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Time-series incubations in a coastal environment illuminates the importance of early colonizers and the complexity of bacterial biofilm dynamics on marine plastics

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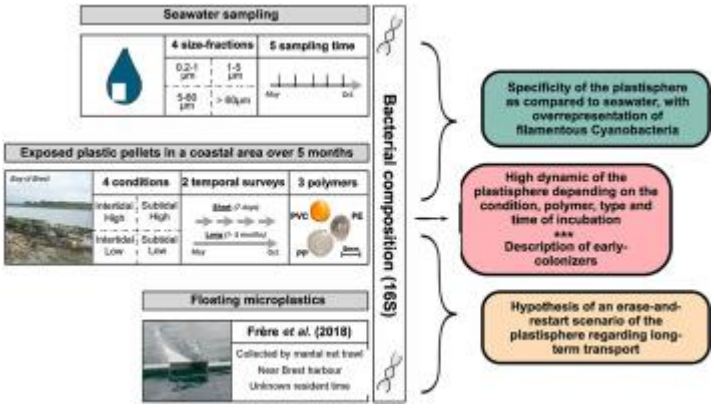
Abstract :

The problematic of microplastics pollution in the marine environment is tightly linked to their colonization by a wide diversity of microorganisms, the so-called plastisphere. The composition of the plastisphere relies on a complex combination of multiple factors including the surrounding environment, the time of incubation along with the polymer type, making it difficult to understand how the biofilm evolves during the microplastic lifetime over the oceans. To better define bacterial community assembly processes on plastics, we performed a 5 months spatio-temporal survey of the plastisphere in an oyster farming area in the Bay of Brest (France). We deployed three types of plastic pellets in two positions in the foreshore and in the water column. Plastic-associated biofilm composition in all these conditions was monitored using 16 S rRNA metabarcoding and compared to free-living and attached bacterial members of seawater. We observed that bacterial families associated to plastic pellets were significantly distinct from the ones found in seawater, with a significant prevalence of filamentous Cyanobacteria on plastics. No convergence towards a unique plastisphere was detected between polymers exposed in the intertidal and subtidal area, emphasizing the central role of the surrounding environment on constantly shaping the plastisphere community diversity. However, we could define a bulk of early-colonizers of marine biofilms such as Alteromonas, Pseudoalteromonas or Vibrio. These early-colonizers could reach high abundances in floating microplastics collected in field-sampling studies, suggesting the plastic-associated biofilms could remain at early development stages across large oceanic scales. Our study raises the hypothesis that most members of the plastisphere, including putative pathogens, could result of opportunistic colonization processes and unlikely long-term transport.

Graphical abstract



Highlights

► Plastic pellets are enriched in Cyanobacteria as compared to seawater. ► Plastisphere is constantly influenced by its surrounding environment. ► Early-colonizers of plastisphere can thrive on floating microplastics. ► Cyanobacteria are possibly inhibited by PVC associated chemical. ► Members of Vibrio are more abundant on seawater large-particles.

1. Introduction

The presence of plastic debris in marine environments has become a global ecological concern, affecting the whole diversity and functioning of the ecosystem, (Hammer et al., 2012; Worm et al., 2017). Since the beginning of the mid-century, plastic production has constantly raised, reaching 368 million tonnes in 2019 (PlasticEurope- The facts, 2020). Overall, 10% of this production ends in the oceans, under the form of small particles of less than 5 mm diameters, called microplastics (Eriksen et al., 2014). The term microplastics describes a complex diversity of particles with different polymer, size, shape and chemical composition among other characteristics (e.g., Rochman et al., 2019). These particles are very resistant to degradation and can last for months to decades in marine environment where they accumulate (Xu et al., 2020). Driven by global marine currents, they spread from near-land zones towards the open ocean, even in the most isolated areas such as the Southern Ocean (Waller et al., 2017) or in deep-sea sediments (Woodall et al., 2014; Kane and Clare, 2019).

In marine environments, microplastics represent a new non-natural substrate for the colonization of a wide diversity of microorganisms that compose the so-called plastisphere (Zettler et al., 2013). These microorganisms, dominated by bacteria and phytoplankton, can rapidly form complex and thick biofilms at the surface of a microplastic (Rummel et al., 2017). Pioneer studies found that bacterial members of the plastisphere are composed of a set of core taxa (Bryant et al., 2016), including heterotrophic members of *Hyphomonadaceae* or *Rhodobacteraceae* families and filamentous *Cyanobacteria*. In addition, harmful species for eukaryotic marine organisms or human populations have been detected within the plastisphere. Particularly, numerous studies reported an enrichment of the *Vibrio* genus on plastic pellets, including potential pathogenic strains (Kirstein et al., 2016, Frère et al., 2018, Zettler et al., 2013). The development of such diverse microorganisms transported by microplastics raises critical questions concerning the impact of the microplastics pollution in marine ecosystem functioning, as well as in global biogeochemical cycles (Zhao et al., 2021), in the transport of resistance/virulence genetic elements (Liu et al., 2021) and in spreading diseases (Kirstein et al., 2016). In addition, this biofilm could change the fate of microplastics by affecting their buoyancy (Lobelle et al., 2021), and bioavailability (Vroom et al., 2017), directly degrading the polymer matrix (Flemming, 2010) or on the contrary serving as a shield from UV light alteration of the plastic (Rummel et al., 2017).

The comprehension of the plastisphere's composition dynamic throughout the life of a microplastic is thus primordial to better anticipate and predict the impact of plastic pollution in marine ecosystems. But despite intensive recent research on the ecology of the plastisphere, major questions remain unanswered (Amaral-Zettler et al., 2020; Wright et al., 2021). Since its first description, there is still no consensus on whether or not members of the plastisphere differ from biofilm-forming bacteria that naturally colonize other substrates (biotic or abiotic) present in the ocean. For instance Oberbeckman et al., (2016) found that a bottle of PET is colonised by similar communities compared to glass and the attached (> 3µm) fraction of seawater, emphasizing that plastics follow natural biofilm colonization processes in seawater and do not select for a specific community. These results however contrast with other studies that define a specific plastic-associated bacterial community compared to glass (Kirstein et al., 2018), wood (Muthukrishnan et al., 2019) or the attached fraction in seawater (Dussud et al., 2018).

Moreover, the underlying processes structuring the bacterial composition of the plastisphere are not well understood. So far, it appears that bacterial composition of plastic-associated biofilms depends on a combination of factors such as the surrounding environment, the seasons of sampling, the position in the water column or the type of polymer, among others (Oberbeckmann et al., 2018). In addition to all these parameters, incubation experiments demonstrated that bacterial members of the plastisphere present different patterns of succession

through time, characteristic of a biofilm set up (Pinto et al., 2019). Classically in marine environment, the plastiphere evolves quickly in number of cells and in its bacterial diversity during the first 1-2 weeks (Dussud et al., 2018; Amaral-Zettler et al., 2020), corresponding to the shift between early-colonizers and secondary members of the biofilm. These first steps of colonization are important as they could influence the community composition over time (Wright et al., 2021). But as we do not know the residence time of collected microplastics, we have little clues on how time influences the plastisphere composition (Wright et al., 2021) as compared to its localisation and thus how the biofilm evolves during the microplastic "lifetime". It could remain stable as the biofilm matures (Oberbeckmann et al., 2014) or constantly adapt to its surrounding environment over long distances and time (Caruso, 2020). For now, the two factors (the temporal assembly and the environment) are rarely investigated simultaneously in incubation experiments (De Tender et al., 2017; Pinto et al., 2019; Xu et al., 2019).

In this study, we first aimed at testing the specificity of plastic-associated bacterial communities of plastic-associated biofilms as compared to a size-gradient of natural particles present in the surrounding seawater (0.22-1µm/1-5µm/5-60µm/>60µm). Secondly, to better characterize the assembly process of the plastisphere, we conducted a five months spatio-temporal in situ experiment in the Bay of Brest. Small plastic pellets were deployed in contrasted environmental conditions, the subtidal and intertidal zones in the foreshore and, within each zone, at two positions in the water column either close to the surface or near the sediments. Three types of plastic polymers, polyethylene (PE), polypropylene (PP) and polyvinyl chloride (PVC) were incubated in these conditions within short and long-term surveys (from May to October). The short-term survey consisted in recurrently incubating polymers for seven days over the five months of incubation and was aimed at better defining early-colonizers of the plastisphere and the influence of the surrounding environment at these first steps. The long-term survey (i.e. monthly collection of pellets placed at the beginning of the experiment) aimed at defining bacterial markers of the temporal assembly process in different conditions. We then investigated the presence of early-colonizers in plastic-associated biofilms whose history is not known by comparing our data to the dataset of Frère et al., which characterized the plastisphere of floating microplastics in the Bay of Brest (Frère et al., 2018). Finally, this experimentation took place in an active oyster park with seasonally induced mortalities notably by pathogenic members of *Vibrio*, in order to investigate the putative role of microplastics as a reservoir for such pathogens.

2. Material & Methods

2.1. Experimental design

Polyethylene (PE,) and polypropylene (PP) plastic pellets (Ø 5mm; spherical and opaque white, oval and translucent respectively) were supplied from GoodFellow while polyvinyl chloride (PVC) pellets (Ø 4mm; spherical and opaque orange) were supplied from Plastic Parts. All pellets were carefully rinsed in ethanol 70% and in sterile water prior to being placed in clean nylon 1mm mesh bags (n=100 pellets per polymer type per nylon bag) on the day of the deployment. A confounding effect of the nylon containers on the microbial colonization of microplastics cannot be ruled out, maintaining small microplastics in situ for days to weeks while ensuring good water flow within each structure (e.g. by regularly replacing biofouled nylon mesh bags) has technical constraints that cannot be easily overcome in any other relevant way. The potential effect would in any case be the same for all polymer types and tidal positions not questioning their comparisons. The nylon mesh bags were then transferred to the study site, an active oyster farming area located in the Bay of Brest (Pointe du Château; 48° 20' 06.19" N, 4°19' 06.37" W; sup. Data 1). The plastic pellets were placed in oyster baskets either (i) fixed on cultivation racks at 70cm off the ground next to the reared oysters or (ii) laying on the sediment in the intertidal growing area; and (iii) fixed to a floating buoy or (iv) laying on sediment in the subtidal growing area. Therefore, four tidal regimes were investigated: intertidal high (water column, IH hereafter), intertidal low (water-sediment interface, IL), subtidal high (water column, SH) and subtidal low (water-sediment interface, SL) (Figure1).
The incubation lasted from May 15th, 2017 to the October 9th, 2017, which corresponds to a period of oyster mortality outbreaks as temperature rises over 16°C (Petton et al., 2015). Previous studies performed in this geographic area reported the poly-microbial aspect of the disease onset, with a role of a phylogenetically coherent virulent population, *Vibrio crassostreae* (Lemire et al., 2015; Bruto et al., 2017), leading to increases of *Vibrio* abundance in seawater (Petton et al., 2019).

2.2. Sample collection and processing

A total of 26 nylon mesh bags and around 7800 plastic pellets were deployed during the experiment. For the long-term survey, 10 bags were deployed in the intertidal area on May 15th, 2017. They were monthly collected (1 nylon mesh bag sampled per intertidal level per collection time) until the end of the experiment on 9th October 2017, leading to various exposition times ranging from 14 to 147 days. In the subtidal growing area, a total of 8 bags were immersed on the 22nd May and collected monthly (one nylon mesh bag per subtidal level per collection time) leading to exposition times ranging from 35 to 140 days.
For the short-term survey, plastics pellets were deployed in the Intertidal environment only, as the subtidal area was accessible only during spring tides. For this survey, 4 bags were progressively exposed over time at the rate of 1 nylon mesh bag deployed per intertidal level per month and collected 7 days later, at the same moment as the bags of the long-term survey

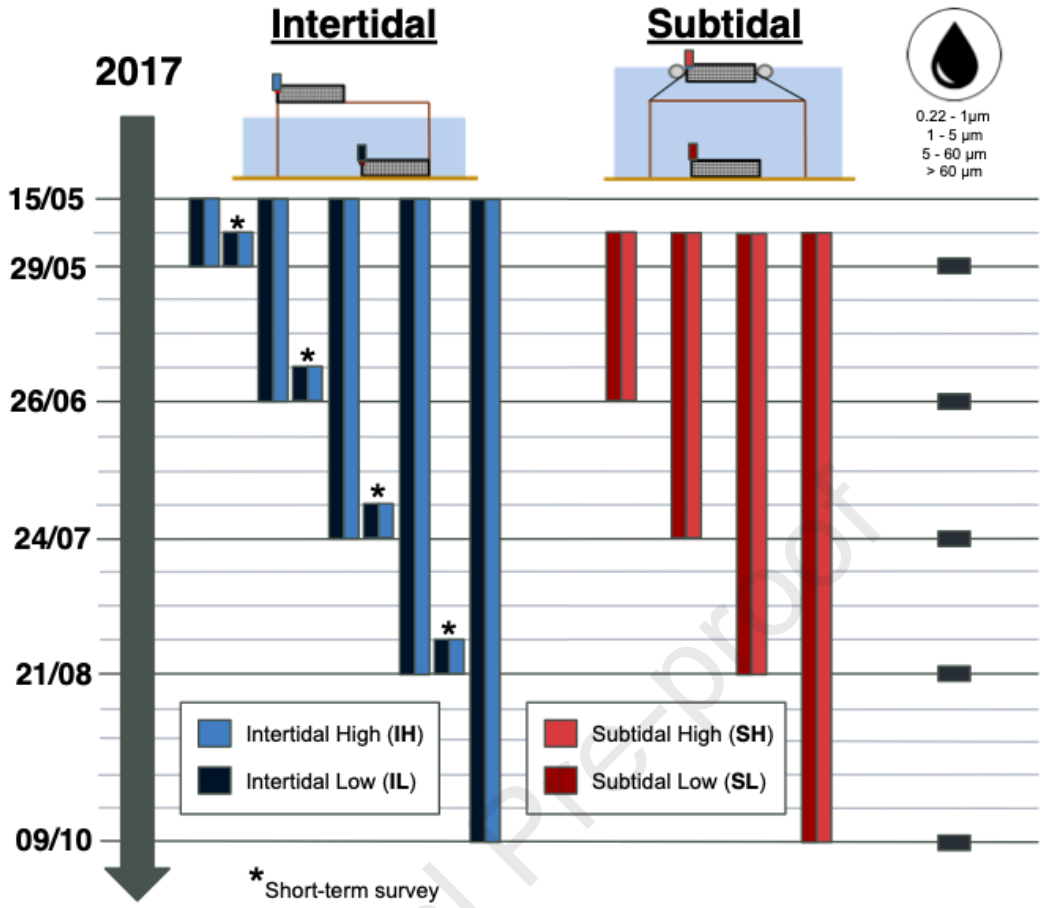


Figure 1: Experimental design of the study. Plastic pellets (PE, PP and PVC) were incubated in an oyster farm. Long-term survey: pellets were incubated on the 15th (for IH and IL) and 22th of May (for SH and SL) and collected monthly in the two areas at the same sampling date (from June onwards). Short-term survey: pellets were incubated 7 days before each sampling date in the intertidal area, as indicated by a *. Seawater sampling: seawater was collected and filtrated into 4 size fractions: 0.22-1µm, 1-5µm, 5-60µm and over 60µm at each sampling date.

As soon as the nylon mesh bags were collected, they were immediately rinsed with sterile sea water 3 times to remove any labile microorganism that was not firmly attached to the pellets, and kept in sealed plastic bags prior to being processed immediately upon arrival at the lab. Each nylon mesh bag was carefully opened on a decontaminated surface and plastic pellets were visually sorted out per polymer type and stored in 15ml sterile falcon tubes kept at -80°C prior to DNA extraction. All benches and materials (sterile forceps and sterile razor blades) used to sort out the pellets were cleaned with ethanol 70° and rinsed in sterile seawater between each sample.

On each sampling date, seawater was also collected at low tide and size fractionated. To collect zooplankton, large phytoplankton and organic particles, a 10 L sample was filtered through a 60 µm plankton net and the collected material was subsequently washed with sterile seawater. We did not expect microplastics to be present in these large fraction-size of seawater as a previous study conducted in the same area showed very low contamination of floating

microplastics in the Bay of Brest (with an average concentration of 0.24 ± 0.35 microplastics per m-3; Frère et al., 2017). Small organic particles and free-living bacterial cells were collected from 2 L water samples pre-filtered through the 60 µm plankton net and sequentially filtered through 5, 1 and 0.22 µm pore size filters. Each membrane filter was cut in half and one section was stored at -20 °C till further DNA extractions. Of note, in the Frère et al. study, floating microplastics were collected at the sea surface in October and December 2015 by mantal net trawl in the area of Brest harbour area (Frère et al., 2018; sup. Data 1).

2.3. Library preparation for bacterial diversity analysis

The study unit considered for bacterial colonization is a pellet. To insure sufficient biological material and replication to take into consideration individual pellet variability in the biofilm composition, the DNA extraction was performed on 3 pools of 10 pellets par polymer type per time point. We extracted DNA of bacterial communities colonizing plastic pellets using the same phenol-chloroform extraction protocol as Frère et al. (2018). Briefly, 10 pellets were pooled in a tube with lysis buffer (Tris pH 8.0, EDTA pH 8.0 and NaCl), SDS 10% and Lauryl sarkosyl 10%, proteinase K (20mg/mL) and were incubated at 55°C for 2 hours. The lysate was then transferred in Matrix B® tubes (filled with 0.1mm silicate beads, MP Biomedicals) and subjected to physical lysis for 5 minutes on a vortex plate. The supernatant was then collected in order to perform a phenol-chloroform extraction using PCI (Phenol, Chloroform Isoamyl alcohol with a ratio of 25:24:1) and a precipitation step with iced ethanol. DNA was resuspended in 30 µL of sterile water. DNA extraction for bacterial community found in the different seawater size fractions was done using Wizard® Genomic DNA Purification Kit (Promega).

As in Frère et al. (2018), Bacterial diversity was assessed by targeting the v4-v5 hypervariable regions of the 16S rDNA gene with the primers 518F (CCAGCAGCYGCGGTAAN) / 926R (CCGTCAATTCNTTTRAGT– CCGTCAATTCTTTGAGT - CCGTCTATTCCTTTGANT) (Nelson et al., 2014), for both plastic and seawater samples. PCR products were purified using Ampure XP® kit and DNA quantity was measured using Picogreen® staining and a plate fluorescence reader (TECAN® infinite M200 Pro). Each sample was diluted to the same concentration and pooled before sequencing in a 2x250 bp paired-end format on an Illumina MiSeq sequencer at the Marine Biological Laboratory (Woods Hole MA, USA).
Altogether, 252 plastic samples and 20 seawater samples were selected for bacterial diversity analysis requiring 3 sequencing plates for a total of 272 samples.

2.4. Bioinformatic analysis

Sequences were processed following the DADA2 pipeline (Callahan et al., 2016). Briefly, reads were quality-filtered with a maximum of 2 expected errors and a tolerance of zero N in the sequence. These high-quality paired-end reads were then merged. Error rates were calculated within each sequencing plate. To allow for comparison with the Frère et al. (2018) dataset, we reprocessed the sequences of their 59 samples using the same DADA2 pipeline. Consequently the 3 sequencing plates of the current study and the 1 sequencing plate of Frère et al., (2018) were combined into a single ASV observation table, for a total of 347 samples. Chimera were detected de novo using the consensus method, and the taxonomy of each ASV was assigned based on Silva 132 (Quast et al., 2013).

2.5. Statistical analysis

All statistical analyses were conducted on RStudio (RStudio Team, 2016) using vegan package (Dixon, 2003) and ggplot2 for graphical displays (Wickham, 2009). For beta diversity analysis, libraries were normalized using their relative abundance. We visualized bacterial commu-

nity structure between the different conditions of incubation using a NMDS based on Bray-Curtis dissimilarities. We used a permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarities and 999 permutations to test the significance of different parameters (tidal level and polymer type) on bacterial community composition associated to plastic pellets. To better understand the influence of the different environmental parameters on the biofilm composition, we investigated the ASVs that were differentially abundant between the different conditions using DESeq2 (Anders and Huber, 2010), with a shrinkage using the apeglm algorithm (Zhu et al., 2019). Significant biomarkers were then filtered according to different criteria: an absolute log fold change above 1, an adjusted pvalue under 0.01 and a relative abundance of 0.5% in at least one sample. Significant differences in bacterial diversity composition between polymers and seawater were only analysed at the family level in order to limit biases due to the use of different DNA extraction protocols for seawater and plastic samples. To better understand how bacterial communities evolve through time within each tidal level, we measured the turnover based on the raw count table (i.e,. the total number of ASVs that vary between two samples) using the function turnover() in codyn R package (Hallett et al., 2016).

For the comparison with the Frère et al. dataset, we only focused on PE and PP polymers of the two studies, to avoid polymer biases. In their study, most of the floating microplastics collected were indeed composed of PE (57%) and PP (21%).

The data produce in this study can be found in https://www.ebi.ac.uk/ena/data/view/PRJEB44493 and all scripts used for this analysis are available at https://github.com/clarilemon/Plastisphere

3. Results

A total of 76,111,862 raw reads were analysed, with a range of 64,940 to 329,750 reads per sample. 63% of the sequences (48,016,784) passed the quality-filtering and merging and were represented by 409,453 Amplicon Sequence Variants (ASVs). This number was reduced to 87,190 ASVs (43,894,497 sequences) after chimera detection. We then removed ASVs affiliated to Eukaryotes (n=100), Archaea (n=875), Mitochondria (n= 982) and Chloroplasts (n=1532), and finally obtained a total of 78,006 ASVs for the 347 samples.

3.1. Bacterial families associated to incubated plastic pellets differ from those in seawater free-living and particular fractions

We used a differential abundance analysis to investigate for specific members of the plastisphere. Families (phylotypes) belonged to Cyanobacteria including *Thermosynechoccaceae, Phormidesmiaceae, Synechococcales or Xenococcaceae*, were significantly more abundant on incubated plastic pellets as compared to different size-fractions of the surrounding seawater (0.22-1µm, 1-5µm, 5-60µm and above 60µm; sup. Data 2). On contrary, different families were not or poorly abundant on plastic polymers compared to seawater such as SAR11 Clade, NS11-12 marine group, the *Rubritaleaceae, Colwelliaceae* or *Desulfobulbaceae* family. The *Vibrionaceae* family was here found significantly more abundant in all seawater fractions as compared to plastic pellets.

Differences in family composition between plastic pellets and the seawater size-fractions decreased as the seawater fraction size increased, as illustrated by the low number of families (n=23) different between the polymers and the highest fraction of seawater, over 60µm (n=64, for the 0.2-1µm fraction; sup. Data 2).

3.2. Influence of tidal regime and plastic type on the plastisphere diversity and composition in the long-term survey

For the long term survey, bacterial community composition on plastic pellets was significantly influenced by the either intertidal or subtidal position (PERMANOVA Pr(>f) =0.001), the position in water column (PERMANOVA Pr(>f) =0.001) in each zone and the type of polymer between PVC and PE or PVC and PP (PERMANOVA Pr(>f) =0.006 and Pr(>f) =0.002 respectively) but not between PE and PP (PERMANOVA, Pr(>f) =0.979) (Figure 2).

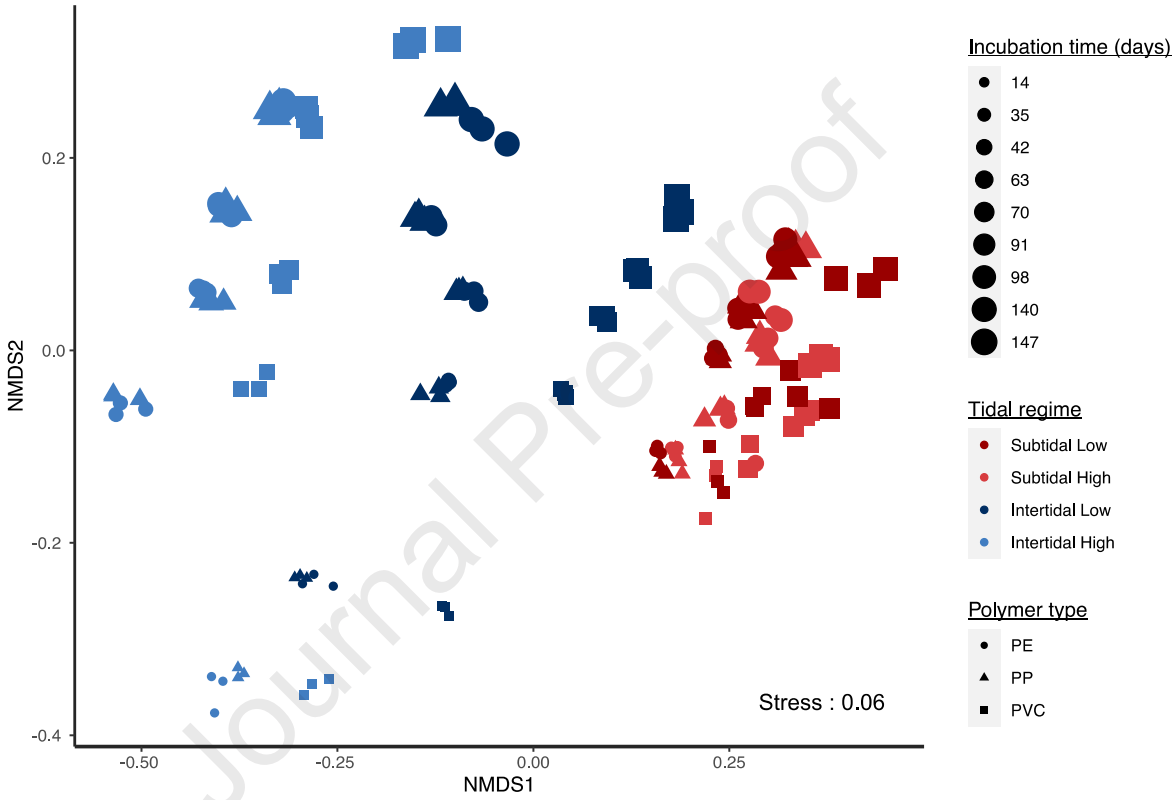


Figure 2: Influence of tidal regime, time and plastic polymer type on the plastisphere communities incubated in coastal environment. NMDS based on Bray-Curtis dissimilarities of the bacterial communities associated to plastic pellets during the temporal survey.

DESeq2 analysis revealed that ASVs affiliated to *Phormidesmeciaceae* (*Phormidesmis* ANT.LACV5.1) were significantly more abundant in PE and PP pellets (sup. Data 3). Overall, the plastic type seemed to influence a much greater number of ASVs for the plastics incubated in the intertidal area (n=26 ASVs and n=50 ASVs for IH and IL respectively) than in the subtidal area (n=1 ASV and n=2 ASV for SH and SL respectively; sup. Data 4).

As bacterial communities differed significantly between PVC on one side and PP&PE on the other side, we thereafter only focused on the PE and PP samples to investigate the spatial (i.e,

tidal regime) and temporal variability of plastisphere composition in the long-term survey. This selection was done to avoid the confounding effect of the polymer type and because PE and PP represent the most produced polymers and the wide majority of plastic debris in the marine environment (GESAMP, 2015). Again, we retrieved ASV affiliated to *Phormidesmiaceae* (*Phormidesmis* ANT.LACV5.1) as significantly more abundant in the intertidal area as compared to the subtidal area (Sup. data 3). In the first, they can represent up to 40% of the bacterial community, but only reach a maximum of 5% in the subtidal zone (Sup. Data 3). ASVs affiliated to the *Rhizobiaceae* (*Pseudahrensia* sp.) were more abundant in the subtidal area. Similarly, the position in the water column seemed to influence a much greater number of ASVs for the plastics incubated in the intertidal area (n=154 ASVs) than in the subtidal area (n=3 ASVs).

3.3. Temporal dynamic of the bacterial biofilm in the long-term survey

The time of incubation significantly influence the bacterial community (PERMANOVA Pr(>f) =0.001). The total turnover (i.e. the proportion of ASVs that appears or disappears between two consecutive time points) decreased with time for all incubation conditions (Figure 3a). After 140 days of incubation, it was higher in the subtidal environment than in the intertidal environment with a mean of 0.7% and 0.6% of community changes, respectively (Figure 3a). In general, rate of community structure change decreased with time as indicated by a decrease of Bray-Curtis dissimilarity between successive time points. However the evolution of Bray-Curtis dissimilarities was different depending on the tidal regime (Figure 3b). This decrease was mainly due to a drop between the first (14-42 days) and second (42-70 days) pairwise comparison for polymers incubated close to the sediments in the intertidal zone. In the subtidal area, dissimilarities were highly variable within the 6 replicates in the water column after 140 days of incubation, indicating the increasing influence of stochastic changes in relative abundance of some members of the community through time.

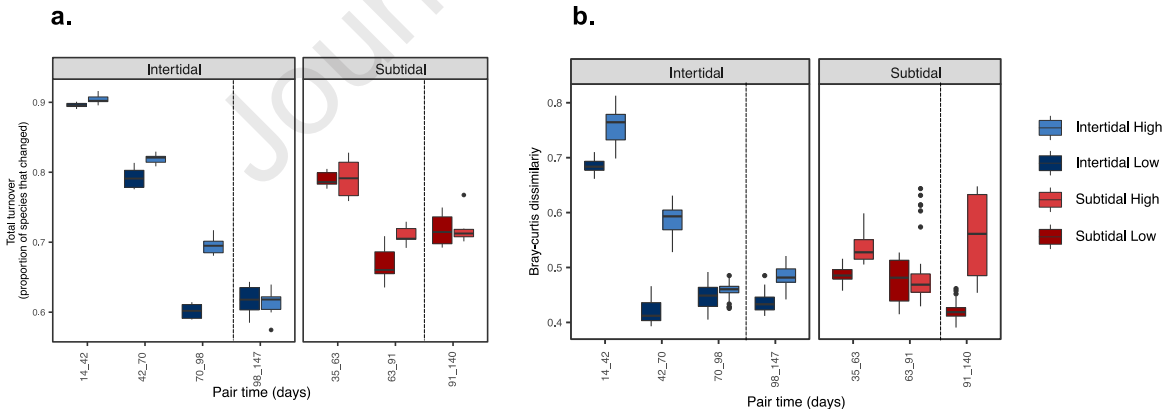


Figure 3: Temporal succession of bacterial communities in the long-term survey. a. Total turnover and b. Bray-Curtis distances between two consecutive time points in the long-term survey for each condition.

3.4. Bacterial communities associated to plastic pellets in the short-term survey

A short-term incubation of polymers was used to define early colonizers, i.e. ASVs that were significantly more abundant in polymers incubated for 7days compared to plastic pellets exhibiting longer incubation times that were collected at the same time (Figure 1). Of note, with a constant 7 days of incubation, we observed that the date of sampling (or deployment in the environment) significantly influenced the composition of the biofilm (PERMANOVA Pr(>f)

=0.001, sup. Data 5). In line with this, a total of 555 ASVs were defined as early colonizer, but none of them was repeatedly found as early colonizer for all the 4 sampling times (sup. Data 6).

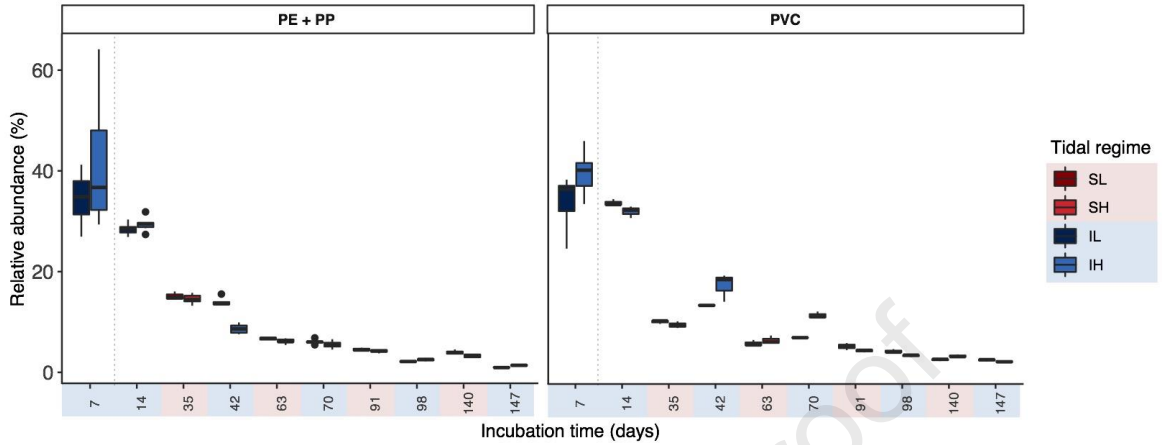


Figure 4: Distribution of early colonizers in the long-term survey
Cumulative relative abundance of the ASVs defined as early colonizers for the different incubation time tested in our experiment, including in the subtidal area

ASVs defined as early-colonizers in the intertidal area presented a clear decrease in relative abundance with longer incubation time in the long-term temporal survey for all the conditions tested, i.e. the different tidal regime and plastic type (Figure 4). Interestingly, some genera were found to exclusively present early colonizers (Sup. Data 6). Among these early-colonizers genera, some were abundant (>1% of the sequences) in the long-term survey at 14 days of incubation and then presented a strong drop after this time. They included genera such as *Nonlabens sp. Polaribacter 4 sp. Alteromonas sp., Oleiphilus sp., Pseudoalteromonas sp.* and un unknown member of *Moraxellaceae* (Sup. Data 7). Noteworthy, the most abundant ASV of Vibrio, ASV5 was defined as early colonizers in our study. It was found significantly more abundant in the plastisphere after 7 days of incubation than after 70 days in polymers collected the 27th of July 2017 (sup Data 6).

3.5. Detection of early bacterial colonizers within floating microplastics collected in the Bay of Brest in 2015

The key bacterial taxa specific of young biofilms defined here were used to better understand the structure of the plastisphere on floating microplastic from which we have no information on their past history in the marine environment. Our results were compared with those of Frère et al. (2018) that focused on the bacterial colonization of floating microplastics in the Bay of Brest. We focused on the polymers commonly studied between both studies, i.e. PE and PP.

Only 15% of the total number of ASVs were shared between polymers collected in the two datasets, but they represented the abundant members of the plastisphere associated to floating plastic pellets as they accounted from 12.7% to 84.5% of the total community (sup. Data 8). Among these shared ASVs, the bacterial communities differed between the two datasets (sup. Data 8). Floating microplastics in the Frère et al., 2008 study were significantly enriched in ASVs affiliated to families typical of anthropic and freshwater systems such as the *Staphylococcaceae* or *Psychromonadaceae*. On the contrary, they presented lower abundance of ASVs affiliated to the *Phormidesmiaceae* family that was typical of the intertidal environment (sup. Data 8).

Interestingly, despite the differences in space and time, some ASVs defined as early colonizers in the present study were strongly enriched in the biofilms from floating microplastics captured in the Bay of Brest (Figure 5). The most abundant were affiliated to *Psychrobacter* (ASV1 representing up to 42% of the plastisphere on sample MP026), *Pseudoalteromonas* (ASV4 representing up to 17% on MP059) and *Alteromonas* (ASV14 representing up to 11% on MP065).

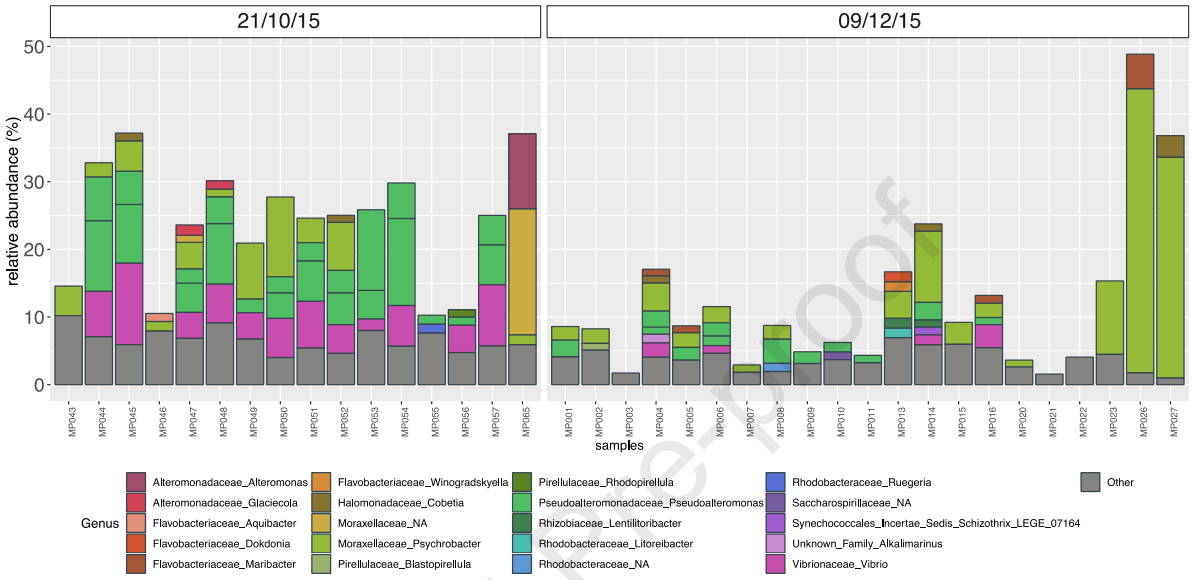


Figure 5: Distribution of early-colonizers in floating microplastics collected for Frère et al. (2018) study. Only the ASVs with a relative abundance above 1% are displayed with their taxonomic affiliation given. Two sampling surveys were conducted in Frère et al on October 21st, 2015 and December 9th, 2015.

3.6. Distribution of Vibrio members in seawater particles, incubated plastic pellets and floating microplastics.

In our study site, mortalities of young oysters occurred between May the 29th and August the 25th. During this period, *Vibrio crassostreae* was detected in all fractions of seawater (Piel et al., in press). Our 16S analysis showed that members of Vibrio sp. were relatively abundant in the particulate fraction of seawater, particularly those above 60µm (Figure 6). But they remained poorly represented in all the 270 incubated plastic samples herein (Figure 6). In addition, our comparative analysis suggests that the dominant ASVs affiliated to Vibrio sp in Frere et al., 2018 were the same as the ones found in the particulate fraction of seawater of the current study, including the most abundant one, ASV5 (Figure 6).

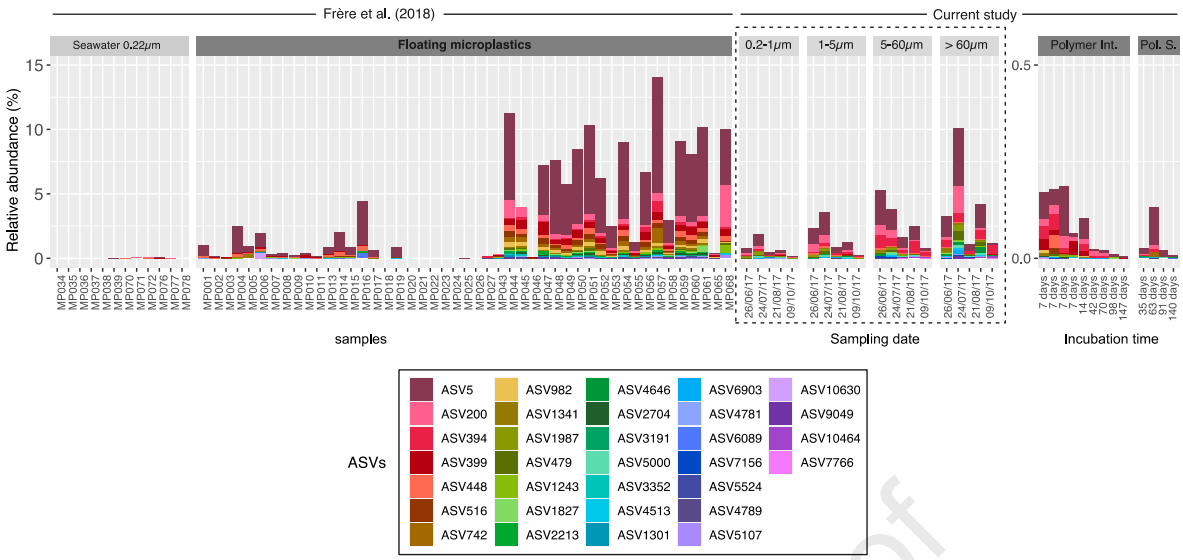


Figure 6: Distribution of *Vibrio* ASVs in Frère *et al*. (2018) floating microplastics and in the different size-fraction of seawater in the current study. The dominant ASVs are the same for seawater, floating microplastics and incubated plastics. A caution must be taken regarding the relative abundances of Vibrio for the different size fractions of seawater as a different DNA extraction protocol was used. Polymer Int. and Pol. S. refers to polymers incubated in the intertidal and subtidal zone respectively. The relative abundance of *Vibrio* ASVs displayed for the polymers of the current study is the mean of the 6 PE, 6 PP and 6 PVC incubated in the two positions in the water column. Note that the y-axis labels changed for these polymers as members of *Vibrios* were much less abundant.

4. Discussion

4.1 Niche partitioning of bacterial families between plastic pellets and different size-fractions of seawater.

Our study showed that plastic pellets harbours different bacterial families than the ones found on particulate matter in coastal seawater. Particularly, in our experimental conditions, plastic pellets were significantly enriched in Cyanobacteria, among which six families (*Thermosynechoccaceae, Phormidesmiaceae, Xenococcaceae, Synechococcales incertae sedis, Cyanobacteraceae* and uncultivated O*xyphotobacteria*), an observation also made in the Western Mediterranean Sea by Dussud et al. study (Dussud et al., 2018). Filamentous Cyanobacteria can use microplastics as a substrate to develop on, with taxa such as the epiphytic *Xenococcaceae* family or the *Phormidesmiaceae* family. The later are widely reported as being able to colonize plastic debris in the marine environment (Zettler et al., 2013; Bryant et al., 2016). Their success at the surface of microplastics can be due to their remarkable ability to withstand transportation across long distances (Curren and Leong, 2019). If this enrichment of such photosynthetic organisms in the plastisphere as compared to the surrounding seawater particles is confirmed in other coastal (as in Harvey et al., 2020), and open ocean waters, this raises the question of microplastics in enhancing ecosystem primary productivity (Amaral-Zettler et al., 2020; Wright et al., 2021). In parallel, filamentous Cyanobacteria can have key implications in the fate of microplastic, as members of the *Phormidesmis* genus are known to degrade hydrocarbons, implying that they can putatively hydrolyse plastic polymers (Yokota et al., 2017).

Similarly, previous studies have identified that bacterial communities colonizing microplastics were significantly distinct from natural seawater particles (Dussud et al., 2018; Kesy et al., 2019). But there, authors distinguished bacteria with a free-living lifestyle (that pass through 3µm filter) and bacteria with an attached lifestyle (that are retained on 3µm filter). This dichotomous view may however not represent the continuum of size and diversity of the particulate matter (Mestre et al., 2017). This great diversity of substrates from the smallest particles (i.e., eukaryotic cells) towards bigger particles (i.e., large zooplankton, macroalgal debris) are known to harbour distinct bacterial communities (Mestre et al., 2017; Grossart, 2010). Our study showed that plastic pellets harbours different bacterial families than the ones found on 3 different size-fractions (1-5µm, 5-60µm and over 60µm) of particulate matter in seawater. However, the specificity of the plastisphere compared to large seawater particles is less clear as the number of different family decreases as the natural particle size fractions increase. This result suggests that in addition to the type of substrate, the available surface size for colonization could be an important driver for biofilm-forming bacterial communities (Catão CP et al., 2021; Hou et al., 2021). The highest size (above 60µm in our study) likely concentrates most of the zooplankton. Some bacteria colonizing zooplankton exoskeleton are rather opportunists, while taking advantage of a rich substrate to settle on (Gerdts et al., 2013). Microplastics in this sense can be similar to seawater particles, as they also represent hotspot of organic matter (Zettler et al., 2013) that can attract common opportunist organisms able to colonize any surface immersed in seawater (Wright et al., 2021). These results highlight the complexity to define the plastic-specific bacterial members, that could be present only in low abundance (Kirstein et al., 2019; Scales et al., 2021) and how understanding the plastisphere requires a better characterization of the attached marine bacterial fraction and their lifestyle in the marine environment (Grossart, 2010).

4.2. The environment is a central parameter in shaping plastic-associated biofilms.

Our study confirms that the composition of the plastisphere is highly dynamic as it is influenced by all the parameters tested: the position in the foreshore (intertidal vs subtidal), the position in the water column (seawater vs sediments), the type of polymer (PE/PP vs PVC) and the time of

incubation (from 14 days to 147 days). Among all the parameters tested, the position in the foreshore was the most prevalent one. The subtidal and intertidal areas of a coastal shore can be considered as two distinct environments that selects different microbial communities (Lee et al., 2014; Weigel and Erwin, 2016), including bacteria biofilms associated to sediments, or sponges (Lv et al., 2016; Weigel and Erwin, 2016). In the intertidal area, plastic pellets in both heights follow repeated cycles of immersion/emersion, thus microorganisms at the surface of the incubated plastics must be adapted to withstand strong fluctuations in hydrodynamic conditions, temperature, desiccation, salinity and UV radiation (Garbary, 2007). In addition to being biomarkers of plastic type, the filamentous Cyanobacteria *Phormidesmis* was here also one of the most important biomarkers of this intertidal area. These organisms present a high capacity to tolerate desiccation (Olsson-Francis et al., 2013 ; Potts, 1999) making them particularly successful in the intertidal zone were they form microbial mats (Decho, 2000).

Interestingly, the intertidal environment seemed to exacerbate the influence of other parameters (i.e. position in water, plastic type) on the biofilm community composition, suggesting that certain parameters could be relevant on the structure of plastic-associated communities solely under particular ambient environmental conditions. Similarly, a previous study found that polymer-specific assemblages between PE and PS were present only under conditions with higher salinity and nutrient concentration (Oberbeckmann et al., 2018). The temporal succession of the biofilm was also influenced by the tidal position, with a strong stabilisation of the plastisphere in the intertidal area not seen in the subtidal. Indeed, the regular fluctuations of the environment by cycles of emersion/immersion in the intertidal area could lead to a better stabilisation of bacterial communities (Nguyen et al., 2021) expected for floating plastics in coastal areas favouring beaching/remobilisation cycles. Overall, our results strongly support the conclusion reached by Caruso (2020) that bacterial communities associated to plastic are continuously influenced by their surrounding environment, and never converge toward a unique plastisphere (Caruso et al., 2020). This also suggests, that the bacterial composition of the plastisphere is likely to evolve in the context of major global changes (Harvey et al., 2020).

4.3. PVC as a potential inhibitor of filamentous Cyanobacteria.

The most common polymers PE and PP that compose microplastics waste in seawater usually harbour similar bacterial communities (Amaral-Zettler et al., 2015; Frère et al., 2018; Basili et al., 2020; Wu et al., 2020). Our results are in line with these observations, as no significant differences in biofilm composition could be found between PE and PP at any time and condition of incubation. Close physical properties of polymers notably as the substrate characteristics of plastics such as its roughness or hydrophobicity have been demonstrated to be among the most relevant factors in driving bacterial colonization (Ogonowski et al., 2018; Caruso, 2020), under certain environmental conditions (Oberbeckmann et al., 2018). However, in the present study, roughness characterized on 300x300µm² surfaces using optical confocal profilometry (Sneox - Sensofar(R)) cannot be an origin since it is far from being similar between PE-PVC (roughness 1-2µm) and PP (180 nm). Interestingly, PVC harboured significantly different communities, which was also observed among a variety of polymers in the North Sea (Kirstein et al., 2018) or in the Adriatic Sea (Pinto et al., 2019). Rosato et al., suggested that these differences could be more likely linked to differences in the chemical load (organic and inorganic additives) between the different polymers (Rosato et al., 2020). Adsorbed contaminants could also influence the biofilm formation and microbial communities (Djaoudi et al., 2021). As PVC can contain 10-60% additives such as phthalates plasticizers or lead-based stabilizers by weight (Markarian, 2007; Teuten et al., 2009; Net et al., 2015) it is likely that those residual compounds can leach upon plastic weathering and either promote (leached additives used as a source of nutrients) or limit (toxicity) the growth of bacteria. A recent study demonstrated experimentally that the leachates of PVC could inhibit growth of the Cyanobacteria, *Prochlorococcus sp.* that did not happen with PE (Tetu et al., 2019). Our differential

abundance analysis showed that the Cyanobacteria belonging to the genus *Phormidesmis* were significantly less abundant in the PVC polymer in the intertidal area where they normally proliferate. This could be due to the greater UV exposure during emersion phases that could have enhanced both particle degradation and chemical leaching from PVC (Suhrhoff and Scholz-Böttcher, 2016). While a consensus emerge on the negligible effect of polymer type on the plastisphere community, the chemical load (additives and adsorbed contaminants) unique to each plastic debris based on its formulation and life history is suspected to exhibit much greater role in shaping the microplastics bacterial communities (Scales et al., 2021). Today, more than 5300 polymer formulations are commercial available, and more than 4000 known chemicals are associated with plastic packaging alone (Groh et al., 2019) which represent a considerable proportion of collected aquatic litter (74.5% in rivers) (Schwarz et al., 2019). This chemical complexity raises the difficulty in assessing plastics toxicity at all biological levels from individuals to communities and ecosystems.

4.4. Specific early-colonizers were identified in the plastisphere

Even though the plastisphere is highly dynamic within short-term incubation, we were able to define a bulk of ASVs that were more abundant in young biofilms compared to more mature biofilm of weeks or months of incubation overall the 4 repeated short-periods. However, none of them was found in all the 4 sampling date, indicating that the attachment of primary colonizers that are shaping the early stages of biofilm development depends on the surrounding community from whom they emerged (Datta et al., 2016) plus stochastic processes (Veach et al., 2016). In a theoretical aspect, these early colonizers are thought to be not well adapted to thrive in the late successional stages of the biofilms (Connell and Slatyer, 1977 ; Brislawn et al., 2019). The most abundant ones detected in our study were affiliated to different genera such as *Nonlabens sp., Oleiphilus sp.,* an unknown member of the *Moraxellaceae* family and members of *Gammaproteobacteria* such as *Alteromonas sp.*, *Pseudoalteromonas sp.* These genera presented an expected temporal dynamic for early colonizers as their relative abundances dropped out after 14 days and for the rest of the survey (up to 147 days in the intertidal area). Besides, these taxa also never reached high abundances in the subtidal environment for which the first sampling started at 35 days of incubation. Our observations are supported by a rich literature: members of the *Gammaproteobacteria* are often described as primary colonizers of a variety of substrates in seawater such as glass, steel (Dang et al., 2008; Salta et al., 2013; Lawes et al., 2016) and plastics (De Tender et al., 2017; Dussud et al., 2018; Pollet et al., 2018). Early colonizers of biofilm are well adapted to colonize new surfaces: they are typical fast-growing opportunists bacteria that can quickly respond to changes in their environment thanks to versatile genomes (Polz et al., 2006). They possess all the required genes to sense, attach and produce a complex matrix of exopolysaccharides that will allow a wider community of bacteria and eukaryote (e.g., larvae) to settle on the biofilm (Steinberg et al., 2002). Such characteristics are present in *Alteromonas sp.* (Sinha et al., 2017), *Pseudoalteromonas sp.* (Saravanan and Jayachandran, 2008) or *Nonlabens sp.* (Guillonneau et al., 2018). Interestingly, along with their capacity to form biofilms, part of them are putative hydrocarbon-degrading bacteria such as *Alteromonas sp.* (Yoon et al., 2012). The latter may find easily available carbon substrates on plastics particle at early time points, such as additives, organic compounds or adsorbed hydrocarbons that progressively deplete over time (Erni-Cassola et al., 2020).

4.5. Microplastics in the environment likely face multiple cycles of colonization.

The results acquired here on incubated microplastics were compared to the bacterial diversity of floating microplastic collected in the Bay of Brest in 2015 (Frère et al., 2018) at fine resolution (i.e., using ASVs which represent the exact same 16S sequence corrected from its sequenc-

ing errors) thanks to the use of identical molecular biology protocols and bioinformatics pipelines. Our ASVs identified as early-colonizers could reached high relative abundances (up to 47% of the entire community) in floating microplastics collected in the Frère et al. (2018) study. This likely reflects the expected short immersion time of floating microplastics in the bay of Brest as suggested by the particle dispersal model showing substantial water renewal of 30% at each tide cycle leading to more than 60% of the particles expelled from the bay after 10 days (Frère et al., 2017).

However, the early-colonizers genera identified in our study were also reported as abundant on microplastics spreading at the surface of the ocean, with a likely much longer incubation time (Zettler et al., 2013). Similarly, in the study of Oberbeckman (2020), two OTUs affiliated to *Alteromonas sp.* were among the most abundant OTUs found associated to plastics in both the Baltic sea and the North Sea (Oberbeckmann and Labrenz, 2020). Finally, in two recent paper, the most abundant ASVs on plastic debris collected in the Mediterranean sea, the Baltic sea and the Sargasso sea were affiliated to *Pseudoalteromonas sp.* and *Alteromonas sp.* (Basili et al., 2020, Scales et al., 2021). These observations support an erase and restart scenario for the plastisphere as suggested by De Tender et al. (2017): biofilms associated to microplastics that spread all over the ocean could hardly reach a permanent stable mature state and rather offers continuous possibilities for early, opportunist colonizers to settle and become dominant members of the plastisphere (De Tender et al., 2017). Indeed, biofilm in this case, probably face unpredictable and fast-changes in their surrounding environment (Sebille et al., 2020), that could modify and alter the biofilm stability, or at least a part of it, on microplastics floating at sea. Hydrodynamic or shear stress could contribute to a significant cell loss in marine biofilm (Sweat et al., 2017) and delay its formation (Schmidt et al., 2018), thus maintaining the biofilm in early development stages (Rochex et al., 2008).

4.6. Questioning microplastics as a new vector of putative pathogens among Vibrio genus.

A key problematic in plastisphere study is the ability of microplastics to carry putative pathogens populations as suggested by numerous report of the *Vibrio* genus being enriched on microplastics in coastal (Kirstein et al., 2016; Frère et al., 2018; Basili et al., 2020), estuarin (Laverty et al., 2020) and offshore environments (Zettler et al., 2013). To test if plastics pellets could enrich Vibrio members and act as a reservoir of such putative pathogens, our study took place in an oyster farming area that experiment recurrent mortalities that can be due to infections of pathogenic Vibrio (Lemire et al., 2015; Bruto et al., 2017). A central actor of these mortalities, *V. crassostreae*, was detected in seawater during the mortality period recovered by our study (Piel et al., in press). In contrast with previous publications, in the context of our study all members of *Vibrio* were found poorly abundant on exposed plastic pellets. Several non-exclusive hypotheses could be suggested to interpret this result.

First, the particularity of our sampling area, an oyster farm with mortalities, could have an opposite effect and attract populations of *Vibrio* against the plastics, as they would find a higher resource of nutrients there. In addition, cycles of emersion/immersion experimented in the intertidal and occasionally the subtidal area may stimulate an attachment instability of some *Vibrios* as instability of *V. crassostreae* has been shown experimentally on polystyrene beads (Foulon et al., 2016). Then, members of this genus were recently described as rather early, opportunist colonizers of microplastics (Kesy et al. 2021) in accordance with our study. The colonization processes of Vibrio in our study site would thus favour natural particles or moribund oysters, instead of exposed plastic pellets covered by mature and competitive biofilms. Accordingly, members of *Vibrio* were abundant in all seawater size fractions, particularly on large particles (over 60µm). This is consistent with their ability to occupy heterogeneous ecological niches (Polz et al., 2006) and marine floating substrates, among them phytoplank-

ton, zooplankton, macroalgae or faecal pellets (Hunt et al., 2008 ; Michotey et al., 2020). Interestingly, the most abundant *Vibrio* ASV (ASV5) detected in seawater particles in our study was also the most abundant one present in floating microplastics collected in the Bay of Brest (Frere et al., 2018). This observation rather comforts the possibility that a single population of *Vibrio* could colonize multiple habitats (Schmidt et al., 2014). To conclude, our data support the hypothesis that microplastics could opportunely enrich early colonizers *Vibrio* from their surrounding environment as observed with natural suspended particles but their ability to efficiently carry these putative pathogens over long period of time and distances remains to be proven.

5. Conclusion

As it is the case when evaluating microplastics toxicity, our study demonstrates that the nature of the plastisphere cannot be generalized based on the polymer type and is instead strongly shaped by the environment and in a second time, by the chemical load (additives and adsorbed contaminants) that is unique to single plastic debris based on their own formulation and life history. Our study also highlighted a specific diversity of early-colonizers that are not present in long-term incubation polymers. The abundance of such early-colonizers in floating microplastics collected in the Bay of Brest as well as in multiple other environments suggests that these plastic particles face multiple microbial colonisations, rather than carrying a unique and stable complex biofilm through time. However, experimental designs with exposed polymers in a static point as in our study are likely not representative of floating microplastics that derive in the oceans. To confirm this erase and restart scenario, future studies should investigate how the displacement of microplastics across contrasted environments (including nutrients, salinity and contaminants gradients for plastics travelling from Rivers to the open Oceans) influences the plastisphere dynamics over time.

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Highlights

- Plastic pellets are enriched in Cyanobacteria as compared to seawater
- Plastisphere is constantly influenced by its surrounding environment
- Early-colonizers of plastisphere can thrive on floating microplastics
- Cyanobacteria are possibly inhibited by PVC associated chemical
- Members of Vibrio are more abundant on seawater large-particles

Authors contributions Statement

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Frédérique Le Roux : Writing - review and editing
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Declaration of 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.