Institute of Biochemistry and Biology

Microbial Gene Exchange on Microplastic Particles

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University of Potsdam

by

María de Jesús Arias Andrés

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eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Potsdam

Von

María de Jesús Arias Andrés

Potsdam, 10.10.2018

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Preface

This scientific work was conducted mainly at the Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), Dept. Experimental Limnology, Alte Fischerhuette 2, D-16775 Stechlin, Germany. This work was carried out from August 2014 until May 2018. Research was conducted independently with the funding from the Leibniz Association (Germany) and partially with funds from COST-European Cooperation in Science and Technology, COST Action ES1403. The Ph.D. of María de Jesús Arias Andrés was supported by a scholarship from Universidad Nacional (Costa Rica).

List of Publications

This thesis is a publication-based dissertation. Three publications constitute the central part of the thesis and are integrated as chapters, each with individual figures and tables numbering, supplementary data and individual reference list. María de Jesús Arias Andrés's share of work is explained for each. The name used for publications is Maria Arias-Andres.

Chapter I

Eckert, E.M., Di Cesare, A., Kettner, M.T., Arias-Andres, M., Fontaneto, D., Grossart, H.-P., and Corno, G. (2018) Microplastics increase impact of treated wastewater on freshwater microbial community. Environ. Pollut. **234**: 495–502. doi:10.1016/j.envpol.2017.11.070.

Maria Arias-Andres participated together with the other co-authors in designing the experiment, performing the analyses and writing the manuscript.

Chapter II

Arias-Andres, M., Klümper, U., Rojas-Jimenez, K., and Grossart, H.-P. (2018) Microplastic pollution increases gene exchange in aquatic ecosystems. Environ. Pollut. **237**: 253–261. doi: 10.1016/j.envpol.2018.02.058

Maria Arias-Andres designed major parts of the experiment with help from HPG and UK, performed most of the analysis with the help of KRJ, and wrote major parts of the manuscript with inputs from all co-authors.

Chapter III

Arias-Andres, M., Kettner M.T., Miki, T., Grossart, H.-P. (2018) Microplastics: New substrates for heterotrophic activity contribute to altering organic matter cycles in aquatic ecosystems. Sci. Total Environ. **635**:1152-1159. doi: 10.1016/j.scitotenv.2018.04.199

Maria Arias-Andres participated in designing the experiment together with TK and HGP, performed the analysis with TK and TM and wrote major parts of the manuscripts with input from all co-authors.

Summary

Plastic pollution is ubiquitous on the planet since several millions of tons of plastic waste enter aquatic ecosystems each year. Furthermore, the amount of plastic produced is expected to increase exponentially shortly. The heterogeneity of materials, additives and physical characteristics of plastics are typical of these emerging contaminants and affect their environmental fate in marine and freshwaters. Consequently, plastics can be found in the water column, sediments or littoral habitats of all aquatic ecosystems. Most of this plastic debris will fragment as a product of physical, chemical and biological forces, producing particles of small size. These particles (< 5mm) are known as "microplastics" (MP). Given their high surface-to-volume ratio, MP stimulate biofouling and the formation of biofilms in aquatic systems.

As a result of their unique structure and composition, the microbial communities in MP biofilms are referred to as the "Plastisphere." While there is increasing data regarding the distinctive composition and structure of the microbial communities that form part of the plastisphere, scarce information exists regarding the activity of microorganisms in MP biofilms. This surface-attached lifestyle is often associated with the increase in horizontal gene transfer (HGT) among bacteria. Therefore, this type of microbial activity represents a relevant function worth to be analyzed in MP biofilms. The horizontal exchange of mobile genetic elements (MGEs) is an essential feature of bacteria. It accounts for the rapid evolution of these prokaryotes and their adaptation to a wide variety of environments. The process of HGT is also crucial for spreading antibiotic resistance and for the evolution of pathogens, as many MGEs are known to contain antibiotic resistance genes (ARGs) and genetic determinants of pathogenicity.

In general, the research presented in this Ph.D. thesis focuses on the analysis of HGT and heterotrophic activity in MP biofilms in aquatic ecosystems. The primary objective was to analyze the potential of gene exchange between MP bacterial communities vs. that of the surrounding water, including bacteria from natural aggregates. Moreover, the thesis addressed the potential of MP biofilms for the proliferation of biohazardous bacteria and MGEs from wastewater treatment plants (WWTPs) and associated with antibiotic resistance. Finally, it seeks to prove if the physiological profile of MP biofilms under different limnological conditions is divergent from that of the water communities. Accordingly, the thesis is composed of three independent studies published in peer-reviewed journals. The two laboratory studies were performed using both model and environmental microbial communities. In the field experiment, natural communities from freshwater ecosystems were examined.

In **Chapter I**, the inflow of treated wastewater into a temperate lake was simulated with a concentration gradient of MP particles. The effects of MP on the microbial community structure and the occurrence of integrase 1 (*int 1*) were followed. The *int 1* is a marker

associated with mobile genetic elements and known as a proxy for anthropogenic effects on the spread of antimicrobial resistance genes. During the experiment, the abundance of *int1* increased in the plastisphere with increasing MP particle concentration, but not in the surrounding water. In addition, the microbial community on MP was more similar to the original wastewater community with increasing microplastic concentrations. Our results show that microplastic particles indeed promote persistence of standard indicators of microbial anthropogenic pollution in natural waters.

In **Chapter II**, the experiments aimed to compare the permissiveness of aquatic bacteria towards model antibiotic resistance plasmid pKJK5, between communities that form biofilms on MP vs. those that are free-living. The frequency of plasmid transfer in bacteria associated with MP was higher when compared to bacteria that are free-living or in natural aggregates. Moreover, comparison increased gene exchange occurred in a broad range of phylogenetically-diverse bacteria. The results indicate a different activity of HGT in MP biofilms, which could affect the ecology of aquatic microbial communities on a global scale and the spread of antibiotic resistance.

Finally, in **Chapter III**, physiological measurements were performed to assess whether microorganisms on MP had a different functional diversity from those in water. General heterotrophic activity such as oxygen consumption was compared in microcosm assays with and without MP, while diversity and richness of heterotrophic activities were calculated by using Biolog® EcoPlates. Three lakes with different nutrient statuses presented differences in MP-associated biomass build up. Functional diversity profiles of MP biofilms in all lakes differed from those of the communities in the surrounding water, but only in the oligo-mesotrophic lake MP biofilms had a higher functional richness compared to the ambient water. The results support that MP surfaces act as new niches for aquatic microorganisms and can affect global carbon dynamics of pelagic environments.

Overall, the experimental works presented in **Chapters I** and **II** support a scenario where MP pollution affects HGT dynamics among aquatic bacteria. Among the consequences of this alteration is an increase in the mobilization and transfer efficiency of ARGs. Moreover, it supposes that changes in HGT can affect the evolution of bacteria and the processing of organic matter, leading to different catabolic profiles such as demonstrated in **Chapter III**. The results are discussed in the context of the fate and magnitude of plastic pollution and the importance of HGT for bacterial evolution and the microbial loop, i.e., at the base of aquatic food webs. The thesis supports a relevant role of MP biofilm communities for the changes observed in the aquatic microbiome as a product of intense human intervention.

Zusammenfassung

Die Plastikverschmutzung ist auf dem Planeten allgegenwärtig, da jährlich mehrere Millionen Tonnen Plastikabfall in die aquatische Ökosystemen gelangen. Darüber hinaus wird erwartet, dass die Menge an produziertem Plastik in naher Zukunft exponentiell ansteigen wird. Die Heterogenität der Kunststoffmaterialien, ihrer Additive und physikalischen Eigenschaften ist typisch für diese neu auftretenden Schadstoffe und beeinflusst deren Umweltverhalten in Meeres- und Süßwasser. Als Folge kann Plastik in der Wassersäule, den Sedimenten oder Küstenlebensräumen aller aquatischen Ökosysteme gefunden werden. Die meisten dieser Plastikabfälle fragmentieren durch das Zusammenspiel physikalischer, chemischer und biologischer Kräfte, wodurch kleine Partikel erzeugt werden. Diese Partikel (<5mm) sind auch bekannt als "Mikroplastik" (MP). Aufgrund ihres hohen Oberflächen-Volumen-Verhältnisses stimuliert MP das Biofouling und somit die Bildung von Biofilmen in aquatischen Systemen.

Aufgrund ihrer einzigartigen Struktur und Zusammensetzung werden die mikrobiellen Gemeinschaften in MP-Biofilmen als "Plastisphäre" bezeichnet. Während es immer mehr Daten über die spezifische Zusammensetzung und Struktur der mikrobiellen Gemeinschaften – die Teil dieser Plastisphäre sind – gibt, existieren hingegen nur wenige Informationen über die Aktivität von Mikroorganismen in MP-Biofilmen. Dieser Lebensstil des Anheftens und Besiedelns von Oberflächen ist oft mit der Zunahme von horizontalem Gentransfer (HGT) unter Bakterien verknüpft. Diese Art der mikrobiellen Aktivität stellt eine besonders relevante Funktion dar und sollte daher in MP-Biofilmen analysiert werden. Der horizontale Austausch von mobilen genetischen Elementen (MGEs) ist ein wesentliches Merkmal von Bakterien. Er ist verantwortlich für die schnelle Evolution dieser Prokaryoten und ihre Anpassungsfähigkeit an verschiedenste Umweltbedingungen. Der Prozess des HGT ist zudem entscheidend für die Verbreitung von Antibiotikaresistenzen sowie für die Entwicklung von Pathogenen, da viele MGEs bekanntermaßen Antibiotikaresistenzgene (ARGs) und genetische Determinanten für Pathogenität enthalten.

Im Allgemeinen konzentriert sich die Forschung in der vorliegenden Dissertation auf die Analyse des HGT und der heterotrophen Aktivität in MP-Biofilmen in aquatischen Ökosystemen. Das Hauptziel besteht darin, das Potenzial des Genaustausches zwischen MP-Bakteriengemeinschaften und dem des umgebenden Wassers, einschließlich der Bakterien in natürlichen Aggregaten, zu analysieren. Darüber hinaus befasst sich diese Doktorarbeit mit dem Potenzial von MP-Biofilmen zur Ausbreitung biologisch gefährlicher Bakterien und MGEs, die aus Kläranlagen stammen und mit Antibiotikaresistenzen assoziiert sind. Schließlich soll bei verschiedenen limnologischen Bedingungen überprüft werden, ob das jeweilige physiologische Profil von MP-Biofilmen von dem der Wassergemeinschaften abweicht. Dementsprechend besteht die Arbeit aus drei unabhängigen Studien, die in

Fachzeitschriften veröffentlicht wurden. In den beiden Laborstudien wurden sowohl mikrobielle Modell- als auch Umwelt-Gemeinschaften betrachtet. Im Freilandexperiment wurden schließlich die natürlichen Gemeinschaften aus Süßwasserökosystemen untersucht.

In Kapitel I wurde der Zufluss von geklärtem Abwasser mit einem Konzentrationsgradienten von MP-Partikeln in einen See der gemäßigten Klimazone simuliert. Dabei wurden die Effekte von MP auf die mikrobielle Gemeinschaftsstruktur und das Auftreten von Integrase 1 (int 1) verfolgt. Int 1 ist ein Marker, der mit mobilen genetischen Elementen assoziiert ist und zur Abschätzung anthropogener Einflüsse auf die Ausbreitung antimikrobieller Resistenzgene verwendet ist. Während des Experiments erhöhte sich das Vorkommen von Int1 in der Plastisphäre mit zunehmender MP-Partikelkonzentration, jedoch nicht im umgebenden Wasser. Darüber hinaus ähnelte die mikrobielle Gemeinschaft auf MP zunehmend der ursprünglichen Abwassergemeinschaft mit steigender Mikroplastikkonzentration. Unsere Ergebnisse zeigen, dass Mikroplastikpartikel tatsächlich die Persistenz von Standardindikatoren mikrobieller anthropogener Verschmutzung in natürlichen Gewässern fördern.

In Kapitel II wurde die Permissivität von aquatischen Bakterien gegen das Modell-Plasmid für Antibiotikaresistenz pKJK5 zwischen Gemeinschaften, die Biofilme auf MP bilden, gegenüber denen, die frei leben, verglichen. Die Häufigkeit des Plasmidtransfers unter den MP-assoziierten Bakterien war höher als unter Bakterien, die frei oder in natürlichen Aggregaten leben. Der verstärkte Genaustausch trat darüber hinaus bei einem breiten Spektrum phylogenetisch diverser Bakterien auf. Die Ergebnisse deuten auf eine unterschiedliche Aktivität von HGT in MP-Biofilmen hin, welche die Ökologie aquatischer mikrobieller Gemeinschaften auf globaler Ebene sowie die Verbreitung von Antibiotikaresistenzen beeinflussen könnten.

Schließlich wurden in Kapitel III physiologische Messungen durchgeführt, um festzustellen, ob Mikroorganismen auf MP eine andere funktionelle Diversität aufwiesen als jene im Wasser. Die generelle heterotrophe Aktivität, wie der Sauerstoffverbrauch, wurde in Mikrokosmentests mit und ohne MP verglichen, während die Diversität und Vielfalt heterotropher Aktivitäten mit Hilfe von Biolog® EcoPlates berechnet wurden. Drei Seen mit unterschiedlichen Nährstoffbedingungen wiesen Unterschiede in der Ausprägung der MP-assoziierten Biomasse auf. In allen Seen unterschieden sich die funktionellen Diversitätsprofile der MP-Biofilme von denen der Gemeinschaften im umgebenden Wasser, aber nur die MP-Biofilme des oligo-mesotrophen Sees hatten eine höhere funktionelle Vielfalt im Verglichen zum Umgebungswasser. Die Ergebnisse verdeutlichen, dass MP-Oberflächen als neue Nischen für aquatische Mikroorganismen fungieren und die globale Kohlenstoffdynamik im Pelagial beeinflussen können.

Insgesamt unterstützen die in den Kapiteln I und II vorgestellten experimentellen Studien ein Szenario, in dem die Umweltverschmutzung durch MP die HGT-Dynamik zwischen aquatischen Bakterien beeinflusst. Zu den Folgen dieser Veränderung gehört eine Erhöhung

der Mobilisierungs- und Übertragungseffizienz von ARGs. Darüber hinaus wird vermutet, dass eine Beeinflussung des HGT die Evolution von Bakterien und die Umsetzung von organischem Material verändern könnte, was zu verschiedenen katabolischen Profilen führt, wie in Kapitel III gezeigt. Die Ergebnisse werden in Zusammenhang mit dem Ausmaß der Plastikverschmutzung sowie der Bedeutung von HGT für die bakterielle Entwicklung und „mikrobielle Schleife“, d. h. an der Basis der aquatischen Nahrungsnetze, diskutiert. Diese Doktorarbeit veranschaulicht die Bedeutung von MP-Biofilmgemeinschaften für die beobachteten Veränderungen des aquatischen Mikrobioms als eine Folge der intensiven anthropogenen Eingriffe.

List of abbreviations

MP: microplastic(s)

HGT: horizontal gene transfer

MGEs: mobile genetic elements

ARGs: antibiotic resistant genes

WWTPs: wastewater treatment plants

FL: free-living bacteria

PA: particle-associated bacteria

FCM: flow cytometry

FACS: fluorescence-activated cell sorting

Introduction

Plastic pollution has recently caught the attention of both the scientific community and the public opinion. Increasing amounts of manufactured plastic polymers end up accumulating on landfills or the natural terrestrial and aquatic environments (Geyer *et al.*, 2017). This waste is easily fragmented and weathered down to particles of less than a few millimeters denominated "microplastics" (MP), whose complete mineralization could take up to centuries (Keswani *et al.*, 2016). These particles are easily transported to freshwater ecosystems and finally to the oceans. In natural environments, MP surfaces represent a new habitat for biofilm formation, where diverse microbial assemblages develop, often referred to as the "plastisphere" (Zettler *et al.*, 2013). However, very few studies have addressed microbial activities of MP biofilms and their repercussions for ecosystem functioning and alteration of water quality.

Our knowledge of microbial biofilms tells us they are essential for both biodiversity and functioning of aquatic ecosystems (Battin *et al.*, 2016). The proximity of prokaryotic and eukaryotic cells together with a polymer matrix to protect them from many environmental stressors (e.g., physical abrasion, UV radiation) (Demeter *et al.*, 2016), provides conditions to maximize interactions between different groups colonizing on MP. The result is a diverse network of metabolic co-operation that transforms organic matter and shape water biogeochemistry, with many ecological implications for the entire aquatic food web. The metabolic diversity in biofilms is also a product of horizontal gene transfer (HGT) among different species, whereby mobile genetic elements (MGEs) are shared integrating, e.g., different degradation pathways (Flemming *et al.*, 2016). The increased proximity of cells on biofilms enhances HGT, an essential driver in the evolution of organismic, particularly of bacterial communities on Earth (Madsen *et al.*, 2012). It is also a very relevant process for the spread of antibiotic resistance genes (ARGs) in the world, a long-recognized threat by the World Health Organization (Balcázar *et al.*, 2015).

This dissertation starts with an introduction to the magnitude of current plastic and MP pollution in the environment and highlights of current knowledge of the microbial communities that make up the plastisphere. Recent knowledge of HGT in biofilm lifestyles is presented to contextualize the relevance of studies on MP biofilms in aquatic ecosystems. Finally, a brief overview of the main techniques used in this work is presented in the context of the primary research hypothesis.

Microplastic pollution and the "Plastisphere"

Plastic is a generic term for materials made out from different polymers, thereby having different physical and chemical properties such as density (Cole *et al.*, 2011). The manufacture of plastic materials in a year represents the consumption of around 8% of the world's oil production, and it was estimated in more than 332 million metric tons (MT) in 2015 (P.C.H. Hollman *et al.*, 2013; PlasticsEurope, 2015). Among the polymers most used are low and high-density polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET) (Ivar do Sul and Costa, 2014). Plastic materials can be easily molded and manufactured, they have a low cost, and display high resistance to different water, chemical, temperature and light conditions (da Costa *et al.*, 2017). These characteristics, and others that are given by different types of additives, enhance their performance and encourage the widespread use of plastics.

An approximate amount of 6300 MT of plastic waste produced by the end of 2015 ended up in aquatic ecosystems, and it is expected that plastic pollution may double by 2050 (Geyer et al., 2017). Physical, chemical, and biological degradation of plastic waste in the environment result in constant inputs of plastic particles, which are not so readily mineralized (Cole et al., 2011; Law and Thompson, 2014). Microplastics (MP) is the term generally used for those particles of less than 5 mm, either product of plastic fragmentation or from the industrial origin (Keswani *et al.*, 2016). Floating plastic debris found in all subtropical gyres is mainly composed of < 1cm diameter particles (Cózar et al., 2014), with estimations of 15-51 trillion plastic particles in the oceans (van Sebille *et al.*, 2015). Nevertheless, MP are also ubiquitous in all forms of freshwaters, whereby substantial amounts stem from inputs of wastewater treatment plants (WWTPs) (Wagner and Lambert, 2018; Leslie *et al.*, 2017; Mintenig *et al.*, 2017).

Although MP pollution can be formed by fragmentation of large plastic litter, it also originates from fibers that pass the WWTPs (Law and Thompson, 2014). The most commonly found MP in the environment include PE, PP, low-density PE (LDPE), PVC (Bakir *et al.*, 2012) and PET (Oberbeckmann *et al.*, 2014). Further, The analysis of persistent organic pollutants (POPs) in marine MP shows that they can concentrate some of these substances such as polychlorinated biphenyls (PCBs) by several orders of magnitude in respect to the surrounding water (Andrady, 2011; Hirai *et al.*, 2011). All these particles have therefore different composition with respect to other types of particulate material in aquatic ecosystems and provide an increased surface-to-volume ratio that makes them attractive for microbial attachment.

Microplastics interact with microorganisms, the base of aquatic food webs. The specific term, the "plastisphere," was coined to refer to all the assemblages in MP particles (Zettler *et al.*, 2013). Several observations in samples of water and sediments found that the microbial colonization of MP particles presents a different structure and compositions to the surrounding habitats in marine and freshwaters, converging towards specific groups

(Harrison *et al.*, 2014; McCormick *et al.*, 2016). After this, there has been increasing calls to research MP-associated microorganisms, and they have been identified as a significant threat to aquatic biota (Kalogerakis *et al.*, 2014; McCormick *et al.*, 2014).

From a microbial perspective, the presence of microbial pathogens as hitchhikers in MP biofilms is a cause for concern (Kirstein *et al.*, 2016). Also, experiments with zooplankton taxa have demonstrated the passage of MP particles with their associated biofilms in the planktonic food web (Setälä *et al.*, 2014). On the other hand, the recently found potential differences in metabolism of MP-associated bacteria (Bryant *et al.*, 2016) could translate into alterations of the biogeochemical cycles and the evolution of bacteria in aquatic systems. One of the reasons this can happen is via changes in the genetic exchange between microorganisms that form part of the MP biofilm communities.

Horizontal gene transfer (HGT) in aquatic ecosystems and MP biofilms

There are different mechanisms for HGT, meaning the sharing of genetic material between organisms of different species or not a parent-offspring relationship (Soucy *et al.*, 2015). Among these, conjugation requires direct intercellular exchange while transduction and transformation are a virus-mediated transport and environmental uptake of DNA, respectively (Drudge and Warren, 2012). All of these mechanisms have in common that they give microorganisms the ability to adapt to changing conditions (McDaniel *et al.*, 2012). Also, the incorporation of those changes to their genome causes the evolution of microbial life (Linz *et al.*, 2007).

The attention towards transfer dynamics of MGEs was triggered by their use in genetic engineering to provide applications useful for human life, and because of its role in the problem of antibiotic resistance in pathogenic bacteria (Aminov, 2011). However, with the advent of the "omics" era, there is a reassessment of the definitions of MGEs and their role in ecosystems (Aminov, 2011). Conjugation by plasmids, double-stranded circular or linear DNA molecules capable of autonomous replication (Carattoli *et al.*, 2014), and the ubiquity of integrons, genetic platforms that acquire exogenous genes in the form of mobile gene cassettes (Gillings, 2017), are two types of MGEs studied in this Ph.D. thesis.

The presence of transferable plasmids and conjugation events has been shown to positively affect biofilm formation (Broszat and Grohmann, 2014). Thereby, plasmid-encoded factors are believed to influence biofilm development (Madsen *et al.*, 2012). Biofilms are aggregations of microorganisms that adhere to a solid surface in a matrix composed of extracellular biopolymers (Dickschat, 2010). They may adhere to an inert or biotic surface or exist as free-floating communities. The reasons why biofilm lifestyles offer suitable characteristics for HGT include the high density that allows for contact between cells, and matrix concentration of chemical compounds that induce gene transfer (Aminov, 2011; Sezonov *et al.*, 2007).

Experimental data provide evidence of enhanced conjugative transfer in biofilms vs. free-living bacteria: more transconjugants can be found after mating on a filter compared with mating in liquid culture (Broszat and Grohmann, 2014). HGT via bacterial conjugation is also a major force driving the recent antibiotic resistance spread (Reisner *et al.*, 2012). Nevertheless, there is less detailed information on the extent of HGT in the spatially structured populations in biofilms (Broszat and Grohmann, 2014) and in particular on pelagic particles including MP in aquatic systems.

Differences in diversity between free-living and particle-associated microorganisms have been documented in both limnic and marine environments (Bižić-Ionescu *et al.*, 2015; Rösel *et al.*, 2012). In the particle associated lifestyle, some features are more common such as chemotaxis, as well as the pressure effects of grazing (Tang *et al.*, 2006). Indeed, MP biofilms in marine systems have shown enrichment in genes associated with chemotaxis, frequent cell-to-cell interactions, and nitrogen fixation (Bryant *et al.*, 2016). The concentration of nutrients in particles of organic matter in water (e.g., oceans) can be several orders of magnitude higher than in the surrounding waters and serves as an anchor point for bacteria. Hence, MP particles are an ideal hotspot for gene exchange, since they provide an environment rich in carbon sources and ideal for bacteria to meet and exchange information (Stewart, 2013).

Objectives and study approach

In this Ph.D. thesis gene exchange was analyzed among bacterial communities in MP biofilms and complemented with the study of microbial composition and heterotrophic activities in the plastisphere. These topics were addressed in three scientific publications with the following research questions:

1. **Chapter I**: Does MP provide an ecological niche for wastewater-derived bacteria and the spread of ARGs by HGT?
2. **Chapter II**:
    a. Does MP surface increase HGT frequency in aquatic ecosystems?
    b. Does bacteria in MP biofilms from aquatic ecosystems are more permissive to plasmid transfer than bacteria in the surrounding water?
3. **Chapter III**: Are MP new niches for enhanced heterotrophic activities in aquatic ecosystems?

The thesis aim is to provide evidence that MP represents a new habitat for bacteria, which alters the rates of horizontal gene exchange and heterotrophic activities in freshwater ecosystems. The study comprises bacteria from natural aggregates and the free-living community. The functional features analyzed include **a)** the spread of the integron class 1, a marker of anthropogenic pollution associated with mobile genetic elements, and known as a

proxy for the spread of antimicrobial resistance genes; **b)** the transfer frequency of a self-conjugative plasmid encoding resistance to a given antibiotic substance; and **c)** the functional diversity profile based on the use of different carbon sources by microbial communities on microplastics.

Traditional experiment-based quantification of HGT has limitations such as growth conditions of bacterial species in a model system, scaling and limited capacity to single cell analysis. Detection limits are around one event every 10^9-10^{10} bacteria exposed (Nielsen *et al.*, 2014). Over the last decade, advances in reporter gene technology have provided new insights into the extent and spatial frequencies of HGT *in vitro* and in natural environments (Reisner *et al.*, 2012). Accordingly, a set of different tools like fluorescent-based flow cytometry (FCM) and cell sorting (FACS), next-generation sequencing, qPCR, and metabolic fingerprint methods were used in this thesis. Notably, the use of FCM equipped with FACS and qPCR were chosen because they are suitable alternatives to cultivation-based methods for routine measurement of plasmid persistence and sorting of single bacterial cells, thereby opening avenues for high-throughput analyses (Loftie-Eaton *et al.*, 2014).

Chapter I

Microplastics increase impact of treated wastewater on freshwater microbial community

Eckert, E.M., Di Cesare, A., Kettner, M.T., Arias-Andres, M., Fontaneto, D., Grossart, H.-P., and Corno, G. (2018) Microplastics increase impact of treated wastewater on freshwater microbial community. Environ. Pollut. 234: 495–502. doi:10.1016/j.envpol.2017.11.070.

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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

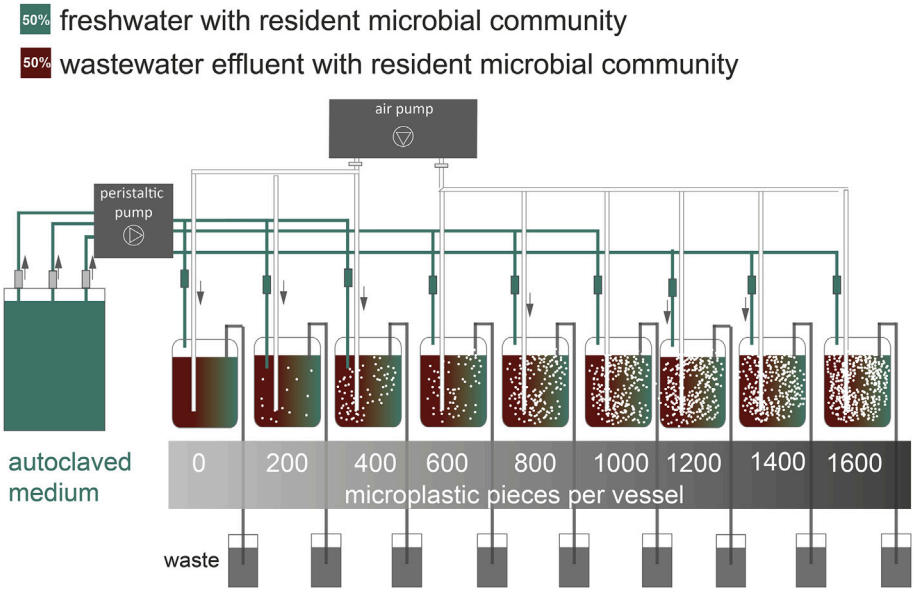

Fig. 1. Schematic representation of the chemostat set-up.

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

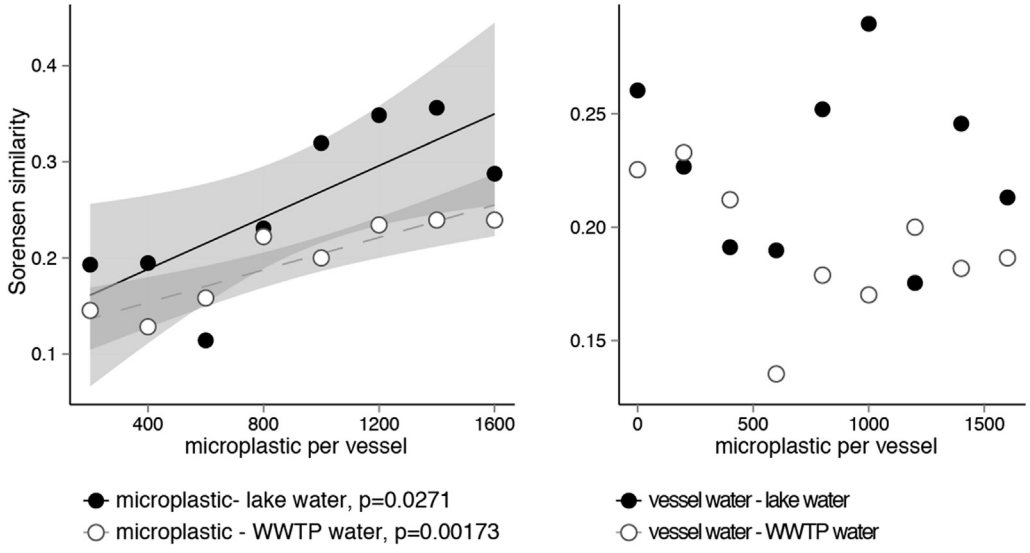

Fig. 2. Relationship between Sorensen similarity of the microbial communities on microplastic (left) and vessel water (right) to the original wastewater and lake water community in dependence of the concentration of microplastic. The regression line, confidence interval and p-values were plotted only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

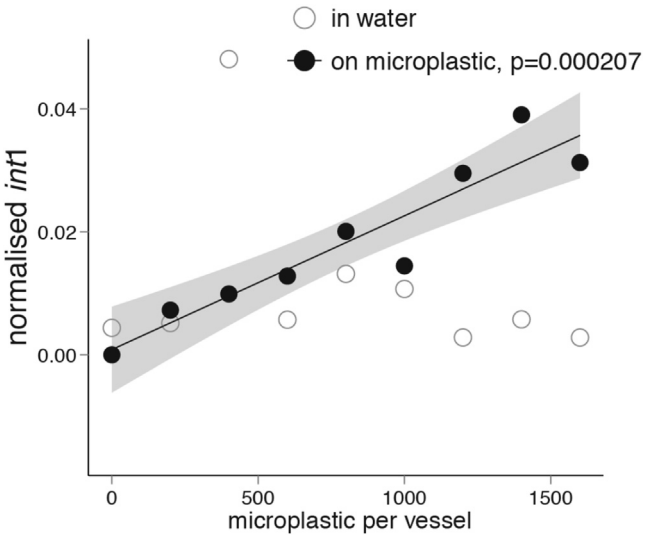

Fig. 3. Relationship between abundance of *int*1 in water (white) and on microplastic (black) with the concentration of microplastic. Abundance values of *int*1 are expressed as arcsin of square root of the proportion between abundance of *int*1 and abundance of 16S rDNA. The regression line, confidence 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.

References

Alberghini, S., Polone, E., Corich, V., Carlot, M., Seno, F., Trovato, A., Squartini, A., 2009. Consequences of relative cellular positioning on quorum sensing and bacterial cell-to-cell communication. FEMS Microbiol. Lett. 292, 149–161.
Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46.
Barnes, D., Milner, P., 2005. Drifting plastic and its consequences for sessile organism dispersal in the Atlantic Ocean. Mar. Biol. 146, 815–825.
Barraud, O., Baclet, M.-C., Denis, F., Ploy, M.-C., 2010. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J. Antimicrob. Chemother. 65, 1642–1645.
Baselga, A., Orme, C.D.L., 2012. betapart: an R package for the study of beta diversity. Methods Ecol. Evol. 3, 808–812.
Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.
Cai, L., Zhang, T., 2013. Detecting human bacterial pathogens in wastewater treatment plants by a high-throughput shotgun sequencing technique. Environ. Sci. Technol. 47, 5433–5441.
Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. Mar. Pollut. Bull. 62, 2588–2597.
Corno, G., Coci, M., Giardina, M., Plechuk, S., Campanile, F., Stefani, S., 2014. Antibiotics promote aggregation within aquatic bacterial communities. Front. Microbiol. 5, 297.
Corno, G., Jürgens, K., 2008. Structural and functional patterns of bacterial communities in response to protist predation along an experimental productivity gradient. Environ. Microbiol. 10, 2857–2871.
Corno, G., Salka, I., Pohlmann, K., Hall, A.R., Grossart, H.-P., 2015. Interspecific interactions drive chitin and cellulose degradation by aquatic microorganisms. Aquat. Microb. Ecol. 76, 27–37.
Corno, G., Villiger, J., Pernthaler, J., 2013. Coaggregation in a microbial predator–prey system affects competition and trophic transfer efficiency. Ecology 94, 870–881.
Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322.
Crawley, M.J., 2013. The R Book Second Edition. John Wiley & Sons.
Di Cesare, A., Eckert, E.M., D'Urso, S., Bertoni, R., Gillan, D.C., Wattiez, R., Corno, G., 2016a. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. Water Res. 94, 208–214.
Di Cesare, A., Eckert, E.M., Teruggi, A., Fontaneto, D., Bertoni, R., Callieri, C., Corno, G., 2015. Constitutive presence of antibiotic resistance genes within the bacterial community of a large subalpine lake. Mol. Ecol. 24, 3888–3900.
Di Cesare, A., Fontaneto, D., Doppelbauer, J., Corno, G., 2016b. Fitness and recovery of bacterial communities and antibiotic resistance genes in urban wastewaters exposed to classical disinfection treatments. Environ. Sci. Technol. 50, 10153–10161.
Di Cesare, A., Luna, G.M., Vignaroli, C., Pasquaroli, S., Tota, S., Paroncini, P., Biavasco, F., 2013. Aquaculture can promote the presence and spread of antibiotic-resistant Enterococci in marine sediments. Plos One 8, e62838.
Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8.
Dris, R., Gasperi, J., Rocher, V., Saad, M., Renault, N., Tassin, B., 2015. Microplastic contamination in an urban area: a case study in Greater Paris. Environ. Chem. 12, 592–599.
Eerkes-Medrano, D., Thompson, R.C., Aldridge, D.C., 2015. Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. Water Res. 75, 63–82.
Eriksen, M., Mason, S., Wilson, S., Box, C., Zellers, A., Edwards, W., Farley, H., Amato, S., 2013. Microplastic pollution in the surface waters of the laurentian great lakes. Mar. Pollut. Bull. 77, 177–182.
Gall, S., Thompson, R., 2015. The impact of debris on marine life. Mar. Pollut. Bull. 92, 170–179.
Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279.
González, J.M., Iriberri, J., Egea, L., Barcina, I., 1992. Characterization of culturability, protistan grazing, and death of enteric bacteria in aquatic ecosystems. Appl. Environ. Microbiol. 58, 998–1004.
Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the Natural environment to infectious diseases. Nat. Rev. Microbiol. 2, 95–108.
Hall, R.M., Collis, C.M., 1995. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol. Microbiol. 15, 593–600.
Keswani, A., Oliver, D.M., Gutierrez, T., Quilliam, R.S., 2016. Microbial hitchhikers on marine plastic debris: human exposure risks at bathing waters and beach environments. Mar. Environ. Res. 118, 10–19.
Kettner, M.T., Rojas-Jimenez, K., Oberbeckmann, S., Labrenz, M., Grossart, H.-P., 2017. Microplastics alter composition of fungal communities in aquatic ecosystems. Environ. Microbiol. 19, 4447–4459.
Kirstein, I.V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., Gerdts, G., 2016. Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic particles. Mar. Environ. Res. 120, 1–8.
Köllner, K.E., Carstens, D., Keller, E., Vazquez, F., Schubert, C.J., Zeyer, J., Bürgmann, H., 2012. Bacterial chitin hydrolysis in two lakes with contrasting trophic statuses. Appl. Environ. Microbiol. 78, 695–704.
Krueger, M.C., Harms, H., Schlosser, D., 2015. Prospects for microbiological solutions to environmental pollution with plastics. Appl. Microbiol. Biotechnol. 99, 8857–8874.
Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2015. Package 'lmerTest'. R Package Version 2.
Law, K.L., 2017. Plastics in the marine environment. Annu. Rev. Mar. Sci. 9, 205–229.
Lehmann, K., Bell, T., Bowes, M.J., Amos, G.C., Gaze, W.H., Wellington, E.M., Singer, A.C., 2016. Trace levels of sewage effluent are sufficient to increase class 1 integron prevalence in freshwater biofilms without changing the core community. Water Res. 106, 163–170.
Lehtola, M.J., Torvinen, E., Kusnetsov, J., Pitkänen, T., Maunula, L., von Bonsdorff, C.-H., Martikainen, P.J., Wilks, S.A., Keevil, C.W., Miettinen, I.T., 2007. Survival of Mycobacterium avium, Legionella pneumophila, Escherichia coli, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. Appl. Environ. Microbiol. 73, 2854–2859.
Lenz, R., Enders, K., Nielsen, T.G., 2016. Microplastic exposure studies should be environmentally realistic. Proc. Natl. Acad. Sci. 113, E4121–E4122.
Leslie, H., Brandsma, S., van Velzen, M., Vethaak, A., 2017. Microplastics en route: field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota. Environ. Int. 101, 133–142.
Li, W., Tse, H., Fok, L., 2016. Plastic waste in the marine environment: a review of sources, occurrence and effects. Sci. Total Environ. 566, 333–349.
Luna, G.M., Dell'Anno, A., Danovaro, R., 2006. DNA extraction procedure: a critical issue for bacterial diversity assessment in marine sediments. Environ. Microbiol. 8, 308–320.
Ma, L., Li, A.-D., Yin, X.-L., Zhang, T., 2017. The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments. Environ. Sci. Technol. 51, 5721–5728.

Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K., Stenström, T., 1993. In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. 59, 2293–2298.
McCafferty, J., Reid, R., Spencer, M., Hamp, T., Fodor, A., 2012. Peak Studio: a tool for the visualization and analysis of fragment analysis files. Environ. Microbiol. Rep. 4, 556–561.
Mincer, T.J., Zettler, E.R., Amaral-Zettler, L.A., 2016. Biofilms on plastic debris and their influence on marine nutrient cycling, productivity, and hazardous chemical mobility. In: The Handbook of Environmental Chemistry. Springer, Berlin, Heidelberg.
Mintenig, S., Int-Veen, I., Löder, M.G., Primpke, S., Gerdts, G., 2017. Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. Water Res. 108, 365–372.
Oberbeckmann, S., Löder, M.G., Labrenz, M., 2015. Marine microplastic-associated biofilms–a review. Environ. Chem. 12, 551–562.
Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J., Suggests, M., 2007. The vegan package. Community Ecol. package 10.
Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290.
PlasticsEurope, 2015. An Analysis of European Plastics Production, Demand and Waste Data.
Ramette, A., 2009. Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. Appl. Environ. Microbiol. 75, 2495–2505.
RCore Team, 2013. R: a Language and Environment for Statistical Computing (Vienna, Austria).
RStudio Team, 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. http://www.rstudio.com/.
Stalder, T., Barraud, O., Jové, T., Casellas, M., Gaschet, M., Dagot, C., Ploy, M.-C., 2014. Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool. ISME J. 8, 768–777.
Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl. Environ. Microbiol. 66, 4605–4614.
Suzuki, S., Pruden, A., Virta, M., Zhang, T., 2017. Antibiotic resistance in aquatic systems. Front. Microbiol. 8, 14.
Van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B.D., Van Franeker, J.A., Eriksen, M., Siegel, D., Galgani, F., Law, K.L., 2015. A global inventory of small floating plastic debris. Environ. Res. Lett. 10, 124006.
Wagner, M., Scherer, C., Alvarez-Muñoz, D., Brennholt, N., Bourrain, X., Buchinger, S., Fries, E., Grosbois, C., Klasmeier, J., Marti, T., 2014. Microplastics in freshwater ecosystems: what we know and what we need to know. Environ. Sci. Eur. 26, 12.
Wanjugi, P., Harwood, V.J., 2013. The influence of predation and competition on the survival of commensal and pathogenic fecal bacteria in aquatic habitats. Environ. Microbiol. 15, 517–526.
Wéry, N., Lhoutellier, C., Ducray, F., Delgenès, J.-P., Godon, J.-J., 2008. Behaviour of pathogenic and indicator bacteria during urban wastewater treatment and sludge composting, as revealed by quantitative PCR. Water Res. 42, 53–62.
Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. Springer Science & Business Media.
Wickham, H., 2012. reshape2: Flexibly Reshape Data: a Reboot of the Reshape Package. R package version 1.
Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the "plastisphere": microbial communities on plastic marine debris. Environ. Sci. Technol. 47, 7137–7146.
Zotina, T., Köster, O., Jüttner, F., 2003. Photoheterotrophy and light-dependent uptake of organic and organic nitrogenous compounds by Planktothrix rubescens under low irradiance. Freshw. Biol. 48, 1859–1872.

Chapter II

Microplastic pollution increases gene exchange in aquatic ecosystems

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Microplastic pollution increases gene exchange in aquatic ecosystems☆

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

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

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

2.1. Strains and culturing

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

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

2.2. Microplastic particles

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

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

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

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

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

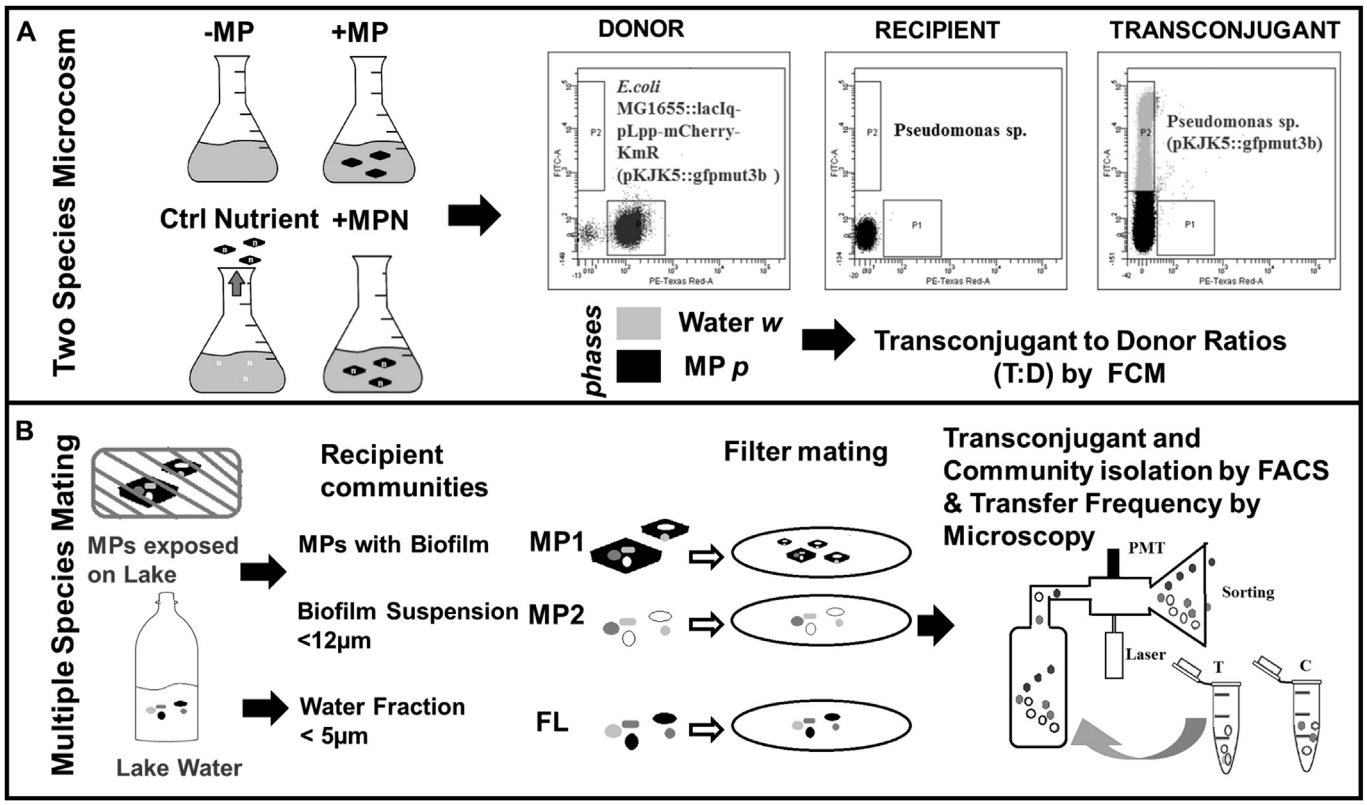

Fig. 1. Experimental design. A) Two Species Microcosm. Treatments without and with microplastics are indicated by -MP and +MP, respectively. Treatment of microplastics pre-exposed to organic matter (+MPN) and a control for nutrient desorption (Ctrl Nutrient) were included. The detection of the donor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

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

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

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

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

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

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

2.5. Molecular and sequence analyses

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

2.6. Data and statistical analyses

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

3. Results

3.1. Experiment 1: two-species microcosm

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

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

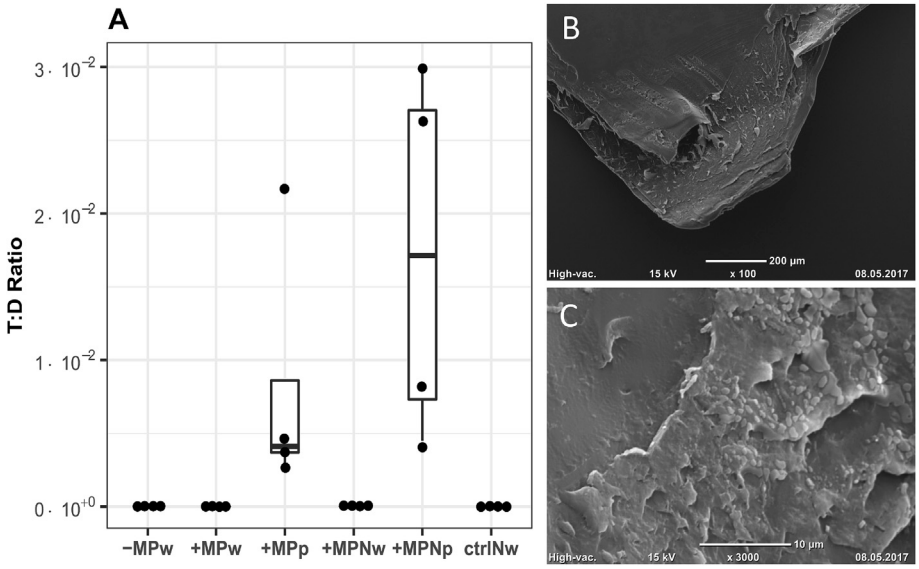

Fig. 2. Results of Two-species microcosm. A) Box plots and dots represent the Transconjugant to Donor ratios (T:D) from four independent flask replicates of bacteria in: i) water phase of treatments without microplastics (-MPw), ii) water and particle phases in treatments with microplastics (+MPw and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

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

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

3.2. Experiment 2: multiple species mating

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

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

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

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

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

4. Discussion

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

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

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

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

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

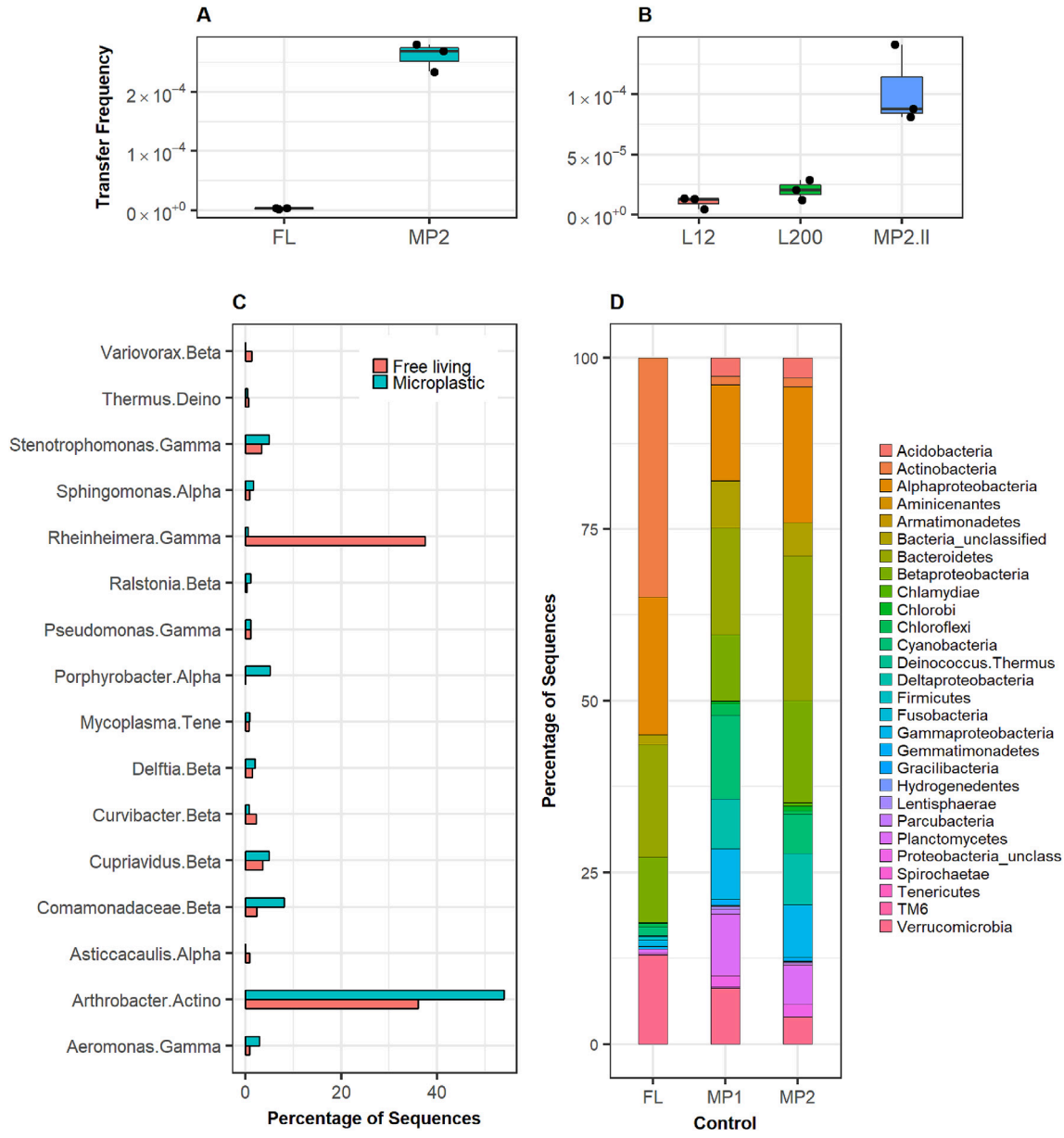

Fig. 3. Results of multiple species matings. Box plots and dots compare the frequency of transfer events from triplicate filter matings with A) free-living bacteria (FL) and microplastic-associated bacteria (MP2) and B) water fractions < 12 μm and <200 μm (L12 and L200, respectively) and microplastic-associated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

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

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

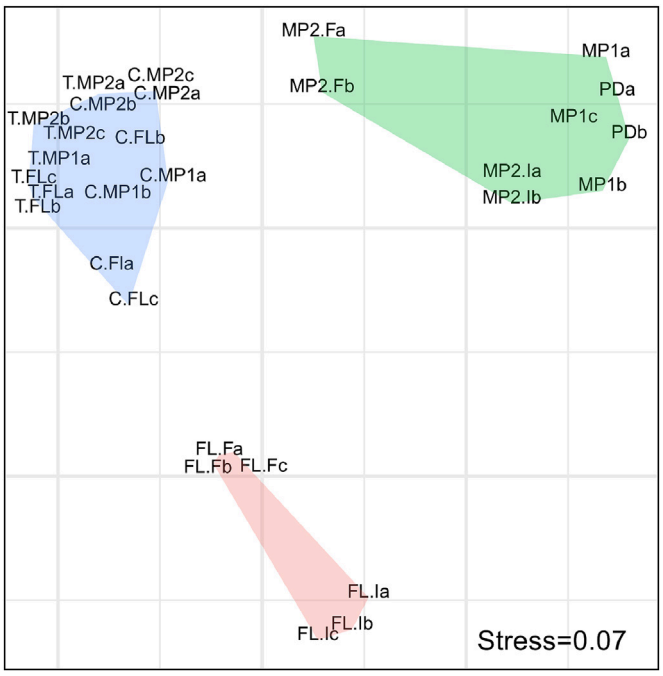

Fig. 4. Non-metric multidimensional scaling plot (nMDS) of samples analyzed by 16S rRNA gene metabarcoding. Samples include: FL = free-living bacteria, MP1 = biofilm on the microplastic particles, MP2 = suspension of microplastic biofilm bacteria, PD = particles post-detachment of MP2. Letters C and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

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

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

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

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

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

5. Conclusions

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

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

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

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

References

Aminov, R.I., 2011. Horizontal gene exchange in environmental microbiota. Front. Microbiol. 2, 158.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., et al., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522.
Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. 303, 298–304.
Carson, H.S., Nerheim, M.S., Carroll, K.a., Eriksen, M., 2013. The plastic-associated microorganisms of the North Pacific Gyre. Mar. Pollut. Bull. 75, 126–132.
Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. Mar. Pollut. Bull. 62, 2588–2597.
Czekalski, N., Berthold, T., Caucci, S., Egli, A., Bürgmann, H., 2012. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into lake Geneva, Switzerland. Front. Microbiol. 3, 1–18.
Drudge, C.N., Warren, L.A., 2012. Prokaryotic horizontal gene transfer in freshwater lakes: implications of dynamic biogeochemical zonation. J. Environ. Prot. (Irvine, Calif) 3, 1634–1654.
Duis, K., Coors, A., 2016. Microplastics in the aquatic and terrestrial environment: sources (with a specific focus on personal care products), fate and effects. Environ. Sci. Eur. 28, 2.
Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.
Free, C.M., Jensen, O.P., Mason, S.A., Eriksen, M., Williamson, N.J., Boldgiv, B., 2014. High-levels of microplastic pollution in a large, remote, mountain lake. Mar. Pollut. Bull. 85, 156–163.
Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3 e1700782.
Grossart, H.-P., Kiorboe, T., Tang, K., Ploug, H., 2003. Bacterial colonization of particles: growth and interactions. Appl. Environ. Microbiol. 69, 3500–3509.
Grossart, H.-P., Thorwest, M., Plitzko, I., Brinkhoff, T., Simon, M., Zeeck, A., 2009. Production of a blue pigment (Glaukothalin) by marine Rheinheimera spp. Int. J. Microbiol. 2009, 1–7.
Heuer, H., Ebers, J., Weinert, N., Smalla, K., 2010. Variation in permissiveness for broad-host-range plasmids among genetically indistinguishable isolates of Dickeya sp. from a small field plot. FEMS Microbiol. Ecol. 73, 190–196.
Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., et al., 2011. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. Mar. Pollut. Bull. 62, 1683–1692.
Jahnke, A., Arp, H.P.H., Escher, B.I., Gewert, B., Gorokhova, E., Kühnel, D., et al., 2017. Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment. Environ. Sci. Technol. Lett. 4, 85–90.
Keswani, A., Oliver, D.M., Gutierrez, T., Quilliam, R.S., 2016. Microbial hitchhikers on marine plastic debris: human exposure risks at bathing waters and beach environments. Mar. Environ. Res. 118, 10–19.
Kesy, K., Oberbeckmann, S., Müller, F., Labrenz, M., 2016. Polystyrene influences bacterial assemblages in Arenicola marina-populated aquatic environments in vitro. Environ. Pollut. 219, 219–227.
Kettner, M.T., Rojas-Jimenez, K., Oberbeckmann, S., Labrenz, M., Grossart, H.-P., 2017. Microplastics alter composition of fungal communities in aquatic ecosystems. Environ. Microbiol. 19, 4447–4459.
Kirstein, I.V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., et al., 2016. Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic particles. Mar. Environ. Res. 120, 1–8.
Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gülay, A., Sørensen, S.J., et al., 2017. Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner. ISME J. 11, 152–165.
Klümper, U., Dechesne, A., Smets, B.F., 2014. Protocol for evaluating the permissiveness of bacterial communities toward conjugal plasmids by quantification and isolation of transconjugants. In: Hydrocarbon and Lipid Microbiology Protocols - Springer Protocols Handbooks, pp. 275–288.
Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., et al., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945.
Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120.
Law, K.L., Thompson, R.C., 2014. Microplastics in the seas. Science (80) 345, 144–145.
Madsen, J.S., Riber, L., Kot, W., Basfeld, A., Burmølle, M., Hansen, L.H., et al., 2016. Type 3 fimbriae encoded on plasmids are expressed from a unique promoter without affecting host motility, facilitating an exceptional phenotype that enhances conjugal plasmid transfer. In: Forestier, C. (Ed.), PLoS One 11 e0162390.
McCormick, A., Hoellein, T.J., Mason, S.A., Schluep, J., Kelly, J.J., 2014. Microplastic is an abundant and distinct microbial habitat in an urban river. Environ. Sci. Technol. 48, 11863–11871.
McCormick, A.R., Hoellein, T.J., London, M.G., Hittie, J., Scott, J.W., Kelly, J.J., 2016. Microplastic in surface waters of urban rivers: concentration, sources, and associated bacterial assemblages. Ecosphere 7 e01556.
Musovic, S., Oregaard, G., Kroer, N., Sorensen, S.J., 2006. Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among Gram-Positive and Gram-Negative bacteria indigenous to the barley rhizosphere. Appl. Environ. Microbiol. 72, 6687–6692.
Naz, I., Hodgson, D., Smith, A., Marchesi, J., Ahmed, S., Avignone-Rossa, C., et al., 2016. Effect of the chemical composition of filter media on the microbial community in wastewater biofilms at different temperatures. RSC Adv. 6, 104345–104353.
Oberbeckmann, S., Osborn, A.M., Duhaime, M.B., 2016. Microbes on a bottle: substrate, season and geography influence community composition of microbes colonizing marine plastic debris. In: Carter, D.A. (Ed.), PLoS One 11 e0159289.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al., 2016. Vegan: Community Ecology Package. R-package version 2.4-0.
Pruesse, E., Peplies, J., Glöckner, F.O., 2012. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28, 1823–1829.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596.
R-Core-Team, 2017. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Ribeiro, M., Monteiro, F.J., Ferraz, M.P., 2012. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. Biomatter 2, 176–194.
Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. In: Hrbek, T. (Ed.), PeerJ 4 e2584.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.
Schuster, A.-K., Szewzyk, U., 2016. Draft genome sequence of Rheinheimera sp. F8, a biofilm-forming strain which produces large amounts of extracellular DNA. Genome Announc. 4 e00082-16.
Selinummi, J., Seppälä, J., Yli-Harja, O., Puhakka, J., 2005. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 39, 859–863.
Setälä, O., Fleming-Lehtinen, V., Lehtiniemi, M., 2014. Ingestion and transfer of microplastics in the planktonic food web. Environ. Pollut. 185, 77–83.
Sezonov, G., Joseleau-Petit, D., D'Ari, R., 2007. Escherichia coli physiology in Luria-bertani broth. J. Bacteriol. 189, 8746–8749.
Smalla, K., Top, E.M., Jechalke, S., 2015. Plasmid detection, characterization, and ecology. Microbiol. Spectr. 3 https://doi.org/10.1128/microbiolspec.PLAS-0038-2014.
De Tender, C.A., Devriese, L.I., Haegeman, A., Maes, S., Ruttink, T., Dawyndt, P., 2015. Bacterial community profiling of plastic litter in the belgian Part of the North sea. Environ. Sci. Technol. 49, 9629–9638.
Viršek, M.K., Lovšin, M.N., Koren, Š., Kržan, A., Peterlin, M., 2017. Microplastics as a vector for the transport of the bacterial fish pathogen species Aeromonas salmonicida. Mar. Pollut. Bull. 125, 301–309.
Westcott, S.L., Schloss, P.D., 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. In: Eren, A.M. (Ed.), PeerJ 3 e1487.
World Economic Forum and Ellen MacArthur Foundation, 2017. The New Plastics Economy – Catalysing Action. http://www.ellenmacarthurfoundation.org/publications.
Zalasiewicz, J., Waters, C.N., Ivar do Sul, J.A., Corcoran, P.L., Barnosky, A.D., Cearreta, A., et al., 2016. The geological cycle of plastics and their use as a stratigraphic indicator of the anthropocene. Anthropocene 13, 4–17.

Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the 'Plastisphere': microbial communities on plastic marine debris. Environ. Sci. Technol. 47, 7137–7146.
Zhang, M., Pereira e Silva, M.D.C., Chaib De Mares, M., van Elsas, J.D., 2014. The mycosphere constitutes an arena for horizontal gene transfer with strong evolutionary implications for bacterial-fungal interactions. FEMS Microbiol. Ecol. 89, 516–526.
Ziajahromi, S., Neale, P.A., Leusch, F.D.L., 2016. Wastewater treatment plant effluent as a source of microplastics: review of the fate, chemical interactions and potential risks to aquatic organisms. Water Sci. Technol. 74, 2253–2269.

Chapter III

Microplastics: New substrates for heterotrophic activity contribute to altering organic matter cycles in aquatic ecosystems

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Microplastics: New substrates for heterotrophic activity contribute to altering organic matter cycles in aquatic ecosystems

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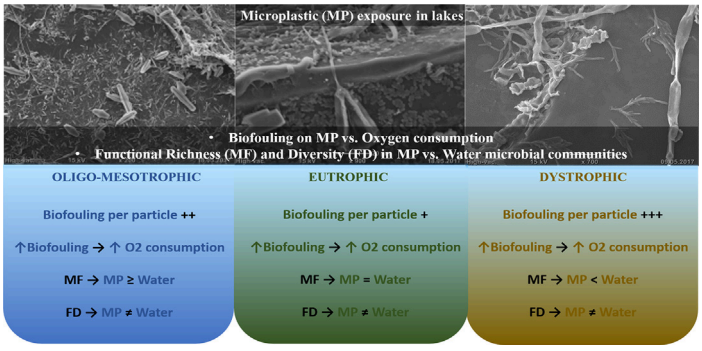
HIGHLIGHTS

- Microbial activities in microplastic (MP) biofilms are poorly described
- Pronounced biofouling on MP in oligo-mesotrophic and dystrophic lakes
- Microbial functional diversity on MP differs from water regardless of nutrients
- Multi-functionality of MP microorganisms is affected by local conditions

GRAPHICAL ABSTRACT



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ABSTRACT

Heterotrophic microbes with the capability to process considerable amounts of organic matter can colonize microplastic particles (MP) in aquatic ecosystems. Weather colonization of microorganisms on MP will alter ecological niche and functioning of microbial communities remains still unanswered. Therefore, we compared the functional diversity of biofilms on microplastics when incubated in three lakes in northeastern Germany differing in trophy and limnological features. For all lakes, we compared heterotrophic activities of MP biofilms with those of microorganisms in the surrounding water by using Biolog® EcoPlates and assessed their oxygen consumption in microcosm assays with and without MP. The present study found that the total biofilm biomass was higher in the oligo-mesotrophic and dystrophic lakes than in the eutrophic lake. In all lakes, functional diversity profiles of MP biofilms consistently differed from those in the surrounding water. However, solely in the oligo-mesotrophic lake MP biofilms had a higher functional richness compared to the ambient water. These results demonstrate that the functionality and hence the ecological role of MP-associated microbial communities are context-dependent, i.e. different environments lead to substantial changes in biomass build up and heterotrophic activities of MP biofilms. We propose that MP surfaces act as new niches for aquatic microorganisms and that the constantly increasing MP pollution has the potential to globally impact carbon dynamics of pelagic environments by altering heterotrophic activities.

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1. Introduction

It is estimated that 6300 million metric tons of plastic waste produced by the end of 2015 ended up in aquatic ecosystems, and even more alarming is the prediction that plastic pollution may double by 2050 (Geyer et al., 2017). Fragmentation of plastic waste into microplastic particles (<5 mm, MP) is of great concern since they interact with organisms at the base of aquatic food webs (Law and Thompson, 2014) or with higher trophic levels (P.C.H. Hollman et al., 2013; Barboza et al., 2018). Microplastics are present in all oceans and recent data indicate they are also ubiquitous in rivers, lakes, reservoirs, and other types of inland water systems (Lambert and Wagner, 2018).

Microplastics, released into the environment in such enormous quantities, provide new surfaces for microbial attachment and biofilm formation. These are the prokaryotic and eukaryotic communities on MP, commonly referred to as the "plastisphere". Although critically important for the health of the aquatic ecosystems, they have been largely overlooked. Some studies have shown them to be diverse and distinct from those in the surrounding water (Zettler et al., 2013; De Tender et al., 2017; McCormick et al., 2014). In addition, MP can act as vectors for the transport of exogenous microbial groups to natural ecosystems. For instance, it was experimentally demonstrated that growing amounts of MP favor the survival of wastewater-derived bacteria (Eckert et al., 2018). Many studies indicate that composition of microbial communities on (micro)plastics in marine and freshwater ecosystems significantly differs from that of the ambient water, or from those on natural aggregates and other substrates (Oberbeckmann et al., 2016; Kettner et al., 2017; Oberbeckmann et al., 2018; Reisser et al., 2014; Hoellein et al., 2014).

Yet, it is not clear to what extent variations in microbial community composition on MP translate to differences in microbial functionality, e.g., activities such as the degradation of organic matter and its transfer to higher trophic levels. Heterotrophic microorganisms play an important role in the mineralization of organic material in aquatic ecosystems (Pernthaler and Amann, 2005) and surface-attached microorganisms, in particular, are a key to understand microbial organic matter cycling (Grossart, 2010; Hunter et al., 2016). Especially, surface-attached bacterial communities in pelagic and benthic zones form versatile metabolic cooperation networks and allow for increased horizontal gene transfer (Kesy et al., 2017). Biofilms are recognized as metabolic hotspots and major sites of dissolved organic carbon (DOM) degradation (Sabater et al., 2011). A pioneering study in marine environments by Bryant et al. (2016) observed an increased abundance of genes involved in xenobiotics degradation in MP biofilms compared to the surrounding water. Consequently, the introduction of MP colonized by diverse and distinct microbial communities may result in altered dynamics of carbon processing in any aquatic ecosystem.

The present study hypothesizes that MP serve as new niches and enhance heterotrophic activities in aquatic ecosystems. Accordingly, we propose the presence of MP leads to changes in the functional diversity of heterotrophs in aquatic ecosystems. In order to test this hypothesis, we examined the heterotrophic activities of microbial communities on MP incubated in three temperate lakes with different trophic statuses and limnological features.

We compared the microbial functional richness, the diversity of carbon substrates utilized, as well as the oxygen consumption, between MP biofilms and microorganisms in the surrounding water. In addition, we estimated the total biomass per particle and examined the surface of individual particles by using scanning electron microscopy (SEM). Our aim is to target the rarely studied aspects of microbial functionality on MP, and their possible ecological consequences for carbon cycling in aquatic ecosystems.

2. Materials and methods

2.1. Lake descriptions and metadata

Microplastics incubation and water sampling were conducted in three temperate lakes of different trophic statuses and limnological features: the oligo-mesotrophic Lake Stechlin (S, coordinates 53°08′36.3″N 13°01′47.4″E), the dystrophic southwest basin of Lake Grosse Fuchskuhle (F, coordinates 53°06′20.3″N 12°59′05.3″E) and the shallow eutrophic Lake Dagow (D, coordinates 53°09′04.7″N 13°03′07.7″E). All lakes are located in Brandenburg, northeast Germany and are representatives of common lake types of temperate regions. Details on their physical-chemical characteristics can be found in Allgaier and Grossart (2006). Temperature, pH, oxygen, and conductivity were measured in the water during sampling with a multiparameter probe YSI 6600 (YSI incorporated, Yellowsprings, USA), and samples were taken in triplicates for analysis of DOC, total N, and total P concentrations.

2.2. Microplastics and sampling

Additive-free and clean polystyrene films were obtained from Norflex® (Nordenham, Germany). The material was cut with a metal multiple punch maker (RW home, Renz, Germany) to produce 4 mm × 4 mm × 0.1 mm squared particles. Microplastics were incubated in the lakes inside seven stainless steel cylinder cages (mesh size of 3 mm, 25 cm length and 10 cm diameter) with respectively 1000, 2000, 4000, 6000, 8000, 10,000 and 12,000 particles. The cages were placed in Lake Stechlin at 5 m depth, in Lake Grosse Fuchskuhle at 0.1 m depth and in Lake Dagow at 2.5 m depth at the end of July 2015. Depths correspond to a similar light intensity of 200–250 μmol m^{-2} s^{-1} measured with a spherical quantum sensor (LI-COR spherical SPQA1307, USA) on a sunny day in July. Every 2 weeks, cages were cleaned on the outside with a brush and MP samples were finally retrieved in November 2015. Cages were opened and plastic pieces were placed on sieves in plastic trays and always kept wet with water from the respective lake and depth. Pieces were stored in sterile 50 mL falcon tubes for transportation to the lab using coolers with ice packs. At the same time, triplicates of 1 L of water from the respective incubation depth were taken with a vertical point sampler and transported in coolers to the lab.

2.3. SEM analysis of biofilms on microplastics

For each lake, samples of MP from cages with 1000, 6000 and 12,000 particles were fixed with 4% formaldehyde and stored at −20 °C for later microscopy. Scanning electron microscopy of MP was performed with a JEOL-6000 instrument (JEOL). Samples were prepared by 60 s sputter time with Gold Palladium (Brunk et al., 1981).

2.4. Total biomass quantification

To estimate the total biomass per MP particle, we used the crystal violet adsorption/desorption method, for which both living and dead cells, as well as extracellular polymeric substances (EPSs) from the biofilm, are stained (Azeredo et al., 2017). For each cage, 10 particles were separated and dried at 60 °C for 60 min for biofilm fixation (Kwasny and Opperman, 2010). One particle per well was placed on a multiple deep-well plate. A solution of 0.3% Crystal Violet stain (Merck) was added to each well and incubated for 15 min. The solution was removed with a pipette and the wells were rinsed with distilled water for 4 times to remove excess crystal violet. Then 1 mL of 95% ethanol was added to each well and mixed gently by pipetting. After 10 min, the liquid was transferred to a separate multiple well plate for optical density estimation at 600 nm (OD_{600}nm) with a Synergy™ 2 (Biotek) multiplate reader.

2.5. Oxygen consumption microcosms

For each lake, 50 MP particles from each cage were transferred into 50 mL glass bottles and filled with water from the lake. Triplicate bottles were prepared with MP from each cage as well as for the only-water control (0). This was done during the first hours after sample collection. Bottles were sealed gas-tight with rubber lids and a crimp top, avoiding any air bubbles. Oxygen was recorded on day 0 and day 8 with a micro fiber optic oxygen meter (Microx TX3, PreSens, Germany). During the entire time, bottles were incubated in the dark at 10 °C. These bottles containing MP and water, or only water, are herein called microcosms.

2.6. Biolog® EcoPlate inoculation

The functional profiles of MP biofilms and microbial communities in the ambient water were determined by incubation in Biolog® EcoPlates. These 96-well plates contain 31 different lyophilized carbon substrates in triplicates and a redox indicator that changes color upon substrate respiration. Three wells without added carbon are also included as a control. The intensity of color change is proportional to the amount of substrate utilized. The list of the 31 carbon substrates is available in the SI. Samples consisted of MP from cages with 6000 particles from each lake, as well as un-filtrated lake water (W) or filtered by 5 μm to obtain the free-living fraction (FL) by removing natural aggregates. Briefly, individual particles (MP) were carefully taken with tweezers, washed by dipping on 0.2 μm filtered water from the lake and placed in individual wells of the EcoPlate. The wells were further filled with 100 μL of 0.2 μm filtered water. Parallel to this, wells of the other plates were filled with 100 μL of un-filtrated lake water (W) or 100 μL of 5 μm-filtrated lake water (FL), respectively. For each sample type (MP, W, FL), two EcoPlates were prepared to result in 6 individual-well replicates per substrate/sample. The plates were incubated in the dark at 10 °C, in a humid chamber to avoid any desiccation. After 6 days, the absorbance of each well was determined with the multiplate reader at 595 nm.

2.7. Data analysis

All statistics and data visualization were conducted with R version 3.4.1 (2017) using RStudio (version 1.0.153 – © 2009–2017 RStudio, Inc.). The absorbance readings from the crystal violet assay (OD_{600}nm) were introduced in a generalized linear model (GLM (RCoreTeam, 2013)), assuming a Gaussian distribution, to test if the estimated total biomass was related with the number of MP in the cages. In addition, the OD_{600}nm absorbance values from different cages were compared by a Kruskal-Wallis test followed by multiple comparisons with Dunn's test, using a probability of $p = 0.05$ and the Benjamini-Hochberg method for *p*-adjustment (Benjamini and Hochberg, 1995) using program "dunn.test" (Dinno, 2017).

The total oxygen consumption within 8 days (mg L^{-1} of O_2) in each microcosm was obtained by subtracting the O_2 concentration in each bottle at day 8 to its initial concentration (mg L^{-1} day 0–mg L^{-1} day 8). The values obtained in bottles with MP were compared to those from the control with only water (0) using a multiple comparison test after Kruskal-Wallis and the function *kruskalmc* "one-tailed" test from program "pgirmess" with a probability of $p = 0.05$ (Giraudoux, 2017). The total oxygen consumed per microcosm was introduced in a generalized linear model (GLM) together with the OD_{600}nm describing the biomass per particle derived from the crystal violet assay. For each lake, a Gaussian family distribution of the oxygen data was assumed, after applying the modified Park Test with the function *park* from package "LDdiag" (Yongmei, 2012). As a goodness-of-fit of the model, the amount of deviance accounted for (adjusted D-squared) was calculated with function *Dsquare* from "modEvA" package (Barbosa et al., 2016).

For each lake, we calculated the richness of functions and determined the functional diversity profile of samples from: a) individual particles (MP) b) the water (W) or c) the free-living bacterial community (FL), using the readings from the EcoPlates. The OD_{595}nm values were first normalized by subtracting the measurements of the control with no carbon substrate. To address the richness of functions we calculated a multi-functionality index (MF = number of substrates utilized). We used a quantile-thresholding method to define if the absorbance at 595 nm measured in the EcoPlate can be classified as presence (1) or absence (0) of carbon substrate metabolism (Byrnes et al., 2014; Miki et al., 2017). This resulted in matrixes of MF values for each sample type (MP, W, and FL) and threshold (0.1 to 0.9). The MF indexes of the samples per threshold were compared first by Kruskal-Wallis test, followed by multiple comparisons with Dunn's Test at $p = 0.05$. The MF values obtained per each sample from all thresholds were also compared by using this test. To analyze the functional diversity profiles, a dissimilarity matrix for each sample was created from the OD_{595}nm measurements of the EcoPlate, using a Bray-Curtis method (Miki et al., 2017). These matrices were compared by permutational multivariate analysis of variance (Permanova) at a probability value of 0.05 and with 999 permutations using the function *adonis* from "vegan" package (Oksanen et al., 2017). *p*-Values from pairwise comparisons were adjusted according to the Benjamini-Hochberg method. Dissimilarity matrices were visualized in a non-metric multidimensional scaling plot using "ggplot2" package (Wickham, 2009).

3. Results

3.1. Microplastic particle colonization depends on the environment

Physical and chemical (including nutrients) characteristics of the three lakes during sampling are summarized in Table 1. Visual inspection already showed that MP from cages with lower particle numbers had a higher biomass. Colonization of MP by green algae was observed in cages from all lakes, but especially noticeable in biofilms from Lake Grosse Fuchskuhle. In this lake, larvae of *Trichoptera* sp. built cases with MP (Fig. S1A). Observation by SEM (Fig. 1) revealed the presence of filaments, bacteria and diatom-like structures in MP biofilms from all lakes. MP biofilms from the oligo-mesotrophic Stechlin (Fig. 1A) and dystrophic brown lake Grosse Fuchskuhle (Fig. 1C) showed dense autotrophic growth (diatoms and green algae structures, Fig. S1B and C). In the eutrophic Lake Dagow, dense bacteria-sized structures frequently occurred directly on the MP surface with numerous diatoms and small filter-feeders (e.g. stalked ciliates) (Fig. 1B).

Table 1
Classification of lakes by trophic status, physical parameters and nutrient concentrations in water samples.

Parameter (unit)	Lake		
	Stechlin	Dagow	Grosse Fuchskuhle SW
Trophy	Oligo-mesotrophic	Eutrophic	Dystrophic
Sampling depth (m)[a]	5	2.5	0.1
Sampling date	Nov 11, 2015	Nov 17, 2015	Nov 24, 2015
pH	8.3	7.8	4.5
Oxygen (mg L^{-1}) (temperature)	11.2 (10.2 °C)	9.4 (8.98 °C)	5.3 (5.65 °C)
Conductivity (uS/cm)	268	559	46
DOC (mg L^{-1})[b]	3.8 ± 0.7	12.5 ± 0.2	34.0 ± 0.2
Total N (mg L^{-1})[b]	0.4 ± 0.0	1.4 ± 0.0	1.8 ± 0.0
Total P (mg L^{-1})[b]	9 ± 2	35 ± 2	62 ± 2

[a] Depth for deployment of cages and water sampling.
[b] Dissolved Organic Carbon results represent mean ± sd of 3 independent water samples.

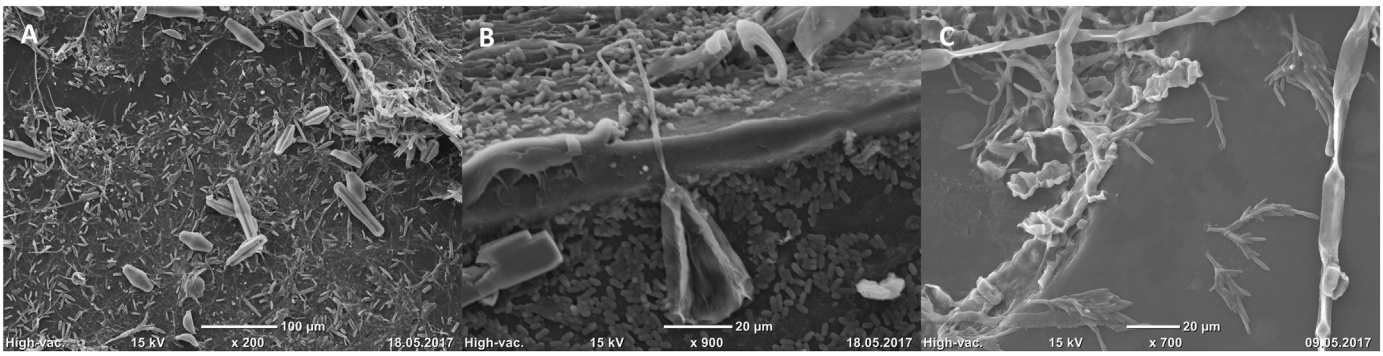

Fig. 1. Scanning electron microscopy images of MP surfaces after three months exposure. MP from A) oligo-mesotrophic Lake Stechlin showing dense colonization with diatoms B) eutrophic Lake Dagow presented dense bacteria-like structure on the surface of MP and bacterivorous ciliates, together with diatoms, and C) dystrophic Lake Grosse Fuchskuhle showed more dense growth by green algae.

3.2. Total biomass per MP particles increases O2 consumption in lake microcosms

There were significant differences in the estimates of total biomass (OD_{600}nm of the crystal violet assay) among the three differing lakes (Kruskal-Wallis chi-squared = 102.6, df = 2, $p < 0.05$). The OD_{600}nm was higher for Lake Grosse Fuchskuhle, followed by Lake Stechlin and lower for Lake Dagow (multiple comparisons by Dunn's test, $p < 0.05$). In all lakes, increased MP number per cage resulted in a lower biomass per particle (Fig. 2; Table S1). High variations among and within cages were observed for samples from Lakes Stechlin and Dagow (Fig. 2; Table S1). The oxygen concentration decreased in all bottle microcosms during the incubation time of 8 days (Fig. S2) indicating active microbial respiration. The magnitude of this reduction differed among the three lakes ranging from 4 ± 1 mg L^{-1} O_2 in Lake Dagow to 2 ± 2 mg L^{-1} O_2 in Lake Stechlin and 1.4 ± 0.6 mg L^{-1} O_2 in Lake Grosse Fuchskuhle (Kruskal-Wallis chi-squared = 33. 6, df = 2, p-value = 5.2e−08, multiple comparisons by Dunn's test).

For each lake, significant differences in oxygen consumption were found among microcosms with particles from different cages (Kruskal-Wallis, Table S2). Microcosms with a higher total biomass per particle consumed more oxygen. This was corroborated in a generalized linear model for each lake, where the biomass per particle explained differences in total oxygen consumption (GLM, family = Gaussian $p < 0.05$). The adjusted D^2 (the equivalent of a R^2 in a least square model) was 0.84 (p = 2.17e−09), 0.31 (p = 0.003) and 0.68 (p = 1.63e−06) for Lakes Stechlin, Dagow and Grosse Fuchskuhle, respectively.

3.3. Functional richness and diversity of microplastic biofilms differs from water

The respiration of carbon substrates, indicated by the optical density measured after 6 days in the EcoPlates (Fig. S3), was in general highest for samples of Lake Grosse Fuchskuhle (0.3 ± 0.4) followed by those of Lake Dagow (0.1 ± 0.2), and lowest for those of Lake Stechlin (0.07 ± 0.20) (mean OD_{595}nm ± sd of the biofilm (MP), un-filtered water (W) and free-living microbial fraction (FL) of each lake; Kruskal-Wallis chi-squared = 257.47, p-value ≤0.001). For each lake, differences among OD_{595}nm values were also found among MP, W and FL (Kruskal-Wallis, $p < 0.05$, Fig. S3).

Besides these differences in the intensity of carbon substrate turnover, MP and water samples (W and FL) also presented differences in their metabolic richness, expressed as the multi-functionality index, MF (i.e. the number of different carbon substrates metabolized in the EcoPlates according to quantile-based thresholds). These differences

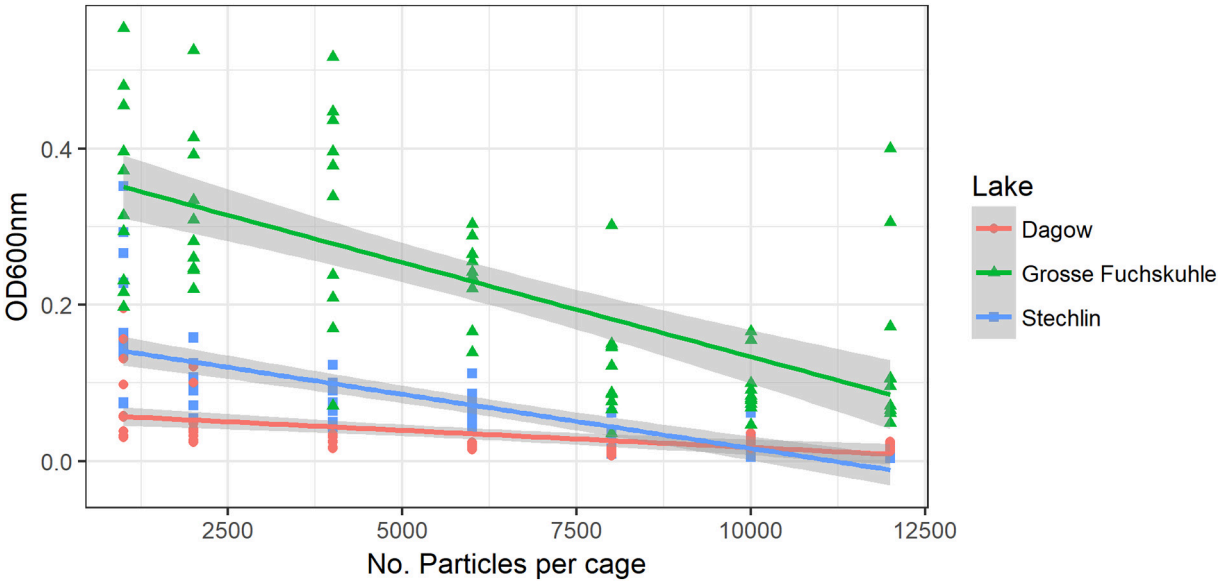

Fig. 2. Effect of MP density in the cage (no. of particles) on biofilm biomass per particle (OD_{600}nm). Trend lines represent the regression analysis of the data for each lake.

among samples from each lake were found (Kruskal-Wallis and later multiple comparisons by Dunn's Test, $p < 0.05$) either for individual thresholds (Fig. 3) or comparing multi-functionality indexes derived with all thresholds (Fig. S4). In oligo-mesotrophic Lake Stechlin (Fig. 3A), MF indexes of MP biofilms were similar to those of W when taking the percentiles 10th to 50th as thresholds, but there were no statistical differences in MF indexes of water sample W and FL fraction with any individual threshold value. When comparing MF indexes obtained with all thresholds, functional richness was, in general, higher for MP biofilms than of W and FL fraction (Fig. S4 A). In eutrophic Lake Dagow (Fig. 3B), the MF indexes of MP biofilms and W were similar to each other and higher than for FL regardless of the threshold value applied. The same result was obtained when comparing MF indexes from all thresholds together (Fig. S4 B). Finally, in dystrophic Lake Grosse Fuchskuhle (Fig. 3C), W showed a higher MF index than FL or MP biofilms in most thresholds (except when using the 80th and 90th percentile) or combining all thresholds (Fig. S4C).

The dissimilarity matrixes allowed us to compare the functional diversity profiles of the microbial communities, based on the pattern of carbon substrates utilized on the EcoPlate (Fig. 4). When analyzing this functional diversity, for Lakes Stechlin (Permanova, $p < 0.01$, Fig. 4A) and Grosse Fuchskuhle (Permanova, $p < 0.001$, Fig. 4C), the

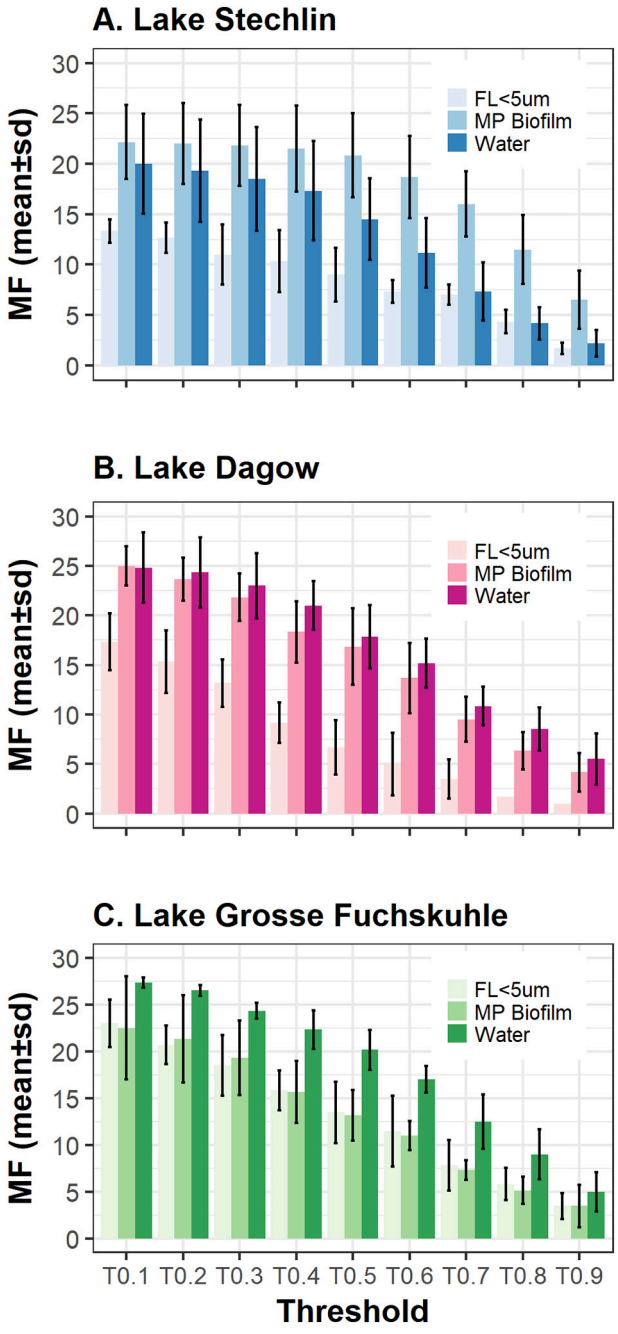

Fig. 3. Multi-functionality in carbon substrate use (MF) of microbial communities of microplastic vs. water samples from Lakes A) Stechlin, B) Dagow and C) Grosse Fuchskuhle. The MF index indicates the number of carbon substrates utilized. Bars represent the mean value of MF calculated from the Biolog EcoPlates readings, applying different quantile-based thresholds. Samples included MP from cages with 6000 particles (MP), the unfiltered water from the lake (W) and the free-living bacteria fraction (FL < 5 μm) of the water. Error bars indicate standard deviation.

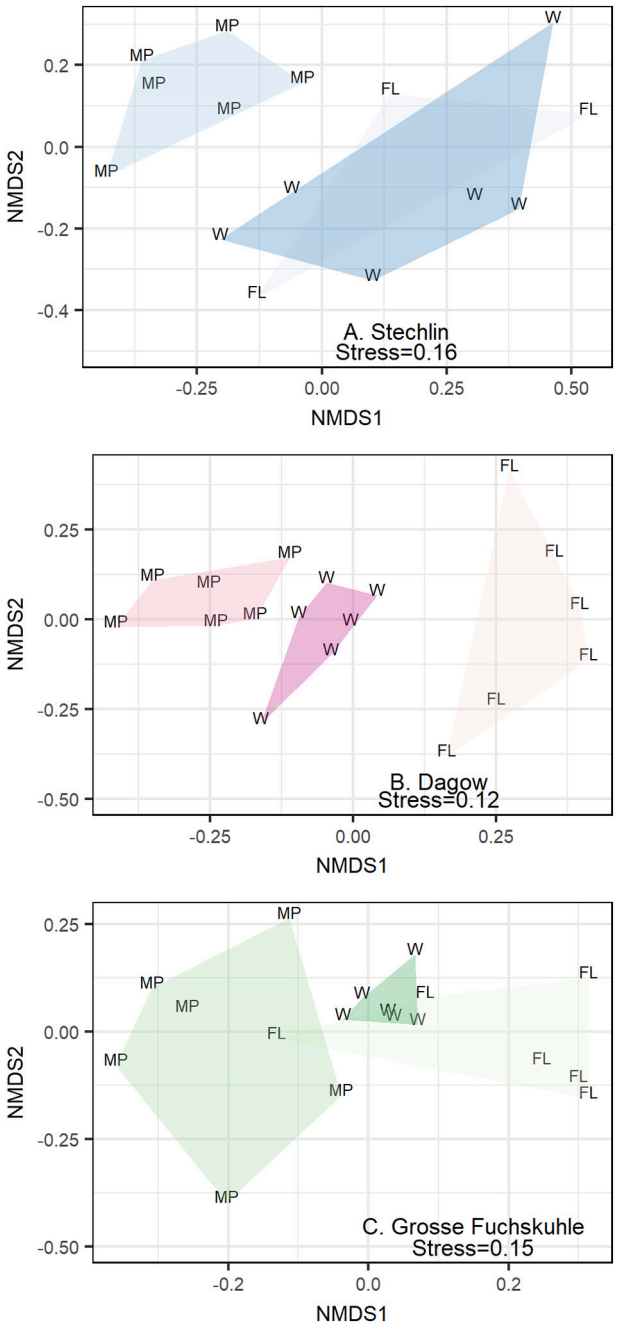

Fig. 4. nMDS plots depict microbial functional diversity of MP biofilms (MP) as well as samples of unfiltered water (W) and free-living bacteria (FL) in water from Lakes A) Stechlin, B) Dagow and C) Grosse Fuchskuhle based on dissimilarity matrixes by Bray Curtis Method from OD_{595}nm measurements in EcoPlates.

physiological profile of MP biofilms was different from that of W and FL. The three kinds of samples (MP, W, and FL) from Lake Dagow significantly differed from each other (Permanova, $p < 0.001$, Fig. 4B). Results of Permanova and pairwise comparisons are summarized in Tables S3 and S4, respectively.

4. Discussion

In the present study, we demonstrated physiological differences between microbial communities on MP and those free-living or in water with natural aggregates, regardless of the trophic state of the studied lakes. The physiological aspects assessed included overall heterotrophic activity in the form of oxygen consumption, and more specifically in the form of respiration of 31 carbon substrates. The results also revealed that microscopic primary producers comprise an important constituent of MP communities in freshwaters, and that, as biofouling increased, so did microbial respiration. While there is growing consensus on a diverse MP colonization (Zettler et al., 2013; Kettner et al., 2017), our results support the notion that MP also represent hotspots for increased heterotrophic activities in different freshwater ecosystems.

4.1. MP biofouling influences the intensity of heterotrophic activity

Biofouling of MP was evident in all lakes, upon visual inspection. This is consistent with previous studies performed in freshwater systems showing distinct bacterial and fungal assemblages growing on MP (Kettner et al., 2017; McCormick et al., 2014, 2016; Hoellein et al., 2014). Colonization of MP by diatoms, microalgae, and cyanobacteria has been also described in marine ecosystems (Yokota et al., 2017; Reisser et al., 2014). In this scenario, autotrophic organisms are the providers of organic matter that can be utilized by heterotrophic microbes of aquatic biofilms, while predators, such as the bacterivorous ciliates observed in Lake Dagow or the insect larvae coated with MP in Lake Grosse Fuchskuhle, seem to play a role in transferring the organic matter to higher trophic levels (Zancarini et al., 2017). Our observations indicate that microbial food webs on MP can include several trophic groups, as also determined for other surface-habitats like the benthic communities (Proia et al., 2012). In this regard, the study of the interconnections existing among different life domains growing on MP deserves further attention.

Microplastics incubated in lakes with high light and lower nutrient availability or more humic substances showed more biofouling per particle by autotrophic microorganisms, such as algae and diatoms. The preference towards living attached to surfaces at nutrient-limited conditions is a well-known phenomenon (Petrova and Sauer, 2012). Moreover, autotrophic microorganisms as those detected on MP are an important source of natural particle materials in aquatic systems, such as transparent exopolymers (TEP; Lemarchand et al., 2006), which facilitate aggregate formation and affect organic carbon transport. The formation of biofouling on MP has been reported to affect sinking time of MP in marine environments (Kaiser et al., 2017). Consequently, it is possible that the observed differences in biofouling and total biofilm biomass in the present study can translate into altered sinking time of MP in freshwaters, depending on the environment, which has influenced the biofouling intensity. However, differences in the MP transport throughout the water column still need to be investigated in detail in freshwaters, considering for instance changes in biofouling that can make the particles buoyant again (Ye and Andrady, 1991) or high turbulence.

Biomass per particle decreased steadily with increasing MP concentration per cage. This decrease can have several reasons related to enclosure conditions within the cages such as light shading between particles limiting algae growth (Schwab et al., 2011), together with physical abrasion of biofilm, in a more extreme way as turbulence mixing limits aggregate size (Colomer et al., 2005). However, the decrease in total biomass per particle explained also the decrease in MP-specific oxygen consumption, used as an indication of general heterotrophic activity. In this regard, although observations by SEM were only qualitative, an intense bacterial colonization of MP from Lake Dagow and in Lake Stechlin was clearly visible under the microscope, whereas much less bacteria-shaped organisms were observed for Lake Grosse Fuchskuhle. Instead, MP biofilms in this dystrophic lake were dominated by photoautotrophic organisms (obvious also from the intense green color of the biofilm), which could explain the comparably lower oxygen consumption.

4.2. Functional diversity in MP differs from water regardless of nutrient status

The use of intact biofilms per particle and undiluted water samples probably explains differences in the intensity of substrate respiration in the EcoPlates (OD_{595}nm). The multi-functional indexes (MF) determined by quantile thresholds and dissimilarity matrixes bases on Bray Curtis method, allowed the comparison of the functional richness and diversity respectively, of both types of samples. The MF indexes suggest that the ability of microbial communities in MP to utilize a different number of carbon substrates than microorganisms in water depends on the environmental context. However, the functional diversity profile (dissimilarity matrixes) of MP biofilms differed from that of the surrounding water - including microorganisms from natural aggregates or only the free-living fraction- irrespective of the lake's trophic status. These results were obtained by analysis of the carbon substrate use in EcoPlates, a recognized tool to study functional diversity and to compare metabolic activities of heterotrophic microbial communities (Lima-Bittencourt et al., 2014; Stefanowicz, 2006; Leflaive et al., 2008). In consequence, environmental conditions can not only lead to a distinct microbial community assembly on MP as previously observed (De Tender et al., 2015; Hoellein et al., 2017), but also to differences in their functional structure.

The mechanisms by which MP microeukaryotic autotrophs alter the qualitative as well as quantitative metabolization of different carbon substrates are not completely clear and require further study. Our current knowledge about natural aquatic biofilms, however, can offer us some clues. For instance, it has been shown that bacterial abundances are closely linked to the presence of algae, consequently boosting extracellular microbial activities, when algal biomass increases in the system (Proia et al., 2012). We suggest that autotrophic microorganisms on MP in the oligotrophic lake provided conditions (e.g., diversity of nutrients in algae exudates) for more heterotrophic activities than those detected in the surrounding water.

The proximity of different microorganisms co-occurring in the MP biofilms could facilitate the formation of micro-consortia for the co-degradation of organic substances (Costerton et al., 1995), and might also explain the higher amount of substrates utilized in MP biofilms than in Stechlin lake water. Further, enhanced horizontal exchange of genes recently described in MP bacterial communities are likely involved in the different metabolic repertoires on MP communities (Arias-Andres et al., 2018). In a similar way, a study on the North Atlantic plastic garbage patch found a potential overrepresentation of degradation metabolic pathways in bacteria from plastic vs. water (Didier et al., 2017). We did not perform a taxonomic assessment of the communities, hence we can only suggest that conditions in Lake Grosse Fuchskuhle, (e.g., low pH and adsorption dynamics of humic substances by MP) could be involved in the lower number of carbon substrates utilized in MP biofilms. These characteristics are known to be selective for bacterial specialists in the same dystrophic ecosystem (Hutalle-Schmelzer et al., 2010).

4.3. MP biofilms can alter carbon cycling in aquatic ecosystems

We propose that biofouling leading to increased heterotrophic activity in freshwater MP probably increases particle-sinking velocity in a

context-dependent way and potentially affects water biogeochemistry in zones where microplastics accumulate. When MP accumulate in sediments (Woodall et al., 2014), the elevated heterotrophic activities as observed on MP in the present study, might facilitate the formation of oxygen-depleted "dead zones" as at the coast and seafloor (Zalasiewicz et al., 2016; Kaiser et al., 2017). On the other hand, an intense accumulation of buoyant particles, together with nutrient pollution, can result in reduced light penetration and the formation of microniches of high heterotrophic activity in the pelagic zones of freshwater ecosystems, similar to marine environments (Bhateria and Jain, 2016; Kamp et al., 2016; Law, 2017).

To our knowledge, this is the first study demonstrating functional differences between MP-associated and pelagic microorganisms in different freshwater lake types. Further experiments are needed to demonstrate the specific consequences of this alteration in organic matter cycling, for example in aquatic food webs. In this direction, a recent publication shows that changes in gut microbiota after exposure to microplastics is coupled with alterations in growth and reproduction, together with changes in isotopic composition of C and N of invertebrates in laboratory exposures (Zhu et al., 2018). On the other hand, another experiment showed increased algal growth, but no effect on *Daphnia magna* survival or reproduction in the presence of microplastics (Canniff and Hoang, 2018).

Although there is a growing interest in studying the diversity of microorganisms that colonize the MP, we addressed the less explored but equally important aspects of their functional capacities. We illustrate how MP – under different nutrient conditions in contrasting freshwater ecosystems – support growth of heterotrophic microorganisms with a highly diverse functional profile. Heterotrophic communities were accompanied by autotrophs that seemingly affected total MP biofilm respiration. Since specific habitats can define microbial life strategies and activities, studying the impact of MP pollution on the ecology of aquatic ecosystems requires a deeper understanding of microbial processes in the plastisphere. Based on our results, and considering the sheer amounts of environmental MP pollution, we believe more studies should investigate the multiple functions performed by microbial communities in the plastisphere in relation to those in the surrounding water. We hope that the techniques used here will open the doors to new opportunities to standardize methods for assessment of disruption of processes at the base of the aquatic food web by plastic pollution.

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Conflict of interests

The authors declare no competing financial interests.

Appendix A. Supplementary information

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.04.199.

References

Allgaier, M., Grossart, H.-P., 2006. Diversity and seasonal dynamics of actinobacteria populations in four lakes in northeastern Germany. Appl. Environ. Microbiol. 72, 3489–3497.
Arias-Andres, M., Klümper, U., Rojas-Jimenez, K., Grossart, H.-P., 2018. Microplastic pollution increases gene exchange in aquatic ecosystems. Environ. Pollut. 237, 253–261.
Azeredo, J., Azevedo, N.F., Briandet, R., Cerca, N., Coenye, T., Costa, A.R., et al., 2017. Critical review on biofilm methods. Crit. Rev. Microbiol. 43, 313–351.
Barbosa, A.M., Brown, J.A., Jimenez-Valverde, A., Real, R., 2016. modEvA: model evaluation and analysis. https://cran.r-project.org/package=modEvA.
Barboza, L.G.A., Vieira, L.R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., et al., 2018. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquat. Toxicol. 195, 49–57.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. 57, 289–300.
Bhateria, R., Jain, D., 2016. Water quality assessment of lake water: a review. Sustain. Water Resour. Manag. 2, 161–173.
Brunk, U., Collins, V.P., Arro, E., 1981. The fixation, dehydration, drying and coating of cultured cells for SEM. J. Microsc. 123, 121–131.
Bryant, J.A., Clemente, T.M., Viviani, D.A., et al., 2016. Diversity and Activity of Communities Inhabiting Plastic Debris in the North Pacific Gyre. In: Jansson, J.K. (Ed.), mSystems. 1(3), e00024-16. https://doi.org/10.1128/mSystems.00024-16.
Byrnes, J.E.K., Gamfeldt, L., Isbell, F., Lefcheck, J.S., Griffin, J.N., Hector, A., et al., 2014. Investigating the relationship between biodiversity and ecosystem multifunctionality: challenges and solutions Freckleton R (ed). Methods Ecol. Evol. 5, 111–124.
Canniff, P.M., Hoang, T.C., 2018. Microplastic ingestion by *Daphnia magna* and its enhancement on algal growth. Sci. Total Environ. 633, 500–507.
Colomer, J., Peters, F., Marrasé, C., 2005. Experimental analysis of coagulation of particles under low-shear flow. Water Res. 39, 2994–3000.
Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M., 1995. Microbial biofilms. Annu. Rev. Microbiol. 49, 711–745.
De Tender, C.A., Devriese, L.I., Haegeman, A., Maes, S., Ruttink, T., Dawyndt, P., 2015. Bacterial community profiling of plastic litter in the Belgian part of the North Sea. Environ. Sci. Technol. 49, 9629–9638.
De Tender, C., Schlundt, C., Devriese, L.I., Mincer, T.J., Zettler, E.R., Amaral-Zettler, L.A., 2017. A review of microscopy and comparative molecular-based methods to characterize 'Plastisphere' communities. Anal. Methods 9, 2132–2143.
Didier, D., Anne, M., Alexandra, T.H., 2017. Plastics in the North Atlantic garbage patch: a boat-microbe for hitchhikers and plastic degraders. Sci. Total Environ. 599-600, 1222–1232.
Dinno, A., 2017. dunn.test: Dunn's test of multiple comparisons using rank sums. https://cran.r-project.org/package=dunn.test.
Eckert, E.M., Di Cesare, A., Kettner, M.T., Arias-Andres, M., Fontaneto, D., Grossart, H.-P., et al., 2018. Microplastics increase impact of treated wastewater on freshwater microbial community. Environ. Pollut. 234, 495–502.
Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3, e1700782.
Giraudoux, P., 2017. pgirmess: data analysis in ecology. https://cran.r-project.org/package=pgirmess.
Grossart, H.-P., 2010. Ecological consequences of bacterioplankton lifestyles: changes in concepts are needed. Environ. Microbiol. Rep. 2, 706–714.
Hoellein, T., Rojas, M., Pink, A., Gasior, J., Kelly, J., 2014. Anthropogenic litter in urban freshwater ecosystems: distribution and microbial interactions. PLoS One 9, e98485.
Hoellein, T.J., McCormick, A.R., Hittie, J., London, M.G., Scott, J.W., Kelly, J.J., 2017. Longitudinal patterns of microplastic concentration and bacterial assemblages in surface and benthic habitats of an urban river. Freshw. Sci. 36, 491–507.
Hollman, P.C.H., Bouwmeester, H., RJB, Peters, 2013. Microplastics in the aquatic food chain; sources, measurement, occurrence and potential health risks. Wgeningen. https://www.wageningenur.nl/en/Publication-details.htm?publicationId=publication-way-343430303039.
Hunter, W.R., Niederdorfer, R., Gernand, A., Veuger, B., Prommer, J., Mooshammer, M., et al., 2016. Metabolism of mineral-sorbed organic matter and microbial lifestyles in fluvial ecosystems. Geophys. Res. Lett. 43, 1582–1588.
Hutalle-Schmelzer, K.M.L., Zwirnmann, E., Krüger, A., Grossart, H.-P., 2010. Enrichment and cultivation of pelagic bacteria from a humic lake using phenol and humic matter additions. FEMS Microbiol. Ecol. 72, 58–73.
Kaiser, D., Kowalski, N., Waniek, J.J., 2017. Effects of biofouling on the sinking behavior of microplastics. Environ. Res. Lett. 12, 124003.
Kamp, A., Stief, P., Bristow, L.A., Thamdrup, B., Glud, R.N., 2016. Intracellular nitrate of marine diatoms as a driver of anaerobic nitrogen cycling in sinking aggregates. Front. Microbiol. 7, 1669.
Kesy, K., Hentzsch, A., Klaeger, F., Oberbeckmann, S., Mothes, S., Labrenz, M., 2017. Fate and stability of polyamide-associated bacterial assemblages after their passage through the digestive tract of the blue mussel *Mytilus edulis*. Mar. Pollut. Bull. 0–1.
Kettner, M.T., Rojas-Jimenez, K., Oberbeckmann, S., Labrenz, M., Grossart, H.-P., 2017. Microplastics alter composition of fungal communities in aquatic ecosystems. Environ. Microbiol. 19, 4447–4459.
Kwasny, S.M., Opperman, T.J., 2010. Static biofilm cultures of gram-positive pathogens grown in a microtiter format used for anti-biofilm drug discovery. Current Protocols in Pharmacology. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 1–27.
Lambert, S., Wagner, M., 2018. Microplastics are contaminants of emerging concern in freshwater environments: an overview. In: Wagner, M., Lambert, S. (Eds.), Freshwater Microplastics. Springer International Publishing, Cham, pp. 1–23.

Law, K.L., 2017. Plastics in the marine environment. Annu. Rev. Mar. Sci. 9, 205–229.
Law, K.L., Thompson, R.C., 2014. Microplastics in the seas. Science 80 (345), 144–145.
Leflaive, J., Danger, M., Lacroix, G., Lyautey, E., Oumarou, C., Ten-Hage, L., 2008. Nutrient effects on the genetic and functional diversity of aquatic bacterial communities. FEMS Microbiol. Ecol. 66, 379–390.
Lemarchand, C., Jardillier, L., Carrias, J.-F., Richardot, M., Debroas, D., Sime-Ngando, T., et al., 2006. Community composition and activity of prokaryotes associated to detrital particles in two contrasting lake ecosystems. FEMS Microbiol. Ecol. 57, 442–451.
Lima-Bittencourt, C.I., Costa, P.S., Reis, M.P., Santos, A.B., Barbosa, F.A.R., Valentin, J.L., et al., 2014. A survey on cultivable heterotrophic bacteria inhabiting a thermally unstratified water column in an Atlantic Rainforest lake Smidt H (ed). PeerJ e478, 2.
McCormick, A., Hoellein, T.J., Mason, S.A., Schluep, J., Kelly, J.J., 2014. Microplastic is an abundant and distinct microbial habitat in an urban river. Environ. Sci. Technol. 48, 11863–11871.
McCormick, A.R., Hoellein, T.J., London, M.G., Hittie, J., Scott, J.W., Kelly, J.J., 2016. Microplastic in surface waters of urban rivers: concentration, sources, and associated bacterial assemblages. Ecosphere 7, e01556.
Miki, T., Yokokawa, T., Ke, P.-J., Hsieh, I.-F., Hsieh, C., Kume, T., et al., 2017. Statistical recipe for quantifying microbial functional diversity from EcoPlate metabolic profiling. Ecol. Res. 3–4.
Oberbeckmann, S., Osborn, A.M., Duhaime, M.B., 2016. Microbes on a bottle: substrate, season and geography influence community composition of microbes colonizing marine plastic debris Carter DA. PLoS One 11, e0159289.
Oberbeckmann, S., Kreikemeyer, B., Labrenz, M., 2018. Environmental factors support the formation of specific bacterial assemblages on microplastics. Front. Microbiol. 8, 2709.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al., 2017. Vegan: community ecology package. R Package Version 2.4–3.
Pernthaler, J., Amann, R.I., 2005. Fate of heterotrophic microbes in pelagic habitats: focus on populations. Microbiol. Mol. Biol. Rev. 69, 440–461.
Petrova, O.E., Sauer, K., 2012. Sticky situations: key components that control bacterial surface attachment. J. Bacteriol. 194, 2413–2425.
Proia, L., Cassió, F., Pascoal, C., Tlili, A., Romaní, A.M., 2012. In: Guasch, H., Ginebreda, A., Geiszinger, A. (Eds.), The Use of Attached Microbial Communities to Assess Ecological Risks of Pollutants in River Ecosystems: The Role of Heterotrophs. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 55–83.
RCoreTeam, 2013. R: A Language and Environment for Statistical Computing.
Reisser, J., Shaw, J., Hallegraeff, G., Proietti, M., Barnes, D.K.A., Thums, M., et al., 2014. Millimeter-sized marine plastics: a new pelagic habitat for microorganisms and invertebrates. PLoS One 9, e100289.
Sabater, S., Tranvik, L.J., Peter, H., Ylla, I., Gudasz, C., Romanı, A.M., 2011. Multifunctionality and Diversity in Bacterial Biofilms. 6. https://doi.org/10.1371/Citation (e-pub ahead of print).
Schwab, F., Bucheli, T.D., Lukhele, L.P., Magrez, A., Nowack, B., Sigg, L., et al., 2011. Are carbon nanotube effects on green algae caused by shading and agglomeration? Environ. Sci. Technol. 45, 6136–6144.
Stefanowicz, A., 2006. The biolog plates technique as a tool in ecological studies of microbial communities. Pol. J. Environ. Stud. 15, 669–676.
Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis.
Woodall, L.C., Sanchez-Vidal, A., Canals, M., Paterson, G.L.J., Coppock, R., Sleight, V., et al., 2014. The deep sea is a major sink for microplastic debris. R. Soc. Open Sci. 1 (140317-140317).
Ye, S., Andrady, A.L., 1991. Fouling of floating plastic debris under Biscayne Bay exposure conditions. Mar. Pollut. Bull. 22, 608–613.
Yokota, K., Waterfield, H., Hastings, C., Davidson, E., Kwietniewski, E., Wells, B., 2017. Finding the missing piece of the aquatic plastic pollution puzzle: interaction between primary producers and microplastics. Limnol. Oceanogr. Lett. 2, 91–104.
Yongmei, N., 2012. LDdiag: link function and distribution diagnostic test for social science researchers. https://cran.r-project.org/package=LDdiag.
Zancarini, A., Echenique-Subiabre, I., Debroas, D., Taïb, N., Quiblier, C., Humbert, J.-F., 2017. Deciphering biodiversity and interactions between bacteria and microeukaryotes within epilithic biofilms from the Loue River, France. Sci. Rep. 7, 4344.
Zalasiewicz, J., Waters, C.N., Ivar do Sul, J.A., Corcoran, P.L., Barnosky, A.D., Cearreta, A., et al., 2016. The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. Anthropocene 13, 4–17.
Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the 'Plastisphere': microbial communities on plastic marine debris. Environ. Sci. Technol. 47, 7137–7146.
Zhu, D., Chen, Q.-L., An, X.-L., Yang, X.-R., Christie, P., Ke, X., et al., 2018. Exposure of soil collembolans to microplastics perturbs their gut microbiota and alters their isotopic composition. Soil Biol. Biochem. 116, 302–310.

Discussion

Synthesis of the dissertation

The scientific contribution of this Ph.D. thesis to understanding the effects of MP pollution on the ecology of aquatic microbial communities can be summarized as follows:

1. **Chapter I** presents experimental evidence that MP affect the structure of microbial communities in aquatic ecosystems by serving as a vector for exogenous, wastewater-derived, microbial colonizers and their mobile genetic elements.

2. **Chapter II** demonstrates how the introduction of MP surfaces into freshwater ecosystems enhances the frequency of horizontal gene transfer of ARGs and selects for a community of bacteria more permissive to plasmid transfer in a model as well as environmental microbial communities.

3. **Chapter III** points to the fact that the functional diversity of MP biofilm communities is different from that of bacteria in the surrounding water, whereby MP is acting as a new locus for various heterotrophic activities in the water column.

The evidence was generated using microcosms and freshwater environmental microbial communities as significant constituents of the experimental design. **Chapter I** analyzed the effect of increasing MP pollution on the prevalence of wastewater-derived bacteria and class 1 integron among bacterial communities in a freshwater microcosm system. Further, studies in **Chapters II** and **III** compared the functional traits of plasmid transfer and functional diversity of carbon substrate use by comparing MP-associated microbial communities vs. those in the surrounding water. The following sections provide a general discussion of the obtained results from an ecological perspective taking the strong influence of human activities on the Earth's microbiome into account.

Human interference on Earth has been hastened after the mid-20th century, with substantial effects on microbial communities, noticeable as pronounced changes in human microbiota, increased antimicrobial resistance or alterations in carbon and nitrogen cycling of natural ecosystems (Gillings and Paulsen, 2014). The exponential increase in the production of plastic polymers and its omnipresence in the environment coincides, among other factors, with this human-induced acceleration period (Zalasiewicz *et al.*, 2016). The experiments performed in this thesis demonstrate how MP could affect microbial community structure, evolution and ecological functions, including the distribution of bacterial mobile genetic elements in aquatic systems. The obtained results reveal significant insight into the many facets of plastic pollution for the observed changes to the planet´s microbiome during the contemporary Anthropocene.

Overall, the thesis addressed the little-explored aspect of gene exchange and heterotrophic activities among MP microbial communities and compared changes following the presence of MP in the aquatic environment. An overview of the microbial communities that colonized MP during the experiments and their potential physiology is given. This general description is based on the results of the metabarcoding and microscopic analysis performed in **Chapters II** and **III**. The role of MP in the transport of bacteria from WWTPs to natural ecosystems is mentioned, and a discussion on the relationship between HGT on MP biofilms and the different types of carbon metabolism of these microbial communities is presented. The possible effects of MP-induced alterations on microbial biodiversity and aquatic food webs, bacterial evolution, and the spread of antibiotic resistance genes are discussed. Suggestions for the challenges and hypotheses for future research are provided. Finally, the general significance of the obtained results for society and microbial ecology is illustrated.

Potential of microbial MP biofilms for generating new ecological interactions

The microbial MP communities analyzed in this thesis originated from wastewater or lakes. Freshwaters are generally close to many sources of plastic pollution, but this situation has been much less studied than in marine areas (Wagner and Lambert, 2018). Freshwaters habitats support a vital share of Earth´s biodiversity but are also among the most human-altered environments (Kopf *et al.*, 2015). For example, eutrophication causes cyanobacterial blooms paralleled with specific changes in the diversity and composition of particle-associated and free-living heterotrophic bacterioplankton (e.g., Woodhouse *et al.*, 2016). Similarly, MP biofilms in Lake Stechlin showed a predominance of Cyanobacteria together with Bacteroidetes and Alphaproteobacteria. These groups were commonly found in other studies on plastics in various aquatic systems (Oberbeckmann *et al.*, 2016; McCormick *et al.*, 2016, 2014; De Tender *et al.*, 2015). Also, some bacterial families were enriched on MP in Lake Stechlin, as observed previously on lake aggregates (Bižić-Ionescu *et al.*, 2015). This notion supports the role of MP for changes of microbial community structure by human

interference. Since Stechlin is a meso-oligotrophic lake, the obtained results indicate the potential of increased MP pollution to cause not only changes in bacterial community composition but also carbon cycling in freshwater ecosystems, namely in a similar mode as the excess supply of nutrients (i.e., eutrophication).

The experiments in **Chapter I** suggest the importance of plastic as a vector for the survival of wastewater microbial communities in natural aquatic ecosystems. The last 100 years, wastewaters have increased the mobilization of large numbers of microbial cells across the globe (Zhu *et al.*, 2017). Ecologists have long emphasized the importance of human activities for microbial dispersal and persistence in both aquatic and terrestrial ecosystems (Wilkinson, 2010; Litchman, 2010). Invasive species are linked to the loss of biodiversity on a global scale and the increased spread of pathogens (Amalfitano *et al.*, 2015; Keswani *et al.*, 2016). Wastewaters are known sources of microbial hazards, that can impede their reuse for human consumption (Schoen and Garland, 2017), and of MP harboring diverse microbial biofilm communities (Ziajahromi *et al.*, 2017). The combined hazard of MP and wastewater can reduce animal fitness and cause infectious disease, e.g., the health of corals can be reduced as MP can affect their integrity and expose them to coral pathogens (Reichert *et al.*, 2017).

Moreover, sequences of the phylum Chlamydiae, to which many pathogens and obligate intracellular bacteria belong, were detected only on controls of MP biofilms in experiments of **Chapter II** (0.1 - 0.4% relative abundance). Since Lake Stechlin is a relatively pristine environment, we expect the detected sequences of this phylum to belong to parasites of amoeba, ciliates or flagellates that may also colonize MP (e.g., Parachlamydiaceae; see Corsaro and Venditti, 2009). However, this notion suggests how MP can select for microorganisms with parasitic needs.

The physiological characteristics of bacterial groups selected on plastic are relevant to understanding the effects of MP on the microbial communities in aquatic environments. In marine waters, it has been described that plastics recruit bacterial groups associated to the degradation of complex molecules, such as hydrocarbon contaminants (Harrison *et al.*, 2014; Keswani *et al.*, 2016). In **Chapter II**, the phylum Acidobacteria was detected (ca. 3% relative abundance) only on MP biofilms of Lake Stechlin, but not in free-living communities (FL; see supporting information of **Chapter II** in Annex section). The physiological diversity and ecological relevance of Acidobacteria are comparable to that of Proteobacteria and Firmicutes (Zimmermann *et al.*, 2012). There is a poor understanding of biofilm formation by this taxon in natural conditions and its ecological role. However, genomes of this group encode for exopolysaccharide (EPS) synthesis and the degradation of different polysaccharides, and at least 50% of genera could use starch, laminarin, and xylan in culture-based experiments (Kielak *et al.*, 2016). This information suggests MP are also potential hotspots for microorganisms with enhanced capacities for the degradation of complex polymeric compounds. However, this needs to be further demonstrated.

In experiments of **Chapters II** and **III**, there was biofouling by algae and diatoms on MP in lakes. While eukaryotic communities were previously reported, for example on marine MP (e.g., Zettler *et al.*, 2013), their associated bacteria and importance in food web dynamics on MP remain poorly analyzed. In this context, pan-genome analyses suggest members of the candidate phylum Parcubacteria, which comprised 0.72% of the relative sequence abundance on MP and 0.05% in FL bacteria of Lake Stechlin (**Chapter II**). These bacteria have been characterized previously as ectosymbionts or parasites (Castelle *et al.*, 2017; Nelson and Stegen, 2015).

Additionally, in microcosms with MP and water of lakes with different nutrient concentrations in **Chapter III**, oxygen consumption increased together with biofouling. MP from lakes with different limnological features showed variations in biofouling intensity, the qualitative composition of microalgae and diatoms, and the richness of carbon substrate respiration compared to FL bacteria in the surrounding water. In this scenario, eukaryotes influence the composition and function of bacterial communities of the MP biofilm. The nature of the biofouling is closely related to environmental parameters (e.g., light, nutrients, and pH) or other specific characteristics of each lake. Therefore, these factors ultimately modulate the effect of MP on the diversity, physiology and hence ecological role of microbial communities in freshwaters.

Microplastics alter HGT and metabolism of aquatic microbial communities

Chapter I shows that increasing MP pollution could influence the abundance and distribution of class 1 integrons in aquatic ecosystems, while **Chapter II** presents evidence that MP can increase the horizontal transfer of a conjugative plasmid containing an antibiotic resistance gene. Both studies indicate MP affect the distribution of mobile genetic elements in aquatic ecosystems. The most studied example of genes introduced by human activities is the spread of ARGs, e.g., by wastewater inflow into natural aquatic systems (Guo *et al.*, 2017). The primary focus when discussing antibiotic resistance spread is how it impacts the fight against human infectious diseases. However, it is estimated that such type of genetic import and shuffle generally affects the diversity and evolution of native microbial communities (Power *et al.*, 2016). In this context, the role of MP is not only the transport of mobile genetic elements *per se* but also their selection, e.g., the selection of bacteria more permissive to plasmid pKJK5 transfer as demonstrated in **Chapter II**.

The increased permissiveness of bacteria for HGT on MP biofilms can affect microbial evolution on Earth since HGT facilitates the widespread distribution of ARGs, clusters of biodegradative pathways, pathogenicity determinants, and bacterial speciation processes (de la Cruz and Davies, 2000). Human intervention on bacterial gene exchange by the current massive plastic pollution is, therefore, similar to that of antibiotics that turn human-pathogenic bacteria resistant to any antibiotic treatment (Stevenson *et al.*, 2018). As with

antibiotics in the environment (see Lopatkin *et al.*, 2016b, 2016a), the mechanisms by which plastic surfaces modulate HGT remain to be elucidated. Incidentally, a study found that as the abundance of a plasmid increases in a natural microbial community, its populations are more permissive to its transfer (Bellanger *et al.*, 2014b). Also, evidence shows that permissiveness for plasmid transfer in individual species is affected by the surrounding community structure and specific environmental settings (de la Cruz-Perera *et al.*, 2013). These facts suggest adaptation towards plasmid acquisition at the community level, as observed in MP communities vs. FL bacteria in the surrounding water (**Chapter II**).

Crucial to the analysis of HGT alterations in the aquatic realm, is how it affects carbon cycling. **Chapter III** presents evidence that MP biofilms have a different metabolic profile for carbon degradation compared to FL bacteria in the surrounding water. The trophy status of the aquatic system, the biofouling of the particle with autotrophic organisms and changes in HGT dynamics, seem to be crucial to the observed differences in microbial physiology on MP biofilms. Given the proportion of plastic pollution, the emergence of this new habitat can reach global consequences for nutrient cycling, like those inflicted by agriculture on nitrogen and methane cycles (Gillings and Paulsen, 2014) or the increase in CO_2 levels leading to climate change (Monroe *et al.*, 2018). Also, multiple feedbacks to microbial dynamics, including those that control greenhouse gas emissions and carbon sequestration could result from altered activities in MP biofilms.

Plastics add significant amounts of allochthonous carbon to aquatic ecosystems. According to studies in seawater, between 260 and 23,600 metric tons of DOC per year were estimated to escape from the 4.8-12.7 x 10^{12} metric tons of plastics entering the ocean in 2010 (Romera-Castillo *et al.*, 2018). Conversely, plastics have a large capacity to adsorb substances from the surrounding water (Hirai *et al.*, 2011) or contain additives incorporated during manufacture (Jahnke *et al.*, 2017). Since the quality of dissolved organic matter (DOM) shape microbial community assembly and activity in aquatic ecosystems (Ruiz-González *et al.*, 2015; Pernthaler, 2017), plastic-derived DOC could partially explain a different profile of carbon substrate utilization by microbes on MP vs. those in the surrounding water. Indeed, MP biofilms showed a different catabolic profile in **Chapter III**. Finally, the released carbon from MP polymers or MP biofilms can influence the free-living bacteria by contributing to their DOC bioavailability as seen in general with particulate organic matter –POM (Zhang *et al.*, 2016).

In conclusion, increased HGT together with exogenous bacteria and mobile genetic elements in MP biofilms can alter the functionality of microbial communities of natural aquatic systems. The lateral exchange of genes in MP biofilms enables new microbial ecotype adaptations in the aquatic habitat, for example by contributing to the assembly of new metabolic pathways (Soucy *et al.*, 2015). Gene exchange occurs among bacteria but can also occur between bacteria and archaea (Fuchsman *et al.*, 2017) or bacteria and eukaryotes (Lacroix and Citovsky, 2016). On the other hand, organic compounds from or transported by plastics potentially enhance HGT as has been seen earlier for organic compounds from

wastewater (Jiao *et al.*, 2017). These combined factors can significantly contribute to alterations in carbon dynamics of natural microbial communities and may be further enhanced by the release of organic matter from MP (Zhang *et al.*, 2016).

Challenges and prospects in the study of MP effects on aquatic microbes

Plastic pollution in aquatic ecosystems can show a high spatial variability with "garbage patches" where it massively accumulates and locations with low MP concentrations where a substantial sampling effort is required (Goldstein *et al.*, 2013). Indeed, the variety of methods to measure MP in environmental samples has improved in the last years, especially in marine systems (Rocha-Santos and Duarte, 2015). However, a complete understanding of MP pollution is far from complete. For example, some studies show that concentrations remain stable in some locations (Beer *et al.*, 2018), while others propose that the problem increases faster than previously expected (Lebreton *et al.*, 2018). In this context, there is an intense and controversial discussion regarding the environmental relevance of the nature and concentration of MP used in experimental studies performed until now, mainly addressing adverse effects on aquatic biota (Lenz *et al.*, 2016; Phuong *et al.*, 2016).

Manipulation in MP concentration to study the activity of their associated microbial communities allows predictions on MP-induced effects on microbial activities and their ecological consequences. Therefore, using different concentrations in **Chapters I** and **III** allowed us to account for the effect of increased MP densities on overall microbial dynamics in aquatic systems (e.g., the distribution of wastewater vs. lake microbial communities, changes in biofouling). The experiments on the lakes permitted the detection of specific effects of MP more directly, since other factors (e.g., MP heterogeneity in shape and composition) may obscure these phenomena in real scenarios. Thereby, the information produced from hypothesis-driven experiments facilitates the search for specific MP-induced effects in natural ecosystems.

Regarding the quantity of MP in laboratory studies, it requires accounting for the amount of material needed for the analysis of microbial activity. For example, in the case of plasmid transfer rates, measured in experiment one in **Chapter II**, the event rate is about once every 1,000 or 1,000,000. Considering: i) a bacterial density between 10^3 to 10^5 cells per mm^2 (Dussud *et al.*, 2018); ii) the shape of the MP used in this work (square particles of 4 x 4 x 0.1 mm); iii) conditions of 50/50% donor/recipient cell concentrations in microcosms of **Chapter II**; and iv) a detachment of 50% of the cells, it would require sampling approximately 23 MP pieces to meet the goal of analyzing transconjugant occurrence after at least 200,000 donor cells in the flow cytometer. This calculation does not take into account additional samples for

DNA extraction or SEM observations and the fact that the flow cytometer cannot analyze the complete volume of each sample.

Fibers are the prevalent form of MP particles reported in the environment, although these are not commercially produced and thus remain less used in microcosm studies (Cole, 2016). A fiber can offer a higher surface to volume ratio and roughness than a bead or the particles used in this thesis. As observed in **Chapter II** and by others, the surface irregularities of MP result in a patchy distribution and activity of microbial communities (Dussud *et al.*, 2018). That would imply that fibers´ irregularities could offer even more places for increased HGT. However, as shown by differences in transconjugant isolation between MP1 and MP2 in **Chapter II**, biofilm communities can show different conjugation dynamics according to the physical biofilm structure. Besides, the potential of a specific donor-plasmid combination to invade a biofilm is the result of different ecological factors in the surrounding environment (Bellanger *et al.*, 2014a).

In the case of MP-associated microbial communities, perhaps the most critical challenge is the interpretation of the relevance of the results obtained from different scales of observation, i.e., from the micro-scale (observations on MP biofilms) up to the macro-scale (e.g., aquatic food webs). Therefore, concerning MP effects for gene exchange and finally carbon metabolism by microbial communities in aquatic ecosystems, follow-up studies of this thesis are required to demonstrate the repercussions to aquatic food webs (Figure 1) and antibiotic resistance spread to the human-microbiome (Figure 2). Below details of relevant aspects to be addressed in the future are summarized.

1. Evidence of MP effects on a broader group of HGT mechanisms, and in the presence of relevant environmental stressors.

Other mechanisms of HGT such as transformation and transduction in MP biofilms were not addressed in this thesis. While conjugation (e.g., by plasmids) is usually mentioned as the most common mechanism of HGT (Lopatkin *et al.*, 2016a), these other processes are also relevant in scenarios where significant MP pollution is expected, e.g., WWTPs. In this regard, studies show the prevalence of virus-like particles in WWTPs and ARG-like genes in the virome of activated sludge, indicating the involvement of bacteriophages in the spread of ARGs in the environment (Tamaki *et al.*, 2012; Balcazar, 2014). Moreover, biofilms are suitable environments for transduction, e.g. of genes that encode for bacterial toxins (Solheim *et al.*, 2013). In addition, surface properties are fundamental in the survival of viruses, and bacteriophage proteins are known to bind to plastics such as polystyrene in laboratory studies (Vasickova *et al.*, 2010; Adey *et al.*, 1995; Bakhshinejad and Sadeghizadeh, 2016). However, there are no current reports analyzing bacteriophages in MP biofilms from the natural environment. Therefore, it is likely that MP pollution might increase the rate of viral infection in natural aquatic systems as well. The topic of bacteriophages constitutes a new, open and relevant field for future investigation.

Finally, limnologic conditions of the lakes influenced MP effects on microbial activity measurements in **Chapter III**. These biogeochemical properties include nutrient availability and influence HGT (Drudge and Warren, 2012). The presence of sheer amounts of MP in marine and freshwater ecosystems coincides with other environmental stressors such as eutrophication. The similarities of MP effects on microbial community structure with those of eutrophication, that cause frequent cyanobacterial blooms, raise great socio-economic concerns. However, the interactions and individualities in MP effects and nutrient excess on microbial community structure and function remain to be clarified in future studies. This information is necessary for a more accurate assessment of MP effects on the Earth´s microbiome.

2. Demonstrate the connection between altered HGT and carbon cycling dynamics on MP biofilms through the aquatic food web.

Following the experimental demonstrations in **Chapter I** and **II**, which highlights that MP can introduce exogenous MGEs and that MP-associated bacteria are more permissive to plasmid transfer, **Chapter III** illustrates how MP microbial communities display a different carbon catabolic profile. Although not demonstrated in this Ph.D. Thesis, specificities of their mobilome, or all MGE present in cells (Siefert, 2009), influence the metabolic differences between MP biofilm and FL bacteria in the surrounding water. Accordingly, the analysis of overall plasmid content, or the MP biofilm 'plasmidome' (Walker, 2012), can shed further light into whether there is a selection of specific plasmids (e.g., with specific clusters of biodegradation genes) in bacterial communities of MP biofilms. Similarly, studies of plasmid metagenomes in WWTPs serving industrial vs. residential areas suggest adaptation at the community level to the microbial composition of wastewaters (Sentchilo *et al.*, 2013). Omics studies combined with physiological methods (for example measurement of specific enzymatic activities) will serve to examine the link between HGT and metabolic diversity in MP biofilms.

In general, the activities of MP biofilm communities are the result of the synergy of organisms belonging to different domains of life, as observed with general heterotrophic activity and biofouling of MP particles with eukaryotic autotrophs in **Chapter III**. Also, heterotrophic eukaryotes, for instance, ciliates (filter feeders) found on MP of Lake Dagow, can also affect the interaction among bacterial populations, including their HGT. For example, multi-trophic interactions via predation can influence the transfer rate of conjugative plasmids among bacterial communities (Cairns *et al.*, 2016). Additionally, changes in the function of the gut microbiome can result after MGEs with new metabolic pathways get transferred from MP-associated bacteria to the gut microbiome of higher organisms (Flint *et al.*, 2012). These changes can lead to alterations in the organism's growth and life traits (e.g., reproduction), as mentioned in **Chapter III**. In this regard, it is necessary to first analyze the microbial networks of different life domains in MP biofilms. Secondly, it is essential to demonstrate the transfer of mobile genetic elements from MP-associated bacteria to the microbiome of aquatic organisms and humans.

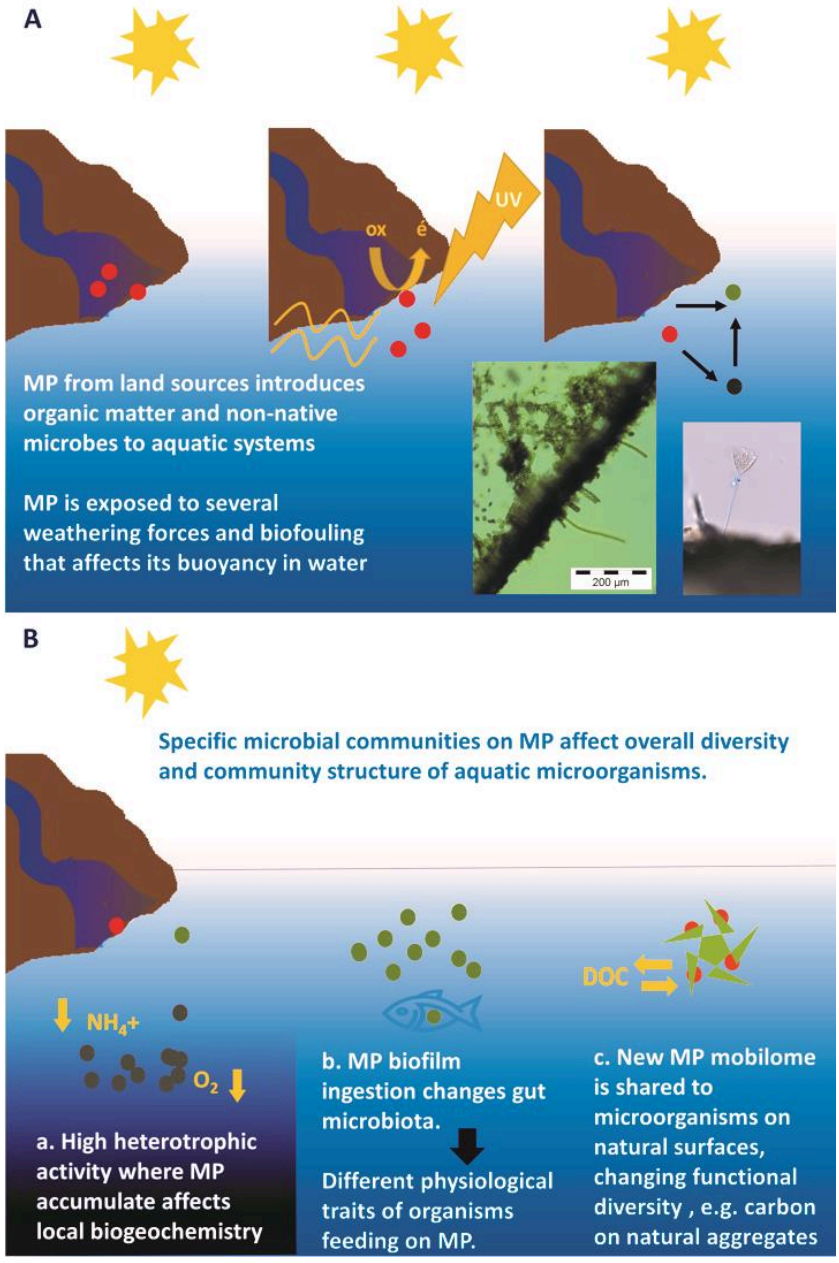


Figure 1. Ecological effects of MP in aquatic ecosystems. Panel A describes changes that MP undergo once they enter aquatic ecosystems, including weathering forces (shearing by water movement, radiation, oxidation processes) and biofouling that affect sinking velocities. Panel B gives the potential effects of MP on higher levels of aquatic ecosystems by alterations in HGT and function of microbial communities (described in points 1 and 2).

3. Describe survival and exchange of ARGs on MP when moving through the aquatic food chains and eventually to the microbiome of humans or farm animals.

This Ph.D. thesis described microbial activities, including HGT, in MP biofilms. The class of MGEs analyzed (an integron and IncP-1ε plasmid) have an important role in the spread of antibiotic resistance of clinical relevance (Gillings *et al.*, 2008; Li *et al.*, 2016). Multiple ARGs in single MGEs result from strong selection exerted by human activity, affecting the

Earth´s microbiome and producing the loss of human lives (Gillings and Paulsen, 2014). In the last decade, there is an urgent need to understand the origins and development of antibiotic resistance in the environment from an ecological perspective (Allen *et al.*, 2010). As discussed throughout the thesis, MP are new surfaces that sustain biofilms with exogenous bacteria and MGEs. In this context, MP-associated bacteria have the potential to surpass the three bottlenecks for horizontal transfer of ARGs, from its original hosts in an aquatic ecosystem to a human or animal pathogen, as described in Martínez *et al.* (2015). These constraints are 1) ecological connectivity, 2) the founder effect, and 3) fitness costs.

The low degradability of the polymers that make up plastic debris and changes in buoyancy provide MP with the opportunity to be mobilized across long distances over prolonged periods of time (Eerkes-Medrano *et al.*, 2015; Ryan, 2015). Therefore, MP biofilms can facilitate connectivity among microorganisms from different ecosystems for the exchange of ARGs, e.g., from an aquaculture pond to an agriculture field and after that, a mangrove in the coast, harvested for bivalves for consumption. When MP are ingested by aquatic biota, for instance by these filter-feeder mollusks, the chance of transfer of ARGs to the human microbiome can be substantially increased.

In the scenario mentioned above, environmental conditions and biological features modulate the barriers and opportunities for continuous selection of ARGs throughout the different ecosystems (Skippington and Ragan, 2011; Madsen *et al.*, 2012). In this sense, chemical substances and organic matter adsorbed or released from MP can diversify the positive selection pressures for a genetic element. For example, the presence of heavy metals in MP together with bacteria in which an ARG is in a cluster with genes for heavy metal tolerance. Co-tolerance would allow an ARG from MP biofilms to invade a habitat where another ARG with a similar substrate profile is already established, thus surpassing the "founder effect." Also, protection from some types of grazing and nutrient availability in MP biofilm settings can provide bacteria with the time and conditions to overcome the fitness costs associated with the incorporation of a new genetic resistance determinant.

To demonstrate these events in MP biofilms require further experimental work and environmental sampling efforts. Moreover, in addition to demonstrating the transfer of an antibiotic genetic determinant from MP biofilms to the rest of the aquatic food web, the resulting phenotype of resistance should be confirmed. To demonstrate antibiotic resistance spread by MP pollution requires a combination of genomic, culturing and physiological approaches. MP present ubiquitous vectors for ARGs contained in plasmids, bacteriophages and integrative elements among others. Therefore, the study of ARGs on MP biofilms provide an excellent opportunity to understand antibiotic resistance from a planetary ecological standpoint.

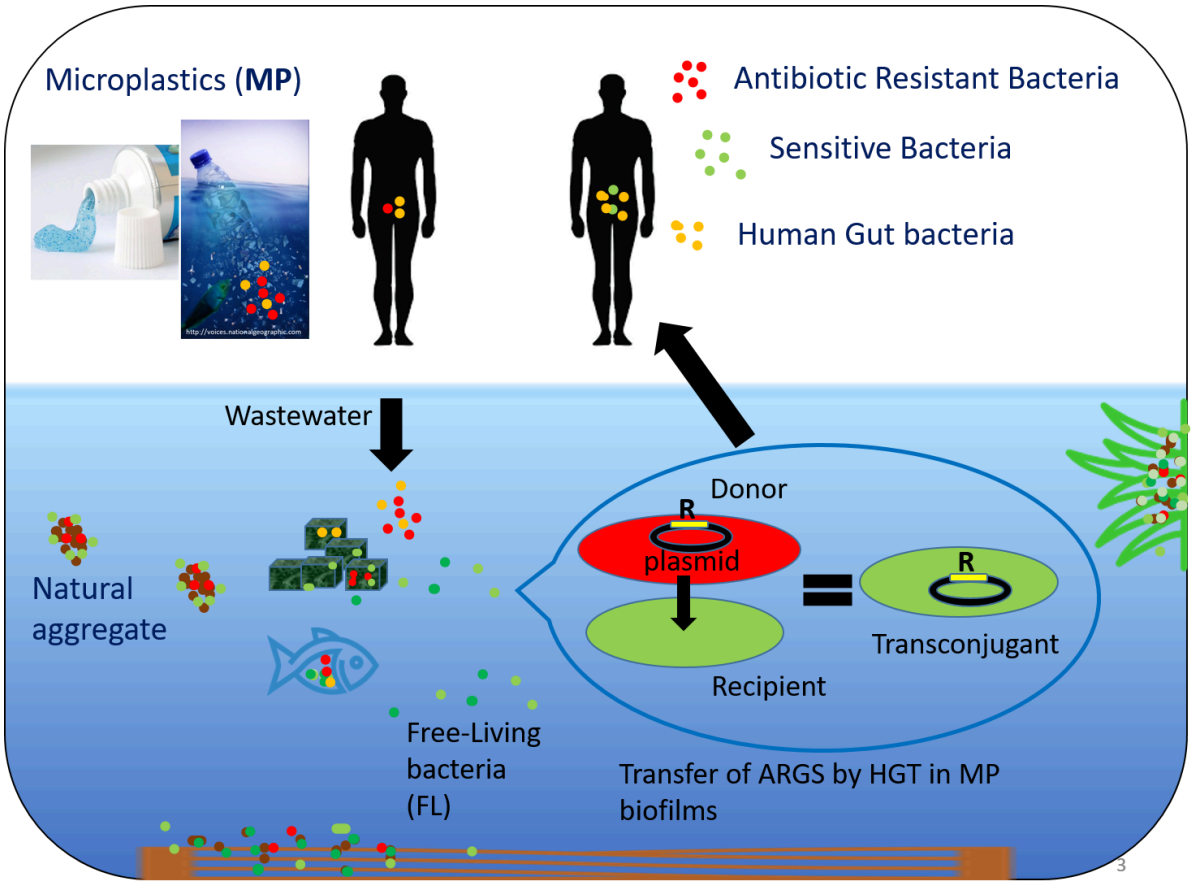


Figure 2. MP affects the distribution of ARGs in aquatic ecosystems and their transfer through the aquatic food web to human populations. Wastewaters can contain both MP and antibiotic-resistant bacteria. Resistant bacteria can transfer plasmid-borne ARGs to aquatic bacteria as they coincide on MP biofilms, in the water or the gut of aquatic biota. Further contact of humans with the aquatic system enables the transfer of ARGs to the human microbiome

Concluding remarks: scientific and social outlook

One of the most significant contributions of this Ph.D. thesis to the understanding of ecological interactions among microbial communities of MP biofilms is the emphasis on their functional capacities, specifically on gene exchange. At the bottom line, this Ph.D. thesis suggests that **the magnitude of MP pollution has the potential to produce long-term and irreversible changes in the microbial world, which can affect the base of all aquatic food webs on the planet**. This conclusion is based on the significant role of HGT among microorganisms in the evolution of life on Earth. Therefore, alterations in this process ultimately change the functioning of biogeochemical cycles on the planet. These, in turn, regulate vital aspects of life for multicellular organisms, for example, air and water quality, nutrient availability and the capacity to adapt to changing conditions. Besides, HGT has a

direct influence on the evolution of microbial symbiotic relationships, including those of parasitism, and the development of pathogens of animals and plants.

The concept was developed throughout three experimental studies and after analysis of recent literature on the ecology of natural biofilms and HGT mechanisms. It is also firmly sustained in the enormous and ubiquitous nature of current MP contamination as described in the introduction. Humans have introduced a disturbance of global magnitude and massive potential for change in a very short period. Indeed, although starting in the 1950´s, most of the accumulated plastic pollution was produced in the last decade (Geyer *et al.*, 2017). The growth of plastic pollution is comparable to the global increase in temperature widely accepted as a human-induced climate change. Throughout a century, CO_2 emissions increased from the industrial revolution between the 18-19th centuries to its peak in the 21st century. Therefore, it is reasonable to predict that the consequences of MP contamination on aquatic microbial communities worldwide will massively increase in the near future.

The current state of MP biofilms studies has many limitations such as the lack of long-term data and the overrepresentation of marine vs. freshwater ecosystems. The first is understandable since MP have been produced and released into nature in a relatively short period. The study of freshwater MP must address the element of the substantial heterogeneity these aquatic systems display (e.g., limnic, lentic, permanent, temporal, depth and trophy status). There are of course numerous restrictions on studying cellular activities in biofilms *in vivo*. Therefore, the Ph.D. thesis demonstrates the need to combine both genomic and physiological approaches to address in detail the multiple aspects of microbial biodiversity and function of MP biofilms.

Up to date, local and national governments are discussing many strategies for mitigation of plastic pollution (e.g., the transfer to a circular economy), and the awareness of the problem is increasing. New ideas emerge from the public every day on how to reduce and reuse plastic products, as well as initiatives to recuperate plastic pollution from the environment (Syberg *et al.*, 2018). Despite such increasing efforts, there is still more to do from the perspectives of economics, management, and regulations, to reduce the number of plastics in aquatic systems to an extent where adverse effects should be negligible. Most of the plastic debris ends up as MP particles, and a great deal is left to understand the long-term repercussions of this difficult-to-handle pollution.

In that sense, international organizations have differences in their view on the MP pollution for aquatic ecosystems. For example, in the European Union, the Water Framework Directive (WFD) does not mention the problem while the Marine Strategy Framework Directive (MSFD) includes it in a legislative proposal (Gago *et al.*, 2016). In the regulatory and legislative context, the information presented in this Ph.D. thesis gives a new perspective of the "true" extent of the MP problem, which is the alteration of microbial ecological interactions through HGT, with a particular emphasis on the potential for spreading antibiotic

resistance. Of course, this perspective needs further research and development to translate into specific risk assessment strategies.

The Ph.D. thesis provides hypothesis based testing of MP effects on the function of microbial communities in aquatic ecosystems. The specific ideas on how to follow up on the results, presented towards the end of the discussion, are meant to look for evidence of the connection between MP biofilm microbial diversity and the changes observed in the Earth´s microbiome. The primary objectives should be to provide scientific-based theories for the alteration of microbial communities and their ecological role by MP with potential effects for human health via severe changes in the Earth's ecosystem services. Finally, this knowledge should be used to decide the fate of plastic polymer use in human activities, and hopefully in more stringent regulations on plastics final disposition.

References

Adey NB, Mataragnon AH, Rider JE, Carter JM, Kay BK. (1995). Characterization of phage that bind plastic from phage-displayed random peptide libraries. *Gene* **156**: 27–31.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol* **8**: 251–259.

Amalfitano S, Coci M, Corno G, Luna GM. (2015). A microbial perspective on biological invasions in aquatic ecosystems. *Hydrobiologia* **746**: 13–22.

Aminov RI. (2011). Horizontal Gene Exchange in Environmental Microbiota. *Front Microbiol* **2**: 158.

Andrady AL. (2011). Microplastics in the marine environment. *Mar Pollut Bull* **62**: 1596–605.

Arias-Andres M, Kettner MT, Miki T, Grossart H-P. (2018). Microplastics: New substrates for heterotrophic activity contribute to altering organic matter cycles in aquatic ecosystems. *Sci Total Environ* **635**: 1152–1159.

Bakhshinejad B, Sadeghizadeh M. (2016). A polystyrene binding target-unrelated peptide isolated in the screening of phage display library. *Anal Biochem* **512**: 120–128.

Bakir A, Rowland SJ, Thompson RC. (2012). Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. *Mar Pollut Bull* **64**: 2782–9.

Balcazar JL. (2014). Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS Pathog* **10**: e1004219.

Balcázar JL, Subirats J, Borrego CM. (2015). The role of biofilms as environmental reservoirs of antibiotic resistance. *Front Microbiol* **6**. e-pub ahead of print, doi: 10.3389/fmicb.2015.01216.

Battin TJ, Besemer K, Bengtsson MM, Romani AM, Packmann AI. (2016). The ecology and biogeochemistry of stream biofilms. *Nat Rev Microbiol* **14**: 251–263.

Beer S, Garm A, Huwer B, Dierking J, Nielsen TG. (2018). No increase in marine microplastic concentration over the last three decades – A case study from the Baltic Sea. *Sci Total Environ* **621**: 1272–1279.

Bellanger X, Guilloteau H, Bonot S, Merlin C. (2014a). Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. *Sci Total Environ* **493**: 872–882.

Bellanger X, Guilloteau H, Breuil B, Merlin C. (2014b). Natural microbial communities supporting the transfer of the IncP-1Î² plasmid pB10 exhibit a higher initial content of plasmids from the same incompatibility group. *Front Microbiol* **5**: 1–8.

Bižić-Ionescu M, Zeder M, Ionescu D, Orlić S, Fuchs BM, Grossart H-P, *et al.* (2015). Comparison of bacterial communities on limnic versus coastal marine particles reveals profound differences in colonization. *Environ Microbiol* **17**: 3500–3514.

Broszat M, Grohmann E. (2014). Antibiofilm Agents. In: Rumbaugh KP, Ahmad I (eds) Springer Series on Biofilms Vol. 8. Springer Berlin Heidelberg: Berlin, Heidelberg, pp 1–32.

Bryant JA, Clemente TM, Viviani DA, Fong AA, Thomas KA, Kemp P, *et al.* (2016). Diversity and Activity of Communities Inhabiting Plastic Debris in the North Pacific Gyre Jansson JK (ed). *mSystems* **1**: e00024-16.

Cairns J, Jalasvuori M, Ojala V, Brockhurst M, Hiltunen T. (2016). Conjugation is necessary for a bacterial plasmid to survive under protozoan predation. *Biol Lett* **12**: 20150953.

Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, *et al.* (2014). In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. *Antimicrob Agents Chemother* **58**: 3895–3903.

Castelle CJ, Brown CT, Thomas BC, Williams KH, Banfield JF. (2017). Unusual respiratory capacity and nitrogen metabolism in a Parcubacterium (OD1) of the Candidate Phyla Radiation. *Sci Rep* **7**: 40101.

Cole M. (2016). A novel method for preparing microplastic fibers. *Sci Rep* **6**: 34519.

Cole M, Lindeque P, Halsband C, Galloway TS. (2011). Microplastics as contaminants in the marine environment: A review. *Mar Pollut Bull* **62**: 2588–2597.

Corsaro D, Venditti D. (2009). Detection of Chlamydiae from freshwater environments by PCR, amoeba coculture and mixed coculture. *Res Microbiol* **160**: 547–552.

da Costa JP, Duarte AC, Rocha-Santos TAP. (2017). Microplastics – Occurrence, Fate and Behaviour in the Environment. In: Vol. 75. *Comprehensive Analytical Chemistry*. pp 1–24.

Demeter MA, Lemire JA, Turner RJ, Harrison JJ. (2016). Biofilm Survival Strategies in Polluted Environments. In: *Biofilms in Bioremediation: Current Research and Emerging Technologies*. Caister Academic Press, pp 43–56.

Dickschat JS. (2010). Quorum sensing and bacterial biofilms. *Nat Prod Rep* **27**: 343–369.

Drudge CN, Warren LA. (2012). Prokaryotic Horizontal Gene Transfer in Freshwater Lakes: Implications of Dynamic Biogeochemical Zonation. *J Environ Prot (Irvine, Calif)* **03**: 1634–1654.

Dussud C, Meistertzheim AL, Conan P, Pujo-Pay M, George M, Fabre P, *et al.* (2018). Evidence of niche partitioning among bacteria living on plastics, organic particles and surrounding seawaters. *Environ Pollut* **236**: 807–816.

Eerkes-Medrano D, Thompson RC, Aldridge DC. (2015). Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. *Water Res* **75**: 63–82.

Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. (2016). Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* **14**: 563–575.

Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**: 289–306.

Fuchsman CA, Collins RE, Rocap G, Brazelton WJ. (2017). Effect of the environment on horizontal gene transfer between bacteria and archaea. *PeerJ* **5**: e3865.

Gago J, Galgani F, Maes T, Thompson RC. (2016). Microplastics in Seawater: Recommendations from the Marine Strategy Framework Directive Implementation Process. *Front Mar Sci* **3**. e-pub ahead of print, doi: 10.3389/fmars.2016.00219.

Geyer R, Jambeck JR, Law KL. (2017). Production, use, and fate of all plastics ever made. *Sci Adv* **3**: e1700782.

Gillings M, Boucher Y, Labbate M, Holmes A, Krishnan S, Holley M, *et al.* (2008). The Evolution of Class 1 Integrons and the Rise of Antibiotic Resistance. *J Bacteriol* **190**: 5095–5100.

Gillings MR. (2017). Class 1 integrons as invasive species. *Curr Opin Microbiol* **38**: 10–15.

Gillings MR, Paulsen IT. (2014). Microbiology of the Anthropocene. *Anthropocene* **5**: 1–8.

Goldstein MC, Titmus AJ, Ford M. (2013). Scales of Spatial Heterogeneity of Plastic Marine Debris in the Northeast Pacific Ocean Hewitt J (ed). *PLoS One* **8**: e80020.

Guo J, Li J, Chen H, Bond PL, Yuan Z. (2017). Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Res* **123**: 468–478.

Harrison JP, Schratzberger M, Sapp M, Osborn a M. (2014). Rapid bacterial colonization of low-density polyethylene microplastics in coastal sediment microcosms. *BMC Microbiol* **14**: 232.

Hirai H, Takada H, Ogata Y, Yamashita R, Mizukawa K, Saha M, *et al.* (2011). Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Mar Pollut Bull* **62**: 1683–1692.

Ivar do Sul J a, Costa MF. (2014). The present and future of microplastic pollution in the marine environment. *Environ Pollut* **185**: 352–64.

Jahnke A, Arp HPH, Escher BI, Gewert B, Gorokhova E, Kühnel D, *et al.* (2017). Reducing Uncertainty and Confronting Ignorance about the Possible Impacts of Weathering Plastic in the Marine Environment. *Environ Sci Technol Lett* **4**: 85–90.

Jiao Y-N, Chen H, Gao R-X, Zhu Y-G, Rensing C. (2017). Organic compounds stimulate horizontal transfer of antibiotic resistance genes in mixed wastewater treatment systems. *Chemosphere* **184**: 53–61.

Kalogerakis N, Arff J, Banat IM, Broch OJ, Daffonchio D, Edvardsen T, *et al.* (2014). The role of environmental biotechnology in exploring, exploiting, monitoring, preserving, protecting and decontaminating the marine environment. *N Biotechnol* **00**: 1–11.

Keswani A, Oliver DM, Gutierrez T, Quilliam RS. (2016). Microbial hitchhikers on marine plastic debris: Human exposure risks at bathing waters and beach environments. *Mar Environ Res* **118**: 10–19.

Kielak AM, Barreto CC, Kowalchuk GA, van Veen JA, Kuramae EE. (2016). The Ecology of Acidobacteria: Moving beyond Genes and Genomes. *Front Microbiol* **7**: 1–16.

Kirstein I V., Kirmizi S, Wichels A, Garin-Fernandez A, Erler R, Löder M, *et al.* (2016). Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic

particles. *Mar Environ Res* **120**: 1–8.

Kopf RK, Finlayson CM, Humphries P, Sims NC, Hladyz S. (2015). Anthropocene Baselines: Assessing Change and Managing Biodiversity in Human-Dominated Aquatic Ecosystems. *Bioscience* **65**: 798–811.

de la Cruz-Perera CI, Ren D, Blanchet M, Dendooven L, Marsch R, Sørensen SJ, *et al.* (2013). The ability of soil bacteria to receive the conjugative IncP1 plasmid, pKJK10, is different in a mixed community compared to single strains. *FEMS Microbiol Lett* **338**: 95–100.

de la Cruz F, Davies J. (2000). Horizontal gene transfer and the origin of species: lessons from bacteria. - PubMed - NCBI. *Trends Microbiol* 128–133.

Lacroix B, Citovsky V. (2016). Transfer of DNA from Bacteria to Eukaryotes. *MBio* **7**: e00863-16.

Lebreton L, Slat B, Ferrari F, Sainte-Rose B, Aitken J, Marthouse R, *et al.* (2018). Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic. *Sci Rep* **8**: 4666.

Lenz R, Enders K, Nielsen TG. (2016). Microplastic exposure studies should be environmentally realistic. *Proc Natl Acad Sci* **113**: E4121–E4122.

Leslie HA, Brandsma SH, van Velzen MJM, Vethaak AD. (2017). Microplastics en route: Field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota. *Environ Int* **101**: 133–142.

Li X, Wang Y, Brown CJ, Yao F, Jiang Y, Top EM, *et al.* (2016). Diversification of broad host range plasmids correlates with the presence of antibiotic resistance genes Smalla K (ed). *FEMS Microbiol Ecol* **92**: fiv151.

Linz S, Radtke A, von Haeseler A. (2007). A likelihood framework to measure horizontal gene transfer. *Mol Biol Evol* **24**: 1312–9.

Litchman E. (2010). Invisible invaders: non-pathogenic invasive microbes in aquatic and terrestrial ecosystems. *Ecol Lett* **13**: 1560–1572.

Loftie-Eaton W, Tucker A, Norton A, Top EM. (2014). Flow cytometry and real-time quantitative PCR as tools for assessing plasmid persistence. *Appl Environ Microbiol* **80**: 5439–46.

Lopatkin AJ, Huang S, Smith RP, Srimani JK, Sysoeva TA, Bewick S, *et al.* (2016a). Antibiotics as a selective driver for conjugation dynamics. *Nat Microbiol* **1**: 16044.

Lopatkin AJ, Sysoeva TA, You L. (2016b). Dissecting the effects of antibiotics on horizontal gene transfer: Analysis suggests a critical role of selection dynamics. *BioEssays* **38**: 1283–1292.

Madsen JS, Burmølle M, Hansen LH, Sørensen SJ. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunol Med Microbiol* **65**: 183–95.

Martínez JL, Coque TM, Baquero F. (2015). What is a resistance gene? Ranking risk in resistomes. *Nat Rev Microbiol* **13**: 116–123.

McCormick A, Hoellein TJ, Mason SA, Schluep J, Kelly JJ. (2014). Microplastic is an

Abundant and Distinct Microbial Habitat in an Urban River. *Environ Sci Technol* **48**: 11863–11871.

McCormick AR, Hoellein TJ, London MG, Hittie J, Scott JW, Kelly JJ. (2016). Microplastic in surface waters of urban rivers: concentration, sources, and associated bacterial assemblages. *Ecosphere* **7**: e01556.

McDaniel LD, Young EC, Ritchie KB, Paul JH. (2012). Environmental factors influencing gene transfer agent (GTA) mediated transduction in the subtropical ocean. *PLoS One* **7**: e43506.

Mintenig SM, Int-Veen I, Löder MGJ, Primpke S, Gerdts G. (2017). Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. *Water Res* **108**: 365–372.

Monroe JG, Markman DW, Beck WS, Felton AJ, Vahsen ML, Pressler Y. (2018). Ecoevolutionary Dynamics of Carbon Cycling in the Anthropocene. *Trends Ecol Evol* **33**: 213–225.

Nelson WC, Stegen JC. (2015). The reduced genomes of Parcubacteria (OD1) contain signatures of a symbiotic lifestyle. *Front Microbiol* **6**: 713.

Nielsen KM, Bøhn T, Townsend JP. (2014). Detecting rare gene transfer events in bacterial populations. *Front Microbiol* **4**: 415.

Oberbeckmann S, Loeder MGJ, Gerdts G, Osborn a M. (2014). Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European waters. *FEMS Microbiol Ecol* **90**: 478–92.

Oberbeckmann S, Osborn AM, Duhaime MB. (2016). Microbes on a Bottle: Substrate, Season and Geography Influence Community Composition of Microbes Colonizing Marine Plastic Debris Carter DA (ed). *PLoS One* **11**: e0159289.

P.C.H. Hollman, H. Bouwmeester, Peters RJB. (2013). Microplastics in the aquatic food chain;. Sources, measurement, occurrence and potential health risks. Wgeningen. https://www.wageningenur.nl/en/Publication-details.htm?publicationId=publication-way-343430303039.

Pernthaler J. (2017). Competition and niche separation of pelagic bacteria in freshwater habitats. *Environ Microbiol* **19**: 2133–2150.

Phuong NN, Zalouk-Vergnoux A, Poirier L, Kamari A, Châtel A, Mouneyrac C, *et al.* (2016). Is there any consistency between the microplastics found in the field and those used in laboratory experiments? *Environ Pollut* **211**: 111–123.

PlasticsEurope. (2015). An Analysis of European Plastics Production, Demand and Waste Data. http://www.plasticseurope.org/en.

Power ML, Samuel A, Smith JJ, Stark JS, Gillings MR, Gordon DM. (2016). Escherichia coli out in the cold: Dissemination of human-derived bacteria into the Antarctic microbiome. *Environ Pollut* **215**: 58–65.

Reichert J, Schellenberg J, Schubert P, Wilke T. (2017). Responses of reef building corals to microplastic exposure. *Environ Pollut* 1–6.

Reisner A, Wolinski H, Zechner EL. (2012). In situ monitoring of IncF plasmid transfer on semi-solid agar surfaces reveals a limited invasion of plasmids in recipient colonies. *Plasmid* **67**: 155–61.

Rocha-Santos T, Duarte AC. (2015). A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. *TrAC Trends Anal Chem* **65**: 47–53.

Romera-Castillo C, Pinto M, Langer TM, Álvarez-Salgado XA, Herndl GJ. (2018). Dissolved organic carbon leaching from plastics stimulates microbial activity in the ocean. *Nat Commun* **9**: 1430.

Rösel S, Allgaier M, Grossart H-P. (2012). Long-term characterization of free-living and particle-associated bacterial communities in Lake Tiefwaren reveals distinct seasonal patterns. *Microb Ecol* **64**: 571–83.

Ruiz-González C, Niño-García JP, Lapierre J-F, del Giorgio PA. (2015). The quality of organic matter shapes the functional biogeography of bacterioplankton across boreal freshwater ecosystems. *Glob Ecol Biogeogr* **24**: 1487–1498.

Ryan PG. (2015). Does size and buoyancy affect the long-distance transport of floating debris? *Environ Res Lett* **10**: 084019.

Schoen ME, Garland J. (2017). Review of pathogen treatment reductions for onsite non-potable reuse of alternative source waters. *Microb Risk Anal* **5**: 25–31.

van Sebille E, Wilcox C, Lebreton L, Maximenko N, Hardesty BD, van Franeker JA, *et al.* (2015). A global inventory of small floating plastic debris. *Environ Res Lett* **10**: 124006.

Sentchilo V, Mayer AP, Guy L, Miyazaki R, Green Tringe S, Barry K, *et al.* (2013). Community-wide plasmid gene mobilization and selection. *ISME J* **7**: 1173–1186.

Setälä O, Fleming-Lehtinen V, Lehtiniemi M. (2014). Ingestion and transfer of microplastics in the planktonic food web. *Environ Pollut* **185**: 77–83.

Sezonov G, Joseleau-Petit D, D'Ari R. (2007). Escherichia coli Physiology in Luria-Bertani Broth. *J Bacteriol* **189**: 8746–8749.

Siefert JL. (2009). Defining the Mobilome. In: M.B. G, J.P. G, Olendzenski LC (eds). *Horizontal Gene Transfer. Methods in Molecular Biology*. Humana Press, pp 13–27.

Skippington E, Ragan MA. (2011). Lateral genetic transfer and the construction of genetic exchange communities. *FEMS Microbiol Rev* **35**: 707–735.

Solheim HT, Sekse C, Urdahl AM, Wasteson Y, Nesse LL. (2013). Biofilm as an Environment for Dissemination of stx Genes by Transduction. *Appl Environ Microbiol* **79**: 896–900.

Soucy SM, Huang J, Gogarten JP. (2015). Horizontal gene transfer: building the web of life. *Nat Rev Genet* **16**. e-pub ahead of print, doi: 10.1038/nrg3962.

Stevenson C, Hall JPJ, Brockhurst MA, Harrison E. (2018). Plasmid stability is enhanced by higher-frequency pulses of positive selection. *Proc R Soc B Biol Sci* **285**: 20172497.

Stewart FJ. (2013). Where the genes flow. *Nat Geosci* **6**: 688–690.

Syberg K, Hansen SF, Christensen TB, Khan FR. (2018). Risk Perception of Plastic Pollution: Importance of Stakeholder Involvement and Citizen Science BT - Freshwater Microplastics : Emerging Environmental Contaminants? In: Wagner M, Lambert S (eds). Springer International Publishing: Cham, pp 203–221.

Tamaki H, Zhang R, Angly FE, Nakamura S, Hong P-Y, Yasunaga T, *et al.* (2012). Metagenomic analysis of DNA viruses in a wastewater treatment plant in tropical climate. *Environ Microbiol* **14**: 441–452.

Tang K, Grossart H, Yam E, Jackson G, Ducklow H, Kiørboe T. (2006). Mesocosm study of particle dynamics and control of particle-associated bacteria by flagellate grazing. *Mar Ecol Prog Ser* **325**: 15–27.

De Tender CA, Devriese LI, Haegeman A, Maes S, Ruttink T, Dawyndt P. (2015). Bacterial Community Profiling of Plastic Litter in the Belgian Part of the North Sea. *Environ Sci Technol* **49**: 9629–9638.

Vasickova P, Pavlik I, Verani M, Carducci A. (2010). Issues Concerning Survival of Viruses on Surfaces. *Food Environ Virol* **2**: 24–34.

Wagner M, Lambert S. (2018). Freshwater Microplastics. Springer International Publishing: Cham.

Walker A. (2012). Welcome to the plasmidome. *Nat Rev Microbiol* **10**: 379–379.

Wilkinson DM. (2010). Have we underestimated the importance of humans in the biogeography of free-living terrestrial microorganisms? *J Biogeogr* **37**: 393–397.

Woodhouse JN, Kinsela AS, Collins RN, Bowling LC, Honeyman GL, Holliday JK, *et al.* (2016). Microbial communities reflect temporal changes in cyanobacterial composition in a shallow ephemeral freshwater lake. *ISME J* **10**: 1337–1351.

Zalasiewicz J, Waters CN, Ivar do Sul JA, Corcoran PL, Barnosky AD, Cearreta A, *et al.* (2016). The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. *Anthropocene* **13**: 4–17.

Zettler ER, Mincer TJ, Amaral-Zettler LA. (2013). Life in the "Plastisphere": Microbial Communities on Plastic Marine Debris. *Environ Sci Technol* **47**: 7137–7146.

Zhang Y, Xiao W, Jiao N. (2016). Linking biochemical properties of particles to particle-attached and free-living bacterial community structure along the particle density gradient from freshwater to open ocean. *J Geophys Res Biogeosciences* **121**: 2261–2274.

Zhu Y-G, Gillings M, Simonet P, Stekel D, Banwart S, Penuelas J. (2017). Microbial mass movements. *Science (80-)* **357**: 1099–1100.

Ziajahromi S, Neale PA, Rintoul L, Leusch FDL. (2017). Wastewater treatment plants as a pathway for microplastics: Development of a new approach to sample wastewater-based microplastics. *Water Res* **112**: 93–99.

Zimmermann J, Portillo MC, Serrano L, Ludwig W, Gonzalez JM. (2012). Acidobacteria in Freshwater Ponds at Doñana National Park, Spain. *Microb Ecol* **63**: 844–855.

Supplementary information Chapter I

Table S1 Effect of microplastic per vessel on numbers of bacterial cells, aggregates and microcolonies in the water of the chemostats. Output results of generalised linear models are given.

	Estimate	Standard Error	t value	P-value
(**A**) Bacterial cell numbers in the ambient water				
(Intercept)	7.92	0.24	32.0	<0.0001
microplastic per vessel	-0.00	0.00	-1.1	0.317
(**B**) Aggregate numbers				
(Intercept)	5.27	0.46	11.4	<0.0001
microplastic per vessel	-0.00	0.00	-0.7	0.503
(**C**) Microcolony numbers in the ambient water				
(Intercept)	7.21	0.09	75.1	<0.0001
microplastic per vessel	-0.00	0.00	-1.7	0.139

Table S2 Number of pieces of microplastic and ARISA peaks detected in the vessel water and on microplastic (MP).

Vessel	Pieces of MP	ARISA peaks water	ARISA peaks MP
1	1600	42	66
2	1400	34	66
3	1200	34	52
4	1000	65	64
5	800	47	50
6	600	57	25
7	400	56	33
8	200	70	34
9	0	66	NA

Table S3 Effect of microplastic per vessel on the OTU richness on (**A**) microplastic and (**B**) in water. Output results of linear models are given.

	Estimate	Std. Error	t value	P-value
(**A**) OUT richness on microplastic				
(Intercept)	3.26	0.17	18.7	1.53e-06
microplastic per vessel	0.00	0.00	3.63	0.011
(**B**) OTU richness in water				
(Intercept)	4.24	0.11	38.22	2.18e-09
microplastic per vessel	-0.00	0.00	-3.45	0.01

Figure S1 Abundance of cells (A), small cell clusters (B) and larger aggregates (C) during the 15 days of experiment in all the chemostat vessels.

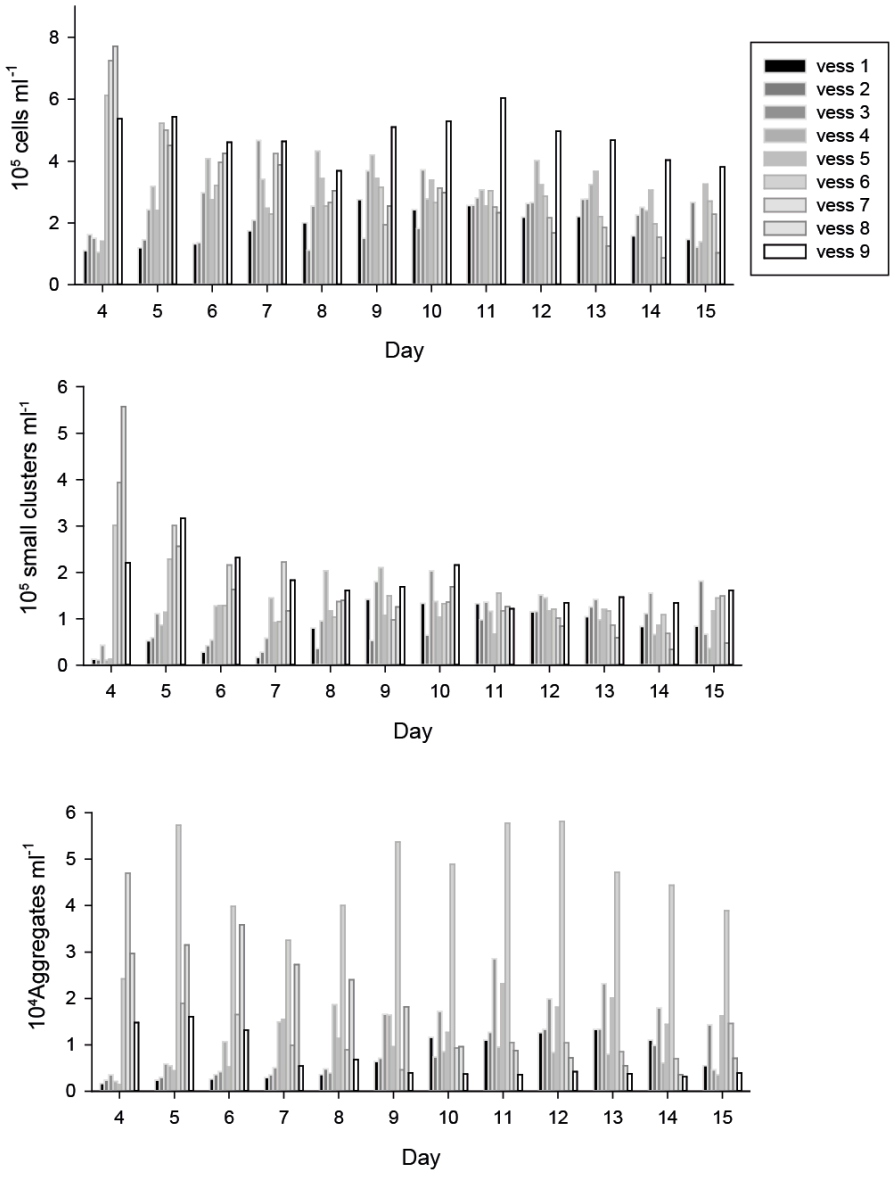
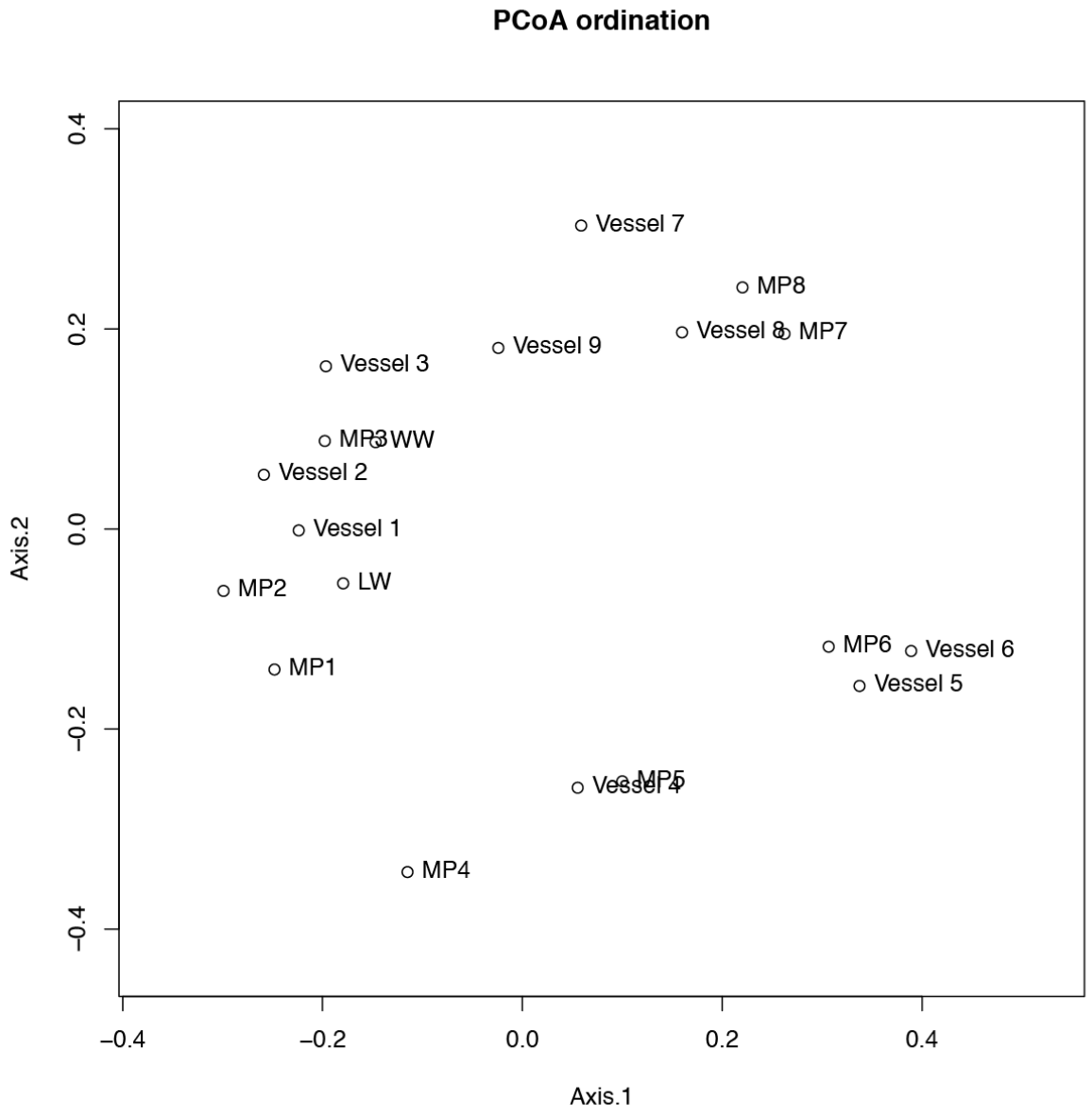


Figure S2 PCoA computet on the Sorensen distance matrix of ARISA peaks in the various samples. Vessel referes to vessel water and are free-water samples. MP refers to microplastic biofilm samples. LW and WW refer to the inoculum communities of lake water and waste water, respectively. Vessel 1 had the highest concentration of microplastic and Vessel 9 had none.



Supplementary Information Chapter II

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

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

4. *FCM and FACS*

Instrument Set Up

A 70 µm nozzle was used at a sheath fluid pressure of 70 psi. Prior to measurement of experimental samples, the proper functioning of the instrument was checked by using the cytometer setup and tracking module (CS&T) with CS&T beads (Becton Dickinson). Before the isolation of cells by FACS, a decontamination procedure for aseptic sorting was followed as described in BD FACSAriaII User´s Guide p187, including the exchange of the 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

Nucleic acid manipulation and sequencing

DNA was extracted from the particles (25 particles per tube), filters (one per tube), and FACS-sorted cells (approx. 20 µL per tube; Table S4), using the REDExtract-N-AmpTM Tissue PCR kit (Sigma). For MPs and filters, we used the recommended protocol for tissues, while for the sorted cells we used the protocol recommended for saliva. DNA concentration was determined using a Quantus™ Fluorometer and stored at 4°C for further processing. The reaction mix for the amplification of the V4 region of the 16S rRNA gene was prepared in a total volume of 50 µl containing MyTaq Red DNA Polymerase (BIOLINE, Germany) and 10

ng of template DNA. It was performed with the following protocol: an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 50°C for 40 sec, extension at 72°C for 1 min, and a 5 min final extension at 72°C. The amplicons were checked in a 1% agarose gel, and then sent for paired-end sequencing by Illumina MiSeq technology.

SI Figures and tables

Figure S1

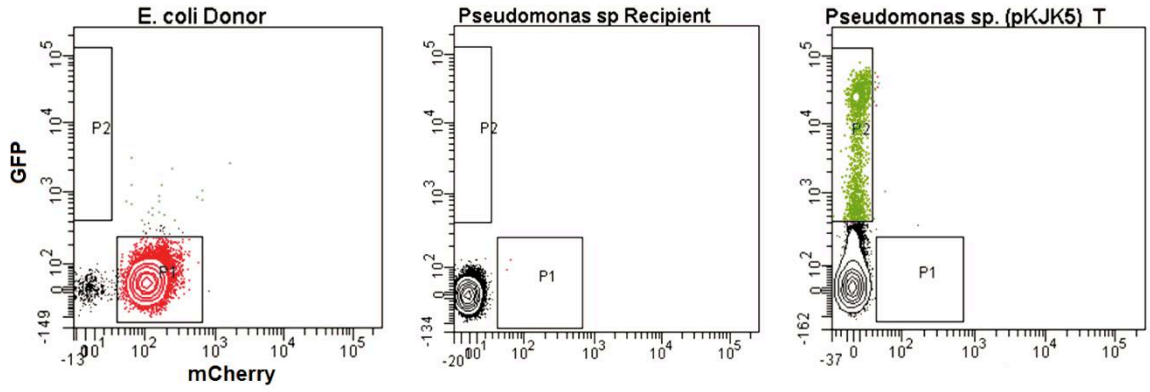


Figure S1. Contour plots in the FCM analysis of the two-species microcosm show the fluorescence pattern (from left to right) from *E. coli* donor strain expressing *mCherry*, *Pseudomonas* sp. recipient strain with no fluorescent expression and *Pseudomonas* sp. after plasmid pKJK5::*gfpmut3* acquisition. 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).

Figure S2

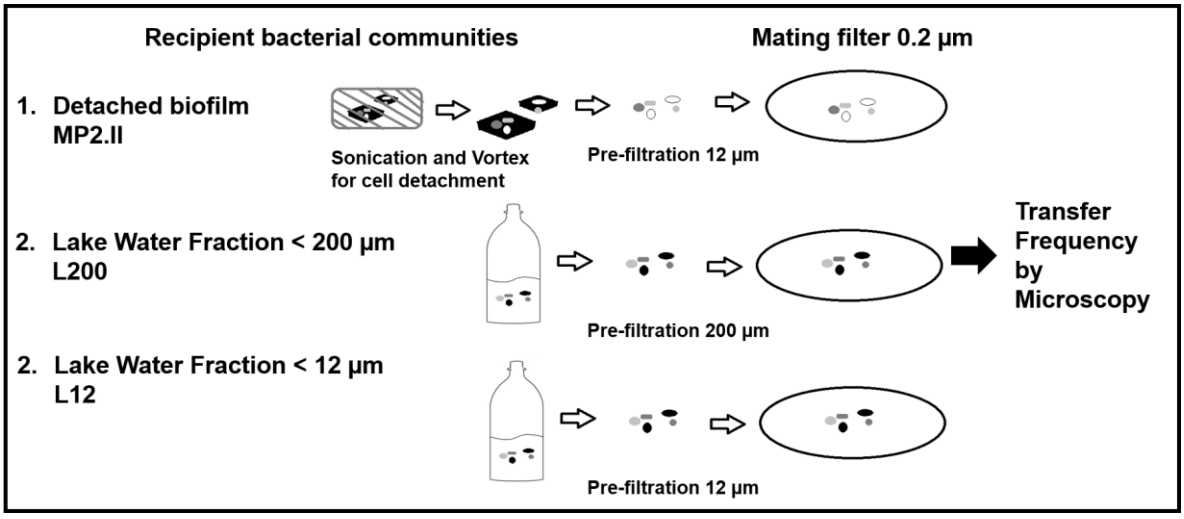


Figure S2. As part of experiment two, transfer frequencies were determined by microscopy for matings of the donor with recipient bacteria originated from microplastic biofilms (MP2.II) and bacteria from lake water pre-filtered by 12µm filter (L12) or 200µm mesh (L200).

Figure S3

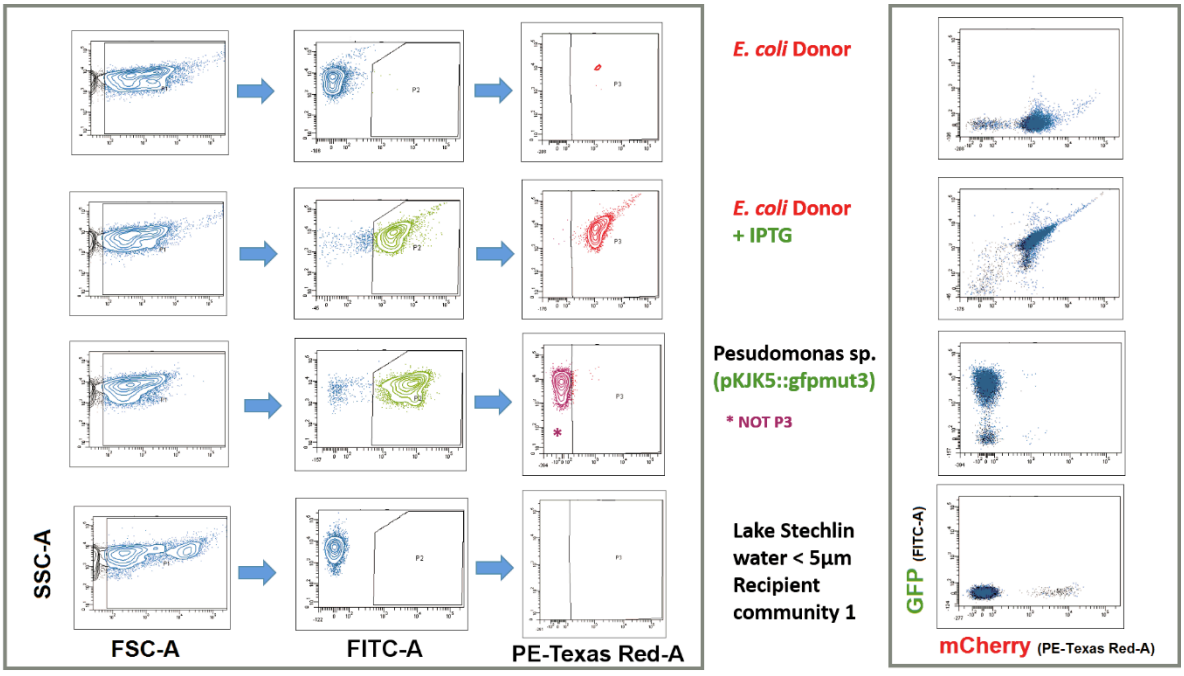


Figure S3. Contour plots on the left exemplify the triple gating procedure for transconjugant FACS-isolation in experiment two. A gate (P1) was set on a bivariate FSC-A vs SSC-A plot for bacterial events using the *E. coli* strain. A second subsequent gate was set on a bivariate FITC-A vs SSC-A plot 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

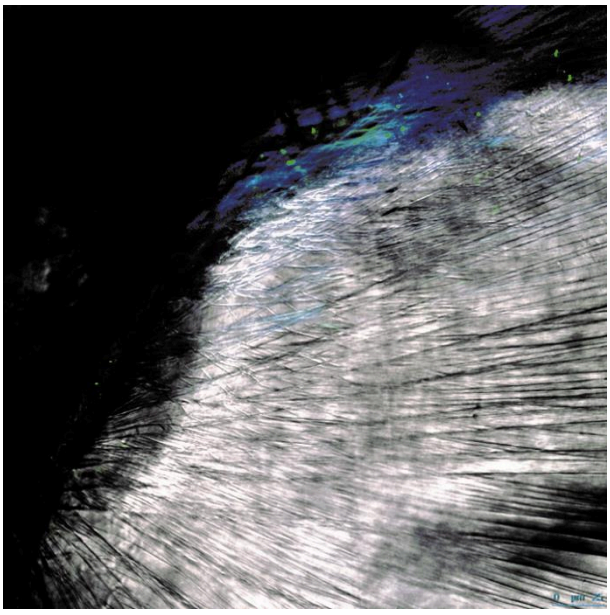


Figure S4. Image of a microplastic biofilm from the two-species microcosm obtained by confocal laser microscopy. Expression of *gfp* in transconjugants is shown in green. Biofilm on the plastic piece was stained with DAPI stain (in blue).

Table S1. Summary of FACS sorted events and filter pooling in Multiple Species 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

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

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)

Table S4. Relative abundances (%) of major phylogenetic groups in the reference 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_unclassifie	0.18	1.78	1.59	1.40

d				
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

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

Table S5. Relative abundance of major phylogenetic groups on reference communities 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

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

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

Supplementary Information Chapter III

Carbon sources included in Biolog EcoPlate™

2-Hydroxy-Benzoic Acid

4-Hydroxy-Benzoic Acid

D,L-α-Glycerol-Phosphate

D-Cellobiose

D-Galactonic Acid γ-Lactone

D-Galacturonic Acid

D-Glucosaminic Acid

D-Malic Acid

D-Mannitol

D-Xylose

Glucose-1-Phosphate

Glycogen

Glycyl-L-Glutamic Acid

i-Erythritol

Itaconic Acid

L-Arginine

L-Asparagine

L-Phenylalanine

L-Serine

L-Threonine

N-Acetyl-D-Glucosamine

Phenylethylamine

Putrescine

Pyruvic Acid Methyl Ester

Tween 40

Tween 80

α-Cyclodextrin

α-D-Lactose

α-Keto-Butyric Acid

β-Methyl-D-Glucoside

γ-Hydroxy-Butyric Acid

Supplementary Table 1. Effect of particle quantity in each cage on the estimation of biofilm biomass per particle (OD_{600nm}) using the crystal violet assay. Output results of generalized linear models (GLM) are given for each lake.

	Estimate	Std. Error	t-value	p-value
Lake Stechlin				
(intercept)	1.55e-01	1.05e-02	14.80	< 2.00e-16
Particles	-1.40e-05	1.45e-06	-9.55	3.53e-14
Lake Dagow				
(intercept)	6.14e-02	6.84e-03	8.98	3.72e-13
Particles	-4.38e-06	9.47e-07	-4.63	1.70e-05
Lake Grosse Fuchskuhle				
(intercept)	3.75e-01	2.32e-02	16.14	< 2.00e-16
Particles	-2.41e-05	3.22e-06	-7.50	1.78e-10

Supplementary Table 2. Output of Kruskal-Wallis tests comparing oxygen consumption (mg) among microcosms with particles from different cages, per lake.

Lake	Chi-squared	p-value
Stechlin	21.39	0.0032
Dagow	22.17	0.0024
Grosse Fuchskuhle	21.17	0.0035

Supplementary Table 3. Permanova results based on Bray-Curtis dissimilarities using OD_{595}nm data in relation to sample type (Microplastic=MP, un-filtered water =W and free-living microbial fraction FL), for a) oligo-mesotrophic Lake Stechlin, b) eutrophic Lake Dagow and c) dystrophic Lake Grosse Fuckskuhle.

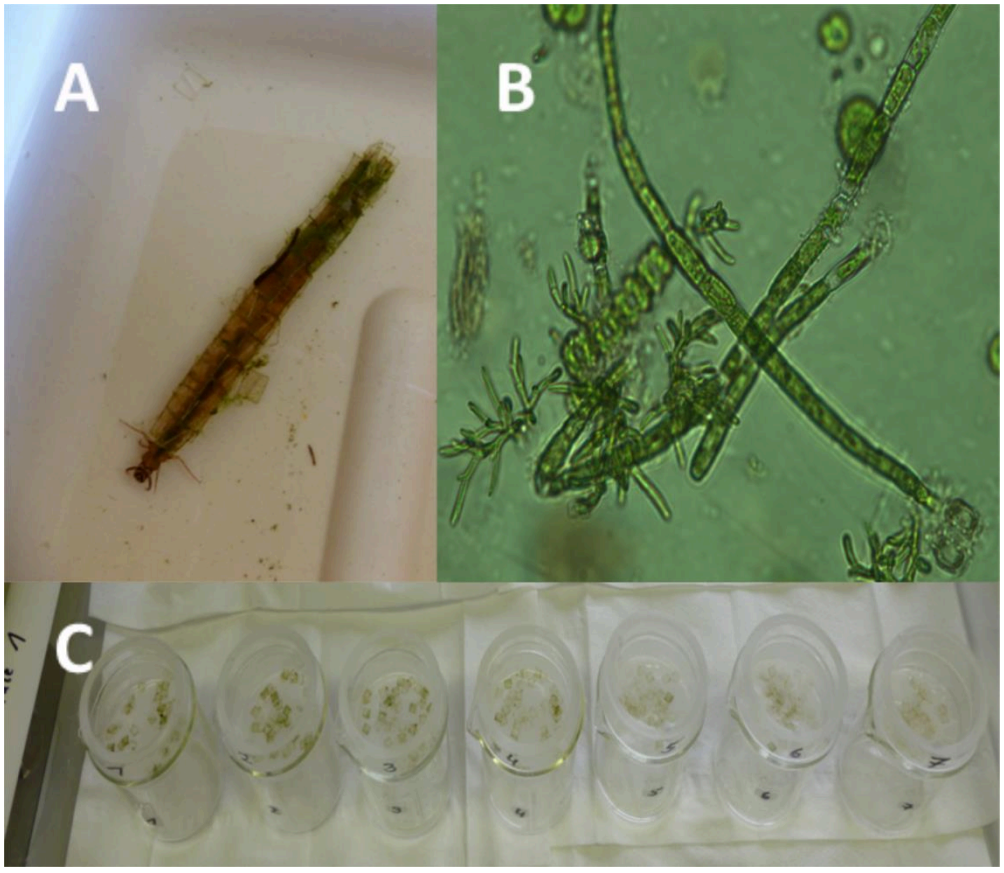
Lake		Df	Sum Sq	F Model	R^2	Pr(>F)
a) Dagow						
	Sample	2	1.08	4.537	0.38	0.001
	Residuals	15	1.79		0.62	
	Total	17	2.87		1.00	
b) Stechlin						
	Sample	2	1.16	2.0684	0.26	0.01
	Residuals	12	3.38		0.74	
	Total	14	4.54		1.00	
c) Grosse Fuchskuhle						
	Sample	2	0.59	3.8667	0.34	0.001
	Residuals	15	1.14		0.66	
	Total	17	1.72		1.00	

p-values based on 999 permutations.

Supplementary Table 4. Results of pairwise comparisons of sample types (Microplastic=MP, un-filtered water =W and free-living microbial fraction FL) after Permanova based on Bray-Curtis dissimilarities using OD_{595}nm data for a) oligo-mesotrophic Lake Stechlin, b) eutrophic Lake Dagow and c) dystrophic Lake Grosse Fuckskuhle.

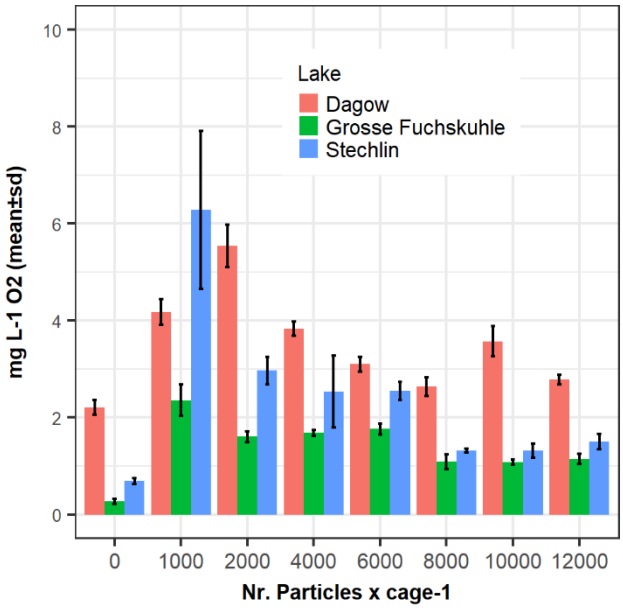
Lake	Pairs	F.Model	R^2	p	p(adjusted)
a) Lake Dagow					
	MP vs W	3. 40	0.25	0.003	0.003
	MP vs FL	6.36	0.39	0.002	0.003
	W vs FL	3.57	0.26	0.001	0.003
b) Lake Stechlin					
	MP vs W	3.27	0.25	0.004	0.012
	MP vs FL	2.677	0.28	0.013	0.0195
	W vs FL	0.427	0.06	0.951	0.951
c) Lake Fuku					
	MP vs W	3.98	0.28	0.001	0.003
	MP vs FL	4.97	0.33	0.005	0.0075
	W vs FL	2.13	0.17	0.06	0.06

Fig.S1



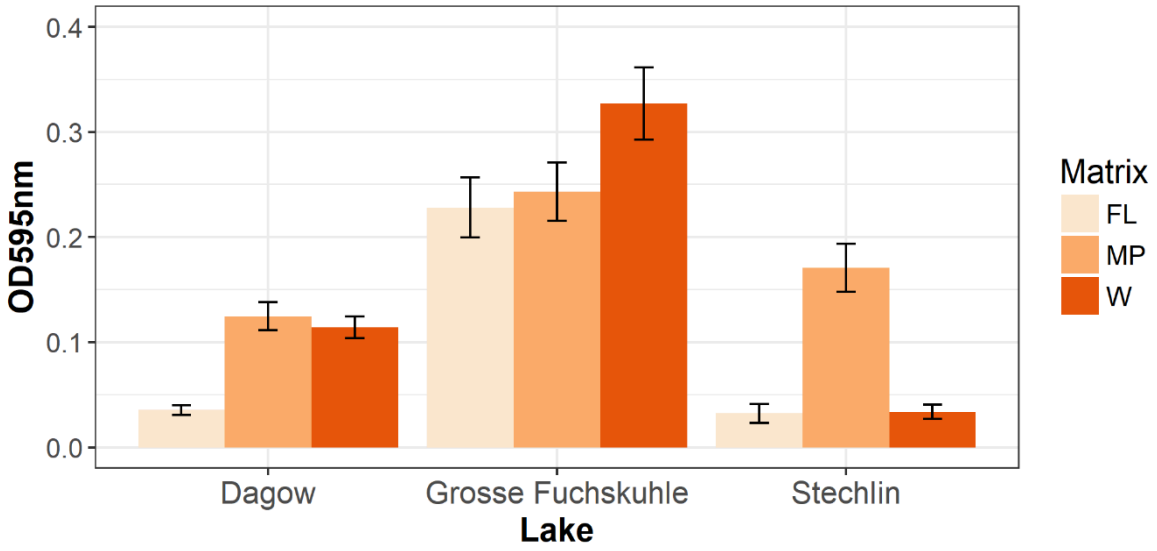
Supplementary Figure 1. A. Larvae of *Trichoptera* sp. used microplastics to build its case. B. Image of green microalgae in a fresh sample of microplastic observed with a light microscopy (40x). C. From left to right, microplastics from cages with least and most particles per cage show highest to lowest biomass, respectively. All images are taken from Lake Grosse Fuchskuhle incubations.

Fig. S2



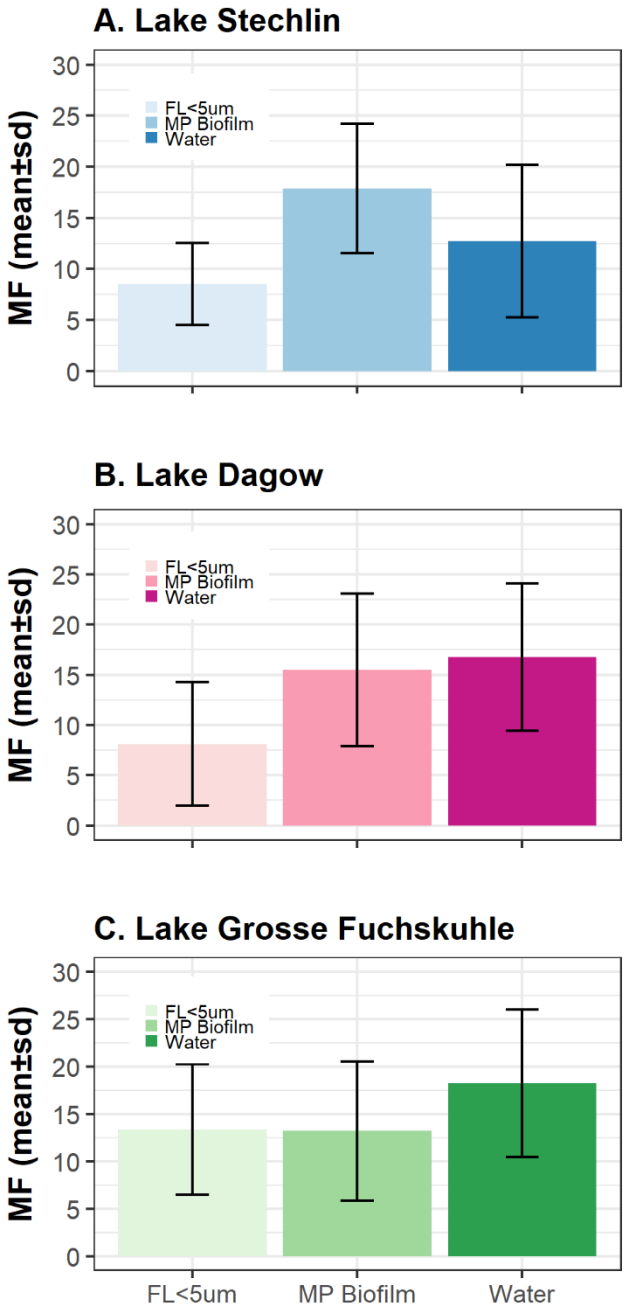
Supplementary Figure 2. Oxygen consumed by microplastic biofilms and water in closed microcosms after 8 days. The Y-axis indicates the O_2 (mg L^{-1}) consumed in 8 days at 10°C and the X-axis indicates number of particles in the cages from the three lakes, from which each 50 microplastics were taken for the microcosm incubation (0 indicates the lake water control with no microplastics).

Fig. S3



Supplementary Figure 3. Bars represent the mean (± standard error) of the optical density measured at 595nm in Biolog EcoPlates inoculated with microplastic particles (MP; all retrieved from cage with 6000 particles) and water samples without (W) and with pre-filtration trough 0.5 µm pore size meshes (FL). Samples from Lakes Stechlin (S), Dagow (D) and Grosse Fuchskuhle (F) were measured after 6 days of EcoPlate incubation. A higher optical density corresponds to a higher respiration, in average, on the EcoPlates.

Fig. S4



Supplementary Figure 4. Average of all multifuntionality indexes MF, or number of substrates used in EcoPlates based on all threshold values (0.1 to 0.9) for OD595nm. The averages are for samples from Lake Stechlin (A), Dagow (B) and Grosse Fuchskuhle (C). "FL<5µm" refers to samples W5, i.e. the free-living bacteria fraction (<5.0 µm). "MP Biofilm" refers to samples from cages with 6000 particles. "Water" refers to samples "W", i.e. the total bacteria fraction (without any pre-filtration). Error bars indicate standard deviation; n=6. Red asterisks indicate different groups at $p = 0.05$.

Declaration

I hereby declare that this thesis and the work presented in it is entirely my own, except where otherwise indicated. I have only used the documented utilities and references.

I certify that this work has not been submitted to any other institution of higher education.

Heredia, 12.05.2018

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María de Jesús Arias Andrés