Water Research 192 (2021) 116852

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Riverine microplastic and microbial community compositions: A field study in the Netherlands

Lapo Mughini-Gras[a,b,1,*], Rozemarijn Q.J. van der Plaats[a,1], Paul W.J.J. van der Wielen[c,d], Patrick S. Bauerlein[c], Ana Maria de Roda Husman[a,b]

[a] Centre for Infectious Disease Control (Clb), National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlandss
[b] Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, Netherlands
[c] KWR Water Research Institute, Nieuwegein, Netherlands
[d] Laboratory for Microbiology, Wageningen University & Research, Wageningen, Netherlands

ARTICLE INFO

Article history:
Received 19 October 2020
Revised 13 January 2021
Accepted 17 January 2021
Available online 19 January 2021

Keywords:
Plastic
Biofilm
Pathogen
Antimicrobial resistance
Microbiome
Surface water

ABSTRACT

Plastic pollution in aquatic environments, particularly microplastics (<5 mm), is an emerging health threat. The buoyancy, hydrophobic hard surfaces, novel polymer carbon sources and long-distance transport make microplastics a unique substrate for biofilms, potentially harbouring pathogens and enabling antimicrobial resistance (AMR) gene exchange. Microplastic concentrations, their polymer types and the associated microbial communities were determined in paired, contemporaneous samples from the Dutch portion of the river Rhine. Microplastics were collected through a cascade of 500/100/10 μm sieves; filtrates and surface water were also analysed. Microplastics were characterized with infrared spectroscopy. Microbial communities and selected virulence and AMR genes were determined with 16S rRNA-sequencing and qPCR. Average microplastic concentration was 213,147 particles/m^3; polyamide and polyvinylchloride were the most abundant polymers. Microbial composition on 100–500 μm samples differed significantly from surface water and 10–100 μm or smaller samples, with lower microbial diversity compared to surface water. An increasingly 'water-like' microbial community was observed as particles became smaller. Associations amongst specific microbial taxa, polymer types and particle sizes, as well as seasonal and methodological effects, were also observed. Known biofilm-forming and plastic-degrading taxa (*e.g. Pseudomonas*) and taxa harbouring potential pathogens (*Pseudomonas*, *Acinetobacter, Arcobacter*) were enriched in certain sample types, and other risk-conferring signatures like the *sul1* and *erm(B)* AMR genes were almost ubiquitous. Results were generally compatible with the existence of taxon-selecting mechanisms and reduced microbial diversity in the biofilms of plastic substrates, varying over seasons, polymer types and particle sizes. This study provided updated field data and insights on microplastic pollution in a major riverine environment.

Introduction

Millions of tons of plastic end up in aquatic environments globally every year (Ivleva et al., 2017). There is particular concern about small plastic particles (<5 mm) termed 'microplastics' originating from different industrial processes, cosmetics and packaging materials, as well as larger plastic debris breaking apart by (micro)biological and physical degradation (Ivleva et al., 2017). Microplastics are long-living and accumulate in food webs, being harmful to several lifeforms, including humans (Sharma and Chatterjee, 2017). Moreover, microplastics provide surfaces for biofilm formation and tend to select for distinctive microbial communities thanks to their hydrophobic hard surface, novel organic polymers, additives and contaminants, as well as secondary biofilm members attaching to primary colonisers (Zettler et al., 2013; Miao et al., 2019; Jiang et al., 2018; McCormick et al., 2016). These biofilms may also harbour pathogens and favour antimicrobial resistance (AMR) gene transfer (McCormick et al., 2016; Arias-Andres et al., 2018; Imran et al., 2019).

* Corresponding author: National Institute for Public Health and the Environment (RIVM), Centre for Infectious Disease Control (Clb), Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, Utrecht, the Netherlands.
E-mail addresses: lapo.mughini.gras@rivm.nl, l.mughinigras@uu.nl (L. Mughini-Gras).
[1] Equal contributors.

https://doi.org/10.1016/j.watres.2021.116852

Freshwater accumulates microplastics from several sources, including land run-off and wastewater treatment plants (WWTPs, McCormick et al., 2016) with rivers transporting microplastics to seas and oceans. Co-emission with wastewater also exposes microplastics to pathogens, antimicrobials and antimicrobial-resistant microorganisms in WWTP effluents, enabling their transport downstream (McCormick et al., 2016). Indeed, high quantities of microplastics promote survival of WWTP-derived microorganisms in freshwater (Eckert et al., 2018). Moreover, the longevity and biofilm-enhanced buoyancy of microplastics favour their long-distance dispersal in water, (McCormick et al., 2016) and biofilms increase the likelihood of microplastic ingestion, (Reisser et al., 2014) deposition (Barnes et al., 2009) and decomposition (Yoshida et al., 2016).

Studies on the abundance, sources and public health relevance of microplastic-associated microbial communities in freshwater are scarce, (Miao et al., 2019; Jiang et al., 2018; McCormick et al., 2016; Blettler et al., 2018) with most research focusing on marine environments. Moreover, studies often use manufactured plastics of known polymers weathered experimentally instead of observational study designs, which reflect more closely the real-world conditions of microplastic pollution. Field data are needed to assess potential health risks and include lotic ecosystems in the global budgets of plastic. The aim of this study was to determine microplastic concentrations, their polymer types and associated microbial communities, including selected virulence and AMR genes, in the river Rhine, an important contributor to microplastic pollution in the North Sea (Mani et al., 2015). The microplastic and microbial community compositions of paired, contemporaneous samples, were thus tested for possible associations in order to determine whether microbial diversity and taxon abundances differed significantly as a function of the respective plastic polymer concentrations. Moreover, seasonal and geographical differences in both microplastic and microbiota profiles were investigated.

Materials and methods

2.1. Sampling

Two sampling sites in the Dutch portion of the Rhine were selected: 'Lobith' (Rhine's entry into the Netherlands) and 'Vianen', close to a drinking water abstraction point where the 'Lekkanaal' provides a separate portion of the Rhine (unmixed with other water bodies) before reaching the North Sea (Fig. 1). At each site, four samplings were performed, two in July 2019 (summer) and two in January 2020 (winter). Both sites were sampled contemporaneously. At each sampling, 450–750 litres of surface water was filtered through a cascade of two metal sieves (Gilson, USA) of respectively 500 and 100 μm, with a 10 μm plankton net (HydroBios, Germany) at the end. For each fraction residue, paired samples were generated for respectively plastic particle and microbiological analyses. The surface water before filtration and the filtrate were also analysed microbiologically. Each fraction residue was transferred into a glass bottle using Milli-Q ultrapure water (MilliporeSigma, USA) and stored at 4°C. Water temperature, pH and turbidity, when measured, were similar within the same season (Table S1).

2.2. Microplastic analyses

Plastic particle analyses were based on already described methods (Mintenig et al., 2020; Löder et al., 2015; Mintenig et al., 2018; Leslie et al., 2017) and focused on 10 and 100 μm residues. Particle numbers larger than 500 μm and those of 10–20 μm were estimated using fitted trend lines (Kooi and Koelmans, 2019). The combined residue suspensions were filtrated over a 10 µm metal

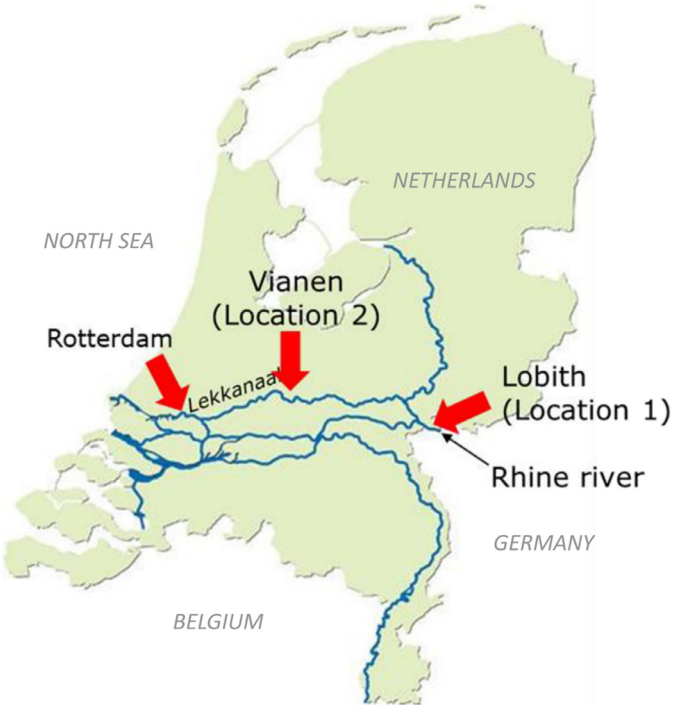

Fig. 1. Map of the Netherlands with indication of the entry of the Rhine river in the country and the two sampling locations ('Lobith' and 'Vianen') and discharge into the North Sea.

mesh and the filter was transferred into a beaker with 10% sodium dodecylsulfonate. After a day, the suspension was filtrated again and the filter was transferred into a beaker with 75 ml 12.5% potassium hydroxide for 5 days at 35 °C. Subsequently, the suspension was re-filtrated, transferred into a beaker with 50 ml 30% hydrogen peroxide for one day at 35 °C and filtrated again. The residues were then transferred into a separation funnel using 100 ml zinc chloride (1.6 g/cm^3). The funnels were shaken and left still to enable settling of denser materials. The settled material was discarded by continuously turning the valve of the funnel to prevent clogging, re-suspension and particle loss. About 10 ml of liquid was allowed to remain in the funnel, which was filtrated again over a metal filter. Using 4 ml ethanol, the retained materials were removed from the filter and transferred into a glass vial. A vortex was created in this suspension to distribute the particles evenly. A subsample (20–100 µm) was put on a microscope slide. The sample was analysed using a Laser Direct Infrared (LDIR) chemical imaging system (Agilent, Germany) to detect and characterise 20–500 µm particles (as 20 μm is the technical limit). Concentrations were expressed as particles/m^3 water. All analyses were performed in duplicate.

2.3. Microbiological analyses

2.3.1. Filtration and dna extraction

Within a day after collection, the samples (surface water, 500, 100, and 10 μm residues, and the filtrate) were filtrated using a vacuum system (Sartorius, Germany) with a 47 mm diameter cellulose nitrate filter membrane (0.45 μm pore diameter) until saturation. Two different filtration methods, termed 'flotation' and 'homogenisation', were applied to each sample. As plastic tends to float, the flotation method consisted of letting the water stand still for 15 min and then filter out only the upper part of the water volume with the vacuum system. For the homogenisation method, the bottles were shaken to homogenise the content, which was then filtrated with the vacuum system. DNA extraction was done using

the DNeasy PowerWater kit (QIAGEN, Germany). All analyses were performed in duplicate.

2.3.2. 16S rRNA gene sequencing

Sequencing of the V4 hypervariable region of the 16S rRNA gene was used to determine the microbial community composition in each sample. The quantity of isolated DNA (bacterial load) was determined using 16S real-time PCR and the samples were diluted to the necessary concentration for sequencing. The 16S rRNA gene was amplified using F515/R806 primers and 30 amplification cycles. The Nextera XT indexes were used to give every sample a unique adaptor barcode sequence to combine the samples into a single 16S rRNA gene pool for sequencing. After the first and second PCR, the amplicons were checked with the QIAxcel Advanced System (QIAGEN) using the QIAxcel DNA High Resolution Kit. The concentration of the pool sample was quantified with the KAPA Library Quantification Kit for Illumina Platforms (Roche, Switzerland). 10% PhiX DNA spike-in control was added to the pool to increase the library diversity. 16S rRNA gene amplicons were sequenced using the in-house Illumina MiSeq (Illumina, USA) platform to sequence 300 bp paired end reads.

2.3.3. Virulence and amr gene detection

The AMR genes *sul1* and *erm(B)* and the virulence genes *stx1* and *stx2* were used as indicators of AMR and virulence in the microbial communities of the collected samples. These genes were selected based on their relevance for public health and the environment. The genes were investigated using qPCR as described elsewhere (Franz et al., 2015). The PCR mix for the *sul1* and *erm(B)* genes included the iQ SYBR Green Supermix 1x (Bio-Rad, the Netherlands) with 400 nM primer concentration and 2 μl DNA sample. The PCR mix was optimised in 20 μl total volume. The Taq polymerase activation was obtained at 95°C for three minutes, amplification with 40 cycles of 15 s at 95°C and at 60°C for 30 s. After the amplification, the melting curve program started at 60°C with an increment of 0.5°C for five seconds until 95°C; amplification and melting curves were then analysed. Gene concentrations were quantified using a gBlock Gene Fragment that contained the genetic code of the *sul1* and *erm(B)* genes (Integrated DNA Technologies, USA). Eight dilutions of the standard were taken in every plate. The presence of *stx1* and *stx2* was tested in 2.5 μl DNA sample with a primer concentration of 400 and 500 nM of the FAM probe, respectively, and 1x iQ Multiplex Powermix (Bio-Rad). The PCR mix was optimized in 25 μl total volume. The Taq polymerase activation was done at 95°C for 10 min, amplification for 40 cycles at 95°C for 15 s and 60°C for 30 s; genes were detected with CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

2.4. Data analyses

2.4.1. Microplastic abundance

Differences in microplastic concentrations between particle sizes (20–100 or 100–500 μm), seasons (summer or winter), and sampling locations (Lobith or Vianen) were tested for statistical significance using generalized linear models (GLMs) with a log link and negative binomial error distribution. Differences in relative abundances of polymer types were tested using GLMs with a logit link and binomial error distribution.

2.4.2. Bioinformatics

The reads obtained from 16S rRNA gene sequencing were transformed into sample-wise relative abundances. To remove noise, only taxa present at a confident level of detection of 0.01% relative abundance over all samples were selected for analysis. Taxa, their patterns and potential differences thereof were studied at the lowest taxonomic level resolution (genus). The raw sequence reads are deposited in the European Nucleotide Archive (ENA) repository under accession number PRJEB41771. Taxonomic identification was performed with the naïve Bayesian classifier in DADA2 (Callahan et al., 2016). The SILVA ribosomal RNA database was used for taxonomic annotation (Quast et al., 2013). Quality of the reads was checked with FastQC and trimming of the adapters was done using Trimmomatic (Bolger et al., 2014).

2.4.3. Microbial diversity

The Shannon index was used to quantify α-diversity of microbial communities. Differences in Shannon index over sample types (surface water, 500, 100, and 10 μm residues, filtrate), sampling sites, seasons, and filtration methods (flotation or homogenisation) were tested using GLMs with an identity link function and Gaussian error distribution; normality of residuals was checked with the Shapiro-Wilk test.

2.4.4. Microbial community composition

Ordination of microbial community composition (taxon relative abundances) in relation to sample type, season, sampling location, and filtration method, as well as polymer type abundances in the respective samples, were visualized with canonical correspondence analysis (CCA). Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity was used to test for differences in microbial communities across the aforementioned variables. The SIMPER method (CLARKE, 1993) based on Bray-Curtis dissimilarity was used to identify taxa primarily responsible for the differences. Significance was tested using GLMs with a logit link and binomial error distribution, also for the associations with polymer type abundances.

2.4.5. General analytics

All GLMs included cluster-robust sandwich variance estimators to account for sample replicates. In all analyses, family-wise Bonferroni correction was applied. Bonferroni-corrected p-values <0.05 were considered statistically significant; when the p-value was very small (*i.e.* <0.001), the 'p-value$<0.001'$ cut-off was reported. Analyses were performed using Stata 16 (StataCorp, USA), PAST 4.03 (Oslo University, Norway), and R studio (RStudio, USA).

Results

Microplastics

Microplastics were found in all sites and samplings. Both the highest and lowest microplastic concentrations were found in Vianen (Table 1), respectively in summer (334,667 particles/m^3) and winter (102,708 particles/m^3). Overall microplastic concentrations were significantly higher in summer *vs.* winter (*p*-value=0.001), and the 20–100 μm fraction was significantly higher than the 100–500 μm one (*p*-value<0.001). The estimated 10–20 µm fraction particle number was in the order of magnitude of 800,000 particles/m^3 (summer) and 500,000 particles/m^3 (winter). In summer, the particle number for the >500 µm fraction was estimated at 2500 particles/m^3, and at 1500 particles/m^3 in winter (Figure S1). No significant differences in microplastic concentrations were found between locations. The average concentration across seasons and locations was 213,147 (95% confidence interval [95%CI]: 76,721–349,572) particles/m^3. Polyamide and polyvinylchloride were the most abundant polymers (30% and 26% of total, respectively). All but two polymer type concentrations (polystyrene and polyurethane) were significantly more represented amongst 20–100 than 100–500 μm particles. The concentrations of polypropylene and polyvinylchloride were those significantly higher in summer *vs.* winter. No significant differences between locations were found (Table S2). When the polymer type

Table 1
Mean microplastic concentrations (particles per 1000 L; averages of duplo samples) per size and polymer type in surface water samples collected at the two sampling locations in summer and winter.

Polymer	Location 1 (Lobith)						Location 2 (Vianen)					
	Summer			Winter			Summer			Winter		
	20 – 100 μm	100 – 500 μm	Total	20 – 100 μm	100 – 500 μm	Total	20 – 100 μm	100 – 500 μm	Total	20 – 100 μm	100 – 500 μm	Total
Isoprene	30,134 (97%)	800 (3%)	30,934 (10%)	24,750 (96%)	1064 (4%)	25,815 (19%)	24,267 (100%)	0 (0%)	24,267 (7%)	16,863 (94%)	1150 (6%)	18,013 (18%)
Polyamide (PA)	73,333 (87%)	10,667 (13%)	84,000 (27%)	46,946 (97%)	1596 (3%)	48,542 (36%)	86,667 (89%)	10,933 (11%)	97,600 (29%)	34,683 (92%)	2922 (8%)	37,605 (37%)
Polyethylene (PE)	10,667 (95%)	534 (5%)	11,200 (4%)	9474 (93%)	745 (7%)	10,219 (8%)	7733 (97%)	267 (3%)	8000 (2%)	2923 (100%)	0 (0%)	2923 (3%)
Polyethylene Terephthalate (PET)	21,067 (92%)	1867 (8%)	22,933 (7%)	13,732 (95%)	798 (5%)	14,531 (11%)	27,734 (94%)	1867 (6%)	29,600 (9%)	11,114 (96%)	479 (4%)	11,593 (11%)
Polypropylene (PP)	6667 (86%)	1067 (14%)	7734 (2%)	3673 (91%)	372 (9%)	4045 (3%)	5867 (85%)	1067 (15%)	6934 (2%)	1629 (92%)	144 (8%)	1773 (2%)
Polystyrene (PS)	10,400 (100%)	0 (0%)	10,400 (3%)	798 (71%)	319 (28%)	1118 (1%)	8000 (100%)	0 (0%)	8000 (2%)	335 (87%)	48 (13%)	383 (0%)
Polyurethane (PU)	14,667 (100%)	0 (0%)	14,667 (5%)	3034 (93%)	213 (7%)	3247 (2%)	13,334 (100%)	0 (0%)	13,334 (4%)	1868 (91%)	192 (9%)	2060 (2%)
Polyvinylchloride (PVC)	93,600 (95%)	4800 (5%)	98,400 (31%)	9953 (96%)	372 (4%)	10,326 (8%)	111,734 (97%)	3200 (3%)	114,934 (34%)	8863 (90%)	958 (10%)	9821 (10%)
Unknown	32,534 (94%)	2134 (6%)	34,667 (11%)	14,584 (84%)	2874 (16%)	17,458 (13%)	30,667 (96%)	1334 (4%)	32,001 (10%)	14,947 (81%)	3593 (19%)	18,540 (18%)
Total	293,066 (93%)	21,867 (7%)	314,933 (100%)	126,946 (94%)	8356 (6%)	135,303 (100%)	316,000 (94%)	18,667 (6%)	334,667 (100%)	93,223 (91%)	9486 (9%)	102,708 (100%)

concentrations were analysed as relative abundances (*i.e.* as the proportions of each polymer type concentrations over the total microplastic concentration in a sample [Figure S2]), the significant differences were as follows: polypropylene was more abundant amongst 100–500 than 20–100 μm particles (p-value=0.009), polyvinylchloride was more abundant in summer than in winter (p-value<0.001), polystyrene was more abundant in Lobith than in Vianen (p-value=0.018).

Microbial communities

The 80 samples (*i.e.* 2 samples x 5 types of sample x 2 filtration methods x 2 sampling locations x 2 seasons) produced 8390,415 high-quality sequence reads in total and resulted in 480 taxa distributed over two kingdoms (99.9% *Bacteria*, 0.1% *Archaea*), 29 phyla (mostly *Proteobacteria* 47%, *Bacteroidetes* 17% and *Cyanobacteria* 14%), 67 classes (mostly *Betaproteobacteria* 21%, *Gammaproteobacteria* 17% and *Chloroplast* 11%), 102 orders (mostly *Burkholderiales* 17%, *Pseudomonadales* 10%, and *Flavobacteriales* 8%), 171 families (mostly *Comamonadaceae* 14%, *Moraxellaceae* 9% and *Sporichthyaceae* 5%), and 307 genera (mostly *Acinetobacter* 7%, *Flavobacterium* 5% and hgcI clade 4%). Fig. 2 shows the mean relative abundances of the top 15 taxa at genus level.

Shannon index

The Shannon index was lowest amongst 500 μm samples and highest amongst 10 μm samples (Fig. 3): α-diversity amongst 500 μm samples was significantly lower than any other sample type (p<0.001); α-diversity did not differ significantly between the other sample types. α-diversity was significantly increased in winter (p-value<0.001) and when using the homogenisation method (p-value<0.001). No significant differences between sampling locations were found. α-diversity was not significantly associated with microplastic concentrations, nor specific polymer types.

Microbiota profiles

The PERMANOVA and CCA (Fig. 4) showed that there were significant differences in microbial community over sample types (overall average dissimilarity=77.56%, p-value<0.001), seasons (dissimilarity=71.92%, p-value<0.001), and filtration methods (dissimilarity=67.10%, p-value=0.004), but not between sampling sites. The *post-hoc* tests after PERMANOVA showed that the significant differences (all p-values=0.001) were those between surface water and 500 μm (dissimilarity=84.20%) and 100 μm (dissimilarity=74.61%) samples, between the filtrates and the 500 μm (dissimilarity=82.55%) and 100 μm (dissimilarity=74.61%) samples, and between the 10 μm and 500 μm (dissimilarity=81.34%) and 100 μm (dissimilarity=69.76%) samples. Therefore, the microbial community composition of 500 and 100 μm samples resembled one another and differed significantly from that of the 10 μm samples, the filtrates, and the surface water, which were similar to each other. The microbial community also seemed to shift towards a more 'water-like' profile as the particles became smaller. For subsequent analyses, therefore, sample types were grouped together into 'small particle/water-like' samples (*i.e.* surface water, filtrates, and 10 μm samples), and into 'large particle' samples (*i.e.* 500 and 10 μm samples).

Only 21 of the observed 480 taxa were responsible for >50% of the dissimilarities in overall microbial community composition between groups (Table 2), with most of these taxa being significantly enriched in specific groups of samples (Figs. 5 and 6). The largest significant differences in relative abundance were found for *Acinetobacter* between sample types and seasons, and for *Flavobacterium* between filtration methods (Fig. 6). Relative abundance data are given in Table S3.

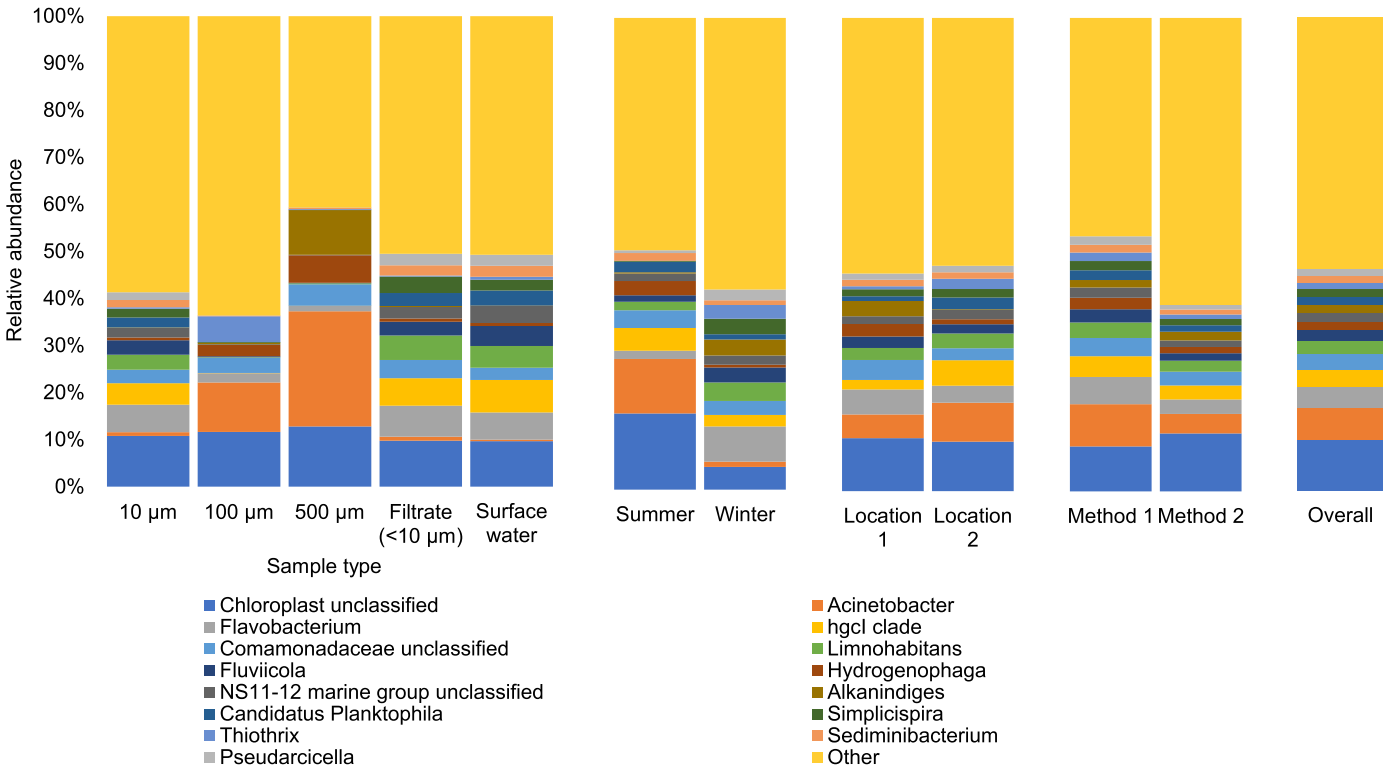

Fig. 2. Mean relative abundance of the most abundant microbial taxa (genus level) overall and per type of sample (500 μm, 100 μm and 10 μm microplastics, filtrates, and surface water), season, sampling location and filtration method.

Table 2
Taxa primarily responsible for the observed dissimilarities in the overall microbial community composition between sample types, seasons, and filtration methods.

Genus	Taxon-specific contribution (%) to the overall average dissimilarity		
	Between sample types(77.56%)	Between seasons (71.92%)	Between filtration methods(67.10%)
Acinetobacter	9.21	7.87	7.68
Chloroplast unclassified	8.55	10.17	9.72
hgcI clade	3.62	3.09	3.21
Flavobacterium	3.19	3.92	3.67
Limnohabitans	2.71	2.24	2.28
Alkanindiges	2.21	1.89	1.99
Fluviicola	2.11	1.82	1.90
Thiothrix	2.08	2.08	1.91
Comamonadaceae unclassified	2.03	1.88	1.97
Hydrogenophaga	1.98	1.78	1.78
Candidatus Planktophila	1.70	1.43	1.48
NS11–12 marine group unclassified	1.67	1.22	1.37
Simplicispira	1.60	1.99	1.68
Acidovorax	1.56	1.31	1.41
Pseudarcicella	1.37	1.35	1.32
Roseateles	1.30	1.12	1.15
Massilia	1.27	1.30	1.21
Sediminibacterium	1.19	0.97	1.04
LD12 freshwater group unclassified	1.15	1.40	1.33
Pseudomonas	1.14	1.07	1.13
Synechococcus	0.97	1.26	1.12
Other (459 taxa)	47.42	48.85	49.65

Microplastics and microbiota

The associations between the differentially abundant taxa and the polymer type relative abundances were investigated in a subset of 32 paired samples of 10 and 100 μm analysed for microbial community composition that were coupled with the analysed plastic particle sizes of 20–100 and 100–500 μm, respectively (Fig. 7). Polyethylene terephthalate was negatively associated with *Chloroplast* (p-value=0.012) and positively associated with *Flavobacterium* (p-value=0.021) and *Simplicispira* (p-value=0.009); polypropylene was positively associated with *Acinetobacter* (p-value<0.001), polystyrene with *Hydrogenophaga* (p-value<0.001), polyethylene with *Pseudomonas* (p-value=0.036), isoprene with *Pseudarcicella* (p-value<0.0001), and polyurethane with *Synechococcus* (p-value <0.001); polyvinylchloride particles were negatively associated with *Flavobacterium* (p-value=0.011) and *Synechococcus* (p-value=0.007), and particles of unknown polymers were positively associated with *Massilia* (p-value=0.035) and negatively associated with *Hydrogenophaga* (p-value=0.007) and *Acinetobacter* (p-value<0.001).

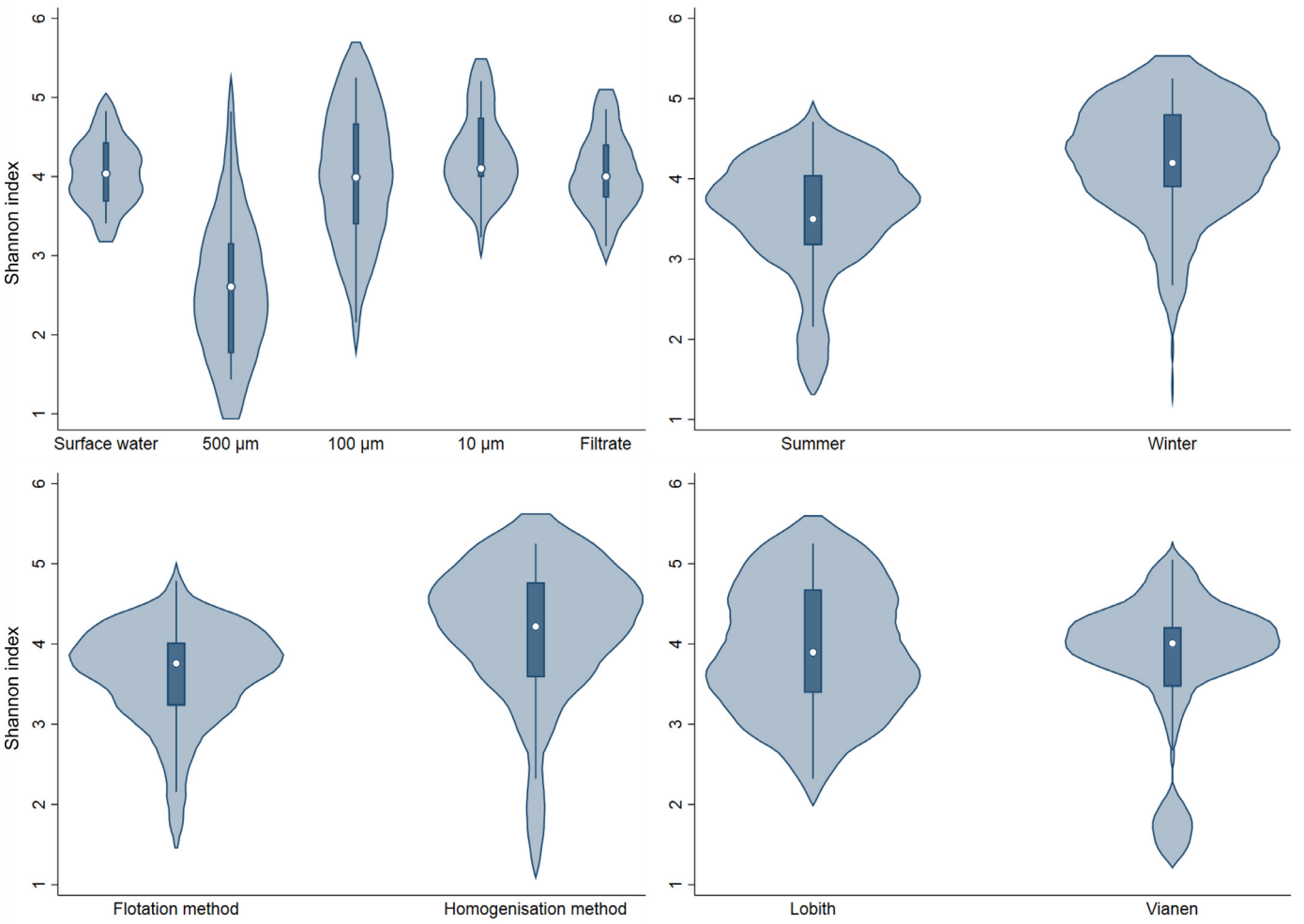

Fig. 3. Shannon index (alpha diversity) at the genus level of the microbial community composition per sample type (500 μm, 100 μm and 10 μm microplastics, filtrates, and surface water), season, sampling location and filtration method.

Pathogens

Focusing on genera detected in the samples and known to harbour pathogenic species, such as *Acinetobacter*, *Pseudomonas*, *Legionella*, *Mycobacterium* and *Arcobacter*, we found large particles to be significantly enriched (p-value$<$0.001) with *Acinetobacter* (relative abundance 14.6%) and *Pseudomonas* (2.0%) as compared to small particle/water like samples (0.7% and 0.8%, respectively), whereas *Legionella* and *Mycobacterium* were not significantly associated with sample type. For *Arcobacter*, significantly higher abundances were found in all sample types (all p-values$<$0.001) as compared to 500 μm samples.

Virulence and amr genes

The virulence genes *stx1* and *stx2* were not detected in any sample, but the AMR genes *erm(B)* and *sul1* were detected in respectively 75 and 79 (out of the 80) samples, resulting in a prevalence of 93.8% (95%CI: 80.9–98.2%) for *erm(B)* and 98.8% (95%CI: 91.2–99.8%) for *sul1*. The 5 *erm(B)*- and 1 *sul1*-negative samples were all 500 μm samples collected in Vianen in both summer ($n=2$) and winter ($n=3$).

Discussion

In this study, the biofilms of large particles (100–500 μm) were found to harbour significantly different microbial communities with decreased diversity as compared to surface water and smaller particles (10–100 μm or smaller). This might be related to the relatively larger attachment surface of the significantly more abundant small *vs.* large particles. Moreover, there was a trend towards a more water-like microbial community as the residue fraction size became smaller, an indication that planktonic microbiota accumulated in the lowest layers of the sieve cascade. We also observed more temporal than spatial heterogeneity, with season influencing the microbial communities significantly.

Taxon diversity on freshwater microplastics is often lower than the water itself or natural substrates (Miao et al., 2019; Jiang et al., 2018; McCormick et al., 2016; McCormick et al., 2014). We also found polypropylene to be significantly more abundant amongst large particles (where diversity was the lowest), and it has indeed been reported that microbial diversity is particularly low on polypropylene (Zettler et al., 2013). Moreover, diversity was lower in summer despite microplastic concentrations were higher in summer. Lower diversity during summertime has been observed in different freshwater environments and seems to be driven by changes in several physicochemical parameters besides temperature (García-Armisen et al., 2014).

Previous studies have shown distinct microbial communities between plastic and water samples (Zettler et al., 2013; Jiang et al., 2018; Ogonowski et al., 2018). These differences are intriguing, as microbial communities exist in close proximity in the river and were collected contemporaneously. amongst the most interesting differences was the relatively high abundance of *Gammaproteobacteria* (especially *Acinetobacter*, *Pseudomonas*, *Thiothrix*, *Alkanindiges*) and *Betaproteobacteria* (especially *Roseateles*,

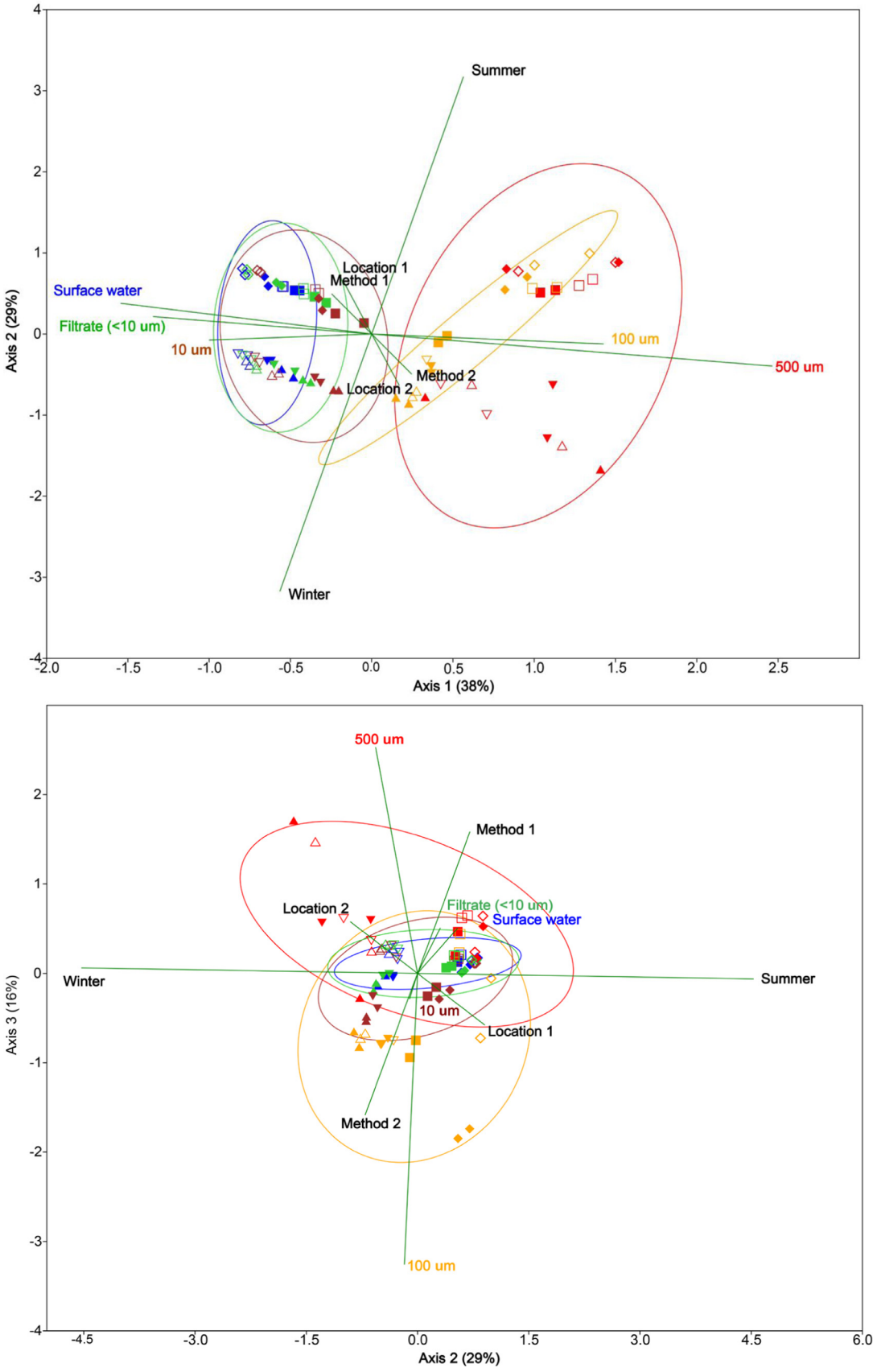

Fig. 4. Canonical correspondence analysis plots of the overall microbial community composition at the genus level in the different types of samples (500 μm, 100 μm and 10 μm microplastics, final filtrate, and surface water), collected at the two sampling locations, in summer and winter, and analysed with two different filtration methods. *Data points represent the different samples (n = 80), which are coloured (together with the 95% ellipses) according to sample type (blue=surface water; red=500 μm; orange=100 μm; brown=10 μm; green=filtrate). Quadrilateral (i.e. four-sided) symbols indicate samples taken in summer from location 1 'Lobith' (squares) or location 2 'Vianen' (diamonds), whereas three-sided symbols indicate samples taken in winter from Lobith (triangle) or Vianen (inverse triangle); colour-unfilled (empty) symbols indicate samples analysed with filtration method 1 (flotation) and filled symbols indicate samples analysed with filtration method 2 (homogenisation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

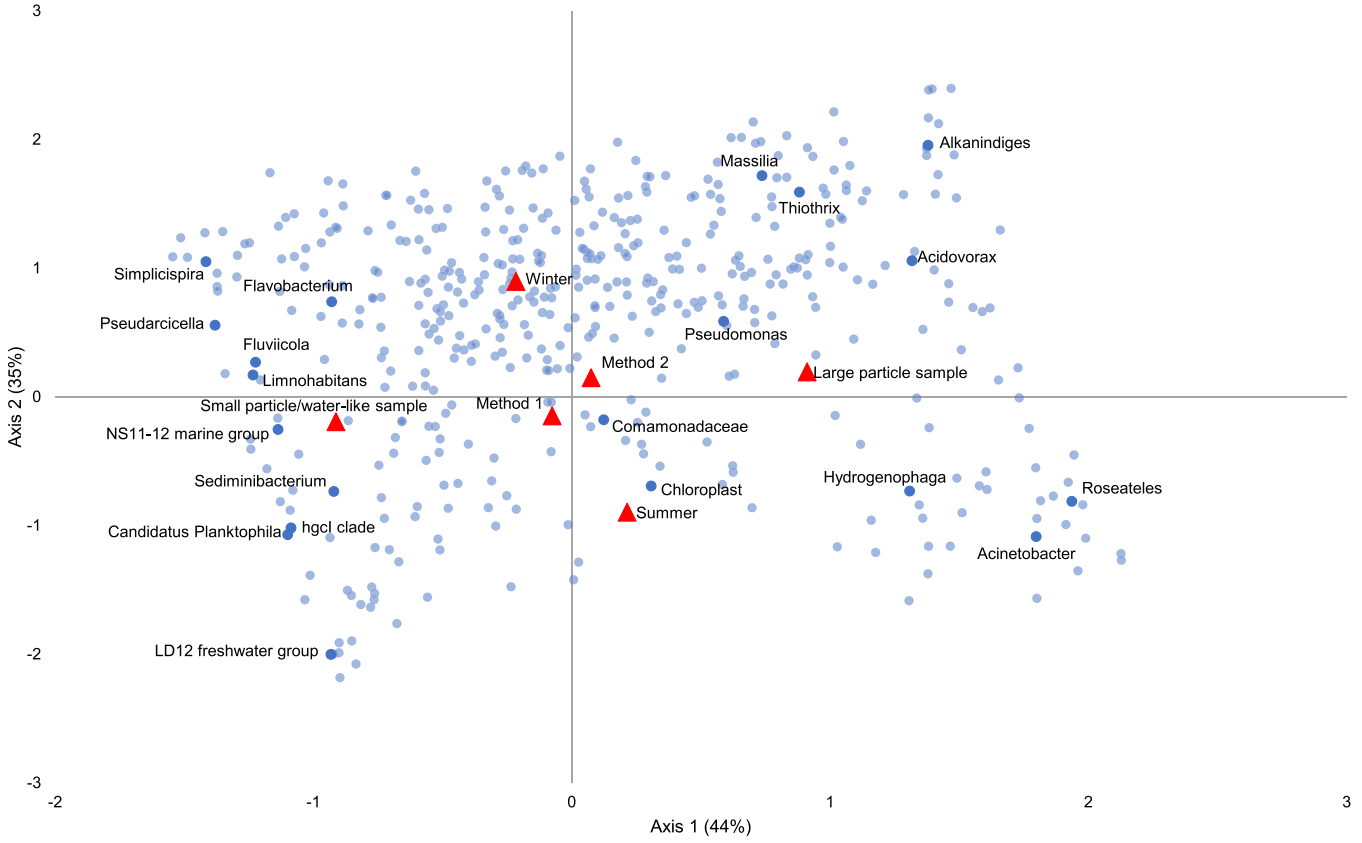

Fig. 5. Canonical correspondence analysis plots of the overall microbial community composition at the genus level with indication of the taxa primarily responsible for the observed dissimilarities between sample types, seasons and filtration methods.
Data points represent the position of the features included in the canonical correspondence analyses, i.e. the most differentially abundant taxa reported in Table 2 (dots), and the variables (triangles) defining the groups of samples, i.e. the sample type, season and filtration method. Differences between sampling locations are not visualized as they are not significant. 'Small particle/water-like' samples include surface water, filtrate and 10 μm microplastic samples, whereas 'large particle' samples include 500 μm and 100 μm microplastic samples. 'Method 1' corresponds to the flotation method and 'method 2' corresponds to the homogenisation method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Massilia, *Hydrogenophaga*, *Acidovorax* on large particles and different *Bacteroidetes* and *Actinobacteria* taxa (especially NS11–12 marine group, hgcI clade, *Pseudarcicella*, *Candidatus Planktophila*) on small particle/water-like samples (Fig. 6). Previous studies reported similar findings, particularly *Gammaproteobacteria* and *Betaproteobacteria* being more abundant on plastic and *Bacteroidetes* and *Actinobacteria* on natural substrates (Zettler et al., 2013; Miao et al., 2019; McCormick et al., 2016; Ogonowski et al., 2018). Certain species belonging to *Gammaproteobacteria* are early biofilm producers on artificial substrates and the genus *Pseudomonas* especially is notorious to thrive on plastics (McCormick et al., 2016; McCormick et al., 2014). Exposure to pathogenic *Pseudomonas* species may cause abrasion and ear infections, (Schets et al., 2014) and two opportunistic human pathogens (*Pseudomonas monteilii* and *Pseudomonas mendocina*) and one plant pathogen (*Pseudomonas syringae*) have been reported to occur exclusively on plastic (Wu et al., 2019). We found *Pseudomonas* to be more abundant in samples with high polyethylene concentrations, and *Pseudomonas* is known to be able to biodegrade plastic polymers, particularly polyethylene (Tribedi et al., 2015). It is worth stressing that plastic polymer composition and microbial community composition were tested for possible associations in separate samples. While such correlative study design allowed us to determine whether the microbial community composition varies depending on the abundance of the different plastic polymers, it does not provide the same level of detail as analysing the microbial assemblages of individual plastic pieces of specific polymer types. Although this study was primarily meant to generate hypotheses rather than testing them, most of the aforementioned results about microbial diversity and the different associations between plastic polymers and microbial taxa were previously observed, meaning that our results were confirmatory in nature, which in turn supports the validity of the analyses in general. It follows, therefore, that our findings support the general notion that microplastics may serve as unique substrates for microbial colonisation and that taxon selection occurs during biofilm development on microplastics, resulting in reduced capacity to support diversity (Miao et al., 2019).

Microplastics are often seen as potential vectors of pathogenic microorganisms (Miao et al., 2019; Jiang et al., 2018; McCormick et al., 2016). In addition to the biofilm-associated and possibly plastic-degrading taxa, taxa that are known to harbour potentially pathogenic species, such as *Pseudomonas, Acinetobacter* and *Arcobacter*, as well as the selected AMR genes, were found. While the absence of the *stx1* and *stx2* genes might be explained by the lack of detectable *Escherichia* and *Shigella* bacteria (although these genes are described in other *Enterobacteriaceae* as well), it can also be hypothesized that selection taking place in aquatic environments plays a role since these genes would give no immediate advantage for bacterial fitness in such environments. Conversely, AMR genes would confer an advantage, as antimicrobial residues are present in surface water, especially wastewater (Arias-Andres et al., 2018; Imran et al., 2019). Moreover, co-selection of multiresistance in microplastic-contaminated environments is an emerg-

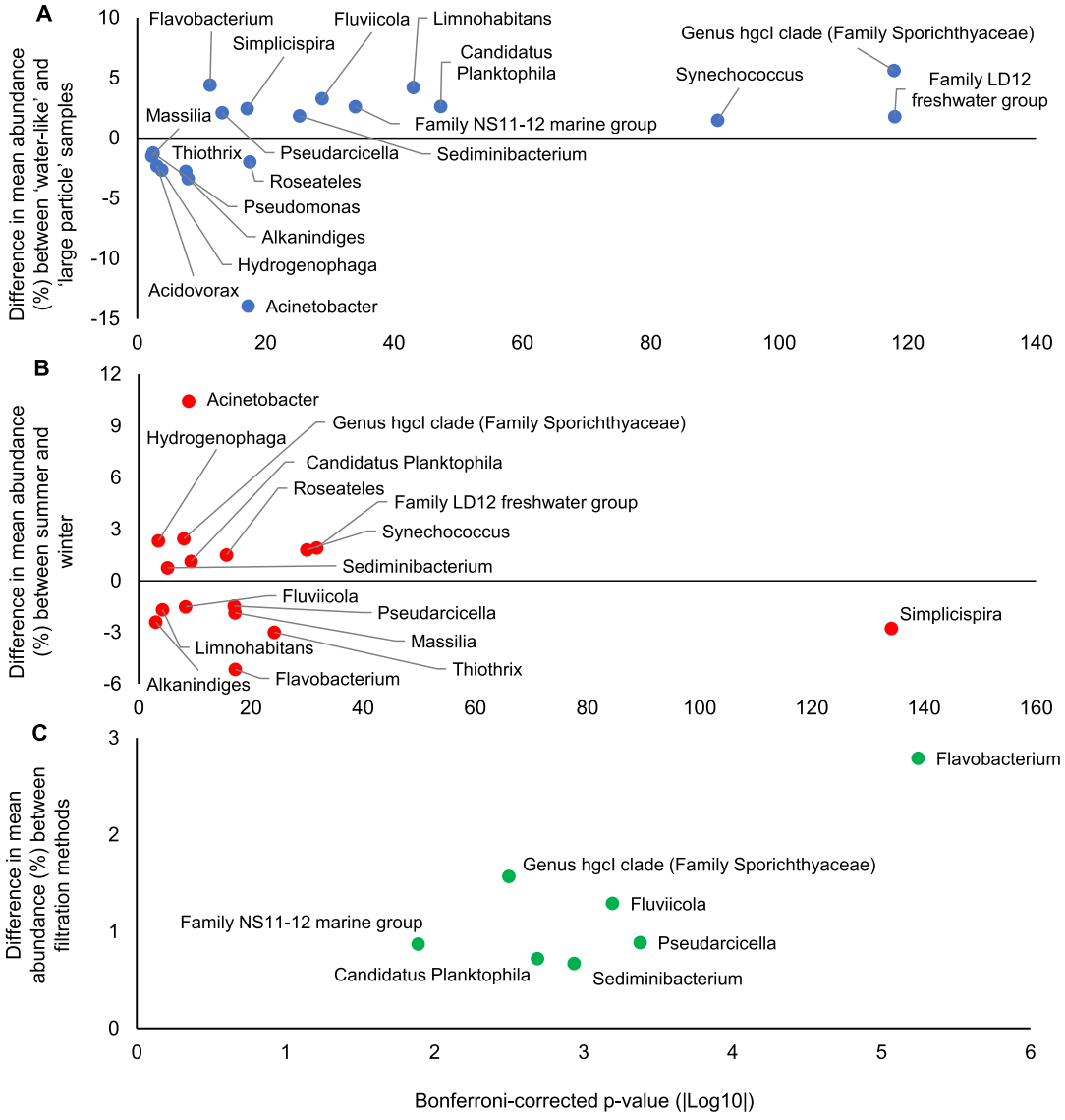

Fig. 6. Differentially abundant taxa between sample types, seasons and filtration methods.
Panel A: the points indicate taxa with statistically significant differences in abundance between 'small particle/water-like' samples (i.e. surface water, filtrates, 10 μm microplastic samples) and 'large particle' samples (i.e. 500 μm and 100 μm microplastic samples). Negative values in the Y axis indicate that the taxa were less abundant in 'small particle/water-like' samples than in the 'large particle' samples, and vice versa for the positive values. Panel B: the points indicate taxa with statistically significant differences in abundance between summer and winter samples. Negative values in the Y axis indicate that the taxa were less abundant in summer than in winter, and vice versa for the positive values. Panel C: the points indicate taxa with statistically significant differences in abundance between the two filtration methods. There are only positive values in the Y axis because all the significant differences pertained to taxa that were more abundant in the samples analysed with the flotation than the homogenisation methods. Only the taxa primarily responsible for the observed dissimilarities in the overall microbial community composition reported in Table 2 were considered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ing risk, (Imran et al., 2019) with microplastics acting as hotspots for emerge and spread. Selective enrichment of certain bacterial pathogens on microplastics has been reported (McCormick et al., 2016). The genera *Acinetobacter* and *Pseudomonas*, which include several potential pathogenic species, were significantly more abundant amongst large particles than surface water or small particle/water-like samples. Moreover, the genus *Arcobacter*, which also includes potential pathogens, were almost as abundant on the particles as in water, but more abundant amongst 100 and 10 μm samples. As our analyses did not allow for species identification, but only for genera that may contain known pathogenic species, further research is needed to confirm these findings. 16S rRNA gene sequencing is a powerful method to determine microbial community structures, but metagenomic sequencing is likely to provide a more detailed picture, including microbial species, virulence and AMR genes, as it is particularly useful to determine the genomic potential of different environments.

This study has several limitations. We were not able to fully differentiate plastic from seston particles in the samples. Unless one manually isolates (relatively large) plastic particles or uses a controlled weathering experiment in which specific plastic particles of known polymer and concentration are exposed to a given aquatic environment, it is hard to isolate and characterize plastic particles from surface water in a way that does not alter or damage their biofilms. Our method, although imperfect, allowed us to analyse directly the samples, both chemically and microbiologically in parallel. Furthermore, although the samples were not made of 100% plastic, the microbial community results were highly comparable with those from studies using exclusively plastic particles in controlled experiments (Miao et al., 2019). This indicated that the

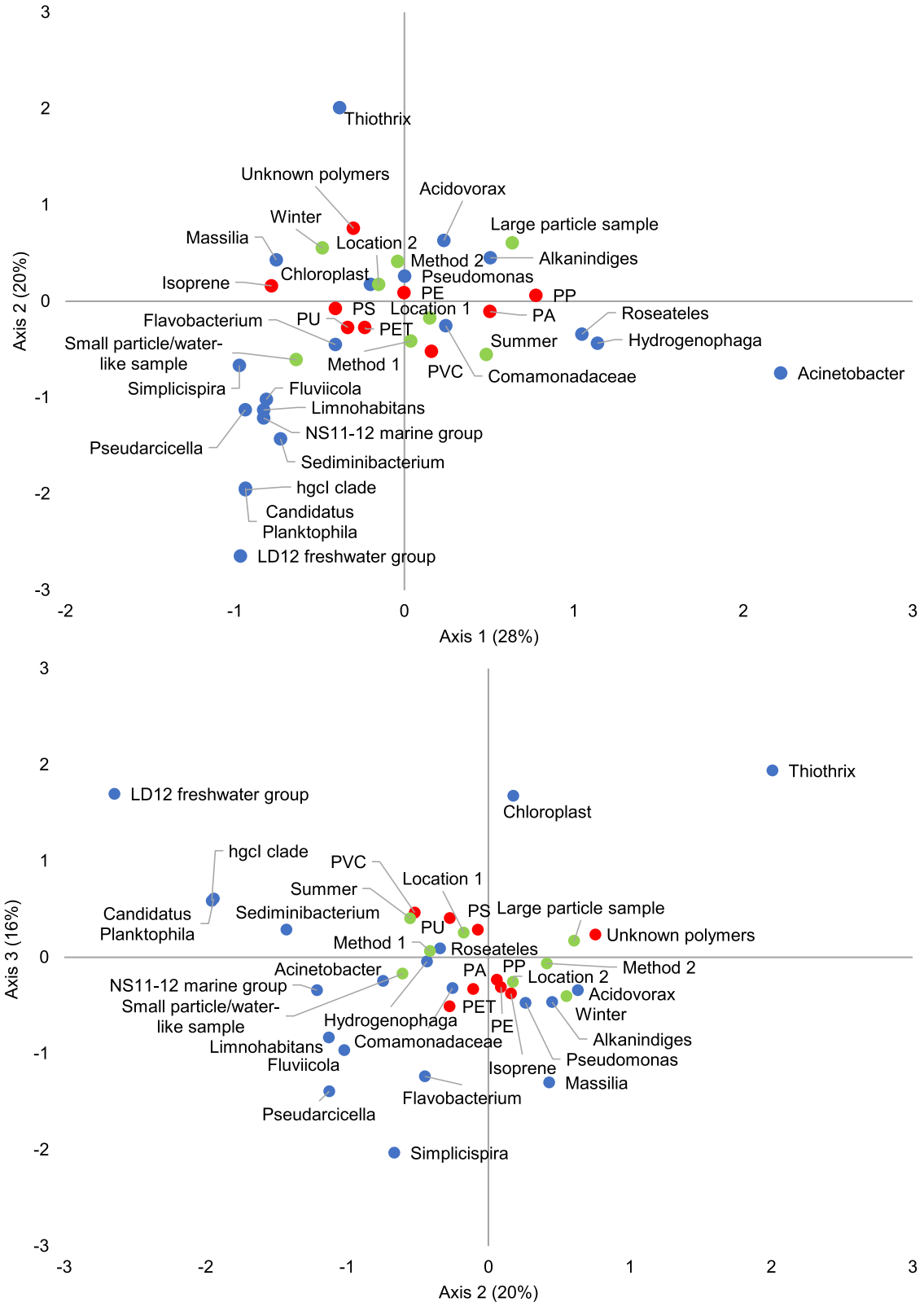

Fig. 7. Canonical correspondence analysis plots of the differentially abundant taxa along with the group variables and relative abundances of microplastic polymer types. *Data points represent the position of the features included in the CCA, i.e. the most differentially abundant taxa reported in Table 2 and Fig. 6 (in blue), the group variables (in green) sample type, season, filtration method and sampling location, and relative abundances of each polymer type (in red). 'Small particle/water-like' samples include surface water, filtrate and 10 μm microplastic samples, whereas 'large particle' samples include 500 μm and 100 μm microplastic samples. Location 1 corresponds to 'Lobith' and location 2 to 'Vianen' (Fig. 1). Filtration method 1 corresponds to the flotation method and filtration method 2 corresponds to the homogenisation method. PA = Polyamide, PE = Polyethylene (PE), PET = Polyethylene Terephthalate, PP = Polypropylene, PS = Polystyrene, PU = Polyurethane, PVC = Polyvinylchloride. Analysis is based on a subset of 32 microplastic samples of 10 μm and 100 μm analysed for microbial community composition that could be coupled meaningfully with the analysed particle sizes of 20–100 μm and 100–500 μm ranges, respectively (Table 1). Analysis is based on all taxa (480), but only those differentially abundant (Table 2 and Fig. 6) are reported to improve visibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

sampling did capture plastic particles and that non-plastic particles had a limited impact, and even if the results were not specific for plastic, they were likely to be driven by the plastic. Differences in microbial communities between filtration methods were also observed. With the flotation method, the fraction of plastic particles that was captured included the floating plastic particles, so the results represented mainly the microbiota associated with (enhanced) microplastic buoyancy and low-density particles. Conversely, the homogenisation method comprised the sediment, with plastic having density exceeding that of water, but also non-plastic residues. Both filtration methods were thus complementary to one another in providing data for the whole microbial community in a sample.

Conclusions

Microplastics were widespread in the analysed samples, with concentrations, polymer types and microbial communities varying mainly by season and particle size, and less by location. Results were compatible with the existence of taxon-selecting mechanisms and reduced microbial diversity in the biofilms of plastic substrates. A number of biofilm-associated and plastic-degrading taxa were enriched in certain types of samples and occurred more abundantly in association with specific plastic polymers, including taxa that may harbour human (entero)pathogens, as well as ubiquitous AMR genes. This information helps understanding microplastic pollution in riverine environments to raise awareness on the potential public and ecosystem health effects, including the hazards of microplastic dispersal via freshwater, as well as the risks of microbial introduction into areas/niches where microplastics can favour the global spread of waterborne diseases and AMR.

Contributor information

LMG, AMdRH and PB conceived and designed the study. PB, RvdP and PvdW acquired the data. LMG, RvdR and PB performed the analyses. All authors interpreted the findings. LMG wrote the first draft of the manuscript. All authors contributed to drafting subsequent versions, critically reviewed and approved the final version.

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.

Ackowledgements

The authors would like to thank Angela van Hoek for support in AMR and virulence gene analyses. This study was supported by the Netherlands Organisation for Health Research (ZonMw). Funders had no role in study design, collection, analysis, interpretation, writing and deciding to publish this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.116852.

References

Arias-Andres, M., Klumper, U., Rojas-Jimenez, K., Grossart, H.P., 2018. Microplastic pollution increases gene exchange in aquatic ecosystems. Environ Pollut 237, 253–261.

Barnes, D.K., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc Lond B Biol Sci 364 (1526), 1985–1998.

Blettler, M.C.M., Abrial, E., Khan, F.R., Sivri, N., Espinola, L.A., 2018. Freshwater plastic pollution: recognizing research biases and identifying knowledge gaps. Water Res. 143, 416–424.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13 (7), 581–583.

CLARKE, K.R, 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18 (1), 117–143.

Eckert, E.M., Di Cesare, A., Kettner, M.T., et al., 2018. Microplastics increase impact of treated wastewater on freshwater microbial community. Environ Pollut 234, 495–502.

Franz, E., van Hoek, A.H., Wuite, M., et al., 2015. Molecular hazard identification of non-O157 Shiga toxin-producing Escherichia coli (STEC). PLoS ONE 10 (3), e0120353.

García-Armisen, T., İnceoğlu, Ö., Ouattara, N.K., et al., 2014. Seasonal Variations and Resilience of Bacterial Communities in a Sewage Polluted Urban River. PLoS ONE 9 (3), e92579.

Imran, M., Das, K.R., Naik, M.M., 2019. Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: an emerging health threat. Chemosphere 215, 846–857.

Ivleva, N.P., Wiesheu, A.C., Niessner, R., 2017. Microplastic in Aquatic Ecosystems. Angewandte Chemie International Edition 56 (7), 1720–1739.

Jiang, P., Zhao, S., Zhu, L., Li, D., 2018. Microplastic-associated bacterial assemblages in the intertidal zone of the Yangtze Estuary. Science of The Total Environment 624, 48–54.

Kooi, M., Koelmans, A.A., 2019. Simplifying Microplastic via Continuous Probability Distributions for Size, Shape, and Density. Environ Sci Technol Lett 6 (9), 551–557.

Löder, M.G.J., Kuczera, M., Mintenig, S., Lorenz, C., Gerdts, G., 2015. Focal plane array detector-based micro-Fourier-transform infrared imaging for the analysis of microplastics in environmental samples. Environmental Chemistry 12 (5), 563–581.

Leslie, H.A., Brandsma, S.H., van Velzen, M.J.M., Vethaak, A.D., 2017. Microplastics en route: field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota. Environ Int 101, 133–142.

Mani, T., Hauk, A., Walter, U., Burkhardt-Holm, P., 2015. Microplastics profile along the Rhine River. Sci Rep 5 (1), 17988.

Miao, L., Wang, P., Hou, J., et al., 2019. Distinct community structure and microbial functions of biofilms colonizing microplastics. Science of The Total Environment 650, 2395–2402.

Mintenig, S.M., Bäuerlein, P.S., Koelmans, A.A., Dekker, S.C., van Wezel, A.P., 2018. Closing the gap between small and smaller: towards a framework to analyse nano- and microplastics in aqueous environmental samples. Environmental Science: Nano 5 (7), 1640–1649.

Mintenig, S.M., Kooi, M., Erich, M.W., et al., 2020. A systems approach to understand microplastic occurrence and variability in Dutch riverine surface waters. Water Res. 176, 115723.

McCormick, A.R., Hoellein, T.J., London, M.G., Hittie, J., Scott, J.W., Kelly, J.J., 2016. Microplastic in surface waters of urban rivers: concentration, sources, and associated bacterial assemblages. Ecosphere 7 (11), e01556.

McCormick, A., Hoellein, T.J., Mason, S.A., Schluep, J., Kelly, J.J., 2014. Microplastic is an Abundant and Distinct Microbial Habitat in an Urban River. Environ. Sci. Technol. 48 (20), 11863–11871.

Reisser, J., Shaw, J., Hallegraeff, G., et al., 2014. Millimeter-sized marine plastics: a new pelagic habitat for microorganisms and invertebrates. PLoS ONE 9 (6), e100289.

Schets, F.M., van den Berg, H.H., Baan, R., Lynch, G., de Roda Husman, A.M., 2014. Pseudomonas aeruginosa on vinyl-canvas inflatables and foam teaching aids in swimming pools. J Water Health 12 (4), 772–781.

Sharma, S., Chatterjee, S., 2017. Microplastic pollution, a threat to marine ecosystem and human health: a short review. Environmental Science and Pollution Research 24 (27), 21530–21547.

Tribedi, P., Gupta, A.D., Sil, A.K., 2015. Adaptation of Pseudomonas sp. AKS2 in biofilm on low-density polyethylene surface: an effective strategy for efficient survival and polymer degradation. Bioresour Bioprocess 2 (1), 14.

Quast, C., Pruesse, E., Yilmaz, P., et al., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41 (Database issue), D590–D596.

Yoshida, S., Hiraga, K., Takehana, T., et al., 2016. A bacterium that degrades and assimilates poly(ethylene terephthalate). Science 351 (6278), 1196–1199.

Ogonowski, M., Motiei, A., Ininbergs, K., et al., 2018. Evidence for selective bacterial community structuring on microplastics. Environ Microbiol 20 (8), 2796–2808.

Wu, X., Pan, J., Li, M., Li, Y., Bartlam, M., Wang, Y., 2019. Selective enrichment of bacterial pathogens by microplastic biofilm. Water Res 165, 114979.

Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the "plastisphere": microbial communities on plastic marine debris. Environ Sci Technol 47 (13), 7137–7146.