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Microplastics increase impact of treated wastewater on freshwater microbial community☆

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ABSTRACT

Plastic pollution is a major global concern with several million microplastic particles entering every day freshwater ecosystems via wastewater discharge. Microplastic particles stimulate biofilm formation (plastisphere) throughout the water column and have the potential to affect microbial community structure if they accumulate in pelagic waters, especially enhancing the proliferation of biohazardous bacteria. To test this scenario, we simulated the inflow of treated wastewater into a temperate lake using a continuous culture system with a gradient of concentration of microplastic particles. We followed the effect of microplastics on the microbial community structure and on the occurrence of integrase 1 (*int*1), a marker associated with mobile genetic elements known as a proxy for anthropogenic effects on the spread of antimicrobial resistance genes. The abundance of *int*1 increased in the plastisphere with increasing microplastic particle concentration, but not in the water surrounding the microplastic particles. Likewise, the microbial community on microplastic was more similar to the original wastewater community with increasing microplastic concentrations. Our results show that microplastic particles indeed promote persistence of typical indicators of microbial anthropogenic pollution in natural waters, and substantiate that their removal from treated wastewater should be prioritised.

1. Introduction

Global production of plastic dramatically and constantly increased in the past 60 years reaching 322 million of tons in 2015 with rising tendencies (PlasticsEurope, 2015). Substantial parts of this huge amount of plastic escape dumping at landfill sites, recycling, or waste treatment and thus enters the environment, where it accumulates, particularly in aquatic habitats (Eriksen et al., 2013; Law, 2017). In the environment, plastic remains almost unchanged for a long time and its complete mineralization has been estimated to require centuries (Barnes and Milner, 2005; Krueger et al., 2015). The term *microplastic* has been coined to describe manufactured microbeads (primary microplastic) or fragments of < 5 mm in diameter that are formed during plastic degradation (secondary microplastic) and their total number floating in the oceans has been estimated to range between 15 and 51 trillion particles in 2014 (Van Sebille et al., 2015). Plastic-derived hazards are well described for numerous aquatic organisms ranging from zooplankton to mammals (Cole et al., 2011; Gall and Thompson, 2015; Li et al., 2016). Although identified as an emerging environmental threat for the oceans, little is known about microplastic in freshwater ecosystems and its ecological consequence (Eerkes-Medrano et al., 2015; Wagner et al., 2014). In particular, wastewater treatment plants (WWTP) effluents represent an important point source for microplastic particles for freshwater environments (Leslie et al., 2017; Mintenig et al., 2017). Although WWTPs remove between 83 and 95% of all microplastic particles (Dris et al., 2015), there is still a substantial quantity; e.g. around 9×10^3 pieces of microplastic m^{-3} were found in the effluent of a German WWTP. Based on the annual effluents of the twelve tested WWTPs, a total discharge of up to

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4×10^9 microplastic particles and fibres per WWTP can be expected to be released into the environment (Mintenig et al., 2017).

One feature of microplastic particles is that they constitute new submerged surfaces for bacterial and eukaryotic colonization, dispersal, nutrient cycling, and biofilm formation (Kettner et al., 2017; Mincer et al., 2016; Oberbeckmann et al., 2015). The fact that microplastic particles host specific assemblages differing from the open waters led to formulate the term *plastisphere* (Zettler et al., 2013). Microplastic particles have been hypothesized to even act as a vector for opportunistic microbial colonisers that otherwise might not be able to proliferate in the surrounding waters (Keswani et al., 2016). For example, the potential pathogen *Vibrio parahaemolyticus* was found on floating microplastic particles (Kirstein et al., 2016). Within the biofilm, such bacteria can be protected from grazing pressure and competition for nutrients is reduced (Corno et al., 2014; Costerton et al., 1999). Another point of concern is that the close vicinity of cells growing in biofilms might increase Horizontal Gene Transfer (HGT) between different bacteria and may thus favour the transfer of pathogenicity and antibiotic resistance in the environment (Costerton et al., 1999).

The here proposed experiment is based on the notion that wastewater effluents contain specific microbial communities, which can include potential human pathogens (Cai and Zhang, 2013; Wéry et al., 2008) and antibiotic resistance genes (ARGs (Di Cesare et al., 2016a),). If microplastic and potential pathogens are released concomitantly, microplastic particles might provide an ecological niche for WWTP-derived pathogens. Moreover, the presumed enhanced HGT in biofilms might facilitate the spread of ARGs (Suzuki et al., 2017). Therefore, we aimed to evaluate the role of microplastic particles in the accumulation of class 1 integrons, which are gene cassettes capture elements (Hall and Collis, 1995) associated with mobile genetic elements involved in the spread of ARGs in the environment (Ma et al., 2017; Stalder et al., 2014). We set up a continuous culture experiment in chemostats with increasing numbers of microplastic particles incubated in different vessels. We used a microbial community from an equimolar mix of waters from a large oligotrophic lake (Lake Maggiore) and from the effluent of the largest municipal WWTP that directly discharges into the lake (Fig. 1). Our experiment mimicked the direct outlet of WWTPs to a receiving aquatic ecosystem such as a lake or a river, where both natural and WWTP waters mix. Since particles and bacterial inoculum were added at the same time, both communities had equal chances of colonizing the microplastic particles.

2. Material and methods

2.1. Experimental set-up

Continuous cultures in chemostats were set up to mimic conditions where water from a WWTP effluent enters into a freshwater system. Therefore, for the inoculum, on September 23rd, 2015, 10 L of lake water were sampled from the shore of Lake Maggiore (WGS84 coordinates: 45.924647° N, 8.545711° E), and concomitantly water was sampled from the municipal WWTP effluent of Verbania (Italy). Both waters were subsequently filtered through 126 μm and 10 μm plankton nets to remove large grazers and particles, but keep the bacterial communities and the smaller eukaryotic predators. Cell numbers were determined immediately by flow cytometry and the waters were mixed to achieve a balanced bacterial community half in cell numbers each from the WWTP effluent and from Lake Maggiore. The starting community consisted of 2.57×10^6 bacterial cells mL^{-1}. Each chemostat vessel was filled with 750 mL of the inoculum solution, including the mixed bacterial communities of the lake and WWTP.

Autoclaved water from the same lake, without any additional bacterial community, was used as a medium during the experiment: 60 L of surface lake water was sampled from the same station as sampled for the inoculum, at the shore of Lake Maggiore (on September 21st, 2015), and pre-filtered over glass microfiber filters (grade GF/C). The medium water was aliquoted into three bottles (18 L), each of them supplemented with chitin from the stock solution (see below), autoclaved, and each bottle used to feed a triplet of running chemostat vessels (Fig. 1).

Chitin was chosen as a supplementary carbon source since this refractory substrate represents one of the most prevalent autochthonous biopolymers in natural aquatic ecosystems (Corno et al., 2015; Köllner et al., 2012). Since medium water was pre-filtered, natural sources of biopolymers, e.g. chitinous body parts of dead zooplankton, were potentially removed and were thus hereby replaced. A final concentration of approximately 4 mg L^{-1} dissolved

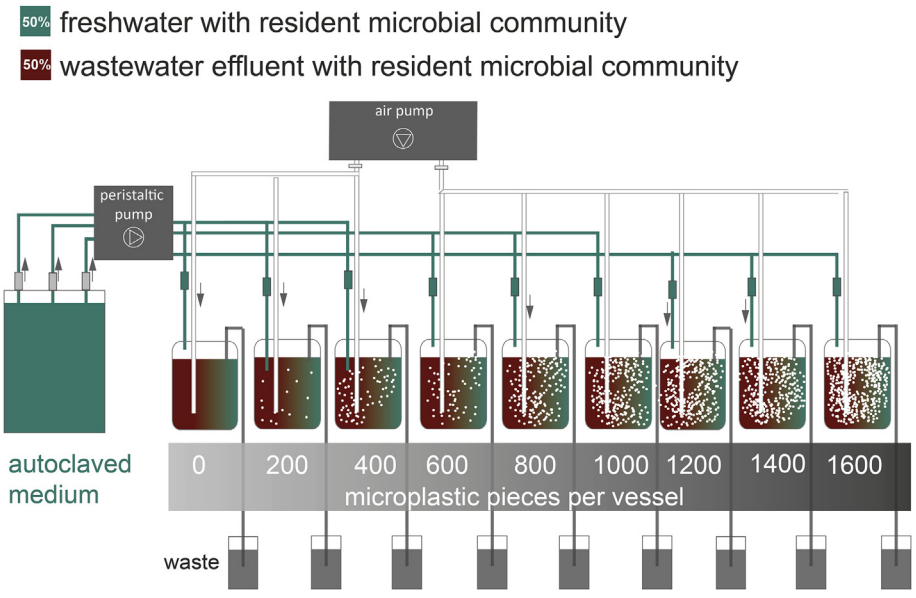

Fig. 1. Schematic representation of the chemostat set-up.

organic carbon (DOC) from chitin was used for the inoculum and for the medium. The stock solution for chitin was prepared by adding 24 g of chitin (from crab shell, practical grade, Sigma Aldrich) to 1200 mL Milli-Q water. The suspension was autoclaved at 121 °C for 20 min after vigorous shaking and subsequently filtered over 5.0 μm polycarbonate filters, and 0.22 μm polyvinylidene fluoride (type GVWP) filters to obtain the dissolved chitin fraction. The filtrate (approx. 900 mL) was autoclaved again and stored at 4 °C.

Microplastic particles were produced from additive-free polystyrene sheets of 0.1 mm thickness obtained from ergo. fol (Norflex, Germany). The sheets were cut with a metal multiple punch maker (RW home, Renz, Germany) to produce 4 mm × 4 mm × 0.1 mm square microplastic particles. Microplastic particles were sterilized by repeated washing with 3% H_2O_2 and sterile MQ water.

The chemostat vessels containing the mixture of inoculum with chitin and microplastic particles were kept at 20 °C in the dark overnight (~16 h) before the chemostat system was switched on in the morning and adjusted to a constant dilution rate of 0.1 d^{-1}, meaning a daily exchange of ~75 mL with fresh, sterile medium. Fine air bubbling kept plastic particles floating in the water column. The continuous cultures were kept at 20 °C in the darkness for 15 days in order to avoid biofilm formation of primary producers on the vessel surfaces.

2.2. Bacterial abundance and size distribution

Starting from day 4 to avoid the fluctuations caused by the initial adaptation of the communities to the new environmental conditions, daily samples (10 mL of water, fixed with formaldehyde, 2% final concentration) for cell counts were taken from each vessel and stored in the dark at 4 °C. Bacterial abundance and size distribution (defined in three groups as: 1. single and doubling cells, 2. small clusters of approximately 3–9 cells, and 3. large aggregates composed by at least 10 cells) were quantified for each sample by flow cytometry (Accuri C6, BD Biosciences) to follow potential shifts from free-living single cells towards larger aggregates, as this indicates a response of the bacterial community to specific ecological factors (predation, competition), or a different composition in species (Corno and Jürgens, 2008). In detail, aliquots of 0.5 mL for each sample were stained with SYBR Green I (final concentration 1%, Life Technologies) for 12 min in the dark. Counts were set to a minimum of 2×10^6 events within the gate designed for single and doubling cells, and 5×10^2 events in the gates of bacterial aggregates (Corno et al., 2013). Flow cytometry counts were confirmed by a random preliminary check and by further epifluorescence microscopic analysis for difficult samples (DAPI and Axioplan microscope; Zeiss, Germany).

2.3. DNA extraction

We sampled the microbial community at the beginning (from WWTP water, lake water, and mixed inoculum) and at the end of the experiment in each vessel (from water and from the biofilms on the microplastic particles). To define the initial WWTP and lake water community composition, duplicate samples of lake water (500 mL), WWTP water (250 mL), and mixed inoculum (250 mL) were filtered on 0.22 μm polycarbonate filters and stored at −20 °C until DNA extraction. At the end of the experiment and from each vessel, duplicate 50 mL of water were filtered onto 0.22 μm polycarbonate filter and twice 50 microplastic particles were retrieved with sterile forceps. Microplastic pieces were rinsed three times with 10 mL sterile Artificial Lake Water (ALW (Zotina et al., 2003),). All samples were stored at −20 °C in cryo-vials before DNA extraction. To break microbial cells, zirconia and glass beads of different sizes (0.1 mm, 0.7 mm, and 1.0 mm) as well as 760 μL extraction buffer (100 mM Tris-HCl, 20 mM Na_2EDTA, 1.6 M NaCl, 1% SDS; pH 8) were added to each sample and subjected to horizontal vortexing (frequency = 30 s^{-1}, 3 min). Additionally, samples were treated with Proteinase K (PCR grade, final concentration of approximately 200 μg mL^{-1}) and incubated at 60 °C for 1 h with short vortexing intervals every 10 min. The liquid phase was then transferred into a new vial where 200 μL CTAB buffer (5% CTAB, 1.6 M NaCl) and 900 μL phenol/chloroform/isoamyl alcohol (25:24:1, Carl Roth) were added. After horizontal vortexing (frequency = 17 s^{-1}, 10 min) and centrifugation (16000*g*, 10 min, 4 °C) the aqueous phase was transferred to a new vial. Then, 900 μL of chloroform/isoamyl alcohol (24:1, Carl Roth) were added, gently mixed and centrifuged (16000*g*, 10 min, 4 °C). The aqueous phase was again transferred, and the contained DNA was precipitated with 40 μL 3 M Na_2-acetate and 1400 μL pure ethanol overnight at 4 °C. The DNA pellet obtained by centrifugation (16000*g*, 90 min, 4 °C) was separated from the supernatant carefully. The pellet was washed with 700 μL ice-cold 70% ethanol and centrifuged (16000*g*, 10 min, 4 °C). After removing the supernatant, the DNA pellet was air-dried under a clean bench and then re-suspended in 40 μL PCR grade water and stored at −20 °C until further processing. The DNA concentration was analysed in a Quantus™ Fluorometer with QuantiFluor ds DNA system (Promega GmbH, Germany).

2.4. Bacterial community pattern: PCR and ARISA

Each DNA extract was amplified by three independent PCRs (technical triplicates) using primers that target the length-variable bacterial ITS region (ITSF and ITSReub as described elsewhere (Ramette, 2009)). The PCR mix contained 1 mM $MgCl_2$ (Bioline), 1x MyTaq™ buffer (Bioline), 0.8 μL–10 μL of extracted DNA (depending on DNA concentration), 0.6 μg μL^{-1} bovine serum albumin (Sigma-Aldrich), 0.3 μM ITSF (5′-GTC GTA ACA AGG TAG CCG TA-3′) 0.3 μM ITSReub (5′-GCC AAG GCA TCC ACC-3′, labelled with HEX™ dye phosphoramidite) and 1.25 units MyTaq™ DNA polymerase (Bioline) in a total of 50 μL with PCR grade water (Roche Applied Science). The PCR cycler program (FlexCycler, Analytic Jena) was set to 94 °C for 3 min for the initial denaturation, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 45 s, elongation at 72 °C for 90 s and a final elongation at 72 °C for 5 min. Amplification success was checked on a 2% agarose gel (55 min, 120 V, in 0.5x TAE buffer) under UV light after staining with Midori Green Advance DNA stain (Nippon Genetics Europe).

PCR products were sent to Services in Molecular Biology (SMB Berlin, Germany) for PCR product purification, standardization of DNA concentration and automated ribosomal intergenic spacer analysis (ARISA). The purified, standardized PCR products mixed with 11 μL Hi-Di formamide and 0.5 μL GeneScan™ 1200 LIZ® size standard were run on the Applied Biosystems 3130 xl Genetic Analyzer. PCR products of different fragment length were separated with capillary electrophoresis (80 cm capillary) under the following conditions: 1.4 kV injection voltage, 25 s injection time, 14.6 kV run voltage, 60 °C oven temperature and a total run time of 4500 s. ARISA electropherograms were evaluated with PeakStudio v2.2 (McCafferty et al., 2012). Automated peak detection was complemented with necessary manual corrections. Each spectrum reached a quality control score between 0.2 and 0.3, as recommended in the user manual (PeakStudio Fodor Lab UNCC (2012)). The operational taxonomic unit (OTU) matrix was created using peaks from 50 to 1000 base pairs and a minimum peak height of 50 fluorescence units and a bin size of 2 base pairs (confirmed as valid by the applying the detection threshold suggested elsewhere (Luna et al., 2006)). Peaks detected in only one replicate were not considered as OTU for downstream analyses. The OTU matrix was converted into a presence/absence table to be used for further

statistical analyses.

2.5. 16SrDNA and int1 quantification

Duplicated DNA extracts from both biofilm on microplastic particles and surrounding water samples in the vessels were used for quantification of 16SrDNA and *int*1 genes by qPCR assays with a CFX Connect Real-Time PCR Detection System (Bio-Rad), using primer pairs Bact1369F/Prok1492R (5′-CGG TGA ATA CGT TCY CGG-3'/5′-GGH TAC CTT GTT ACG ACT T-3′, annealing T 55 °C) (Di Cesare et al., 2015; Suzuki et al., 2000) and intI1LC1/intI1LC5 (5′-GCC TTG ATG TTA CCC GAG AG-3'/5′-GAT CGG TCG AAT GCG TGT-3′, annealing T 60 °C (Barraud et al., 2010)), respectively. The specificity of reaction was evaluated by the melting profile analysis using the PRECISION MELT ANALYSIS Software 1.2 built in CFX MANAGER Software 3.1 (Bio-Rad), and the amplicon size was confirmed by electrophoresis. Detection limits were determined according to Bustin et al. (2009) and yielded 232 and 40 copy μL^{-1} for 16SrDNA and *int*1, respectively. Average ± standard deviation of detection efficiencies and coefficients of regression for all runs of both genes were 109.175 ± 13.877 and 0.989 ± 0.007, respectively. A qPCR inhibition test was carried out by the dilution method (Di Cesare et al., 2013) and resulted in a negligible inhibition; always less than 1 threshold cycle was calculated. Concentrations were then converted to copy μL^{-1} (Di Cesare et al., 2013) and *int*1 was normalised per copy of 16SrDNA.

2.6. Statistical analyses

All statistics were conducted with *R* 3.1.2 (RCore Team, 2013) using RStudio (RStudio Team, 2015). The *R* package *reshape2* v1.4 (Wickham, 2012) was used for data handling. All figures and graphs were made with *ggplot2* v2.2.1 (Wickham, 2009) and additionally processed in Adobe Illustrator CS5.

The impact of the concentration of microplastic particles on bacterial cell counts at the end of the experiment was evaluated applying generalized linear models (GLMs) considering a quasi-poisson distribution, due to over-dispersion of the count data (Crawley, 2013).

Differences in bacterial OTU composition between different samples (Beta-diversity) were evaluated by Sørensen's similarity index (βsor) in the R package *betapart* v1.3 (Baselga and Orme, 2012) on a presence/absence matrix of the OTUs obtained from ARISA data. Principal coordinate decomposition (PCoA, package *ape* v3.4 (Paradis et al., 2004)) was computed for the βsor similarity distance matrix for graphical depiction of the sample similarity. The similarity of the bacterial community of the samples was analysed in relationship to the corresponding vessel and environment the bacteria lived on/in (i.e. water or microplastic) and their interaction (vessel*growth environment) by permutational multivariate analysis of variance of the dissimilarity matrix with the *adonis* command in the R package *vegan* v2.2-1 with 9999 permutations (Anderson, 2001; Oksanen et al., 2007).

In addition, it was assessed whether the communities at the end were closer to the original WWTP water or lake water community. The pair-wise similarity of the chemostat communities (of βsor) of both water and microplastic to the original communities (WWTP water or lake water) was analysed in relationships to the increasing concentration of microplastic particles using linear models (LMs) (Crawley, 2013). This means that we tested whether the specific community patterns of the vessel water and of the microplastic were more similar to the WWTP or lake water community with increasing microplastic concentrations.

The impact of the concentration of microplastic on *int*1/16S gene abundances was assessed first by addressing the effect of the quantity of microplastic, the growth environment (water or microplastic), and their interaction (microplastic concentration*-growth environment) on the total abundance of *int*1 in each vessel. The statistical model used for these analyses was a Linear Mixed Effect Model (LMEM), with the chemostat vessel identity included in the error structure to avoid pseudoreplication (R package: *lmerTest* v2.0-20 (Kuznetsova et al., 2015)). In case of a significant interaction between the growth environment (water or microplastic) and the concentration of microplastic, Linear Models (LM) (Crawley, 2013) were performed separately for the microplastic and the water fraction to test whether the *int*1/16S gene abundances were influenced by the concentrations of microplastic particles. Given that *int*1/16S data are proportions, the raw values were transformed by the arcsin of the square root (Crawley, 2013) to improve model fit.

3. Results

3.1. Cell numbers and phenotypic distribution

At day 8, after adaptation to the chemostat conditions, the number of single cell or doubling free-living bacteria in the water was on average $2.8 \pm 0.9 \times 10^6$ cells mL^{-1} (range: 1.1–4.2 × 10^6 cells mL^{-1}, Fig. S1). The number of small clusters of 3–9 cells and of large aggregates of more than 10 cells was $1.2 \pm 0.5 \times 10^5$ mL^{-1} and $1.3 \pm 1.2 \times 10^4$ mL^{-1}, respectively. Despite temporal fluctuations in each vessel, similar concentrations were found at the end of the experiment on day 15 ($2.2 \pm 1 \times 10^6$ free-living bacteria mL^{-1}, $1.1 \pm 0.5 \times 10^5$ small clusters mL^{-1}, $1.2 \pm 1.1 \times 10^4$ large aggregates mL^{-1}, Fig. S1). In the presence of microplastics, however, abundances of the different cell phenotypes at the end of the experiment did not significantly change in relation to the microplastics concentration (GLM: free-living cells: $t = -1.1$, $p = 0.317$, small clusters: $t = -1.7$, $p = 0.139$, large aggregates: $t = -0.7$, $p = 0.503$, Table S1), even though the highest number of free-living cells was observed in the treatment without microplastics.

3.2. Bacterial community patterns

The bacterial community composition was not different between biofilm and free-living communities (PCoA, Fig. S2). At the end of the 15-days experiment, the bacterial community composition was significantly influenced by differences between the individual vessels (71% of variance, Table 1), with very little differences between the growth environment, either in water or on microplastic (6% of variance). We then compared whether distances of the community profiles in terms of Beta-diversity changed with increasing microplastic concentrations by comparing the samples to initial WWTP and lake water community patterns. Comparison of bacterial community composition at the end of the experiment to the initial inoculum derived from WWTP and lake water did not reveal significant differences between bacterial communities in the water fraction in relationship with the concentration of microplastic particles (Table 2, Fig. 2). On microplastics, however, the similarity to the initial WWTP community increased with increasing microplastic, and it increased more than the similarity to the original lake water community (Table 2, Fig. 2). The fact that similarities to lake and to WWTP original communities increased, even if differently, is explained by the OTU richness on microplastics, which significantly increased with microplastic concentration (Table S2&S3, $t = 3.6$, $p = 0.011$) and consequently, at the end of the experiment more WWTP as well as lake water genotypes resided on microplastics. In the surrounding water, however, OTU richness significantly decreased with increasing microplastic concentration (Table S3, $t = -3.5$, $p = 0.011$).

Table 1
Effect of differences in chemostat identity (vessel) and in growth environment (GE; microplastic particles/water) on the variance of the distance matrix of Sorensen beta diversity of the ARISA profiles. Output results of a permutational multivariate analysis of variance are given.

	Degrees of freedom	Sums Of Squares	Mean Squares	F-value	R^2	P-value
Vessel	8	2.58	0.32	2.7	0.7107	0.001
GE	1	0.23	0.23	2.0	0.0639	0.028
Residuals	7	0.82	0.12		0.2254	
Total	16	3.64	1.00			

Table 2
Effect of the number of microplastic particles per vessel on the β-Sorensen similarity of bacterial communities in vessel water and the inoculum from lake water (LW, **A**) or WWTP (WW, **B**) and on microplastic and the inoculum of with LW (**C**) and WW (**D**) bacterial community patterns. Output results of linear models are given.

	Estimate	Std. Error	t value	P-value
(**A**) βsor distance vessel water to LW community				
(Intercept)	0.231e-01	0.0249	9.3	0.00003
microplastic per vessel	0.000005	0.00003	−0.2	0.845
(**B**) βsor distance vessel water to WW community				
(Intercept)	0.211	0.0176	12	0.000006
microplastic per vessel	−0.00003	0.00002	−1.3	0.221
(**C**) βsor distance microplastic to LW community				
(Intercept)	0.134	0.04.68	2.8	0.0283
microplastic per vessel	0.0001	0.00005	2.9	0.0271
(**D**) βsor distance microplastic to WW community				
(Intercept)	0.120	0.0159	7.5	0.0003
microplastic per vessel	0.00008	0.00002	5.4	0.00173

3.3. Integrase 1 occurrence

The mean normalised abundance of *int1* was ~20 times lower in the original lake water (3.05×10^{-3}) than in the original WWTP water (6.68×10^{-2}). After mixing lake and WWTP waters for inoculation, the mean abundance of *int*1/16SrDNA gene copy was 2.33×10^{-2}, the same order of magnitude of abundances measured at the end of the experiment: 4.1×10^{-2} in water and 2.9×10^{-2} on microplastic particles (Fig. 3). Overall, the vessel water and microplastic *int*1/16SrDNA gene copy was not affected by microplastics concentration (LMEM: $t = -1.1$, $p = 0.306$, Table 3). There was, however, a significant effect of the interaction between the growth environment on which the *int1* gene was measured (i.e. microplastic or water) and microplastics concentration ($p = 0.011$, Table 3). The significant interaction suggests a differential response of the *int*1 concentrations, thus we tested the abundance of *int*1 separately for each growth environment. Whereas no effect was obvious in water (LM: $t = -0.8$, $p = 0.455$, Table 4, Fig. 3), a significant and positive effect of microplastics concentration on *int*1 abundance was found on microplastics ($t = 7.0$, $p < 0.001$, Table 4, Fig. 3).

4. Discussion

4.1. Exchange of microbes between microplastic and surrounding water

We mixed microbial communities from treated WWTP water and natural lake water to simulate a WWTP effluent, and to follow the survival of WWTP bacteria in the plastisphere. The most similar communities were those from the same chemostat. This suggests a heterogeneous and different community assembly trajectory in each vessel, with differences in the growth environment (microplastic and surrounding water) only explaining 6% of the observed variance in bacterial community composition. Bacterial cell numbers and morphologies in the water determined by flow cytometry did not significantly change with increasing microplastic concentration. As the bacterial abundance on small clusters and in large aggregates did not significantly differ with increasing microplastic concentration, we assume that microplastic had little effects on biofilm shedding (Donlan, 2002). It is thus unlikely that the similarities found between the water and microplastic are due

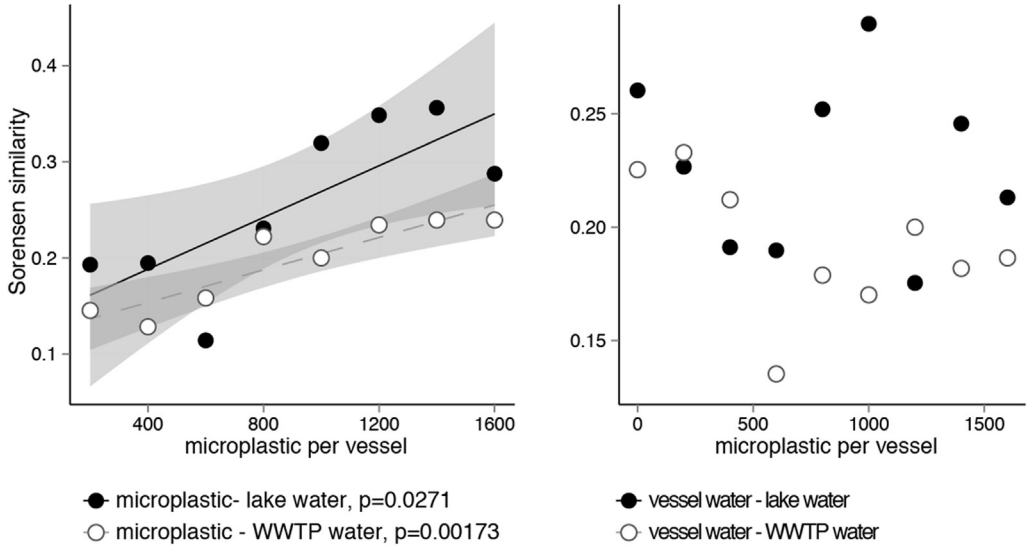

Fig. 2. Relationship between Sorensen similarity of the microbial communities on microplastic (left) and vessel water (right) to the original wastewater and lake water community in dependence of the concentration of microplastic. The regression line, confidence interval and p-values were plotted only for changes in similarity that gave a statistically significant result in the linear model (Table 2).

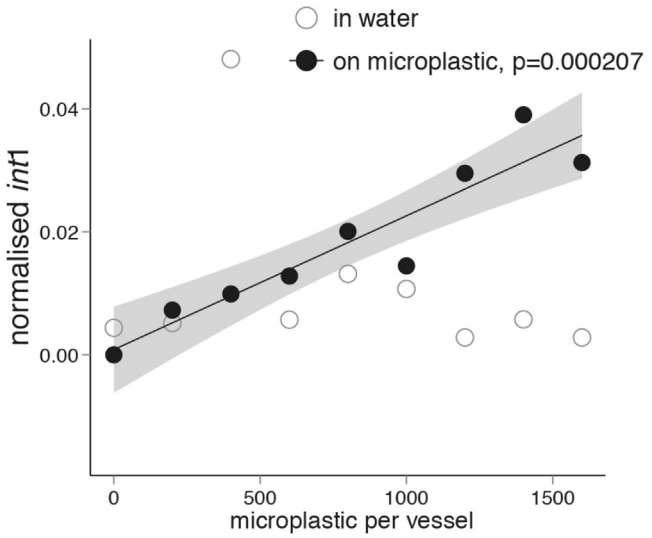

Fig. 3. Relationship between abundance of *int*1 in water (white) and on microplastic (black) with the concentration of microplastic. Abundance values of *int*1 are expressed as arcsin of square root of the proportion between abundance of *int*1 and abundance of 16S rDNA. The regression line, confidence interval and p-values were plotted only for measurements done with the growth environment that gave a statistically significant result in the linear model (Table 4).

Table 3
Effect of the quantity of microplastic (MP), the growth environment (GE, water or microplastic) and their interaction on the abundance of *int*1. Output results of a linear mixed effect model with vessel identity in the error structure are given.

	Estimate	Standard Err	Degrees of freedom	t-value	p-value
(Intercept)	0.16	0.067	14	2.5	0.0243
MP	−0.00007	0.00007	14	−1.1	0.3058
GE	−0.161	0.0951	14	−1.7	0.1120
MP: GE	0.0002	0.0001	14	2.9	0.0109

Table 4
Effect of microplastic per vessel on abundance of *int*1 in (**A**) water and on (**B**) microplastic. Output results of linear models are given.

	Estimate	Standard Error	t value	P-value
(A) In water				
(Intercept)	0.169	0.0904	1.9	0.102
microplastic per vessel	−0.00007	0.00009	−0.8	0.455
(B) On microplastic				
(Intercept)	0.0008	0.0002	0.288	0.782022
microplastic per vessel	0.00002	0.000003	7.024	0.000207

to detached pieces of biofilm. It is more likely that the pattern in bacterial community composition in the plastisphere is substantially influenced by the local surrounding water (Zettler et al., 2013).

4.2. Dose dependent effect of microplastic on persistence of OTUs and int1

The more microplastic particles were present in the chemostats, the more similar was the pattern of the microbial community of the plastisphere to the one of the WWTP. At the same time, although to a lesser extent, the higher microplastic particle concentration leads to an increased similarity between microbial communities on microplastic and lake water, demonstrating a generally greater richness in the plastisphere with increasing microplastic particle concentration. As it has been previously suggested, biofilm formation on natural and artificial surfaces including microplastic particles increases the likelihood for survival of allochthonous bacteria, e.g. from WWTP, in natural aquatic environments (Lehtola et al., 2007; Manz et al., 1993). In the case of WWTP derived bacteria, this might be due to the protection from grazing by protists, which is one of the major causes of mortality of such bacteria in natural water bodies (González et al., 1992; Wanjugi and Harwood, 2013).

Similarly, a significant relationship was found between the increase in microplastic concentration and the relative abundance of *int*1/16SrDNA gene copies within the microbial community in the plastisphere. The closer physical proximity between bacteria on microplastic favours the contact between surface-attached bacteria and thus may trigger the mobilization of *int*1, presumably in association with mobile genetic elements (Gillings et al., 2015). However, taking together that both *int*1 abundance and bacterial richness on microplastic increase with increasing concentration in the vessel hints to an important role of the recruitment of *int*1 positive planktonic bacteria into the microbial community of the biofilm (Donlan, 2002). Detachment and reattachment of bacteria from biofilms is an essential part of any biofilm development (Hall-Stoodley et al., 2004). Moreover, increased similarity to the community pattern of WW was not observed in the surrounding water suggesting that such bacteria could only survive for short time periods in open waters. Biofilm forming and *int*1 containing bacteria might thus benefit from higher microplastic particle abundance in the vessels since it increases the probability for free-floating bacteria to encounter a new piece of microplastic for colonization. The finding of other particles to inhabit might even be triggered by quorum sensing. Also here, it is more likely for a bacterium to sense the signal if the biofilm is close by, since the signal strongly diffuses with distance (Alberghini et al., 2009).

WWTPs often release *int*1 into the surrounding environment (Di Cesare et al., 2016a; Di Cesare et al., 2016b). According to an earlier mesocosm study, even small amounts of sewage effluent can significantly increase *int*1 prevalence in freshwater biofilms without any changes in the free-living microbial communities (Lehmann et al., 2016). Thus, there might be a potential connection between the survival and spread of WWTP derived bacteria and increasing abundances of *int*1 within the plastisphere.

4.3. Differences of experimental set-up to nature

Regarding its comparability to conditions in nature, this experiment has certain limitations: Concentrations of microplastic used in this experiment were very high (Lenz et al., 2016). This was due to the fact that the surface of the microplastic should have exceeded the surface of the chemostat vessel in the highest concentration. Moreover, we kept the chemostat in the dark to overcome potential confounding factors of biofilms formed by primary producers on the vessel surface. Most WWTP effluents discharged directly in lakes are released into deep waters where there is no light, but others (especially when the receiving environment is a river, or an artificial channel) are released into shallow waters, where light plays an important role in shaping microbial communities. Third, when microplastics are discharged from a WWTP, they are likely already colonised by WWTP inhabiting bacteria, whereas here we used clean microplastic particles. The latter implies that our results might even underestimate the consequent similarity of microplastic-attached communities to initial WWTP communities. As a further step, systematic studies with environmental samples are needed to observe the survival rates of WWTP bacteria and *int*1 abundance on microplastic under fully natural conditions.

5. Conclusions

In conclusion, this study hints at an additional threat posed by the emerging pollutant microplastic, namely the favouring of survival of WWTP-derived bacteria including genes that are involved in the spread of antibiotic resistance genes such as the class 1 integrons in natural freshwater environments. With conventional wastewater treatment, however, an adequate removal of microplastic particles and associated bacteria carrying *int*1 - possibly associated with ARGs - cannot be guaranteed. Consequently, an improved treatment should be considered for the safe reuse of wastewater in order to reduce the risk of spreading both *int*1 and ARGs in the environment through microplastic.

Disclaimer

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Conflicts of interest

None.

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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.2017.11.070.

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