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Microplastic pollution increases gene exchange in aquatic ecosystems☆

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ABSTRACT

Pollution by microplastics in aquatic ecosystems is accumulating at an unprecedented scale, emerging as a new surface for biofilm formation and gene exchange. In this study, we determined the permissiveness of aquatic bacteria towards a model antibiotic resistance plasmid, comparing communities that form biofilms on microplastics vs. those that are free-living. We used an exogenous and red-fluorescent *E. coli* donor strain to introduce the green-fluorescent broad-host-range plasmid pKJK5 which encodes for trimethoprim resistance. We demonstrate an increased frequency of plasmid transfer in bacteria associated with microplastics compared to bacteria that are free-living or in natural aggregates. Moreover, comparison of communities grown on polycarbonate filters showed that increased gene exchange occurs in a broad range of phylogenetically-diverse bacteria. Our results indicate horizontal gene transfer in this habitat could distinctly affect the ecology of aquatic microbial communities on a global scale. The spread of antibiotic resistance through microplastics could also have profound consequences for the evolution of aquatic bacteria and poses a neglected hazard for human health.

1. Introduction

It is estimated that 12,000 Mt of plastic waste will be released into the environment by 2050 (Geyer et al., 2017). Millions of tons of microplastic particles (<5 mm) from many industrial products (Keswani et al., 2016), but also resulting from the physical, chemical, and biological degradation of plastic waste, are constantly released into aquatic systems worldwide (Cole et al., 2011; Law and Thompson, 2014). This environmental problem is becoming more serious, given the steady increase in plastics production, which is currently estimated at 300 million tons per year (Zalasiewicz et al., 2016). Furthermore, the amount of plastic pollution is so significant that its footprint on the planet is now considered an indicator of the Anthropocene (Duis and Coors, 2016; Zalasiewicz et al., 2016).

Microplastics constitute highly recalcitrant pollutants and act as long-lasting reactive surfaces, containing additives and/or absorbing organic matter and chemical substances, such as heavy metals, antibiotics, pesticides, and other xenobiotics (Hirai et al., 2011; Jahnke et al., 2017). Additionally, microplastics can be colonized by different microbial communities from natural surface-attached and free-living microbial communities (Kettner et al., 2017; Oberbeckmann et al., 2016; Zettler et al., 2013). Consequently, they form specific niches for microbial life and are collectively known as "The Plastisphere" (Keswani et al., 2016).

Although there is a growing interest in studying the problem of plastics in aquatic habitats, relatively little is known on the effect of microplastic pollution in freshwater ecosystems. The few available measurements indicate that microplastics can reach high quantities, even in remote ecosystems in areas of low population densities (Free et al., 2014), while it was shown that in urban areas, waste-

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water treatment plants constitute, for example, important sources of microplastics, releasing up to several million pieces per day (McCormick et al., 2016). Microplastics in all kinds of aquatic systems can be transported over long distances (horizontally), and through the water column, after changes in biofouling that affect particle density (vertically), thus serving as vectors for the selection and spread of attached pathogenic bacteria, harmful algae and invasive species (Keswani et al., 2016; Kirstein et al., 2016; Zalasiewicz et al., 2016).

A rarely explored feature of microplastic biofilms is their potential as so-called "hot-spots" of horizontal gene transfer (HGT), as they display areas of increased nutrient availability and high cell densities of microbial cells, allowing for intense interactions (Aminov, 2011; Sezonov et al., 2007). Conjugation is the main mechanism of directed HGT, a process in which two bacteria in close contact can exchange genetic information via plasmid transfer from a donor to a recipient cell (Drudge and Warren, 2012). This process can occur even between distantly related taxa, affecting bacterial evolution and the spread of multiple phenotypic traits, such as antibiotic or heavy metal resistance genes (Carattoli, 2013).

We hypothesize that pollution by microplastics in aquatic ecosystems favors higher transfer frequencies of plasmids carrying antibiotic resistance genes. Because of the relevance of microplastics and antibiotic resistance genes as contaminants worldwide, a better understanding of the HGT of antibiotic resistance genes within microplastic-associated communities is timely. The analysis of gene exchange events in the Plastisphere can broaden our understanding of the effects of plastic pollution on the ecology of aquatic ecosystems, bacterial evolution, and the emerging risks to environmental and human health.

2. Materials and methods

The hypothesis was tested with two experiments. In the first, plasmid transfer frequency between two bacterial species was determined in a microcosm study, in the presence or absence of microplastics. Water from the meso-oligotrophic Lake Stechlin was used as media. As donor, we used a red-fluorescently tagged *E. coli* strain with the self-transmissible, green-fluorescently tagged, plasmid pKJK5, encoding resistance to trimethoprim. The green fluorescence protein is repressed in donor cells while active upon plasmid transfer in transconjugant cells (bacteria incorporating the plasmid via conjugation). Accordingly, donor (red), recipient (non-fluorescent) and transconjugant (green) fluorescent protein expression allowed comparison of transconjugant to donor ratios by means of flow cytometry (FCM).

In the second experiment, we incubated microplastics directly in Lake Stechlin, and harvested bacteria from colonizing biofilms on microplastics, free-living bacteria and from natural aggregates. Subsequently, standardized filter matings of each community against the exogenous donor strain were performed on polycarbonate filters, to evaluate their permissiveness towards plasmids. Fluorescence-activated Cell Sorting (FACS) was performed for the isolation of transconjugant cells and further analysis of the community composition.

2.1. Strains and culturing

E. coli MG1655 tagged chromosomally with a *lacIq-Lpp-mCherry-km*^{R} gene cassette into the chromosomal *att*Tn7 site, which conferred red fluorescence and a lacI^q repressor, and the IncP-1ε broad host range (BHR) plasmid pKJK5::*gfpmut3* (Klümper et al., 2017) was used as a donor-plasmid system. A *Pseudomonas* sp. isolate from Lake Stechlin was used as a recipient strain.

Strains were cultured on nutrient broth DEV (10 g/L Meat Peptone, 10 g/L Meat Extract, 5 g/L NaCl) for experiment one and in LB medium (10 g/L Tryptone, 5/L Yeast Extract, 5 g/L NaCl) for experiment two. Antibiotics (Kanamycin Km 50 μg/mL, Trimethoprim TMP 30 μg/mL) were added to the medium used to support the donor strain. For information on supplier of chemicals also see SI.A culture of *Pseudomonas* sp. carrying the plasmid was also prepared in LB medium with TMP 30 μg/mL. Finally, as a control during FACS gating in the second experiment, a culture of the *E. coli* strain was supplemented with IPTG to induce GFP expression. Cells were harvested by centrifugation (10,000 × g at 4 °C for 10 min), washed and finally resuspended in 0.9% NaCl sterile solution, to eliminate media and antibiotics. Cell densities of *E. coli* and *Pseudomonas* sp. suspensions were estimated after DAPI stain using the CellC software (Selinummi et al., 2005) prior to inoculation of experiments.

2.2. Microplastic particles

Additive-free polystyrene films were obtained from Norflex® (Nordenham, Germany). The material was cut with a metal multiple puncher to produce 4 mm × 4 mm x 0.1 mm square particles. These particles were treated with 70% ethanol, 3% H_2O_2 and sterile ultrapure water (MQ) for disinfection and to eliminate residual organic matter contamination.

2.3. Set-up of experiment 1 (two-species microcosm)

Each microcosm consisted on 100 ml of 0.2 μm filtered water from Lake Stechlin (SLW) in pre-combusted 300 ml flasks (Fig. 1A). Four treatments were assayed: a) without microplastics (-MP); b) with microplastics (+MP); c) with microplastics pre-soaked in nutrient broth (+MPN) and d) a control for nutrient desorption (Ctrl Nutrient). We used 50 microplastic particles per microcosm in treatments b, c and d. Prior to the start of the experiment, particles of the +MP treatment were incubated for three days in MQ water, while in the +MPN treatment for three days in nutrient broth DEV (refer to the SI for details) and then washed with MQ water. In the control for nutrient desorption, microplastics were treated as in +MPN, incubated for additional 24 h in filter-sterile lake water, and then separated by decantation prior bacterial inoculation.

Each microcosm (four replicates per treatment) was inoculated with donor and recipient suspension of 5×10^6 cells mL^{-1} (D:R ratio = 1:1). We also included two controls for contamination consisting of non-inoculated filtered lake water with and without microplastics. The microcosms were incubated at 20 °C for 72 h in dark conditions and constant agitation at 150 rpm, followed by 4 °C for 48 h, to allow proper folding of GFP (Klümper et al., 2014). Thereafter, MP particles were washed with 0.9% sterile NaCl solution and five were preserved for confocal and scanning electron microscopy analysis, while the rest (n = 45) were vortexed for 1 min in 1 mL of sterile pyrophosphate (50 mM $Na_4O_7P_2$) -Tween80 (0.05%) buffer solution for biofilm detachment. A sample of 10 mL of water was taken from each flask with a sterile pipette.

Donor and transconjugant cells from the water (w) and particle (p) phases of each replicate were analyzed by flow cytometry using a FACSAriaII instrument and BD FACSDiva TM software v6 (Becton Dickinson Biosciences, San Jose, CA). The instrument had a 488 nm laser (100 mW) connected to a green fluorescent detector at 500–550 nm, and a 532 nm (150 mW) laser connected to a red fluorescent detector at 600–620 nm. Side scatter threshold was set at 300. A gate for bacterial events using both strains was set on a bivariate FSC-A vs. SSC-A plot. Gates for donor, recipient and transconjugant were set in a second gate on a bivariate FITC-A vs. PE-Texas Red-A plot with cell suspensions from each strain (Fig. S1). Event rate was <3000 e/sec. Donor and transconjugant events were

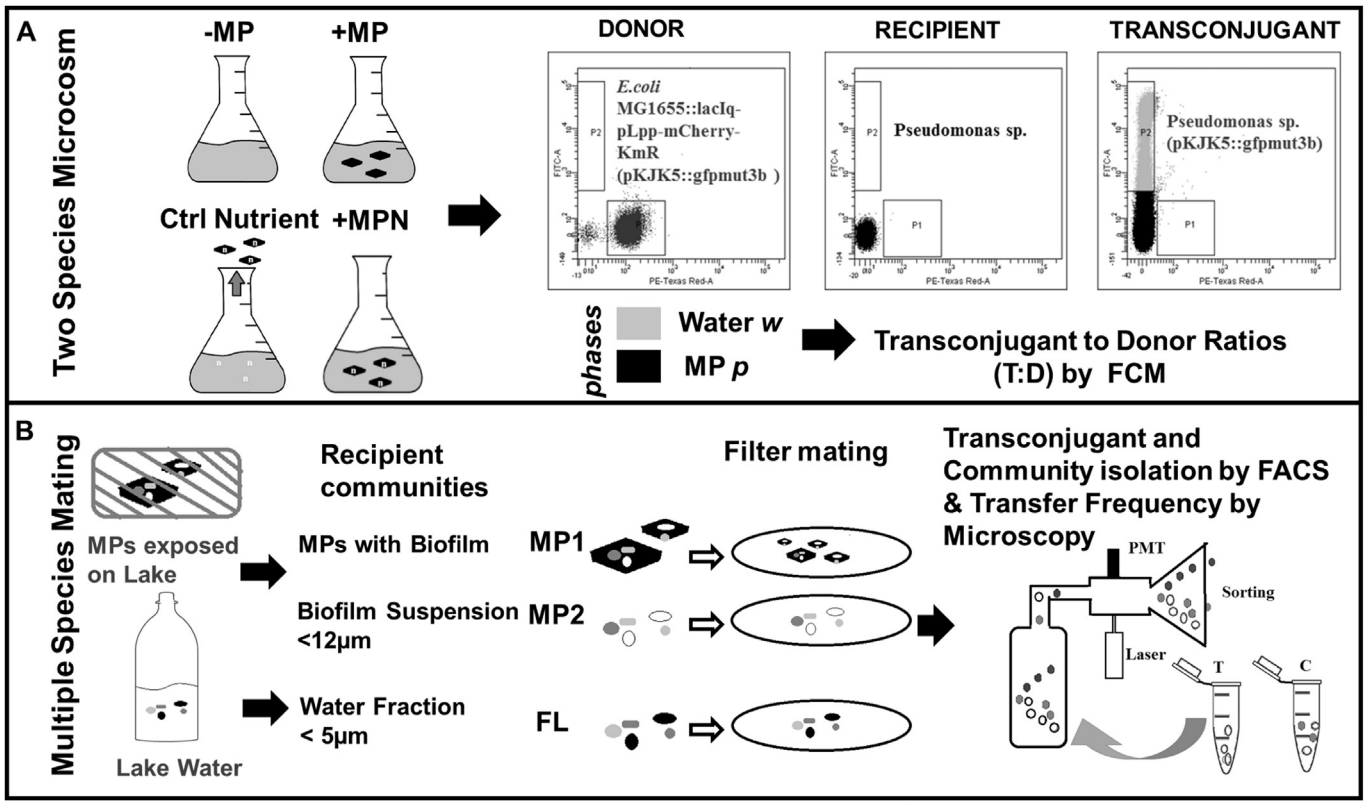

Fig. 1. Experimental design. A) Two Species Microcosm. Treatments without and with microplastics are indicated by -MP and +MP, respectively. Treatment of microplastics pre-exposed to organic matter (+MPN) and a control for nutrient desorption (Ctrl Nutrient) were included. The detection of the donor (P1 gate), recipient and transconjugant (P2 gate) populations was performed by flow cytometry, based on their fluorescent protein expression patterns, in FITC vs. Texas Red A plots (for transconjugant-green vs. donor-red fluorescence detection respectively). In each flask, bacteria both from water (w) and attached to microplastics (p) were screened, and the Transconjugant per Donor ratios were calculated for each phase-treatment. B) Multiple Species Matings. Recipient bacteria originate from microplastic biofilms and the free-living (FL) bacterial communities of lake water. The biofilm was obtained both as direct biofilm on microplastics (MP1) and as detached bacteria suspension (MP2). Transfer frequencies were determined by microscopy for matings of the donor with MP2 and FL. FACS isolated transconjugant (T) and bacterial community (C) cells were isolated from matings against MP1, MP2 and FL, and were used for metabarcoding using 16S rRNA gene markers.

recorded simultaneously, with 200,000 donor events as a stopping gate on all water phase samples and the biofilm suspension of +MPN. For the +MP biofilm suspension 20,000 donor events were recorded. Frequency of plasmid transfer was calculated as the ratio of *Pseudomonas* sp. transconjugant cells per *E. coli* plasmid donor cell (T:D ratio). Cell densities were estimated as before in water samples taken at the beginning and end of the experiment.

2.4. Set-up of experiment 2 (multispecies species matings)

In the second experiment (Fig. 1B), microplastic particles were incubated directly in Lake Stechlin using mesh-sealed stainless steel cylinders cages (mesh size of 3 mm, 25 cm length and 10 cm diameter). Five cages, with ~1500 particles per cage, were placed in the lake mesolimnion (6 m depth), and incubated for four weeks, starting in mid-July 2016. Filter matings consisted of three recipient community treatments: a) biofilm formed on the particles, washed with 0.9% NaCl (MP1); b) cell suspension from the biofilm (MP2), obtained by vortexing and sonication of ca. 500 microplastic particles per cage in ice-cold pyrophosphate-Tween 80 buffer. Cell suspensions were pooled and pre-filtered through a 12 µm filter to remove larger organisms in this sample; c) the free-living bacteria (FL), obtained after 5 µm pre-filtration of lake water taken with a vertical point sampler at a depth of 6 m. Multispecies matings were performed on 0.2 µm black PC filters, 25 mm diameter (Whatman, UK) as described previously (Klümper et al., 2014). A 1:1 donor:recipient ratio (3.38×10^7 cells of each; density estimation as in Experiment 1) was used, except for treatment MP1 that consisted of 14 particles per filter, containing an unknown number of recipient cells on intact biofilms. Mating filters were incubated onto agar plates made with SLW at 20 °C for 72 h in dark conditions, followed by 4 °C for 48 h. In a second trial (Fig. S2), as recipient cells we used a suspension derived from biofilms associated to microplastics incubated for six weeks (MP2.II), and bacteria from lake water pre-filtered through a 200-µm mesh (L200) or a 12-µm filter (L12).

Donor (red) and transconjugant (green) microcolonies (objects larger than 7 μm^2) on mating filters (n = 3) with MP2 and FL were visualized using an Axio Imager Z1 fluorescence microscope equipped with a Plan-Apochromat 10x/0.45 M27 objective, a 10x eyepiece, AxioCamMR3 monochrome camera, and AxioVision software v4.9.1.0 (all from Zeiss). Red (mCherry) and green (GFP) fluorescence detection was based on excitation at 545/25 nm with emission at 605/70 nm, and excitation at 475/40 nm with emission at 530/50, respectively. ImageJ v1.49 software was used for image analysis of 40 randomly chosen microscopic fields of 0.6 mm^2 per image. Transfer frequencies on whole filters (triplicates) were calculated as in Klümper et al. (2014).

For cell isolation of transconjugants and recipients, mating filters or particles of the same treatment were pooled (Table S1) and vortexed in 15 ml Falcon tubes with 0.9% NaCl. The suspension from treatment MP1 was filtered by 12 µm. Transconjugants were separated using FACS, using a sequential gating procedure

as in the protocol by Klümper et al. (2014) with some modifications. Briefly, a first gate for size was set on a bivariate FSC-A vs SSC-A plot. The second gate was set on a bivariate FITC-A vs SSC-A plot for cells expressing green fluorescence. Finally, a third gate was set on a bivariate SSC-A vs. PE-Texas Red-A plot to exclude cells with red fluorescence (Fig. S3). Recipient cells (including transconjugants) were collected after gating first on a bivariate FSC-A vs SSC-A plot, followed by gating on a bivariate SSC-A vs. PE-Texas Red-A plot to exclude red fluorescence. Event rate was <20,000 e/sec and SSC threshold was set at 300. A first sort was performed in yield mode (≥20,000 events). Cells were then passed again through the instrument, with the same gating procedure and sorted using the purity mode. Cells were collected in 0.9% NaCl and centrifuged at 10,000 × g for 45 min at 4 °C. The resulting 20 μL pellets were stored at −80 °C for DNA extraction.

2.5. Molecular and sequence analyses

DNA was extracted from particles, filters and FACS-sorted cells, using the REDExtract-*N*-AmpTM Tissue PCR kit (Sigma). We amplified the V4 region of the 16S rRNA gene with primers 515F and 806R (Caporaso et al., 2011) and sequenced it with Illumina MiSeq technology. The sequence data was deposited at the NCBI Sequence Read Archive (BioProject PRJNA384132, BioSample accessions: SAMN06829022- SAMN06829051). The sequence reads were paired and quality filtered using MOTHUR 1.37.6 following the SOP tutorial (Kozich et al., 2013; Schloss et al., 2009). Subsequent processing included alignment against the SILVA v123 data set (Quast et al., 2012), pre-clustering (1 mismatch threshold), chimera removal with UCHIME (Edgar et al., 2011), and taxonomic classification. Sequences were assigned to OTUs using a split method based on taxonomy (Westcott and Schloss, 2015). For this step, sequences were clustered at the genus level and were then assigned to OTUs according to the Vsearch method with a 0.03 distance cut-off (Rognes et al., 2016). We further performed a manual curation using the RDP and SILVA reference databases, implemented in the SINA Alignment and Classify service (Pruesse et al., 2012).

2.6. Data and statistical analyses

Data processing, visualizations, and statistical analyses were performed in R 3.4.1 (R-Core-Team, 2017). Transconjugant to donor ratios (T:D) in all microcosms were calculated for each replicate and phase of each treatment. We used the Kruskal-Wallis non-parametrical test to compare bacterial growth and T:D ratios of treatment-phase combinations. A Mann-Whitney-Wilcoxon Test was used to compare T:D of water and particle phases within a treatment or to compare each of these to the T:D of the treatment with no microplastics. Mann-Whitney-Wilcoxon Test was used to compare the values of the transfer frequencies between water and biofilm communities in the multiple species matings. We used the Vegan package (Oksanen et al., 2016) to perform the nMDS ordinations, Permanova (adonis), pairwise adonis (with Benjamini and Hochberg adjustment), and Analysis of Multivariate Homogeneity of group dispersions on Hellinger-transformed data.

3. Results

3.1. Experiment 1: two-species microcosm

Plasmid transfer frequency in each microcosm was calculated as the ratio of *Pseudomonas* sp. cells that acquired the green-fluorescent plasmid (transconjugant cells) per *E. coli* donor cell (T:D ratio, Fig. 1A). Within each treatment, the T:D ratio was calculated for both microplastic particles (p), and the water phase (w). Ratios measured from bacteria on pure microplastics (+MPp, ratio: $8.2 \pm 9.0 \times 10^{-3}$, mean ± SD) were three orders of magnitude higher than those of bacteria in the surrounding water of the same treatment (+MPw, $2.5 \pm 2.9 \times 10^{-6}$), or bacteria from the treatment without microplastics (-MPw, $7.5 \pm 2.9 \times 10^{-6}$). These differences in transfer frequency were highly significant (Kruskal-Wallis, $H = 18.726$, $p = 0.002$, Fig. 2 and Table S2).

In the treatment with microplastics pre-incubated in a protein-rich medium, the ratio was higher on microplastic (+MPNp, $1.7 \pm 1.3 \times 10^{-2}$) than in the surrounding water (+MPNw, $3.8 \pm 4.8 \times 10^{-6}$) or in the water from the treatment without

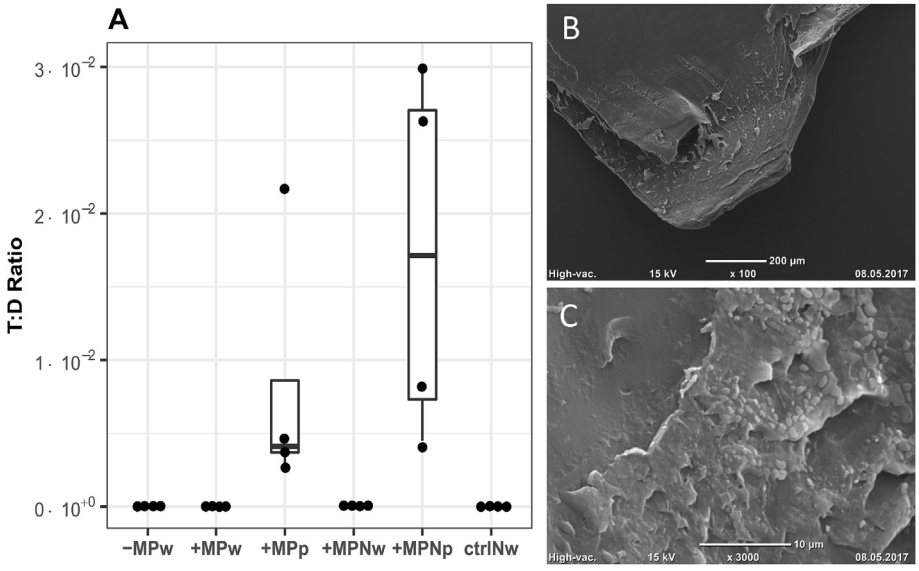

Fig. 2. Results of Two-species microcosm. A) Box plots and dots represent the Transconjugant to Donor ratios (T:D) from four independent flask replicates of bacteria in: i) water phase of treatments without microplastics (-MPw), ii) water and particle phases in treatments with microplastics (+MPw and +MPp), iii) water and particle phases in treatments with microplastics pre-treated with organic matter (+MPNw and +MPNp) and iv) water phase of the nutrient desorption control. SEM images of: B) Microplastics showing roughened edges and corners. C) Bacterial colonization of microplastics during the experiment in plastic from +MPN treatment.

microplastics. We did not detect any significant difference in the T:D ratios of the two treatments containing microplastics (Table S2); however, the approximate number of total cells (events gated in the FSC vs. SSC) detached from the organic matter-enriched particles was two times higher than from untreated particles (~2500 cells mL^{-1} and ~1200 cells mL^{-1}, respectively).

The proportion of events that were classified as donor cells using FCM (i.e., inside the donor cell gate) varied ~10 times between water ($40 \pm 2\%$) and particles ($4 \pm 0.2\%$). For all treatments and controls we observed similar increases in cell density in water (~30% increase in cells per mL) from the start to the end of the experiment, including the control of nutrient desorption (Kruskal-Wallis, $H = 0.89576$, $p = 0.83$). Finally, observations of microplastics with fluorescence microscopy confirmed the presence of transconjugants (Fig. S4), while scanning electron microscopy images indicated a patchy bacterial colonization mainly at the more roughened edges (Fig. 2B and C).

3.2. Experiment 2: multiple species mating

We performed standardized filter matings of natural bacteria from Lake Stechlin against a donor strain carrying the model plasmid pKJK5, and analyzed transfer frequencies by fluorescence microscopy. First, we compared microplastic-associated bacteria to the free-living community (Fig. 1B, MP2 and FL recipient communities respectively) and later, to communities including bacteria from natural organic matter aggregates (Fig. S1, L200 and L12).

Uptake frequency of plasmid pKJK5 by bacteria from microplastic biofilms (transconjugant colonies per initial recipient cell number) was two orders of magnitude higher (MP2, mean ± SD: $2.6 \pm 0.2 \times 10^{-4}$) than of free-living bacteria (FL, $3.0 \pm 1.3 \times 10^{-6}$, Fig. 3A). A difference of an order of magnitude was observed when comparing uptake frequencies of microplastic bacteria (MP2.II, $1.0 \pm 0.3 \times 10^{-4}$) with FL bacteria together with cells from aggregates of <200 μm and <12 μm (L200: $2.1 \pm 8.2 \times 10^{-5}$ and L12: $1.1 \pm 5 \times 10^{-5}$, respectively, Fig. 3B). Altogether, biofilm bacteria on microplastics presented higher permissiveness ($1.8 \pm 0.9 \times 10^{-4}$, MP2 + MP2II) than did bacteria from the surrounding water ($1.1 \pm 0.9 \times 10^{-5}$), irrespective of the bacterial size fraction tested (Mann-Whitney *U* Test, $W = 54$, $p = 0.0004$).

Transconjugants and associated recipient communities from MP1, MP2 and FL were sorted using FACS, and subsequently identified by 16S rRNA gene sequencing. The pool of transconjugants comprised 802 OTUs (97% sequence similarity) assigned to 16 major phylogenetic groups, of which Actinobacteria, Gammaproteobacteria and Betaproteobacteria were the most abundant, representing 41.9%, 33.9% and 14.9% of all sequences, respectively. We detected 34 main genera present in both microplastic-associated and free-living communities, comprising nearly 90% of all transconjugant sequences (Fig. 3C, Table S3). However, we observed that some genera, such as *Rheinheimera* displayed large differences in relative abundance between the two communities (0.65% and 37.4%, respectively).

Cluster differentiation observed in the multivariate analyses (Fig. 4) was consistent with results of the statistical tests, revealing significant differences (Permanova, $F = 12.17$, $df = 2$, $p = 0.001$) in bacterial composition of the three main clusters. Communities derived from the matings against *E. coli* comprised the first group. When analyzing community composition within this cluster, we also detected significant differences (Permanova, $F = 3.52$, $df = 1$, $p = 0.003$) between microplastic and free-living communities. The second cluster grouped samples from the natural free-living communities, which were dominated by members of Actinobacteria, Alphaproteobacteria, and Bacteroidetes. The third cluster consisted of the reference community of microplastic-associated bacteria, which was dominated by Bacteroidetes, Alphaproteobacteria, and Cyanobacteria (Fig. 3D, Table S4). Within the transconjugant bacteria, *Arthrobacter* (Actinobacteria) was the most abundant genus in both microplastic-associated and free-living communities, representing 53.9% and 36% of all sequences, respectively (Fig. 3C).

The relative abundances of major phylogenetic groups from MP2, MP1 and particles after mechanical detachment of biofilm (PD), show similarities between them, and more differences to FL (Table S4). Composition of reference communities after incubation (FL.F and MP2.F in Fig. 4), and an overview of sequences assigned to Bacteria are given in Tables S5 and Table S6, respectively.

4. Discussion

T:D ratios in water and microplastic-associated bacteria in the first experiment showed an increased frequency of recipients acquiring the plasmid on pure microplastic surfaces, with up to one transconjugant per 46 donor cells on the microplastics as compared to one transconjugant per 100,000 cells in the surrounding water. Notably, increased plasmid transfer occurred in the absence of selective pressure by antibiotics. This indicates that microplastics, as such, represent an artificial and persistent surface for bacterial colonization, development of intense interactions, and gene exchange via HGT. Furthermore, we observed that organic matter adsorption to microplastic particles also increased plasmid transfer frequencies, simulating expected natural activities under conditions of high dissolved organic carbon, as shown for natural organic matter aggregates (Grossart et al., 2003).

High transfer frequencies on microplastics occurred despite low initial densities of the donor strain compared to water. Moreover, the slow growth rate of bacteria in our medium suggests that the majority of transconjugants originated from single horizontal transfer events, rather than from vertical transmission of the plasmid during clonal expansion. The spatial differentiation observed in microbial particle colonization might resemble effects of increased weathering of plastic over time on HGT, since this material can suffer from physical and chemical abrasion, leading to patchy zones of biofilm colonization. This has been seen previously on the coarsened surfaces of prosthetic plastic implants (Ribeiro et al., 2012), and on microplastics collected in the environment (Carson et al., 2013).

In the second experiment, natural lake communities formed on microplastics were consistently more permissive to plasmid transfer than free-living bacteria, or bacteria on natural aggregates. For this experiment, we prevented differences in plasmid uptake related to dissimilarities in plasmid-donor invasiveness, by using the same surface matrix, and a low-nutrient medium. We also used high donor densities, to ensure maximized possible contact with potential recipient cells. Additionally, we standardized the initial number of recipient bacteria in matings with MP2 and FL, which allowed us to report transfer frequency independent of growth through microscopy (Klümper et al., 2014).

The broad range of aquatic bacterial taxa permissive to plasmids in microplastic-associated communities is consistent with previous results showing a high diversity of soil bacteria acquiring plasmids (Klümper et al., 2015, 2017; Musovic et al., 2006). Concentration of most of the transconjugant sequences in certain genera also support previous reports showing that plasmid transfer in soils is dominated by a core of super-permissive recipients (Klümper et al., 2015). Moreover, the community composition of aquatic bacteria associated with microplastics at high taxonomic levels that we observed was similar to the results of previous studies (De Tender et al., 2015; McCormick et al., 2014, 2016; Kesy et al., 2016).

We highlight that plasmid transfer from our *E. coli* donor strain to a phylogenetically distant bacterium such as *Arthrobacter*

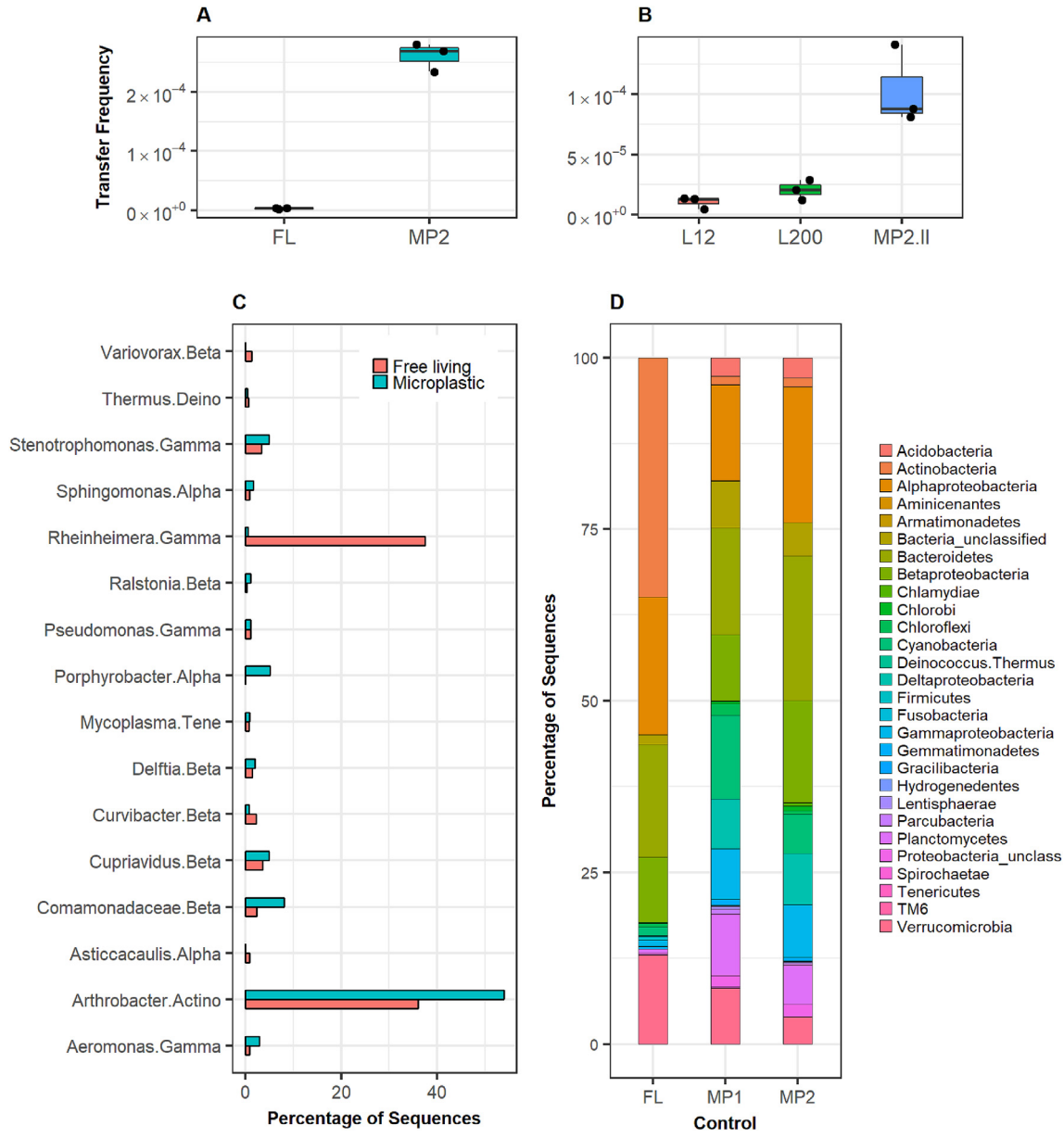

Fig. 3. Results of multiple species matings. Box plots and dots compare the frequency of transfer events from triplicate filter matings with A) free-living bacteria (FL) and microplastic-associated bacteria (MP2) and B) water fractions < 12 μm and <200 μm (L12 and L200, respectively) and microplastic-associated bacteria (MP2.II). C) Abundance distribution and taxonomy (genus and class) of the most abundant transconjugant sequences resulting from filter matings against free-living and microplastic-associated bacteria of Lake Stechlin. D) Overview on bacterial community composition of reference samples of free-living (FL), microplastic biofilm (MP1) and the suspension of microplastic biofilm (MP2) at the beginning of the experiment.

(Actinobacteria) can not only occur, but it can be a frequently occurring process within a natural aquatic community, as previously observed in terrestrial environments (Klümper et al., 2017; Musovic et al., 2006). The fact that most transconjugant sequences of this genus were assigned to a single OTU indicates the extremely high plasmid uptake capacity of this actinobacterial phylotype. The genus *Rheinheimera* (Gammaproteobacteria) has often been assigned as environmental bacteria, capable of forming biofilms, using a wide range of carbon substrates and producing pigments displaying antimicrobial activities (Grossart et al., 2009; Naz et al., 2016; Schuster and Szewzyk, 2016). In addition, *Rheinheimera* isolates obtained from sediments of a lake used for human drinking water were shown to grow on media supplemented with sulfamethoxazole-TMP-streptomycin (Czekalski et al., 2012). However, to our knowledge, ours is the first study to demonstrate the frequent occurrence of plasmid transfer events within this genus and to reveal the possible mechanism for acquisition of its antibiotic resistance profiles.

Overall, we show that a phylogenetically diverse core of natural aquatic bacteria is highly permissive towards acquisition of plasmid pKJK5. This can be seen in both microplastic-associated and free-living communities from the pelagic zone of Lake Stechlin and in the absence of any selective pressure, i.e., known exposure to antibiotics. Here, we demonstrate that bacterial permissiveness, also measured as plasmid transfer frequencies, is significantly greater on microplastics than in the surrounding water with or without cells from natural aggregates. This indicates that plastic biofilms provide favorable conditions for community interactions and hence

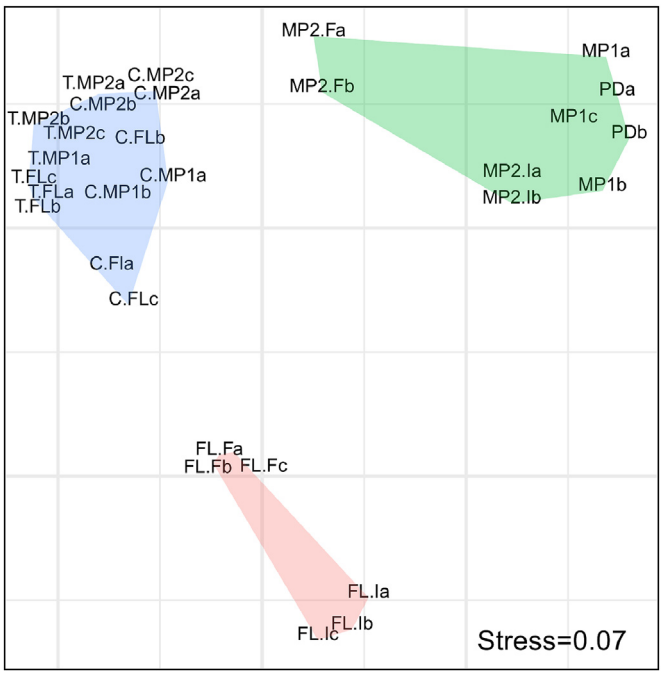

Fig. 4. Non-metric multidimensional scaling plot (nMDS) of samples analyzed by 16S rRNA gene metabarcoding. Samples include: FL = free-living bacteria, MP1 = biofilm on the microplastic particles, MP2 = suspension of microplastic biofilm bacteria, PD = particles post-detachment of MP2. Letters C and T before each sample type refer to the recipient community and transconjugant FACS-isolated bacterial cells from mating filters, respectively. Letters I and F refer to reference bacterial communities of reference samples at the beginning (I) and the end of the mating (F) incubations. Lower letters a, b and c represent replicates of each sample and/or community.

for plasmid acquisition, but it also indicates that permissive bacteria preferentially colonize microplastic biofilms in aquatic ecosystems. The exposure of communities to nutrients or metals has led to communities with increased plasmid transfer frequencies, without strong changes in the taxonomic composition of the transconjugant pools (Heuer et al., 2010; Klümper et al., 2017; McCormick et al., 2014; Smalla et al., 2015).

The combination of 1) a new surface with low degradability that allows for closer contact and thus plasmid conjugation (by a factor of up to 1000), and 2) the selection of more conjugation permissive bacteria (by a factor of up to 100 according to plasmid uptake determined in filter matings), could lead to an exponential (100,000-fold) increase in the transfer of antibiotic resistance genes in aquatic environments. Although this estimate is an oversimplification of conjugation rates in nature, our data support a reasonable hazard potential posed by microplastics.

An enhanced plasmid transfer might provide plasmids the opportunity to establish themselves in new hosts, triggering different evolutionary processes and increasing the capacity to occupy new ecological niches. As a result, a host-plasmid combination, including potential pathogens carrying plasmids that harbor antibiotic resistance genes, can persist in the long term (Madsen et al., 2016; Zhang et al., 2014), in particular when microplastics are present. Considering that plastic pollution in aquatic systems is increasing and may soon surpass the total fish biomass in the ocean (World Economic Forum and Ellen MacArthur Foundation, 2017), further studies on their colonization by bacteria and subsequent transfer of genetic elements are urgently required.

Many compartments of pelagic environments show cell aggregations and nutrient distributions that are favorable for increased gene transfer (Drudge and Warren, 2012). In our study, we observed a similar increase in transfer frequencies in matings when compared to communities with natural aggregates. However, it is important to emphasize that microplastics differ from natural particles in many aspects, especially with respect to their extremely low biodegradability, long-distance transport dynamics and accumulation, as well as their associated microbial community composition (Drudge and Warren, 2012; Kettner et al., 2017; Zettler et al., 2013).

Finally, our results imply that microplastic biofilms provide new hot spots for spreading antibiotic resistance genes by HGT in natural aquatic ecosystems. Tons of microplastics in sites like wastewater treatment plants, that get colonized by a multitude of microorganisms including pathogenic bacteria from humans or animals (Viršek et al., 2017; Ziajahromi et al., 2016), pose a tremendous potential for antibiotic resistance spreading by HGT. The high density and close physical contact between cells of biofilms facilitate bacterial conjugation and consequently the transfer of plasmids containing antibiotic resistance genes. We show that resistant strains in plastic biofilms frequently transfer resistance genes to a broad range of species. Effluents of wastewater treatment plants often flow into natural aquatic ecosystems, where some of the original pathogenic species may persist in the floating biofilm (McCormick et al., 2014). During the transit through these aquatic ecosystems, processes of horizontal and vertical gene transfer on the associated bacteria can occur continuously. Multiple encounters between the microplastics-associated bacterial community and various natural populations are likely given that plastic particles remain present in the environment for extremely long periods, resulting even in their transfer to the gut microbiota of organisms feeding on microplastics (Setälä et al., 2014).

5. Conclusions

This is the first report examining interactions between microplastic contaminants in aquatic ecosystems, their associated bacterial biofilms, and their horizontal transfer of antibiotic resistance genes. From different scientific and socio-economic perspectives, these results, together with previous observations of microplastic biofilm communities have profound implications. First, microplastics provide favorable conditions for the establishment of groups of microorganisms that differ from those in the surrounding water or on natural aggregates, thereby altering the structure and composition of microbial communities in aquatic environments. Second, on plastics, an increased permissiveness towards plasmids carrying antibiotic resistance genes and eventually other genes facilitates the establishment of novel traits in bacterial communities by evolutionary changes at the species and population levels. Finally, the high recalcitrance and often low density of microplastics provide ideal conditions for collection, transport and dispersion of microorganisms and their associated mobile genetic elements over long distances, which could even reach a global scale. This poses increasing but greatly neglected hazards to human health because pathogens can invade new localities and natural, non-pathogenic microorganisms can potentially acquire and thus rapidly spread antibiotic resistance.

This study highlights the magnitude and complexity of problems related to microplastic pollution are likely larger than previously thought. Our data supports the need for more research regarding the spread of mobile genetic elements on microplastics in the environment. It also raises serious concerns that the plastic-dependent lifestyle of modern societies causes tremendous and often unknown effects on aquatic ecosystems and the Earth more generally. The conclusions of our work highlight the need for a more responsible use of plastics by modern societies and demand for more stringent regulations for production, handling, and disposal of these long lasting materials.

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.02.058.

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