Microplastic pollution increases gene exchange in aquatic ecosystems

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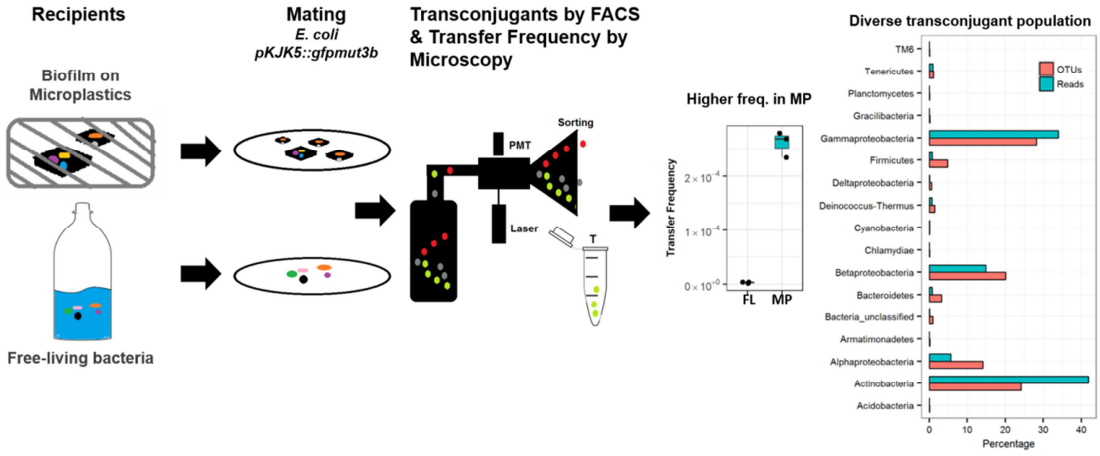
Abstract

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.

Capsule: Increased horizontal gene transfer via microplastic particles

Keywords: Microplastics, Aquatic Ecosystems, Biofilm, Horizontal Gene Transfer, Antibiotic Resistance

Graphical abstract



Highlights:

- Higher ratios of plasmid transfer in microplastic-associated bacteria than in free-living bacteria
- Gene exchange occurred in a broad range of phylogenetically-diverse bacteria
- Spread of antibiotic resistance through microplastics can affect aquatic ecology and evolution, but also human health

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-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.

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.

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, 10g/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 x 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.

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 x 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.

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

145 microplastics (-MP); b) with microplastics (+MP); c) with microplastics pre-soaked in
146 nutrient broth (+MPN) and d) a control for nutrient desorption (Ctrl Nutrient). We used 50
147 microplastic particles per microcosm in treatments b, c and d. Prior to the start of the
148 experiment, particles of the +MP treatment were incubated for three days in MQ water,
149 while in the +MPN treatment for three days in nutrient broth DEV (refer to the SI for
150 details) and then washed with MQ water. In the control for nutrient desorption,
151 microplastics were treated as in +MPN, incubated for additional 24 hours in filter-sterile
152 lake water, and then separated by decantation prior bacterial inoculation.

153 Each microcosm (four replicates per treatment) was inoculated with donor and
154 recipient suspension of $5x10^6$ cells mL^{-1} (D:R ratio = 1:1). We also included two controls
155 for contamination consisting of non-inoculated filtered lake water with and without
156 microplastics. The microcosms were incubated at 20°C for 72 h in dark conditions and
157 constant agitation at 150 rpm, followed by 4°C for 48 h, to allow proper folding of GFP
158 (Klümper *et al.*, 2014). Thereafter, MP particles were washed with 0.9% sterile NaCl
159 solution and five were preserved for confocal and scanning electron microscopy analysis,
160 while the rest (n=45) were vortexed for 1min in 1mL of sterile pyrophosphate (50 mM
161 $Na_4O_7P_2$) -Tween80 (0.05%) buffer solution for biofilm detachment. A sample of 10 mL of
162 water was taken from each flask with a sterile pipette.

163 Donor and transconjugant cells from the water (w) and particle (p) phases of each
164 replicate were analyzed by flow cytometry using a FACSAriaII instrument and BD
165 FACSDiva TM software v6 (Becton Dickinson Biosciences, San Jose, CA). The instrument
166 had a 488 nm laser (100mW) connected to a green fluorescent detector at 500-550 nm, and
167 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 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.

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, 25mm diameter (Whatman, UK) as described previously (Klümper *et al.*, 2014). A 1:1

191 donor:recipient ratio (3.38 x 10^7 cells of each; density estimation as in Experiment 1) was
192 used, except for treatment MP1 that consisted of 14 particles per filter, containing an
193 unknown number of recipient cells on intact biofilms. Mating filters were incubated onto
194 agar plates made with SLW at 20°C for 72 h in dark conditions, followed by 4°C for 48 h.
195 In a second trial (Fig. S2), as recipient cells we used a suspension derived from biofilms
196 associated to microplastics incubated for six weeks (MP2.II), and bacteria from lake water
197 pre-filtered through a 200-µm mesh (L200) or a 12-µm filter (L12).

198 Donor (red) and transconjugant (green) microcolonies (objects larger than 7 μm^2) on
199 mating filters (n=3) with MP2 and FL were visualized using an Axio Imager Z1
200 fluorescence microscope equipped with a Plan-Apochromat 10x/0.45 M27 objective, a 10x
201 eyepiece, AxioCamMR3 monochrome camera, and AxioVision software v4.9.1.0 (all from
202 Zeiss). Red (mCherry) and green (GFP) fluorescence detection was based on excitation at
203 545/25 nm with emission at 605/70 nm, and excitation at 475/40 nm with emission at
204 530/50, respectively. ImageJ v1.49 software was used for image analysis of 40 randomly
205 chosen microscopic fields of 0.6 mm^2 per image. Transfer frequencies on whole filters
206 (triplicates) were calculated as in Klümper *et al.* (2014).

207 For cell isolation of transconjugants and recipients, mating filters or particles of the
208 same treatment were pooled (Table S1) and vortexed in 15 ml Falcon tubes with 0.9%
209 NaCl. The suspension from treatment MP1 was filtered by 12 µm. Transconjugants were
210 separated using FACS, using a sequential gating procedure as in the protocol by Klümper et
211 al. (2014) with some modifications. Briefly, a first gate for size was set on a bivariate FSC-
212 A vs SSC-A plot. The second gate was set on a bivariate FITC-A vs SSC-A plot for cells
213 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 x g for 45 min at 4°C. The resulting 20 µL pellets were stored at -80°C for DNA extraction.

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).

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.

Results

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,

258 2.5±2.9 x 10^{-6}), or bacteria from the treatment without microplastics (-MPw, 7.5±2.9 x 10^{-6}). These differences in transfer frequency were highly significant (Kruskal-Wallis, H = 18.726, p = 0.002, Fig. 2 and Table S2).

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

268 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 ±2%) and particles (4 ±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 2C).

276 *Experiment 2: multiple species mating*

277 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-

280 living community (Fig. 1B, MP2 and FL recipient communities respectively) and later, to
281 communities including bacteria from natural organic matter aggregates (Fig.S1, L200 and
282 L12).

283 Uptake frequency of plasmid pKJK5 by bacteria from microplastic biofilms
284 (transconjugant colonies per initial recipient cell number) was two orders of magnitude
285 higher (MP2, mean ± SD: $2.6\pm0.2 \times 10^{-4}$) than of free-living bacteria (FL, $3.0\pm1.3 \times 10^{-6}$,
286 Fig. 3A). A difference of an order of magnitude was observed when comparing uptake
287 frequencies of microplastic bacteria (MP2.II, $1.0\pm0.3 \times 10^{-4}$) with FL bacteria together with
288 cells from aggregates of < 200 µm and < 12 µm (L200: $2.1\pm8.2 \times 10^{-5}$ and L12: $1.1\pm5 \times 10^{-5}$
289 , respectively, Fig. 3B). Altogether, biofilm bacteria on microplastics presented higher
290 permissiveness ($1.8\pm0.9 \times 10^{-4}$, MP2 + MP2II) than did bacteria from the surrounding
291 water ($1.1\pm0.9 \times 10^{-5}$), irrespective of the bacterial size fraction tested (Mann-Whitney U
292 Test, W = 54, p = 0.0004).

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

303 Cluster differentiation observed in the multivariate analyses (Fig. 4) was consistent
304 with results of the statistical tests, revealing significant differences (Permanova, F=12.17,
305 df=2, p = 0.001) in bacterial composition of the three main clusters. Communities derived
306 from the matings against *E. coli* comprised the first group. When analyzing community
307 composition within this cluster, we also detected significant differences (Permanova,
308 F=3.52, df=1, p = 0.003) between microplastic and free-living communities. The second
309 cluster grouped samples from the natural free-living communities, which were dominated
310 by members of Actinobacteria, Alphaproteobacteria, and Bacteroidetes. The third cluster
311 consisted of the reference community of microplastic-associated bacteria, which was
312 dominated by Bacteroidetes, Alphaproteobacteria, and Cyanobacteria (Fig. 3D, Table S4).
313 Within the transconjugant bacteria, *Arthrobacter* (Actinobacteria) was the most abundant
314 genus in both microplastic-associated and free-living communities, representing 53.9% and
315 36% of all sequences, respectively (Fig. 3C).

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

321 **Discussion**

322 T:D ratios in water and microplastic-associated bacteria in the first experiment showed an
323 increased frequency of recipients acquiring the plasmid on pure microplastic surfaces, with
324 up to one transconjugant per 46 donor cells on the microplastics as compared to one
325 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* (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 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 over-simplification 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

418 bacterial conjugation and consequently the transfer of plasmids containing antibiotic
419 resistance genes. We show that resistant strains in plastic biofilms frequently transfer
420 resistance genes to a broad range of species. Effluents of wastewater treatment plants often
421 flow into natural aquatic ecosystems, where some of the original pathogenic species may
422 persist in the floating biofilm (McCormick *et al.*, 2014). During the transit through these
423 aquatic ecosystems, processes of horizontal and vertical gene transfer on the associated
424 bacteria can occur continuously. Multiple encounters between the microplastics-associated
425 bacterial community and various natural populations are likely given that plastic particles
426 remain present in the environment for extremely long periods, resulting even in their
427 transfer to the gut microbiota of organisms feeding on microplastics (Setälä *et al.*, 2014).

428

429 **Conclusions**

430 This is the first report examining interactions between microplastic contaminants in
431 aquatic ecosystems, their associated bacterial biofilms, and their horizontal transfer of
432 antibiotic resistance genes. From different scientific and socio-economic perspectives, these
433 results, together with previous observations of microplastic biofilm communities have
434 profound implications. First, microplastics provide favorable conditions for the
435 establishment of groups of microorganisms that differ from those in the surrounding water
436 or on natural aggregates, thereby altering the structure and composition of microbial
437 communities in aquatic environments. Second, on plastics, an increased permissiveness
438 towards plasmids carrying antibiotic resistance genes and eventually other genes facilitates
439 the establishment of novel traits in bacterial communities by evolutionary changes at the
440 species and population levels. Finally, the high recalcitrance and often low density of

441 microplastics provide ideal conditions for collection, transport and dispersion of
442 microorganisms and their associated mobile genetic elements over long distances, which
443 could even reach a global scale. This poses increasing but greatly neglected hazards to
444 human health because pathogens can invade new localities and natural, non-pathogenic
445 microorganisms can potentially acquire and thus rapidly spread antibiotic resistance.

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

454

455 **Acknowledgments**

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462

Conflict of interest

The authors declare no competing financial interests.

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603

Figure captions

Figure 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.

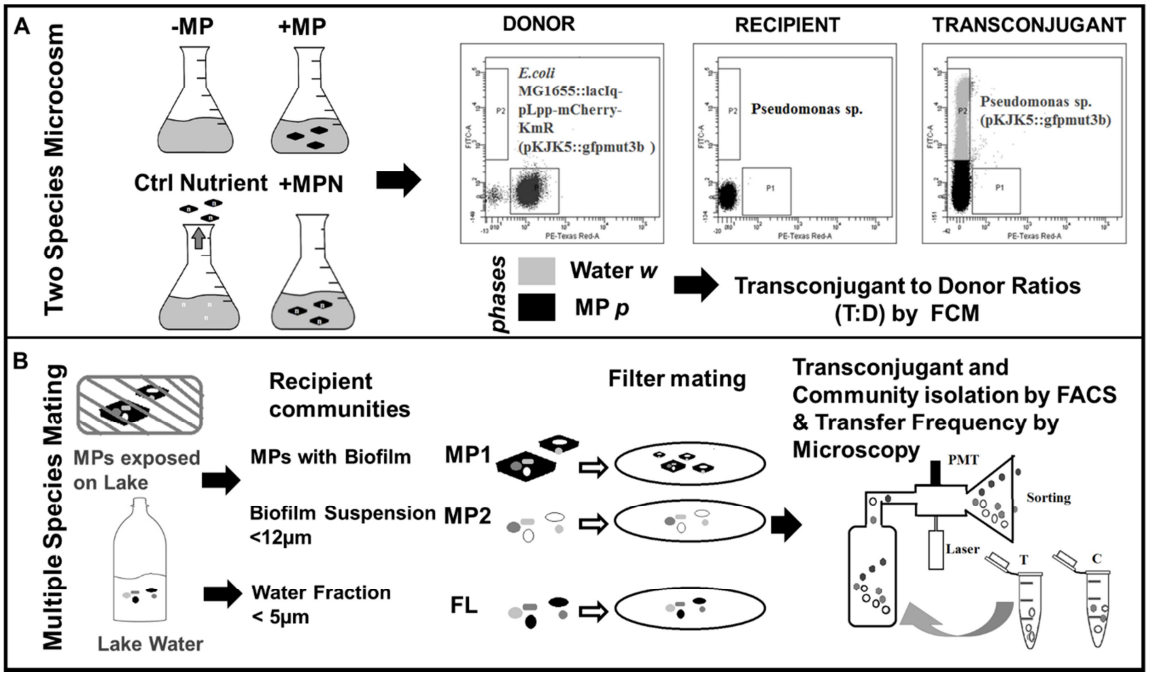
Figure 2. Results of Two-species microcosm. A) Box plots and dots represent the Transconjugant to Donor ratios (T:D) from four independent flask replicates of bacteria in: i) water phase of treatments without microplastics (-MPw), ii) water and particle phases in treatments with microplastics (+MPw and +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.

Figure 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 < 12µm 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.

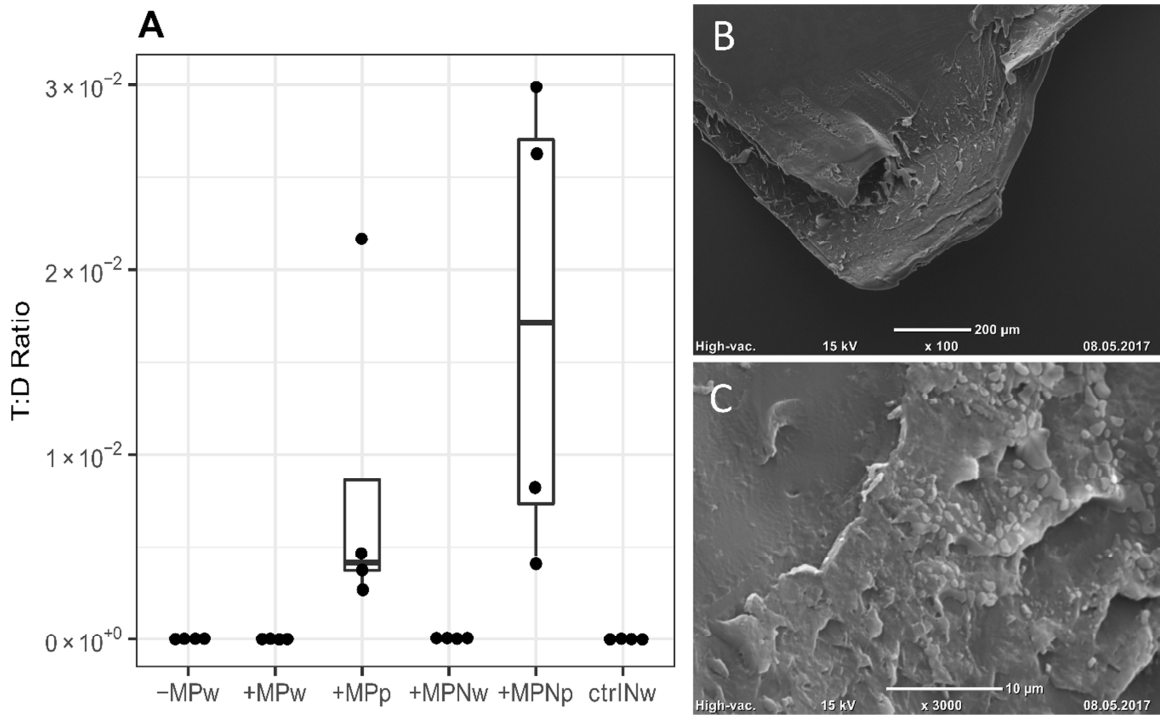
Figure 4. Non-metric multidimensional scaling plot (nMDS) of samples analyzed by 16S rRNA gene metabarcoding. Samples include: FL= free-living bacteria, MP1= biofilm on the microplastic particles, MP2=suspension of microplastic biofilm bacteria, PD= particles post-detachment of MP2. Letters C and T before 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.

Figure 1


A
Two Species Microcosm
-MP
+MP
Ctrl Nutrient
+MPN
DONOR
RECIPIENT
TRANSCONJUGANT
E.coli MG1655::lacIq-pLpp-mCherry-KmR (pKJK5::gfpmut3b)
Pseudomonas sp.
Pseudomonas sp. (pKJK5::gfpmut3b)
phases
Water w
MP p
Transconjugant to Donor Ratios (T:D) by FCM
B
Multiple Species Mating
MPs exposed on Lake
Lake Water
Recipient communities
MPs with Biofilm
Biofilm Suspension <12µm
Water Fraction < 5µm
Filter mating
MP1
MP2
FL
Transconjugant and Community isolation by FACS & Transfer Frequency by Microscopy
PMT
Sorting
Laser
T
C

652 **Figure 2**

653



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655

656

Figure 3

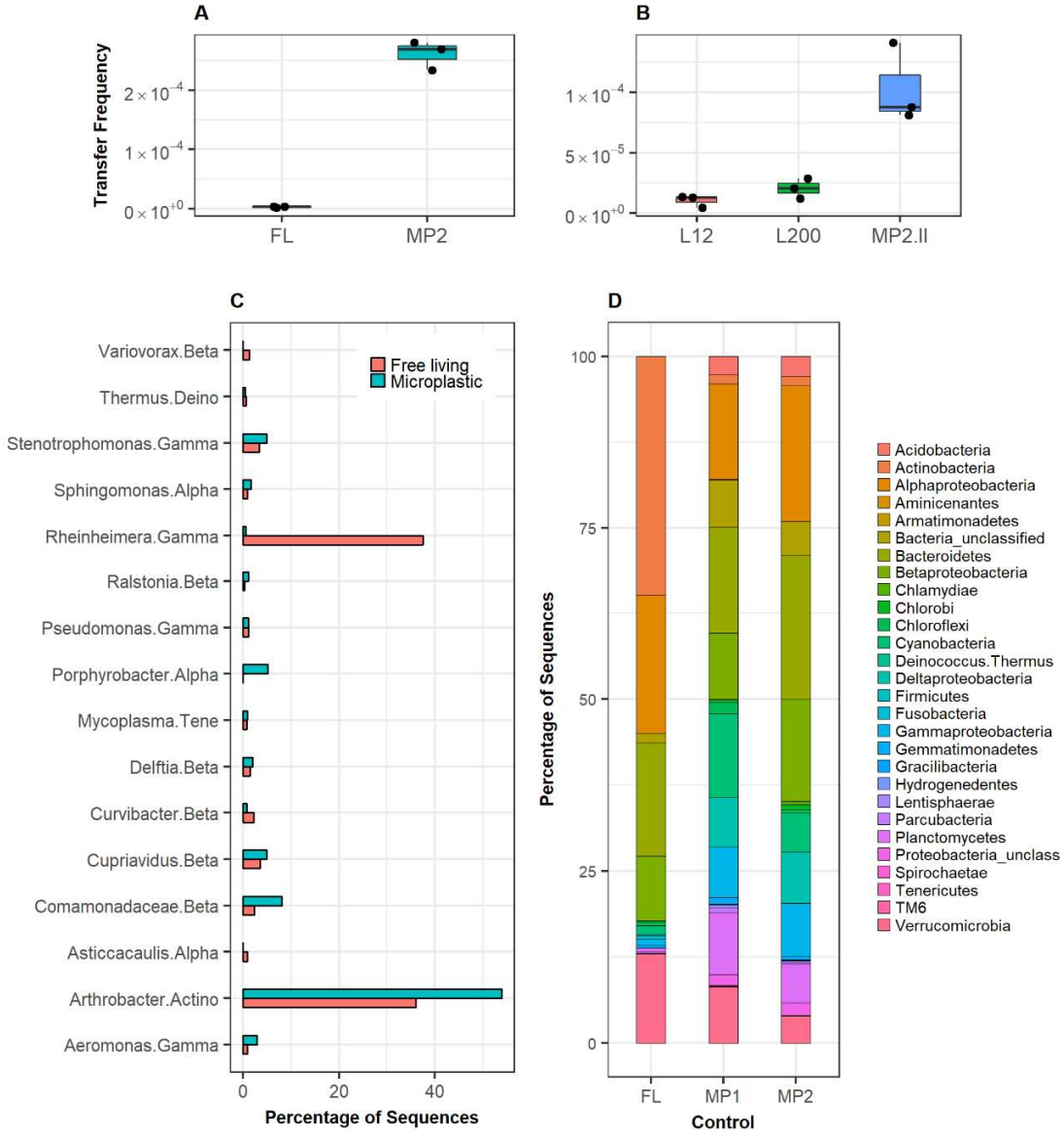
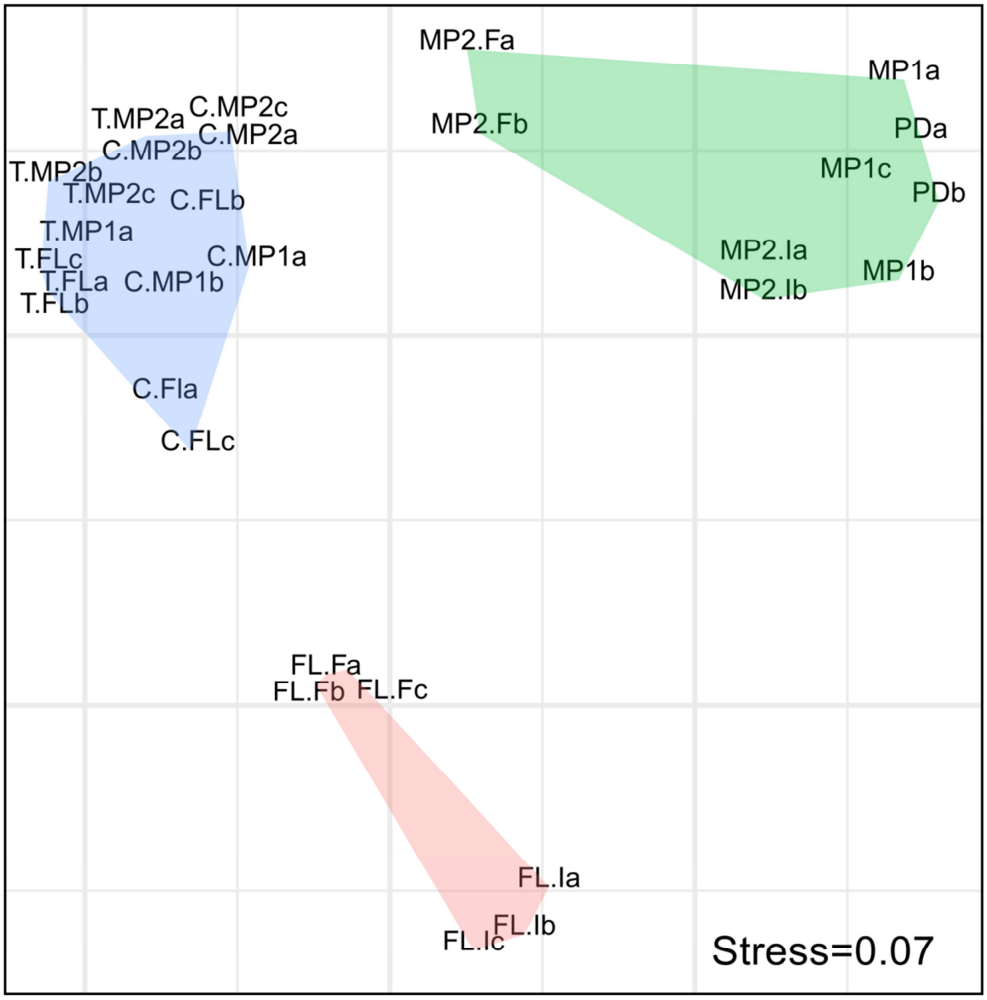

A
Transfer Frequency
FL
MP2
B
L12
L200
MP2.II
C
Free living
Microplastic
Variovorax.Beta
Thermus.Deino
Stenotrophomonas.Gamma
Sphingomonas.Alpha
Rheinheimera.Gamma
Ralstonia.Beta
Pseudomonas.Gamma
Porphyrobacter.Alpha
Mycoplasma.Tene
Delftia.Beta
Curvibacter.Beta
Cupriavidus.Beta
Comamonadaceae.Beta
Asticcacaulis.Alpha
Arthrobacter.Actino
Aeromonas.Gamma
Percentage of Sequences
D
Percentage of Sequences
FL
MP1
MP2
Control
Acidobacteria
Actinobacteria
Alphaproteobacteria
Aminicenantes
Armatimonadetes
Bacteria_unclassified
Bacteroidetes
Betaproteobacteria
Chlamydiae
Chlorobi
Chloroflexi
Cyanobacteria
Deinococcus.Thermus
Deltaproteobacteria
Firmicutes
Fusobacteria
Gammaproteobacteria
Gemmatimonadetes
Gracilibacteria
Hydrogenedentes
Lentisphaerae
Parcubacteria
Planctomycetes
Proteobacteria_unclass
Spirochaetae
Tenericutes
TM6
Verrucomicrobia

Figure 4


MP2.Fa
MP1a
MP2.Fb
PDa
MP1c
PDb
MP2.Ia
MP1b
MP2.Ib
T.MP2a
C.MP2c
C.MP2a
C.MP2b
T.MP2b
T.MP2c
C.FLb
T.MP1a
T.FLc
C.MP1a
T.FLa
C.MP1b
T.FLb
C.Fla
C.FLc
FL.Fa
FL.Fb
FL.Fc
FL.Ia
FL.Ib
FL.Ic
Stress=0.07

664 **Supplementary information**

665 **Microplastic pollution increases gene exchange in aquatic ecosystems**

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687

SI Materials and Methods

1. Materials and strain growth conditions

- Reagent providers: Meat Peptone (Carl Roth), Meat Extract (Sigma-Aldrich), NaCl (ChemSolute, TH Geyer), Tryptone (Carl Roth), Yeast Extract (MP Biomedicals), Sodium, Sodium Pyrophosphate Tetrabasic Decahydrate (Fluka Analytical, Sigma-Aldrich), Tween 80 (Carl Roth). Antibiotics were provided by Sigma-Aldrich.
- Stechlin Lake Water media (SLW) for the two-species microcosm (experiment one) and for the agar plates in the multispecies filter matings (experiment two) was prepared by filtering water from Lake Stechlin with GF/F filter and a 0.2 µm filter in a pre-combusted (to eliminate residual organic matter) glass bottle, under 400 mbar pressure. Dissolved organic carbon (DOC) in the lake water after filtration (SLW) - used for the Two-species microcosm - was 3.8 mg/L. SLW plates used for the Multispecies mating, contained 2% agar, which was autoclaved and distributed in 47 cm petri dishes. Plates were kept at 4°C until the next day for the mating procedure. One mating filter was placed per filter.
- Strain growth in Two-species microcosm: strains were grown in 2 mL of DEV, with antibiotics (for strains carrying plasmid) or without (*Pseudomonas* sp.), at 30°C for 4 h. The cultures were transferred (1:40) to 20 mL of fresh media and incubated overnight at 30°C and 100 rpm. A second transfer (1:20) was made in 50 mL of media, and cultures were incubated for 5 hours at 30°C and 100 rpm.
- Strain growth in Multispecies mating: strains were grown on 2 mL of LB, with antibiotics and/or IPTG, at 37°C for 4 h. The culture was transferred (1:40) to fresh medium and incubated overnight at 30°C and 100 rpm.

2. *Treatment of microplastic particles:*

- Cleaning procedure: Approximately 100 MP were placed in 15 mL sterile falcon tubes with 3 mL of 70% Ethanol (HPLC grade Ethanol and autoclaved MQ water) for about 1 h for disinfection. Plastic was then washed in 3 mL of MQ water, and finally manually agitated and vortexed for 1 min in 3 mL of 3% H2O2 solution.
- For the Two-species microcosm, the plastic was placed for 3 days either in 5 mL of Nutrient Broth (DEV) or MQ water. After this period, plastic pieces were washed 2 times with MQ water. MQ water was added to the tubes with particles and allowed to rest at room temperature for 5 hours. After this period, the plastic was washed again, water was removed with a pipette, and particles were stored overnight at 4°C. Four replicates containing 50 plastic pieces previously incubated on nutrient rich media were placed in flasks with 100 mL of SLW for 24 h in the dark at 20°C (later used as Nutrient control).
- Scanning electron microscopy of microplastics from the microcosms was performed with a JEOL-6000 instrument. Samples were prepared by 60 sec sputter time with Gold Palladium.

3. *Transfer frequency determined by microscopy*

Image J 1.49v software was used for image analysis. The functions “contrast enhancement” and “background subtraction” were used in each image, and objects larger than 7 μm^2 were manually counted, based on optimization experiments and the protocol by Klümper et al. (2014). The same procedure was performed for control filters of only either MP2 or FL. For each replicate and treatment combination, the green fluorescent objects were averaged. The average count of fluorescent objects measured in the control particles or filters of MP2 and

734 FL was subtracted. The transfer frequencies calculations for the whole filters were done as
735 in Klümper et al. (2014):

736 Transfer frequency = Transconjugant events per picture * filter area (μm^2) / picture area
737 (μm^2) * recipients introduced originally

738 *4. FCM and FACS*

739 *Instrument Set Up*

740 A 70 µm nozzle was used at a sheath fluid pressure of 70 psi. Prior to measurement of
741 experimental samples, the proper functioning of the instrument was checked by using the
742 cytometer setup and tracking module (CS&T) with CS&T beads (Becton Dickinson).
743 Before the isolation of cells by FACS, a decontamination procedure for aseptic sorting was
744 followed as described in BD FACSAriaII User´s Guide p187, including the exchange of the
745 0.2 µm filter unit. The following voltages (V) were used during analysis:

Detector	Experiment 1	Experiment 2
SSC-A	248	300
FSC-A	320	500
BP filter 525/50 nm	536	508
BP filter 610/20 nm	356	500

746

747 *Nucleic acid manipulation and sequencing*

748 DNA was extracted from the particles (25 particles per tube), filters (one per tube), and
749 FACS-sorted cells (approx. 20 µL per tube; Table S4), using the REDExtract-N-AmpTM
750 Tissue PCR kit (Sigma). For MPs and filters, we used the recommended protocol for
751 tissues, while for the sorted cells we used the protocol recommended for saliva. DNA
752 concentration was determined using a Quantus™ Fluorometer and stored at 4°C for further

753 processing. The reaction mix for the amplification of the V4 region of the 16S rRNA gene
754 was prepared in a total volume of 50 µl containing MyTaq Red DNA Polymerase
755 (BIOLINE, Germany) and 10 ng of template DNA. It was performed with the following
756 protocol: an initial denaturation step at 94°C for 2 min, followed by 35 cycles of
757 denaturation at 94°C for 40 sec, annealing at 50°C for 40 sec, extension at 72°C for 1 min,
758 and a 5 min final extension at 72°C. The amplicons were checked in a 1% agarose gel, and
759 then sent for paired-end sequencing by Illumina MiSeq technology.

760

SI Figures and tables

Figure S1

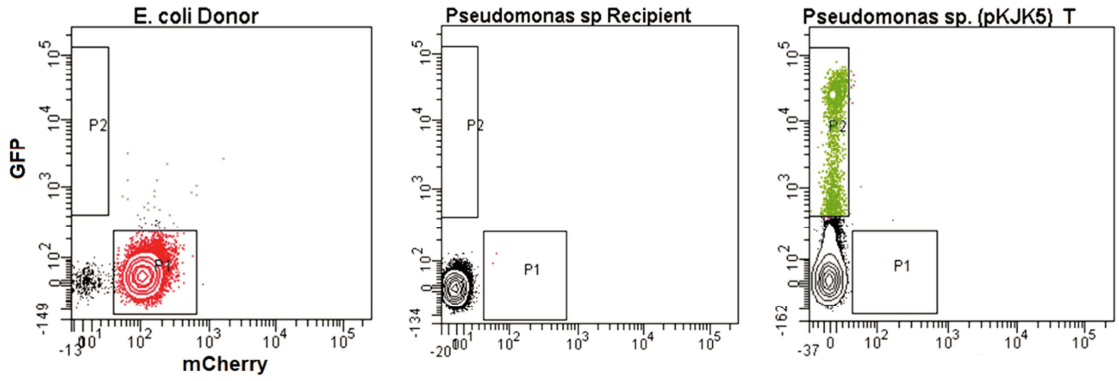
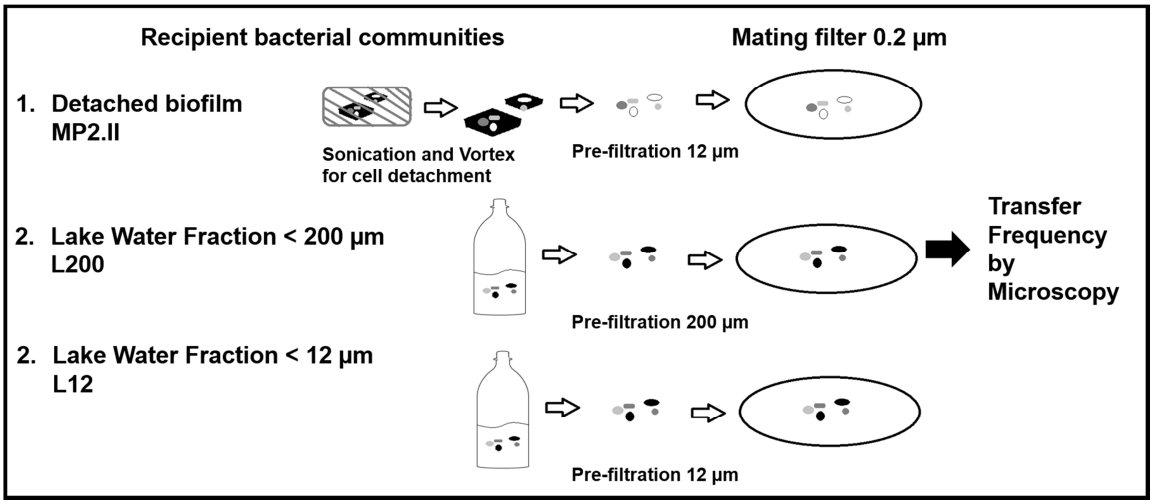


Figure S1. Contour plots in the FCM analysis of the two-species microcosm show the fluorescence pattern (from left to right) from *E. coli* donor strain expressing *mCherry*, *Pseudomonas* sp. recipient strain with no fluorescent expression and *Pseudomonas* sp. after plasmid pKJK5::*gfpmut3* acquisition. Gates for donor strain (P1) and transconjugant (P2) detection are indicated on the plots and events depicted with red and green color, respectively. Plots represent 20 000 events. X-axis indicates mCherry (FITC-A in original plot) and Y-axis indicates GFP expression (PE-Texas Red-A in original plot).

774 **Figure S2**



775

776 **Figure S2**. As part of experiment two, transfer frequencies were determined by microscopy
777 for matings of the donor with recipient bacteria originated from microplastic biofilms
778 (MP2.II) and bacteria from lake water pre-filtered by 12µm filter (L12) or 200µm mesh
779 (L200).

Figure S3

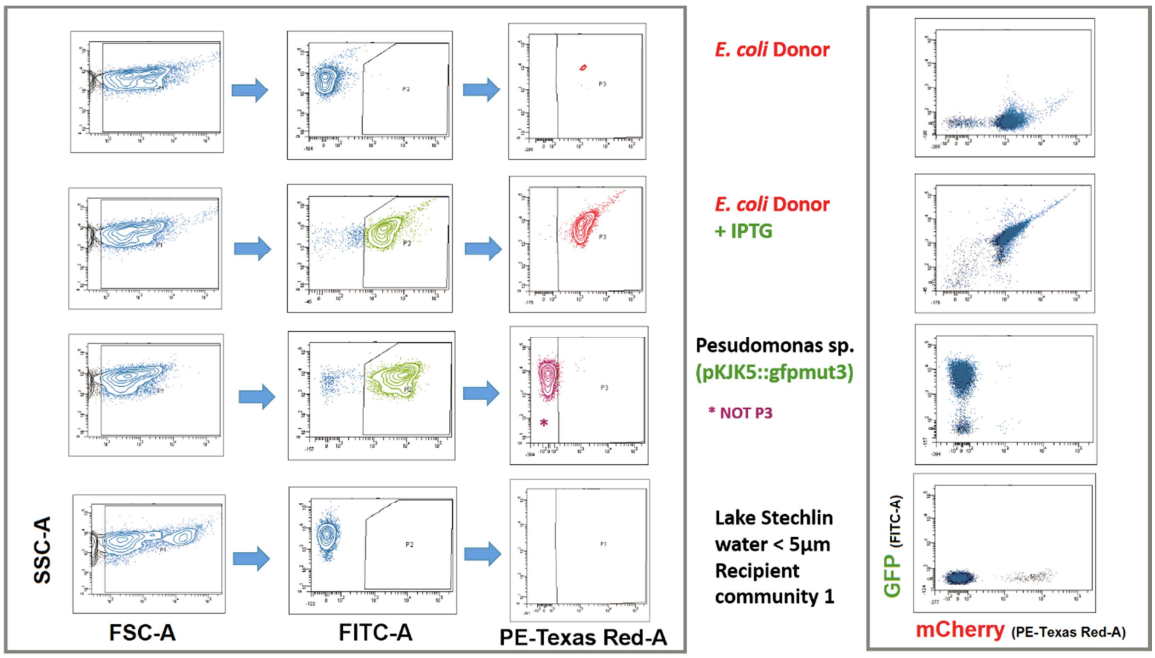


Figure S3. Contour plots on the left exemplify the triple gating procedure for transconjugant FACS-isolation in experiment two. A gate (P1) was set on a bivariate FSC-A vs SSC-A plot for bacterial events using the *E. coli* strain. A second subsequent gate was set on a bivariate FITC-A vs SSC-A plot including events from the *E. coli* strain grown with IPTG and the *Pseudomonas* sp. strain with plasmid pKJK5::*gfpmut3* (both expressing *gfp*, the first one expressing *mCherry*). Then a third gate (P3) was set on a bivariate FSC-A vs. PE-Texas Red-A plot to include all events from the *E. coli* donor grown with IPTG (expressing *mCherry* and *gfp*) and a NOT-P3 gate in the same plot included all events from the *Pseudomonas* sp. with plasmid pKJK5::*gfpmut3* (transconjugants). Contour plots on the right show the GFP vs. mCherry (FITC-A vs. PE-Texas Red-A on original plots) expression pattern of each sample. Plots represent 10 000 events.

Figure S4

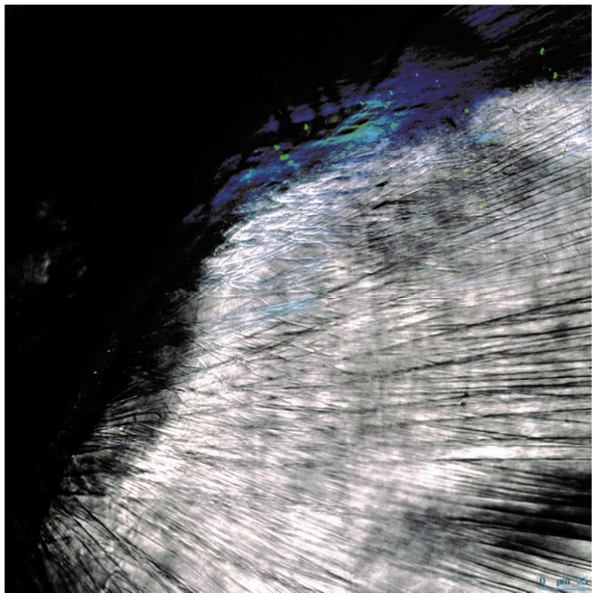


Figure S4. Image of a microplastic biofilm from the two-species microcosm obtained by confocal laser microscopy. Expression of *gfp* in transconjugants is shown in green. Biofilm on the plastic piece was stained with DAPI stain (in blue).

800 **Table S1.** Summary of FACS sorted events and filter pooling in Multiple Species
801 Experiment.

Sample	Replicate	No. mating filters Pooled	No. events sorted Gate NOT P3	No. events sorted Gate NOT P4
Mating with MP2	1	2	16 085	50 000
	2	2	15 044	50 000
	3	2	18 071	28 394
Mating with FL	1	4	30 000	121 1386
	2	4	30 000	115 704
	3	4	23 037	126 135
Mating with MP1	1	16[a]	4040[b]	73 127
	2	16[a]	1651[b]	43 346

802 [a] indicate 16 filters with ca. 14 particles each. The letter [b] indicates that these two parallels were combined in
803 one tube for centrifugation and further DNA extraction. Gate NOT P3 was used for isolation of
804 Transconjugants and Gate NOT P4 for Community Cells.

805

Table S2. Transconjugant to Donor Ratios (T:D) in the Two-Species Microcosm.

Treatment or Ctrl	-MPw[a]	+MPw	+MPp	+MPNw	+MPNp	Crtl Nw[a]
Phase	water	water	particle	water	particle	water
Replicate 1	1.00×10^{-5}	5.00×10^{-6}	2.17×10^{-2}	0	3.02×10^{-2}	0.00×10^{-6}
Replicate 2	5.00×10^{-6}	0	4.25×10^{-3}	0	4.52×10^{-3}	0.00×10^{-6}
Replicate 3	1.00×10^{-5}	5.00×10^{-6}	4.00×10^{-3}	5.00×10^{-6}	8.26×10^{-3}	0.00×10^{-6}
Replicate 4	5.00×10^{-6}	0	2.85×10^{-3}	1.00×10^{-5}	2.60×10^{-2}	0.00×10^{-6}
Mean ± SD	$7.50 \pm 2.89 \times 10^{-6}$	$2.50 \pm 2.89 \times 10^{-6}$	$8.20 \pm 9.02 \times 10^{-3}$	$3.75 \pm 4.79 \times 10^{-6}$	$1.72 \pm 1.27 \times 10^{-2}$	$1.25 \pm 2.50 \times 10^{-6}$

The letter [a] indicates microplastics were not present in the flask of the respective treatment or control after bacteria inoculation. Treatments or Ctrl are i) water phase of treatments without microplastics (-MPw), ii) water and particle phases in treatments with microplastics (+MPw and +MPp, respectively), iii) water and particle phases in treatments with microplastics pre-treated with organic matter (+MPNw and +MPNp, respectively) and iv) water phase of the nutrient desorption control (Crtl Nw). A cero indicates there were not events in the transconjugant gate after measuring at least 200,000 donor events during the FCM analysis.

Table S3. Transconjugant genera detected on both FL and MP2. Numbers indicate the relative abundances (%) of sequences of this genera in the transconjugant (T) and recipient communities (C) isolated by FACS, and in filters of reference samples (I), from FL, MP1 and MP2.

	FL			MP1			MP2		
Phylum - *Genera*	**T**	**C**	**I**	**T**	**C**	**I**	**T**	**C**	**I**
Actinobacteria									
Acaricomes	0.21	0.00	0.00	0.28	0.45	0.04	0.32	0.01	0.00
Arthrobacter	35.99	35.11	0.89	48.79	46.25	0.65	53.86	49.06	0.91
Renibacterium	0.02	0.06	0.00	0.14	0.03	0.00	0.06	0.01	0.00
Rhodococcus	0.17	0.03	0.02	0.71	0.11	0.00	0.06	0.13	0.00
Alphaproteobacteria									
Aminobacter	0.25	0.28	0.02	0.57	0.36	0.00	0.76	0.75	0.00
Bradyrhizobium	0.27	0.09	0.00	0.43	0.03	0.00	0.19	0.18	0.00
Caulobacter	0.29	0.12	0.03	0.71	0.64	0.16	0.51	0.73	0.29
Methylobacterium	0.23	0.09	0.05	0.14	0.25	0.02	0.32	0.13	0.07
Sphingomonas	0.99	6.20	0.02	3.97	1.03	0.07	1.71	1.54	0.15
Betaproteobacteria									
Aquabacterium	0.19	0.15	0.03	0.28	0.73	0.43	0.13	0.21	0.54
Comamonas	0.08	0.09	0.02	0.00	0.03	0.00	0.06	0.03	0.15
Cupriavidus	3.67	3.01	0.12	2.70	2.66	0.00	4.99	6.22	0.07
Curvibacter	2.24	3.16	0.03	5.25	2.63	0.02	0.88	1.00	0.04
Delftia	1.49	1.60	0.02	2.13	1.84	0.00	2.02	2.32	0.00
Hydrogenophaga	0.02	0.21	0.00	0.00	0.25	0.02	0.25	0.10	0.07
Pelomonas	0.15	0.03	0.00	0.28	0.11	0.00	0.25	0.04	0.04
Ralstonia	0.33	0.31	0.02	0.57	0.45	0.00	1.20	0.51	0.00
Undibacterium	0.25	0.34	0.03	0.00	0.36	0.02	0.06	0.03	0.07
Gammaproteobacteria									
Acidibacter	0.10	0.18	0.64	0.28	0.14	0.34	0.19	0.16	0.00
Acinetobacter	0.10	1.01	0.00	0.14	0.28	0.00	0.32	0.01	0.00
Aeromonas	0.93	3.22	0.02	0.85	1.51	0.02	2.97	0.15	0.36
Coxiella	0.04	0.12	0.00	0.00	0.00	0.02	0.06	0.06	0.07
Escherichia-Shigella	0.02	12.28	0.03	0.00	1.09	0.00	0.06	5.25	0.00
Halomonas	0.04	0.00	0.00	0.14	0.00	0.09	0.06	0.03	0.11
Pseudomonas	1.12	2.21	0.03	1.42	4.81	0.02	1.14	0.39	0.00
Rheinheimera	37.44	4.63	0.02	8.94	10.40	0.02	0.63	0.01	0.15
Shewanella	0.06	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.07
Stenotrophomonas	3.44	2.33	0.05	5.25	2.66	0.02	4.99	4.66	0.00
Bacteroidetes									

Hydrotalea	0.02	0.00	0.00	0.00	0.00	0.02	0.06	0.03	0.15
Sediminibacterium	0.17	0.09	3.80	0.14	0.11	0.04	0.19	0.30	0.47
Deinococcus-Thermus									
Thermus	0.68	0.55	0.02	1.13	0.78	0.00	0.51	0.87	0.00
Firmicutes									
Atopostipes	0.02	0.03	0.00	0.14	0.00	0.00	0.06	0.01	0.00
Streptococcus	0.02	0.00	0.02	0.00	0.08	0.00	0.13	0.00	0.00
Tenericutes									
Mycoplasma	0.79	1.93	0.02	1.84	0.73	0.00	0.95	4.95	0.00
No. Replicates	3	3	3	1	2	3	3	3	2
Relative Abundance	91.8 (4827)	79.4 (4329)	5.9 (5979)	87.2 (705)	80.8 (3578)	2.0 (4454)	80.0 (1582)	79.9 (8186)	3.8 (2756)

819

820

821 **Table S4.** Relative abundances (%) of major phylogenetic groups in the reference
822 bacterial communities at the beginning of the mating experiment (I).

Phylogenetic Group	FL	MP2	MP1	PD
Acidobacteria	0.00	2.98	2.65	5.55
Actinobacteria	**34.87**	1.34	1.26	0.72
Aminicenantes	0.00	0.00	0.02	0.00
Armatimonadetes	0.02	0.00	0.07	0.05
Bacteria_unclassified	1.40	4.90	6.89	7.34
Bacteroidetes	**16.42**	**21.08**	**15.56**	**19.10**
Chlamydiae	0.00	0.44	0.09	0.03
Chlorobi	0.15	0.73	0.36	0.33
Chloroflexi	0.50	0.44	1.62	1.03
Cyanobacteria	1.27	5.73	**12.26**	**12.94**
Deinococcus-Thermus	0.02	0.07	0.00	0.03
Elusimicrobia	0.00	0.00	0.00	0.06
Firmicutes	0.40	0.15	0.02	0.09
Fusobacteria	0.00	0.00	0.04	0.02
Gemmatimonadetes	0.33	0.58	0.94	0.98
Gracilibacteria	0.02	0.04	0.04	0.00
Hydrogenedentes	0.00	0.11	0.11	0.09
Latescibacteria	0.00	0.00	0.00	0.02
Lentisphaerae	0.00	0.25	0.38	0.12
Microgenomates	0.00	0.00	0.00	0.02
Parcubacteria	0.02	0.25	0.72	0.42
Planctomycetes	0.57	5.62	8.98	7.28
Alphaproteobacteria	**20.05**	**19.70**	**13.92**	12.09
Betaproteobacteria	9.48	**14.88**	9.61	6.99
Deltaproteobacteria	0.20	7.33	7.18	3.92
Gammaproteobacteria	1.02	7.58	7.27	6.92
Proteobacteria_unclassified	0.18	1.78	1.59	1.40
Spirochaetae	0.03	0.11	0.18	0.06
TA06	0.00	0.00	0.00	0.02
Tenericutes	0.02	0.00	0.02	0.00
TM6	0.05	0.04	0.02	0.05
Verrucomicrobia	12.96	3.88	8.17	**12.34**
No. Replicates	3 filters	2 filters	3 x 50 particles	2 x 50 particles
No. sequences	5979	2756	4454	6428

823 FL= water fraction of <5µm; MP2= suspension of the biofilm after detachment of microplastic and filtration
824 by 12µm; MP1= biofilm directly on microplastic; PD= biofilm left in particles after detachment procedure to
825 produce MP2. In **bold** the 3 groups with higher relative abundances within each sample type

826

827 **Table S5.** Relative abundance of major phylogenetic groups on reference communities
828 of FL and MP2 at the end of the mating experiment (F).

Phylogenetic Group	FL	MP2
Acidobacteria	0.00	0.02
Actinobacteria	11.73	2.39
Alphaproteobacteria	**21.76**	**17.66**
Armatimonadetes	0.01	0.05
Bacteria_unclassified	1.16	0.62
Bacteroidetes	3.43	5.71
Betaproteobacteria	**22.47**	**10.34**
Chlamydiae	0.00	0.02
Chlorobi	0.13	0.05
Chloroflexi	0.09	0.00
Cyanobacteria	0.42	0.62
Deinococcus-Thermus	0.00	0.02
Deltaproteobacteria	0.04	0.05
Firmicutes	0.01	0.07
Fusobacteria	0.00	0.02
Gammaproteobacteria	**35.85**	**59.25**
Gemmatimonadetes	0.08	0.00
Hydrogenedentes	0.00	0.02
Lentisphaerae	0.00	0.02
Microgenomates	0.00	0.02
Parcubacteria	0.01	0.02
Planctomycetes	0.17	2.14
Proteobacteria_unclassified	0.41	0.52
Spirochaetae	0.00	0.02
Verrucomicrobia	2.20	0.32
No. Replicates	3 filters	2 filters
No. sequences	7578	4061

829 FL= water fraction of <5µm; MP2= suspension of the biofilm after detachment of microplastics and
830 filtration through 12 µm. Filters of reference samples after incubation were saved for DNA extraction to
831 assess changes in general bacterial community composition due to incubation conditions. In **bold** the 3
832 groups with higher relative abundances within each sample type.

833

834 **Table S6.** Overview of OTUs and sequences assigned to Bacteria after Illumina sequencing

835 on experiment two

Samples	OTUs	Sequences
All samples	9932	54,463
Transconjugant cells sorted by FACS (all treatments)	802	7114
From matings with FL	546	4827
From matings with MP1	161	705
From matings with MP2	257	1582
Recipient cells sorted by FACS (all treatments)	1837	16,093
From matings with FL	723	4329
From matings with MP1	667	3578
From matings with MP2	1114	8186
Reference FL (filters)	4378	5979
Reference MP1 (particles)	2282	4454
Reference MP2 (filters)	1414	2756

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837