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# Microplastic polymer properties as deterministic factors driving terrestrial plastisphere microbiome assembly and succession in the field

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## Abstract

Environmental microplastic (MP) is ubiquitous in aquatic and terrestrial ecosystems providing artificial habitats for microbes. Mechanisms of MP colonization, MP polymer impacts, and effects on soil microbiomes are largely unknown in terrestrial systems. Therefore, we experimentally tested the hypothesis that MP polymer type is an important deterministic factor affecting MP community assembly by incubating common MP polymer types in situ in landfill soil for 14 months. 16S rRNA gene amplicon sequencing indicated that MP polymers have specific impacts on plastisphere microbiomes, which are subsets of the soil microbiome. Chloroflexota, Gammaproteobacteria, certain Nitrososphaerota, and Nanoarchaeota explained differences among MP polymers and time points. Plastisphere microbial community composition derived from different MP diverged over time and was enriched in potential pathogens. PICRUSt predictions of pathway abundances and quantitative PCR of functional marker genes indicated that MP polymers exerted an ambivalent effect on genetic potentials of biogeochemical cycles. Overall, the data indicate that (i) polymer type as deterministic factor rather than stochastic factors drives plastisphere community assembly, (ii) MP impacts greenhouse gas metabolism, xenobiotic degradation and pathogen distribution, and (iii) MP serves as an ideal model system for studying fundamental questions in microbial ecology such as community assembly mechanisms in terrestrial environments.

## INTRODUCTION

The rise of plastic began in the 1930s and its success was based in parts on its versatility at a low price. Plastic has been a key factor for the comfort of modern life and in a plethora of milestones in science and technology (Plastic Europe, 2019). Ninety years later, we live in a plasticized world where plastic production is continuously increasing, resulting in huge amounts of plastic waste that is often dumped into the environment. Nowadays, around 100 Mt of such mismanaged plastic waste enters the environment with a projected increase of 50% by 2060 posing potential hazards (Jambeck et al., 2015; Lebreton & Andrady, 2019). The harmful effects of macroscopic plastic debris in the environment on larger animals such as sea turtles are well

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documented (Marn et al., 2020). Mechanical and chemical stresses cause fragmentation of plastic debris into smaller plastic particles, defined as microplastic (MP) when the diameter is less than 5 mm (Law & Thompson, 2014). This MP fraction, mainly consisting of polyethylene (PE), polypropylene (PP), polystyrene (PS), polyamide (PA) and polyethylene terephthalate (PET), has recently gained attention (Ivleva et al., 2017; Rabnawaz et al., 2017). Ecological impacts such as reduced plant root biomass, lowered C/N ratios or reduced microarthropod abundance in MP-treated soils indicate adverse effects of MP on ecosystems (Lin et al., 2020; Souza Machado et al., 2019). Such negative effects have also been reported in mammals, but recent studies provide information that MP itself is not as toxic as previously thought (Kwon et al., 2017; Deng et al., 2017; Braeuning, 2019; Stock et al., 2019). Nevertheless, further risk assessment including impacts on soil microbiomes and their function is required due to the potential alteration of soil functions, adsorbed xenobiotics and environmental pathogens.

In general, prokaryotes of the genera *Bacillus*, *Pseudomonas*, *Rhodococcus* and *Ideonella* putatively degrade petroleum-based polymers such as PET and possibly PE (Gambarini et al., 2021; Jacquin et al., 2019; Yoshida et al., 2016) and may be important in the plastisphere. MPs are readily colonized by microorganisms, leading to alteration of the microbial communities in the surrounding soil (Dudek et al., 2020; Esan et al., 2019; Gkoutselis et al., 2021; Zafar et al., 2013) Previous studies report that plastics in marine habitats harbour communities of potentially harmful bacterial strains (Silva et al., 2019; Zettler et al., 2013). Increasing amounts of MP are entering the food web and are likely to threaten humans and wildlife health (Huerta Lwanga et al., 2017; Wang et al., 2019). It is likely that such MP effects are modified by biofilms due to a strong change in surface properties. Therefore, it is of great interest to investigate the MP-colonizing microbial communities and how these communities assemble. Biofilm formation and colonization need to be studied in order to obtain a valid appraisal of possible health and ecological risks. Microbial biofilms support diverse bacterial communities in different habitats as they can protect the assemblages from stressors, such as desiccation or toxins (Elias & Banin, 2012). Research on biofilm on plastic surfaces has focused on marine ecosystems in recent decades, where an eco-corona of microorganisms adhering to the plastic surface has been investigated (Ramsperger et al., 2020). Furthermore, biofilms harbour microorganisms that can colonize very specific biological niches (Yin et al., 2019). MP represents an interesting niche, as an accumulation of potential plastic-metabolizing microorganisms within the 'plastisphere'. Mercier et al. (2017), Ogonowski et al. (2018), and Roager and Sonnenschein (2019) reported a decrease of Frankiales and Micrococcales for PE, PP and PS compared to glass and cellulose controls, while Kirstein et al. (2016) found an enrichment of *Vibrio* species on plastic particles in aquatic systems. Although microbial colonization can be non-specific, a previous study revealed a clear link between plastic types and associated microbial communities with different functional genetic potential in the marine environment (Zettler et al., 2013).

Large amounts of MP in the environment allow MP-associated communities to influence not only the immediate microenvironment but possibly also global processes (Lorenzo, 2018). For instance, the biogeochemical N-cycle is predominated by prokaryotes, that keep it in balance (Falkowski et al., 2008). Recent studies have found that MP exposure altered the abundances of marker genes for relevant functions related to the nitrogen or carbon cycle in sediments (Seeley et al., 2020; Zhang et al., 2020).

In the terrestrial environment, municipal landfills serve to manage the plastic waste generated and are globally widespread (Nizzetto et al., 2016; Vaverková et al., 2018). These dump sites constitute an anthropogenically influenced niche with long-term plastic pollution, and a largely unexplored potential enrichment zone for 'plastiphilic' microorganisms (Mersiowsky et al., 1999; Xie et al., 2006), suggesting landfills as ideal model systems to study the previously unknown effects of MP polymer types on the soil microbiome (Meyer-Dombard et al., 2020).

Changes in functional potentials between polluted and non-polluted soils might be caused by differential enrichment of plastisphere (i.e. MP and soil impacted by MP) microbial communities that have been poorly explored. The plastisphere is defined in this study as the combination of direct MP microbiome and soil microbiome from the surrounding soil matrix. Many studies in the past have focused on randomly collected plastic with undefined characteristics in aquatic environments (Vaksmaa et al., 2021; Zettler et al., 2013). Systematic studies with defined MP in soil are lacking so far. Despite the importance of MP colonization, the mechanisms of community assembly in the plastisphere are largely unknown, as are the potential effects on the functional potentials of microbiomes. Thereby, fundamental questions of microbial community ecology, that is, how specific microbiomes are composed of a larger pool of species, deserve attention (Chase, 2003; Orrock & Watling, 2010). A recurring theme in this context is the impact of deterministic and stochastic factors on community assembly. Thus, we tested the hypothesis that MP polymer type is an important deterministic factor impacting MP community assembly, succession and biofilm formation in the plastisphere. Our objective was to explore the assembly of the microbial community on five defined MP polymers buried in the soil of a dumping site over a 14-month period. In particular, we addressed the specific effects of MP polymer type on colonization, succession and biofilm formation, as well

as on the genetic functional potential, employing a range of molecular and imaging methods.

## EXPERIMENTAL PROCEDURES

### In situ incubation and sampling

Field incubations were carried out from January 2020 until March 2021 at the Kolenfeld dump site (aha, Hannover, Germany; 52°21′59.9″ N; 9°25′52.5″ E). The site is located in Northern Germany in the warmer temperate zone. Soil parameters including acidity (pH), nutrient contents and moisture content are given in Table S1. A 1.5 m deep pit was excavated on the slope of the dump site with 5–8 years old waste material. Up to 50 g soil from the pit was mixed with approximately 400 mg PE, PP, PS, PA or PET particles (size: 400–600 μm) (Table 1). MP particles with rough surfaces and undefined shapes were carefully compacted with the soil matrix. The supplemented soil was then transferred into mesh bags made of durable polyester mesh fabric SEFAR PETEX 07–400/48 (Sefar GmbH, Eding, Germany) with a pore size of 400 μm, preventing visible permeation of MP through the mesh fabric, and sealed with adhesive tape. Mesh bags with unamended soil served as controls (NoMP). Three mesh bags per treatment were placed in the pit and covered with around 1 m of the previously excavated loose soil material. After incubation periods of 5, 9 and 14 months, the mesh bags were destructively sampled and the soil in the mesh bags was filled into 50 ml flasks. Additionally, soil material from the pit 30 cm away from mesh bags was transferred into a flask at each time point as bulk soil control. Samples were stored at 4°C for subsequent analysis of soil characteristics, microscopy and nucleic acid extraction within 10 days.

### Soil characterization

Soil parameters such inorganic nitrogen, nutrient and soil water content and acidity (pH) were determined for soil samples taken at timepoint 0 and 9 months. $NO_3^-$, $NO_2^-$ and $NH_4^+$ were extracted using $CaCl_2$ extraction buffer according to DIN 17745:2005-6. Subsequently, samples were stored at −20°C until analysis via an ICS 90 Dionex Ion chromatograph (Thermo Fisher, Waltham, MA, USA). The same extract was used for pH measurements using a pH 7110 pH-metre (Xylem analytics, Weilheim, Germany). To overcome inhomogeneity of the soil matrix, the material was manually divided into five different fractions namely: inorganics, soil, hard plastics, fibres and rubber-like material. After fractionation, each fraction was weighed before and after drying at 70°C until weight stability (about 4 days) to calculate the moisture content. The fractions were then ground using a Retsch 200 MM mill (Retsch GmbH, Haan, Germany) with liquid $N_2$ for plastic and rubber fractions. The resulting fine powder was analysed in an elemental analyser (Isotope Cube®, Elementar Analysesysteme GmbH, Hanau, Germany). The final contents were calculated according to the percentage of dry weight respective fractions. Concentrations of Al, Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn were examined using the Mehlich 3 extraction method coupled with ICP-OES (Ultima 2, Horiba Jobin-Yvon, Longjumeau, France) (Mehlich, 1984).

### Scanning electron microscopy

The integrity of the plastic surfaces and biofilm formation was examined by SEM. Individual MP particles from mesh-bag incubations and pristine particles as a reference were collected and transferred to 1.5 ml reaction tube, washed by vigorous shaking with 1 ml $ddH_2O$ and subsequently dried at 70°C for 16 h prior to analysis. The washed particles were fixed on conductive adhesive carbon tape and coated with a thin gold layer (≈ 10 nm) by sputtering. The surface appearance and bacterial attachments were visually characterized using a scanning electron microscope (Zeiss EVO MA 10; Carl Zeiss, Jena, Germany) equipped with a wolfram filament and applying an electron beam voltage of 6 kV and a beam current of 3 pA.

**TABLE 1** MP properties

| MP type | Raw plastic material (manufacturer[a]) | Mol. weight (g $mol^{-1}$)[b] | Zeta potential (pH = 7) (mV) | Glass transition/melting temperature (°C) |
|---|---|---|---|---|
| PE (semi-crystalline) | Lupolen 1800P-1 | 273,000 | −70 | −100/105 |
| PP (semi-crystalline) | Moplen HP526J | 420,000 | | −10–0/158 |
| PA | Ultramid A27 E | 70,000 | | 60–75/260 |
| PS (amorphous) | 158N/L Styrolution | 280,000 | −55 | 100/na |
| PET (amorphous) | CleanPET WF | 80,000 | −65 | 80°/250–260 |

[a]PE&PP: Lyondellbasell; Rotterdam, Netherlands; PA: BASF, Ludwigshafen, Germany; PS: Ineos; London, United Kingdom; PET, Aubervilliers, France.
[b]Mean molecular weight of MP was either determined by the provider of the particles or by the manufacturer of the raw material.
Abbreviation: na, not available.

## Biofilm analysis by confocal laser scanning microscopy

CLSM following fluorescent staining addressed the formation of extracellular polymeric substances and biological colonization of plastic surfaces. For fluorescent staining, three MP beads were added to 20 µl of a solution containing 10 µg $mL^{-1}$ of peanut, soybean and wheat germ agglutinin—Alexa Fluor™ (647, 555 and 488) conjugates and 25 µM Syto™ 40 (Invitrogen, Carlsbad, CA, USA) targeting saccharides (e.g. lipopolysaccharides [LPS]) and nucleic acids, respectively. The mixtures were then incubated in the dark at room temperature for 20 min. The autofluorescence controls for the T9 and T14 samples were incubated accordingly in water without fluorophores. MP fragments were then washed three times in sterile tap water droplets, and CLSM was performed using a Leica SPE confocal microscope (Leica Microsystems, Wetzlar, Germany) with 10× magnification (NA = 0.3). All generated 3D image stacks were blind deconvoluted using the AutoQuantTM deconvolution algorithm implemented in the LASX software (Leica Microsystems). 3D image Z stacks of individual fluorescence channels were projected merged into RGB images using FIJI software (GitHub, San Francisco, CA, USA). GIMP software (GIMP V 2.1.0, GNU Image Manipulation Program, freeware, http://www.gimp.org) was used to equally augment brightness and enhance contrast by linear histogram stretching for all images preserving comparability.

## Nucleic acid extraction

DNA was extracted in five replicates from one mesh bag per treatment using a modified phenol extraction method (Griffiths et al., 2000; Töwe et al., 2011) with following additional modifications: Samples were transferred to 2 ml screw cap tubes filled with Ø 0.1 mm and Ø 0.5 mm zirconia beads (250 mg each) and 1 glass bead. Initial cell lysis was performed using a BeadBlaster24 homogenizer (Benchmark Scientific, Sayreville, NJ, USA) at 2 × 30 s at 5.5 m $s^{-1}$ with an intermitted cooling step on ice. The upper phase was washed with chloroform (chloroform:isoamyl-alcohol, 24:1, Carl Roth, Karls, Germany) and precipitated overnight at 4°C with 1:2 PEG 6000 (30 [w/v] in 1.6 M NaCl). Samples were centrifuged with 16.000 × *g* for 45 min at 4°C to obtain DNA pellets, which were washed twice with 70% ice-cold ethanol. Air-dried pellets were resuspended in 60 µl sterile $ddH_2O$, and DNA concentration and quality were determined using a spectrophotometer (DeNovix, Wilmington, DE, USA). The DNA extracts were stored at −20°C until analysis.

## Quantification of functional genes by quantitative

Each DNA extract was diluted 1:50 and 1:5000 with sterile $ddH_2O$ for analysis of functional marker and 16S rRNA genes, respectively (Table S2). 10 µl reactions contained 2 µl DNA extract, 5 µl Luna Universal 2X qPCR Mastermix (New England Biolabs, Ipswich, USA), gene-specific primers, dNTPs and $MgCl_2$/bovine serum albumin solution (Table S3 and S4). In total, five extracts per treatment were measured in technical duplicates. For negative controls, $ddH_2O$ instead of extracts was applied for each assay. Standards were obtained by serial dilution of M13 PCR amplicons ($10^1$ to $10^7$ copies $\mu l^{-1}$) from plasmids containing the gene of interest. PCR runs were conducted in a CFX96 Connect Real-Time PCR System (Bio-Rad; Hercules, United States) followed by melting curve analysis to determine amplicon specificity.

## 16S rRNA amplicon sequencing, data processing and further downstream analyses

For community analysis, DNA extracts were processed according to the protocol provided by Illumina (San Diego, USA) using the primer pair: 515F–806R (Tables S2 and S4) to amplify archaeal and bacterial 16S rRNA gene fragments. Purification of 16S rRNA gene amplicons was performed using MagSi-NGS$^{PREP}$ Plus magnetic beads (Steinbrenner, Wiesenbach, Germany) following the manufacturer's instructions. Sequencing libraries were prepared after indexing the amplicon PCR products with Nextera XT v2 Primer Kit (www.illumina.com) and sequenced on the iSeq-100 NGS instrument (www.illumina.com). Sequencing data were demultiplexed using Illumina's Local Run Manager. 16S rRNA gene sequence data were analysed using the QIIME2 bioinformatic package as described by Mlinar et al. (2020). Briefly, 16S rRNA gene short reads (150 bp R1 reads) were denoised using the DADA2 algorithm, and taxonomic classification (based on Oren & Garrity, 2021) was performed using classifiers trained on 16S rRNA gene sequences (99% identity threshold, trimmed to the amplicon region excluding PCR primers) from the SILVA 138 database (www.arb-silva.de).

## Data processing and downstream analyses

Alpha diversity of communities was examined using the plot_richness function based on the package 'phyloseq' in R version 4.1.0 (McMurdie & Holmes, 2013). Beta diversity analyses were based on the Aitchison compositional distance metric available in the DEICODE plugin

for QIIME2 (Martino et al., 2019). Taxonomic trait rankings and log-ratios of abundances between different sample groups were visualized using the packages vegan, 'phyloseq' and ggplot2 in R version 4.1.0, R Core Team 2012 (McMurdie & Holmes, 2013; Villanueva & Chen, 2019). Rarefaction curves were also created using the 'vegan' package. Indicator analysis was conducted using the 'indicspecies' package (Cáceres & Legendre, 2009) in R with the multipatt function with 'r.g.' and 9999 permutations. Differential abundance analysis based on the negative binomial distribution was carried out using the DESeq2 library with the fittype settings 'parametric' in R (Love et al., 2014). In addition, a phylogenetic investigation of bacterial communities by reconstruction of unobserved states (PICRUSt) analysis implemented in QIIME2 (Douglas et al., 2020) was performed and metabolic pathways were manually assigned using the Metacyc database (Caspi et al., 2020). The sequences for all samples were processed and aligned using the 16S Pathogenic Identification Process (16SPIP) (Miao et al., 2017). Processing was done as previously reported (Li et al., 2021; Weig et al., 2021). In brief, database hits with a greater than 99% similarity to reference sequences were identified as taxa of potential concern to human health. The abundance of these taxa in amplicon libraries was calculated based on the sequence data described in the previous section. In vivo pathogenicity of these identified taxa was not tested.

## Statistical analysis

Significant differences were calculated using SigmaPlot 13.0 (Systat Software Inc. San Jose, CA, USA) in case the analyses did not yield significant data internally. If neither the initial model nor the model transformed with ln-, x^2 or sqrt(x)-function met normality assumption, the Dunn's post hoc test was applied after the non-parametric ANOVA on the ranks, while the others were analysed using the parametric one-way ANOVA with Tukey's post hoc test. To assess beta diversity differences, the QIIME2 pipeline was used R 4.1.0 for visualization. The qPCR results were compared with the PICRUSt predictions by performing a spearman correlation using the cor.test function in the 'ggpubr' package in R (https://CRAN.R-project.org/package=ggpubr) (Kassambara & Kassambara, 2020). All values were divided by the mean of the respective soil sample after 5 months to factorize the values.

# RESULTS

## Colonization and surface structure of MP

Untreated MP prior to incubation in the soil matrix showed rough surface structures devoid of any microbial attachment for all MP and only little autofluorescence (Figure 1). Bacterial colonization along with attachment of fungal mycelia and thus biomodification of the polymer surfaces was observed after 5 months of incubation and increased with incubation time (Figure 1A). The attachment of soil-derived particulate organic matter to MP was detected after 9 months of incubation (Figure 1A). Abrasions or cracks in the immediate proximity of the attached microbial structures were not visible.

Microbial colonization was corroborated by the detection of nucleic acids and LPS (blue and green

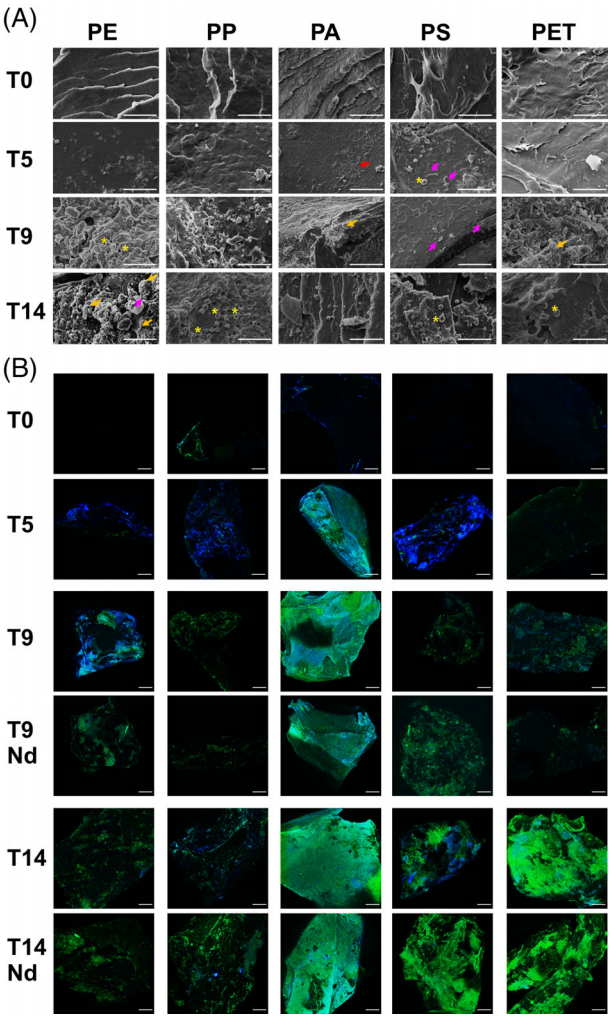


**FIGURE 1** Scanning electron microscopy (SEM) (A) and confocal laser scanning microscopy (CLSM) (B) images of microplastic (MP) incubated in mesh bags buried in dump site soil. PE, polyethylene; PA, polyamide; PET, polyethylene terephthalate; PP, polypropylene; PS, polystyrene. T5, T9, and T14 indicate incubation for 5, 9 and 14 months, respectively (A and B). (A) Arrows indicate examples of bacteria (magenta), soil matrix particles (orange), and mycelia (red); yellow asterisks indicate examples of conidia. Scale bars reflect 10 μm. (B) MPs stained with lectin fluorescent dye conjugates targeting glucoconjugates including lipopolysaccharides, and SYTO 40 targeting DNA was observed with confocal microscopy at 10× magnification. Nucleic acids and lectin bound LPS show blue and green fluorescence, respectively. Nd, no dye—control samples without fluorescent dyes. Scale bars reflect 100 μm.

fluorescence, respectively) (Figure 1B). PE and PS beads were densely colonized, but LPS were hardly detected after 5 months. LPS was more pronounced on PA (Figure 1B). PET was less colonized than the other MP types after 5 months of incubation (Figure 1B). After 9 months, PET surfaces showed considerable biofilm formation. The highest colonization densities were observed for all MP beads after 14 months of incubation, with highest colonization and LPS densities for PA, PET and PS beads. Controls without dye were characterized by autofluorescence after 9 and 14 months of incubation particularly for PA, PS and PET. Considering the absence of autofluorescence of unstained MP, such data suggest coating of soil organic compounds to the MP surface after 9 and 14 months in addition to LPS formation (Figure 1B). Fluorescence was heterogeneously distributed on MPs (Figure 1B), indicating the development of a patchy coating consisting of bacteria, LPS, and soil organic carbon on all MPs within 14 months of in situ incubation.

## Classification of amplicon sequence variants and alpha diversity

The development of biofilms on MP and development of an eco-corona over time was demonstrated in this study, prompting research into MP impacts on the diversity of recruited plastisphere-inhabiting microbes and microbial succession. 16S rRNA gene amplicon sequencing of 105 samples yielded an average of $5.4 \times 10^4$ reads per sample (minimum $2.3 \times 10^4$ reads per sample) and 564 ASVs in total (547 and 17 ASVs assigned to bacteria and archaea, respectively) with sufficient sequencing depth as indicated by the rarefaction curves (Figure S1). The dominant bacterial phyla, accounting for more than 80% of all sequences were Pseudomonadota, Chloroflexota, Bacteroidota, and Actinomycetota (Figure 2A, Figure S2A). Anaerolineae-associated SBR1031 (phylum Chloroflexota) and Methylococcales (Figure S3) were the most abundant bacterial classes. Archaea comprised 0.5% of the total reads. The most prominent archaeal phyla were Haloarchaeota, Nitrososphaerota and Nanoarchaeota accounting for more than 80% of the archaeal sequences (Figure 2B and Figure S2B). Nitrososphaeria (phylum Nitrososphaerota) along with Nanoarchaia (Woeseachaeales, phylum Nanoarchaeota) were the most abundant archaeal classes with ecologically relevant families belonging to Nitrosopumilaceae and Nitrososphaeraceae, as well as Methanomicrobiaceae and Methanosarcinaceae, being known ammonia oxidizers and methanogens, respectively (Figures S4 and S5). The predominant classes varied in relative abundance depending on the treatment, but some MP types resulted in strong enrichments of specific classes that were not as abundant in control soil samples (Figure S3). For instance, Actinomarinales together with other Actinomycetota became dominant classes in the incubation of PA, PE and PP samples. Bacillales were enriched in PS and PET samples, Limnochordales in PET and Xanthomonadales in PP. These changes were often most pronounced after 5 months of incubation.

The alpha diversity measures of the investigated communities differed between treatments and were variable among time points (Figure 3, Table S5). Microbiome alpha diversities of all polymer treatments except NoMP, PE and PA differed significantly from the bulk soil microbiome according to Fisher index ($p < 0.05$; Figure 3). PET, PP and PS plastispheres had a lower alpha diversity than bulk soil. Incubation time (9 and 14 months) had a significant effect ($p < 0.05$) on alpha diversity that tended to increase with time, with the highest values reached after 9 months of incubation.

## Beta diversity

Effects of polymer types and time points were reflected in the Principal Coordinate Analysis (PCoA) biplot (Figure 4A). The PC1 and PC2 axes together explained 96% of total variance. Bacterial taxa that correlated best with the separation of the samples were *Methylocaldum*, Gammaproteobacteria, Anaerolinea and Streptosporangiaceae.

MP type- and time-dependent community formation were also observed for archaea (Figure S5). Woesearchaeales families (phylum Nanoarchaeota) dominated in the soil and NoMP samples, especially after 5 and 9 months of incubation, their presence was strongly reduced in MP-supplemented samples. These samples were characterized by a high relative abundance of methanogens within the Halobacterota (Methanosarcinales) and the Thermoplasmatota (Methanomassilicoccales) (Figures S4 and S5). These differences were reflected in the PCoA biplot, where the two axes explained more than 80% of the variance and the differences were largely explained by the relative abundance of Nanoarchaeota, Nitrososphaerota and Haloarchaeota (Figure 4B).

PCoA indicated that bacterial communities originating from specific treatments were clearly clustered and distinguished between different time points, but only to a small extent for archaea (Figures S6 and S7). PC1 and PC2 together explained more than 80% of the variability in the data sets. Among others, Planctomycetota were important in explaining the time-dependent variability in bulk soil and NoMP communities, while Anaerolinea Gammaproteobacteria including methylotrophic species, and Chryseolinea explained most of the time-dependent variability in the plastisphere. Time-

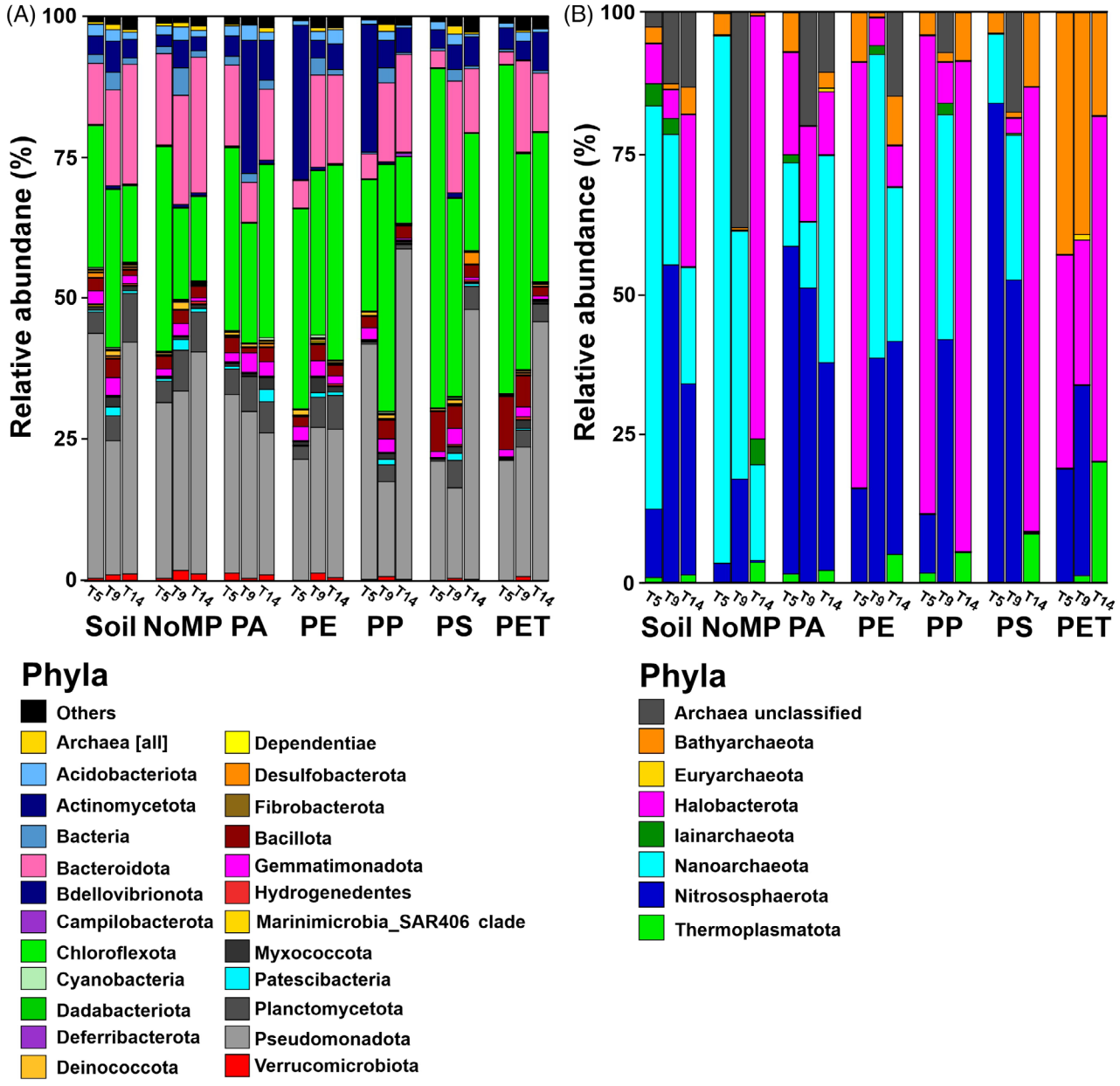


**FIGURE 2** Effect of microplastic (MP) polymers on relative abundances of 16S rRNA gene derived bacterial phylum amplicon sequence variants (ASVs) and total archaeal abundance (A) and only archaeal phylum ASVs (B). Phyla with less than 2% relative abundance or visible detection in amplicon sample were grouped as 'Others'. T5, T9, and T14 indicate incubation for 5, 9 and 14 months, respectively.

dependent distances in the archaeal community were mainly explained by Woesearchaeales and Nitrosopumilaceae including *Nitrosocosmicus* sp. and methanogenic Halobacterota (Figure S7).

Beta diversity distances of polymer-specific plastispheres and NoMP to bulk soil communities increased over time, except for the NoMP, PS and PET microbiomes, which had the greatest distance to soil communities after 5 months of incubation (Figure 5). Distances of plastisphere to soil communities were consistently greater than NoMP samples after 14 months of incubation. Time-dependent community changes were likewise reflected in beta diversity analysis.

## Differential relative abundance analysis of taxa

Key family-level taxa that were differentially abundant in the plastisphere compared to combined bulk soil and NoMP controls as assessed by DESeq2 analysis (Figure 6). Several families were either enriched (plastisphere specific) or depleted (bulk soil/NoMP specific) in the plastisphere. The samples were primarily clustered according to incubation time. Based on the patterns, the clustering of bacterial and archaeal taxa resulted in six and two groups for bacteria and archaea, respectively. The pattern for Group 1 bacterial taxa, which

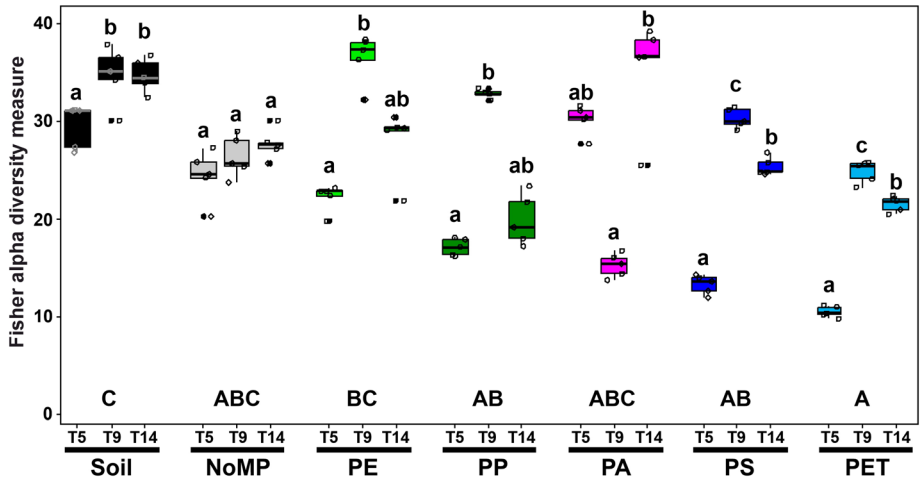


**FIGURE 3** Fisher's alpha diversity of mesh bag and bulk soil communities over time. Each box represents five replicates and the 50% of central data. The central line represents the median and whiskers the data range excluding outliers. Lower-case letters indicate significant differences between time points of one treatment, whereas capital letters indicate significant differences between the treatments over the course of the experiment. T5, T9, and T14 indicate incubation for 5, 9 and 14 months, respectively.

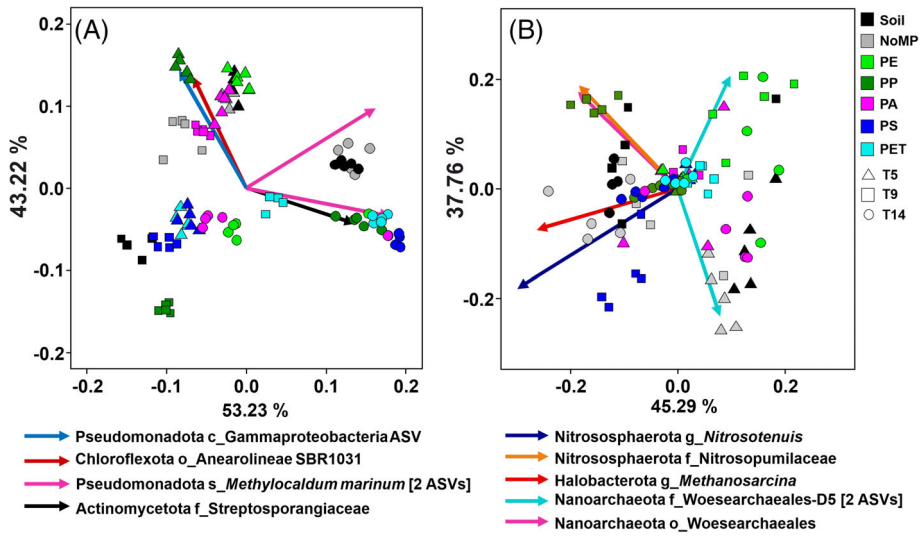


**FIGURE 4** Principal coordinate analysis (PCoA) biplot based on robust Aitchison beta diversity measures calculated for bacteria (A) and archaea (B). Arrows represent Euclidian distances from the origin and indicate most important features. Classification was done to the lowest possible taxonomic level. Lower-case letters indicate taxonomic rank as follows: c, class; o, order; f, family and g, genus and s, species. Ambiguous family assignments were as follows: D5, SCGC_AAA011-D5. T5, T9, and T14 indicate incubation for 5, 9 and 14 months, respectively. ASV, amplicon sequence variant.

comprise Shewanellaceae and Aeromonadaceae was time-dependent, showing depletion in the plastisphere at T9 and enrichment at T14 (Figure 6A). Group 4 contained taxa related to the plastisphere while Groups 2, 3 and 5 contained more taxa related to bulk soil and NoMP treatment. Archaeal group 1, accommodating methanogenic families as well as members of Bathyarchaeota were substantially enriched in the plastisphere compared to the control treatments (Figure 6B). In contrast Group 2 taxa, consisting primarily of Woesearchaeales and Iainarchaeales were consistently depleted. The effects of MP on the Group 2 taxa Nitrososphaeraceae and Nitrosopumilaceae were less pronounced. Generally, samples from T5 and T9 appeared to have more related patterns, in contrast to most samples from T14.

## Indicator species analysis

Multiple taxa specifically associated with certain polymer types were identified after 5, 9 and 14 months of in situ incubation (Table S6). Anaerolineae SBR1031 (PA, PE, PP, PET + PS), Methylococcaceae (*Methylocaldum*

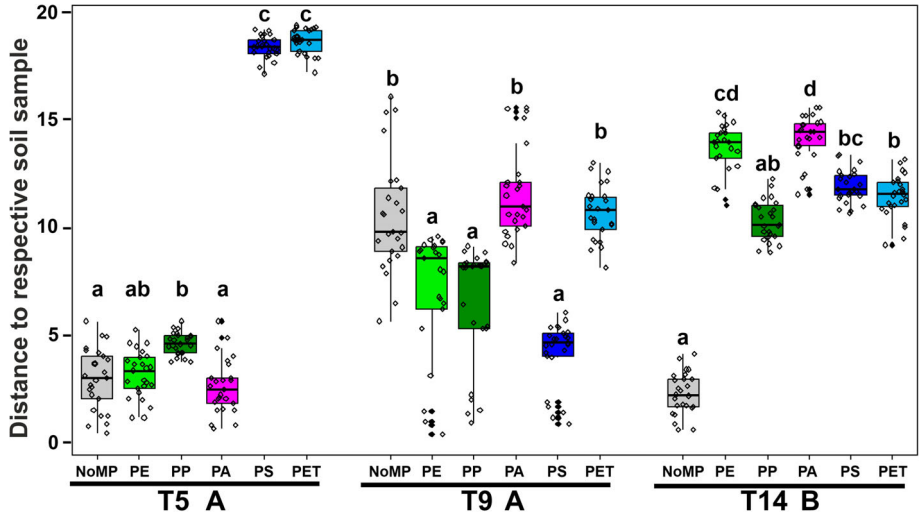


**FIGURE 5** Mean robust Aitchison distances of mesh bag to soil communities. The robust Aitchison distance between all replicates of each treatment and all replicates of the respective soil control (5 × 5) were displayed as boxplots. Each box represents five replicates and the 50% of central data. The central line represents the median and whiskers the data range excluding outliers. Error bars indicate standard deviation and lower-case letters indicate significant differences ($p < 0.05$) within one time point, whereas capital letters indicate significant differences between time points. T5, T9, and T14 indicate incubation for 5, 9 and 14 months, respectively.

PA + PET, PET + PS, PET + PP), Streptosporangiaceae (PET, PET + PS) and actinomycetotal as well as gammaproteobacterial families preferentially occurred in the plastisphere (Table S6). Many additional indicator taxa, including methylotrophic and Gram-positive taxa, were detected at each time point. Nitrosopumilaceae (*Nitrosotenuis*) and Nitrososphaeraceae (*Nitrosocosmicus*) were indicative for the PA and PS plastisphere soils sampled after 5 and 9 months of incubation, while Methanosarcineae, Methanomicrobiaceae and Methanoassiliicoccaceae were characteristic of specific time points of PET, PE + PP, PET +PS and PP + PS (Table S7). Woesearchaeales were more characteristic for soil and NoMP controls.

## Analysis of potential pathogens

ASVs assigned to 17 pathogenic bacteria were detected in the plastisphere and in control treatments (Figure 7A). Overall, there was a trend towards increased relative abundance of pathogen-assigned ASVs in the plastisphere compared to bulk soil and NoMP control ($p < 0.059$). ASVs related to *Staphylococcus epidermidis*, *Nocardia farcinia*, *N. asteroids*, *Providencia alcalifaciens* and *Serratia marcenscens* had, on average, higher relative abundance in the plastisphere compared to controls, in particular after 14 months of incubation. Plastisphere accumulation effects were significant after 14 months of incubation for ASVs assigned to *Aeromonas hydrophila* (PP), *Vibrio fluvialis* (PP) and *Pseudomonas aeruginosa* (PET; Figure 7A). Some individual replicates of the PP plastisphere had a relative abundance of ASVs related of *A. hydrophila* of >10% (data not shown).

## Functional potentials by qPCR of marker genes

Polymer properties influenced soil microbial community structure in the plastisphere and biofilm formation. The consequences of such MP effects on microbially mediated processes such as greenhouse gas metabolism and nitrogen transformation were assessed by quantifying functional marker genes and displayed either directly or processed with DEseq2 (Table S2). Functional genes that determine the genetic potential for bacterial (B-*amoA*) and archaeal ammonia oxidation (A-*amoA*), nitrate reduction including denitrification and nitrous oxide reduction (*napA*, *narG*, *nirK*, *nirS*, *nosZ*I, *nosZ*II), methane oxidation (*pmoA*), methanogenesis (*mcrA*) and anaerobic hydrogen production (*hydA*) were represented in the plastisphere, soil and NoMP controls (Figures S9 and S10). *pmoA* was most abundant in the bulk soil, NoMP and plastisphere samples, and exceeded *amoA* gene abundance by two orders of magnitude. The *pmoA* gene abundances decreased over time, while the functional gene abundances associated with nitrate reduction, denitrification and nitrous oxide reduction, namely *narG*, *napA* and *nosZI/II*, tended to increase (Figure S8/S9). Significant differences between polymer types at specific time points were found for most of the genes addressed, for example, soil to PS (*mcrA*) at T5 or soil to PA (A-*amoA*) at T9 (Figure S8/S9).

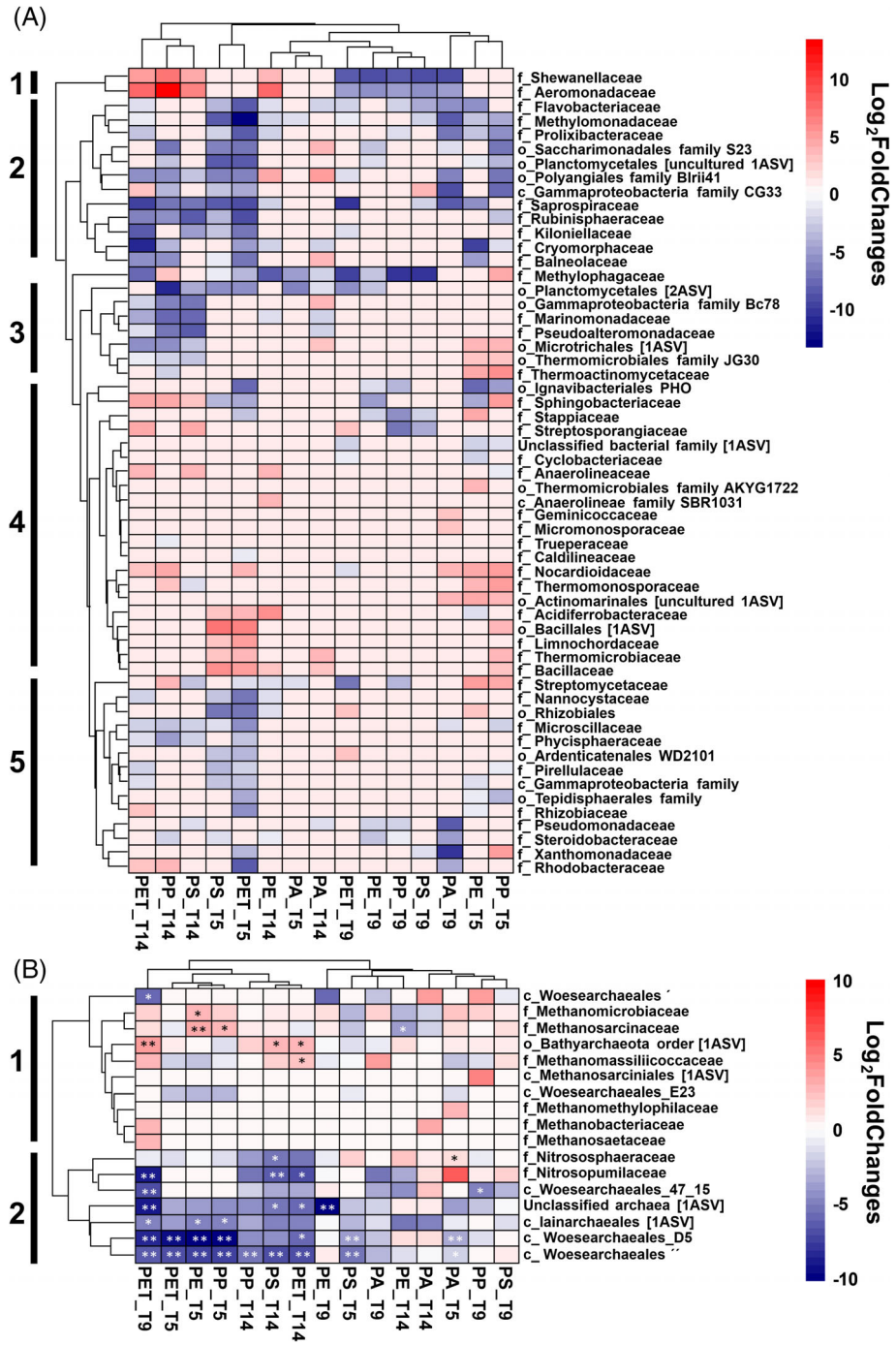


**FIGURE 6** Effect of MP polymers on bacterial (A) and archaeal (B) families (or lowest applicable classification) relative to combined soil and NoMP controls. The heat map represents log2fold changes (colour-coded) calculated by DEseq2. Positive and negative values indicate that taxa were significantly enriched or depleted, respectively, in a specific polymer sample at a given time relative to the combined soil and NoMP control samples. Hierarchical clustering was applied. Only taxa were displayed with a significant higher or lower log2fold change than 2 or −2, respectively, and an average relative abundance above 0.2% in all samples (A). All taxa are displayed and significant differences are indicated by asterisks (*$p < 0.05$; **$p < 0.01$) for archaea (B). Lower-case letters indicate taxonomic rank as follows: c, class; o, order; f, family and g, genus. Ambiguous taxonomic assignments were as follows: D5, SCGC_AAA011-D5; E23, SCGC_AAA286-E23; 47_15, GW2011_GWC1_47_15; Woesearchaeles´ and Woesearcheales´´ were assigned to two different families without further resolution. CG33, 1013-28-CG33; Bc78, EPR3968-O8a-Bc78; Jg30, JG30-KF-CM45; PHO, PHOS-HE36. The first letters in sample names on the x-axes indicate MP polymers and numbers after the 'T' reflect in situ incubation time in months. Numbers on the left site indicate clusters. ASV, amplicon sequence variant.

Equally, the ratios of gene abundance often differed among polymers and time points (Figure S10). The polymer effects were variable, and the time effects occurred most clearly between T5 and T9 or T14 (Figure S10). The most significant effects were observed for the ratio of bacterial to archaeal *amoA* that

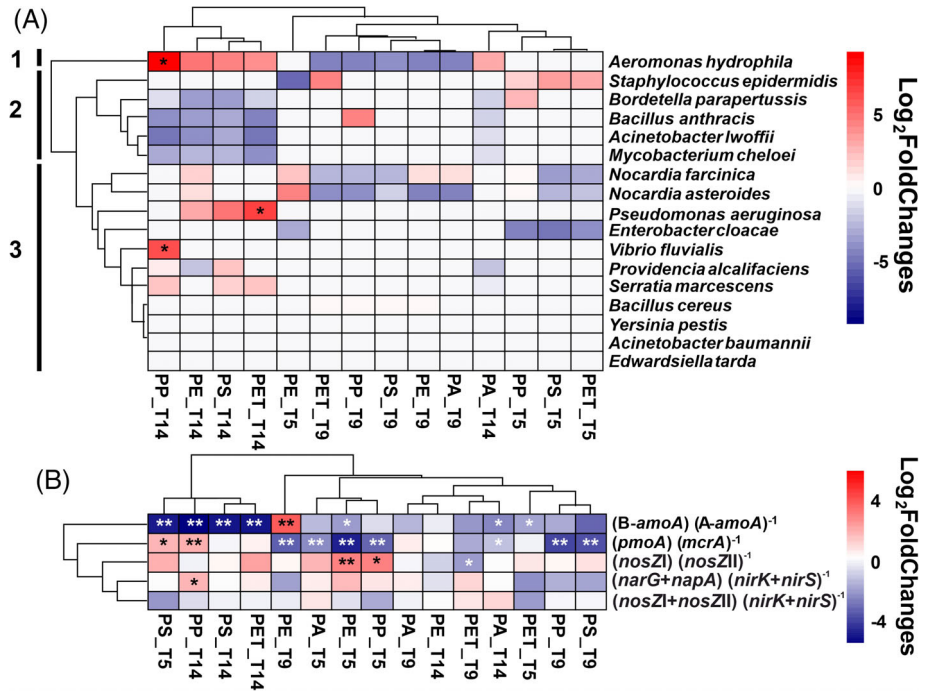


**FIGURE 7** Effect of MP polymers on putative bacterial pathogens based on relative abundance data of ASVs (A) and functional gene ratios determined by quantitative PCR (B). The heat map represents log2fold changes (colour coded) calculated by DEseq2. Positive and negative values indicate taxa (A) or gene ratios (B) that were significantly enriched or depleted, respectively, in a specific polymer sample at a given time relative to the combined Soil and NoMP samples. Hierarchical clustering was applied. Significant differences are indicated by asterisks (*$p < 0.05$; **$p < 0.01$). The first letters in sample names on the x-axes indicate MP polymers and numbers after the 'T' reflect in situ incubation time in months. Numbers on the left site indicate clusters.

tended to decrease over time, indicating an increasing predominance of archaeal over bacterial ammonia oxidizers, particularly in the PP, PS and PET plastisphere compared to controls (Figure 7B).

The dissimilatory nitrate-to-nitrite reduction potential indicated by the ratio of nitrate (*narG* + *napA*) to nitrite (*nirS* + *nirK*) reductase genes was close to 1 and was hardly affected by polymer type, as observed for nitrous oxide reductase (*nosZ*I+ *nosZ*II) to nitrite (*nirS* + *nirK*) gene ratios (Figure 7B and S10). The ratio of *pmoA* to *mcrA* genes appeared to decrease by two orders of magnitude over time (Figure S10). *pmoA* to *mcrA* gene ratios were significantly lower in most PA, PE and PP plastispheres compared to controls after 5 and/or 9 months of incubation (Figure 7B) Thus, the MP polymer and time had an effect on the abundance of functional genes.

## PICRUST predicted metabolic functions

Many metabolic functions related to xenobiotic or aromatic compound degradation were detected in the controls and the plastisphere (Figure S11). Catechol, toluene and octane degradation pathways were important and occurred particularly frequently in the plastisphere. Degradation of the nylon-6 oligomer, which is closely associated with plastic-degradation capabilities, was also predicted. Pathways related to catechol, gallate and toluene degradation were indicative for PA + PS; PE + PS and PET + PP, respectively (Table S8 and S9). The abundance of methanogenesis pathways correlated well with *mcrA* gene abundance (Figure S12A,B), while that of the predicted denitrification pathways showed no significant correlation with the abundance of denitrification-associated genes (Figure S12C,D).

## DISCUSSION

MP is ubiquitous in the marine and terrestrial environment. However, most research to date focused on marine environments, although up to 20 times more plastic is released into the terrestrial environment (Horton et al., 2017). The persistence of MP in the environment and its high potential to alter microbiomes (Kirstein et al., 2016) necessitates research on the effects of MP in the terrestrial plastisphere (i.e. soil polluted with plastics) to identify the risks to humans and nature associated with increasing MP accumulation. In addition, the plastisphere is an ideal system to study mechanisms of microbial community development, that is, the factors that shape the plastisphere microbiomes.

Colonization of MP in the terrestrial environment was consistent with previous studies on biofilm formation on plastic in soil (Gkoutselis et al., 2021; Mumtaz et al., 2010), sea water (Lobelle & Cunliffe, 2011) and aquatic fresh water environment (Ramsperger et al., 2020). We have extended such studies and demonstrated differential colonization of five different plastic types by microbes and adhesion of fluorescent compounds (soluble organic carbon) from the soil environment. Fungal spores and hyphal biomass, yeasts, green algae, as well as certain prokaryotes are known to cause autofluorescence in soils and may have contributed to the detected autofluorescence of MP beads (Bhatta et al., 2006; Hahn et al., 1993; Nam & An, 2017). Nevertheless, the findings are consistent with the postulated formation of an 'eco-corona' on all polymers, which substantially alters the surface properties of polymers (Wheeler et al., 2021).

However, differential development of biofilms on different types of microplastic was observed at earlier time points during early stage biofilm formation (T5 and T9; Figure 1), which is consistent with correlations observed between different microbial adhesion behaviours depending on polymer properties and bacterial strains (Cai et al., 2019; Hansen et al., 2021). After overcoming electrostatic repulsion, bacteria start to increase adhesin production and form components of the extracellular polymer matrix, including LPS, to enhance adhesion and prevent entropy-driven detachment (Cappitelli et al., 2014). At this early stage, the organic coating of plastic surfaces plays an important role as it determines the electrostatic potential and hydrophobicity of the surface (Rummel et al., 2021). Specific adhesins or the secretion of surfactants play a crucial role in the specificity of whether or not a bacterium attaches on a certain surface (Christova et al., 2004; Klemm & Schembri, 2000). Previous studies reported differential attachment depending on shore hardness, surface structure, and hydrophobicity (Bhagwat et al., 2021; Cai et al., 2019; Francone et al., 2021; Hossain et al., 2019). Indeed, hydrophilicity is a factor that correlates with biofilm formation and increasing in the order of PP < PE < PS < PET < PA (Sanni et al., 2015), which is consistent with intense eco-corona formation on the more hydrophilic PA and PET polymer surfaces (Figure 1B). Thus, hydrophilicity of MP is a factor likely to influence community development in the plastisphere. On top of that, additives of polymers can have a strong effect on polymer-derived effects (Lear et al., 2021), though these effects are expected to be low due to low additive quantities in the applied plastic samples.

Indeed, the MP polymer type impacted the microbiome and community succession in the plastisphere (Figure 4). The distances of the communities in the plastisphere among the treatments were smaller than distances to bulk soil controls (Figure 5), suggesting that deterministic factors related to the MP played a major role in the formation of the plastisphere communities (Figure 5). Such findings are in line with the assumption that the initial bare plastic surface has strong effects on the formation of the prokaryotic community due to the idiosyncratic surface properties. After the initial colonization and adsorption of soil organic carbon, that is, the formation of an eco-corona, the soil microbiome is exposed to the eco-corona rather than the MP surface itself. At this stage, it is only of minor importance whether the strains are able to attach to or thrive on the plastic, but rather how they thrive on the formed eco-corona. This trend has also been proposed for marine systems and reviewed recently (Wright et al., 2020). However, this implies a convergence of communities on the different plastispheres, which is contrary to our findings. A likely explanation is plastic type-specific eco-coronas that affect the microbial community even at a later stage of development. Generally, there is a strong debate about whether, and to what extent, stochastic or deterministic factors are important for community development. In many studies from aquatic environments, stochastic factors appear to dominate to a varying degree (Jones & Bennett, 2017; Kane et al., 2020; Roguet et al., 2015; Zhou et al., 2014). Deterministic factors generally appear to be more important for the development of microbial community at later stages in soil when stable levels of selection are reached (Dini-Andreote et al., 2015). The data from this study suggest that deterministic factors are more important for community development in the terrestrial plastisphere at smaller scales pointing towards specific MP polymers as rather homogeneous selective environments.

Highlighting specific changes in the microbiome development showed that in all communities studied, the class SBR1031 of the order Anaerolineales, was most dominant and enriched in the plastisphere (Figures 6, S3), most likely due to large anoxic microsites in the soil where MP was buried and interactions with the hydrophobic MP surface (Rohe et al., 2021). Anaerolineales have been reported to possess metal resistance genes that may help them thrive in metal-rich habitats such as certain municipal waste dump sites and were abundant in TNT-contaminated sediments (Janßen et al., 2021; Miranda et al., 2018). Anaerolineae SBR1031 have been reported to occur on plasticizer amended PVC microplastic in soil (Yan et al., 2021), are also well known inhabitants of anoxic biogas digesters, and recent records suggest a high potential for attachment to hydrophobic surface through the synthesis of adherence proteins (Xia et al., 2016). Thus, Anaerolineales are thought to be important plastisphere organisms in anoxic soil niches.

Previous studies on plastisphere colonization have detected various taxa such as Alteromonadales, Caulobacterales, Chitinophagales, Cytophagales, Flavobact

eriales, Frankiales, Saprospirales, Rhodospirillales, and Vibrionales that readily colonize plastic surfaces in freshwater, marine and soil environments (Dudek et al., 2020; Hansen et al., 2021; MacLean et al., 2021; Ogonowski et al., 2018; Vaksmaa et al., 2021; Weig et al., 2021; Xie et al., 2021; Zettler et al., 2013). Combined indicator analysis data of PS + PET showed that Bacillales are indicative of PS and PET treatments and might include plastic-degrading taxa (Gambarini et al., 2021). As in this study Gemmatimonadetes, Bacteroidetes and Nitrospirae were detected particularly in the plastisphere of mulch film contaminated crop fields and are thought to cope especially well with hydrophobic surfaces (Qian et al., 2018). In concordance with previous studies, we showed that certain methanogens within the Methanomicrobiaceae, Methanosarcinaceae and Methanomassilicoccaceae are moderately enriched in the plastisphere (Figures 6B and S5). Thus, the diversity of taxa detected in the plastisphere is high due to the multiple interactions between polymers and environmental microbiomes.

Significant differences between plastic types within a time point and between time points were found for multiple gene abundances (Figures S8 and S9). The largest plastic type and time point dependent variations were detected for archaeal and bacterial *amoA*, *napA*, and *nirS*. Seeley et al. (2020) likewise detected positive effects of polyurethane and polylactic acid on abundances of *amoA* and *nirS* and negative effects of polyvinyl chloride in sediment sludge mesocosms. The ratio of bacterial to archaeal *amoA* decreased with just one exception in the plastisphere compared to the soil (Figure 7B), suggesting a preference towards ammonia-oxidizing archaea, as expected during constant mineralization of organic matter, which takes place in landfill soils (Hink et al., 2017; Lubberding et al., 2012; Prosser et al., 2020). MP supplementation increases mineralization rates in soil mesocosms, which may contribute to the ratio shifts either by increasing the surface area of biofilms or by other plastic-specific effects (Gao et al., 2021). Deviating results for PE after 9 months are most likely derived from seasonal or microhabitat effects. Given the demonstrated effects of plastic on the genetic potential of *narG*, *narH* and *norB* as crucial genes for denitrification in marine environments (Bryant et al., 2016), MP is expected to alter N-cycling activities. Despite these expectations, our study is partly in contrast, as the abundances of denitrification and $H_2$ production marker genes were not as strongly affected as the abundances of ammonia oxidation and methane metabolism associated genes. Quantification of *pmoA* and *mcrA* genes suggest an impact of MP on methane cycling, that is, a lower potential for methane oxidation on PE, PS and PA, particularly at earlier time points (Figure 7B), an observation consistent with the increase of methanogenic taxa compared to soil and indicator species analysis (Figure 6B, Table S7). Thus, MP has the potential to increase net methane emissions. Also, the predominance of *Methylocaldum*, a methanotroph identified as an indicator of some MP, is not unexpected in a landfill cover soil being a methane hotspot (Kaupper et al., 2022). Thus, MP may influence the microorganisms involved in the methane cycle, potentially affecting the net methane emission in landfill covers.

The indicator analysis of metabolic pathways predicted by PICRUSt showed the potential of MP and soil communities to degrade certain xenobiotic and/or aromatic compounds, as well as biopolymers such as chitin (Figure S11). The functional potentials for degrading aromatic compound were particularly more important in the plastisphere than in the soil (Tables S8 and S9). Catechol, toluene, and gallate degradation pathways as indicators of PA, PE, PET, PP and PS suggest high xenobiotic degradation capabilities in the plastisphere, which is particularly important for dump site soils where polyaromatic hydrocarbons and similar compounds are released from anthropogenic waste through weathering and UV oxidation (Cerniglia, 1984; Sørensen et al., 2021).

The capability to attach is a recognized pathogenicity factor (Pakbin et al., 2021), suggesting that MP in general might select for pathogens. Indeed, a few ASVs related to known pathogens were enriched in the terrestrial PP and PET plastispheres (Figure 7A). The time-dependent enrichment of *Aeromonas hydrophila* was most evident. *A. hydrophila* is known to cause skin infections, ranging from mild wound infestations to fatal tissue necrosis supported by adhesion abilities and the secretion of exotoxins structurally similar to those of *Vibrio cholerae* (Zhiyong et al., 2002). *P. aeruginosa* was also found to be enriched in the plastisphere and has gained public attention due to its frequent occurrence during hospitalization and its multi-drug resistances (Aloush et al., 2006). Indeed, *Pseudomonas* spp. including *P. aeruginosa* are commonly found in the terrestrial, freshwater and marine plastisphere on PS, PE, PP, and PET, suggesting that such organisms are widespread in the plastisphere (Metcalf et al., 2022). The third enriched taxon was *Vibrio fluvialis*, which is known as a food-borne pathogen predominantly from rivers and coastal areas causing diarrhoea (Ramamurthy et al., 2014). In general, *Vibrio* spp. are commonly encountered in the plastisphere from other habitats including marine systems (Kirstein et al., 2016; Metcalf et al., 2022). Other detected potentially pathogenic candidates with only low or negligible enrichment in the plastisphere were *S. epidermidis*, *N. farcinica*, *N. asteroides*, *P. alcalifaciens* and *S. marcescens*, which have been reported to play a role in nosocomial catheter infection (Vuong & Otto, 2002), pleural, cutaneous and systemic nocardiosis (Corti & Villafañe Fioti, 2003; Torres et al., 2000), diarrhoea (Albert et al., 1992) and nosocomial pneumatic infection

(Hejazi & Falkiner, 1997). Health problems with MP, where the plastic can act as carrier for either pathogens or for antibiotic resistance, are supported by previous studies (Gkoutselis et al., 2021; Kirstein et al., 2016; Zhu et al., 2022). These findings point to the need to further investigate MP-derived pathogens and highlight a health risk potential that has not been sufficiently explored.

## CONCLUSION

Our findings provide new insights into the colonization pattern of MP particles in a municipal landfill and the functional consequences. MP particles are differentially colonized and attract organic material from the surrounding soil, forming an MP type-specific eco-corona. Plastic surfaces and their organic coating induce a specific MP-associated microbiome with a dominance of Anaerolinea and Pseudomonadota for bacterial, and Halobacterota and Thermoplasmatota for archaeal ASVs. Polymer type was shown to be a deterministic factor shaping community development in the plastisphere. Plastisphere microbiomes have a higher potential for greenhouse gas methane emissions and represent a previously overlooked terrestrial reservoir of potential bacterial pathogens that are subject to continuous change. Although these genetic potentials identified by qPCR and PICRUSt need to be confirmed in future studies at the process level, our study shows that the impact of MP is more severe than previously thought. Lastly, our study highlights putative risks resulting from the pathogenic potentials of plastispheres, which give rise to future research.

### AUTHOR CONTRIBUTIONS

S.R. and G.G carried out laboratory work and wrote the original draft. Sequence data processing was done by L.H., A.W. and S.R. CLSM analyses weredesigned and supervised by M.O. SEM analyses were conducted by S.R. and A.D. A.H., L.H., G.R. and M.A.H. revised the manuscript. A.H. supported the design of the study. M. A.H. and G.R. acquired fundig, designed the study, and supervised the project.

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### CONFLICT OF INTEREST

The authors declare no competing interests.

### DATA AVAILABILITY STATEMENT

Amplicon sequencing data have been deposited in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the Bioproject PRJNA858406. All parameters used for each step in the Qiime2 workflow can be accessed via the provenance tab of the electronic data (.qza and .qzv data files available upon request) via view.qiime2.org.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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