Jendrossek and Handrick, 2002; Sudhakar et al., 2008; Tokiwa and Calabia, 2004; Volova et al., 2010; Volova et al., 2017). However, extensive and deep characterizations of communities associated to PHAs using modern techniques such as high-throughput sequencing methods have been done on a short timescale (Dussud et al., 2018; Pinnell and Turner, 2019). In view of a possible large use of PHBvs in coastal marine applications, it is therefore extremely interesting to know whether the entering of these plastics in marine sediments and coastal dunes

may affect local microbial communities and more in general coastal ecosystems. In this study, we investigated in mesocosm over a long term (27 months) (i) the succession of the plastisphere microbial community developing on PHBV and PHBV/PO manufacts buried in seabed sediment and dune sand and (ii) the impact of these manufacts on the microbes present in the surrounding sediment by using high-throughput sequencing methods. Qualitative data on the behavior of PHBV and PHBV/PO manufacts in these compartments were also provided.

2. Materials and methods.

2.1. Seabed and dune sand mesocosm and plastic material.

Mesocosms were established at the INVE Aquaculture Research Center at Rosignano Solvay (Italy) in June 2016. Seabed mesocosms consisted of containers (45 cm x 20 cm, 15 cm height) filled with a mixture (1: 1) of commercial fine sand (organic matter < 0.01%) and carbonate sand collected for the seabed near to the dune area around the center. The containers were placed on the bottom of an outdoor tank (7000 L) equipped following previously established protocols (Balestri and Lardicci, 2012, Balestri and Lardicci 2006). Briefly, a seawater circulating system taken the water from the open sea in front of the Aquaculture center (Ligurian Sea, 43° 23' 00" N, 10° 26' 00" E). Before entering the tank, the seawater flowed to mechanical filters that removed marine debris. The water inlet and outlet were positioned so to create a circular water flow within the tank. The seawater in the tank was renewed continuously at a rate of approximately 2 m^3/h. Seawater chemical/physical variables such as temperature, oxygen levels, nutrients concentrations and salinity were dependent on natural environmental conditions. Seawater temperature ranged from 11 to 28 °C, pH was 8.0–8.2, and salinity varied between 37.6 and 38.2 over the experimental period.

Dune sand mesocosms consisted of the same containers as described above. Mesocosms were filled with a mixture (1: 1) of commercial sand and sand collected from mobile dunes and placed outdoor, about 1 m apart each other in a back-dune area at the Aquaculture Center where they were exposed

to natural environmental conditions (e.g. air temperature, salt spray and atmospheric precipitations). Mean air temperature ranged from 4.6 to 29.8 °C over the experimental period.

Manufacts (Haake III type dog-bone tensile bars: width 10 mm, width in the narrow section 4.8 mm, thickness 1.35 mm, length 90 mm) made of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) and on PHBV with 20% fibres of *Posidonia oceanica* (PHBV/PO) were produced as described elsewhere (Seggiani et al., 2017; 2018). Dog-bones were dried overnight at 35 °C and individually weighted using a high-precision balance. They were sterilized by washing with 70% ethanol immediately before the onset of the experiment (June 2016), individually placed in a sterilized plastic net and then buried in sand in mesocosms at approximately 5 cm of depth. In each mesocosm, 13 dog-bones made of PHBV or PHBV/PO were buried. There were three replicates mesocosm for each type of dog-bone. Three seabed and three dune sand mesocosms containing only sediment but no dog-bones were also established and used as controls. In total, there were nine seabed mesocosms and nine dune sand mesocosms. The experiment was run for 27 months (June 2016 to September 2018). All mesocosms were weekly reallocated in randomly chosen positions during the experimental period.

2.2. Sampling.

Sediment samples were collected at the onset of the experiment in June 2016 (T0), i.e. immediately before marine and dune exposure, for initial microbial community characterization. Sediment and manufact samples were then retrieved from mesocosms in September 2016 (T1), i.e. at the end of the first summer season, and in June 2017 (T2), i.e., at the end of the first year of exposure to marine and dune sediments, taking into account for the degradation rate of PHBVs under natural conditions reported in previous studies (in Meereboer et al., 2020). Since at this stage all manufacts did not show substantial visible signs of degradation, the period of exposure of remaining samples in mesocosms was prolonged until September 2018 (T3) so to entirely cover the second summer season of sediment incubation. This is because in summer the high temperatures reached in the

Mediterranean Sea and dunes are favourable to biodegradation while in winter and spring the temperatures are often lower than 5 °C and thus below the optimal conditions for biodegradation (Mergaert, et al., 1995). At each sampling, three PHBV and three PHBV/PO dog bones were collected (one sample for mesocosm) and individually placed in sterile Falcon tubes together with the surrounding sediments for microbial community characterization. Only sediments were collected from control mesocosms. Immediately after sampling, sediments were separated from dog-bone manufacts. Dog-bones surface was mildly washed, in order to discharge any non-stuck material. From each dog-bone, a piece corresponding to a 1 x 1 cm surface was cut and immediately treated for Scanning Electron Microscopy observation (see below). Residues of manufacts and sediments were separately stored at -20° C for DNA extraction. For qualitative degradation assessment, PHBV and PHBV/PO dog-bone samples were retrieved monthly from each of the three mesocosms during the period from T0 to T1, and every two-three months during the subsequent period until the occurrence of manufact fragmentation. Three virgin dog-bones maintained in laboratory were also sampled each time and used as control.

2.3. Electron microscopy and qualitative degradation assessment of plastics.

For Scanning Electron Microscopy observation, the 1 x 1 cm pieces cut from dog-bones were fixed in 2% OsO4, dehydrated in ethanol and, after critical point drying, coated with gold and observed with a JEOL/JSM-5410 scanning electron microscope. In order to assess the degradation status, in terms of weight loss, each collected dog-bone was washed with sterile seawater, dried overnight at 35 °C and weighted. The weight loss of each manufact was calculated as the difference between the dry weight recorded at each sampling time (residual weight) and the weight before the start of the experiment (T0) and expressed as percentage.

2.4. SSU rRNA gene amplification and sequencing.

From each dog-bone, a portion corresponding to a 1.5 x 1 cm surface was cut and total genomic DNA was extracted using the DNeasy PowerSoil Kit (Qiagen). Total genomic DNA extraction was performed also on 10 gr of sediments (manufact-associated sediments and control sediments) using the DNeasy PowerMax Soil Kit (Qiagen). For amplification, a concentration of 1000 ng/mL of tgDNA was used. Replicas of each treatment were pooled before amplification. PCR was performed using the KAPA HiFi HotStart Ready Mix with the prokaryotic primer set for the V3–V4 regions of the SSU rRNA gene suggested by Klindworth and colleagues (2013). The Illumina overhang adapter sequences added to the forward and reverse primers were 5′-TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG-3′ and 5-GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAG-3′, respectively (Illumina protocol, Part # 15044223, Rev. B). Amplicons were barcoded, thus obtaining a sequencing library from each sample. They were then pooled, and sequenced on the Illumina MiSeq platform (2 × 300 paired-end sequencing) by IGATech (Udine, Italy).

2.5. Sequence analysis.

Obtained sequences (raw reads) have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under study accession number PRJEB39187. Raw reads of prokaryotic V3–V4 regions obtained by Illumina MiSeq were analysed using the Quantitative Insights Into Microbial Ecology version 2 (QIIME2, https://qiime2.org) software package (Bolyen et al., 2019). Reads were truncated at 260 bp length to remove the lower-quality last base calls. After that, quality filtering, primer trimming, and pair-end read merging were performed with DADA2 (Callahan et al., 2016), with de novo chimera removal. Only unique sequence variants represented by 10 sequences or more and detected in at least two samples were retained. Sequence variants were then aligned using MAFFT (Katoh and Standley, 2013) and a phylogenetic tree was inferred with FastTree (Price et al., 2010). Three long-branching sequence variants were manually inspected and removed as clearly recognized as additional, non-detected chimeras. Release 132 of the Silva database (Quast et al.,

2013) was used for taxonomic assignment of sequence variants. A Naive Bayes classifier was trained extracting the regions of interest from SSU rRNA representative sequences (99% similarity clustered Operational Taxonomic Unit) as recommended by Werner and colleagues (2012). Sequence variants identified as mitochondria or chloroplasts were removed before further data processing. Bar plots, heatmaps and calculation of core community between couples of samples were also produced using QIIME2. The presence of bacterial genera formerly reported as PHA/PHB-degrading was checked using two specific databases as reference: http://pmbd.genome-mining.cn/home/ (Gan and Zhang, 2019) and http://www.ded.uni-stuttgart.de/ (Knoll et al., 2009).

2.6. Statistical analysis.

For statistical analysis of prokaryotic community data, all samples were normalized randomly extracting an equal number of sequences from each library (corresponding to the number of merged, quality-filtered reads in the smallest library). Rarefaction curves, alpha-diversity and beta-diversity analyses were performed using QIIME2. Alpha-diversity was estimated by calculating four different indexes: sequence variant number, Faith's Phylogenetic Diversity (Faith-PD, a qualitative index using phylogenetic information), and Shannon's (quantitative, non-phylogeny-based index) for richness and Pielou's Evenness for evenness. Comparison among index values for different communities was performed by the Kruskal-Wallis non-parametric test. Different metrics (Bray-Curtis and Jaccard for quantitative and qualitative data, respectively, and Uni-Frac distances, both weighted and unweighted, to assess the impact of phylogeny) were calculated and used for assessing beta-diversity by multivariate PCoA and Permanova. Vectors associated to the 23 OTU most correlated with the ordination axes were superimposed onto the PCoA plots. Vector correlation was calculated by Pearson correlations, and the length and the direction of each vector indicate the strength and sign, respectively, of the relationship between that OTU and the PCO axes (Anderson et al., 2008). PCoAs were carried out in PRIMER v6 (Primer-E Ltd., Plymouth) with PERMANOVA add-on software (Anderson et al., 2008; Clarke and Gorley, 2006). The R software

package was used to produce Venn diagrams between sediments and PHBVs manufacts for seabed and dune sand environments, in order to highlight the number of shared sequence variants between the two habitats.

3. Results.

3.1. PHBV and PHBV/PO degradation in seabed and dune sand mesocosm.

The trend of the residual weight of PHBV and PHBV/PO dog-bones expressed as percentage relative to the original weight as a function of burial times in the seabed and dune sediments is presented in Figure 1. During the first two months of burial, the weight of manufacts remained substantially unchanged. After this lag time, it decreased gradually indicating that the degradation process occurred slowly. On average, the total percentage of weight loss relative to the original weight of PHBV manufacts was smaller than that of PHBV/PO regardless of the sediment environment. After 12 months (July 2017), the total mean weight loss of PHBV/PO manufacts buried in seabed was larger (about 23%) than that of manufacts buried in dune sediments (about 9%). The recording of weight ceased in July 2017 for the manufacts exposed to seabed sediment and in September 2017 for those exposed to dune sediment due to their subsequent fragmentation and the difficulty in distinguishing the pieces belonging to the different manufacts present in the sediment. However, the degradation process did not end at this stage. Indeed, plastic fragments of samples retrieved for microbial characterization were still present in the subsequent year (September 2018).

3.2. Seabed and dune sand microbial community composition.

Seabed microbial communities.

Final dataset of seabed samples, after quality filtering and chimera check, consisted of 1,407,895 sequences, with 74,099 ± 23,910 mean sequences per library. The library containing most sequences was obtained from the PHBV/PO manufacts (126,696), the one with fewest from the

PHBV-associated sediments (30,333), both collected at T2. Number of sequence variants and values of calculated indexes are reported in Table 1. Rarefaction curves reached plateaus, thus confirming that sequencing depth was sufficient to sample all sequence variants in the libraries (data not shown). In all bacterial communities, the most represented phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria* (Figure 2). Despite the use of bacterial primers, a small percentage of sequences affiliated to *Archaea* was retrieved (relative abundance per sample up to 0.78%). Among bacteria formerly known as PHA/PHB-degrading bacteria (Gan and Zhang, 2019; Knoll et al., 2009), only bacteria belonging to the genera *Aestuariibacter* and *Ruegeria* were detected in microbial communities colonizing manufacts buried in seabed, with very low relative abundances (0.04% to 0.10%). SEM observations on seabed-buried manufacts revealed a conspicuous and rich bacterial biofilm coverage on both PHBV and PHBV/PO, at all time-points (Figure 3). Diatom frustules (frequently) and fungal hyphae (rarely) were also observed.

Dune sand microbial communities.

Obtained dataset for dune sand samples comprised 1,669,743 sequences (87,881 ± 31,981 mean sequences per library). The highest number of sequences was obtained from library of PHBV/PO manufacts at T2 (156,757), the lowest from library of PHBV-associated sands at T3 (31,991). Number of sequence variants and values of calculated indexes are reported in Table 1. Rarefaction curves reached plateaus, thus confirm that sequencing depth was sufficient to sample all sequence variants in the libraries (data not shown). Again, the most represented phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria* (Figure 2). Bacteria belonging to the genus *Skermanella* (*Alphaproteobacteria*, *Proteobacteria*) were substantially represented in libraries from dune sand samples (percentage abundance ranging from 0.99% to 34.7%). A negligible fraction of sequences affiliated to *Archaea* was also retrieved (relative abundance per sample up to 0.16%). Several genera or species previously reported as PHA/PHB-degrading bacteria were detected in microbial communities colonizing manufacts buried in dune sand, like *Pseudomonas*, *Acidovorax*,

Altererythrobacter, *Brevundimonas*, and *Paucimonas* (Gan and Zhang, 2019; Knoll et al., 2009). SEM observations on dune sand-buried manufacts showed the presence of a partial and patchy microbial biofilm coverage on PHBV and PHBV/PO, with occurring fungal hyphae (Figure 3).

3.3. Comparison among different microbial communities.

The difference in environmental matrix (seabed *versus* dune sand) clearly turned out to be the strongest factor driving microbial community composition. Indeed, when analyzing the entire data set, differences between dune sand and seabed data were always the strongest and clearest. When all samples were plotted together in a PCoA analysis, the two groups clearly emerged (Figure S1). This striking difference was also supported by Permanova tests (Table S1A), which always gave highly significant results ($p<0.01$), when comparing seabed and dune sand bacterial communities. Kruskal-Wallis test, on the contrary, gave a barely significant value ($p<0.05$) when comparing Faith-Pd indexes, and no significance when comparing number of sequence variants as well as Evenness and Shannon (Table S2A).

When considering separately data from dune sand and seabed, sampling time emerged as the most important factor in the first matrix, while microhabitat (manufact surface or sediment) was the most important in the second. Indeed, while communities from PHBVs and sediments in seabed diverge early in time, this is not true in dune sand. The different trends are clearly visible in PCoA analyses performed on the two datasets (Figure 4) and are supported by inferential statistic results (Table S1B-E, S2B-E). For dune sand samples, Permanova always gave highly significant values when comparing different sampling times ($p<0.01$), while p values were only barely significant when comparing different microhabitats ($p<0.01$ only with Bray-Curtis distance matrix). Similarly, p values of Kruskal-Wallis tests on sequence variance number and diversity indexes were more significant for different sampling times than for different microhabitats. For seabed samples, Permanova results were always highly significant ($p<0.01$), for different sampling times as well as

for microhabitats. Probability values from Kruskal-Wallis tests were always significant ($p<0.05$ or $p<0.01$) for different microhabitats, but never ($p>0.05$) for different sampling times.

No significant differences ever emerged when comparing communities colonizing different kind of manufacts (PHBV Vs PHBV/PO, Table S1F-G, S2F-G) or when comparing communities from manufact-associated sediments with those from control samples (Table S1H-I, S2H-I, Figure S2).

Sequence variants mostly correlated with the ordination axes in all the analysed sample groups display a heterogeneous taxonomic affiliation, even at the phylum level (Table S3, Figures 4, S1, S2).

Calculation of core community between couple of samples (manufact-colonizing community and community of manufact-associated sediment at each time point) revealed an average percentage of 49.67% ± 6.18% shared sequence variants for seabed samples (49.03% ± 6.82% for PHBV and 44.30% ± 5.67% for PHBV/PO) and of 61.19% ± 13.19% for dune sand samples (55.91% ± 18.82% for PHBV and 61.10% ± 7.71% for PHBV/PO). Venn diagrams calculation showed that 76.96% of sequence variants colonizing PHBVs in seabed are shared with sediments. The same value is 83.62% for PHBVs in sand dune (Figure 5).

Heatmaps with the 100 most abundant taxa (at genus-level) among those colonizing manufact surfaces were produced for seabed and dune sand samples, separately. In both habitats most of the taxa are present in both manufact-colonizing and in sediment communities. Nevertheless, the fraction of taxa which are totally absent in sediment samples is higher in seabed (Figure 6, Figure S3, Tables S4, S5).

4 Discussion

4.1. PHBV- and PHBV/PO-colonizing microbial communities in seabed and dune sand.

The bacterial communities colonizing PHBV and PHBV/PO manufacts differ from those of the surrounding sediments and their richness is generally lower. As shown by the number of sequence variants and index values, in both the environmental matrices the manufact surface constituted a

more selective microhabitat if compared to sediments. A similar trend was already found for bacterial communities colonizing plastics in aquatic environments (Amaral-Zettler et al., 2020; Harrison et al., 2014; Ogonowski et al., 2018; Zettler et al., 2013). Few studies have been performed up to now in sediments and a clear trend did not emerged yet. Nevertheless, in past studies similar levels of richness were reported between communities of plastics and of sediments (De Tender et al., 2015; Woodall et al., 2018). Recently, a lower number of bacterial components has been also reported in stranded microplastics with respect to sediments (Wu et al., 2020). In the present study, plastic manufacts, originally sterile, were steadily buried in sediments. Therefore, our finding of a lower richness on plastic manufacts actually reflects a colonization process that occurred entirely *in situ*. Anyway, more studies are still required to further confirm an eventual higher selectivity of plastic surfaces as bacterial microhabitat in marine sediments.

Calculated core communities, together with Venn diagrams and produced heatmaps, show that the microbial taxa colonizing manufacts are largely recruited from the surrounding sediments. This is in line with previous studies, reinforcing the hypothesis that plastic-colonizer bacteria largely come from their surrounding environment (Jiang et al., 2018). Nevertheless, it is worthy of mention that both Venn diagrams and heatmaps of taxa dominating plastic-colonizing communities indicate that the contribution of communities from the seawater could be not negligible, especially for manufacts buried in seabed. A wider range of potential colonizers is therefore available for plastics in seabed, and this could represent an additional factor explaining the faster degradation rate observed in seabed with respect to dune sand (see section 4.2).

The length of the performed experiment allowed us to observe the succession of the bacterial communities over 27 months, an exceptionally long period, if compared to most of the previously performed studies (Amaral-Zettler et al., 2020; Jacquin et al., 2019). Several studies focusing on plastic-colonizing community succession have been performed mainly in seawater (Dang and Lovell, 2000; De Tender et al., 2017; Salta et al., 2013), but not in marine sediment. In these studies, changes in biofilm succession on plastics over short periods of time have been already

observed (see for example Pinto et al., 2019). We found here that changes in the microbial communities colonizing manufacts occurred in both environments up to one year, as outlined by PCoA graphs and Permanova tests (Figure 4, S1, Tables S1, S2). Long-term changes in the community composition of microorganisms that colonize plastics have been reported in a one-year study in seawater (Xu et al., 2019). Our results depict these bacterial communities as highly dynamics and continuously modifying in sediments, too. Moreover, we were also able to highlight a difference in succession length and process between seabed and dune sand matrices. Indeed, while in seabed communities colonizing PHBVs diverge early in time from those living in surrounding sediments (already after three months), this is not true in dune sand environment (see Figure S1). In addition, significant changes no longer occurred after one year in manufact-colonizing community, succession seems to go on at least up to the end of the experiment (more than two years) in dune sand. This difference could be due to a higher heterogeneity in abiotic parameters for dune sand environment, where, for example, temperature variations are more pronounced, thus preventing an overall stabilization of the bacterial community.

4.2. Degradation and impact of PHBV and PHBV/PO in seabed and dune sand.

The research in the area of the biodegradation of PHA-based bio-composites in natural marine and coastal environments is still limited. The changes of weight of dog-bone samples buried in marine and dune sediments over time detected in our study indicate that PHBV manufacts made with lignocellulosic fibres of *P. oceanica* (PHBV/POs) degraded faster and to a larger extent than those without fibres (PHBVs). This finding is in agreement with results of previous studies on the behaviour of other PHA bio-composites in seawater, providing further evidence that the addition of natural fibres, such as cellulose and hemicellulose, into the PHA polymer matrix accelerates the biodegradation process (Meereboer et al. 2020). This is probably because the presence of fibres promotes water uptake and allows microbial enzyme permeation into the amorphous fractions (Meereboer et al. 2020). Our study also revealed that the degradation of PHBV/PO and PHBV

samples proceeded faster in the seabed than in the dune sand. This could be due to the constant presence of seawater, the mechanical abrasion of the plastic surface caused by sediments under seawater flow and a higher general stability of abiotic conditions in the seabed than in dune sediments. For instance, seawater temperature did never decrease below 10° C while the temperature in dune sand in winter often decreased below 5 °C, and it is known that the biodegradation of PHAs at this temperature is negligible (Mergaert, et al., 1995). The degradation process observed here occurred more slowly than as observed in previous studies on tensile samples made with PHBV/corn starch composites. Indeed, these latter achieved a mass loss of 80-100% after about one year of immersion in coastal tropical waters (Imam et al., 1999). However, this different degradation behavior could be related to a variety of factors, including environmental conditions, alongside the different formulation of the composites.The present study focused on whole microbial communities, more than on specific microbial components, nevertheless we compared our datasets with known information on PHA/PHB-degrading bacteria (Gan and Zhang, 2019; Knoll et al., 2009). Only a few genera of bacteria previously described as PHA/PHB-degrading were detected in communities colonizing manufact surfaces, some of which were previously reported in biofilms grown on poly(3-hydroxybutyrate-co-3-hydroxyhexanoate, PHBHHx, Morohoshi et al., 2013). Nevertheless, their number and relative abundances were low and even negligible for what concerns manufacts buried in seabed sediments. Moreover, many of these genera did not increase their relative abundance in time, on the contrary in many cases their abundances decreased up to a complete depletion from datasets. This could indicate a change over time in the degrading roles inside the community and underlines the importance of long-term studies in order to get a complete picture of the degrading-community dynamics. However, it is important to keep in mind that high caution must be used when inferring functions only from taxonomic assignment (Amaral-Zettler et al., 2020; Knight et al., 2018). Furthermore, many PHA/PHB-degrading bacteria might be still undiscovered (Gan et al., 2019) and the hypothesis that complex microbial communities, rather than single bacterial components, are responsible for these

processes is increasingly supported (Jacquin et al., 2019). The heterogeneity in taxonomic affiliation found even within sequence variant groups mostly contributing to spatial distribution of samples in multivariate analysis seems to support this view.

Comparison between communities colonizing the two polymers did not outline any significant difference. The structure and composition of these communities differed, of course, greatly between seabed and dune sand environments and among different sampling times, but the kind of polymer (PHBV Vs PHBV/PO) did not play a fundamental role. Therefore, observed differences in degradation time between PHBV and PHBV/PO cannot be explained by substantial differences in the colonizing bacterial communities. The dissimilar intrinsic characteristics of the two formulates (Seggiani et al. 2017) surely play an important role, even assuming all the rest being absolutely equal, bacterial community composition included. Indeed, the presence of cellulose fibres *per se* favours the fragmentation (and hence the degradation) by increasing water uptake (Ferrero et al., 2015; Khiari et al., 2011; Le Duigou et al., 2014). Absence of material selectivity by plastic-colonizing bacteria has been previously shown for some non-biodegradable polymers (Oberbeckmann et al., 2016; Witt et al., 2011), and the differences in physical-chemical properties of the material have been proposed as the main factor influencing their degradability (Xu et al., 2019). Data here presented confirm this finding when comparing two different formulations of PHBVs, both biodegradable. Selectivity has been previously demonstrated for biodegradable *versus* non-biodegradable polymers (Dussud et al., 2018; Pinnell and Turner, 2019), which could indeed constitute a fundamental discriminating aspect. Moreover, material selectivity has been shown to decrease over time, being higher especially at the very beginning of colonization (i.e. in the first week, see Pinto et al., 2019).

One of the most important, still open, question concerning the dispersion of plastic in the marine habitats is its impact on microbial community. As, up to now, most of the studies performed by microbiologists focused mainly on plastic-degraders or plastic-colonizers, the matter is still largely unknown (Tetu et al., 2020). Here, we always observed the absence of significant differences

between communities of control and of manufact-associated sediments, both in dune sand and seabed. Therefore, in both environmental matrices, no relevant impact by the PHBV and PHBV/PO manufacts on natural bacterial communities of sediments could be observed. This finding, along with the previously documented absence of interaction with seagrasses and dune plants (Balestri et al., 2019), confirms these materials, especially the faster-degrading PHBV/PO, as suitable for use in coastal habitat restoration.

Conclusions.

Data here presented show that bacterial communities colonizing PHBV and PHBV/PO manufacts in seabed sediments and dune sands differ from those of the surrounding sediments. In particular, they display a lower richness, hence connoting manufacts as a selective microhabitat for sediment microbes. Moreover, in line with previous findings, we found that recruitment of colonizers on these plastics occurs largely from bacteria present in the surrounding sediments, although the contribution from the water column bacterial pool could be not negligible for manufacts buried in seabed.

The exceptional length of the performed test (more than two years) allowed to observe succession of plastisphere community over a previously unexplored time scale. We found that time plays a major role in structuring the microbial community, especially in dune sand, probably because of a wider range in abiotic parameter variations. Possibly for the same reason, succession appears highly dynamic in dune sand even after two years, while in seabed the community structure seems to reach a certain stability after one year. Obtained data highlight the importance of performing long-term studies when trying to characterize composition and dynamics of plastisphere bacterial communities.

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Figure legends.

Fig 1. Residual weight percentage graphs and pictures of dog-bones buried in seabed and dune sand mesocosms during the experiment. Seabed data are re-elaborated from Seggiani et al. 2018. The test was stopped in July 2017 for seabed and in September 2017 for dune sand due to the subsequent fragmentation of dog-bones and the difficulty in distinguishing among the manufacts present in the sediment.

Fig 2. Relative abundances of bacterial phyla in the obtained libraries from seabed and dune sand mesocosms are shown in the bar plot. Proteobacteria is always the dominant phylum. Ten most abundant phyla are shown in the color legend.

Fig. 3. Scanning electron microscopy pictures showing dog-bone surfaces in seabed and dune sand mesocosms at different times during the experiment. Bars: 100 micrometers.

Fig. 4. Principal component analysis (PCoA) graph of prokaryotic communities from seabed and dune sand mesocosms (Bray-Curtis distances). Similar results were produced using different metrics (weighted Uni-Frac, unweighted Uni-Frac, and Jaccard). Correlation vectors of the top 23 contributive sequence variants to the sample distribution are also represented (see Table S3 for taxonomic identities and vector lengths). PCoA graphs of all samples together as well as of sediment samples only were also produced (Figures S1, S2).

Fig.5. Venn diagrams showing overlaps between sequence variants retrieved in sediments and those retrieved on PHBVs (here intended as both PHBV and PHBV/PO manufacts).

Fig. 6. Heatmaps of the 100 most abundant PHBV-colonizing taxa in seabed and dune sand mesocosm samples. An ordered list of the taxa for each of the two heatmaps is presented in Tables S4 and S5. A beclustered version of the two heatmaps is reported in Figure S3.

Table 1. Number of sequence variants and values of calculated indexes in seabed and dune sand samples. (Sb) seabed, (DS) dune sand, (C) control sediments, (S-PHBVs) manufact-associated sediments, (PHBVs) manufact. PHBVs is here intended as both PHBV and PHBV/PO.

	Seabed					Dune sand				
Index	Sb (total)	Sb	Sb-C	Sb-S-PHBVs	Sb-PHBVs	DS (total)	DS	DS-C	DS-S-PHBVs	DS-PHBVs
Sequence variants (n)	15,40	810 ± 410	1,054 ± 543	1,013 ± 225	503 ± 132	11,27	593 ± [illegible]	640 ± 344	703 ± 316	470 ± 163
Faith-PD	-	63.60 ± 26.90	73.53 ± 37.21	74.45 ± 20.65	51.50 ± 12.52	-	41.8[illegible] ± 18.[illegible]2	43.25 ± 23.71	45.95 ± 20.71	37.76 ± 12.64
Shannon	-	7.55 ± 1.78	8.20 ± 2.01	8.81 ± 0.48	6.00 ± 0.75	-	7.1[illegible] ± 0.98	7.30 ± 1.08	7.64 ± 0.79	6.45 ± 0.96
Pielou's Evenness	-	0.79 ± 0.12	0.82 ± 0.13	0.88 ± 0.03	0.67 ± 0.07	-	0.79 ± 0.07	0.80 ± 0.06	0.82 ± 0.04	0.73 ± 0.07

Declaration of competing interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT author statement.

Claudia Vannini: Conceptualization, Formal analysis, Resources, Data Curation, Writing - Original Draft, Visualization, Supervision.

Alessia Rossi: Validation, Formal analysis, Investigation, Resources, Data curation, Writing - Review & Editing.

Flavia Vallerini: Validation, Investigation, Resources, Visualization.

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Graphical abstract

Highlights

- Microbial richness is lower on PHBVs than in sediments, both in seabed and dune sand.
- Degradation rates of different PHBVs are due to intrinsic characteristics of the materials.
- PHBVs have no impact on the microbial community of the surrounding sediments.
- Recruitment of plastic colonizers occurs largely from the surrounding sediments.
- Succession appears highly dynamic even after two years, especially in dune sand.