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Fungal Diversity and Dynamics during Long-Term Immersion of Conventional and Biodegradable Plastics in the Marine Environment

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Abstract: Plastics are associated with a worldwide pollution crisis, with strong negative impacts on both terrestrial and aquatic ecosystems. In marine environments, various organisms are colonizing plastic debris, but few studies have focused on fungal communities despite their non-trivial ecological roles in the marine environment. In this study, different types of plastics (biodegradable and conventional) immersed in marine natural environments and under laboratory controlled settings were collected after long-term colonization. Using a metabarcoding approach targeting two genetic markers, namely, the ITS2 region and the V4 hypervariable region of the 18S rRNA gene, we highlighted that fungal communities associated with plastic polymers were distinct from those found in the surrounding seawater. They also differed significantly between sampling locations and the nature of immersed polymers, indicating that fungal colonization was impacted by the sites and types of plastics, with clear dissimilarities between conventional and biodegradable polymers. Specifically for the conventional PVC polymer (Polyvinyl chloride), we also observed the successive stages of biofilm development and maturation after long-term immersion in seawater. A noticeable change in the fungal communities was observed around 30–40 days in natural settings, suggesting a colonization dynamic likely associated with a transition from biofilm formation to distinct communities likely associated with biofouling. Overall, this study strengthens the idea that the fungal kingdom is an integrated part of the "plastisphere".

Keywords: plastisphere; marine fungi; diversity; colonization dynamics; biodegradable vs. conventional plastics

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1. Introduction

Global plastic production almost doubled from 2002 to 2020, now reaching 367 million tons per year [1]. Plastic mismanagement, estimated at around 30 million tons per year [2], generates, inter alia, plastic leakage into the natural environment. Recent studies estimated that 3–10% of annual mismanaged plastic waste ends up in the marine environment [3,4]. Marine plastic litter poses complex multifaceted issues for ecosystems, human health, the economy, and even the climate, as it affects the oceans' ability to sequester carbon [5]. Land-based sources are recognized as the main cause of marine plastic pollution, accounting for

approximately 80% of total marine debris, largely composed of single-use plastics (~50%), while sea-based sources, primarily composed of fishing gear, account for around 20% [6,7]. Plastic is now considered a marker of the Anthropocene Epoch [8], an informal unit of geological time that marks the start of human impact on geology and ecosystems, and has also led to the term "Plastic age" [9].

Microplastics (MPs), mainly resulting from the physical, chemical, and biological fragmentation of larger plastic wastes, dominate the marine plastic debris found on the ocean surface and are generally composed of conventional polymers: polyethylene (PE, ~55%), polypropylene (PP, ~15%), or polystyrene, (PS, ~10%) [10]. These polyolefins appear refractory to biodegradation because of their hydrophobic nature and high molecular weight [11]. This is also the case for PE with pro-oxidant additives (OXO), which has been shown to be poorly biodegradable, or even not biodegradable, in the marine environment [12,13]. On the other hand, biodegradable plastics, still far from replacing the global demand for conventional ones, with a global production volume of 3.8 million tons, representing ~1% of the total plastic production [14,15], do exist. These biodegradable plastics can be classified into two categories: (i) those derived from fossil resources but biodegradable under some specific conditions, such as poly(1,4-butylene adipate-co-1,4-butylene terephthalate) (PBAT) and polycaprolactone (PCL), and (ii) those bio-based, such as polylactic acid (PLA) and poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) (PHBV) [16,17]. PLA is efficiently decomposed under specific conditions such as industrial composting environments, requiring temperatures of >50 °C, while its biodegradation in seawater is negligible [18]. PHBV shows efficient biodegradability in the ocean, with films being completely disintegrated after 9 months in seawater [19] or with a ~90% biodegradation after 45 days [20]. Other authors have found different PHBV biodegradation rates, with the observed variations being attributed to different polymer molecular weights and/or different immersion sites (characterized by different temperatures, different microbial communities, etc.) [19]. PCL also exhibits high biodegradability in numerous environments, including the marine environment, with biodegradation rates similar to those of PHBV [21,22].

Pieces of plastic debris, either biodegradable or not, which end up in the oceans are rapidly covered by organic matter, forming an ecocorona, a unique layered structure that alters the hydrophobicity of the plastic surfaces, and which allows for microbial attachment and colonization [23–25]. Marine microbial communities colonizing marine plastic debris, known as the "plastisphere" [26], rapidly form a complex biofilm within minutes/hours [25] to 15–30 days, usually leading to a mature biofilm [27,28]. The literature on the colonization dynamic in terms of prokaryotic communities fluctuates with research studies highlighting Alphaproteobacteria at early stages and Gammaproteobacteria at later ones [23], while others revealed an opposite trend [29,30]. Recently, Latva et al. (2022) [25] detected the importance of the Bacteroidetes as a pioneering phylum (min/h), then outreached by Gammaproteobacteria, and later by Alphaproteobacteria. A recent meta-analysis of 35 studies specifically targeting conventional plastic matrices clearly demonstrated that environmental variables, rather than polymer type, appear as key factors affecting the prokaryotic composition of the plastisphere [31].

The microeukaryotic component of the plastisphere has been largely overlooked to date. Most metabarcoding studies targeting microeukaryotes have targeted the V4, V7, or V9 regions of the 18S rRNA gene and highlighted the occurrence of diatoms (Stramenopiles) as early colonizers, while high occurrence of dinoflagellates (Alveolata), algal species, and even metazoan taxa represent a significant fraction of the eukaryotic communities [26,32–36]. Still, co-occurrence networking analysis suggests that microeukaryotes play a limited role in the mature biofilm compared to bacteria [34]. Kettner et al. (2017) [37] targeted the V4 region of the 18S rRNA gene to assess the relative part of fungal contribution to microeukaryotic communities. Around 1/5th of all taxa was affiliated with the fungal kingdom and, more precisely, to the Ascomycota, Basidiomycota, and Chytridiomycota phyla, differing from fungal communities in the surrounding water, among sampling sites and polymer types. Other studies targeting the ITS2 genetic marker (i.e., [38]) detected mainly

Ascomycota and Basidiomycota on PE samples without specific shifts, either temporal or geographical, in community composition. Using conventional plastic samples collected from surface waters of the western South Atlantic and Antarctic Peninsula, Lacerda et al. (2020) [39] highlighted similar trends in terms of community composition, with dominance of Ascomycota and Basidiomycota, and no distinct geographical differences. However, there is still a scientific gap for marine fungal communities associated with biodegradable plastics. As the bacterial and fungal abundance and diversity vary greatly between conventional and biodegradable plastics under terrestrial conditions [40], it is possible to hypothesize that the architecture of fungal communities will also differ in the marine environment, as it was likewise proven for marine bacterial communities (e.g., [18,41]).

Culture-based approaches have also been used to delve deeper into the colonization of plastics by fungi, with recent results supporting some molecular-based data showing two thirds ascomycetes and one third basidiomycetes, with some taxa being different compared to sediment fungal communities [42]. The latter study also highlighted the biodegradation potential of the fungal plastisphere, with some detected taxa already known as able to biodegrade plastic polymers (e.g., PE and polyurethane, PU). To date, certain marine fungal isolates appear able to efficiently degrade plastics, such as, for example, *Zalerion maritimum* ATCC34329 [43] or *Alternaria alternata* FB1 [44] on PE.

In this study, our aim was to characterize the fungal communities associated with different plastics polymers in various marine environments using a multi-marker metabarcoding approach targeting the ITS2 region and the V4 hypervariable region of the 18S rRNA gene. We intend to better understand the influence of (i) plastic type (i.e., conventional—PE, aged PE (PEO), OXO, PP, and PVC—vs. biodegradable—PCL and PHBV—polymers), (ii) geographical location (Atlantic ocean, Indian ocean, Mediterranean Sea), and (iii) immersion time (from 1 to 365 days) on fungal colonization dynamics.

2. Materials and Methods

2.1. Sample Collection

Two types of plastic material immersions were performed. The first type corresponded to immersion in natural settings in several French coasts (i) in the Toulon (43°06′25″ N; 5°55′41″ E), and the Banyuls-sur-Mer (42°29′300″ N; 3°08′700″ E) bays in the northwestern Mediterranean Sea, (ii) in the bay of Lorient (Kernevel Harbor, 47°43′8.0178″ N; 3°22′7.2048″ W) along the south coast of Brittany, (iii) in the bay of Brest (Pointe du Château; 48°20′06.19″ N, 4°19′06.37″ W) in the Atlantic Ocean, and (iv) in the Reunion Island (Le Port, 20°56′03″ S, 55°17′0.6″ E) in the Indian Ocean (complementary information described in [29,45,46] (Figure 1A, and Supplementary Table S1). The second type corresponded to immersion in controlled conditions in aquariums in the dark, with direct circulation to the sea by pumping seawater from 30 m from the coast and 4 m deep in the Banyuls Bay (complementary information described in Odobel et al. 2021 [47]) (Figure 1A). Depending on the site, various polymers among PVC (as panels), PE, PP, PEO, OXO, PCL, and PHBV (as polymer films), were incubated for specific durations with various sampling times (Figure 1B).

As a special note, the experimental design for plastic immersion in the Bay of Brest also integrated the effect of different tidal regimes on microbial colonization, as detailed in Lemonnier et al. (2022) [46]. Briefly, PE, PP, and PVC pellets were deployed in clean nylon mesh bags at different tidal positions in the Bay of Brest in order to test the influence of two parameters on microbial community composition: (i) the position in the foreshore (subtidal versus. intertidal, the latter being influenced by regular cycles of immersion and emersion) and the position in the water column (water column versus water–sediment interface).

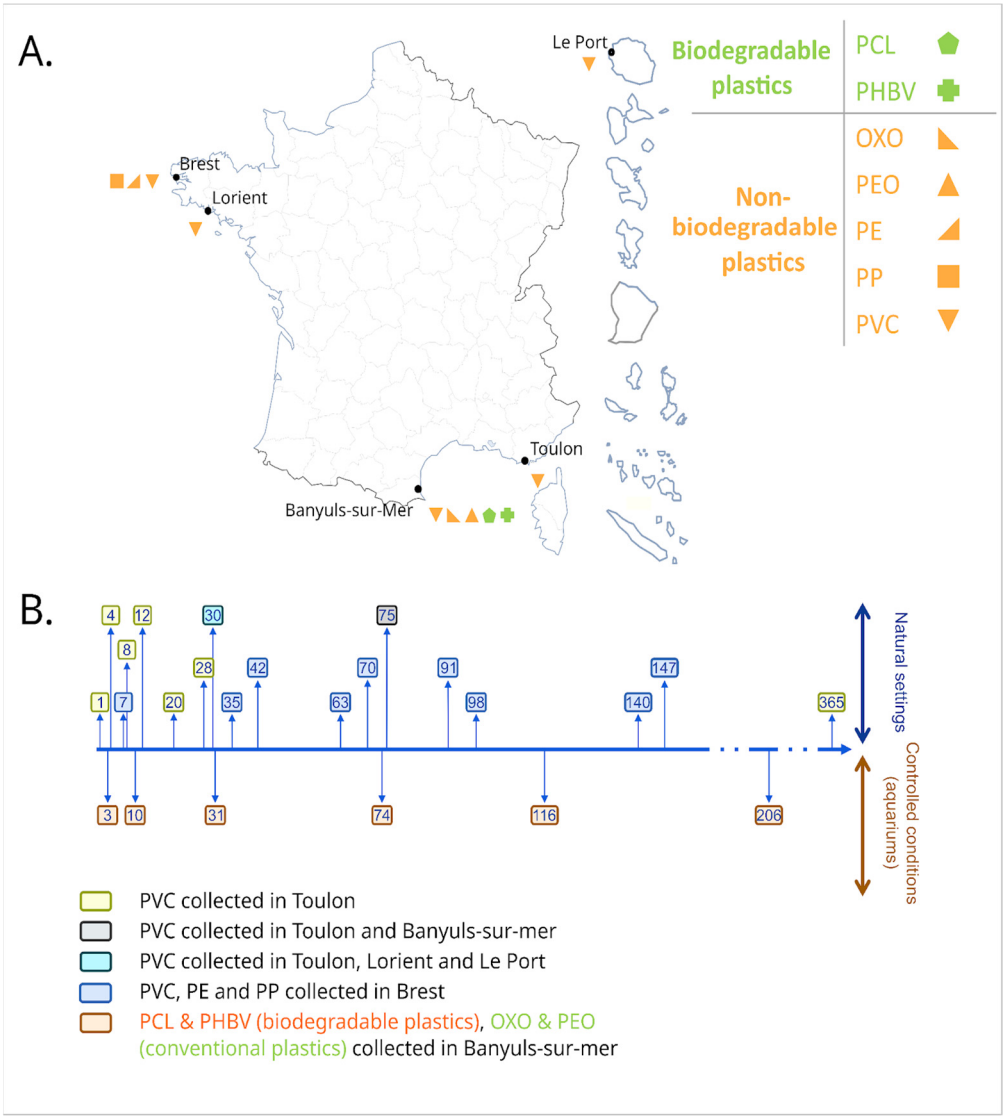

Figure 1. (**A**) Map of France and French territories presenting the sampling locations and the studied plastic types. (**B**) Timeline representing incubation times (days) for the different studied plastic types with the corresponding immersion methods. Meaning of polymer acronyms: PCL (Polycaprolactone), PHBV (Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), OXO (Oxodegradable-Polyethylene), PEO (Oxidized Polyethylene), PE (Polyethylene), PP (Polypropylene), and PVC (Polyvinyl chloride).

2.2. DNA Extraction, Amplification and Sequencing

DNA extraction was performed from both the polymers and surrounding seawater (as reference) at all sampling times using (i) the PowerBiofilm DNA isolation kit (Qiagen, Courtaboeuf, France) for Toulon, Banyuls-sur-mer, Lorient, and Le Port PVC samples, as detailed in Catao et al. (2021) [45] or (ii) a phenol-chloroform extraction method for polymers immersed in the Bay of Brest, as detailed in Lemonnier et al. (2022) [46], and those incubated in an aquarium with direct seawater circulation from the Banyuls Bay, as detailed in Odobel et al. (2021) [47]. DNA extracts were used as template for individual semi-nested PCR amplification of either the ITS2 rRNA region or the V4 region of the 18S rRNA gene. Fungal ITS2 region amplicons were generated using the 5.8S-Fun (5′-AACTTTYRRCAAYGGATCWCT-3′) and ITS4-Fun (5′-AGCCTCCGCTTATTGATATGCTTAART-3′) primers and customized thermocycling parameters [48] after a first PCR amplification using the fungal-specific primer ITS1f (5′)-CTTGTCATTTAGAGGAAGTAA-3′)) and the general primer ITS4 (5′)-TCCTCCGCTTATTGATATGC-3′)). A similar strategy

was used to generate fungal 18S amplicons using a semi-nested PCR approach with the use of primers nu-ssu-0817 (5′-TTAGCATGGAATAATRRAATAGGA-3′) and nu-ssu-1536 (5′-TCTGGACCTGGTGAGTTTCC-3′) for the first PCR and nu-ssu-0817 (5′-TTAGCATGG AATAATRRAATAGGA-3′) and nu-ssu-1196 (5′-TCTGGACCTGGTGAGTTTCC-3′) [49] for the second amplification following customized parameters [50]. PCR products were visualized by agarose gel electrophoresis and iTAG sequenced at Eurofins Genomics using Illumina MiSeq PE300 chemistry.

2.3. Bioinformatics and Statistical Analyses

Raw data were analyzed with the SAMBA V3.0.1 workflow (https://github.com/ifremer-bioinformatics/samba, accessed on 4 March 2023) [51], a standardized and automatized metabarcoding analysis tool developed by the Ifremer Bioinformatics Department (SeBiMER). SAMBA was developed using the NextFlow workflow manager [52] and consists of three main parts: data integrity checking, bioinformatics processes, and statistical analyses. This workflow uses QIIME 2 [53] and DADA2 (Divisive Amplicon Denoising Algorithm) [54] with default parameters (unless indicated) and was processed here to analyze the generated ITS2 and the V4 region of the 18S fungal iTags. Raw data were first filtered (primer removal, removal of reads with incomplete or incorrect primer sequences) using Cutadapt [55]. The ASV (Amplicon Sequence Variants) clustering method was then applied using DADA2 [54] following a four steps approach: filtering (quality, sequencing error correction), pairwise read merging, inference of sample ASVs, and chimera identification. The first stage assesses the quality of the data and executes quality filtering by cutting the endpoints of the reads in order to obtain the highest percentage of data in the samples. As the iTag datasets were sequenced in numerous runs, including ITS2 and 18S runs, an optimization step was performed to determine the best threshold (in terms of length of read trimming) for each run (Supplementary data). To counteract the known overestimation of diversity generated by DADA2, a complementary step of ASV clustering was performed using dbOTU3 [56]. The generated ASVs were then taxonomically assigned using a Naive Bayesian method against the SILVA V138 [57] and UNITE V8 [58] databases. As suggested by McMurdie et al. (2014) [59], the DESeq2 method [60] was chosen as another data normalization alternative to rarefaction. Additionally, the statistical framework called ANalysis of COmposition of Microbiomes ANCOM was used for the comparison of the microbiome composition [61]. The R phyloseq object generated by the SAMBA workflow was used for all diversity analyses. The alpha diversity, for intraspecific diversity comparisons, was investigated using four indices: Chao1, Shannon, Simpson's inverse, and Pielou. Bubble plots were performed using R software version 3.6.1 (ggplot2 package) and used to depict the taxonomic assignment of ASVs at the phylum and genus levels. An interspecific comparison of samples was also performed as part of the beta diversity analysis using an ordination analysis (i.e., Principal Coordinate Analysis -PCoA-) and a hierarchical ascendant classification analysis.

3. Results

3.1. Long-Term Immersion of PVC in Natural Settings

The following results were obtained for a single type of polymer, namely PVC, that was immersed under natural conditions in different marine environments for a duration of up to one year. The fungal communities, as revealed by ITS2 and the V4 region of the 18S genetic markers, were found to be more diverse on plastic compared to those in the surrounding seawater and were mainly structured by the geographic location.

For the 48 studied PVC samples (panels and pellets), Illumina Miseq DNA sequencing generated 7,779,430 matched sequences for ITS2 and 1,667,500 for the V4 region of the 18S rRNA gene. The ITS2 data set contained 3954 fungal ASVs, while the V4 region of the 18S data set contained only 769 fungal ASVs.

3.1.1. Analysis Based on the ITS2 Dataset

The alpha diversity was measured using the Chao1, Pielou, Shannon, and Simpson indices (Supplementary Table S2). Among all evaluated factors, 'Location' (Toulon, Banyuls-sur-mer, Lorient, Brest, and Le Port), 'Incubation time' (from 1 to 365 days) and 'Environment' (plastic vs. seawater), the first two were very highly significant for all diversity indices (Figure 2A,B). For the Shannon and Pielou indices (Figure 2C), the 'Environment' variable indicated a significant difference, distinguishing free fungal populations in seawater from those linked to plastics (only 38 ASVs in common between the plastic-associated communities and the free living ones, meaning ~1% of the total ASVs). Lower index values were observed in the surrounding seawater samples (108 unique ASVs, representing less than 3% of the total ASVs) compared to the plastic samples (3808 unique ASVs), indicating a lower diversity and equitability of fungal communities in seawater compared to plastic-associated ones. Banyuls-sur-mer had the highest diversity of fungal communities, followed by Brest, Toulon, Lorient, and Le Port (Figure 2A). Toulon and Banyuls-sur-Mer, both in the Mediterranean Sea, presented the highest number of shared ASVs (n = 95), with other locations having less than 55 ASVs in common.

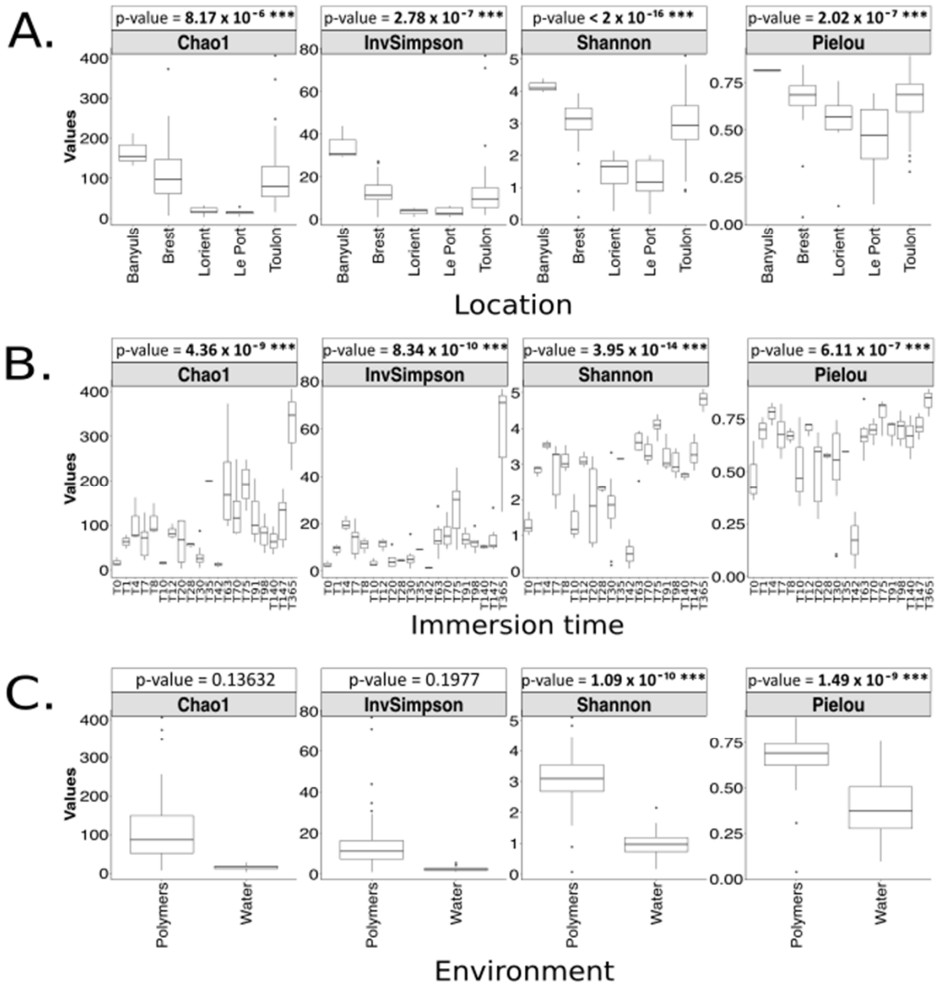

Figure 2. Alpha diversity indices and their associated significance, based on the ITS2 dataset for the variables (**A**) "Location", (**B**) "Immersion time" (days), and (**C**) "Environment". "***" represents a very highly significant influence of the evaluated factor.

Immersion time also affected the richness and diversity of fungal communities. A sinusoidal-like trend can be observed on Figure 2B with increasing diversity values from 0 to 7 days, followed by a reduction from 8 to 30 days and then an increasing amplitude, with accentuated crest values at days 75 and 365. The highest number of shared ASVs

throughout the different immersion durations was only found at later immersion stages, i.e., between days 75 and 365 (77 ASVs shared), followed by days 63 and 70 (52 ASVs shared) and days 91 and 147 (51 ASVs in common), suggesting a putative stabilization of fungal communities attached to PVC after several weeks of immersion.

For beta diversity, the three factors 'Location' (p-value = 0.0001), 'Incubation time' (p-value = 0.0001), and 'Environment' (p-value = 0.0081) showed highly significant differences between samples (Figure 3). For the 'Location' variable, explaining 11% of the variation, samples collected in Brest, Lorient, and Le Port differed from those collected in Banyuls-sur-Mer and Toulon (Figure 3A). As a result, fungal communities from the Atlantic/Indian Ocean (Lorient, Brest/Le Port) and the Mediterranean Sea (Toulon and Banyuls-sur-mer) differed significantly. Furthermore, the communities sampled in the Atlantic Ocean also differed between Brest and Lorient. For the 'Immersion duration' variable, explaining 27.6% of the variation, normalized PCoA and hierarchical clustering (Figure 3B,D) showed that there was also a distinction between the time point samples, in addition to the differentiation by location. Indeed, for the location with the highest number of time points, i.e., Toulon (9 time point samples from 1 to 365 days), a colonization dynamic was observed with a clear dissimilarity between fungal communities from 1 to 30 days compared to 75 and 365 days. Regarding PVC samples immersed in Brest (8 time point samples from 7 to 147 days), no such trend was observed, suggesting that different immersion conditions (immersion site influenced by tide in Brest site contrary to Toulon) affect the colonization dynamic and unbalance the architecture of fungal communities. According to DESeq2, normalized PCoA based on Bray–Curtis dissimilarities for the 'Environment' variable (Figure 3C), representing 1.6% of the variation, samples taken from polymers differed from those retrieved from surrounding seawater.

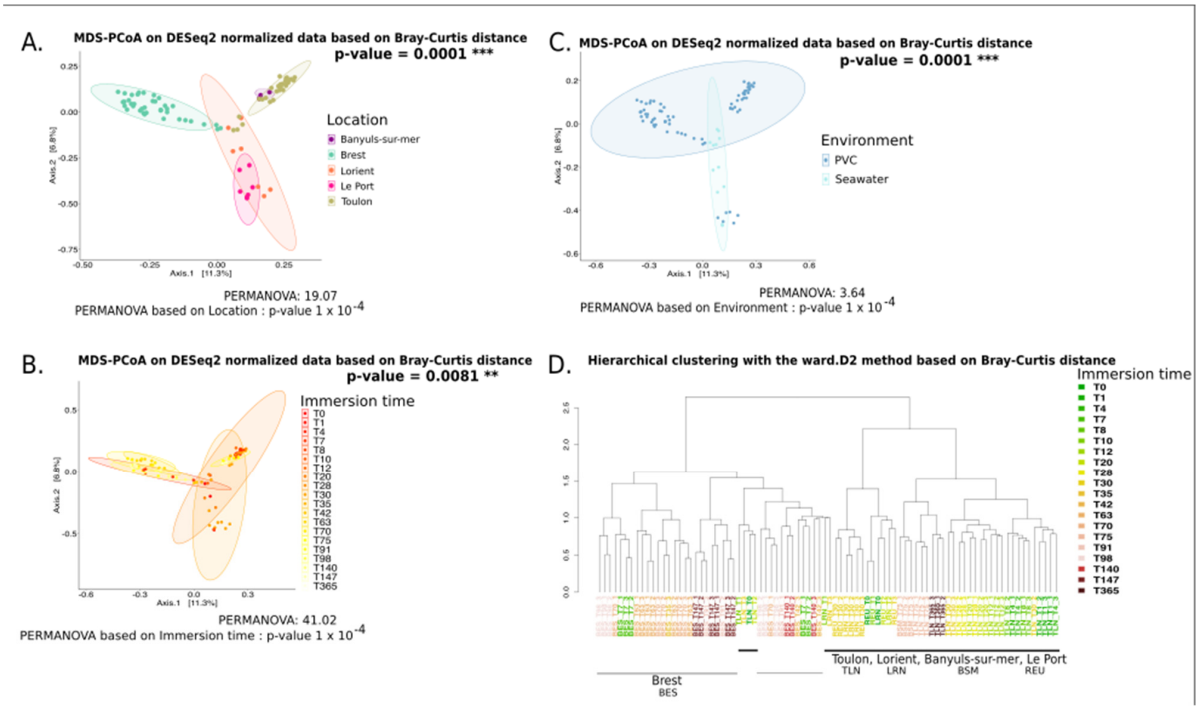

Figure 3. Principal Coordinate Analysis (PCoA) of the similarity in fungal community composition, based on the ITS2 dataset, showing the relationship between the variables "Location" (**A**), "Immersion time" (days), (**B**) and "Environment" (**C**), and the significance of each variable. (**D**) Hierarchical clustering of the samples based on the variable "Incubation time"."**" and "***" represent a highly and a very highly significant influence of the evaluated factor, respectively. The PCoA was generated using Bray–Curtis dissimilarity as the distance measure, with each point representing a sample. Ellipses connect groups of samples that were subjected to the same conditions.

A bubble plot for the 'Location' variable was used to display taxonomic diversity at the phylum and genus levels (Figure 4A,B). Ascomycota, Chytridiomycota and Basidiomycota were present in all sites, albeit at different proportions. Ascomycota was the most common phylum in Toulon (60.6%), Banyuls-sur-Mer (58.5%), followed by Le Port (32%), Brest (26.5%) and Lorient (20%). ASVs affiliated with the Basidiomycota phylum accounted for 20.4% of the community in Le Port, 13.7% in Banyuls-sur-Mer, 13.4% in Toulon, and 4.7% in Brest and Le Port. Brest had the highest concentrations of ASVs identified as Chytridiomycota (18.1%) and Rozellomycota (11.1%). ASVs affiliated with the phylum Chytridiomycota accounted for 5.1% of the fungal population in Lorient, 4.8% in Banyuls-sur-Mer, 3.8% in Le Port, and 3.5% in Toulon. All taxa appeared distributed unevenly between sites (Figure 4A). Brest had the highest diversity (52.1%), followed by Toulon (39.2%), Banyuls-sur-mer (5.6%), Lorient (1.6%), and Le Port (1.5%).

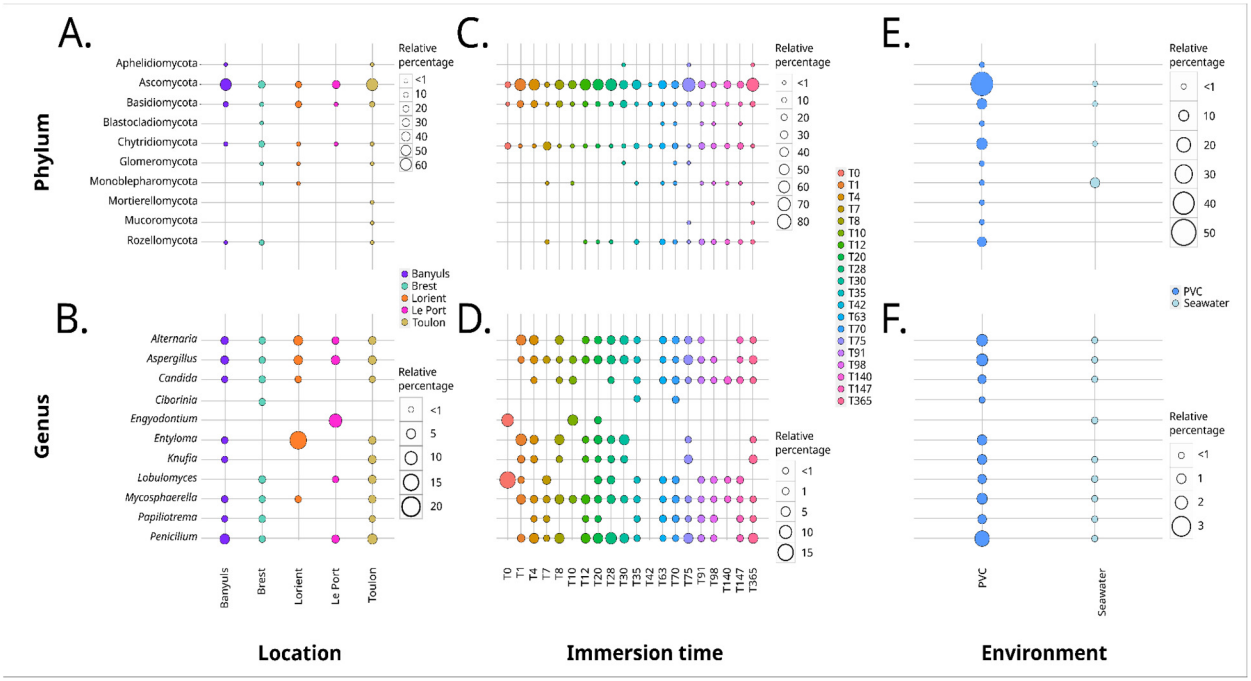

Figure 4. Bubble-plot representing the proportions of phyla or genus, based on the ITS2 dataset, according to the variables (**A**,**B**) "Location". (**C**,**D**) "Immersion time" (days). (**E**,**F**) "Environment". The listed genera correspond to the 10 most abundant genera, also containing 2 genera identified as differentially abundant using ANCOM (*Alternaria* and *Engyodontium*), and one extra genus that was also highlighted by ANCOM (*Ciborinia*).

Alternaria appeared to be the most ubiquitous taxon based on its detection in all sites, albeit at different proportions in Lorient (4.5%), followed by Banyuls-sur-Mer (2.2%), Toulon (2.2%), Le Port (1.7%), and Brest (0.9%). ASVs affiliated with *Penicillium* (5.1%), *Aspergillus* (2.9%), *Knufia* (2.6%), *Alternaria* (2.2%), *Mycosphaerella* (2%), *Entyloma* (2%), *Lobulomyces* (0.6%), *Papiliotrema* (0.3%), and *Candida* (0.2%) were mostly retrieved in Toulon. Banyuls-sur-Mer had the same composition but different abundances, with the exception of *Lobulomyces* (Figure 4B). For this site, *Penicillium* (5.4%) was the most common genus, followed by *Aspergillus* (2.8%), *Alternaria* (2.2%), *Entyloma* (0.9%), *Mycosphaerella* (0.9%), *Candida* (0.4%), *Knufia* (0.3%), and *Papiliotrema* (0.2%). *Entyloma* was the most common genus (16.1%) in Lorient. *Engyodontium* was exclusively found in Le Port (10.1%). For this site, *Aspergillus* and *Penicillium* were retrieved at close proportions, i.e., 3.9% and 2.5% respectively. At the genus level, Brest was dominated by *Lobulomyces* (1.4%), *Papiliotrema* (1.2%), *Candida* (1.0%), *Alternaria* (0.9%), *Mycosphaerella* (0.8%), *Penicillium* (0.5%), and *Aspergillus* (0.4%).

The Ascomycota phylum was present at all immersion times, with a higher relative abundance proportion of ASVs at 75 (70.2%) and 365 (67.4%), then at days 1 (57.3%), 4

(53.3%), 12 (52.7%), 20 (52.8%), and 28 (56.5%), and thus was globally consistent with the crests of the sinusoidal-like trend that was previously highlighted (Figure 4C). The Basidiomycota phylum was present at all immersion times, with higher abundance at days 1 (23.2%), 4 (23.9%), and 8 (21.4%), as was the Chytridiomycota phylum, but with lower abundance from days 0 to 30 (mean = 8.6% and median = 4.9%) and higher abundance from days 35 to 365 (mean = 12.2 and median = 14%). Despite low abundance during the first days of immersion (until 30 days of immersion), the Monoblepharomycota and Rozellomycota phyla were mostly present in the latter stages, from 35 to 365 days of immersion. Blastocladiomycota were exclusively found at days 63, 70, 91, 98, and 147. The Aphelidiomycota and Glomeromycota phyla were only present at days 30 and 75, respectively, and 70 for Glomeromycota and 365 for Aphelidiomycota. Mucoromycota were present at days 75 and 365, while Mortierellomycota were present only at the end of the immersion period (365 days). Two genera were identified as differentially abundant based on immersion times using ANCOM. The first is the genus *Alternaria*, which was more prevalent during the early stages of colonization (mean from days 0 to 30 = 2.3% vs. mean from days 35 to 365 = 0.8%) (Figure 4D). The second genus is *Ciborinia*, which was only found at days 35 (1.0%) and 70 (0.2%). *Penicillium citrinum* was observed at days 75 and 365, whereas *Chaetomium globosum* was identified at days 12 and 365. *Aureobasidium pullulans* was detected at various times (days 0 to 4, then from 8 to 35, followed by days 63 to 75, and day 365).

According to the 'Environment' variable (i.e., PVC vs. surrounding seawater), all phyla showed a contrasting proportion (Figure 4E). Ascomycota accounted for more than a one-third of the overall diversity (41.6%), with a relative proportion on plastic of 42% and in surrounding seawater of 0.3%. The Chytridiomycota phylum followed, with a total diversity of more than 11.2%, found mainly on plastics (11.2%) rather than in surrounding saltwater (0.1%). The Basidiomycota phylum, which accounted for 8.8% of the global diversity, had a higher relative proportion on plastics (8.9%) than in seawater (0.04%). Rozellomycota (7.1%), Blastocladiomycota (0.1%), Mucoromycota (0.1%), Aphelidiomycota (0.04%), Glomeromycota (0.03%), and Mortierellomycota (0.01%) were identified solely on plastics and with low relative abundance.

The *Penicillium* (2.6% vs. 0.03%), *Alternaria* (1.5% vs. 0.01%), *Aspergillus* (1.6% vs. 0.004%), *Knufia* (1.0% vs. 0.01%), and *Mycosphaerella* (1.3% vs. 0.01%) genera were all found in higher abundance on PVC compared to the surrounding seawaters (Figure 4F). The *Entyloma* genus was identified only on plastics (1.1%). On the other hand, the *Engyodontium* genus, on the basis of ANCOM, was identified as a differentially abundant taxon and was exclusively abundant in the surrounding seawater (0.1%). At the species level, *Aureobasidium pullulans*, *Chaetomium globosum*, and *Penicillium citrinum* were detected as almost exclusively associated with PVC.

Data on environmental variables (temperature, pH, and salinity) were all available for Banyuls-sur-Mer, Lorient, Le Port, and Toulon. Using this dataset, the 'Temperature' variable indicated a significant difference across samples in terms of beta diversity visualization (p-value = 0.0001) (Supplementary Figure S1). For the Brest location, for which only the environmental variable "Temperature" was available, the Shannon index was exclusively significant (p-value = 0.000169).

Additionally, for the Brest location, beyond PVC, PE, and PP were also immersed, but no difference was observed between these three conventional plastic polymers in terms of alpha and beta diversity (Supplementary Figure S2A,B), suggesting that in the tested conditions, the type of plastic matrix had a negligible impact on fungal colonization. Interestingly, the fungal community composition on plastic pellets was significantly influenced by the tidal regimes. As shown in Supplementary Figure S2C,D, fungal communities associated with PVC pellets deployed in the intertidal area and height-positioned (water column), which were thus strongly influenced by fluctuations in temperature, UV radiation, and desiccation, differed from those associated with plastic pellets deployed in subtidal areas or intertidal areas but maintained in the seawater–sediment interface.

3.1.2. Analysis Based on the V4 Region of the 18S rRNA Gene

Results generated using this genetic marker were largely consistent with those related to the ITS2, with significant effects of location, environment, and immersion time on alpha and beta diversity. Detailed results developed below highlight slight difference, mainly due to the smaller number of total fungal ASVs obtained using the V4 region of the 18S rRNA gene data set (769 ASVs compared to the 3954 ASVs for ITS2).

The Chao1, Pielou, Shannon, and Simpson indices were used to calculate alpha diversity (Supplementary Table S3). All diversity indices were very highly significant for the 'Location' (Toulon, Banyuls-sur-mer, Lorient, Brest, and Le Port), 'Incubation time' (from days 1 to 365), and 'Environment' (plastic vs. surrounding seawater) variables (Figure 5). Banyuls-sur-mer showed the greatest richness, followed by Toulon, Brest, Lorient, and Le Port. This trend aligns with the results from the ITS2 analysis, albeit that Toulon presented in this case higher richness than Brest, a difference that may be attributed to the smaller number of total fungal ASVs obtained in the 18S data set, as shown in Figure 5A. Indeed, the fact that the V4 region of the 18S rRNA gene data set generated 5 times fewer ASVs compared to the ITS2 data set may have resulted in lower representation of some specific ASVs.

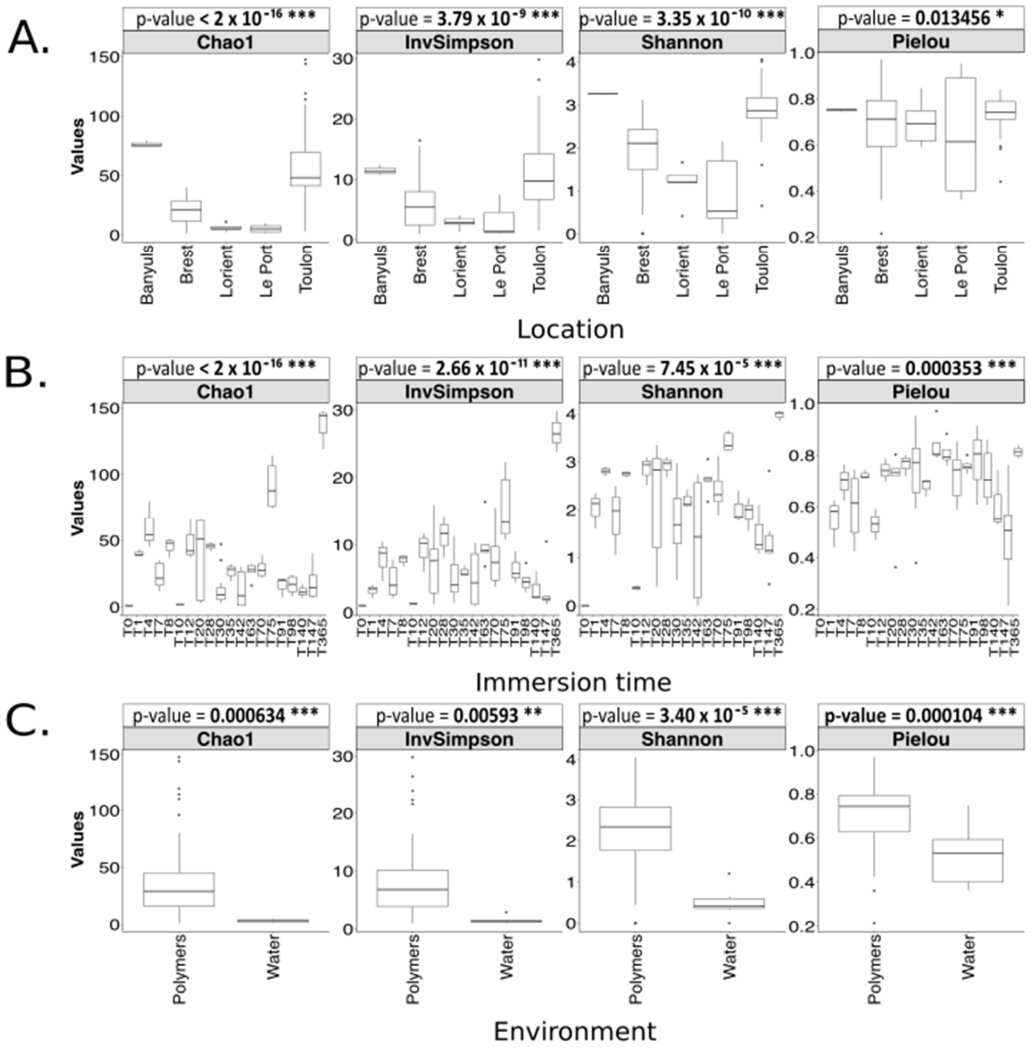

Figure 5. Alpha diversity indices and their associated significance, based on the V4 region of the 18S rRNA gene dataset for the variables (**A**) "Location", (**B**) "Immersion time", (days) and (**C**) "Environment". "*", "**", and "***" represent a significant, highly significant, and very highly significant influence of the evaluated factor, respectively.

Toulon and Banyuls-sur-Mer, both in the Mediterranean Sea, presented the highest number of common ASVs (n = 77). The other sites had fewer than 30 ASVs in common, consistent with the ITS2 dataset. The period of immersion also influenced the diversity of fungal populations, as shown in Figure 5B, showing a similar sinusoidal trend as obtained with the ITS2 dataset, albeit less pronounced. The highest number of shared ASVs was found at the later stages of immersion, i.e., between days 75 and 365 (24 shared ASVs), but also between days 4 to 8 (11 shared ASVs), indicating an early colonization and then a putative stabilization of the fungal communities associated with PVC after several weeks of immersion, as detected for the ITS dataset. The 'Environment' variable revealed a significant difference, distinguishing free fungal populations in seawater from those linked with plastics (Figure 5C). Interestingly, no ASV was shared by the polymer-related and the so-called free-living communities. The diversity indices were lower in the surrounding seawater samples (18 unique ASVs) compared to the plastic samples (751 unique ASVs), indicating that fungal communities in seawater were less diverse and unequal than those associated with PVC.

In terms of beta diversity, the 'Location' (p-value = 0.0001) and 'Incubation time' (p-value = 0.0001) variables were very highly significant, and the 'Environment' variable to a lesser degree (p-value = 0.0380) (Figure 6). Samples collected in Brest, Lorient, and Le Port appeared different from those collected in Banyuls-sur-Mer and Toulon for the 'Location' variable, which explained 19% of the variation (Figure 6A). As a result, the fungal communities in the Atlantic/Indian Ocean (Lorient, Brest/Le Port) and the Mediterranean Sea (Toulon and Banyuls-sur-mer) differed greatly, which is consistent with the ITS2 dataset. Samples collected from plastics differed from those obtained from the surrounding seawater, according to the DESeq 2 standardized PCoA based on Bray–Curtis dissimilarities for the 'Environment' variable (Figure 6C), accounting for 1.4% of the variation.

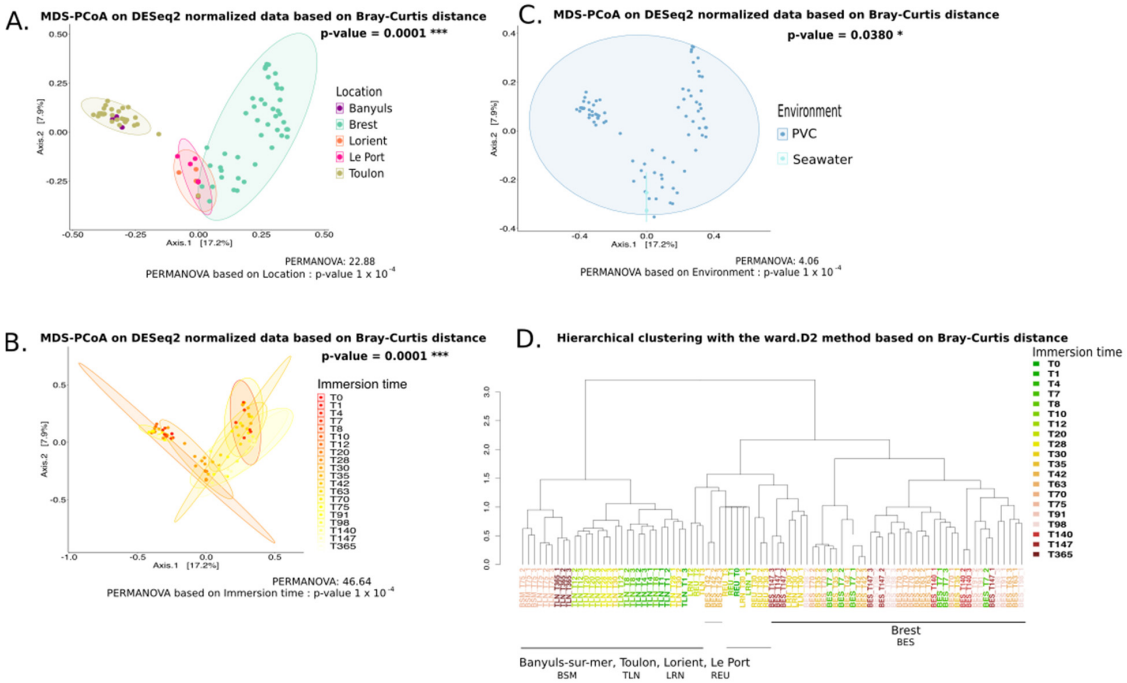

Figure 6. Principal Coordinate Analysis (PCoA) of the similarity in fungal community composition, based on the V4 region of the 18S rRNA gene dataset, showing the relationship between the variables "Location" (**A**), "Immersion time" (days), (**B**) and "Environment" (**C**), and the significance of each variable. (**D**) Hierarchical clustering of the samples based on the variable "Incubation time". "*", and "***" represent a significant, and a very highly significant influence of the evaluated factor, respectively.The PCoA was generated using Bray–Curtis dissimilarity as the distance measure, with each point representing a sample. Ellipses connect groups of samples that were subjected to the same conditions.

To represent the taxonomic diversity at the phylum and genus levels, a bubble plot for the variable 'Location' was created (Figure 7). Although both ASVs affiliated with the phyla Ascomycota and Basidiomycota were found, only those affiliated with Ascomycota predominated at each location (Figure 7A). Ascomycota accounted for 77.4% of the population in Le Port, 76.9% in Banyuls-sur-Mer, 71.9% in Toulon, 64.4% in Lorient, and 43.0% in Brest. Lorient (35.6%), Toulon (22.1%), Banyuls-sur-mer (19.7%), Brest (15.2%), and Le Port (5.5%) had the highest concentrations of Basidiomycota. Chytridiomycota, which was absent in Lorient, and made up 36.3% of the population in Brest, 17.0% in Le Port, 4.5% in Toulon, and 3.1% in Banyuls-sur-Mer. Mucoromycota was only found in Toulon (0.8%) and Brest (0.1%), while Cryptomycota was detected in Brest (5.5%), Banyuls-sur-mer (0.4%), and Toulon (0.3%). Using ANCOM, the genera *Lobulomyces*, LKM11, and *Cladosporium* were identified as differentially abundant taxa, indicating their significant abundance in at least one location (Figure 7B). Consistent with results generated using the ITS2 dataset, taxa appeared irregularly distributed among locations, with Toulon accounting for 58.1% of the whole taxa, followed by Brest (32.1%), Banyuls-sur-Mer (7.6%), Le Port (1.1%), and Lorient (1.0%).

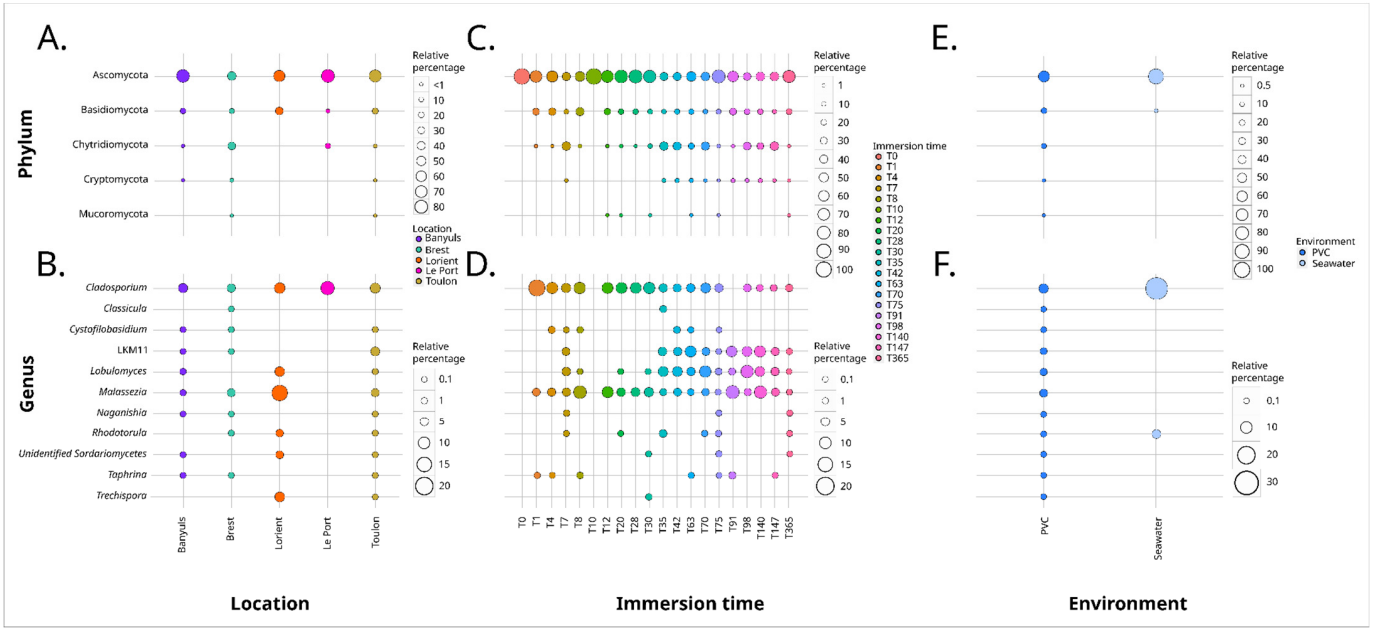

Figure 7. Bubble-plot representing the proportions of phyla or genus according to the variables (**A**,**B**) Location, (**C**,**D**) "Immersion time" (days), and (**E**,**F**) "Environment". The listed genera correspond to the nine most abundant genera, also containing three genera identified as differentially abundant using ANCOM (*Cladosporium*, *LKM11* and *Lobulomyces*), and two extra genera that were also highlighted by ANCOM (*Cystofilobasidium* and *Naganishia*).

At all immersion times, the phylum Ascomycota was present (Figure 7C), with a higher relative abundance between days 1 and 30 (mean of 72.2%) compared to days 35 to 365 (mean of 50.6%). The Basidiomycota phylum, although absent at days 0 and 10, was present in comparable proportions from day 1 to 30 (mean of 19.4%), and days 35 to 365 (mean of 17.3%). The Mucoromycota phylum was only present in low abundance at several incubation times (days 12, 20, 30, 63, 75, with a mean of 0.6%), but accounted for 1.0% and 1.5% of the relative abundance at days 75 and 365, respectively, consistent with the ITS2 data set. The Cryptomycota phylum was mainly present from days 35 to 365 (mean of 5.2%), with only a presence at day 7 (4.1%) in the early incubation stage. The Chytridiomycota phylum was also prevalent in the late immersion times (days 35 to 365), with a mean relative abundance of 26.6%.

On the basis of ANCOM, the *Lobulomyces*, *Cladosporium*, *Cystofilobasidium*, and *Naganishia* genera and the environmental clade LKM11 were identified as differentially abundant taxa, showing that these genera are significantly abundant at certain immersion times (Figure 7D). The *Lobulomyces* genus (mean from days 0 to 30 of 0.6% vs. mean between days 35 and 365 of 5.3%), as well as the environmental clade LKM11 (mean from days 0 to 30 of 0.4% vs. mean between days 35 and 365 of 5.0%) were more prevalent in later immersion times. *Cystofilobasidium* was more abundant in the early stages of incubation (mean of 0.4%) than in the later stages (mean of 0.3%) at days 4, 7, and 8, as well as other genera such as *Cladosporium* (mean from days 0 to 30 of 7.5% vs. mean between days 35 and 365 of 3.4%).

The 'Environment' variable (comparison of plastics vs. surrounding seawater) revealed that all phyla had a differential proportion (Figure 7E). The Ascomycota accounted for about two-thirds of the overall diversity (63.0%), with a 62.8% relative proportion on plastics and a 95.1% relative proportion in the surrounding seawater. The Basidiomycota phylum, although weakly represented, showed a larger relative proportion on plastics (19.7%) than in the surrounding seawater (4.9%). The Chytridiomycota (14.8%), Cryptomycota (1.9%), and Mucoromycota (0.5%) phyla were only detected on PVC samples.

Although results generated using the genetic marker ITS2 and those using the V4 region of the 18S rRNA gene were largely consistent, some genera identified using ITS2 were not detected in the 18S dataset (for example, *Alternaria*, *Aspergillus*, *Knufia*, *Mycosphaerella*), a difference that may be due to significantly lower richness and diversity compared to the ITS2 dataset (Figure 7F). The *Rhodotorula* genus was identified in higher concentrations in the surrounding water compared to PVC samples (4.9% vs. 0.5%). The low diversity and richness reported in the surrounding seawater may explain why many genera were only detected on plastics using the V4 region of the 18S rRNA gene, namely, *Malassezia* (3.6%), *Lobulomyces* (2.5%), the environmental clade LKM11 (1.9%), *Cladosporium* (0.03%), unidentified Sordariomycetes (0.3%), *Taphrina* (0.3%), *Trechispora* (0.1%), and *Classicula* (0.1%).

3.2. Long-Term Immersion of Plastics in Controlled Conditions

A second set of results was obtained for different plastic types, either conventional (PEO and OXO) or biodegradable (PCL and PHBV), immersed in aquariums in Banyuls-sur-Mer, with direct circulation to the Mediterranean Sea in darkness. A fungal community structure was highlighted and appeared unique and relatively complex, depending on the considered plastic type and dynamic.

Illumina Miseq DNA sequencing generated 1,059,789 paired sequences for the 24 polymer film samples incubated in direct-to-sea aquariums. These iTAGs generated 138 fungal ASVs (Supplementary Table S4). The tested variables were 'Environment' (plastics vs. seawater), 'Type of plastics' (conventional vs. biodegradable), and 'Incubation time' (from 3 to 206 days) (Figure 8). No difference was observed for the 'Type of plastics' and 'Incubation time' variables (Figure 8B,C); only the 'Environment' variable showed significant difference for the Chao1 index (p-value = 0.0396), while the InvSimpson index appeared close to 0.05 (p-value = 0.0511), indicating a clear difference between the free fungal communities in seawater and those associated with plastic films (Figure 8A). The Chao1, Pielou, Shannon, and InvSimpson indices, which were used to assess alpha diversity, showed clear trends of lower values for fungal communities associated with plastics compared to those in surrounding seawater samples, indicating lower richness and equitability. There were significant disparities in the amount of fungal ASVs found in samples taken from plastics and surrounding seawater (Figure 8A). The seawater samples had an average of 14.66 fungal ASVs, which was higher than the average of 7.43 fungal ASVs in plastic samples, and just 13 ASVs appeared to be shared across the two environments, hence representing about 9% of the total ASVs.

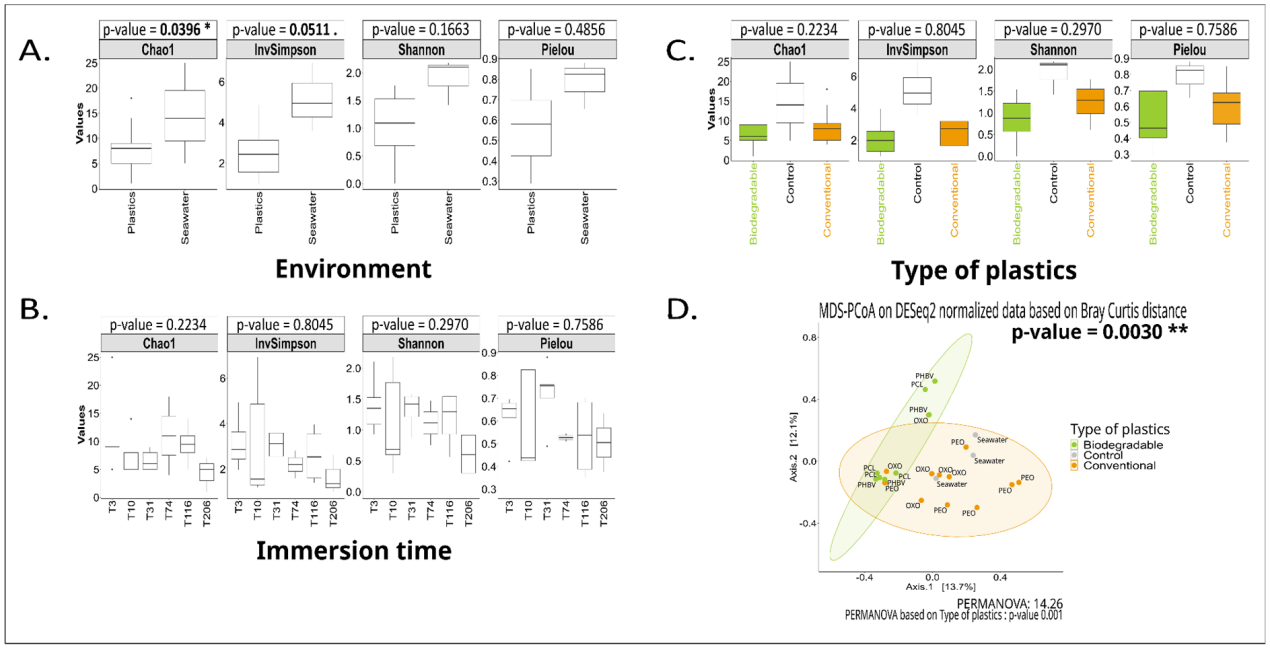

Figure 8. Significance of alpha diversity indices for the variable (**A**) "Environment", (**B**) "Immersion time" (days), and (**C**) "Type of plastics". (**D**) The PCoA demonstrates the relationship between the similarity in fungal community composition and the variable "Type of plastics". "*" and "**" represent a significant, and a highly significant influence of the evaluated factor, respectively. The PCoA was generated using Bray–Curtis dissimilarity as the distance measure, with each point representing a sample. Ellipses connect groups of samples that were subjected to the same conditions.

For beta diversity visualization, the PCoA method normalized by DESeq 2 and based on Bray–Curtis dissimilarities was used for each variable. The 'Type of plastics' variable was the only one that showed significant difference between samples (p-value = 0.0030) (Figure 8D) and thus appeared as the main parameter structuring the fungal communities. Indeed, while alpha-diversity analyses did not reveal intraspecific difference between fungal communities associated with biodegradable (PCL and PHBV) and conventional (PEO and OXO) plastics, beta-diversity analyses strongly indicated a significant interspecific difference. Sixty-one ASVs were unique to conventional plastics and 32 to biodegradable plastics. Six ASVs were shared by the two plastic types. Conventional plastics had four ASVs in common with the surrounding seawater, while biodegradable plastics only had two. Remarkably, fungal communities in seawater exhibit a higher degree of similarity with those found on conventional plastics.

The Ascomycota phylum was found in greater number in surrounding seawater (77.3%) than on plastics (73.6%) as Chytridiomycota (1.5% vs. 0.3%) and Basidiomycota (9.1% vs. 3.1%), in contrast to Mucoromycota, which was only observed on plastics (2.6%) (Figure 9A,B).

A bubble plot for the 'Type of plastics' variable was used to visualize taxonomic diversity at the phylum level (Figure 9B,C). Ascomycota was the most commonly detected and abundant phylum associated with conventional plastics (70.6%) in contrast to Basidiomycota, which was the most frequently detected and abundant phylum associated with biodegradable plastics (71.6%). The phylum Mucoromycota was exclusively present on conventional plastics, while the phylum Chytridiomycota was only present on biodegradable plastics. Taxonomic diversity associated with conventional and biodegradable plastics was similar at the genus level. *Penicillium* was the most shared genus between the two types of plastics (biodegradable 43.8% vs. conventional 56.2%). Fewer ASVs (34.3%) were found on biodegradable plastics, compared to 65.7% on conventional plastics. While some genera were predominant on biodegradable plastics compared to conventional plastics (e.g., *Dipodascus*, 70.8% vs. 29.2%), others were only present on conventional plastics (e.g., *Burgoa*,

Candida, *Mucor,* and *Engyodontium*). The latter genus was characterized as a differentially abundant taxon on the basis of ANCOM, clearly showing that this genus is significantly abundant on conventional plastics. Other genera, such as *Angustimassarina* and *Hannaella*, were only found on one plastic polymer (OXO and PHBV, respectively).

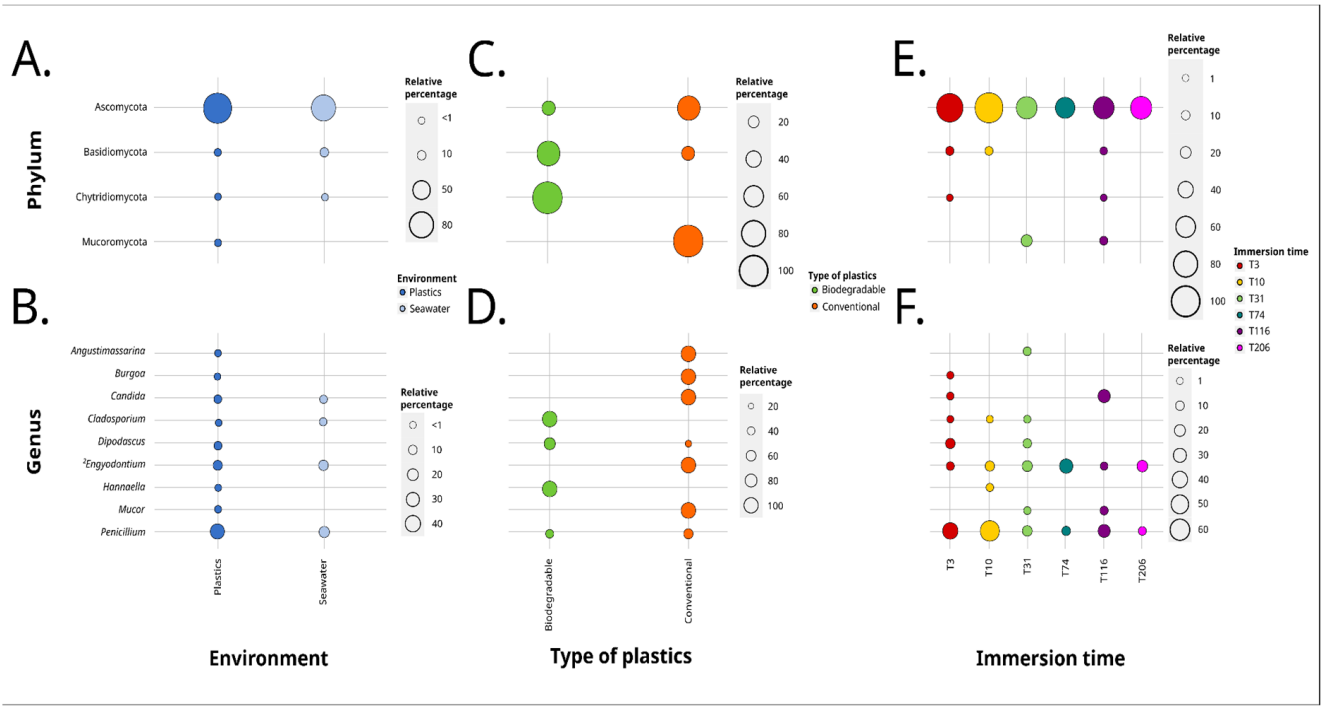

Figure 9. Bubble-plot representing the proportions of phyla or genus according to the variables (**A**,**B**) "Environment", (**C**,**D**) "Type of plastics", and (**E**,**F**) "Incubation time" (days).

4. Discussion

Our analyses provide original information to better understand fungal diversity and ecology on different polymers immersed in marine natural environments and under laboratory-controlled settings. The main conclusions correspond to (i) the distinction between polymer and free-living fungal communities, and the impact of (ii) the type of plastics (conventional vs. biodegradable), (iii) the location and associated environmental parameters, and (iv) the immersion duration on the fungal colonization. Our findings demonstrate that plastics provide a novel ecological niche for surface-associated fungi in the marine environment and that these fungi are actual members of the plastisphere.

4.1. Plastics Influence Marine Fungal Communities

Fungal community structure differed between plastics and the surrounding seawater regardless of the polymer type, either in natural settings or in controlled conditions (aquariums), and based on either ITS2 or the V4 region of the 18S rRNA gene datasets. It has been well established that microbial communities associated with marine plastics strongly diverge from those living free in surrounding seawaters [23]. Since the discovery of the unique microbial communities, referred to as the "plastisphere", harbored by marine plastic debris [26], a large majority of studies depicted significantly higher bacterial community richness on plastics compared to seawater (e.g., [32,34,38,41,62]. Moreover, our DNA extracts were the same used in Catao et al. (2021) [45] that also showed specific bacterial biofilm communities compared to planktonic ones. This pattern was not confirmed for aquatic microeukaryotes in the Baltic Sea, where PE and PS samples showed significantly lower richness than surrounding water samples [63], a specific trend that was also shown for fungi on the same samples [37]. Using a culture-dependent approach, a lower fungal species richness on uncharacterized MPs was also highlighted compared to the surround-

ing sediments [42], suggesting a selection phenomenon exerted by the plastic matrix. Our findings on PVC immersed in natural settings thus appear to be in stark contrast to these previous works, but are still in line with a recent study highlighting higher fungal community richness on PVC compared to seawater [64]. This strongly suggests that microbial colonization of plastics in natural settings is a multidimensional process that depends on many parameters, the environmental factors of each studied habitat being the most important one [65]. Thus, as depicted for bacterial communities [28,31], fungi associated with plastic are also strongly influenced by their surrounding environment and do not converge toward a standardized plastisphere. However, the influence of plastic polymers, more precisely their intrinsic features, cannot be totally ignored. Indeed, previous studies have characterized PVC as a putative inhibitor of specific bacterial communities, more precisely filamentous cyanobacteria [46], because of plastic-weathering-generating leachates, such as phthalates, known to limit the colonization of specific bacterial taxa (e.g., on *Prochlorococcus*, as described in Tetu et al. (2019) [66], albeit under laboratory conditions). This process may thus indirectly promote the establishment of fungal communities on some specific plastics due to the inhibition of certain bacterial communities.

A distinct contrast was noted under controlled conditions in aquariums in darkness, as fungal diversity and richness appeared lower when compared to seawater. This trend appears to be inconsistent with previous studies targeting bacterial communities that have used a comparable metabarcoding approach but found bacterial richness to be higher on plastic samples compared to the free-living fraction [41]. This may be explained by the competitive exclusion principle, more precisely, competition for space as microorganisms colonize a surface leading to low fungal richness on a plastic surface when bacterial richness is high and higher fungal richness when some bacterial taxa are constrained. When examining the entire dataset, which includes both natural settings and controlled conditions, our results suggest that light may also be an important parameter structuring fungal communities associated with plastics. Wallbank et al. (2022) [67] highlighted that a 50% decline in light intensity with a depth from 20 to 60 cm beneath the water surface had no significant impact on plastic-associated fungal community composition. Here, we can hypothesize that presence of light, instead of light intensity, represents a non-trivial parameter influencing fungal abundance on marine plastics, either directly or indirectly through the settlement of biofouling macro- and microorganisms. Light definitely triggers the colonization of photosynthetic organisms on plastic surfaces in natural environments, and this may explain the presence of specific fungal taxa that are known to interact with these photosynthetic macro- and microorganisms in our data sets. Indeed, in natural settings, on PVC, while certain organisms of the Chytridiomycota phylum were found in both seawater and on plastics, others from the Rozellomycota or Aphelidiomycota phyla were only found on this polymer. Taxa from these phyla have been demonstrated to be parasites of many algal groups, including diatoms [68,69], and could parasitize dense microalgal biofilms on the surface of plastics [33,70]. In order to emphasize the relative complexity of fungal communities associated with plastics, in terms of diversity, richness, but also known lifestyles, some putative plastic-degraders were also detected here. At the genus level, ASVs affiliated with *Alternaria* were detected on PVC in all sites, underlining its ubiquitous nature. Knowing that a recent study highlighted the ability of the marine isolate *Alternaria alternata* FB1 to degrade PE [44], and that plastic degraders occur mainly during the first stages of colonization as pioneer microorganisms [23], the presence of this genus mostly during the early stages of colonization of PVC appears consistent in terms of both time and space. At the species level, the *Aureobasidium pullulans*, *Chaetomium globosum*, and *Penicillium citrinum* species, almost exclusively present on PVC in this study, have already been reported to show PVC degradation potential [71]. The presence of such putative plastic-degraders definitely paves the way for further studies to better understand the functions of plastic-associated marine fungi and their real abilities to utilize plastic polymers as a carbon source.

4.2. The Biodegradable and Conventional Nature of Polymers Impacts the Plastic-Associated Fungal Community

Based on the two types of plastic material immersion, either in natural settings or in controlled conditions (aquariums), our results clearly show that there are no significant differences in terms of community composition among polymers of a similar type (conventional or biodegradable), but that fungal communities associated with biodegradable plastics are significantly different from those associated with conventional ones. Our results thus appear consistent with previous studies highlighting no distinct communities on plastics related to the same type, either for bacterial communities [34,72,73], microeukaryotes [27,34], or specifically, fungi [37,74]. The abundance and diversity of bacteria were shown to vary greatly between biodegradable and conventional plastics [75,76]. Similar results were obtained on bacterial and microeukaryotic communities when comparing PE and PP to PBAT/PLA [77]. Differences in terms of community composition are related to intrinsic features such as hydrophobicity, surface roughness, and density, which vary significantly between biodegradable and conventional polymers [77,78]. The type of polymers (biodegradable vs. conventional polymers) definitely impacts the microbial community composition of the plastisphere. As observed by Li et al. (2023) [79], conventional plastics have greater richness and diversity than biodegradable plastics. Conventional plastics with a poor biodegradability and more hydrophobic surface may thus favor random microbial colonization and are more likely to house a broader diversity of bacteria in the absence of particular selection [80]. This has been confirmed by Mercier et al. (2017) [81], revealing that the surface of PET had higher bacterial operational taxonomic units (OTUs) and Shannon index compared to the surfaces of three other biodegradable polymers. Therefore, compared to biodegradable plastics, conventional plastics seem to favor more bacterial and fungal communities [40]. Here, no significant differences were observed for fungal communities in terms of alpha diversity between biodegradable and conventional plastics. However, as only seven kinds of plastic polymers were used, it is quite difficult to compare these results to previous studies. Clear differences in terms of beta-diversity between biodegradable and conventional plastics allowed us to highlight specific taxa being related to one type of plastics. Using the ITS2 genetic marker, only biodegradable plastics harbored the *Dipodascus* genus, more specifically, *Dipodascus australiensis*, a fungus already reported as dominant in corn silage kept in biodegradable films [82]. Using the ANCOM tool, *Engyodontium* was emphasized as specifically associated with conventional plastics, which appears consistent with a previous study describing *Engyodontium* as plastic degraders, more precisely, through its ability to biodegrade pretreated PP [83].

4.3. Geographical Location Impacts the Plastic-Associated Fungal Community

Besides the intrinsic features of plastics, the immersion location and associated environmental parameters have a major influence on microbial communities [72,84]. According to recent research targeting the bacterial and microeukaryotic populations associated with plastics, location, followed by time, and then plastic type, appeared as the main factors structuring microbial communities [77].

Our results clearly confirmed that the structure of fungal communities associated with plastics varied significantly based on their geographic location, and specifically, a clear dichotomy between open ocean sites (Brest, Lorient and Le Port) versus a semi-enclosed basin (Mediterranean Sea). Knowing that the studied open ocean sites are eutrophic and the semi-enclosed ones are mesotrophic [45], we can hypothesize that the trophic level is another major parameter structuring fungal communities associated with plastics. The observed dichotomy between sites appears consistent with several field-sampling studies. Indeed, using plastic litter samples of the North Sea, location was shown to impact the bacterial colonization of plastics [85], a pattern that was also observed for eukaryotic communities in North and Baltic seas [86]. Characterization of the plastisphere from PP and PE samples collected off the coast of Italy led to the conclusion that geographical location (sampling sites) was the main structuring factor [87]. However, no difference in terms of

fungal colonization was found between plastics collected in the western South Atlantic Ocean compared to those harvested in the Antarctic Peninsula [39]. Such discrepancy may be due to a better stabilization of fungal communities, meaning a kind of convergence of biofilm after long drift in the marine environment and highlights the limitations of field sampling, representing snapshots of plastic colonization at uncertain times.

Our results also strongly highlighted the importance of the 'Temperature' parameter, with higher richness when sites were characterized by temperatures between 21 and 26 °C, in line with the global thermal preference of marine fungi [88,89], strengthening the importance of the location and associated environmental parameters on the colonization potential of plastics by fungi. Stages of microbial colonization definitely depend on the growth rate of the biofilm, which itself is determined by numerous factors including temperature, salinity, light, nutrient availability, etc. [23,28,90]. Our results thus tend to reinforce such statements. The trends observed for bacteria and specific protists associated with plastics are likely to hold true for fungi as well.

4.4. Immersion Duration Impacts the Plastic-Associated Fungal Community

The dynamic of microbial colonization of plastic matrices in the marine environment is a non-trivial question, and recent surveys come to the conclusion that if strong shifts occur during the early stages of microbial colonization, communities converge as stable mature biofilms [23]. However, the stochastic colonization of pioneering microorganisms, along with the continuous influence of the surrounding environment may yet limit communities from converging toward a unique standardized plastisphere, as previously shown for bacteria [46,91].

Our results regarding PVC immersed in natural settings clearly highlighted a fungal colonization dynamic with a shift between the first 30 to 40 days of immersion, characterized by early stages of colonization and biofilm formation, followed by a second stage with distinct communities likely associated with biofouling. At this particular stage, it is important to take a step back and acknowledge the limitations of our approach. Our study involved the integration of results from multiple research projects, each using different PVC matrices (pellets versus panels) and DNA extraction methods (kit versus phenol-chloroform). As such, the data visualization we present may be subject to biases. However, we have taken measures to control for these biases by conducting individual analyses (Supplementary Figure S3) that demonstrated consistent trends across different PVC matrices and sample sites (albeit less pronounced for samples collected in Brest), using either ITS2 or 18S data sets. While our results appear robust, but still not free from biases, we acknowledge the need for further analyses to confirm their validity.

A previous immersed-based study focusing on the colonization of PE by fungal communities however concluded that no clear temporal profile could be established [38], illustrating once again the multiparametric process of fungal colonization and calling for more studies on fungi to better understand the colonization success and dynamics. Our results were consistent with previous research on bacteria, which showed that biofilms on conventional plastics matured after approximately 30 days under controlled conditions [29,47,92]. After this first stage of biofilm formation, our results highlighted a second stage that may be correlated to the implementation of photosynthetic macro and microorganisms as part of the biofouling communities [28,93]. Consistent with such a hypothesis, at late immersion sampling time points, the Chytridiomycota, Rozellomycota, Aphelidomycota, and Cryptomycota phyla, which are described as parasites of numerous algal groups, were present, and thus may suggest an interaction with biofouling macroorganisms. This raises important ecological questions, knowing that plastic waste is hypothesized to act as a vector for toxic algal bloom species in marine habitats, with the potential to disturb the oceanic carbon pump and vertical nutrient flows [63].

Finally, and specifically for the PE, PP, and PVC samples immersed in Brest, the temporal succession of the biofilm along with the effect of tidal influence were shown as significant structuring parameters. Plastic samples specifically deployed in an intertidal area, which

were impacted by regular fluctuations of the environment by repeated cycles of emersion and immersion, harbored significantly different fungal communities compared to those immersed in subtidal areas. In addition, fungal communities evolved through time within this specific intertidal level, consistent with previous results on bacterial communities on the same samples [46]. The specific pattern showing that bacterial communities associated with plastics are dramatically and continuously influenced by the environment [91] can thus be extended to the fungal kingdom.

5. Conclusions

Overall, through a metabarcoding analysis of a broad set of samples, which allowed for a determination of the diversity and distribution patterns of fungal communities associated with plastics immersed in natural and controlled settings, we were able to demonstrate that (i) the fungal Kingdom is an integrated part of the plastisphere, (ii) plastic-associated fungal communities are relatively complex and distinct from those living in surrounding seawaters, and (iii) locations and inherent environmental parameters, type of plastics (conventional vs. biodegradable), and immersion duration have strong influence on fungal colonization. Our findings pave the way for further studies to delve deeper into the biodegradation capabilities and functionalities of these fungal communities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15040579/s1, Figure S1: Significance of alpha diversity indices for the variable “Temperature”; Figure S2: Alpha and beta-diversity analyses for the variables "Polymers”, “Tidal location” and “Immersion time”. Figure S3: Alpha diversity indices and their associated significance for the variable “Immersion time”. Table S1: Summary of the information regarding samples and methods used in each PVC-dedicated study. Table S2: Number of reads before and after processing, number of ASVs, and alpha diversity index values for each sample for the ITS2 dataset (natural settings). Table S3: Number of reads before and after processing, number of ASVs, and alpha diversity index values for each sample for the 18S dataset (natural settings). Table S4: Number of reads before and after processing, number of ASVs, and alpha diversity index values for each sample for the ITS dataset (controlled conditions).

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