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.

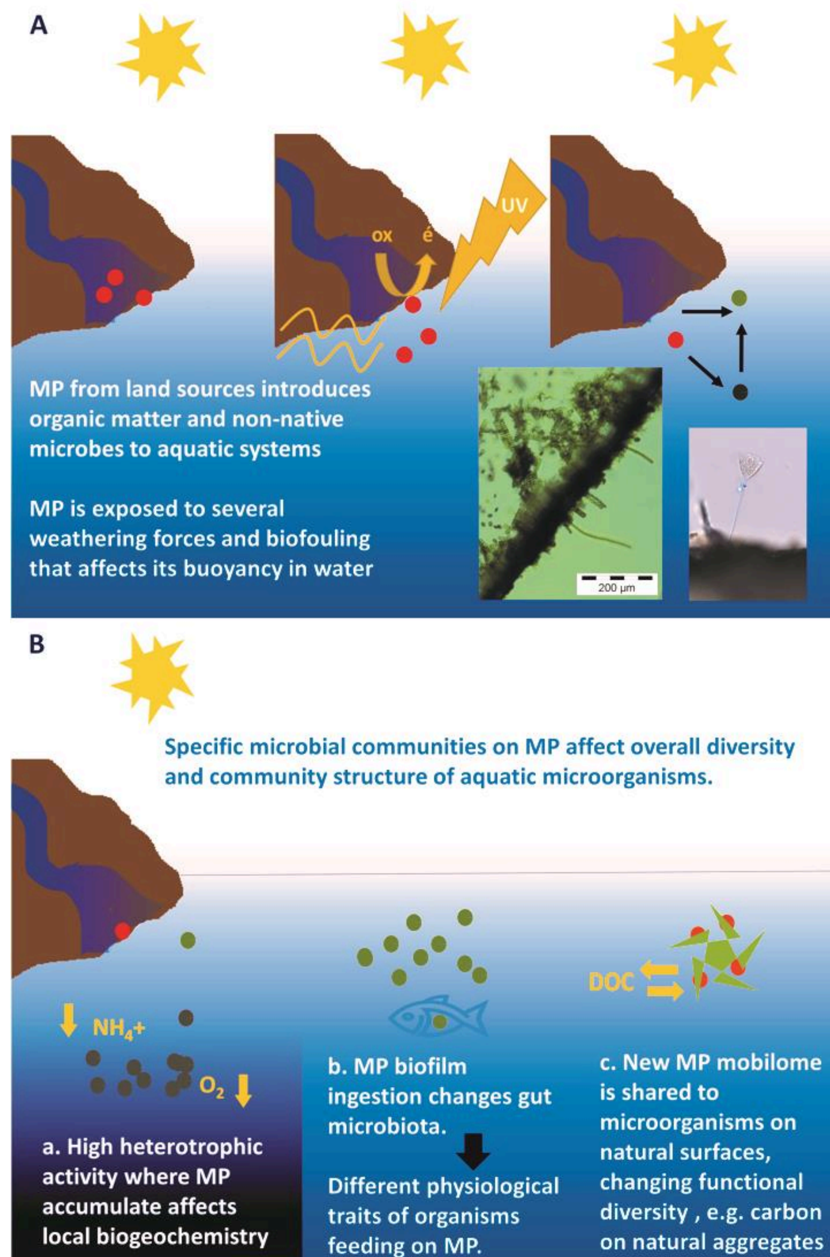

Figure 1. Ecological effects of MP in aquatic ecosystems. Panel A describes changes that MP undergo once they enter aquatic ecosystems, including weathering forces (shearing by water movement, radiation, oxidation processes) and biofouling that affect sinking velocities. Panel B gives the potential effects 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.

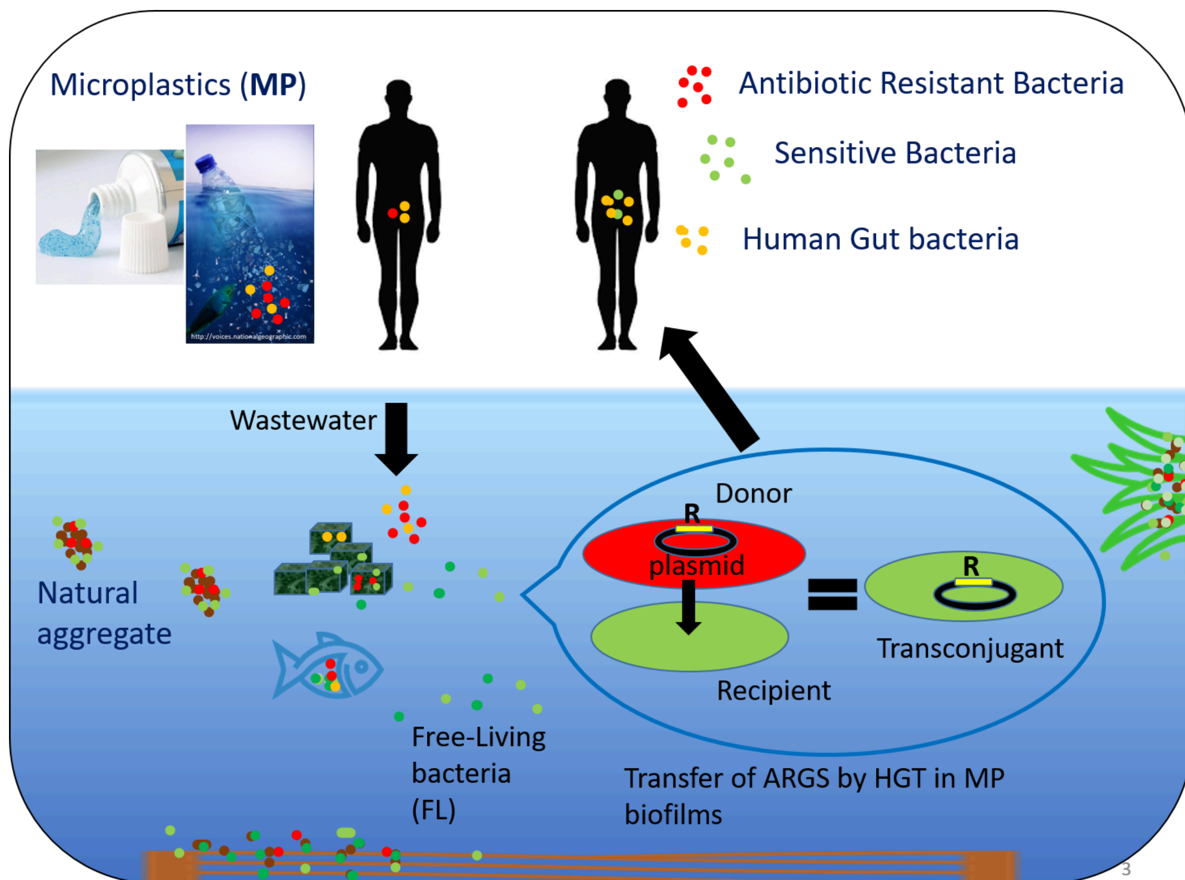

Figure 2. MP affects the distribution of ARGs in aquatic ecosystems and their transfer through the aquatic food web to human populations. Wastewaters can contain both MP and antibiotic-resistant bacteria. Resistant bacteria can transfer plasmid-borne ARGs to aquatic bacteria as they coincide on MP biofilms, 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.

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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.

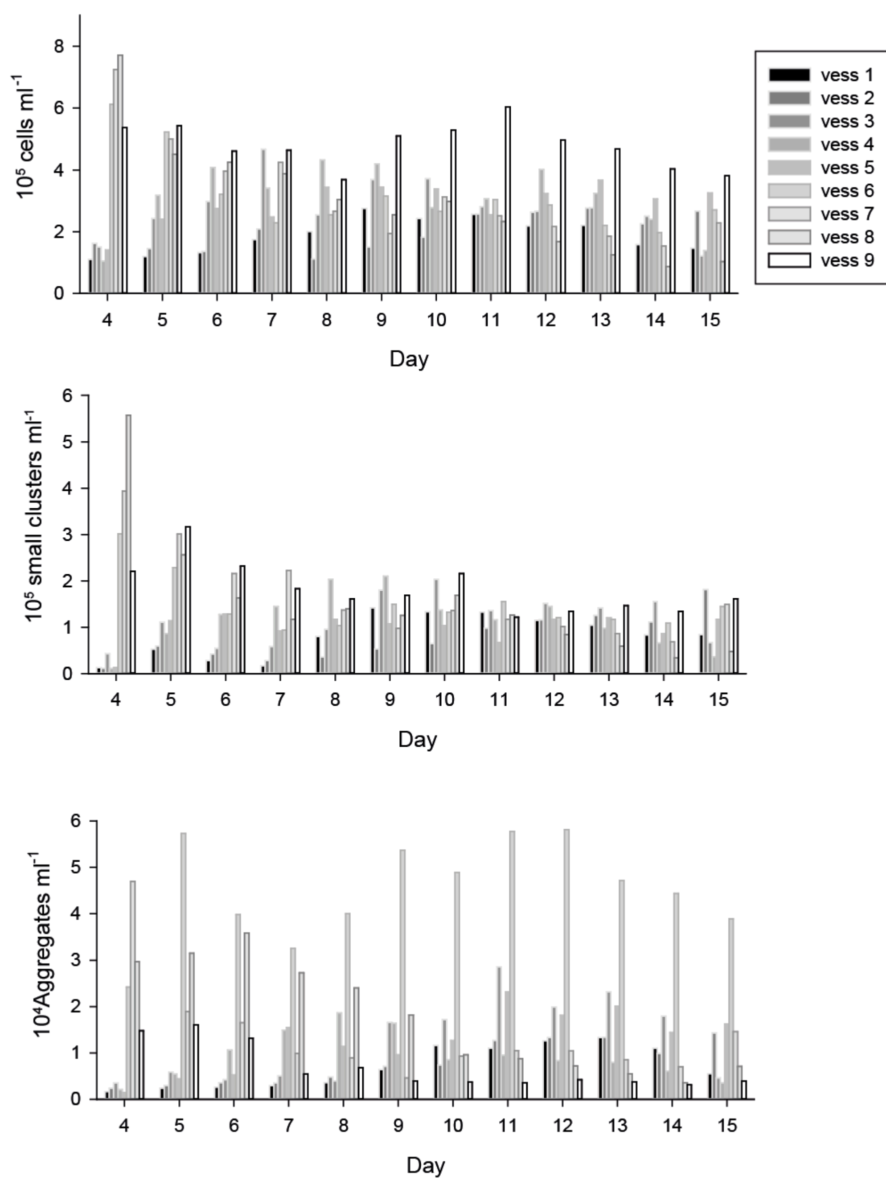
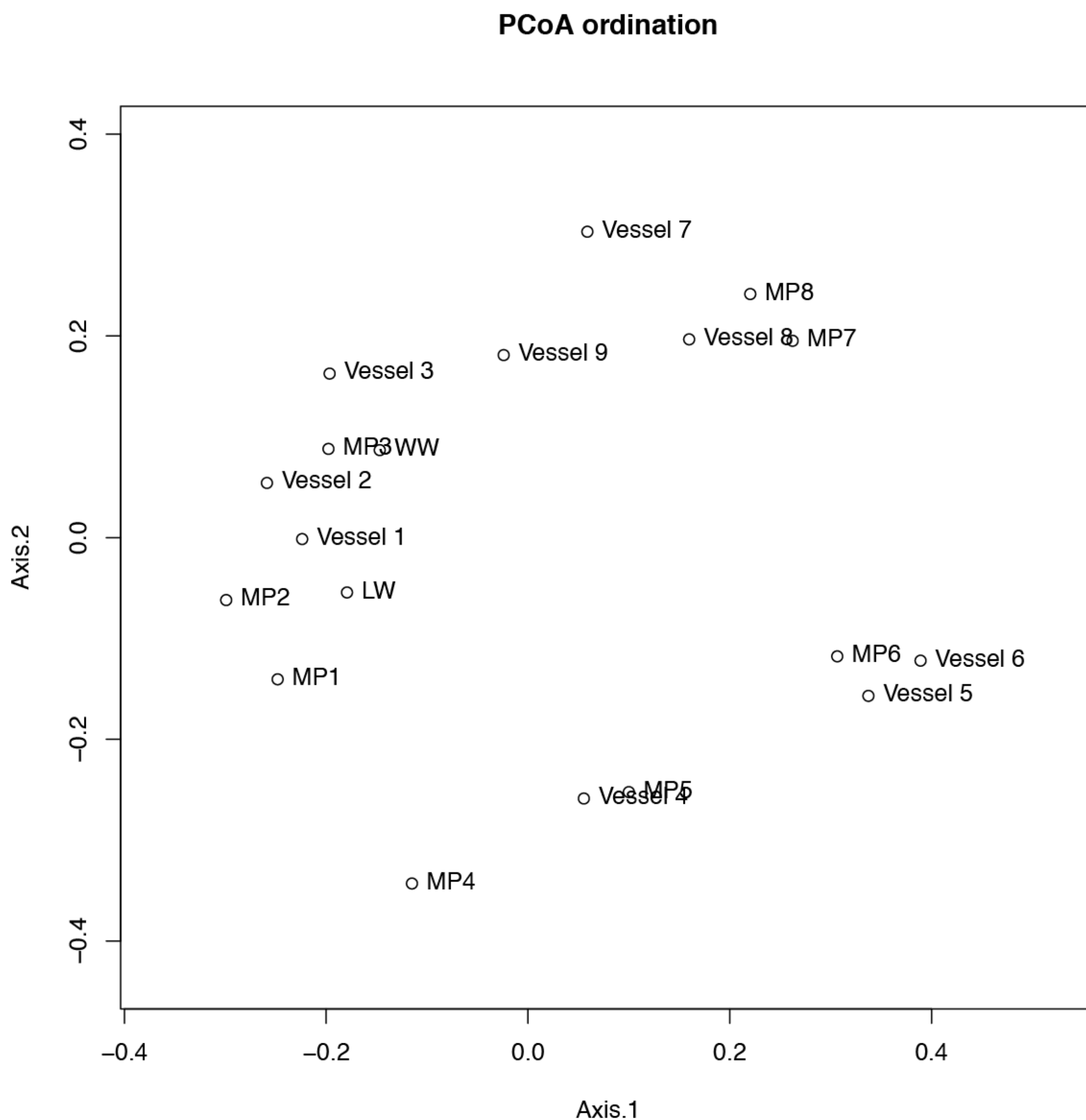


Figure S2 PCoA computet on the Sorensen distance matrix of ARISA peaks in the various samples. Vessel referes to vessel water and are free-water samples. MP refers to microplastic biofilm samples. LW and WW refer to the inoculum communities of lake water and waste water, respectively. Vessel 1 had the 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

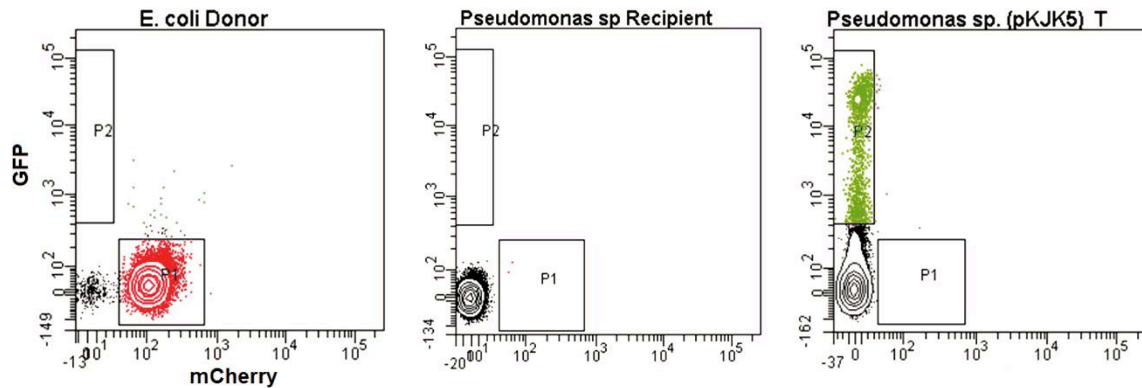


Figure S1. Contour plots in the FCM analysis of the two-species microcosm show the fluorescence pattern (from left to right) from *E. coli* donor strain expressing *mCherry*, *Pseudomonas* sp. recipient strain with no fluorescent expression and *Pseudomonas* sp. after plasmid pKJK5::*gfpmut3* acquisition.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

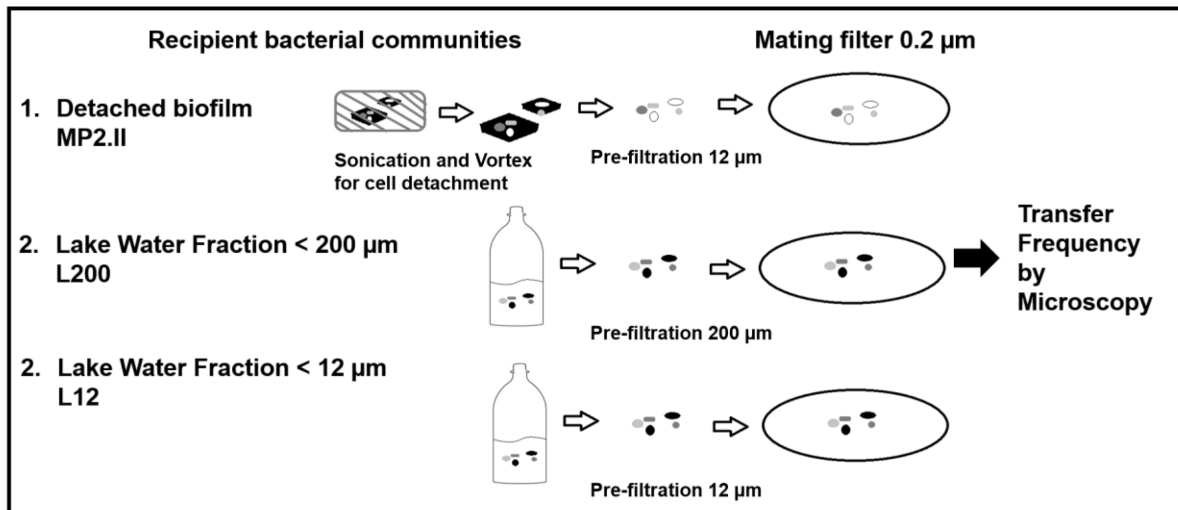


Figure S2. As part of experiment two, transfer frequencies were determined by microscopy for matings of the donor with recipient bacteria originated from microplastic biofilms (MP2.II) and bacteria from lake water pre-filtered by 12µm filter (L12) or 200µm mesh (L200).

Figure S3

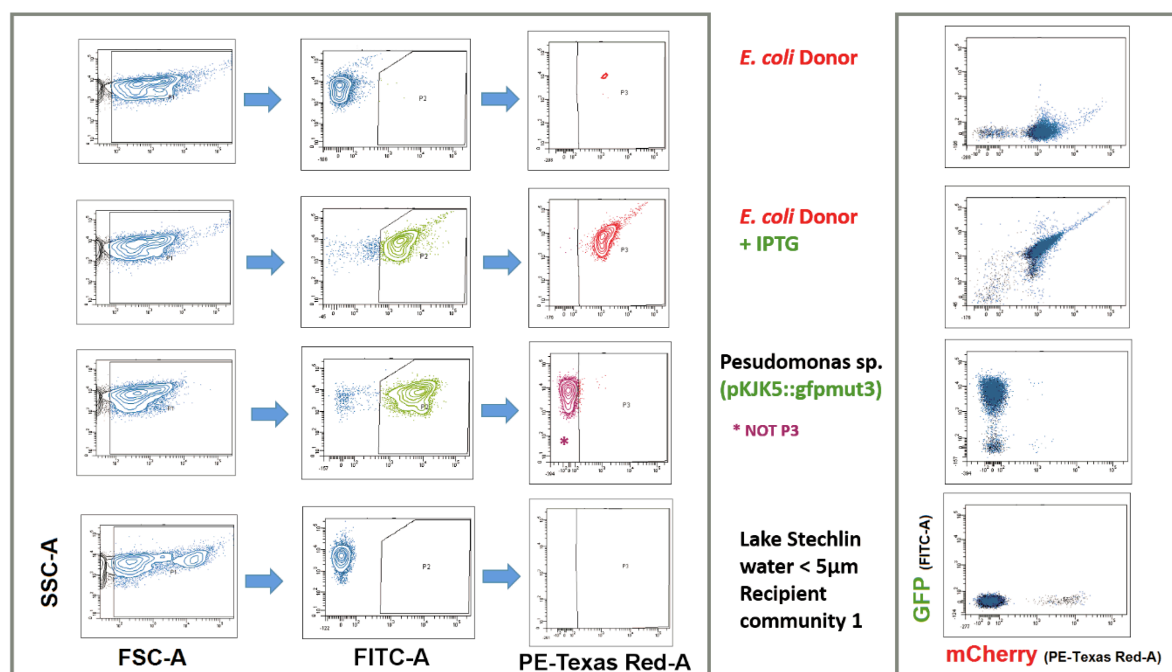


Figure S3. Contour plots on the left exemplify the triple gating procedure for transconjugant FACS-isolation in experiment two. A gate (P1) was set on a bivariate FSC-A vs SSC-A plot for bacterial events using the *E. coli* strain. A second subsequent gate was set on a bivariate FITC-A vs SSC-A plot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

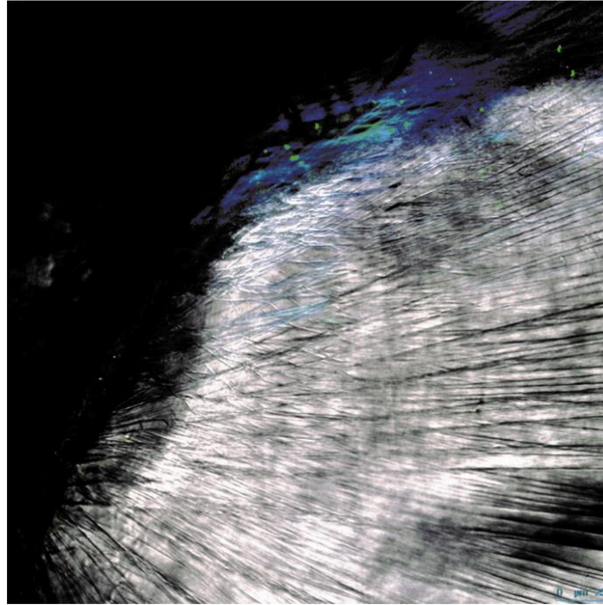


Figure S4. Image of a microplastic biofilm from the two-species microcosm obtained by confocal laser microscopy. Expression of *gfp* in transconjugants is shown in green. Biofilm on the plastic piece was stained with DAPI stain (in blue).

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 $x10^{-5}$	5.00 $x10^{-6}$	2.17 $x10^{-2}$	0	3.02 $x10^{-2}$	0.00 $x10^{-6}$
Replicate 2	5.00 $x10^{-6}$	0	4.25 $x10^{-3}$	0	4.52 $x10^{-3}$	0.00 $x10^{-6}$
Replicate 3	1.00 $x10^{-5}$	5.00 $x10^{-6}$	4.00 $x10^{-3}$	5.00 $x10^{-6}$	8.26 $x10^{-3}$	0.00 $x10^{-6}$
Replicate 4	5.00 $x10^{-6}$	0	2.85 $x10^{-3}$	1.00 $x10^{-5}$	2.60 $x10^{-2}$	0.00 $x10^{-6}$
Mean ± SD	7.50 ± 2.89 $x10^{-6}$	2.50 ± 2.89 $x10^{-6}$	8.20 ± 9.02 $x10^{-3}$	3.75 ± 4.79 $x10^{-6}$	1.72 ± 1.27 $x10^{-2}$	1.25 ± 2.50 $x10^{-6}$

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.

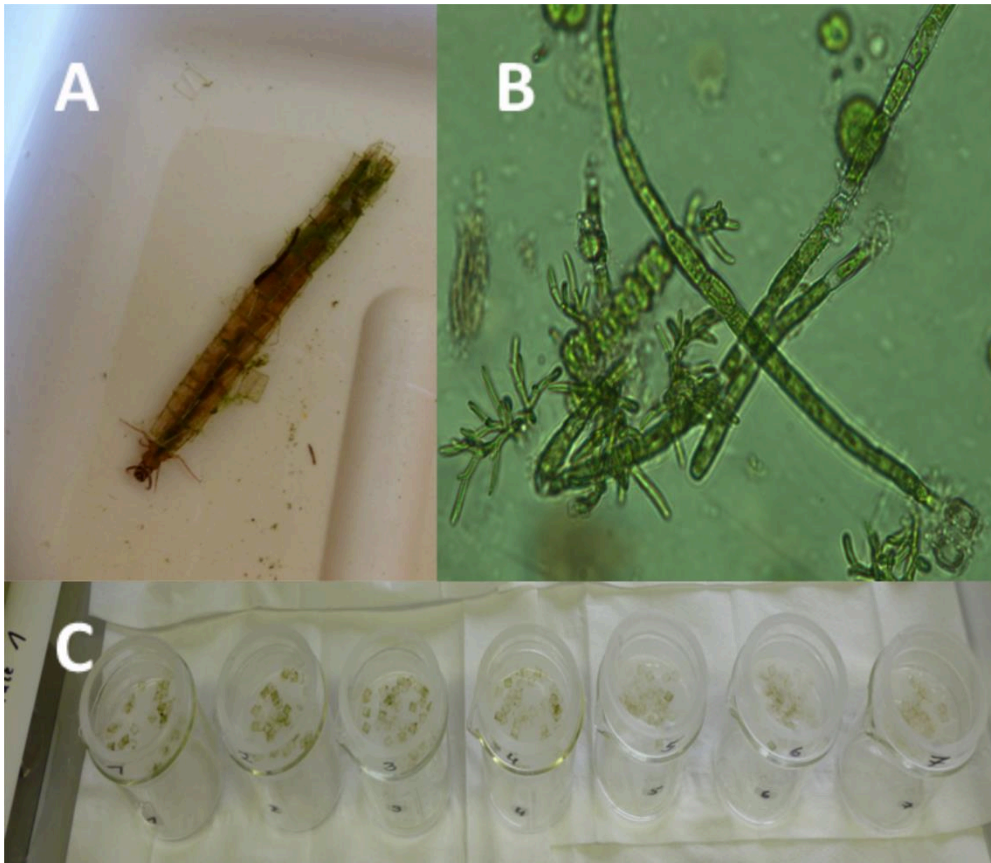
Lake		Df	Sum Sq	F Model	R^2	Pr(>F)
a) Dagow						
	Sample	2	1.08	4.537	0.38	0.001
	Residuals	15	1.79		0.62	
	Total	17	2.87		1.00	
b) Stechlin						
	Sample	2	1.16	2.0684	0.26	0.01
	Residuals	12	3.38		0.74	
	Total	14	4.54		1.00	
c) Grosse Fuchskuhle						
	Sample	2	0.59	3.8667	0.34	0.001
	Residuals	15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

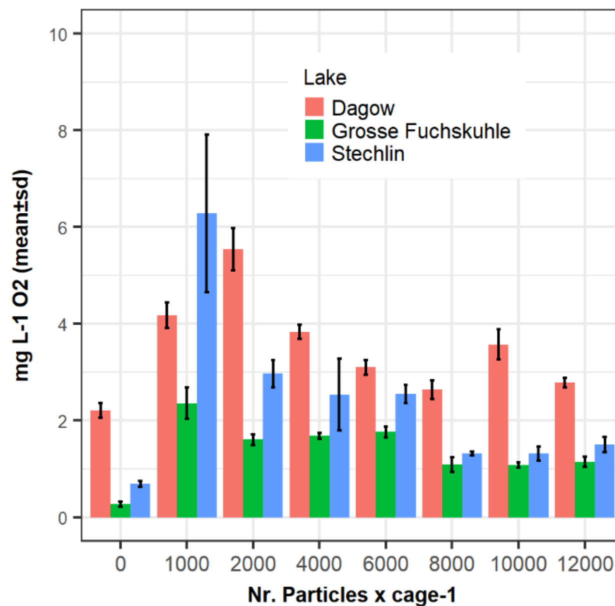
Lake	Pairs	F.Model	R^2	p	p(adjusted)
a) Lake Dagow					
	MP vs W	3. 40	0.25	0.003	0.003
	MP vs FL	6.36	0.39	0.002	0.003
	W vs FL	3.57	0.26	0.001	0.003
b) Lake Stechlin					
	MP vs W	3.27	0.25	0.004	0.012
	MP vs FL	2.677	0.28	0.013	0.0195
	W vs FL	0.427	0.06	0.951	0.951
c) Lake Fuku					
	MP vs W	3.98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



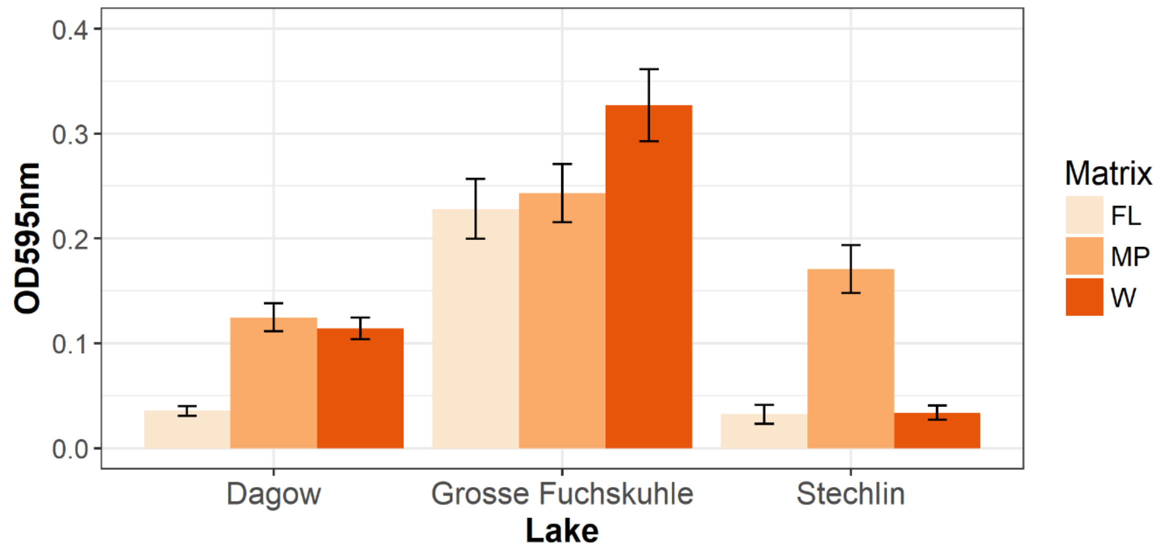
Supplementary Figure 1. A. Larvae of *Trichoptera* sp. used microplastics to build its case. B. Image of green microalgae in a fresh sample of microplastic observed with a light microscopy (40x). C. From left to right, microplastics from cages with least and most particles per cage show highest to lowest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

Fig. S2



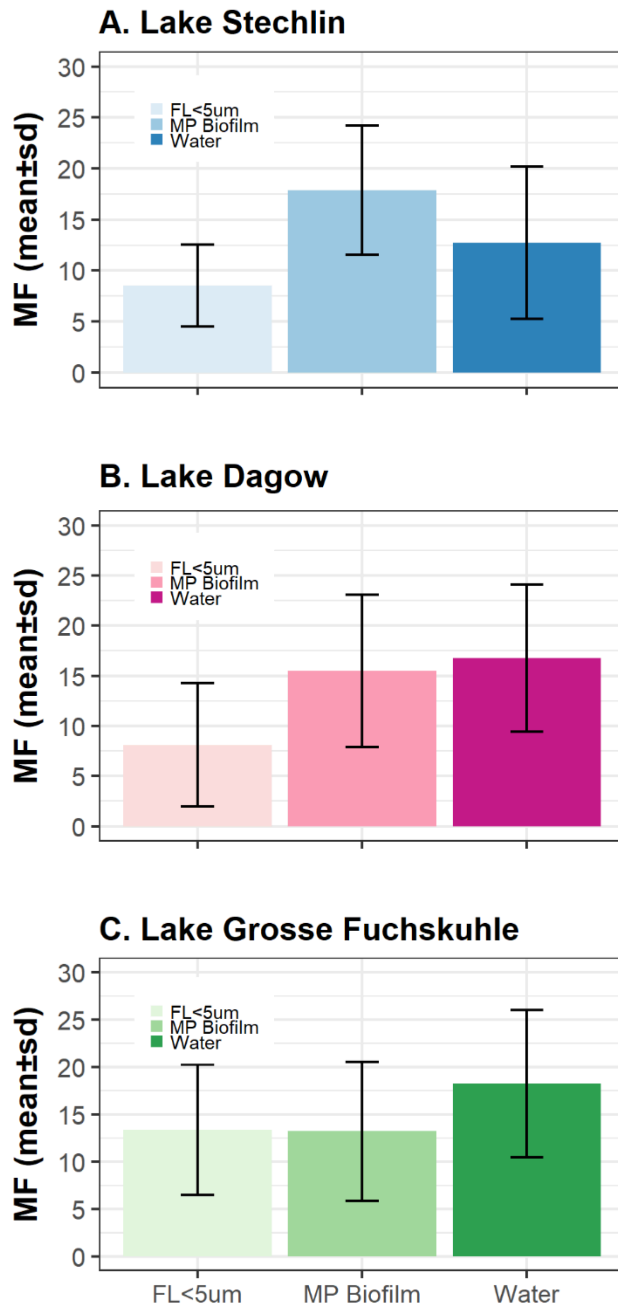
Supplementary Figure 2. Oxygen consumed by microplastic biofilms and water in closed microcosms after 8 days. The Y-axis indicates the O_2 (mg L^{-1}) consumed in 8 days at 10°C and the X-axis indicates number of particles in the cages from the three lakes, from which each 50 microplastics were taken 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