Microbial colonization of microplastic particles in aquatic systems

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Es handelt sich bei dieser Arbeit um meinen ersten Versuch.

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Summary

The continuously increasing pollution of aquatic environments with microplastics (plastic particles < 5 mm) is a global problem with potential implications for organisms of all trophic levels. For microorganisms, trillions of these floating microplastics particles represent a huge surface area for colonization. Due to the very low biodegradability, microplastics remain years to centuries in the environment and can be transported over thousands of kilometers together with the attached organisms. Since also pathogenic, invasive, or otherwise harmful species could be spread this way, it is essential to study microplastics-associated communities. So far, scientists focused on the bacterial colonization of plastics from marine and freshwater habitats. Within the frame of this dissertation, eukaryotic communities on microplastics were analyzed for the first time in brackish environments, and they were compared systematically with communities in the surrounding water and on the natural substrate wood ("study I"). For this purpose, two types of microplastics – polyethylene and polystyrene – as well as wood pellets were incubated for two weeks at seven different locations. These stations were located at the costal Baltic Sea, the lower course of the River Warnow and in a wastewater treatment plant.

With Illumina MiSeq high-throughput sequencing, which gives a resolution down to the genus level, more than 500 different eukaryotic taxa were detected on the microplastics samples. Particularly abundant were the following eukaryotes: the dinoflagellate *Pfiesteria*, the green alga *Ulva*, fungal-like protists of the order Rhinosporideacae or the genera *Rhizidiomyces* and *Pythium*, as well as different ciliates (e. g. *Zoothamnium*), fungi (e. g. *Chytridium*) and small metazoans such as nematodes and rotifers. Hence, there were primary producers and consumers, but also destruents and potential parasitic organisms, which highlights the complexity of microplastics biofilms. This idea was supported by network analyses, which revealed positive correlations of various prokaryotes with eukaryotes, prokaryotes with other prokaryotes and eukaryotes with further eukaryotes. These correlations indicate numerous interaction possibilities in microplastics biofilms.

Despite the high number of different eukaryotes, the biodiversity was clearly lower on microplastics in comparison to the diversity on wood or in the surrounding water. Furthermore, eukaryotic community compositions on microplastics significantly differed from those on wood and in water. Beside the substrate-dependence, a strong impact of the location on colonization patterns was observed, probably resulting from different environmental conditions prevailing at each of the stations.

Overall, the study demonstrated how complex and unique the microbial life on microplastics can be. Especially the high abundance of the genus *Pfiesteria* – which could include fish

pathogenic and bloom forming toxigenic species – indicates that also harmful organisms could enrich on microplastics.

In another experiment ("study II"), the effect of increasing microplastics concentrations on bacterial communities was tested. A situation was simulated in which treated wastewater containing microplastics was introduced into a freshwater lake. The development of bacterial assemblages – which were equally composed of microorganisms from the wastewater treatment plant effluent and the lakewater – was followed over a period of two weeks. With increasing microplastics concentrations, the resulting bacterial communities became more similar to those from the treated wastewater. Moreover, the abundance of integrase I increased together with rising concentrations of microplastics. Integrase I is often used as a marker for anthropogenic environmental pollution and is further linked to genes conferring, e.g., antibiotic resistance. Thus, high concentrations of microplastics promote the survival of wastewater-derived bacteria and biological indicators of undesired anthropogenic impacts. The presence of microplastics can consequently increase the probability of negative effects of wastewater treatment plant effluents on natural aquatic ecosystems.

This dissertation gives detailed insights into prokaryotic and eukaryotic communities on microplastics in brackish and freshwater systems. Even though microplastics provide novel microhabitats for various microorganisms, their colonization can also pose potential threats to humans and the environment. That comprises in particular the enrichment of *Pfiesteria*, *Pythium*, *Rhizidiomyces,* Rhinosporideacae and wastewater-derived bacteria, since they might include toxigenic, pathogenic, antibiotic-resistant or parasitic organisms. Further studies are vitally needed to give explanations about specific risks linked to the spread of harmful, microplastics-associated species, and to assess global consequences for aquatic ecosystems. The clear distinction between microplastics and natural substrate types regarding their community compositions could additionally hint at different microbial interactions and ecological functionalities. Considering the ever-increasing amount of plastic debris, possible effects on the flow of energy and matter as well as on aquatic food-web dynamics have to be examined. Ultimately, – beside intensified research on these topics – an international strategy needs to be developed to minimize effectively the global pollution of the environment with plastics and microplastics and hence to avert further damage to ecosystems and human health.

Zusammenfassung

Die stetig steigende Verschmutzung der Gewässer mit Mikroplastik (Plastikteilchen < 5 mm) ist ein weltweites Umweltproblem und wirkt sich potentiell auf Organismen aller trophischen Ebenen aus. Für Mikroorganismen stellen Billionen dieser schwimmenden Mikroplastik-partikel eine riesige Fläche zur Besiedlung dar. Aufgrund der sehr schlechten Abbaubarkeit verbleibt Mikroplastik Jahre bis Jahrhunderte in der Umwelt und kann samt der angehefteten Organismen über mehrere Tausend Kilometer weit transportiert werden. Da sich darüber auch pathogene, invasive oder anderweitig gefährliche Arten verbreiten könnten, ist es essentiell, die Mikroplastik-assoziierten Gemeinschaften zu untersuchen. Bisher lag der Forschungsschwerpunkt auf der bakteriellen Besiedlung von Plastik aus marinen oder Süßwasser-Habitaten. Im Rahmen dieser Doktorarbeit wurden erstmals die eukaryotischen Gemeinschaften auf Mikroplastik in Brackwasser-Habitaten analysiert und mit Gemeinschaften aus dem umgebenden Wasser und auf dem natürlichen Substrat Holz verglichen („Studie I“). Dazu wurden zwei verschiedene Mikroplastiktypen – Polyethylen und Polystyrol – sowie Holzpellets für zwei Wochen an sieben verschiedenen Standorten inkubiert. Die Stationen lagen im Küstenbereich der Ostsee, im Unterlauf der Warnow und in einem Klärwerk.

Über Illumina MiSeq Hochdurchsatz-Sequenzierungen, welche eine Auflösung bis auf Gattungs-Ebene ermöglichen, wurden über 500 verschiedene eukaryotische Taxa auf den Mikroplastikproben identifiziert. Besonders häufig vorkommende Eukaryoten waren der Dinoflagellat *Pfiesteria*, die Grünalge *Ulva*, pilzähnliche Protisten der Ordnung Rhinosporideacae oder der Gattungen *Rhizidiomyces* und *Pythium*, sowie verschiedene Ciliaten (z. B. *Zoothamnium*), Pilze (z. B. *Chytridium*) und kleine Metazoen wie Nematoden und Rädertierchen. Damit sind sowohl Primärproduzenten und Konsumenten also auch Destruenten sowie eventuelle parasitäre Organismen vertreten, was die Komplexität von Mikroplastikbiofilmen verdeutlicht. Unterlegt wurde diese Idee durch Netzwerkanalysen, die aufzeigten, dass das Vorkommen verschiedenster Prokaryoten mit Eukaryoten, Prokaryoten mit anderen Prokaryoten und Eukaryoten mit weiteren Eukaryoten untereinander positiv korreliert waren. Dies ließ auf eine Vielzahl von Interaktionsmöglichkeiten in Mikroplastikbiofilmen schließen.

Trotz der hohen Anzahl verschiedener Eukaryoten war die Biodiversität auf Mikroplastik deutlich geringer als auf Holz bzw. im umgebenden Wasser. Zudem unterschieden sich die Zusammensetzungen der Eukaryoten-Gemeinschaften auf Mikroplastik signifikant von jenen auf Holz und im Wasser. Neben der Substrat-Abhängigkeit konnte auch ein großer Einfluss des Standorts auf die Besiedlung festgestellt werden, was wahrscheinlich auf die unterschiedlichen Umweltbedingungen an den jeweiligen Stationen zurückzuführen ist.

Insgesamt zeigte die Studie, wie komplex und einzigartig das mikrobielle Leben in Mikroplastikbiofilmen sein kann. Insbesondere deutet die hohe Abundanz der Gattung *Pfiesteria* – zu der fischpathogene und toxische Algenblüten-bildende Arten gehören könnten – darauf hin, dass sich auch gefährliche Organismen auf Mikroplastik anreichern können.

In einem weiteren Experiment („Studie II“) wurde untersucht, welche Auswirkung steigende Mikroplastikkonzentrationen auf Bakteriengemeinschaften haben. Hierbei wurde die Situation simuliert, dass Mikroplastik-haltiges Wasser aus dem Kläranlagenablauf in einen See eingeleitet wird. Über einen Zeitraum von zwei Wochen wurde die Entwicklung der Bakteriengemeinschaften, die zu Beginn jeweils zur Hälfte aus Mikroorganismen aus dem Kläranlagenablauf und Seewasser zusammengesetzt waren, verfolgt. Je höher die Mikroplastikkonzentration war, desto ähnlicher waren die resultierenden Bakteriengemeinschaften zu jenen aus dem Klärwerk. Zudem stieg mit der Konzentration das Vorkommen von Integrase I auf Mikroplastik an. Integrase I wird häufig als genetischer Marker für anthropogene Umweltverschmutzung eingesetzt und ist darüber hinaus mit Genen verknüpft, die z. B. Antibiotika-Resistenzen übertragen können. Hohe Konzentrationen von Mikroplastik fördern folglich das Überleben von Bakterien, die aus Kläranlagen stammen und Indikatoren für unerwünschte anthropogene Einflüsse darstellen. Somit erhöht Mikroplastik die Wahrscheinlichkeit von negativen Auswirkungen von Klärwerksabläufen auf natürliche aquatische Ökosysteme.

Diese Doktorarbeit konnte tiefe Einblicke in prokaryotische und eukaryotische Gemeinschaften auf Mikroplastik in Brack- und Süßwassersystemen gewähren. Auch wenn Mikroplastikpartikel neuartige Mikrohabitate für verschiedenste Mikroorganismen repräsentieren, so birgt ihre Besiedlung potentielle Gefahren für Mensch und Umwelt. Hier ist vor allem die Anreicherung von *Pfiesteria*, *Pythium*, *Rhizidiomyces,* Rhinosporideacae oder Bakterien aus Kläranlagen zu nennen, denn zu ihnen könnten toxische, pathogene, antibiotika-resistente oder parasitäre Organismen gehören. In Zukunft sind dringend Studien notwendig, die Aufschluss über spezifische Risiken durch die Verbreitung von gefährlichen, Mikroplastik-assoziierten Arten geben und weltweite Folgen für aquatische Lebensräume abschätzen. Die deutlichen Unterschiede in der Gemeinschaftszusammensetzung zwischen Mikroplastik und natürlichen Substraten könnten außerdem auf verschiedenartige mikrobielle Interaktionen und ökologische Funktionalitäten hinweisen. In Anbetracht der stetig steigenden Menge an Plastikmüll, müssen die möglichen Effekte auf Energie- und Stoffflüsse sowie Dynamiken in aquatischen Nahrungsnetzen untersucht werden. Schlussendlich sollte jedoch, neben der verstärkten Erforschung dieser Themen, eine internationale Strategie entwickelt werden, um die globale Verschmutzung der Umwelt mit Plastik und Mikroplastik effektiv zu minimieren und somit weitere Gefahren für Ökosysteme und die menschliche Gesundheit abzuwehren.

Introduction

What are microplastics, where do they come from and where do they end up?

Plastic pollution represents one of the biggest environmental issues of the 21st century. Future archeologists will find plastic waste in the sediments and soil layers. Analogous to the Stone Age or Bronze Age our century might be termed the Plastic Age (Thompson *et al.*, 2009; Osborn and Stojkovic, 2014). Plastic is a collective term for a large number of different artificial polymers primarily made from fossil oil. The most commonly produced plastic types are polypropylene (PP, 24% of global production), low-density and high-density polyethylene (LDPE, 21% and HDPE, 17%), polyvinyl chloride (PVC, 19%) thermoplastic polyester (PET, 7%) and polystyrene (PS, 6%) (Andrady, 2011). Moreover, the majority of plastic types contains a variety of additives such as flame-retardants, pigments for coloring, stabilizers and softeners. After the invention of the first synthetic polymer Bakelite in 1907, the global market offers now a diversity of more than 5000 different grades of plastic materials (Leslie *et al.*, 2017, updated on www.campusplastics.com). Today, plastic products appear to be indispensable in our daily lives, but along with the exponential increase of plastics in the past decades, the environmental pollution is constantly growing (Galloway *et al.*, 2017; Geyer *et al.*, 2017). In 2016, more than 335 million tons of plastic were produced worldwide (Plastics Europe, 2017), and it has been estimated that 4.8 to 12.7 million tons of plastic could enter the oceans every year (Jambeck *et al.*, 2015).

A topic of increasing interest in the public and scientific community is microplastics, which is reflected not only by a steep increase in scientific publications, but also by a rising number of reports in newspapers, the television and online media. So-called microplastics are plastic particles with a size up to 5 mm. They are further distinguished between primary and secondary microplastics (Cole *et al.*, 2011). Primary microplastics are directly industrially manufactured as microbeads, which are used, e.g., as scrubbing agents in cosmetics or air-blasting media or as a starting material for the production of other plastic products. Secondary microplastics derive from the breakdown of larger plastic items. In the environment, where plastic is exposed to UV-light, wind or wave action, the material gets brittle due to the formation of microcracks on the surface and consequently fragments into smaller pieces (Andrady, 2011). These microplastics are ubiquitous and can be detected in the oceans and seas, in lakes including remote mountain lakes, in rivers, in arctic sea ice, in coastal and deep sea sediments, at beaches close to and far away from human civilization, in soils, in the atmosphere, and even in the guts of different animals (Claessens *et al.*, 2011; Van Cauwenberghe *et al.*, 2013; Free *et al.*, 2014; Lechner *et al.*, 2014; Obbard *et al.*, 2014; van Sebille *et al.*, 2015; Eerkes-Medrano *et al.*, 2015; Gall and Thompson, 2015; Stolte *et al.*, 2015; Dris *et al.*, 2016; Duis and Coors, 2016; Lavers and Bond, 2017). Sources of (micro)plastics in aquatic environments are the direct spillage of industrial

resin pellets, illegal dumping of waste at sea or the loss of fishing-gear (Law, 2017). Indirectly, plastics on land can enter water bodies during strong wind or flooding events, for instance from improperly managed landfills and farmlands with agricultural foils or microplastic-contaminated sewage sludge (Zubris and Richards, 2005; Duis and Coors, 2016). The majority of microplastics, which arrives as cosmetic microbeads or fibers of synthetic textiles at wastewater treatment plants (WWTP), ends up in the sewage sludge (Talvitie *et al.*, 2015; Carr *et al.*, 2016; Mahon *et al.*, 2017). However, the remaining microplastics are emitted with the effluent water, emphasizing WWTPs as relevant point sources of microplastic in aquatic ecosystems (Browne *et al.*, 2011; McCormick *et al.*, 2014; Mintenig *et al.*, 2017). Of particular importance are also rivers, which transport approximately 1.15 to 2.41 million tons of plastic to the oceans every year (Lebreton *et al.*, 2017). The pollution rates seem to correspond positively with factors such as population density, but in general, the distribution of (micro)plastics is very heterogeneous in the environment. Researchers estimated that up to 51 trillion microplastic particles could float at the ocean surface, accumulating especially in the large gyres (van Sebille *et al.*, 2015). The latter two studies focused merely on buoyant plastic, but the abundance of microplastics can be up to four orders of magnitude higher in sediments, leading to the assumption that the deep sea constitutes a major sink (Woodall *et al.*, 2014). Additionally, an unknown proportion of plastic debris is trapped in artic ice or washed ashore (Browne *et al.*, 2011; Obbard *et al.*, 2014). Beside marine environments, also freshwaters around the globe are polluted and again, river and lake sediments as well as the shorelines often have higher concentrations of microplastics than the surface water, supporting the notion that sediments are possible sinks for these pollutants (Dris *et al.*, 2015; Eerkes-Medrano *et al.*, 2015, Wendt-Potthoff *et al.,* 2017). Owing to the fact that plastics are very persistent towards degradation – estimations range from years to centuries (Shah *et al.*, 2008; Barnes *et al.*, 2009) – and since they rather fragment than getting mineralized (Kubowicz and Booth, 2017), its global distribution and accumulation in the environment has reached dimensions raising concerns about negative impacts on wild life and humans.

Interactions of aquatic organisms with microplastics

Microplastics have the potential to interact with organisms of all trophic levels within the aquatic food webs, from small bacteria to large whales, (Wright *et al.*, 2013; Gall and Thompson, 2015; Harrison *et al.*, 2018) with yet unforeseeable consequences on whole ecosystem level.

Interactions of higher organisms with microplastics

In laboratory studies as well as in nature it has been found that zooplankton, including krill, ingest microplastics (Desforges *et al.*, 2015; Cole *et al.*, 2016). Especially aged microplastics, which were subjected to weathering and biofouling, can be preferred over pristine

microplastics (Vroom *et al.*, 2017). Ingestion of high amounts of microplastics can even lead to immobilization of daphnids (Rehse *et al.*, 2016). Since zooplankton represents a vital link in aquatic food webs, it has been hypothesized and demonstrated in laboratory experiments that zooplankton can act as a vector for transferring microplastics to organisms of higher trophic levels (Setälä *et al.*, 2014). In other laboratory studies, the trophic transfer was observed from mussels to crabs (Farrell and Nelson, 2013) and for lobsters fed with fishes that were exposed to microplastics beforehand (Murray and Cowie, 2011). In the English Channel, 2.9% of the wild fish larvae (Steer *et al.*, 2017) and 36.5% of the fishes caught had ingested microplastics (Lusher *et al.*, 2013). Also in the North and Baltic Sea, 5.5% of all sampled fishes had plastics, predominantly microplastics, in their gastrointestinal tracts (Rummel *et al.*, 2016). It has been observed for several animals that large plastic items (macroplastics) can block the digestive tract leading to false satiation or injuries, which results in an overall reduced fitness and sometimes death (Gall and Thompson, 2015). In contrast, much less is known about the physiological impact of the incorporation of microplastics (reviewed in Wright *et al.*, 2013). After ingestion, microplastics can accumulate in the gut, translocate from the gut to the circulatory system and potentially to other organs (Browne *et al.*, 2008) or they can be egested (Murray and Cowie, 2011; Cole *et al.*, 2016). As long as the particle is not metabolized, it remains available after excretion and can be ingested again by other organisms. Finally, microplastics have been detected also in seafood such as *Mytilus edulis* and *Crassostrea gigas* (Van Cauwenberghe and Janssen, 2014), raising questions regarding the safety for human consumption (Santillo *et al.*, 2017).

Interactions of microorganisms with microplastics

Beside the just mentioned impact of microplastics on organisms in the middle and at the top of aquatic food webs, microplastics affect also microorganisms, which form the basis of all food webs. As soon as microplastics enter aquatic systems, they are rapidly – within hours – colonized by microorganisms (Harrison *et al.*, 2014). Most likely, organic nutrients, which attached to the plastic surface, have attracted these organisms; a phenomenon described for inert submerged surfaces called ZoBell-effect (Zobell, 1943). Surface-attached microbes often produce extracellular polymeric substances providing a matrix for further organisms and easing their settlement (Costerton *et al.*, 1987). This formation of biofilms on surfaces in water is a natural process and is generally beneficial to the inherent organisms since they are embedded in a matrix, which protects them to a certain extent from antimicrobial agents, grazing pressure, UV radiation, shear stress et cetera (Costerton *et al.*, 1987; Hall-Stoodley *et al.*, 2004; Park *et al.*, 2011). The proximity of cells further facilitates interactions such as social cooperation (Flemming *et al.*, 2016), horizontal gene transfer (Aminov, 2011) or the formation of digestive consortia for efficient *in-situ* element cycling (Costerton *et al.*, 1987). The life-styles of

microorganisms in biofilms often differ substantially from those of free-living organisms (Flemming *et al.*, 2016). Thus in the first place, microplastics in environments represent novel, man-made microhabitats to microorganisms. These microplastics biofilms are also termed the "plastisphere" (Zettler *et al.*, 2013) or the "ecocorona" (Galloway *et al.*, 2017). The first scientific report on microbial colonization appeared in 1972, wherein Carpenter mentioned the attachment of various diatoms and hydroids onto plastic particles in the Sargasso Sea (Carpenter and Smith, 1972). At that time, the topic of plastic biofilms received little attention and was revived only in the 21st century with publications for instance from Masó *et al.* (2003) or Zettler *et al.* (2013), who hypothesized that floating plastic debris could serve as a vector for harmful algal bloom species or potential pathogens of the bacterial genus *Vibrio*. Especially the application of high-throughput sequencing methods allowed for a steep increase of knowledge regarding the bacterial colonization of microplastics. Several members of the phyla Bacteroidetes, Proteobacteria, Verrucomicrobia and Cyanobacteria are commonly represented in marine and freshwater microplastics biofilms (Zettler *et al.*, 2013; Hoellein *et al.*, 2014; De Tender *et al.*, 2015; Oberbeckmann *et al.*, 2018). The repeated occurrence of certain bacterial taxa across different habitats and biofilm ages lead to the idea that plastics could have a core microbiome (De Tender *et al.*, 2015, 2017; Bryant *et al.*, 2016). Interestingly, microplastics might even select for specific bacterial taxa, for instance for *Hyphomonas* and *Erythrobacter*, which were exclusively occurring on microplastics and were neither detectable in the surrounding water nor on another natural surface (Oberbeckmann *et al.*, 2018). Beside a distinct taxonomic composition in comparison to water, plastic-attached bacteria can have a different repertoire of functional genes, in particular a higher abundance of genes associated with degradation of xenobiotics, conjugation of plasmid DNA or chemotaxis (Bryant *et al.*, 2016).

In contrast to bacteria, much less attention has been paid to the eukaryotic colonization, though some studies mention eukaryotic organisms in microplastics biofilms, but predominantly using methods with lower throughput and lower taxonomic resolution such as microscopy. These studies revealed that microplastics from marine environments were colonized by different diatoms (as the most often mentioned group among the eukaryotes), dinoflagellates, coccolithophores, radiolarians as well as by small metazoans such as hydrozoans, crustaceans, bryozoans, barnacles or insect eggs (Carpenter and Smith, 1972; Masó *et al.*, 2003; Carson *et al.*, 2013; Zettler *et al.*, 2013; Reisser *et al.*, 2014; Bryant *et al.*, 2016). Only few studies – and still with a main focus on bacteria – used sequencing methods to target also the eukaryotic communities on microplastics (Zettler *et al.*, 2013; Bryant *et al.*, 2016; Debroas *et al.*, 2017) or macroplastics (Oberbeckmann *et al.*, 2016), showing a dominance of green algae and the SAR supergroup (SAR = Stramenopiles, Alveolata and Rhizaria). In general,

the composition of microplastics-associated communities can differ clearly from assemblages in water or on other organic materials and it is further influenced by seasonal variation and sampling location (Hoellein *et al.*, 2014; McCormick *et al.*, 2014; Amaral-Zettler *et al.*, 2015; De Tender *et al.*, 2015; Oberbeckmann *et al.*, 2016, 2018).

The colonization represents also the initial necessary step for a potential bio-degradation of microplastics. Plastics are typically hydrophobic, have a high molecular mass and as solids, they are insoluble in water explaining their extremely low bioavailability (Krueger *et al.*, 2015). Under laboratory conditions, a (partial) bio-degradation of specific plastic types by some microorganisms has been reported as reviewed by Shah *et al.* (2008). The presence of microorganisms known to degrade hydrocarbons and/or xenobiotics as well as the observation of bacteria in pits and grooves on plastic surfaces from marine samples lead to the question, whether the attached microbes were able to degrade it (Zettler *et al.*, 2013; Bryant *et al.*, 2016; Oberbeckmann *et al.*, 2016; De Tender *et al.*, 2017). However, very few studies have directly addressed biodegradation potentials of plastics, e.g., of polyethylene carrier bags, under environmental conditions, but signs of degradation are often lacking and a mineralization to carbon dioxide, water and/or methane could not be proven so far (Tosin *et al.*, 2012; Eich *et al.*, 2015; Nauendorf *et al.*, 2016; Pauli *et al.*, 2017). And although fungi – known for their high metabolic diversity – are of particular importance regarding the breakdown of complex organic matter including plastics (Krueger *et al.*, 2015, 2016; Grossart and Rojas-Jimenez, 2016; Paço *et al.*, 2017), only two research groups have examined, which fungi actually occur on plastics in aquatic environments (Oberbeckmann *et al.*, 2016; De Tender *et al.*, 2017).

A last relevant point in regards to interactions with microorganisms is the role of plastic as a dispersal vector. Since plastic particles can travel with water currents up to several thousand kilometers (Law and Thompson, 2014), they can simultaneously transport the attached organisms including also potential pathogens and invasive species, which could threaten the global marine biodiversity (Barnes, 2002). Beside the horizontal transport, microplastics undergo also a vertical transport. Not only shape, size and physical density of plastic particles, but also their biofilms influence the sinking behavior in the water column (Fazey and Ryan, 2016; Kaiser *et al.*, 2017). By altering sedimentation rates of organic matter, microplastics might have the potential to change element cycles, i.e. the biological pump (Ducklow *et al.*, 2001), in aquatic ecosystems. Thus, on one hand microplastics have an effect on microbial colonization patterns, while on the other hand the attached organisms also influence the physical properties of microplastics. Nevertheless, the numerous interaction possibilities between microplastics and microorganisms as well as the resulting ecological implications are still far away from being understood and – in the light of increasing plastic pollution – explain the need for further research in the field of microplastics colonization.

Motivation and Thesis outline

Bacteria and small eukaryotes represent the vast majority of life on earth. They are the most important organisms, influencing all global cycles of carbon, nutrients and energy. Moreover, microorganisms have an extremely high diversity regarding their phylogenies, metabolisms and morphologies (Lozupone and Knight, 2007; Keeling and Campo, 2017). As they fulfill many vital roles in aquatic ecosystems from primary production to decomposition, we need to understand how microbial communities are affected by microplastics pollution.

In my dissertation, I will study the impact of microplastics on prokaryotic and eukaryotic communities in different brackish and freshwater environments. I will focus on two different aspects, which are addressed in two separate studies. The aim of the first study (herein called "study I") is to unveil the composition and diversity of eukaryotic communities on microplastic particles while paying special attention to differences to other substrate types and among study sites (Manuscript I). Since fungi have been widely neglected in microplastics studies – despite their importance as decomposers and parasites – I will dedicate an extra chapter to the fungal communities (Manuscript II). The second study (herein called "study II") targets a more specific question, i.e. whether increasing microplastic concentrations result in higher abundances of integrase I, which is proposed as a possible marker for anthropogenic pollution and linked, e.g., to antibiotic resistance genes (Manuscript III). For both studies, I will discuss possible implications and the relevance of microbial plastic colonization for aquatic ecosystems.

Introduction to study I – The exposure experiments

The majority of studies on (micro)plastics biofilms were conducted in marine environments (Masó *et al.*, 2003; Zettler *et al.*, 2013; Carson *et al.*, 2013; Reisser *et al.*, 2014; Harrison *et al.*, 2014; Oberbeckmann *et al.,* 2014, 2016; Amaral-Zettler *et al.*, 2015; De Tender *et al.*, 2015, 2017; Bryant *et al.*, 2016; Debroas *et al.*, 2017). Much less is known about microplastic colonization in freshwater ecosystems (Hoellein *et al.*, 2014, 2017, McCormick *et al.*, 2014, 2016) and even less in brackish water bodies (Oberbeckmann *et al.*, 2018). The Baltic Sea is one of the largest brackish ecosystems in the world suffering already from many anthropogenic pressures such as overfishing, intense shipping traffic, eutrophication and chemical contamination (Snoeijs-Leijonmalm and Andrén, 2017). In addition, marine litter – whereof in the Baltic Sea around 70% are plastic materials – is rated as a special concern by the Baltic Marine Environment Protection Commission (HELCOM, 2017). Due to the high population density, semi-enclosed location, small water volume and low water renewal time, the Baltic Sea has presumably a higher vulnerability to microplastics pollution than the open ocean.

Studying the colonization patterns in the Baltic Sea and its catchment area will help to identify organisms of special interest, such as potentially harmful organisms, and hence hypotheses can

be developed regarding the impact of colonized microplastics. It will further give new insights into the complexity and diversity of the eukaryotic life in microplastics biofilms.

To address this, an exposure experiment was conducted at five stations from the River Warnow to the coastal Baltic Sea in northeast Germany (Figure 1). For a period of 15 days, two types of microplastics – polystyrene (PS) and polyethylene (PE) – and a natural reference substrate (wood) were incubated there (Figure 2). In addition, water samples were retrieved at each station.

Figure 1. Map showing the exposure locations of the first experiment of study I. The five stations are located at the Pier Heiligendamm (station 1), in the mouth (stations 2 and 3) and in the lower reaches of the River Warnow (stations 4 and 5).

Figure 2. Polystyrene (PS, left) and polyethylene (PE, middle) particles used for the exposure experiments. Wood pellets (right) were used as a natural reference surface.

Since microplastics enter rivers also via wastewater treatment plant (WWTP) effluents, the colonization was further examined in a second exposure experiment at two stations inside a WWTP (Figure 3; name and location of WWTP remain anonymous as pre-agreed with the operators). Both exposure experiments were conducted within the MikrOMIK project, which

studies the role of microplastics as vector for microbial populations in the ecosystem of the Baltic Sea (see also https://www.io-warnemuende.de/mikromik-home.html). With this in mind, DNA was extracted from all samples and sequenced on the Illumina MiSeq platform, to study the eukaryotic community composition and diversity, while comparing differences among substrate types (PS, PE, wood, water) and locations (stations 1 to 7). Network analyses, including both eukaryotic and prokaryotic taxa, were carried out to reveal interaction possibilities among the organisms in PE-, PS- and wood-associated biofilms. All hypotheses (H) and research questions (RQ) for these exposure studies are given below.

Figure 3. Pictures showing the exposure locations of the second experiment of study I inside a WWTP. Station 6 (left) is situated after the last sedimentation tank and station 7 (right) after the final treatment with an oxygenated biofilm reactor.

Hypotheses and research questions for study I

- H I.1) The eukaryotic/fungal community compositions on microplastics (here polyethylene and polystyrene) differ from those in the surrounding water and those on wood.
- H I.2) The eukaryotic/fungal community compositions differ among the sampling locations.
- *RQ I.1)* How similar are eukaryotic/fungal communities across different substrate types and sampling locations?
- *RQ I.2)* Are eukaryotic/fungal communities more, likewise or less diverse than communities in the surrounding water or on wood?
- *RQ I.3)* How many eukaryotic/fungal taxa can be found on microplastics?
- *RQ I.4)* Which eukaryotic/fungal kingdoms and taxa are the most common on microplastics?
- *RQ I.5)* Which eukaryotic/fungal taxa are specifically associated with a certain substrate type?

- *RQ I.6)* Considering both prokaryotic and eukaryotic taxa in biofilms on microplastics and wood, which organisms are positively correlated and might interact with each other?

Specific hypotheses and research questions regarding the fungal communities

- H I.3) The relative abundance of fungal reads on solid substrates is higher than in water.
- H I.4) The fungal diversity on wood is higher in comparison to the diversity on other substrate types.
- *RQ I.7)* Is there a difference regarding the fungal diversity between the sampling locations?
- H I.5) The fungal community composition is location-dependent resulting in the identification of location-specific fungal operational taxonomic units (OTUs).

Introduction to study II – A chemostat experiment

WWTP and rivers do not only introduce microplastics into the seas and oceans, but also into freshwater lakes (Eriksen *et al.*, 2013; Hoellein *et al.*, 2014, 2017). Test objects for study II were Lake Maggiore in northern Italy and an adjacent WWTP (Figure 4). While walking along the lakeshore, we sometimes found large amounts of styrofoam (foamed PS) suggesting a pollution of Lake Maggiore with microplastics (Figure 5). Only recently, a research group has confirmed this and reported an average concentration of 45000 particles per square kilometer in the surface water of Lake Maggiore, wherein foamed PS made up 9% of the identified polymer types (Sighicelli *et al.*, 2018).

Figure 4. Picture of the Italian city Intra at Lake Maggiore and parts of the WWTP facility (lower right-hand corner) emitting treated wastewater into the river, which flows directly into the lake.

Study II was supported within the frame of the EU Nereus COST-action dealing with emerging challenges associated with treated wastewater and in particular also with antibiotic-resistant bacteria and their genes. We suggested therein microplastics as one of the most critical emerging challenges.

Combining the facts that first, WWTPs emit microplastics (Talvitie *et al.*, 2015; Mintenig *et al.*, 2017) while releasing also antibiotic-resistant bacteria (Munir *et al.*, 2011; Di Cesare *et al.*, 2016); second, gene transfer is enhanced among bacteria living in biofilms (Costerton *et al.*, 1987); and third, microplastics can travel with their attached biota (Barnes and Milner, 2005), the emission and colonization of microplastics from WWTPs could result in an increased risk of spreading antibiotic-resistant bacteria in aquatic environments. This would pose an additional threat to the already ascertained occurrence of potentially pathogenic *Vibrio* spp. on microplastics (Kirstein *et al.*, 2016).

Figure 5. Styrofoam along the shoreline of Lago Maggiore. Please note that all white spots in the picture represent plastic debris, whereas water is only visible in the upper left-hand corner.

Figure 6. The setup of the chemostat system in a climatic chamber for study II.

For study II, the impact of treated wastewater on freshwater bacterial communities was examined under increasing concentrations of PS micro-particles. Therefore, an equal mixture of microbial communities from Lake Maggiore and effluent water of a close-by WWTP (Figure 4) were incubated in continuous-culture chemostat systems (Figure 6) for 15 days with PS concentrations from 0 to 1600 particles per vessel. Quantitative polymerase chain reaction (qPCR) for respectively both communities, developed in the vessel water and on microplastic surfaces, was applied to assess the relative abundances of integrase I. Integrase I, found in diverse mobile genetic elements, has been previously suggested as a proxy for anthropogenic

pollution since it is linked, e.g., to genes for antibiotic resistance (Gillings *et al.*, 2015). It was further tested – using automated ribosomal intergenic spacer analysis (ARISA) – whether the communities at the end of the experiment were more similar or dissimilar to the respective initial communities from Lake Maggiore and WWTP effluent. The hypothesis and research questions are summarized below.

Hypothesis and research questions for study II

- H II.1) The abundance of integrase I increases with increasing MP concentration.
- *RQ II.1)* In which direction do bacterial communities develop under increasing MP concentration, when the inoculum was an equal mixture of freshwater- and WWTP-derived microorganisms?
- *RQ II.2)* Are bacterial assemblages on MP more, likewise or less diverse than communities in the surrounding water?

Declaration of contributions to Manuscripts

Manuscript I

Marie Therese Kettner, Sonja Oberbeckmann, Matthias Labrenz, Hans-Peter Grossart.
The eukaryotic life on microplastics in brackish ecosystems.
submitted to Environmental Microbiology on 5th of April 2018

Study design: SO and ML; Sampling: SO and MTK; Sample processing: MTK; Data evaluation: MTK; Writing: MTK with implementation of suggestions by SO, ML and HPG

Manuscript II

Marie Therese Kettner, Keilor Rojas-Jimenez, Sonja Oberbeckmann, Matthias Labrenz, Hans-Peter Grossart, 2017. *Microplastics alter composition of fungal communities in aquatic ecosystems.* Environmental Microbiology (2017), 19(11), 4447–4459.
doi:10.1111/1462-2920.13891

Study design: SO and ML; Sampling: SO and MTK; Sample processing: MTK; Data evaluation: MTK and KRJ; Writing: MTK with implementation of suggestions by KRJ, SO, ML and HPG

Manuscript III

Ester Maria Eckert, Andrea Di Cesare, Marie Therese Kettner, Maria Arias-Andrés, Diego Fontaneto, Hans-Peter Grossart, Gianluca Corno, 2018. *Microplastics increase impact of treated wastewater on freshwater microbial community.* Environmental Pollution (2018), 234, 495-502.
https://doi.org/10.1016/j.envpol.2017.11.070

Study design: EME, GC, HPG, MTK and MAA; Realization of chemostat experiment: equally MTK, MAA, EME and GC; Sampling and sample processing: equally MTK, MAA, EME, GC and ADC; Data evaluation: EME, ADA, DF, MTK, MAA and GC; Writing: EME and ADC, who implemented suggestions by MTK, MAA, DF, HPG and GC

Contributions confirmed by Prof. Dr. Hans-Peter Grossart _____

Compendium of Manuscripts

Manuscript I

Marie Therese Kettner, Sonja Oberbeckmann, Matthias Labrenz and Hans-Peter Grossart

The eukaryotic life on microplastics in brackish ecosystems.

submitted to Environmental Microbiology on 5th of April 2018

For Supplementary Information for Manuscript I see Appendix starting from page 88.

The eukaryotic life on microplastics in brackish ecosystems.

Summary

Microplastics (MP) constitute an emerging contaminant all over the globe. Rivers and wastewater treatment plants (WWTP) transport annually several million tons of MP into lakes, estuaries and oceans, where it provides an increasing artificial surface for microbial colonization. As knowledge on MP-attached communities is insufficient for brackish ecosystems, we conducted exposure experiments in the coastal Baltic Sea, an in-flowing river and a WWTP within the drainage basin. With Illumina MiSeq sequencing, we analyzed the eukaryotic colonization of two types of MP, polyethylene and polystyrene, and compared it to communities in the surrounding water and on a natural surface (wood). More than 500 different taxa across almost all kingdoms of the eukaryotic tree of life were identified on MP, dominated by Alveolata, Metazoa and Chloroplastida. The eukaryotic community composition on MP was significantly distinct from wood and water communities. Network analyses revealed co-occurrence patterns among eukaryotes of different trophic levels as well as between bacteria and eukaryotes, hinting at many possibilities for microbial interactions on MP. This first report on total eukaryotic communities on MP in brackish environments highlights the complexity of MP-associated biofilms, potentially leading to altered microbial activities and the enrichment and dispersal of harmful microorganisms via MP.

Introduction

Along with the exponential increase of plastic products in the past decades, the environmental pollution with plastic is constantly growing (Galloway *et al.*, 2017; Geyer *et al.*, 2017). Nowadays, “microplastics” (MP, plastic particles with a size below 5 mm) can be found in almost all aquatic environments (Eerkes-Medrano *et al.*, 2015; Law, 2017), where it interacts with organisms ranging from bacteria, algae and zooplankton to birds, fishes, seals and many more (Zettler *et al.*, 2013; Gall and Thompson, 2015; Clark *et al.*, 2016). An important aspect of plastic pollution is that the material provides a large surface for microbial colonization, and drifting MP can function as a vector for (micro)organism dispersal (Keswani *et al.*, 2016). For a long time it is known that various eukaryotes such as diatoms and hydroids settle on MP (Carpenter and Smith, 1972). However, it took more than 30 years until the colonization of plastic received more scientific attention. Questions were raised whether MP could facilitate the spread of harmful algae (Masó *et al.*, 2003), pathogens (Kirstein *et al.*, 2016; Viršek *et al.*, 2017) or invasive species threatening global biodiversity (Barnes, 2002). The majority of studies in the field are focused on bacterial MP colonization (Hoellein *et al.*, 2014, 2017, McCormick *et al.*, 2014, 2016), whereas eukaryotic communities were often considered secondarily or analyzed solely microscopically, allowing for a rather low coverage and

taxonomic resolution (Masó *et al.*, 2003; Carson *et al.*, 2013; Zettler *et al.*, 2013; Oberbeckmann *et al.*, 2014; Reisser *et al.*, 2014; Bryant *et al.*, 2016). Though MP biofilms comprise a high number of different (micro)eukaryotes, which are presumably of at least the same ecological importance as bacteria, systematic and detailed studies, using e.g. advanced sequencing methods, are rare (Oberbeckmann *et al.*, 2016; Debroas *et al.*, 2017; Kettner *et al.*, 2017). This scientific field is still at an early stage in describing the occurrence of (micro)eukaryotes on this new artificial habitat and we are far away from understanding the ecological consequences, neither on local communities nor on global ecosystem scales. Hence, this holistic knowledge is required to better understand ecosystem and health related issues emerging from plastic pollution.

It has been shown that location, based on variances in environmental conditions, is one significant factor influencing the microbial community composition on MP (Hoellein *et al.*, 2014; Oberbeckmann *et al.*, 2014), emphasizing the need that studies should cover a wide range of ecosystems around the globe. Beyond our project (Kettner *et al.*, 2017; Oberbeckmann *et al.*, 2018), prokaryotic and eukaryotic MP colonization in brackish ecosystems has not been investigated in detail. Hence, we dedicate our study to one of the world's largest brackish seas – the Baltic Sea – as well as one of its in-flowing tributaries – the River Warnow – and a municipal wastewater treatment plant (WWTP). Rivers and WWTPs play crucial roles in introducing and transporting MP to lakes, seas and oceans (Lechner *et al.*, 2014; Talvitie *et al.*, 2015; McCormick *et al.*, 2016; Lebreton *et al.*, 2017; Mintenig *et al.*, 2017). Researchers estimated that rivers transport 1.15 to 2.41 million tons of plastic waste into the world's oceans every year, with a positive correlation between population density in the watershed and the amount of introduced waste (Lebreton *et al.*, 2017). The Baltic Sea's catchment area includes 14 countries with approximately 200 rivers and 85 million people. As the surface area of the Baltic Sea is four times smaller than its drainage area and since the average water residence time is three to four decades, its ecosystem suffers eminently from severe anthropogenic pressures such as eutrophication, chemical contamination, overfishing and intense shipping traffic (Snoeijs-Leijonmalm and Andrén, 2017). Today, the Baltic Sea ranks among the most polluted seas worldwide (HELCOM, 2010) and MP emerge as additional anthropogenic pressure. Quantitative information on MP pollution in the Baltic Sea and its drainage basin, however, is scarce (Talvitie *et al.*, 2015; Setälä *et al.*, 2016; Lebreton *et al.*, 2017). MP including synthetic fibers seem to be nearly omnipresent in samples from Baltic beaches in Germany (Stolte *et al.*, 2015), Poland (Graca *et al.*, 2017) and the Kaliningrad region (Esiukova, 2017). In the surface water of the River Warnow and Baltic Coast around Rostock, Germany, polyethylene (PE) and polystyrene (PS) are among the most commonly found types of MP (pers. comm., Leibniz Institute for Baltic Sea Research Warnemünde).

With this study, we focus on the examination of MP colonization and discuss its possible implications for aquatic microbial ecology. In particular, we aim at elucidating the eukaryotic diversity on two types of MP, namely PE and PS, after 15 days of incubation in the Baltic Sea, the River Warnow and a wastewater treatment plant (WWTP). We use results from Illumina MiSeq sequencing to test our hypothesis that eukaryotic MP-attached communities are distinct from communities on a natural surface (wood) or the surrounding water. Further, we analyze if the beta diversity is different on these tested substrate types and we evaluate whether eukaryotic assemblages differ among the seven incubation sites. We perform network analyses comprising prokaryotic and eukaryotic taxa to reveal which organisms co-occur and might interact with each other. Our detailed view into community compositions will provide new insights into the microbial life on MP in aquatic ecosystems.

Results

Eukaryotic communities across different substrate types and locations

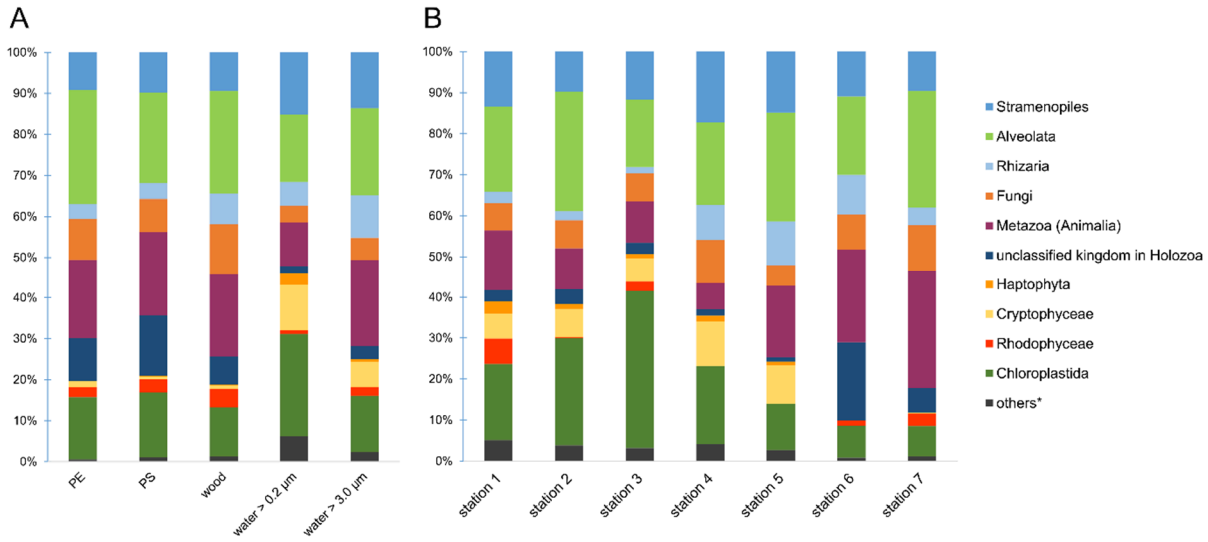

Fig. 1. Eukaryotic community composition on kingdom level across different substrate types (A) and locations (B). Proportions in bar charts are based on read counts after a Hellinger-transformation. * others = Amoebozoa, Discicristoidea, "Incertae Sedis" and an unclassified kingdom in Eukaryota

From all 95 samples with more than 3.67 million reads, we were able to identify 738 different eukaryotic taxa. On PE and PS, we detected 426 and 433 different taxa, respectively. The 738 taxa were assigned to 14 different kingdoms. Common representatives of our samples were from the SAR supergroup (Stramenopiles + Alveolata + Rhizaria), Fungi, Holozoa including Metazoa, and different algae, especially Chloroplastida. The composition of kingdoms varied across the substrate types and locations (Fig. 1). For instance, water samples had a higher proportion of Cryptophyceae, whereas PE and PS had more reads assigned to a kingdom within Holozoa, mainly from the order Rhinosporideacae. Compared to samples from the River

Warnow and the Baltic Sea (stations 1 to 5), WWTP samples (stations 6 and 7) had almost no Cryptophyceae and Haptophyta, fewer Chloroplastida, but more Holozoa.

We compiled a list of the 20 most abundant taxa (by read counts) for a rough description of eukaryotic communities of the different substrate types (Table S1). These top 20 taxa comprise ca. 70% to 81% of the respective community. Among the top 20 taxa on PE and PS, we found organisms from different trophic levels. Green algae from the genus *Ulva* (sea lettuce) and the class Trebouxiophyceae were common primary producers in MP biofilms. As primary or secondary consumers, we detected different ciliates assigned to Peritrichia, the ConThreeP group, specifically *Zoothamnium* and the suctorian *Ephelota*. Consumers from the kingdom Metazoa (Animalia) were the rotifers Adinetida and Ploimida, the nematodes Diplogasterida and Rhabditida, the mollusk Caenogastropoda and the crustacean Podocopida. As a potential mixotroph we found the dinoflagellate *Pfiesteria*. We further identified fungi from Chytridiomycota, for instance *Chytridium*, as well as fungal-like organisms such as Rhinosporideacae, *Rhizidiomyces* and *Pythium*, which can have saprotrophic or parasitic life styles. When comparing the top 20 taxa across the different substrate types (Table S1), we often observe omnipresent taxa, but also taxa that were differentially abundant on one substrate or another. Common organisms from the smaller water fraction (0.2 µm to 3.0 µm) were picoeukaryotic green algae such as *Ostreococcus* and *Micromonas*, the cryptophytes *Leucocrytos* and *Teleaulax*, or the heterotrophic *Picomonas*, which were all rare on the solid substrates. In comparison to PE and PS, the larger water fraction (> 3 µm) contained more phototrophs such as the diatoms *Skeletonema* and *Thalassiosira* or the green algae *Scenedesmus*. The compilation of the top 20 taxa on wood were similar to those on PE and PS, but included the fungus LKM11 among the dominant organisms.

Effects of substrate type and location on eukaryotic community composition

When comparing the eukaryotic communities at the deepest classified taxon level (ideally genus), we observed a significant impact of the factors substrate type ($p = 0.001$; $R^2 = 0.14$), location ($p = 0.001$; $R^2 = 0.47$) and their interaction ($p = 0.001$; $R^2 = 0.27$) on the community composition. All results of the permutational multivariate analysis of variance, short PERMANOVA, are shown in Table S2. A homogeneous data dispersion among the factor groups, which is necessary for a valid interpretation of PERMANOVA results, was given (Table S3). We tested further with pairwise PERMANOVA (Table S4), which substrates and locations differed from each other. The eukaryotic community composition on all substrate types was significantly different ($p < 0.04$; Table S4), with the exception of PE and PS ($p = 0.942$; Table S4). Communities on both MP types displayed a Bray-Curtis similarity of 78.7 % (Table S4). The lowest Bray-Curtis similarities (41.8% to 46.2%; Table S4) were observed between communities on the solid substrates PE, PS and wood vs. the surrounding water communities

(size fraction 0.2 µm to 3.0 µm). Also, each location had a significantly different community composition than any other station ($p \leq 0.005$; Table S4). We found the highest Bray-Curtis similarities between stations that were geographically close to each other, namely station 4 and 5 (70.4%; Table S4), the two estuary stations 2 and 3 (65.5%; Table S4) and both WWTP stations 6 and 7 (66.1%; Table S4). The Bray-Curtis similarity matrix for individual samples is illustrated in a non-metric multidimensional scaling (NMDS) plot (Fig. 2). The plot visualizes a grouping by location, a separation between the communities associated with solid substrates and water (lower similarity) and the proximity of MP-associated communities (higher similarity).

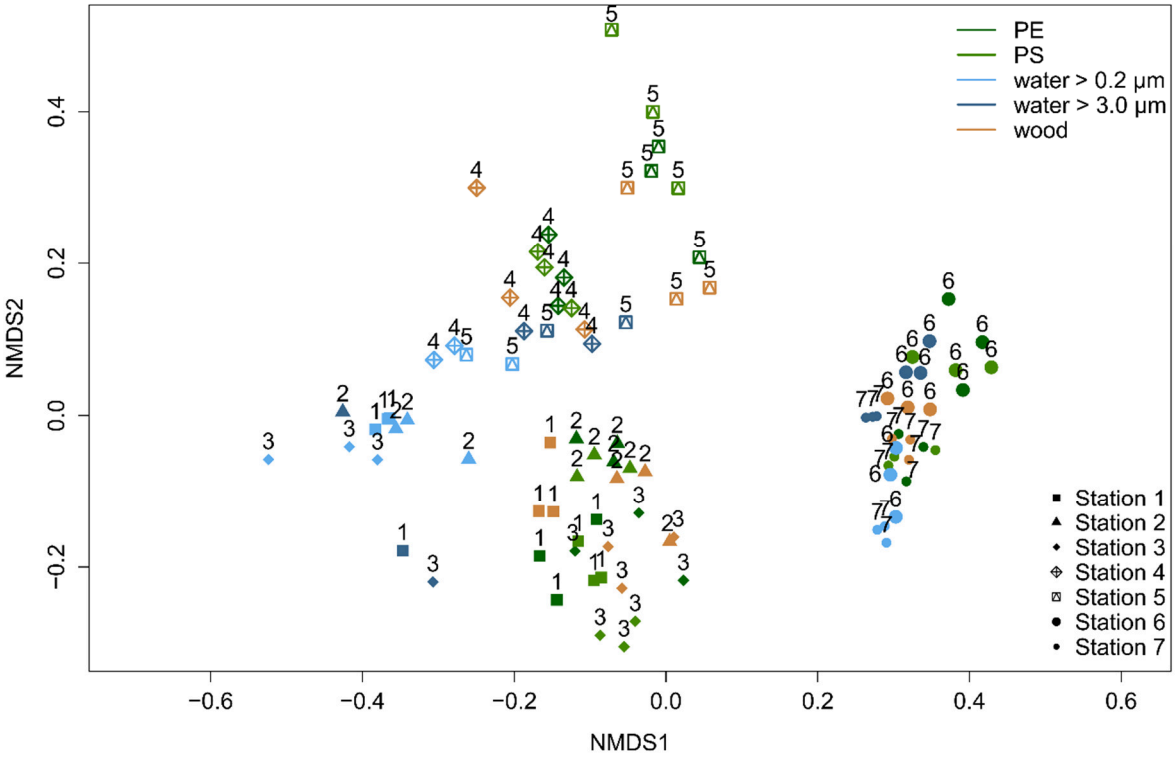

Fig. 2. Non-metric multidimensional scaling (NMDS) plot visualizing Bray-Curtis similarities of eukaryotic communities among individual samples (stress value = 0.15). With increasing similarity, the points have a closer proximity. Colors represent different substrate types. Symbols and numbers indicate different locations.

Diversity of eukaryotes on different substrate types

Continuous diversity profiles for the different substrate types are depicted in Fig. 3, with "ChaoJost" as the estimated diversity ($^{q}\dot{D}$) over the diversity order q (Chao and Jost, 2015). These profiles allow for a quick comparison of diversities, since e.g. non-overlapping graphs indicate a higher diversity of the upper graph, i.e. the respective substrate type. The continuous diversity profiles comprise further three classical diversity estimators as special cases along the graph (as explained in legend of Fig. 3). When comparing the estimated taxon richness (q = 0), both water size fractions were more diverse than the solid substrates PE, PS and wood. This ranking changes when we follow the profiles with increasing q, while the

influence of rare taxa on diversity estimations decreases. For q ≥ 2, we still observe a lower taxon diversity of PE and PS, but the smaller size fraction in water (between 0.2 µm and 3.0 µm) had a similarly low diversity, whereas wood had an even higher diversity than the larger size fraction of water (> 3.0 µm). Since diversity profiles and their 95% confidence intervals of the water size fraction > 3.0 µm and those of PE and PS never overlapped, we can conclude that eukaryotic communities in water (> 3 µm) had a significantly higher diversity than on MP.

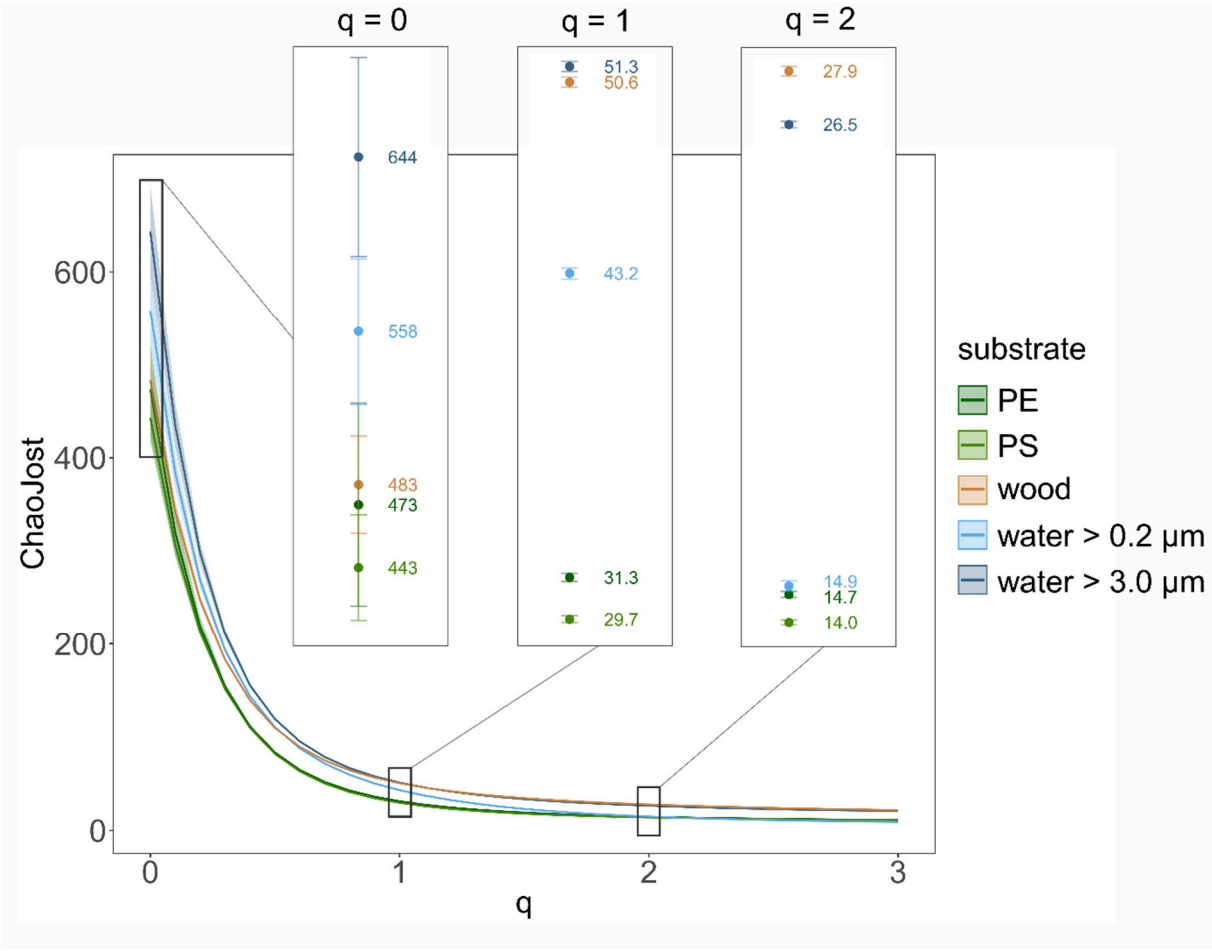

Fig. 3. Eukaryotic taxon diversity presented as continuous diversity profiles with 95% confidence intervals for the substrates PE, PS and wood as well as the small (between 0.2 µm and 3.0 µm) and large water size fraction (> 3.0 µm). Diversity estimator "ChaoJost" (as proposed by Chao and Jost, 2015) on the y-axis and diversity order q on the x-axis. Estimated richness (q = 0), Shannon diversity (q = 1, exponential of Shannon entropy) and Simpson diversity (q = 2, inverse Simpson concentration) are enlarged in boxes.

Potential eukaryotic key taxa

We conducted an "indicator species analysis" (De Cáceres and Legendre, 2009) to identify eukaryotic taxa, which were significantly more abundant on certain substrate types. Only one taxon each was associated with PE and PS, a rotifer and a chlorophyte (Table S5). Wood-associated taxa were assigned mainly to Fungi from the phylum Ascomycota or to Alveolata from the phylum Ciliophora (Table S5). The lists of taxa that were associated with water were

substantially longer and comprised a greater variety of eukaryotic kingdoms. Logically, several small eukaryotes were associated with the smaller water fraction (between 0.2 µm and 3.0 µm), such as *Ochromonas* (Ochrophyta), *Picomonas* (Picozoa), *Micromonas* and *Ostreococcus* (both Chlorophyta), or *Geminigera* and *Teleaulax* (both Cryptomonadales) (Table S5). Associated with the larger water fraction (> 3.0 µm) were a number of nematodes and arthropods, many different chlorophytes, for instance *Monoraphidium* and *Scenedesmus*, or ochrophytes such as *Thalassiosira*, *Cyclotella*, and *Nannochloropsis*, as well as many other taxa across different phyla (Table S5).

Pfiesteria was the most abundant genus (by reads counts) on PE and the second most abundant on PS. It was detected mainly at the stations 4 and 5 (together 99.7%; Table S6 and S7). Read counts from *Pfiesteria* originated with more than 88% from MP (PE+PS) and less than 2% from water (Table S6). This signifies a strong enrichment of *Pfiesteria* on MP and indicates a preference towards these substrate types. To obtain more information about potential relatives of *Pfiesteria*, a representative sequence (most abundant read; get.oturep function in mothur) was picked and loaded into the NCBI's blastn program (BLASTN 2.6.1, default settings; Zhang *et al.*, 2000; Morgulis *et al.*, 2008). The top 50 search results are presented in Table S8. Among those, 10 hits were *Pfiesteria piscicida*, with 100% query coverage and 99% identity to our representative sequence.

Co-occurrence networks

To evaluate interaction possibilities among taxa within communities on the different solid substrate types PE, PS and wood, we calculated co-occurrence networks, which contain not only eukaryotic but also prokaryotic taxa (Fig.4 and Fig. S1 with nodes labelled with taxon names). Each node of a network represents a different taxon and the edges are significant positive correlations between them. For all substrate types, we observed numerous positive correlations among prokaryotes, among eukaryotes as well as between prokaryotic and eukaryotic taxa, particularly in the WWTP (Fig. 4, Fig. S1, Table 1). Beside the variety of bacterial taxa, eukaryotic taxa of the kingdoms Chloroplastida, Alveolata and Stramenopiles dominate within the co-occurrence networks. Especially on PE and PS, bacteria appear to be highly interconnected with eukaryotes (Fig. 4 A-D), whereas on wood bacteria are primarily correlated to other bacteria (Fig. 4 E). Fungi are represented more often on wood and all substrate types inside the WWTP (Fig. 4 B, D, F), than in networks of PE or PS in the Baltic Sea and River Warnow (Fig. 4 A, C). Archaea occur very rarely and exclusively in WWTP networks (Fig. 4 B, D). All networks are highly heterogeneous and are on average rather decentralized, meaning that only few nodes have a central position within the network (Table 1). This is also reflected in the formation of several clusters (denser grouping of nodes) within networks, resulting often in entirely dis-connected clusters (See Table 1 for number of connected

components, wherein a connected component is defined as a cluster in which all nodes are directly or indirectly, i.e. via further nodes, connected to each other.). For instance in the WWTP, both PE and PS networks formed two large dis-connected clusters and additionally some smaller clusters (Fig. 4 B, D). When looking at the respective taxa within these large clusters, it became apparent that those taxa in the left cluster for PE (Fig. 4 B, Fig. S1 B) and in the lower cluster for PS (Fig. 4 D, Fig. S1 D) were more abundant in station 6, whereas the other taxa dominated in stations 7. The same location-dependent formation of clusters holds true for the other networks. Finally, what all networks have in common is that they are very complex and contain co-occurring taxa of different trophic levels across several kingdoms in the tree of life, reflecting diverse possibilities for microbial interactions.

Table 1. Topological parameters of co-occurrence networks for PE, PS and wood from both incubation experiments I (Baltic Sea to River Warnow, stations 1 to 5) and II (WWTP, stations 6 and 7). Networks were analyzed in cytoscape version 3.5.1 (Shannon et al., 2003) with the NetworkAnalyzer tool release 2.7 (Assenov et al., 2008).

	PE stations 1-5	**PE stations 6-7**	**PS stations 1-5**	**PS stations 6-7**	**wood stations 1-5**	**wood station 6-7**
input taxa	188	225	188	239	195	228
number of nodes (output taxa)	134	196	144	207	144	208
number of edges	314	700	416	823	451	776
clustering coefficient	0.38	0.48	0.38	0.41	0.45	0.46
connected components	4	10	7	11	4	4
network diameter	15	10	13	12	13	16
network centralization	0.06	0.12	0.17	0.10	0.10	0.06
network heterogeneity	0.67	0.85	0.88	0.85	0.74	0.69
shortest paths	16012	15000	13050	17148	18376	40608
shortest paths (in percent)	89%	39%	63%	40%	89%	94%
average shortest path length	5.98	3.63	4.13	3.58	5.02	6.19
average number of neighbors	4.69	7.14	5.78	7.95	6.26	7.46

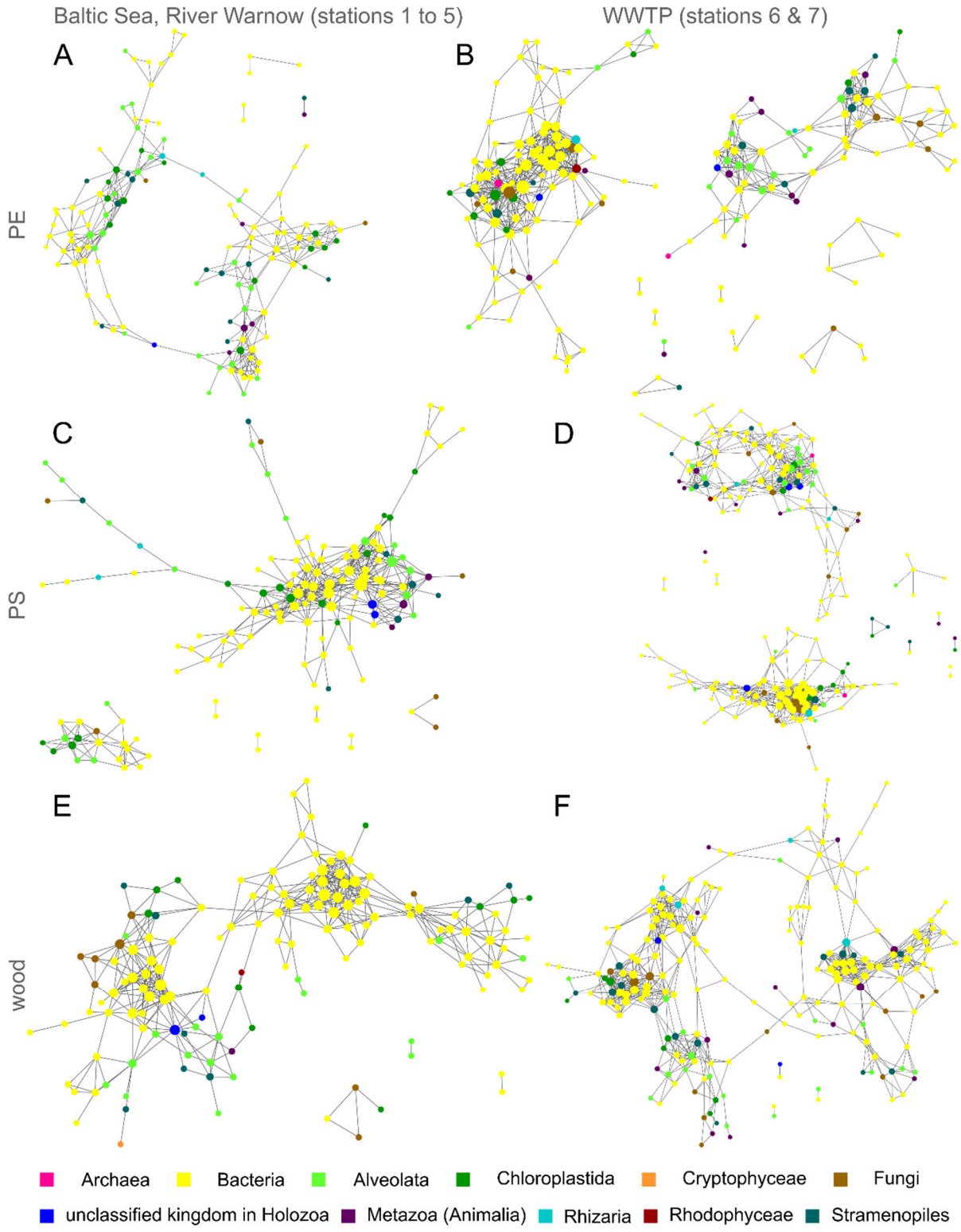

Fig. 4. Co-occurrence networks of prokaryotic and eukaryotic taxa for PE, PS and wood from both incubation experiments I (Baltic Sea to River Warnow, stations 1 to 5) and II (WWTP, stations 6 and 7). Each node is a taxon and the node diameter increases with the number of direct neighbors. Edges represent significant positive correlations ($p < 0.05$) between nodes. Colors indicate different kingdoms.

Discussion

A plastic item, with a mass of one gram, floating in the open sea can harbor significantly more organismic biomass compared to one thousand liter of surrounding seawater (Mincer *et al.*, 2016). The current global increase of plastics and microplastics pollution in the environment (Galloway *et al.*, 2017) demands more detailed knowledge on MP-colonizing organisms and how these differ from natural communities, to better assess the multifaceted impact of MP on aquatic ecosystems.

Effect of substrate type and location on eukaryotic communities

Our results support the hypothesis that substrate type has a significant impact on eukaryotic community composition in aquatic systems. Eukaryotic communities on MP differed from those on floating wood and in the surrounding water. The lowest similarities were found between communities of the smaller water size fraction (0.2 µm to 3.0 µm) and solid substrates (PE, PS and wood) and might be a result of the lifestyle of certain picoeukaryotes dominating in these water samples. Small organisms such as *Ostreococcus* and *Micromonas* have a high surface to volume ratio, which is advantageous for the uptake of nutrients, i.e. they are well adapted for living freely in the water column and this could explain their lower abundances on the solid substrates. Clear differences to communities in water and on natural surfaces have been shown previously also for bacterial communities on MP, suggesting that plastic affects both prokaryotic and eukaryotic community compositions (McCormick *et al.*, 2014, 2016, Oberbeckmann *et al.*, 2016, 2018; Kettner *et al.*, 2017). No significant differences were detected between the eukaryotic communities on PE and PS, which is in accordance with other studies comparing microbial communities between plastic and other hard substrates (Hoellein *et al.*, 2014; Oberbeckmann *et al.*, 2016). Despite low sample replication, other studies, however, hint at distinct microbial colonization patterns among different plastic/polymer types (Zettler *et al.*, 2013; Debroas *et al.*, 2017). Plastic-associated communities also differ from organic substrates (Hoellein *et al.*, 2014; McCormick *et al.*, 2014, 2016), which is confirmed by our comparison of communities on MP vs. wood. Beside the outlined effect of substrate type, we identified a strong impact of location on eukaryotic community composition. Location-dependency of plastic-associated communities has been observed previously (Hoellein *et al.*, 2014; Oberbeckmann *et al.*, 2014, 2016, 2018; Amaral-Zettler *et al.*, 2015; Kettner *et al.*, 2017) and as it is generally accepted that local environmental factors are influencing microbial colonization patterns, we will not further discuss this in more detail. In general, the existing studies on microbial plastic colonization are difficult to compare, especially due to differences in sampling environments, seasons, plastic types, biofilm age or approaches to identify species. Nevertheless, we can see several similarities, i.e. the occurrence of diatoms as early colonizers, a high frequency of organisms from the SAR-group

including dinoflagellates and the suctorian *Ephelota*, as well as different algae and holozoans (Carpenter and Smith, 1972; Masó *et al.*, 2003; Carson *et al.*, 2013; Zettler *et al.*, 2013; Hoellein *et al.*, 2014; Bryant *et al.*, 2016; Oberbeckmann *et al.*, 2016; Debroas *et al.*, 2017). Despite the small particle sizes of MP, we identified numerous organisms assigned to the kingdom Metazoa/Animalia such as nematodes, rotifers or annelids suggesting they attach to MP mainly as eggs, larvae and juveniles, or their environmental DNA (eDNA) adsorbed to the particle. We assume that the retrieved high read abundances of metazoans might rather reflect their multicellularity than the actual number of individuals. In contrast to earlier studies mentioned above, we found a remarkably higher diversity and frequency of fungal taxa (Kettner *et al.*, 2017), and reported for the first time a *Pfiesteria*-related dinoflagellate as the dominant taxon on MP. The high sequencing depth and sample number allowed us to capture a higher eukaryotic diversity and at the same time, we obtained a deeper taxonomic resolution than in those previous studies.

Diversity and co-occurrence patterns of microorganisms on MP

Taking together all MP samples, we identified more than 500 different eukaryotic taxa and the majority of eukaryotic kingdoms from the tree of life were present. Yet, the eukaryotic diversity was significantly lower on MP than on wood or in the surrounding water (> 3.0 µm). Also in other studies, MP communities were found to be less diverse than in water (Zettler *et al.*, 2013; Debroas *et al.*, 2017). As we detected respectively solely one taxon that was specifically associated with PE or PS and due to the lower diversity, we assume that MP was colonized mainly by opportunistic eukaryotes. Possibly, PE and PS rather excluded organisms than attracting a specialized MP community. The lower diversity on PE and PS might have been the result of a short exposure time, since longer incubation would lead to more mature biofilms with possibly more micro-niches for a number of additional organisms. On the other side, wood was exposed over the same time span, but showed the highest eukaryotic diversity. The higher attractiveness of wood for microeukaryotic colonization compared to MP may have been caused by its rougher surface facilitating microbial cell attachment. Additionally, bacteria and fungi can utilize wood as a food source, which renders nutrients available also to other organisms and thus increases eukaryotic diversity. Other researchers provide hints, that also plastic might be degraded to a certain degree by the attached bacterial and fungal community (Zettler *et al.*, 2013; McCormick *et al.*, 2016; Debroas *et al.*, 2017). Although we did not check for MP bio-degradation, we assume it is an inferior process, since several better nutrient and carbon sources are available in the MP biofilms.

The biofilms on MP and wood harbored various organisms simultaneously, which suggests a number of possible interactions such as symbiosis, predator-prey relationships, infections or the collective degradation of organic matter. Indeed, our network analyses revealed many

positive correlations among eukaryotes and especially among bacteria, as well as between bacteria and different eukaryotic kingdoms. For instance, the numerous positive correlations between fungal and bacterial taxa on wood could support the idea of a collective metabolization of this substrate. *Amoebophyra*, a dinoflagellate known to infect other dinoflagellates (Kim *et al.*, 2008), was actually positively correlated to the occurrence of Suessiaceae on PE and another unclassified dinoflagellate on PS, which could indicate a parasitic relationship. The positive correlations on PE and PS of the suctorian *Ephelota* and different bacterial taxa might be explained by ectosymbiosis, which has been previously observed microscopically by Zettler *et al.* (2013); interestingly even with a sulfite-oxidizing bacterium as proposed by them. A significant co-occurrence can unfortunately only indicate but not prove a microbial interaction (Faust and Raes, 2012). Certainly, many organisms simply co-existed together as they prefer similar environmental niches, e.g. the photosynthetic bacterium *Erythrobacter*, which occurred on PE and PS together with the algae *Picochlorum*. Although PE- and PS- associated community compositions had a high Bray-Curtis similarity, it seems challenging to spot just as many similarities in their co-occurrence patterns. This might give a first hint that other interaction possibilities existed on PE and PS, even though their biofilm communities were overlapping. Another relevant observation from the PE, PS and wood co-occurrence networks was the obvious cluster-formation by locations. This highlights that not merely the substrate type, but rather the locations – i.e. the local environmental factors – influenced the community compositions as well as the according co-occurrence patterns, meaning interaction possibilities.

Relevance of MP colonization for aquatic ecosystems

The colonization of floating MP can have various implications for aquatic ecosystems. First, we observed a strong enrichment of *Pfiesteria* reads on MP in comparison to the surrounding water. The respective sequence was closely related to the species *Pfiesteria piscicida*, which is able to produce toxins (Moeller *et al.*, 2007) and associated with harmful blooms and major fish kills (Glasgow *et al.*, 2001). Toxic *Pfiesteria* strains can harm fishes by the toxin release itself, but also by physically attacking the fish's epidermis (Burkholder and Marshall, 2012). Further, *Pfiesteria piscicida* is able to form resting stages (Coyne *et al.*, 2006), which is particularly interesting for the survival and transport on MP to habitats far away. Also the dinoflagellate *Heterocapsa* was present on MP. Certain species within the *Heterocapsa* genus are able to form toxic red tides, which can cause mass mortality of bivalves (Horiguchi, 1995). Moreover, we detected numerous potentially parasitic eukaryotes such as zoosporic fungi (Kettner *et al.*, 2017) or the Peronosporomycetes (former Oomycetes) *Pythium* and *Lagenidium*. Unfortunately, the short fragment length required for Illumina amplicon sequencing does not allow for a resolution to species or strain level and is not providing any

information on the life stage, making prove of toxicity or predictions of infection potentials difficult. Nevertheless, we show that various potentially harmful eukaryotes or their close relatives can colonize and even enrich on MP. Consequently, our results support findings from Masó *et al.* (2003), who suggested plastic debris as a vector for harmful algal bloom species in marine environments.

Second, floating MP introduces a long lasting surface (substantially longer than naturally occurring organic particles such as fecal pellets, algal aggregates or driftwood) for settlement and passive dispersal, since plastic has extremely low degradation rates (Shah *et al.*, 2008; Andrady, 2011) and thus can be transported with ocean currents over thousands of kilometers (Law *et al.*, 2014). Plastic offers a new dispersal medium for the attached communities, including harmful organisms such as potential human pathogens (Kirstein *et al.*, 2016), fish pathogens (Viršek *et al.*, 2017), or bloom-forming dinoflagellates (Masó *et al.*, 2003). An enrichment of parasites and pathogens together with the increased ability for dispersal, presumably pose a serious threat to aquacultures (Horiguchi, 1995; Moestrup *et al.*, 2014) and – more importantly – wildlife and humans. Barnes (2002) estimated that human litter, whereof the majority is plastic, more than doubles the rafting opportunities for all kinds of organisms and therefore holds a high potential to threaten the global marine biodiversity. For the Baltic Sea, maritime transport is the most important today's factor for the introduction of non-indigenous species (Oesterwind *et al.*, 2016). Our study highlights that also plastic has to be considered as an additional and even more frequent transport medium for numerous species in the Baltic Sea. Its impact on aquatic biodiversity, however, still needs to be resolved.

Third, floating colonized MP has the potential to change carbon, nutrient and energy dynamics in the aquatic realm. Recently, Yokota *et al.* (2017) reported on increased photosynthetic activities of cyanobacteria on MP. Since we detected numerous different algae on MP, it seems obvious to also assume an increase in eukaryotic photosynthetic activity following MP colonization. Bryant *et al.* (2016) concluded that MP creates net autotrophic hot spots in the oligotrophic sea. Furthermore, the colonization of MP (Kaiser *et al.*, 2017) as well as the incorporation of MP into algal aggregates (Long *et al.*, 2015) or zooplankton fecal pellets (Cole *et al.*, 2016) alters the overall load, leading either to enhanced sinking or floating of particles. Since organic aggregates represent the main vehicles for transport of organic matter from the sea surface to the bottom (Ducklow *et al.*, 2001), MP has the potential to affect the oceanic carbon pump and vertical fluxes of nutrients.

Conclusion

Our study demonstrates that MP biofilms in brackish and freshwater ecosystems comprise complex communities representing several trophic levels and interaction possibilities of not

only bacteria, but also eukaryotes ranging from protists to metazoans. Current research indicates that solely the addition of a surface could increase microbial productivity, suggesting MP as important hotspots for aquatic carbon and nutrient cycling. How plastic pollution can affect the global microbial productivity and biochemical cycling of organic carbon and nutrients and thus oceanic carbon pump efficiency through altered vertical migration of particles, yet, remains unknown. When judging the risk of dispersal of invasive or harmful organisms, the complexity and dynamic nature of MP biofilms have to be considered, especially in terms of their location-dependency. Our study amongst others provides increasing evidence that potential pathogens and parasites can thrive and even enrich on plastic surfaces. In the future, studies on survival rates during MP migration as well as systematic risk assessments regarding the impact of MP on biodiversity and infection potentials are vitally needed.

Experimental Procedures

Incubation experiments

Polyethylene particles (PE, ExxonMobil™ HDPE HTA 108, ExxonMobil Chemical Europe, Belgium, diameter 3-5 mm, density 0.96 g cm^{-3}), polystyrene particles (PS, Polystyrol 143 E, BASF, Germany, diameter 3-5 mm, density 1.04 g cm^{-3}) and wood particles (1Heiz® Holzpellets, 1Heiz® Pellets AG, Germany, density 1.12 g cm^{-3}) were exposed in triplicates to natural aquatic communities at seven different stations in north-east Germany. Particles were sampled after an incubation for 15 days in surface water (1 to 3 m depth) in containers surrounded by a nylon mesh with 500 µm mesh size. Additionally, we retrieved water samples on day 15 at each station for comparing the eukaryotic communities on the plastic substrates (PE, PS), the natural substrate wood and the natural eukaryotic communities in the surrounding water. Water samples (1 to 3 replicates à 1 to 2 l) were filtered onto 3 µm pore-size membranes (Whatman® Nuclepore Track-Etch Membrane, polycarbonate, GE Healthcare, Germany) to concentrate the eukaryotes. The filtrate (2 to 3 replicates à 0.3 to 0.5 l) was subsequently filtered onto 0.22 µm pore-size membranes (Durapore® membrane filters, polyvinylidene fluoride, Merck Millipore Ltd., Ireland) to detect also the picoeukaryotes and eventual environmental DNA. Samples were stored at -80 °C until further processing. We conducted the first incubation experiment in August/September 2014 at stations 1 to 5. Station 1 is located at the pier Heiligendamm in the coastal Baltic Sea. Station 2 and 3 are situated close to the estuary mouth of the River Warnow, thereby station 2 is in the canal Alter Strom and station 3 in a marina on the other side of the estuary. Station 4 and 5 are located ca. 8 and 12 km, respectively, upstream in the River Warnow. The second incubation experiment was conducted in April/May 2015 in an anonymous wastewater treatment plant (WWTP). Station 6 is in the outlet of the last sedimentation treatment, where conventional WWTPs would

discharge into the receiving waters. This WWTP has an additional treatment stage with an oxygenated biofilm reactor. Station 7 is located at the outlet of that reactor. Further details on the incubation experiments and sampling locations including environmental parameters, coordinates and a map are given by Kettner *et al.* (2017).

DNA extraction, PCR amplification, sequencing and sequence processing

DNA extraction from PE, PS, wood and filtered water samples was carried out based on a protocol of Nercessian *et al.* (2005), which was optimized for our samples. The procedure includes a chemical, mechanical and enzymatic cell lysis step, followed by phenol-chloroform extraction and an ethanol precipitation of extracted nucleic acids. DNA was amplified by PCR using the universal eukaryote primers Eu565F and Eu981R (Stoeck *et al.*, 2010; with addition of the bases -TGA at the 3' end of the reverse primer according to LGC Genomics, Berlin, Germany), which target the highly variable V4 region of the 18S rRNA gene. Allowing for one mismatch, these primers cover 77.4 % of all Eukaryota in the SILVA database v128 while excluding Bacteria and Archaea (Table S9). PCR amplifications and subsequent sequencing on the Illumina MiSeq platform (2*300 bp paired end, MiSeq reagent kit V3) were performed by LGC Genomics, Berlin, Germany. Raw Illumina reads were demultiplexed, then barcodes, adapters and primers were clipped. Reads were further processed in mothur v1.39.5 (Schloss *et al.*, 2009; released in March 2017) following the mothur MiSeq SOP adapted to our target region (Kozich *et al.*, 2013; url: https://www.mothur.org/wiki/MiSeq_SOP; online access May 2017). Processed reads were classified in mothur using SILVA's non-redundant small subunit rRNA database v128 (Quast *et al.*, 2013; released in September 2016). Taxonomy was based on the current SILVA taxonomy (Yilmaz *et al.*, 2014; database v128) with the deepest possible taxonomic resolution at the genus level. Eukaryotic taxa are herein named according to their genus (for instance *Ostreococcus*) or – if no genus could be assigned – after the next higher classified level with the prefix "unclassified" (for instance unclassified Rhinosporideacae). Further details on methods from DNA extraction to sequence processing were described previously by Kettner *et al.* (2017). Raw reads were made available under BioSample accessions from SAMN06806566 to SAMN06806660 of the BioProject PRJNA383789 at the Short Read Archive (SRA) of NCBI.

Data evaluation and statistics

The final output for the 95 samples is a read-abundance-table with all 22 taxonomic levels. The lists of the top 20 taxa per substrate were created based on relative abundances. After a Hellinger-transformation of the data (Legendre and Gallagher, 2001), bar charts were compiled on kingdom level for the different substrates types (PE, PS, wood, water > 3.0 µm and 3.0 µm > water >0.2 µm) and locations (station 1, 2, 3, 4, 5, 6 and 7). Second, we

statistically evaluated the read-abundance-table for the deepest taxonomic levels using R (version 3.3.1, R core team, 2016) and the R package vegan 2.4-1 (Oksanen *et al.*, 2016). To test whether the factors "substrate type" and "location" had a significant effect ($p < 0.05$) on the eukaryotic community composition, we performed a permutational multivariate analysis of variance (PERMANOVA) and pairwise PERMANOVA (adonis function in vegan, 999 permutations). Prior to that, the table was Hellinger-transformed (Legendre and Gallagher, 2001) and converted into a Bray-Curtis similarity matrix. A prerequisite for correct interpretation of PERMANOVA results is to check for homogeneity of dispersion, which we did with the betadisper and permutest function in vegan (999 permutations). A two-dimensional NMDS plot was created to visualize the Bray-Curtis similarity among the 95 samples. In addition, we calculated the Bray-Curtis similarity between each pair of substrates and stations, respectively. The eukaryotic diversity on different substrate types was calculated with the "ChaoJost" estimator for continuous diversity profiles (Chao and Jost, 2015) applying the Diversity function of the R package SpadeR version 0.1.1 (Chao *et al.*, 2016). Before that, read abundances were added up for each substrate type and rarefied to 483071 reads to assure for comparability of diversity among substrates. Continuous diversity profiles (function ${}^{q}\dot{D}$, see Chao and Jost (2015)) and specific Hill numbers (richness for q=0, Shannon diversity for q=1 and Simpson diversity for q=2) were plotted with 95% confidence intervals using the R package ggplot2 version 2.2.1 (Wickham, 2009). To check if specific taxa were significantly associated with a single substrate type, we performed an "indicator species analysis" (R package indicspecies 1.7.6; De Cáceres and Jansen, 2016; De Cáceres and Legendre, 2009). Obtained p-values from multiple testing in pairwise PERMANOVA and indicator species analysis were adjusted according to Benjamini and Hochberg (1995).

Network analysis

For the network analyses of PE-, PS and- wood-associated biofilms, we combined both prokaryote (Oberbeckmann *et al.*, 2018) and eukaryote datasets, which were independently Hellinger-transformed (Legendre and Gallagher, 2001) beforehand. Water samples were not included, as the objective was herein to characterize interaction possibilities within biofilm communities only. With regard to the strong differences in community composition, we calculated the networks separately for experiment I (River Warnow to Baltic Sea, stations 1-5, in total 15 samples per substrate type) and experiment II (WWTP, station 6 and 7, in total 6 samples per substrate type). Exclusively abundant taxa, which occurred in at least half of the samples and had a relative abundance of more than 0.2% within the dataset, were used for the analysis. Network analyses were conducted in Cytoscape version 3.5.1 (Shannon *et al.*, 2003) with the CoNet 1.1.1. beta application following the recommendations of Faust and Raes (2016). Taxa correlations were validated running networks with 1000 iterations. As we

focused on possible co-occurrences, we chose only positive edges for network visualization. Topological parameters of co-occurrence networks were analyzed with the NetworkAnalyzer tool release 2.7 (Assenov *et al.*, 2008) in Cytoscape.

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Conflict of Interest

The authors declare no conflict of interest.

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Manuscript II

Marie Therese Kettner, Keilor Rojas-Jimenez, Sonja Oberbeckmann, Matthias Labrenz, and Hans-Peter Grossart

Microplastics alter composition of fungal communities in aquatic ecosystems.

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Microplastics alter composition of fungal communities in aquatic ecosystems

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Summary

Despite increasing concerns about microplastic (MP) pollution in aquatic ecosystems, there is insufficient knowledge on how MP affect fungal communities. In this study, we explored the diversity and community composition of fungi attached to polyethylene (PE) and polystyrene (PS) particles incubated in different aquatic systems in north-east Germany: the Baltic Sea, the River Warnow and a wastewater treatment plant. Based on next generation 18S rRNA gene sequencing, 347 different operational taxonomic units assigned to 81 fungal taxa were identified on PE and PS. The MP-associated communities were distinct from fungal communities in the surrounding water and on the natural substrate wood. They also differed significantly among sampling locations, pointing towards a substrate and location specific fungal colonization. Members of Chytridiomycota, Cryptomycota and Ascomycota dominated the fungal assemblages, suggesting that both parasitic and saprophytic fungi thrive in MP biofilms. Thus, considering the worldwide increasing accumulation of plastic particles as well as the substantial vector potential of MP, especially these fungal taxa might benefit from MP pollution in the aquatic environment with yet unknown impacts on their worldwide distribution, as well as biodiversity and food web dynamics at large.

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Introduction

Microplastics (MP), widely defined as plastic particles < 5 mm in size, have been detected in aquatic systems all over the globe, including oceans, freshwaters, polar environments and also pristine mountain lakes or at beaches of remote and uninhabited islands (Ivar do Sul *et al.*, 2011; Free *et al.*, 2014; Obbard *et al.*, 2014; Wagner *et al.*, 2014; Eerkes-Medrano *et al.*, 2015; Lavers and Bond, 2017; Law, 2017). MP can enter the aquatic environments directly via the spillage of industrial resin pellets or through the effluent of wastewater treatment plants as cosmetic microbeads or fibres of synthetic clothing (Browne *et al.*, 2011; Cole *et al.*, 2011). In addition, MP derives from the breakdown of larger plastic items originating for instance from illegal dumping, lost fishing-gear or improperly managed landfills (Law, 2017). Approximately 51 trillion particles with a weight of 236 000 metric tons are estimated to float in the oceans (van Sebille *et al.*, 2015), but these numbers will likely rise due to increasing plastic production, fragmentation and extremely low biodegradability (Barnes *et al.*, 2009). An unknown fraction of plastic debris is washed ashore or sinks to the seafloor (Barnes *et al.*, 2009; Tekman *et al.*, 2017). MP provide artificial and persistent surfaces, which can be colonized by numerous microorganisms as reviewed by Oberbeckmann *et al.* (2015). As buoyant plastic particles can be transported over long distances (Law and Thompson, 2014), along with their attached biota, they also serve as vectors for various microorganisms including potentially pathogenic bacteria (Kirstein *et al.*, 2016) and harmful algal species (Masó *et al.*, 2003). Assuming one spherical particle per m^3 in the ocean with a diameter of 1 mm, MP could provide a surface area of 4.2 million km^2, which is around twice as much as the area of Greenland (Charette and Smith, 2010; Hidalgo-Ruz *et al.*, 2012; Eriksen *et al.*, 2014). This represents a potentially large surface available for microbial colonization on MP, which may alter community compositions and functions with possible consequences for diversity and biogeochemical cycles.

Table 1. Comparison of eukaryotic to fungal reads and taxa for the incubation experiments in the River Warnow to Baltic Sea and in the wastewater treatment plant.

	Total ($n = 95$)	River Warnow to Baltic Sea Stations 1, 2, 3, 4, 5 ($n = 65$)	Wastewater treatment plant Stations 6, 7 ($n = 30$)
Number of eukaryotic reads	4 108 183	2 322 700	1 785 483
Number of fungal reads	154 906	65 898	89 008
Percentage of fungal reads	3.8%	2.8%	5.0%
Number of eukaryotic taxa	793	655	501
Number of fungal taxa	173	121	137
Percentage of fungal taxa	21.8%	18.5%	27.3%

n = number of samples.

Although this research field has recently been pushed forward (Zettler *et al.*, 2013), we are still far from understanding the ecological effects of microbial MP colonization.

Within the microbial communities of plastic biofilms, fungi have been almost completely overlooked. Fungi hold many important ecological roles in aquatic ecosystems, that is, as decomposers of organic matter (Bärlocher and Boddy, 2016; Grossart and Rojas-Jimenez, 2016), parasites of several algae or other organisms (Gleason *et al.*, 2008; Wurzbacher *et al.*, 2010), and as food source for higher trophic levels (Kagami *et al.*, 2007). Moreover, fungi can be predators, endophytes, symbionts or pathogens (Wurzbacher *et al.*, 2011). The application of next generation sequencing has led to an increasing discovery rate of fungal taxa in aquatic ecosystems, especially within the early diverging lineages such as Chytridiomycota and Cryptomycota (Richards *et al.*, 2015; Comeau *et al.*, 2016; Grossart *et al.*, 2016). Investigations of the functional roles of these lineages have recently led to fundamental revisions in the concept of aquatic food web structures, emphasizing their profound relevance in aquatic ecosystems (Kagami *et al.*, 2014; Grossart and Rojas-Jimenez, 2016). In addition, fungi are of special interest for potential plastic decomposition in the environment, due to their vast metabolic potential and ability to degrade recalcitrant structures (Krueger *et al.*, 2015; Grossart and Rojas-Jimenez, 2016). However, to date, a single study has reported on the presence of fungi on plastic bottles made of polyethylene terephthalate (PET), but insights into fungal diversity and their ecological meaning remained peripheral (Oberbeckmann *et al.*, 2016).

We applied Illumina sequencing of the hypervariable V4 region of the 18S rRNA gene to analyse the fungal communities on MP and compared them to communities in the surrounding water and to the natural substrate wood. MP and wood particles were incubated for 15 days at seven stations located in north-east Germany with five stations along the lower course of the River Warnow up to the coast of the Baltic Sea (Fig. 1), and two stations inside a wastewater treatment plant (WWTP). The Baltic Sea is a brackish and highly anthropogenically influenced ecosystem (Conley *et al.*, 2009), and rivers in densely populated areas can be important pathways for transporting plastic litter to the open sea (Lechner *et al.*, 2014), while WWTPs are considered as relevant point sources of MP (Mintenig *et al.*, 2017). The polymers in our study – high-density polyethylene (PE) and polystyrene (PS) – rank among the most commonly produced plastic polymers and have been frequently found as pollutants in aquatic environments (Claessens *et al.*, 2011; Wagner *et al.*, 2014).

With this work, we aimed to unveil the fungal diversity within MP biofilms and examined the effects of substrate type and location on fungal diversity, community composition and their abundance in relation to other eukaryotes on MP. We hypothesized that fungi are frequent members of MP-attached communities, occurring preferentially on solid substrates compared to living freely in the surrounding water. As numerous fungal species are capable of degrading wood, but only very few might be able to use plastic polymers as an energy source (Krueger *et al.*, 2015), we expected to see a clear preference of certain fungi towards wood, resulting in a comparably higher fungal abundance on wood and a distinction between MP and wood-associated communities. Lastly, we hypothesized that also location significantly alters fungal community composition, allowing to determine location-specific fungal OTUs. Herein, we provide the first detailed report about MP-colonizing fungi while pointing out differences to water and wood communities as well as among study sites.

Results

Abundance of fungal reads

Approximately 4% of the more than four million eukaryotic reads were classified as Fungi and 22% of all detected 793 eukaryotic taxa were assigned to Fungi (Table 1). Up to 51 different fungal taxa could be identified on a single PS sample and up to 36 on PE. Across all 95 samples, the relative abundance of reads classified as fungi varied from

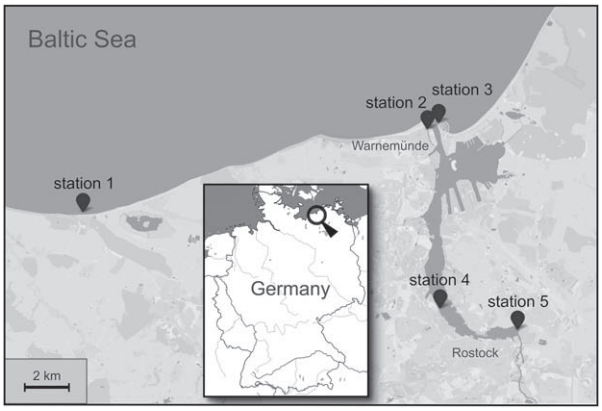

Fig. 1. Map showing the study locations for the incubation experiments, starting in the Baltic Sea (station 1) and ending in the River Warnow (station 5). Station 6 and 7 inside the wastewater treatment plant do not appear on the map to preserve anonymity. Map created using R package leaflet (Cheng *et al.*, 2017) and maps from © OpenStreetMap. Map of Germany from d-maps.com, URL http://d-maps.com/m/europa/germany/allemagne/allemagne07.gif

0.01% to 46% and the number of taxa ranged from 4 to 67. PE, PS and wood samples from the River Warnow to the Baltic Sea (station 1, 2, 3, 4 and 5; Fig. 1) had a significantly higher relative abundance of fungal reads than water samples (Kruskal–Wallis rank sum test, post-hoc Dunn's test, $p < 0.05$, Supporting Information SI1 Table S1 and Fig. 2A). In the WWTP (station 6 and 7), no significant differences among substrates were detected, although several wood samples displayed high fungal abundances (Fig. 2A). Comparing all locations, station 4 (close to a WWTP outlet), 6 and 7 had significantly higher fungal abundances than stations 3 and 5 (Kruskal–Wallis rank sum test, post-hoc Dunn's test, $p < 0.05$, Supporting Information SI1 Table S1 and Fig. 2B).

Fungal communities on PE and PS

Taking all PE and PS samples together, 347 different OTUs assigned to 81 fungal taxa could be determined (Fig. 3 and Supporting Information SI1 Table S2). The majority of fungal reads on MP were assigned to the phylum Chytridiomycota with 41% relative abundance, followed by Ascomycota, unclassified fungi and Cryptomycota with 18%, 17% and 15% respectively. A smaller proportion of reads was assigned to the phyla LKM15 (6%), Basidiomycota (2%) or was of uncertain taxonomic placement (0.3%, 'Incertae Sedis', all of them assigned to the order Mortierellales). Ascomycota, Basidiomycota and Chytridiomycota respectively contained 43, 22 and 10 different taxa. A high OTU richness was often observed for groups with a low taxonomic resolution, for example, Chytridiomycota with 40 OTUs (Fig. 3). The most abundant fungal genus on both PE and PS was *Chytridium* with 22% and 19% of the fungal reads respectively.

Fungal communities across substrates and locations

In Fig. 4, the composition of fungal phyla is illustrated across all observed substrates (Fig. 4A) and locations (Fig. 4B). From the River Warnow to the Baltic Sea, PE and PS samples were dominated by Chytridiomycota,

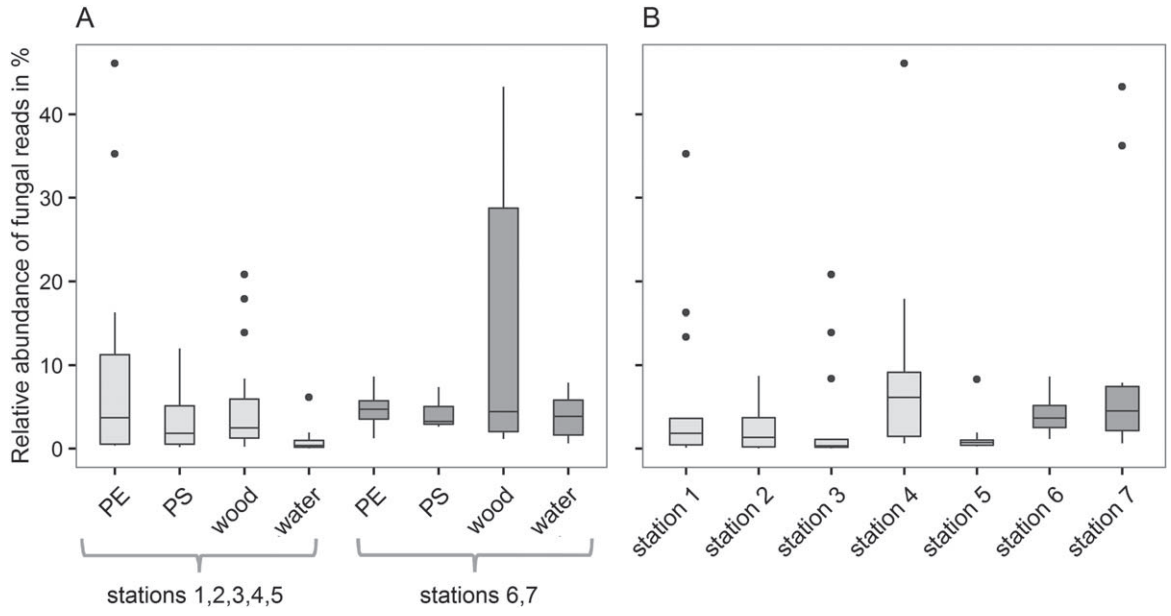

Fig. 2. Box-Whisker-Plots of the relative fungal read abundances for the factors substrate type (A) and location (B).

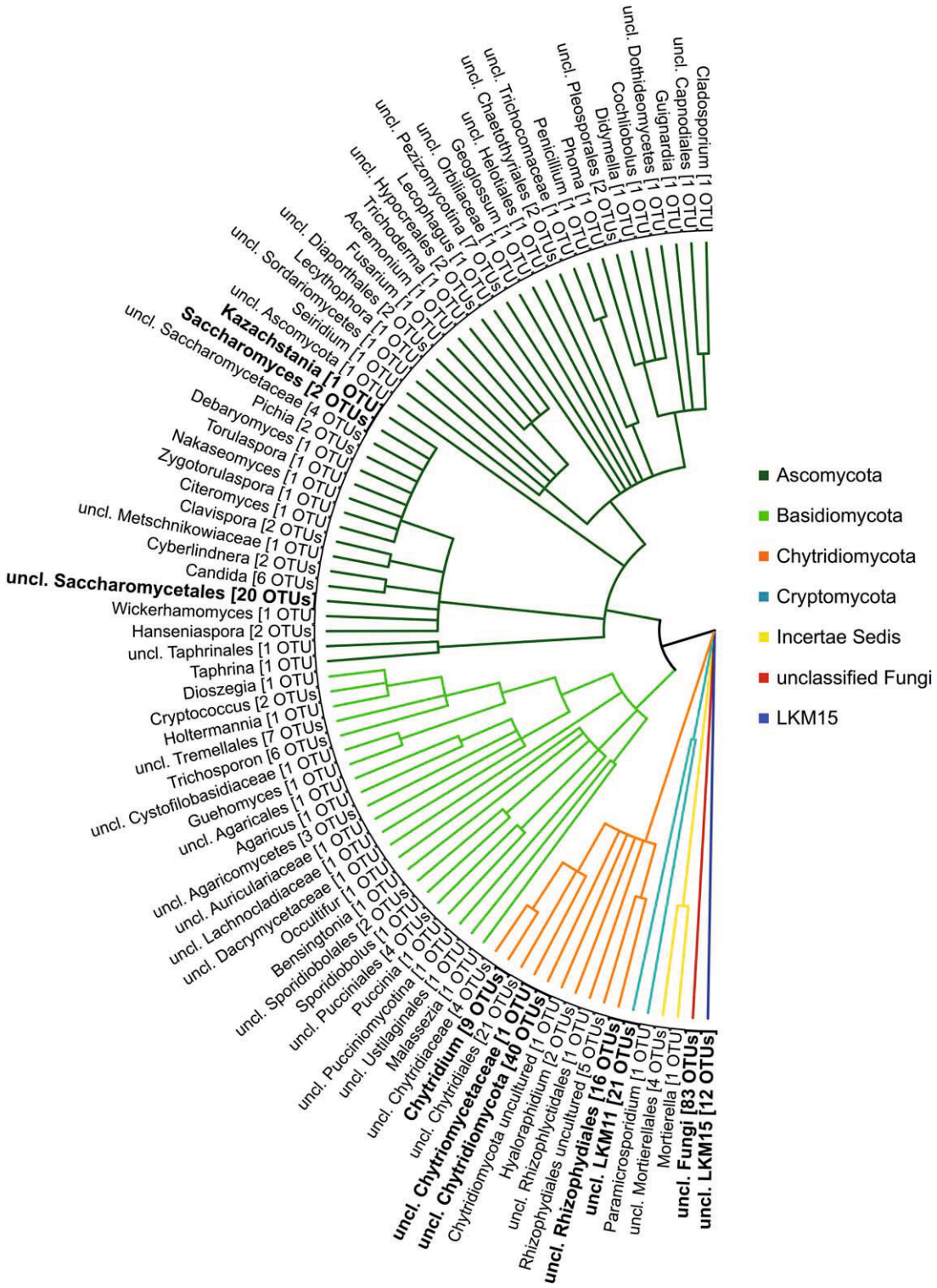

Fig. 3. Taxonomic tree containing all fungal taxa on both microplastics PE and PS, while excluding OTUs with only one read. In bold are the top 10 taxa which represent 91% of the fungal reads on MP. The tree is structured from kingdom (root), subkingdom, phylum, subphylum, class, order and family to genus (leaves). Unclassified (prefix uncl.) genera are named after the next higher classified taxonomic level. [Colour figure can be viewed at wileyonlinelibrary.com]

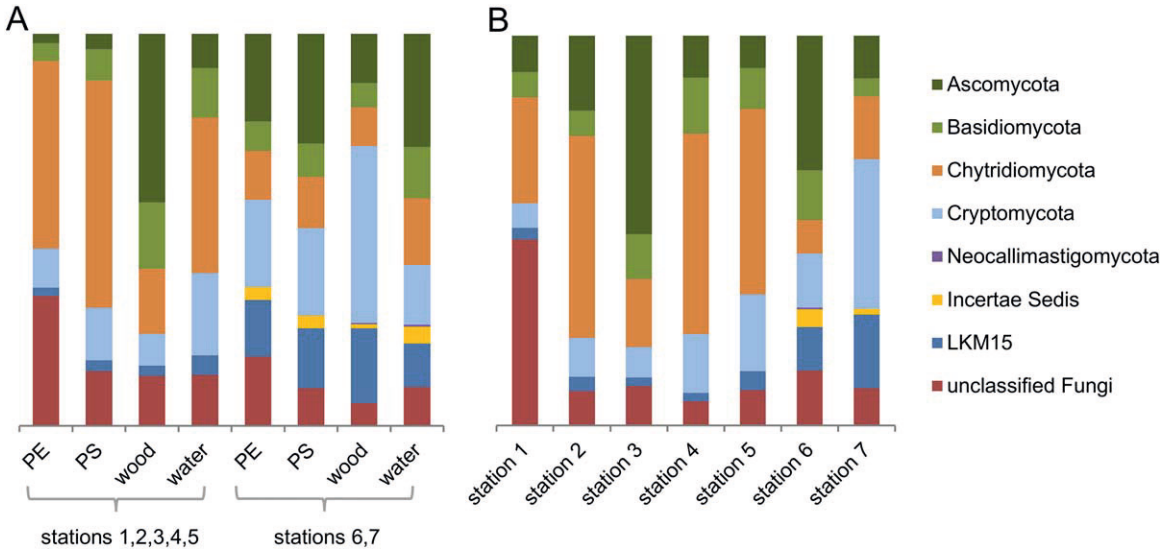

Fig. 4. A. Relative read abundance of fungal phyla on different substrates. First four bars from River Warnow to Baltic Sea (station 1, 2, 3, 4 and 5) and last four bars from wastewater treatment plant (station 6, 7).
B. Relative read abundance of fungal phyla at different locations. Abundance data has been Hellinger-transformed. [Colour figure can be viewed at wileyonlinelibrary.com]

Cryptomycota and unclassified Fungi, while Ascomycota were more abundant on wood samples. In contrast, the distribution of fungal phyla over different substrates in the WWTP was more even. In this study, Ascomycota were mainly composed of the genera *Saccharomyces*, *Candida* and *Kazachstania*. Basidiomycota were mostly assigned to *Cryptococcus* and *Trichosporon*, while Chytridiomycota and Cryptomycota were dominated by *Chytridium* and the lineage LKM11 respectively.

The effects of substrate type and location on fungal community composition

Substrate type as well as location had a highly significant effect on the composition of fungal OTUs for samples from the River Warnow to the Baltic Sea (PERMANOVA, $p = 0.001$, Supporting Information SI1 Table S3). Pairwise comparisons revealed significant differences between each substrate (pairwise PERMANOVA, $p = 0.001$, Supporting Information SI1 Table S4), except for the two MP types PE and PS. The Bray–Curtis similarity was the highest (58%) between PE and PS, and the lowest between wood and water (20%) (Supporting Information SI1 Table S4). The fungal community composition varied significantly between stations (pairwise PERMANOVA, $p = 0.001$, Supporting Information SI1 Table S4), except for stations 2 and 3. Stations 2 and 3 had a Bray–Curtis similarity of 43%, whereas the lowest similarity was observed between station 3 and 5 with 12% (Supporting Information SI1 Table S4). In the WWTP, different substrate types led to a weak differentiation regarding the fungal community compositions (Supporting Information SI1 Tables S3 and S4). Community composition was significantly different before (station 6) and after (station 7) the final biological treatment of the WWTP (PERMANOVA, $p = 0.001$, Supporting Information SI1 Table S4). Data dispersion among the factor groups was homogeneous for almost all cases (Supporting Information SI1 Table S5).

An illustration of similarities regarding the fungal community composition is shown in a NMDS plot (Fig. 5 and Supporting Information SI1 Fig. S1). It visualizes a

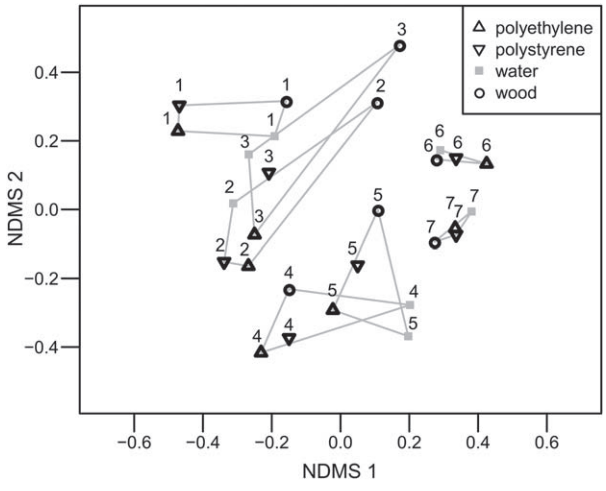

Fig. 5. NMDS of fungal community composition analysed by substrate type and location (stress value 0.20, pooled sample replicates). Higher similarity in fungal community composition results in closer ordination of according points. Symbols represent different substrate types (PE, PS, wood and water). Lines connect samples from the same location (numbers for stations 1 to 7).

grouping by location with overlapping of the more similar stations (station 2 and 3 as well as station 4 and 5) and the similarity between PE and PS per location. Stations 6 and 7 from the wastewater treatment plant separate along the first axis from the other five stations in the River Warnow and the Baltic Sea. The higher Bray–Curtis similarities and lower differentiation by substrate type of these two WWTP stations (Supporting Information SI1 Table S4) are reflected by a closer position of the respective data points in the NMDS plot (Fig. 5).

Fungal OTUs associated with specific substrate types or locations

The indicator species analysis (Supporting Information SI1 Table S6) revealed that one OTU, that is, the second most abundant fungal OTU within the dataset, was significantly associated with MP (PE + PS) ($p = 0.007$, Supporting Information SI1 Table S6). This OTU was assigned to the genus *Chytridium*. A much larger number of OTUs was associated with wood or water. The majority of OTUs that were significantly more abundant on wood were assigned to *Candida* and other fungi of the order Saccharomycetales (Ascomycota). OTUs assigned to the genera *Ogataea* and *Kuraishia* (Ascomycota) occurred exclusively on wood samples. In contrast, water-associated OTUs were exclusively found in the Basidiomycota, Chytridiomycota, Cryptomycota and unclassified Fungi. OTUs from the genus *Rozella* (Cryptomycota) and an unclassified Exobasidiales (Basidiomycota) were almost exclusively found in water.

Numerous fungal OTUs were also significantly more abundant at certain locations (Supporting Information SI1 Table S6), especially at station 1 in the Baltic Sea as well as in the WWTP. Fewer OTUs were associated with locations in the River Warnow stations 4 and 5 and very few to the estuary stations 2 and 3. OTUs that were significantly more abundant at station 1 were assigned mainly to Chytridiomycota and unclassified Fungi. Many OTUs associated with station 6 derived from different yeasts like *Trichosporon* (Basidiomycota) and *Candida* (Ascomycota), whereas OTUs associated with station 7 belonged in most cases to Chytridiomycota, Cryptomycota, LKM15 and unclassified Fungi.

The effects of substrate type and location on fungal alpha diversity

An estimation of fungal alpha diversity (OTU richness, Pielou evenness and Simpson's diversity index) on different substrates and at different locations is shown in Fig. 6. PE and PS samples from the River Warnow and the Baltic Sea had a significantly lower OTU richness, Pielou evenness and Simpson diversity than water and wood (Kruskal–Wallis rank sum test, post-hoc Dunn's test, $p < 0.05$, Supporting Information SI1 Table S7). For the WWTP samples, no significant differences were detected between substrate types. Due to differences in sequencing depth (Supporting Information SI2 Fig. S2), the diversity is difficult to compare between locations of the two incubation experiments. Within each single incubation experiment, no differences were detected among the locations regarding their OTU richness, Pielou evenness and Simpson diversity.

Discussion

This is one of the first reports showing that the presence of MP alters the composition of eukaryotic microbial communities in aquatic ecosystems. MP were easily colonized by different microorganisms, including an abundant diversity of fungi. This recalcitrant surface could be considered a new hotspot for the growth of fungal communities whose structure and composition differed significantly from that found in the surrounding water or other natural solid substrate such as wood. Furthermore, MP-associated fungal communities were shown to vary significantly among sites, which may have profound environmental and ecological implications. For example, microplastic contaminants could collect, transport and disperse a number of non-resident microorganisms including pathogens from WWTPs along their transit from land-based to marine systems.

Previously, Oberbeckmann *et al.* (2016) examined the presence of prokaryotic and eukaryotic microorganisms on PET bottles in the North Sea, and reported only 24 fungal OTUs assigned to Ascomycota, Basidiomycota and Chytridiomycota. In our study, the observed fungal richness and diversity were significantly higher with up to 136 OTUs per sample, mostly assigned to Chytridiomycota and Cryptomycota, and to a lesser extend to Ascomycota and Basidiomycota. Ascomycota were previously assumed to be the most prevalent group within aquatic fungal communities (Shearer *et al.*, 2007; Jones and Pang, 2012). However, recent studies based on culture-independent methods are revealing high proportions of Chytridiomycota in various aquatic ecosystems (Richards *et al.*, 2015; Comeau *et al.*, 2016), which is consistent with our findings. Interestingly, in the WWTP the two most dominant taxa were assigned to LKM11 (Cryptomycota) and LKM15. The predominance of LKM11 in a WWTP has been reported before (Matsunaga *et al.*, 2014), but the ecological role of members of this lineage is still unclear (Grossart *et al.*, 2016).

In our study, we detected a clear differentiation between the fungal communities in the water column and those associated with solid substrates. Distinctions between plastic and water communities have also been found in studies of other microorganisms (Zettler *et al.*, 2013;

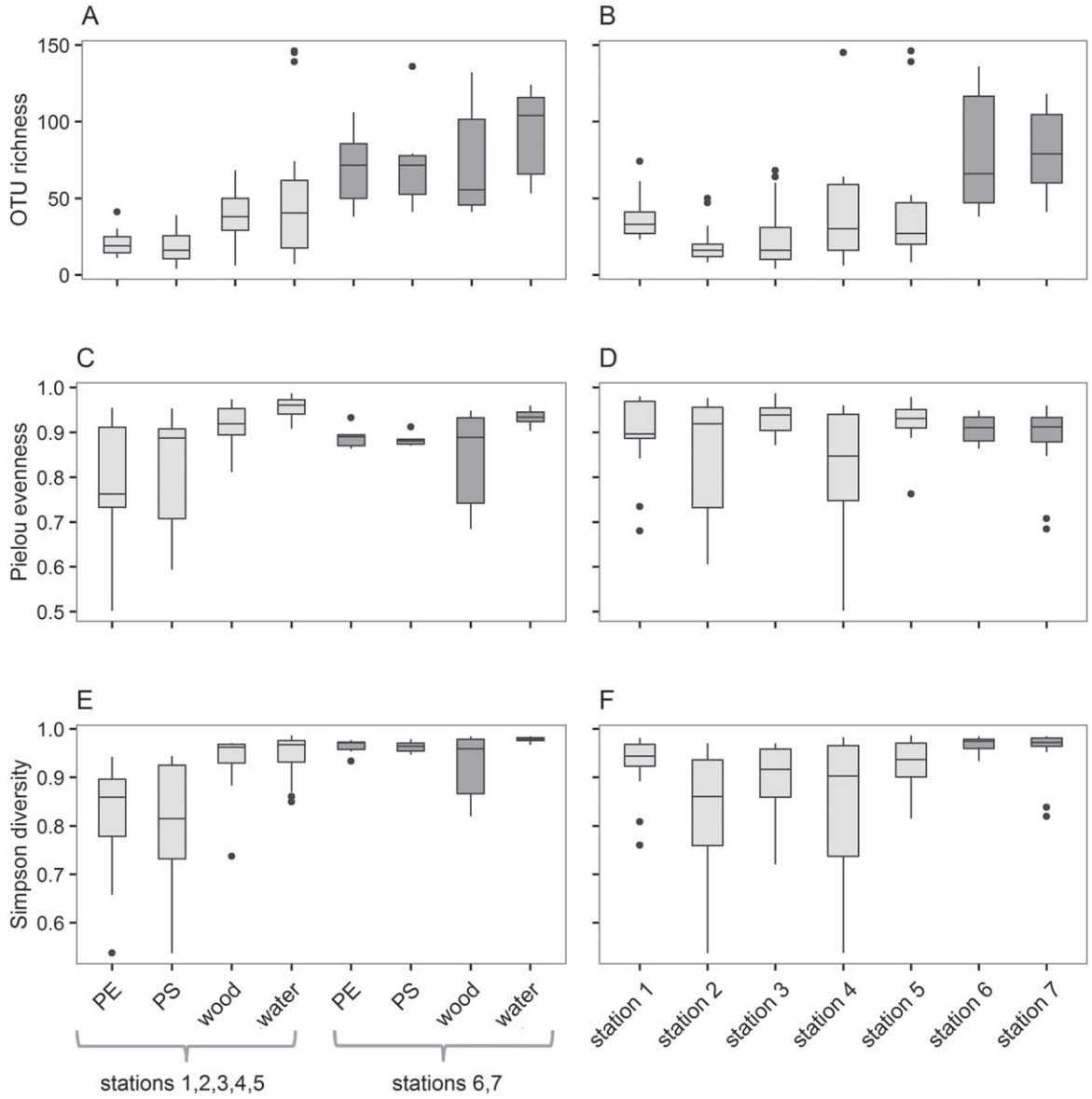

Fig. 6. Boxplots of OTU richness (A,B), Pielou evenness (C,D) and Simpson's diversity index (E,F) for different substrate types (A,C,E) and locations (B,D,F).

Hoellein *et al*., 2014; McCormick *et al*., 2014; Oberbeckmann *et al*., 2016). The higher relative abundance of fungi on PE, PS and wood is likely a result of the higher affinity of certain fungal species to attach to solid surfaces (Richards *et al*., 2012) or to establish specific relationships with other substrate-attached organisms, as might be the case for some parasitic chytrids (Wurzbacher *et al*., 2010; Gleason *et al*., 2014). Potential hosts could be some of the numerous eukaryotes that were present in the MP biofilms, for example, Chlorophyta, Rhodophyceae, Dinophyceae, Diatomea, Nematoda, Rotifera and Crustacea (Kettner *et al*. in preparation). The observed differences between MP- and wood-associated communities, sampled from the River Warnow and the Baltic Sea, further indicate that there are specific preferences for certain substrate types. For example, yeasts such as *Candida* or *Ogataea* exhibited a strong preference for wooden substrates, as they are probably able to degrade wood constituents like xylose and cellulose (Schauer and Hanschke, 1999). High numbers of Ascomycota on decaying wood have also been found by Rämä *et al*. (2014). In contrast to wood and MP, we did not detect significant distinctions between PE- and PS-associated communities. Compared to natural surfaces in aquatic environments, for example, river snow or plant-litter, both MP types appear to have rather similar surface attributes, like a high hydrophobicity and a smooth surface,

which are relevant factors for cell adhesion and biofilm formation (Donlan, 2002; Renner and Weibel, 2011). Nevertheless, fungal communities on PE and PS had a Bray–Curtis similarity of only 58%, hinting at an influence of plastic type on colonization as shown by Zettler *et al.* (2013).

Beside the differences in community composition between water and solid substrates, the NMDS plot illustrates a superior grouping of samples by location. It suggests that the surrounding water – including its local environmental parameters and source communities – were shaping fungal colonization patterns on PE, PS and wood. These results furthermore expand the notion of possible habitat and location specificity of fungi (Monchy *et al.*, 2011; Panzer *et al.*, 2015; Wurzbacher *et al.*, 2016). Also in our study, each location was significantly different from any other location regarding their fungal community composition, with the exception of stations 2 and 3, which are located geographically close to each other. One of the possible factors influencing the presence of fungi might be the nutrient concentration, as we detected the highest relative fungal abundances at stations located inside a WWTP or close to the discharge point of a WWTP. These stations had elevated amounts of nitrate (Supporting Information SI1 Table S8), which may have altered fungal growth (Ferreira *et al.*, 2006).

It is moreover possible that a shift in taxonomic composition can be accompanied by changes in functionality (Allison and Martiny, 2008; Shade *et al.*, 2012). For example, the abundant presence of Chytridiomycota could result in different kinds of parasitic or saprotrophic relationships with the primary producers also present in the MP biofilms, mediating the carbon, nutrient and energy transfer into the food webs (Kagami *et al.*, 2007; 2014; Agha *et al.*, 2016). In addition, fungi could be attracted by organic substances adsorbed to the plastic surface, which form a so-called conditioning surface film (Donlan, 2002). In this regard, several saprotrophic fungi would be able to feed on those organic substances or on detritus that emerged within the biofilm including complex molecules (Wurzbacher *et al.*, 2011; Richards *et al.*, 2012).

In contrast to natural biofilms on wood or algal aggregates, which will disintegrate over time, MP may persist for years, decades or even centuries (Shah *et al.*, 2008; Andrady, 2011) while carrying its attached biota. However, it is not clear how stable the MP-associated microbial communities are; some are likely to change along with changing environmental factors, whereas others might remain more resilient within their micro-niches, and others could even switch to resting stages waiting for more favourable conditions to reactivate. As buoyant MP can be transported over very long distances (Barnes, 2005; Law and Thompson, 2014), MP could facilitate the spread of fungi to new habitats, possibly even at the global scale. Consequently, our work raises an additional warning about the problem of increasing accumulation of plastic contaminants in aquatic ecosystems, as MP provide selective advantages for the colonization of certain groups of microorganisms, but also serve as highly persistent vehicles for their dispersion. As MP introduces huge surfaces for colonization and biofilms are hotspots for microbial activities (Costerton *et al.*, 1987), further research is necessary to better elucidate interactions between the microorganisms on MP, their metabolic capabilities and the resulting impacts on biodiversity, food web structures and overall ecosystem functioning.

Conclusions

Despite their high phylogenetic diversity and versatile metabolic potentials, fungi gained very little attention in previous studies on MP colonization. Here, we provide a first in-depth report about fungal taxa that occurred on MP in different aquatic ecosystems. We observed that location and substrate type significantly influenced fungal community compositions. Fungal communities on MP differed from those in water and on the natural substrate wood. The ecological consequences of selected microbial communities on MP, however, remain largely unknown, though the diverse and abundant occurrence of fungi on plastic points towards their high relevance on this artificial habitat. Fungi – as parasites, pathogens, symbionts or saprobes – could substantially influence community dynamics and cycling processes within MP biofilms. Our findings can now serve as a basis for future studies aimed at unveiling ecological functions by targeting more specific fungal groups of interest such as algal parasites or potential plastic degraders.

Experimental procedures

Incubation experiments and sampling

To examine effects of the factors 'substrate type' and 'location' on the fungal community composition and diversity, three different substrates for microbial colonization – plus water – and seven different incubation sites were studied (Fig. 1 and Supporting Information SI1 Table S8 for brief description of study sites including environmental parameters). The three substrates were high density polyethylene particles (PE, ExxonMobil^{TM} HDPE HTA 108, ExxonMobil Chemical Europe, Belgium, diameter 3–5 mm, density 0.96 g cm^{-3}), polystyrene particles (PS, Polystyrol 143 E, BASF, Germany, diameter 3–5 mm, density 1.04 g cm^{-3}) and wood pellets (1Heiz® Holzpellets, 1Heiz® Pellets AG, Germany, density 1.12 g cm^{-3}). Wood pellets were made of pine (approximately 90%), spruce (approximately 10%) and could contain traces of other tree species. Wood served as a natural reference substrate in comparison to the artificial substrates PE and PS.

The substrates were incubated at seven different locations at a water depth of 1 to 3 m in separate, custom-made cages (cylindrical shape, diameter 7 cm, height 8 cm). Nine cages were deployed at each station (three substrate types à three

replicates). To provide comparable surface areas for particle colonization, cages were loaded with 60 g PE, 54 g PS and 35 g wood respectively. The lateral surface of these cages consisted of a nylon mesh with 500 μm mesh size, to allow exchange with the surrounding water. Two incubation experiments were performed. The first experiment was conducted at stations one to five (from the pier Heiligendamm in the Baltic Sea into the River Warnow, approximately 12 km upstream) in 2014, from 19 to 20 August until 3–4 September. The second experiment was conducted in a municipal WWTP in 2015, from 28 April until 12 May. Station 6 was located after the last sedimentation tank, where conventional WWTPs would discharge. Station 7 was situated after the additional treatment with an oxygenated biofilm reactor.

After the incubation period of 15 days, PE, PS and wood particles were washed three times with 0.2 μm-filtered, autoclaved water from the corresponding station to remove loosely attached organisms. On the same day when particles were retrieved, water samples were collected at the respective stations. To collect the microorganisms, 1 to 2 l of each water sample (1 to 3 replicates) were filtered onto 3 μm pore-size membranes (Whatman® Nuclepore Track-Etch Membrane, polycarbonate, GE Healthcare, Germany) and 0.3 to 0.5 l of the filtrate (2 to 3 replicates) onto 0.22 μm pore-size membranes (Durapore® membrane filters, polyvinylidene fluoride, Merck Millipore, Ireland). Particles and filter membranes were rapidly frozen in liquid nitrogen and stored at −80°C until further processing.

Additionally, three water samples were retrieved at each location for determining concentrations of phosphate, nitrate, nitrite, ammonium and silicon dioxide according to standard methods (Hansen and Koroleff, 1999). Temperature and salinity were measured *in situ* with a portable meter (HQ40d, Hach, Germany). Water samples for analysis of nutrient concentrations as well as temperature and salinity measurements were obtained at the start and end of each incubation experiment. In Supporting Information SI1 Table S8 average data of the measurements are shown.

DNA extraction, amplification and sequencing

DNA was extracted from PE, PS, wood and water samples using a modified protocol (Nercessian *et al.*, 2005) optimized and refined for biofilms attached to MP. The procedure starts with a chemical and mechanical cell disruption (bead beating in a lysis solution containing Tris, EDTA, NaCl and SDS) followed by an enzymatic treatment with proteinase K and addition of a CTAB buffer. DNA was extracted from the solution first with phenol/chloroform/isoamyl alcohol and a then with chloroform/isoamyl alcohol. DNA was precipitated with ethanol and sodium acetate, subsequently washed in ethanol, dried, re-suspended in pure water and stored at −20°C. All DNA samples were sent to LGC Genomics, Berlin, Germany for purification, PCR amplification, equimolar mixing and Illumina MiSeq sequencing (2*300 bp paired end, Miseq reagent kit V3). The eukaryote-specific primers Eu565F and Eu981R (Stoeck *et al.*, 2010; with slight modification according to LGC Genomics, Berlin, Germany; see Supporting Information SI1 Table S9 for primer specificity), targeting the highly variable V4 region of the 18S rRNA gene, were used for PCR amplification. More information on DNA extraction and primers are given in SI1.

Sequence processing

Raw Illumina Miseq reads were demultiplexed, then barcodes, adapters and primers were clipped (pre-processing by LGC Genomics, Berlin, Germany). Reads were further processed in mothur v1.37.6 (Schloss *et al.*, 2009) according to mothur MiSeq SOP (Kozich *et al.*, 2013; online access April 2016), which included quality filtering of joined reads, alignment, chimera removal and classification of remaining reads using the non-redundant SILVA database v123 (Quast *et al.*, 2013) complemented with the online SINA Alignment Service v.1.2.11 (Pruesse *et al.*, 2012). Raw reads are available at the Short Read Archive (SRA) of NCBI under BioSample accessions from SAMN06806566 to SAMN06806660 of the BioProject PRJNA383789.

After extracting the fungal reads from the data set, two approaches were chosen for data evaluation as explained below and they are herein called 'taxon-based approach' and 'OTU-based approach' (OTU = operational taxonomic unit). The taxon-based approach is based on a data matrix containing all fungal taxa and their read abundances per sample. Fungal taxa were named according to the SILVA taxonomy (Yilmaz *et al.*, 2014) and often resolved down to the genus level. In case fungal taxa could not be classified on genus level, they were named after the next higher classified taxonomic level (family, order, class, subphylum, phylum or kingdom). This imbalanced taxonomic resolution can cause skewed results when comparing community composition and diversity among samples. To overcome this potential bias, operational taxonomic units (OTUs) were calculated. At first, the taxonomic information from the classified fungi is used to split the dataset into distinct taxonomy groups (here on genus level). Then, OTUs were clustered within these groups by applying a 3% distance threshold. This 'OTU-based approach' improves the comparability of samples by generating a more consistent taxonomic resolution. Detailed methods and rarefaction curves (Fig. S2) are presented in Supporting Information SI2.

Data evaluation and statistical methods

Statistical calculations were performed in R version 3.3.1 (R core team, 2016) including the R package vegan (Oksanen *et al.*, 2016). All 95 samples were assigned to the corresponding substrate type (polyethylene, polystyrene, wood or water) and location (station 1, 2, 3, 4, 5, 6 or 7).

Relative fungal read abundance was calculated by dividing the number of fungal reads by the number of eukaryotic reads for each sample. A tree was calculated for fungal taxa and OTUs, excluding those containing only one single read, from all MP samples (PE and PS) using the R package phangorn (Schliep, 2011). The tree was plotted with iTOL v3 (Letunic and Bork, 2016). Data tables with taxon or OTU abundances per sample were Hellinger-transformed (Legendre and Gallagher, 2001) as advised by Ramette (2007). After conversion into a Bray–Curtis similarity matrix, the effects of substrate type and location on the fungal community composition were

evaluated with non-parametric multivariate analysis of variance (PERMANOVA) and pairwise PERMANOVA (adonis function in vegan, 999 permutations). The homogeneity of group dispersions was checked for the factors substrate and location with the betadisper and (pairwise) permutest function in vegan (999 permutations). To further illustrate differences in fungal community composition, Bray–Curtis similarities were calculated between each factor group pair. Additionally, the Bray–Curtis similarity matrix of sample replicates and all 95 samples was represented in a two-dimensional NMDS plot. To evaluate which OTUs contributed to differences in fungal community composition, an indicator species analysis (De Cáceres and Legendre, 2009) was performed (R package indicspecies; De Cáceres, 2013), which indicates when certain OTUs are significantly associated with specific substrates or locations. To assess the fungal alpha diversity, three classical indices were calculated as described in Borcard *et al*. (2011): richness *N* (number of different OTUs), Simpson's index 1-D (diversity function in vegan, index = simpson) and Pielou evenness *J* ($J = H/\log(N)$; with H = Shannon's index). Kruskal–Wallis rank sum tests were used to check for overall significant differences among factor groups for the relative fungal read abundance, *N*, 1-D and *J*. When results were significant, pairwise comparisons were tested using pairwise test for multiple comparisons of mean rank sums (Dunn's test) in the R package PMCMR (Pohlert, 2014). *P*-values from multiple testing (Dunn's test, pairwise PERMANOVA and indicator species analysis) were adjusted according to Benjamini and Hochberg (1995).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Results of Kruskal-Wallis test and posthoc Dunn's test for comparison of relative fungal read abundance on different substrate types and at different locations.
Table S2. Overview of all fungal taxa found on microplastic (polyethylene and polystyrene) across all samples. Taxa with only one read are not presented herein. Taxonomic information is based on SILVA v128 (Yilmaz *et al*., 2014).
Table S3. PERMANOVA results after 999 permutations for the factors substrate and location as well as their interaction term.
Table S4. Results of pairwise PERMANOVA (999 permutations) and Bray-Curtis (BC) similarity for different substrate types and locations. P-adjustment according to Benjamini and Hochberg, 1995.
Table S5. Results of permutation tests for homogeneity of multivariate dispersions for different substrate types and locations. If dispersion was not homogeneous, the results of pairwise comparisons are also given. df = degrees of freedom, Sq = squares.
Table S6. Results of indicator species analysis with OTUs associated to each group or combination of groups for substrate type and location. For each OTU the assigned taxon, phylum, indicator value, adjusted p-value (according to Benjamini and Hochberg, 1995) and number of reads are given.
Table S7. Results of Kruskal-Wallis test and posthoc Dunn's test for comparison of fungal OTU richness, Pielou

evenness and Simpson's index, each on different substrate types and at different locations. Subscript numbers behind the substrate indicate study stations.

Table S8. Incubation sites with short description, coordinates, incubation time, and averaged values of temperature, salinity, phosphorous, nitrate, nitrite, ammonium and silicon dioxide. To preserve anonymity, coordinates of the wastewater treatment plant (WWTP) are not given. * Salinity values were provided by personnel of the wastewater treatment plant. DD = decimal degrees.

Table S9. Primer specificity tested with Silva TestPrime1.0 (Klindworth *et al.*, 2013). List of taxa and their according coverage in the Silva Taxonomy browser (database v128). Coverage is given in percent allowing zero and one mismatch.

Fig. S1. NMDS of fungal community composition analyzed by substrate type and location (stress value 0.24). Ellipses surround samples from the same location at a confidence interval of 80%. Shapes and numbers indicate stations. Colors represent the different substrate types polyethylene, polystyrene, wood and water.

Fig. S2. Rarefaction curves illustrating the number of OTUs over the number of reads for each sample.

SI1 (pdf file) contains tabular overview of full taxonomic information for fungi on MP, an alternative NMDS plot with individual samples, results of statistical tests (KruskalWal-lis test, Dunn's test, PERMANOVA, pairwise PERMANOVA, permutation test for homogeneity of multivariate dispersion), results of indicator species analysis, description of study sites and primer specificity. SI1 includes Tables S1 to S9 and Fig. S1.

SI2 (pdf file) contains a detailed description of the methods DNA extraction, PCR amplification, sequence processing, data evaluation and statistical methods as well as a brief discussion about methodological limitations. SI2 includes also rarefaction curves (Fig. S2).

Manuscript III

Ester Maria Eckert, Andrea Di Cesare, **Marie Therese Kettner**, Maria Arias-Andrés, Diego Fontaneto, Hans-Peter Grossart and Gianluca Corno

Microplastics increase impact of treated wastewater on freshwater microbial community.

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Microplastics increase impact of treated wastewater on freshwater microbial community☆

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ABSTRACT

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

1. Introduction

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

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

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

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

2. Material and methods

2.1. Experimental set-up

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

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

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

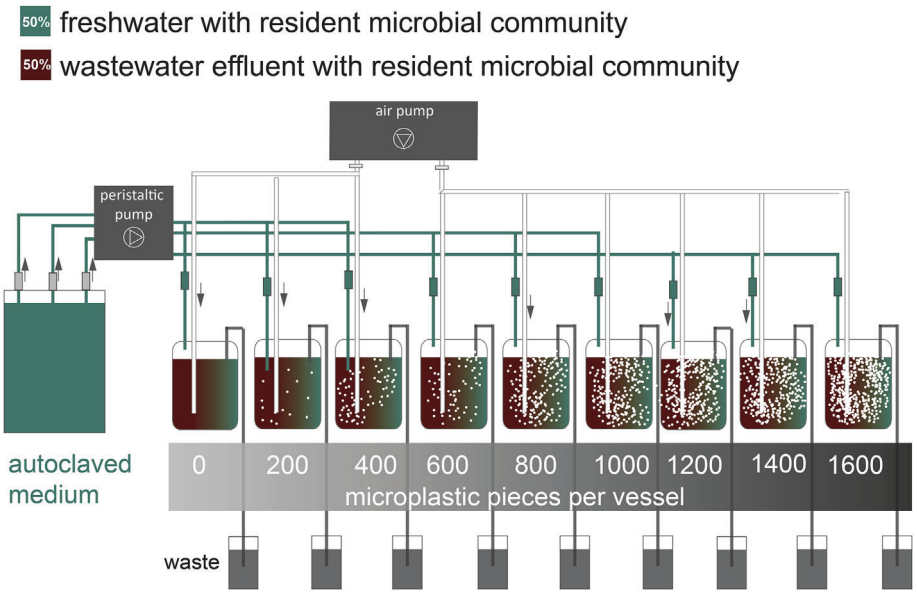

Fig. 1. Schematic representation of the chemostat set-up.

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

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

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

2.2. Bacterial abundance and size distribution

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

2.3. DNA extraction

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

2.4. Bacterial community pattern: PCR and ARISA

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

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

statistical analyses.

2.5. 16SrDNA and int1 quantification

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

2.6. Statistical analyses

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

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

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

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

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

3. Results

3.1. Cell numbers and phenotypic distribution

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

3.2. Bacterial community patterns

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

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

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

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

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

3.3. Integrase 1 occurrence

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

4. Discussion

4.1. Exchange of microbes between microplastic and surrounding water

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

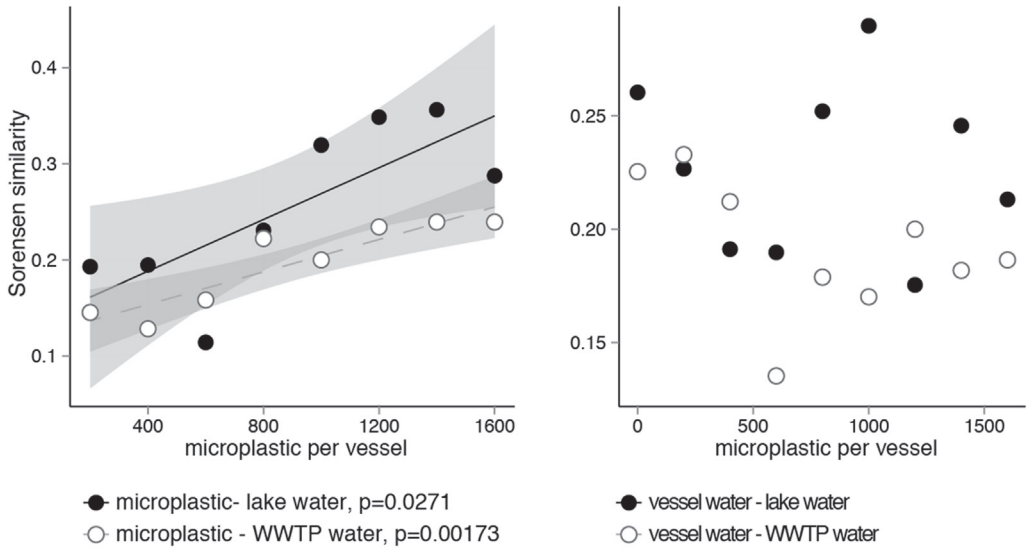

Fig. 2. Relationship between Sorensen similarity of the microbial communities on microplastic (left) and vessel water (right) to the original wastewater and lake water community in dependence of the concentration of microplastic. The regression line, confidence interval and p-values were plotted only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

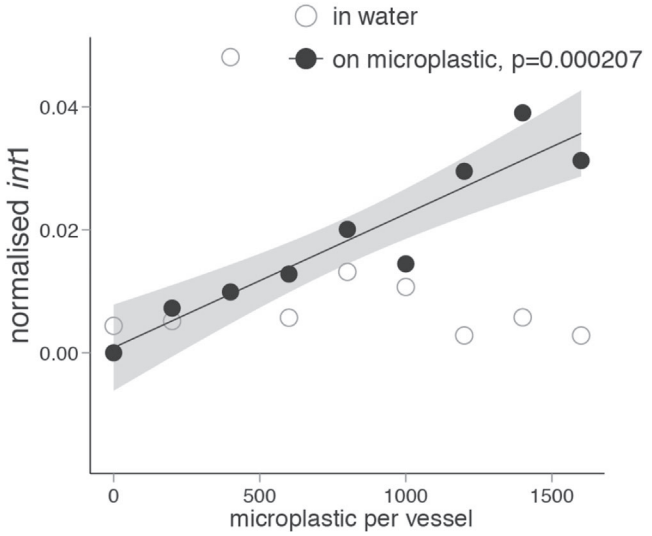

Fig. 3. Relationship between abundance of *int*1 in water (white) and on microplastic (black) with the concentration of microplastic. Abundance values of *int*1 are expressed as arcsin of square root of the proportion between abundance of *int*1 and abundance of 16S rDNA. The regression line, confidence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

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

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

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

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

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

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

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

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

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

4.3. Differences of experimental set-up to nature

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

5. Conclusions

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

Disclaimer

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Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.11.070.

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Brief summary of the results

With study I (Kettner *et al.*, 2017, Kettner *et al.,* submitted), I could confirm the hypothesis (H I.1) that the eukaryotic/fungal community compositions on microplastics (PE and PS) significantly differ from those on wood and in the surrounding water. Further, they were clearly distinct among the sampled stations (H I.2). I observed the highest similarities in community compositions between PE and PS (as sole pair, which did not significantly differ) and, generalized, the lowest similarities between all solid substrates PE, PS and wood versus water (*RQ I.1*). The eukaryotic diversity was lowest on both microplastics and similarly high on wood and in water (*RQ I.2*). Nevertheless, I detected 505 different eukaryotic taxa, including 110 fungal taxa, on microplastics (*RQ I.3*). Common taxa on microplastics were, e.g., *Pfiesteria*, *Ulva*, *Ephelota* or *Chytridium* (*RQ I.4*). I could barely identify eukaryotic organisms that were specifically associated with either PE or PS (*RQ I.5*). Instead, several indicator organisms were found for wood and water (*RQ I.5*). My network analyses – including both prokaryotic and eukaryotic taxa – revealed that numerous organisms, also from different trophic levels, were positively correlated to each other, which indicated diverse microbial interaction possibilities on microplastics (*RQ I.6*). Regarding fungi, I detected a higher relative fungal abundance on solid substrates compared to water, as expected (H I.3). The fungal diversity was higher on wood than on microplastics, but did not differ significantly from the diversity in the surrounding water (*RQ I.2* and H I.4). I could neither determine differences in fungal diversities among sampling stations from the first nor the second exposure experiment (*RQ I.7*). In contrast, I could prove that the fungal community compositions differed significantly among substrate types (H I.1) and locations (H I.2), resulting in a distinct set of indicator organisms for microplastics, wood and water (*RQ I.5*) as well as for each of the seven stations (H I.5).

In study II (Eckert *et al.*, 2018), we focused specifically on the impact of WWTP-derived communities on freshwater assemblages with increasing microplastics concentrations. As expected, the abundance of integrase I – a marker associated with mobile genetic elements – increased in microplastics biofilms along with rising microplastics concentrations (H II.1). At the end of the experiment, bacterial communities on microplastics were more similar to the initial communities from treated wastewater, although the inoculum was an equal mixture of freshwater and WWTP-derived communities (*RQ II.1*). With increasing microplastics concentrations, we observed an increasing bacterial OTU richness in microplastics biofilms but a decreasing richness in the surrounding vessel water (*RQ II.2*).

Discussion

Microplastics-associated organisms

Within the Leibniz SAW MikrOMIK project (study I), we were able to identify more than 1500 prokaryotic and 500 eukaryotic taxa across all microplastics samples (Kettner *et al.*, submitted; data for prokaryotes provided by Sonja Oberbeckmann; see also Oberbeckmann *et al.,* 2018). With respect to the rather short incubation time of 15 days, our data highlights the diverse colonization of microplastics in aquatic ecosystems. To our knowledge, this was the first microplastics colonization study conducted in brackish environments. In contrast to other sequencing studies covering plastics-associated eukaryotes (Zettler *et al.*, 2013; Oberbeckmann *et al.*, 2016; De Tender *et al.*, 2017), we could present an in-depth report about the eukaryotic life on microplastics, including the widely neglected kingdom of fungi (Kettner *et al.*, 2017, Kettner *et al.,* submitted). Especially fungi contributed strongly to the overall diversity of microplastics biofilms. While fungal reads represented only 4% of all eukaryotic reads, they made up a fifth of all eukaryotic taxa (Kettner *et al.*, 2017). Solely two other studies reported on the presence of fungi – though not on microplastics but on larger plastic debris – in pelagic and littoral zones of the North Sea (Oberbeckmann *et al.*, 2016; De Tender *et al.*, 2017). In both studies, Ascomycota and Basidiomycota dominated the fungal communities, but also Chytridiomycota were found. Although our study was conducted at other locations and used different materials and methods, we identified many fungal taxa from these three phyla as well, albeit Chytridiomycota was the most dominant phylum on microplastics (Kettner *et al.*, 2017). Furthermore, we were able to detect taxa across almost all fungal phyla and revealed for the first time, that also Cryptomycota (predominantly LKM11) and LKM15 were relevant biofilm members. As demonstrated with high numbers of OTUs within the phyla Chytridiomycota, Cryptomycota and LKM15 (Kettner *et al.*, 2017), we can expect a high diversity hidden in these early diverging lineages of the fungal kingdom. Despite increasing evidence for their phylogenetic diversity, little is known about their ecological functions (Richards *et al.*, 2012; Grossart *et al.*, 2016). Many species of the early diverging lineages are parasitic fungi, which can play crucial roles in food web dynamics, as for instance chytrids are infecting cyanobacteria or algae and consequently make this biomass available to other organisms in the food web (Haraldsson *et al.*, 2018). Possibly, parasitic chytrids have also the potential to influence algal and cyanobacterial abundances in microplastics biofilms. On the other side, Ascomycota, Basidiomycota and further fungi are commonly known for their importance in nutrient cycling, since they decompose dead organic matter and even recalcitrant substances (Bärlocher and Boddy, 2016; Grossart and Rojas-Jimenez, 2016). Due to their versatile enzymatic machinery, fungi are suggested as potential candidates for the biodegradation of plastics in aquatic environments (Krueger *et al.*, 2015, 2016; De Tender *et al.*, 2017; Paço *et al.*, 2017).

Nevertheless, fungal colonization of environmental plastic samples has been described in only three studies so far (Oberbeckmann *et al.*, 2016; De Tender *et al.*, 2017; Kettner *et al.*, 2017) and yet, it is not understood, which specific functions they fulfill. The ecology of fungi in plastics biofilms therefore deserves much more scientific attention.

Besides fungi, we identified numerous taxa all across the eukaryotic tree of life from unicellular protists to multicellular metazoans. Microplastics biofilms contained organisms from different trophic levels, i.e. primary producers such as green algae (*Ulva* or class Trebouxiophyceae) and red algae (*Polysiphonia*), as well as primary and secondary consumers such as different ciliates (ConThreeP including sessile filter-feeder *Zoothamnium* or suctorian *Ephelota*), crustaceans, rotifers, nematodes and other small animals (Kettner *et al.,* submitted). The highly abundant dinoflagellate *Pfiesteria* is mixotrophic and consequently may act as primary producer and/or primary consumer. Lastly, also saprotrophs and potential parasites such as various fungi (Kettner *et al.*, 2017) and fungal-like organisms (*Pythium*, *Rhizdiomyces* or order Rhinosporideacea) were commonly detected on microplastics (Kettner *et al.,* submitted). Again, no microplastics studies in the Baltic Sea environment exist for a direct comparison of our results. In accordance with studies in oligotrophic marine environments, we also found high abundances of Chloroplastida, the SAR supergroup (SAR = Stramenopiles, Alveolata and Rhizaria) and a great variety of metazoans (Zettler *et al.*, 2013; Reisser *et al.*, 2014; Bryant *et al.*, 2016; Oberbeckmann *et al.*, 2016; Debroas *et al.*, 2017). Diatoms, which have been observed very frequently on plastics by microscopy (Carpenter and Smith, 1972; Carson *et al.*, 2013; Zettler *et al.*, 2013; Eich *et al.*, 2015), were represented in our study with 44 different taxa, but made up less than 1.3% of the overall read abundance. Bryant *et al.* (2016) revealed a similarly low proportion of diatom reads and explained this result with their comparably low biomass. The presence of some eukaryotic genera in microplastics biofilms – for instance *Ephelota* – has been reported in previous studies (Zettler *et al.*, 2013; Debroas *et al.*, 2017). With our study, however, we could reveal the occurrence of many more eukaryotes that have not been detected on microplastics before, e.g. *Pfiesteria*, *Ulva*, *Chytridium*, or *Pythium* (Kettner *et al.*, 2017, Kettner *et al.,* submitted).

The indicator species analysis, which tested if certain organisms were specifically associated with PE, PS, wood or water, showed that almost no organisms significantly occurred on PE or PS (Kettner *et al.*, 2017, Kettner *et al.,* submitted). This supports the hypothesis that microplastics are mainly colonized by opportunistic microbes (Oberbeckmann *et al.,* 2014; Keswani *et al.*, 2016). Nonetheless, microplastics biofilms hold distinct communities with diverse possibilities for microbial interactions, which I will present in the following chapters.

Influence of microplastics on microbial community composition and diversity

In study I, the eukaryotic community compositions on both PE and PS differed significantly from those on wood and in the surrounding water (Kettner *et al.*, 2017, Kettner *et al.,* submitted). This indicates substrate-specificity of eukaryotes and highlights that in brackish environments, biofilms on artificial microplastics are distinct from biofilms on the natural substrate wood. Similar patterns have been identified in freshwaters, though for bacteria, by Hoellein *et al.* (2014) and McCormick *et al.* (2014, 2016). They found that bacterial communities on microplastics were distinct to those on other organic substrates and in water. In marine environments, also plastics-associated eukaryotic communities and specifically fungal assemblages differ from the surrounding water (Oberbeckmann *et al.*, 2016; De Tender *et al.*, 2017). However, we were not able to detect significant differences between eukaryotic communities on both microplastic types, i.e. PE and PS (Kettner *et al.*, 2017, Kettner *et al.,* submitted). Generally, hard substrates such as plastics, glass or aluminum seem to reveal much less pronounced differences regarding their associated communities (McCormick *et al.*, 2014; Oberbeckmann *et al.*, 2016), but more studies are required to verify or falsify this notion.

Secondly, we observed a clear location-dependence of the eukaryotic community compositions (Kettner *et al.*, 2017, Kettner *et al.,* submitted). The location (R^2=0.467, $p < 0.001$, PERMANOVA) seemingly had a stronger impact on colonization patterns than the substrate type (R^2=0.140, $p < 0.001$, PERMANOVA). This is in line with findings by Oberbeckmann *et al.* (2016) for pro- and eukaryotes in the North Sea. Most likely, the local environmental conditions – such as salinity, light availability, temperature, carbon, nutrient and oxygen concentrations etc. – influence the resident microbial communities in the water, which in turn affect the communities on microplastics floating in that water. Possibly, the concentration of microplastics in the water can further alter colonization patterns, as we have seen in study II (Eckert *et al.*, 2018). With high concentrations, we observed that microplastics-associated bacterial communities became more similar to the initial assemblages of treated wastewater (Eckert *et al.*, 2018). The reason remains speculative but conceivably, wastewater-derived bacteria may preferentially live surface-attached, since nutrients can be markedly enriched on surfaces (Zobell, 1943). Since in our experiment, mixed microbial communities grew in rather nutrient-poor lake water medium, wastewater-derived bacteria – which are used to comparably higher nutrient concentrations – increase their chance of survival when attaching to microplastics (Eckert *et al.*, 2018). This gives them a selective advantage in comparison to lake water bacteria that are adapted to live in nutrient poor environments. The higher abundance of integrase I with increasing microplastics concentration, additionally points out that high amounts of plastic debris seem to facilitate the survival of not only wastewater-derived bacteria, but also of bacteria containing genes that are linked to anthropogenic

pollution and involved in the spread of antibiotic resistance (Gillings *et al.*, 2015; Eckert *et al.*, 2018).

Next to the described differences in community composition, we also found variations regarding the eukaryotic and bacterial diversities. While the eukaryotic diversity was rather similar across all sampled locations in study I, we detected significant differences among the materials (Kettner *et al.*, 2017, Kettner *et al.,* submitted). Microplastics-associated eukaryotic communities were much less diverse than those on wood and in the surrounding water. To our knowledge, comparisons of the entire eukaryotic diversity on microplastics in reference to natural particles have not been performed previously. In comparison to water, however, microplastics from marine environments have a lower eukaryotic diversity (Debroas *et al.*, 2017). Other research groups found that likewise, the bacterial diversity was significantly lower on plastics than in the surrounding water or on organic matter (Zettler *et al.*, 2013; McCormick *et al.*, 2014, 2016). Contrary to that, we observed in study II that in half of the vessels microplastics biofilms had a higher bacterial diversity than the surrounding water (Eckert *et al.*, 2018). This contradiction can be explained by the cultivation technique. We used chemostat systems in study II, which were set to a constant dilution rate of 10% water volume every day. As a consequence, bacteria that are not reproducing fast enough, are flushed out of the chemostat vessels. Only bacteria attaching to microplastics – which were retained in the vessel during the entire experiment – are not exposed to this artificial selection pressure and can thus develop a more diverse community. We further noted an increasing bacterial diversity of microplastics biofilms with increasing microplastics concentration (Eckert *et al.*, 2018). Along with higher particle concentrations, a higher surface area is available for bacterial colonization and the chance of a free-living bacterium to attach to microplastics increases. The resulting increase in diversity (Eckert *et al.*, 2018) is thus similar to findings from Goldstein *et al.* (2014), who identified a significant increase in taxon diversity with increasing available surface area of plastic items. In addition, Debroas *et al.* (2017) found that mesoplastics (5 mm – 200 mm) have a higher prokaryotic and eukaryotic diversity than microplastics.

In our studies, we did not investigate all aspects that can be relevant for diversity and colonization patterns of microplastics, such as the hydrophobicity and roughness, the adsorption of nutrients, heavy metals and persistent organic pollutants to the material's surface, or the pioneer colonizers influencing which organisms settle next in the biofilms (Artham *et al.*, 2009; Ashton *et al.*, 2010; Rochman *et al.*, 2013; Harrison *et al.*, 2018). Since microniches and gradients develop in mature biofilms (Flemming *et al.*, 2016), the age of the biofilm as well as seasonal changes of environmental parameters play further important roles for the microplastics-associated assemblages (Artham *et al.*, 2009; Harrison *et al.*, 2014; Oberbeckmann *et al.*, 2014, 2016). If animals ingest microplastics, the gut passage can

additionally alter microbial communities on the particle (Kesy *et al.*, 2016). Basically, all biotic and abiotic factors can affect the community composition and diversity, but location, meaning the local environmental parameters, apparently have the strongest impact (Hoellein *et al.*, 2014; Amaral-Zettler *et al.*, 2015; Oberbeckmann *et al.*, 2016; Kettner *et al.*, 2017; Kettner *et al.,* submitted). The material type and its properties play a lesser but still significant role, especially regarding the diversity (Kettner *et al.*, 2017, Kettner *et al.,* submitted).

Horizontal transport of microplastics – Dispersal of organisms

The fact that microplastics are found in ocean gyres or artic sea ice, which are hundreds to thousands kilometers away from the land-based pollution sources, demonstrate how far floating particles can travel (Obbard *et al.*, 2014; van Sebille *et al.*, 2015). This opens up entirely new possibilities for the transport of plastic-attached organisms. The rafting on plastic can extend the geographical range of certain organisms beyond the active or passive dispersal of their reproductive propagules (Aliani and Molcard, 2003). Spreading of organisms via rafting happens also on natural objects such as wood, seaweed or seeds (Jokiel, 1990). But the crucial difference to natural particles is the higher persistence as well as the widespread and increasing abundance of (micro)plastics in aquatic ecosystems. Barnes (2002) has estimated that human litter more than doubles the rafting opportunities for attached organisms. As we could prove that microplastics-associated communities were significantly distinct to wood-associated communities (Kettner *et al.*, 2017, Kettner *et al.,* submitted), we can also expect differences regarding the kind of organisms transported via natural and artificial rafts, respectively. For example, we observed a strong enrichment of the dinoflagellate *Pfiesteria* on microplastics in comparison to wood and the surrounding water (Kettner *et al.,* submitted). Some *Pfiesteria* strains are able to kill fishes by producing potent toxins or by physically attacking the fish's gills or epidermis (Burkholder and Marshall, 2012). As microplastics have been detected not only in the intestines of fishes but also in their gills (Lu *et al.*, 2016), an enriched occurrence of *Pfiesteria* could likely increase the infection risk. In addition, the bacterial fish pathogen *Aeromonas salmonicida* has been recently identified on microplastics (Viršek *et al.*, 2017). Together with results from Kirstein *et al.* (2016), who detected potentially human pathogenic *Vibrio* spp. on microplastics, we can infer that floating plastic debris with its associated organisms could pose a threat to animals and human beings. Yet, sufficient knowledge for establishing comprehensive risk assessments is lacking (Keswani *et al.*, 2016). First models, however, illustrated that the infection potential in coral reefs increases from 4% to 89% when the corals were in contact with plastics (Lamb *et al.*, 2018). Moreover, several scientists expect that plastic pollution will have a negative impact on the global marine biodiversity by reducing the fitness of animals that ingested or got entangled in plastic debris (Gall and Thompson, 2015) and additionally by facilitating the dispersal of invasive, non-native species (Barnes, 2002;

Moore, 2008; Rech *et al.*, 2016). One might think that the biodiversity could also increase with the transport of microplastics since numerous organisms would arrive to new locations, but this aspect has not been addressed so far. On the other hand, our and others studies revealed that microplastics-associated biofilms were significantly less diverse than communities in the surrounding water (McCormick *et al.*, 2014; Kettner *et al.*, 2017; Kettner *et al.,* submitted), which lowers the chance for microplastics to act as a vector for diversification.

When WWTPs discharge microplastics into rivers and lakes, the resulting microplastics biofilms can harbor typical WWTP-related microorganisms, which possibly include human pathogens and fecal indicator organisms (Keswani *et al.*, 2016). We could show that bacterial assemblages on microplastics became more similar to initial WWTP effluent communities – although they were cultivated in sterile lakewater medium and equally mixed with lakewater communities – when particle concentrations increased (Eckert *et al.*, 2018). Furthermore, the abundance of integrase I increased significantly in microplastics biofilms with rising microplastics concentrations (Eckert *et al.*, 2018). Both points underline that higher plastic pollution results in a stronger impact of WWTP-derived microbes on freshwater communities, since microplastics seem to favor the survival of plastic-attached bacteria, which stem from treated wastewater and carry typical marker genes for anthropogenic pollution (Eckert *et al.*, 2018). The occurrence of integrase I is further linked, e.g., to genes conferring antibiotic resistance (Gillings *et al.*, 2015). Our project partners in study I could further observe on WWTP samples the abundant colonization of microplastics by bacteria that are usually associated with antibiotic resistance (Oberbeckmann *et al.*, 2018). Consequently, results from study I (Oberbeckmann *et al.*, 2018) and study II (Eckert *et al.*, 2018) indicate the risk that microplastics might facilitate the spread of antibiotic resistant bacteria from WWTP effluents. All previous studies just captured a snapshot of the microplastics-associated communities. However, biofilms are highly dynamic systems and the community composition is subjected to constant changes (Costerton *et al.*, 1987). The dynamic nature of biofilms makes it very difficult to predict the survival and spread of invasive, pathogenic, antibiotic resistant or other harmful organisms in aquatic ecosystems through drifting microplastics. Recently, researchers have investigated how microplastics-associated communities shift from a river-site close to and further away from the outlet of a WWTP, and compared them to bacterial assemblages in water and on natural seston (Hoellein *et al.*, 2017). With a higher distance, bacterial communities on microplastics seemed to develop from rather “WWTP-like” towards “stream-like” assemblages (Hoellein *et al.*, 2017). Also microplastics biofilms in ocean gyres are apparently more influenced by the local environment than by their location of origin (Amaral-Zettler *et al.*, 2015). Still, the composition of microplastics-associated communities remained distinct to those on natural substrates and in the surrounding water (Hoellein *et al.*, 2017). Another important

aspect, that needs to be considered when judging the dispersal of organisms via microplastics, is the ability of many prokaryotic and eukaryotic microbes to form resting stages (Ellegaard and Ribeiro, 2018). For example, cysts of *Alexandrium taylori* – a dinoflagellate species that can form harmful blooms – have been frequently observed on floating plastic debris (Masó *et al.*, 2003). Likewise, *Pfiesteria* species are able to produce cyst stages and incidences have been reported, in which cyst germination was linked to bloom initiation (Coyne *et al.*, 2006). While the community composition in microplastics biofilms is likely adapting to changing environmental conditions during the transportation of the particle, certain members of the communities might also form resting stages allowing them to survive longer and to revive again under more favorable conditions. As the dispersal of harmful organisms on highly persistent plastic items can entail serious consequences, future studies should address more specifically how floating debris and the attached biota affects wildlife and human health.

Vertical transport of microplastics – Impact on carbon and nutrient cycles

The abundance of diverse algae, bacteria, ciliates, fungi and tiny animals (Kettner *et al.*, 2017, Kettner *et al.,* submitted; Oberbeckmann *et al.,* 2018) hint at phototrophic and heterotrophic activities in microplastics biofilms. Indeed, plastic items floating in the ocean have been suggested as net autotrophic "hot spots" in oligotrophic (Bryant *et al.*, 2016) and pelagic environments (Pauli *et al.*, 2017). Because of the positive primary production, floating plastic could be a local source of oxygen and act as a carbon sink (Pauli *et al.*, 2017). A dense colonization of microplastics can further increase the sinking velocities of the particles (Kaiser *et al.*, 2017), possibly leading to an export of carbon and nutrients to the sediment. On the other side, rapid defouling of sinking plastics can render them buoyant again (Ye and Andrady, 1991), resulting in a "yo-yo" up and down transport through the water column (Ryan, 2015). Microplastics embedded in algal aggregates (Lagarde *et al.*, 2016) or zooplankton fecal pellets (Cole *et al.*, 2016) can additionally alter sedimentation rates, since their densities can greatly differ from natural aggregates. Yet, it is not understood, whether microplastics have a net positive or net negative effect on organic matter export to sediments.

In addition to the intensity of phototrophic and heterotrophic activities, i.e. oxygen production and respiration, plastics are also influencing the gene expression in the attached organisms (Bryant *et al.*, 2016; Pauli *et al.*, 2017), pointing at distinct metabolic processes. For instance, genes for nitrogen fixation, denitrification, photosynthesis and xenobiotic degradation were significantly more abundant in plastic-associated communities than in picoplankton communities in the surrounding water (Bryant *et al.*, 2016). Taking together changes in quantity and quality of metabolic activities as well as the incorporation into other natural aggregates, microplastics have the potential to alter carbon and nutrient cycling dynamics in aquatic

environments. Especially if the plastic pollution continues to increase, it may even affect important processes such as the ocean carbon pump.

Microplastics biofilms as places for microbial interactions

The simultaneous presence of various prokaryotes and eukaryotes of different trophic levels suggests that there are numerous possibilities for microbial interactions on microplastics biofilms (Kettner *et al.*, 2017, Kettner *et al.,* submitted; Oberbeckmann *et al.,* 2018). In general, biofilms are known as hotspots for interactions such as predation, grazing, competition, parasitism, symbiosis, communication, or cooperation for the joint breakdown of organic matter (Faust and Raes, 2012; Flemming *et al.*, 2016). With our network analyses, we identified several hundred significant positive correlations among prokaryotes, among eukaryotes as well as between prokaryotes and eukaryotes (Kettner *et al.,* submitted), which support the idea of intense and diverse inter-microbial interactions on microplastics. As a general pattern, we observed that networks on microplastics seem to differ from those on wood, since wood-associated networks were dominated by inter-bacterial interactions, whereas on microplastics, bacteria were correlated more often to the kingdoms Alveolata and Chloroplastida (Kettner *et al.,* submitted). Furthermore, the cluster formation within networks indicated that interaction possibilities were strongly defined by the location, which is probably connected to the fact that also the community composition is location-dependent (Kettner *et al.,* submitted). To give an example for a potential interaction, we saw that some bacteria were positively correlated with certain algae (Kettner *et al.,* submitted). These bacteria could have possibly consumed algal exudates (Brock and Clyne, 1984). Further, networks contained several correlations among bacteria and fungi (Kettner *et al.,* submitted), especially in the WWTP, hinting at a potential interaction for the synergistic degradation of organic matter (Romaní *et al.*, 2006). Zettler *et al.* (2013) presented a network of putative hydrocarbon-degrading bacteria, and hypothesized that microbial consortia on microplastics could be able to collectively degrade recalcitrant carbon substrates. Moreover, researchers found an increased abundance of genes involved in xenobiotic degradation on microplastics compared to the surrounding water (Bryant *et al.*, 2016; Debroas *et al.*, 2017; Syranidou *et al.*, 2017). While some interpret this as a hint for a possible bio-degradation of plastics (Zettler *et al.*, 2013; Oberbeckmann *et al.*, 2016; Syranidou *et al.*, 2017), others link it to the presence of dyes or organic pollutants – such as polycyclic aromatic hydrocarbons –, which were adsorbed to the plastics surface (De Tender *et al.*, 2017; Debroas *et al.*, 2017). On microplastics, we also found many fungal and fungal-like organisms, i.e. Rhinosporideacae, which can have parasitic lifestyles, pointing at potential host-parasite interactions (Kettner *et al.*, 2017, Kettner *et al.,* submitted). This assumption could be supported by results of other studies, in which genes were found to be enriched on microplastics that are involved in infection and virulence, such as genes for type IV and type VI

secretion systems (Bryant *et al.*, 2016; Harrison *et al.*, 2018). In addition, viruses might be able to infect prokaryotes or eukaryotes as suggested by Mincer *et al.* (2016), but the presence of viruses in microplastics biofilms has not been investigated yet.

Figure 7 summarizes the most important interaction possibilities of microorganisms on microplastics as well as interactions of a microplastics particle with its biotic and abiotic environment. The illustration is based on our results (Kettner *et al.*, 2017; Kettner *et al.*, submitted; Eckert *et al.*, 2018), on general knowledge about biofilms (Costerton *et al.*, 1987, 1995; Flemming *et al.*, 2016) and microplastics (Andrady, 2011; Mincer *et al.*, 2016; Harrison *et al.*, 2018).

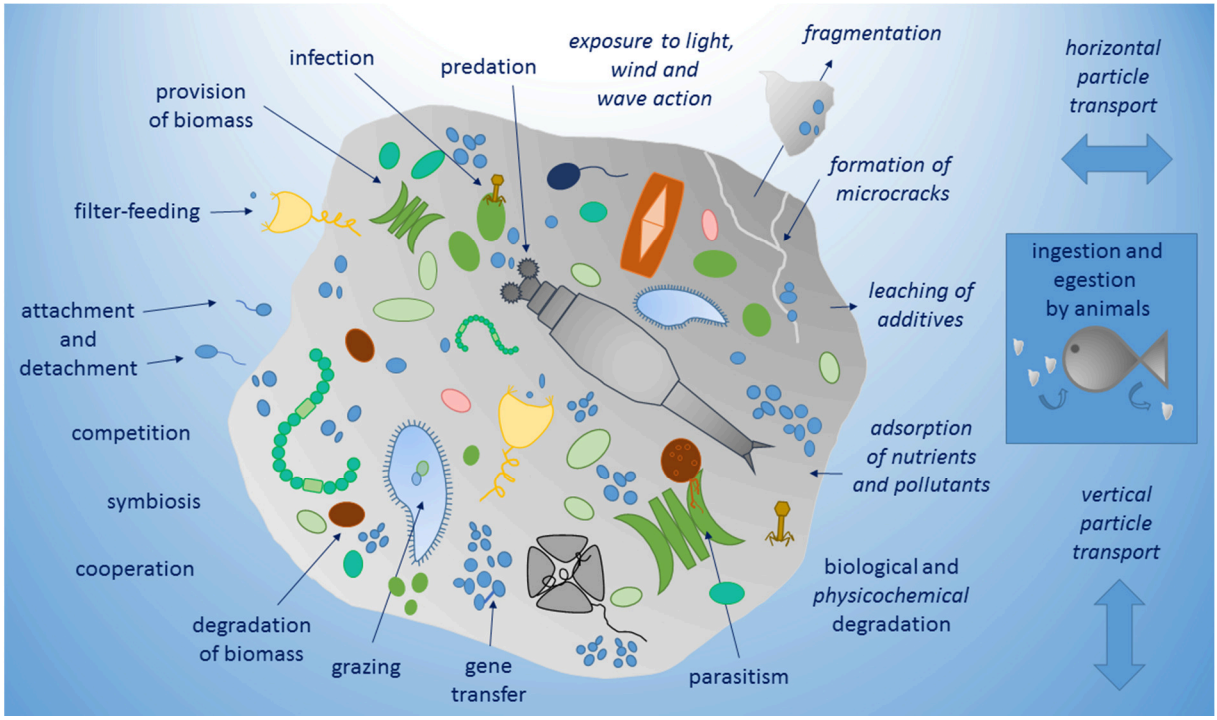

Figure 7. Schematic illustration of a colonized microplastic particle and possible interactions among the attached organisms as well as physicochemical (in italic letters) and biological interactions with the surrounding environment.

Nevertheless, revealing potential microbial interactions on microplastics has just started (Zettler *et al.*, 2013; Debroas *et al.*, 2017; Kettner *et al.*, submitted), and we are still far away from fully understanding them. Compared to the history of life, the presence of microplastics in aquatic environments is a quite recent event, pointing out microplastics as novel habitats. Future research will give new insights into interactions in these small ecosystems.

One highly relevant interaction in biofilms is the exchange of genetic material among microbes (Flemming *et al.*, 2016). In study II, we could observe a significantly increased abundance of integrase I in microplastics biofilms with increasing microplastics concentration (Eckert *et al.*, 2018). Since integrase I is often located on mobile genetic elements (Gillings *et al.*, 2015), it

suggests a possible increase of horizontal gene transfer between bacteria on microplastics. Gene transfer plays a fundamental role in the adaptation, speciation and evolution of prokaryotes (Gogarten *et al.*, 2002). Consequently, microplastics biofilms might act as novel hotspots for microbial evolution. As discussed above, the increased abundance of integrase I could also pose a threat, since integrase I is linked to antibiotic resistance, and the dispersal of microplastics could possibly facilitate the spread of resistant bacteria. Only recently, researchers could confirm that microplastics lead to a significant increase of the horizontal gene transfer of a plasmid encoding for resistance to the antibiotic trimethoprim (Arias-Andres *et al.*, 2018). Bacteria from many different classes were able to acquire the plasmid and transfer rates were up to 100 times higher on microplastics compared to free-living bacterial communities in the surrounding water (Arias-Andres *et al.*, 2018). As the global spread and increase of antibiotic resistances is one of the major challenges of the current century (Kumarasamy *et al.*, 2010; WHO, 2015), the occurrence of resistant microbes on microplastics requires urgently intensified research.

Conclusion and Outlook

Microplastics are not simply another anthropogenic pollutant in aquatic environments. Not only can the chemical composition of plastics interfere with aquatic organisms, but also the provision of new floating surfaces for microbial colonization can have effects in various ways. In comparison to natural surfaces such as wood, feathers or algal aggregates, microplastics are highly persistent – lasting for years or centuries – and can travel around the globe including the attached organisms. Therefore, it is necessary to understand the colonization patterns of microplastics and resulting consequences for aquatic ecosystems. With my dissertation, I could give detailed information about eukaryotic communities on microplastics including diverse interactions possibilities with prokaryotes, highlighting the complexity of microbial life within microplastics biofilms. I could further show that community compositions are strongly dependent on the location and that microplastics-associated communities significantly differ from those in the surrounding water and on the natural surface wood. These results for brackish environments support findings of other studies conducted in freshwater and marine ecosystems. Moreover, the herein observed differences in community compositions, diversities and networks can hint at distinct activities. As microplastics seemingly influence microbial activities in multiple different ways, this topic requires more research to elucidate the ecological functionality within the biofilm and its close surrounding, but also the impacts of colonized plastic particles on element cycling and energy flows on a global scale.

With our studies, we detected for the first time, high abundances of *Pfiesteria*-like dinoflagellates and fungal-like organisms Rhinosporideacae, *Pythium*, *Rhizdiomyces* on microplastics. Those might include toxigenic, parasitic or pathogenic strains. Further, high

concentrations of microplastics seem to favor the survival of wastewater-derived bacteria and possibly enhance genetic exchange as indicated by the increased abundance of integrase I, which is linked to anthropogenic pollution and antibiotic resistance. This points out once more that microplastics biofilms can harbor and possibly transport undesired microorganisms. The complexity of communities on microplastics with several trophic levels makes it very difficult to judge the risk of dispersing harmful species; since on the one hand, they could find suitable microniches for survival or reproduction, but on the other hand, they might be consumed, infected or outcompeted by other organisms of the microplastics biofilm. The research on plastic-related dispersal of pathogenic, antibiotic resistant, invasive or otherwise harmful organisms should be prioritized in the future.

More importantly, we have to ask ourselves, how the world will look like for the future generations, if we do not stop plastic pollution. Will the increase in human population and wealth result in a growing demand for plastic products? Will we move towards a sustainable circular economy and will we minimize environmental pollution or will we continue business as usual? Will floating plastic waste support the formation of harmful algal blooms including cyanobacterial blooms? Could it increase infection potentials as it carries pathogens and antibiotic resistant microbes? Will it cause a decrease in global biodiversity? Many very relevant questions are open, but if no actions are taken, there will be a tipping point when the mass of planktonic plastics could outweigh the biomass of phyto- and zooplankton or fishes. Most likely, this will have adverse effects on whole food-web dynamics. In particular, the evolution – especially of “higher” organisms – might be too slow to cope with the rapidly increasing plastic pollution.

For sure, the everyday life of human beings is more convenient with the huge variety of plastic products. The resulting pollution is the price that not only we pay, but also many other organisms on this planet. In the light of the potential harmful effects of microplastics pollution on humankind and wildlife, policy-makers, society and industries have to find urgently effective ways to minimize the current environmental contamination with plastic.

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Appendix

Supplementary Information for Manuscript I

The eukaryotic life on microplastics in brackish ecosystems

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Table S1. Lists of top 20 eukaryotic taxa on polyethylene (PE), polystyrene (PS), wood, and both water size fractions (water > 0.2 µm and water > 3.0 µm). Taxa are ranked by their read counts. Proportion of taxon reads to total reads (of the according substrate) are given in percent.

PE	reads	percent	PS	reads	percent	wood	reads	percent
Pfiesteria	117566	14.5%	unclassified Rhinosporideacae	114099	13.8%	Ephelota	45773	9.5%
unclassified Peritrichia II	116312	14.3%	Ulva	106131	12.8%	unclassified ConThreeP	31940	6.6%
Ulva	85737	10.6%	Pfiesteria	98750	11.9%	unclassified Peritrichia II	26399	5.5%
unclassified Adinetida	55483	6.8%	unclassified Adinetida	93331	11.3%	unclassified Diplogasterida	26133	5.4%
unclassified Rhinosporideacae	51142	6.3%	unclassified Peritrichia II	42753	5.2%	Pfiesteria	25825	5.3%
Ephelota	26003	3.2%	unclassified Haplotaxida	30754	3.7%	unclassified LKM11	24621	5.1%
unclassified ConThreeP	21229	2.6%	unclassified Diplogasterida	27636	3.3%	unclassified Adinetida	22996	4.8%
Zoothamnium	20661	2.5%	unclassified ConThreeP	27516	3.3%	unclassified Bilateria	18389	3.8%
unclassified Rhabditida	20114	2.5%	Ephelota	19009	2.3%	unclassified Haplotaxida	17974	3.7%
unclassified Bilateria	18713	2.3%	Zoothamnium	17836	2.2%	unclassified Trebouxiophyceae II	15767	3.3%
unclassified Diplogasterida	18590	2.3%	Rhizidiomyces	16204	2.0%	Rhogostoma	15264	3.2%
unclassified Ploimida	16176	2.0%	unclassified Podocopida	15004	1.8%	unclassified Rhinosporideacae	12944	2.7%
unclassified Haplotaxida	14599	1.8%	unclassified Bilateria	14559	1.8%	Ulva	9708	2.0%
Pythium	14242	1.8%	unclassified Ploimida	10690	1.3%	unclassified Stramenopiles	8755	1.8%
unclassified Caenogastropoda	14203	1.8%	unclassified Myoida	8171	1.0%	unclassified Podocopida	7492	1.6%
Chytridium	10650	1.3%	unclassified Trebouxiophyceae II	7115	0.9%	unclassified Rhabditida	6974	1.4%
unclassified Fungi II	10463	1.3%	Rhogostoma	7032	0.8%	unclassified Perkinsidae II	6953	1.4%
unclassified Trebouxiophyceae II	9841	1.2%	Chytridium	6499	0.8%	unclassified Intramacronucleata	6536	1.4%
unclassified Chytridiomycota II	8637	1.1%	unclassified Bacillariophyceae	6122	0.7%	unclassified Alveolata	5788	1.2%
uncultured Choreotrichia	7109	0.9%	unclassified LKM11	5456	0.7%	unclassified Trebouxiophyceae	5497	1.1%
sum of top 20	**657470**	**81.1%**	**sum of top 20**	**674667**	**81.4%**	**sum of top 20**	**341728**	**70.7%**
total	810599	100.0%	total	829213	100.0%	total	483071	100.0%

water > 0.2 µm	reads	percent	water > 3.0 µm	reads	percent
Ostreococcus	156615	22.4%	unclassified ConThreeP	68193	8.1%
unclassified Trebouxiophyceae II	43472	6.2%	unclassified Copepoda	63372	7.5%
Micromonas	35637	5.1%	Neoceratium	62110	7.3%
unclassified Trebouxiophyceae	30333	4.3%	unclassified Diplogasterida	60845	7.2%
unclassified MAST-6	25435	3.6%	unclassified Peritrichia II	51969	6.1%
unclassified Syndiniales III	18883	2.7%	unclassified Cercozoa	39179	4.6%
unclassified Spongillida	17241	2.5%	unclassified Rhabditida	36147	4.3%
Ulva	16163	2.3%	Skeletonema	32120	3.8%
Leucocryptos	14657	2.1%	unclassified Adinetida	27094	3.2%
Teleaulax	14535	2.1%	Ostreococcus	25587	3.0%
unclassified Cercozoa	14012	2.0%	Rhogostoma	22011	2.6%
Amoebophrya	13939	2.0%	unclassified Intramacronucleata	20140	2.4%
unclassified Stramenopiles	12412	1.8%	unclassified Trebouxiophyceae II	16843	2.0%
FV18-2G7	12339	1.8%	unclassified Cyclopoida	13512	1.6%
Ochromonas	12197	1.7%	Thalassiosira	11675	1.4%
unclassified Bilateria	11904	1.7%	unclassified Sphaeropleales	9840	1.2%
Picomonas	10058	1.4%	unclassified Bilateria	8672	1.0%
unclassified Haptoria	9886	1.4%	unclassified Ploimida	8657	1.0%
unclassified ConThreeP	9341	1.3%	Scenedesmus	8343	1.0%
Pseudopedinella	9222	1.3%	unclassified Calanoida	7839	0.9%
sum of top 20	**488281**	**69.9%**	**sum of top 20**	**594148**	**70.3%**
total	698248	100.0%	total	845688	100.0%

Table S2. Results of permutational multivariate analysis of variance (PERMANOVA) after 999 permutations for the factors substrate and location as well as their interaction term. df = degrees of freedom, Sq = squares

PERMANOVA (999 permutations)						
	df	sum Sq	mean Sq	F-Model	**R^2**	**p-value**
substrate	4	3.614	0.903	16.715	**0.140**	**0.001**
location	6	12.030	2.005	37.095	**0.467**	**0.001**
substrate:location	24	6.876	0.286	5.301	**0.267**	**0.001**
Residuals	60	3.243	0.054		0.126	
Total	94	25.763			100.0	

Table S3. Results of permutation tests for homogeneity of multivariate dispersions for different substrate types and locations. P-values over 0.05 indicate a homogenous dispersion. df = degrees of freedom, Sq = squares

Dispersion levels for substrates types					
Average distance to median:					
PE	PS	wood	water > 0.2 µm	water > 3.0 µm	
0.490	0.498	0.478	0.448	0.462	
Permutation test for homogeneity of multivariate dispersions (999 permutations):					
	df	sum Sq	mean Sq	F-Model	**p-value**
Groups	4	0.032	0.008	0.961	0.454
Residuals	90	0.749	0.008		
Dispersion levels for locations					
Average distance to median:					
Station 1	Station 2	Station 3	Station 4	Station 5	Station 6	Station 7
0.407	0.365	0.415	0.377	0.383	0.324	0.311
Permutation test for homogeneity of multivariate dispersions (999 permutations):					
	df	sum Sq	mean Sq	F-Model	**p-value**
Groups	6	0.131	0.022	1.858	0.110
Residuals	88	1.036	0.012		

Table S4. Results of pairwise PERMANOVA (999 permutations) and Bray-Curtis (BC) similarity for different substrate types and locations. P-adjustment according to Benjamini and Hochberg, 1995.

Pairwise PERMANOVA for different substrate types					
pairs	**F-Model**	**R^2**	**p-value**	**p adjusted**	**BC similarity**
PE vs PS	0.418	0.010	0.942	0.942	78.7%
PE vs wood	2.157	0.051	0.028	**0.035**	68.1%
PE vs water>0.2µm	6.455	0.145	0.001	**0.003**	42.1%
PE vs water>3.0µm	3.080	0.088	0.006	**0.012**	54.8%
PS vs wood	2.185	0.052	0.035	**0.039**	69.5%
PS vs water>0.2µm	6.764	0.151	0.001	**0.003**	41.8%
PS vs water>3.0µm	3.506	0.099	0.004	**0.010**	53.7%
wood vs water>0.2µm	6.107	0.138	0.001	**0.003**	46.2%
wood vs water>3.0µm	2.728	0.079	0.015	**0.021**	59.4%
water>0.2µm vs water>3.0µm	3.828	0.113	0.013	**0.021**	56.2%
Pairwise PERMANOVA for different locations					
Station 1 vs Station 2	5.895	0.197	0.001	**0.001**	54.3%
Station 1 vs Station 3	4.422	0.156	0.001	**0.001**	56.4%
Station 1 vs Station 4	7.936	0.249	0.001	**0.001**	46.0%
Station 1 vs Station 5	9.547	0.285	0.001	**0.001**	43.0%
Station 1 vs Station 6	20.657	0.443	0.001	**0.001**	20.7%
Station 1 vs Station 7	19.091	0.423	0.001	**0.001**	24.7%
Station 2 vs Station 3	3.693	0.133	0.005	**0.005**	65.5%
Station 2 vs Station 4	7.866	0.247	0.001	**0.001**	48.8%
Station 2 vs Station 5	10.631	0.307	0.001	**0.001**	43.3%
Station 2 vs Station 6	21.691	0.455	0.001	**0.001**	24.8%
Station 2 vs Station 7	21.345	0.451	0.001	**0.001**	28.0%
Station 3 vs Station 4	8.106	0.252	0.001	**0.001**	42.1%
Station 3 vs Station 5	11.190	0.318	0.001	**0.001**	39.4%
Station 3 vs Station 6	18.635	0.418	0.001	**0.001**	24.6%
Station 3 vs Station 7	18.012	0.409	0.001	**0.001**	25.7%
Station 4 vs Station 5	4.360	0.154	0.003	**0.003**	70.4%
Station 4 vs Station 6	21.961	0.458	0.001	**0.001**	27.3%
Station 4 vs Station 7	23.208	0.472	0.001	**0.001**	27.9%
Station 5 vs Station 6	18.550	0.416	0.001	**0.001**	29.2%
Station 5 vs Station 7	19.253	0.425	0.001	**0.001**	30.3%
Station 6 vs Station 7	6.198	0.181	0.001	**0.001**	66.1%

Table S5. Results of indicator species analysis with taxa associated to each substrate type. For each taxon the corresponding phylum, kingdom, indicator value and adjusted p-value (according to Benjamini and Hochberg, 1995) are given. Taxonomic information is based on SILVA (Yilmaz *et al.*, 2014) database v128.

Eukaryotic taxa associated to substrate types				
taxon	**phylum**	**kingdom**	**indicator value**	**p-value adjusted**
PE (polyethylene)				
unclassified Monogononta	Rotifera	Metazoa (Animalia)	0.549	0.033
PS (polystyrene)				
unclassified Ulvophyceae II	Chlorophyta (unclassified phylum)	Chloroplastida	0.547	0.025
wood				
Candida	Ascomycota	Fungi	0.843	0.005
Ogataea	Ascomycota	Fungi	0.787	0.005
Kuraishia	Ascomycota	Fungi	0.724	0.005
unclassified Saccharomycetales III	Ascomycota	Fungi	0.669	0.005
unclassified Cyrtophoria	Ciliophora	Alveolata	0.630	0.007
Pseudocohnilembus	Ciliophora	Alveolata	0.618	0.005
Wickerhamomyces	Ascomycota	Fungi	0.588	0.007
Choricystis	Chlorophyta (unclassified phylum)	Chloroplastida	0.585	0.019
Saccharomycopsis	Ascomycota	Fungi	0.577	0.005
Pichia	Ascomycota	Fungi	0.558	0.010
Clavispora	Ascomycota	Fungi	0.524	0.017
unclassified Cystofilobasidiaceae II	Basidiomycota	Fungi	0.522	0.010
Condylostoma	Ciliophora	Alveolata	0.470	0.050
water > 0.2 µm				
Ochromonas	Ochrophyta	Stramenopiles	0.797	0.005
E222	Ochrophyta	Stramenopiles	0.786	0.005
Paraphysomonas	Ochrophyta	Stramenopiles	0.779	0.005
unclassified MAST-4D	MAST-4	Stramenopiles	0.764	0.005

unclassified Pedinellales II	Ochrophyta	Stramenopiles	0.750	0.005
Bolidomonas	Ochrophyta	Stramenopiles	0.746	0.005
unclassified DH147-EKD10	DH147-EKD10	Eukaryota (unclassified kingdom)	0.745	0.005
unclassified Chrysophyceae	Ochrophyta	Stramenopiles	0.734	0.005
unclassified MAST-2	MAST-2	Stramenopiles	0.723	0.005
unclassified Mamiellophyceae	Chlorophyta (unclassified phylum)	Chloroplastida	0.711	0.005
Chrysochromulina	Prymnesiophyceae	Haptophyta	0.708	0.005
Picomonas	Picozoa	Eukaryota (unclassified kingdom)	0.707	0.005
Mamiella	Chlorophyta (unclassified phylum)	Chloroplastida	0.705	0.005
Micromonas	Chlorophyta (unclassified phylum)	Chloroplastida	0.704	0.005
unclassified Cryptomonadales	Cryptomonadales	Cryptophyceae	0.704	0.005
Ostreococcus	Chlorophyta (unclassified phylum)	Chloroplastida	0.704	0.005
Geminigera	Cryptomonadales	Cryptophyceae	0.701	0.005
Paragymnodinium	Dinoflagellata	Alveolata	0.697	0.005
unclassified Prymnesiales	Prymnesiophyceae	Haptophyta	0.696	0.005
Hemiselmis	Cryptomonadales	Cryptophyceae	0.693	0.005
unclassified Mamiellales	Chlorophyta (unclassified phylum)	Chloroplastida	0.681	0.005
unclassified Syndiniales Group I	Protalveolata	Alveolata	0.679	0.005
unclassified Chromulinales	Ochrophyta	Stramenopiles	0.677	0.005
Teleaulax	Cryptomonadales	Cryptophyceae	0.668	0.005
uncultured Cryptomonadales	Cryptomonadales	Cryptophyceae	0.668	0.005
Spumella	Ochrophyta	Stramenopiles	0.665	0.005
unclassified LG21-05	Ochrophyta	Stramenopiles	0.664	0.005
unclassified Chloroplastida	Chloroplastida (unclassified phylum)	Chloroplastida	0.653	0.005
unclassified A31	Protalveolata	Alveolata	0.650	0.005
unclassified Dictyochophyceae	Ochrophyta	Stramenopiles	0.650	0.005
Amoebophrya	Protalveolata	Alveolata	0.650	0.007
unclassified Syndiniales III	Protalveolata	Alveolata	0.645	0.007
Leucocryptos	Kathablepharidae	Cryptophyceae	0.641	0.005
unclassified Spongillida	Porifera	Metazoa (Animalia)	0.641	0.005
uncultured Oligotrichia	Ciliophora	Alveolata	0.639	0.007

FV18-2G7	Cryptomonadales	Cryptophyceae	0.636	0.005
Prasinoderma	Chlorophyta (unclassified phylum)	Chloroplastida	0.632	0.005
Tiarina	Ciliophora	Alveolata	0.623	0.005
Uroglena	Ochrophyta	Stramenopiles	0.621	0.005
unclassified Cryptomonadales II	Cryptomonadales	Cryptophyceae	0.618	0.005
unclassified MAST-6	MAST-6	Stramenopiles	0.618	0.007
unclassified OLI11255	OLI11255	Alveolata	0.614	0.007
unclassified Exobasidiales	Basidiomycota	Fungi	0.609	0.005
unclassified Anthoathecata	Cnidaria	Metazoa (Animalia)	0.605	0.010
Chaetoceros	Ochrophyta	Stramenopiles	0.602	0.010
Pseudopedinella	Ochrophyta	Stramenopiles	0.600	0.007
unclassified Chlorophyta	Chlorophyta (unclassified phylum)	Chloroplastida	0.598	0.005
Gyrodinium	Dinoflagellata	Alveolata	0.591	0.007
unclassified Choreotrichia	Ciliophora	Alveolata	0.588	0.007
Diaphanoeca	Choanoflagellida	Holozoa (unclassified kingdom)	0.579	0.005
unclassified MAST-3F	MAST-3	Stramenopiles	0.577	0.007
unclassified MAST-3E	MAST-3	Stramenopiles	0.576	0.007
unclassified Pedinellales	Ochrophyta	Stramenopiles	0.574	0.013
Prymnesium	Prymnesiophyceae	Haptophyta	0.568	0.010
unclassified Ctenostomatida	Bryozoa	Metazoa (Animalia)	0.559	0.015
Pseudochattonella	Ochrophyta	Stramenopiles	0.556	0.005
unclassified MAST-12A	MAST-12	Stramenopiles	0.553	0.005
unclassified Oligotrichia	Ciliophora	Alveolata	0.552	0.027
unclassified Hydrozoa	Cnidaria	Metazoa (Animalia)	0.549	0.010
Vermamoeba	Tubulinea	Amoebozoa	0.546	0.007
SS1-E01-69	Ochrophyta	Stramenopiles	0.543	0.005
Pirsonia	Cercozoa	Rhizaria	0.524	0.038
Pycnococcus	Chlorophyta (unclassified phylum)	Chloroplastida	0.523	0.005
unclassified P34-45	Ochrophyta	Stramenopiles	0.519	0.015
unclassified Sarcinochrysidales	Ochrophyta	Stramenopiles	0.518	0.005
Imantonia	Prymnesiophyceae	Haptophyta	0.513	0.005

unclassified Phyllodocida	Annelida	Metazoa (Animalia)	0.505	0.017
unclassified LG01-09	Ochrophyta	Stramenopiles	0.494	0.019
OLI16029	Prymnesiophyceae	Haptophyta	0.482	0.007
unclassified Prasinophytae II	Chlorophyta (unclassified phylum)	Chloroplastida	0.466	0.044
Poterioochromonas	Ochrophyta	Stramenopiles	0.463	0.023
Gymnophrys	Cercozoa	Rhizaria	0.437	0.048
Phaeocystis	Prymnesiophyceae	Haptophyta	0.424	0.015
unclassified Syndiniales Group III	Protalveolata	Alveolata	0.423	0.019
Sarcinochrysis	Ochrophyta	Stramenopiles	0.402	0.044
Dictyocha	Ochrophyta	Stramenopiles	0.354	0.038
water > 3.0 µm				
Trachydiscus	Ochrophyta	Stramenopiles	0.743	0.005
Aphanomyces	Peronosporomycetes	Stramenopiles	0.728	0.005
Pteromonas	Chlorophyta (unclassified phylum)	Chloroplastida	0.728	0.005
unclassified Conoidasida	Apicomplexa	Alveolata	0.714	0.005
Hydrodictyon	Chlorophyta (unclassified phylum)	Chloroplastida	0.695	0.005
Discostella	Ochrophyta	Stramenopiles	0.685	0.005
Oocystis	Chlorophyta (unclassified phylum)	Chloroplastida	0.672	0.005
unclassified Mediophyceae	Ochrophyta	Stramenopiles	0.669	0.005
unclassified Sphaeropleales	Chlorophyta (unclassified phylum)	Chloroplastida	0.659	0.005
unclassified Cyclopoida	Arthropoda	Metazoa (Animalia)	0.652	0.007
Monodus	Ochrophyta	Stramenopiles	0.648	0.005
Symbiodinium	Dinoflagellata	Alveolata	0.643	0.005
Cyclotella	Ochrophyta	Stramenopiles	0.642	0.005
Thalassiosira	Ochrophyta	Stramenopiles	0.638	0.029
uncultured Rhizophydiales	Chytridiomycota	Fungi	0.624	0.005
unclassified Ochrophyta	Ochrophyta	Stramenopiles	0.622	0.015
Desmodesmus	Chlorophyta (unclassified phylum)	Chloroplastida	0.619	0.005
Scenedesmus	Chlorophyta (unclassified phylum)	Chloroplastida	0.615	0.005
unclassified Pezizomycotina II	Ascomycota	Fungi	0.614	0.005
Marvania	Chlorophyta (unclassified phylum)	Chloroplastida	0.611	0.005

Rhogostoma	Cercozoa	Rhizaria	0.610	0.017
unclassified Eustigmatales II	Ochrophyta	Stramenopiles	0.609	0.005
unclassified Eustigmatales	Ochrophyta	Stramenopiles	0.603	0.017
unclassified Chlorophyceae	Chlorophyta (unclassified phylum)	Chloroplastida	0.603	0.005
unclassified Chlorellales	Chlorophyta (unclassified phylum)	Chloroplastida	0.599	0.010
Volvox	Chlorophyta (unclassified phylum)	Chloroplastida	0.597	0.005
unclassified Chlamydomonadales	Chlorophyta (unclassified phylum)	Chloroplastida	0.589	0.023
Micractinium	Chlorophyta (unclassified phylum)	Chloroplastida	0.586	0.005
unclassified Peronosporomycetes II	Peronosporomycetes	Stramenopiles	0.582	0.010
Golenkinia	Chlorophyta (unclassified phylum)	Chloroplastida	0.575	0.007
Monoraphidium	Chlorophyta (unclassified phylum)	Chloroplastida	0.575	0.021
unclassified Chlorophycea	Chlorophyta (unclassified phylum)	Chloroplastida	0.574	0.013
unclassified Arthropoda	Arthropoda	Metazoa (Animalia)	0.570	0.005
Tetracystis	Chlorophyta (unclassified phylum)	Chloroplastida	0.570	0.007
Stephanodiscus	Ochrophyta	Stramenopiles	0.569	0.013
Synedra	Ochrophyta	Stramenopiles	0.568	0.007
Polytoma	Chlorophyta (unclassified phylum)	Chloroplastida	0.567	0.017
Aulacoseira	Ochrophyta	Stramenopiles	0.559	0.007
unclassified Rhabditida	Nematoda	Metazoa (Animalia)	0.557	0.013
unclassified Chromadorida	Nematoda	Metazoa (Animalia)	0.556	0.005
Ophryocystis	Apicomplexa	Alveolata	0.556	0.005
unclassified Limnomedusae	Cnidaria	Metazoa (Animalia)	0.546	0.029
unclassified Monhysterida	Nematoda	Metazoa (Animalia)	0.545	0.019
Mychonastes	Chlorophyta (unclassified phylum)	Chloroplastida	0.544	0.048
Nannochloropsis	Ochrophyta	Stramenopiles	0.542	0.015
Chlorococcum	Chlorophyta (unclassified phylum)	Chloroplastida	0.541	0.010
unclassified Chytridiales	Chytridiomycota	Fungi	0.539	0.015
Saprolegnia	Peronosporomycetes	Stramenopiles	0.539	0.023
Blastocystis	Incertae Sedis	Stramenopiles	0.538	0.007
Synura	Ochrophyta	Stramenopiles	0.537	0.010
Stichococcus	Chlorophyta (unclassified phylum)	Chloroplastida	0.536	0.017

unclassified Hypocreales II	Ascomycota	Fungi	0.530	0.029
unclassified Calanoida	Arthropoda	Metazoa (Animalia)	0.530	0.040
Chlamydomonas	Chlorophyta (unclassified phylum)	Chloroplastida	0.522	0.021
unclassified Chromadorea	Nematoda	Metazoa (Animalia)	0.515	0.019
Fragilaria	Ochrophyta	Stramenopiles	0.514	0.015
Debaryomyces	Ascomycota	Fungi	0.509	0.029
unclassified Nematoda	Nematoda	Metazoa (Animalia)	0.506	0.015
Eustigmatos	Ochrophyta	Stramenopiles	0.505	0.010
Gomphonema	Ochrophyta	Stramenopiles	0.501	0.027
unclassified Chytridiaceae II	Chytridiomycota	Fungi	0.477	0.019
Carteria	Chlorophyta (unclassified phylum)	Chloroplastida	0.463	0.025
Cryptomonas	Cryptomonadales	Cryptophyceae	0.453	0.015
unclassified Mortierellales	Incertae Sedis	Fungi	0.449	0.023

Table S6. *Pfiesteria* read counts and percentages from different substrates and locations.

substrate	reads	percent	location	reads	percent
PE	117566	47.9%	**Station 1**	54	0.0%
PS	98750	40.2%	**Station 2**	462	0.2%
wood	25825	10.5%	**Station 3**	83	0.0%
water > 0.2 µm	681	0.3%	**Station 4**	41794	17.0%
water > 3.0 µm	2629	1.1%	**Station 5**	203051	82.7%
			Station 6	6	0.0%
			Station 7	1	0.0%
sum	245451	100.0%	sum	245451	100.0%

Table S7. *Pfiesteria* read counts from the substrate replicates at station 4 and 5.

substrate	station 4 replicate a	station 4 replicate b	station 4 replicate c	station 5 replicate a	station 5 replicate b	station 5 replicate c
PE	2149	1990	4606	39234	22490	46730
PS	12684	10921	6041	37222	30381	1337
wood	1209	1460	99	13466	5925	3629
water > 0.2 µm	4	515	NA	36	96	NA
water > 3.0 µm	66	50	NA	2338	167	NA

Table S8. Top 50 hits from NCBI's Web BLAST service (BLASTN 2.6.1, default settings, (Zhang *et al.*, 2000; Morgulis *et al.*, 2008)) of the most abundant sequence that was assigned to the genus *Pfiesteria*.

Description	Max score	Total score	Query cover	E value	Identity	Accession number
Pfiesteriaceae sp. *masanensis* isolate VIMS 1050 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	688	688	100%	0.0	99%	EU048553.1
Pfiesteriaceae sp. *masanensis* isolate VIMS 1041 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	688	688	100%	0.0	99%	EU048552.1
Dinophyceae sp. Lucy-3 18S small subunit ribosomal RNA gene, complete sequence	688	688	100%	0.0	99%	AY251289.1
Pfiesteria-like dinoflagellate 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain Jeju Lucy-200504	688	688	100%	0.0	99%	AM050345.1
Pfiesteria-like dinoflagellate 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain Masan Lucy-200505	688	688	100%	0.0	99%	AM050344.1
Pfiesteria-like sp. clone POC-8 small subunit ribosomal RNA gene, complete sequence	688	688	100%	0.0	99%	AY121856.1
Pfiesteria-like dinoflagellate 18S small subunit ribosomal RNA gene, partial sequence	688	688	100%	0.0	99%	AY033487.1
Pfiesteria-like sp. HR1NovC5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	688	688	100%	0.0	99%	AY590482.1
Pfiesteria-like sp. CCMP1835 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	688	688	100%	0.0	99%	AY590477.1
Uncultured eukaryote clone WS073.008 18S ribosomal RNA gene, partial sequence	682	682	100%	0.0	99%	KP404756.1
Stoeckeria sp. SSSC09 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, strain SSSC09	682	682	100%	0.0	99%	HG005132.1

Uncultured eukaryote clone KRL09E10 18S ribosomal RNA gene, partial sequence	682	682	100%	0.0	99%	KC315836.1
Stoeckeria sp. SSMS0806 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain SSMS0806	682	682	100%	0.0	99%	FN557541.1
Uncultured dinoflagellate clone ssu14 18S ribosomal RNA gene, partial sequence	682	682	100%	0.0	99%	AY628342.1
Dinophyceae sp. Shepard's Crook 18S small subunit ribosomal RNA gene, complete sequence	682	682	100%	0.0	99%	AY251291.1
Dinophyceae sp. Shepherd's Crook small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	682	682	100%	0.0	99%	AY590479.1
Pfiesteria-like sp. HR1SSeptA5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	682	682	100%	0.0	99%	AY590483.1
Pfiesteria-like sp. NC Lucy-V27 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	680	680	100%	0.0	99%	AY590485.1
Uncultured eukaryote clone 282A09 small subunit ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	KJ925512.1
Uncultured marine eukaryote clone I-9-MC884-OTU-47 18S ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	KC771146.1
Uncultured eukaryote clone AI5F14RJ2E07 18S ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	GU824700.1
Uncultured eukaryote clone AI3F14RJ1C03 18S ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	GU824602.1
Uncultured eukaryote clone AI5F14RJ1G07 18S ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	GU824184.1
Pfiesteria piscicida clone Ppi+Rhod_cDNA221 18S ribosomal RNA, partial sequence	676	676	100%	0.0	99%	FJ600090.1
Uncultured alveolate partial 18S rRNA gene, clone 4-H5	676	676	100%	0.0	99%	FN690233.1
Pfiesteria piscicida strain PPSB27 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	DQ991382.1

Pfiesteria piscicida 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene (partial), ITS1 and ITS2, strain PPMS0507	676	676	100%	0.0	99%	AM231033.1
Pfiesteria piscicida 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene (partial), ITS1 and ITS2, strain PPBS0507	676	676	100%	0.0	99%	AM231028.1
Paulsenella vonstoschii 18S rRNA gene	676	676	100%	0.0	99%	AJ968729.1
Uncultured dinoflagellate clone ssu34 18S ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	AY628348.1
Pfiesteria piscicida small subunit ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	AY112746.1
Pfiesteria piscicida isolate NCSU B-125-4(12/9) 18S small subunit ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 23S large subunit ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	AF330600.1
Pfiesteria piscicida 18S small subunit ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	AY033488.1
Pfiesteria piscicida small subunit ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	AY245693.1
Pfiesteria piscicida small subunit ribosomal RNA gene, partial sequence	676	676	100%	0.0	99%	AF149793.1
Pfiesteria piscicida small subunit ribosomal RNA gene, complete sequence	676	676	100%	0.0	99%	AF077055.1
Uncultured eukaryote clone 268D02 small subunit ribosomal RNA gene, partial sequence	675	675	100%	0.0	99%	KJ925188.1
Uncultured eukaryote clone KRL03E38 18S ribosomal RNA gene, partial sequence	675	675	100%	0.0	99%	KC315816.1
Uncultured dinoflagellate clone SSU1_M0 18S ribosomal RNA gene, partial sequence	675	675	100%	0.0	99%	AY628349.1

Table S9. Primer specificity tested with Silva TestPrime1.0 (Klindworth et al., 2013). Forward primer: Eu565F CCAGCASCYGCGGTAATTCC, reverse primer: Eu981R ACTTTCGTTCTTGATYRATGA (Stoeck *et al.*, 2010; with addition of the bases -TGA by LGC Genomics, Berlin, Germany). List of taxa and their according coverage in the Silva Taxonomy browser (database v128). Coverage is given in percent allowing zero and one mismatch.

Taxon	coverage allowing zero mismatch	coverage allowing one mismatch	in our data-set
Eukaryota	55.9%	77.4%	yes
Chloroplastida	79.9%	86.9%	yes
Rhodophyceae	88.6%	100.0%	yes
Glaucophyta	50.0%	94.3%	no
Cryptophyceae	85.7%	96.0%	yes
Amoebozoa	56.3%	77.5%	yes
Haptophyta	0.9%	94.6%	yes
Incertae Sedis kingdom			
phylum Ancyromonadida	100.0%	100.0%	yes
unclassified kingdom in Holozoa			
phylum Choanoflagellida	86.0%	95.2%	yes
phylum Ichthyosporea	21.7%	88.3%	yes
Metazoa (Animalia)	62.1%	81.3%	yes
Excavata	2.8%	13.2%	no
Centrohelida	94.7%	95.8%	no
Discicristoidea	60.4%	89.6%	yes
Fungi	19.2%	59.1%	yes
Stramenopiles	87.8%	93.7%	yes
Alveolata	73.4%	88.0%	yes
Rhizaria	0.2%	53.5%	yes
unclassified kingdom in Eukaryota			
phylum Picozoa	89.5%	92.1%	yes
phylum DH147-EKD10	72.7%	90.9%	yes
phylum SA1-3C06	0.0%	100.0%	yes
Bacteria	0.0%	0.0%	no
Archaea	0.0%	0.0%	no

Fig. S1. Co-occurrence networks for PE (A, B), PS (C, D) and wood (E, F) for both incubation experiments I (Baltic Sea to River Warnow, stations 1 to 5) and II (WWTP, stations 6 and 7). Networks were calculated in Cytoscape 3.5.1 (Shannon *et al.*, 2003) with the CoNet app version 1.1.1. beta (Faust and Raes, 2016). Nodes are labelled with taxon name and relative node sizes correspond to number of neighbor nodes. Colors indicate different kingdoms.

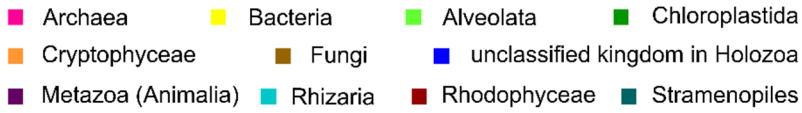


Fig. S1 A) PE co-occurrence network (stations 1 to 5)

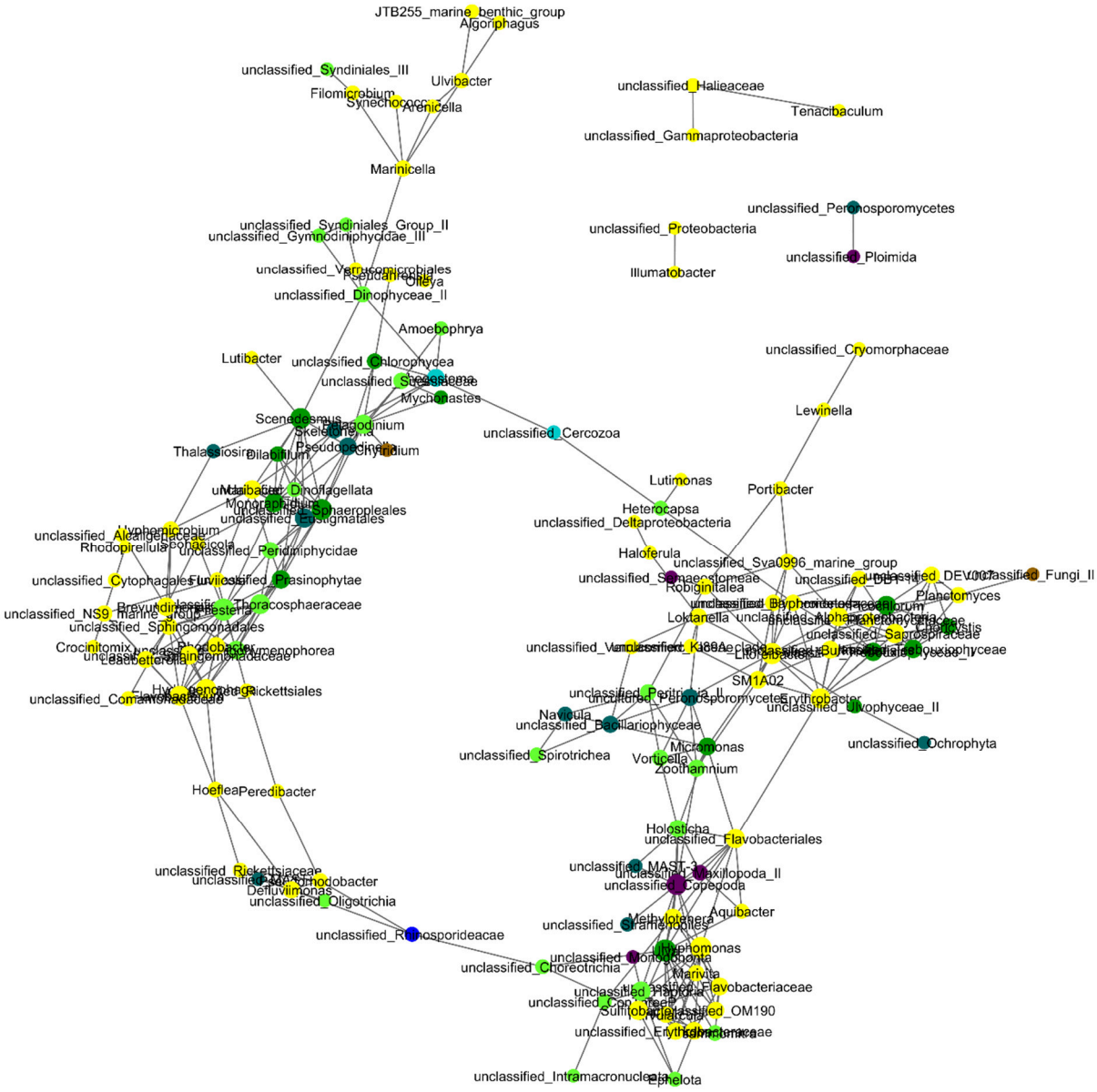


Fig. S1 B) PE co-occurrence network (stations 6 and 7)

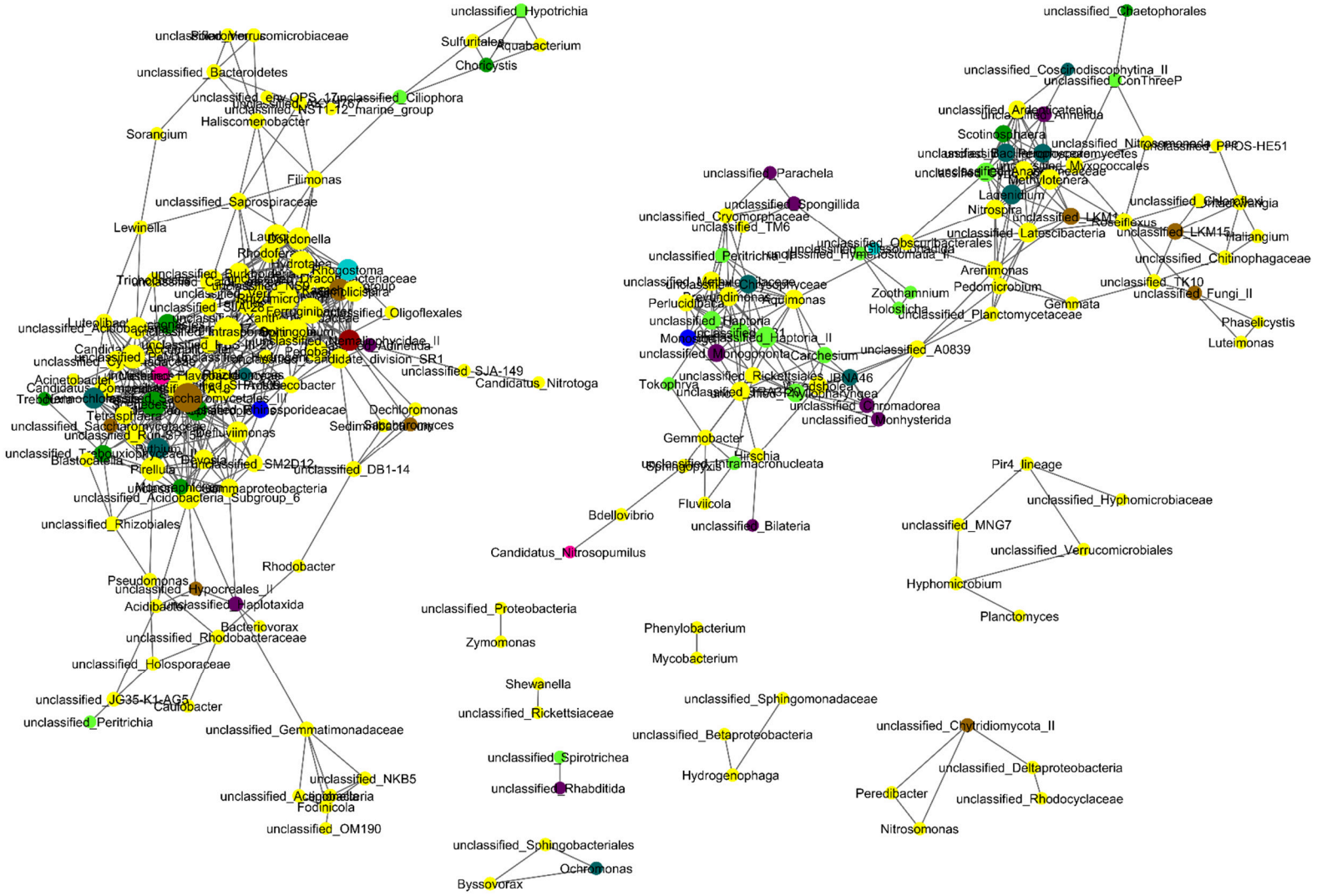


Fig. S1 C) PS co-occurrence network (stations 1 to 5)

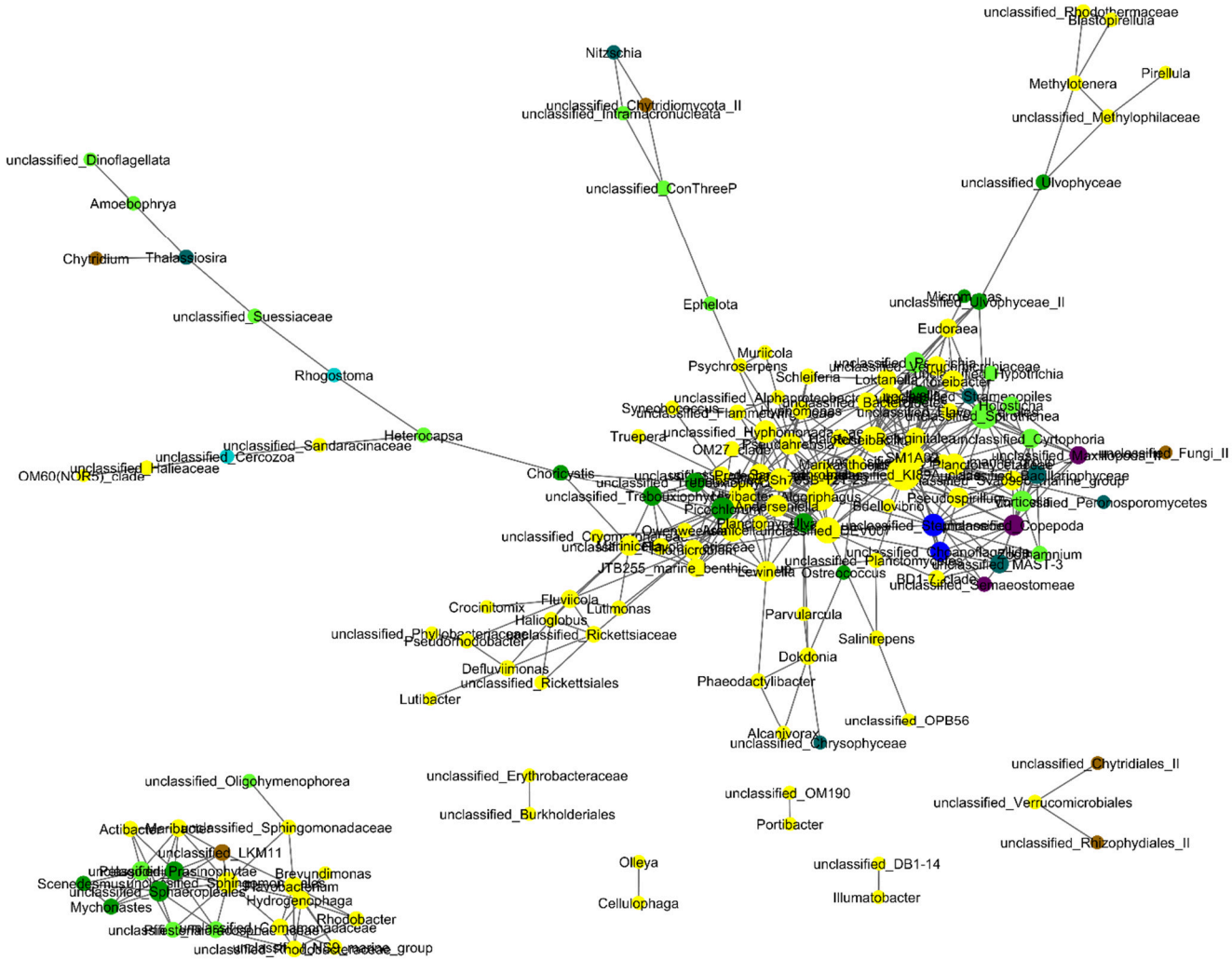


Fig. S1 D) PS co-occurrence network (stations 6 and 7)

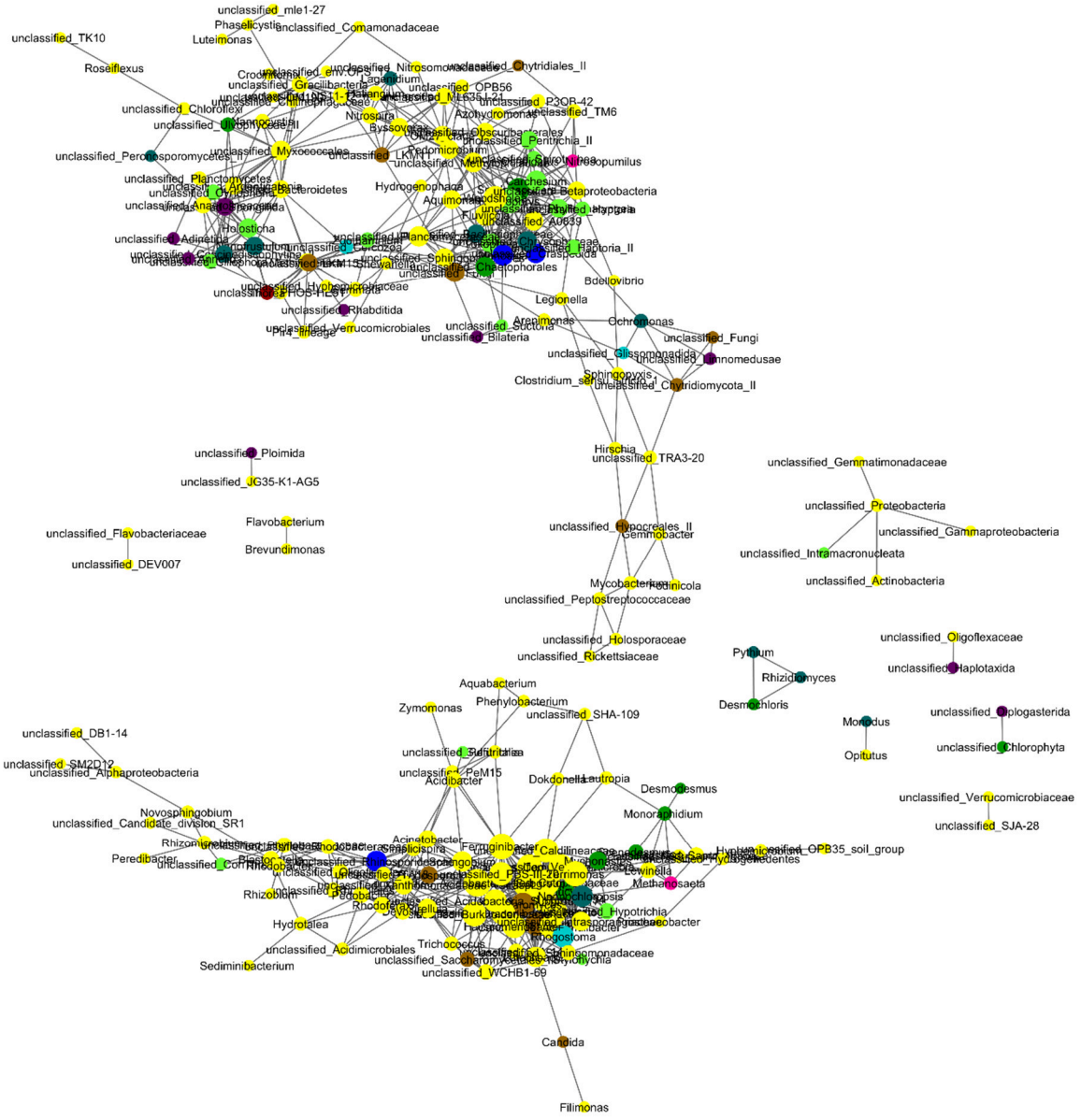


Fig. S1 E) wood co-occurrence network (stations 1 to 5)

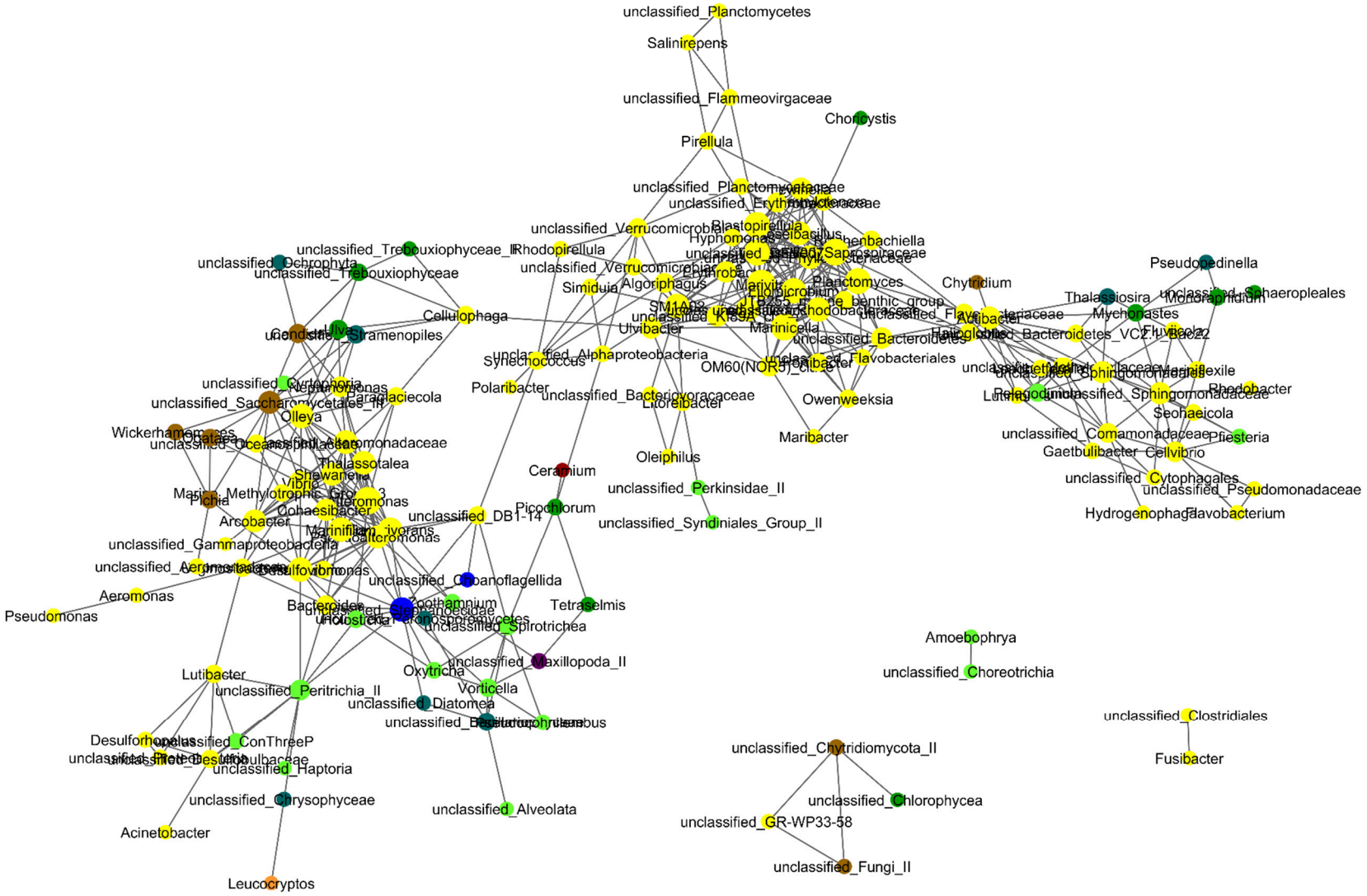
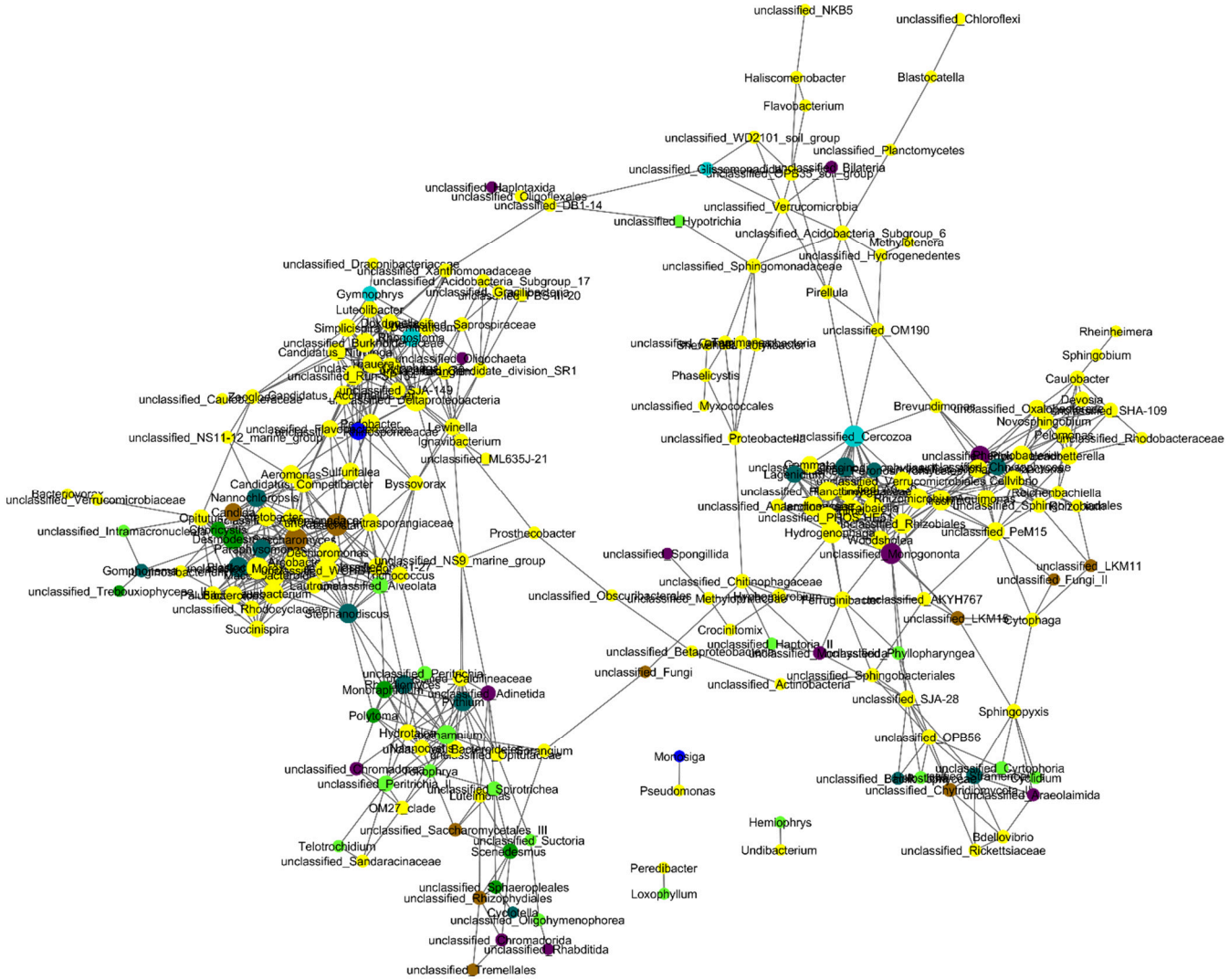


Fig. S1 F) wood co-occurrence network (stations 6 and 7)



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Supplementary Information for Manuscript II (SI1)

Microplastics alter composition of fungal communities in aquatic ecosystems

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Table S1. Results of Kruskal-Wallis test and posthoc Dunn's test for comparison of relative fungal read abundance on different substrate types and at different locations.

Comparison of relative fungal read abundance on different substrate types

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 27.72, df = 7, p-value = 0.001

Pairwise comparisons using Dunn's-test for multiple comparisons of independent samples, p adjustment according to Benjamini and Hochberg, 1995

	PE $_{1,2,3,4\&5}$	PS $_{1,2,3,4\&5}$	wood $_{1,2,3,4\&5}$	water $_{1,2,3,4\&5}$	PE $_{6\&7}$	PS $_{6\&7}$	wood $_{6\&7}$
PS $_{1,2,3,4\&5}$	0.680	-	-	-	-	-	-
wood $_{1,2,3,4\&5}$	0.787	0.787	-	-	-	-	-
water $_{1,2,3,4\&5}$	0.004	0.033	0.008	-	-	-	-
PE $_{6\&7}$	0.787	0.564	0.691	0.006	-	-	-
PS $_{6\&7}$	0.884	0.680	0.787	0.009	0.916	-	-
wood $_{6\&7}$	0.787	0.534	0.680	0.006	0.966	0.884	-
water $_{6\&7}$	0.966	0.680	0.787	0.006	0.787	0.884	0.787

Comparison of relative fungal read abundance at different locations

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 22.30, df = 6, p-value = 0.001

Pairwise comparisons using Dunn's-test for multiple comparisons of independent samples, p adjustment according to Benjamini and Hochberg, 1995

	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6
Station 2	0.544	-	-	-	-	-
Station 3	0.222	0.544	-	-	-	-
Station 4	0.260	0.075	0.015	-	-	-
Station 5	0.223	0.544	0.960	0.015	-	-
Station 6	0.383	0.116	0.020	0.841	0.020	-
Station 7	0.285	0.078	0.015	0.930	0.015	0.911

Table S2. Overview of all fungal taxa found on microplastic (polyethylene and polystyrene) across all samples. Taxa with only one read are not presented herein. Taxonomic information is based on SILVA v128 (Yilmaz *et al.*, 2014).

phylum	class	order	family	genus
Ascomycota	uncl. Ascomycota	uncl. Ascomycota	uncl. Ascomycota	uncl. Ascomycota
Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Guignardia
Ascomycota	Dothideomycetes	Capnodiales	uncl. Capnodiales	uncl. Capnodiales
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium
Ascomycota	Dothideomycetes	uncl. Dothideomycetes	uncl. Dothideomycetes	uncl. Dothideomycetes
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Cochliobolus
Ascomycota	Dothideomycetes	Pleosporales	uncl. Pleosporales	uncl. Pleosporales
Ascomycota	Dothideomycetes	Pleosporales	Incertae Sedis	Phoma
Ascomycota	Eurotiomycetes	Chaetothyriales	uncl. Chaetothyriales	uncl. Chaetothyriales
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	uncl. Trichocomaceae
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium
Ascomycota	Geoglossomycetes	Geoglossales	Geoglossaceae	Geoglossum
Ascomycota	Leotiomycetes	Helotiales	uncl. Helotiales	uncl. Helotiales
Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	uncl. Orbiliaceae
Ascomycota	Pezizomycetes	Pezizales	uncl. Pezizales	Lecophagus
Ascomycota	uncl. Pezizomycotina	uncl. Pezizomycotina	uncl. Pezizomycotina	uncl. Pezizomycotina
Ascomycota	Sordariomycetes	Diaporthales	uncl. Diaporthales	uncl. Diaporthales
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma
Ascomycota	Sordariomycetes	Hypocreales	uncl. Hypocreales	uncl. Hypocreales
Ascomycota	Sordariomycetes	Hypocreales	Incertae Sedis	Acremonium
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium
Ascomycota	Sordariomycetes	Sordariales	Coniochaetaceae	Lecythophora
Ascomycota	Sordariomycetes	uncl. Sordariomycetes	uncl. Sordariomycetes	uncl. Sordariomycetes
Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seiridium
Ascomycota	Saccharomycetes	Saccharomycetales	Metschnikowiaceae	uncl. Metschnikowiaceae
Ascomycota	Saccharomycetes	Saccharomycetales	Metschnikowiaceae	Clavispora
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Saccharomyces
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Kazachstania
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	uncl. Saccharomycetaceae
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Pichia
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Debaryomyces
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Torulaspora
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Nakaseomyces
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Citeromyces
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Zygotorulaspora
Ascomycota	Saccharomycetes	Saccharomycetales	uncl. Saccharomycetales	uncl. Saccharomycetales
Ascomycota	Saccharomycetes	Saccharomycetales	Incertae Sedis	Candida
Ascomycota	Saccharomycetes	Saccharomycetales	Incertae Sedis	Cyberlindnera
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycodaceae	Hanseniaspora
Ascomycota	Saccharomycetes	Saccharomycetales	Wickerhamomyceteae	Wickerhamomyces
Ascomycota	Taphrinomycetes	Taphrinales	Taphrinaceae	Taphrina

Ascomycota	Taphrinomycetes	Taphrinales	uncl. Taphrinales	uncl. Taphrinales
Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	Agaricus
Basidiomycota	Agaricomycetes	Agaricales	uncl. Agaricales	uncl. Agaricales
Basidiomycota	Agaricomycetes	uncl. Agaricomycetes	uncl. Agaricomycetes	uncl. Agaricomycetes
Basidiomycota	Agaricomycetes	Auriculariales	Auriculariaceae	uncl. Auriculariaceae
Basidiomycota	Agaricomycetes	Russulales	Lachnocladiaceae	uncl. Lachnocladiaceae
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	Guehomyces
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	uncl. Cystofilobasidiaceae
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Dioszegia
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Holtermannia
Basidiomycota	Tremellomycetes	Tremellales	uncl. Tremellales	uncl. Tremellales
Basidiomycota	Tremellomycetes	Tremellales	Trichosporonaceae	Trichosporon
Basidiomycota	Dacrymycetes	Dacrymycetales	Dacrymycetaceae	uncl. Dacrymycetaceae
Basidiomycota	Agaricostilbomycetes	Agaricostilbales	Agaricostilbaceae	Bensingtonia
Basidiomycota	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	Occultifur
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	Sporidiobolus
Basidiomycota	Microbotryomycetes	Sporidiobolales	uncl. Sporidiobolales	uncl. Sporidiobolales
Basidiomycota	Pucciniomycetes	Pucciniales	Pucciniaceae	Puccinia
Basidiomycota	Pucciniomycetes	Pucciniales	uncl. Pucciniales	uncl. Pucciniales
Basidiomycota	uncl. Pucciniomycotina	uncl. Pucciniomycotina	uncl. Pucciniomycotina	uncl. Pucciniomycotina
Basidiomycota	Exobasidiomycetes	Malasseziales	Incertae Sedis	Malassezia
Basidiomycota	Ustilaginomycetes	Ustilaginales	uncl. Ustilaginales	uncl. Ustilaginales
Chytridiomycota	Incertae Sedis	Chytridiales	Chytridiaceae	Chytridium
Chytridiomycota	Incertae Sedis	Chytridiales	Chytridiaceae	uncl. Chytridiaceae
Chytridiomycota	Incertae Sedis	Chytridiales	uncl. Chytridiales	uncl. Chytridiales
Chytridiomycota	Incertae Sedis	Chytridiales	Chytriomycetaceae	uncl. Chytriomycetaceae
Chytridiomycota	Incertae Sedis	uncl. Chytridiomycota	uncl. Chytridiomycota	uncl. Chytridiomycota
Chytridiomycota	Incertae Sedis	uncult. Chytridiomycota	uncult. Chytridiomycota	uncult. Chytridiomycota
Chytridiomycota	Incertae Sedis	Monoblepharidales	Incertae Sedis	Hyaloraphidium
Chytridiomycota	Incertae Sedis	Rhizophlyctidales	uncl. Rhizophlyctidales	Rhizophlyctidales
Chytridiomycota	Incertae Sedis	Rhizophydiales	uncl. Rhizophydiales	uncl. Rhizophydiales
Chytridiomycota	Incertae Sedis	Rhizophydiales	uncult. Rhizophydiales	uncult. Rhizophydiales
Cryptomycota	Incertae Sedis	Incertae Sedis	Incertae Sedis	Paramicrosporidium
Cryptomycota	uncl. LKM11	uncl. LKM11	uncl. LKM11	uncl. LKM11
uncl. Fungi	uncl. Fungi	uncl. Fungi	uncl. Fungi	uncl. Fungi
Incertae Sedis	uncl. Mucoromycotina	Mortierellales	Mortierellaceae	Mortierella
Incertae Sedis	uncl. Mucoromycotina	Mortierellales	uncl. Mortierellales	uncl. Mortierellales
LKM15	uncl. LKM15	uncl. LKM15	uncl. LKM15	uncl. LKM15

Table S3. PERMANOVA results after 999 permutations for the factors substrate and location as well as their interaction term.

PERMANOVA results (999 permutations)	for fungal OTUs from the River Warnow to Baltic Sea (Station 1, 2, 3, 4, 5)	for fungal OTUs from the wastewater treatment plant (Station 6, 7)
substrate	**p = 0.001**	**p = 0.024**
	R^2 = 0.164	R^2 = 0.158
location	**p = 0.001**	**p = 0.001**
	R^2 = 0.119	R^2 = 0.272
substrate*location	**p = 0.001**	**p = 0.001**
	R^2 = 0.067	R^2 = 0.140

Table S4. Results of pairwise PERMANOVA (999 permutations) and Bray-Curtis (BC) similarity for different substrate types and locations. P-adjustment according to Benjamini and Hochberg, 1995.

Pairwise PERMANOVA for different substrate types					
Comparison of substrates types for OTUs from stations 1, 2, 3, 4 and 5					
pairs	**F-Model**	**R^2**	**p-value**	**p adjusted**	**BC similarity**
PE vs PS	0,753	0,026	0,634	0,634	58 %
PE vs wood	5,891	0,174	0,001	0,001	25 %
PE vs water	3,295	0,090	0,001	0,001	22 %
PS vs wood	5,540	0,165	0,001	0,001	27 %
PS vs water	2,969	0,083	0,001	0,001	26 %
wood vs water	5,989	0,154	0,001	0,001	20 %
Comparison of substrates for OTUs from stations 6 and 7					
PE vs PS	0,854	0,079	0,577	0,577	58 %
PE vs wood	1,645	0,141	0,080	0,134	45 %
PE vs water	1,823	0,102	0,058	0,134	49 %
PS vs wood	1,153	0,103	0,309	0,371	49 %
PS vs water	1,660	0,094	0,089	0,134	53 %
wood vs water	2,228	0,122	0,030	0,134	39 %
Pairwise PERMANOVA for different locations					
Comparison of locations for OTUs from stations 1, 2, 3, 4 and 5					
1 vs 2	6,730	0,219	0,001	0,001	22 %
1 vs 3	4,244	0,150	0,001	0,001	21 %
1 vs 4	9,613	0,286	0,001	0,001	15 %
1 vs 5	10,475	0,304	0,001	0,001	14 %
2 vs 3	1,569	0,061	0,113	0,113	43 %
2 vs 4	4,855	0,168	0,001	0,001	26 %
2 vs 5	7,073	0,228	0,001	0,001	17 %
3 vs 4	4,033	0,144	0,001	0,001	19 %
3 vs 5	5,369	0,183	0,001	0,001	12 %
4 vs 5	4,839	0,168	0,001	0,001	42 %
Comparison of locations for OTUs from stations 6 and 7					
6 vs 7	10,481	0,272	0,001	0,001	34 %

Table S5. Results of permutation tests for homogeneity of multivariate dispersions for different substrate types and locations. If dispersion was not homogeneous, the results of pairwise comparisons are also given. df = degrees of freedom, Sq = squares

Dispersion level for substrates types (with OTUs from stations 1, 2, 3, 4 and 5)					
Average distance to median:					
PE $_{1,2,3,4\&5}$	PS $_{1,2,3,4\&5}$	wood $_{1,2,3,4\&5}$	water $_{1,2,3,4\&5}$		
0.571	0.581	0.496	0.599		
Permutation test for homogeneity of multivariate dispersions: (999 permutations)					
	df	sum Sq	mean Sq	F-Model	p-value
Groups	3	0.100	0.033	3.020	0.027
Residuals	61	0.670	0.011		
Pairwise comparisons: (Observed p-value below diagonal, permuted p-value above diagonal)					
	PE $_{1,2,3,4\&5}$	PS $_{1,2,3,4\&5}$	wood $_{1,2,3,4\&5}$	water $_{1,2,3,4\&5}$	
PE $_{1,2,3,4\&5}$	-	0.663	0.149	0.233	
PS $_{1,2,3,4\&5}$	0.647	-	0.087	0.351	
wood $_{1,2,3,4\&5}$	0.155	0.093	-	0.028	
water $_{1,2,3,4\&5}$	0.252	0.371	0.015	-	
Dispersion level for locations (with OTUs from stations 1, 2, 3, 4 and 5)					
Average distance to median:					
Station 1	Station 2	Station 3	Station 4	Station 5	
0.481	0.537	0.602	0.514	0.483	
Permutation test for homogeneity of multivariate dispersions: (999 permutations)					
	df	sum Sq	mean Sq	F-Model	p-value
Groups	4	0.128	0.032	2.316	0.077
Residuals	60	0.832	0.014		
Dispersion level for substrate types (with OTUs from stations 6 and 7)					
Average distance to median:					
PE $_{6\&7}$	PS $_{6\&7}$	wood $_{6\&7}$	water $_{6\&7}$		
0.430	0.418	0.438	0.438		
Permutation test for homogeneity of multivariate dispersions: (999 permutations)					
	df	sum Sq	mean Sq	F-Model	p-value
Groups	3	0.002	0.001	0.249	0.857
Residuals	26	0.062	0.002		
Dispersion level for locations (with OTUs from stations 6 and 7)					
Average distance to median:					
Station 6	Station 7				
0.403	0.394				
Permutation test for homogeneity of multivariate dispersions: (999 permutations)					
	df	sum Sq	mean Sq	F-Model	p-value
Groups	1	0.001	0.001	0.109	0.747
Residuals	28	0.146	0.005		

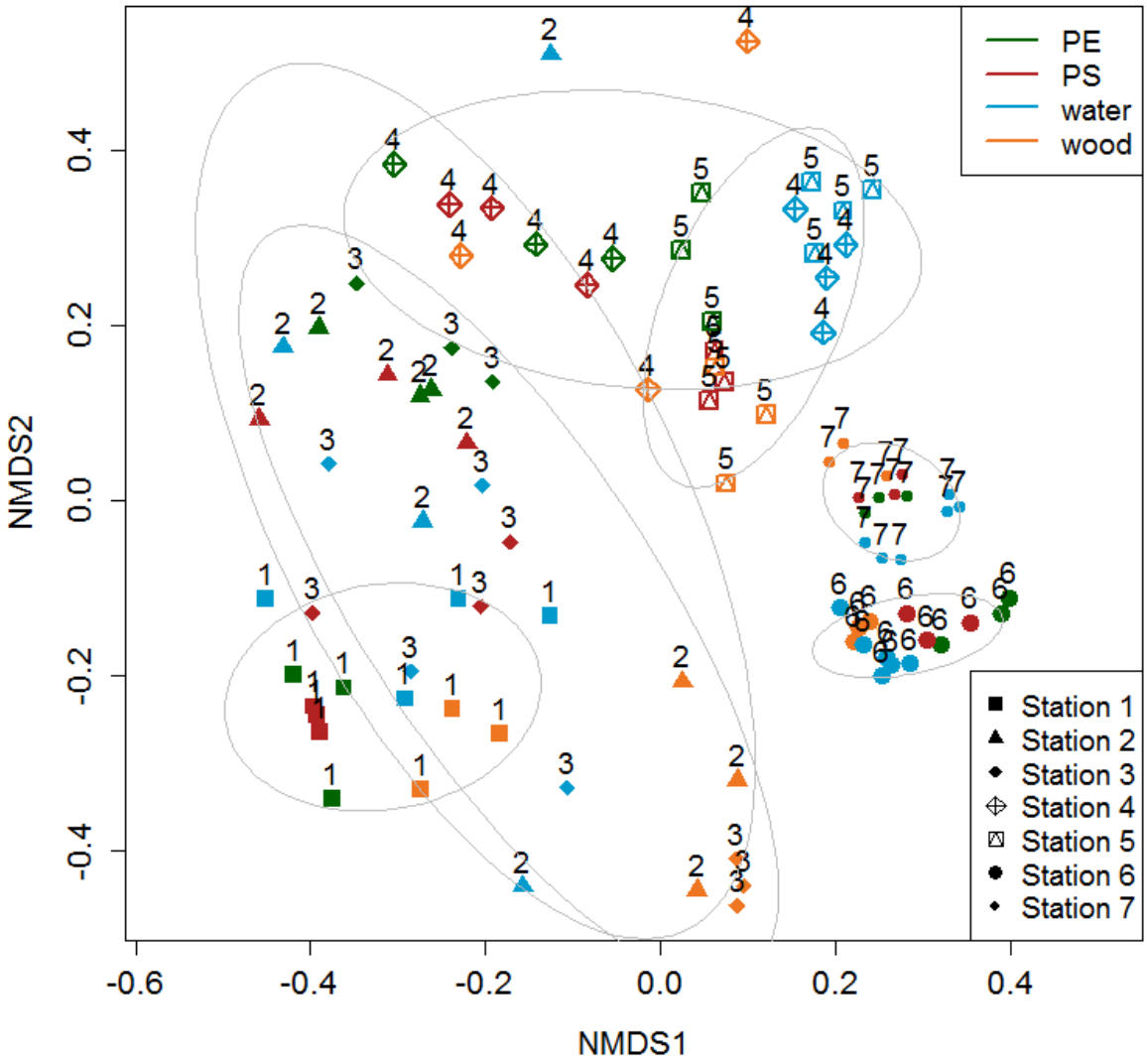

Fig. S1. NMDS of fungal community composition analyzed by substrate type and location (stress value 0.24). Ellipses surround samples from the same location at a confidence interval of 80%. Shapes and numbers indicate stations. Colors represent the different substrate types polyethylene, polystyrene, wood and water.

Table S6. Results of indicator species analysis with OTUs associated to each group or combination of groups for substrate type and location. For each OTU the assigned taxon, phylum, indicator value, adjusted p-value (according to Benjamini and Hochberg, 1995) and number of reads are given.

Fungal OTUs associated to substrate types						
OTU	**assigned taxon**	**assigned phylum**	**indicator value**	**adjusted p-value**	**reads of OTU on all substrates**	**reads of OTU on this substrate**
Microplastic (PE+PS)						
OTU 0002	Chytridium	Chytridiomycota	0.580	0.007	16977	16390
PE (polyethylene)						
none						
PS (polystyrene)						
none						
wood						
OTU 0021	Candida	Ascomycota	0.924	0.007	1225	1224
OTU 0024	Candida	Ascomycota	0.845	0.007	1045	1045
OTU 0017	Candida	Ascomycota	0.843	0.007	1358	1357
OTU 0033	Ogataea	Ascomycota	0.787	0.007	807	807
OTU 0032	Candida	Ascomycota	0.780	0.007	833	832
OTU 0041	unclassified Saccharomycetales	Ascomycota	0.754	0.007	577	547
OTU 0043	Candida	Ascomycota	0.724	0.007	551	551
OTU 0056	Kuraishia	Ascomycota	0.724	0.007	320	320
OTU 0048	unclassified Saccharomycetales	Ascomycota	0.690	0.007	401	401
OTU 0051	unclassified Saccharomycetales	Ascomycota	0.690	0.007	389	389
OTU 0058	unclassified Saccharomycetales	Ascomycota	0.690	0.007	310	310
OTU 0062	Wickerhamomyces	Ascomycota	0.690	0.007	245	245
OTU 0087	unclassified Saccharomycopsidaceae	Ascomycota	0.655	0.007	151	151
OTU 0181	unclassified Saccharomycetales	Ascomycota	0.655	0.007	38	38
OTU 0169	unclassified Saccharomycetales	Ascomycota	0.617	0.007	41	41

OTU 0187	Wickerhamomyces	Ascomycota	0.617	0.007	33	33
OTU 0092	Clavispora	Ascomycota	0.585	0.007	140	106
OTU 0189	unclassified Saccharomycetales	Ascomycota	0.577	0.007	33	33
OTU 0091	unclassified Cystofilobasidiaceae	Basidiomycota	0.538	0.020	143	129
OTU 0113	Hanseniaspora	Ascomycota	0.535	0.018	96	36
OTU 0167	unclassified Saccharomycetales	Ascomycota	0.519	0.007	43	42
OTU 0141	Candida	Ascomycota	0.486	0.020	61	53
OTU 0163	Meyerozyma	Ascomycota	0.479	0.018	46	38
OTU 0219	unclassified Saccharomycetales	Ascomycota	0.436	0.048	25	25
water						
OTU 0027	unclassified Exobasidiales	Basidiomycota	0.685	0.007	878	878
OTU 0034	uncultured Rhizophydiales	Chytridiomycota	0.591	0.007	806	795
OTU 0127	unclassified Chytridiomycota	Chytridiomycota	0.530	0.007	78	78
OTU 0191	unclassified Rhizophydiales	Chytridiomycota	0.530	0.007	32	32
OTU 0068	unclassified Chytridiomycota	Chytridiomycota	0.509	0.020	206	200
OTU 0114	unclassified Fungi	unclassified Fungi	0.500	0.007	96	96
OTU 0128	unclassified Rhizophydiales	Chytridiomycota	0.500	0.013	78	78
OTU 0036	unclassified Chytridiales	Chytridiomycota	0.484	0.013	665	662
OTU 0047	Rozella	Cryptomycota	0.468	0.020	426	426
OTU 0061	unclassified Rhizophydiales	Chytridiomycota	0.468	0.020	265	265
OTU 0183	unclassified Fungi	unclassified Fungi	0.468	0.013	35	35
OTU 0185	unclassified Chytridiomycota	Chytridiomycota	0.467	0.044	35	26
OTU 00938	Rozella	Cryptomycota	0.433	0.048	169	169
OTU 0155	unclassified Fungi	unclassified Fungi	0.433	0.048	49	49
OTU 0171	Exobasidium	Basidiomycota	0.433	0.037	40	40
OTU 0179	unclassified Chytridiomycota	Chytridiomycota	0.433	0.041	39	39
OTU 0195	unclassified LKM11	Cryptomycota	0.433	0.025	30	30
OTU 0197	unclassified Fungi	unclassified Fungi	0.433	0.034	30	30

Fungal OTUs associated to locations

OTU	assigned taxon	assigned phylum	indicator value	adjusted p-value	reads of OTU at all locations	reads of OTU at this location
Station 1 (Baltic Sea coast, pier Heiligendamm)						
OTU 0095	unclassified Chytridiales	Chytridiomycota	0.961	0.002	128	128
OTU 0023	unclassified Rhizophydiales	Chytridiomycota	0.920	0.002	1048	1048
OTU 0089	unclassified Fungi	unclassified Fungi	0.892	0.002	148	147
OTU 0076	unclassified Chytridiales	Chytridiomycota	0.867	0.002	186	181
OTU 0123	Chytridium	Chytridiomycota	0.832	0.002	82	82
OTU 0125	unclassified Fungi	unclassified Fungi	0.775	0.002	79	76
OTU 0045	unclassified Fungi	unclassified Fungi	0.734	0.002	457	456
OTU 0046	unclassified Chytridiomycota	Chytridiomycota	0.708	0.002	441	429
OTU 0102	Dioszegia	Basidiomycota	0.690	0.002	115	103
OTU 0156	unclassified Chytridiomycota	Chytridiomycota	0.679	0.002	49	49
OTU 0003	unclassified Fungi	unclassified Fungi	0.668	0.002	8835	8772
OTU 0037	unclassified Fungi	unclassified Fungi	0.663	0.002	622	620
OTU 0081	unclassified Saccharomycetales	Ascomycota	0.652	0.002	170	103
OTU 0132	unclassified Chytridiomycota	Chytridiomycota	0.620	0.002	71	71
OTU 0192	unclassified Chytridiales	Chytridiomycota	0.614	0.002	31	30
OTU 0217	Chytridium	Chytridiomycota	0.540	0.002	25	20
Station 2 (Estuary, canal Alter Strom)						
OTU 0124	unclassified Fungi	unclassified Fungi	0.555	0.002	80	65
Station 3 (Estuary, Marina)						
OTU 0067	unclassified Chytridiales	Chytridiomycota	0.679	0.002	213	213
OTU 0214	unclassified Fungi	unclassified Fungi	0.480	0.008	26	26
Station 4 (River Warnow, close to a WWTP outlet)						
OTU 0101	unclassified Rhizophydiales	Chytridiomycota	0.602	0.002	118	111
OTU 0148	unclassified Chytridiales	Chytridiomycota	0.588	0.002	56	51
OTU 0182	unclassified Chytridiomycota	Chytridiomycota	0.587	0.002	36	35

OTU 0099	unclassified Chytridiales	Chytridiomycota	0.555	0.004	121	121
OTU 0199	unclassified Chytridiaceae	Chytridiomycota	0.491	0.008	29	24
Station 5 (River Warnow, before city center of Rostock)						
OTU 0079	unclassified Chytridiomycota	Chytridiomycota	0.784	0.002	172	172
OTU 0007	unclassified Chytridiomycota	Chytridiomycota	0.749	0.002	3969	3907
OTU 0098	unclassified Chytridiales	Chytridiomycota	0.722	0.002	126	124
OTU 0109	uncultured Rhizophydiales	Chytridiomycota	0.620	0.002	103	103
OTU 0138	unclassified Chytridiomycota	Chytridiomycota	0.599	0.002	64	40
OTU 0186	unclassified Fungi	unclassified Fungi	0.456	0.022	34	32
Station 6 (WWTP, before final biological treatment)						
OTU 0053	Trichosporon	Basidiomycota	0.846	0.002	375	373
OTU 0029	unclassified Saccharomycetales	Ascomycota	0.835	0.002	866	862
OTU 0009	unclassified Fungi	unclassified Fungi	0.829	0.002	2608	2550
OTU 0025	unclassified Saccharomycetales	Ascomycota	0.821	0.002	1007	865
OTU 0110	unclassified Saccharomycetaceae	Ascomycota	0.821	0.002	102	100
OTU 0035	Trichosporon	Basidiomycota	0.784	0.002	725	543
OTU 0094	Candida	Ascomycota	0.725	0.002	129	121
OTU 0028	Candida	Ascomycota	0.723	0.002	868	688
OTU 0106	unclassified Tremellales	Basidiomycota	0.683	0.002	110	110
OTU 0137	unclassified Saccharomycetales	Ascomycota	0.683	0.002	65	65
OTU 0144	unclassified Pezizomycotina	Ascomycota	0.683	0.002	58	58
OTU 0083	unclassified Rhizophydiales	Chytridiomycota	0.671	0.002	158	129
OTU 0064	unclassified Fungi	unclassified Fungi	0.643	0.002	230	226
OTU 0209	uncultured Rhizophydiales	Chytridiomycota	0.642	0.002	27	23
OTU 0171	Exobasidium	Basidiomycota	0.632	0.002	40	40
OTU 0118	unclassified Saccharomycetales	Ascomycota	0.615	0.002	90	87
OTU 0135	unclassified Tremellales	Basidiomycota	0.598	0.002	68	62
OTU 0213	unclassified Pezizomycotina	Ascomycota	0.580	0.002	26	23
OTU 0150	unclassified Trichocomaceae	Ascomycota	0.532	0.005	53	50
OTU 0072	unclassified Fungi	unclassified Fungi	0.516	0.008	198	198

OTU 0115	Mortierella	Incertae Sedis	0.516	0.002	95	95
OTU 0164	unclassified Rhizophydiales	Chytridiomycota	0.516	0.005	44	44
OTU 0057	unclassified Saccharomycetales	Ascomycota	0.504	0.009	318	153
OTU 0070	unclassified Chytridiomycota	Chytridiomycota	0.501	0.002	203	202
OTU 0190	Cyberlindnera	Ascomycota	0.493	0.009	33	30
OTU 0196	unclassified Saccharomycetales	Ascomycota	0.485	0.008	30	28
OTU 0168	unclassified Pucciniomycotina	Basidiomycota	0.447	0.026	43	43
OTU 0173	unclassified LKM11	Cryptomycota	0.447	0.026	40	40
OTU 0130	unclassified Mortierellales	Incertae Sedis	0.414	0.049	76	75
Station 7 (WWTP, after final biological treatment)						
OTU 0013	unclassified Chytriomycetaceae	Chytridiomycota	0.978	0.002	1583	1580
OTU 0015	unclassified LKM15	LKM15	0.938	0.002	1563	1524
OTU 0031	unclassified Chytridiales	Chytridiomycota	0.935	0.002	835	830
OTU 0054	unclassified LKM15	LKM15	0.923	0.002	364	349
OTU 0049	unclassified LKM11	Cryptomycota	0.916	0.002	400	373
OTU 0008	unclassified LKM11	Cryptomycota	0.881	0.002	3553	3253
OTU 0014	unclassified Chytridiomycota	Chytridiomycota	0.862	0.002	1578	1388
OTU 0077	unclassified Fungi	unclassified Fungi	0.856	0.002	184	184
OTU 0071	unclassified Chytridiomycota	Chytridiomycota	0.840	0.002	200	198
OTU 0022	Paramicrosporidium	Cryptomycota	0.830	0.002	1219	1136
OTU 0160	unclassified Chytridiomycota	Chytridiomycota	0.816	0.002	47	47
OTU 0059	unclassified Fungi	unclassified Fungi	0.808	0.002	277	267
OTU 0075	unclassified Fungi	unclassified Fungi	0.730	0.002	186	186
OTU 0112	unclassified Fungi	unclassified Fungi	0.730	0.002	99	99
OTU 00541	unclassified LKM15	LKM15	0.723	0.002	436	337
OTU 0194	unclassified Fungi	unclassified Fungi	0.690	0.002	31	29
OTU 0111	unclassified Fungi	unclassified Fungi	0.690	0.002	99	96
OTU 0096	unclassified Fungi	unclassified Fungi	0.689	0.002	127	114
OTU 0157	unclassified LKM15	LKM15	0.683	0.002	48	48
OTU 00813	unclassified LKM15	LKM15	0.617	0.002	198	157

OTU 0221	unclassified Fungi	unclassified Fungi	0.611	0.002	25	24
OTU 0085	unclassified Fungi	unclassified Fungi	0.594	0.002	157	155
OTU 0142	unclassified Fungi	unclassified Fungi	0.577	0.002	60	60
OTU 0153	unclassified Fungi	unclassified Fungi	0.577	0.002	50	50
OTU 0129	unclassified Fungi	unclassified Fungi	0.536	0.004	78	77
OTU 0093	Lecophagus	Ascomycota	0.516	0.004	130	130
OTU 0108	unclassified Chytridiales	Chytridiomycota	0.516	0.005	106	106
OTU 0166	unclassified Chytridiomycota	Chytridiomycota	0.516	0.007	44	44
OTU 0203	unclassified Chytridiales	Chytridiomycota	0.516	0.008	28	28
OTU 0212	unclassified Rhizophydiales	Chytridiomycota	0.516	0.005	27	27
OTU 0218	unclassified Chytridiomycota	Chytridiomycota	0.495	0.009	25	24
OTU 0100	unclassified Fungi	unclassified Fungi	0.487	0.007	121	93
OTU 0103	unclassified Chytridiomycota	Chytridiomycota	0.482	0.005	114	97
OTU 0205	Chytridium	Chytridiomycota	0.478	0.008	27	26

Table S7. Results of Kruskal-Wallis test and posthoc Dunn's test for comparison of fungal OTU richness, Pielou evenness and Simpson's index, each on different substrate types and at different locations. Subscript numbers behind the substrate indicate study stations.

Comparison of richness on different substrate types

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 49.78, df = 7, p-value = 0.001

Pairwise comparisons using Dunn's-test for multiple comparisons of independent samples, p adjustment according to Benjamini and Hochberg, 1995

	PE $_{1,2,3,4\&5}$	PS $_{1,2,3,4\&5}$	wood $_{1,2,3,4\&5}$	water $_{1,2,3,4\&5}$	PE $_{6\&7}$	PS $_{6\&7}$	wood $_{6\&7}$
PS $_{1,2,3,4\&5}$	0.855	-	-	-	-	-	-
wood $_{1,2,3,4\&5}$	0.086	0.039	-	-	-	-	-
water $_{1,2,3,4\&5}$	0.038	0.015	0.878	-	-	-	-
PE $_{6\&7}$	0.005	0.003	0.147	0.166	-	-	-
PS $_{6\&7}$	0.003	0.002	0.105	0.123	0.878	-	-
wood $_{6\&7}$	0.005	0.003	0.147	0.166	0.996	0.878	-
water $_{6\&7}$	0.001	0.001	0.005	0.005	0.523	0.669	0.523

Comparison of richness at different locations

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 43.22, df = 6, p-value = 0.001

Pairwise comparisons using Dunn's-test for multiple comparisons of independent samples, p adjustment according to Benjamini and Hochberg, 1995

	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6
Station 2	0.097	-	-	-	-	-
Station 3	0.160	0.815	-	-	-	-
Station 4	0.752	0.212	0.335	-	-	-
Station 5	0.828	0.144	0.212	0.815	-	-
Station 6	0.026	0.001	0.001	0.008	0.015	-
Station 7	0.014	0.001	0.001	0.004	0.008	0.815

Comparison of Pielou evenness on different substrate types

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 44.46, df = 7, p-value = 0.001

Pairwise comparisons using Dunn's-test for multiple comparisons of independent samples, p adjustment according to Benjamini and Hochberg, 1995

	PE $_{1,2,3,4\&5}$	PS $_{1,2,3,4\&5}$	wood $_{1,2,3,4\&5}$	water $_{1,2,3,4\&5}$	PE $_{6\&7}$	PS $_{6\&7}$	wood $_{6\&7}$
PS $_{1,2,3,4\&5}$	0.861	-	-	-	-	-	-
wood $_{1,2,3,4\&5}$	0.016	0.042	-	-	-	-	-
water $_{1,2,3,4\&5}$	0.001	0.001	0.079	-	-	-	-
PE $_{6\&7}$	0.861	0.891	0.156	0.005	-	-	-
PS $_{6\&7}$	0.891	0.891	0.102	0.002	0.891	-	-
wood $_{6\&7}$	0.714	0.861	0.246	0.011	0.891	0.861	-
water $_{6\&7}$	0.013	0.033	0.891	0.156	0.129	0.079	0.191

Comparison of Pielou evenness at different locations

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 8.58, df = 6, p-value = 0.199

Comparison of Simpson's index on different substrate types

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 54.26, df = 7, p-value = 0.001

Pairwise comparisons using Dunn's-test for multiple comparisons of independent samples, p adjustment according to Benjamini and Hochberg, 1995

	PE $_{1,2,3,4\&5}$	PS $_{1,2,3,4\&5}$	wood $_{1,2,3,4\&5}$	water $_{1,2,3,4\&5}$	PE $_{6\&7}$	PS $_{6\&7}$	wood $_{6\&7}$
PS $_{1,2,3,4\&5}$	0.989	-	-	-	-	-	-
wood $_{1,2,3,4\&5}$	0.010	0.010	-	-	-	-	-
water $_{1,2,3,4\&5}$	0.001	0.001	0.503	-	-	-	-
PE $_{6\&7}$	0.006	0.006	0.456	0.837	-	-	-
PS $_{6\&7}$	0.010	0.010	0.613	0.949	0.881	-	-
wood $_{6\&7}$	0.024	0.024	0.837	0.837	0.753	0.837	-
water $_{6\&7}$	0.001	0.001	0.010	0.040	0.299	0.191	0.085

Comparison of Simpson's index at different locations

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 29.66, df = 6, p-value = 0.001

Pairwise comparisons using Dunn's-test for multiple comparisons of independent samples, p adjustment according to Benjamini and Hochberg, 1995

	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6
Station 2	0.120	-	-	-	-	-
Station 3	0.339	0.526	-	-	-	-
Station 4	0.310	0.562	0.926	-	-	-
Station 5	0.926	0.110	0.293	0.267	-	-
Station 6	0.112	0.001	0.007	0.007	0.120	-
Station 7	0.120	0.001	0.008	0.007	0.146	0.926

Table S8. Incubation sites with short description, coordinates, incubation time, and averaged values of temperature, salinity, phosphorous, nitrate, nitrite, ammonium and silicon dioxide. To preserve anonymity, coordinates of the wastewater treatment plant (WWTP) are not given. * Salinity values were provided by personnel of the wastewater treatment plant. DD = decimal degrees.

Station	short site description	latitude DD	longitude DD	incubation season (à 15 days)	T °C	S psu	PO_4^{3-} µmol l^{-1}	NO_3^- µmol l^{-1}	NO_2^- µmol l^{-1}	NH_4^+ µmol l^{-1}	SiO_2 µmol l^{-1}
1	Baltic Sea coast, pier Heiligendamm	54.14620	11.84343	summer 2014	17.4	14.7	0.26	0.03	0.07	0.43	5.2
2	Estuary, canal Alter Strom	54.18053	12.08722	summer 2014	18.3	13.5	1.23	2.67	0.35	3.38	17.8
3	Estuary, Marina	54.18308	12.09540	summer 2014	17.0	13.5	0.82	3.84	0.17	2.28	11.1
4	River Warnow, close to a WWTP outlet	54.10603	12.09618	summer 2014	18.0	10.1	2.14	48.76	0.59	7.55	147.0
5	River Warnow, before city center of Rostock	54.09725	12.15130	summer 2014	17.6	6.6	3.31	6.03	0.49	5.47	40.0
6	WWTP, before final biological treatment	-	-	spring 2015	15.2	1.1*	0.35	751.35	11.42	24.95	155.2
7	WWTP, after final biological treatment	-	-	spring 2015	14.8	1.1*	2.03	701.80	0.45	1.23	148.9

Table S9. Primer specificity tested with Silva TestPrime1.0 (Klindworth *et al.*, 2013). List of taxa and their according coverage in the Silva Taxonomy browser (database v128). Coverage is given in percent allowing zero and one mismatch.

Taxon		coverage allowing zero mismatch	coverage allowing one mismatch
Eukaryota		55.9%	77.4%
Opisthokonta		45.8%	73.0%
Nucletmycea		19.3%	59.3%
Fungi		19.2%	59.1%
	Ascomycota	23.5%	43.3%
	Basidiomycota	0.2%	75.8%
	Blastocladiomycota	75.0%	96.9%
	Chytridiomycota	72.0%	85.6%
	Cryptomycota	81.0%	89.9%
	Entomophthoromycota	0.0%	46.5%
	Glomeromycota	79.9%	89.3%
	Incertae Sedis	47.0%	51.7%
	LKM15	69.6%	95.7%
	Microsporidia	0.0%	0.0%
	Neocallimastigomycota	81.0%	90.5%
	TE101H	66.7%	66.7%
	uncultured Fungi	47.1%	82.4%
Bacteria		0.0%	0.0%
Archaea		0.0%	0.0%

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Supplementary Information for Manuscript II (SI2)

Microplastics alter composition of fungal communities in aquatic ecosystems

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Detailed Methods

DNA extraction

Zirconia and glass beads of different sizes (1 mm, 0.7 mm and 0.1 mm) and 400 µl extraction buffer containing 100 mM Tris-HCl, 20 mM Na_2EDTA, 1.6 M NaCl and 1% SDS were added to each sample (per sample: 70 PE particles, 70 PS particles, 1 ml wood particles or one filter membrane). Samples were then subjected to mechanical disruption (2*1 minute, f = 30 Hz, Mixer Mill MM 400, Retsch, Germany) and enzymatic treatment with proteinase K (8 µl with concentration of 10 mg ml^{-1}) for 1 h at 60 °C while shaking at 350 rpm. The liquid phase (~ 800 µl) was transferred to a new reaction tube to which 200 µl of 5% CTAB and 1.6 NaCl and 1 vol. of Roti®-phenol/chloroform/isoamyl alcohol (ratio 25:24:1, pH 7.5-8, Carl Roth, Germany) were added. After horizontal vortexing (10 min, room temperature, 1 000 rpm) and centrifugation (10 min, 4 °C, 16 000 g) the aqueous phase was transferred and 1 vol. of Roti®-C/I (ratio 24:1, Carl Roth, Germany) was added. Liquids were mixed by manual shaking and

separated again by centrifugation (10 min, 4 °C, 16 000 g). The aqueous phase was transferred and 1/10 vol. of 3 M Na-acetate and 2-3 vol. of 100% ethanol were added. After gentle mixing, DNA was precipitated overnight at 4 °C and subsequently pelleted by centrifugation (90 min, 4 °C, 17 000 g). The DNA pellet was washed with 70% ethanol, dried in the Eppendorf Concentrator plus (Eppendorf, Germany) and re-suspended in 40 µl PCR grade water (Roche Applied Science, Germany) (10 min, 37 °C, 350 rpm). Extracted DNA was stored at -20 °C. DNA concentration was measured using the Quantus™ Fluorometer with the according kit QuantiFluor® dsDNA System (Promega, Germany).

Primers for amplification

The eukaryote-specific primers Eu565F (5'-CCAGCA(G/C)C(C/T)GCGGTAATTCC-3') and Eu981R (5'-ACTTTCGTTCTTGAT(C/T)(A/G)ATGA-3') (Stoeck *et al.*, 2010; with bases TGA added by LGC Genomics, Berlin, Germany) were used for PCR amplification. Their coverage was evaluated with *in silico* PCR using TestPrime v1.0 (Klindworth *et al.*, 2013; URL https://www.arb-silva.de/search/testprime/; online access December 2016). Allowing one mismatch, the primer pair covers 77.6% of all eukaryote sequences, 59.6% of all fungal sequences and no prokaryote sequences in the SILVA SSU database v128. Results for fungal phyla are provided in Table S9 in SI2.

Sequence processing

Two approaches were chosen for data evaluation as explained below and they are herein called "taxon-based approach" and "OTU-based approach" (OTU = operational taxonomic unit).

Pre-processing of raw Illumina Miseq reads was carried out by LGC Genomics, Berlin, Germany. That comprised demultiplexing and clipping of barcodes, adapters and primers. The demultiplexing step was run in bcl2fastq 1.8.4 software (Illumina Inc., San Francisco, USA), allowing up to two mismatching bases in the index sequence. After sorting reads according to their inline barcodes, barcodes (allowing no more than one mismatch), Illumina TruSeq™ adapters (allowing no more than one mismatch) and primers (allowing up to three mismatches) were clipped.

All further processing was done with mothur v1.37.6 (Schloss *et al.*, 2009) according to mothur's MiSeq SOP (Kozich *et al.*, 2013; URL https://www.mothur.org/wiki/MiSeq_SOP;

online access April 2016). Briefly, forward and reverse reads were paired and quality filtered (minimum quality score = 25, no ambiguous bases, maximum number of homopolymers = 8, minimum length = 300 bp) leading to 5.5 million paired reads with a median length of 375 bp. Unique sequences were aligned to the non-redundant SILVA database v123 containing full-length bacterial, archaeal and eukaryotic sequences (Quast *et al.*, 2013). This reference database was customized in mothur by reducing it to the eukaryotic reference sequences and to the region of interest (start position = 13870, end position = 22116). Chimeras were identified (~ 3.3% of the reads) and removed with the UCHIME algorithm (Edgar *et al.*, 2011). Remaining reads were classified with a minimum bootstrap value of 80 using the non-redundant SILVA database v123 including all 22 taxonomic levels down to the genus (GitHub repositories; URL https://github.com/rec3141/diversity-scripts/blob/master/convert_silva_taxonomy.r; user rec3141, 2016). All reads belonging to the kingdom Fungi and LKM15 (a taxon that was not considered part of the fungi in the SILVA database v123, but is classified as fungus since version 128) were extracted from the dataset using the get.lineage command in mothur. This generated the data table for the "taxon-based approach" and contains the number of reads per taxon for all samples.

For the "OTU-based approach" the fungal subset was split on the deepest taxon level (genus) and operational taxonomic units (OTUs) were calculated within these levels applying the cluster.split command in mothur. OTUs were clustered with a 3% distance cut-off using VSEARCH within mothur (abundance-based greedy clustering; GitHub repositories; URL https://github.com/torognes/vsearch; user torognes, 2015). OTUs were assigned to a consensus taxonomy allowing a confidence threshold of 80 and using again the customized non-redundant SILVA database v123 including all 22 taxonomic levels. This created the data table for the "OTU-based approach" with the number of reads per OTU for all samples. Unclassified taxa or OTUs at higher taxonomic ranks (kingdom level and above) with at least 100 reads, were checked individually with the online SINA Alignment Service v.1.2.11 (Pruesse *et al.*, 2012) using default settings. The SINA aligner compares sequences to the databases SILVA v128, RDP, greengenes, LTP and EMBL. If the classification could be improved at least to phylum level, the according reads were added to the taxon- and OTU-based data tables. If taxonomies changed from SILVA v123 to v128, they were updated in our dataset.

Data evaluation and statistical methods

Samples from both incubation experiments (River Warnow to Baltic Sea: station 1, 2, 3, 4, 5 with 65 samples and WWTP: station 6, 7 with 30 samples) were prepared and sequenced under comparable conditions but not in the same Illumina sequencing run. Due to differences in sequencing depths between the two runs (Fig. SI1), datasets from both experiments were analyzed separately for comparisons of fungal community composition and alpha diversity. All water samples were treated as substrate type "water" for later statistical tests, regardless of the size-fraction, since the fungal community compositions of both size-fractions (water > 3 µm and water > 0.2 µm) were not significantly different (pairwise PERMANOVA, $p > 0.05$, data not shown).

Methodological limitations

Studies applying next generation sequencing methods may suffer from a range of biases from sampling, over DNA extraction, primer selection, PCR amplification and all steps involved during sequencing to bioinformatics and choice of databases etc. (Stoeck *et al.*, 2010; Bonder *et al.*, 2012; Santamaria *et al.*, 2012; Brooks *et al.*, 2015; Padilla *et al.*, 2015; Schirmer *et al.*, 2015). While processing all samples equally, we can assure a high comparability within our dataset, however, not all biases can be avoided. Calculated proportions of fungi in eukaryotic communities could be biased, since our "universal" eukaryote primer set did not cover 100% of the fungi and eukaryotes. Read abundances gave us a good indication about presence, absence and importance of organisms, but they do not precisely reflect the real biological abundances of organisms (Amend *et al.*, 2010) and should ideally be verified with additional techniques (Grossart and Rojas-Jimenez, 2016). A general limitation of DNA-based sequencing studies is the inability to distinguish between dead and active cells. The rather young age of the biofilm and the significant enrichment of fungi on PE, PS and wood in comparison to the surrounding water make us assume that fungi were primarily active members within the biofilm communities. Fungal taxa with low read abundances might contribute to a lesser extent to the overall microbial activity on MP, but rare taxa could play important roles as potential seed banks (Lennon and Jones, 2011).

Many rarefaction curves (Fig. SI2) did not reach saturation, indicating that a greater sampling effort could improve our diversity estimations. But one major constraint remains, namely the low representation of fungi with a deep taxonomic resolution in reference databases (Nilsson

et al., 2016). This specifically applies to aquatic fungi and the early diverging lineages. It might explain the high number of unclassified fungi and the low resolution for the phyla Chytridiomycota and Cryptomycota in comparison to, e.g., Basidiomycota. In addition to that, fungal taxonomy is under constant revision (Bai and Boekhout, 2015) and moves from a morphology-based towards a sequence-based classification (Hibbett *et al.*, 2016). Taxonomic information presented herein is up to date, but a re-analysis of our dataset in some years will most likely change and improve the classification. For instance, members of the genus *Candida* might be assigned to another genus, since some *Candida* species have been re-classified recently (Kurtzman, 2016). With our study we were nevertheless able to capture a detailed view into fungal diversity and community composition on different substrates and locations.

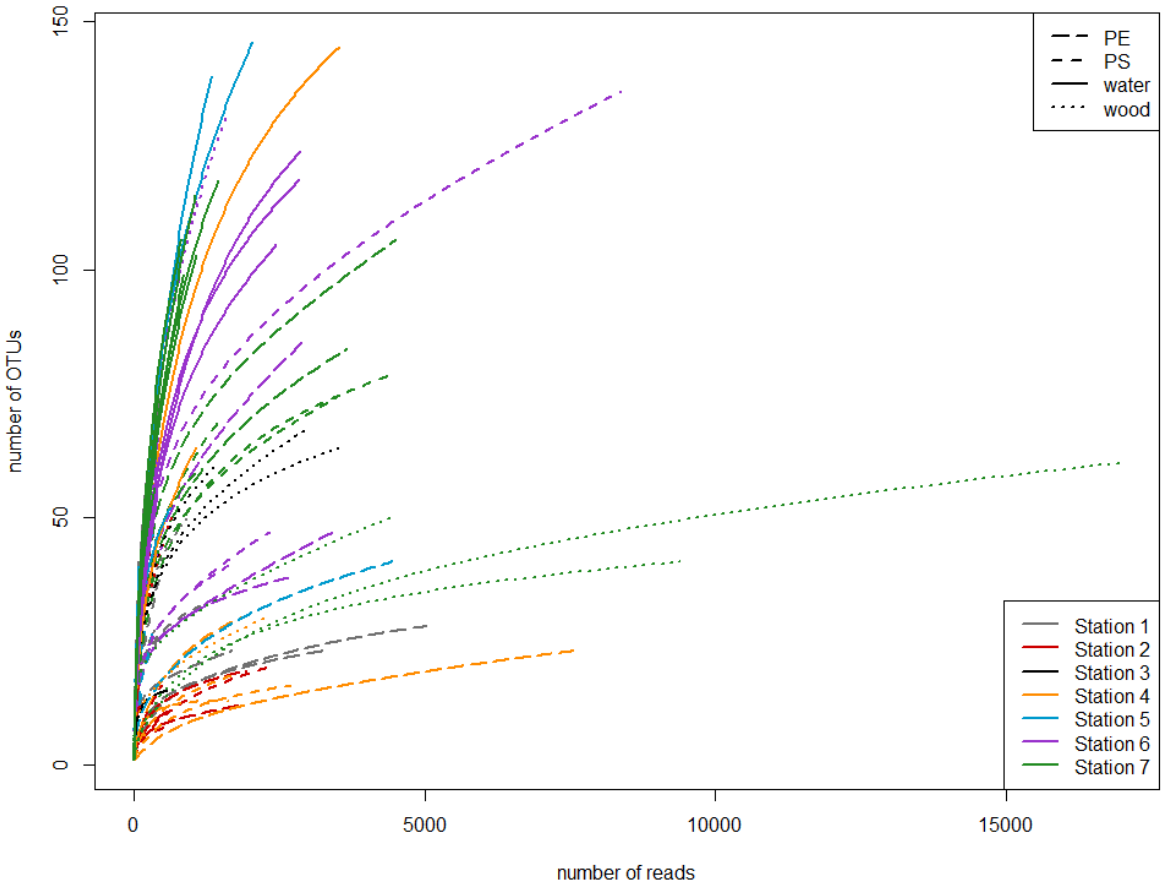

Fig. SI2. Rarefaction curves illustrating the number of OTUs over the number of reads for each sample.

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Supplementary Information for Manuscript III

Microplastics increase impact of treated wastewater on freshwater microbial community

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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**) OTU 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.

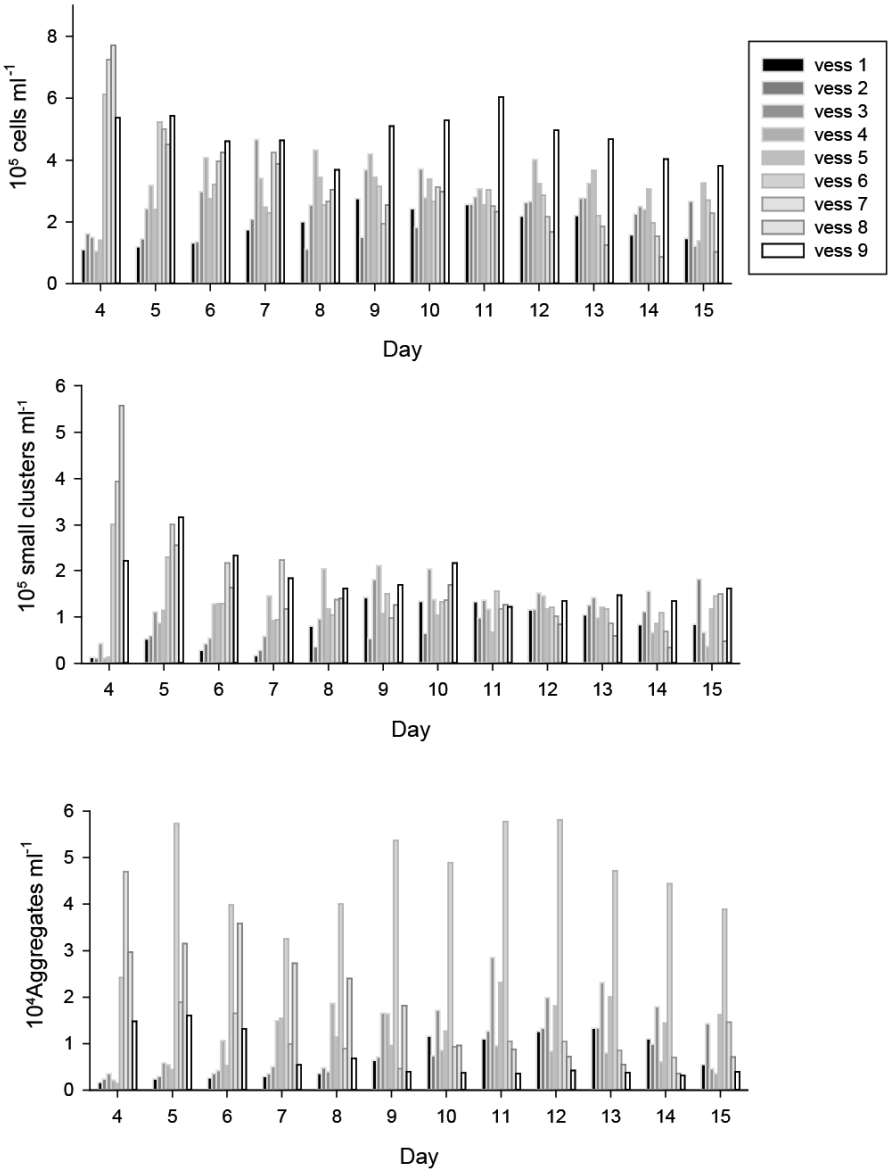

Figure S2 PCoA computet on the Sorensen distance matrix of ARISA peaks in the various samples. Vessel referes to vessel water and are free-water samples. MP refers to microplastic biofilm samples. LW and WW refer to the inoculum communities of lake water and waste water, respectively. Vessel 1 had the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