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Diversity and predicted inter- and intra-domain interactions in the Mediterranean Plastisphere☆

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ABSTRACT

This study investigated the biogeography, the presence and diversity of potentially harmful taxa harbored, and potential interactions between and within bacterial and eukaryotic domains of life on plastic debris in the Mediterranean. Using a combination of high-throughput DNA sequencing (HTS), Causal Network Analysis, and Scanning Electron Microscopy (SEM), we show regional differences and gradients in the Mediterranean microbial communities associated with marine litter, positive causal effects between microbes including between and within domains of life, and how these might impact the marine ecosystems surrounding them. Adjacent seas within the Mediterranean region showed a gradient in the microbial communities on plastic with non-overlapping endpoints (Adriatic and Ligurian Seas). The largest predicted inter-domain effects included positive effects of a novel red-algal Plastisphere member on its potential microbiome community. Freshwater and marine samples housed a diversity of fungi including some related to disease-causing microbes. Algal species related to those responsible for Harmful Blooms (HABs) were also observed on plastic pieces including members of genera not previously reported on Plastic Marine Debris (PMD).

1. Introduction

The presence of plastic marine debris (PMD) in the world ocean is receiving increasing attention (Rochman, 2020), and the impacts on marine organisms from marine mammals to fish due to ingestion and entanglement are well documented (Fossi et al., 2018; Kuhn & van Franeker, 2020; Laist, 1997). PMD is also known to transport non-indigenous and potentially harmful species (Aliani & Molcard, 2003; Derraik, 2002; Masó et al., 2003). In contrast to the visible impact of larger pieces of PMD, the role of smaller plastic pieces on marine ecosystems and the transport of microbial taxa between biomes is still relatively unknown (Amaral-Zettler et al., 2020). Microplastics (defined as pieces < 5 mm in maximum dimension) may be too small to impact larger animals directly, but more and more organisms are being discovered that ingest them. Microplastics are the most abundant form of PMD, and also serve as an attachment surface for a wide diversity of

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microbes suggesting that microbe-plastic interactions may represent the major ecosystem impact of plastic litter, with the potential to ripple up the food chain and impact nutrient and carbon cycles (Zhao et al., 2020).

The first published reports of plastic debris in the Mediterranean documented pre-production plastic pellets from beaches in the eastern Mediterranean (Shiber, 1979), and plastic debris floating in the central Mediterranean (Morris, 1980). With a restricted exchange with the open ocean, and a surrounding population of about 500 million people, surveys confirm widespread distribution and high concentrations of floating microplastic in the Mediterranean (Suaria et al., 2018), making it one of the most polluted seas in the world, with PMD concentrations comparable to those found in the major oceanic gyres (Cozar et al., 2014; van Sebille et al., 2015).

When a piece of plastic enters the ocean, microbes colonize it within hours (Harrison et al., 2014). Over time the microbial community and other microscopic organisms on the surface of the plastic, sometimes referred to as the "Plastisphere" (Zettler, Mincer, & Amaral-Zettler, 2013), becomes more diverse and the composition changes, effectively forming independent islands of biodiversity. Numerous studies have demonstrated that the microbial community attached to plastic is very different from the community in the surrounding water, and the bacterial Plastisphere community in the Mediterranean is different from particle-attached microbes (Dussud et al., 2018b). Many organisms found attached to PMD are common fouling organisms that will attach to any surface, but there is also evidence that plastic is preferentially colonized by certain microbes that form a "core" Plastisphere community (Frère et al., 2018; Kirstein et al., 2019). Some members of these microbial communities such as diatoms produce secondary metabolites, including dimethyl sulfide, shown to influence ingestion of plastic in birds, fishes, sea turtles and possibly even marine mammals (Savoca, 2018; Savoca et al., 2016).

In the Mediterranean, interactions with PMD have been reported for over 100 species (Anastasopoulou & Fortibuoni, 2019) including invertebrates, fish, sea turtles, and birds, as well as threatened species such as marine mammals (Fossi et al., 2018) in the Pelagos Sanctuary, a Specially Protected Area of Mediterranean Importance (SPAMI; MedPAN and RAC/SPA, 2019). The impact of the microplastic and its associated microbial community on animals that ingest it is not well understood. HAB species attached to plastic debris were first reported from the Mediterranean (Masó et al., 2003), and the Plastisphere's role in harboring and transporting other harmful microbes is receiving increased attention as potential pathogens for marine animals and humans have been reported on PMD in the Atlantic (Zettler et al., 2013), the North Sea (Kirstein et al., 2016), as well as the Mediterranean (Dussud et al., 2018b; Virsek et al., 2017).

Bacterial studies dominate existing information about the microbial communities associated with plastic debris, with very few studies targeting eukaryotes (Wright et al., 2020). However, as is evidenced by these and microscope-based studies (e.g. Carson et al., 2013; Masó et al., 2016), microbial eukaryotes are well-represented on PMD. Studies employing high-throughput sequencing of eukaryotes on plastic are even more limited, though as pointed out by Bryant et al. (2016), eukaryotic reads can dominate the community attached to plastic debris, and these eukaryotes are important in the nutrient and carbon cycling of the biofilm. There have been some studies of eukaryotes on plastic including fungi (Kettner et al., 2017) that can be parasites or pathogens and also potentially contribute to the breakdown of plastic. Understanding the role of eukaryotes and their interactions with bacteria is crucial to understanding the role of microbes in the fate of plastic debris in the ocean (Kettner et al., 2019). However, so far there are very few HTS studies that looked at bacteria and eukaryotes on the same plastic samples (reviewed in Amaral-Zettler et al., 2020; Davidov et al., 2020), and only two looked at communities on samples of environmental plastic (Bryant et al., 2016; Debroas et al., 2017).

Geographic location and environmental parameters appear to be the primary influences shaping Plastisphere communities, but studies using environmentally collected samples are rare. Environmental samples show that ocean-basin scale differences exist in Plastisphere communities from the North Pacific and North Atlantic gyres (Amaral-Zettler et al., 2015), but the question of whether these large-scale biogeographic differences extend to other areas or to smaller scales within regional seas is still an open question. The multiple basins in the Mediterranean region provide an opportunity to test hypotheses about the biogeography of microbial interactions with PMD. We hypothesized that 1) the Mediterranean Plastisphere varies regionally, and 2) that rivers, known to be an important source of plastic debris to marine systems, contain very distinct Plastisphere communities from those in the open sea, and the presence of freshwater microbes in the marine environment can represent markers of their origin. We tested these hypotheses using both bacterial and eukaryotic targeted amplicon sequencing, supplemented by SEM imaging. Other authors have speculated that eukaryotes play an important role in the degradation and fate of PMD (Dudek et al., 2020; Schlundt et al., 2020), but to our knowledge this study represents the first comprehensive bacteria/eukaryote study using environmental samples (63 plastic pieces with both bacterial and eukaryotic sequences), also providing new insights into the biogeographic differences and microbial interactions in the Plastisphere.

2. Material and methods

2.1. Sample collection

A total of 131 net tows were collected to sample PMD from June–August 2017 (Tyrrhenian, Ionian, Adriatic seas) and July/August 2018 (Tyrrhenian and Ligurian seas) as part of Expédition MED Citizen Science surveys and the Pelagos Plastic Free project (see Fig. 1a and Table S1 for details of sampling dates and locations). A manta net (0.6 m × 0.2 m with a 330 μm mesh) was towed from a boom that kept the net 2–3 m off the side of the boat and out of the wake. The net was towed for 30 min at 2–3 knots in sea conditions 3 or lower on the Beaufort Scale. Trawl length was calculated using GPS locations at the start and end of the tow. After each tow, the cod end was emptied into a bucket sterilized with 70% ethanol and rinsed 5x with seawater from the sampling site. A single plastic piece from the bucket was selected for microbial community analysis and transferred with sterile forceps into a sterile jar containing 0.2 μm-filtered seawater from that site and swirled to rinse off non-attached organisms. The selected piece was photographed and measured and then subdivided for DNA, SEM, and chemical analyses. Plastic for DNA analyses was placed in Qiagen cell lysis solution (QIAGEN Benelux B.V., Venlo, The Netherlands) and frozen at −18 °C. Plastic for microscopy was preserved in 4% paraformaldehyde at 5 °C; then transferred to 50:50 PBS:Ethanol within 22 h and stored at −20 °C. Plastic for ATR-FTIR spectroscopic analyses was folded into clean aluminum foil and preserved at room temperature. All remaining plastic pieces from each trawl were concentrated over a 200-μm mesh, preserved with 70% ethanol (2017) or 20% hydrogen peroxide (2018) and stored at room temperature for subsequent counting and chemical analyses.

Samples for comparison of the microbial communities in the water were taken during one manta trawl per week. Each time, 0.5–1 L of seawater was filtered through a 0.2 μm Sterivex filter cartridge (Merck Millipore, Darmstadt, Germany). The cartridge was then flooded with Qiagen cell lysis solution and frozen until extraction.

Another 40 plastic debris items (>5 mm) were collected from shore at four rivers and ports, along with filtered water samples (Fig. 1 and Table S1). These samples were processed as described above for samples collected from the vessel.

2.2. Plastic quantification and chemical composition

In the laboratory, samples from net tows were rinsed with fresh water and manually separated from organic matter using a

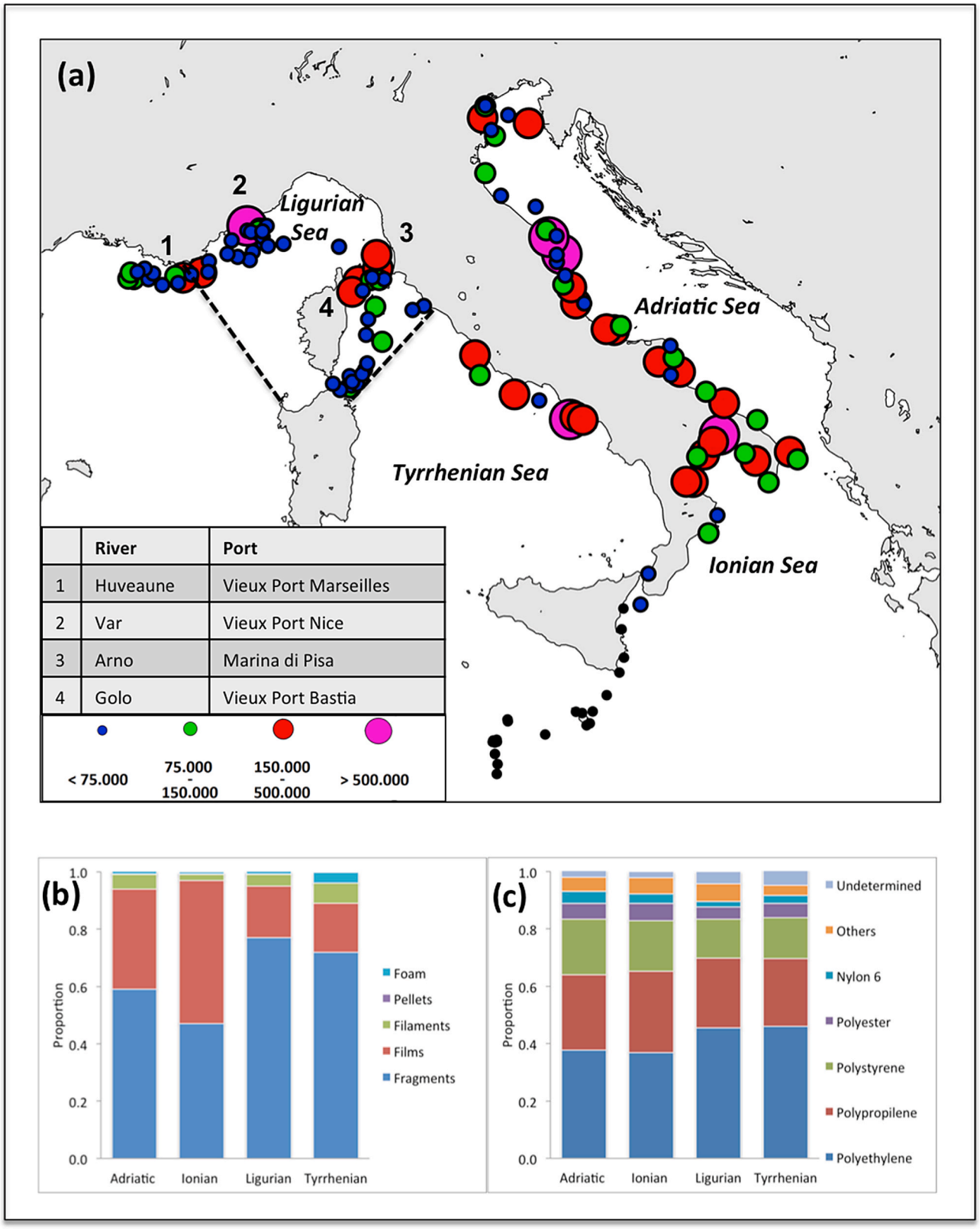

Fig. 1. Distribution, abundance, and types of plastic. (a) Map of the central-western Mediterranean Sea showing the location of sampling stations and concentrations (items per km^2, before wind correction) of plastic pieces between 1 and 5 mm (MP_{1-5mm}), and outline of the Pelagos Sanctuary (black dashed lines). The size and color of the circles are proportional to measured concentration values. Black dots indicate locations for which abundance data are not shown in this paper. Numbers 1–4 indicate locations of sampled ports and rivers as indicated in the legend table. **(b)** Regional differences in the percentage of the 5 different categories of plastic pieces for MP_{1-5mm}. **(c)** Regional differences in the percentage of polymer types for MP_{1-5mm} (subset of 5500 out of 24,630 pieces or 22% of the total). The category "others" includes: Polyamides, Polyurethane, Polyacrylic, Polyvinyl chloride, Cellophane, Poly(methyl methacrylate), Polycarbonate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

stereomicroscope (10–40× magnification). After drying at 50 °C, plastic pieces were counted and sorted into categories (fragment; film; line; pellet; foam) as suggested by GESAMP (Tahir et al., 2019). All pieces used for microbial community analysis, and 22% of the total plastic pieces collected (5500 of 24,630 pieces) were analyzed using an Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectrometer (Nicolette 6700 spectrophotometer (Thermo Scientific)) to identify plastic polymer type. All spectra were recorded in the absorbance mode in the 4000-600 cm^{-1} region with 4 cm^{-1} resolution and 12 scans. Each piece of plastic was placed onto the germanium diamond cell (ATR Golden Gate) to obtain a better-quality spectrum. Sample spectra were compared to reference spectra (instrument library and http://www.ftir-polymers.com/soon.htm) in order to identify the chemical nature of the plastic pieces (Pedrotti et al., 2016) using a matching factor ≥0.7. A subset representing 22% of the total samples (5500 of 24,630 total plastic pieces) was analyzed for polymer type. Samples were selected using a proportional random sampling strategy. In particular, plastic pieces were divided into homogeneous subgroups using the manual Quartering Method (Gerlach et al., 2002) and using a board to delineate subgroups so that the sample could be divided precisely. In this study, plastic pieces smaller than 1 mm were not considered, so we use the abbreviation $MP_{1–5mm}$ to refer to the concentration we measured. Plastic pieces that were removed for microbial analysis were included in the final count of total plastic pieces.

2.3. DNA extraction, sequencing, and analysis

We extracted total genomic DNA from individual pieces using a commercial kit (Gentra Puregene Kit, Qiagen, Valencia, CA) as previously reported (Amaral-Zettler et al., 2015). We performed genomic DNA amplifications using specific primers targeting the V6 bacterial (967F and 1064R, Sogin et al., 2006) and V4 eukaryotic (EukV4F and EukV4R, Balzano et al., 2015) hypervariable regions of SSU rRNA genes. Amplicon generation (with negative controls) and sequencing was performed on an Illumina NextSeq (bacteria) or MiSeq (eukaryotes) platform following protocols at the Marine Biological Laboratory W. M. Keck Ecological and Evolutionary Genetics Facility in Woods Hole, MA, USA (details in the Supporting Information).

All sequences were demultiplexed using unique barcodes, and then merged for quality control retaining only pairs with complete overlap and no mismatches (Amaral-Zettler et al., 2015). Reads were then dereplicated using vsearch v.2.8.0 (Rognes et al., 2016) with the –derep_full length option. Of the original 131 samples, 87 yielded both bacterial and eukaryotic sequences, but only 75 were retained after eliminating samples with insufficient sequencing depth (63 plastic and 12 water samples). The analysis yielded 374,400 unique bacterial sequences and 1,194,277 unique eukaryotic sequences across these 75 samples. Unique sequences were clustered into SWARM Operational Taxonomic Units (OTUs) using Swarm v2.2.2 (Mahé et al., 2015), hereafter referred to as OTUs, with the fastidious option selected to link nearby low abundance swarms with the minimum difference (*d*) between swarms set to 1. Bacterial clustering generated 55,247 OTUs with the largest OTU swarm 7032 in size (max generations = 15) and eukaryotic data yielded 166, 318 OTUs with the largest swarm of 45,052 (max generations = 20). Representative sequences were then subjected to chimera detection with vsearch v2.8.0 uchime-denovo option, resulting in the removal of 2435 chimeras for the bacteria and 19,467 chimeras for the eukaryotic OTUs.

Sequence data was processed using methods specific to the different objectives including taxonomic assignment, network analysis, and eukaryotic phylogenetic placement (see Supporting Information for details). Raw sequence data have been deposited in the NCBI Sequence Read Archive in the BioProject PRJNA721966.

2.4. Causal effect analysis

For causal effect analysis we retained OTUs found in at least 30% of the samples. This filtering resulted in 718 OTUs for the bacteria and 153 OTUs for the eukaryotes. To exclude any OTUs potentially originating from chloroplast or mitochondrial sequences, representative sequences of bacteria were subjected to BLAST (Altschul et al., 1990) against the Silva SSU Ref v132 database (Quast et al., 2013) and sequences with top hits to chloroplast or mitochondrial sequences were removed. The remaining bacterial and eukaryotic OTU tables were merged and then transformed with variance stabilizing transformation (Anders & Huber, 2010) to render OTU abundances approximately Gaussian distributed. This data was used for causal effect estimation following the aIDA approach (Taruttis et al., 2015) using 100 sub-samples of 2/3 of the samples each (75 samples in total) and alpha = 0.5. aIDA first reconstructs a partially directed graph (network) from the observational abundance data based on conditional independence tests. The local structure of the graph is then used to estimate causal effects, that is the predicted change in abundance of an OTU, following a change in abundance of the OTU predicted as cause. Because of the underlying network structure, causal effects are less prone to predict spurious interactions than correlation. Causal effects were sorted and the strongest 20 positive effects, the ones with highest probability of being true positives (Taruttis et al., 2015), were displayed for each of bacterial on bacterial, bacterial on eukaryotic, eukaryotic on bacterial, and eukaryotic on eukaryotic OTUs using Cytoscape v3.4.0.

2.5. SEM analyses

To quantify biofilm coverage, diatoms, and bacteria/archaea, we SEM-imaged all of the plastic pieces that were used for the molecular analyses. Samples were dehydrated on ice through an ethanol series, then critical point dried and sputter coated (10–20 nm of platinum or gold). Coated samples were imaged on a JEOL-JSM-6700F (JEOL Ltd., Tokyo, Japan). The surface was imaged across the major axis of each piece of plastic for at least 6 locations (Fig. S1). The percent coverage of the plastic surface by biofilm was estimated using a scale from 0 to 4, where 0 corresponds to no biofilm, 1 is approximately 25% coverage, 2 is approximately 50% coverage, 3 is approximately 75% coverage, and 4 is approximately 100% coverage (Fig. S2). Counts of individual diatoms (at 500x) and bacteria/archaea (at 1500x) were made in each field to calculate concentrations per mm^2 for 2017 samples.

2.6. Multivariate analyses

Non-metric multidimensional scaling (NMDS) was used to investigate differences between microbial communities on PMD from different regions. Analyses were run in R (R-Core-Team, 2020) with the packages 'vegan_2.5–6', 'plot3D_1.3, and 'car_3.0–7, and using metaMDS for the NMDS analysis, anosim, and pairwise.adonis for the comparisons between groups, and scatter3d for the visualization.

3. Results and discussion

3.1. Plastic debris concentrations, distributions, size classes, and characteristics

Plastic pieces were present at all 131 stations (Fig. 1a) with a mean abundance of 236 pieces per tow (SD: 358; median: 129) corresponding to a mean total concentration of 1.7×10^5 plastic pieces per km^2. The concentrations of floating microplastic ($MP_{1–5mm}$) pieces varied regionally, and $MP_{1–5mm}$ represented on average 87% of the total number of plastic pieces per tow. Average $MP_{1–5mm}$ concentration was significantly higher ($P < 0.05$) in the Ionian and Adriatic Seas than in the Ligurian and Tyrrhenian Seas (Kruskal-Wallis chi-squared = 9.3632, df = 3, P = 0.02483). Samples collected in the Pelagos Sanctuary for marine mammals (n = 49) had an $MP_{1–5mm}$ average concentration of 1.2×10^5 items km^{-2}. Previous studies have reported contrasting results with areas of highest concentration of $MP_{1–5mm}$ collected in nets in the

Pelagos Sanctuary and the lowest in the Adriatic Sea (Suaria et al., 2016). Most of our samples were taken relatively close to shore, and research has shown that boundary currents influence accumulation of floating plastic marine debris from the coast to 10 km offshore (Pedrotti et al., 2016). The spatial and temporal variability of PMD in the Mediterranean is well established (Cozar et al., 2014; G. Suaria et al., 2016; Caldwell et al., 2020), and modeling results suggest that in the Mediterranean Sea high temporal and spatial variability in ocean currents results in temporary accumulation zones for macro litter (Mansui et al., 2020; Mansui et al., 2015) and micro litter (Fossi et al., 2017).

The most common form of PMD we encountered was fragments, followed by films (Fig. 1b), as has been reported by other researchers for this region (Cozar et al., 2014), though in our samples plastic films were the most abundant category in the Ionian Sea. Foam in our samples was mostly expanded polystyrene (EPS) and was particularly abundant in two manta net trawls from the Gulf of Naples (22% of 649 pieces, and 50.9% of 446 pieces). Pre-production pellets were rare, but in one manta net near Vasto, in the Adriatic Sea, there were 12 pellets representing 2.5% of the total pieces in the tow. A dozen different polymer materials were identified (Fig. 1c), with only 239 pieces out of 5503 scanned that could not be identified (4.3% that may not be plastic). Polyethylene and polypropylene were the most common polymers in all regional seas. EPS and Nylon (both associated with fishing activities) were more abundant in the Ionian and Adriatic Sea.

3.2. Biogeography of Mediterranean bacterial and eukaryotic plastisphere communities

Using combined bacterial and eukaryotic datasets, we found overlap between Plastisphere communities of the different seas around Italy, but also recognizable differences corresponding to biogeographic zonation (Fig. 2a). The distribution of sample points in the multidimensional space defined by the NMDS shows that there is overlap in Plastisphere communities between adjacent seas, but the communities at each end of this geographic gradient (Adriatic Sea and Ligurian Sea) do not overlap. ANOSIM and pairwise comparisons showed significant differences between all seas except the adjacent Ionian-Tyrrhenian, and Tyrrhenian-Ligurian (Table S2). It must be noted that our samples were collected sequentially over two summers, so some of the effects are undoubtedly due to seasonal and interannual factors. Nevertheless, the overall pattern corresponded well with distinct Mediterranean hydrodynamic provinces and their role in structuring marine populations (Rossi, et al., 2014). Numerous publications have documented differences in communities on plastic and the surrounding ambient water (reviewed in Amaral-Zettler et al. (2020)), but our study extends marine comparisons to rivers and ports where we found clear differences but also some overlap (Fig. 2b). Microbial communities from river water and river plastic were distinct from each other and from marine counterparts, with port water and plastic clustering between river and marine systems. A recent study in Italian fresh waters (Di Pippo et al., 2020) also showed differences between communities on plastic and in the surrounding lake water. Understanding connectivity between Plastisphere communities in freshwater and marine systems is an important step in understanding the contribution of rivers to attached microbial communities in marine systems. Future studies should expand on this theme to study changes in the microbial community as plastic moves from lakes to rivers, and onward to the sea.

3.3. Regional distribution patterns and HABs

Fig. 3 provides an overview of the community differences that we observed between Plastisphere and water-associated communities from different marine sites, rivers, and ports. Alphaproteobacteria, Gammaproteobacteria, and Cyanobacteria dominated Plastisphere bacterial communities, while Alphaproteobacteria and other Proteobacteria often dominated water samples (shown in blue) in seawater (XMD_0082A,

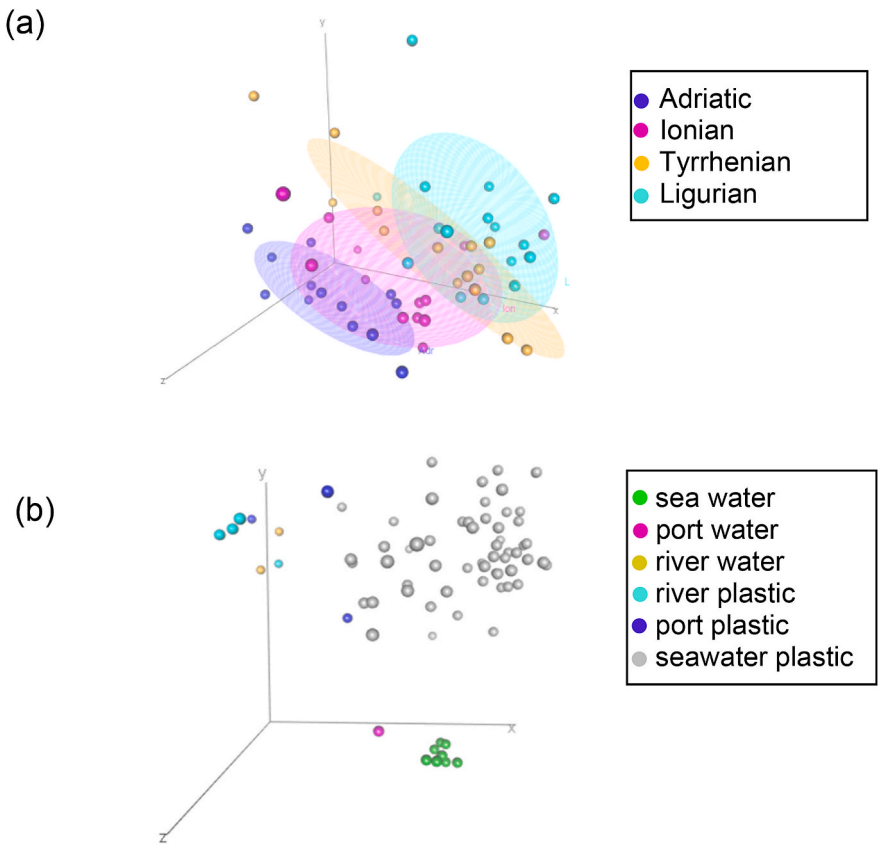

Fig. 2. Plastisphere community gradients. Three dimensional NMDS analyses of microbial communities in different geographic regions and from different environments: **(a)** Plastisphere microbial communities (combined bacteria and eukaryotes) showing a gradient from the Adriatic (blue) through the Ionian (pink), Tyrrhenian (yellow), and Ligurian (aqua) seas; **(b)** Microbial communities (combined bacteria and eukaryotes) in water and on plastic from marine, port, and river locations. Communities in seawater (green) and port water (pink) are distinct from communities on plastic. Communities in river water (gold) and Plastisphere communities of river plastic (aqua) and port plastic (blue) are distinct from the communities on seawater plastic (gray). Axes were rotated to clarify separation of different groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

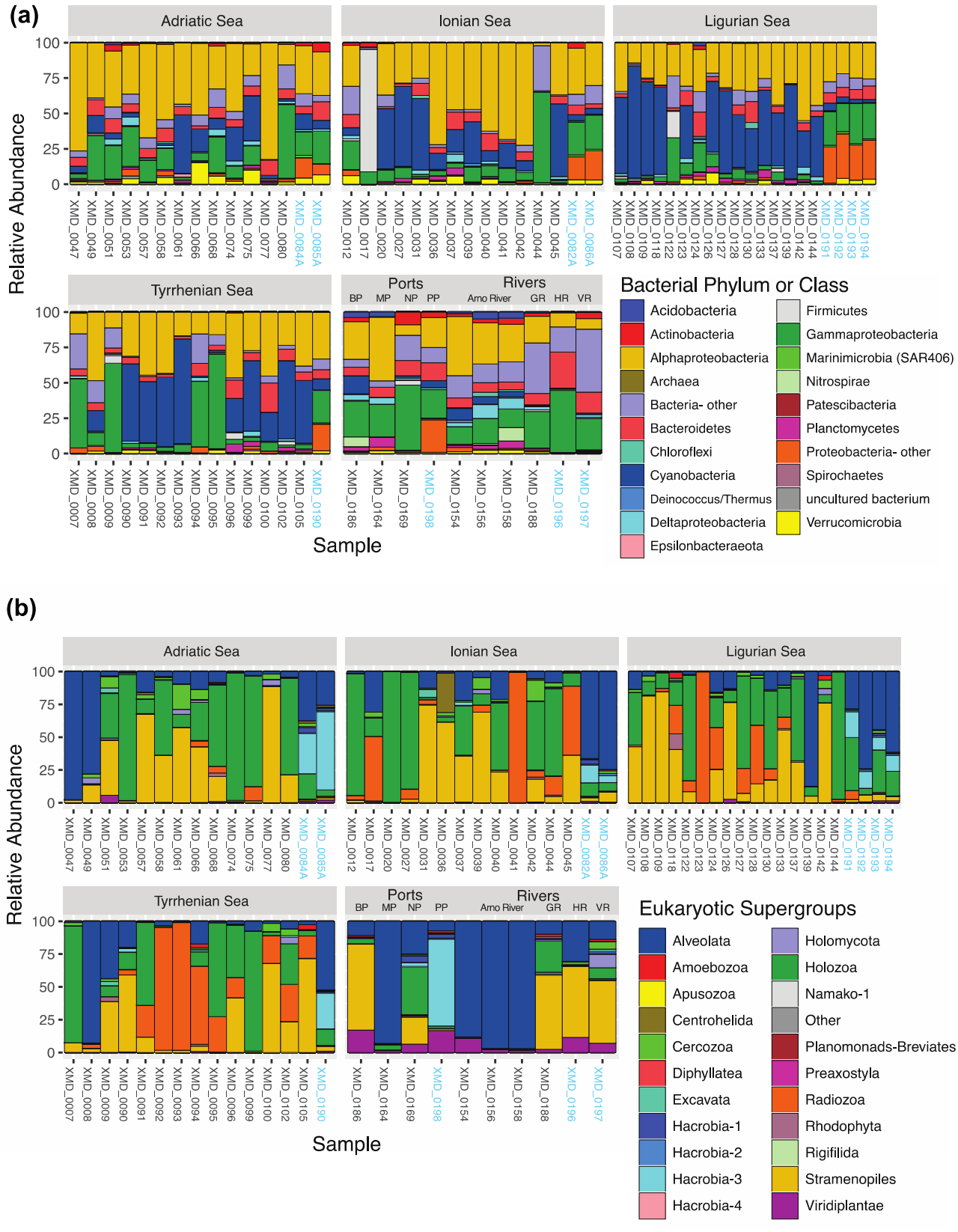

Fig. 3. Summary of taxonomic groups. Overview of broad-scale **(a)** bacterial and **(b)** eukaryotic taxonomic representation in Expédition MED (XMD) plastic samples (in black) and water samples (in blue). Abbreviations are as follows: BP (Bastia Port); MP (Marseilles Port); NP (Nice Port); PP (Marina di Pisa Port); GR (Golo River); HR (Huveaune River); VR (Var River). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

XMD_0084A, XMD_0085A, XMD_0086A, XMD_00190–00194) and ports (XMD_0198), while rivers (XMD_0196 and XMD_0197) contained larger percentages of "other" bacterial groups including Comamonadaceae, Enterobacteriaceae, and *Arcobacter* (Fig. 3a). The Plastisphere filamentous cyanobacteria included putative HAB species *Anabaena*, *Annamia* (Golo River, Bastia Port, Adriatic), Chroococcales (Ligurian, Ionian, Tyrrhenian), *Cyanobium*, *Geitlerinema* (Nice Port), *Limnothrix* (Ionian, Adriatic), *Lyngbya*, (Tyrrhenian), *Nodularia* (Nice Port), *Snowella* (Bastia Port), as well as HAB OTUs assigned to Nostocaceae, Oscillatoriaceae, Phormidiaceae (including *Planktothrix*), Pseudanabaenaceae (Bastia Port), and Synechococcaceae cyanobacterial families. Firmicutes bacteria (*Alkalibacillus, Salimicrobium, Marinococcus, Salinicoccus*) dominated one sample from the Ionian Sea (XMD_0017) identified as an aromatic polyamide (e.g. Kevlar) while another sample (XMD_0122) identified as a wax from the Ligurian Sea also displayed larger proportions of Firmicutes (with contributions from Ruminococcaceae, *Acidaminococcus*, *Megasphaera*, *Anaerovibrio*, and *Propionispira*).

Dominant eukaryotic supergroups varied widely across sampled plastic debris (Fig. 3b). For example, Alveolata dominated certain plastic samples in the Adriatic (XMD_0047, XMD_0049), Ligurian (XMD_0139), and Tyrrhenian Seas (XMD_0008), but alveolate taxa in a given sample varied from predatory suctorian ciliates (e.g. *Ephelota* or *Acineta*) on marine plastic to Petrichous ciliates (e.g. Sessilida) on riverine and port plastic (Arno River – XMD_0154,-0156,-0158; Marseilles Port – XMD_0164). In contrast, Alveolata-dominated water samples were more often dinoflagellates as in the Ligurian Sea sample (XMD_0192). Hacrobia (cryptomonads and haptophytes) were mostly encountered in water samples: both marine (Adriatic XMD_0085: *Chrysochromulina*: Tyrrhenian: XMD_0190: *Chrysochromulina* and *Phaeocystis*) and ports (Marina di Pisa Port XMD_0198: *Chrysochromulina, Prymnesium, Tisochrysis*). Other eukaryotic supergroups that dominated reads were Holozoa, Stramenopiles, and Radiozoa. Holozoan (animal) supergroups were often dominated by decapod crustaceans (Adriatic XMD_0074), medusazoans (Ionian XMD_0020), hydrozoans (Ligurian XMD_0144) or turbellarian worms (Tyrrhenian XMD_0099). Samples dominated by stramenopiles included chrysophytes, diatoms and labyrinthulids (Adriatic XMD_0077), chrysophytes and diatoms, (Tyrrhenian XMD_0105), and diatoms (Ionian XMD_0031; Ligurian XMD_0109). Radiozoa were dominated by colonial radiolaria that are associated with open ocean environments. Aside from any harmful effects due to the microbes themselves, there is increasing recognition of microbial biofilms on plastic debris influencing colonization by invertebrates, ingestion of plastic, plastic breakdown, sinking, and potential transport of non-indigenous species and pathogens (Caruso, 2015).

HABs cause periodic problems with human health in the Mediterranean (Harmful Algal Event Database, http://haedat.iode.org/, Region 11) and have been associated with mass mortality events of marine mammals that are also prone to other microbial diseases caused by viruses, bacteria, and protists (Bossart, 2011). In the Pelagos Sanctuary, marine litter is considered to be a major threat for marine mammals (Fossi et al., 2018), and some of our samples were collected in fin whale-preferred feeding habitats (Druon et al., 2012). HAB-associated taxa included some dinoflagellates found only on our plastic samples, mainly attributed to *Amphidinium* species including *A. massartii* (100% identity), *A. carterae* (99.5%), *A. klebsii* (99.5%), and *Amphidinium* sp. (97.4–100%) that are known to produce bioactive compounds with toxicological effects (Murray et al., 2015). We also detected OTUs with high percent identity to *Gonyaulax spinifera* (99.7%), *Azadinium poporum* (99%), *Azadinium caudatum* (96%), and *Ostreopsis* sp. (97.6%), only the last of these has been previously identified on PMD (Masó et al., 2003). We also detected a broader diversity of dinoflagellate OTUs that were found in both water and plastic-associated samples, including members of the genera *Karenia, Polykrikos, Dinophysis, Alexandrium, Amphidoma, Heterocapsa, Karlodinium, Prorocentrum* and *Margalefidinium* confirmed against the DINOREF database (Mordret et al., 2018). While we did find molecular signatures of microbes potentially harmful to marine mammals including potential pathogens and HAB species, based on marker gene detection alone, we cannot prove that any of the organisms we report are harmful. Also, our molecular methods only provide relative abundance data and not absolute numbers. Finally, levels of toxin production are often variable in HABs, so at this point the risk associated with ingestion of plastic as a source of harm to marine animals remains unquantified.

3.4. Network analysis, causal effect estimation, and polymer/water-associated distribution patterns

One of the motivations for our study was to gain deeper insights into potential inter and intra-domain microbial interactions on plastic debris. We chose a causal discovery approach for this aim as this allows for inferring directed effects, distinguishing cause and effect. The reconstructed partially directed network is not interpreted as such but used to estimate causal effects of one OTU on another. As such, the approach differs from traditional correlation-based network reconstruction approaches, where the network is interpreted. Here, the question is: If a specific OTU changes in abundance, what will happen to the abundance of another OTU? We interpret the largest estimated causal effects as potential interactions, but these potential interactions should be confirmed using more empirical approaches.

A previous study examining cross-domain associations on mesoplastic and pooled microplastic pieces from the North Atlantic concluded that eukaryotes play a minor role in the biofilm (Debroas et al., 2017). Nevertheless, diatoms and a number of important eukaryotic groups including predators such as suctorians and cercozoa were identified in their study. Our multi-domain network reconstruction and causal effect estimation, using a much larger data set of individual plastic pieces in the Mediterranean predicted strong relationships between OTUs from different domains. Fig. 4 (Table S3) highlights the top 20 positive estimated causal effects for each pairing of bacteria-bacteria, bacteria-eukaryote, eukaryote-bacteria and eukaryote-eukaryote OTUs. Bacteria-bacteria causal effects were fairly evenly distributed between Freshwater/Seawater/Plastic (Fig. 4a). In contrast, Bacterial- > Eukaryotic, Eukaryotic- > Bacterial, and Eukaryotic- > Eukaryotic effects were largest on Plastic, suggesting that Eukaryotes play an important role in structuring the Plastisphere ecosystem. *Filomicrobium* (circles Filomicr_497, Filomicr_dc9) dominated Bacterial- > Bacterial causal effects (Fig. 4a) on plastic followed by *Loktanella* (Loktan_efc), a member of the Rhodobacteraceae. *Filomicrobium* has been reported on PVC pipes (Liu et al., 2014), and is a member of the Hyphomicrobiaceae that commonly occur on plastic surfaces. *Loktanella* is a member of the Rhodobacterales, primary bacterial surface colonizers (Dang et al., 2008). Debroas et al. (2017) described members of the Rhodobacteriales, and Rhizobiales as keystone species from their network analyses – so both analyses seem to confirm their important role in the Plastisphere. What is interesting is that *Filomicrobium* and *Loktanella*, both members of the Alphaproteobacteria, are predicted to influence the presence of two different Flavobacteraceae (Flavo_7a9 and Flavo_31f), another family with a key functional role in marine biofilm formation (Pollet et al., 2018).

Bacteria-eukaryote causal effects on eukaryotic phototrophs were among the strongest in plastic-associated causal-effect relationships (Fig. 4b, Table S3). These were often Alphaproteobacteria (Filomicr_dc9, Filomicr_15e, Hypho_949, Hypho_535, Hypho_3f1) influencing phototrophs including diatoms (Navic_3ac, Nitzs_5ab), chlorophytes (Chloro_d95), rhodophytes (Rhodo_f7e), photosynthetic chlorachniophytes (Bigelo_864), and photosynthetic dinoflagellates (Dino_089, Tripos_230). These bacteria may be providing an essential growth factor (e.g. vitamin B12) for the associated phototrophs – a well-established phenomenon in microalgal culture. We also observed a large effect between Saprospiraceae bacteria (Sapro_d26) and a Labyrinthulomycete *Aplanochrytrium* (Aplano_8b3) that may be an example of a eukaryote scavenging after a bacterium known to breakdown

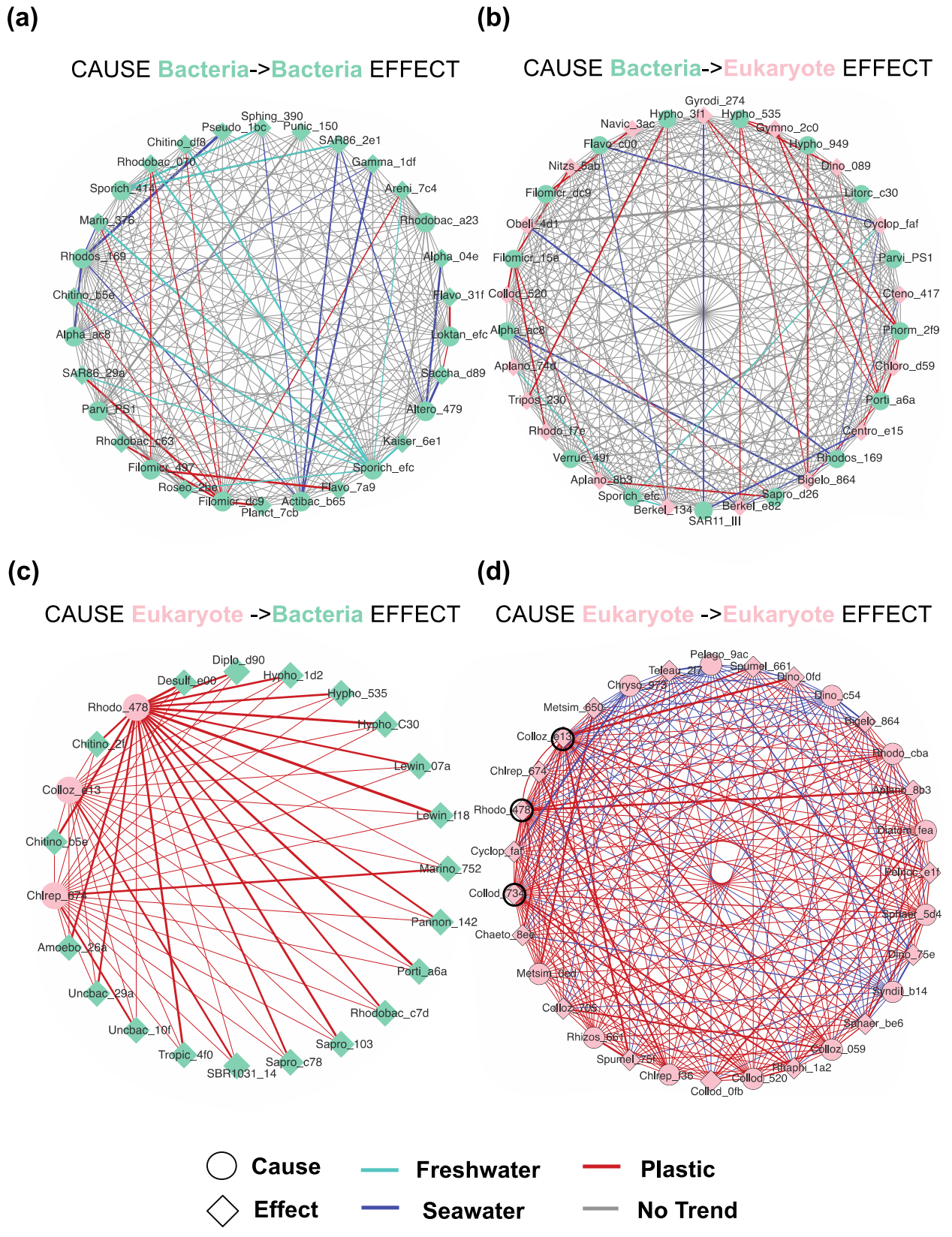

Fig. 4. a-d Microbial interactions. The top 20 cause-effect pairs for each domain interaction illustrated as circular networks rendered in Cytoscape. The approach predicts the impact of a change in abundance of one organism (Cause: OTU-circles) on the abundance of the other organisms (Effect: OTU- diamonds) in the community. Line thickness represents the strength of the effect, and the color of the line denotes whether the relationship occurred preferentially in a given environment (e.g. Freshwater, Seawater, Plastic, No Trend). See main text and Supporting Information online for abbreviation codes and effect size interaction numerical values. In (d) Colloz_e13, Rhodo_734 and Collod_734, demonstrated both cause and effect behavior in the top 20 Eukaryote-Eukaryote interactions (indicated with black circle around pink diamond). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

complex carbon substrates (Raghukumar & Damare, 2011). This particular association is very interesting from an ecological perspective since *Aplanochrytrium* is a known saprotroph and can associate with brown seaweeds as endophytes (Sathe-Pathak et al., 1993). This type of relationship might explain the possible co-occurrence of both Saprospiraceae bacteria and *Aplanochrytrium* as members of algal microbiomes. A final noteworthy predicted causal effect was between the cyanobacterium *Phormidium* (Phorm_2f9) and dinoflagellates *Gyrodinium* (Gyrodi_274) and *Gymnodinium* (Gymno_2c0), pairings that may be related to nitrogen-fixing capacity in the cyanobacterium (Bergman et al., 1997) in an oligotrophic environment like the Mediterranean.

Estimated causal effects from eukaryotes on bacteria (Fig. 4c, Table S3) were dominated by a Chorodactylon Rhodophyte (Rhodo_478) on a large variety of bacteria. The exact phylogenetic affiliation of the Rhodophyte OTU remains to be determined since its top BLAST hit was only a 78% match to a Rhodophyte called *Tsunamia transpacifica* identified from plastic in the Japanese Tsunami debris of 2011 (West et al., 2016). The Rhodophyte-bacteria effects were among the strongest positive causal effects we observed in our data and included effects on bacterial OTUs of Saprospiraceae, Hyphomonadacea, Desulfobacteraceae, Rhodobacteraceae, Diplorickettsiaceae, Amoebophilaceae, Stappiaceae, and Anaerolineae bacterial families. Among the Saprospiraceae we found members of the *Portibacter* and *Lewinella* genera, the latter also reported in Oberbeckmann et al. (2016) who found them to be exclusively present on PET and absent from water samples in the North Sea. As these authors note, *Lewinella* constitutes a "defining member" of red algal biofilms and microbiomes (Miranda et al., 2013) – lending further support for our microbiome hypothesis. Causal effect analysis identified the red alga as the "cause" and *Lewinella* as the "effect" that would not be possible with other networking approaches. Other strong estimated effects included the cercozoan *Chlorarachnion reptans* (Chlrep_674) with *Marinobacter* (Marino_752) – a member of the Alteromonadales and known biofilm former, and *Collozoum* sp. (Colloz_e13), a colonial radiolarian (Radiozoa) that is exclusively planktonic with a chloroflexus bacterium "SBR1031" that may be taking advantage of the breakdown of radiolarian biomass concentrated on PMD.

Considering potential Eukaryote-Eukaryote interactions (Fig. 4d, Table S3), the Rhodo_478 OTU also had strong positive effects on *Aplanochytrium* (Aplano_8b3) and a diatom (Cyclop_faf). Note that certain OTUs (Colloz_e13, Collod_734, Rhodo_478) were both cause and effects, so they are displayed with a modified symbol. We hypothesize that this Rhodophyte is carrying its own microbiome of bacteria and microbial eukaryotes. *Aplanochytrium* (Aplano_8b3) could be a parasite of the diatom (Cyclop_faf) since *Aplanochytrium* is known to parasitize/prey on diatom hosts (Hamamoto & Honda, 2019). It is noteworthy that Debroas et al. (2017) also detected Cercozoa with significant linkages to Chrysophyceae, Labyrinthulida and Diatoms in their network analyses and speculate that they may serve a functional role in the plastisphere as bacterivores, predators and detritovores. Confirming these observations was beyond the scope of this study but will hopefully fuel future work. Radiolarian Colloz_e13 dominated the top 20 Eukaryotic- > Eukaryotic causal effects with a positive effect on a Syndiniales Group II dinoflagellate (Dino_0fd) suggesting a host-parasite relationship. It also had effects on another colonial radiolarian species *Rhaphidozoum* sp. (Rhaphi_1a2), multiple spumellarian radiolaria (Spumel_75f, Spumel_661), a schyphozoan (Pelnoc_e1f), and a diatom (Chaeto_8ee). The prevalence of radiolarians associated with PMD is not a novel observation but reported in previous work (Zettler et al., 2013; Bryant et al., 2016). We speculate that gelatinous colonial radiolaria may become entangled with MP when they are concentrated into Langmuir circulation induced windrows where they encounter higher concentrations of other radiolarian species and PMD. Radiolarian colonies may also be capturing small plastic pieces as "prey" since they are generalist predators that can reach meters in length. Preliminary data on microplastic from fish-guts suggest that this is not merely an artifact of collecting plastic in nets, because radiolarian DNA signatures are still evident on plastics recovered from fish guts (Unpublished results). Radiolaria associated with PMD, may also have important implications for the transport and fate of PMD sinking to the depths (Gutierrez-Rodriguez et al., 2019), a critical issue with respect to the on-going "missing plastic" debate. Still, the fact that biofilms in general are also reservoirs for environmental DNA (eDNA) means that we need to interpret these associations with caution and test these putative relationships using alternative validation methods such as *in-situ* hybridization (Schlundt et al., 2020).

Some of the strong cause-and-effect pairs included representatives of freshwater communities, reflecting river inputs (indicated with aqua lines in Fig. 4), particularly in the Bacteria-Bacteria effects. Both positive and negative effects were evident, suggesting beneficial and antagonistic interactions, but we've chosen to highlight positive effects here for space limitations. Table S3 provides examples of negative causal effects, a key to OTU abbreviation taxonomic assignment, and estimated effect size values.

There were also differences between the eukaryotic communities found on plastic only, vs. those found in water and on plastic, and those found in water only as shown in Fig. 5 that summarizes the distribution of Eukaryotic supergroups. As suggested by the causal effect analysis, this broad-scale comparison shows that Radiozoa, of which radiolaria are a member, were more frequently detected in plastic samples and in water-and-plastic samples than in water samples alone. Cercozoans were also enriched in plastic-and-water samples. We saw noteworthy differences among the Holomycota, or true fungi, with increasing diversity found on plastic (Fig. 5a) versus water (Fig. 5b). Holymycota can degrade refractile compounds and are of growing interest to the field of plastic debris research. Many are also parasitic on other taxonomic groups from protists to metazoa (including mammals) and have been recorded as abundant on marine plastic in other studies (Kirstein et al., 2018). Plastic also included a larger diversity of Apusozoa and Amoebozoa than water or water and plastic. Supporting Information Fig. S3 provides a more in-depth overview of Holomycota that were found only on plastic vs. water-and-plastic vs. only water. Figure S4 provides a more in-depth summary of the phylogentic placement of the fungi we detected in our samples versus those of other studies. Some fungi were found in both marine and freshwaters, suggesting that they may be euryhaline and capable of surviving on plastic transferred from rivers into the ocean. At least one example of a Microsporidian (XMD_df73b0f) (Fig. 4Sb) occurred in the Golo River and also Bastia Port – both located within the Pelagos Sanctuary.

3.5. Insights from microscopic observations of bacterial and eukaryotic coverage

Quantitative counts of bacteria and diatom cells on environmental plastic are rare. Our counts of bacteria/archaea and diatoms on film and fragments (2017 only) yielded counts from 0 to 49,023 bacteria/archaea cells mm^{-2}, with a mean of 5038 cells mm^{-2}, in the same range as a previous study in the Mediterranean Sea (Dussud et al., 2018a). Diatoms were found on 62 of 68 pieces imaged and ranged from 0 to 4293 cells mm^{-2}, with a mean of 509 cells mm^{-2}. We categorized diatoms into 8 distinct morphotypes (Fig. S5). Biofilm coverage was patchy, but in our randomized fields of view averaged about 40% coverage, higher than previously reported by Dussud et al. (2018a). However, we selected plastic pieces with visible biofilms when possible, so our results are biased toward plastic with higher coverage. In addition, their estimates were based on cell DNA, so extracellular polymeric substances and dead cell materials that were not fluorescent may not have shown up. Only 11% of our samples were in the highest coverage categories (3–4); this may be a consequence of more fouled samples sinking below the surface (Gundogdu et al., 2017; Holmström, 1975; Ye & Andrady, 1991). Diatom and bacterial/archaea counts were generally correlated with each other and there were regional differences in the diversity and types of diatoms found on plastic, with some generalists found throughout the

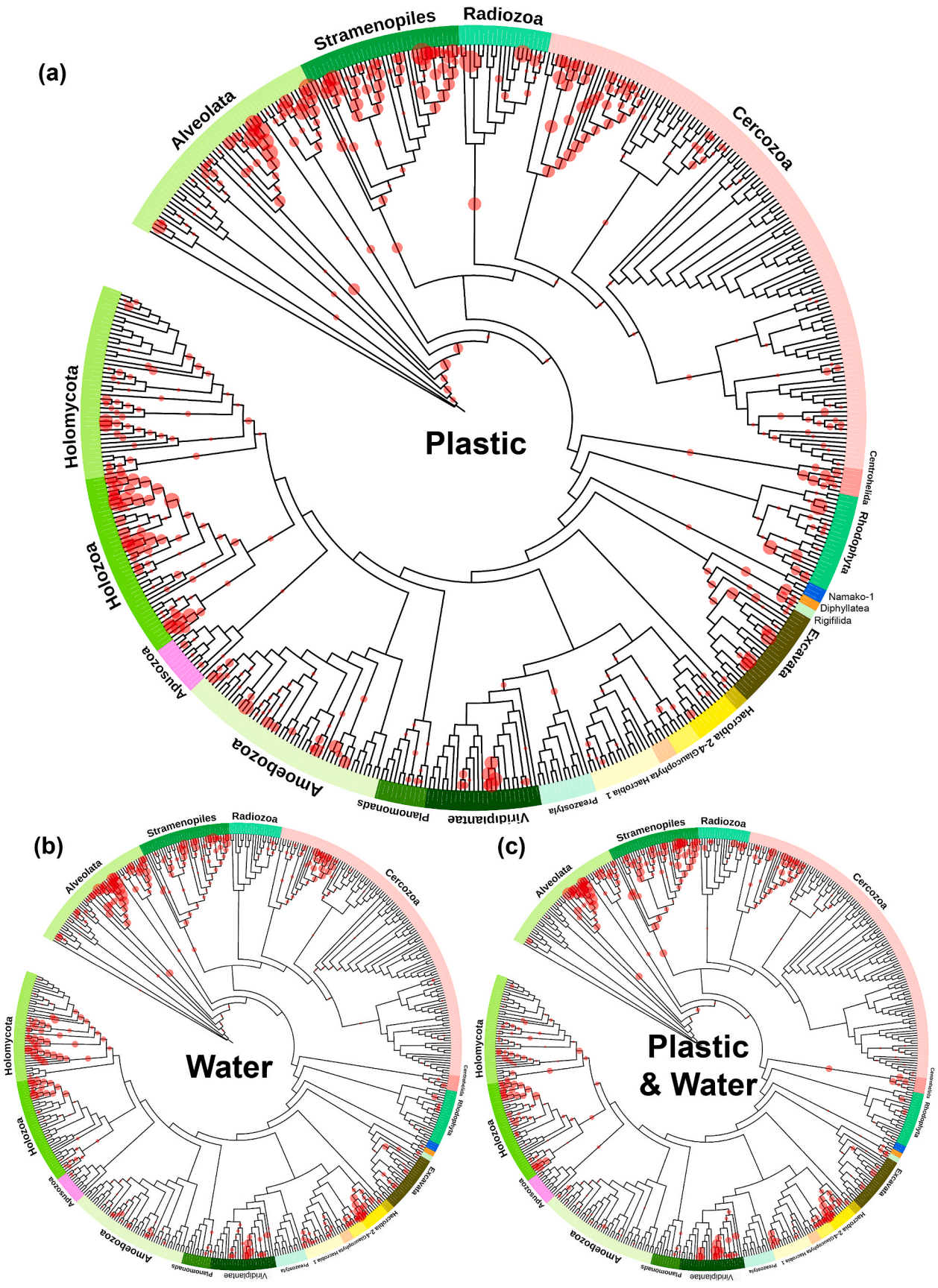

Fig. 5. Eukaryotic taxa on plastic vs. water. Comparison of phylogenetic placements of eukaryotic OTUs on a taxonomically constrained global eukaryotic tree (derived from Mahé et al., 2017) according to origin. Branching patterns are not necessarily reflective of those obtained from more rigorous phylogenomic methods. Relative sizes of the circles indicate the relative contributions of a given OTU (normalized per tree) associated with **(a)** plastic-only, **(b)** water-only, and **(c)** plastic and water.

sampling areas, while others occurred in only some areas (Fig. S6). These differences in the Plastisphere community probably derive from the known differences in oceanographic conditions and plankton communities in different areas of the Mediterranean Sea (Siokou-Frangou et al., 2010). Other eukaryotes identified in the SEM images include dinoflagellates, choanoflagellates, ciliates, fungi, bryozoans, hydroids, and copepods.

4. Conclusions

Much remains unknown about the Plastisphere in the Mediterranean, a global change hot spot that is heavily impacted by humans of many countries, but also important to fisheries, tourism, and ecosystem services. The goal of this study was to begin to fill some of the gaps in our understanding of how bacteria and eukaryotes interact on plastic marine debris and how that might impact the fate of plastic and the surrounding ecosystems. Analysis of combined bacterial and eukaryotic datasets showed regional biogeographic patterns within the Mediterranean Sea, as well as clear differences between Plastisphere communities in rivers, ports, and seas. Causal network analyses suggested that eukaryotes (and their microbiomes) are an integral part of the Plastisphere that can affect community development and interactions, settlement by metazoans, and ballasting that leads to sinking. We identified a number of potential HAB algae and fungal parasites, including some not associated with plastic before, but toxicity/pathogenicity cannot be established from our approach, and any risk to animals from ingested microbes on plastic remains unquantified. Future research should investigate actual interactions between microbes on plastic, as well as ascertain whether any of the “potential” pathogens and HABs are truly harmful.

Author statement

All authors contributed to and approved the final form of this publication and take responsibility for the accuracy of the data and analysis. Sampling and analyses were performed according to required permits and in compliance with the Nagoya Protocol.

Data accessibility

- Plastic abundance and distribution data will be submitted to the European Marine Observation and Data Network (EMODnet), https://www.emodnet.eu/.
- Images of plastic used for sequencing can be found in Supporting Material.
- Sequence data from this study have been deposited in the National Center for Biotechnology Information’s Sequence Read Archive under accession number PRJNA721966 (https://www.ncbi.nlm.nih.gov/genbank/) and are available through the VAMPS Plastisphere portal (vamps2.mbl.edu).

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117439.

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