Plastics as vectors of microorganisms in the aquatic environment

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PREFACE

This dissertation is submitted for the degree of Doctor of Philosophy in the University of Alcalá, Madrid. The research described in this Thesis was carried out at the Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering or the University of Alcalá and the Department of Biology of Universidad Autónoma de Madrid during the period March 2017 – June 2022 under the supervision of Dr. Roberto Rosal and Dr. Francisco Leganes Nieto, professors at the universities of Alcalá and Autónoma de Madrid, respectively.

The results obtained during the elaboration of this Thesis have been published in three peer-reviewed internationals journals, namely Desalination and Water Treatment, Science of the Total Environment and Ecotoxicology and Environmental Safety. One more paper is being prepared at the time of writing this report. Moreover, some of the results included in this work have been presented in national and international conferences and workshops related with ecology and the effects of plastics in the environment.

All the results described in this Ph.D. dissertation are original, except where due reference has been made to the work of others. No part of this dissertation, or any similar to it, has been, or is currently being, submitted for any degree of other qualification at any other university.

Sergio Martínez-Campos Gutiérrez

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Para mi padre
Sin tus enseñanzas y tu esfuerzo no habría llegado aquí

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La tesis que a continuación se presenta ha sido fruto de un arduo trabajo, pero que no podría haber sido llevado a cabo sin esas personas que, aunque no figuren en la tesis ni en los papers, han supuesto un apoyo incondicional para su realización. En este grupo entraría mi madre y mi hermano, que han tenido que soportar horas y horas oyéndome hablar del mundo de los plásticos, me han dado un abrazo cuando más lo necesitaba y me han dado su punto de vista desde una perspectiva "no científica".

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SUMMARY

The development of plastics has been one of the largest technological breakthroughs in the history of mankind. Since the middle of the 20th century, the production of plastics has been increasing, according to the multiplication of their uses. Plastics, once used, represent a huge volume of waste that may end up dumped in the environment. The high persistence of plastics makes it very difficult to eliminate them naturally, so plastics tend to accumulate in environmental compartments. This problem has been of great concern to the scientific community, which is paying a growing attention to all the possible effects that plastics could have on aquatic ecosystems.

One of the most unknown impacts derives from the ability of microorganisms to attach to plastics. Eventually, a broad range of microorganisms can colonize plastics, forming communities that become more and more complex until they constitute new ecosystems. This new type of ecosystem is called "plastisphere". Many factors influence the communities constituted on plastics, such as geographic location, type of material, or the length of time this material has been exposed in the environment. Many plastic materials have already been colonized during their use, such as reverse osmosis membranes or greenhouse plastics, and it is unknown how these microorganisms may affect ecosystems once they are abandoned at the end of their useful life. In addition, the mobility of plastics, especially the smaller fractions, commonly known as microplastics (MPs), can lead to the mobilization of pathogenic microorganisms, bacteria with antibiotic resistance genes (ARGs) and invasive organisms with the capacity to alter the communities of the receiving ecosystems.

In the last few years, numerous studies have focused on the fate of plastics in marine ecosystems. However, there is an important knowledge gap about freshwater ecosystems. It has been proven that one of the main routes for plastic entering the oceans are rivers or intermittent waterways. Moreover, wastewater treatment plants are a hotspot for MPs, antibiotics and ARGs. The interaction between the afore mentioned factors must be considered since it could cause microplastics to act as a reservoir of antibiotic resistance potentially representing a risk for the environment and also to human health.

The overall main objective of this Doctoral Thesis is to characterize the potential of plastics as vectors of microorganisms in aquatic environments, especially in freshwater ecosystems. Based on these objectives, the thesis is organized into a series of chapters to achieve the objective.

Chapter 1 introduces the latest research on the impact of plastics to the environment. It highlights the importance of plastic for our society, its massive use, and the huge waste it

generates. Subsequently, the plastic cycle is introduced, including the way plastic reaches the environment and moves through it, eventually reaching any place on the planet. Afterwards, the impact of plastics depending on their size is discussed. Finally, the introduction focuses on the plastisphere, how it is constituted, the factors that determine its composition, and the different types of microorganisms associated to it. In addition, the risk posed by the plastisphere to humans and the environment due to the possible transportation of pathogenic organisms, ARGs, and invasive species.

Hypothesis and objectives define the hypothesis underlying the development of this doctoral thesis and details its objectives

Chapter 2 analyzes the bacterial and fungal communities formed in reverse osmosis membranes during their useful life. Reverse osmosis membranes are made of different polymers layers. The external one shows a tendency to become coated with different types of microorganisms in a biofilm, commonly denoted as biofouling, that reduces membrane lifetime. Noticeably, the viability of these microorganisms, once membranes are discarded, is essentially unknown. The chapter focuses on whether key microorganisms exist in reverse osmosis membranes from different origins as well as on the microbial viability and the extent of biofilm formation. Three reverse osmosis membranes were selected, two of them from a salty aquifer treatment system, and one from a seawater desalination plant. The results showed that the origin of treated water strongly affects the microbial community but also that there were common genera in the different membranes, which could play a common role in biofilm formation in reverse osmosis membranes and other plastic substrates.

Chapter 3 focuses on the early bacterial colonization of MPs in contact with the effluent of two wastewater treatment plants with emphasis on the presence of two ARGs (*sulI* and *tetM*). Wastewater treatment plants are considered one of the main entry points for MPs and ARGs into the aquatic environment. Despite this fact, there is a lack of knowledge on the capacity of MPs to act as a reservoir of ARGs. The results showed that the location (sampling site), and the properties of the plastic (hydrophobicity and roughness) played an important role in the early bacterial colonization phase. Furthermore, specific genera were detected for each type of polymer, suggesting that polymer type determines the early attachment of bacteria. Although the tested effluents waters contained both ARGs (*sulI* and *tetM* were detected) MPs concentrated only *sulI* gene.

Chapter 4 studies the prokaryotic and eukaryotic community found in four commonly used plastics allowed to colonize for one year in two sections of the same river. Both locations were characterized by very different environmental conditions, one located

in a section with natural land use and the other downstream of a wastewater treatment plant. The purpose was to mimic the fate of plastic debris, which could be trapped in the same area for a long time. The influence of the type of plastic substrate and the specific conditions of the river location on the attached microbial community were studied. Specifically, one of the sampling sites was located close to the discharge of a WWTP to assess the role of wastewater effluents on the ability of plastics to act as a reservoir of ARGs. The results confirmed that the location and type of substrate strongly conditioned the prokaryotic and eukaryotic community developed on plastics, while contact time played a less significant role. The results showed a correlation between the abundance of resistance genes and the concentration of their respective antibiotics.

Chapter 5 explores the ability of greenhouse plastics to act as vectors of bacteria once they are abandoned in the environment at the end of their useful life. Discarded agricultural plastics are often abandoned creating an environmental problem in agricultural areas, and in nearby riverbeds and seas. In this research, greenhouse plastics were collected from greenhouses, and found abandoned near greenhouses, in dry riverbeds, in the river, and in the sea. Therefore, the full path followed by greenhouse plastics from use to the sea was tracked. The results showed a significant difference in the microbial communities attached to plastics taken from the river and the sea in comparison with those taken from the soil, denoting an evolution in the microbial community attached to the plastics. Nevertheless, the presence of several genera attached to the plastic independently of the sampling location confirmed the role of plastics as vectors of microorganisms.

Chapter 6 is a General Discussion which summarizes the results of the doctoral thesis, discussing the relationship between the different chapters and with the objectives stated in this document. Finally, the **General Conclusions** outlines the conclusions obtained in this Doctoral dissertation.

RESUMEN

El desarrollo de los plásticos ha sido uno de los mayores avances tecnológicos de la historia de la humanidad. Desde mediados del siglo XX, la producción de plásticos ha ido en aumento, a la vez que se multiplicaban sus usos. Los plásticos, una vez desechados, generan un enorme volumen de residuos acaban en el medio ambiente. La elevada persistencia de los plásticos dificulta enormemente su eliminación natural, por lo que los plásticos tienden a acumularse en los distintos ecosistemas. Este problema es motivo de preocupación para la comunidad, que muestra un creciente interés por los posibles efectos negativos que los plásticos puedan tener en los ecosistemas acuáticos.

Uno de los impactos más desconocidos en este ámbito es la capacidad de los microorganismos para adherirse a los plásticos, empleándolos como soporte para su desarrollo. Con el tiempo, las comunidades fijadas al plástico se vuelven más complejas según se van uniendo microorganismos más variados, llegando a constituir un nuevo ecosistema. Este nuevo tipo de ecosistema ha sido denominado como "plastisfera".

Son muchos los factores que limitan las comunidades fijadas en la plastisfera como la situación geográfica, el tipo de plástico o el tiempo de exposición al medio ambiente. Además, muchos de los materiales plásticos han sido ya colonizados durante su uso, como las membranas de osmosis inversa o los plásticos de invernadero y se desconoce cómo pueden afectar estos microorganismos a los ecosistemas una vez que son abandonados en el medio ambiente al final de su vida útil. Además, la movilidad de los plásticos, especialmente de las fracciones más pequeñas, comúnmente conocidas como microplásticos (MPs), puede provocar la movilización de microorganismos patógenos, bacterias con genes de resistencia a los antibióticos (GRA) y organismos invasores con capacidad para alterar las comunidades de los ecosistemas receptores.

En los últimos años se ha dedicado un gran esfuerzo al estudio de la presencia y efectos de los plásticos en los ecosistemas marinos. Sin embargo, existe una importante falta de conocimiento sobre su desarrollo los ecosistemas de agua dulce. Esto resulta llamativo ya que se ha comprobado que una de las principales vías de entrada de plásticos en los océanos son los ríos o los cursos de agua intermitentes. Además, las plantas de tratamiento de aguas residuales son una vía de entrada importante para los MP, los antibióticos y los ARG. Es importante considerar la interacción entre estos factores ya que es posible que los MPs actúen como un reservorio de resistencia a los antibióticos representando potencialmente un riesgo para el medio ambiente y también para la salud humana.

El objetivo principal de esta Tesis Doctoral es caracterizar el potencial de los plásticos como vectores de microorganismos en ambientes acuáticos, especialmente en ecosistemas de agua dulce. En base a este objetivo, la tesis se organiza en una serie de capítulos para lograr satisfacer este objetivo.

El **Capítulo 1** presenta las últimas investigaciones sobre el impacto de los plásticos en el medio ambiente. Destaca la importancia del plástico para nuestra sociedad, su uso masivo y el enorme volumen de residuos que genera. Posteriormente, se introduce el ciclo del plástico, incluyendo la forma en que llega al medio ambiente y se mueve a través de él, alcanzando finalmente cualquier lugar del planeta. Después, se discute el impacto de los plásticos en función de su tamaño. Por último, la introducción se centra en la plastisfera, cómo está constituida, los factores que determinan su composición y los diferentes tipos de microorganismos asociados a ella. Además, se analiza el riesgo que supone la plastisfera para el ser humano y el medio ambiente debido al posible transporte de organismos patógenos, GRAs y especies invasoras.

Los **Objetivos Generales** definen la hipótesis en la que se basa el desarrollo de esta tesis doctoral y especifica los objetivos que se persiguen de forma más detallada.

El **Capítulo 2** analiza las comunidades bacterianas y fúngicas que se forman en las membranas de ósmosis inversa durante su vida útil. Las membranas de ósmosis inversa están formadas por diferentes capas de polímeros. La externa muestra una tendencia a recubrirse con diferentes tipos de microorganismos en una biopelícula, comúnmente denotada como bioensuciamiento que reduce la vida útil de la membrana. Cabe destacar que la viabilidad de estos microorganismos, una vez desechadas las membranas, es esencialmente desconocida. El capítulo se centra en determinar si existen microorganismos comunes en las membranas de ósmosis inversa de diferentes orígenes, así como en la viabilidad microbiana y el grado de formación de la biopelícula. Se seleccionaron tres membranas de ósmosis inversa, dos de ellas procedentes de un sistema de tratamiento de acuíferos salinos y una de una planta desalinizadora de agua de mar. Los resultados mostraron que el origen del agua tratada afecta en gran medida a la comunidad microbiana, pero también la existencia de géneros comunes entre las distintas membranas, que podrían desempeñar un papel común en la formación de biopelículas en las membranas de ósmosis inversa y en otros sustratos plásticos.

El **Capítulo 3** se centra en la colonización bacteriana temprana de MPs en contacto con el efluente de dos plantas de tratamiento de aguas residuales, haciendo hincapié en la presencia de dos ARG (*sulI* y *tetM*). Las plantas de tratamiento de aguas residuales se consideran uno de los principales puntos de entrada de MPs y GRAs en el medio acuático.

A pesar de este hecho, existe un notable grado de desconocimiento sobre la capacidad de las MP para actuar como reservorio de ARGs. Los resultados mostraron que la ubicación (lugar de muestreo) y las propiedades del plástico (hidrofobicidad y rugosidad) desempeñaron un papel importante en la fase temprana de la colonización bacteriana. Además, se detectaron géneros específicos para cada tipo de polímero, lo que sugiere que el tipo de polímero determina la adhesión temprana de las bacterias. Aunque las aguas de los efluentes analizados contenían ambos GRAs (se detectaron *sulI* y *tetM*), los MPs concentraban sólo el gen *sulI*.

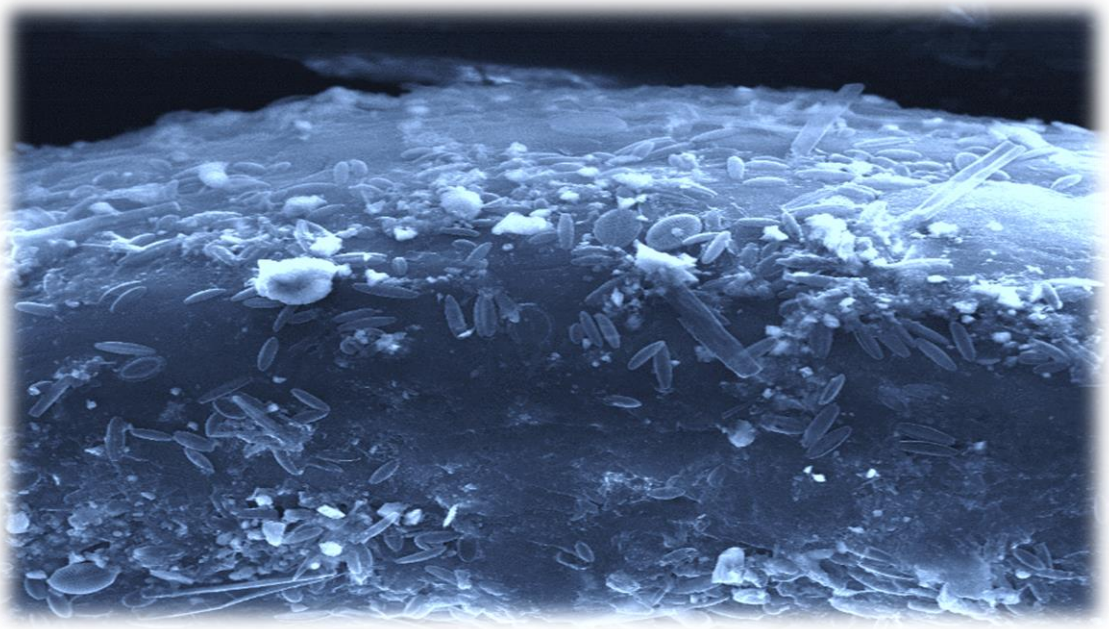
El **Capítulo 4** estudia la comunidad procariota y eucariota desarrollada en cuatro plásticos de uso habitual que se dejaron colonizar de forma natural en dos secciones del mismo río. Ambas localizaciones se caracterizaban por unas condiciones ambientales muy diferentes, una situada en un tramo con uso natural del suelo y la otra agua abajo de una planta de tratamiento de aguas residuales. El objetivo fue estudiar el proceso natural de colonización de residuos plásticos, que pueden quedar atrapados en una misma zona durante mucho tiempo. Se estudió la influencia del tipo de sustrato plástico y las condiciones específicas de la ubicación del río en la comunidad microbiana adherida. En concreto, uno de los lugares de muestreo se situó cerca del vertido de una EDAR con el fin de evaluar la influencia de sus efluentes sobre la capacidad de los plásticos para actuar como reservorio de GRAs. Los resultados confirmaron que la ubicación y el tipo de sustrato condicionaron fuertemente la comunidad procariota y eucariota desarrollada sobre ellos. El tiempo de contacto desempeñó un papel menos significativo. Los resultados mostraron una correlación entre la abundancia de los genes y la concentración de sus respectivos antibióticos.

El **Capítulo 5** explora la capacidad de los plásticos de invernadero para actuar como vectores de bacterias una vez que son abandonados en el medio ambiente al final de su vida útil. Los plásticos agrícolas se desechan a menudo de forma incontrolada, creando un problema medioambiental en las zonas agrícolas y en los cauces de los ríos y mares cercanos. En esta investigación se recogieron plásticos de invernadero, abandonados cerca de los mismos, en los cauces secos de los ríos, en el río y en el mar, siguiendo todo el recorrido que seguirían en un proceso normal de diseminación. Los resultados mostraron una diferencia significativa en las comunidades microbianas adheridas a los plásticos tomados del río y del mar en comparación con los tomados del suelo, lo que indica una evolución en la comunidad microbiana adherida a los plásticos. No obstante, la presencia de varios generos comunes adheridos al plástico independientemente del lugar de muestreo confirmó que los plásticos actúan como vectores de microorganismos.

El **Capítulo 6** es una Discusión General que resume los resultados de la Tesis Doctoral, discutiendo la relación entre los diferentes capítulos y con los objetivos fijados en este documento. Por último, las **Conclusiones Generales** exponen las conclusiones obtenidas en la Tesis Doctoral.

CHAPTER 1

INTRODUCTION



1. PLASTICS, FROM PROMISE TO REALITY

The term plastic refers to a wide range of organic materials, commonly known as synthetic polymers, which at some stage of manufacture, can be molded, extruded, melted, or spun (Thompson et al., 2009a). The first functional synthetic plastic developed was Bakelite, invented in 1907 by Leo Baekeland (Royappa, 1996). Initially, Bakelite was created as a substitute to cover the commercial demand of a resin called shellac, which was produced at a high cost from insects belonging to the Kerriidae family. Bakelite demonstrated better properties that natural lacquers, such as high resistance to chemicals, heat, and scratching. This plastic was used until the middle of the twentieth century, due to the development of new plastics, such as polyvinyl chloride and polystyrene, which competed in cost and eventually replaced it (Crespy et al., 2008). The development of this new generation of plastics marks the beginning of the Plastics Age.

The popularity of plastics is mainly due to its versatility. Plastics are resistant to light and chemicals, possess electrical insulation properties, withstand a wide range of temperatures, are relatively strong, and, due to their malleability, can be processed in almost all kinds of shapes and sizes (Thompson et al., 2009b). Since the 1950s, many types of synthetic polymers have been developed to satisfy different kinds of needs, including modified natural polymers, thermosetting plastics, thermoplastics and, more recently, biodegradable plastics and bioplastics (Andrady and Neal, 2009). Synthetic polymers can be classified into plastics and rubbers. Plastics are rigid at working temperatures while rubbers are elastic. Plastics can be either thermoplastics if they can be melt-processed or thermosets, the chains of which are cross-linked into a rigid shape. Lately, some types of thermoplastic elastomers have entered the market although the general classification is still valid (Gilbert, 2017). Currently, the most produced worldwide types of plastics include polypropylene (PP) (23%), low-density polyethylene (LDPE) and linear low-density polyethylene (LLDPE) (17%), polyvinyl chloride (PVC) (16%), high-density polyethylene (HDPE) (15%), polystyrene (PS) and expandable polystyrene (6%), polyethylene terephthalate (PET) (7%) and polyurethane (PU) (6%) (Statistica, 2022).

As a result, the demand for plastics has been steadily increasing. In 2019, global plastics production reached its peak value, manufacturing 368 million tons (Mt) (PlasticsEurope, 2021). If the increase in demand for plastics continues, it is expected that plastics production could double in 20 years to satisfy the industry and consumers' necessities (Lebreton and Andrady, 2019). In addition, it is estimated that the production of plastics represents 6% of global oil consumption that could increase to 20 % by 2050. To achieve

this production, 6% of the oil produced globally is needed, which could increase to 20% by 2050 if the current trend continues (WEF, 2016).

A major problem associated to plastics is that, after fulfilling their function, many of them are improperly discarded. Most of them are designed to be durable but a significant number of plastic goods have short lifetimes such as those used for packaging (which represented about one quarter of the volume of all plastics used) (Lebreton and Andrady, 2019). It has been estimated that in 2020, 29.5 Mt were collected as post-consumer waste in the Europe (EU) plus United Kingdom (UK), Norway and Switzerland through official schemes, equivalent to roughly half of the plastic produced in the same countries. Approximately 17.8 Mt of this amount corresponds to single-use packaging waste (short-life products, data from 2018). Only 34.6% was recycled, and 23.4% was sent directly to the landfill (PlasticsEurope, 2021, 2020) .This is of particular concern in developing countries, where landfills are the main way of plastic disposal (Agamuthu, 2012). Eventually, a considerable amount of plastic ends up in the environment. Furthermore, plastics accumulates in the environment because the rate at which plastic waste enters exceeds the natural rate of disappearance, which ranges from decades to centuries for common plastics (Chamas et al., 2020). Most methods of plastic removal from the environment such as periodic clean-up actions are ineffective in remote locations where plastics tend to accumulate. Accordingly, plastic can be classified as a "poorly reversible pollutant" (MacLeod et al., 2021). In this context, microplastics (MPs) plastics with their largest dimension between 1000 nm and 5 mm, see below) would be considered to readily meet the criteria for very persistent (vP) substances for different environmental compartments in Annex XIII of REACH (ECHA, 2015)

To solve this problem, the European Union, the United States, and other countries around the world have developed measures and policy initiatives aimed at establishing an integrated plastic waste management (Lebreton and Andrady, 2019). Such policies align with the concepts of circular economy, and involve the design of reusable plastics, or the use of biodegradable materials, which in the case of single-use plastics would alleviate the problem of waste generation (Moshood et al., 2021; Watkins et al., 2017). Among biodegradable polymers, there are currently two major categories. Firstly, there are the biodegradable polymers that originated from oil derivatives, such as polycaprolactone (PCL) or poly(butylene succinate) (PBS) (Tokiwa et al., 2009). Secondly, there are the bioplastics. Bioplastics are a good alternative because they are functionally similar to synthetic plastics. Bioplastics are produced from biomass, which includes vegetables such as potatoes, cotton,

corn, plant tissues or different types of microorganisms and fungi. This reduces dependence on the use of crude oil and its derivatives for the production of plastics (Thakur et al., 2018). Some of the most commonly used biopolymers include polylactic acid (PLA), polyhydroxybutyrate (PHB), polyhydroxyalkanoate (PHA) and various starch-based polymers (Atiwesh et al., 2021). These environmental policies and new bioplastics are still in the early stages of development, which means that the plastic problem does not still have a long-term solution (Wydra et al., 2021).

2. THE PLASTIC CYCLE

Plastics, once discarded in the environment, do not remain in the place where they have been abandoned. The discovery of large plastic islands in the Atlantic Ocean (Moore et al., 2001), and the occurrence of plastics in remote locations such as Antarctica (González-Pleiter et al., 2020; Jambeck et al., 2015; Lacerda et al., 2019), and high mountain lakes (Free et al., 2014), demonstrate the mobility of plastics between different ecosystems. This process is described in recent studies as similar to a biogeochemical cycle (such as those of water, phosphorus, or nitrogen). Plastics would thus not only move between different habitats but also have direct interactions with living beings. These considerations can be rationalized in a conceptual scheme model known as the plastic cycle, which is summarized in **Figure 1** (Bank and Hansson, 2019; Lecher, 2018).

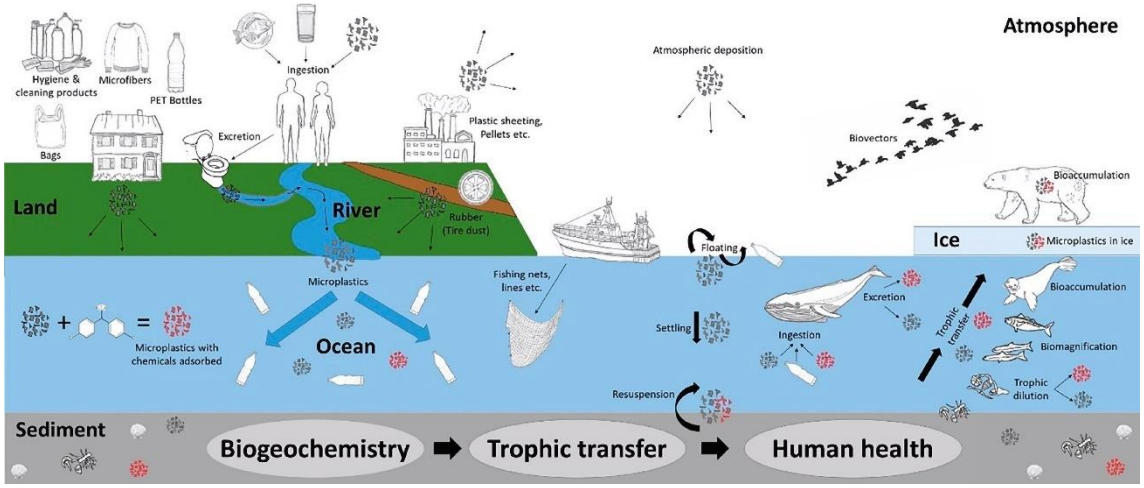


Figure 1. Conceptual model of plastic pollution cycle and the interactions between biogeochemistry, trophic transfer, and human health and exposure. Taken from Bank and Hansson (2019).

According to the available data, the plastic cycle has the ocean as the planet's ultimate sink for plastics (Bank and Hansson, 2019). There is considerable variability in the estimated volume of plastic reaching the ocean, though the consensus is that most of it are Macroplastics (MaPs). Borrelle et al. (2020) estimated, in 2019, between 19 to 26 Mt of MaPs waste reached the ocean and considered that these volumes would increase to 53 million

metric tons per year by 2030. Onink et al. (2021) estimated 1.15–12.7 Mt of plastic enter the ocean per year. Jambeck et al., (2015) estimated about 8 Mt of MaPs and 1.5 Mt of primary MPs entering the ocean annually. Cózar et al. (2014) was conservative, dividing the oceanic stock of 14.4 kt by a best MPs flux estimate of 6.1 kt $year^{-1}$ yields residence times around 2.4 years for the floating stock at the ocean surface.

The determination of the exact amount of plastic in the oceans is challenging and current studies oscillate by several orders of magnitude (Hardesty et al., 2017). Cózar et al. (2014) used the data from samples collected from Malaspina's circumnavigation cruise to make a global estimation of the load of plastic debris in oceans' surface ranging from 6.6 to 35.2 kt. Eriksen et al. (2014) used data from a set of 24 expeditions in different world's oceans to estimate the global plastic load in five trillion plastic particles with a weight of 269 kt. Once in the ocean, plastic is highly mobile, so it can be found in different areas, including surface, the entire water column, the ocean floor, sediments in estuaries and coasts, and even the land ice in polar areas (GESAMP, 2016). Specifically, ocean gyres are considered to be hotspots of plastic pollution and locations where the concentration of plastic can even exceed that of zooplankton (Barnes et al., 2009).

The main sources of plastics in the ocean are of land-based origin (approximately 80% of plastics), while 20% comes from ship waste including the fishing industry and intentional or accidental waste dumping from ships (Jambeck et al., 2015; Wayman and Niemann, 2021). The land-based sources include the natural waterways (rivers and seasonal streams), sewage/drainage systems, wind or plastic abandoned in coastal areas (Amaral-Zettler et al., 2020; Wayman and Niemann, 2021).

The differences in local catchment areas make it difficult to calculate the amount of plastic contributed by rivers (Jambeck et al., 2015). Conservative analysis estimates suggest that between 1.15 to 2.41 Mt of plastic waste enters the ocean each year from rivers and streams (Lebreton et al., 2017). This flow is highly variable and depends on multiple factors, including the season of the year, the land uses close to the river (including agriculture activities), and the proximity of nearby urban areas. (Jambeck et al., 2015). The input of plastics into rivers is conditioned by natural processes such as wind or surface runoff or direct discharges, including those from wastewater treatment plants (WWTPs) or mismanaged dumping sites (Barros and Seena, 2021; Edo et al., 2020).

It had been previously thought that most plastics come from only a few rivers but Meijier et al., (2022) found that rivers emitted around 1 Mt/year in 2015 but small rivers play

a significant role, and the top ten emitting rivers would contribute with 18% of the global load (compared with estimations ranging from 56% to 91% of previous studies).

Terrestrial ecosystems also participate in the plastic cycle. Fuller and Gautam (2016) detected concentrations of up to 6.7% of plastics in an industrial soil. Current research points to different origins for terrestrial plastics such as poor landfill management, use of contaminated soil amendments like sewage sludge and compost, plastic mulches, and greenhouse plastics, among others (Bläsing and Amelung, 2018; Edo et al., 2020).

The mobility of plastic is not only limited to terrestrial or aquatic transport. Recently the presence of small plastic fibers and fragments in the atmosphere has been reported even at high altitude, which may explain the presence of this material in totally pristine environments (Allen et al., 2019; Bergmann et al., 2019; González-Pleiter et al., 2021a; Janice et al., 2020; Melanie et al., 2022). Janice et al. (2020) studied the atmospheric deposition of plastics in several protected areas of the United States and found an average deposition rate of 132 plastics per square meter per day. González-Pleiter et al., (2021a) showed the atmospheric concentrations of MPs were higher in urban areas and models showed that MPs could be dispersed up to 400 km away. Bergmann et al. (2019) finds a large number of MPs in snow, in locations ranging from continental Europe to the Arctic, suggesting long-range movement of PMs at the atmospheric level.

3. PLASTIC SIZE CONDITIONS THE EFFECT OF PLASTICS TO ECOSYSTEMS

As illustrated in the plastic cycle (**Figure 1**), plastics break into bulk fragments of multiple sizes within the ecosystem. The large variety of sizes found in environmental samples, makes more relevant its classification by size classes instead of type or material (Frias and Nash, 2019; Ramkumar et al., 2021; Roch et al., 2021). It is important to note that plastics do not always come in small sizes because of environmental deterioration but may also have been created in smaller sizes for their functionality. In the last case, they are known as primary MPs, while those that have been downsized by environmental weathering are referred to as secondary MPs (Ramkumar et al., 2021).

In terms of size classification, there are different frameworks and classifications, so that there is not a fully agreed international standard (Lechthaler et al., 2020). The largest plastics are known as megaplastics and are those whose size exceeds one meter (GESAP, 2019). They are followed by the MaPs, which are plastic items with sizes between 1 m and 25 mm. After that, the mesoplastics, with size dimensions between 25 mm and 5 mm while plastics with their largest dimension between 1000 nm and 5 mm are called MPs (GESAP,

2019). The smallest particles, denoted as nanoplastics (NPs), are those plastics with <1000 nm in any other dimensions (GESAP, 2019).

The impact of MaPs on the environment is of great concern because of the evident interactions of live organisms with plastics (Lechthaler et al., 2020). Firstly, a large number of cases of wildlife trapped by large plastics have been recorded, affecting up to 243 species including sea turtles, sea mammals, birds and fishes (Gall and ThoMPsson, 2015). The entanglement with plastics can exert different types of wildlife damage consequences, including death by drowning or severe lacerations. Plastic entanglement may also reduce mobility, making it easier for the individuals to be trapped as prey or, on the contrary, to prevent them from hunting (Li et al., 2016). Secondly, animals can directly ingest large volumes of plastics. In most situations, this will not cause direct death, but can cause intestinal obstruction, affect digestive enzymes and hormones, and even reduce feeding stimulation (Lechthaler et al., 2020; Li et al., 2016). Another risk from MaPs is that they include different additives introduced during their manufacturing process that can be released in the open environment (Koelmans et al., 2014). Chemical risk is not limited to additives, because MaPs can adsorb various toxic compounds in contact with them once in the environment such as persistent organic pollutants, chlorinated pesticides, and polycyclic aromatic hydrocarbons (PAHs) or heavy metals (Nakashima et al., 2012; Rios et al., 2010). Eventually, macroplastics can break down under solar radiation and through mechanical fragmentation into MPs and NPs, resulting in new problems and making their removal from the environment difficult or even impossible (Kalogerakis et al., 2017).

The risks of MPSs are higher than the ones reported for MaPs since their smaller size facilitates their movement between ecosystems, which amplifies their associated risks (Shamskhany et al., 2021). Moreover, fauna promotes MPs mobility, thereby becoming biovectors, because they can transport them associated with parts of their bodies, as it has been shown for birds and bees (Bourdages et al., 2021; Edo et al., 2021). MPs can also be ingested by wildlife (Wesch et al., 2016). MPs can accumulate in individuals resulting in higher concentrations than those existing in the surrounding environment (Gobas and Morrison, 2000). Plastic pollution may increase through the food chain, generating bioaccumulation or biomagnification; in other words, organisms with higher positions in the food chain accumulate a greater number of plastics (Solomon et al., 2013). Humans, generally located at the end of the food chain , are also acceptors of MPs (Smith et al., 2018). In addition, MPs have a higher specific surface area than MaPs, which allows them to adsorb much higher concentrations of chemicals from the surrounding environment (Mato et al., 2001; Verdú et

al., 2022, 2021). MPs can also degrade under the effects of light, oxygen, temperature and mechanical erosion (Vighi et al., 2021). Furthermore, it has been demonstrated that invertebrates can promote this breakdown, leading to secondary NPs (Mateos-Cárdenas et al., 2020).

The effect of NPs on the environment is under-explored compared to that of MPs (Liuwei Wang et al., 2021). Despite this, the current hypothesis suggests that they may pose a greater risk (Vighi et al., 2021). The toxicological effect of NPs has been proved at the bacterial level, indicating that even the simplest organisms are at risk (Sun et al., 2018; Tamayo-Belda et al., 2021). NPs have also been shown capable to uptake nutrients and affect the development of plants (Liuwei Wang et al., 2021). Regarding human health, the presence of NPs in lung tissue has recently been discovered, indicating that some types of particles are bioavailable for our assimilation (Amato-Lourenço et al., 2021).

4. THE PLASTISPHERE AS AN EMERGING ECOSYSTEM

Until recently, the ability of microorganisms to colonize plastic waste and their possible impact on the environment was unknown. The first study describing the process date back to the 1970s, and revealed the presence of bacteria and diatoms attached to polystyrene spheres in coastal waters by microscope techniques (Carpenter et al., 1972). However, the complexity of this process would not be clarified until much later.

Zettler et al. (2013) identified the presence of a complex microbial community consisting of different microorganisms including autotrophs, heterotrophs, and predators on plastic fragments from marine ecosystems. Therefore, microorganisms not only colonize plastics but can develop their life cycle on the plastic, which results in the holistic concept referred to as "plastisphere" (Zettler et al., 2013). Nonetheless, the abundance of studies analyzing the plastisphere is still limited. Most of the research dedicated to the plastisphere is focused on coastal and marine environments (Agostini et al., 2021; Delacuvellerie et al., 2019; Du et al., 2022; Vannini et al., 2021). Studies on the plastisphere in other environments, such as freshwater (Barros and Seena, 2021; Di Pippo et al., 2020; Wang et al., 2021) or soil ecosystems (Li et al., 2021; MacLean et al., 2021; C. Wang et al., 2022)are steadily increasing.

In general, there are two strategies for the study of the plastisphere in aquatic environments, one of which involves experiments of microorganisms colonization in laboratory conditions or in the field and the other involves environmental sampling (Du et al., 2022). Laboratory studies offer the advantage of creating environmental conditions artificially, excluding the variability of natural environments, and help to develop accurate colonization models (Seeley et al., 2020). Field sampling, in contrast, despite its complexity

and higher cost, particularly in difficult to access locations (González-Pleiter et al., 2021b; Krause et al., 2020), allows obtaining more representative data (Du et al., 2022). The differences in experimental designs and the complexity of natural environments make it difficult to compare results between both types of studies but sheds light on the most common organisms found in the plastisphere. (De Tender. et al., 2017).

4.1. THE PLASTISPHERE FORMATION ONTO PLASTICS PRESENT IN AQUATIC ECOSYSTEMS

The colonization of plastics in aquatic ecosystems (graphically described in **Figure 2**) begins with the adhesion of microorganisms from the surrounding environment. The hard and persistent surface of plastic materials provides a suitable environment for microbial colonization (De Tender. et al., 2017). However, there are several characteristics of the plastic surface that affect its colonization, such as crystallinity, surface free energy, and particle shape and roughness (Rummel et al., 2017). Specifically, roughness, characterized by the small defects in the plastic developed during its manufacture, provide a surface with holes and grooves (Švorčík et al., 2006), which can be amplified by exposure to environmental conditions (Katsikogianni and Missirlis, 2004) and may promote the adherence of microorganisms (Hossain et al., 2019). On the contrary, the high hydrophobicity of most plastics complicates the initial attachment of microorganisms on the plastic surface (Wright et al., 2020). Besides the water column is loaded with organic matter and inorganic components, which may also adhere to the plastic surface. This first layer, called "ecocorona", significantly decreases surface hydrophobicity, allowing the pioneer microorganisms to adhere to the plastic substrate (Galloway et al., 2017).

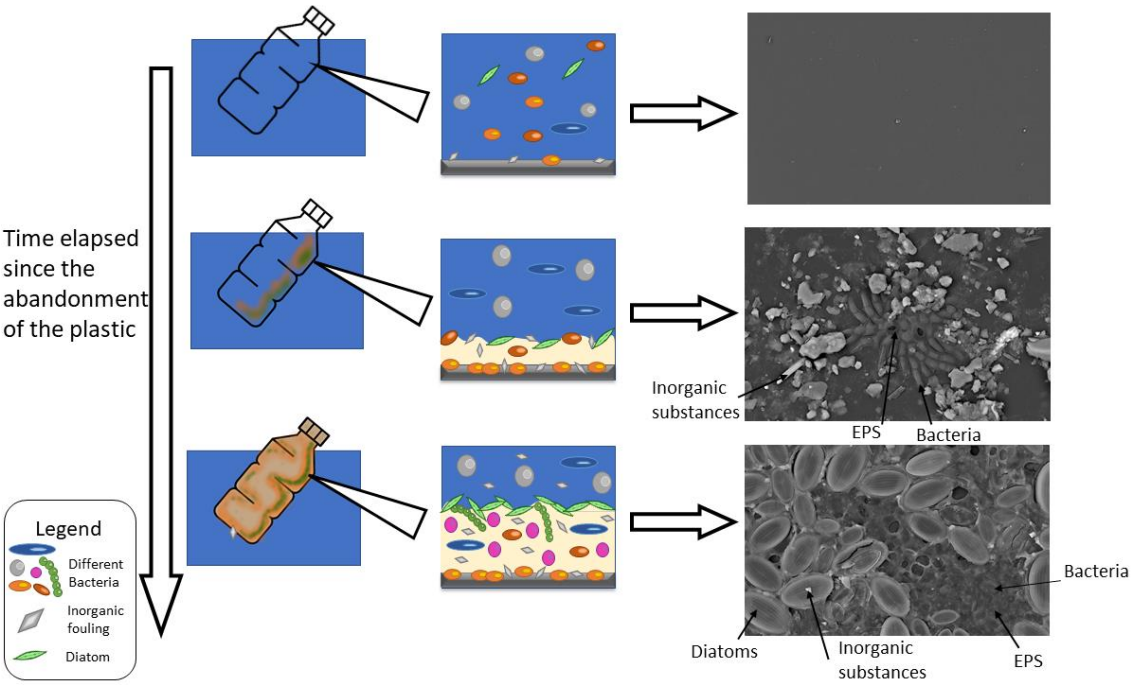


Figure 2. Microbial colonization of a plastic item in an aquatic environment. In the beginning, only pioneer organisms, which develop best on hard surfaces, are attached. Over time, the Extracellular Polymeric Substances (EPS) generated by the pioneer organisms allows the attachment of other microorganisms. In the end, a complex microbial community is formed. Photosynthetic microorganisms such as cyanobacteria and diatoms are distributed on the surface. The rest of the microorganisms are located in the biofilm according to their ecological niche. Some microorganisms in the water (shown in grey) do not attach to the plastisphere. Source: Author's elaboration

Pioneer microorganisms can form a reversible binding, resulting in different effects on the plastisphere during adhesion (Du et al., 2022). First, they decrease the hydrophobicity of the plastic surface (Chen et al., 2020; Lobelle and Cunliffe, 2011). Second, they generate Extracellular Polymeric Substances (EPS), which further facilitate the colonization by new microorganisms (Bhagwat et al., 2021; Kumar et al., 2020). The first colonizers can influence the following ones playing a role in selecting the type of community formed (Rummel et al., 2017). These pioneer microorganisms are commonly members of the classes Alphaproteobacteria and Gammaproteobacteria (De Tender et al., 2017; Dussud et al., 2018a; Quero and Luna, 2017), with the genera *Alteromonas*, *Thalassobius, Neptuniibacter* and *Poseobacter* prominent within these classes (Zhang et al., 2022). Several studies also reported the presence of diatoms and cyanobacteria in this first phase (Amaral-Zettler et al., 2015).

Secondary microorganisms, that follow the first colonizers, cause the irreversible attachment of the microbial community to the plastic by several strategies, such as the synthesis of more EPS, the generation of adhesion proteins, or the use of cellular structures such as pili (Dussud et al., 2018a). This process also expands the colonizable surface and attracts more microorganisms to the plastisphere (Lorite et al., 2011). These microorganisms commonly belong to the class Bacteroidetes, especially the family Flavobacteriaceae (Quero

and Luna, 2017; Zhang et al., 2022). The community attached to the plastic matures when the different processes of competitiveness or synergy produced among the species stabilize, reducing the changes in the community (Lorite et al., 2011; Wright et al., 2020).

The timescale for this process is still not entirely elucidated; some studies report that the process starts only a few minutes after the plastic comes into contact with water (Quero and Luna, 2017). This first phase can be prolonged for a couple of days (Erni-Cassola et al., 2020) to a few weeks (Chen et al., 2020). The intermediate phases of colonization can extend over months and the precise timing is not known; it is only apparently that the whole process strongly depends on the environmental conditions (Du et al., 2022).

4.2. The diversity of the plastisphere

Several studies have shown that the communities embedded in plastic are different from those on natural particles of other materials (such as glass, wood or rock) located in the same environment either in marine or freshwater ecosystems (Barros and Seena, 2021; Dussud et al., 2018a; Kirstein et al., 2019). They are also different from the surrounding water (Amaral-Zettler et al., 2015; Frère et al., 2018; Zettler et al., 2013) and, eventually, from communities formed on other plastic materials colonized in the proximity (Dussud et al., 2018a; Witt et al., 2011). However, to date, no study has demonstrated the presence of taxa exclusive from the plastisphere and absent from the surrounding environment (Amaral-Zettler et al., 2020).

In general, the richness of the communities attached to the MPs and MaPs tend to be lower than the surrounding environment (Amaral-Zettler et al., 2015; Dussud et al., 2018a; Zettler et al., 2013). Hoellein et al. (2014) suggested that the plastisphere could have a major diversity in eutrophic environments. The limited diversity would explain the specificity of certain microorganisms to colonize plastics and, although the rate of biodegradation of plastics is very low, the presence of specific taxa capable of using plastic as a carbon source, such as Oceanospirillales and Alteromonadales, has been frequently described (Chen et al., 2019; Wright et al., 2021). Most studies are focused on prokaryotic rather than eukaryotic microorganisms, which may be due to the low number of 18S rRNA gene copies obtained during metabarcoding studies (Amaral-Zettler et al., 2020).

The few studies that analyze beta diversity attributed the differences observed in the microbial community attached to plastics to a variety of factors. Recent studies have shown that local variables play the most important role to determine the microbial community developed on plastics. Wright et al., (2021) performed a meta-analysis that included 35 studies examining the plastisphere by 16S rRNA sequencing. The results confirmed that geographic

location followed by salinity were the most important factors conditioning plastic colonization. Furthermore, environmental factors such as oxygen available, light, temperature, nutrients and the presence of contaminants play important roles in the development of plastic colonizing community (Chen et al., 2019; Hoellein et al., 2014; Wagner et al., 2014).

The physical and chemical properties of the substrates (hydrophobicity, roughness, size, type of polymer) may select the microorganisms that attach to them (Battin et al., 2016). In particular, the size of the plastic plays a fundamental role (**Figure 3**). Larger plastics, such as MaPs, have sufficient surface to allow the development of complex and complete biofilms (Rogers et al., 2020). Moreover, fully developed biofilms can attract macroinvertebrates that use them as food, many of which are slow-moving or practically immobile (De-la-Torre et al., 2021). Smaller plastics, such as MPs, allow the development of smaller biofilms but due to their smaller size, mobility of microorganisms is increased. (Rogers et al., 2020). In the case of even smaller sizes, such as NPs, microorganisms do not have sufficient surface to attach. On the contrary, NPs are expected to become embedded in the matrix of EPS generated by the bacteria, which facilitates their aggregation, although they do not serve as support (Rogers et al., 2020).

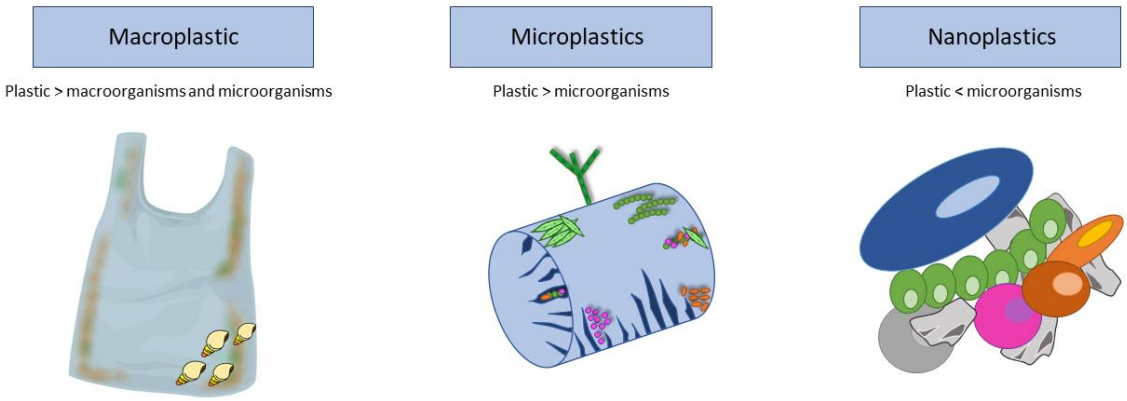


Figure 3. Plastic size determines the colonizing organisms. Macroplastics and mesoplastics allow large organisms, such as invertebrates or macroalgae, to attach to the plastics. Microplastics, which are smaller, serve as a substrate only for microorganisms. Ultimately, nanoplastics are too small serve as support even for single cell organisms, but they can bind to microbial EPS. Adapted from Rogers et al., (2020).

4.3. MICROBIAL GROUPS IN THE PLASTISPHERE

The most recent studies showed that once the microbial community developed on plastics has matured, a complex micro-ecosystem is established that depends mainly on the synergies and relationships of the attached organisms as well as on the resources available in the environment (Amaral-Zettler et al., 2020; Li et al., 2021). The idea that microorganisms can use plastics as a carbon source until their mineralization is not generally consistent with the persistence of these materials (Oberbeckmann and Labrenz, 2020). Therefore, the plastisphere depends on external water, nutrients, and energy to grow. Aquatic environments are not water-limited, but will depend on nutrients and energy from the environment. (Wright et al., 2020). To optimize resources, the microorganisms developed in the plastisphere diverge and assume well-defined ecological niches organized in primary producers (such as phototrophs), predators, decomposers and heterotrophs (Amaral-Zettler et al., 2020). **Figure 4** shows some of these microorganisms and the roles that they perform.

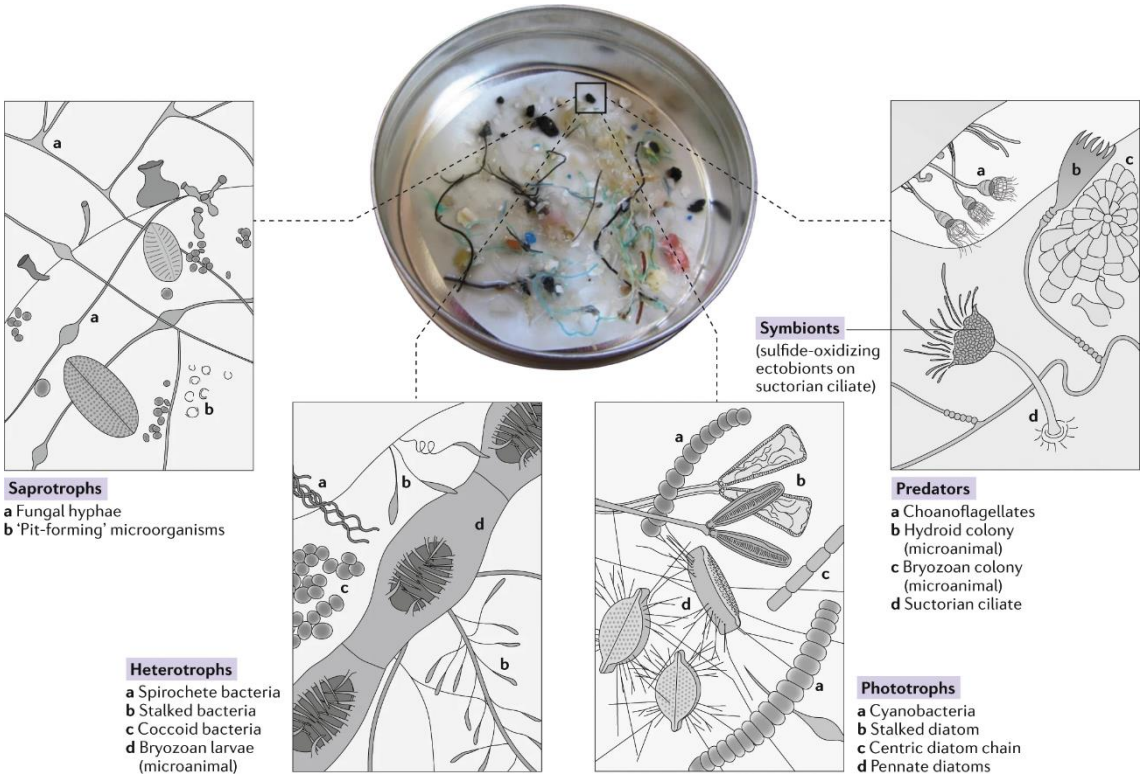


Figure 4. Conceptual model of the microbial community associated with the plastisphere in the open ocean. These ecosystems include different microbial organisms such as bacteria, protists and animals that play very diverse roles, such as primary producers, herbivores, predators, heterotrophs or organisms capable of developing different types of symbiotic relationships. Taken from Amaral-Zettler et al. (2020)

The most dominant of all phototrophic organisms are diatoms, as long as the plastics are exposed to light (Amaral-Zettler et al., 2020). The studies that describe them report their occurrence in the early stages of biofilm formation and as predominant in more advanced stages of colonization (Kettner et al., 2019; Oberbeckmann et al., 2014). The genera

identified on plastics included *Sellaphora*, *Amphora*, *Mastogloia* and *Nitzschia* (Muthukrishnan et al., 2019). In addition to diatoms, cyanobacteria are another group of phototrophs usually found in the plastisphere assemblage. Their abundance in MPSs was found to be higher than that of the surrounding water column. (Bryant et al., 2016). Cyanobacteria are generally located in the outer layers of the biofilm embedded in the EPS matrix. Their development facilitates the generation of organic macromolecules produced by photosynthesis, which helps the development of heterotrophic organisms located in the lower layers in the biofilm (Di Pippo et al., 2020).

Regarding heterotrophic organisms, the presence of photoheterotrophs, such as those from the genera *Erythrobacter* and *Roseobacter*, is especially remarkable. Some species of these genera produce bacteriochlorophyll, which fixes carbon dioxide without producing oxygen (Amaral-Zettler et al., 2020). Within the organisms commonly considered heterotrophs, some studies highlighted the presence of *Pseudomonas* and *Azotobacter* (Amaral-Zettler et al., 2020). The importance of heterotrophic organisms is interesting for several reasons. Some of them, such as *Pseudomonas*, are known for their high capacity to generate EPS, which enhances the biofilm matrix and its capacity to degrade and metabolize plastics (Wilkes and Aristilde, 2017). Other organisms need the collaboration of heterotrophs to degrade plastics, resulting in microorganism consortiums. Wang et al., (2022) detected a decrease in alpha diversity in plastisphere attached to PE mulching film over time, as the community adapted to use the plastic as a carbon source. The microorganisms that formed this consortium included members of the genera *Pseudomonas*, *Methylobacillus*, *Methylotenera*, *Acinetobacter* and *Sphingopyxis*. Joshi et al., (2022) obtained higher degradation of LDPE after isolating the microbial community attached to plastics from the seafloor than using common organisms used in biodegradation or individual cultures of the same microorganisms. Cameron et al., (2022) detected the presence of genes encoding enzymes for the degradation of the main components of PET in an environmental consortium dominated mainly by species of the genera *Bacillus* and *Pseudomonas*.

Some microorganisms can produce exoenzymes capable of altering the polymer surface thereby reducing hydrophobicity, increasing bioavailability and allowing other organisms to colonize and degrade the plastic (Tu et al., 2020). This process has been studied in the case of PET under the influence of the bacteria *Ideonella sakaiensis* and the filamentous fungus *Fusarium oxysporum* (Yoshida et al., 2016). Furthermore, the proximity between phototrophic and heterotrophic organisms allows creating a nutrient cycle between them,

which promotes the development of the plastisphere community (Bryant et al., 2016; Di Pippo et al., 2020).

The growth of the microbial community gives the opportunity for different predators to join the plastisphere. Within this group, several studies have recognized the presence of ciliates (such as the genus *Ephelota*), choanoflagellates, radiolaria, and small flagellates such as *Micromonas* (Bryant et al., 2016; Zettler et al., 2013).

4.4. Risks associated to the plastisphere

The scientific interest in the plastisphere is not only based on the need to understand its ecology but also on the risks that colonizing organisms may pose for the environment and for human health. This problem is mainly due to the ability of plastics to move between different ecosystems, which allows them to act as vectors for certain harmful organisms. Within them, three distinct categories can be differentiated: invasive species, pathogenic organisms, and antibiotic-resistant bacteria (ARBs), the latter being carriers of antibiotic resistance genes (ARGs) (Barros and Seena, 2021; Wu et al., 2022).

4.4.1. Invasive species

Invasive species are organisms that move into an ecosystem in which they are non-native to occupy pre-existing ecological niches. Invasive species are capable to compete with local species, to alter the ecosystem's food webs, and to reduce biodiversity (Didham et al., 2005). It has been stated that the global diversity of marine species could decrease by 58% in a scenario of a global biotic mixing (Derraik, 2002).

Accordingly, the bryozoan species *Membranipora tuberculata* has been reported to travelled from Australia to New Zealand, crossing the Tasman Sea attached to plastic litter (Gregory, 1978). *Electro tenella*, another bryozoan was found on plastic wastes on the Florida Coast, EE.UU, and they could increase their population by drifting in plastic from the Caribbean (Derraik, 2002; Winston et al., 1997). Pinochet et al., (2020) showed that bryozoan larvae preferably colonized marine plastics rather than other natural substrates such as wood, which facilitates their subsequent dispersal in the ocean (Minchin, 1996).

Most confirmed cases of mobility of invasive organisms involve sessile species, which attach to plastics during their larval stage. Węsławski and Kotwicki (2018) proved the presence of the barnacle *Lepas anatifera* associated with MaPs found on the coasts of Svalbard, an allochthonous organism not documented before in that area.

Garcia-Vazquez et al. (2018) showed the presence of several non-native species attached to different plastic debris such as the barnacle *Amphilabanus improvisus*, the sea snail *Crepidula fornicata*, the oyster *Magallanas gigas* and the alga *Chorda filum* in the Gothenburg

region of Sweden. Specifically, the latter two species were also found in nearby rocky substrates, which could indicate a subsequent dispersal.

These results confirm that MPs and MaPs can potentially serve as vectors for invasive organisms, but more studies are required to assess the risk that this may pose. **Table 1** summarizes some of these studies.

Table 1.
Studies that have detected possible invasive species associated to plastic transport

Plastic substrate	Environment	Results	Location	Reference
Virgin plastic pellets of PE and PP	Beach, sea	The bryozoan specie *Membranipora tuberculate* is associated with several of these pellets on New Zealand beaches. This specie is typical of tropical and subtropical environments, as it was found associated with plastic pellets in Australia, suggesting a possible transport between both places	New Zealand	Gregory (1978)
Undefined plastic trash	Beach, sea	The bryozoan specie *Electro tenella* was the most abundant bryozoan on plastic trash in Florida coast, possible travelling from Bermuda	Florida, EE. UU	Winston et al. (1997)
Different types of large plastic objects (MaPs)	Coast, sea	The barnacle *Lepas anatifera*, never described in the area, is identified, associated with different types of MaPs	Svalbard, Artic	Węsławski and Kotwicki (2018)
Different types of large plastic litter (mesoplastics and MaPs)	Coast, sea	Several allochthonous species were identified in the region such as the barnacle specie *Amphilabanus improvisus*, the sea snail specie *Crepidula fornicata*, the oyster specie *Magallanas gigas* and the specie *Chorda filum*	Gothenburg, Sweden	Garcia-Vazquez et al. (2018)

4.4.2. PATHOGENIC AND TOXIC SPECIES

The first report on the possibility that plastics may facilitate the dispersal of pathogens and algae/bacteria capable of releasing toxins was delivered before the concept of plastisphere was established. Masó et al., (2003) detected the presence of two dinoflagellate species (*Coolia sp.* and *Ostropsis sp.*) with the potential to release toxins attached to plastic litter in the Mediterranean Sea. After that, the possibility that plastics may facilitate the dispersal of pathogenic microorganisms and/or algae/bacteria capable of releasing toxins was recognized as a major scientific concern (Bowley et al., 2021).

The initial studies focused on the detection of the genus *Vibrio* (Bowley et al., 2021). This genus is characterized by growing mainly in coastal environments (Bowley et al., 2021). Most *Vibrio* species are harmless, although some are known to cause diseases in humans and wildlife including mollusks, fish and crustaceans (Lafferty et al., 2015). Numerous studies confirmed the presence of *Vibrio* attached to plastics in the middle of the ocean (Frère et al., 2018; Kirstein et al., 2016; Sun et al., 2020; Zhang et al., 2020). In contrast to their usual coastal habitats, these findings suggest that floating plastics are capable of displacing *Vibrio* over long distances. (Bowley et al., 2021). Other potentially pathogenic microorganisms found associated with plastics in marine environments include the species *Aeromonas salmonicida* or *Arcobacter spp.* (Amaral-Zettler et al., 2020; De Tender et al., 2015; Frère et al., 2018; Kirstein et al., 2016).

The detection of possibly pathogenic microorganisms in the plastisphere also includes freshwater environments (Barros and Seena, 2021). The presence of *Vibrio*, although generally endemic to marine environments, has also been found in freshwater environments (Laverty et al., 2020; Xue et al., 2020). Specifically, Laverty et al. (2020) isolated three pathogenic species: *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio cholera* from three MPs (PS, PE, and PP) recovered from the Elizabeth River estuary, in the United States. Other possibly pathogenic genera that appeared in freshwaters associated with plastics include *Pseudomonas* (McCormick et al., 2016; Wu et al., 2019; Xue et al., 2020)*, Acinetobacter* (McCormick et al., 2016; Xue et al., 2020)*, Arcobacter* (McCormick et al., 2014, 2016)*, Tenacibaculum* (McCormick et al., 2016)*, and Aeromonas* (Shi et al., 2021). Specifically, Wu et al., (2019) identified three pathogenic *Pseudomonas* species: *Pseudomonas mendocina* (induce nosocomial infections), *Pseudomonas monteilii* (cause hypersensitivity pseumonitis and bronchiectasis) and *Pseudomonas syringae* (exclusive plant pathogen) in MPs biofilms but not in biofilms formed on natural substrates.

The most recent studies indicate that WWTP effluents are one of the main pathways for the release of MPs colonized by pathogenic microorganisms to the environment (Junaid et al., 2022). Most of the genera detected are consistent with those previously detected in freshwater plastics such as *Vibrio*, *Pseudomonas*, *Acinetobacter* and *Arcobacter* (Junaid et al., 2022). Furthermore, the genus *Bacillus*, specifically the species *Bacillus anthracis* was detected in an abundance higher than 1% in MPs incubated in domestic wastewater from Shanghai, China, showing an anthropogenic enrichment of pathogenic microorganisms in plastics delivered to the environment (Shi et al., 2021).

Concerning microorganisms capable of releasing toxic compounds into the environment, microalgae attached to plastics have a relevant role in this process (Caruso, 2019). Studies in this field are still limited, although they show evidence on plastics colonization by taxonomic groups relevant for toxin production, such as cyanobacteria and dinoflagellates (Casabianca et al., 2019; Masó et al., 2003; Oberbeckmann et al., 2014). Casabianca et al. (2019) identified the presence of the harmful algal species *Pseudo-nitzschia multiseries*, *Pseudo-nitzschia australis*, *Pseudo-nitzschia seriata,* and *Pseudo-nitzschia multistriata* in several plastic samples collected from the Mediterranean Sea. These plastics, if ingested, cannot only represent a risk to the environment but also to food chains and might end up causing human health problems (Casabianca et al., 2019). **Table 2** summarizes the results of some of these studies.

Table 2.
A summary of the studies that have identified pathogen species and toxin-generating species associated to plastic transport

Plastic substrate	Environment	Results	Location	Reference
MPs obtained from the fragmentation of marine aquaculture nets	Marine aquaculture area, sea	The potentially pathogen genus *Vibrio* appeared significantly abundant after 3 hours of colonization and was kept until 21 days after colonization	Sungo Bay, China	Sun et al. (2020)
Collection of different types of MPs including PE, PS and PP	Sea	The *Vibrio* genus was found on the collected MPs. Specifically, the species *Vibrio splendidus*, a pathogen of oyster appeared in high relative abundance	Bay of Brest (France)	Frère et al. (2018)
Different kinds of plastic debris collected from seven sampling sites	River	Occurrence of several potentially pathogenic genera attached to the plastics such as *Pseudomonas*, *Acinetobacter* and *Vibrio*	Urumqi river (China)	Xue et al. (2020)
MPs collected and subsequently identified as PE, PP and PS	Estuarine water	Three types of pathogenic *Vibrio*: *Vibrio cholerae*, *Vibrio vulnificus* and *Vibrio parahaemolyticus* were cultured from the collected samples	Elizabeth river (EEUU)	Laverty et al. (2020)
MPs obtained from landfill leachates, overall PS	Landfill leachates	Several human pathogenic species associated with plastics were detected, such as *Acinetobacter lwoffii*, *Afipia broomeae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and pathogenic *Escherichia coli*	Shanghái, China	Shi et al. (2020)
Floating plastic litter	Sea	Potential toxin-generating species *Coolia sp.* and *Ostropsis sp.* appear attached to the plastics	Mediterranean Sea	Masó et al. (2003)
Floating plastic litter	Shoreline	The harmful algal species *Pseudo-nitzschia multiseries*, *Pseudo-nitzschia australis*, *Pseudo-nitzschia seriata*, and *Pseudo-nitzschia* multistriata are associated with floating plastic litter	Mediterranean Sea	Casabianca et al. (2019)

4.4.3. Persistence and propagation of ARGs in the plastisphere

WWTP effluents are not only the main entry points of MPs and NPs into aquatic environments but also one of the main hotspots for ARBs and cognate ARGs and (Liu et al., 2021; Sathicq et al., 2021). The reason for this is the massive use of antibiotics in human and veterinary medicine, animal farming and agro-industrial production, which causes their subsequent release through water treatment facilities and, therefore, the development of antibiotic resistance by the microbiota (Guo et al., 2017; Taylor et al., 2011). The ability of ARBs to colonize plastics can provide them with a unique habitat that allows them to survive and to colonize new habitats as the plastic moves (Oberbeckmann et al., 2018; Sathicq et al., 2021).

ARBs, once attached to the plastic, can transfer their ARGs to the rest of the microbial community through horizontal gene transfer (Syranidou and Kalogerakis, 2022). Conjugation is the most important process of ARGs transfer (Zhang et al., 2021). ARGs are usually part of mobile genetic elements such as plasmids or integrons, especially in the case of commonly used antibiotics (Zhang et al., 2021). Moreover, class 1 integron-integrase genes (*intI*1) have been detected in higher abundances on the surface of MPs than in the surrounding environment (Wang et al., 2020). Consequently, plastics can serve as a hotspot for ARGs, promoting gene transfer between the plastisphere and the environment and causing the proliferation of ARBs in the open environment (Imran et al., 2019).

The factors promoting the development of the so-called antibiotic resistome (collection of all genes that directly or indirectly contribute to antibiotic resistance) in plastic substrates are currently under investigation (Syranidou and Kalogerakis, 2022). ARGs have been described in plastics from different environments subject to anthropogenic contamination including soil (Yan et al., 2020), freshwater (Li et al., 2021) and seawater environments (Karkanorachaki et al., 2021). A correlation has been found between certain taxa and specific ARGs. The phylum Firmicutes and the genus *Bacillus* correlated with the abundance of the *tetA* (resistance to tetracycline) and *sul1* (resistance to sulfonamide) genes. In contrast, *Pseudomonas* correlated with the *copA* (copper resistance) and *zntB* (zinc resistance) genes (Guo et al., 2020).

On the contrary, there are factors whose influence on the development of the resistome in the plastisphere has not yet been entirely clarified (Syranidou and Kalogerakis, 2022). The presence and concentration of antibiotics is a determining factor in the development of ARGs, even though it is not known whether an antibiotic promotes the development of its specific resistance gene, which could imply a co-selection among different antibiotics present in the same environment (Zhao et al., 2020). Bengtsson-Palme et al. (2016) found no correlation but co-selection occurred in ARGs found in effluents of Swedish WWTPs.

The type of polymer is another variable with unclear influence (Syranidou and Kalogerakis, 2022). Parrish and Fahrenfeld (2019) observed a significant abundance of *sul1* (resistance to sulfonamide) gene in MPs but did not detect significant differences among substrates. On the contrary, Guo et al. (2020) detected a higher relative abundance of ARGs in PE compared to PP, PS, PET or PVC particles.

Despite all the information gathered in recent years, there is still much to be studied about the development of the resistome in the plastisphere. Specifically, It is still unclear the

way ARGs abundance changes with environmental factors or with the concentration of antibiotics (Syranidou and Kalogerakis, 2022). **Table 3** summarizes the results of some of these studies.

Table 3.
A summary of ARGs identified in plastics

Plastic substrate	Environment	Results	Location	Reference
PE plastic film cut in MPs fragments	River, estuary and marine waters (mesocosms)	There was an increase in *intI1* and *intI2* genes (gene transfer operators) in the MPs, responsible for ARG transfer. There was also an enrichment of *sul1* (sulfonamide resistance), *tetC* (tetracycline resistance), *tetX* (tetracycline resistance) and *ermE* (macrolide resistance) genes in the MPs. MPs accumulated more ARGs in freshwaters	Lab	Wang et al. (2020)
Plastic samples with biofilm associated and identified as PS, PET, PP, PE and PVC	Estuary	The genes *intI1* (gene transfer operator), *sul1* (sulfonamide resistance), *tetA* (tetracycline resistance), *tetW* (tetracycline resistance), *aac(6')-Ib* (fluoroquinolones resistance), *Chl* (chloramphenicol resistance), *copA* (copper resistance) and *zntB* (zinc resistance) showed higher abundance in plastics than the surrounding water and the collected sediment	Yangtze Estuary (China)	Guo et al. (2020)
Five types of incubated MPs (PE, PP, PS, PE-fiber and PE-fiber-PE)	River	ARGs detected in MPS conferred resistance to almost all major classes of antibiotics commonly used for humans and animals. Furthermore, anthropogenic influence had the greatest effect on ARG enrichment in the MPs.	Beilun River (China)	Li et al. (2021)
MPs of PS and PE	Batch reactors	Differences between particles were detected even though no significant *sul1* gene differences were detected with the reactor water.	Lab	Parrish and Fahrenfeld (2019)

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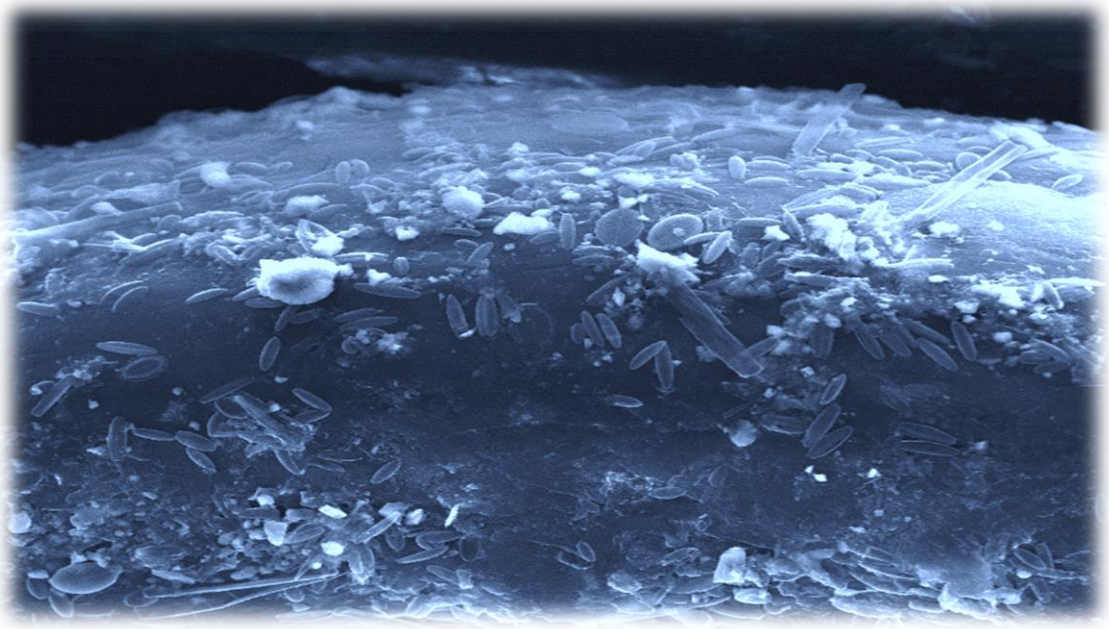
HYPOTHESIS AND OBJECTIVES



The plastisphere is a novel ecosystem that has attracted the interest of the scientific community working on the impact of plastics in the environment. There are still many questions to be answered about the role of plastics as a new niche for microorganisms. This new microbial habitat might already be performing a role at the ecological level. Most studies have been done in marine environments with few tackling freshwater systems and soils. Some studies indicate that geography and season are the main factors in shaping microbial communities in plastics; however, more research is necessary in order to know whether the plastisphere really differs between different sites or even latitudes. In addition, the complexity of the plastisphere may depend on the timing of microorganisms' colonization and this should be studied more deeply. In this regard, early-stage development as well as long-stablished plastisphere communities are not well known. In addition, the type of plastic might select for specific types of microbial communities and there might be important differences between biodegradable and non- biodegradable plastics. The size and ageing of plastics released into the environment might also select for specific microorganisms. Microorganisms forming part of the plastisphere might be pathogens and/or carry antibiotic resistance genes (ARGs); so that environmental plastics might act as vector for the disseminations of pathogens and/or ARGs. The main goal of this Ph.D. thesis is to address some of the above cited knowledge gaps on plastisphere research, as well as to shed light on the potential of plastics to act as reservoirs and vectors of ARGs.

The initial hypothesis is that the communities of microorganisms attached to plastics might be different among the different tested plastics, particularly between biodegradable and non-biodegradable ones, and different to free-living water or soil bacteria and to those colonizing other natural or artificial substrates. Furthermore, we hypothesized that those bacteria colonizing plastics might act as reservoirs and eventually as vectors of ARGs contributing to their global spread. We also hypothesized that other factors, besides the type of polymer, such as colonization time, geographical location or other environmental conditions may affect the development and complexity of the plastisphere.

SPECIFIC OBJECTIVES:

1. Characterization of the plastisphere developed on plastics of different type (biodegradable as well as non-biodegradable plastics), size (micro and macroplastics) and use (reverse osmosis membranes, greenhouse plastics and everyday use plastic items).
2. Identification of specific plastic-core microbiomes by comparing the plastisphere to microbial colonization on non-plastic substrates or to free-living water or soil microorganisms.
3. Identification of factors that might affect the formation of the plastisphere, considering location (WWTPs effluents, rivers, greenhouse, soil and sea), colonization time, intrinsic plastic properties (roughness and hydrophobicity), plastic ageing in the environment and different environmental conditions (salinity, pH, water chemical parameters such as nutrients and antibiotic concentrations).
4. Characterization of the role of plastics as vectors for certain bacteria allowing their transfer between different environmental compartments by studying greenhouse plastic-associated bacterial community changes during its lifecycle from its initial point of use towards receiving environments.
5. Assessment of the role of plastics as reservoirs of ARGs and eventually as ARGs vectors contributing to their global spread.

CHAPTER 2 *CHARACTERIZATION OF MICROBIAL COLONIZATION AND DIVERSITY IN REVERSE OSMOSIS MEMBRANE AUTOPSY*

ABSTRACT

Biofouling can cause serious problems in reverse osmosis membranes (RO membranes) reducing module performance and their useful life. The main goal of this study was to gain insight into microbial colonization of used RO membranes with different feed water and inorganic fouling. We studied three RO membranes. Two were collected from the same desalination plant, fed with brackish water. These membranes belonged to two consecutive phases of the desalination process. The third one was from a seawater desalination plant. A three-tiered approach was proposed: The first-tiered approach was the use of SEM to detect fouling and presence of adhered microorganisms on the RO membranes. The second-tiered approach was to use specific stains, which indicated viable cells and the presence of extracellular biofilm matrix due to microbial colonization; ATR-FTIR was used to better determine the chemical nature of the matrix. The third-tiered approach was Illumina sequencing to study microbial composition and diversity. The study helped identifying key microorganisms (bacteria and fungi) as biofilm formers and the extent of the biofilm matrix; this knowledge may be useful for new antifouling treatments.

1. INTRODUCTION

Reverse Osmosis (RO) membranes are the most used technology for water desalination (Burn et al., 2015). Despite their widespread use, RO membranes have some important problems like the high energetic cost (Karagiannis and Soldatos, 2008) or an easy deterioration by oxidizing agents (Zhao et al., 2001). However, the biggest problem of RO membranes is fouling. Fouling is the accumulation of unwanted material on the membrane. Fouling produces a decrease of obtained permeated and a reduction of ionic rejection (Baker, 2005). There are four types of fouling: inorganic (produced by precipitation of salts), organic (composed by humic acid), colloidal (suspended particles) and biofouling (generated by microorganism such as bacteria, fungi, algae that usually form biofilms on the membrane) (Guo et al., 2012). Feed water chemistry or intrinsic membrane properties may greatly affect membrane fouling (Ke et al., 2013).

Biofouling affects more than one-third of RO membranes (Chesters et al., 2013) and, normally, it is only detected but not fully characterized. For this reason, only general strategies exist to eliminate or prevent biofouling like chlorination, changes in membrane surface properties (hydrophobicity and roughness) or a chemical cleaning (Chesters et al., 2013; Khan et al., 2015; Landaburu-Aguirre et al., 2016; Oh et al., 2018; Subramani and Hoek, 2010). However, these techniques are not entirely efficient, for example, chlorination cannot eliminate initial biofilm formed because bacteria can be resistant to chemical stress or can grow after the treatment (Subramani and Hoek, 2010). Thus, strategies for microbial antifouling have to rely on knowledge of the potential causes and monitoring of biofilm formation should be implemented.

Studies on biofilm development in RO membranes have evolved from culture-dependent methods, genetic clone libraries, fluorescence in situ hybridization to –omics (for a comprehensive review, see Sánchez (2018). Many of these studies involved advanced wastewater treatments, effluents from industrial or water purification plants or laboratory–scale RO systems and only a few addressed RO membranes from desalination plants (Al Ashhab et al., 2014; Ben-Dov et al., 2016; Levi et al., 2016; Oh et al., 2018); –omics studies overcome the limitation of biased-selectivity of culture-dependent methods and facilitates a deeper knowledge of the real microbial composition of RO membranes biofilms. Mostly, pyrosequencing platforms have been used (Al Ashhab et al., 2014; Ayache et al., 2013; Ferrera et al., 2015; Khan et al., 2015, 2013; Kim et al., 2014) and in a few cases Illumina sequencing has been used (Chamberland et al., 2017; Zodrow et al., 2014). Studies have dealt with bacterial identification and diversity, paying less or no attention to fungal diversity (Al

Ashhab et al., 2014, 2017). Within bacteria, the phylum Proteobacteria has been found to be dominant in all studies; within Proteobacteria, family Sphingomonadaceae, particularly genus *Sphingomonas*, seems to be involved in biofilm initiation while family Rhodobacteracea seems to be associated with mature biofilms (Al Ashhab et al., 2014, 2017; Bereschenko et al., 2011, 2008; Chen et al., 2004). With regards to fungi, Al Ashhab et al., (2014) found that phyla Ascomycota and Basidiomycota were dominant in RO membranes from filtered treated wastewaters.

The objective of this study was to gain insight into microbial colonization of used RO membranes with different feed water and inorganic fouling. We studied three RO membranes, two were collected from the same desalination plant, fed with brackish water and the third was from a seawater desalination plant. A three-tiered approach was performed: Firstly, the presence of inorganic fouling and biofouling was detected by Scanning Electron Microscopy (SEM). Secondly, we determined cell viability of microorganisms of the biofilm using the stain Filmtracer™ LIVE/DEAD® biofilm viability kit. The presence and extension of biofilm matrix was evaluated by the stain Filmtracer™ SYPRO® Ruby biofilm matrix Stain; ATR-FTIR was used to better determine the chemical nature of the matrix. Finally, microbial composition and diversity (bacteria and fungi) was studied by Illumina sequencing. Our results give information about biofouling development in different RO membranes and allows identifying key microorganisms that might be useful to understand better this fouling process.

2. MATERIAL AND METHODS

2.1. SAMPLING RO MEMBRANES

Genesys Membrane Products, S.L., provided membrane samples used for this study. **Table 1** includes some characteristics of the three membranes (A, B and C) used for the study. Selected membranes were mainly chosen considering two main factors: Nature of feed water: brackish water (membranes A and B) vs. seawater (Membrane C) and nature of the fouling: mainly inorganic vs. mainly organic. It should be noted that the three membranes corresponded to polyamide-polysulphone commercial models although brands were different. Besides, on the samples with inorganic fouling (brackish water membranes), samples showed also different inorganic components: colloidal matter vs. scaling.

Sampling of the membranes was carried out during conventional autopsies and membranes coupons were obtained from the middle length of each module.

No data about operation time is available. By the way the three membranes were autopsied due to a significant presence of fouling which was producing failures in plant.

Fouling detected on each sample is very common for the kind of water and membrane position. Besides the samples described in **Table 1**, a conventional polyamide-polysulphone membrane was used as reference for some of the analyses carried out during the study.

A fourth RO membrane unused (named D) was used as control membrane in all experiments.

All the samples were delivered in fragments of 20×20 cm and conserved in sealed bags to avoid air exposure and reduce environmental contamination.

Table 1
Membrane samples details

	Brackish water membranes (BW)		**Sea water membrane (SW)**
	Membrane A	**Membrane B**	**Membrane C**
Membrane model	TORAY TM720-400	TORAY TM720-400	DOW FILMTEC SW30XHR-440i
Membrane position	1st membrane – 1st stage	Last membrane – 2nd stage	1st membrane
Feedwater	Coastal well water (Ibiza, Balearic Islands, Spain)		Sea water (Muscat, Oman)
Organic content	13%	12%	87%
Inorganic content	87%	88%	13%
Inorganic component	Aluminosilicates-colloidal matter and particles of iron-chromium as main components	Calcium carbonate as main component	Aluminosilicates-colloidal matter, magnesium, calcium, phosphorus, sulphur

2.2. SEM

The morphological characterization of RO membrane surface was performed using SEM. All samples were dissected in the different layers that composed the RO membrane (polyester layer, polyamide layer, mesh spacer and permeate carrier) checking biofouling formation in each layer.

Samples were fixed with a solution of glutaraldehyde 5% (v/v) in sodic cacodylate 0.2 M (pH 7.2) for 1 h. Afterwards, the fixer was removed with two washes with sodium cacodylate 0.2 M (pH 7.2). Subsequently, samples were dehydrated by immersing them in solutions with increasing concentrations of ethanol in periods up to 10 min to a concentration of 100 % (v/v). At this moment, a solution of acetone (100%) was used for the immersion of samples for 10 min. With this, critical drying point was achieved in samples using a sample dryer by critical point Polaron model CPD7501.

When the samples were dry, they were metallized with a gold layer of 30 mm using a metallizer Polaron model SC7640. Then, the RO membrane layers were observed with a

scanning electron microscope Zeiss DSM 950, using Quartz PCI software for analysis and image capture. The images obtained were coloured using GIMP v. 2.8.22.

2.3. Attenuated total reflection–Fourier transform infrared (ATR–FTIR) spectral analysis

ATR–FTIR spectra were recorded on a Thermo Nicolet IS10 spectrometer (ThermoFisher Scientific Inc., Massachusetts, USA) using an ATR-FTIR accessory (smart iTR) and the OMNIC™ software version 9.1.26 (ThermoFisher Scientific Inc., Massachusetts, USA). Spectra were collected in absorbance mode (log 1/R). For each measure, 16 scans were accumulated. The resolution was 4, the window aperture was at medium resolution, the gain was 2 and the optical velocity was 0.4747. At these parameters, good quality spectra with less spectral noise were obtained. 0.5 cm^2 of the RO membrane were measured between the range 1800-800 cm^{-1}. Between samples, the ATR-crystal was cleaned with isopropanol and the background was updated. For each RO membrane, 3 random spots were analyzed. Data were saved as. spa and .csv files.

The analysis of results and their graphical plots were performed with the software SigmaPlot 12.0 (Systat Software, San Jose, CA).

2.4. Application of specific stains to study biofilm cellular viability and biofilm matrix

Cellular viability and the presence of biofilm matrix were checked using stains applied to the polyamide layer. Bacterial viability assays were performed using Filmtracer™ LIVE/DEAD® biofilm viability kit (ThermoFisher Scientifc Inc., Massachusetts, USA). This kit allows discrimination between live and dead cells; it is based on a cell permeable dye for staining live cells (green fluorescence; SYTO 9) and a cell impermeable dye (red fluorescence, propidium iodide, PI) for staining dead and dying cells which are characterized by compromised cell membranes. For the staining of the polyamide layer, samples were cut in fragments of 0.5 cm^2 under sterile conditions and 50 μL of Filmtracer stain (a mixture of SYTO 9 and PI in DMSO, following the manufacturer's recommendations) were used. The incubation was performed in the dark for 15 min at room temperature. Then, samples were observed using confocal microscope (Confocal SP5 Leica Microsystems). For green fluorescence (SYTO 9), excitation was performed at 480 nm and emission at 500 nm. For red fluorescence (PI, dead cells), the excitation/emission wavelengths were 490 nm and 635 nm, respectively.

For the visualization of the extracellular polymeric matrix, samples were cut in fragments of 0.5 cm^2 under sterile conditions. 200 μL were stained with Filmtracer™

SYPRO® Ruby (Molecular Probes, Invitrogen) per sample, incubated in the dark for 30 min at room temperature, and rinsed with distilled water. Filmtracer™ SYPRO® Ruby stained most classes of proteins, including glycoproteins, phosphoproteins, lipoproteins, calcium binding proteins, fibrillar proteins and other proteins that constituted the biofilm matrix. Then, they were observed using confocal microscope (Confocal SP5, Leica Microsystems) with excitation/emission wavelengths of 450 nm and 610 nm, respectively.

In addition, several controls were included to check the performance of the stain in the presence of salt and also of a true bacterial biofilm. For all these controls, membrane D was initially taken and sterilized in the autoclave at 120°C in a short and dry cycle of 20 min. For the first control, a layer of crystals of NaCl salt was allowed to be formed on membrane D to check if the salts could interfere with the fluorochromes. For this, membrane was bathed in a solution of 1 M NaCl and then allowed to dry in an oven at 50°C until the salt crystal layer was formed. In a second control, *Pseudomonas putida*, which is a reference bacteria for biofilm formation, was cultured in a liquid medium and afterwards put into contact with membrane for 24 h, time enough for biofilm formation.

2.5. MICROBIAL DIVERSITY ANALYSIS

2.5.1. DNA EXTRACTION

A square of 1 cm^2 was cut from every RO membrane, including all layers. The feed layer was separated and crushed with a mortar using liquid nitrogen to reduce the layer to powder while the rest of the layers were cut in smaller fragments. The DNA of the entire sample was extracted using the FastDNA® Spin Kit for Soil (MP Biomedicals) and subsequent stored at -80°C until sequencing.

The procedure was the same for all samples. Three independent replicates were done for each RO membrane for reproducibility.

2.5.2. DNA SEQUENCING

PCR amplifications of the regions V3-V4 of the 16S rDNA and the ITS2 regions were carried out by the Genomics service of the Parque Científico de Madrid (Madrid, Spain) using the primers described in **Table 2**.

PCR products were purified and Miseq (Illumina) were prepared according to manufacturer's instructions. DNA libraries were checked for size, concentration and integrity using a Bioanalyzer (Agilent). Amplicon sequencing was performed using an Illumina Miseq sequencer. Paired-end reads (2×300) were generated according to manufacturer's instructions obtaining at least 100000 reads per replicate.

Table 2
Description of the primers used to perform DNA amplification. The regions which were amplified, and the sequences of the primers are indicated. The primer tail is indicated in bold

Region	Reference number	Sequence
16S	16SV3-V4-CS1	**ACACTGACGACATGGTTCTACA**CCTACGGGNGGCWGCAG
	16SV3-V4-CS2	**TACGGTAGCAGAGACTTGGTCTG**ACTACHVGGGTATCTAATCC
ITS	ITS4-CS1	**ACACTGACGACATGGTTCTACA**TCCTCCGCTTATTGATATGC
	ITS86F-CS2	**TACGGTAGCAGAGACTTGGTCTG**TGAATCATCGAATCTTTGAA

2.5.3. Data analysis

16S rDNA (bacterial) and ITS (fungi) profiling was determined using QIIME v. 1.8.0 (Caporaso et al., 2010) following the protocols (Pylro et al., 2014a, 2014b) described in de Brazilian Microbiome Project (https://www.brmicrobiome.org).

Briefly, reads were quality filtered and trimmed by Trimmomatic v. 0.32 (Bolger et al., 2014). First reads were paired and filtered to remove low quality pairs and singletons. In the case of ITS reads, an additional step using ITSx (Bengtsson-Palme et al., 2013) as carried out to remove non-fungal sequences. USEARCH v7 (Edgar, 2010) was employed to calculate operational taxonomic units (OTUs) at a 97% similarity level using the UPARSE v. 9 algorithm (Edgar, 2013) and to remove chimeric OTUs using UCHIME algorithm (Edgar et al., 2011). Taxonomic assignation was performed by the Uclust method (Edgar, 2010) using Greengenes v13_8 (DeSantis et al., 2006) for 16S sequences and UNITE v12_11 (Pyle, 2004) for ITS sequences.

Diversity metrics as CHAO1 (Chao, 1984) and Unifrac (Lozupone and Knight, 2005) were calculated to determine alpha and beta diversity respectively. Unweighted Unifrac values were used to represent sample variability by PCoA. Shannon–Weaver Index (Shannon, 2001) was calculated as an estimate of the fungal and bacteria diversity.

2.5.4. Accession numbers

Sequences used in this study were submitted to the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) under Sequence Read Archive (SRA) accession number: SRP131637.

3. Results and discussion

3.1. Visual RO membrane observations, SEM

Visual examination of the polyamide membrane in all used RO membranes (membranes A, B and C) showed fouling on the membranes. In general, this accumulation was produced in the valley areas and located in bands of deposits (not shown). These bands were established in the contact area between the spacer and the membrane (de Roever and

Huisman, 2007; Fernandez-Álvarez et al., 2010) As shown below by using scanning electron microscopy (SEM), it was possible to appreciate the presence of microorganisms and differentiate between fouling and biofouling because of the properties of the RO membrane made the perfect environment for the growth of microorganisms on the polyamide surface (Kwak et al., 1999).

In membrane A (shown in **Figure 1a**), the polyamide surface was covered by a series of crystalline and round morphology particles. These structures have a heterogeneous distribution, so there are areas where large, compacted crystals of 10 μm are formed and in other areas smaller crystals up to 2 μm can be observed. Although inorganic fouling is the main one in this sample, there were also microorganisms which could be seen between the compact crystals of the fouling. This distribution was somewhat irregular, and microorganism were not very abundant.

In membrane B (**Figure 1b**), fouling was composed of a thick layer of highly compacted crystals which were homogeneously distributed throughout the membrane. The size of each of these crystals was much greater than in the case of membrane A. This could be due to the fact that the concentrated water leaving membrane A was used as feed water for this membrane to increase the efficiency (Khan et al., 2015). The water was more concentrated in salts and that facilitated the formation of a fouling layer of greater thickness with larger crystals. In the case of microorganisms, the few microorganisms that could be visualized were settled on the salt crystals and not in the matrix holes. Also, some microorganisms grew in the spacer and not only in the polyamide layer. Two types of microorganisms based on their shapes (coccoid and bacillar) were seen over the salt crystals.

Inorganic fouling in membrane C was significantly smaller than that of A and B membranes (**Figure 1c**). It was only seen in the form of small incrustations in the surface of the polyamide layer. Conversely, biofouling was homogeneously distributed throughout this layer. It was appreciated throughout the whole membrane that there was a mucilaginous substance that covered the crystals and that surrounded the microorganisms. These microorganisms were visible both below this mucilage layer and above, when this occurred, the microorganisms appeared embedded in this layer. The morphology of these microorganisms was more varied than those found in the A and B membranes, appearing structures with coccoid shape of small size (0.2 μm) along with bacilli of heterogeneous sizes.

Visualization of control membrane (membrane D) in **Figure 1d** shows the normal appearance of an unused RO membrane surface. The surface had a morphology of ridge-

and-valley structures due the two monomers constituting the layer of polyamide 1-4-benzenediamine bound to terephthaloyl chloride (de Roever and Huisman, 2007).

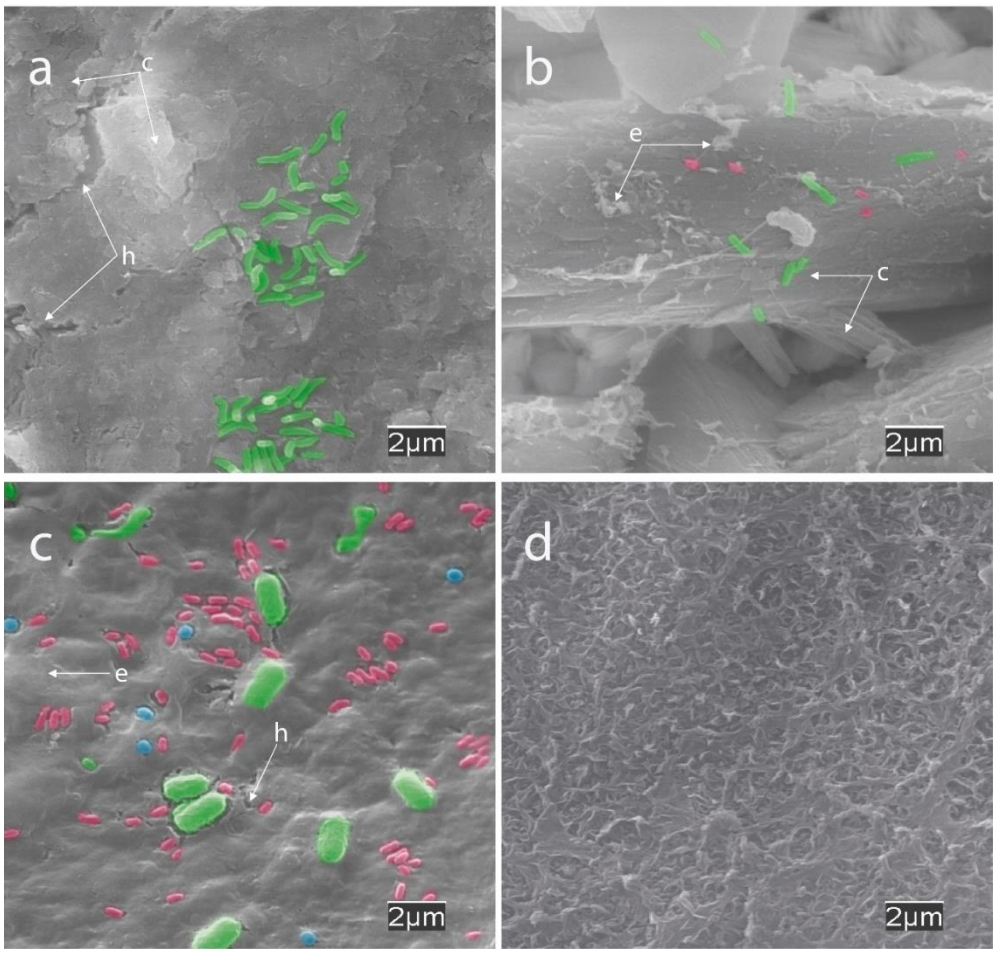


Figure 1. Distribution of fouling / biofouling in polyamide layer in each membrane sample. The microorganisms are marked in color with GIMP v. 2.8.22 for better visualization. Each color indicated different sizes and morphological shapes. a) Distribution of bacilli-shaped microorganisms between the fouling crystals in membrane A. b) Distribution of microorganisms on the fouling crystals in Sample B. c) Microorganisms embedded in membrane C. d) Control membrane D. Legend of acronyms: c: crystals, e: EPS, h: holes.

To summarize, in membranes A and B, inorganic fouling predominated, corroborating data in **Table 1**. The thicker layer of crystals was found in membrane B that operates at the last position from the second stage of the brackish water desalination plant. Microorganisms were not very abundant in any of the two membranes, being less abundant in membrane B; no clear extracellular polymeric substance (EPS) matrix could be visualized by SEM in these membranes. Membrane C was characterized by mostly organic fouling; the biofouling layer was much more evident than that of the RO membranes from brackish water. Differences in fouling between membranes A, B and membrane C are probably related to the different feed water, brackish *vs.* seawater.

SEM allowed to visualize fouling and biofouling in all three membranes. This is a technique commonly used in RO membranes studies and autopsies. Depending on feed

water, pre-treatment, and chemical structures of the membranes, fouling and biofouling has been visualized in many RO membranes (Al Ashhab et al., 2017, 2014; Bereschenko et al., 2011; Fernandez-Álvarez et al., 2010). Nevertheless, to study in detail biofouling confocal laser scanning microscopy (CLSM) which specific stains and FTIR analyses were performed.

3.2. VIABILITY OF MICROORGANISMS AND PRESENCE OF BIOFILM MATRIX BY USING CLSM AND ATR-FTIR

The visualization of the membranes using CLSM allowed checking cell viability and their distribution on the membrane surface using the Filmtracer™ LIVE/DEAD® biofilm viability kit. Bacterial colonization does not consist only in the adhesion of free bacteria onto the membrane. The microorganisms, once adhered, are embedded in an extracellular polymeric substance (EPS) forming a biofilm. The EPS provides stability to the biofilm (Flemming and Wingender, 2010). The biofilm matrix was visualized using the Filmtracer™ SYPRO® Ruby biofilm matrix stain that stains mostly EPS proteins.

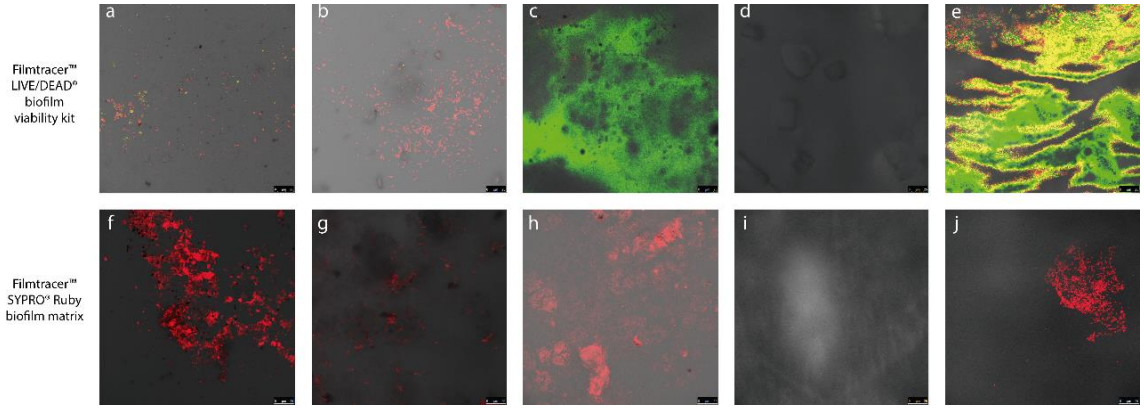


Figure 2. CLSM images. Staining with Filmtracer™ LIVE / DEAD® biofilm viability kit was used in the first row (a–e) and Staining with Filmtracer™ SYPRO® Ruby biofilm matrix Stain is shown in the second row (f–j). a and f corresponded to sample A, b and g are taken of sample B and sample C was shown in c and h. The other four photographs corresponded to the controls performed in membrane D. The stains made on the sterile membrane with a layer of NaCl are shown in d and i. Images e and j correspond to the biofilm formed with *Pseudomonas putida*. Legend: membrane A (a, f), membrane B (b, g), membrane C (c, h), control with a layer of NaCl (d, i) and control with *Pseudomonas putida* biofilm (e, j).

Membrane A (**Figure 2a**) had few cells distributed throughout the membrane. Although there was a high percentage of dead cells (red fluorescence), many microorganisms remained alive. The biofilm matrix in membrane A can be seen in **Figure 2f**. The matrix was much more distributed on the membrane than could be initially observed with the Filmtracer™ LIVE/DEAD® biofilm viability kit.

In membrane B, as can be seen in **Figure 2b**, a larger number of microorganisms with a clearly defined shape could be seen, although mostly dead. **Figure 2g** shows that biofilm matrix accumulated in small clusters. This situation was very different from that of membrane A despite being part of the same desalination plant. This is because plants that

treat brackish water use two parallel RO membranes. In this way, the water rejected in membrane A serves as feed water for the RO membrane B to increase process throughput (Greenlee et al., 2009).

Microorganisms adhered on membrane C (**Figure 2c**) were very abundant and were spread evenly throughout the membrane. No red fluorescence was observable meaning that microorganisms were alive probably due to the fact that it barely had salt deposits on the membrane that could affect the biofilm. **Figure 2h** shows that the biofilm matrix was spread throughout the sample, although there were areas in which a larger fluorescence was observed due to a higher concentration of extracellular proteins. This result confirmed the SEM images previously shown for this membrane.

To demonstrate the validity of the results, two controls were performed. The first control consisted of arranging a layer of salts (composed of NaCl) on membrane D to check if the salts interacted with the performance of the stains. As shown in **Figure 2d** and **Figure 2i**, no fluorescence was observed meaning that the stains do not interact with NaCl crystals so that no false positives can be attributed to sample staining. In the second control, a *Pseudomonas putida* culture was grown for 24 h on the polyamide layer of membrane D because of its great ability to rapidly form biofilms. In **Figure 2e** most of the cells are stained green because most of the bacteria were viable. The cells that showed a yellow color may be slightly damaged (Hu et al., 2017; Ibarra-Trujillo et al., 2012). For this reason, yellow cells were generally considered viable, while orange cells could be considered severely damaged (Boulos et al., 1999). **Figure 2j** shows the matrix of the *Pseudomonas putida* biofilm.

The control experiment with the *Pseudomonas putida* biofilm indicates that both stains were valid for biofilm visualization and can be used regularly in RO membranes.

These stains had certain advantages over SEM because they gave clearer results and did not interfere with the inorganic incrustations that existed in fouled membranes.

ATR-FTIR was applied to further analyze fouling/biofouling of the three membranes (**Figure 3**).

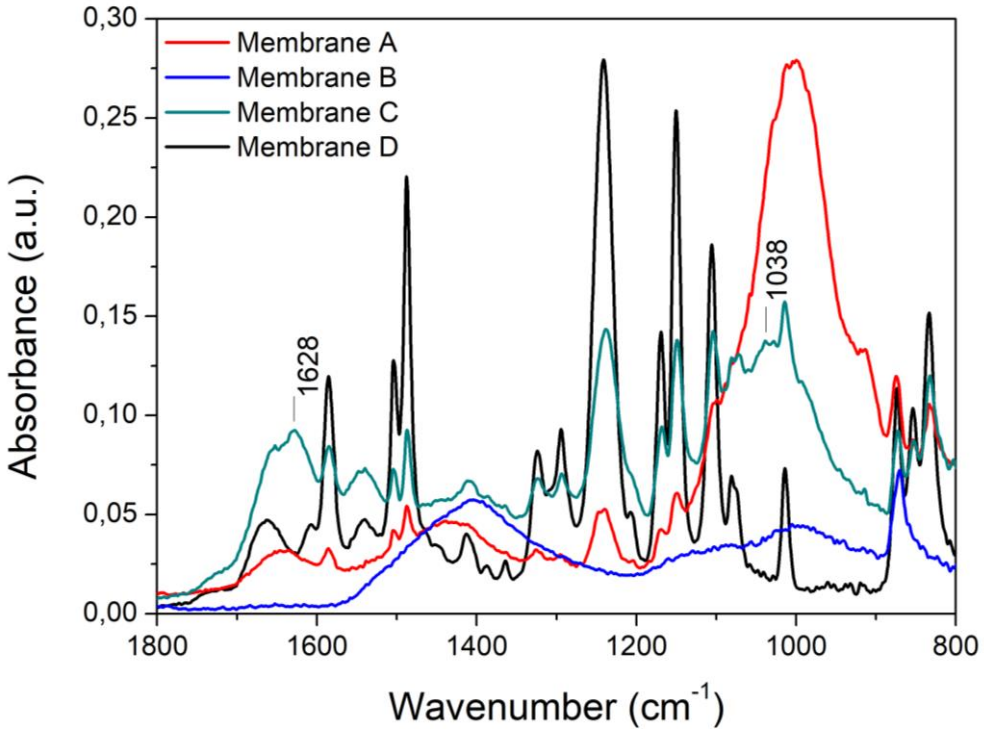


Figure 3. FTIR spectra of the control membrane D (black trace), membrane A (red trace), membrane B (blue trace) and membrane C (green trace).

The black trace in the figure corresponds to a characteristic polyamide-polysulphone membrane surface spectrum. This spectrum was used as reference to verify the presence of fouling/biofouling on the membranes sample surface.

Thus, IR spectra from membrane A (red trace in **Figure 3**) and membrane B surface (blue trace in **Figure 3**) do not show any of the characteristic bands from membrane composition, which demonstrates the significant presence of fouling on their surface (Tang et al., 2007). Fouling bands obtained from these membranes are characteristic of the components previously identified (**Table 1**): Aluminosilicates on membrane A (peak at around 1000 cm^{-1}) (Gabelich et al., 2006) and calcium carbonate on membrane B (peak around 1400 cm^{-1}) (Yang et al., 2008). Peaks indicating chemical bonds related to EPS matrix such as those around 1650 cm^{-1} and 1540 cm^{-1} assigned to C=O and N-H (Al Ashhab et al., 2017; Quilès et al., 2010; Tran et al., 2007; Yang et al., 2008), respectively, indicative of proteins were not identified; also those assigned to polysaccharides (peaks around 1000 cm^{-1}) (Tran et al., 2007; Yang et al., 2008) are masked by inorganic fouling.

On the other side, membrane C spectrum (green trace in **Figure 3**) shows bands from fouling/biofouling, but also many bands from membrane composition (thinner fouling than previous samples). Fouling bands appear at wavelengths characteristic of aluminosilicates (**Figure 3** - 1.000 cm^{-1}) but there is a distinctive peak at 1038 cm^{-1} which

may be assigned to P=O, COO and C-O-C stretching vibrations present in phosphodiesters and rings in polysaccharides (Quilès et al., 2010). The peak at 1628 cm^{-1} was assigned to C=O (amide bond) (Quilès et al., 2010) that could be related to protein derivatives commonly related to the presence of biofilm.

CSLM stains and ATR-FTIR corroborated that biofouling was present mostly in seawater membrane C, where adhered microorganisms were highly abundant and viable; the biofilm matrix was well developed as indicated by the Filmtracer™ SYPRO® Ruby biofilm matrix stain and ATR-FTIR clearly indicated the presence of proteins in the matrix. Regarding membranes A and B from brackish water, mostly inorganic fouling was found and adhered microorganisms were in lesser abundance and many were dead.

3.3. Analysis of microbial composition and diversity

A metagenomics approach using next generation sequencing techniques (Illumina platform) was carried out to determine microbial composition and diversity on RO membranes.

3.3.1. Bacterial composition and diversity

The most representative phylum was Proteobacteria that was present in all samples in a range between 64.3% and 53.1% (**Figure 4**; see also **Table S1 in Supplementary Material 1** that shows the relative abundance at the genus level for all three membranes). This result fits with previous studies demonstrating the dominance of this phylum in the Mediterranean Sea (Acinas et al., 1999) and in the Arabic Sea (Fuchs et al., 2005). Other studies have also demonstrated the dominance of this phylum over the microbial communities adhered to RO membranes (Al Ashhab et al., 2017, 2014; Ben-Dov et al., 2016; Bereschenko et al., 2008, 2007; Levi et al., 2016).

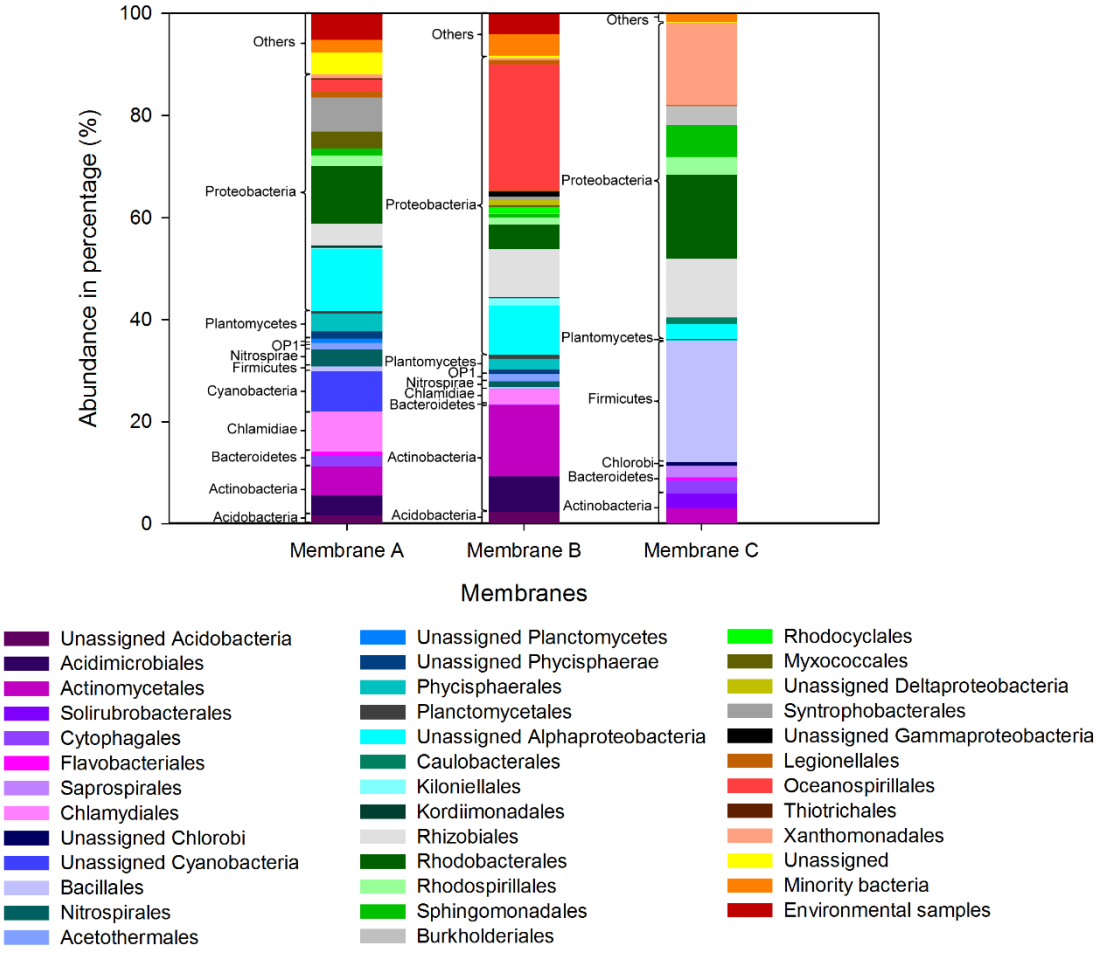


Figure 4. Relative abundance of prokaryotic communities at the order level in used membranes. To the left of the bars the orders are grouped in phyla. Minorities are OTUs whose representation is less than 0.5%; unassigned are those sequences that have only been identified as bacteria and lastly the Environmental Sample refer to those sequences that have not been recognized at any taxonomic level.

In membrane C, the main phylum after Proteobacteria was Firmicutes, with a representation of 23.5%; within this phylum, family Paenibacillaceae and genus *Brevibacillus* were the most abundant. This phylum has been observed in other systems of RO membranes as one of the most important biofilm formers (Nagaraj et al., 2017) and was the main group in biofilms from milk processing membranes (Chamberland et al., 2017).

Phylum Bacteroidetes abundance was higher in membrane C (5.63%) than in membranes A 3.13%) or B (0.20%). The most representative families were Cytophagaceae and Flavobacteriaceae. Phylum Bacteroidetes has also been found to be abundant in RO membranes from seawater like membrane C or secondary effluents from WWTPs (Al Ashhab et al., 2014; Ferrera et al., 2015; Khan et al., 2015; Zhang et al., 2011).

The rest of the phyla and their relative percentages varied greatly among the samples, although no phylum reached the importance of Proteobacteria. Membrane A presented also the phyla Actinobacteria (9.5%), Chlamydiae (7.8%) and Cyanobacteria (7.8%). In membrane B the phylum Actinobacteria (20.9%) was more abundant with respect to membrane A

(9.6%). Within this phylum, genus *Mycobacterium* was the one that increased its relative abundance the most (in membrane A 2.60% and in membrane B 6.37%) although *Mycobacterium* grows slowly, it is capable of tolerating saline environment (Chen et al., 2004; Santos et al., 2015). All these phyla have also been reported in RO membrane biofilms although at low abundance (Bereschenko et al., 2011, 2008; Chamberland et al., 2017; Chen et al., 2004).

Within Proteobacteria, the Alphaproteobacteria class was dominant in membranes A and C (32.1% in A and 42.23% in C); The relative abundance of Alphaproteobacteria was 27.7% in membrane B. Gammaproteobacteria abundance was higher in membrane B (27.6%) as compared to membrane A (5.1%) and C (16.6%). The differences of Gammaproteobacteria abundance between RO membrane A and RO membrane B might mainly be due to the salinity changes that occurred in the feed water, as Gammaproteobacteria can increase their population in biofilms under saline conditions during the late stages of biofilm maturation (Zhang et al., 2014). Deltaproteobacteria and Betaproteobacteria were in significant lower proportions: 9.9% in membrane A, 2% in B and absent in C for Deltaproteobacteria; absent in membrane A, 0.7% in B and 3.5% in C for Betaproteobacteria.

Alphaproteobacteria and Betaproteobacteria have been usually found in RO membrane biofilms (Al Ashhab et al., 2017, 2014). Regarding Gammaproteobacteria, Al Ashhab et al. (2014) found that this class predominated in RO membranes after a cleaning cycle while Betaproteobacteria were almost completely excluded after cleaning. Deltaproteobacteria was found at very low abundance in RO membranes, in agreement with the results reported here (Al Ashhab et al., 2014; Chen et al., 2004). Alpha-, Beta- and Gammaproteobacteria have been suggested to be involved in initial colonization and biofilm development (Al Ashhab et al., 2014; Bereschenko et al., 2010; Hörsch et al., 2005; Pang and Liu, 2007); in fact, Alphaproteobacteria have been claimed as responsible for the biofouling in RO membranes (Chen et al., 2004).

Within Alphaproteobacteria, the order Rhizobiales predominated in membrane B (9.4%), while in sample C it represented 11.5% and in membrane A only represented 5.6%. Family Hyphomicrobiaceae with genera *Devosia* and *Hyphomicrobium* was dominant particularly in membranes A and B (brackish water). This order has been found as dominant in biofilms from RO membranes (Ayache et al., 2013; Bereschenko et al., 2008; Pang and Liu, 2007). Pang and Liu (2007) found that Rhizobiales were metabolically versatile under aerobic conditions which might be an important advantage in environments with limited

nutrients input like RO membranes. Some members of this order have been found to degrade organic contaminants and to secrete glycosphingolipids which have been suggested to play a relevant role in the initial colonization of RO membranes as well as in the production of EPS during biofilm maturation (Skorupska et al., 2006). In addition, within Alphaproteobacteria, order Rhodobacterales predominated in membranes A and C (11.3% in A and 16.5% in C), which was represented mainly by the family Rhodobacteraceae (10.9% in A and in C 15.5%); the members of this family such as *Rhodobacter* have been found to be associated with mature biofilms (Khan et al., 2013). Family Sphingomonadaceae is also frequently found in RO membranes and in particular, genus *Sphingomonas*, also known to produce sphingolipids (Pollock, 1993; Pollock and Armentrout, 1999), has been reported as initial colonizers of biofilms (Bereschenko et al., 2010; Gutman et al., 2014). Bereschenko et al. (2010) reported that the unique capability of *Sphingomonas* for spreading and producing a layer of EPS may outcompete other microorganisms such as *Pseudomonas* that may exist as floating aggregates in feed water. This family was present in all three membranes although it was less abundant than family Rhodobacteraceae. Rhizobiales may replace family Sphingomonadaceae during the process of biofilm (Bereschenko et al., 2010).

Within Betaproteobacteria, order Burkholderiales was the most abundant with families Comamonadaceae, Rhodocydaceae and Alcaligenaceae as majoritarian. These families have been found as abundant in the biofilms of membrane bioreactors (MBRs) used for wastewater treatment (Lim et al., 2012; Oh et al., 2018; Xia et al., 2010). Family Comamonadaceae was also found to participate in denitrification processes within the biofilm (Wu et al., 2013).

In the case of the Gammaproteobacteria, the order Oceanospirillales was predominant in membrane A (2.4%) and membrane B (24.6%), whose main family, Oceanospirillaceae, was also predominant in both samples, although in different percentages (membrane A was 1.8% and membrane B was 18%). The family Oceanospirillaceae is characterized for being marine microorganisms (Satomi and Fujii, 2014).

In membrane C the most abundant order was Xanthomonadales (16.2%), whose only representative in this case was the family Xanthomonadaceae (16.2%). The most abundant genus of this family, *Pseudoxanthomonas* (8.4%), is remarkable for its ability to metabolize recalcitrant metabolite substances, so they are often used in biofilters (Nopcharoenkul et al., 2013). Their great abundance might imply that these microorganisms can metabolize unconventional carbon sources that reach RO membranes, serving their products as

substrates for other microorganisms in the biofouling community, facilitating their development.

The absence of the Pseudomonadaceae in all membranes (representing less than 0.1% of the community in C and absent from the rest of membranes) is a relevant fact. This family encompasses the genus *Pseudomonas*, a genus widely investigated and used in trials for its great ability to form biofilms as it is able to produce large amounts of EPS (Herzberg et al., 2009; Herzberg and Elimelech, 2008; Tseng et al., 2013). Many studies have reported the presence of this genus in RO membranes (Ayache et al., 2013; Baker and Dudley, 1998; Bereschenko et al., 2010, 2008; Ferrera et al., 2015; Khan et al., 2015; Kim et al., 2014; Ridgway et al., 1983; Zodrow et al., 2014). Although this is not the first time that the absence of the genus *Pseudomonas* in biofouling of RO membranes has been observed (Acinas et al., 1999), this genus seems to be more frequent in RO membranes from wastewater treatments (Al Ashhab et al., 2017).

The rest of the phyla and their relative percentages varied greatly among the samples, although no phylum reached the importance of Proteobacteria. Membrane A presented also the phyla Actinobacteria (9.5%), Chlamydiae (7.8%) and Cyanobacteria (7.8%). In membrane B the phylum Actinobacteria (20.9%) was more abundant with respect to membrane A (9.6%). Within this phylum, genus *Mycobacterium* was the one that increased its relative abundance the most (in membrane A 2.60% and in membrane B 6.37%) although Mycobacterium grows slowly, it is capable of tolerating saline environment (Santos et al., 2015).

The Shannon-Weaver index was calculated to evaluate α-diversity (**Table 3**). The diversity was high in the three samples, but membrane A and membrane B presented a high value in comparison with that of membrane C. This could be very relevant, since a greater microbial diversity implies a greater resistance to diverse factors of stress and the development of diverse metabolic pathways among the microorganisms that make up the community (Briones and Raskin, 2003).

Table 3
α-diversity Shannon-Weaver Index. The index was calculated using the relative abundance of the detected genera in each DNA region

Region	Membrane A	Membrane B	Membrane C
16S RNA	3.44	3.28	2.89
ITS	2.7	2.33	2.11

The results obtained with β-diversity allowed to statistically differentiate between the three samples. Distances were represented through Principal Coordinates Analysis 2D-Plots that are shown in **Figure S1 in Supplementary Material 1**. Significant differences were

found between membrane A, membrane C (with a *p*-value of 9.95×10^{-15}), membrane B, and membrane C (*p*-value = 1.57×10^{-15}). Results showed less significant differences between membrane A and membrane B (*p*-value of 6.32×10^{-7}). This statistically significant differences might be explained by the facts that membranes A and B had different feed water than membrane C (brackish *vs.* seawater); that membranes A and B showed mostly inorganic fouling while that of membrane C was mainly organic; and also, although the three studied membranes corresponded to polyamine-polysulphone commercial models, they were from different companies. Although biofouling is a problem that develops in all RO membranes independently of their origin (Darton and Fazel, 2001), the composition of the community of microorganisms seems to vary depending on the location, inorganic fouling, salinity and even membrane brand. Thus, this kind of analysis is important to prepare site-specific treatments to diminish or delay biofouling.

3.3.2. FUNGAL COMPOSITION AND DIVERSITY

Unlike prokaryotes, in the case of fungi, there was a large percentage of OTUs that could not be identified (the average of the three membranes was 21.3%) or only were identified as fungi (22.6%) because the generation of unintentional chimeras during PCR amplification is frequent. These chimeras have been detected even in the UNITE database (included 1825 chimeras) because detecting chimeras was a challenge (Nilsson et al., 2015).

The fungal communities identified in the three membranes were classified mainly in the Ascomycota and Basidiomycota phyla (shown in **Figure 5**; **Table S2 in Supplementary Material 1** shows the relative abundance at the genus level for all three membranes).

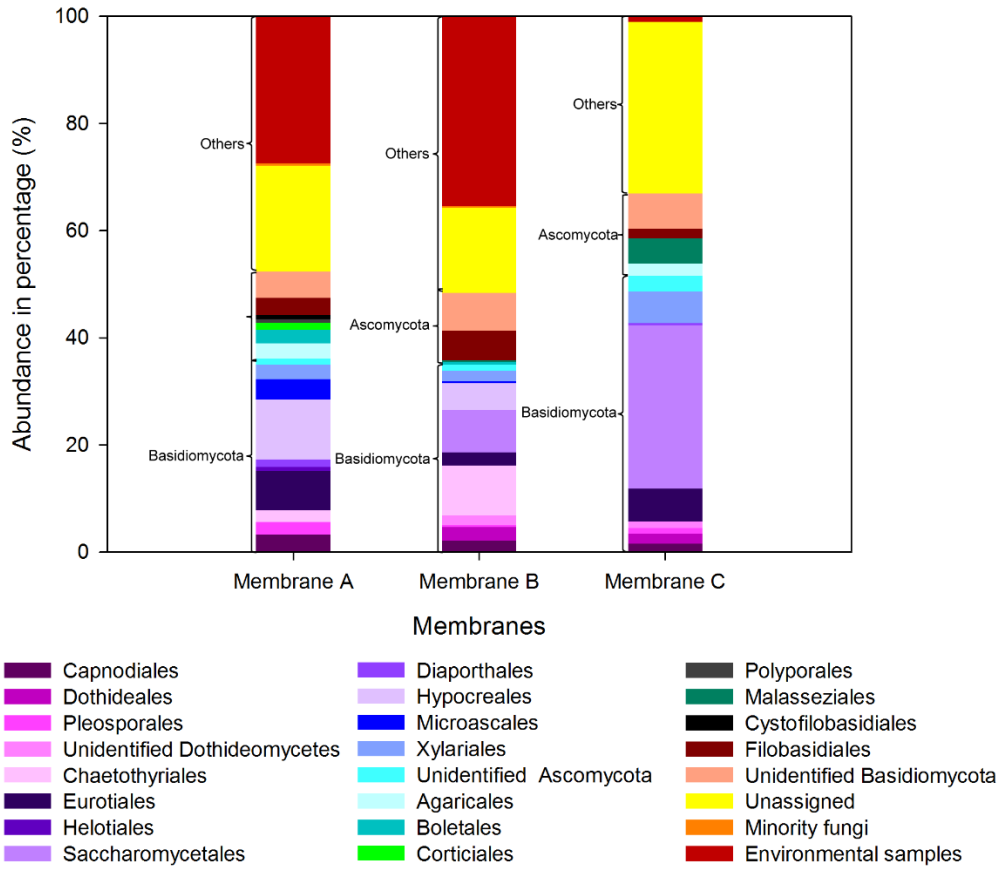


Figure 5. Relative abundance of fungi at the order level in used membranes. To the left of the bars the orders are grouped in phyla. Minorities are OTUs whose representation is less than 0.5%; unassigned are those sequences that have only been identified as fungi and lastly the Environmental Sample refer to those sequences that have not been recognized at any taxonomic level.

The Ascomycota phylum was more abundant (in membrane A it represented 36.6%, membrane B 35.3% and membrane C 51.6%) than the phylum Basiodiomycota (membrane A: 16.13%, membrane B: 13.4% and membrane C: 15.3%).

In membrane A and B, the classes Sordariomycetes (membrane A: 19.6% and membrane B: 7.4%) and Eurotiomycetes (membrane A: 9.5% and membrane B: 11.6%) were predominant in Ascomycota. Within Eurotiomycetes there was a divergence between families depending on the membrane. In membrane A, the family Trichocomaceae (7.4%) was most abundant while in membrane B it was Chaetothyriaceae with a representation of 9.3%.

In membrane C, the Ascomycota phylum was mainly represented by the genus *Candida* (55%), the rest being microorganisms of the class Saccharomycetes (1.2%). *Candida* constituted by unicellular fungi, had already been identified previously in other RO membranes (Al Ashhab et al., 2014). This fungus is also able to form biofilms as a way to develop resistance to antifungal products (Kumamoto, 2002), which, together with other

microorganisms that constitute biofouling, causes a greater difficulty in elimination and must be considered for the development of more effective cleaning of RO membrane.

Fungal diversity in RO membranes was low. The values obtained with the Shannon-Weaver index (**Table 3**) were all below three for RO membranes. As with prokaryotes, the fungal diversity was higher in membrane A and B than in membrane C. This could be due to the apparent low diversity of fungi in saline environments (Richards et al., 2011).

Contrary to what happens with prokaryotic communities, fungi have hardly been studied in RO membranes. The only study that considered them analyzed a water treatment system in which RO membranes functioned as a tertiary treatment system, concluding that most fungi were Ascomycota, as found in our study (Al Ashhab et al., 2014). Within Ascomycota, family Capnodoaceae, has been reported to form biofilms in hard substrates such as rocks (Ruibal et al., 2009); in our study, order Capnodiales was present at percentages ranging from 0.7% (Membrane C) to 3.3% in membrane A; but within this order, family Capnodaceae was not found. In a more recent study, Al Ashhab et al. (2017) also found that Ascomycota and Basidiomycota were dominant, although Ascomycota was found at higher abundance, but after a cleaning procedure there was a significant shift with Ascomycota predominating in cleaned RO membranes and Basidiomycota dominating control biofilms. Authors also reported that the community composition of Ascomycota at the beginning and at the end of the cleaning procedure changed but considered that there is remarkable lack of information regarding fungal community members and further research is needed. Thus, the lack of studies about the presence of fungi in biofilms developed in RO membranes must be considered as an important limitation for biofilm prevention and elimination.

The results obtained with β-diversity allowed to statistically differentiate between the three membranes. Distances were represented through Principal Coordinates Analysis 2D-Plots, which are shown in **Figure S2 in Supplementary Material 1**. The fungal communities established in the three RO membranes were significantly different between them: membrane A and membrane B with a *p*-value of 6.6×10^{-3}, membrane B and C with *p*-value of 3×10^{-3} and finally membrane A and membrane C with a *p*-value of 8.88×10^{-6}. As stated above for bacterial diversity, these statistically significant differences might be explained by the different feed water, inorganic fouling and even membrane brand and location.

4. CONCLUSIONS

A three-tiered approach that might be useful for RO membranes autopsies was proposed in the study that included the determination of inorganic fouling and biofouling by SEM; biofilm cell viability and biofilm matrix presence by specific stains for CLSM and FTIR analysis and Illumina sequencing to study microbial composition and diversity.

SEM may be used as a first-tiered approach as it provides clear information about inorganic fouling and may detect microorganisms attached to the membrane surfaces but it cannot give information about the viability of these organisms or the extension and nature of the biofilm matrix. The second-tiered approach should be the use of specific stains like the Filmtracer™ LIVE/DEAD® biofilm viability kit and the Filmtracer™ SYPRO® Ruby biofilm matrix to detect viable cells and matrix extension by CSLM, respectively. ATR-FTIR analysis might be useful to provide information about the chemical nature of the biofilm matrix; this is relevant because cleaning procedures such as conventional chemical treatments have been found to fail in removing developed biofilms in RO membranes. Once biofouling has been detected, the third-tiered approach is the study of microbial composition and diversity with the objective of identifying key microorganisms in the process of biofouling; this information may be useful for the development of advanced antibiofouling treatments for the desalination industries. This approach may take advantage of techniques of massive DNA sequencing like the Illumina platform used in this study.

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6. SUPPLEMENTARY MATERIAL 1

CONTENTS:

Figure S1: Principal component analysis of the bacterial diversity in: membrane A (blue circles), membrane B (orange triangles) and membrane C (red squares). Each sample was represented three times, one for replicate. The Y axis explained 82.48 % and Y axis 11.41 % of the variability in the data for the bacterial groups.

Figure S2: Principal component analysis of the fungal diversity in: membrane A (blue circles), membrane B (orange triangles) and membrane C (red squares). Three replicates were made per membrane except for membrane C which only two replicates were considered due to insufficient reads for the third replicate. The Y axis explained 21.41 % and Y axis 34.15 % of the variability in the data for the fungal groups.

Table S1: Relative abundance of microorganisms identified to genus level by region 16S.

Table S2: Relative abundance of fungi identified up to genus level by region ITS.

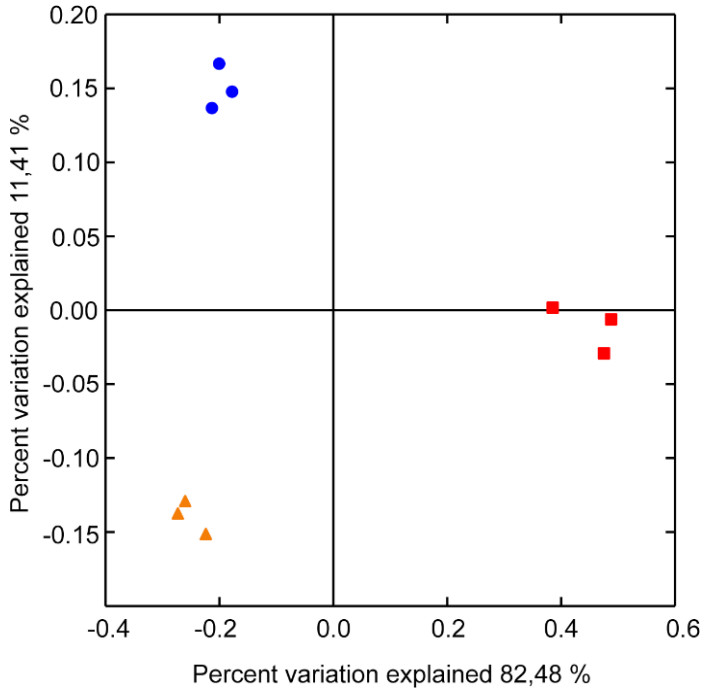

Figure S1. Principal component analysis of the bacterial diversity in: membrane A (blue circles), membrane B (orange triangles) and membrane C (red squares). Each sample was represented three times, one for replicate. The Y axis explained 82.48 % and Y axis 11.41 % of the variability in the data for the bacterial groups.

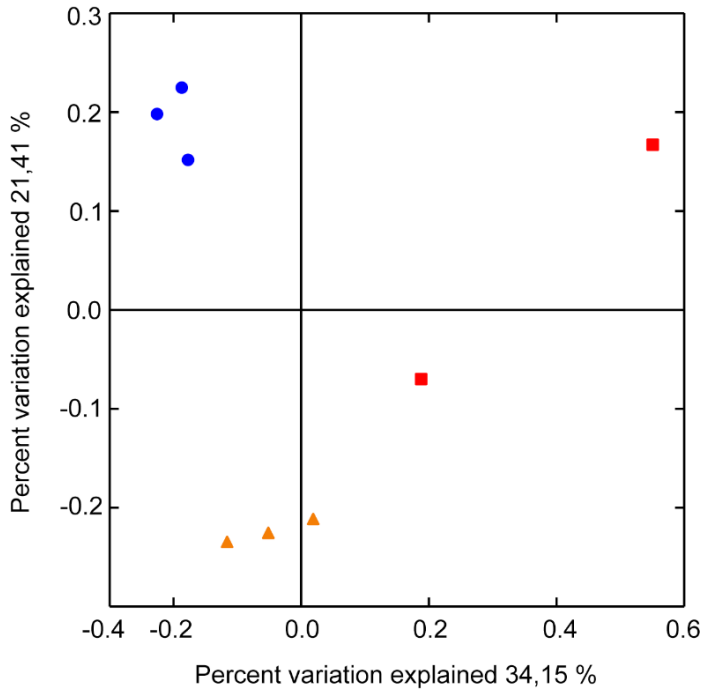

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Table S1
Relative abundance of microorganisms identified to genus level by region 16S

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Unassigned	Other	Other	Other	Other	Other	5.83%	4.87%	0.83%
Archaea	Crenarchaeota	Thaumarchaeota	Cenarchaeales	Cenarchaeaceae	*Nitrosopumilus*	0.30%	0.47%	0.00%
Bacteria	Acidobacteria	AT-s2-57	Unassigned	Unassigned	Unassigned	0.00%	0.33%	0.00%
Bacteria	Acidobacteria	Holophagae	Holophagales	Unassigned	Unassigned	0.07%	0.10%	0.00%
Bacteria	Acidobacteria	Solibacteres	Solibacterales	PAUC26f	Unassigned	0.03%	0.00%	0.00%
Bacteria	Acidobacteria	Sva0725	Sva0725	Unassigned	Unassigned	1.67%	2.33%	0.00%
Bacteria	Acidobacteria	[Chloracidobacteria]	RB41	Ellin6075	Unassigned	0.00%	0.00%	0.00%
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Unassigned	Unassigned	3.30%	6.20%	0.17%
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111	Unassigned	0.07%	0.10%	0.00%
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Iamiaceae	*Iamia*	0.47%	0.63%	0.00%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Cellulomonas*	0.00%	0.00%	0.00%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Gordoniaceae	*Gordonia*	0.03%	0.03%	0.33%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Unassigned	0.00%	0.00%	0.00%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Agromyces*	0.00%	0.00%	0.07%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Leucobacter*	0.00%	0.00%	0.10%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Microbacterium*	0.00%	0.00%	0.10%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	*Mycobacterium*	2.60%	6.37%	1.57%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	*Nocardia*	0.10%	0.00%	0.00%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Unassigned	1.87%	7.07%	0.63%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	*Pimelobacter*	0.20%	0.03%	0.00%
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	*Pseudonocardia*	0.77%	0.33%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Unassigned	Unassigned	0.00%	0.00%	0.03%
Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Unassigned	Unassigned	0.00%	0.00%	2.80%
Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Conexibacteraceae	*Conexibacter*	0.00%	0.00%	0.00%
Bacteria	BHI80-139	Unassigned	Unassigned	Unassigned	Unassigned	0.07%	0.00%	0.00%
Bacteria	BRC1	NPL-UPA2	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	BRC1	PRR-11	Unassigned	Unassigned	Unassigned	0.03%	0.00%	0.00%
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	Unassigned	0.00%	0.00%	0.00%
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Other	0.00%	0.00%	0.00%
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Unassigned	1.73%	0.03%	0.00%
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Leadbetterella*	0.00%	0.00%	2.67%
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Flammeovirgaceae	Unassigned	0.40%	0.07%	0.00%
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Flammeovirgaceae	*Fulvivirga*	0.00%	0.00%	0.00%
Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Flammeovirgaceae	*Reichenbachiella*	0.00%	0.00%	0.00%
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Unassigned	Unassigned	0.07%	0.10%	0.10%
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Unassigned	1.80%	17.97%	0.00%
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Unassigned	0.47%	0.00%	0.00%
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	0.03%	0.00%	0.00%
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	0.00%	0.00%	0.43%
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Muricauda*	0.23%	0.00%	0.00%
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Robiginitalea*	0.03%	0.00%	0.00%
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Unassigned	0.10%	0.00%	0.07%
Bacteria	Bacteroidetes	VC2_1_Bac22	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Bacteroidetes	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	Unassigned	0.07%	0.00%	0.00%
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Unassigned	0.00%	0.00%	2.30%
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Saprospiraceae	Unassigned	0.00%	0.00%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Other	Other	5.00%	1.03%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Unassigned	Unassigned	1.87%	0.23%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Parachlamydiaceae	Other	0.27%	1.37%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Parachlamydiaceae	*Candidatus Protochlamydia*	0.03%	0.00%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Rhabdochlamydiaceae	*Candidatus Rhabdochlamydia*	0.07%	0.10%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Simkaniaceae	Other	0.40%	0.03%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Waddliaceae	Other	0.00%	0.00%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Waddliaceae	Unassigned	0.20%	0.00%	0.00%
Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Waddliaceae	*Waddlia*	0.00%	0.10%	0.00%
Bacteria	Chlorobi	OPB56	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.67%
Bacteria	Chloroflexi	Anaerolineae	SBR1031	A4b	Unassigned	0.00%	0.03%	0.00%
Bacteria	Chloroflexi	TK17	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Chloroflexi	Thermomicrobia	JG30-KF-CM45	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Cyanobacteria	4C0d-2	MLE1-12	Unassigned	Unassigned	7.87%	0.10%	0.00%
Bacteria	Cyanobacteria	ML635J-21	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	*Anabaena*	0.00%	0.03%	0.00%
Bacteria	Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	*Nostoc*	0.00%	0.00%	0.03%
Bacteria	Firmicutes	Bacilli	Bacillales	Unassigned	Unassigned	0.40%	0.03%	0.03%
Bacteria	Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	*Alicyclobacillus*	0.00%	0.00%	0.87%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Unassigned	0.03%	0.00%	0.00%
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	0.43%	0.10%	0.07%
Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Brevibacillus*	0.07%	0.07%	22.60%
Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Cohnella*	0.00%	0.00%	0.07%
Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*	0.00%	0.00%	0.17%
Bacteria	Firmicutes	Bacilli	Bacillales	Thermoactinomycetaceae	Unassigned	0.00%	0.00%	0.00%
Bacteria	Gemmatimonadetes	Gemm-2	Unassigned	Unassigned	Unassigned	0.00%	0.20%	0.00%
Bacteria	Gemmatimonadetes	Gemm-3	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.13%
Bacteria	Gemmatimonadetes	Gemm-4	Unassigned	Unassigned	Unassigned	0.00%	0.13%	0.00%
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	0.00%	0.00%	0.33%
Bacteria	NKB19	Unassigned	Unassigned	Unassigned	Unassigned	0.00%	0.07%	0.00%
Bacteria	NKB19	noFP_H4	Unassigned	Unassigned	Unassigned	0.47%	0.27%	0.00%
Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Unassigned	1.23%	1.10%	0.00%
Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	2.03%	0.03%	0.00%
Bacteria	OD1	ZB2	Unassigned	Unassigned	Unassigned	0.00%	0.07%	0.00%
Bacteria	OP1	[Acetothermia]	[Acetothermales]	Unassigned	Unassigned	1.30%	1.40%	0.00%
Bacteria	Planctomycetes	C6	MVS-107	Unassigned	Unassigned	0.00%	0.37%	0.00%
Bacteria	Planctomycetes	OM190	CL500-15	Unassigned	Unassigned	0.20%	0.10%	0.00%
Bacteria	Planctomycetes	OM190	agg27	Unassigned	Unassigned	0.83%	0.00%	0.00%
Bacteria	Planctomycetes	Phycisphaerae	CCM11a	Unassigned	Unassigned	1.47%	0.93%	0.00%
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales	Unassigned	Unassigned	2.67%	0.83%	0.17%
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	Unassigned	0.83%	1.23%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Isosphaeraceae	Unassigned	0.00%	0.00%	0.13%
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	Unassigned	0.00%	0.10%	0.00%
Bacteria	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	A17	0.00%	0.00%	0.07%
Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	*Planctomyces*	0.40%	0.77%	0.17%
Bacteria	Proteobacteria	Unassigned	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Other	Other	Other	0.00%	0.00%	0.13%
Bacteria	Proteobacteria	Alphaproteobacteria	Unassigned	Unassigned	Unassigned	12.20%	9.73%	2.90%
Bacteria	Proteobacteria	Alphaproteobacteria	BD7-3	Unassigned	Unassigned	0.23%	0.13%	0.17%
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Other	0.00%	0.00%	0.73%
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Unassigned	0.00%	0.00%	0.27%
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Mycoplana*	0.00%	0.00%	0.03%
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium*	0.00%	0.00%	0.27%
Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Unassigned	Unassigned	0.00%	0.83%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae	Unassigned	0.17%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae	*Thalassospira*	0.00%	0.53%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Kordiimonadales	Kordiimonadaceae	Unassigned	0.50%	0.20%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Other	Other	0.10%	0.10%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Unassigned	Unassigned	0.03%	0.00%	1.53%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	*Chelatococcus*	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	0.00%	0.00%	0.03%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Unassigned	0.10%	0.67%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Devosia*	0.43%	2.27%	0.50%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Parvibaculum*	0.10%	4.50%	0.10%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Rhodoplanes*	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Other	0.00%	0.00%	6.80%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Unassigned	0.07%	0.03%	1.37%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Nitratireductor*	0.00%	0.10%	0.87%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	0.00%	0.03%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Kaistia*	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	*Xanthobacter*	0.00%	0.00%	0.13%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	Unassigned	0.00%	0.00%	0.93%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	*Hyphomonas*	0.03%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	*Maricaulis*	0.43%	1.10%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Other	0.00%	0.07%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unassigned	10.90%	3.67%	15.50%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Rhodobacter*	0.00%	0.00%	0.10%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Unassigned	0.00%	0.03%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Unassigned	1.97%	1.17%	3.23%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	*Azospirillum*	0.03%	0.07%	0.10%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	*Rhodovibrio*	0.00%	0.03%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Other	Other	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Unassigned	Unassigned	0.10%	0.00%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Other	0.33%	0.13%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Unassigned	0.80%	0.13%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Other	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Unassigned	0.03%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.00%	0.00%	0.17%
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingopyxis*	0.10%	0.40%	6.10%
Bacteria	Proteobacteria	Betaproteobacteria	Unassigned	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Other	Other	0.00%	0.00%	0.07%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Unassigned	0.00%	0.00%	1.03%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*	0.00%	0.00%	2.07%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Pigmentiphaga*	0.00%	0.00%	0.03%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unassigned	0.03%	0.00%	0.23%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Delftia*	0.00%	0.00%	0.03%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Hydrogenophaga*	0.00%	0.00%	0.10%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Limnobacter*	0.00%	0.13%	0.00%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Ralstonia*	0.00%	0.03%	0.00%
Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	Unassigned	0.00%	0.00%	0.10%
Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	*Nitrosovibrio*	0.00%	0.00%	0.03%
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Unassigned	0.07%	1.23%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	*Bacteriovorax*	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	*Bdellovibrio*	0.00%	0.07%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Unassigned	Unassigned	2.13%	0.20%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Haliangiaceae	Unassigned	0.00%	0.17%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	*Plesiocystis*	1.13%	0.00%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	NB1-j	JTB38	Unassigned	0.10%	0.00%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	NB1-j	MND4	Unassigned	0.00%	0.03%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	NB1-j	NB1-i	Unassigned	0.00%	0.93%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Unassigned	6.53%	0.77%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	34P16	Unassigned	Unassigned	0.00%	0.07%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Unassigned	0.00%	0.03%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	HB2-32-21	0.10%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	HTCC2188	HTCC	0.03%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Idiomarinaceae	Unassigned	0.03%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	J115	Other	0.00%	0.03%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	J115	Unassigned	0.03%	0.07%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Other	Other	0.17%	0.13%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Unassigned	Unassigned	0.00%	0.20%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Unassigned	0.07%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Unassigned	0.00%	0.00%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Proteobacteria	Gammaproteobacteria	HTCC2188	HTCC2089	Unassigned	0.07%	0.93%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Unassigned	Unassigned	0.33%	0.10%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	Unassigned	0.23%	0.13%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Other	0.13%	0.00%	0.27%
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Unassigned	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	*Legionella*	0.33%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Unassigned	Unassigned	0.47%	0.20%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	*Alcanivorax*	0.17%	6.47%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Unassigned	1.80%	17.97%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unassigned	0.00%	0.00%	0.07%
Bacteria	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Unassigned	0.23%	0.90%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Unassigned	0.67%	0.37%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Other	0.00%	0.00%	0.97%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Arenimonas*	0.10%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Dokdonella*	0.00%	0.00%	0.00%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Pseudoxanthomonas*	0.00%	0.00%	8.40%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	0.00%	0.00%	5.70%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Thermomonas*	0.00%	0.00%	1.10%
Bacteria	Proteobacteria	Gammaproteobacteria	[Marinicellales]	[Marinicellaceae]	Unassigned	0.00%	0.03%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Bacteria	Proteobacteria	TA18	CV90	Unassigned	Unassigned	0.00%	0.23%	0.00%
Bacteria	SBR1093	EC214	Unassigned	Unassigned	Unassigned	0.10%	0.37%	0.00%
Bacteria	SBR1093	VHS-B5-50	Unassigned	Unassigned	Unassigned	0.00%	0.10%	0.00%
Bacteria	Spirochaetes	[Leptospirae]	[Leptospirales]	Leptospiraceae	*Turneriella*	0.00%	0.00%	0.00%
Bacteria	TM6	SJA-4	Unassigned	Unassigned	Unassigned	4.27%	0.43%	0.07%
Bacteria	TM6	SJA-4	YJF2-48	Unassigned	Unassigned	0.00%	0.00%	0.00%
Bacteria	Verrucomicrobia	Opitutae	Puniceicoccales	Puniceicoccaceae	Other	0.00%	0.00%	0.00%
Bacteria	Verrucomicrobia	Opitutae	Puniceicoccales	Puniceicoccaceae	Unassigned	0.00%	0.00%	0.00%
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Prosthecobacter*	0.00%	0.00%	0.07%
Bacteria	WPS-2	Unassigned	Unassigned	Unassigned	Unassigned	0.00%	0.17%	0.00%

Table S2
Relative abundance of fungi identified up to genus level by region ITS

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
No blast hit	Other	Other	Other	Other	Other	27.40%	35.37%	1.00%
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Cladosporium*	3.30%	1.03%	0.77%
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Septoria*	0.00%	1.10%	0.80%
Fungi	Ascomycota	Dothideomycetes	Dothideales	Dothideaceae	*Endoconidioma*	0.00%	2.53%	0.00%
Fungi	Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	unidentified	0.00%	0.00%	1.90%
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Incertae_sedis	*Phoma*	0.00%	0.33%	0.00%
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	unidentified	0.00%	0.00%	0.97%
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Chalastospora*	0.87%	0.00%	0.00%
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Lewia*	1.47%	0.00%	0.00%
Fungi	Ascomycota	Dothideomycetes	unidentified	unidentified	unidentified	0.00%	1.87%	1.30%
Fungi	Ascomycota	Eurotiomycetes	Chaetothyriales	Chaetothyriaceae	*Cyphellophora*	2.17%	6.40%	0.00%
Fungi	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Exophiala*	0.00%	2.87%	0.00%
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	1.87%	0.00%	1.87%
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Eupenicillium*	0.00%	0.00%	0.00%
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Paecilomyces*	4.30%	1.77%	0.00%
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*	1.23%	0.63%	4.27%
Fungi	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	unidentified	0.70%	0.00%	0.00%
Fungi	Ascomycota	Leotiomycetes	unidentified	unidentified	unidentified	0.00%	0.40%	0.00%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae_sedis	*Candida*	0.00%	2.30%	28.87%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Pichiaceae	*Pichia*	0.00%	5.20%	1.60%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	0.00%	0.40%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Fungi	Ascomycota	Sordariomycetes	Diaporthales	Gnomoniaceae	*Gnomonia*	0.00%	0.00%	0.37%
Fungi	Ascomycota	Sordariomycetes	Diaporthales	Valsaceae	*Valsa*	1.43%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Engyodontium*	1.47%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Hypocrea*	2.10%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	1.07%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Incertae_sedis	*Acremonium*	4.17%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Cosmospora*	0.63%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	0.80%	2.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Haematonectria*	1.03%	2.97%	0.00%
Fungi	Ascomycota	Sordariomycetes	Melanosporales	Ceratostomataceae	*Sphaerodes*	0.27%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Microascales	Halosphaeriaceae	*Sigmoidea*	1.10%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Pseudallescheria*	2.70%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Wardomycopsis*	0.00%	0.47%	0.00%
Fungi	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	*Truncatella*	0.00%	0.00%	1.60%
Fungi	Ascomycota	Sordariomycetes	Xylariales	Diatrypaceae	*Eutypella*	0.53%	0.00%	0.00%
Fungi	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Xylaria*	2.27%	1.97%	4.33%
Fungi	Ascomycota	unidentified	unidentified	unidentified	unidentified	1.13%	1.10%	2.93%
Fungi	Basidiomycota	Agaricomycetes	Agaricales	Lyophyllaceae	*Lyophyllum*	0.00%	0.00%	0.83%
Fungi	Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	*Hypholoma*	2.77%	0.00%	0.00%
Fungi	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	*Clitocybe*	0.00%	0.00%	1.40%
Fungi	Basidiomycota	Agaricomycetes	Boletales	Rhizopogonaceae	*Rhizopogon*	1.27%	0.00%	0.00%
Fungi	Basidiomycota	Agaricomycetes	Boletales	Sclerodermataceae	*Astraeus*	0.00%	0.43%	0.00%
Fungi	Basidiomycota	Agaricomycetes	Boletales	Suillaceae	*Suillus*	1.30%	0.00%	0.00%

(Continued)

Kingdom	Phylum	Class	Order	Family	Genus	Membrane A	Membrane B	Membrane C
Fungi	Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae	unidentified	1.27%	0.00%	0.00%
Fungi	Basidiomycota	Agaricomycetes	Polyporales	Steccherinaceae	*Irpex*	0.70%	0.00%	0.00%
Fungi	Basidiomycota	Incertae_sedis	Malasseziales	Incertae_sedis	*Malassezia*	0.00%	0.27%	4.77%
Fungi	Basidiomycota	Tremellomycetes	Cystofilobasidiales	unidentified	unidentified	0.80%	0.00%	0.00%
Fungi	Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Cryptococcus*	3.13%	5.57%	1.83%
Fungi	Basidiomycota	unidentified	unidentified	unidentified	unidentified	4.90%	7.10%	6.50%
Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	19.86%	15.93%	32.10%
						100.00%	100.00%	100.00%

CHAPTER 3

EARLY AND DIFFERENTIAL BACTERIAL COLONIZATION ON MICROPLASTICS DEPLOYED INTO THE EFFLUENTS OF WASTEWATER TREATMENT PLANTS

ABSTRACT

Microbial colonization of microplastics (MPs) in aquatic ecosystems is a well-known phenomenon; however, there is insufficient knowledge of the early colonization phase. Wastewater treatment plant (WWTP) effluents have been proposed as important pathways for MPs entry and transport in aquatic environments and are hotspots of bacterial pathogens and antibiotic resistance genes (ARGs). This study aimed at characterizing bacterial communities in the early stage of biofilm formation on seven different types of MPs deployed in two different WWTPs effluents as well as measuring the relative abundance of two ARGs (*sulI* and *tetM*) on the tested MPs. Illumina Miseq sequencing of the 16S rRNA showed significant higher diversity of bacteria on MPs in comparison with free-living bacteria in the WWTP effluents. β-diversity analysis showed that the in-situ environment (sampling site) and hydrophobicity, to a lesser extent, had a role in the early bacterial colonization phase. An early colonization phase MPs-core microbiome could be identified. Furthermore, specific core microbiomes for each type of polymer suggested that each type might select early attachment of bacteria. Although the tested WWTP effluent waters contained antibiotic resistant bacteria (ARBs) harboring the sulI and tetM ARGs, MPs concentrated ARBs harboring the *sulI* gene but not *tetM*. These results highlight the relevance of the early attachment phase in the development of bacterial biofilms on different types of MP polymers and the role that different types of polymers might have facilitated the attachment of specific bacteria, some of which might carry ARGs.

1. INTRODUCTION

Plastics have been widely used since 1950 and their use is increasing (Drzyzga and Prieto, 2019). These plastics usually have a short product lifetime and because of their persistence, accumulate in the environment, especially in aquatic ecosystems (Duis and Coors, 2016; Ivleva et al., 2017).

Plastics interact with co-occurring organisms (from mammals to microorganisms) in aquatic ecosystems in different ways (Kettner et al., 2019; Macreadie et al., 2017). One important impact of this pollution is that plastics provide an artificial, hard and persistent surface for microbial colonization (Miao et al., 2019; Rummel et al., 2017). The attached microbial communities on plastic surface are termed as "plastisphere" (Amaral-Zettler et al., 2020; Zettler et al., 2013). Thus, plastics have emerged as novel ecological habitats, that are usually constituted by microbial communities significantly different to those living in the surrounding environment (De Tender et al., 2015).

According to NOAA's definition, plastics fragments below 5 mm are considered microplastics (Gago et al., 2016). They are easily transported between environmental compartments (Law and Thompson, 2014), including freshwaters, oceans, polar environments and pristine mountain lakes (Free et al., 2014; Kettner et al., 2017; Oberbeckmann et al., 2018; Waller et al., 2017), staying in the environment for long periods of time serving as a vector for the dispersal of invasive species, including pathogens but also antibiotic resistance bacteria (ARBs) carrying antibiotic resistance genes (ARGs) (Arias-Andres et al., 2018; Kirstein et al., 2016; Laganà et al., 2019; McCormick et al., 2014; Oberbeckmann et al., 2018). Furthermore, MPs provide a large surface area that increases the available space for microbial colonization (Hidalgo-Ruz et al., 2012).

Microbial colonization of MPs in freshwater environments is poorly known in comparison with marine environments (Jacquin et al., 2019). Recent studies reported that wastewater treatment plants (WWTPs) is one of the principal pathways of MPs entering into freshwater and marine ecosystems (Edo et al., 2020; McCormick et al., 2014). The MPs that end up in the WWTPs not only come from the degradation of macroplastics, but many are a common formulation in cosmetics and other personal care products (Carr et al., 2016). Although WWTPs usually have the capacity to remove 99% of the MPs, a small but significant fraction of MPs ends up in the effluent with the potential to interact with the river biota (Murphy et al., 2016). In this context, recent studies have performed colonization experiments in rivers and in locations close to the discharge of WWTP (Kettner et al., 2019; Kettner et al., 2017; Oberbeckmann et al., 2018). However, these studies analyzed microbial

communities established on the MPs after two weeks of in situ incubation. Peng et al. (2018) studied early (24–48 h) biofilm colonization on polypropylene (PP) large bio-cords deployed downstream of a WWTP outlet but, specifically on MPs, early colonization studies seem to be lacking although the first hours or days of biofilm formation affects the subsequent maturation of the biofilm (Goecke et al., 2010; Peng et al., 2018).

Furthermore, numerous previous studies have recognized that WWTPs are one of the most important hotspots for propagation of pathogens and ARBs and their cognate ARGs in the environment (Bouki et al., 2013; Guo et al., 2017; Hendriksen et al., 2019; Pärnänen et al., 2019). So far, only few studies have addressed the potential of MPs as vectors of pathogens and ARGs mostly in marine systems (Wang et al., 2020; Yang et al., 2019); regarding freshwaters, Oberbeckmann et al. (2018) detected certain bacteria commonly associated with antibiotic resistance downstream of a WWTP. Arias-Andres et al. (2018) stablished the capacity of MPs to be "hot-spots" of horizontal gene transfer (HGT).

In this study, we characterized, for the first time, early bacterial colonization on seven types of MPs [three biodegradable plastics, namely polylactic acid (PLA), poly-3-hydroxybutyrate (PHB), polycaprolactone (PCL), and four non-biodegradable plastics, namely polyethylene terephthalate (PET), low-density polyethylene (LDPE), polystyrene (PS) and polyoxymethylene (POM)]. These MPs were deployed during 48 h into the effluents of two WWTPs with different water treatments, different water sources and located in different towns. We hypothesized that early MP-biofilm forming bacteria might be different among the tested MPs and different to free-living water bacteria and to those colonizing another artificial substrate (borosilicate spheres). Furthermore, we hypothesized that MPs-colonizing bacteria might act as vectors of ARGs and contribute to their spread.

2. MATERIAL AND METHODS

2.1. STUDY SITE

Two full-scale activated sludge WWTPs in Spain were selected for this study. Cantoblanco (Universidad Autónoma de Madrid) wastewater plant, denoted as WWTP1, processes approximately 931 m^3 per day from the university facilities, various research institutes located in the campus, a hospital, and an elderly nursing home. The Guadalajara wastewater treatment plant, denoted as WWTP2, processes approximately 45,000 m^3 per day. It treats domestic and industrial water from the city of Guadalajara (medium-size city with about 86,000 inhabitants). The operational variables and treatments performed in each WWTP is depicted in **Table S1 in Supplementary Material 1**. The location of the WWTPs is shown in **Figure S1a in Supplementary Material 1**.

2.2. PLASTIC SUBSTRATES USED FOR MICROBIAL COLONIZATION AND CHARACTERIZATION OF THEIR SURFACE PROPERTIES

Seven types of polymers were considered; the biodegradable polylactic acid (PLA), poly-3-hydroxybutyrate (PHB) and polycaprolactone (PCL) and the non-biodegradable but in widespread use, polyethylene terephthalate (PET), polyoxymethylene (POM), polystyrene (PS) and low-density polyethylene (LDPE); the size range of all tested MPs was 3–5 mm. Borosilicate spheres (BS) were used as non-plastic substrate control (size range between 2 and 8 mm). All substrates were commercial and additive-free. The most important information of these substrates is detailed in **Table S2 in Supplementary Material 1**.

The surface properties of the materials used as substrates for microbial colonization were studied by contact angle measurements. Contact angles were determined with an optical contact angle meter (Krüss DSA25 Drop Shape Analysis System) at room temperature using the sessile drop technique. Contact angles were measured using drops of MilliQ water, glycerol and diiodomethane delivered by the built-in syringe. Contact angle measurements were taken at least at three different positions for each solvent and material and analysed using the software Drop Shape Analysis (DSA4) release 2.1. Surface tension was calculated using the procedure by Van Oss (2007). The procedure allowed obtaining the free energy of interaction between two identical surfaces immersed in a liquid, ΔG_{SWS}, which is a measure of the hydrophobicity or hydrophilicity of the surface. If $\Delta G_{SWS} > 0$, the surface is hydrophilic, whereas if $\Delta G_{SLS} < 0$, it is hydrophobic. The different calculated parameters are shown in **Table S3 in Supplementary Material 1**.

The microtexture of all substrate materials was evaluated using a high-resolution 3D microscope with interferometry and profilometry model Leica DCM 8 with the analysis mode in confocal mode (green LED). The software used to process the result is Leica Scan version 6.5. The areas considered were 649 μm × 488 μm using three measurements per particle and three different particles. The measured parameters were the developed interfacial area ratio (Sdr) and kurtosis value (Sku). The Sdr parameter is expressed as the percentage of additional surface area contributed by the texture as compared to the planar definition area, the Sdr of a completely level surface is 0, but when a surface has any slope, its Sdr value becomes larger. The Sku value is a parameter of the sharpness of the surface height: height normal distribution has a value of 3; a value of Sku less than 3 indicates that height distribution is skewed above the mean plane; on the contrary, Sku values higher than 3 indicates that its height distribution is spiked. (high Sku values indicated a spiky surface, low Sku values indicates a bumpy surface) (Blunt and Jiang, 2003).

2.3. Design of the colonization experiment

The substrates were sterilized according to their properties: PLA, PHB, POM, PET and BS were sterilized by autoclave (120 °C, 20 min); PCL, LDPE and PS, because of their low melting temperature, were sterilized using 10% hydrochloric acid 1 min and cleaning with sterilized Milli-Q water. Approximately, 5 g of each polymer type pellet and BS were introduced into sterilized metallic cages with 1 mm holes by triplicate. These cages were deployed during 48 h at a depth of 20 cm at the exit of the WWTP secondary clarifiers, separated from each other by 15 cm. (see **Figures S1b–f in Supplementary Material 1** for details on the colonization experiment). WWTP1 incubation was carried out on October 19th-21st 2017, WWTP2 on March 14th-16th, 2018.

After the incubation, all MP pellets and BS were carefully extracted from the metallic cages to avoid the destruction of the biofilm and the residual water of the sample dried with sterilized filter paper. Dried MP pellets and BS were put into sterile tubes, frozen in liquid nitrogen and finally stored at −20 °C until DNA extraction.

In order to obtain a representative sample of the bacterial community in surrounding water, 1 L of water was sampled in wide mouthed polyethylene bottles and kept cool in the dark. Water was filtered by 0.22 μm membrane Millipore filter. Filters were frozen in liquid nitrogen and stored at −20 °C until DNA extraction.

Environmental properties of WWTP effluent waters were analysed at the beginning of the experiment (0 h) and at the end of the incubation time (48 h) (**Table S4 in Supplementary Material 1**). Dissolved oxygen, temperature, pH and conductivity were measured in situ using an oxygen portable meter ProfiLine Oxi 3310 (WTW), an electrical conductivity meter CDTM 523 and a microprocessor pH Meter pH 96 (WTW), respectively. Nutrient (nitrate, nitrite, ammonium and phosphate) concentrations were determined by duplicate using colorimetric methods as previously described (Perona et al., 1999). The chemical oxygen demand (COD) was measured using the COD cell Test kit (Merck Millipore).

2.4. Microbial diversity analysis

2.4.1. DNA extraction

Phenol:chloroform method was essentially carried out as previously described (Debeljak et al., 2017). Total DNA was extracted from all frozen MP pellets and frozen BS and water filters in triplicate. Pellets of each substrate were distributed in three 2 ml Eppendorf tubes. Water filters were cut into small fragments with sterilized scissors and

distributed in three 1.5 mL Eppendorf tubes. The procedure started with the addition of Tris-HCL 10 mM, EDTA 0.1 mM pH 7.5, 0.05% SDS (W/V) and 0.01% of silica pellets (W/V). After that, 0.5 volumes of hot phenol ultrapure pH 7.9 (65 °C) was added, and the samples were vortexed and warmed to 65 °C for 1 min three times to fully release the DNA from the biofilms developed in the samples. After that, 0.5 volume of chloroform was added, and the samples were vortexed and frozen again six times. Finally, samples were centrifuged at 13,000 rpm at 4 °C for 20 min. The supernatant of the samples was transferred to a new Eppendorf tube and 1 volume of hot phenol pH 7.9 (65 °C) was added to wash the sample which was subsequently centrifuged at 13,000 rpm at 4 °C for 20 min. The process was repeated twice. Finally, all supernatants that belonged to the same sample were pooled and 2 volumes of absolute ethanol was added, the sample was mixed and frozen at −20 °C overnight to precipitate the DNA. Samples were subsequently centrifuged at 13000 rpm at 4 °C for 20 min. The supernatant was discarded, and the pellet was washed with 1 volume of ethanol 70% to remove the salts. Samples were further centrifuged at 13000 rpm at 4 °C for 2 min. Finally, samples were dried, and the DNA was resuspended in 40 µL of Milli-Q water. All samples were stored at −20 °C.

2.4.2. DNA SEQUENCING

PCR amplifications of the regions V3-V4 of the 16S rRNA of each of the three replicates of each microplastic plus three replicates of BS and water effluent filters (54 sequenced samples) were carried out by the Genomics service of the Parque Científico de Madrid (Madrid, Spain). The primers used are shown in **Table S5 in Supplementary Material 1**. DNA libraries and amplicon sequencing were performed as previously described (Martínez-Campos et al., 2018).

2.4.3. DATA ANALYSIS

16S rRNA profiling was determined using Quantitative Insights Into Microbial Ecology 2 (QIIME 2) v. 2019.4 (Bolyen et al., 2019) (https://docs.qiime2.org/2019.1). The complete pipeline of the process can be found in the end of **Supplementary Material 1**.

Briefly, the quality of the reads (fastq format) was evaluated with FastQC 0.11.18 (Bioinformatics, 2011) and with the q2-demux plugin. The reads, cleaned and trimmed paired ends, were filtered and denoised using DADA2 (Callahan et al., 2016) via q2-dada2. Identified amplicon sequence variants (ASVs) were aligned using MAFFT (Katoh et al., 2002) via q2-alignment, and used to construct a phylogeny with FastTree2 (Price et al., 2010). Rarefaction curves were estimated via q2-diversity to 71,940 lectures depth per sample. α-

diversity methods, that includes Shannon index (Shannon and Weaver, 1949) Chao1 index (Chao and Lee, 1992) and Pielou's evenness (Pielou, 1966), were estimated via q2-diversity and the differences between samples were evaluated using Kruskal-Wallis statistics method (Kruskal and Wallis, 1952).

ASVs were taxonomically assigned using the q2-feature classifier plugin (Bokulich et al., 2018) based on classify-sklearn naïve Bayes taxonomy classifier using Silva 128, 99% OTUs database (Quast et al., 2012). A specific classifier for the amplified 16S region was trained using the primers specified above and a maximum fragment size of 300 nts.

For β–diversity analysis, two types of analysis were performed. Between-treatment variability was analysed with principal coordinate Analysis (PCoA) based in ASV abundance (Bray-Curtis dissimilarity) (Sorenson, 1948) and visualized using EMPeror (Vázquez-Baeza et al., 2013). Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was applied to test significant differences between sites and substrates considering 999 permutations.

Redundancy analysis (RDA) was performed to establish a correlation between environmental and intrinsic plastic factors (site, roughness, and hydrophobicity) and the bacterial community established in each substrate. The relative abundance of the microbial groups at genus level in each sample was used as "species data", filtering out genera with a relative abundance less than 0.5%. Environmental variables were transformed using log (x + 1) to avoid the differences in scale (binary data were not transformed, and hydrophobicity was transformed to positive values). A Monte-Carlo permutation test with 999 permutations was carried out to test the significance of the environmental parameters in relation to distribution pattern of samples. The analysis was performed using vegan package in Rstudio.

To identify differentially attached taxa among the different substrates and water at both WWTPs, the linear discriminant analysis effect size method (LEfSe) (Segata et al., 2011) was used. This was performed with the LEfSe online tool in the Galaxy framework, using all default settings for data formatting and LDA (Linear Discriminant Analysis) effect size. The factors "substrate" and "location" were set as classes. Non-transformed relative abundance was used and the strategy for multi-class analysis "one-against-all" was performed.

2.4.4. Accession numbers

Sequences used in this study were submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under the Bioproject accession number: PRJNA543601.

2.5. Relative abundance of ARGs

The relative abundance of two ARGs (*sulI* and *tetM*) in the bacterial community attached to the tested substrates was compared to the relative abundance of the two genes in free-living water bacteria using quantitative PCR (qPCR). *sulI* confers resistance to sulphonamides which are a class of antibiotics for which resistance is a worldwide problem and has been documented in wastewater impacted environments (Garner et al., 2018). *tetM* provides a high level resistance to tetracycline (Morse et al., 1986), a class of antibiotics used to treat a number of human infections such as cholera, brucellosis, plague, malaria, and syphilis; the *tetM* gene has also been documented in urban sewage (Hendriksen et al., 2019; Pärnänen et al., 2019).

qPCR assays were carried out in a LightCycler® 480 (Roche; USA) system using 2.5 ng of template DNA and using. LightCycler® 480 SYBR Green I Master (Roche; USA). The primers for amplification of the *sulI* and *tetM* genes are depicted in **Table S6 in Supplementary Material 1**. Three technical replicates were run for each gene and each sample obtaining in each one a detectable cycle threshold (Ct) value. Both positive and negative controls were included in every run.

The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the 16S gene of the same sample.

3. Results

3.1. Characterization of substrates

Surface properties were shown in **Table 1**. The Gibbs free energy of interaction, ΔG_{SWS}, gives a measure of surface hydrophobicity, which was, in increasing order: PHB < PS < PCL < POM < LDPE < PET < BS < PLA. Surface topography was visualized using 3D microscopy.

Figure S2 in Supplementary Material 1 revealed substrate-dependent differences. LDPE displayed the highest roughness (expressed as Sdr, see **Table 1**) mostly with ridge-and-valley appearance. PHB, PCL and POM displayed intermediate roughness and uneven surfaces. PS and PET had the flattest surface roughness with Sdr values close to BS. Besides, PET, PLA, PHB, POM, LDPE and PCL with kurtosis values (Sku > 3; **Table 1**) showed spiked surfaces, while BS and PS were softer.

Table 1
Surface properties of the materials.

	ΔG_{sws} (mJ/m²) [a]	Sdr (%) [b]	Sku [c]
PLA	−54.5 ± 8.1	24.1 ± 13.4	6.9 ± 2.8
PHB	−20.4 ± 4.5	41.5 ± 3.9	4.9 ± 2.2
PCL	−34.6 ± 2.1	37.7 ± 8.7	3.6 ± 0.8
PET	−45.5 ± 3.9	8.2 ± 1.7	7.4 ± 7.1
LDPE	−42.4 ± 2.3	84.6 ± 30.7	3.8 ± 0.4
POM	−41.5 ± 5.2	22.4 ± 41.0	4.1 ± 1.7
PS	−29.0 ± 3.9	8.2 ± 3.4	2.9 ± 0.4
BS	−45.6 ± 5.8	3.8 ± 1.1	2.9 ± 0.3

[a] ΔG_{sws} is the Gibbs free energy of interaction. The more negative, the more hydrophobic is the surface.
[b] Sdr is the developed interfacial area ratio defined as the percentage of additional area due to texture if compared to planar area (zero represents a flat surface).
[c] Sku: kurtosis of roughness profile; Sku > 3: spiked distribution with numerous high peaks and low valleys; Sku < 3: means few peaks and low valleys.

3.2. Taxonomical annotation

About 7,111,208 reads were obtained using Illumina sequencing. After quality filtration, reads merging and chimera removal using DADA2, 5,620,437 sequences remained (79.0% of the total reads) which were assigned to 9075 ASVs. 3970 ASVs were identified in WWTP1 while 6293 ASVs were identified in WWTP2.

The rarefaction plot (**Figure S3 in Supplementary Material 1**) reached the plateau with the current sampling effort in all samples, pointing out that the bacterial libraries were adequately sampled. In order to validate the statistics results, the sequence depth used to evaluate the α- and β- diversity was 70,940 reads per sample.

3.3. α-diversity analysis

Bacteria diversity was estimated using the alpha components, namely diversity (Shannon Index), evenness (Pielou's evenness) and bacterial richness (Chao1 Index). These indexes are represented in **Figure 1** according to location (WWTP1 or 2) and substrates.

The WWTP1 samples had significant lower values of Shannon index (Global Kruskal Wallis p-value = 2.9×10^{-10}) (**Figure 1a**), Pielou evenness (Global Kruskal Wallis p-value = 2.8×10^{-10}) (**Figure 1b**) and Chao1 (global Kruskal Wallis p-value = 0.0004) (**Figure 1c**) than WWTP2 samples. This indicated a higher bacterial diversity in WWTP2 than in WWTP1, underpinning an important difference in species richness between both locations that could be related both to the operational conditions of both WWTPs (**Table S1 in Supplementary Material 1**) as well as nutrient loads which are higher in WWTP1 (**Table S4 in Supplementary Material 1**).

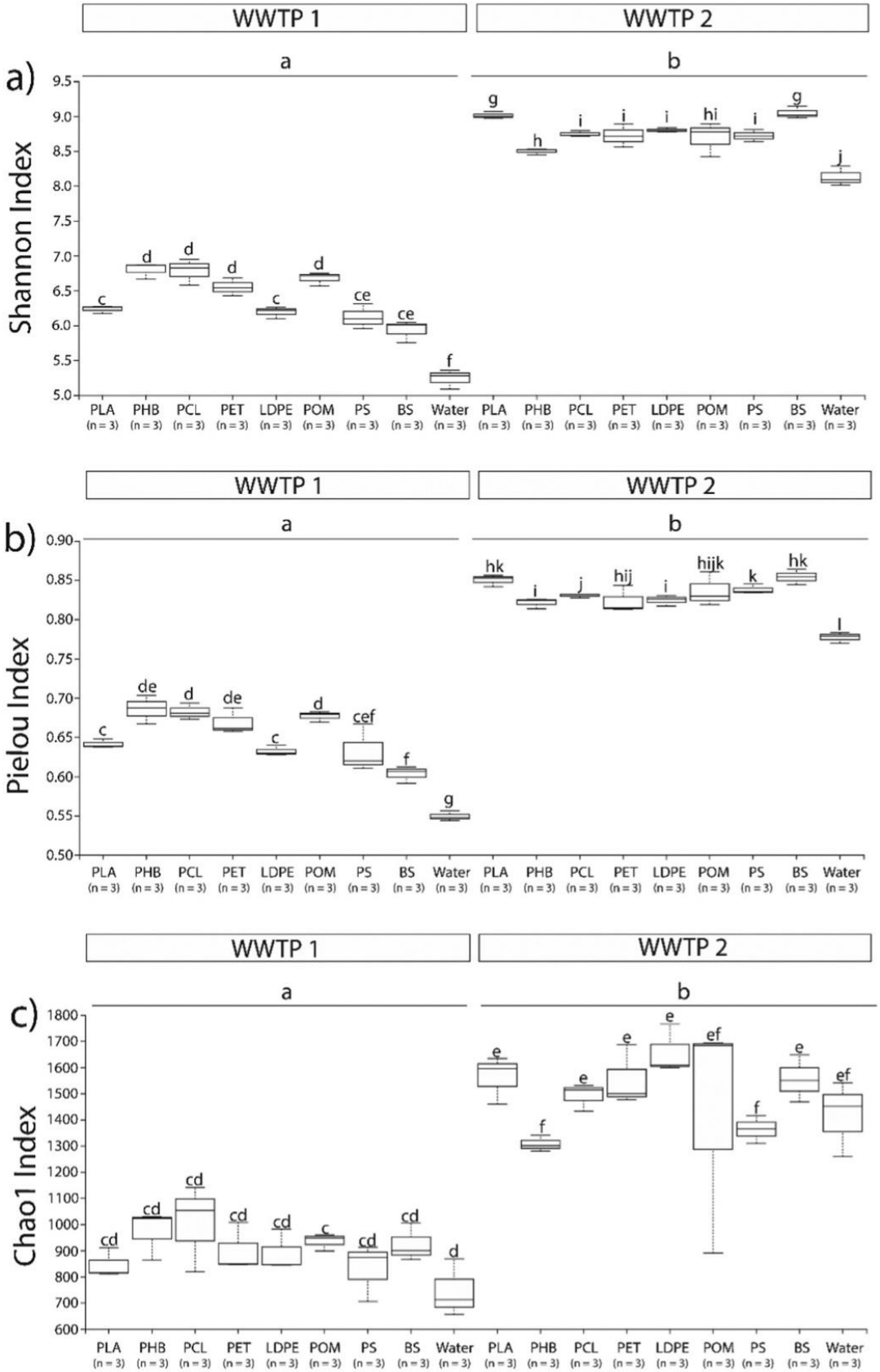

Figure 1. Boxplots of ASVs representing α-diversity using (a) the Shannon-Wienner index, (b) Pielou Evenness index and (c) Chao 1 index in the different substrates in WWTP1 and WWTP2. Lowercase letters indicated significant differences in the Kruskal-Wallis analysis. Statistical significance was a p-value < 0.05. Polylactic acid (PLA), poly-3-hidroxybutirate (PHB), polycaprolactone (PCL), polyethylene terephthalate (PET), low-density polyethylene (LDPE), polystyrene (PS) and polyoxymethylene (POM), borosilicate spheres (BS).

The comparison among the studied substrates revealed that effluent water, independently of the WWTP, presented significant lower Shannon Index value (pairwise Kruskal Wallis *p*-value < 0.05) (**Figure 1a**), and Pielou evenness value (pairwise Kruskal Wallis *p*-value < 0.05) (**Figure 1b**) than all the other tested substrates, revealing a less diverse bacterial community than those present in MPs and BS.

The sampling site had a significant role on bacterial diversity on the different tested substrates: PHB and PCL presented significant higher diversity and evenness in WWTP1 (pairwise Kruskal Wallis *p*-value < 0.05) while PLA and BS presented a slightly higher diversity in WWTP2 (pairwise Kruskal Wallis *p*-value < 0.05) (**Figures 1a, 1b**). These results might suggest that, in general, bacterial assemblages attached to biodegradable MPs were more diverse on these than on the rest of substrates (with the exception of BS in WWTP2).

3.4. BACTERIAL COMMUNITY COMPOSITION

Fifty-one bacterial phyla divided in 188 classes and 2 Archaea phyla divided in 6 classes were identified in the whole sample set (**Supplementary Material 2**).

Taxonomic analysis showed that the majority of the reads in the sample set were associated with the phyla Proteobacteria with 59.9% relative abundance followed by Bacteroidetes (14.7%), Actinobacteria (6.6%), Chloroflexi (5.1%), Firmicutes (4.2%), Saccharibacteria (1.4%) and Planctomycetes (1.3%) (**Supplementary Material 2**). Proteobacteria (60.72%), Bacteirodetes (16.17%), Actinobacteria (6.6%) were the most abundant phyla in MPs biofilms¸ Proteobacteria (55.75%), Bacteroidetes (11.6%), Antinobacteria (10.5%) in BS biofilms and Proteobacteria (58.25%), Parcubacteria (11.04%), Firmicutes (7.1%) in free-living bacteria in water.

The most abundant classes were Betaproteobacteria (23.1%), Alphaproteobacteria (21.6%) and Gammaproteobacteria (11.8%) in the phylum Proteobacteria, Sphingobacteria (8.5%) in the phylum Bacteroidetes, Acidimicrobia (3.9%) in the phylum Actinobacteria and Clostridia (2.5%) in the phylum Firmicutes (**Supplementary Material 2**). Betaproteobacteria (24.24%), Alphaproteobacteria (21.39%), Gammaproteobacteria (12.04%) were the most abundant classes in MPs biofilms; Alphaproteobacteria (26.20%), Betaproteobacteria (18.5%), Shingobacteriia (8.34%) in BS biofilms and Betaproteobacteria (19.42%), Alphaproteobacteria (18.11%), Gammaproteobacteria (14.14%) in free-living bacteria in water.

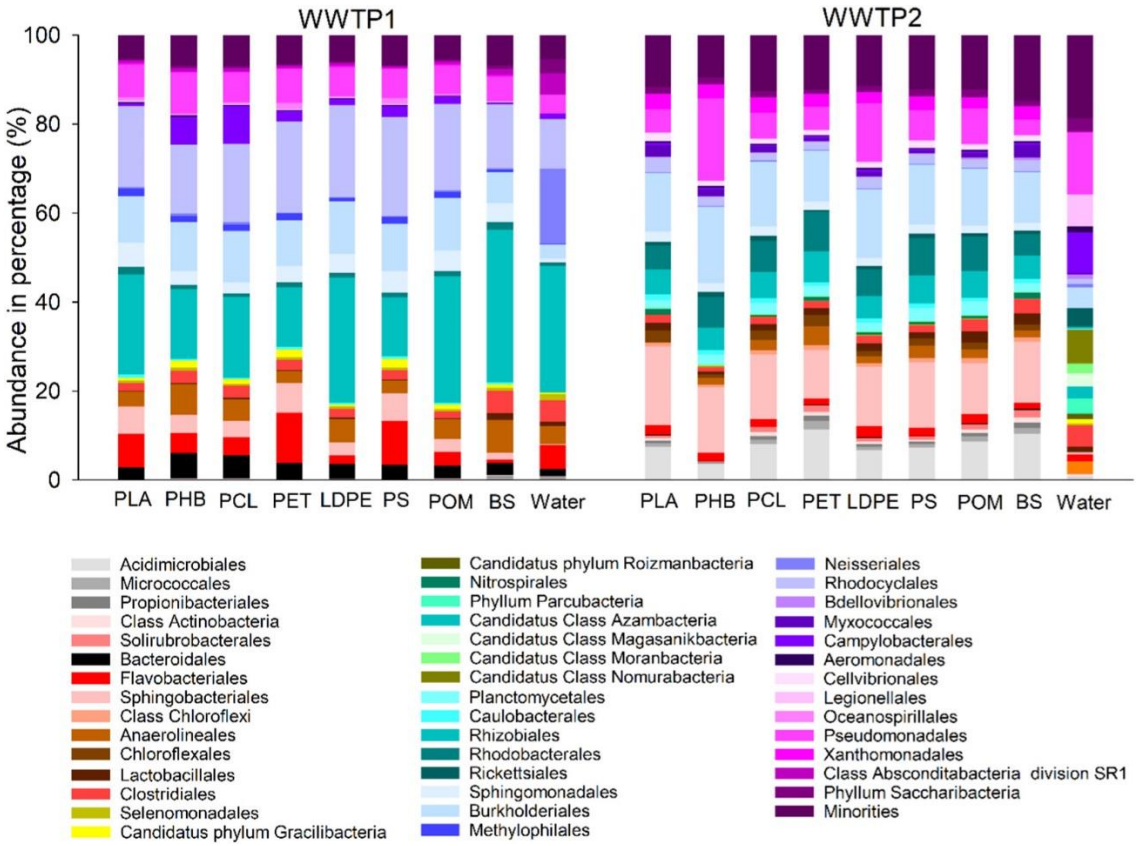

Figure 2. Relative abundance of bacteria communities at the order level associated to the different substrates in WWTP1 and WWTP2. Minority are orders whose representation is less than 1%.

The bar chart represented in **Figure 2** shows the bacterial distribution at the order level associated to the tested substrates and WWTPs. Firstly, microbial community composition at his level was clearly different between the two WWTPs. WWTP1 was characterized by a high abundance of the orders Rhizobiales (22.3%), Rhodocyclales (17.8%), Burkholderiales (9.8%), Pseudomonadales (6.7%) and Flavobacteriales (5.5%). In addition to these shared orders, Neisseriales (16.9%) was dominant in the free-living bacteria in water samples, while Sphingobacteriales (4.7%) dominated in the MPs-attached biofilms. In contrast, Anaerolineales (7.5%) and Clostridiales (5.0%) were more abundant in BS.

In contrast, Sphingobacteriales (12.6%), Burkholderiales (12.8%), Pseudomonadales (8.7%), Acidimicrobiales (7.4%) and Rhodobacterales (6.3%) characterized the distribution of bacterial order abundance in WWTP2. The abundance of the orders Campylobacterales (9.2%) Legionallales (7.1%) and Rickettsiales (4.0%) was higher in in the free-living bacteria in comparison with BS- and MPs-attached biofilms. Conversely, the order Rizhobiales dominated both BS (5.2%) and MP (5.8%) biofilms. There were not clear differences at the order level between the different tested substrates (MPs and BS).

However, at family-level resolution (**Supplementary Material 2**), there were differences in the relative abundance with respect to the tested substrates. Comamonadaceae

(11.9%), Rhodocyclaceae (10.1%), Moraxellaceae (7.5%), Hyphomicrobiaceae (4.8%) and Rhodobacteraceae (4.3%). displayed a higher relative abundance on MPs compared to BS and water samples, independently of the location. Considering the location, family Campylobacteraceae (7.1%), specifically the genus Arcobacter, was found as predominant in PHB (6.0%) and PCL (8.1%) in WWTP1. In comparison, the unassigned family JG35-K1-AG5 (23.25%) dominated in BS samples and Neisseriaceae (17.0%) dominated in WWTP1 effluent water. Regarding WWTP2, Saprospiraceae (10.4%) predominated in MPs and BS assemblages in comparison with free-living bacteria in the effluent water; families Campylobacteraceae (9.1%) and Legionellaceae (7.12%) were more abundant in the effluent water.

3.5. β-DIVERSITY

The data suggest that there are significant differences in bacterial composition between the two WWTPs and between MPs and BS and water; to further explore this, β-diversity metrics was used. A Principal Coordinate Analysis (PCoA) (**Figure 3**) was performed to determine the relevance of the site factor (WWTP1 or WWTP2) or tested substrate (MP, BS or effluent water). The statistical relevance of factors was analysed by PERMANOVA tests (**Table 2**). The LEfSe analysis was subsequently used to confirm which taxa, if any, were significantly more abundant in each group.

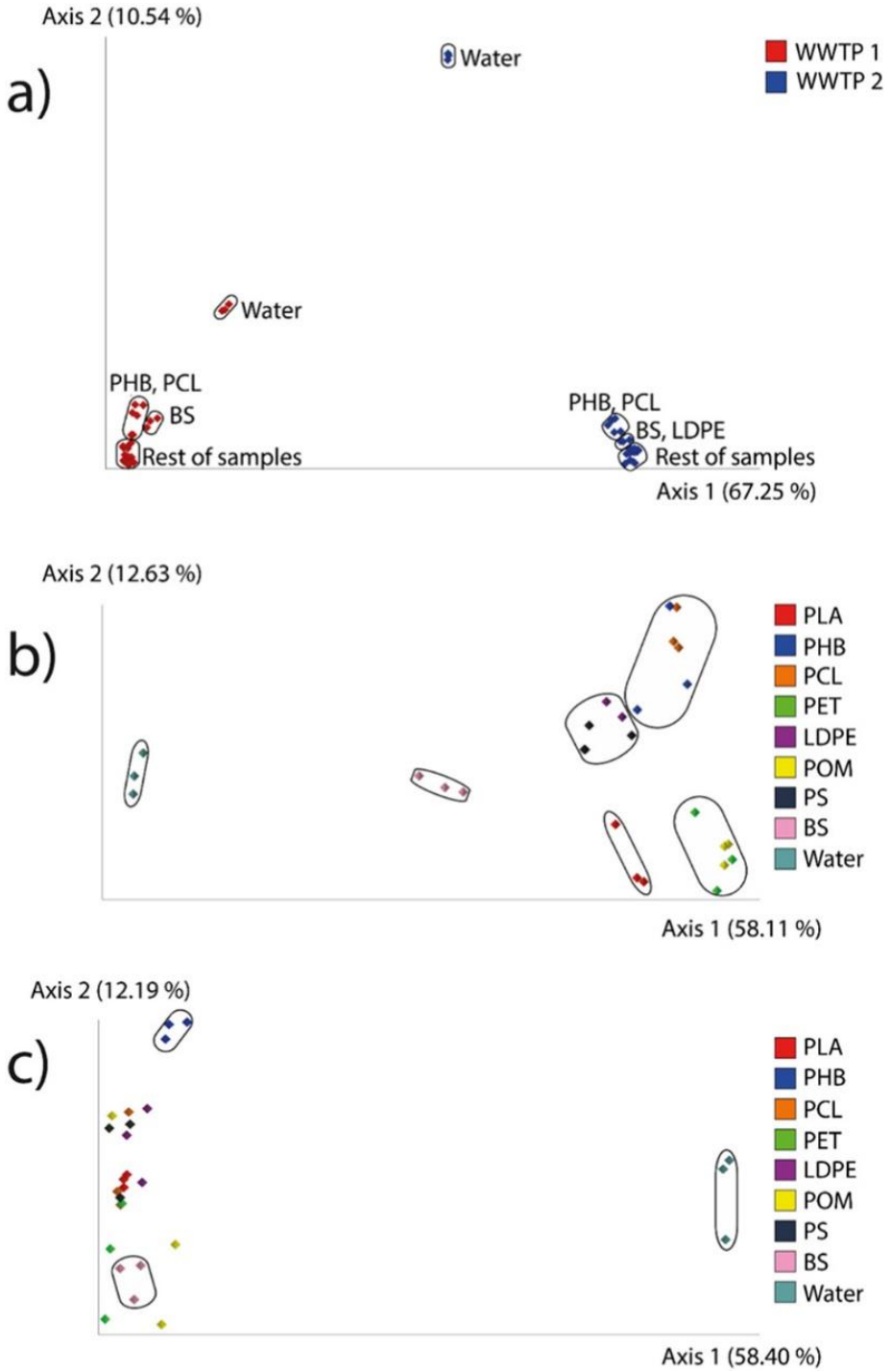

Figure 3. PCoA analysis of the microbial composition in samples based on Bray-Curtis dissimilarity. (a) Global analysis of all samples, (b) analysis of WWTP1 samples, (c) analysis of WWTP2 samples. Percentage in axes represent % of variation explained by that axis.

Site (*in situ* environment) (**Figure 3**) had a highly significant effect on the bacterial community (PERMANOVA; p-value < 0.05). The Bray-Curtis PCoA plot revealed an important pattern of clustering structure according to the sampling location, finding a very clear differentiation in the distance on the first axis, which explained the 65.2% of the difference between clusters. It should be noticed that water samples were clearly separated

from BS and MPs according to the second axis coordinate, which explained only 10.5% of the difference between clusters. The significant differences among the two WWTPs was confirmed by PERMANOVA tests (PERMANOVA; *p*-value < 0.05, **Table 2**). In addition; LEfSe analysis revealed significant differences in the abundance of different bacterial taxa among WWTP1 and WWTP2 (**Table 3**) highlighting the taxa Rhodocyclaceae, Hyphomicrobiaceae, Rhizobiales JG35 K1 AG5 *Fluviicola*, Sphingomonadaceae, *Arcobacter*, *Aquabacterium*, *Zoogloea*, *Paludibacter*, Uncultured Anaerolineaceae, Uncultured Sphingobacteriales, *Acidovorax* and *Pseudomonas* in WWTP1 and Uncultured Saprospiraceae, *Acinetobacter*, Rhodobacteraceae, Comamonadaceae, *Microthrix*, *Leeia*, Rhodocyclaceae 12up, Acidimicrobiaceae, *Roseiflexus*, Saccharibacteria, *Variovorax*, *Terrimonas*, *Dokdonella*, Chloroflexi ambiguous taxa, *Iamia* and *Mycobacterium* in WWTP2.

Table 2
Global and pairwise PERMANOVA analysis.

	Groups	PERMANOVA	
		Pseudo-F	***p*-value**
Global		36.34	< 0.01
Pairwise	WWTP1 - WWTP2	97.37	< 0.01
	Water - MP	5.67	< 0.01
	Water - BS	2.75	0.07
	MP - BS	0.95	0.20
	Water WWTP1 - Water WWTP2	96.70	0.10
	BS WWTP1 - BS WWTP2	59.67	0.08
	MP WWTP1 - MP WWTP2	195.34	< 0.01
	MP WWTP1 - Water WWTP1	30.67	< 0.01
	MP WWTP1 - BS WWTP1	8.17	< 0.01
	Water WWTP1 - BS WWTP1	39.81	0.10
	MP WWTP2 - Water WWTP2	36.72	< 0.01
	MP WWTP2 - BS WWTP2	38.84	0.07
	Water WWTP2 - BS WWTP2	39.43	0.10
	PLA - PHB	0.51	0.58
	PLA - PCL	0.42	0.58
	PLA - PET	0.28	0.57
	PLA - LDPE	0.31	0.57
	PLA - POM	0.24	0.57
	PLA - PS	0.26	0.58
	PLA - BS	0.45	0.57
	PLA - Water	3.22	0.08

(Continued)

	Groups	PERMANOVA	
		Pseudo-F	***p*-value**
Pairwise	PHB - PCL	0.26	0.57
	PHB - PET	0.57	0.57
	PHB - LDPE	0.38	0.56
	PHB - POM	0.41	0.57
	PHB - PS	0.42	0.57
	PHB - BS	0.95	0.56
	PHB - Water	3.05	0.08
	PCL - PET	0.33	0.58
	PCL - LDPE	0.27	0.57
	PCL - POM	0.29	0.58
	PCL - PS	0.24	0.58
	PCL - BS	3.04	0.06
	PCL - Water	3.04	0.06
	PET - LDPE	0.43	0.57
	PET - POM	0.14	0.64
	PET - PS	0.29	0.60
	PET - BS	0.66	0.58
	PET - Water	3.38	0.01
	LDPE - POM	0.38	0.57
	LDPE - PS	0.16	0.67
	LDPE - BS	0.54	0.57
	LDPE - Water	3.15	0.09
	POM - PS	0.32	0.59
	POM - BS	23.69	0.10
	POM - Water	3.43	0.00
	PS - BS	76.46	0.09
	PS - Water	48.27	0.11
	PS - BS	2.78	0.08

Table 3.
Differential bacterial taxa abundance comparing WWTP1 and WWTP2 samples by linear discriminant analyses (using LEfSe). Taxa with the highest Log LDA score in each group are listed.

Sampling point	Taxa	Log LDA score
WWTP1	Rhodocyclaceae	4.79
	Hyphomicrobiaceae	4.56
	Rhizobiales JG35 K1 AG5	4.43
	Fluviicola	4.24
	Sphingomonadaceae	4.06
	Arcobacter	4.01
	Aquabacterium	3.92
	Zoogloea	3.84
	Paludibacter	3.82
	Uncultured Anaerolineaceae	3.80
	Uncultured Sphingobacteriales	3.78
	Acidovorax	3.74
	Uncultured Gracilibacteria	3.68
	Rhizobiales	3.65
	Pseudomonas	3.55
WWTP2	Uncultured Saprospiraceae	4.50
	Acinetobacter	4.14
	Rhodobacteraceae	4.11
	Comamonadaceae	4.10
	Microthrix	4.08
	Leeia	4.01
	Rhodocyclaceae 12up	3.86
	Acidimicrobiaceae	3.78
	Roseiflexus	3.73
	Saccharibacteria	3.71
	Variovorax	3.66
	Terrimonas	3.62
	Dokdonella	3.57
	Chloroflexi ambiguous taxa	3.50
	Iamia	3.48
	Mycobacterium	2.78

RDA analysis (**Figure 4**) further confirmed a significant influence of the in situ environment in the community diversity, factor that was strongly correlated with the first RDA axis that explained 70.11% of the variation. The physicochemical substrate properties hydrophobicity and roughness were highly correlated with the second RDA axis that only explained 2.89%. Results of Monte-Carlo test showed that the influence of site (p-value =

0.001) and hydrophobicity (p-value = 0.015) was significant although roughness was not (p-value = 0.094).

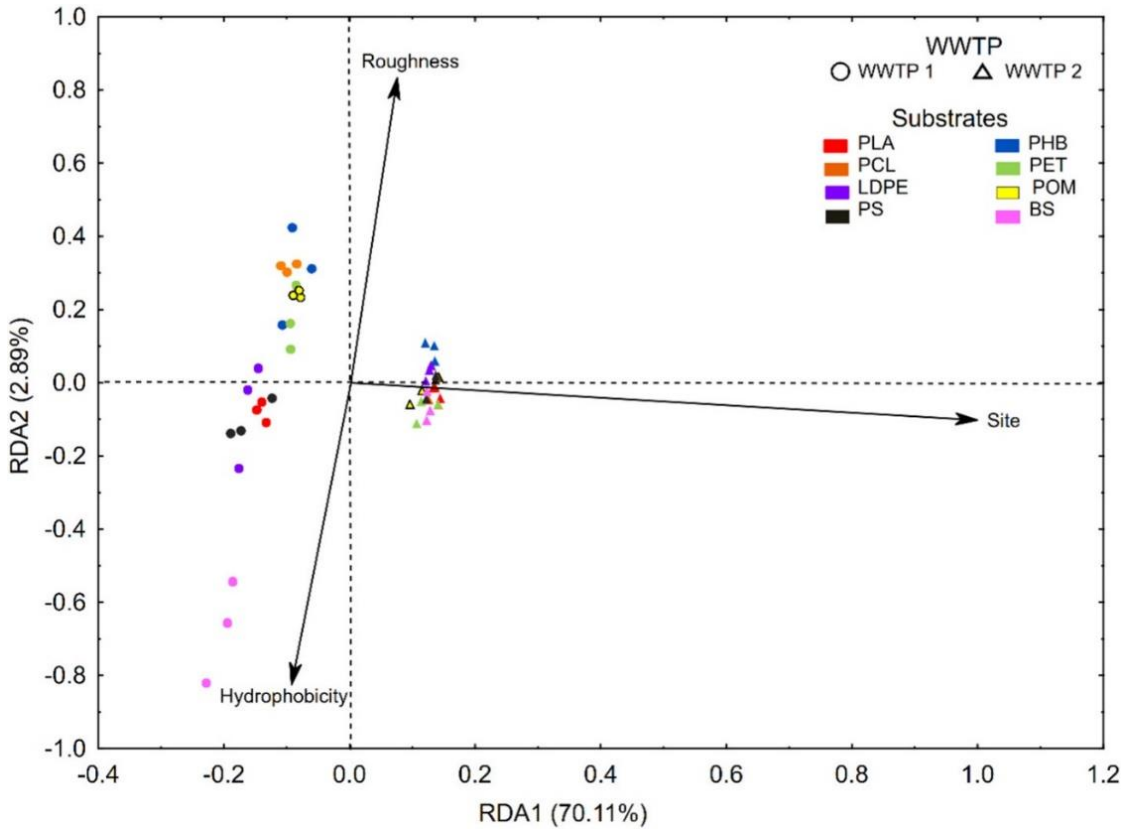

Figure 4. Redundancy analysis plot (RDA) of bacterial diversity in relation to site (*in situ* environment) and substrate surface proprietaries (roughness and hydrophobicity).

Although pairwise PERMANOVA tests did not detect significant differences among bacterial communities when comparing, as a whole, water-MPs, water-BS and MPs-BS (PERMANOVA; p-value < 0.05) (**Table 2**), when the samples were globally analysed by LEfSe analysis, certain taxa were significantly more abundant in MPs compared to water and BS (**Table 4**). In this context, uncultured Saprospiraceae, Comamonadaceae, Rhodobacteraceae, *Aquabacterium*, *Zoogloea*, *Acidovorax*, *Sphaerotilus*, Uncultured Sphingobacteriales, Acidimicrobiaceae, *Variovorax*, *Roseiflexus*, *Terrimonas*, *Dodonella*, *Pseudomonas* and *Perludibaca* might constitute the MP "core microbiome" in the studied WWTP effluents. BS selected for quite different taxa, including Rhizobiales, Sphingomonadaceae and photosynthetic ones like cyanobacteria. Effluent water free-living bacteria included, among others, Enterobacteriaceae which were not found in MPs. Nevertheless, when considering each WWTP separately, LEfSe analysis revealed some differences in the MPs core microbiome which might be due to the clear differences in the performance and characteristics of each WWTP (**Tables S7 and S8 in Supplementary Material 1**).

Table 4
Differential bacterial taxa abundance comparing MP-associated assemblages to borosilicate-associated assemblages and water sample bacterial communities by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed.

Substrate	Taxa	Log LDA score
MPs	Uncultured Saprospiraceae	4.35
	Comamonadaceae	4.33
	Rhodobacteraceae	4.04
	Aquabacterium	3.80
	Zoogloea	3.77
	Acidovorax	3.69
	Sphaerotilus	3.65
	Uncultured Sphingobacteriales	3.63
	Acidimicrobiaceae	3.56
	Variovorax	3.53
	Roseiflexus	3.47
	Terrimonas	3.41
	Dokdonella	3.38
	Pseudomonas	3.37
	Perludibaca	3.32
BS	Uncultured Anaerolineaceae	4.17
	Rhizobiales	3.95
	Sphingomonadaceae	3.85
	Chistensenellaceae 7 group	3.65
	Uncultured Aeroccaceae	3.20
	Leucobacter	3.16
	Paucibacter	3.14
	Chlorella sp. CC Bw 9	3.03
	Ignatzschineria	3.01
	Proteiniclasticum	3.88
	Holdemania	3.87
	Caldisericum	3.87
	Paucisalibacillus	3.82
	Dermacoccus	3.80
	Cyanobacteria subsection IV Family I	3.78

(Continued)

Substrate	Taxa	Log LDA score
Water	Rhizobiales JG35 K1 AG5	4.95
	Leeia	4.85
	Rhodocyclaceae	4.65
	Flavobacterium	4.38
	Unculture candidate division SR1	4.28
	Saccharibacteria	3.93
	Alcaligenaceae GK98 freshwater group	3.53
	Methylocystaceae	3.50
	Uncultured Veillonaceae	3.33
	Enterobacteriaceae	3.28
	Dialister	3.23
	Saccharofermentans	3.22
	Uncultured compost bacterium Saccharibacteria	3.11
	Alistipes	3.08
	Bifidobacterium	3.03

Table 5
Differential bacterial taxa abundance comparing the different MP-associated assemblages by linear discriminant analyses (using LEfSe). Taxa with the highest Log LDA score in each group are listed.

Plastic	Taxa	Log LDA score
PLA	Uncultured Saprospiraceae	4.46
	Uncultured Sphingobacteriales	3.84
	Dokdonella	3.56
	Spongiibacteraceae BD1 7clade	3.47
	Comamonas	3.38
	Aeromonas	3.24
	Flavobacteriales NS9 marine group	3.18
	Xanthomonadaceae uncultured	3.16
	Bacteroidetes	3.15
	Sphingomonadales	3.12
	Thauera	3.03
	Dechloromonas	3.02
	Sphingobacteriales	3.02
	Chitinophagaceae	2.97
	Sorangium	2.95

(Continued)

Plastic	Taxa	Log LDA score
PHB	*Acinetobacter*	4.59
	Aquabacterium	4.12
	Pseudomonas	3.65
	Lautropia	3.35
	Ferruginibacter	3.32
	Vibrio	2.84
	Gracilibacteria	2.60
PCL	*Sphaerotilus*	3.88
	Variovorax	3.77
	Terrimonas	3.60
	Simplicispira	3.24
	Sphingobium	3.01
	Cyanobacteria	2.73
PET	Rhodobacteraceae	4.15
	Thermomonas	3.25
	Xantomonadales Incertae Sedis	3.16
	Agitococcus lubricus group	3.09
	Betaproteobacteria SC I 84	3.02
	Ferribacterium	2.97
	Uncultured Rhizobiales A08329	2.91
	Uncultured Sphingobacteriales	2.79
	Acetobacteraceae	2.67
	Reyranella	2.62
LDPE	Comamonadaceae	4.40
	Zooglea	3.98
	Ernhydrobacter	3.24
	Betaproteobacteria	3.18
	Candidatus Competibacter	2.60
POM	Sphingobacteriales OPS17	3.11
	Uncultured Fimbrimonadaceae	3.01
	Uncultured Verrucomicrobiaceae	3.00
	Gammaproteobacteria	2.74
	Bdellovibrio	2.65
	Deltaproteobacteria SAR324 glade marine group B	2.59
	Prosthecobacter	2.50
PS	*Acidovorax*	3.81
	Hydrogenophaga	3.15

LEfSe analyses also reported differential abundance of certain taxa in each specific MP, regardless of the WWTP as shown in **Table 5**. Of the tested MPs, PLA showed the higher diversity with fifteen taxa with the highest scores, followed by PET with ten taxa; PS MPs showed the lowest diversity. In general, the tested MPs did not share taxa suggesting that each MP might select different attached bacteria.

3.6. Relative abundance of the ARGs *tetM* and *sulI*

Figure 5 shows the relative abundance of ARGs *tetM* and *sulI* on the tested substrates and WWTP water effluents. Pairwise Kruskal Wallis test for significant differences among substrates and water in the two WWTPs is shown in **Table S9 in Supplementary Material 1**.

The relative abundance of the *sulI* gene changed significantly depending on the WWTP. In WWTP1 no significant differences were found among MPs and water; however, BS had a significant lower relative abundance of the *sulI* gene compared to water (p-value < 0.05).

Regarding WWTP2, the *sulI* gene was detected in a significantly higher relative abundance attached to POM and PS MPs as well as on BS than in water (p-value <0.05). In general, significantly less *tetM* was detected in MPs and BS than in water in both WWTPs.

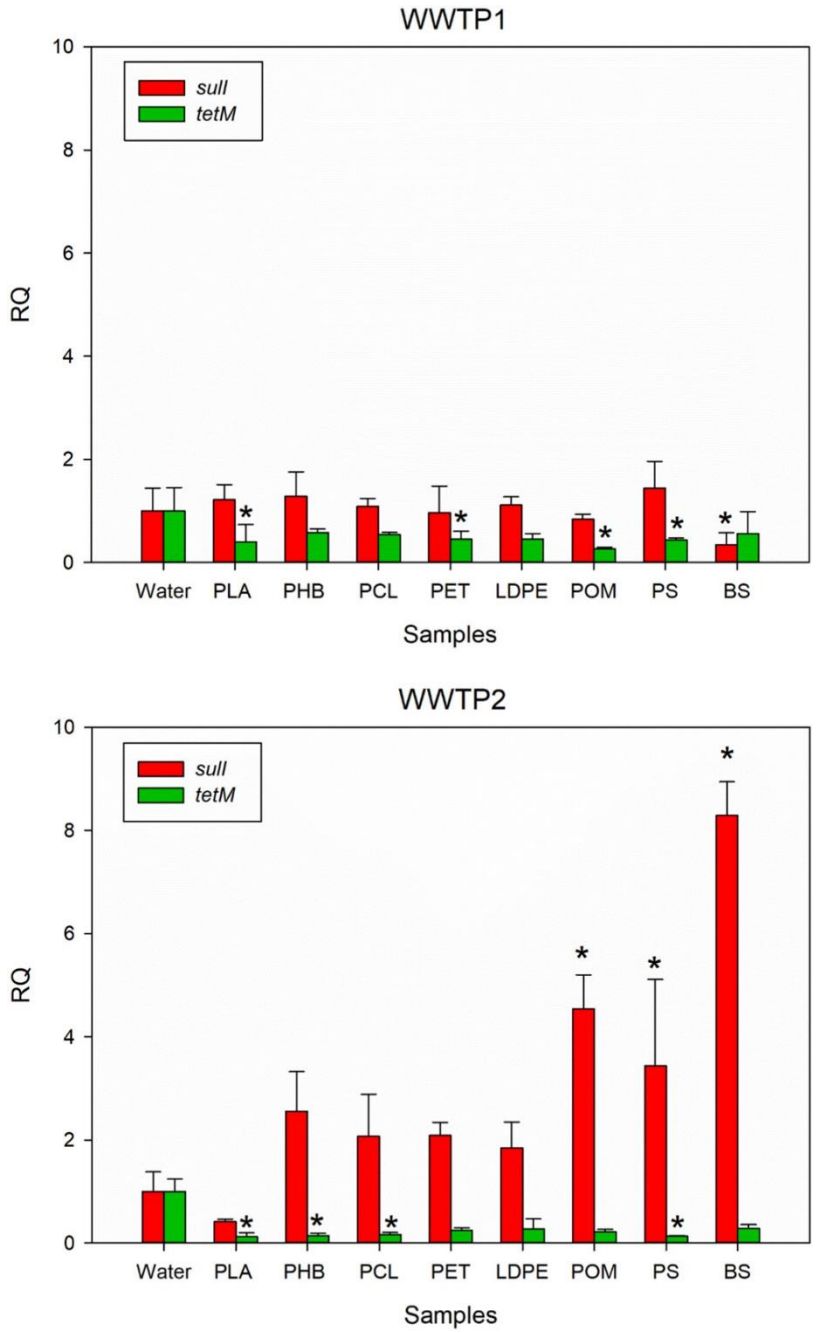

Figure 5. Relative abundance of *sulI* and *tetM* genes measured in the different substrates and effluent water in WWTP1 and WWTP2. Error bars indicate standard deviations of triplicates. Asterisk (*) denotes a statistically significant difference between the relative abundance of *sulI* and *tetM* genes in substrates and effluent water in each WWTP (Kruskal-Wallis test; p value < 0.05).

4. Discussion

The present study provides relevant information about bacterial community assemblages in different MPs exposed for a short time (48 h) to WWTP effluents in situ. To our knowledge, this is the first study to do so in seven different types of polymers including biodegradable (PLA, PHB, PCL) as well as non-biodegradable (PET, LDPE, PS, POM) MPs. Marine plastic debris is mainly composed of PE, PP and PS; in this context, most marine

studies have focused on the use of these polymers for the colonization studies as well as PET or polycarbonate (PC) (De Tender et al., 2017; Dussud et al., 2018; Oberbeckmann et al., 2016; Ogonowski et al., 2018). Dussud et al. (2018) besides virgin PE, have used artificially aged PE as well as the biodegradable polyester PHBV. Marques et al. (1997) also used PHBV. Lee et al. (2014) used PS and PVC plates deployed on a cold seep in the Red Sea. Regarding freshwater systems, Hoellein et al. (2014) compared hard and soft substrata including plastics deployed on a river, a pond and recirculating laboratory streams. Oberbeckmann et al. (2018) analysed the colonization of HDPE and PS pellets incubated for 14 days at sampling stations in the estuary of the river Warnow (including WWTP discharge) and in the Baltic Sea. McCormick et al. (2014, 2016) did not perform colonization experiments but collected plastics from surface river waters and WWTPs effluent and analysed the microbial assemblages on the collected MPs.

WWTPs have been revealed as one of the main hotspots for the release of MPs in freshwater (Edo et al., 2020; Magnusson and Norén, 2014; McCormick et al., 2014), as well as pathogens and ARBs (Pazda et al., 2019). MPs can interact with sewage-related microorganisms, including pathogens and ARBs, and transport them downstream, ending up in the oceans (McCormick et al., 2014; Oberbeckmann et al., 2018; Oberbeckmann et al., 2014). In this context, Hoellein et al. (2014) suggested that plastic biofilms might be more stable and remain intact longer and transport biofilms further compared to natural surfaces like wood or other natural particles.

The signs of the existence of the plastisphere were denoted for the high diversity in the MPs in comparison with the water, independently of the WWTP. These results were novel in comparison with previously studies that evaluated the bacterial biofilm formation in WWTP effluent. Peng et al. (2018) analysed the early biofilm formation (24–48 h) in a WWTP effluent using a bio-cord of PP fine fiber as substrate and reported a diversity bacterial richness much lower in the water than in the biofilm. McCormick et al. (2014) recollected MPs from the WWTPs effluent and showed a higher diversity in MPs than in WWTP effluent water. Our results reported that MPs had a higher bacterial diversity than WWTP effluent water free-living bacteria, that could be explained because the early colonization that occurs on bare substrates implies active adhesion capacities for pioneer bacterial species, and these pioneers facilitate the adhesion of new species from water column in the first hours (Lyautey et al., 2005).

The factor in situ environment sampling site was the most significant explaining the bacterial diversity in the different tested MPs. Oberbeckmann et al. (2018) demonstrated

that the degree of specificity of the marine microbiome on MPs depended on the environmental conditions and they only observed significant differences between MP microbiomes in areas with lower nutrients; they refer the term plastisphere, proposed by Zettler et al. (2013), to be used in certain environmental conditions such as "lower nutrients, high salinity". In this context, the two WWTPs of this study showed significant operational differences (**Table S1 in Supplementary Material 1**): In WWTP1, the treatment is based on a contact-stabilization process, unable to remove nutrients efficiently presenting difficulty in generating a stable effluent of good quality. On the contrary, in WWTP2, the secondary treatment was based in the A2O method, which removed nutrients efficiently using two anaerobic ponds and an anoxic pond as well as an oxic pond allowing a high-quality effluent; also A/A/O (A/O) systems, as compared to other systems such as membrane bioreactors (MBRs), usually show higher Simpson's diversity index and evenness index meaning also a higher bacterial diversity (Hu et al., 2012) as also found in this study. This could be related to the chemical parameters of each WWTP effluents, with WWTP1 effluent showing a higher nutrient load than WWTP2 particularly regarding PO_4^{3-}, NH_4^+ and COD (**Table S4 in Supplementary Material 1**). Previous studies confirmed that the microbial communities adhered to the MPs depended mainly on the location (Amaral-Zettler et al., 2015; McCormick et al., 2014; Oberbeckmann et al., 2018; Oberbeckmann et al., 2014).

Rummel et al. (2017) defined hydrophobicity and roughness as the two principal superficial parameters of polymers that can affect the colonization of MPs. This information suggests that the first phases of colonization might be dependent on the MP surface properties.

In this study, MP superficial parameters were secondary to the factor in situ environment, nevertheless, hydrophobicity had a significant role although smaller and roughness did not play any significant role. Oberbeckmann et al. (2018) found that *in situ* environment was the major factor in their two-week experiment. Ogonowski et al. (2018), in a two-week study also, found that substrate hydrophobicity strongly correlated with bacterial composition across all tested substrate. Clearly, more studies on how changes in surface properties of the same material over time affect colonization process are needed before reaching significant conclusions in this matter.

Illumina sequencing data highlighted significant differences among bacterial assemblages on MPs, BS and bacterial communities in WWTP effluent water samples after 48 h of colonization. However, most studies have shown that the microbial community in plastics is similar to that in other substrates (glass, metal, organic particulate matter) although

clearly different to that of free-living microorganisms in the water column or marine sediment (Bryant et al., 2016; Dussud et al., 2018; Harrison et al., 2014; Hoellein et al., 2014; McCormick et al., 2014; McCormick et al., 2016). A few studies, however, found significant changes in microbial diversity depending on polymer type (De Tender et al., 2017; Ogonowski et al., 2018; Webb et al., 2008). Most of these studies considered colonization data over a week. In this context, Hoellein et al. (2014) and Oberbeckmann et al. (2016, 2018) suggested that future experiments on MP biofilms should include the colonization phase of the first few hours to days because difference in microbial diversity between substrate types might be stronger during early stages of biofilm formation on MPs. Biofilms are envisaged as an effective strategy for microbes to survive in unfavorable environments. The formation of a biofilm is a dynamic sequence of events, which, for better understanding, has been divided into distinct developmental stages: it is initiated by planktonic bacteria that first attach to each other (cell-to-cell attachment, termed as cohesion). Then, they attach themselves reversibly to a surface usually through physical forces and in real time, a number of the reversibly adsorbed cells remain immobilized and become irreversibly adsorbed onto the surface (physical appendages of bacteria such as fimbriae or pili as well as adhesins have a predominant role in this phase). Once adsorbed, they form microcolonies and produce the extracellular polymeric substance (EPS), the glue that holds the microbial community together and acts as a barrier to chemicals (containing exopolysaccharides, proteins, nucleic acids and other bacterial detritus). In the final stage, the biofilm disperses, and the free microbes look for new niches to be established (Hall-Stoodley et al., 2004). During biofilm initiation, nutrients, and dissolved organic matter (DOM, which may facilitate the formation of a surface organic layer on the substrate) and bacterial input form the surrounding water will affect the microbial communities and their interaction.

Phyla Proteobacteria [Betaproteobacteria (24.24%), Alphaproteobacteria (21.39%), Gammaproteobacteria (12.04%)], Bacteirodetes and Actinobacteria dominated MPs biofilms in this study. Members of alpha and gammaproteobacteria as well as Bacteroidetes and Firmicutes are characteristic of early biofilm colonization and are known to produce the EPS (Dang and Lovell, 2000). Peng et al. (2018) in their study on early biofilm formation on a PP bio-cord deployed downstream of a WWTP outlet found that Alphaproteobacteria dominated the biofilm and that this class showed "biofilm-specific" property, suggesting that the ability of colonization was more relevant in the very early stage of biofilm formation; also Actinobacteria may contribute significantly to organic matter processing. Some members of Bacteroidetes are reported to have a role in initial biofilm formation as they can degrade

biopolymers to low molecular weight DOM that helps in biofilm conditioning (Kirchman, 2002).

At the family level, Comamonadaceae, Rhodocyclaceae, Moraxellaceae, Hyphomicrobiaceae and Rhodobacteraceae predominated on MPs compared to BS and water samples. Comamonadaceae, has been found as dominant in MPs collected from urban rivers and associated WWTP effluents (McCormick et al., 2014, 2016). Family Rhodobacteraceae and Flavobacteriaceae were found as dominant in MPs colonized in marine waters (Bryant et al., 2016; De Tender et al., 2017; Oberbeckmann et al., 2018; Zettler et al., 2013).

An interesting question is whether a MPs-core microbiome can be identified. De Tender et al. (2017) identified 25 bacterial core OTUs on both plastic sheets and dolly ropes deployed in a harbor in Belgian part of the North Sea. Oberbeckmann et al. (2018) reported a marine MPs-microbiome core where Hyphomonadaceae and Erythrobacteraceae were dominant. Ogonowski et al. (2018) in their colonization experiment using PE, PP and PS in the Baltic Sea (brackish system) reported that Alphaproteobacteria, Bacteroidetes and Plantomycetes predominated in plastics compared to non-plastic substrates. Regarding freshwaters, McCormick et al. (2014) identified 46 OTUs that accounted for more than 60% variation between plastic and non-plastic substrates, the most common taxa on plastics were Pseudomonadaceae, Proteobacteria and Campylobacteraceae, other relevant taxa were *Arcobacter* and *Aeromonas*. In a similar but more recent study, McCormick et al. (2016) identified Pseudomonadaceae, Gammaproteobacteria and Comamonadaceae in MPs collected also from urban rivers; other relevant taxa more abundant on collected MPs were *Pseudomonas* and *Aquabacterium*. Peng et al. (2018) identified 44 OTUs as dominant in the plastic biofilms deployed in the effluent of a WWTP; these OTUS corresponded to members of the Alphaproteobacteria, Gammaproteobacteria, Firmicutes, and Bacteroidetes. In this study, we have been able to identify a core microbiome of fifteen taxa that have colonized MPs deployed into the effluent of two quite different WWTP effluents; it is noteworthy that there were some coincidences with those taxa described by McCormick et al. (2014, 2016) like Comamonadaceae, *Aquabacterium* or *Pseudomonas* and also with some taxa described by Peng et al. (2018) such as Rhodobacteraceae or *Pseudomonas*. Despite the coincidences, there are many differences that might suggest that the specific environment (site) is the parameter that might select the indicator species. More studies in a range of different environments are necessary before reaching a conclusion about MPs-core microbiomes.

It is noteworthy that some of the genera found as dominant in MPs such as *Pseudomonas*, *Variovorax*, *Aquabacterium* or *Acidovorax* have species with the capacity to metabolize recalcitrant substances, including plastics. *Pseudomonas* has already been previously described as one of the first colonizers of the plastisphere (McCormick et al., 2014; Wu et al., 2019), it is one of the main producers of exopolysaccharide (EPS), that facilitates the adhesion of new bacteria (Chien et al., 2013) and also provides protection against harmful substances, such as heavy metals (Pal and Paul, 2008). In addition, it can metabolize plastics such as PE, PET and PS to some extent as a source of carbon and energy under laboratory conditions (O'Leary et al., 2005; Ronkvist et al., 2009; Yoon et al., 2012). Likewise, some species of the genus *Acidovorax* can accumulate PHB inside (Schulze et al., 1999). Morohoshi et al. (2018) detected the presence of this genus associated to biofilms that degraded PHB. Some species of the genus *Aquabacterium* are able to metabolize plasticizers used in PVC (Kalmbach et al., 1999); this genus has been identified as dominant in biofilms attached to plastics in drinking water plants (Kalmbach et al., 2000). The isolation of these strains could be very important to establish new metabolic pathways that favour the biodegradation of plastics. The genus *Variovorax* is able to degrade several aquatic pollutants such as trichloroethylene, linuron and arsenite (Satola et al., 2013).

The high relative abundance of the genus *Roseiflexus*, on MPs, whose only representative species is the photosynthetic *Roseiflexus castenholzii* (Hanada et al., 2002), indicates the importance of microbial primary producers other than cyanobacteria associated with MPs (Yokota et al., 2017).

An issue with MPs colonization is the presence of pathogenic bacteria. Genus *Pseudomonas* include species that are opportunistic pathogens to humans such as *Pseudomonas aeruginosa*, which has already been found in WWTPs with multiple resistance to antibiotics (McCormick et al., 2014; Slekovec et al., 2012). Within family *Campylobacteraceae*, genus *Arcobacter*, which also contains some opportunistic pathogenic members which are known to cause human gastrointestinal infections, has also been found attached to MPs and remarkably, it has been found in both freshwaters and marine habitats (Harrison et al., 2014; McCormick et al., 2014). In this study, *Arcobacter* was found in MPs biofilms specifically in WWTP1, which has a higher organic load than WWTP2. Interestingly, also in this study, the well-known human pathogenic genus *Mycobacterium* (belonging to the *Mycobacteriaceae* family, Actinobacteria phylum) was also found in bacterial assemblages on MPs in WWTP2. Other studies have found *Vibrio* spp. on MPs which also has some pathogenic species for man and aquatic fauna (Kirstein et al., 2016; Zettler et al., 2013) or fish pathogens such as *Aeromonas*

(McCormick et al., 2014). The fact that some of these pathogens may be early MP colonizers and could be transported from WWTPs to rivers and even oceans may raise some concerns on potential risk to human health. However, at present, the role of plastics in general as vectors of pathogenic microorganisms is unknown. Future studies should examine the survival rates of the bacteria adhered to the MPs as they drift along the river to the sea.

In this study LEfSe Analysis allowed the identification of early bacterial colonizers on each of the seven tested MPS; this implies that the type of polymer might select for such early colonizers. This finding is not reported in most studies because it might be possible that this is mostly evident only in early colonization studies (Oberbeckmann et al., 2018). However, Ogonowski et al. (2018) found differences in bacterial colonization of PE, PP and PS in their two-week study of colonization in brackish waters from the Baltic Sea, with PS being the substrate with a higher diversity.

It is noteworthy that the biodegradable MPs used in this study, PLA, PHB and PCL, showed a significant abundance of genera with potential pathogenic members: *Pseudomonas*, *Comamonas*, *Aeromona*s and *Vibrio*. Does this mean that biodegradable MPs might be vectors of pathogenic bacteria in aquatic environments? This is an issue to be further investigated and clarified. Biodegradable MPs also were enriched on potential degrading taxa such as *Aquabacterium* and *Pseudomonas* in PHB and *Variovorax* in PCL.

Regarding non-biodegradable plastics, (PET, LDPE, POM and PS), genus *Ferribacterium* was selected in PET, this taxon has previously been reported as a characteristic microorganism in sewage sludge (Luo et al., 2020) and as an early colonizer attached to PP bio-cords deployed in a WWTP outlet (Peng et al., 2018). Genus *Zooglea*, very abundant in LDPE, has a crucial role in aerobic wastewater treatments due to its ability to degrade organic carbon and promote floc formation (Dris et al., 2015). Although it is usually more frequent in wastewater effluent water, it has already been found in MP assemblages in freshwater environment (McCormick et al., 2014) and related to the early formation of biofilm at PP bio-cords in WWTP effluents (Peng et al., 2018). In PS, the most abundant genus was the potential plastic *Acidovorax*, which is very frequent in activated sludge (Heylen et al., 2008).

There is also a growing concern that MPs, in general, may be reservoirs of ARBs and cognate ARGs. ARBs may survive in the presence of one or more antibiotics and that might be a potential threat for human health (Proia et al., 2016). Most ARGs are located on broad-host range conjugative plasmids or other mobile elements that can be transferred to nearby receptors leading to global spread of resistance (Sultan et al., 2018; Wang et al., 2020). The

main source of ARBs/ARGs is to be found in urban sewage as has been proved by global monitoring of antibiotic resistance (Hendriksen et al., 2019; Pärnänen et al., 2019).

The role of plastic biofilm as ARG reservoirs has been seldom studied. Yang et al. (2019) in an in-situ study found 64 ARG subtypes of 11 ARG types and 47 MRG subtypes in microbes on plastic particles in the North Pacific Gyre and Wang et al. (2020) under laboratory conditions using river water collected from the pristine headwater zone of the Taihu Lake, China, and sea water collected from the East Sea of China found that PE MPs concentrated most ARGs from the surrounding water including *sulI*, *tetA*, *tetC*, *tetX*, *ermE* and *ermF*. Our study showed that the *sulI* gene was already present in WWTP effluents and that it was present in MPs at the same level than in effluent water in MPs deployed in WWTP1 and at higher abundances in POM, PS and BS than in effluent water in MPs in WWTP2. Proia et al. (2016) found a significant abundance of *sulI* in biofilms situated after a WWTP effluent. However, Yang et al. (2019) that did not detect the presence of sulphonamide resistance genes in marine MPs, stating that sulphonamide resistance is associated with anthropic environments and not with relatively pristine environments such as marine sediments or lakes. On the contrary, *tetM* abundance was significantly lower in MPs than in effluent water, meaning that MPs do not seem to concentrate ARBs, which harbor this gene in particular. *SulI* genes are part of the 3' conserved segments of Class 1 integrons. In this context, the *sulI* gene is usually considered as a marker of the presence of this class of integrons associated with resistance to sulphonamides and quaternary compounds. Class 1 integrons is the one most frequently detected in *Enterobacteriaceae*, including *Campylobacte*r spp., *Escherichia coli*, and *Salmonella enterica* serotype Typhimurium (Lucey et al., 2000; Carattoli, 2001; Zhao et al., 2001). The environmental relevance of this class of integrons is that it as a primary source of resistance genes and is suspected to serve as reservoirs of antimicrobial resistance genes within microbial populations (Carattoli, 2001). Regarding plastics, Wang et al. (2020) found a significant correlation between ARGs and class 1 integron integrase gene (*intI1*) suggesting that *intI1* might facilitate the transmission of *sulI*, *tetX*, *ermE* and *ermF* between water and MPs through horizontal gene transfer which might underpin the role of MPs as conveyors of microbial resistance in aquatic environments.

This study is the first to evaluate seven different types of MPs as potential vectors of *sulI* and t*etM* finding that they could be conveyors of *sulI* but not *tetM*. High throughput studies should analyze more globally the ability of MPs to accumulate ARBs and cognate ARGs and the possible impact on the environment and human health.

5. CONCLUSIONS

This study addresses for the first time the early bacterial colonization phase of seven different types of MPs including biodegradable and non-biodegradable ones deployed in WWTP effluent water.

In situ environment (sampling site) along with hydrophobicity to a lesser extent were the factors explaining bacterial diversity in the tested MPs.

The MPs clearly showed a different bacterial diversity when compared to that of WWTP effluent water or borosilicate glass. An early colonization phase MPs-core microbiome was identified. Furthermore, LEfSe analysis allowed identifying core microbiomes specific for each type of polymer suggesting that each type might select early attachment of bacteria.

It is of concern that some of the taxa identified on MPs could have pathogenic members and be a threat to human health. The fact that these taxa are found in biodegradable MPs suggests that the capacity of the MPs to act as vector of potentially pathogenic taxa may be facilitated by their biodegradability.

The tested WWTP effluent waters contained ARBs harboring the *sulI* and *tetM* ARGs, MPs concentrated the ARBs harboring the *sulI* gene, particularly those deployed in WWTP2, but not *tetM*. This might have to do with the specific sites and/or the ARG-carrying bacteria present in the site and their ability to attach to different MP polymers. This merits further study before claiming that MPs may act as global vectors of ARGs.

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7. SUPPLEMENTARY MATERIAL 1

CONTENTS:

Table S1.
Operational variables of the two wastewater treatment plants (WWTP) evaluated in this study.

Operation variable	WWTP1	WWTP2
Location	Cantoblanco (Madrid, Spain)	Guadalajara (Castilla La Mancha, Spain)
Coordinate (DG)	Longitude: 40.5442 Latitude: -3.6845	Longitude: 40.6211 Latitude: -3.1909
Type of sewage	Domestic and hospital	Industrial and domestic
Discharge (m^3/d)	931	45000
Population equivalent	5927	91600
Total suspended solids (mg/L)	265	300
BOD_5 (mg/L)	382	350
TKN (mg/L)	54.1	50
TP (mg/L)	12.7	12
Pre-treatment	Bar screens Grit removal	Bar screens Grit removal Fat and grease removal
Primary treatment	No	Three primary tanks sedimentation
Secondary treatment	Aerobic system by contact	A2O

Table S2.
Principal characteristics of the different substrates used in this study.

Name	Abbreviation	Biodegradability	Manufacturer	Shape	Diameter (mm)	Density (g/cm2)
Polylactic acid	PLA	Yes	Goodfellow	Pellet	3	1.24
Polyhydroxybutyrate	PHB	Yes	Goodfellow	Pellet	5	1.25
Polycaprolactone	PCL	Yes	Aldrich Chemistry	Pellet	3 - 5	1.15
Polyethylene terephthalate	PET	No	Goodfellow	Pellet	4	1.39
Low-density polyethylene	LDPE	No	Goodfellow	Pellet	3.5	0.92
Polyoxymethylene	POM	No	Goodfellow	Pellet	5	1.41
Polystyrene	PS	No	Goodfellow	Pellet	3	1.05
Borosilicate glass pearls	SS	No		Sphere	2	2.23
Borosilicate glass pearls	MS	No		Sphere	5	2.23
Borosilicate glass pearls	BS	No		Sphere	8	2.23

Table S3.
Contact angle measurements and surface free energy components.

Material	Contact angle (°)			Surface free energy components (mJ/m2)					
	Water	Glycerol	Diiodomethane	gSLW	gS(+)	gS(-)	gSAB	gS	ΔGSWS
PLA	86.7 ± 7.0	64.5 ± 9.4	62.5 ± 5.1	27.13	3.22	0.82	3.25	30.38	-54.5 ± 8.1
PHB	69.3 ± 7.1	46.8 ± 8.7	76.5 ± 7.6	19.34	9.52	6.10	15.24	34.58	-20.4 ± 4.6
PCL	74.6 ± 2.1	49.8 ± 4.1	63.1 ± 4.9	26.80	6.39	2.80	8.47	35.26	-34.6 ± 2.1
PET	84.1 ± 2.9	64.3 ± 4.8	68.6 ± 7.4	23.65	3.81	1.92	5.42	29.06	-45.5 ± 3.9
LDPE	95.0 ± 3.4	54.5 ± 3.1	74.3 ± 2.9	20.09	8.73	0.00	0.00	20.09	-42.4 ± 2.3
POM	81.7 ± 3.3	65.6 ± 2.2	68.9 ± 7.0	23.47	3.03	3.68	6.67	30.15	-41.5 ± 5.2
PS	77.1 ± 3.5	59.3 ± 2.9	78.3 ± 7.0	18.37	6.24	4.99	11.16	29.53	-29.0 ± 3.9
BS	74.0 ± 2.4	62.3 ± 3.7	41.0 ± 4.47	39.11	0.43	7.51	3.60	42.70	-45.6 ± 5.8

Table S4.
Physical and chemical parameters in the two WWTP effluents.

Location	WWTP1		WWTP2	
Time	0 h	48 h	0 h	48 h
Temperature (°C)	13.7	12.4	19.3	19.6
pH	7.40	7.52	7.27	7.06
Oxygen (mg/L)	1.52	2.09	4.62	4.06
Oxygen (%)	13.9	20.6	53.5	47.6
Salinity (μs/cm)	622	649	903	970
PO_4^{3-} (mg/L)	6.95	9.20	4.80	5.70
NO_2^- (mg/L)	0.04	0.05	0.02	0.03
NO_3^- (mg/L)	0.25	0.20	35.7	21.8
NH_4^+ (mg/L)	69.9	55.2	0.35	0.35
COD (mg/L)	85.0	50.0	8.5	14.0

Table S5.
Description of the primers for 16S rRNA Illumina sequencing. The amplified region and the sequences of the primers are indicated. The primer tail is shown in bold.

Region	**Reference number**	**Sequence**
16S	16SV3-V4-CS1	**ACACTGACGACATGGTTCTACA**CCTACGGGNGGCWGCAG
	16SV3-V4-CS2	**TACGGTAGCAGAGACTTGGTCT**GACTACHVGGGTATCTAATCC

Table S6.
qPCR primers for specific detection and quantification of ARGs.

Target gene	**Primer**	**Sequence (5′ - 3′)**	**References**
16S rRNA	F1048	GTGSTGCAYGGYTGTCGTCA	Pei et al. (2006)
	R1194	ACGTCRTCCMCACCTTCCTC	
sulI	sul(I)-FX	CGCACCGGAAACATCGCTGCAC	Pei et al. (2006)
	sul(I)-RX	TGAAGTTCCGCCGCAAGGCTCG	
tetM	tetM-FW	ACAGAAAGCTTATTATATAAC	Mao et al. (2015)
	tetM-RV	TGGCGTGTCTATGATGTTCAC	

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Table S7.
Differential bacterial taxa abundance comparing microplastic-associated assemblages to borosilicate-associated assemblages and water sample bacterial communities in WWTP1 by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed.

Substrate	Taxa	Log LDA score
MPs	Rhodocyclaceae	4.82
	Hyphomicrobiaceae	4.58
	Fluviicola	4.33
	Arcobacter	4.17
	Comamonadaceae	4.08
	Aquabacterium	4.08
	Zooglea	4.03
	Uncultured Sphingobacteriales	3.92
	Acidovorax	3.91
	Sphaerotilus	3.77
	Paludibacter	3.74
	Pseudomonas	3.70
	Uncultured Gracilibacteria	3.64
	Perludibaca	3.61
	Comamonas	3.54
BS	Uncultured Anaerolineaceae	4.19
	Sphingomonadaceae	4.17
	Rhizobiales	4.10
	Christensenellaceae 7 group	3.66
	Rhodobacteraceae	3.62
	Trichococcus	3.58
	Ottowia	3.53
	Gammaproteobacteria WN HWB 116	3.53
	Peptostreptococcaceae	3.52
	Alkanindiges	3.42
	Uncultured Verrumicrobia LD1 PB3	3.26
	Methylotenera	3.25
	Clostridium	3.24
	Leucobacter	3.20
	Cyanobacteria Subsection IV family I	3.18

(Continued)

Substrate	Taxa	Log LDA score
Water	*Leeia*	4.92
	Rhizobiales JG35 K1 AG5	4.89
	Rhodocyclaceae 12 up	4.62
	Flavobacterium	4.38
	Unculture candidate division SR1	4.32
	Saccharibacteria	4.13
	Alcaligenaceae GK98 freshwater group	3.59
	Methylocystaceae	3.51
	Uncultured Veillonellaceae	3.32
	Dialister	3.25
	Enterobacteriaceae	3.23
	Uncultured compost bacterium *Saccharibacteria*	3.22
	Bifidobacterium	3.16
	Streptococcus	3.16
	Ruminococcaceae UCG 014	3.15

Table S8.
Differential bacterial taxa abundance comparing microplastic-associated assemblages and water sample bacterial communities in WWTP2 by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed. BS is not included as LEfSe analysis did not find any taxa clearly more abundant in BS with respect to MPs and water

Material	Taxa	LDA effect score
MPs	Uncultured Saprospiraceae	4.61
	Comamonadaceae	4.46
	Rhodobacteraceae	4.27
	Candidatus *Microthrix*	4.18
	Acidimicrobiaceae	3.90
	Variovorax	3.79
	Roseiflexus	3.78
	Terrimonas	3.74
	Dokdonella	3.68
	Chloroflexi	3.62
	Iamia	3.57
	Rhodobacter	3.54
	Lautropia	3.49
	Sphaerotilus	3.46
	Pirellula	3.45
	Mycobacterium	2.85

(Continued)

Substrate	Taxa	Log LDA score
Water	Rhizobiales JG35 K1 AG5	5.05
	Leeia	4.84
	Rhodocyclaceae 12up	4.65
	Flavobacterium	4.39
	Unculture candidate division SR1	4.24
	Uncultured Anaerolineaceae	4.21
	Rhodocyclaceae	3.94
	Rhizobiales	3.80
	Christensenellaceae 7 group	3.65
	Saccharibacteria	3.51
	Methylocystaceae	3.51
	Alcaligenaceae GK98 fresh water group	3.46
	Arcobacter	3.45
	Uncultured Veillonellaceae	3.45
	Hyphomicrobiaceae	3.43

Table S9.
Pairwise Kruskal Wallis test.

WWTP	Gene	Comparison	Difference of Means	Test statistic	*p*-value
WWTP1	*sulI*	PLA *vs.* Water	0.154	0.048	0.827
		PHB *vs.* Water	0.225	0.429	0.513
		PCL *vs.* Water	0.024	0.048	0.827
		PET *vs.* Water	-0.099	0.429	0.513
		LDPE *vs.* Water	0.051	0.196	0.658
		POM *vs.* Water	-0.218	0.429	0.513
		PS *vs.* Water	0.382	1.190	0.275
		BS *vs.* Water	-0.719	3.857	0.050
	tetM	PLA *vs.* Water	-0.811	-20.000	0.020
		PHB *vs.* Water	-0.476	-4.333	0.504
		PCL *vs.* Water	-0.516	-6.000	0.335
		PET *vs.* Water	-0.777	-20.000	0.002
		LDPE *vs.* Water	-0.604	-9.667	0.136
		POM *vs.* Water	-0.790	-21.000	0.001
		PS *vs.* Water	-0.624	-11.333	0.080
		BS *vs.* Water	-0.495	-9.667	0.136

(Continued)

WWTP	Gene	Comparison	Difference of Means	Test statistic	p-value
WWTP2	*sulI*	PLA *vs.* Water	-0.636	-3.333	0.564
		PHB *vs.* Water	1.506	10.000	0.083
		PCL *vs.* Water	1.015	6.000	0.299
		PET *vs.* Water	1.036	6.667	0.248
		LDPE *vs.* Water	0.793	5.167	0.423
		POM *vs.* Water	3.492	15.167	0.019
		PS *vs.* Water	2.384	12.333	0.033
		BS *vs.* Water	7.240	18.167	0.005
	tetM	PLA *vs.* Water	-0.901	-19.833	0.002
		PHB *vs.* Water	-0.877	-18.000	0.050
		PCL *vs.* Water	-0.858	-15.833	0.015
		PET *vs.* Water	-0.858	-8.677	0.181
		LDPE *vs.* Water	-0.744	-10.333	0.111
		POM *vs.* Water	-0.802	-10.000	0.123
		PS *vs.* Water	-0.889	-19.000	0.003
		BS *vs.* Water	-0.736	-6.333	0.328

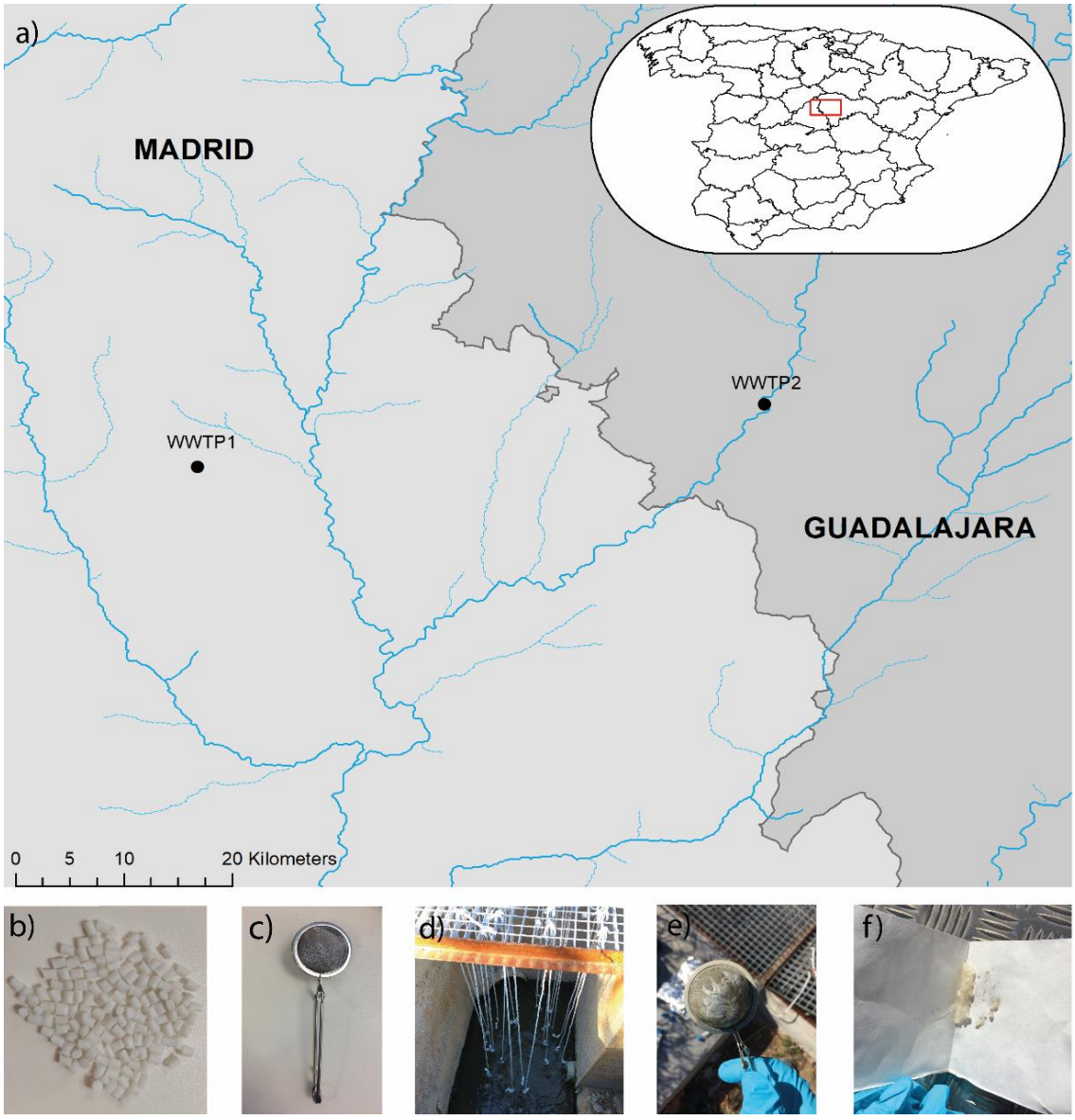

Figure S1. Sampling sites and details of the colonization experiments. a) Spain map showing location of WWTP1 and WWTP2, b) Virgin MPs before the colonization experiment, c) Metal cage with MPs inside, d) Deployment of metal cages with MPs into WWTP effluent, e) Cages after 48 h of colonization, f) Drying of the colonized MPs onto sterilized filter paper.

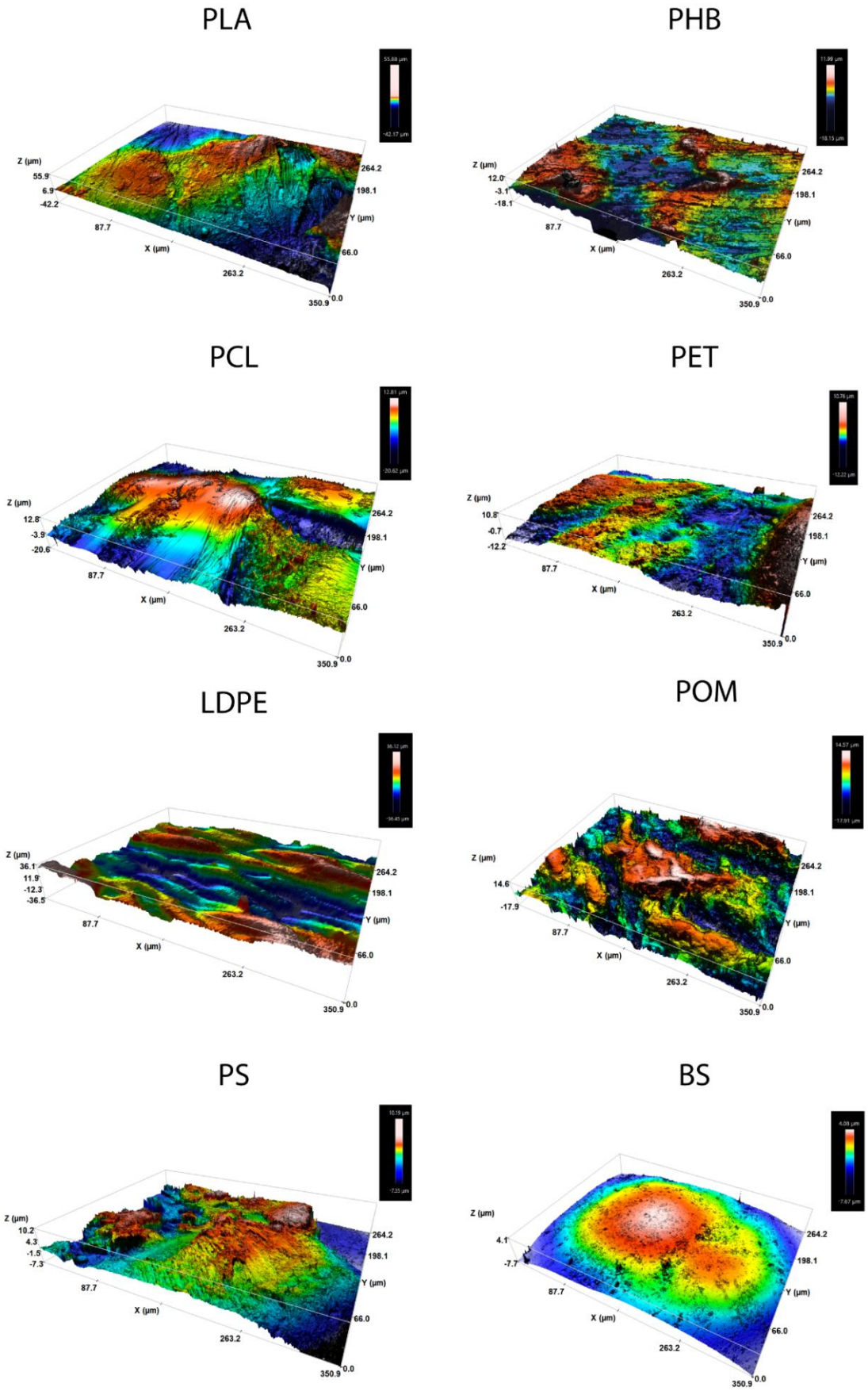

Figure S2. Images of the surface of each substrate obtained by 3D microscopy. Red color represents roughness crests and blue color represents the sunken areas.

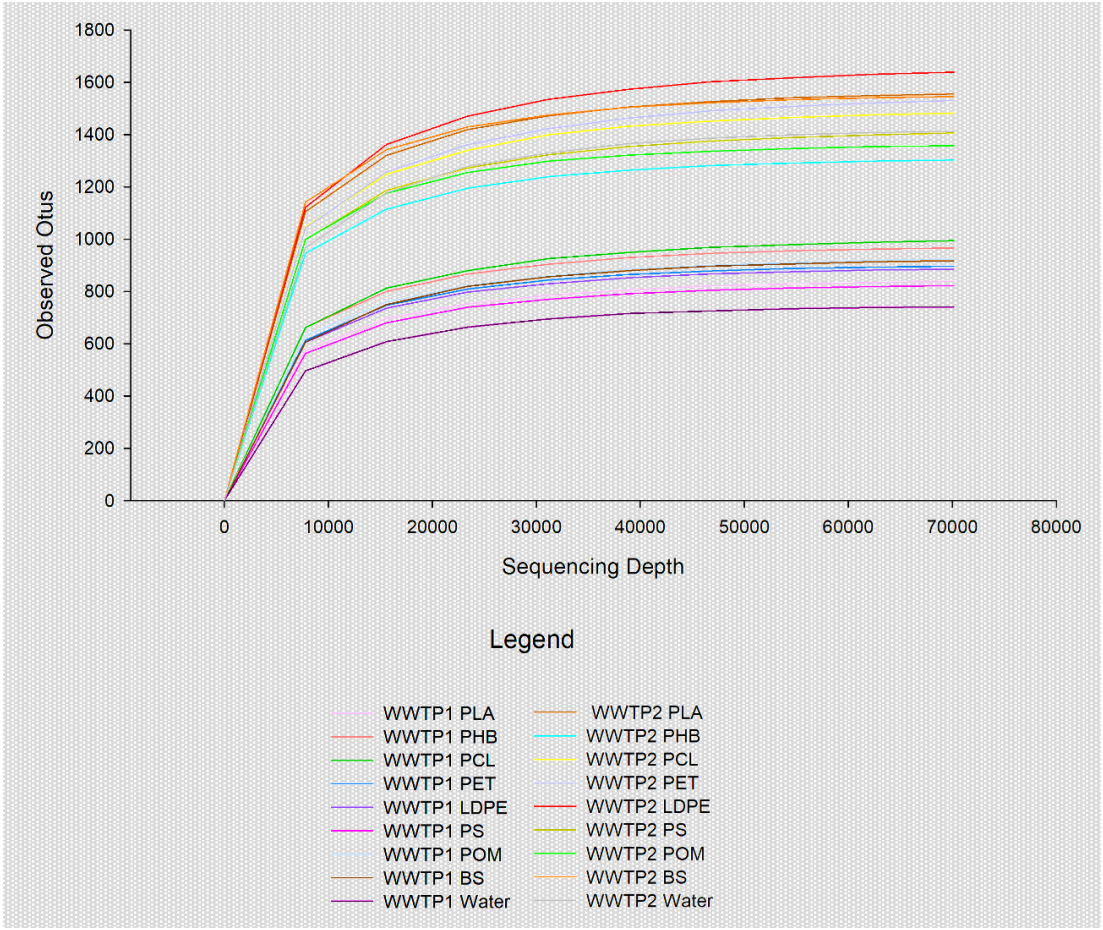

Figure S3. Rarefaction curve that compares the observed ASVs index in comparison with number of reads for each sample (sequencing depth).

Metagenomics pipeline.

The guide of this information can be found in the QIIME 2 user documentation (https://docs.qiime2.org/2019.10/)

#1 Group the files within the same folder

#2 Import data to QIIME2

```
qiime tools import –type SampleData[PairedEndSequencesWithQuality]'
--input-path lecturas
--input-format CasavaOneEightSingleLanePerSampleDirFmt
--output-path GuadaUAMjunto.qza
```

#3 Check the quality of the samples according to QIIME2

```
qiime demux summarize
--i-data GuadaUAMjunto.qza
--o-visualization calidadsecuenciasmicroplastics.qzv
```

#4 Use of dada2 to denoises single-end sequences, dereplicates them, and filters chimeras. According to the quality obtained before, the lectures are trimmed and truncate

```
qiime dada2 denoise-single
--i-demultiplexed-seqs GuadaUAMjunto.qza
--p-trim-left 20
--p-trunc-len 240
--o-representative-sequences microplasticsdada2.qza
--o-table microplasticstable-dada2.qza
--o-denoising-stats microplasticstats-dada2.qza
```

#5 Create metadata file and validate with Keemei

#6 Generate a summarise table of the content

```
qiime feature-table summarize
--i-table microplasticstable-dada2.qza
--o-visualization microplasticstable.qzv
--m-sample-metadata-file microplasticosrevision.tsv
```

#7 Generate tabular view of feature identifier to sequence mapping, including links to BLAST each sequence against the NCBI nt database

```
qiime feature-table tabulate-seqs
--i-data microplasticsdada2.qza
--o-visualization microplasticsrep-seqs.qzv
```

#8 Create a sequence alignment using MAFFT. The result is used to infer a phylogenetic tree

```
qiime phylogeny align-to-tree-mafft-fasttree
--i-sequences microplasticsdada2.qza
--o-alignment microplasticsaligned-rep-seqs.qza
--o-masked-alignment microplasticsmasked-aligned-rep-seqs.qza
--o-tree microplasticsunrooted-tree.qza
--o-rooted-tree microplasticsrooted-tree.qza
```

#9 Generate interactive alpha rarefaction curves considerating the "min_depth" and the "max_depth"

```
qiime diversity alpha-rarefaction
```

```
--i-table microplasticstable-dada2.qza
--i-phylogeny microplasticsrooted-tree.qza
--p-max-depth 70139
--m-metadata-file microplasticosrevision.tsv
--o-visualization microplasticsalpha-rarefaction.qzv
```

#10 Applies a collection of diversity metrics (including Shannon Index and Bray-Curtis matrix)

```
qiime diversity core-metrics-phylogenetic
--i-phylogeny microplasticsrooted-tree.qza
--i-table microplasticstable-dada2.qza
--p-sampling-depth 70139
--m-metadata-file microplasticosrevision.tsv
--output-dir core-metrics-results
```

#11 Compare visually and statistic the alpha diversity by Shannon index, Pielou evenness and Chao 1 index

```
mkdir alpha
qiime diversity alpha-group-significance
--i-alpha-diversity core-metrics-results/shannon_vector.qza
--m-metadata-file microplasticosrevision.tsv
--o-visualization alpha/GuadalajaraUAMshannongroup.qzv
qiime diversity alpha
--i-table microplasticstable-dada2.qza
--p-metric pielou_e
--o-alpha-diversity alpha/microplasticsallpielou.qza
qiime diversity alpha-group-significance
--i-alpha-diversity alpha/microplasticsallpielou.qza
--m-metadata-file microplasticosrevision.tsv
--o-visualization alpha/microplasticsallpielou.qzv
qiime diversity alpha
--i-table microplasticstable-dada2.qza
 --p-metric chao1
 --o-alpha-diversity alpha/microplasticsallchao1.qza
qiime diversity alpha-group-significance
--i-alpha-diversity alpha/microplasticsallchao1.qza
--m-metadata-file microplasticosrevision.tsv
--o-visualization alpha/microplasticsallchao1.qzv
```

#12 Grouping of samples and comparison of statistics PERMANOVA and PERMDISP using Bray Curtis distance matrix

```
mkdir beta
qiime diversity beta-group-significance
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Materialplace
 --p-method permanova
  --p-pairwise
 --p-permutations 999
 --o-visualization beta/permanovaMaterialplace
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column WWTP
 --p-method permanova
 --p-pairwise
```

```
 --p-permutations 999
 --o-visualization beta/permanovaWWTP
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Paper
 --p-method permanova
 --p-pairwise
 --p-permutations 999
 --o-visualization beta/permanovaPaper
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Material
 --p-method permanova
  --p-pairwise --p-permutations 999
 --o-visualization beta/permanovaMaterial
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Materialplace
 --p-method permdisp
 --p-pairwise
 --p-permutations 999
 --o-visualization beta/permdispMaterialplace
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column WWTP
--p-method permdisp
--p-pairwise
--p-permutations 999
--o-visualization beta/permdispWWTP
qiime diversity beta-group-significance
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column Paper
--p-method permdisp
--p-pairwise
--p-permutations 999
--o-visualization beta/permdispPaper
qiime diversity beta-group-significance
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column Material
--p-method permdisp
--p-pairwise
--p-permutations 999
--o-visualization beta/permdispMaterial
```

#13 Train the classifier Silva 128 at 99 % similitude with the primers.

```
qiime tools import
--type 'FeatureData[Sequence]'
 --input-path 99_otus_16S.fasta
 --output-path 99_otus_16S.qza
qiime tools import
--type 'FeatureData[Taxonomy]'
```

```
--input-format HeaderlessTSVTaxonomyFormat
--input-path consensus_taxonomy_7_levels.txt
--output-path consensus_taxonomy_7_levels.qza
qiime feature-classifier extract-reads
--i-sequences 99_otus_16S.qza
--p-f-primer CCTACGGGNGGCWGCAG
--p-r-primer GACTACHVGGGTATCTAATCC
--o-reads consensus_taxonomy_7_levelsref-seqs.qza
qiime feature-classifier fit-classifier-naive-bayes
--i-reference-reads consensus_taxonomy_7_levelsref-seqs.qza
 --i-reference-taxonomy consensus_taxonomy_7_levels.qza
 --o-classifier SILVA_128_99_classifier.qza
```

#14 Assign taxonomy using the classifier Silva 128 at 99 % similitude. After that, generate a taxa bar plot interactive

```
mkdir taxonomy
qiime feature-classifier classify-sklearn
--i-classifier SILVA_128_99_classifier.qza
--i-reads  microplasticsdada2.qza
--o-classification microplasticsalltaxonomysilva.qza
qiime metadata tabulate
--m-input-file microplasticsalltaxonomysilva.qza
--o-visualization microplasticsalltaxonomysilvavisualizationtaxonomysilva.qza
qiime taxa barplot
--i-table microplasticstable-dada2.qza
--i-taxonomy microplasticsalltaxonomysilva.qza
--m-metadata-file  microplasticosrevision.tsv
--o-visualization microplasticosrevisiontaxa-bar-plotsSilva.qzv
```

#15 Group the replicates and create a taxa bar plot using a new metadata file

```
qiime feature-table group
--i-table microplasticstable-dada2.qza
--p-axis sample
--p-mode sum
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column Paper
--o-grouped-table Papergrouptable.qza
qiime taxa barplot
--i-table Papergrouptable.qza
--i-taxonomy microplasticsalltaxonomysilva.qza
--m-metadata-file microplasticosrevisionPaper.tsv
--o-visualization taxonomy/microplasticsPaper-bar-plotsSilvasimple.qzv
```

#16 Convert the archives in txt to use in Lefse. For this process, if it is necessary to collapse the taxa results at speciess level, export the data and convert to txt format.

```
qiime taxa collapse
--i-table microplasticstable-dada2.qza
--i-taxonomy microplasticsalltaxonomysilva.qza
--p-level 7
--o-collapsed-table microplastics_collapsedspecies.qza
qiime tools export
--input-path microplastics_collapsedspecies.qza
--output-path speciesslefsetable
biom convert
```

```
--i speciesslefsetable/feature-table.biom
--o feature-tablespecies.txt
--header-key “taxonomy” --to-tsv
```

8. SUPPLEMENTARY MATERIAL 2

The following supplementary material accompanies which details the taxonomic classification of all samples obtained from the sequencing of the gene region 16S rRNA can be downloaded from https://zenodo.org/record/6519048#.YnLi5-jP1D8

CHAPTER 4

TIME-COURSE BIOFILM FORMATION AND PRESENCE OF ANTIBIOTIC RESISTANCE GENES ON EVERYDAY PLASTIC ITEMS DEPLOYED IN RIVER WATERS UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

ABSTRACT

Plastics, once in the environment, may become a habitat for different organisms. This new ecosystem is known as the plastisphere. The plastisphere has been widely studied in the oceans; however, there is a knowledge gap regarding freshwater ecosystems, particularly about how it changes along time. Here, we have characterized along one year the evolution of the eukaryotic and bacterial communities attached to four everyday plastics items deployed into two sites with different levels of anthropogenic impact in the same river. α-diversity analyses showed that the sampling site had a significant role in bacterial and eukaryotic diversity. The most impacted site 2 showed higher values of the Shannon diversity index. β-diversity analyses showed that sampling site explained most of the sample variation followed by substrate type and time of colonization; in this regard, core microbiomes/biomes in each plastic at 1, 3, 6 and 12 months could be identified at genus level, giving a global overview of the evolution of the plastisphere along time. The concentration of antibiotics onsite also affected the development of the bacterial community. The relative abundance of several types of antibiotic resistance genes (ARGs) in the plastics was determined. The highest abundance of ARGs was recorded at site 2, just downstream of the WWTP effluent, which also had the highest concentration of antibiotics. In general, positive correlations were observed between the concentration of each type of antibiotic and cognate ARGs on plastics. These results provide relevant information on the evolution along time of the plastisphere in freshwater ecosystems and on the most relevant factors shaping it. The positive correlation between the abundance of ARGs and antibiotic concentrations emphasizes the potential role of plastics in the global spreading of antibiotic resistance.

1. INTRODUCTION

The unique properties of plastics, such as durability, weight, versatility, and malleability, have made it one of the most widely manufactured material since its invention in the mid-19th century (Li et al., 2021; Thompson et al., 2009). This is reflected in the global plastic production, which reached its higher value in 2019 with 368 million tons (excluding polyethylene terephthalate (PET) -fibers, polyamide (PA) -fibers, and polyacryl-fibers) and stabilizing in 2020 (PlasticsEurope, 2021). In Europe (referred to EU27 plus Norway, Switzerland, and the United Kingdom), plastics are used mainly in packaging (40.5 %), building and construction (20.4%), automotive (8.8%), electrical and electronics (6.2%), household, leisure and sports (4.3%) and agriculture (3.2%) (PlasticsEurope, 2021). Among the most commonly used polymers are low and high-density polyethylene (LDPE and HDPE), polypropylene (PP), polyvinyl chloride (PVC), PET, polyurethane (PUR) and polystyrene (PS) (PlasticsEurope, 2021). Subsequently, its poor management means that at least 60% of the plastics produced end up in landfills or the environment without proper treatment (Chamas et al., 2020; Wang et al., 2021). In the environment, plastics can be transported from the soil and wastewater treatment plants (WWTPs) to the rivers and consequently to the oceans (Jambeck et al., 2015; Martínez-Campos et al., 2022). In fact, the major entry-point of plastic to the oceans is by riverine input (Meijer et al., 2021).

Currently, scientific studies focus on the potential impact that plastics cause on aquatic ecosystems (Chae and An, 2017; Thushari and Senevirathna, 2020; Vighi et al., 2021). However, the effects of plastics on rivers environment, despite their key role in the plastics life cycle, are poorly known in comparison with the marine environment (Azevedo-Santos et al., 2021). Thus, recent studies have reported evidence of plastic ingestion by freshwater organisms (Azevedo-Santos et al., 2021) and their potential risk to carry other harmful elements on their surface such as toxic additives (Bolívar-Subirats et al., 2021). These harmful effects have been previously described in marine ecosystems (Barboza et al., 2019).

The concept of plastics as a novel new biotope denoted as plastisphere, is considered a unique ecosystem in which organisms use plastics as a support for their growth (Zettler et al., 2013). The interest of the scientific community in the plastisphere has increased in the last decade (Barros and Seena, 2021). Most of the research analyzing the plastisphere has been conducted in marine environments (Agostini et al., 2021; Keswani et al., 2016; Zhang et al., 2022). These studies proved that the organisms constituting the microbial communities attached to the plastic are remarkably different from those that are found in the surrounding environment (Xu et al., 2019; Zettler et al., 2013). Organisms that can be potential pathogens

(Guo et al., 2017), invasive (Barnes, 2002), or carrying antibiotic resistance genes (ARGs) may be part of the plastisphere implying a risk to ecosystems and human health (Yang et al., 2019). Previous studies have shown significant variations in the microbial community between plastic (Oberbeckmann et al., 2018) and non-plastic materials (Kirstein et al., 2019), as well as the effect of geographical site on the microbial community attached to plastic (Wright et al., 2021b). Regarding plastisphere in freshwater ecosystems, there is limited scientific knowledge and the study of plastisphere in river ecosystems has only recently become of interest (Martinez-Campos et al; 2021; Barros and Seena, 2021; Kettner et al., 2019; McCormick et al., 2014, 2016; Oberbeckmann et al., 2018). Plastics can remain in the same stretch of river for months to years (Newbould et al., 2021). The communities attached to these plastics could evolve and be significantly influenced by the site in which it remains, as in marine ecosystems (Vannini et al., 2021). Furthermore, the proximity to WWTPs, considered to be one of the main hotspots of antibiotics in the environment (Guo et al., 2017), could facilitate the attachment of antibiotic resistance bacteria (ARB) carrying cognate antibiotic resistance genes (ARGs) on plastics and their subsequent spread as plastics move along the river.

Another factor that influences the communities that constitute the plastisphere is the time of colonization. The community attached to the plastisphere exhibits a clear ecological succession during the early stages of colonization (Galloway et al., 2017; Rummel et al., 2021; Wright et al., 2020) as these communities gradually adapt to the new ecosystem over time (Chen et al., 2020; Du et al., 2022; Lorite et al., 2011). A similar phenomenon occurs with the plastic resistome, Yang et al., (2020) analyzed the temporal evolution of ARGs for 30 days, detecting an evolution in their concentration and determining the presence of pioneer, intermediate and persistent ARGs during that month. Nevertheless, there is a considerable gap in the understanding of how the plastisphere evolves over long temporal periods.

In this study, we characterized the evolution along time of the bacterial and eukaryotic community attached on four types of everyday plastic items: a LDPE bag (used in packaging), a PET bottle (used in household), a PS dish (used in household) and a PVC pipe (used in construction). These everyday plastics were incubated for 1 month, 3 months, 6 months and 1 year into two different sites (site 1 and site 2) with different levels of anthropogenic impact in the same river. Site 1 is in an area characterized by natural land use away from relevant urban areas and site 2 is located in an urban area located 50 meters downstream of a WWTP effluent discharge. We hypothesized that the type of polymer might select for specific biofilm forming microorganisms different to the biofilms formed on the

non-plastic substrates and free-living water microorganisms and that colonization time may have a profound effect on the plastisphere complexity. We also tested the hypothesis that these everyday plastic items might host bacteria carrying ARGs and that this could be related to antibiotic contamination in the study area. We also measured several environmental factors in an effort to shed light on main factors affecting biofilm formation on plastics.

2. Material and methods

2.1. Study Area

This study was performed in the Henares River located in the Tagus River Basin (Spain). Two sampling sites (**Figure S1 in Supplementary Material 1**) were selected covering two levels of anthropogenic impact: Site 1, located in the upper reach of the Henares river (Site 1: 40° 50' 10.94" N; 3° 7' 14.23" W) was mainly surrounded by natural areas; and Site 2, located approximately 50 m downstream of the point of discharge of wastewater treatment plant (WWTP) (west) of Alcala de Henares (Madrid, Spain; Site 2: 40° 27' 58.15" N; 3° 24' 55.12" W), was characterized by a high agricultural impact and moderate urban impact (Arenas-Sánchez et al., 2019; Rico et al., 2019).

2.2. Plastic substrates and non-plastic substrates used for microbial colonization

Four types of commercially available everyday plastic items were acquired from local supermarkets (Madrid, Spain): LDPE bag, PET drinking water bottle, PS dish and PVC pipe. Glass microscope slides and limestone rocks were used as chemically inert non-plastic substrate controls. More details about the substrates used in this study are shown in **Table S1 in Supplementary Material 1**.

2.3. Design of the colonization experiment and sampling methods

LDPE bags, PET bottles and PS dishes were pre-treated prior to the experiment: LDPE bags were cut with sterilized scissors to produce 8 cm × 25 cm plastic sheets, discarding coloured areas; PET bottle (height of 33 cm and diameter of 8 cm) bases were punctured to avoid the accumulation of sediments inside the plastic container and labels were discarded; PS dishes were divided into two parts using sterilized scissors producing 10.5 cm × 10.5 cm sheets. PVC pipe, with a diameter of 5 cm and length of 8 cm, did not receive previous treatment.

Two units of each substrate (6 units in the case of rocks) were properly attached inside a stainless-steel cage with flanges and submerged in the middle section of the river. More details about the deployment of the substrates inside the cage are shown in **Figure S2**

in Supplementary Material 1. Four cages were incubated at each sampling site. One cage was collected from each sampling site after one month (20/06/2018; T1), three months (04/09/2018; T3), six months (21/11/2018; T6) and twelve months (21/05/2019; T12) after the experiment started (22/05/2018; T0). The cages were fixed in the river using chains and ropes to avoid being dragged by the river. Immediately after sampling, all samples were transported to the laboratory, where half of the substrates were kept frozen at −20°C until DNA extraction. The rest of samples was stored at 4°C to be used for other analyses as explained below.

In order to obtain a representative sample of the microbial community in the surrounding water, 3 L of water were sampled in wide-mouthed polyethylene bottles and kept cool in the dark. 1 L water was filtered by 2.7 μm glass Millipore filter to collect the particulate material in suspension. Subsequently, 250 mL of the filtered water was further filtered by 0.22 μm membrane Millipore filter to collect the free-living microbial community. Filters were frozen in liquid nitrogen and stored at −20°C until DNA extraction.

2.4. Nutrients and physicochemical parameter analyses

Sampling site waters were characterized at the beginning of the incubation (T0) and at the moment of collecting each cage (T1, T3, T6 and T12). Water temperature, pH, dissolved oxygen (DO, expressed in % and mg/L) and conductivity were measured *in situ* using a portable multimeter probe (HANNA Instruments, Woonsocket, RI, USA, model HI98194). Basic hydrological parameters (water depth and water flow) were measured using a flowmeter. During each sampling, 1 L of water was taken in the middle section of each sampling site for analysis of nutrients and total organic carbon (TOC). Ammonium (NH_4^+), nitrate (NO_3^-), Total Kjeldahl Nitrogen (TKN), orthophosphate (PO_4^{3-}) and total phosphorus were measured according to the methods described in the Standard Methods for the Examination of Water and Wastewater (Chambers, 2019). TOC concentration was measured on a Shimadzu TOC-VCSH/CSN coupled to an ASI-V autosampler (Shimadzu Corporation, Kyoto, Japan).

2.5. Antibiotic concentrations measurements

1 L of water was taken at T0, T1, T3, T6 and T12 in the middle section of each sampling site in amber glass bottles and kept frozen at −20 °C until further analysis. In total, 10 antibiotics were analysed: amoxicillin, azithromycin, ciprofloxacin, clarithromycin, erythromycin, lincomycin, metronidazole, sulfamethoxazole, ofloxacin and trimethoprim. Antibiotic selection was based on the pharmaceuticals detected in the same river by Rico. et al., (2019). Antibiotic concentration was quantified by liquid chromatography using an HPLC system (Agilent 1200 Series, Agilent Technologies) coupled to an Agilent 6495 triple quadrupole (QQQ) mass spectrometer (LC-MS/MS). Further details of the analytical procedure were provided in Rico et al., (2019).

2.6. Scanning electron microscopy (SEM) analysis

For qualitative assessment of biofilm structure, a random collection of three areas per substrate was chosen for scanning electron microscopy (SEM) analysis. Virgin non-exposed substrates were used as controls. Rocks were not considered in the analysis. The selected areas were cut, preventing damage to the biofilm. Afterwards, the fragments were fixed with a solution of glutaraldehyde 5 % (v/v) in sodic cacodylate 0.2 M (pH 7.2) for 1 hour and then washed two times with sodium cacodylate 0.2 M (pH 7.2). Subsequently, samples were dehydrated in a stepwise increasing ethanol series of 10 minutes' immersion in 25 % ethanol, 50 % ethanol, 75 % ethanol, 90 % ethanol and absolute ethanol. Then, samples were dried at 50°C for 24 hours. The dry samples were metalized with a chromium layer of 15 mm using a sputter Quórum model Q150T-S. Then, the substrate surfaces were analysed using a Scanning Electron Microscope Hitachi S-3000N.

2.7. Microbial diversity analysis

2.7.1. DNA extraction

DNA was extracted from the microbial community attached to the exposed plastics, rocks, BS glass and surrounding water filters. For that, samples were divided into three fragments cutting them with sterilized scissors (in the case of rocks, each rock was considered as one replicate). After that, all the sample surfaces, except filters, were scratched using a sterilized scalpel, separating the biofilm from the substrate, which was transferred and divided into various 2 mL tubes, according to the biomass volume. Water filters were cut into small fragments and transferred to 2 mL tubes. DNA extraction was performed using phenol/chloroform method extraction followed by absolute ethanol precipitation according to the protocol by Martínez-Campos et al. (2021).

2.7.2. DNA METABARCODING SEQUENCING

PCR amplification and Miseq Illumina sequencing of the regions V3-V4 of the 16S rRNA and the region V4-V5 of the 18S rRNA of each of the three replicates of each sample (192 sequenced samples) were carried out by the Genomics Service of the Parque Científico de Madrid (Madrid, Spain). The used primers are shown in **Table S2 in Supplementary Material 1.** DNA libraries and amplicon sequencing were performed as previously described in Martinez-Campos, et al., (2018).

2.7.3. DATA ANALYSIS

16S rRNA and 18S rRNA profiling was performed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) v.2020.8 using a modified pipeline described in Martinez-Campos, et al. (2021).

Quality filtering of reads (the quality was previously checked using the q2-demux plugin), trimming paired ends and denoising process was performed using DADA2 (Callahan et al., 2016) via q2-dada2 plugin. All Amplicon Sequence Variants (ASVs) were aligned using MAFFT (Katoh et al., 2002) and used to construct a phylogeny with FastTree2 (using q2-phylogeny) (Price et al., 2010).

For α-diversity analysis, Shannon-Wiener diversity Index (Shannon, 1948) was calculated via q2-diversity after samples were rarefied (subsampled without replacement) to 46242 sequences per sample. The Kruskal Wallis test was used to determine if Shannon diversity indexes were significantly different between samples (pairwise comparison) and between the different treatments (sampling site, time and substrates). Taxonomy was assigned to ASVs via q2-feature classifier plugin (Bokulich et al., 2018) classify-sklearn naïve Bayes taxonomy classifier against the SILVA 132, 99 % OTUs database (Quast et al., 2013) previously trained via q2-feature plugin (Bokulich et al., 2018) using the region of the target sequences that were sequenced for 16S rRNA and 18S rRNA.

For β-diversity analysis, an unweighted-pair group method with arithmetic mean (UPGMA) dendrogram was performed based on Bray-Curtis dissimilarity matrix (Sorensen, 1948) using ASV abundance. The UPGMA dendrogram was obtained via "hclust" function of the stats package (Team et al., 2013) in R Studio (RStudio, 2020). PERMANOVA (Permutational multivariate analysis of variance) test (Anderson, 2001) was applied to test significant differences between samples considering 999 permutations.

To determine the influence of the sampling site, time, substrate (comparison between surrounding water and tested substrates) a distance-based redundancy analysis (dbRDA) (Legendre and Anderson, 1999) was performed based on the Bray-Curtis dissimilarity matrix

(Sorensen, 1948). In the 16S rRNA samples, the analysis also included the antibiotics detected with the highest concentration (macrolides, sulphonamides, quinolones and trimethoprim). The dbRDA was performed using the "dbrda" function from the vegan package (Dixon, 2003). The "anova.cca" function of the vegan package (Dixon, 2003) with 999 permutations was used to perform the significance test of dbRDA. All regression coefficients (R2) were adjusted for multiple testing. Db-RDA graph was performed using the Statistica 13 Software.

To identify differentially attached taxa among the different substrates, the linear discriminant analysis effect size method (LEfSe) (Segata et al., 2011) was used. This was performed with the LEfSe tool v. 1.1.2 available through Bioconda (Grüning et al., 2018), using all default settings for data formatting and LDA (Linear Discriminant Analysis) effect size. The input data included non-transformed relative abundance genera and the strategy for multi-class analysis "one-against-all" was performed.

2.7.4. Accession number

Sequence data obtained in this study were submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under the Bioproject accession number PRJNA783293 for 16s rRNA sequences and PRJNA783563 for 18s rRNA sequences.

2.8. Analysis of plastic surface alterations

One-year colonized plastics samples were softly brushed and washed with deionized water to eliminate as much adhered material possible, dried at 35°C for 24h in an oven and stored in a desiccator. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was applied to assess the potential alteration of the plastic surface on five randomly selected places in each plastic. Spectra were collected in absorbance mode using a Thermo Nicolet IS10 spectrometer with a Smart iTR-Diamond ATR module using the OMNIC software version 9.1.26. The spectral range was at wavenumber 3500–650 cm^{-1} and for each measurement, 16 scans were accumulated. The spectral resolution was 4 cm^{-1}, window aperture was at medium resolution, gain was two and optical velocity 0.4747. These parameters allowed obtaining good quality spectra with low spectral noise.

The hydroxyl index was calculated for each polymer as a measure of the hydroxyl groups formed during their environmental oxidation (Brandon et al., 2016). The index was obtained by dividing the maximum absorption in the 3300–3400 cm^{-1} region by the absorption of a reference peak. The reference taken was the stretching vibration of C-H bonds, which has been shown as relatively insensitive to the transformations due to polymer

ageing (Brandon et al., 2016). The following equations summarize the calculations performed for each plastic:

- Hydroxyl index (LDPE bag) = $\frac{\text{Absorption corresponding to the hydroxyl group } (3300-3400\ cm^{-1})}{\text{Reference peak in the main stretching vibration of } -CH_2\ (2920\ cm^{-1})}$
- Hydroxyl index (PET bottle) = $\frac{\text{Absorption corresponding to the hydroxyl group } (3300-3400\ cm^{-1})}{\text{Reference peak in the C}-\text{H stretching } (2970\ cm^{-1})}$
- Hydroxyl index (PS dish) = $\frac{\text{Absorption corresponding to the hydroxyl group } (3300-3400\ cm^{-1})}{\text{Reference peak in the C}-\text{H aliphatic stretching of } -CH_2\ (2900\ cm^{-1})}$
- Hydroxyl index (PVC pipe) = $\frac{\text{Absorption corresponding to the hydroxyl group } (3300-3400\ cm^{-1})}{\text{Reference peak in the C}-\text{H stretching } (2900\ cm^{-1})}$

2.9. Relative abundance of ARGs

The relative abundance of four ARG genes (*ermF*, *sul1*, *dfrA1*, *qnrSrtF11A*) was compared between the plastics substrates, the non-plastic substrates (BS glass and rock) and the free-living bacterial community in water using quantitative PCR (qPCR). The selection of ARGs was based on the most abundant antibiotics detected in the two sampling sites (see below) and their wide distribution and high abundance in European wastewater treatment plants (Pärnänen et al., 2019). qPCR experiments were carried up by the Genomics Service of the Parque Cientifico de Madrid (Madrid, Spain). qPCR assays were performed using 1 ng of template DNA and LightCycler® 480 SYBR Green I Master (Roche; USA) in a LightCycler® 480 system (Roche; USA). The primers for amplification of the genes are detailed in **Table S3 in Supplementary Material 1**. Thermal cycling details were as described in Pärnänen et al., 2019. Two technical replicates were run for each gene and each sample obtaining in each one a detectable cycle threshold (Ct) value. Both positive and negative controls were included in every run.

The $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the 16S gene of the same sample. Student-Newman-Keuls tests were used to see if there were significant differences between times and substrates in the relative abundance of each of the genes. Spearman correlations were developed to test whether there was a relationship between the antibiotic concentration at each of the sampling sites and the $2^{-\Delta CT}$ values obtained for each substrate.

3. RESULTS

3.1. ENVIRONMENTAL PARAMETERS

The interpretation of the environmental data was divided into physicochemical parameters (**Table S4 in Supplementary Material 1**), nutrients (**Table S5 in Supplementary Material 1**) and antibiotics (**Table S6 in Supplementary Material 1**). The statistical analysis of these parameters between both sampling sites is reported in **Table S7 in Supplementary Material 1**.

3.1.1. PHYSICOCHEMICAL CHARACTERIZATION OF THE SAMPLING SITES ALONG THE TIME COURSE OF THE COLONIZATION EXPERIMENT

The main physicochemical parameters of water are shown in **Table S4 in Supplementary Material 1**. Although samples were taken at regular intervals for one year, seasonality was clearly observed in water temperature and flow rate, with higher temperature and lower water velocity in spring and summer. The percent saturation of DO was in the 70–100 % range in both sampling sites, meaning that no remarkable oxygen depletion occurred along the sampling period as established in Arenas-Sánchez et al. (2019). However, DO levels were slightly but significantly lower in site 2 with respect to site 1 (p-value < 0.05; **Table S7 in Supplementary Material 1**) due to the influence of the WWTP, which is located 50 m upstream from the sampling site. pH values were in the range of 7.1–8.3, that is considered a regular range for freshwater (Bundschuh et al., 2016). Water depth was significantly higher in site 2 with respect to site 1 (p-value < 0.05; **Table S7 in Supplementary Material 1**).

3.1.2. NUTRIENTS

The influence of the WWTP effluent discharge on inorganic nutrients concentrations and TOC (shown in **Table S5 in Supplementary Material 1**) was significant, showing the highest values in the more anthropogenically impacted site 2 (p-value < 0.05; **Table S7 in Supplementary Material 1**). In fact, the concentration of inorganic nutrients in site 2 corresponds to a moderately impacted site (Poikane et al., 2019). N-nitrate and, particularly, N-ammonium levels were higher in the more impacted site 2 (**Table S5 in Supplementary Material 1**). The difference between the two sampling sites was even more striking concerning phosphate concentration. Phosphate concentration was two orders of magnitude higher in sampling site 2 than in sampling site 1 (**Table S5 in Supplementary Material 1**) exceeding the local threshold for poor ecological water status (Poikane et al., 2019).

3.1.3. OCCURRENCE OF ANTIBIOTICS

Regarding the occurrence of antibiotics, ten antibiotics were measured (**Table S6 in Supplementary Material 1**). All of them were detected in site 2 ranging from 1.2 ng L^{-1} (lincomycin) to 7282 ng L^{-1} (azithromycin) whereas in site 1 only seven antibiotics were found (azithromycin, ciprofloxacin and ofloxacin were below quantification limits) with concentrations ranging from 0.20 ng L^{-1} (erythromycin) to 211 ng L^{-1} (metronidazole). Seasonality did not have any clear effect except for azithromycin and ofloxacin in site 2, which fluctuated widely over time. Antibiotics as well as other pharmaceuticals are considered as point source contaminants; the significantly higher levels of antibiotics in site 2, located downstream of a WWTP, namely ciprofloxacin, clarithromycin, erythromycin, sulfamethoxazole, azithromycin, ofloxacin and trimethoprim (p-value < 0.05; **Table S7 in Supplementary Material 1**) confirmed the role of wastewater discharge in the emission of antibiotics to rivers (Osorio et al., 2012).

3.2. MICROBIAL COLONIZATION OF PLASTICS

A visual exam of collected plastics at the different incubation times (**Figure S3 in Supplementary Material 1**) showed that their surface was covered by microorganisms. To assess microbial colonization, the surface of plastics and BS glass was inspected using SEM microscopy (**Figure 1**, and **Figure S4 in Supplementary Material 1**).

A detailed analysis showed that the surface of non-incubated substrates (T0) was smooth, and no depressions or cracks could be observed, except for the LDPE bag, which presented an irregular surface in some small areas (**Figure S4 in Supplementary Material 1**). After the initial first month of colonization, large substrate areas covered with biofouling were observed, mostly diatoms were seen; some inorganic fouling (crystalline and inorganic particles) was also observed particularly in substrates incubated in the anthropogenically impacted site 2. After 3 months of incubation, the formation of a thick biofilm overall plastic surfaces was confirmed. Furthermore, in some areas, no clear individual cells could be observed, which might imply that extracellular polymeric substances (EPS) secreted by microorganisms enabled microbes and suspended particles in water to clump together, an indication of biofilm maturity. In the last phases of incubation (6 and 12 months), the biofouling layer on the plastic surface increased its thickness, showing a clear multilayer biofilm with diverse types of microorganisms, such as diatoms or bacteria, clumped between inorganic particles.

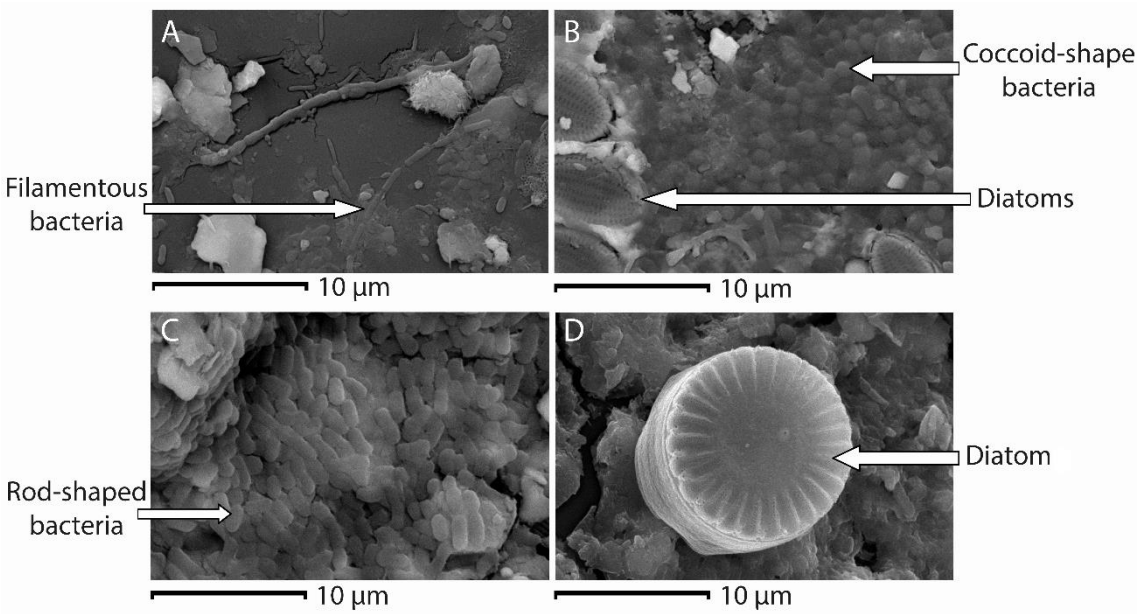

Figure 1. SEM images showing the different microorganism morphologies found colonizing the plastic surface along the incubation time: A) filamentous bacteria detected on PET bottle after 1 month of colonization; B) coccoid-shape bacteria and pennate diatoms identified on PS dish after 3 months of incubation; C) rod-shaped bacteria over the PVC pipe surface after 6 months of colonization; D) a centric diatom located on LDPE bag after 12 months of incubation.

3.3. Taxonomical annotation

In total, 12175631 reads (6426961 reads corresponding to 16S rRNA gene and 5748670 reads corresponding to 18S rRNA gene) were obtained using Illumina sequencing. After quality filtration, reads merging and chimera removal using DADA2, 9334841 sequences remained (4470467 reads of 16S rRNA gene and 4864374 reads of 18S rRNA gene). Based on 99% sequence similarity, these reads were clustered into 16943 ASVs for bacteria and 11129 ASVs for eukaryotes. The rarefaction curves for all samples (**Figure S5** for 16S rRNA and **Figure S6** for 18S rRNA **in Supplementary Material 1**) approached the saturation plateau, pointing out that the libraries were adequately sampled. To validate the statistics results, the sequencing depth used to evaluate the α- and β- diversity was 14953 reads per sample for 16S rRNA and 10263 reads per sample for 18S rRNA.

3.4. Diversity analysis

Microbial α-diversity was estimated using the Shannon Index. Diversity plots for the different substrates, incubation times and sampling sites for 16S rRNA and 18S rRNA are shown in **Figure 2.** The diversity of bacterial and eukaryotic communities differed according to site, but also according to incubation time and substrate (Global p-value < 0.05; **Table S8** and **Table S9 in Supplementary Material 1**). The sampling site had a significant role in bacterial and eukaryotic α-diversity. Sampling site 1 samples had significantly lower values of

eukaryotic and bacterial α-diversity (according to the Shannon Index) than samples from sampling site 2 (*p*-value < 0.05).

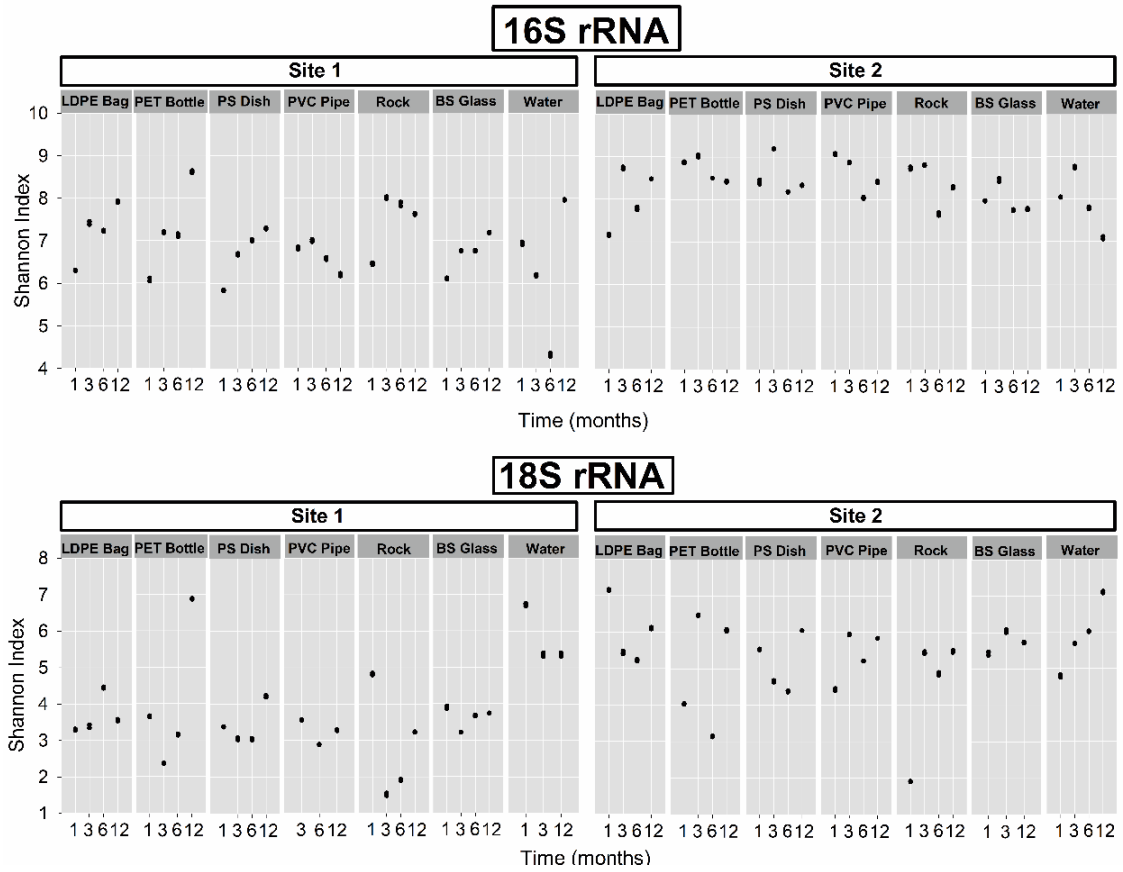

Figure 2. Shannon Index was used as an estimator of α-diversity on plastics (LDPE bag, PET bottle, PS dish and PVC pipe), BS glass, rock and surrounding water (2.7 to 0.22 μm) at the two sampling sites after 1 month, 3 months, 6 months and 1 year of the colonization experiment. Kruskal-Wallis significance analysis is shown in **Table S8** and **Table S9 in Supplementary Material 1**.

Shannon diversity index average values of the bacterial communities in both sampling sites on LDPE bag, PET bottle, PS dish, PVC pipe, Rock, BS glass and water were 7.62 ± 0.74, 7.98 ± 1.05, 7.61 ± 1.05, 7.62 ± 1.05, 7.93 ± 0.71, 7.35 ± 0.75 and 7.13 ± 1.32 respectively. Regarding bacteria, α-diversity was lower on plastics than in the free-living community (*p*-value < 0.05) after 1 month of colonization on both sites (**Table S8 in Supplementary Material 1**). After three months of incubation, bacterial α-diversity increased significantly in all plastic substrates (*p*-value < 0.05) except site 2 PVC pipe which slightly decreased (**Table S8 in Supplementary Material 1**). Water free-living bacteria community diversity from site 1 significantly decreased (*p*-value < 0.05) after 3 months and more markedly after 6 months (*p*-value < 0.05), probably due to the heavy rain that preceded the sampling; similarly, water free-living bacterial diversity also decreased after 6 months in site 2 (*p*-value < 0.05; **Table S8 in Supplementary Material 1**). The heavy rain event probably also explained that a similar decreasing trend, although not so accentuated as with

free-living bacteria in water, was found after 6 months of incubation in all tested substrates, including non-plastic ones: BS glass and rock (p-value < 0.05) except for PS dish from site 1, whose diversity increased (p-value < 0.05; **Table S8 in Supplementary Material 1**). In general, after 12 months of incubation there was an increase in diversity in all tested substrates in site 1 except in the PVC pipe (**Figure 2**). This trend was not so clear in the more impacted site 2 where diversity, except for the LDPE bag, was, in general, lower than after 1 month of colonization in all tested substrates, including also free-living bacteria (**Figure 2 in Supplementary Material 1**).

The eukaryotic mean Shannon diversity average index values in both sampling sites on LDPE bag, PET bottle, PS dish and PVC pipe, Rock, BS glass and water were 4.43 ± 1.00, 4.65 ± 1.65, 4.14 ± 0.96, 4.08 ± 1.49, 4.08 ± 1.56, 4.43 ± 1.03 and 6.24 ± 0.7 respectively. After 1 month of incubation, the Shannon diversity index showed a significantly lower value in substrates in comparison with free-living eukaryotic community (p-value < 0.05; **Table S9 in Supplementary Material 1**) in site 1. On the contrary, in site 2, the eukaryotic communities in some substrates (LDPE bag, PS dish, BS glass) showed higher diversity than water free-living eukaryotic communities (p-value < 0.05; **Table S9 in Supplementary Material 1**). After 3 months of incubation, eukaryotic diversity from site 1 significantly decreased in all samples (p-value < 0.05) with the exception of LDPE bag that increased (**Table S9 in Supplementary Material 1**). In site 2, only the eukaryotic community α-diversity in LDPE bag and PS dish decreased (p-value < 0.05) in contrast with the eukaryotic community α-diversity on the rest of substrates that increased (**Table S9 in Supplementary Material 1**). At 6 months of incubation, eukaryotic community α-diversity from LDPE bag and PET bottle from site 1 decreased significantly (p-value < 0.05) as in all plastic samples from site 2 (**Table S9 in Supplementary Material 1**). Finally, after 12 months of incubation, the eukaryotic community α-diversity in all plastics, except the LDPE bag, significantly increased in site 1 (p-value < 0.05; **Table S9 in Supplementary Material 1**). In the more impacted site 2, similar to what was found for bacterial diversity, there were some fluctuations but not a clear increase in diversity was found in any plastic substrate (**Figure 2**). In the case of non-plastic substrates, the diversity on BS glass significantly decreased (p-value < 0.05); interestingly an increase was observed in the diversity of free-living eukaryotes in site 2 (**Table S9 in Supplementary Material 1**). In conclusion, there were significant changes in the diversity of both bacteria and eukaryotes colonizing plastic substrates along time that differed between the two sampling sites and differed from the diversity of those attached to rocks and BS glass and that of the free-living microorganisms

(**Figure 2**). Furthermore, general changes in the eukaryotic diversity of plastic-attached communities due to incubation times and sampling sites were different from free-living water communities, but not distinct from rock-associated or BS glass-attached communities (**Figure 2**).

3.5. Composition of bacterial communities on plastics

Fifty-two bacterial phyla divided into 150 classes were identified in all the samples (**Supplementary Material 2**). Five archaeal phyla were also identified, classified into 8 classes in all the sample set. The relative abundance of the two domains was markedly unequal, with bacteria representing more than 99.9% of the relative abundance in the sample set, while archaea constituted less than 0.1%. Therefore, the following analysis includes the most abundant taxa in the bacterial community.

Taxonomic analyses showed a bacterial community dominated by the phylum Proteobacteria followed by phyla Bacteroidetes and Cyanobacteria independently of the collected substrate/environment (plastic, BS glass, rock, or water), sampling site and month of collection.

At the class level, the analyses confirmed the specificity of the plastisphere compared to the bacterial communities on BS glass, rock and freshwater, significantly influenced by the sampling site (**Supplementary Material 2**). In sampling site 1, the plastisphere was dominated by the classes Alphaproteobacteria, Oxyphotobacteria and Gammaproteobacteria, similarly to the bacterial community associated with rocks. BS glass-attached bacterial communities were dominated by Alphaproteobacteria, Gammaproteobacteria and Bacteroidia. The greatest change in the bacterial community was detected in the water, highlighting the abundance of the classes Bacteroidia, Gammaproteobacteria and Actinobacteria. In contrast, in sampling site 2 the more abundant attached bacterial classes were Gammaproteobacteria, Alphaproteobacteria and Bacteroidia, independently of tested substrates or free-living bacteria in the water column.

At lower taxonomic levels, such as order (**Figure 3**) and family, the temporal evolution of the bacterial community associated with plastic in both sites can be followed. Thus, early colonizers (after 1 month of incubation), intermediate colonizers (after 3 months of incubation) and late colonizers (after 6-12 months of colonization) can be recognized as the bacterial community stabilizes over time at each of the sampling sites. At both sites, early colonizers of the plastisphere were Betaproteobacteriales (mostly represented by the family Burkholderiaceae), Rhodobacterales (family Rhodobacteraceae), Rhizobiales and Sphingomonadales (family Sphingomonadaceae), independently of the selected sampling

site. These orders were followed in abundance by the orders Methanomassiliicoccales (family Methylophilaceae) and Chitinophagales (family Saprospiraceae) at sampling site 1 and the orders Betaproteobacteriales (family Rhodocyclaceae) and Methylococcales (family Methylomonaceae) at site 2, denoting some variability in the bacterial community according to site.

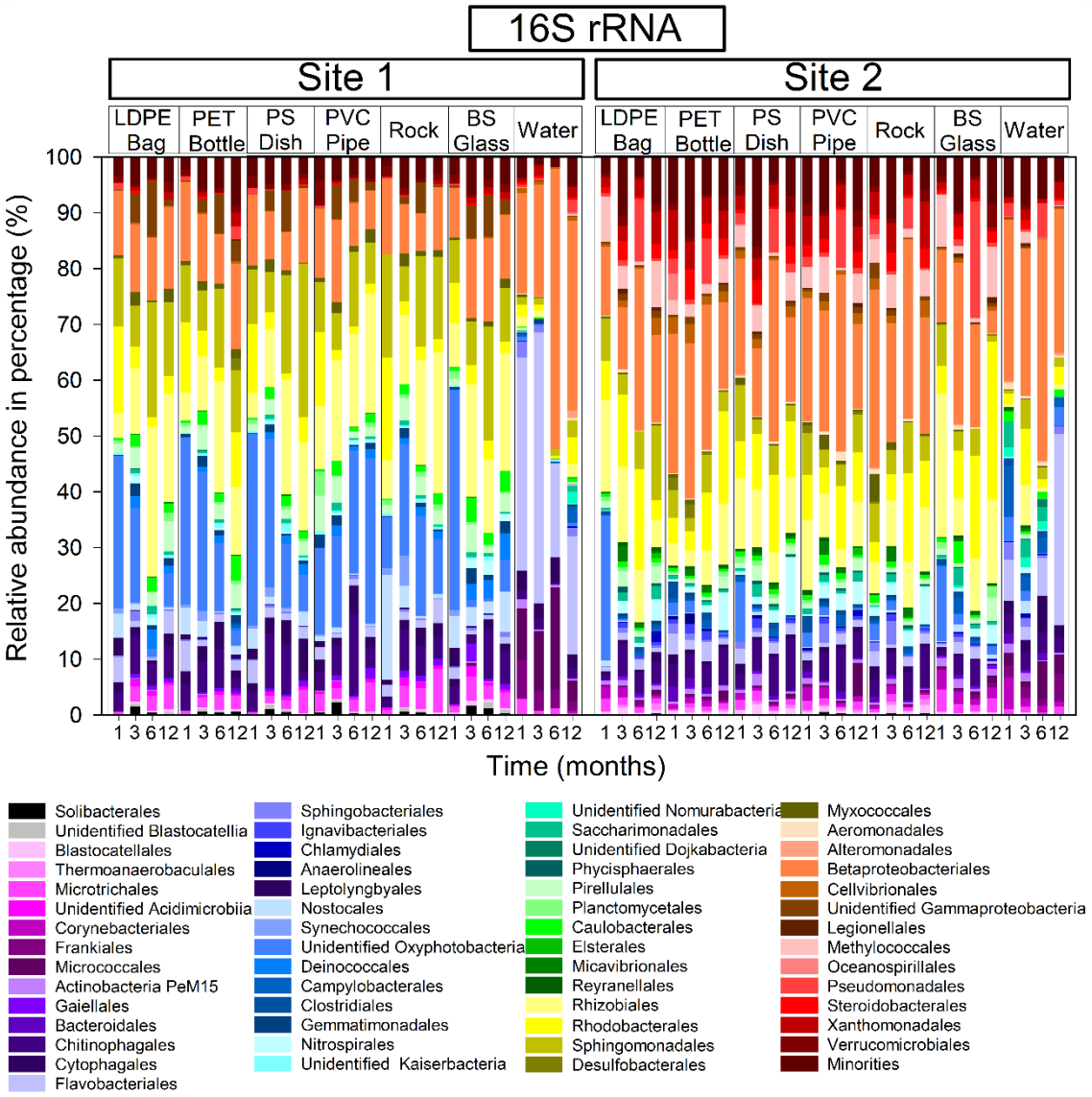

Figure 3. Relative abundance of bacterial community at the order level associated with the different substrates incubated in both sampling sites along increasing times of incubation (1 month, 3 months, 6 months and 12 months). Minorities are orders whose representation is less than 1 %.

After 3 months of incubation, a considerable change takes place in the most abundant bacteria. In the first place, the overall relative abundance of all identified taxa decreased (**Figure 3**; **Supplementary Material 2**). However, the orders Betaproteobacteriales (mostly represented by the family Burkholderiaceae) and Rhizobiales are still the dominant orders in both sites. In addition to these, the order Chitinophagales (mostly represented by the family Saprospiraceae on site 1 and by the family Chitinophagaceae on site 2) was another abundant

order in both sampling sites. At each site, the plastisphere at sampling site 1 shows a high abundance of the orders Sphingomonadales (family Sphingomonadaceae) and Pirellulales (family Pirellulaceae). On the other hand, at site 2, the orders Betaproteobacteriales (family Rhodocyclaceae) and Methylococcales (family Methylomonaceae) were still dominant.

After 6 months of incubation, the bacterial community attached to the plastisphere seems to be settled with no further significant changes (**Figure 3**; **Supplementary Material 2**). Therefore, the orders Rhizobiales (represented mostly by the family Hyphomicrobiaceae in the plastisphere of site 1 as well as by the family Rhizobiaceae in site 2) and Betaproteobacteriales (represented mostly by the family Burkholderiaceae) and Chitinophagales (represented by the family Chitinophagaceae in site 1 and by the family Saprospiraceae in site 2) are the most abundant orders. Order Sphingomonadales (mostly represented by the family Sphingomonadaceae) becomes again dominant in both sites. Some of the most abundant orders at this time of colonization are only relevant in each site, with the order Microtrichales, represented mainly by the family Microtrichaceae, in site 1. At site 2, the order Rhodobacterales is the most abundant in the bacterial community, with the major representative family Rhodobacteraceae.

After 1 year of colonization, there are not any further significant changes in the bacterial community at the order level (**Figure 3**; **Supplementary Material 2**). The orders with the highest abundance at both sampling sites include Rhizobiales (family Rhizobiaceae), Sphingomonadales (family Sphingomonadaceae), Betaproteobacteriales (family Burkholderiaceae) and Chitinophagales (represented mostly by the family Saprospiraceae at site 1 and by the family Chitinophagaceae at site 2). Moreover, the abundance of Rhodobacterales (family Rhodobacteraceae) increases in both sites. However, there are some orders whose relative abundance is higher according to the site and incubation time, such as the order Cytophagales (family Hymenobacteraceae) at site 1 and Nitrospirales (family Nitrospiraceae) at site 2.

3.6. Composition of eukaryotic communities on plastics

Full taxonomic assignment obtained using SILVA 132 database can be found in **Supplementary Material 3**. All 18S rRNA sequences were identified as eukaryotes. The eukaryotic organisms identified do not exclusively consist of microorganisms but include multicellular organisms that can also colonize the plastisphere. Most of the sequences collected from the sample set were identified as the clades Opisthokonta, SAR or Harosa (represented mainly by the group Stramenopiles), and Archaeplastida (constituted primarily

by Chloroplastida). 6.3% of the sequences were identified only as eukaryotic, with no further assignment.

At lower taxonomic levels the effect of sampling site on the sample set is more prominent. Samples collected at sampling site 1 showed the dominance of the phylum Ochrophyta, specifically of the class Diatomea. Another highlighted phylum was Platyhelminthes, represented mainly by the class Gastropoda and Rhabditophora. In the case of sampling site 2, a higher diversity of taxa was found. The dominant phyla were Bryozoa (highlighting the presence of the class Phylactolaemata), Annelida (mainly the class Clitellata) and Platyhelminthes (represented mainly by the class Gastropoda).

The taxonomic analysis detected changes in relative abundance in all samples at the order (**Figure 4**) and family level. As with bacteria, an ecological succession of the eukaryotic community attached to the plastisphere could be observed at both sites. As an exception, the order Achnanthales (site 1), specifically, most of these sequences were as the genus *Cocconeis* of the family Cocconeidaceae was found on all substrates (plastics, BS glass, and rock), representing approximately 50% of the relative abundance of taxa found in these samples, regardless of colonization time.

Regarding temporal succession, potential early eukaryotic colonizers of the plastisphere could be identified after the first month of incubation (**Figure 4**; **Supplementary Material 3**). In this first phase, the eukaryotic orders with the highest relative abundance differ widely between both sites. Only the superorder Heterobranchia (unassigned family and order) showed a high relative abundance at both sites. At site 1, the orders with the highest relative abundance in the plastic assemblage were Tricladida (family Planariidae) and the orders of photosynthetic organisms Chaetophorales (mostly represented by the family Chaetophoraceae), Cymbellales (family Gomphonemataceae) and Ulvales (family Monostromataceae). Instead, at site 2, several types of multicellular organisms of the order Haplotaxida (such as the family Naididae), Diptera, Catenulida (family Stenostomidae) as well as the protist order Tectofilosida dominated.

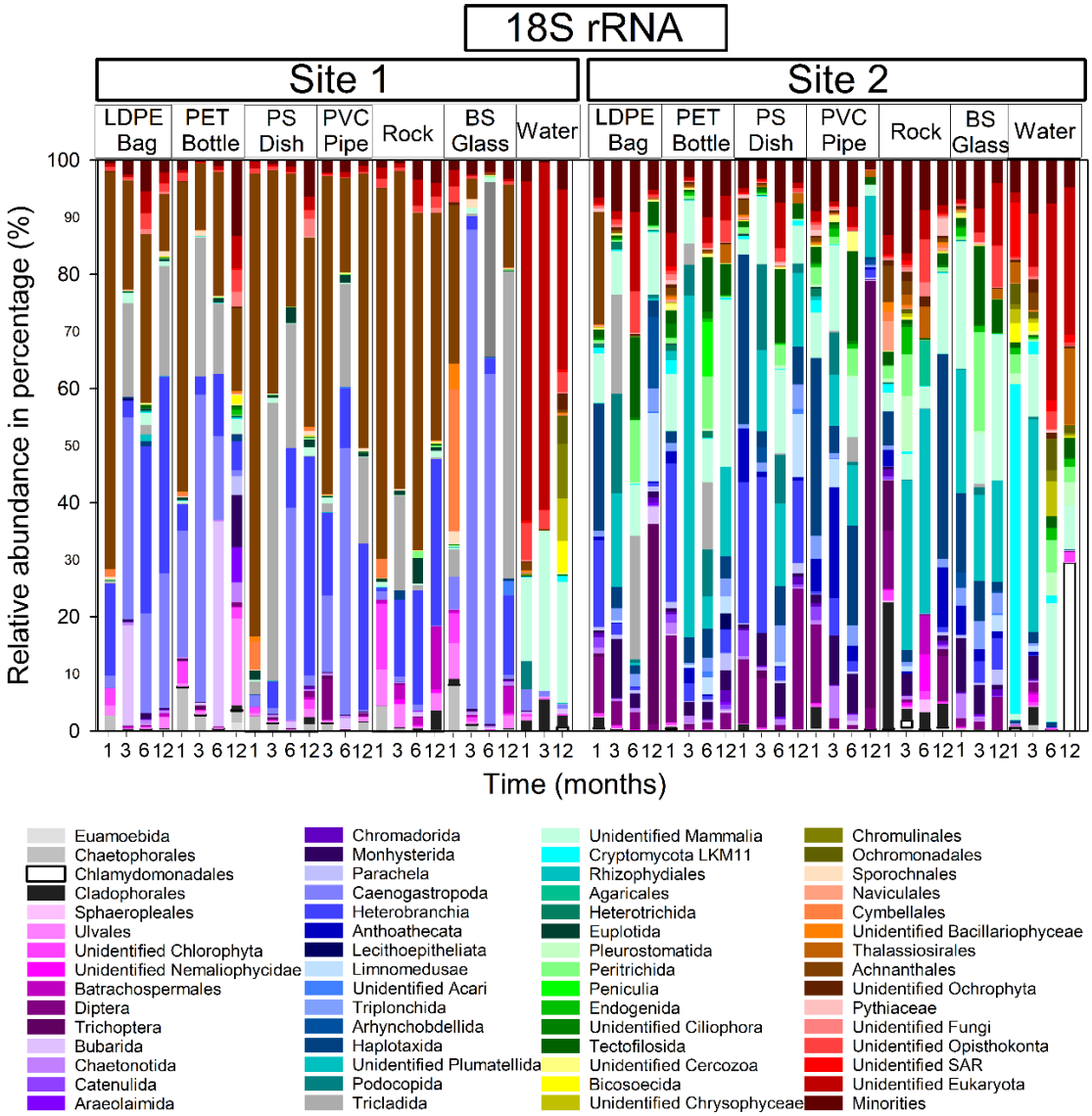

Figure 4. Relative abundance of eukaryotic community at the order level associated with the different substrates incubated in both sampling sites along increasing times of incubation (1 month, 3 months, 6 months and 12 months). Minorities are orders whose representation is less than 1 %.

After 3 months of incubation, the eukaryotic taxa with the highest relative abundances were clearly different to those found after 1 month of colonization and could be considered as intermediate colonizers. (**Figure 4**; **Supplementary Material 3**). Superorder Heterobranchia, the order Tricladida (family Planariidae) and Diptera are the most abundant. At site 1, photosynthetic organisms still play a major role in the community, with the algae of the order Chaetophorales (represented mainly by the family Chaetophoraceae) again prominent. The most abundant novel taxa at this time of colonization included the order Bubarida (whose most abundant family is Scopalinidae) and Caenogastropoda (represented mainly by the family Caecidae). At sampling site 2, the order Haplotaxida (family Naididae) was quite abundant. Other relevant taxa were the ostracod order Podocopida (family

Cypprididae), the bryozoan order Plumatellida (family unidentified) and the nematode order Monhysterida.

After six months of incubation, as already found with the bacterial community, the eukaryotic community in the plastisphere at the order level was already settled since previously detected taxa remained (**Figure 4**; **Supplementary Material 3**). At site 1, the superorder order Bubarida (whose most abundant family is Scopalinidae) and Caenogastropoda (represented mainly by the family Caecidae) were still established. In addition, the relative abundance of the order Euplotidae (represented mainly by the family Aspidiscidae) increased markedly. At sampling site 2, the orders Plumatellida, Monhysterida and Podocopida (family Cypprididae) were still very abundant. The orders Haplotaxida and Tectofilosida that were already prominent in the early stage of colonization, increase their relative abundance after 6 months of incubation. However, some new orders with high relative abundances appeared such as the order Peritrichida (family Opisthonectidae) and Triplonchida. Specifically, the order Tricladida (represented mainly by the family Planariidae) showed a high relative abundance at both sites.

After one year, there were no further significant changes in the eukaryotic community (**Figure 4**; **Supplementary Material 3**). The superorder Heterobranchia still remained at both sites in high abundance. At site 1, the order Tricladida (family Planariidae) and the order Caenogastropoda (family Caecidae) have been retained from the mid-stage of colonization. The orders Ulvales (family Monostromataceae) and Chaetophorales increased their abundance at this stage. In addition, the nematode order Monhysterida increased in relative abundance to a considerable degree in this late phase of colonization. At site 2, the orders Tectophilosida, Plumatellida, Haplotaxida, and Triplonchida are retained from the intermediate stage of colonization. The order Diptera was also found at this stage and several taxa increased their abundance at this stage, such as Trichoptera and Arhynchobdellida (family Erpobdellidae).

3.7. β-DIVERSITY

The db-RDA analysis (based on the Bray-Curtis distance matrix using ASVs) revealed a similar clustering structure for both bacterial and eukaryotic communities (**Figure 5**). The distribution of the samples is mainly based on their site (sampling site), finding a very clear differentiation in the distance on the X-axis (which explained the 26.42 % of the difference between clusters in bacteria and 22.63 % of that difference in eukaryotes). The samples from site 1 are mostly distributed around the Y-axis in a homogenous fashion according to the substrate and to a lesser extent to colonization time. Samples from site 2 showed a more disperse distribution, although there was a certain homogenous pattern of distribution along the Y-axis (which explained the 10.26 % difference between clusters in bacteria and 7.77 % of that difference in eukaryotes) according to the type of the substrate and the time of the colonization. The bacterial and eukaryotic community in water was distinctly different from those substrates at both sites, which is illustrated in the clustering of these samples far apart on the X-axis from the rest of the samples.

The distribution of the samples hierarchized by UPGMA dendrograms (**Figure S7** for bacteria and **Figure S8** for eukaryotes **in Supplementary Material 1**) confirms these results. Furthermore, the dendrograms clearly show that the clusters are ordered first by sampling site, then by substrate type and lastly by the time of colonization.

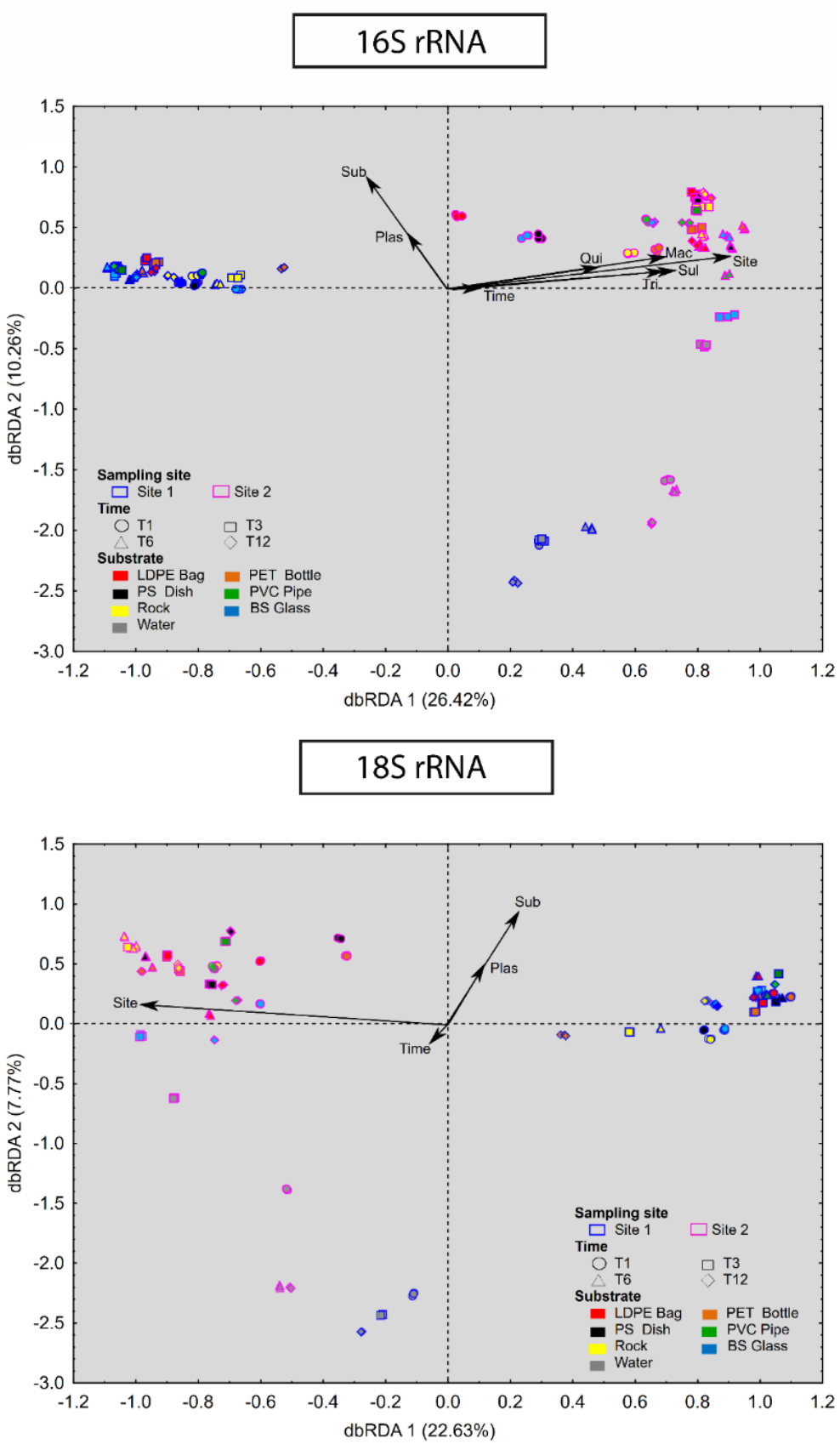

Figure 5. Distance-based redundancy analysis (dbRDA) ordination based on Bray-Curtis dissimilarity of 16S rRNA and 18S rRNA. Each point in the ordination plot represents the community in each sample. The factor abbreviations are Sub (Substrate); Plas (plastic); Qui (Quinolones); Mac (Macrolides); Tri (Trimethoprim); Sul (Sulfamide).

A Monte-Carlo permutation test (999 unrestricted permutations) was performed to better explain the potential influence of the parameters considered in this study regarding bacterial and eukaryotic communities; the parameters were: sampling site, colonization time, substrate and within substrates only plastic; in the case of bacterial communities, the concentration of antibiotics was also added (**Table 1**). The analysis confirmed a significant influence of the sampling site, type of substrate, plastic and colonization time in eukaryotes and concentration of the antibiotics (p-value < 0.05) in the case of bacteria. This analysis confirmed the previous ones as the factor that explained most of the variation in the microbial communities was the sampling site (24.64% for bacteria and 22.15% for eukaryotes). In the bacterial communities, the second factor explaining most of the variation was the concentration of antibiotics, namely, sulphonamides (17.6%), trimethoprim (16.4%), macrolides (16.8%); third factor was the type of substrate (11.4%), followed by the concentration of quinolones (9.2%), whether the material is plastic (3.63%) and lastly, the colonization time (3.62 %). In eukaryotes, the order of the factors explaining the variation was similar with type of substrate accounting for 8.4 % of the difference, plastic 3.19 % and colonization time 3.14 %. This model explains 56.2 % of the differences between samples in the bacterial communities and 32.7 % in the eukaryotic communities, because several factors are already correlated as found in the db-RDA model.

Table 1.
Results of Monte-Carlo permutation tests (999 unrestricted permutations) and percent variation explained for variables considered in the db-RDA analysis.

Gene	Factor	Sum of Squares	F	p-value	Proportion of explained variation (%)
16S rRNA	Site	13.99	93.99	0.001	24.64
	Time	2.05	13.82	0.001	3.62
	Substrate	6.51	43.69	0.001	11.45
	Plastic	0.55	3.71	0.004	3.63
	Quinolones	2.17	14.57	0.001	9.19
	Sulphonamides	2.49	16.76	0.001	17.65
	Trimethoprim	2.98	20.00	0.001	16.45
	Macrolides	2.37	15.88	0.001	16.82
	Residual	23.68	-----	-----	-----
	Model	33.13	27.80	0.001	56.22
18S rRNA	Site	13.48	52.02	0.001	22.15
	Time	1.91	7.38	0.001	3.14
	Substrate	4.94	19.08	0.001	8.37
	Plastic	0.66	2.55	0.007	3.19
	Residual	39.92	-----	-----	-----
	Total	21.00	20.26	0.001	32.77

The differences in the microbial community between samples were confirmed using global and category-based PERMANOVA in this study: sampling site, colonization time and substrate (p-value < 0.05; **Table S10** and **Table S11 in Supplementary Material 1**). In contrast, pairwise comparisons were not significant in either bacterial or eukaryotic communities regarding sampling site, colonization time or substrate (p-value > 0.05; **Table S10** and **Table S11 in Supplementary Material 1**). As pairwise PERMANOVA tests did not detect significant differences among microbial communities, linear discriminant analyses (LEfSe) were subsequently used to further confirm whether certain taxa were significantly more abundant in each substrate considering sampling sites and colonization time (**Table S12** in **Supplementary Material 1** and **Table S13 in Supplementary Material 1**).

The presence of these taxa in both the bacterial and eukaryotic communities of each plastic at each sampling point at different times at each sampling point allows for defining a core microbiome. This core microbiome can be categorized in each plastic according to early colonizers (after one month of colonization), intermediate colonizers (after 3 months of colonization), and late colonizers (after 6-12 months of colonization, although LefSe analyses allowed to identify specific core microbiomes for both time periods).

Identified early colonizers composing the bacterial community (**Table S12 in Supplementary Material 1**) at sampling site 1 in the LDPE bag were *Pseudorhodobacter, Calothrix*, *Porphyrobacter*, *Lacihabitans*, *Silvanigrella* and the family Flavobacteriaceae. In PET bottle, the genera *Streptococcus*, *Pseudorhodobacter* and *Stigeoclonium* were dominant. In PS dish, the genera with highest abundance were *Rhodopirellula*, *Gemella*, *Haemophilus* and *Rothia*. In PVC, the most characteristic genera were *Gemmatimonas, Pirellula, Fluviicola* and *Limnobacter*.

In intermediate periods (corresponding to 3 months of incubation) the dominant taxa differed for each type of plastic (**Table S12 in Supplementary Material 1**). In the LDPE bag, the characteristic genera were *Rhizobacter, Maribacter, Blastopirellula, Imbriiglobus* and *Sandaracinus* after 3 months of incubation. The genera *Pleurocladia*, *Rhodopirellula, Nannocystis, Neochloris, Oligoflexus* and *Ferrovibrio* were the most abundant in the PET bottle after 3 months of incubation. In terms of the PS dish, the taxa Methylophilaceae, Rhodocyclaceae and *Snodgrassella* were dominant after 3 months. In contrast, the genera *Schizothrix*, *Paludibaculum*, *Bryobacter* and *Rhodopirellula* were the most dominant in PVC pipe after 3 months of incubation.

In the last stage of colonization (6-12 months), after 6 months of incubation, the microbiome core in the LDPE bag was characterized by the genera *Hyphomicrobium*, *Amoebophilus*, *Luteolibacter* and *Gallionella*, in the PET bottle, the genera the genera

Hymenobacter, Hyphomonas, Hirschia, Acidibacter, Leptothrix, Dongia and *Rhodobacter* were predominant; in the PS dish, *Roseibacillus* was the most abundant genus and in the PVC pipe, the core microbiome was constituted by the genera *Taeseokella, Pajaroellobacter, Polyangium* and *Cytophaga*.

After 12 months of colonization, the taxa that constituted the core microbiome in the LDPE bag were the genera *Pirellula, Fimbriiglobus, Massilia, Bdellovibrio, Lacibacter* and *Peridibacter*. In the PET bottle, the most abundant genera were *Hymenobacter, Hyphomonas, Hirschia, Acidibacter, Leptothrix, Dongia* and *Rhodobacter*. The genera *Pleurocapsa, Sphingorhabdus, Haliangium, Rickettsia, Deinococcus* and *Hymenobacter* dominated in the PS dish. *Ilumatobacter* was the most abundant genus in the PVC pipe.

In the sampling site 2 (**Table S12 in Supplementary Material 1**) the taxa that constituted the core microbiome at the different times of colonization differed significantly. Among the early colonizers, in the LDPE bag, the dominant genera were *Tychonema, Amoebophilus* and *Desulfatitalea*. In PET bottle, the genera *Streptococcus*, *Pseudorhodobacter* and *Stigeoclonium* were dominant. In PS dish, the genera with highest abundance were *Inhella, Verrucomicrobium, Lacunisphaera, Cellvibrio* and *Bdellovibrio* and in PVC pipe, the genera, which constituted the core microbiome, were *Sphingomonas, Altererythrobacter, Competibacter, Propionivibrio* and *Rhizobacter*.

In the LDPE bag, the most abundant genera after 3 months of incubation were *Defluviimonas, Chryseobacterium, Aeromonas, Blastopirellula, Peredibacter* and *Nitratireductor* and, after 6 months of incubation the taxa were *Gemmobacter, Paracoccus, Thiothrix, Acetobacterium, Brachymonas, Dialister* and *Actibacter*. The genera *Thiobacillus, Pseudomonas*, *Dechlromonas, Roseomonas, Desulfobacter, Competibacter* and *Crenothrix* were more abundant in PET botte after 3 months of colonization, shifting to the genera *Arenimona*s, *Acetoanaerobium*, *Acinetobacter*, *Rhodoferax*, *Tolumonas* and *Thermomonas* after 6 months of incubation. In the PS dish, the genera more representative after 3 months of incubation were *Reyranella, Dinghuibacter, Luteitalea, Rickettsia* and *Planctopirus* which were replaced by the genera *Lautropia*, *Staphylococcus*, *Lawsonella*, *Comamonas*, *Pirellula* and *Pedobacter* after 6 months of incubation. In PVC pipethe genera *Competibacter, Permianibacter, Nitrosomonas* and *Chloroflexi* dominated after 3 months of incubation while the genera *Corynebacterium*, *Chthoniobacter*, *Luteolibacter*, *Leeia* and *Bacteriovorax* were the most characteristic after 6 months of incubation.

After 1 year, the most abundant genera in each substrate shifted. In the LDPE bag, the genera *Methyloparacoccus, Terrimicrobium, Finegoldia, Pirellula, Paracaedibacter* and *Anaerococcus* were dominant. In the PET bottle, the most abundant genera were *Nitrotorga, Tahibacter,*

Lautropia, Nitrotoga, Vogesella and *Schlesneria*. The genera *Chthoniobacter, Pseudoduganella, Chromobacterium, Alysiosphaera* and *Citrobacter* dominated in the PS dish. *Corynebacterium, Chthoniobacter, Luteolibacter, Rhodovastum, Atopostipes* and *Leeia* were the most abundant genera in the PVC pipe in comparison with the rest of substrates.

In contrast, the eukaryotic communities did not differ too much between substrates (**Table S13 in Supplementary Material 1**). For this reason, the LEfSe analysis did not significantly detect a specific taxon in some substrates or detected a low number of taxa. Moreover, some of the taxa detected belonged to multicellular organisms, so in the case of eukaryotes, it is more appropriate to refer to a plastic core biome. In site 1, after 1 month of colonization (early colonizers), only specific organisms were detected in the PET bottle (dominated by the genera *Aphanochaete* and *Chaetopeltis*) and PS dish (the taxa Poales and *Cocconeis*). Later on, the core biome changes remarkably. In LDPE bag, after 3 months of incubation, the characteristic genera were *Marsiela*, *Catenula*, *Daptonema* and *Pseudourostyla* and the taxa *Rheum*, *Oenothera*, *Synchaeta* and Haptoria were most abundant after 6 months of colonization. In the PET bottle, the genera most abundant were the taxa Contienticola and *Schmidtea* after 3 months of colonization and the order Bubarida after 6 months. In the PS dish, a core biome could be identified only after 6 months of colonization, mainly composed by the genus *Sialis*. In the PVC pipe, the order Mermithida was the most abundant after 3 months of incubation and the genera *Eucapnosis*, *Taphrina* and *Vorticella* constituted the biome core after 6 months.

After 1 year, the most characteristic genus in LDPE bag was *Paulinella*. In the PET bottle, the most abundant genera were *Angulamoeba*, *Filamoeba*, *Dictyamoeba*, *Copromyxa* and *Rhizamoeba*. The taxa Fabales, *Plantago*, *Erynia* and *Navicula* were dominated in the PS dish. Eimeriidae was the only family most abundant in the PVC pipe.

In sampling site 2, within the early colonizers, the most abundant taxa in the LDPE bag were *Nematostelium*, Caryophyllales, Poales, *Tetraselmis*, *Chaetomium* and *Stentor*. In the PET bottle, the taxa Clevelandellida, *Pelagothrix*, *Epalxella* and *Plagiopyla* were the most abundant. *Stenostomum* was the most abundant genus in the PS dish. In contrast, the genera *Entamoeba*, *Algulamoeba*, Leptomyxida and *Stenostomum* were the most abundant in the PVC pipe community. After 3 and 6 months of incubation, the dominant taxa differed for each type of plastic (**Table S13 in Supplementary Material 1**). In the LDPE bag, the most characteristic taxa were *Scotinosphaera*, Chlorellales, Ephemeroptera and *Adineta* after 3 months of incubation and *Flabellula*, *Pelodera*, *Rhabditis*, *Garardia* and *Geotrichum* predominated after 6 months of incubation. The genera *Rhizoclonium*, *Haltidytes*, *Bullera* and *Euplotia* were

the most abundant in the PET bottle after 3 months of incubation and, after 6 months of incubation, the biome core was characterized by the taxa *Actinidia*, Ichthyosporea, *Schistonchus* and *Candona*. In terms of the PS dish, the genera *Pterocystis*, *Dorylaimida*, *Cyprodopsis* and *Cryptosporidium* were the most dominant after 3 months of incubation and *Mononchoides*, *Caenorhabditis*, *Tripylella* and *Candida* was the most abundant genus after 6 months of incubation. In contrast, the genera most dominant in PVC pipe after 3 months of incubation were *Saccamoeba*, *Radix*, *Hydra*, *Placorhynchus*, *Urospora* and *Stentor* and, after 6 months of incubation, the core biome was constituted by the genera Ptolemeba, Apodibius, Haplotaxida, Cyclopoidia and *Epistylis*.

After 1 year of colonization, the core biome in the LDPE bag was formed by taxa Pinophyta, Pinustaeda, Chromadorida, *Caenorhabditis* and *Parachela*. In the PS dish, the most abundant taxa were Rhabditida, Macrostomida, *Limnohalacarus* and *Geotrichum* Rhabditida, Macrostomida, *Limnohalacarus* and *Geotrichum*. The taxa *Hydroptila, Brevibucca* and Herpotrichiellaceae dominated in the PVC pipe.

3.8. PLASTIC POLYMER ALTERATIONS

Plastic samples incubated in the two sampling sites were characterized by ATR-FTIR analysis at the end of the experiment (12 months) and compared with virgin, non-incubated plastics, as shown in **Figure 6**. There were clear changes in chemical structure with time, as evidenced by the formation of new functional groups as a result of environmental aging of plastics in comparison with non-incubated plastics (**Figure 6**). Some differences in the spectra of PS dish were observed between sampling sites, but no significant changes were noticed between the spectra of LDPE bags, PET bottles and PVC pipes deployed at the two different sampling sites (**Figure 6**).

Most of the aged plastic samples were characterized by the appearance of new absorption bands in the regions of 3300–3305 cm^{-1} and 1745–1635 cm^{-1} corresponding to the formation of hydroxyl and carbonyl groups respectively (**Figure 6**). In LDPE bags, two significant peaks appeared in the 1000–1200 cm^{-1} region, which could be attributed to the formation of carbon-oxygen bonds (**Figure 6**). Furthermore, the presence of a new absorption band around 1640 cm^{-1} may be assigned to unconjugated C=C, previously described and considered characteristic of the degradation process of LDPE (Otake et al., 1995).

The results for the evolution of hydroxyl indices (**Table S14 in Supplementary Material 1**) revealed that all deployed plastics underwent certain degradation after 1 year of incubation in both sites. The degradation process showed some differences depending on the type of plastic and the sampling site (**Table S14 in Supplementary Material 1**). Hydroxyl index was higher in LDPE bag and PET bottle from sampling site 1 in comparison with sampling site 2 (**Table S14 in Supplementary Material 1**). In contrast, hydroxyl index was higher in PS dish and PVC pipe from sampling site 2 in comparison with sampling site 1 (**Table S14 in Supplementary Material 1**).

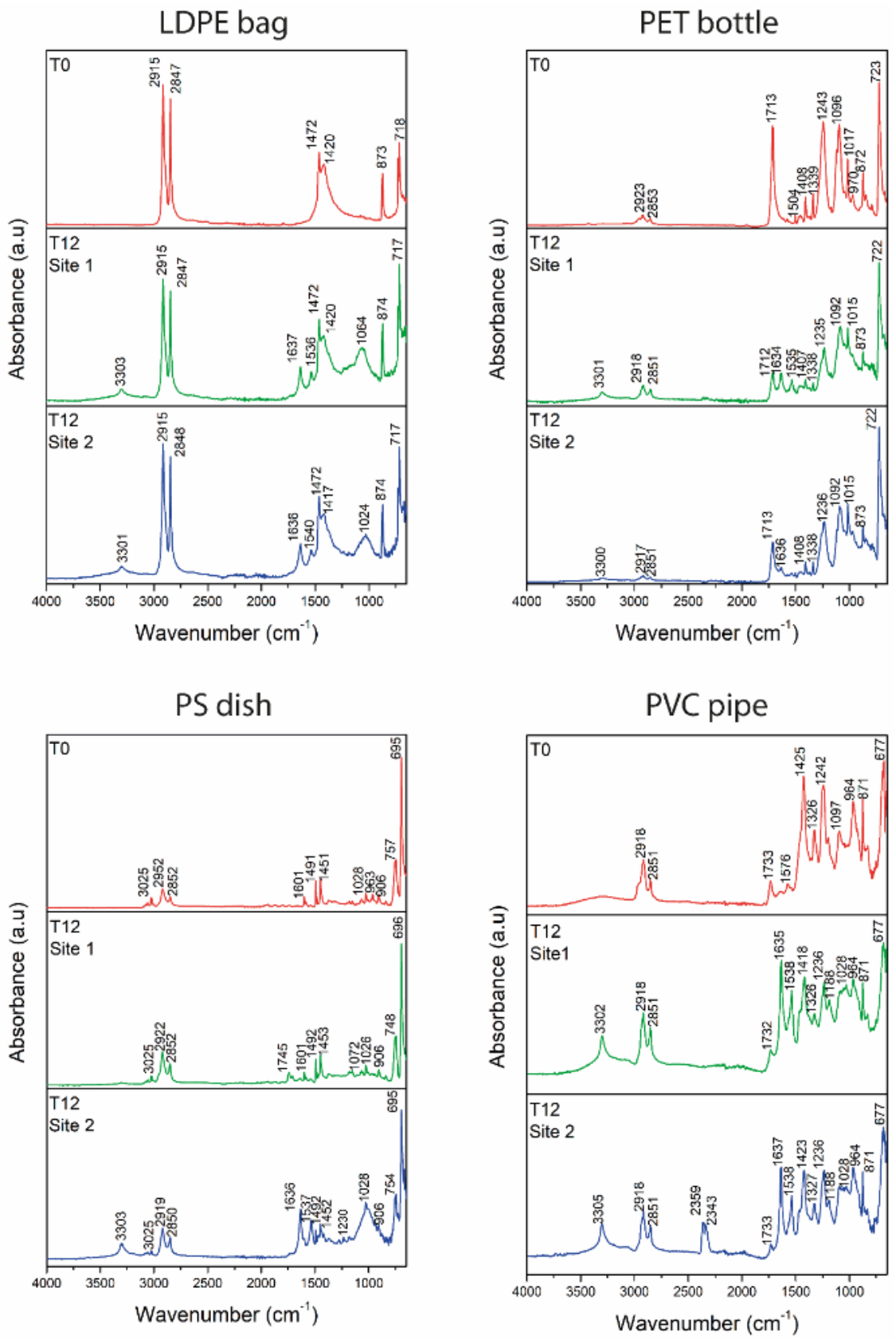

Figure 6. ATR—FTIR comparative spectra of each plastic surface after 1 year of colonization (T12) in the two sites compared with the virgin, non-incubated plastic (T0) treated with the same cleaning protocol.

3.9. ANTIBIOTIC RESISTANCE GENES (ARGS) DETECTED ON PLASTICS

In general, the relative abundance of all ARGs in water was significantly higher than in any of the substrates at sampling site 1 (*p*-value < 0.05, **Tables S15 to S18 in Supplementary Material 1**). In the case of the *sul1* gene, the highest $2^{-\Delta ct}$ values in water were detected after 3 months (0.15) and 12 months of incubation (0.08), significantly exceeded the 2-Δct values detected at the same times of colonization in the plastics ($2^{-\Delta ct}$ values of 1.1×10^{-5} and 3.1×10^{-5} respectively) as well as in the rest of the substrates (*p*-value < 0.05, **Table S15 in Supplementary Material 1**). Regarding the *ermF* gene, the highest relative abundances of the gene were identified after 1 month and 12 months of colonization ($2^{-\Delta ct}$ values of 6.1×10^{-4} and 9.5×10^{-4} respectively, **Figure 7**). The values for the other substrates were indeed low in comparison to water (*p*-value < 0.05, **Table S16 in Supplementary Material 1**). The genes *dfrA* and *qnrSrtF11A* exhibited their highest relative abundance in water at the final stages of incubation (after 6 months and 12 months of incubation) reaching respectively a $2^{-\Delta ct}$ value of 3.1×10^{-5} and 1.1×10^{-4} in the gene *dfrA* and 1.2×10^{-4} and 1.2×10^{-3} in the gene *qnrSrtF11A*. Moreover, in both cases, the abundance of these genes was significantly higher in water than in the other substrates at all times (*p*-value < 0.05, **Table S17** and **Table S18 in Supplementary Material 1**).

At sampling site 2, characterized by elevated antibiotic concentrations in water (**Table S6 in Supplementary Material 1**), a higher abundance of ARGs was detected not only in water but also in plastics, glass, and rock (**Figure 7**), although, in general, neither of the tested ARGs were more abundant in the substrates than in the surrounding water implying that no substrate (plastic, BS glass or rock) concentrated any of them. In the case of *sul1*, this ARG was relative more abundant in water throughout all colonization times (*p*-value < 0.05, **Table S15 in Supplementary Material 1**), except for the 3-month colonization time period. In this period, the relative abundance of the gene in the BS glass was significantly higher ($2^{-\Delta ct}$ value of 0.022) than in the water ($2^{-\Delta ct}$ value of 0.008; *p*-value < 0.05, **Table S15 in Supplementary Material 1**). The relative abundance of the *sul1* gene in the plastics was always lower than in the surrounding water, but significantly higher than in the rock and glass after 6 months and 12 months of incubation (*p*-value < 0.05; **Table S15 in Supplementary Material 1**).

Regarding the *ermF* gene, its relative abundance is only higher in water after the first month of colonization ($2^{-\Delta ct}$ value of 0.006, **Figure 7**; *p*-value < 0.05; **Table S16 in Supplementary Material 1**). After the first 3 months of colonization, the abundance of this

gene in BS glass far surpasses the abundance in rest of the substrates ($2^{-\Delta ct}$ value of 0.006, **Figure 7**; *p*-value < 0.05; **Table S16 in Supplementary Material 1**). In contrast, in the later incubation periods (6 and 12 months of incubation) the relative abundance of this gene in the plastic is the highest among substrates ($2^{-\Delta ct}$ value of 9.5×10^{-3} and 1.8×10^{-3} respectively), although it is only significantly higher at six months of colonization (*p*-value < 0.05; **Table S16 in Supplementary Material 1**).

Concerning *dfrA*, the relative abundance of this gene in the water free-living bacteria was higher than in any of the substrates used, regardless of incubation time (*p*-value < 0.05; **Table S16 in Supplementary Material 1**). The highest value of $2^{-\Delta ct}$ in water was detected after 1 month of incubation (3.5×10^{-4}) (**Figure 7**).

The *qnrSrtF11*A gene was relatively more abundant in the water in comparison with the rest of substrates at most incubation times, except for the three-month incubation (*p*-value < 0.05; **Table S16 in Supplementary Material 1**). The highest relative abundance of the gene in water was detected at 6 months, with a value of $2^{-\Delta ct}$ of 0.002 (**Figure 7**). After 3 months of Incubation, the *qnrSrtF11*A gene was more abundant both in plastic ($2^{-\Delta ct}$ value of 8.12×10^{-4}) and BS glass ($2^{-\Delta ct}$ value of 7.85×10^{-4}) than in rock and water (*p*-value < 0.05; **Table S18 in Supplementary Material 1**).

To confirm whether there was a relationship between the concentration of antibiotics in the water and the relative abundance of genes in each of the substrates, a correlation analysis was performed. Spearman's correlation analysis (**Table S19 in Supplementary Material 1**) confirmed a significant correlation between antibiotic concentration and the abundance of the corresponding ARG at both sampling sites and colonization time (*p*-value < 0.05) independently of the substrate. However, most correlations were not significant if only site 1 was considered because there was little change in the antibiotic concentration in the water (**Table S19 in Supplementary Material 1**). The correlations obtained in all cases were positive, although their strength varied depending on the substrate and each particular ARG (**Table S19 in Supplementary Material 1**). The strongest correlations of *sul1* (0.89) and *dfrA* genes (0.97) were with plastic, of the *ermF* gene with rock (0.78) and of the *qnrSrtF11A* gene with BS glass (0.83; **Table S19 in Supplementary Material 1**). In general, the weakest correlations were in the water, the *sul1* (0.5) and *qnrSrtF11A* (0.54) genes had the strongest correlation values with water (**Table S19 in Supplementary Material 1**).

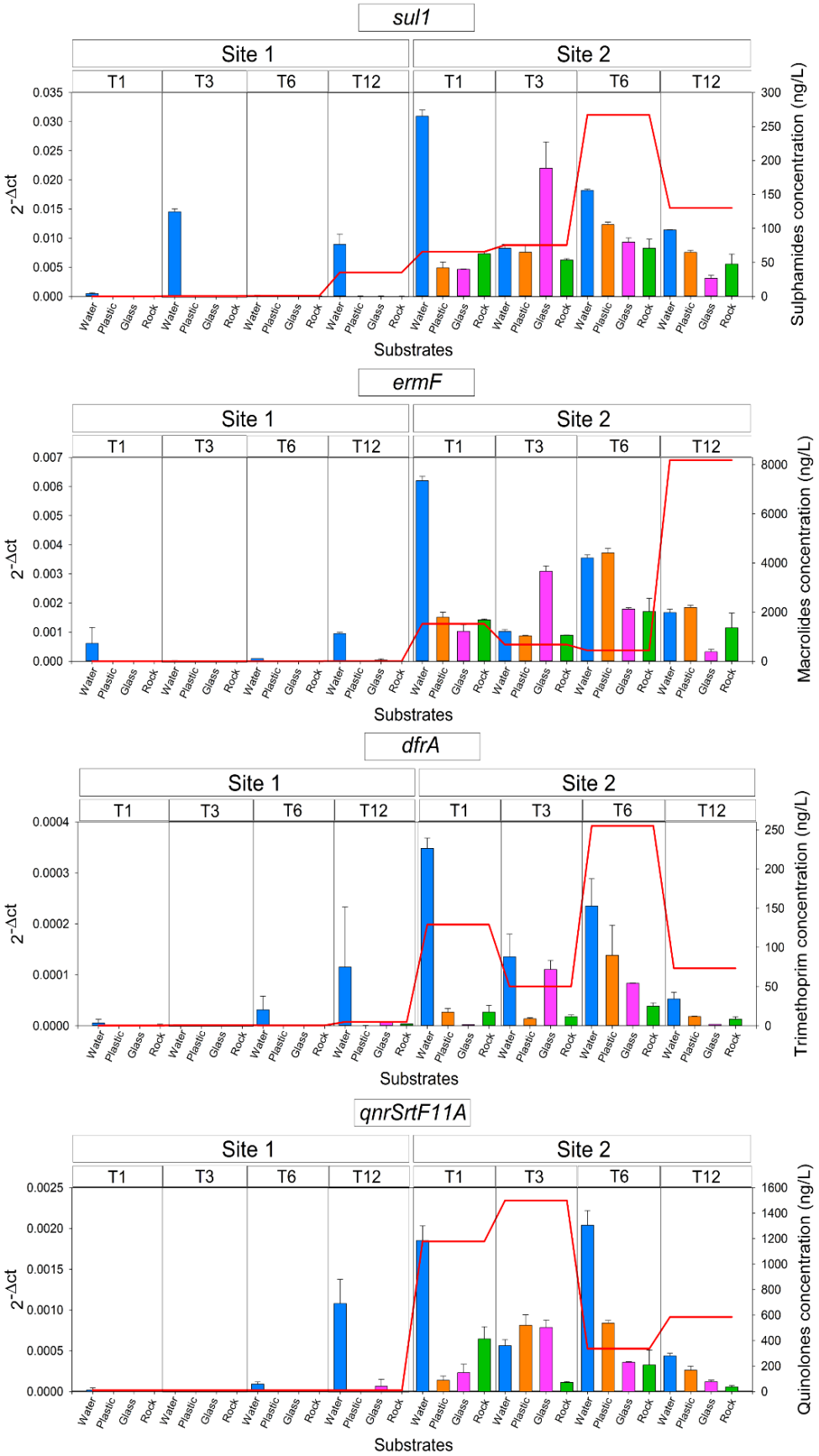

Figure 7. Relative abundance ($2^{-\Delta ct}$) of *sul1*, *ermF*, *dfrA* and *qnrSrtF11A* genes in comparison with the concentration of sulphamides, macrolides, trimethoprim, and quinolones respectively in both sampling sites at the different incubation times. The color of the graph bar corresponds to the type of substrate: blue: water; orange: plastic; pink: BS glass; green: rock.

4. DISCUSSION

This study represents a time-course evaluation of the evolution of the eukaryotic and bacterial communities developed on everyday plastic items over a year in two sites with different levels of anthropogenic impact in the same river. The evaluation of these three factors (site, type of substrate, and incubation time) is essential to understand which organisms form the plastisphere and therefore, the environmental impact they may cause.

The results show that site (sampling site) is the factor mostly influencing the microbial diversity of the different substrates used. Previous studies both in freshwater and marine ecosystems, at different times of plastic colonization and with different types of plastics have reported site as the main factor determining bacterial communities in the plastisphere (Barros and Seena, 2021; Di Pippo et al., 2020; Wright et al., 2021b, Martinez-Campos et al, 2021). The most comprehensive report to date was performed by Wright et al., (2021b) and included meta- analysis of 16S rRNA sequencing results from more than 30 studies developed in a variety of environments (terrestrial, freshwater, and marine water) as well as different plastics, including those used in this study (LDPE, PET, PS and PVC). Wright et al., (2021b) concluded that site is the decisive factor in the constitution of the bacterial community, although the heterogeneity of the experiments hinders a clear conclusion and reports that are more specific are required to obtain clearer conclusions. In our analysis, the two sampling sites selected showed different environmental conditions: sampling site 1, located in a natural area, was characterized by a low concentration of both, nutrients, and antibiotics, as well as good oxygenation, close to saturation. In sampling site 2, the high concentration of nutrients and antibiotics was due to the upstream site of a WWTP. The increase of nutrients (Hendriks and Langeveld, 2017) and antibiotics have been previously detected in effluents from European WWTPs, specifically for the macrolides, sulphamides, trimethoprim, and quinolones classes (Wang et al., 2020) as our study has also determined and this is clearly a relevant factor that may shape bacterial communities in the plastisphere. Previous studies have also shown that WWTPs affect the biodiversity of receiving rivers, in some cases increasing it (Bondarczuk and Piotrowska-Seget, 2019; Price et al., 2018). This could explain why alpha diversity values are significantly higher on all substrates at site 2. Consequently, our assay confirms that the site is the factor that mostly affects the development of plastisphere regarding both bacterial as well as in eukaryotic communities.

In this research, substrate type is the second most influential factor shaping microbial diversity. In addition, within substrate types (plastic, BS glass, rock) and surrounding water,

plastic explains most of the variation regarding microbial diversity. Most of the current studies comparing different substrates (wood, glass, or rock) with plastic have found no significant differences between substrates, although there are clear differences with the surrounding water (Dussud et al., 2018; Oberbeckmann et al., 2016). On the contrary, other studies found differences between the tested substrates as well as a distinctive microbiome core in each plastic, either between different types of plastics (Martínez-Campos et al., 2021; Xu et al., 2019) or concerning other artificial surfaces (Mieczan, 2020). Furthermore, the differences in the morphology of the plastics used in the study could also explain the changes in the eukaryotic and attached bacterial community as Cheng et al. (2021) suggested, indicating that the morphology of the plastics could promote the development of certain specific taxa.

The colonization time was the least significant factor influencing the development of the eukaryotic and bacterial community attached to the plastics in our experiment. Along time, the surface of the plastic begins to suffer a certain degree of degradation as indicated by the hydroxyl index values obtained at both sites. Subsequently, the plastisphere matures as the time progresses and the plastic-associated community tends to converge and become more similar over time, reducing the differences between microbial communities in different substrates (Mincer et al., 2019). This explains the decreasing difference between the substrates in the db-RDA analysis. Secondly, the season of the year promotes the growth and development of certain organisms in the environment, which is relevant for the constitution of the plastisphere, as it has been previously evidenced in marine ecosystems (Amaral-Zettler et al., 2020) and freshwater ecosystems in short-time periods (Mieczan, 2020). In this report and concerning the differential taxa identified throughout the 12 months of the colonization experiment, we could identify an early stage (1 month of incubation), an intermediate stage (corresponding to 3 months of incubation) and a late stage of colonization (corresponding to 6-12 months of incubation).

In the early stage of development of the plastisphere, the pioneer organisms that attach to the plastic generate EPS, decreasing the hydrophobicity and roughness of the material (Yang et al., 2020). In our study, the families Rhodobacteraceae and Sphingomonadaceae probably played these roles. These families have previously shown their ability to attach to different plastic substrates without showing any type of preference, producing exopolysaccharides and surface-adhesion proteins (Balkwill et al., 2006; Di Pippo et al., 2020; Kviatkovski and Minz, 2015). In addition, the family Sphingomonadaceae is characterized by its high capacity to form biofilms in aquatic environments and its ability to

degrade a wide range of organic compounds (Di Gregorio et al., 2017). Another family found in the early phase was Burkholderiaceae characterized by several generalist genera with the ability to degrade different organic compounds, as well as to develop under different nutrient concentrations and be widely distributed in different aquatic environments (Balkwill et al., 2006). Regarding the most abundant eukaryotic taxa in the early phase, the order Achnanthales, specifically the genus *Cocconeis* (family Cocconeidaceae) was found in all substrates and was maintained throughout the entire year of colonization. The dominance of this order of diatoms could explain the vast abundance of diatoms observed by SEM analysis, particularly in plastics deployed at site 1 where they were found covering the surface of all materials. The presence of the genus *Cocconeis* has been previously reported in the marine plastisphere (Dudek et al., 2020; Oberbeckmann et al., 2014). Khan et al., (2020) showed the ability of diatoms to colonize different plastic surfaces. Initially, they do so due to the roughness of the material, but later, they do so with the help of the exopolymers generated by previous pioneer microorganisms. Although diatoms were not the only primary producers attached to plastics, the abundance of the families Chaetophoraceae, Gomphonemataceae and Monostromataceae was also remarkable in the early stage of colonization, confirming the importance of photosynthetic organisms in early shaping of the community that constitutes the plastisphere (Yokota et al., 2017).

In the mid-phase of the plastisphere colonization, which includes the colonization phase after 3 months of incubation, the presence of biofilm-forming organisms is still prominent, although bacteria with defined roles within the microbial community develop. A family that became important during this phase, although it had already appeared in an early phase, is the Burkholderiaceae family. The family Burkholderiaceae is also frequently found as part of the plastisphere in different aquatic environments (Nguyen et al., 2021; Wen et al., 2020). The interest of this family lies in its great metabolic capacity able of degrade polymers such as polyhydroxyalkanoate (PHA) (Ma et al., 2022), or different organic complex substances (van der Zaan et al., 2012). This could explain the formation of hydroxyl and carbonyl groups associated with all the plastics used in this study. Another important family at this phase is the family Saprospiraceae. The family Saprospiraceae, such as family Sphingomonadaceae, is also capable of producing exopolysaccharides, and can utilize products generated in the biofilm as a source of carbon and energy (Yun et al., 2008). The family Microtrichaceae had been previously detected as an intermediate colonizer (4 months of incubation) in marine environments (Tu et al., 2020). This family is generalist, so it also can metabolize plastic carbon, using different types of plastics as substrates in oligotrophic

environments (Agostini et al., 2021). Regarding eukaryotic organisms, the presence of certain families of multicellular organisms, such as Caecidae, Planariidae, Cyprididae, or Diptera, was remarkable. These organisms play roles as primary consumers or predators, and when they are consolidated in the plastisphere, a complex food web is being developed (Amaral-Zettler et al., 2020). These findings are in line with other previous reports and indicate that many multicellular organisms can use plastics as safe refuges. This has already been demonstrated in plastic litter in the ocean (De-la-Torre et al., 2021). Furthermore, De-la-Torre et al., (2021) reported the presence of various organisms that have so far been considered invasive and others that, although not invasive, could become invasive if the plastics drift through the ecosystems.

In the late stages of plastisphere formation (6-12 months of colonization), many of the previously described families are already consolidated, so there are not substantial changes in the families with the highest relative abundances, although there are some exceptions. This is the case of the family Hymenobacteraceae, which has been previously described in association with greenhouse plastics in rivers (Martínez-Campos et al., 2022). The Nitrospirales family, characterized by its participation in the nitrogen cycle, also occupies the plastisphere of site 2, which may be an adaptation of the community attached to the plastisphere to the nitrogen compounds (Baskaran et al., 2020) released by the WWTP effluent. In the case of the family Hiphomicrobiaceae, its abundance increases after 6 months of colonization; some members of the family, such as the genus *Hyphomicrobium*, are restricted facultative methylotrophs, growing on C1 components, such as methanol but not compounds with three or more carbon atoms (Liu et al., 2014). These bacteria could therefore take up these compounds from other organisms already developed in the biofilm. With respect to eukaryotes, the changes in the community are also minor. The case of the order Ulvales is particularly remarkable as it appears again in great abundance in this phase. It has been previously recognized as a colonizer of different artificial substrates such as plastic and may colonize the inner side of packaging items, in our study it developed inside the PET bottle (Bravo et al., 2011). The order Trichoptera is also relevant at this stage. This is noteworthy, considering that Gallitelli et al., (2021) showed that certain macroinvertebrates, such as the larvae of Trichoptera, in freshwater systems choose to use microplastics, compared to other natural substrates, to build their refuges.

LEfSe analyses allowed the identification of differential genera colonizing each of the tested plastics in the different colonization times, this allowed the identification of plastic core microbiomes (biomes in the case of eukaryotic taxa) in each plastic substrate at the

different stages of colonization. Some of the genera found in the core microbiome/biome of each one of the plastics have relevant ecological implication or could pose a risk to human health or the environment.

Specifically, in the LDPE bag microbiome core, several bacterial genera had already been reported in previous studies. *Lacihabitans* was previously found attached to plastics and was characterized by their ability to degrade compounds such as cellulose (Szabó et al., 2021). *Nitratireductor*, which appears at site 2, is a nitrate-reducing bacteria, indicating that plastics and the associated biofilms might influence nitrogen cycling in the marine environment (Ashar et al., 2020). *Caloxtrix* is notable for its ability to produce toxins, which are dangerous to humans (Shardlow, 2021). *Aeromonas*, a potential pathogen for humans and fish, also was relatively abundant in this plastic. (Amaral-Zettler et al., 2020). Other bacterial genera previously detected as attached to LDPE plastics in river water include *Pseudorhodobacter*, *Porphyrobacter* (Martínez-Campos et al., 2022). Regardi ng eukaryotes, the presence of different types of plants such as *Marsiela* or *Pinophyta* could result in the input of organic matter and compounds such as cellulose on the plastics, which can be used by certain bacteria such as *Lacihabitans* (Szabó et al., 2021). *Daptonema* showed a tendency to colonize artificial surfaces after a few days in a water column. (Fonsêca-Genevois et al., 2006). *Nematostelium* was reported to develop in aquatic biofilms, feeding on bacteria attached to the biofilm (Lindley et al., 2007).

In the PET bottle, some of the bacteria found in the associated microbiome core had been previously described as part of the community associated with the plastisphere in aquatic environments such as *Streptococcus* (Oberbeckmann et al., 2014), *Ferrovibrio* (Zhu et al., 2022), *Hymenobacter* (Martínez-Campos et al., 2022) and *Hyphomonas* (Zettler et al., 2013). *Pseudomonas*, found in the core microbiome of the PET bottle at site 2 after 3 months of incubation is widely known for its ability to produce exopolymeric substances that aid in the formation of biofilms (Chien et al., 2013). In addition, this genus has a high metabolic capacity, which enables it to degrade highly complex substances such as plastics, like PET (Vague et al., 2019). *Roseomonas*, which is significantly abundant at different incubation times, is known to have members that are opportunistic pathogens for humans (Rihs et al., 1993). As far as eukaryotic core biome is concerned, a significant abundance of *Aphanochaete* has already been reported in other types of plastics in aquatic environments (Chia et al., 2020). Several species of the genus *Rhizoclonium* have shown a tendency to colonize artificial substrates such as glass rather than natural substrates (Danilov and Ekelund, 2001).

In the PS dish core microbiome, the genus *Pirellula* was found previously colonizing PS in different ecosystems (Purohit et al., 2020). Other associated genera identified in the PS dish which have been found in the plastisphere in previous studies were *Pleurocapsa* (Rogers et al., 2020), *Sphingorhabdus* (Di Pippo et al., 2020) and *Hymenobacter* (Martínez-Campos et al., 2022). Also noteworthy is the presence of the genus *Rhodopirellula*, a genus with the ability to degrade hydrocarbons (de Araujo et al., 2021). *Rickettsia* is known to cause waterborne infectious diseases (Walker et al., 2003). The genus *Staphylococcus* could resist various antibiotics such as β- lactams. (Fuda et al., 2005). Among eukaryotic biome taxa in PS dish, the genus *Ploimidia* appeared attached to plastic litter in different aquatic ecosystems (Kettner et al., 2019). *Cryptosporidium* is a parasite that requires removal from drinking water, so its attachment to plastic could pose a risk to human health (Gómez-Couso et al., 2010). The genus *Candida* is characterized as a potential multi-antibiotic resistant pathogen (Spivak et al., 2022) and some species of this genus also have the potential to degrade polymeric substances (Zahari et al., 2021).

The most abundant bacterial genera in PVC pipe included *Fluviicola* and *Chthoniobacter*, previously described as plastic colonizers (Cappello et al., 2021; Rummel et al., 2021). *Sphingomonas,* which is already present during the first month of colonization, is characterized as a pioneer species in biofilm formation (Bereschenko et al., 2010). This genus has been reported as a dominant colonizer on PVC surfaces since it could participate in the degradation of PVC (Z. Wang et al., 2021; Wright et al., 2021a). The genus *Bryobacter*, also very abundant, has been reported as having members which are multi-resistant bacteria to several antibiotics in wastewater (Zhao et al., 2021). Regarding the eukaryotic biome core, only the genus *Radix* has been detected associated to the plastisphere; some members of this genus prefer to attach to plastics in comparison with other natural substrates (Vosshage et al., 2018).

The scientific community is worried about the increase of antibiotic-resistant bacteria (ARBs) and subsequent implications for human health. Plastics may have an important role in this problem because plastics can function as a reservoir of ARBs and cognate ARGs in marine ecosystems (Liu et al., 2021; Moore et al., 2020; Yang et al., 2019). WWTPs are considered to be one of the major hotspots for ARGs and microplastics which may favour their interaction (Syranidou and Kalogerakis, 2022). Martínez-Campos et al. (2021) showed an enrichment of microorganisms carrying the *sul1* gene in different types of plastics after 48 hours of incubation in the effluent of a WWTP. Yang et al. (2020) studied the temporal

dynamics of 64 antibiotic resistance genes over one month in urban waters showing an increase in ARGs over time.

Our study has found a higher concentration of ARGs in the plastics colonized at sampling site 2, downstream of the WWTP, than at site 1 located in a natural area. In spite of this, the surrounding water shows the highest relative abundance of all tested ARGs in both sampling sites. There is one exception with the gene *ermF* which is more abundant in plastic than in water after 6 months of incubation. Wang et al. (2020) found similar results regarding this gene in different environments (river and estuary) suggesting the possibility that the integrase gene, *intI1*, could play an important role in the transmission of the *ermF* gene from bacteria in the surrounding water to bacteria attached to plastics which would explain its increasing abundance over time on plastics.

In this context, some of the bacterial taxa found in the plastisphere in the present study have been found to carry ARGs such as the Burkholderiaceae family which is a primary carrier of ARGs in situations of high antibiotic concentrations (Cao et al., 2021), such as those occurring in site 2. The genus *Acinetobacter*, which is part of the core microbiome detected in the PET bottle after 6 months of colonization in site 2, is responsible for the persistence of macrolide resistance ARGs in WWTP effluents, which would also explain the higher relative abundance of *ermF* in this sites and colonization phase (April et al., 2022).

Our results reveal that the concentration of antibiotics in the environment is a factor to be considered since there is a positive correlation between it and the presence of ARGs on plastics. This correlation is stronger for ARGs on plastics than for the other substrates analysed in this study, especially in the case of both *sul1* and *dfrA* genes. Therefore, site 2, located downstream of a WWTP, indicates that the antibiotics released by the WWTP may facilitate the selection of ARBs on the plastisphere of near-by plastics and these could, therefore, function as a reservoir for ARGs. On the contrary, site 1, which is characterized by almost undetectable antibiotic concentrations, does not show this correlation between ARG and the concentration of antibiotics in any substrate. The correlation between antibiotics and bacteria-associated ARGs had been previously analysed in freshwater environments (Luo et al., 2010). Our findings are in line with the results obtained by Wang et al., (2020), who proposed that the concentration of ARGs on the surface of microplastics increased through the interaction with the surrounding environment.

5. Conclusions

This study addresses for the first time the long-time colonization (up to one year) of four different types of commonly used plastics deployed in two sampling sites in the same

river with different anthropogenic impact. Three main factors (sampling site, type of substrate and colonization time) explained most of the variation in the microbial communities, thus these factors were relevant in shaping the plastisphere.

The LEfSe analyses allowed to identify core microbiomes/biomes at the genus level along time, three stages regarding time-course evolution of the plastisphere could be identified as early or initial (1 month of incubation), intermediate (3 months) and late colonizers (6-12 months). Some of the identified taxa attached to the plastics could be potential pathogens and pose a risk to human health and the environment. Others could be potential plastic degraders. Different types of higher organisms were also identified which could use the plastics for shelter and be transported to other habitats in drifting plastics. The presence of certain bacteria and eukaryotes could suggest the possibility of complex interactions, such as food webs or the involvement of plastics in biogeochemical cycles.

The concentration of antibiotics in the surrounding water was a crucial factor in the ability of plastics to be reservoirs of antibiotic resistance genes (ARGs). Positive correlations were observed between the concentration of each type of antibiotic and cognate ARGs on plastics, this emphasizes a potential role of plastic in the spreading of antibiotic resistance.

6. References

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7. SUPPLEMENTARY MATERIAL 1

CONTENTS:

Table S15. Global and multiple comparisons of $2^{-\Delta ct}$ values for the *sul1* gene using Student-Newman-Keuls test.

Table S16. Global and multiple comparisons of $2^{-\Delta ct}$ values for the *ermF* gene using Student-Newman-Keuls test.

Table S17. Global and multiple comparisons of $2^{-\Delta ct}$ values for the *dfrA* gene using Student-Newman-Keuls test.

Table S18. Global and multiple comparisons of $2^{-\Delta ct}$ values for the *qnrSrtF11A* gene using Student-Newman-Keuls test.

Table S19. Spearman correlations between the $2^{-\Delta ct}$ values of each ARG in each substrate and the antibiotic concentration measured in water at the two sampling sites after 1 year of the incubation experiment. The antibiotic concentrations were those measured in the water (values reported in **Table S6)**.

Figure S1. Map with sampling stations defined as site 1 and site 2.

Figure S2. Details of the colonization process a) Virgin substrates before the colonization experiment, b) Distribution of the different substrates in the metal cage, c) Deployment of metal cages with substrates inside the river, d) State of the substrates after 1 month of colonization.

Figure S3. Evolution of the state of substrates during the colonization experiment.

Figure S4. SEM images showing microbial colonization onto the different substrates, sites, and times.

Figure S5. Rarefaction curve that compares the observed features (corresponding to ASVs in previous version of QIIME2) in comparison with number of reads for each sample (sequencing depth) in the 16S rRNA gene.

Figure S6. Rarefaction curve that compares the observed features (corresponding to ASVs in previous version of QIIME2) in comparison with number of reads for each sample (sequencing depth) in the 18S rRNA gene.

Figure S7. UPGMA dendrogram obtained from 16s rRNA cluster analysis of samples, using the Bray-Curtis distance measure.

Figure S8. UPGMA dendrogram obtained from 18s rRNA cluster analysis of samples, using the Bray-Curtis distance measure.

Table S1
Characterization of the different used substrates

Name	Material	Size	Company	Manufacturer
Plastic bag	Low-density polyethylene	32 cm × 44 cm	Mercadona	Plasbel (Valencia, Spain)
Plastic bottle	Polyethylene terephthalate	9 cm × 33.5 cm × 9 cm	Aguadoy	Fuente Arevalillo S.L. (Toledo, Spain)
Plastic dish	Polystyrene	21 cm × 21 cm × 0.5 cm	Bosque Verde	SP BERNER PLASIC GROUP S.L. (Valencia, Spain)
Plastic pipe	Polyvinyl chloride	Diameter: 5 cm × Length: 8 cm (thickness: 3.5 mm)	Leroy Merlin	Leroy Merlin
Rock	Limestone	6/10 cm × 6/10 cm	Leroy Merlin	Deocantera Sl. (Barcelona, Spain)
Borosilicate slides	Borosilicate glass	7.6 cm × 2.6 cm × 0.1 cm	Fisher Scientific	Fisher Scientific

Table S2
Primers for 16S rRNA and 18S rRNA Illumina sequencing. The amplified region and the sequences of the primers are indicated. The primer tail is shown in bold

Gene	Primers	Sequence
16S rRNA	CS1-341F	**ACACTGACGACATGGTTCTACA**CCTACGGGNGGCWGCAG
	CS2-805R	**TACGGTAGCAGAGACTTGGTCT**GACTACHVGGGTATCTAATCC
18S rRNA	563f-CS1	**ACACTGACGACATGGTTCTACA**GCCAGCAVCYGCGGTAAY
	1132R-CS2	**TACGGTAGCAGAGACTTGGTCT**CCGTCAATTHCTTYAART

Table S3
qPCR primers for specific detection and quantification of ARGs

Gene identification	Antibiotic group	Forward Sequence	Reverse Sequence
ermF	Macrolides	CAGCTTTGGTTGAACATTTACGAA	AAATTCCTAAAATCACAACCGACAA
sul1	Sulphonamides	GCCGATGAGATCAGACGTATTG	CGCATAGCGCTGGGTTTC
dfrA1	Trimethoprim	GGAATGGCCCTGATATTCCA	AGTCTTGCGTCCAACCAACAG
qnrSrtF11a	Quinolones	GACGTGCTAACTTGCGTGAT	TGGCATTGTTGGAAACTTG
16S rRNA	Housekeeping gene	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG

Table S4.
Physicochemical parameters measured in the sampling sites

Site	Date	Month	pH	DO[a] (sat %)	DO[a] (mg/L)	Conductivity (μS/cm)	Temperature (◦C)	Water Flow (m/s)	Water Depth (cm)
Site 1	22/05/2018	0	8.76	84.5	7.75	1480	15.5	0.41	40
	20/06/2018	1	8.18	98.5	8.81	1269	16.8	0.82	59
	04/09/2018	3	8.05	83.2	7.28	1008	17.7	0.40	42
	21/11/2018	6	8.28	98.0	9.91	1468	10.4	0.46	46
	21/05/2019	12	8.22	100.7	10.46	1357	12.5	0.33	36
Site 2	22/05/2018	0	8.57	73.9	6.16	1110	21.2	0.45	67
	20/06/2018	1	7.71	92.0	7.68	573	21.6	0.43	92
	04/09/2018	3	7.72	78.5	6.04	926	24.5	0.21	76
	21/11/2018	6	8.28	85.7	8.20	1135	7.6	0.21	88
	21/05/2019	12	7.10	86.6	7.54	1255	18.4	0.10	66

[a] DO: dissolved oxygen

Table S5
Nutrients and organic matter in the sampling sites

Site	Date	Month	$N(NH_4^+)$ (mg/L)	$N(NO_3^-)$ (mg/L)	TKN[a] ($NO_3^- + NH_4^+$) (mg/L)	PO_4^{3-} (mg/L)	Total phosphorus (P) (mg/L)	TOC[b] (mg/L)
Site 1	22/05/2018	0	0.17	3.87	4.04	< 0.001	< 0.001	3.41
	20/06/2018	1	0.01	2.78	2.79	0.007	0.002	2.53
	04/09/2018	3	0.01	3.06	3.07	0.015	0.005	2.54
	21/11/2018	6	0.01	2.88	2.88	< 0.001	< 0.001	2.14
	21/05/2019	12	0.02	2.01	2.03	0.024	0.008	3.87
Site 2	22/05/2018	0	1.45	5.05	6.50	0.623	0.203	7.08
	20/06/2018	1	0.46	4.55	5.01	0.494	0.161	7.87
	04/09/2018	3	0.25	4.62	4.87	0.402	0.131	6.93
	21/11/2018	6	0.32	3.79	4.11	0.371	0.121	4.89
	21/05/2019	12	0.67	3.92	4.59	0.641	0.209	7.90

[a] TKN is the Total Kjeldahl Nitrogen

[b] TOC is the Total Organic Carbon

Table S6.
Concentration of the antibiotics detected in the sampling sites (concentration in ng/L)

Site	Site 1					Site 2				
Month	0	1	3	6	12	0	1	3	6	12
Amoxicillin	3.40	2.60	1.60	1.90	5.70	9.70	3.10	3.20	11.70	13.90
Azithromycin	< 0.25[a]	< 0.25[a]	< 0.25[a]	< 0.25[a]	< 0.25[a]	6172.00	885.00	231.00	293.00	7282.00
Ciprofloxacin	< 5.00[a]	< 5.00[a]	< 5.00[a]	< 5.00[a]	< 5.00[a]	191.00	255.00	173.00	190.00	286.00
Clarithromycin	2.00	0.40	1.20	1.90	1.50	237.00	462.00	393.00	98.00	832.00
Erythromycin	0.60	0.40	0.80	0.20	1.30	67.10	177.00	50.80	48.90	72.90
Lincomycin	5.60	0.90	2.10	3.70	6.00	8.20	1.50	16.60	9.10	1.20
Metronidazole	3.20	2.10	0.40	0.50	211.00	66.00	85.60	47.70	191.00	66.30
Sulfamethoxazole	0.54	< 0.05	0.48	0.66	35.20	231.00	65.70	75.40	267.00	130.00
Ofloxacin	< 5.00[a]	< 5.00[a]	< 5.00[a]	< 5.00[a]	< 5.00[a]	1046.00	924.00	1326.00	146.00	299.00
Trimethoprim	1.48	0.48	0.73	0.53	4.94	489.00	129.00	50.20	255.00	73.30

[a] Below quantification limit

Table S7.
One-way ANOVA test for physicochemical parameters, concentrations of nutrients, organic matter and antibiotics between both sampling sites

Parameters	ANOVA test	
	F	***p*-value**
pH	2.234	0.17
DO	3.826	0.09
Conductivity	4.642	0.06
Temperature	1.614	0.24
Water Flow	3.495	0.10
Water Depth	25.160	< 0.05
$N(NH_4^+)$	7.129	< 0.05
$N(NO_3^-)$	14.996	< 0.05
TKN	15.907	< 0.05
PO_4^{3-}	80.212	< 0.05
Total phosphorus	80.212	< 0.05
TOC	40.452	< 0.05
Amoxicillin	5.161	0.05
Azithromycin	3.692	< 0.05
Ciprofloxacin	96.140	< 0.05
Clarithromycin	10.539	< 0.05
Erythromycin	12.000	< 0.05
Lincomycin	1.493	0.25
Metronidazole	0.950	0.36
Sulfamethoxazole	12.534	< 0.05
Ofloxacin	10.853	< 0.05
Trimethoprim	6.008	< 0.05

Table S8.
Kruskal-Wallis test based on Shannon index for 16S rRNA samples. Colonization times are indicated by the following abbreviations: T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization).

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Global	-----			166.74	< 0.05
	Site	---	---	88.89	< 0.05
	---	Time	---	12.44	< 0.05
	---	---	Substrate	8.41	< 0.05
Pairwise	Site 1	T1	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	1.19	0.275
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 2		Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T1	Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 1	T3	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 1	T3	Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 2		Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	0.05	0.8272
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 1	T6	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 1	T6	Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 2		Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	0.43	0.5126
			Bag - Water	0.43	0.5126
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T6	Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	1.19	0.275
	Site 1	T12	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 2		Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T12	Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 1	T1 - T3	Bag	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Bottle	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Dish	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 1	T1 - T3	Pipe	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Glass	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		0.43	0.512
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Rock	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		1.19	0.275
	Site 1	T1 - T3	Water	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 2	T1 - T3	Bag	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T1 - T3	Bottle	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 2	T1 - T3	Dish	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 2	T1 - T3	Pipe	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 2	T1 - T3	Glass	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		1.19	0.275
	Site 2	T1 - T3	Rock	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T1 - T3	Water	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1 - Site 2	T1	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	3.86	< 0.05
			Glass	3.86	< 0.05
			Rock	3.86	< 0.05
			Water	3.86	< 0.05
		T3	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	3.86	< 0.05
			Glass	3.86	< 0.05
			Rock	3.86	< 0.05
			Water	3.86	< 0.05
		T6	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	3.86	< 0.05
			Glass	3.86	< 0.05
			Rock	3.86	< 0.05
			Water	3.86	< 0.05
		T12	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	3.86	< 0.05
			Glass	3.86	< 0.05
			Rock	3.86	< 0.05
			Water	3.86	< 0.05

Table S9.
Kruskal-Wallis test based on Shannon index for 18S rRNA samples. colonization times are indicated by the following abbreviations: T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization).

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Global	-----			157.77	< 0.05
	Site	---	---	45.32	< 0.05
	---	Time	---	10.75	< 0.05
	---	---	Substrate	35.35	< 0.05
Pairwise	Site 1	T1	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	-----	-----
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	-----	-----
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	-----	-----
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	-----	-----
			Pipe - Glass	-----	-----
			Pipe - Water	-----	-----
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 2		Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	0.05	0.8272
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T1	Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 1	T3	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 1	T3	Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 2		Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	0.43	0.5126
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 1	T6	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	-----	-----

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 1	T6	Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	-----	-----
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	-----	-----
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	-----	-----
			Rock - Glass	3.86	< 0.05
			Rock - Water	-----	-----
			Glass - Water	-----	-----
	Site 2		Bag - Bottle	2.33	0.1266
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	-----	-----
			Bag - Water	3.86	< 0.05
			Bottle - Dish	0.43	0.5126
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	-----	-----
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	-----	-----
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	-----	-----
			Pipe - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T6	Rock - Glass	-----	-----
			Rock - Water	3.86	< 0.05
			Glass - Water	-----	-----
	Site 1	T12	Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05
			Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 2		Bag - Bottle	3.86	< 0.05
			Bag - Dish	3.86	< 0.05
			Bag - Pipe	3.86	< 0.05
			Bag - Rock	3.86	< 0.05
			Bag - Glass	3.86	< 0.05
			Bag - Water	3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T12	Bottle - Dish	3.86	< 0.05
			Bottle - Pipe	3.86	< 0.05
			Bottle - Rock	3.86	< 0.05
			Bottle - Glass	3.86	< 0.05
			Bottle - Water	3.86	< 0.05
			Dish - Pipe	3.86	< 0.05
			Dish - Rock	3.86	< 0.05
			Dish - Glass	3.86	< 0.05
			Dish - Water	3.86	< 0.05
			Pipe - Rock	3.86	< 0.05
			Pipe - Glass	3.86	< 0.05
			Pipe - Water	3.86	< 0.05
			Rock - Glass	3.86	< 0.05
			Rock - Water	3.86	< 0.05
			Glass - Water	3.86	< 0.05
	Site 1	T1 - T3	Bag	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Bottle	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Dish	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		1.19	0.2752
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 1	T1 - T3	Pipe	-----	-----
		T1 - T6		-----	-----
		T1 - T12		-----	-----
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Glass	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Rock	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1	T1 - T3	Water	3.86	< 0.05
		T1 - T6		-----	-----
		T1 - T12		3.86	< 0.05
		T3 - T6		-----	-----
		T3 - T12		3.86	< 0.05
		T6 - T12		-----	-----
	Site 2	T1 - T3	Bag	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T1 - T3	Bottle	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 2	T1 - T3	Dish	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		2.33	0.1266
		T6 - T12		3.86	< 0.05
	Site 2	T1 - T3	Pipe	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		3.86	< 0.05
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 2	T1 - T3	Glass	3.86	< 0.05
		T1 - T6		-----	-----
		T1 - T12		3.86	< 0.05
		T3 - T6		-----	-----
		T3 - T12		3.86	< 0.05
		T6 - T12		-----	-----
	Site 2	T1 - T3	Rock	3.86	< 0.05
		T1 - T6		2.33	0.1266
		T1 - T12		0.43	0.5126
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		1.19	0.275

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	Shannon Index	
				H	*p*-value
Pairwise	Site 2	T1 - T3	Water	3.86	< 0.05
		T1 - T6		3.86	< 0.05
		T1 - T12		0.43	0.512
		T3 - T6		3.86	< 0.05
		T3 - T12		3.86	< 0.05
		T6 - T12		3.86	< 0.05
	Site 1 - Site 2	T1	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	-----	-----
			Glass	3.86	< 0.05
			Rock	3.86	< 0.05
			Water	3.86	< 0.05
		T3	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	3.86	< 0.05
			Glass	3.86	< 0.05
			Rock	3.86	< 0.05
			Water	3.86	< 0.05
		T6	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	3.86	< 0.05
			Glass	-----	-----
			Rock	3.86	< 0.05
			Water	-----	-----
		T12	Bag	3.86	< 0.05
			Bottle	3.86	< 0.05
			Dish	3.86	< 0.05
			Pipe	3.86	< 0.05
			Glass	3.86	< 0.05
			Rock	3.86	< 0.05
			Water	3.86	< 0.05

Table S10.
Global and pairwise PERMANOVA analysis based on Bray-Curtis distance matrix for prokaryotes. Colonization times are indicated by the following abbreviations: T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization).

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Global	-----			528.87	< 0.05
	Site	---	---	54.28	< 0.05
	---	Time	---	8.13	< 0.05
	---	---	Substrate	4.88	< 0.05
Pairwise	Site 1	T1	Bag - Bottle	33.44	0.0988
			Bag - Dish	25.79	0.0981
			Bag - Pipe	79.36	0.1025
			Bag - Rock	59.18	0.0974
			Bag - Glass	93.67	0.0993
			Bag - Water	142.87	0.1005
			Bottle - Dish	32.42	0.1002
			Bottle - Pipe	50.64	0.1003
			Bottle - Rock	40.81	0.0948
			Bottle - Glass	74.15	0.1004
			Bottle - Water	121.75	0.0973
			Dish - Pipe	70.86	0.1037
			Dish - Rock	61.78	0.0979
			Dish - Glass	112.79	0.1024
			Dish - Water	142.71	0.1025
			Pipe - Rock	53.48	0.1031
			Pipe - Glass	88.88	0.0962
			Pipe - Water	115.40	0.1036
			Rock - Glass	100.14	0.1046
			Rock - Water	113.17	0.0964
			Glass - Water	134.92	0.0987
	Site 2		Bag - Bottle	23.50	0.1021
			Bag - Dish	13.68	0.1014
			Bag - Pipe	17.42	0.1053
			Bag - Rock	22.76	0.1025
			Bag - Glass	13.78	0.1016
			Bag - Water	86.82	0.0964

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T1	Bottle - Dish	13.38	0.0993
			Bottle - Pipe	10.22	0.0988
			Bottle - Rock	10.37	0.0999
			Bottle - Glass	21.71	0.1023
			Bottle - Water	40.98	0.1032
			Dish - Pipe	13.30	0.1015
			Dish - Rock	11.22	0.1041
			Dish - Glass	12.39	0.0976
			Dish - Water	51.60	0.0977
			Pipe - Rock	13.09	0.0976
			Pipe - Glass	16.23	0.0996
			Pipe - Water	47.24	0.0980
			Rock - Glass	19.26	0.0996
			Rock - Water	42.08	0.1046
			Glass - Water	66.90	0.0981
	Site 1	T3	Bag - Bottle	29.85	0.0994
			Bag - Dish	18.95	0.1019
			Bag - Pipe	14.55	0.1010
			Bag - Rock	21.79	0.0993
			Bag - Glass	30.55	0.0980
			Bag - Water	165.46	0.0965
			Bottle - Dish	22.48	0.0968
			Bottle - Pipe	40.59	0.1012
			Bottle - Rock	40.74	0.0941
			Bottle - Glass	33.70	0.0972
			Bottle - Water	192.35	0.0952
			Dish - Pipe	26.36	0.0978
			Dish - Rock	40.85	0.0969
			Dish - Glass	25.35	0.1019
			Dish - Water	217.05	0.0987
			Pipe - Rock	24.21	0.0976
			Pipe - Glass	37.55	0.1011
			Pipe - Water	187.64	0.0974

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 1	T3	Rock - Glass	43.67	0.0967
			Rock - Water	96.99	0.0995
			Glass - Water	254.30	0.0962
	Site 2		Bag - Bottle	14.06	0.0965
			Bag - Dish	10.42	0.0978
			Bag - Pipe	9.29	0.0996
			Bag - Rock	10.78	0.1006
			Bag - Glass	19.44	0.1023
			Bag - Water	23.20	0.0974
			Bottle - Dish	14.97	0.0983
			Bottle - Pipe	9.91	0.0959
			Bottle - Rock	10.12	0.0971
			Bottle - Glass	16.51	0.1041
			Bottle - Water	23.97	0.0952
			Dish - Pipe	14.89	0.0997
			Dish - Rock	15.75	0.1028
			Dish - Glass	25.41	0.0974
			Dish - Water	35.07	0.1038
			Pipe - Rock	4.41	0.1001
			Pipe - Glass	15.70	0.1069
			Pipe - Water	21.52	0.1018
			Rock - Glass	16.73	0.0983
			Rock - Water	22.53	0.0998
			Glass - Water	36.73	0.1011
	Site 1	T6	Bag - Bottle	43.23	0.1002
			Bag - Dish	53.59	0.0999
			Bag - Pipe	54.81	0.1010
			Bag - Rock	29.24	0.1042
			Bag - Glass	52.15	0.1056
			Bag - Water	325.56	0.1089

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 1	T6	Bottle - Dish	33.70	0.0987
			Bottle - Pipe	25.84	0.1036
			Bottle - Rock	27.46	0.0995
			Bottle - Glass	22.08	0.0939
			Bottle - Water	207.72	0.0967
			Dish - Pipe	25.83	0.0984
			Dish - Rock	45.20	0.097
			Dish - Glass	27.59	0.0989
			Dish - Water	391.55	0.1032
			Pipe - Rock	39.55	0.0996
			Pipe - Glass	25.27	0.1025
			Pipe - Water	194.44	0.1003
			Rock - Glass	41.15	0.1024
			Rock - Water	137.16	0.0938
			Glass - Water	346.65	0.1010
	Site 2		Bag - Bottle	11.50	0.1035
			Bag - Dish	9.40	0.1030
			Bag - Pipe	12.65	0.0983
			Bag - Rock	9.33	0.0984
			Bag - Glass	11.76	0.0966
			Bag - Water	61.23	0.0991
			Bottle - Dish	10.87	0.1011
			Bottle - Pipe	10.43	0.1030
			Bottle - Rock	10.39	0.0962
			Bottle - Glass	12.35	0.1024
			Bottle - Water	40.20	0.1012
			Dish - Pipe	6.01	0.1053
			Dish - Rock	88.61	0.0969
			Dish - Glass	11.12	0.0965
			Dish - Water	225.56	0.0998
			Pipe - Rock	9.15	0.0995
			Pipe - Glass	13.17	0.1045
			Pipe - Water	29.98	0.0978

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T6	Rock - Glass	11.85	0.1066
			Rock - Water	50.14	0.0982
			Glass - Water	61.75	0.1016
	Site 1	T12	Bag - Bottle	25.86	0.0980
			Bag - Dish	23.81	0.0957
			Bag - Pipe	32.68	0.0990
			Bag - Rock	23.00	0.0971
			Bag - Glass	17.94	0.0985
			Bag - Water	83.11	0.1013
			Bottle - Dish	35.20	0.0984
			Bottle - Pipe	52.13	0.0995
			Bottle - Rock	38.19	0.1013
			Bottle - Glass	43.90	0.1003
			Bottle - Water	62.79	0.1029
			Dish - Pipe	89.97	0.1017
			Dish - Rock	58.27	0.1001
			Dish - Glass	43.27	0.0972
			Dish - Water	108.06	0.1008
			Pipe - Rock	42.78	0.0986
			Pipe - Glass	54.88	0.0992
			Pipe - Water	203.71	0.0951
			Rock - Glass	43.65	0.1011
			Rock - Water	100.07	0.0966
			Glass - Water	123.65	0.1013
	Site 2		Bag - Bottle	19.78	0.1009
			Bag - Dish	15.86	0.0988
			Bag - Pipe	17.59	0.0956
			Bag - Rock	16.47	0.0988
			Bag - Glass	104.70	0.1034
			Bag - Water	52.86	0.0995

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T12	Bottle - Dish	7.96	0.0970
			Bottle - Pipe	18.62	0.1018
			Bottle - Rock	11.90	0.0990
			Bottle - Glass	39.53	0.1044
			Bottle - Water	70.73	0.0944
			Dish - Pipe	15.90	0.0983
			Dish - Rock	8.80	0.1054
			Dish - Glass	28.48	0.0995
			Dish - Water	62.52	0.0999
			Pipe - Rock	20.30	0.0965
			Pipe - Glass	44.61	0.0996
			Pipe - Water	89.70	0.1029
			Rock - Glass	126.77	0.1016
			Rock - Water	66.31	0.0997
			Glass - Water	48.80	0.0977
	Site 1	T1 - T3	Bag	116.66	0.1004
		T1 - T6		291.26	0.0985
		T1 - T12		80.41	0.0972
		T3 - T6		58.75	0.0998
		T3 - T12		34.45	0.0978
		T6 - T12		48.51	0.0996
	Site 1	T1 - T3	Bottle	74.34	0.1011
		T1 - T6		104.76	0.0990
		T1 - T12		62.70	0.0967
		T3 - T6		55.30	0.0999
		T3 - T12		41.44	0.0983
		T6 - T12		39.75	0.1011
	Site 1	T1 - T3	Dish	130.14	0.1039
		T1 - T6		273.79	0.1006
		T1 - T12		148.57	0.1031
		T3 - T6		64.55	0.1011
		T3 - T12		92.31	0.0981
		T6 - T12		120.96	0.0960

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 1	T1 - T3	Pipe	77.06	0.0968
		T1 - T6		108.80	0.1002
		T1 - T12		122.08	0.0950
		T3 - T6		46.59	0.0969
		T3 - T12		50.52	0.0942
		T6 - T12		38.53	0.0973
	Site 1	T1 - T3	Glass	209.60	0.1033
		T1 - T6		237.61	0.0989
		T1 - T12		162.30	0.1043
		T3 - T6		54.76	0.1014
		T3 - T12		83.24	0.1013
		T6 - T12		61.25	0.1005
	Site 1	T1 - T3	Rock	64.76	0.1009
		T1 - T6		83.10	0.0983
		T1 - T12		93.20	0.0984
		T3 - T6		44.15	0.0970
		T3 - T12		24.81	0.1026
		T6 - T12		27.36	0.0971
	Site 1	T1 - T3	Water	47.82	0.1014
		T1 - T6		54.40	0.0975
		T1 - T12		36.22	0.1006
		T3 - T6		126.32	0.1010
		T3 - T12		53.89	0.1011
		T6 - T12		68.84	0.1061
	Site 2	T1 - T3	Bag	35.93	0.1019
		T1 - T6		53.89	0.0969
		T1 - T12		40.32	0.1016
		T3 - T6		35.05	0.1057
		T3 - T12		21.73	0.0998
		T6 - T12		38.18	0.1064

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T1 - T3	Bottle	22.22	0.0973
		T1 - T6		115.43	0.0970
		T1 - T12		19.76	0.0968
		T3 - T6		79.70	0.1007
		T3 - T12		13.84	0.0977
		T6 - T12		53.44	0.1020
	Site 2	T1 - T3	Dish	35.07	0.0932
		T1 - T6		33.00	0.1006
		T1 - T12		28.73	0.1065
		T3 - T6		35.07	0.0993
		T3 - T12		21.98	0.0991
		T6 - T12		25.82	0.1032
	Site 2	T1 - T3	Pipe	17.95	0.1012
		T1 - T6		25.37	0.1016
		T1 - T12		29.49	0.1017
		T3 - T6		22.81	0.0963
		T3 - T12		27.91	0.0948
		T6 - T12		35.11	0.0968
	Site 2	T1 - T3	Glass	35.82	0.1015
		T1 - T6		46.81	0.0992
		T1 - T12		54.92	0.0980
		T3 - T6		31.89	0.1003
		T3 - T12		46.59	0.0971
		T6 - T12		52.31	0.1000
	Site 2	T1 - T3	Rock	19.48	0.0961
		T1 - T6		25.58	0.0980
		T1 - T12		22.61	0.1003
		T3 - T6		23.84	0.0994
		T3 - T12		17.80	0.0990
		T6 - T12		24.62	0.0954

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T1 - T3	Water	64.22	0.0997
		T1 - T6		54.07	0.1046
		T1 - T12		55.51	0.0993
		T3 - T6		68.14	0.1027
		T3 - T12		78.94	0.1033
		T6 - T12		65.69	0.1017
	Site 1 - Site 2	T1	Bag	72.66	0.0960
			Bottle	56.06	0.0990
			Dish	64.75	0.1032
			Pipe	56.88	0.0988
			Glass	82.86	0.1014
			Rock	53.13	0.1013
			Water	61.90	0.0997
		T3	Bag	58.35	0.1003
			Bottle	44.89	0.1030
			Dish	95.90	0.1020
			Pipe	51.64	0.0954
			Glass	76.79	0.0959
			Rock	39.55	0.0987
			Water	87.85	0.1003
		T6	Bag	117.70	0.0999
			Bottle	62.29	0.0988
			Dish	102.92	0.0964
			Pipe	63.95	0.0983
			Glass	116.53	0.1009
			Rock	55.71	0.0985
			Water	108.77	0.0976
		T12	Bag	58.67	0.0927
			Bottle	49.51	0.1020
			Dish	76.60	0.1016
			Pipe	139.09	0.1049
			Glass	172.68	0.0990
			Rock	73.09	0.0993
			Water	36.18	0.0987

Table S11.
Global and pairwise PERMANOVA analysis based on Bray-Curtis distance matrix for eukaryotes. colonization times are indicated by the following abbreviations: T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization).

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Global	-----			528.876	< 0.05
	Site	---	---	44.55	< 0.05
	---	Time	---	5.86	< 0.05
	---	---	Substrate	3.65	< 0.05
Pairwise	Site 1	T1	Bag - Bottle	546.35	0.1011
			Bag - Dish	987.93	0.0977
			Bag - Pipe	-----	-----
			Bag - Rock	707.91	0.0991
			Bag - Glass	347.85	0.1009
			Bag - Water	130.30	0.0994
			Bottle - Dish	962.67	0.1029
			Bottle - Pipe	-----	-----
			Bottle - Rock	888.02	0.0996
			Bottle - Glass	407.25	0.1038
			Bottle - Water	134.48	0.1027
			Dish - Pipe	-----	-----
			Dish - Rock	820.09	0.0975
			Dish - Glass	636.83	0.1042
			Dish - Water	135.42	0.1035
			Pipe - Rock	-----	-----
			Pipe - Glass	-----	-----
			Pipe - Water	-----	-----
			Rock - Glass	499.80	0.0968
			Rock - Water	122.32	0.1035
			Glass - Water	126.26	0.1013
	Site 2		Bag - Bottle	284.38	0.0950
			Bag - Dish	283.51	0.0964
			Bag - Pipe	292.25	0.1024
			Bag - Rock	388.06	0.0990
			Bag - Glass	366.34	0.1032
			Bag - Water	131.90	0.0949

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T1	Bottle - Dish	156.52	0.1028
			Bottle - Pipe	211.27	0.1029
			Bottle - Rock	237.75	0.1011
			Bottle - Glass	257.63	0.1012
			Bottle - Water	436.97	0.0978
			Dish - Pipe	188.16	0.1062
			Dish - Rock	391.07	0.1008
			Dish - Glass	373.89	0.0954
			Dish - Water	748.07	0.1010
			Pipe - Rock	155.23	0.1013
			Pipe - Glass	328.45	0.1026
			Pipe - Water	126.57	0.1003
			Rock - Glass	808.65	0.0972
			Rock - Water	808.65	0.0972
			Glass - Water	623.15	0.0984
	Site 1	T3	Bag - Bottle	737.81	0.0972
			Bag - Dish	1037.67	0.0986
			Bag - Pipe	1586.17	0.0963
			Bag - Rock	1056.22	0.0973
			Bag - Glass	1392.16	0.1070
			Bag - Water	634.02	0.0965
			Bottle - Dish	2320.39	0.1012
			Bottle - Pipe	2720.38	0.0940
			Bottle - Rock	599.74	0.1042
			Bottle - Glass	1715.55	0.0997
			Bottle - Water	786.35	0.1037
			Dish - Pipe	1165.66	0.1001
			Dish - Rock	4404.42	0.0995
			Dish - Glass	1413.97	0.0947
			Dish - Water	756.99	0.0963
			Pipe - Rock	2637.47	0.0969
			Pipe - Glass	926.36	0.0969
			Pipe - Water	734.51	0.0954

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 1	T3	Rock - Glass	3376.17	0.1000
			Rock - Water	748.39	0.0974
			Glass - Water	721.10	0.1022
	Site 2		Bag - Bottle	1682.85	0.0969
			Bag - Dish	1095.85	0.0988
			Bag - Pipe	397.35	0.0963
			Bag - Rock	736.55	0.0957
			Bag - Glass	267.02	0.1008
			Bag - Water	729.79	0.1060
			Bottle - Dish	488.00	0.1017
			Bottle - Pipe	722.99	0.1020
			Bottle - Rock	304.93	0.0963
			Bottle - Glass	312.06	0.1019
			Bottle - Water	321.55	0.0977
			Dish - Pipe	729.84	0.0984
			Dish - Rock	437.45	0.1036
			Dish - Glass	382.40	0.0987
			Dish - Water	431.59	0.1011
			Pipe - Rock	229.18	0.1006
			Pipe - Glass	386.83	0.1021
			Pipe - Water	447.34	0.0971
			Rock - Glass	195.31	0.1007
			Rock - Water	244.09	0.1023
			Glass - Water	119.69	0.0955
	Site 1	T6	Bag - Bottle	448.60	0.1014
			Bag - Dish	458.41	0.1025
			Bag - Pipe	643.71	0.1003
			Bag - Rock	1179.35	0.1006
			Bag - Glass	323.23	0.0980
			Bag - Water	-----	-----

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 1	T6	Bottle - Dish	572.33	0.0972
			Bottle - Pipe	1643.98	0.0957
			Bottle - Rock	2044.43	0.0973
			Bottle - Glass	970.16	0.0984
			Bottle - Water	-----	-----
			Dish - Pipe	1642.17	0.0983
			Dish - Rock	874.12	0.0978
			Dish - Glass	1207.55	0.1018
			Dish - Water	-----	-----
			Pipe - Rock	2159.61	0.0967
			Pipe - Glass	1148.43	0.0991
			Pipe - Water	-----	-----
			Rock - Glass	2737.82	0.1061
			Rock - Water	-----	-----
			Glass - Water	-----	-----
	Site 2		Bag - Bottle	86.45	0.1066
			Bag - Dish	174.31	0.1019
			Bag - Pipe	151.68	0.1035
			Bag - Rock	137.28	0.1024
			Bag - Glass	-----	-----
			Bag - Water	172.21	0.0995
			Bottle - Dish	237.08	0.0994
			Bottle - Pipe	142.00	0.0955
			Bottle - Rock	184.33	0.1009
			Bottle - Glass	-----	-----
			Bottle - Water	233.19	0.1061
			Dish - Pipe	162.80	0.0973
			Dish - Rock	203.02	0.1007
			Dish - Glass	-----	-----
			Dish - Water	282.89	0.0995
			Pipe - Rock	117.57	0.1042
			Pipe - Glass	-----	-----
			Pipe - Water	265.84	0.0940

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T6	Rock - Glass	-----	-----
			Rock - Water	278.43	0.1007
			Glass - Water	-----	-----
	Site 1	T12	Bag - Bottle	216.36	0.0948
			Bag - Dish	340.07	0.1005
			Bag - Pipe	413.67	0.1050
			Bag - Rock	578.77	0.1050
			Bag - Glass	560.00	0.0989
			Bag - Water	322.86	0.1027
			Bottle - Dish	189.84	0.1020
			Bottle - Pipe	217.31	0.1016
			Bottle - Rock	238.16	0.1063
			Bottle - Glass	957.49	0.0968
			Bottle - Water	179.45	0.0984
			Dish - Pipe	258.59	0.0978
			Dish - Rock	845.61	0.1021
			Dish - Glass	379.15	0.0980
			Dish - Water	307.81	0.1013
			Pipe - Rock	700.17	0.0983
			Pipe - Glass	358.16	0.0980
			Pipe - Water	341.87	0.0975
			Rock - Glass	668.49	0.0975
			Rock - Water	348.01	0.1006
			Glass - Water	315.21	0.1016
	Site 2		Bag - Bottle	440.40	0.1014
			Bag - Dish	835.65	0.1025
			Bag - Pipe	689.51	0.0964
			Bag - Rock	327.84	0.1034
			Bag - Glass	980.08	0.1008
			Bag - Water	678.06	0.1018

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T12	Bottle - Dish	304.41	0.1010
			Bottle - Pipe	669.41	0.1046
			Bottle - Rock	62.43	0.1013
			Bottle - Glass	322.56	0.0978
			Bottle - Water	436.09	0.1011
			Dish - Pipe	1774.95	0.0983
			Dish - Rock	168.90	0.0959
			Dish - Glass	692.88	0.0983
			Dish - Water	672.45	0.1073
			Pipe - Rock	422.71	0.0983
			Pipe - Glass	1027.90	0.0993
			Pipe - Water	832.99	0.0974
			Rock - Glass	277.82	0.0974
			Rock - Water	352.60	0.1047
			Glass - Water	541.86	0.0979
	Site 1	T1 - T3	Bag	2170.06	0.0967
		T1 - T6		486.53	0.0968
		T1 - T12		1142.91	0.0950
		T3 - T6		636.15	0.1046
		T3 - T12		531.05	0.1036
		T6 - T12		232.74	0.1050
	Site 1	T1 - T3	Bottle	2586.69	0.1009
		T1 - T6		1521.24	0.1047
		T1 - T12		261.98	0.1052
		T3 - T6		1953.88	0.0931
		T3 - T12		280.37	0.0960
		T6 - T12		263.07	0.0967
	Site 1	T1 - T3	Dish	2022.32	0.0986
		T1 - T6		2668.35	0.1053
		T1 - T12		993.68	0.1011
		T3 - T6		1591.80	0.0996
		T3 - T12		856.16	0.0981
		T6 - T12		974.52	0.0990

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 1	T1 - T3	Pipe	-----	-----
		T1 - T6		-----	-----
		T1 - T12		-----	-----
		T3 - T6		1734.36	0.1022
		T3 - T12		648.97	0.0974
		T6 - T12		1119.54	0.1025
	Site 1	T1 - T3	Glass	1311.14	0.1004
		T1 - T6		615.36	0.0947
		T1 - T12		891.80	0.1021
		T3 - T6		335.51	0.0994
		T3 - T12		329.58	0.1061
		T6 - T12		246.53	0.1028
	Site 1	T1 - T3	Rock	1619.64	0.0999
		T1 - T6		1834.06	0.0984
		T1 - T12		1063.55	0.1006
		T3 - T6		1014.90	0.1050
		T3 - T12		3020.33	0.0974
		T6 - T12		2211.54	0.1010
	Site 1	T1 - T3	Water	57.74	0.0972
		T1 - T6		-----	-----
		T1 - T12		47.30	0.0984
		T3 - T6		-----	-----
		T3 - T12		115.45	0.0999
		T6 - T12		-----	-----
	Site 2	T1 - T3	Bag	772.59	0.0971
		T1 - T6		328.27	0.0981
		T1 - T12		1065.26	0.0996
		T3 - T6		206.82	0.0995
		T3 - T12		1008.16	0.0961
		T6 - T12		339.40	0.0963

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	*p* value
Pairwise	Site 2	T1 - T3	Bottle	478.28	0.0970
		T1 - T6		279.68	0.1012
		T1 - T12		257.23	0.0982
		T3 - T6		448.66	0.1008
		T3 - T12		528.22	0.0974
		T6 - T12		271.03	0.0996
	Site 2	T1 - T3	Dish	721.08	0.0996
		T1 - T6		598.79	0.1043
		T1 - T12		564.66	0.0981
		T3 - T6		591.30	0.1057
		T3 - T12		864.63	0.1034
		T6 - T12		567.59	0.1034
	Site 2	T1 - T3	Pipe	730.76	0.1032
		T1 - T6		400.34	0.1028
		T1 - T12		1133.79	0.0955
		T3 - T6		460.50	0.0994
		T3 - T12		1898.64	0.1008
		T6 - T12		845.63	0.0997
	Site 2	T1 - T3	Glass	408.48	0.1002
		T1 - T6		-----	-----
		T1 - T12		573.47	0.1028
		T3 - T6		-----	-----
		T3 - T12		256.98	0.1003
		T6 - T12		-----	-----
	Site 2	T1 - T3	Rock	487.53	0.0953
		T1 - T6		578.61	0.0986
		T1 - T12		343.77	0.0965
		T3 - T6		310.75	0.0984
		T3 - T12		244.38	0.0962
		T6 - T12		201.61	0.1011

(Continued)

Type of test	Type of comparison			Statistic	
	Site	Time	Substrate	PERMANOVA	
				Pseudo-F	***p* value**
Pairwise	Site 2	T1 - T3	Water	602.36	0.0977
		T1 - T6		244.12	0.1007
		T1 - T12		422.12	0.1058
		T3 - T6		216.88	0.0999
		T3 - T12		427.32	0.1020
		T6 - T12		162.55	0.1019
	Site 1 - Site 2	T1	Bag	1490.03	0.1035
			Bottle	548.06	0.1012
			Dish	1255.76	0.0978
			Pipe	-----	-----
			Glass	1060.23	0.0999
			Rock	1003.79	0.0987
			Water	128.16	0.1042
		T3	Bag	1437.64	0.1050
			Bottle	3498.80	0.0957
			Dish	1638.69	0.1026
			Pipe	1681.06	0.0967
			Glass	790.62	0.0972
			Rock	1129.29	0.1012
			Water	374.69	0.1052
		T6	Bag	386.59	0.1034
			Bottle	766.25	0.0983
			Dish	1182.30	0.0994
			Pipe	1096.06	0.1014
			Glass	-----	-----
			Rock	1281.56	0.1024
			Water	-----	-----
		T12	Bag	1614.20	0.0986
			Bottle	262.66	0.0945
			Dish	1104.16	0.1064
			Pipe	3402.96	0.0972
			Glass	771.99	0.1064
			Rock	552.18	0.0993
			Water	155.00	0.0975

Table S12.
Differential bacterial taxa abundance comparing LDPE bag, PET bottle, PS dish, PVC pipe, BS glass, rock, and water between sampling sites along time by linear discriminant analyses (LEfSe). Taxa with the highest Log LDA score in each group are listed. Colonization times are indicated by the following abbreviations: T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization).

Site	Time	Substrate	Taxa	LDA score
Site 1	T1	LDPE bag	*Pseudorhodobacter*	4.41
			Calothrix KVSF5	4.32
			Leptolyngbyaceae uncultured bacterium	4.02
			Flavobacteriaceae	4.02
			Porphyrobacter	3.88
			Methylophilaceae UBA6140	3.80
			Moraxellaceae	3.71
			Lacihabitans	3.27
			Silvanigrella	3.18
			Nostocaceae	3.02
			Sphingobacteriales	2.81
			Salinirepens	2.79
			Pseudendoclonium	2.78
			Vampirovibrionales uncultured bacterium	2.55
		PET bottle	*Streptococcus*	3.57
			Pseudorhodobacter uncultured bacterium	3.45
			Sandaracinaceae uncultured	3.29
			Roseateles	2.52
			Stigeoclonium	2.05
		PS dish	*Rhodopirellula*	4.21
			Bacteroidia	3.34
			Gemella	2.91
			Haemophilus	2.62
			Pasteurellaceae	2.32
			uncultured Rhizobiales bacterium	2.29
			Rothia	2.24

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T1	PVC pipe	Nostocaceae uncultured	4.73
			Rubinisphaeraceae SH PL14	4.02
			Verrucomicrobiaceae uncultured	3.99
			Gemmatimonas unculturedbacterium	3.89
			Microtrichaceae uncultured	3.85
			Pirellula	3.76
			Fluviicola uncultured bacterium	3.56
			Verrucomicrobiaceae uncultured	3.52
			Rubinisphaeraceae uncultured	3.51
			Armatimonas unculturedbacterium	3.32
			Rubinisphaeraceae uncultured	3.27
			Rickettsiales SM2D12	3.26
			Limnobacter	3.26
			Bdellovibrionaceae OM27clade	3.24
			Crocinitomicaceae	3.10
			Phaeodactylibacter uncultured bacterium	3.03
			Ilumatobacteraceae	2.93
			Kineosporiaceae	2.83
			Rubinisphaeraceae uncultured	2.77
			Armatimonadales	2.59
			Rubinisphaeraceae	2.28
		BS Glass	Oxyphotobacteria	4.11
			Kaiserbacteria uncultured organism	3.22
			Oxyphotobacteria SepB 3 uncultured cyanobacterium	3.22
		Rock	*Flavobacterium*	5.03
			Phaeodactylibacter	3.49
			Chamaesiphon PCC 7430	3.41
			Schizothrix LeGe07164 uncultured cyanobacterium	3.27
			Planoglabratella	2.90
			Myxococcales bacteriap25	2.23
			Bryobacter	2.19
			Synura sp. LO234Ke	2.13

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T1	Water	Sporichthyaceae hgcIclade	4.03
			Sporichthyaceae	3.95
			Sphingobacteriales AKYH767 uncultured bacterium	3.86
			Flavobacterium	3.68
			Solitalea	3.63
			Methylopumilus	3.50
			Planktophila	3.47
			uncultured Cryptomonadaceae	3.30
			Algoriphagus	3.29
			Cryomorphaceae uncultured	3.24
			Fluviicola	3.23
			Blastococcus	3.04
			Micrococcaceae	2.92
			Solirubrobacter	2.84
			Skermanella	2.79
			Luteolibacter	2.72
			Opitutus	2.61
			Phycisphaeraceae CL500 3 uncultured bacterium	2.57
			Solitalea uncultured bacterium	2.56
			Limnoluna	2.55
			Pseudonocardia	2.52
			Haliangium	2.49
			Sporichthyaceae	2.42
			Yersinia	2.37
			Rubrobacter	2.31
			Agromyces	2.23
			Sporichthyaceae hgcI clade	2.12
			Pedobacter	2.05

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T3	LDPE bag	*Rhizobacter*	3.98
			Maribacter	3.49
			Blastopirellula	3.27
			imbriiglobus	2.91
			Planctomycetes OM190	2.50
			Sandaracinus	2.39
			Pedosphaeraceae uncultured bacterium	2.38
			Planctomycetes vadinHA49 wastewater	2.16
		PET bottle	Saprospiraceae	4.09
			Pleurocladia	3.43
			Rhodopirellula	3.26
			Rhodanobacteraceae uncultured bacterium	3.24
			Hyphomonadaceae UKL13 1	3.21
			Planctomycetes OM190 uncultured bacterium	2.90
			Nannocystis	2.89
			Silvanigrella	2.82
			Neochloris	2.56
			Oligoflexus	2.37
			Micavibrionaceae unculturedbacterium	2.19
			Ferrovibrio	2.16
		PS dish	Methylophilaceae	3.84
			uncultured Cytophagales bacterium	3.52
			Rhodocyclaceae	2.80
			Snodgrassella	2.34
		PVC pipe	*Schizothrix* LeGe07164	4.27
			Paludibaculum	3.80
			Saccharimonadales uncultured bacterium	3.53
			Myxococcales mle1 27	3.45
			Bryobacter	3.42
			Beijerinckiaceae uncultured	3.16
			Rhodopirellula	2.97
			Polyangiaceae	2.63
			Hyphomicrobiaceae uncultured	2.07

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T3	BS Glass	*Pedomicrobium*	3.96
			Phormidesmis ANT LACV5 1	3.44
			Verrucomicrobiaceae	3.39
			Schizothrix LeGe07164	3.29
			Planctomycetacia	3.26
			Rubinisphaeraceae SH PL14	3.12
			Oligoflexales 0319 6G20	3.09
			Acaryochloris MBIC11017	3.06
			Parcubacteria uncultured organism	2.90
			Phaselicystis	2.74
		Rock	*Fluviicola*	5.01
			Hyphomonadaceae uncultured	3.88
			Gaiellales uncultured	3.86
			Gemmatimonas	3.79
			Gaiellales uncultured	3.54
			Calditerrivibrio	3.41
			Blastocatella	3.38
			Rhizobiales Incertae Sedis uncultured bacterium	3.30
			Lautropia	3.22
			Bacillus	3.21
			Acidibacter	3.19
			Pirellula	3.11
			Microtrichaceae uncultured	3.08
			Pirellulaceae	2.95
			Ilumatobacteraceae uncultured	2.92
			Blastopirellula uncultured bacterium	2.92
			Ilumatobacteraceae uncultured bacterium	2.82
			Ferribacterium	2.78
			uncultured Conexibacteraceaebacterium	2.73
			Pseudorhodoplanes	2.71
			Demequina	2.68
			Terrimonas	2.67

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T3	Rock	Tepidisphaeraceae uncultured bacterium	2.49
			uncultured Verrucomicrobia bacterium	2.43
			Thermomicrobiales JG30 KF CM45	2.30
			Sandaracinaceae uncultured deltaproteobacterium LX33	2.27
			Paenisporosarcina	2.24
			Methyloligellaceae uncultured	2.22
			Gaiella	2.20
			Phytoplasma	2.19
			Rickettsiaceae uncultured bacterium	2.03
		Water	Rhizobiales Incertae Sedis uncultured	4.89
			Aquiluna	3.84
			Cryomorphaceae uncultured	3.59
			Gemmatimonadaceae uncultured	3.33
			Sediminibacterium	2.79
			Solitalea	2.68
			Limnohabitans	2.49
			Steroidobacteraceae uncultured bacterium	2.35
			Lacunisphaera	2.27
			Gallionella	2.22
			Gemmatimonadaceae uncultured prokaryote	2.22
			Prolixibacteraceae uncultured	2.19
			Prolixibacteraceae uncultured soil bacterium	2.16
	T6	LDPE bag	*Hyphomicrobium*	3.98
			Amoebophilus	3.84
			Lautropia	3.65
			Anaerolineae RBG 13 54 9	2.84
			Luteolibacter	2.53
			Gallionella	2.40
			Ketogulonicigenium	2.01

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T6	PET bottle	*Lacihabitans*	4.09
			Verrucomicrobiae uncultured	2.96
			Permianibacter uncultured bacterium	2.73
			Steroidobacter	2.57
			Microtrichales uncultured	2.54
			Schleiferia	2.21
			Bdellovibrionaceae OM27clade uncultured soil bacterium	2.02
			Rhodothermaceae uncultured	2.00
		PS dish	Saprospiraceae uncultured	4.42
			Kaiserbacteria	3.46
			Micavibrionales	3.11
			Bdellovibrionaceae OM27clade	2.82
			Roseibacillus	2.79
		PVC pipe	Chitinophagaceae	4.40
			Taeseokella	3.65
			Chitinophagales	3.55
			Acidimicrobiia uncultured	3.35
			Spirosomaceae uncultured ephemeradanica	2.97
			Pajaroellobacter	2.78
			Polyangium	2.69
			Nannocystaceae	2.66
			Microscillaceae uncultured	2.37
			Opitutaceae IMCC26134	2.33
			Fibrobacteraceae uncultured	2.18
			Microtrichaceae uncultured	2.13
			Cytophaga	2.05
		BS Glass	*Nitrospira*	3.49
		Rock	*Limnohabitans*	5.21
			Ferruginibacter	3.46
			Spirosomaceae	3.10
			Verrucomicrobiales DeV007	3.09
			Xanthomonadales	3.06
			Armatimonadales	2.81

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T6	Rock	Burkholderiaceae AAP99_ uncultured bacterium	2.60
			Planctomicrobium	2.57
			Rickettsiales SM2D12	2.53
			Methylobacterium	2.30
			Niastella	2.10
			Phycisphaeraceae AKYG587	2.09
		Water	Microbacteriaceae	4.92
			Hydrogenophaga	4.65
			Methylotenera	3.89
			Sporichthyaceae hgcIclade	3.15
			Gemmataceae uncultured	2.93
			Opitutaceae IMCC26134	2.58
			Niveispirillum	2.53
			Marinospirillum	2.47
			Holophagaceae marine group	2.35
			Phycisphaeraceae CL500 3	2.28
	T12	LDPE bag	Rhodobacteraceae	4.11
			Sandaracinaceae uncultured bacterium	3.66
			Pirellula	3.66
			Janthinobacterium	3.43
			Fimbriiglobus	2.98
			Sphingobacteriales env OPS17	2.85
			Massilia	2.72
			Bdellovibrio	2.65
			Rhodospirillales uncultured	2.62
			Lacibacter	2.56
			Peredibacter	2.52
			Acanthopleuribacter	2.49
		PET bottle	*Hymenobacter*	4.30
			Hyphomonas	3.83
			Hirschia	3.69
			Acidibacter	3.59
			Leptothrix	3.37
			Chamaesiphon PCC 7430	3.34

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T12	PET bottle	*Dongia*	3.30
			Fimbriiglobus	3.26
			Psychrobacter	3.24
			Sulfuricurvum	3.14
			Rhodobacter	3.12
			Geobacter	3.11
			Sphingopyxis	3.01
			Sideroxydans	2.98
			Sideroxydans	2.91
			Fusibacter	2.85
			Silanimonas	2.83
			Sulfurospirillum	2.83
			Oligoflexus	2.82
			Phaselicystis	2.78
			Nannocystis	2.73
			Gemmatimonas	2.72
			Mangroviflexus	2.66
			Blastopirellula	2.59
			Paucibacter	2.39
			Stenotrophomonas	2.38
			Lacunisphaera	2.34
		PS dish	*Pleurocapsa* PCC 7319	3.83
			Sphingorhabdus	3.83
			Haliangium	3.76
			Rickettsia	3.52
			Deinococcus	3.48
			Hymenobacter	3.46
			Nocardioides	3.27
			Novosphingobium	3.27
			Pseudahrensia	2.35
		PVC pipe	*Ilumatobacter*	4.29
			Absconditabacteriales SR1	2.61
			Myxococcales Blfdi19	2.61

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T12	BS Glass	*Pleurocapsa* PCC 7319	4.48
			Microtrichaceae	3.84
			Rhizobiales	3.79
			Hyphomicrobium	3.53
			Bdellovibrio	2.77
			Verrucomicrobiaceae uncultured bacterium	2.72
			Haliangium	2.49
			Emticicia	2.24
		Rock	*Truepera*	4.33
			Hyphomonadaceae uncultured organism	3.93
			Saprospiraceae uncultured bacterium	3.68
			Nitrosomonadaceae	3.18
			Hyphomicrobiaceae uncultured	3.08
			Nannocystis	2.93
			Undibacterium	2.64
			Armatimonas	2.42
			Acidibacter	2.42
			Confluentibacter	2.42
			Rhodanobacteraceae uncultured	2.25
			Herpetosiphon	2.23
			uncultured Cytophaga	2.20
			Chroococcidiopsis PCC 6712	2.15
		Water	Burkholderiaceae	4.44
			Rheinheimera	3.82
			Rhodoferax	3.13
			Isosphaeraceae uncultured	3.07
			Polynucleobacter	3.03
			Shewanella	2.99
			Pseudohongiella	2.84
			Sulfurimonas	2.70
			Deefgea	2.68
			Caulobacter	2.64
			Epipyxis PR26KG	2.61

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T12	Water	*Albimonas*	2.43
			Chitinibacter	2.34
			Vogesella	2.33
			Flavobacterium	2.28
			Peredibacter	2.08
			Arenimonas	2.01
Site 2	T1	LDPE bag	*Tychonema* CCAP1459 11B	3.63
			Burkholderiales bacterium JOSHI 001	3.31
			Amoebophilus	3.21
			Solirubrobacteraceae uncultured	3.16
			Methylotenera uncultured soil bacterium	2.46
			Caenarcaniphilales microbial mat	2.36
			Desulfatitalea	2.15
		PET bottle	*Arenimonas*	4.16
			Pseudomonas	3.82
			Clostridiales Family XIII	3.73
			Pseudohongiella	3.58
			Desulfomicrobium	3.23
			Lentimicrobiaceae	3.19
			Sulfuritalea	3.11
			Methyloversatilis	3.05
			Desulfobulbus	2.93
			Accumulibacter	2.90
			Leptolinea	2.89
			Treponema 2	2.88
			A*naerovorax*	2.82
			Ruminiclostridium 1	2.81
			Paludibacter	2.79
			Geobacter	2.76
			Sulfurovum	2.73
			Cytophaga	2.67
			Thiobacillus	2.66
			Desulfatiferula	2.63
			Desulfoprunum	2.58

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	PET bottle	Rikenellaceae uncultured	2.57
			Lentimicrobium	2.54
			Adhaeribacter	2.53
			Erysipelothrix	2.49
			Desulfococcus	2.48
			Syntrophus	2.45
			Ruminiclostridium	2.38
			Flavobacterium	2.37
			Oligoflexus	2.29
			Leptospira	2.21
			Bdellovibrio	2.20
			Paludibacter	2.16
		PS dish	*Inhella*	3.35
			Verrucomicrobium	2.97
			Lacunisphaera	2.77
			Cellvibrio	2.76
			Bdellovibrio	2.68
			Pseudohongiella	2.58
			Spirosomaceae uncultured	2.46
			Spirogyramaxima	2.44
			Alkanindiges	2.40
			Lentisphaera	2.31
		PVC pipe	*Sphingomonas*	3.51
			Altererythrobacter	3.08
			Competibacter	3.05
			Saccharofermentans	2.93
			Pseudoxanthomonas	2.92
			Veillonellaceae uncultured	2.79
			Propionivibrio	2.70
			Rhizobacter	2.66
			Desulfobulbus	2.65
			Saccharofermentans	2.60
			Flavihumibacter	2.50
			Lactobacillus	2.48

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	PVC pipe	*Dokdonella*	2.45
			Solirubrobacter	2.37
			Desulfovibrio	2.16
			Smithella	2.13
			Streptomyces	2.12
			Pontibacter	2.10
			Desulfopila	2.03
		BS Glass	*Hyphomicrobium*	4.63
			Phreatobacter	3.79
			Microtrichaceae IMCC26207	3.65
			Hyphomicrobium	3.57
			Crenothrix	3.31
			Methylomonas	3.29
			Bacillaria	3.29
			Methylotenera	3.29
			Romboutsia	3.11
			Clostridium sensu stricto 1	3.07
			Pedomicrobium	3.05
			Ferritrophicum	2.89
			Leucobacter	2.61
			Intestinibacter	2.46
			Turicibacter	2.31
			Desulfurivibrio	2.12
			Streptococcus	2.08
		Rock	*Nitrosomonas*	4.38
			Aquabacterium	3.85
			Myxococcales	3.55
			Sphingobium	3.34
			Devosia	3.25
			Cellvibrio	3.25
			Nitrosomonas	3.13
			Caulobacteraceae	2.98
			Curvibacter	2.95
			Haliangium	2.94

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	Rock	Gallionellaceae	2.94
			Anaerovibrio	2.75
			Clostridium sensu stricto 9	2.73
			Pseudolabrys	2.70
			Rubrivivax	2.65
			Sulfurifustis	2.61
			Methylobacter	2.58
			Chryseolinea	2.54
			Mucilaginibacter	2.54
			Phaselicystis	2.47
			Propionispira	2.46
			Crocinitomix	2.45
			Methylophilus	2.44
			Thiobacillus	2.44
			Cytophaga	2.44
			Dyadobacter	2.17
		Water	*Arcobacter*	4.49
			Mycobacterium	4.26
			Chitinivorax	3.94
			Sediminibacterium	3.90
			Polynucleobacter	3.73
			Aquaspirillum	3.59
			Bacteroides	3.58
			Aquabacterium	3.54
			Simplicispira	3.46
			Fodinicola	3.36
			Hypnocyclicus	3.13
			Aquimonas	2.95
			Tolumonas	2.72
			Prosthecobacter	2.58
			Anaerosinus	2.54
			Limnothrix	2.54
			Subdoligranulum	2.50
			Paludisphaera	2.49

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	Water	*Proteocatella*	2.49
			Ignavibacterium	2.46
			Megamonas	2.45
			Agathobacter	2.44
			Aeromicrobium	2.42
			Bifidobacterium	2.36
			Blautia	2.34
			Laribacter	2.33
			Kaistia	2.30
			Phaselicystis	2.29
			Xanthobacter	2.28
			Fluviicola	2.26
			Enterococcus	2.26
			Collinsella	2.24
			Aquaspirillum	2.20
			Formivibrio	2.07
	T3	LDPE bag	*Defluviimonas*	3.80
			Chryseobacterium	3.34
			Phycisphaeraceae SM1A02	3.33
			Aeromonas	3.12
			Blastopirellula	3.09
			Peredibacter	2.98
			Paraclostridium	2.98
			Nitratireductor	2.89
			Gaiella	2.80
			Weeksellaceae	2.77
			Planctomicrobium	2.74
			Tropicimonas	2.66
			Roseimicrobium	2.63
			uncultured *Verrucomicrobium*	2.62
			Caedibacter	2.61
			Dinghuibacter	2.45
			Aquicella	2.38
			Diplorickettsiaceae uncultured	2.37

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T3	LDPE bag	*Chthonobacter*	2.30
			Labrys	2.24
			Pirellula	2.14
			Fluviicola	2.07
			Chthoniobacter	2.03
			Plesiomonas	2.01
		PET bottle	*Thiobacillus*	3.91
			Dechloromonas	3.65
			Roseomonas	3.39
			Desulfobacter	3.39
			Competibacter	3.17
			Ignavibacterium	3.03
			Crenothrix	3.01
			Sulfurisoma	2.91
			Desulfatiglans	2.85
			Leptolinea	2.84
			Desulfococcus	2.78
			Anaerolineaceae	2.72
			Spirochaeta 2	2.65
			Geothermobacter	2.57
			Ignavibacterium	2.56
			Desulfatiferula	2.55
			Chlorobium	2.54
			Desulfobacterium	2.54
			Leptolinea	2.53
			Desulfobulbus	2.53
			Desulfomonile	2.52
			Thermoanaerobaculum	2.52
			Sphingobacteriales	2.51
			Anaerolinea	2.49
			Thiobacillus	2.46
			Acinetobacter	2.45
			Treponema	2.44
			Anaerolinea	2.43

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T3	PET bottle	*Smithella*	2.41
			Sterolibacterium	2.37
			Bdellovibrio	2.31
			Amaricoccus	2.26
			Caldisericum	2.26
			Methylosarcina	2.26
			Longilinea	2.25
			Sulfurimonas	2.23
			Desulforhabdus	2.17
			Bacteriovorax	2.10
			Woeseia	2.09
			Pirellula	2.08
		PS dish	*Reyranella*	3.55
			Planktothrix NIVA CYA15	3.42
			Rhodanobacteraceae	3.38
			Dinghuibacter	3.37
			Caldilineaceae uncultured	3.30
			Luteitalea	3.19
			Dinghuibacter	2.85
			Nordella	2.80
			Rickettsia	2.76
			Microtrichales	2.69
			Acidaminobacter	2.65
			Cetobacterium	2.62
			Chthonobacter	2.58
			Planctopirus	2.53
			Rubripirellula	2.44
			Thiocapsa	2.43
			Deefgea	2.38
			Peredibacter	2.20
			Desulfobacca	2.14
			Defluviicoccus	2.14
			Chloroflexi	2.07
			Runella	2.06

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T3	PS dish	Caldilineaceae	2.06
			Fimbriiglobus	2.05
			Aquicella	2.05
			Rhizobiales KF JG30 B3	2.03
			Anaerolinea	2.01
		PVC pipe	*Methylibium*	2.69
			Competibacter	2.67
			Permianibacter	2.66
			Nitrosomonas	2.62
			Obscuribacterales	2.59
			Pajaroellobacter	2.59
			Amphiplicatus	2.55
			Chloroflexi OLB14	2.34
			Proteocatella	2.31
			Taibaiella	2.28
			Propionivibrio	2.23
			Steroidobacter	2.01
		BS Glass	*Brevundimonas*	3.89
			Phenylobacterium	3.75
			Sandaracinaceae uncultured	3.71
			Chitinivorax	3.65
			Limnohabitans	3.46
			Armatimonas	3.35
			Sterolibacterium	3.35
			Tabrizicola	3.21
			Bdellovibrio	3.10
			Nitrospira	3.09
			Undibacterium	2.92
			Pseudonocardiaceae	2.90
			Cetobacterium	2.87
			Chroococcidiopsis PCC 6712	2.77
			Ralstonia	2.75
			Rhabdochlamydia	2.70
			Rivicola	2.69

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T3	BS Glass	*Brevundimonas*	2.66
			Chitinimonas	2.62
			Alkanindiges	2.61
			Proteiniclasticum	2.56
			Brachymonas	2.54
			Pirellula	2.26
			Taibaiella	2.16
		Rock	*Denitratisoma*	3.67
			Thermomonas	3.24
			Herpetosiphon	3.10
			Hyphomonadaceae SWB02	2.91
			Brocadia	2.76
			Myxococcales Blfdi19	2.74
			Nitrosomonas	2.74
			Moraxellaceae uncultured	2.74
			Bdellovibrio	2.64
			Meiothermus	2.60
			Methylophilaceae uncultured	2.47
			Bealeia uncultured bacterium	2.45
			Sulfuricellaceae	2.35
			Pelagibium	2.31
			Meiothermus	2.29
			Caldimonas	2.27
			Flavobacterium	2.21
			Roseomonas	2.21
			Jidaibacter	2.18
			Anaerolinea	2.10
			Defluviicoccus	2.09
			uncultured *Chloroflexus*	2.03
			Micavibrionaceae uncultured	2.02
			Myxococcales uncultured	2.00

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T3	Water	Nomurabacteria	3.56
			Xenococcaceae	3.39
			Pleurocapsa PCC 7319	3.31
			Methylophilaceae uncultured	3.05
			Bdellovibrio	2.92
			Parachlamydiaceae	2.69
			Oligoflexaceae uncultured	2.65
			Nostocales	2.62
			Chlamydiales	2.56
			Megaira	2.53
			Alsobacter	2.53
			Rhizobacter	2.42
			Arcobacter	2.37
			Bacteriovorax	2.27
			Fluviicoccus	2.27
			Dinghuibacter	2.07
	T6	LDPE bag	*Zoogloea*	4.69
			Gemmobacter	3.13
			Paracoccus	2.96
			Thiothrix	2.90
			Chthoniobacteraceae	2.68
			Ruminococcaceae NK4A214 group	2.64
			Acetobacterium	2.53
			Synergistaceae uncultured	2.44
			Cloacibacillus	2.44
			Brachymonas	2.35
			Dialister	2.34
		PET bottle	*Actibacter*	3.83
			Arenimonas	3.13
			Acetoanaerobium	2.96
			Leptotrichiaceae	2.92
			Hyphomonadaceae UKL13	2.87
			Acinetobacter	2.78
			Chromatiaceae uncultured	2.72

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T6	PET bottle	*Rhodoferax*	2.54
			Anaeromyxobacter	2.50
			Tolumonas	2.48
			Vitreoscilla	2.36
			Rhodocyclaceae uncultured	2.36
			Accumulibacter	2.18
		PS dish	*Thermomonas*	3.85
			Saprospiraceae uncultured	3.78
			Lautropia	3.30
			Staphylococcus	3.09
			Lawsonella	2.91
			Comamonas	2.80
			Pirellula	2.51
			Pedobacter	2.47
			Fusobacteriaceae	2.46
			Fusobacterium	2.29
			Ruminococcus 2	2.23
			Anaerococcus	2.10
		PVC pipe	*Zoogloea*	4.01
			Thauera	4.00
			Terrimonas	3.81
			Aeromonas	3.75
			Acidovorax	3.73
			Cloacibacterium	3.68
			Ottowia	3.52
			Azospira	3.47
			Haliscomenobacter	3.34
			Macellibacteroides	3.25
			Enterobacteriaceae	3.20
			Devosiaceae	3.07
			Acidovorax	2.96
			Paludibaculum	2.92
			Haliangium	2.85
			Pedobacter	2.85

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T6	PVC pipe	*Lautropia*	2.83
			Microvirga	2.75
			Sphingobacteriaceae	2.72
			Comamonas	2.70
			Runella	2.69
			Prosthecobacter	2.69
			Agitococcus	2.58
			Rubellimicrobium	2.51
			Stella	2.40
			Spironemaculicis	2.17
			Parabacteroides	2.13
		BS Glass	*Acinetobacter*	4.82
			Enhydrobacter	3.76
			Trichococcus	3.33
			Acinetobacter	3.07
			Tetrasphaera	3.04
			Methylocystis	2.90
			Uruburuella	2.87
			Chryseobacterium	2.30
			Ruminococcus	2.29
			Vitreoscilla	2.09
		Rock	*Sphaerotilus*	4.45
			Rhizobiaceae uncultured	3.73
			Zoogloea	3.30
			Chitinophagaceae uncultured	2.87
			Babeliales	2.67
			Emticicia	2.59
			Mycoplasma	2.46
			Pirellula	2.18
			Sebaldella	2.09

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T6	Water	*Pseudarcicella*	4.41
			Fodinicola	3.63
			Gordonia	3.59
			Rhodocyclaceae C39	3.35
			Bdellovibrio	2.99
			Lachnospiraceae	2.98
			Rivicola	2.97
			Agitococcus	2.83
			Geothrix	2.78
			Aeromonas	2.74
			Faecalibacterium	2.68
			Turneriella	2.60
			Desulfovibrio	2.47
			Fusicatenibacter	2.42
			Diplorickettsiaceae uncultured	2.42
			Propionivibrio	2.42
			Ottowia	2.24
			Leptotrichia	2.24
			Prevotellaceae	2.19
			Bacteroides	2.02
			Pleomorphomonas	2.00
			Sphingobacteriales LiUU 11 161	2.00
	T12	LDPE bag	*Methyloglobulus*	3.95
			Rhodanobacteraceae uncultured	3.64
			Thermoanaerobaculaceae Subgroup10	3.54
			Chlamydiales cve6	3.48
			Methyloparacoccus	3.22
			Simkaniaceae	2.90
			Terrimicrobium	2.73
			Finegoldia	2.70
			Diplorickettsiaceae uncultured	2.69
			Phycisphaeraceae SM1A02	2.69
			Fodinicurvataceae uncultured	2.69
			Pirellula	2.69

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T12	LDPE bag	Chlamydiaceae	2.65
			Desulfarculaceae	2.65
			Moraxellaceae uncultured	2.64
			Paracaedibacter	2.62
			Anaerococcus	2.60
			Simkaniaceae uncultured bacterium	2.57
			Arenimonas	2.56
			Anaerococcus uncultured bacterium	2.55
			Aquicella	2.55
			Neisseriaceae uncultured	2.55
			Coxiella	2.51
			Pirellula	2.48
			Legionella	2.43
			Coxiella	2.35
			Planctopirus	2.35
			Rubellimicrobium	2.31
			Myxococcales BIrii41	2.31
			Sandaracinus	2.25
			Legionellaceae uncultured	2.22
			Gemmata	2.21
			Babeliaceae uncultured bacterium	2.17
			Pirellula	2.16
			Chitinophagales 37 13 uncultured soi lbacterium	2.09
			Paracaedibacteraceae	2.08
			Anaerococcus	2.07
			Amphiplicatus	2.02
		PET bottle	*Piscinibacter*	3.62
			Nitrotoga	3.51
			Tahibacter	3.21
			Rickettsiaceae	3.03
			Lautropia	2.90
			Nitrotoga	2.90
			Reyranellaceae uncultured	2.64
			Vogesella	2.33

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T12	PET bottle	*Schlesneria*	2.24
			Clostridium sensu stricto 13	2.21
			Roseiflexaceae uncultured soil bacterium	2.20
			Thermoanaerobaculaceae Subgroup10	2.02
			Runella	2.01
			Silvanigrella	2.01
		PS dish	*Nitrospira*	4.42
			Xanthobacteraceae	3.44
			Microscillaceae uncultured	3.16
			Chthoniobacter	3.10
			Pseudoduganella	2.88
			Chromobacterium	2.75
			Thermoanaerobaculaceae Subgroup10	2.63
			Alysiosphaera	2.55
			Gemmatimonadaceae uncultured	2.48
			Holosporaceae uncultured	2.47
			Steroidobacteraceae	2.35
			Citrobacter	2.29
			Chitinophagales uncultured	2.26
			Fluviicola	2.24
			Hyphomonadaceae uncultured bacterium	2.23
		PVC pipe	*Candidimonas*	3.38
			Corynebacterium 1	3.36
			Chthoniobacter	3.03
			Luteolibacter	2.87
			Rhodovastum	2.83
			Corynebacteriaceae	2.80
			Atopostipes	2.76
			Leeia	2.71
			Verticia	2.65
			Bacteriovorax	2.65
			Caenimonas	2.53
			Aquicella	2.53

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T12	PVC pipe	*Ottowia*	2.53
			Alysiosphaera	2.46
			Syntrophorhabdus	2.43
			Moraxella	2.41
			Peptoniphilus	2.39
			Roseobacter	2.39
			Lactobacillus	2.36
			Paenibacillus	2.34
			Cutibacterium	2.34
			Methyloparacoccus	2.30
			Caenimonas	2.28
			Trachydiscusminutus	2.27
			Hydrogenophilus	2.22
			Dietzia	2.15
		BS Glass	*Rhodobacter*	4.91
			Tabrizicola	4.17
			Luteolibacter	4.00
			Beijerinckiaceae	3.84
			Methylovulum	3.79
			Defluviimonas	3.73
			Legionella	3.64
			Bosea	3.50
			Thoreahispida	3.34
			Verrucomicrobiaceae uncultured	3.30
			Iamia	3.27
			Beijerinckiaceae	3.25
			Rhodococcus	3.22
			Kumanoa	3.08
			Pirellulaceae	3.07
			Verrucomicrobiaceae uncultured	3.05
			Gemmataceae uncultured	3.04
			Crenothrix	2.98
			Xanthobacteraceae uncultured	2.92
			Microthrix	2.89

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T12	BS Glass	*Pirellula*	2.84
			Planctomycetales uncultured	2.83
			Verrucomicrobiaceae uncultured	2.81
			Aminobacter	2.79
			Pirellulaceae uncultured	2.71
			Polymorphobacter	2.67
			Isosphaeraceae	2.67
			Peredibacter	2.66
			Bauldia	2.60
			Luteitalea	2.44
			Amaricoccus	2.44
			Turicibacter	2.42
			Nakamurella	2.37
			Prosthecomicrobium	2.36
			Fimbriiglobus	2.34
			Planctopirus	2.32
			Desulfobacca	2.31
			Anammoximicrobium	2.29
			Blastopirellula	2.18
			Planctomicrobium	2.17
			Oligoflexales 0319 6G20	2.16
			Desulfomoniles	2.14
			Acetobacteraceae uncultured	2.13
			Neochlamydia	2.09
		Rock	*Rhizobiaceae*	4.14
			Lysobacter	3.79
			Blastocatellaceae	3.47
			Dokdonella	3.35
			Steroidobacteraceae uncultured	2.91
			Blastocatella	2.69
			Chthoniobacter	2.55
			Sphingobacteriales AKYH767	2.50
			Saprospiraceae uncultured	2.45

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T12	Water	*Flavobacterium*	4.39
			Rhodoferax	4.35
			Thiothrix	3.52
			Intrasporangiaceae	3.24
			Sporichthyaceae uncultured	3.09
			Malikia	2.97
			Pleomorphomonadaceae uncultured	2.32
			Pirellulaceae uncultured	2.31
			Rikenellaceae uncultured	2.23
			Rhodoferax	2.19

Table S13.
Differential eukaryotic taxa abundance comparing LDPE bag, PET bottle, PS dish, PVC pipe, BS glass, rock, and water between sampling sites along time by linear discriminant analyses (LEfSe). Taxa with the highest Log LDA score in each group are listed. Colonization times are indicated by the following abbreviations: T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization).

Site	Time	Substrate	Taxa	LDA score
Site1	T1	PET bottle	*Aphanochaete*	3.92
			Chaetopeltis	3.22
			Rhizophydiales uncultured	2.31
			Eustigmatales	2.01
		PS dish	*Cocconeis*	5.55
			Poales	2.35
		BS glass	Chlorophyta	4.71
			Monostroma	4.45
			Ulvophyceae	3.38
			Minerva	2.18
		Rock	*Rhoicosphenia*	4.76
			Gomphonema	4.67
			Aphanochaete	4.35
			Bacillariophyceae	4.27
			Sporochonus	3.95
			Chaetophora	3.35
			Achnanthidium	3.24
			Chlorochytrium	2.86

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T1	Water	Podocopida	4.32
			Teleaulax	2.87
			Echinocoleum	2.80
			Gregarina	2.74
			uncultured Chytridiomycota	2.67
			Rozella	2.57
			Fusarium	2.48
			Corylopsis	2.45
			Aphelidea uncultured fungus	2.41
			Prymnesiales	2.35
			Teleostei	2.34
			Melanopsichium	2.29
			Lentinus	2.28
			Cyrtolophosis	2.14
			Choreotrichia uncultured	2.09
			Pfiesteria	2.06
			Nautococcus	2.05
	T3	LDPE bag	*Pseudourostyla*	3.19
			Continenticola	3.06
			Daptonema	2.65
			Catenula KL 2009	2.36
			Lobulomycetaceae	2.30
			Marsilea	2.28
			Oxytricha	2.21
			Peregriniidae	2.09
		PET bottle	*Schmidtea*	3.18
			Continenticola	2.74
		PVC pipe	*Mermithida*	3.85
		BS glass	*Blidingia*	4.08
		Rock	*Caecum*	5.57
			Neoptera	3.66
			Bodomorpha	2.32

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T3	Water	*Cladophora*	4.30
			Perkinsidae uncultured eukaryote	2.82
			Lolium	2.14
	T6	LDPE bag	*Penardia* NVam1	3.07
			uncultured Rhizophydiales	3.05
			Oenothera	2.55
			Fagales	2.52
			Debaryomycetaceae	2.50
			Lobulomycetaceae uncultured	2.49
			Cyrtophoria uncultured eukaryote	2.47
			Brassicales	2.43
			Rheum	2.41
			uncultured Rhizaria	2.35
			Arthrinium	2.34
			Camptobasidium	2.33
			Microascaceae	2.33
			Chlamydonellopsis	2.33
			uncultured Eimeriidae	2.32
			Scuticociliatia	2.31
			Synchaeta	2.30
			Vampyrellida V1ld4	2.29
			Nucletmycea	2.22
			Gyromitus	2.15
			Thecofilosea uncultured	2.13
			Haptoria	2.08
			Fungi LKM15	2.05
			Heteromita	2.00
		PET bottle	Bubarida	5.17
		PS dish	*Sialis*	2.41
		PVC pipe	*Vorticella*	3.60
			Eucapnopsis	3.04
			Perkinsidae	2.70
			Oxytrichidae	2.53
			Taphrina	2.50

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T6	BS glass	*Aspidisca*	4.32
			Carchesium	3.66
			Dysteria	2.84
			Peritrichia	2.52
			Cercozoa B134	2.48
		Rock	*Populus*	3.60
			Ephemeroptera	2.90
			Malpighiales	2.57
			Cylindrocapsa	2.29
			Cyperus	2.24
	T12	LDPE bag	*Paulinella*	2.54
		PET bottle	*Jaoa*	4.77
			Plectus	4.39
			Parachela	4.02
			Copromyxa PKD2011	3.69
			Monhysterida	3.40
			Amoebozoa	3.31
			Sorodiplophrys	3.20
			Hypotrichia	3.20
			Aplanochytrium	3.09
			Suctoria	3.04
			Ptolemeba	2.95
			Pseudovorticella	2.93
			Chaetonotida	2.93
			Dilabifilum	2.89
			Scyphidia 11010803	2.78
			Eufolliculina uncultured eukaryote	2.77
			Dictyamoeba uncultured eukaryote	2.69
			Cercomonas	2.69
			Salpingoecidae	2.68
			Characium	2.63
			Filamoeba	2.60
			Strobilidium	2.54
			Chytridiaceae PML 2015	2.52

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T12	PET bottle	*Blepharisma*	2.49
			Angulamoeba	2.47
			Sarocladium	2.47
			Microdiaphanosoma	2.46
			Lobulomycetaceae	2.46
			Gibellulopsis	2.40
			Ulotrichales	2.39
			Labyrinthula	2.37
			Carcinomyces uncultured fungus	2.33
			Vishniacozyma	2.32
			Chytridiaceae uncultured eukaryote	2.32
			Choanoflagellida	2.31
			Parabirojimia	2.31
			Enoplida uncultured eukaryote	2.31
			Frontonia	2.27
			Stephanopyxisturris	2.27
			Oedogonium	2.26
			Apobryophyllum	2.25
			Oligohymenophorea	2.25
			Freshwater Choanoflagellates 2	2.24
			Gymnophrys	2.19
			Salpingoeca	2.17
			Craticula	2.14
			Craspedida uncultured eukaryote	2.14
			Rhizamoeba	2.07
			Uncultured Cercozoa	2.04
			Protaspidae	2.01
			Paracercomonas	2.01
			Loxophyllum	2.00
		PS dish	Heterobranchia	5.24
			Phaeosphaeriaceae	3.93
			Tetracladiummarchalianum	3.28
			Pleosporales	3.24
			Rhynchosporium	3.22

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T12	PS dish	*Erynia*	3.02
			Pleosporaceae	2.90
			Orbiliaceae	2.58
			Salpingoecidae	2.49
			Tremellales	2.46
			Pseudochilodonopsis	2.38
			Cothurnia	2.35
			Cystofilobasidium	2.34
			Fabales	2.29
			Navicula	2.25
			Plantago	2.20
		PVC pipe	uncultured Eimeriidae	2.33
		BS glass	*Batrachospermum* MCO 2011	4.72
			Anguillospora	3.73
			Zoothamnium	3.33
			Trichoderma	3.14
			Zoothamnium	2.95
			uncultured Chytridiomycota	2.38
			Pseudovorticella	2.22
		Rock	*Phagocatavitta*	5.39
			Atractides HPHyd018	4.00
			Coleoptera	3.42
			Macrostomida	3.17
			Phasmarhabditis eM434	3.12
			Angulamoeba	2.42
			Pseudostaurosiropsis D 07	2.31
			Paraphelidium	2.30
		Water	*Ochromonas*	4.18
			Siluania	3.91
			Cryptomonas	3.57
			Perkinsidae	3.49
			Armillaria	3.14
			Apicomplexa	3.00
			Choricystis NIeS 2342	3.00

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 1	T12	Water	*Paraphysomonas*	2.94
			Chlamydomonadales	2.84
			Cercozoa	2.68
			Chromulinales	2.65
			Chrysophyceae	2.61
			Cryptomonas	2.59
			Stylonychia	2.57
			Geminigera	2.56
			Tetrahymena	2.53
			Rozella	2.51
			Crustomastix	2.44
			Kathablepharidae	2.40
			Cupressus	2.32
			Mychonastes	2.30
			Fragilariales	2.11
Site 2	T1	LDPE bag	Poales	3.07
			Caryophyllales	2.72
			Tricholomataceae	2.60
			Barnettozyma	2.55
			Hyphochytriales	2.45
			Stentor	2.34
			Chaetomium	2.33
			Oligohymenophorea CV1 2A 17	2.24
			Loxophyllum	2.23
			Tetraselmis	2.21
			Chytridiomycetes	2.19
			Cryptomycota LKM11	2.02
			Nematostelium	2.00
		PET bottle	Rhizophydiales	3.83
			Cryptomycota D 5 LKM11	3.83
			Sorodiplophrys	3.41
			Lecythium	3.35
			Thecofilosea	3.22
			Peronosporomycetes	3.15

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	PET bottle	*Pelagothrix*	3.14
			Metopus	2.85
			Spirostomum	2.75
			Plagiopyla	2.71
			Vampyrellidae	2.68
			Pleuronema	2.66
			Paraurostyla	2.61
			Enoplia	2.61
			Rigifilida	2.61
			Harpacticoida	2.57
			Micronuclearia	2.48
			Clevelandellida	2.43
			Epalxella	2.42
			Gregarinasina BAQA40	2.37
			Mortierella	2.37
			Cyclidium	2.33
			Exocolpoda	2.32
			Euamoebida BOLA868	2.31
			Trebouxiophyceae	2.29
			Breviata	2.28
			Diplophrys ATCC 50360	2.28
			Psalteriomonas	2.26
			Salpingoecidae	2.26
			Cymbopleura	2.25
			Surirella	2.21
			Heteromita	2.14
			Colpodida	2.11
			Spizellomycetales	2.07
			Paramicrosporidium	2.06
		PS dish	*Stenostomum*	4.15
			Epidorylaimus	2.71
			Ploimida	2.51
			Pichia	2.25
			Scuticociliatia uncultured	2.05

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	PVC pipe	*Telotrochidium*	3.70
			Stenostomum	3.68
			Holosticha	3.25
			Monocystis	3.03
			Tetrahymena	2.93
			Tausonia	2.61
			Sphaeropleales	2.60
			Ustilaginaceae	2.53
			Nuclearia	2.50
			Entamoeba	2.50
			Sellaphora	2.48
			Ochroconis	2.47
			Aphelidium	2.47
			Heteromita	2.28
			Amphileptus	2.27
			Phymatotrichopsis	2.22
			Entomophthora	2.21
			Angulamoeba	2.17
			Leptomyxida	2.14
			Brevimastigomonas	2.14
		BS glass	*Cladophora*	4.85
			Navicula	4.34
			Arnoldiella	4.01
			Dorylaimida	3.76
			Nowakowskiella	3.43
			Tribonematales	3.41
			Chromadorida	3.10
			Surirella	3.04
			Lulwoana	3.03
			Cryptomycota	2.91
			Thecofilosea	2.89
			Pinnularia	2.71
			Nitzschia	2.63
			Rhizophydium	2.55

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	BS glass	Amorosiaceae	2.53
			Oedogonium	2.45
			Nudifila	2.45
			Paramicrosporidium	2.44
			Litonotus	2.40
			Rhinosporideacae	2.38
			Mucor	2.35
			Anurofeca	2.28
			Ischnamoeba	2.25
			Monoblepharidales	2.22
			Melampsora	2.18
			Salpingoecidae metagenome	2.16
			Spumella	2.14
			Paramicrosporidium	2.03
			uncultured *Pichia*	2.03
		Rock	Haplotaxida	5.05
			Pythium	4.16
			Minchinia	3.36
			Thaumatomonas	3.27
			Protosporangium	3.14
			Aphanochaete	2.99
			Rhabdiopoeus	2.88
			Cyclidium	2.71
			Hemiurosomoida	2.68
			Cercomonadidae	2.67
			Pleurothecium	2.66
			Colpodea	2.59
			Sorodiplophrys	2.49
			Mnium	2.48
			Nudifila	2.41
			Oxytricha	2.30
			Conioscypha	2.27
			Exuviaella	2.27
			Ballistosporomyces	2.23

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T1	Rock	*Gymnophrys*	2.21
			Pterocystis	2.12
			Pyronemataceae	2.05
		Water	Chrysophyceae	3.96
			Cyrtophoria	3.63
			Bicosoecida	3.37
			Haptoria	3.16
			Synura	2.90
			Pedinomonas	2.81
			Teloschistaceae	2.70
			Hypotrichia	2.59
			Carteria	2.57
			Neocallimastigaceae	2.38
			Suigetsumonas	2.32
			Chaetonotus	2.29
			Paratrimastix	2.29
			Chromadorea	2.28
			Sellaphora	2.22
			Blastocystis Ambiguous	2.16
			Scuticociliatia	2.09
			Chromulinales	2.01
	T3	LDPE bag	*Monhystera*	4.16
			Blepharisma	3.73
			Cladosporium	3.44
			Nitokra	3.34
			Adineta	3.30
			Ploimida	3.16
			Prostoma	2.81
			Aspergillaceae	2.70
			uncultured Stramenopile	2.63
			Scotinosphaera	2.53
			Lagenidium	2.44
			Magnoliophyta	2.42
			Candida	2.41

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T3	LDPE bag	Chlorellales	2.24
			Ephemeroptera	2.22
			Lobulomycetaceae	2.11
		PET bottle	Plumatellida	5.45
			Haltidytes	2.72
			Malpighiales	2.62
			Rhizoclonium	2.55
			Plagiopylida	2.50
			Paulinella	2.46
			Euplotia	2.41
			Paraphysomonas	2.22
			Bullera	2.16
		PS dish	*Cypridopsis*	4.02
			Dorylaimida	3.24
			Acanthocystidae	2.46
			Cryptosporidium	2.46
			Pterocystis	2.26
		PVC pipe	*Hydra*	4.89
			Telotrochidium	3.59
			Radix	3.51
			Stentor	3.49
			Placorhynchus	3.24
			Cyphoderia	3.18
			Pseudovorticella	2.43
			Urospora	2.42
			Vampyrella	2.38
			Euglypha	2.32
			Saccamoeba	2.25
		BS glass	*Loxophyllum*	4.69
			Acineta	4.08
			Peritrichia	3.86
			uncultured Chlamydomonadales	3.73
			Oedocladium	3.72
			Chytridiomycetes	3.63

(Continued)

Site	Time	Substrate	Taxa	LDA score
			Philodinida	3.57
			Obertrumia	3.55
			Chrysophyceae	3.42
			Chloromonas	3.30
			Phascolodon	3.29
			Cryptocaryon	3.19
			Zosterodasys	3.17
			Cryptomonas	3.12
			Rhabditidoides	3.12
			Codosigidae	3.09
			Paraphysomonas	3.06
			Viridiraptor	3.00
			Peronosporomycetes	2.97
			Vampyrellidae	2.94
			Protorhabditis	2.82
			Panagrolaimus	2.61
Site 2	T3	BS glass	*Metaurostylopsis*	2.60
			Pseudochilodonopsis	2.51
			Ulnaria	2.49
			Pyrenomonas	2.46
			Paramecium	2.44
			Oligotrichia	2.37
			Orbiliaceae	2.37
			Encyonema	2.35
			Mediophyceae	2.34
			Pleuronema	2.28
			Wislouchiella	2.28
			Glissomonadida	2.23
			Bicosoecida LG08 10	2.22
			Chroomonas	2.21
			Perkinsidae	2.10
			Pseudopirsonia	2.06
			Cladochytrium	2.01

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T3	Rock	Monhysterida	3.97
			Olisthanella	3.45
			Stuckenia	3.45
			Psammorhynchus	3.35
			Tetrahymena	2.70
			Hypotrichia	2.67
			Ptygura	2.65
			Ichthydium	2.39
			Epalxella	2.25
			Aspidisca	2.21
			Colpoda	2.16
			Rhizophydium	2.14
		Water	*Cryptomycota*	3.59
			Tylenchida	3.49
			Paramonas	3.49
			Rosa	3.03
			Ulvella	2.74
			Dimorpha	2.50
			Crustomastix	2.41
			Cryptovalsa	2.37
			Chrysophyceae	2.37
			Apiognomonia	2.36
			Colpodella	2.33
			Aphelidea	2.27
			Rozella	2.26
			Oedogoniales	2.21
			Chloromonas	2.21
			Synurales	2.21
			Oscheius	2.07
			Heteromita	2.06
	T6	LDPE bag	*Girardia*	5.00
			Diplogasterida	3.60
			Pelodera	3.54
			Telotrochidium	3.00

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T6	LDPE bag	*Strelkovimermis*	2.91
			Geotrichum	2.74
			Saprochaete	2.58
			Filobasidiaceae	2.50
			Naganishia	2.50
			Rhabditis	2.49
			Flabellula	2.46
			Cutaneotrichosporon	2.46
		PET bottle	*Frontonia*	4.64
			Candona	4.36
			Floscullariacea	3.82
			Spirostomum	3.67
			Harpacticoida	3.63
			Aeroglyphus	3.15
			Anoetus	3.15
			Peritrichia	2.75
			Remanella	2.63
			Peziza	2.57
			Trichodina	2.53
			Actinidia	2.53
			Schistonchus	2.51
			Ichthyosporea	2.48
			Rhodotorula	2.48
			Glissomonadida	2.41
			Metschnikowia	2.40
			Balantidion	2.38
			Pyrus	2.37
			Saccharomyces	2.30
			Caryophyllidea	2.30
			Peritrichia	2.14
			Cercomonas	2.13

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T6	PS dish	*Tripylella*	4.38
			Myzocytiopsis	3.87
			Aphanomyces	2.87
			Podocopida	2.82
			Mortierella	2.77
			Caenorhabditis	2.77
			Spirotrichea	2.51
			Candida	2.39
			Mononchoides	2.37
			Monogononta	2.21
			Zoothamnium	2.12
		PVC pipe	Haplotaxida	4.90
			Rhogostoma	4.84
			Cercozoa	4.19
			Epistylis	3.57
			Cyclopoida	3.53
			Caudiholosticha	3.25
			Plasmodium	2.96
			Acaulopage	2.65
			Apodibius	2.40
			Vorticella	2.37
			Trichosporon	2.26
			Bicosoeca	2.11
		Rock	*Telotrochidium*	4.88
			Triplonchida	3.69
			Adinetida	3.63
			Bdelloidea	3.48
			Charophyta	3.31
			Fictor	3.21
			Chlorophyceae	2.88
			Rozella	2.82
			Arboramoeba	2.75

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T6	Rock	Collembola	2.72
			Leptomyxida	2.62
			Sporobolomyces	2.46
			Cutaneotrichosporon	2.34
		Water	Chrysophyceae P34 45	4.28
			Ochromonas	4.26
			Cryptomycota	3.86
			Paramecium	3.55
			Hymenostomatia uncultured	3.43
			Acineta	3.29
			Scuticociliatia	2.99
			Pythium	2.97
			Paraphysomonas	2.92
			Chlorophyceae	2.80
			Heteromita	2.78
			Spumella	2.73
			Trithigmostoma	2.71
			Phytomyxea	2.70
			Trimyema	2.66
			Stentor	2.62
			Hypocoma	2.57
			Tetracladium	2.53
			Peritrichia	2.48
			Cryphonectria	2.44
			Euplotes	2.42
			Cordycipitaceae	2.34
			Paratrimastix	2.33
			Trichostomatia	2.28
			Tetrahymena	2.28
			Hafniomonas	2.27
			Bodomorpha	2.26
			Chlamydomonadaceae	2.25
			Paracineta	2.21
			Cryptomonas	2.18

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T6	Water	uncultured Trebouxia	2.18
			Phytophthora	2.16
			Oligohymenophorea	2.15
	T12	LDPE bag	*Erpobdella*	4.77
			Limnomedusae	4.71
			Hypsibius	3.57
			Mononchida	3.52
			Bresslauilla	3.12
			Baetis	3.08
			Acotyledon	3.00
			Trinema	2.74
			Paraphanolaimus	2.68
			Aphelenchoides	2.54
		PET bottle	*Prorhynchus*	4.11
			Parachela	4.07
			Chromadorida	3.98
			Neophaeosphaeria	3.74
			Triplonchida	3.62
			Solanales	3.27
			Adelina	3.09
			Caenorhabditis	3.07
			Knufia	3.00
			Pinus	2.94
			Sclerotinia	2.93
			Hyaloperonospora	2.81
			Pinophyta	2.62
			Copepoda	2.56
			Planothidium	2.49
			Corythion	2.45
		PS dish	Diptera	5.01
			Limnohalacarus	3.51
			Macrostomida	3.26
			Uncultured Thaumatomonadida	2.80
			Rhabditida	2.62

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T12	PS dish	*Lagenidium*	2.57
			Diploneis	2.48
			Geotrichum	2.11
			Saccharomycetaceae	2.02
		PVC pipe	*Hydroptila*	5.49
			Brevibucca	2.20
			Herpotrichiellaceae	2.67
		BS glass	*Hypsizygus*	4.56
			Nemaliophycidae	4.46
			Thorea	4.40
			Sphaeropleales	3.91
			Chlamydopodium	3.76
			Malassezia	3.61
			Aspergillaceae	3.37
			Botryococcus	3.34
			Monactinus	3.18
			Amphora	3.16
			Myrica	3.11
			Pinophyta	2.99
			Pirum	2.96
			Opephoraguenter	2.85
			Orciraptor	2.84
		Rock	*Craspedacusta*	4.08
			Araeolaimida	2.76
			Sistotrema	2.55
			Cryptomycota	2.52
			Cercozoa	2.22
			Paraphelidium	2.16
		Water	*Gonium*	4.93
			Cyclotella	4.71
			Tetracystis	4.28
			Cryptocaryon	3.32
			uncultured *Amoebophrya*	2.59
			Pedinellales	2.55

(Continued)

Site	Time	Substrate	Taxa	LDA score
Site 2	T12	Water	*Telotrochidium*	2.53
			Oxytrichidae	2.49
			Chlamydomonas	2.48
			Amphileptus	2.42
			Stokesia	2.42
			Opisthonecta	2.40
			Elongatocystis	2.30
			Sorosphaerula	2.02

Table S14.
Hydroxyl indices for each tested before and after 1 year of incubation in river water.

Plastic	Hydroxyl Index		
	T0	Site 1	Site 2
LDPE bag	0.03	0.10	0.11
PET bottle	0.48	1.55	1.25
PS dish	0.25	0.24	0.91
PVC pipe	0.46	0.68	0.74

Table S15.
Global and multiple comparisons of $2^{-\Delta ct}$ values for the *sul1* gene using Student-Newman-Keuls test

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
-----			93.23	Yes
Site	---	---	48.35	Yes
---	Time	---	3.75	No
---	---	Substrate	21.58	Yes
Site 1	1 month	Water - Plastic	4.32	Yes
		Water - BS Glass	3.08	Yes
		Water - Rock	3.58	Yes
		Plastic - BS Glass	3.58	Yes
		Plastic - Rock	3.08	Yes
		BS Glass - Rock	2.16	No
Site 2		Water - Plastic	4.43	Yes
		Water - BS Glass	3.84	Yes
		Water - Rock	2.77	Yes

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	**Time**	**Substrate**	**Test statistic**	***p*-value < 0.05**
Site 2	1 month	Plastic - BS Glass	3.16	No
		Plastic - Rock	3.70	No
		BS Glass - Rock	0.93	No
Site 1	3 months	Water - Plastic	3.79	Yes
		Water - BS Glass	4.32	Yes
		Water - Rock	2.78	Yes
		Plastic - BS Glass	2.78	Yes
		Plastic - Rock	2.78	Yes
		BS Glass - Rock	3.79	Yes
Site 2		Water - Plastic	1.54	No
		Water - BS Glass	3.39	Yes
		Water - Rock	3.37	Yes
		Plastic - BS Glass	3.37	Yes
		Plastic - Rock	3.39	Yes
		BS Glass - Rock	4.32	Yes
Site 1	6 months	Water - Plastic	3.79	Yes
		Water - BS Glass	4.32	Yes
		Water - Rock	2.78	Yes
		Plastic - BS Glass	2.78	Yes
		Plastic - Rock	2.78	Yes
		BS Glass - Rock	3.79	Yes
Site 2		Water - Plastic	2.78	Yes
		Water - BS Glass	4.22	Yes
		Water - Rock	4.00	Yes
		Plastic - BS Glass	3.39	Yes
		Plastic - Rock	3.37	Yes
		BS Glass - Rock	1.54	No

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
Site 1	12 months	Water - Plastic	3.70	Yes
		Water - BS Glass	3.79	Yes
		Water - Rock	3.84	Yes
		Plastic - BS Glass	1.85	No
		Plastic - Rock	2.53	No
		BS Glass - Rock	1.85	No
Site 2		Water - Plastic	2.78	Yes
		Water - BS Glass	4.32	Yes
		Water - Rock	3.79	Yes
Site 2	12 months	Plastic - BS Glass	3.79	Yes
		Plastic - Rock	2.77	Yes
		BS Glass - Rock	2.77	Yes
Site 1 - Site 2	1 month	Water	4.64	Yes
		Plastic	4.54	Yes
		BS Glass	4.63	Yes
		Rock	4.76	Yes
Site 1 - Site 2	3 months	Water	3.39	Yes
		Plastic	4.64	Yes
		BS Glass	5.14	Yes
		Rock	2.77	Yes
Site 1 - Site 2	6 months	Water	4.65	Yes
		Plastic	4.87	Yes
		BS Glass	4.65	Yes
		Rock	4.22	Yes
Site 1 - Site 2	12 months	Water	3.09	Yes
		Plastic	4.96	Yes
		BS Glass	3.79	Yes
		Rock	4.26	Yes

Table S16.
Global and multiple comparisons of $2^{-\Delta ct}$ values for the *ermF* gene using Student-Newman-Keuls test

Type of comparison			Student–Newman–Keuls test	
Site	**Time**	**Substrate**	**Test statistic**	***p*-value < 0.05**
-----			92.35	Yes
Site	---	---	11.40	Yes
---	Time	---	3.51	No
---	---	Substrate	8.98	Yes
Site 1	1 month	Water - Plastic	3.16	Yes
		Water - BS Glass	4.48	Yes
		Water - Rock	4.32	Yes
		Plastic - BS Glass	4.01	Yes
		Plastic - Rock	0.31	No
		BS Glass - Rock	2.95	No
Site 2		Water - Plastic	5.14	Yes
		Water - BS Glass	4.65	Yes
		Water - Rock	5.29	Yes
		Plastic - BS Glass	3.47	Yes
		Plastic - Rock	1.23	No
		BS Glass - Rock	3.85	No
Site 1	3 months	Water - Plastic	2.78	Yes
		Water - BS Glass	3.79	Yes
		Water - Rock	4.32	Yes
		Plastic - BS Glass	2.77	Yes
		Plastic - Rock	3.79	Yes
		BS Glass - Rock	2.77	Yes
Site 2		Water - Plastic	3.69	Yes
		Water - BS Glass	2.77	Yes
		Water - Rock	2.93	Yes
		Plastic - BS Glass	4.40	Yes
		Plastic - Rock	2.47	No
		BS Glass - Rock	3.90	Yes

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
Site 1	6 months	Water - Plastic	3.79	No
		Water - BS Glass	3.36	No
		Water - Rock	4.62	No
		Plastic - BS Glass	0.92	No
		Plastic - Rock	0.92	No
		BS Glass - Rock	1.26	No
Site 2		Water - Plastic	2.47	No
		Water - BS Glass	5.46	Yes
		Water - Rock	3.27	No
		Plastic - BS Glass	4.32	Yes
		Plastic - Rock	3.76	Yes
		BS Glass - Rock	0.93	No
Site 1	12 months	Water - Plastic	4.65	Yes
		Water - BS Glass	4.42	Yes
		Water - Rock	5.28	Yes
		Plastic - BS Glass	3.16	No
		Plastic - Rock	0.92	No
		BS Glass - Rock	3.70	No
Site 2		Water - Plastic	2.78	Yes
		Water - BS Glass	4.32	Yes
		Water - Rock	3.70	No
		Plastic - BS Glass	4.65	Yes
		Plastic - Rock	4.40	Yes
		BS Glass - Rock	3.16	No
Site 1 - Site 2	1 month	Water	4.52	Yes
		Plastic	4.16	Yes
		BS Glass	4.52	Yes
		Rock	5.36	Yes
Site 1 - Site 2	3 months	Water	4.32	Yes
		Plastic	3.90	Yes
		BS Glass	5.05	Yes
		Rock	4.81	Yes

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	**Time**	**Substrate**	**Test statistic**	***p*-value < 0.05**
Site 1 - Site 2	6 months	Water	4.98	Yes
		Plastic	4.40	Yes
		BS Glass	3.89	Yes
		Rock	5.69	Yes
Site 1 - Site 2	12 months	Water	3.16	No
		Plastic	4.64	Yes
		BS Glass	2.77	Yes
		Rock	4.65	Yes

Table S17.
Global and multiple comparisons of $2^{-\Delta ct}$ values for the *dfrA* gene using Student-Newman-Keuls test

Type of comparison			Student–Newman–Keuls test	
Site	**Time**	**Substrate**	**Test statistic**	***p*-value < 0.05**
-----			98.83	Yes
Site	---	---	47.44	Yes
---	Time	---	2.49	No
---	---	Substrate	20.20	Yes
Site 1	1 month	Water - Plastic	3.87	Yes
		Water - BS Glass	2.88	No
		Water - Rock	1.89	No
		Plastic - BS Glass	3.70	Yes
		Plastic - Rock	4.42	Yes
		BS Glass - Rock	2.77	No
Site 2		Water - Plastic	4.01	Yes
		Water - BS Glass	4.64	Yes
		Water - Rock	4.01	Yes
		Plastic - BS Glass	4.64	Yes
		Plastic - Rock	0.31	No
		BS Glass - Rock	3.67	No

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
Site 1	3 months	Water - Plastic	4.32	Yes
		Water - BS Glass	2.78	Yes
		Water - Rock	3.79	Yes
		Plastic - BS Glass	3.79	Yes
		Plastic - Rock	2.78	Yes
		BS Glass - Rock	2.78	Yes
Site 2		Water - Plastic	4.003	Yes
		Water - BS Glass	1.54	No
		Water - Rock	3.37	Yes
		Plastic - BS Glass	4.21	Yes
		Plastic - Rock	2.78	Yes
		BS Glass - Rock	3.395	Yes
Site 1	6 months	Water - Plastic	4.48	Yes
		Water - BS Glass	4.85	Yes
		Water - Rock	3.70	No
		Plastic - BS Glass	1.54	No
		Plastic - Rock	3.37	Yes
		BS Glass - Rock	3.39	Yes
Site 2		Water - Plastic	2.78	Yes
		Water - BS Glass	3.79	Yes
		Water - Rock	4.80	Yes
		Plastic - BS Glass	4.43	Yes
		Plastic - Rock	4.42	Yes
		BS Glass - Rock	3.70	No
Site 1	12 months	Water - Plastic	4.89	Yes
		Water - BS Glass	4.26	Yes
		Water - Rock	4.54	Yes
		Plastic - BS Glass	4.32	Yes
		Plastic - Rock	3.79	Yes
		BS Glass - Rock	2.78	Yes

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
Site 2	12 months	Water - Plastic	3.70	No
		Water - BS Glass	5.19	Yes
		Water - Rock	4.43	Yes
		Plastic - BS Glass	4.65	Yes
		Plastic - Rock	2.77	Yes
		BS Glass - Rock	4.32	Yes
Site 1 - Site 2	1 month	Water	5.28	Yes
		Plastic	5.30	Yes
		BS Glass	3.16	No
		Rock	3.74	No
Site 1 - Site 2	3 months	Water	4.39	Yes
		Plastic	4.65	Yes
		BS Glass	4.91	Yes
		Rock	4.65	Yes
Site 1 - Site 2	6 months	Water	4.26	Yes
		Plastic	4.84	Yes
		BS Glass	4.91	Yes
		Rock	3.16	No
Site 1 - Site 2	12 months	Water	3.16	Yes
		Plastic	4.87	Yes
		BS Glass	3.79	Yes
		Rock	3.79	Yes

Table S18.
Global and multiple comparisons of $2^{-\Delta ct}$ values for the *qnrSrtF11A* gene using Student-Newman-Keuls test

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
			93.11	Yes
Site	-	-	10.40	Yes
	Time	-	1.15	No
-	-	Substrate	14.05	Yes

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
Site 1	1 month	Water - Plastic	4.16	Yes
		Water - BS Glass	3.58	Yes
		Water - Rock	2.16	No
		Plastic - BS Glass	2.77	Yes
		Plastic - Rock	4.00	Yes
		BS Glass - Rock	3.09	Yes
Site 2		Water - Plastic	4.16	Yes
		Water - BS Glass	4.01	Yes
		Water - Rock	2.78	Yes
		Plastic - BS Glass	2.16	Yes
		Plastic - Rock	3.79	Yes
		BS Glass - Rock	3.09	Yes
Site 1	3 months	Water - Plastic	0.93	No
		Water - BS Glass	1.26	No
		Water - Rock	4.63	Yes
		Plastic - BS Glass	0.93	No
		Plastic - Rock	3.79	Yes
		BS Glass - Rock	4.53	Yes
Site 2		Water - Plastic	3.76	No
		Water - BS Glass	3.85	Yes
		Water - Rock	2.77	Yes
		Plastic - BS Glass	0.62	No
		Plastic - Rock	3.76	Yes
		BS Glass - Rock	4.53	Yes
Site 1	6 months	Water - Plastic	2.78	Yes
		Water - BS Glass	4.32	Yes
		Water - Rock	3.79	Yes
		Plastic - BS Glass	3.79	Yes
		Plastic - Rock	2.77	Yes
		BS Glass - Rock	2.77	Yes

(Continued)

Type of comparison			Student–Newman–Keuls test	
Site	Time	Substrate	Test statistic	*p*-value < 0.05
Site 2	6 months	Water - Plastic	2.77	Yes
		Water - BS Glass	4.43	Yes
		Water - Rock	3.84	Yes
		Plastic - BS Glass	3.70	No
		Plastic - Rock	3.16	No
		BS Glass - Rock	0.93	No
Site 1	12 months	Water - Plastic	4.22	Yes
		Water - BS Glass	2.78	Yes
		Water - Rock	4.00	Yes
		Plastic - BS Glass	3.39	Yes
		Plastic - Rock	1.54	No
		BS Glass - Rock	3.37	Yes
Site 2		Water - Plastic	2.77	Yes
		Water - BS Glass	3.79	Yes
		Water - Rock	4.32	Yes
		Plastic - BS Glass	2.77	Yes
		Plastic - Rock	3.79	Yes
		BS Glass - Rock	2.77	Yes
Site 1 - Site 2	1 month	Water	4.78	Yes
		Plastic	4.52	Yes
		BS Glass	4.52	Yes
		Rock	4.52	Yes
Site 1 - Site 2	3 months	Water	3.87	Yes
		Plastic	4.32	Yes
		BS Glass	5.85	Yes
		Rock	4.65	Yes
Site 1 - Site 2	6 months	Water	4.65	Yes
		Plastic	4.65	Yes
		BS Glass	4.54	Yes
		Rock	4.80	Yes
Site 1 - Site 2	12 months	Water	2.77	Yes
		Plastic	4.91	Yes
		BS Glass	2.77	No
		Rock	4.22	Yes

Table S19.
Spearman correlations between the $2^{-\Delta ct}$ values of each ARG in plastics, rock, BS glass and water, and the antibiotic concentration measured in water at the two sampling sites after 1 year of the incubation experiment. The antibiotic concentrations were those measured in the water (values reported in **Table S6)**.

Gene	Antibiotic	Site	Sustrate	Correlation coefficient	*p*-value
sul1	Sulphamides	Both sites	Global	0.66	> 0.05
			Water	0.50	> 0.05
			Plastic	0.89	> 0.05
			BS glass	0.75	> 0.05
			Rock	0.75	> 0.05
		Site 1	Global	-0.04	0.81
			Water	0	0.97
			Plastic	0.24	0.54
			BS glass	-0.04	0.88
			Rock	-0.24	0.53
		Site 2	Global	0.25	0.15
			Water	-0.19	0.62
			Plastic	0.88	> 0.05
			BS glass	0	0.98
			Rock	0.15	0.71
ermF	Macrolides	Both sites	Global	0.72	> 0.05
			Water	0.77	> 0.05
			Plastic	0.74	> 0.05
			BS glass	0.77	> 0.05
			Rock	0.78	> 0.05
		Site 1	Global	0.037	0.84
			Water	0.36	0.35
			Plastic	0.03	0.93
			BS glass	0.92	> 0.05
			Rock	0.33	0.39
		Site 2	Global	-0.34	> 0.05
			Water	0	0.98
			Plastic	-0.19	0.62
			BS glass	-0.78	> 0.05
			Rock	-0.15	0.71

(Continued)

Gene	Antibiotic	Site	Sustrate	Correlation coefficient	*p*-value
dfrA	Trimethoprim	Both sites	Global	0.73	> 0.05
			Water	0.81	> 0.05
			Plastic	0.97	> 0.05
			BS glass	0.66	> 0.05
			Rock	0.91	> 0.05
		Site 1	Global	0.37	0.04
			Water	0.49	0.21
			Plastic	0.781	> 0.05
			BS glass	0.59	0.10
			Rock	0.59	0.10
		Site 2	Global	0.29	0.11
			Water	0.59	0.10
			Plastic	0.97	> 0.05
			BS glass	-0.39	0.321
			Rock	0.68	> 0.05
qnrSrtF11A	Quinolones	Both sites	Global	0.70	> 0.05
			Water	0.54	> 0.05
			Plastic	0.79	> 0.05
			BS glass	0.83	> 0.05
			Rock	0.81	> 0.05
		Site 1	Global	-----	-----
			Water	-----	-----
			Plastic	-----	-----
			BS glass	-----	-----
			Rock	-----	-----
		Site 2	Global	0.01	0.96
			Water	-0.29	0.46

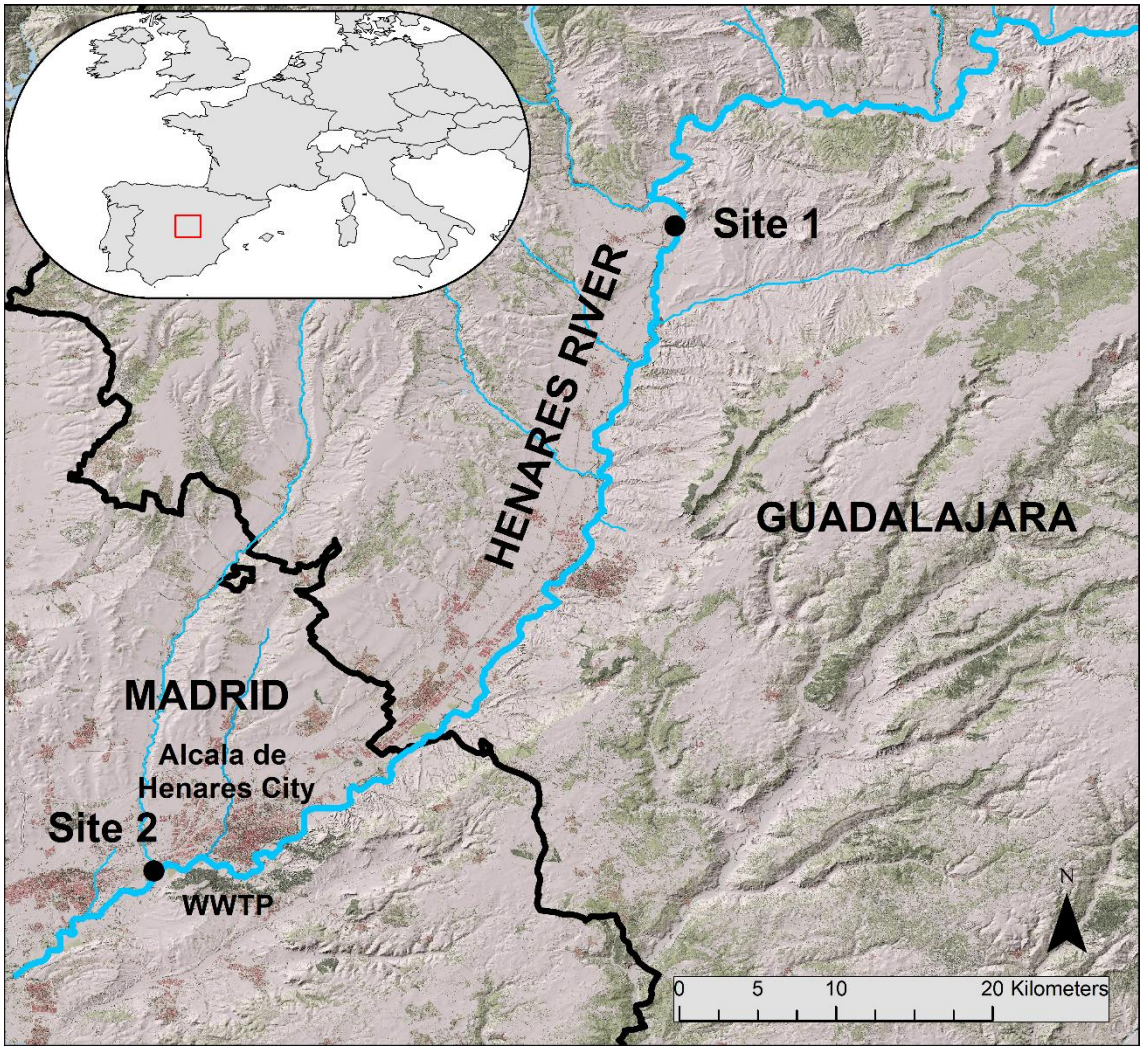

Figure S1. Map with sampling station defined as site 1 and site 2.

Figure S2. Details of the colonization process: a) Virgin substrates previous to the colonization experiment, b) Distribution of the different substrates in the metal cage, c) Deployment of metal cages with plastics inside the river, d) Condition of the substrates after 1 month of colonization.

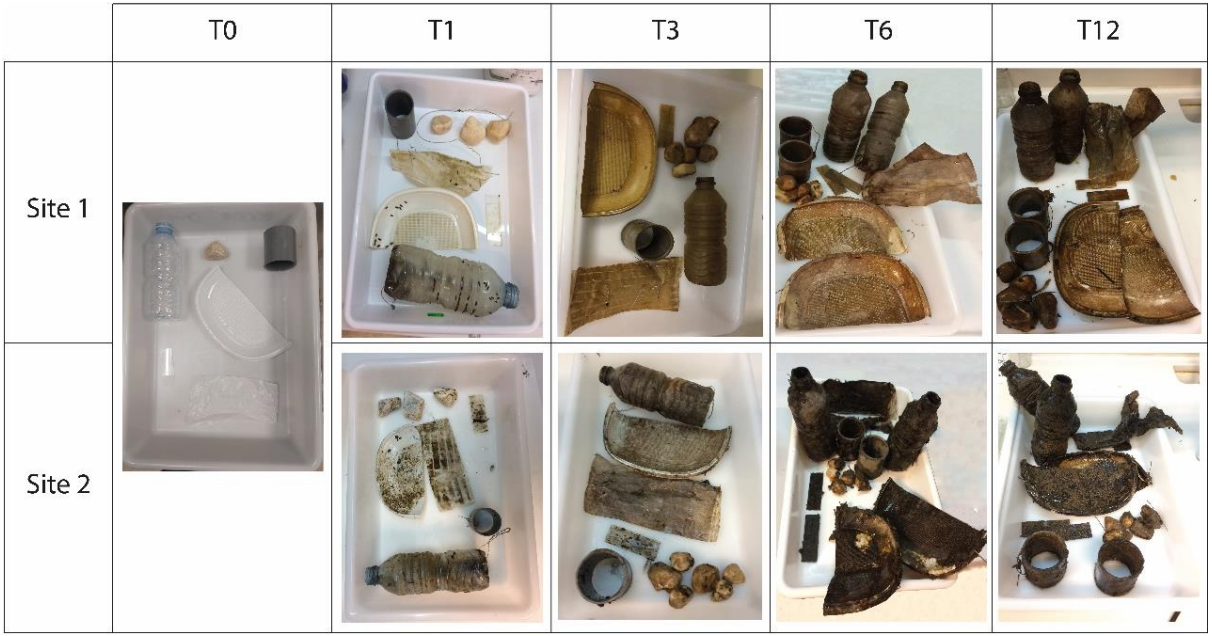

Figure S3. Evolution of the state of substrates during the colonization experiment.

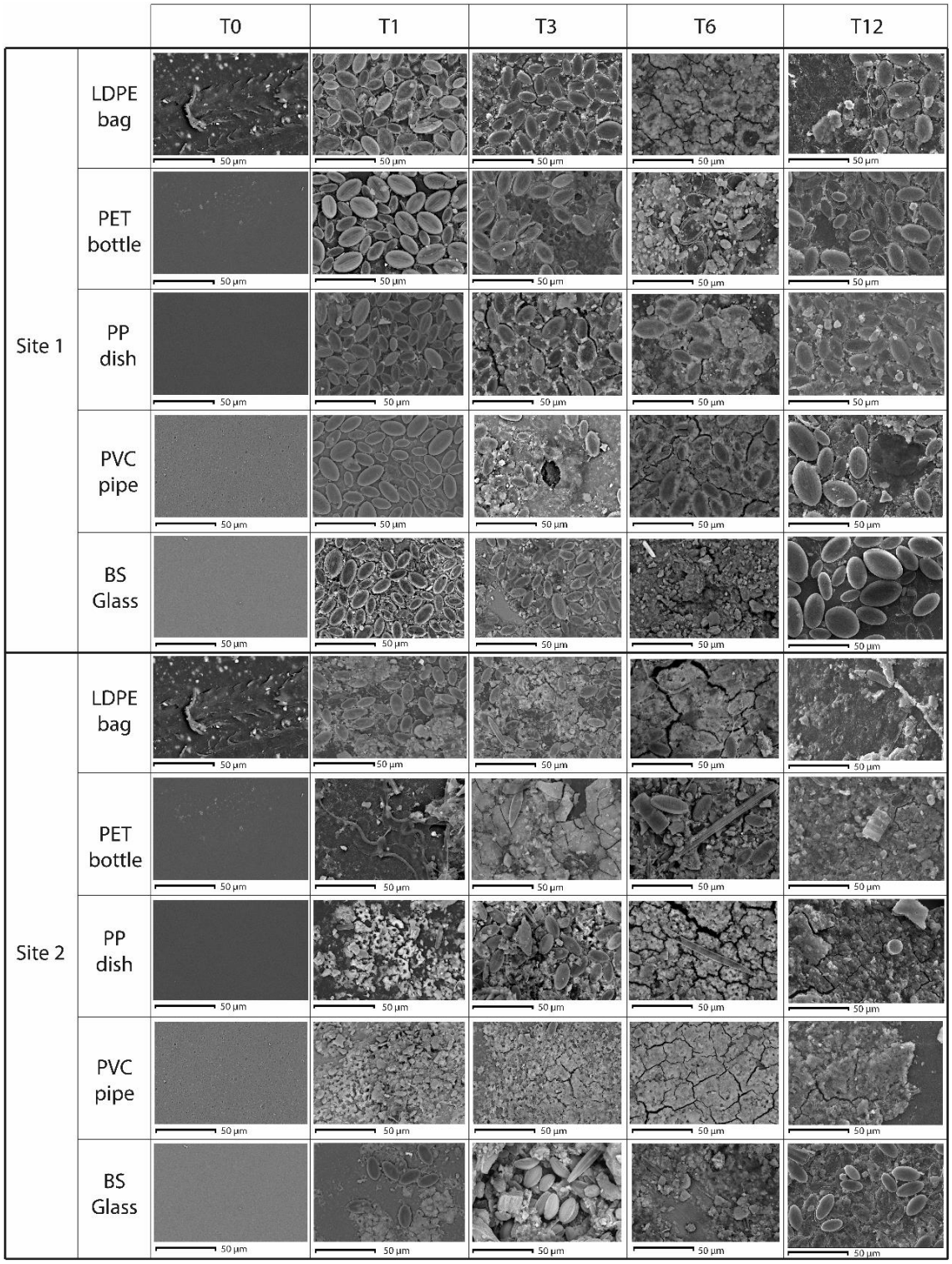

Figure S4. SEM image showing microbial colonization on the different substrates in both sites along incubation time.

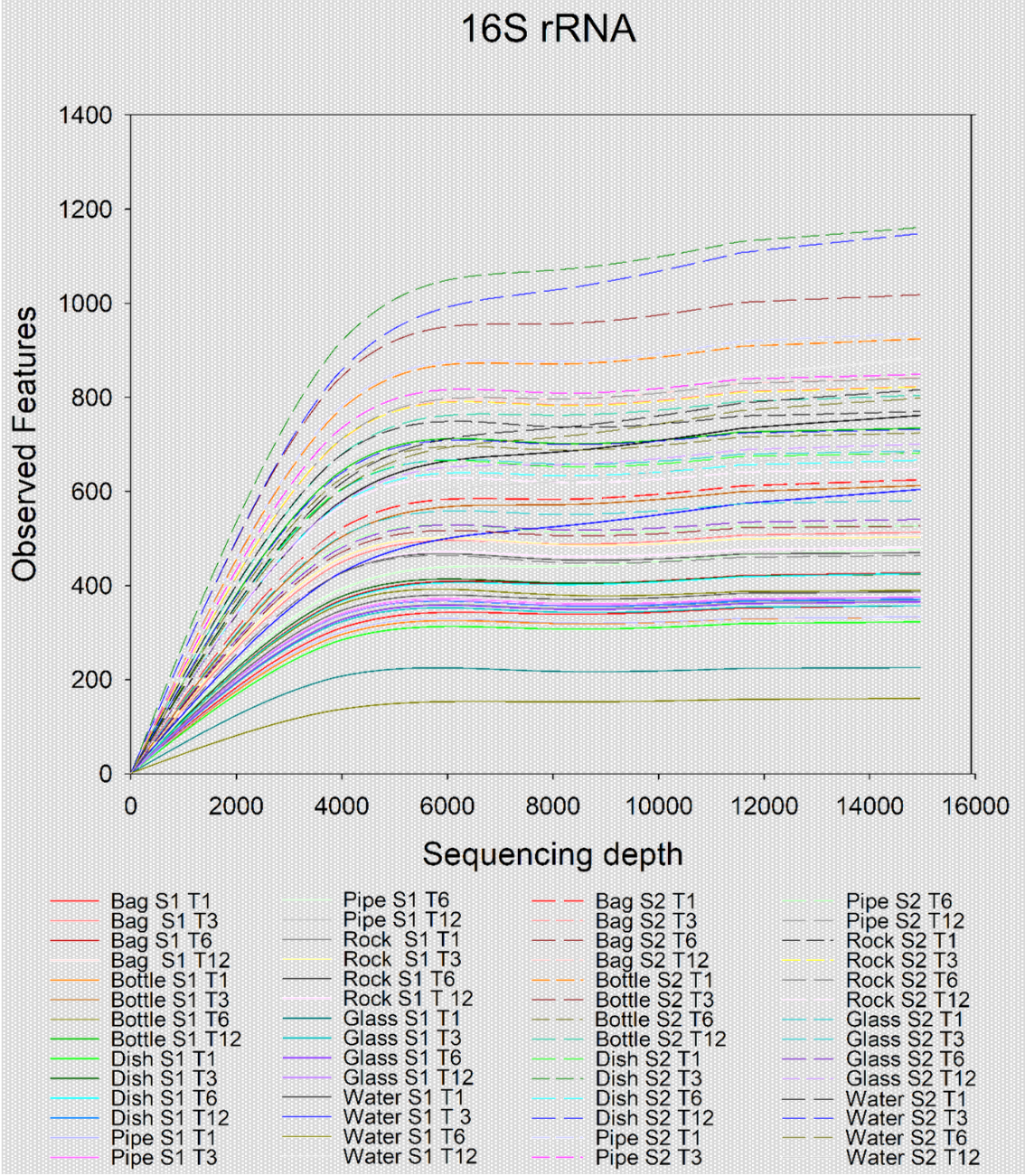

Figure S5. Rarefaction curve that compares the observed features (corresponding to ASVs in previous version of QIIME2) in comparison with number of reads for each sample (sequencing depth) in the 16S rRNA gene.

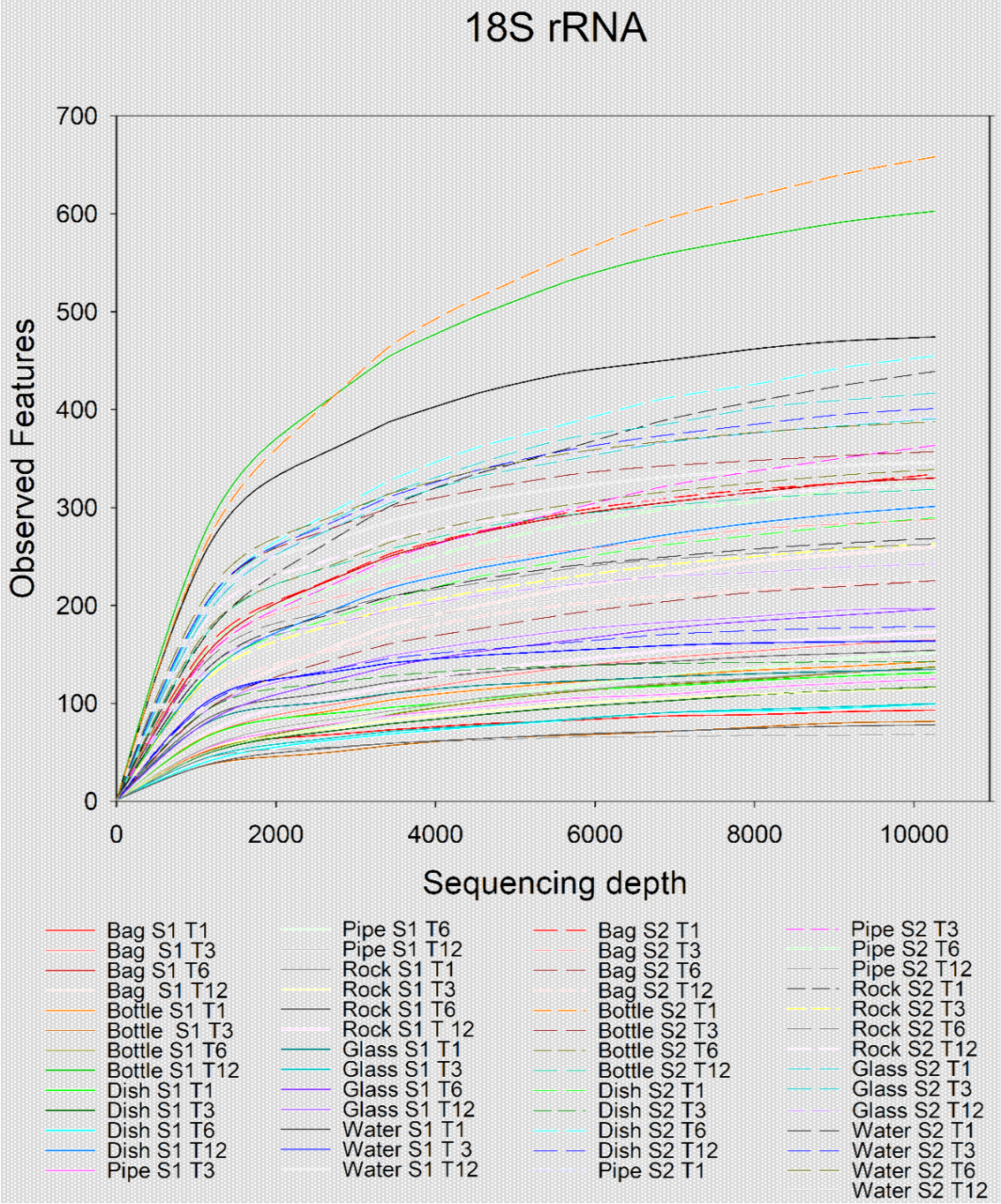

Figure S6. Rarefaction curve that compares the observed features (corresponding to ASVs in previous version of QIIME2) in comparison with number of reads for each sample (sequencing depth) in the 18S rRNA gene.

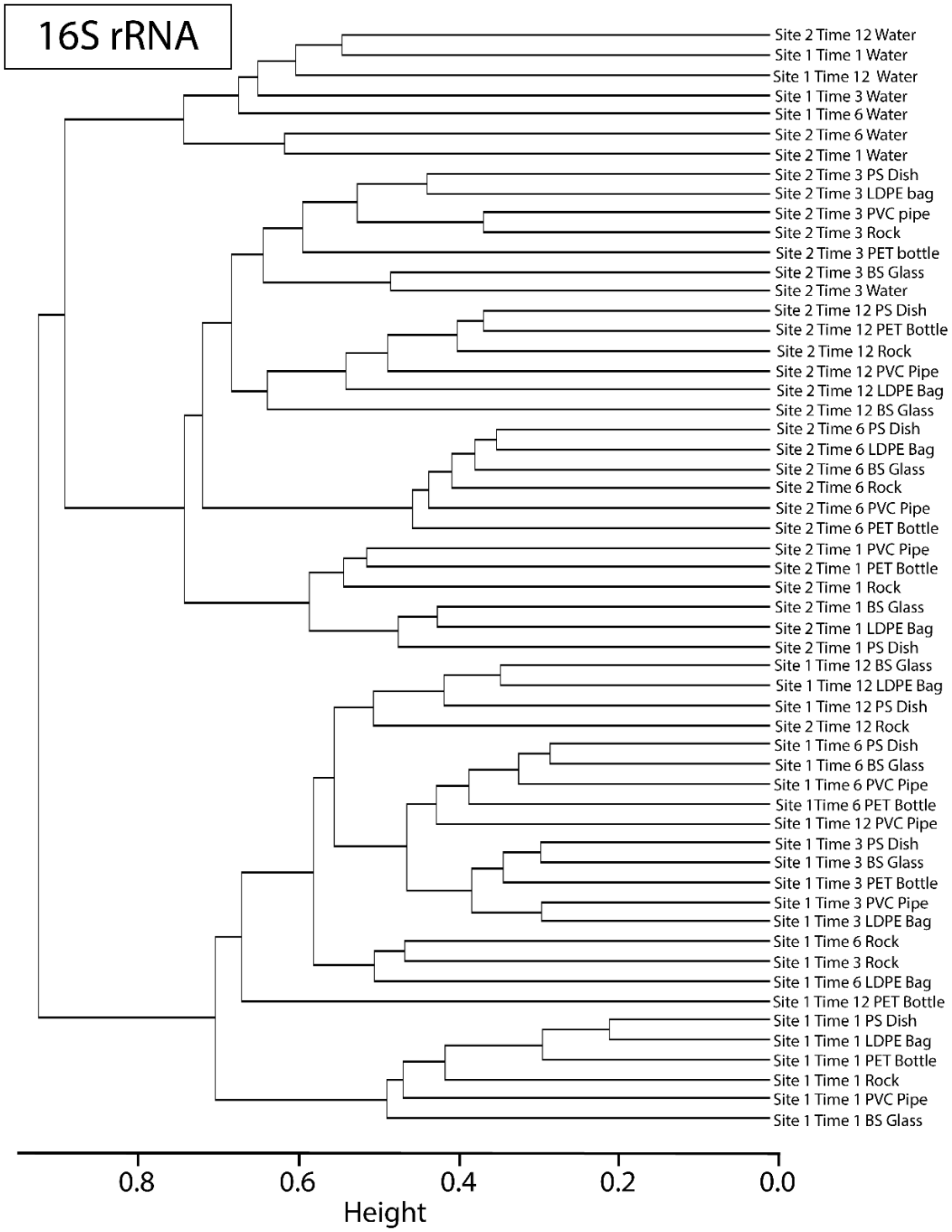

Figure S7. UPGMA dendrogram obtained from 16s rRNA cluster analysis of samples, using the Bray-Curtis distance measure.

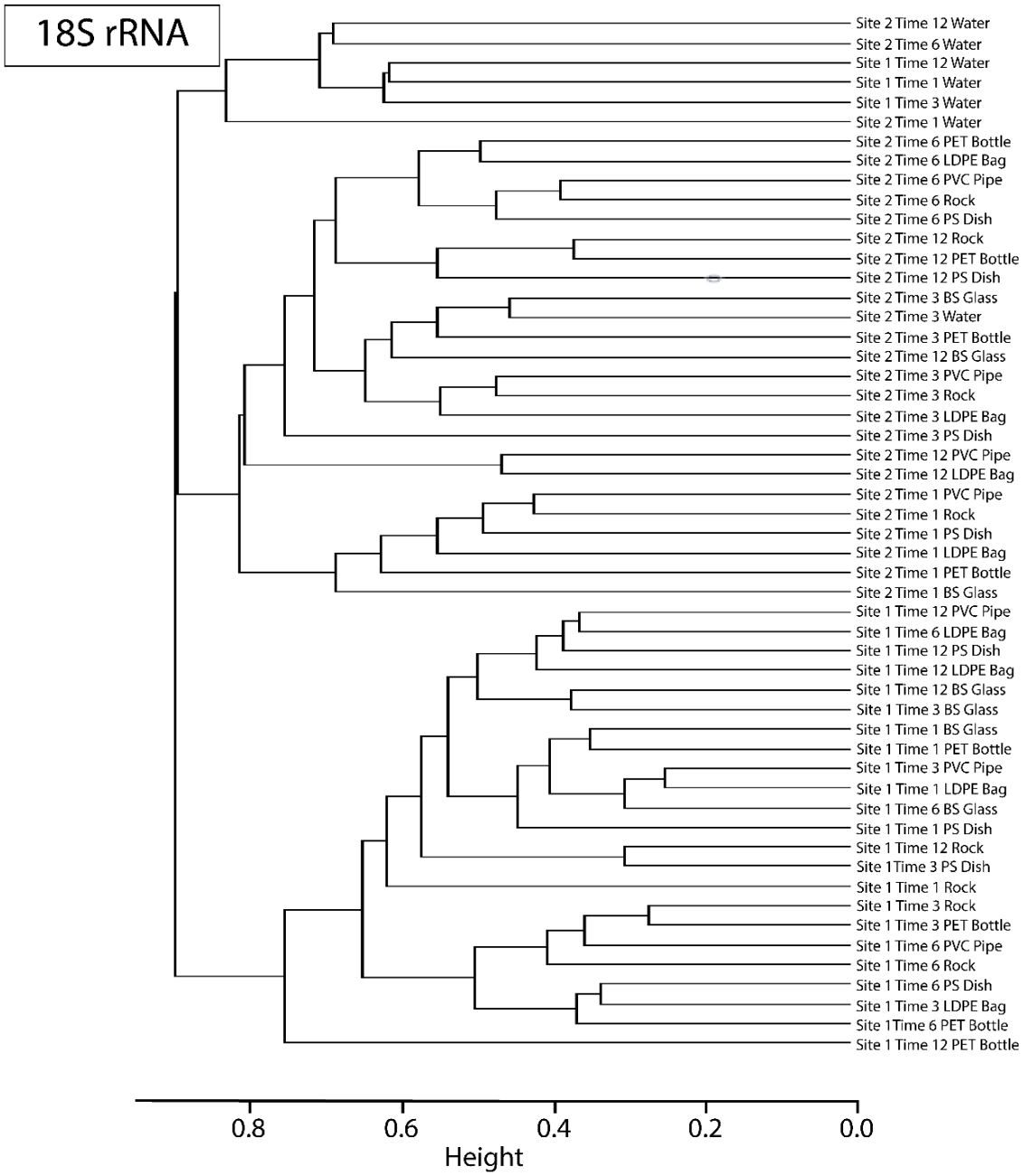

Figure S8. UPGMA dendrogram obtained from 18s rRNA cluster analysis of samples, using the Bray-Curtis distance measure.

8. Supplementary Material 2

The following supplementary material accompanies which details the taxonomic classification of all samples obtained from the sequencing of the gene region 16S rRNA can be downloaded from https://zenodo.org/record/6563214#.YoYUNqjP1D8

9. Supplementary Material 3

The following supplementary material accompanies which details the taxonomic classification of all samples obtained from the sequencing of the gene region 18S rRNA can be downloaded from https://zenodo.org/record/6563539#.YoYxuqjP1D8

CHAPTER 5 *EVOLUTION OF PROKARYOTIC COLONISATION OF GREENHOUSE PLASTICS DISCARDED INTO THE ENVIRONMENT*

ABSTRACT

Current knowledge on the capacity of plastics as vectors of microorganisms and their ability to transfer microorganisms between different habitats (i.e. air, soil and river) is limited. The objective of this study was to characterise the evolution of the bacterial community adhered to environmental plastics [low-density polyethylene (LDPE)] across different environments from their point of use to their receiving environment destination in the sea. The study took place in a typical Mediterranean intermittent river basin in Larnaka, Cyprus, characterised by a large greenhouse area whose plastic debris may end up in the sea due to mismanagement. Five locations were selected to represent the environmental fate of greenhouse plastics from their use, through their abandonment in soil and subsequent transport to the river and the sea, taking samples of plastics and the surrounding environments (soil and water). The bacterial community associated with each sample was studied by 16S rRNA metabarcoding; also, the main physicochemical parameters in each environmental compartment were analysed to understand these changes. The identification and chemical changes in greenhouse plastics were tracked using Attenuated Total Reflection Fourier Transform Infra-red spectroscopy (ATR-FTIR). Scanning Electron Microscope (SEM) analysis demonstrated an evolution of the biofilm at each sampling location. β-diversity studies showed that the bacterial community adhered to plastics was significantly different from that of the surrounding environment only in samples taken from aqueous environments (freshwater and sea) (p-value > 0.05). The environmental parameters (pH, salinity, total nitrogen and total phosphorus) explained the differences observed at each location to a limited extent. Furthermore, bacterial community differences among samples were lower in plastics collected from the soil than in plastics taken from rivers and seawater. Six genera (*Flavobacterium*, *Altererythrobacter*, *Acinetobacter*, *Pleurocapsa*, *Georgfuchsia* and *Rhodococcus*) were detected in the plastic, irrespective of the sampling location, confirming that greenhouse plastics can act as possible vectors of microorganisms between different environments: from their point of use, through a river system to the final coastal receiving environment. In conclusion, this study confirms the ability of greenhouse plastics to transport bacteria, including pathogens, between different environments. Future studies should evaluate these risks by performing complete sequencing metagenomics to decipher the functions of the plastisphere.

1. INTRODUCTION

The rapid development of synthetic polymers, the main constituent of plastics, caused revolutionary progress in the past century (Andrady and Neal, 2009). Plastics vary in chemical structure and can be manufactured in various shapes to meet the demand of multiple uses, including packaging, building, automotive, electronic, household and agriculture. A total amount of 368 million tons of plastics were produced in the world in 2019 to cover this demand, 9 million more than in 2018 (PlasticsEurope, 2020). The widespread use of plastic and improper post-consumer management disseminates plastic debris into the environment. Plastic debris acts as a persistent pollutant in receiving environments (Pazienza and De Lucia, 2020), such as terrestrial (Rillig and Lehmann, 2020; Baho et al., 2021), freshwater (Li et al., 2021d; Zhang et al., 2017) and marine ecosystems (Pattiaratchi et al., 2021; Lebreton et al., 2018). Low-density polyethylene (LDPE) is particularly interesting among thermoplastics since it is widely used for agricultural purposes. The European demand for LDPE is estimated at 8.85 million tons, the second most used plastic after polypropylene (PlasticsEurope, 2020). LDPE is the primary material used for protected cultivation in greenhouse plastics since it has relatively good mechanical and optical properties, extended useful life and a low price (Briassoulis, 2005). Greenhouse plastics are widely used in the Mediterranean, facilitating the all-year cultivation of vegetables (Saltuk, 2018). They fragment during in-service conditions making them functional for 1–4 seasons (Dehbi et al., 2017; Dilara and Briassoulis, 1998). The improper management of end of use greenhouse plastics generates high volumes of waste that usually get disposed of in fields, near water bodies or simply burnt. The problem arises when greenhouses are dismantled, producing a vast amount of plastic waste, estimated to have reached more than 850 million metric tons in 2019 globally (Afxentiou et al., 2021; Scarascia-Mugnozza et al., 2012). Ultimately, discarded greenhouse plastic debris finds its way to riverbeds and is eventually dragged into the sea.

The plastic interactions with receiving environments and when moving between environmental compartments are not fully understood yet. In this context, Bank and Hansson (2019) use the terms "biogeochemical cycle" and "plastic cycle" to describe the processes occurring when plastics move between compartments of the receiving environment. Understanding the "plastic cycle" is pivotal to identifying potential risks posed to the ecosystems from the trophic transfer of plastics (Cox et al., 2019; Latchere et al., 2021). Plastics are hydrophobic and are known to adsorb and then transport toxic chemicals such as PCBs, PBDEs, PAHs and DDTs (Wang et al., 2018). By modifying their structure, plastics

retain nutrients and microorganisms adhered to them, leading to an increasing C:N ratio in the long term due to their decom- position in the soil (Rillig et al., 2019). Plastics create a new type of habitat for the biota, mainly microorganisms, also known as the plastisphere (Zettler et al., 2013), which is prone to changes when moving between environmental compartments (Li et al., 2021a). Some studies examined the colonisation of plastics in soil (Puglisi et al., 2019); however, very limited information about the plastisphere continuum exists (Latchere et al., 2021).

This study aims to describe the bacterial greenhouse plastisphere during its lifecycle by characterising the evolution of the community from the time the greenhouse plastic is in use, discarded and transferred between soil, river and sea environment. Specifically, the bacterial community of LDPE is compared via microscopy and metabarcoding to the one of surrounding environments at (1) the point of use; (2) soils; (3) a riverbed at various distances from the point of use (both dry and water-covered riverbed) and (4) a sea site. To investigate whether plastics act as vectors of bacteria between environmental compartments, we hypothesise that the plastisphere differs from the bacterial communities of the receiving environments at each sampling location. To the authors' knowledge, no previous study investigates how the plastic-associated bacterial community changes during its lifecycle from its initial point of use towards receiving environments. This is the first study confirming that greenhouse plastics act as a vector for certain bacteria, thereby allowing the transfer of microorganisms between different environmental compartments.

2. MATERIAL AND METHODS

2.1. STUDY AREA AND SAMPLING STRATEGY

An intensive agricultural region representative of Mediterranean agricultural locations in the Larnaka district, Cyprus, was selected. It is located between Maroni and Zygi villages, with extensive greenhouse plastic use. Agriculture accounts for 2% of gross domestic product and 13.5% of national exports in 2019, an important part of the country's economy (Adamides, 2020). Cyprus has a typical Mediterranean climate, so farming methods are adapted to the high summer temperatures and limited water supply favouring its location by rivers and the prevalence of small and fragmented farm holdings, which promotes the development of small greenhouse exploitations primarily used for early horticultural crops (Adamides, 2020). In particular, the area selected (shown in **Figure S1 in Supplementary Material 1**) has an estimated cultivating area of 78.3 ha in 2016 and an estimated greenhouse plastic use of approximately 250 tons (Afxentiou et al., 2021). Sampling was carried out in

the Maroni river basin, a typical intermittent Mediterranean river, during the dry phase of the river.

Five sampling locations were selected to assess the changes of the bacterial communities during the greenhouse plastics transfer to the sea. G1 is a sampling location where greenhouse plastic is in use. CG2 is located 20 m from the greenhouse and 30 m from the river, where many greenhouse plastics were discarded. The R3 sampling location is 1.5 km downstream of CG2 in the dry riverbed. The R4 sampling location is 400 m downstream from R3 in the river delta next to the sea, where water was still available. Finally, the S5 sampling location is in the sea, 50 m from R4. More information about the location, type of sample collection and images of each sampling location is given in **Table S1 in Supplementary Material 1** and **Figure S1 in Supplementary Material 1**.

Large fragments of greenhouse plastics were present at the sampling locations. Three fragments (denoted as replicates) were randomly selected in each location and collected using sterile gloves. They were cut into smaller pieces using sterile scissors and stored in four sterile tubes. Plastics collected from soil (G1, CG2, and R3 plastics) were rinsed to remove soil particles using sterile Milli-Q water.

To evaluate the differences between the microbial communities of greenhouse plastics and the surrounding environment, samples were taken according to the following procedure: At G1, CG2, and R3, approximately 100 g of soil adjacent to plastics were taken and placed in sterile tubes for the metabarcoding analyses. For the rest of the analyses, 1 kg of soil was sampled and stored in a sterile plastic bag for further processing in the laboratory. At R4 and S5, 3 L of water were collected in sterilised glass bottles and kept in the dark. All the samples were collected on the same day (July 15th, 2019).

Immediately after sampling, all samples were transported to the laboratory at 4 ºC using cooling boxes. 1 L water was filtered through 2.7 μm glass Millipore filters to retain the particulate material in suspension. Subsequently, 250 mL of the filtered water was further filtered by 0.22 μm sterile membrane Millipore filters to collect the free-living microbial community. The process was repeated three times to obtain three replicates. Two tubes containing plastics were kept frozen at −20 ºC until performing DNA extraction, along with soil and filter samples. The two tubes were stored at 4 ºC to be used for further analyses, as explained below.

2.2. Nutrients and physicochemical analysis

In water samples (R4 and S5), the pH, temperature and conductivity were measured *in situ* using an ExStik II multiparameter probe (pH/conductivity EC500, Extech

Instruments, USA). Dissolved oxygen was measured using a Hanna HI98193 oximeter (Hanna Instruments, USA). Water from R4 and S5 was analysed for nutrients. Nitrate (NO_3^-), nitrite (NO_2^-), ammonium ions (NH_4^+) and orthophosphate ions (PO_4^{3-}) concentrations were measured using Spectroquant Tests (Merck Millipore, USA) following the instructions indicated by the manufacturer with a Spectroquant Pharo 100 spectrophotometer (Merck Millipore, USA). Total inorganic nitrogen of water samples (TIN) was calculated by summing NO_3^-, NO_2^- and NH_4^+ values. From G1, CG2 and R3 soils, pH, conductivity, bulk density, Total Organic Carbon (TOC), the total nitrogen (TN) and total phosphorus (TP) concentrations, and soil texture (which includes % sand, % silt, and % clay) were determined. The bulk density was measured *in situ*, collecting the sample in an aluminium tube of a given volume and measuring its weight. Soil samples of 1 kg were passed through a 2 mm sieve to remove large particles. In addition, 10 g of soil dispersed into 25 mL of Milli-Q water were used for pH measurements. A similar procedure was followed for conductivity, evaluating a soil/water suspension, but the ratio between soil and water was 1:4. The soil texture was assessed using particle size analysis based on the hydrometer method (Bouyoucos, 1962); the total organic carbon (TOC) was calculated using the loss on ignition method (Heiri et al., 2001); the total nitrogen was measured by the Kjeldahl method (Bremner, 1960), and the phosphorus concentration was determined using the sulfomolybdo-phosphate method (Tan, 1996).

2.3. Identification and assessment of weathering of greenhouse plastics using ATR-FTIR

The chemical composition of plastics was assessed using ATR-FTIR to ensure that the plastics collected at the different sampling locations were LDPE from greenhouses. The organic matter covering plastic specimens was removed by digestion with H_2O_2 (33% w/v) and heating at 60 ºC for 24 h. ATR-FTIR spectra were obtained using a ThermoScientific Nicolet iS10 apparatus with a Smart iTR-Diamond ATR module. Spectra were taken in the 4000–650 cm^{-1} range with a resolution of 4 cm^{-1} (data spacing of 0.483 cm^{-1}). A minimum of five spectra were taken per specimen at five different points. The spectra were compared with the library provided by the OMNIC Spectra software v 9.1.26 using Pearson's correlation (Aldrich and Goodfellow library, ThermoFisher Scientific Inc., USA). The minimum matching for identification was set to 80% (Rios Mendoza et al., 2018).

Three indexes were used to estimate the possible weather-related change in plastics between sampling locations according to previous research (Brandon et al., 2016): carbonyl, carbon-oxygen and hydroxyl index. These indices were calculated as the quotient of the peak

height of carbonyl groups (1550–1810 cm^{-1}), carbon-oxygen (1000–1200 cm^{-1}) and hydroxyl groups (region of 3300–3400 cm^{-1}) to a reference peak (2920 cm^{-1}), which corresponds to the C–H asymmetric stretching vibration (Brandon et al., 2016). Before calculating indexes, the spectral baselines were corrected (OMNIC Spectra software v 9.1.26).

2.4. Scanning electron microscopy analysis

The qualitative assessment of the biofilm structure and cellular integrity on greenhouse plastics was performed using SEM. Briefly, one randomly selected piece of plastic of 3 cm^2 (from the sterile tubes described in Section 2.1) was cut into smaller pieces and immersed in 4% paraformaldehyde solution for 30 min to fix the biofilm. Afterwards, the supernatant was removed and washed three times with 1X phosphate-buffered saline. Three replicates per sample were dried at room temperature overnight. The samples were gold-sputtered (32 nm thick films) using an SC7640 Sputter coater (Quorum Technologies, UK) and evaluated using a Quanta 200 microscope (FEI, USA).

2.5. Microbial diversity analysis

2.5.1. DNA extraction

Plastics of 10 cm^2 and water filters were cut into smaller pieces and transferred to 2-mL tubes. DNA extraction was performed based on a phenol:chloroform extraction method followed by absolute ethanol precipitation as previously described by Martínez-Campos et al. (2021). Briefly, 400 μL of Tris 10 mM – EDTA 0.1 mM (7.5 pH), 0.010 g of silica beads, 20 μL of 10% SDS and 250 μL hot ultrapure phenol (pH 8, 65 ºC) were added in each tube. The samples were then vortexed for 1 min and heated to 65 ºC for 1 min in three repeating cycles. 250 μL chloroform were added, and samples were vortexed and frozen 6 times. Finally, samples were centrifuged at 13,000 min^{-1} at 4 ºC for 20 min. The supernatant was transferred to a new Eppendorf 1 mL hot phenol (pH 8, 65 ºC) was added, and the tubes were centrifuged at 13,000 min^{-1} at 4 ºC for 5 min. This step was repeated once. Next, the supernatant was placed in a new Eppendorf, and 1 mL chloroform was added. The sample was mixed by shaking 10 times and centrifuged at 13,000 min^{-1} at 4 ºC for 5 min. Finally, the supernatant of the Eppendorf tubes that belonged to the same sample were mixed, and ethanol was added (double volume of ethanol than supernatant). The sample was then mixed and frozen at –20 ºC overnight to precipitate the DNA. The following day, the samples were centrifuged at 13,000 min^{-1} at 4 ºC for 20 min. Samples were dried, and 40 μL of Milli-Q water was added to resuspend the DNA, the concentration of which was measured spectrophotometrically (NanoDrop™ 1000 Spectrophotometer, Thermo-Scientific, USA).

2.5.2. METABARCODING

Twenty-three samples were used for DNA metabarcoding, including 15 greenhouse plastics (3 samples from each sampling location) and 8 samples from surrounding environments (soil, freshwater, and seawater). Library preparation was performed as instructed by the Illumina workflow at AVVA Pharmaceuticals (de Muinck et al., 2017). Briefly, two consecutive PCR reactions were performed using KAPA HiFi HotStart (KAPA Biosystems, USA). During PCR1, PCR amplicon was produced using 12.5 ng of DNA template and the following primers, including adaptor sequences: 16 S Amplicon PCR Forward Primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTA CGGGNGGCWGCAG) and 16 S Amplicon PCR Reverse Primer (5'GTCTCGTGGGC TCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) to amplify the 16 S V3 and V4 regions, respectively. PCR2 was performed by attaching dual indices and Illumina sequencing adaptors using the Nextera XT Index Kit. PCR clean-up was performed between PCR reactions using AMPure XP beads (Beckman Coulter, UK) according to the manufacturer's instructions. The final pool was sequenced on an Illumina MiSeq paired-end 2x250bp V3 sequencing programme.

2.5.3. BIOINFORMATICS AND DATA ANALYSIS

The analysis of the Illumina MiSeq results was performed using the DADA2 pipeline, which uses the amplicon sequence variants (ASV) (Callahan et al., 2017, 2016) using R v 3.6.2 (Rstudio, 2020). Briefly, quality profiles of the reads were evaluated using the plotQualityProfile function. Quality filtering, denoising, merging and removing chimeric sequences were applied to the dataset. Taxonomic assignment was performed using the Silva 132 99% OTU Database with a bootstrap threshold of 75% (Callahan, 2018).

α-diversity analysis, including the Gini Index (Gini, 1912) and Shannon Diversity Index (Shannon, 1948), was performed via alpha-Diversity function from the otuSummary package (Yang, 2018). The Gini coefficient is a ratio between 1 and 0, measuring the inequality, whereas the Shannon index calculates species uniformity. The differences found between samples were estimated using the Kruskal-Wallis statistic method, and results were plotted using ggplot2 v 3.3.2 function of the tidyverse package (Wickham et al., 2019).

For β-diversity analysis, two methods were employed to compare the similarity of bacterial communities among samples. First, a hierarchical treemap based on the Bray-Curtis similarity matrix (Beals, 1984) was combined with a heatmap based on ASV abundance using the hclust function from the stats package (Team, 2013) to identify the most similar samples. The significant differences between samples (confidence interval 95%) were assessed using

permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001), considering 999 permutations.

Distance-based redundancy analysis (db-RDA) was performed to establish the correlation between environmental parameters and the bacterial community attached to each substrate using the dbrda function of the vegan package (Oksanen et al., 2013). The analysis was performed based on the Bray-Curtis similarity matrix. The environmental factors considered were nitrogen, phosphorus, salinity and pH. To perform a linear regression analysis, the function envfit of the vegan package was used. Envfit shows the maximum correlations between environmental variables and the ordination configuration. The length of the vectors represents the strength of the correlations (Oksanen et al., 2013). The "anova.cca" function of the vegan package (Oksanen et al., 2013) with 999 permutations was used to perform the significance test of db-RDA. The linear discriminant analysis effect size method (LEfSe) (Segata et al., 2011) was used to determine the differentially more abundant taxa (up to genus level) in sampled plastics and their surrounding environments. This analysis was performed with the LEfSe online tool available in the Galaxy framework, using default settings for data formatting. LDA (Linear discriminant analysis) effect size was performed using the strategy for multi-class analysis one-against-all.

Venn diagrams mine the plastics' common and unique bacterial genera at different sampling locations. The same method was applied to evaluate bacterial communities' changes between plastic substrates and their surrounding environments at each sampling location using the "Bioinformatics & Evolutionary Genomics" tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

2.5.4. Accession number

The sequences data obtained in this study were submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under the Bioproject accession number: PRJNA747817.

3. Results

3.1. Spectroscopic analyses

Except for plastics taken directly from the greenhouse at G1, the selection of plastics was carried out in situ by visual inspection. The identification of the plastics was confirmed using ATR-FTIR spectroscopy. All spectra (**Figure 1**) showed characteristic absorption bands at 2915 cm^{-1} and 2848 cm^{-1} (CH_2 asymmetrical and symmetrical stretching), at ~ 1460 cm^{-1} (CH_2 bending), a small absorption band at 1370 cm^{-1} (bending of $-CH_3$ terminal groups

that only appeared in LDPE) and a double band in the region of ~ 728 cm^{-1} and ~ 718 cm^{-1} (corresponding to CH_2 rocking deformation in the amorphous phase and crystalline phase respectively). These are the native bonds present in LDPE (Rajandas et al., 2012). Pearson correlations (**Table S2 in Supplementary Material 1**) confirmed this result with a matching of over 80% in all samples. Some small peaks between ~ 1550–1810 cm^{-1} correspond to carbonyl stretching vibration. A broad absorption band of vibrations at 1037–1012 cm^{-1} indicates a C–O stretching vibration. Both result from the oxidation of the backbone of LDPE.

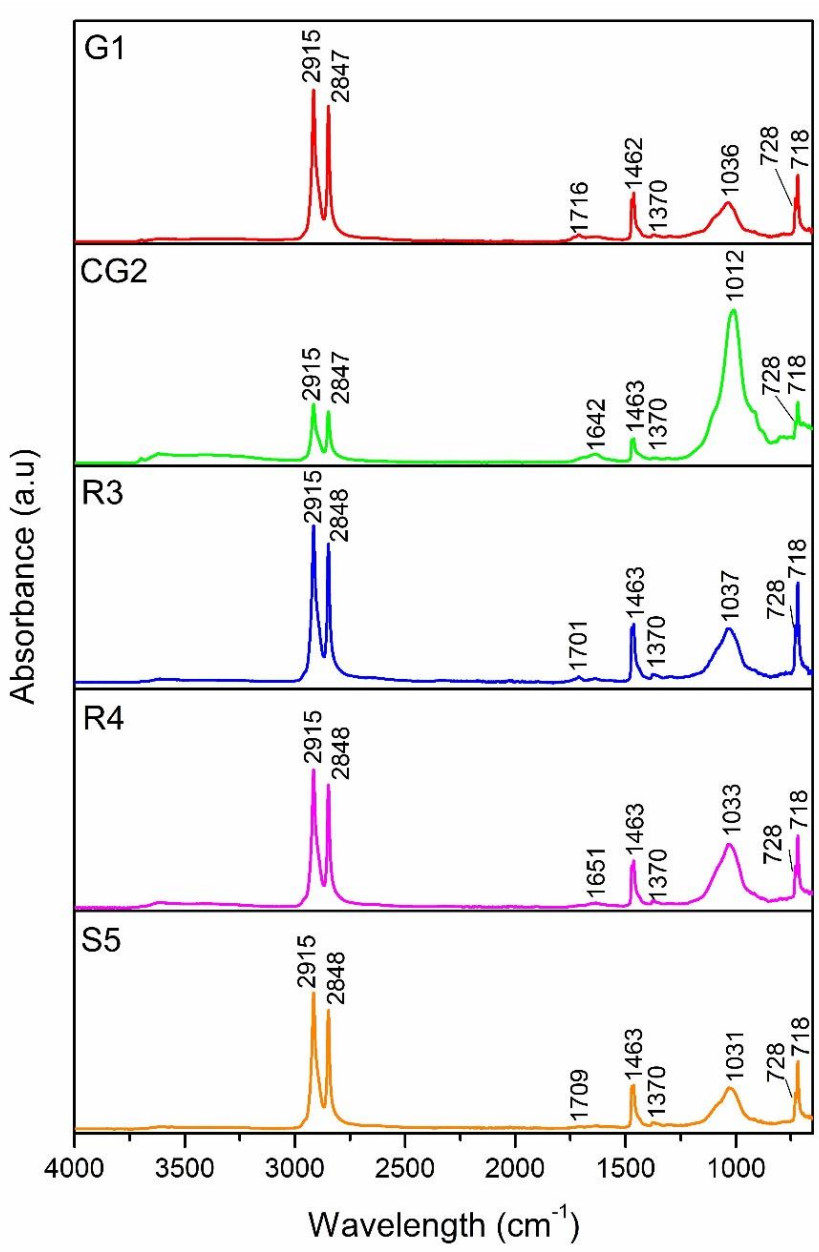

Figure 1. ATR—FTIR spectra of plastics collected in each sampling location: G1: greenhouse sampling location; CG2: sampling location close to the greenhouses; R3: dry riverbed; R4: end of the river near the sea; S5: sea, near the shoreline.

The weathering indices (carbonyl groups, carbon-oxygen, hydroxyl, shown in **Table S2 in Supplementary Material 1**) indicate major photo-oxidation of CG2 plastics (summation of three indices = 1.97) followed by R4 and R3 plastics (0.79 and 0.66, respectively). S5 and G1 plastics were the least photo-oxidated (0.56 and 0.5, respectively). However, the hydroxyl index does not indicate a substantial plastic degradation, in contrast with the carbonyl groups and carbon-oxygen ratios.

3.2. VISUALISATION OF BACTERIAL COMMUNITIES ONTO GREENHOUSE PLASTICS

A detailed examination using Scanning Electron Microscopy (**Figure 2**) confirmed the presence of microbial communities and intact microorganisms on the surface of the plastics. Collected plastics showed fouling covering the surface (**Figure S3 in Supplementary Material 1**). The fouling on plastics increased as the distance from G1 increased, from G1 plastics to the plastic sample in the sea (S5 plastics). At the same time, the abundance of microbes and their distribution on the plastics changed substantially between sampling locations. In addition, a smooth surface primarily characterised G1 plastics with the scattered presence of crystalline structures and diatoms. A true biofilm was not observed, but coccoid- and rod-shaped bacteria embedded in extracellular polymeric substances (EPS) could be seen in hollows around the crystals. Moreover, CG2 plastics had a higher number of crystals, and a clear biofilm spread over the entire surface of the plastic. The biofilm density prevented the clear visualisation of microorganisms embedded in it. R3 plastics had thick inorganic fouling covering all surfaces. The presence of biofilm was limited to the cracks and holes generated in this inorganic fouling. A very dense microbial community was present on the surface of submerged R4 and S5 plastics. Rod-shaped bacteria, diatoms and fungal hyphae dominated R4 plastics. The inorganic fouling forming small crystals was more significant over the biofilm. S5 plastics had a mature biofilm, with a major dominance of Vibrio-shaped bacteria embedded in EPS with a relatively rough surface. The overall biofilm extent was more significant in the greenhouse plastics submerged in water (R4 and S5 plastics) compared to plastics collected from soil (riverbed, CG2 and R3 plastics) or in use (G1 plastics).

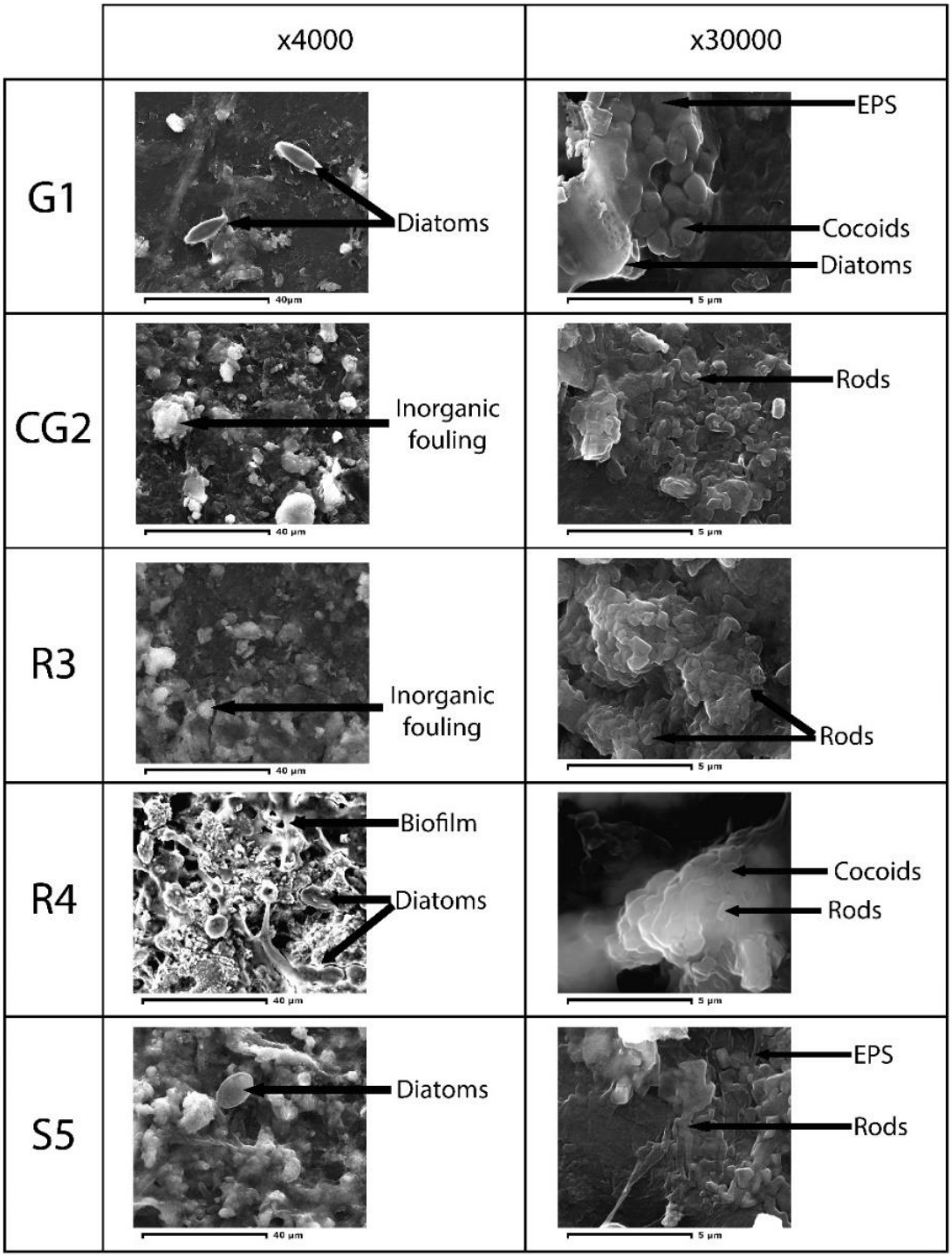

Figure 2. Scanning electron microscope images of plastics collected in each sampling location. The first column shows lower magnification to appreciate the development of the biofilm. The second column showed the presence of micro-organisms in larger magnification. Legend of sampling locations: G1: greenhouse sampling location; CG2: sampling location close to the greenhouse; R3: dry riverbed; R4: end of the river near of the sea; S5: sea near the shoreline. Abbreviations meaning: EPS, extracellular polymeric substances.

3.3. METABARCODING OF GREENHOUSE PLASTIC BACTERIAL COMMUNITIES AND SURROUNDING ENVIRONMENTS

3.3.1. SEQUENCING DATA PRE-PROCESSING AND ASVs ASSIGNMENT

Each sample produced at least 65,023 reads after Illumina sequencing, with a total of 3409,254 reads for all samples. After removing the adaptors, filtration of the sequences, merging, and removal of possible chimeras, 1,148,609 high-quality reads remained. The remaining sequences were associated with 8829 ASVs.

3.3.2. α-DIVERSITY ANALYSIS

Bacteria diversity was initially assessed using the Gini coefficient and Shannon index (**Figure 3**). Gini coefficient results were higher than 0.95 (the lower value corresponding to freshwater with 0.96 ± 0.01), indicating that specific taxa dominated the bacterial community. Global ANOVA indicated significant differences between samples (p-value < 0.05) but the pairwise comparison only demonstrated significant differences (ANOVA p-value < 0.05; **Table S3 in Supplementary Material 1**) between R4 plastics and freshwater. This suggests a lower bacterial community diversity associated with plastics than the surrounding freshwater environment. The values obtained by the Shannon index were, in general, slightly lower for plastics (average value of 4.28 ± 0.53) compared to the surrounding environment (average value of 4.78 ± 0.68). Despite that, no significant differences were found in the global ANOVA (p-value > 0.05) or the pairwise ANOVA tests (**Table S4 in Supplementary Material 1**).

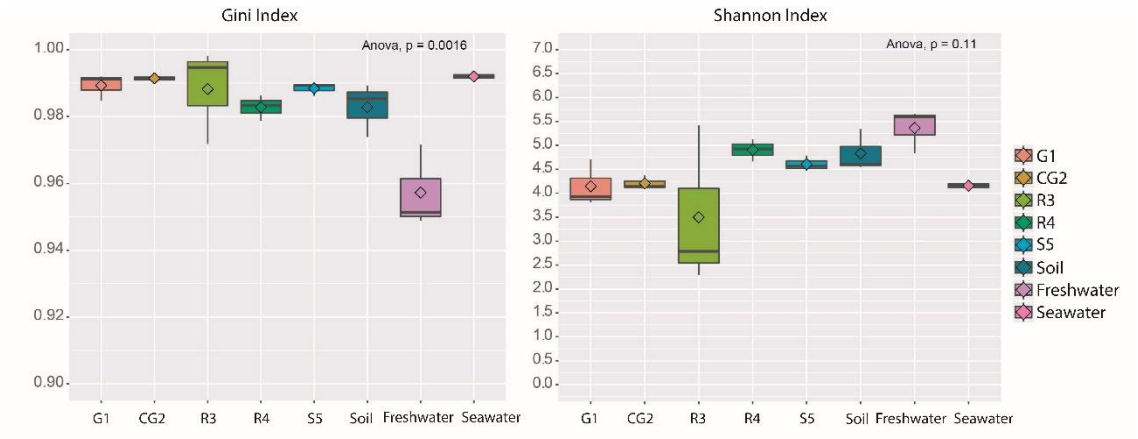

Figure 3. Results of the α-diversity analysis using Gini Index and Shannon Index in the plastics collected from each sampling location (G1, CG2, R3, R4, and S5) in comparison with the surrounding environment of each sampling location: soil (from G1, CG2, and R3), river freshwater (R4) and seawater (S5).

3.3.3. BACTERIAL COMMUNITY COMPOSITION

All obtained ASVs were compared with SILVA 132 database to obtain its taxonomy classification. Forty-two bacterial phyla classified in 85 classes were identified in the whole sample set. The complete taxonomical assignation can be found in **Supplementary Material 2**.

Bacterial taxonomy distribution at the phylum level in all samples is presented in **Figure 4**. The most abundant phyla, independent of the type of sample (plastic, soil, or water) or sampling location, were Proteobacteria (43.6%) followed by Bacteroidetes (16.3%), Cyanobacteria (13.6%), and Actinobacteria (10.9%). Although Proteobacteria were dominant in most collected plastics (G1, CG2, R4, and S5 plastics), Cyanobacteria predominated on R3 plastic (40.6%). Regarding surrounding environmental samples, the soil had Proteobacteria (36.87%), Cyanobacteria (17.3%), and Bacteroidetes (15.8%). The phyla Proteobacteria (35.49%), Bacteroidetes (26.5%), and Pastecibacteria (23.22%) were more abundant in freshwater. The most abundant phyla in seawater were Proteobacteria (56.92%), Bacteroidetes (26%), and Epsilonbacteraeota (7.6%).

The most abundant classes detected in plastics, independently of the sampling location, were Alphaproteobacteria (39%), Oxyphotobacteria (16.7%), Actinobacteria (14.6%), and Bacteroidia (11.33%). Alphaproteobacteria (19.1%), Oxyphotobacteria (17.2%), Bacteroidia (15%), and Gammaproteobacteria (14.4%) were dominant in the soil samples. Bacteroidia (26.3%), Gammaproteobacteria (18.6%), Alphaproteobacteria (15.45%) and Parcubacteria (15.45%) were dominant in freshwater. In seawater, the classes with the highest abundance were Alphaproteobacteria (34%), Bacteroidia (25.5%), Gammaproteobacteria (22.8%), and Campylobacteria (7.6%).

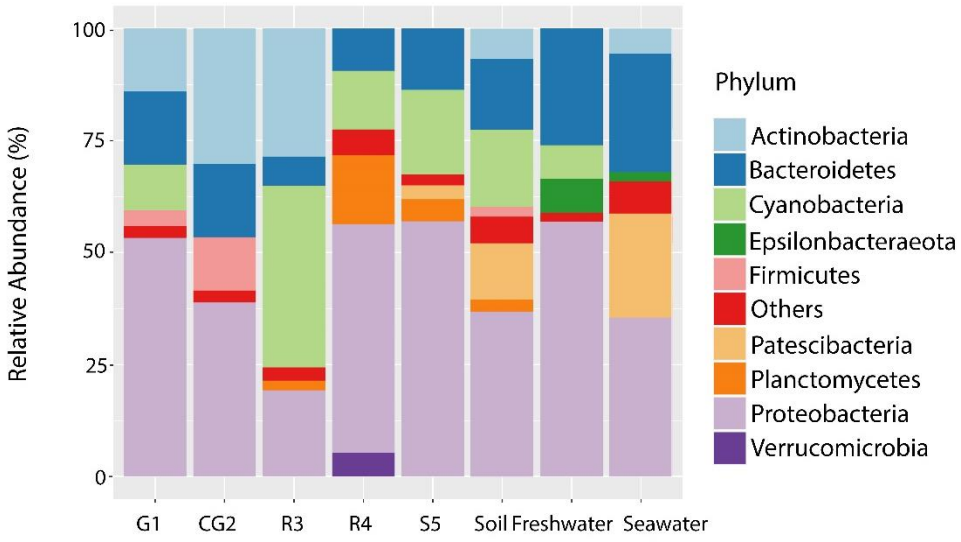

Figure 4. Relative abundance of prokaryotic community based on 16S rRNA metabarcoding at the phylum level.

At the order- and the family- levels, the bacterial distribution differed between the plastics and the surrounding environments at each location. G1 plastics were colonised by the orders Rhizobiales (28.4%; represented mainly by the family Beijerinckiaceae with a 26.9% abundance), Sphingomonadales (14.5%; family Sphingomonadaceae), and Cytophagales (13.4%) represented by the families Hymenobacteraceae (11.81%) and Spirosomaceae (1.6%). On CG2 plastics, the orders with the highest abundance were Cytophagales (15.6%; family Hymenobacteraceae represented 15.5% of total abundance), Micrococcaceae (14.8%; Micrococcaceae represented 13.0%), and Sphingomonadales (12.8%) represented primarily by Sphingomonadaceae (12.8%). The orders Kineosporiales (17.0%; represented by family Kineosporiaceae), Frankiales (5.3%; family Geodermatophilaceae: 4.8%), Sphingomonadales (5.1%; totally represented by the family Sphingomonadaceae) had a higher relative abundance on R3 plastics. The surrounding soil environment was dominated by Saccharimonadales (7.7%), Rhodobacterales (6.9%; represented by the family Rhodobacteraceae: 6.8%), and Flavobacteriales (6.8%; represented by Flavobacteriaceae: 5.1%). On R4 plastics, a substantial change could be observed in the taxonomical distribution at the order level, with the higher abundances of Rhodobacterales (25.2%; family Rhodobacteraceae: 25.24%), Sphingomonadales (13.3%; family Sphingomonadaceae: 13.3%), Pirellulales (12.0%; family Pirellulaceae: 11.9%). In freshwater, the orders with the highest presence were Flavobacteriales (19.5%; family Cryomorphaceae: 12.8%), Rhodobacterales (10.36%; Rhodobacteraceae: 10.4%), and Betaproteobacteriales (9.7%). The bacterial community of S5 plastics was represented by Rhodobacterales (36.8%; family Rhodobacteraceae: 36.8%), Rhizobiales (10.1%; family Rhizobiaceae: 8.8%), and Phormidesmiales (9.8%; family Phormidesmiaceae: 9.8%). In seawater, the orders with significant abundance were Flavobacteriales (25.1%; family Flavobacteriaceae: 18.9%), Vibrionales (13.9%; family Vibrionaceae: 13.9%), and Rhodospirillales (11%).

3.3.4. β-DIVERSITY ANALYSIS

The differential bacterial taxonomy distribution suggests significant variations between the plastics at each location and between the plastics and their surrounding environments (soil, freshwater, or seawater). The distribution of the samples is presented in the db-RDA ordination plot (**Figure 5**). In general, the plastics were more distant between different sampling locations than their surrounding environment, showing a consistent pattern depending on where the plastics were collected (soil, freshwater, or seawater). Global PERMANOVA analysis (**Table S5 in Supplementary Material 1**) confirmed significant differences between all samples (p-value < 0.05). Furthermore, the PERMANOVA test

comparison between the plastics and the surrounding environments (PERMANOVA *p*-value < 0.05) also confirmed significant differences. Plastics collected from soil showed minor differences, distributed along the second axis (8.9%). Replicates from G1 and CG2 plastics were ordinated together, constituting the same cluster without significant differences between them (pairwise PERMANOVA test *p*-value = 0.07), but significantly different from R3 plastic (pairwise PERMANOVA test *p*-value < 0.05). R4 and S5 plastics were distributed along the first axis (15.3%), denoting a major difference in comparison with the greenhouse plastics collected from soil (pairwise PERMANOVA test *p*-value < 0.05) and significantly different between them (pairwise PERMANOVA test *p*-value < 0.05). Furthermore, pairwise PERMANOVA comparison between plastic and their corresponding environment indicated that G1, CG2, and R3 plastic bacterial communities were not significantly different from soil (pairwise PERMANOVA *p*-value > 0.05). In contrast, R4 and S5 plastics had significantly different bacterial communities than their surrounding environment (*p*-value < 0.05). The hierarchical clustering tree based on the Bray-Curtis matrix (**Figure S4 in Supplementary Material 1**) confirmed these results. To explain the possible influence of environmental parameters in the evolution of the bacterial community adhered to greenhouse plastics, the environmental variables measured in the soil (**Table S6 in Supplementary Material 1**) and the water (**Table S7 in Supplementary Material 1**): pH, salinity, total nitrogen, and total phosphorus were used in the db-RDA analysis. In general, locations in dry conditions were characterised by higher values of salinity and pH (G1, CG2, and R3), in contrast with the R4 and S5 with higher values of nutrients (total nitrogen and phosphorus). The high nitrogen values at R4 explained the eutrophication observed during the sampling day. The analysis (**Table 1**) confirmed a significant influence of the pH, TN, salinity, and TP (*p*-value < 0.05). The environmental parameter with the most significant influence was pH (7.9% of explained variation), followed by TN (3.75%), salinity (3.6%), and TP (1.8%). The model only explained 19.6% of the variation, suggesting a low correlation between samples (summation of the explained variables had comparable values).

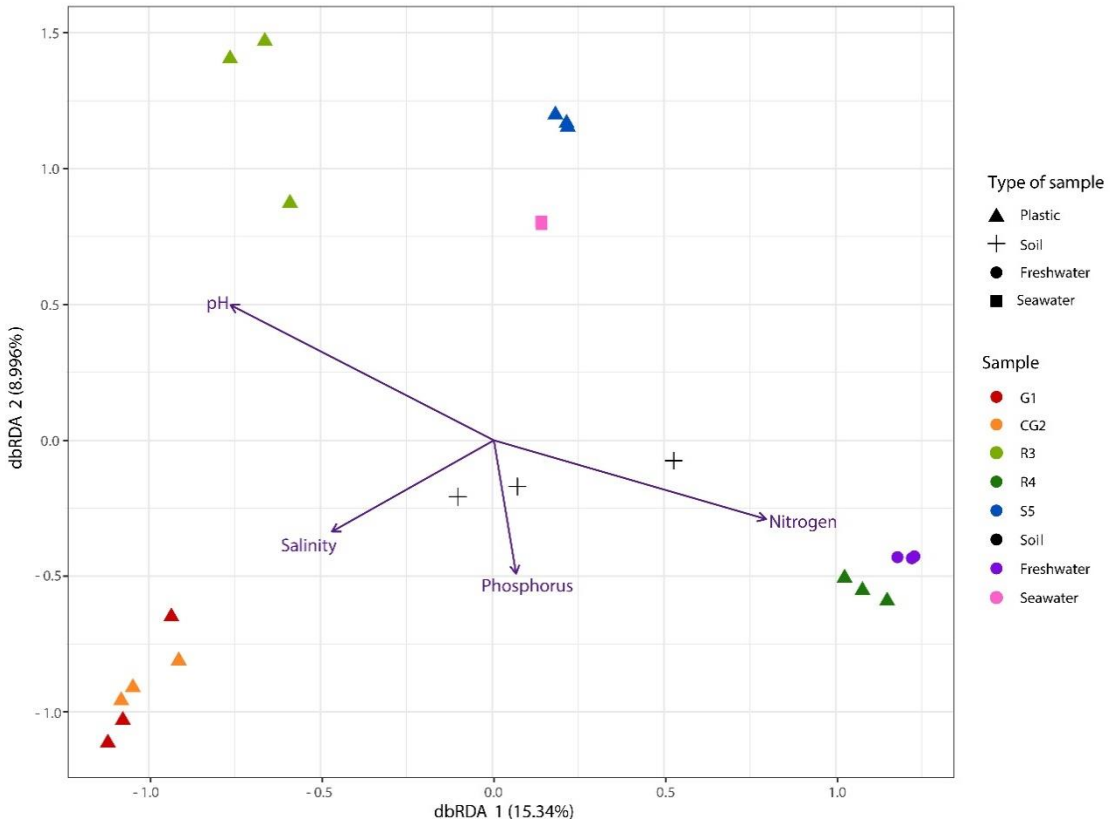

Figure 5. Distance-based redundancy analysis (dbRDA) ordination plot based on Bray-Curtis dissimilarity of 16S rRNA metabarcoding and environmental variables between the different environments selected in this study (soil and water). Each point in the ordination plot represents the community in a given sample.

Table 1

Adjusted percentage of proportion variation explained by each variable in separate db-RDA analysis (gross effects). The total consideration in a single db-RDA model includes all variables (pure effects). The significance of explained variation was tested using the Monte-Carlo test with 999 permutations.

Environmental factor	Df	Sum. of squares	F	*p*-value	The proportion of explained variation adjusted (%)
Salinity	1	0.78	2.18	0.009	3.57
pH	1	1.12	3.10	0.001	7.93
Phosphorus	1	0.58	1.61	0.05	1.76
Nitrogen	1	0.99	2.48	0.001	7.50
Residual	18	6.51	---	------	-----
Total	22	-----	---	------	19.61

LEfSe analysis of the plastic bacterial communities at each sampling location (**Table S8 in Supplementary Material 1**) revealed significant differences in the abundance of some genera. G1 plastics were dominated by *Methylobacterium*, *Sphingomonas*, *Frigobacterium*, *Pantoea*, *Weisella*, *Corynebacterium*, *Bacillus*, *Turicibacter*, *Curtobacterium, Jeotgalicoccus,* and *Clostrium_sensu_stricto_1*. The genera *Hymenobacter*, *Arthrobacter*, *Massilia*, *Kocuria*, *Paracoccus*, *Planomicrobium*, *Modestobacter*, *Kineococcus*, *Kineosporia* and *Rhizorhapis* were more abundant on the CG2 plastic. On R3 plastic, the genera with higher abundance were *Geodermatophilus*, *Nocardiopsis, Marmoricola, Quadrisphaera*, *Roseomonas*, *Blastococcus*, *Skermanella*, *Tepidisphaera*, *Pseudomonas* and *Actinomycetospora*. Plastics collected from the freshwater aquatic environment (R4 plastics) had a higher abundance of specific taxa, including *Porphyrobacter*, *Rhodopirellula*, *Tabrizicola*, *Rubribacterium*, *Ketogulonicigenium*, *Luteolibacter*, *Sandaracinobacter, Sandarakinorhabdus, Germmobacter, Terrimicrobium, Rhodobacter, Legionella* and *Runella*. This result coincides with that obtained in the beta diversity analysis, which shows a greater difference in the bacterial community R4 plastic than the other plastic samples. In contrast, on S5 plastics, the genera *Rubrivirga*, *Maribius, Loktanella, Lewinella, Pseudahrensia, Parvularcula, Erythrobacter, Algimonas, Truepera* and *Granulosicoccus* were dominant.

Furthermore, LEfSe analysis was used to determine differentially abundant genera between each plastic and its surrounding environment. The bacterial community attached to G1 plastic (**Table S9 in Supplementary Material 1**) was characterised by *Rhizorhapis, Jeotgalicoccus, Fructobacillus, Romboutsia, Aureimonas, Turicibacter, Emticicia,* and *Rhodococcus*. Genera *Hymenobacter, Arthrobacter, Methylobacterium, Planococcus, Sphingomonas, Planomicrobium, Roseomonas, Modestobacter, Kineococcus, Geodermatophilus* and *Marmoricola* were enriched in biofilms colonised on CG2 plastic (**Table S10 in Supplementary Material 1**). On R3 plastic (**Table S11 in Supplementary Material 1**), the characteristic genera were *Geodermatophilus, Methylobacterium, Nocardiopsis, Marmoricola, Hymenobacter, Roseomonas, Fiedmanniela, Arthrobacter, Aquipuribacter, Blastococcus* and *Rhodococcus*. In soil locations, the genera *Acinetobacter*, *Micrococcus*, *Delftia* and *Acidibacter* were more abundant (**Tables S9–S11 in Supplementary Material 1**).

The plastics collected in the aquatic environments showed a significant number of specific genera constituting the plastisphere than the soil plastic. Genera *Porphyrobacter, Rhodopirellula, Tabrizicola, Rubribacterium, Algoriphagus, Ketogulonicigenium, Luteolibacter, Sandaracinobacter* and *Roseomonas* were significantly more abundant on the R4 plastic (**Table S12 in Supplementary Material 1**). In contrast, the characteristic taxa in freshwater were *Fluviicola, Sediminibacterium, Limnobacter, Hydrogenophaga, Rheinheimera, Arcobacter, Perlucidibaca*, *Vogesella, Flavobacterium, Marivivens* and *Vibrio*. Bacterial communities in S5 seawater (**Table**

S13 in Supplementary Material 1) were dominated by *Vibrio, Arcobacter, Formosa, Catenococcus, Nereida, Shimia, Phaedactylibacter, Marinomonas, Reichenbachiella* and *Fluviicola*. In contrast, S5 plastics were dominated by *Rubrivirga, Maribius, Loktanella, Lewinella, Perudabrensia, Parvularcula, Erythrobacter, Aquimarina, Algimonas and Nonlabens.*

3.3.5. Plastic-associated bacterial genera

The Venn diagram presents the number of specific genera and those shared among the greenhouse plastics (**Figure 6**). The results demonstrate that many genera are unique to a single plastic, indicating that the bacterial community attached to the plastic in each sampling location was different. Additionally, some genera were shared between the different sampling locations. Specifically, 29 genera (**Table S14 in Supplementary Material 1**) were common between the dry stations (G1-CG2-R3), highlighting the presence of *Kineococcus, Fibrella, Blastocatella, Novosphingobium, Rhodocytophaga, Dyadobacter, Aureimonas, Solirubrobacter, Rathayibacter, Pseudoclavibacter, Pantoea, Streptococcus, Friedmanniella, Staphylococcus, Stenotrophomonas, Bacillus, Aeromicrobium, Rhizorhapis, Variovorax, Lactobacillus* and *Salana* as dominant.

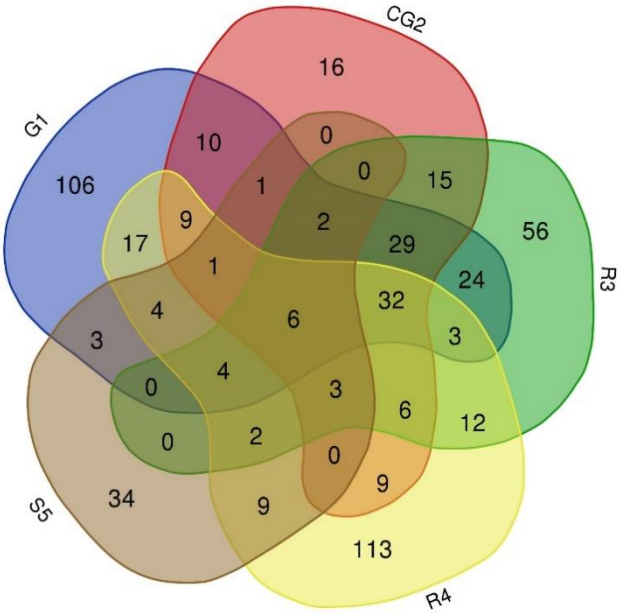

Figure 6. Venn diagram obtained using the taxonomy assignment at the genus level. The figure is the analysis chart of the five plastics in the different sampling locations. Legend of sampling locations: G1: greenhouse sampling location; CG2: sampling location close to the greenhouse; R3: dry riverbed; R4: end of the river near the sea; S5: sea, near the shoreline.

Thirty-one genera were shared between G1, CG2, R3, and R4 plastics, including *Brevundimonas*, *Pedomicrobium*, *Lamia*, *Chryseobacterium, Nocardiopsis, Roseomonas, Pseudomonas, Blastococcus, Spirosoma, Luteimonas, Sphingomonas, Geodermatophilus, Deinococcus, Kocuria, Paracoccus, Modestobacter, Pedobacter, Microvirga, Massilia, Arthrobacter, Rubellimicrobium, Pseudorhodobacter,*

Skermanella, Hymenobacter and *Devosia*. The S5 plastics had a lower number of shared genera. Only six genera were identified in all the plastics samples, constituting the plastic core bacteriome between the sampling locations. These genera were *Flavobacterium, Georgfuchsia, Acinetobacter, Pleurocapsa_PCC-7319, Altererythrobacter* and *Rhodococcus*.

Furthermore, the Venn diagrams detect the common genera between plastics with their surrounding environment (**Figures S4–S8 in Supplementary Material 1**). There was a high percentage of common genera between the plastics and their surrounding environments at G1, CG2, R3, and R4 except for S5 plastics with only 19 genera in common with seawater (**Table S15 in Supplementary Material 1**).

4. DISCUSSION

Our study provides novel information about the evolution of the bacterial assemblages on greenhouse plastics along their life cycle: from the time of use to the time the plastic ends up in the sea. Previously, a meta-study investigated the bacterial assemblages present on microplastics in different environments, collecting the data from previous experiments (Wright et al., 2021). As suggested by the authors, the evident problem was that the experimental process, such as DNA extraction, selection of primers and the time of plastic incubation in the environment, can impact the final reported results, making comparisons very challenging. Furthermore, the studies that include different habitats where incubation experiments are performed (Martínez-Campos et al., 2021, De Tender et al., 2017, Puglisi et al., 2019) use artificially aged plastics (Dussud et al., 2018) or the origin of the plastic is unknown (Wu et al., 2020, Puglisi et al., 2019).

The weathering of plastics was assessed using the degradation indices defined above. The appearance of a broad band centred at 1030 cm^{-1} relates to the oxidation reactions under natural weathering facilitated by the loss of polymer stabilisers (Scoponi et al., 2000). The degradation indexes implied higher degradation for CG2 plastic. This is consistent with the loss of stabilisers in LDPE discarded after its useful life. The fact that specimens collected along the riverbed and in the shoreline displayed lower degradation bands may be explained because, once in the environment, the plastic loses superficial layers. This assumption is supported by data showing that microorganisms from soil can biodegrade the superficial layers of plastics (Chamas et al., 2020, Li et al., 2021a) and the backbone photochemical oxidation induced by the ultraviolet radiation from sunlight can favour the biodegradation of plastics (Tribedi and Dey, 2017).

The capacity of ATR-FTIR to penetrate the samples is typically 0.5–2.0 μm (Mirabella, 1992). Accordingly, the loss of superficial layers can expose less weathered plastic

explaining the lower indices for samples exposed to the environment for a longer time. In their journey to the sea, as SEM images detected, the plastics may be covered with soil and later by water. For this reason, the degradation increased between sampling locations, although it did not yield a higher value than that of CG2 plastic. SEM images detected a significant inorganic layer covering the plastic surface.

Recent studies suggest that the photosynthetic degradation of LDPE in aquatic environments releases microplastics and other chemical compounds, dependent on the possible additives associated with the plastics with a substantial toxicity effect on the environment (Walsh et al., 2021). Our findings indicate a significant degradation of LDPE in terrestrial ecosystems. This process, combined with the fact that plastics are transported to rivers and later to the sea, can act synergistically, contributing significantly to the release of harmful substances to aquatic ecosystems. Additionally, our study confirms that biofilm development slows the degradation produced by sunlight, confirming the hypothesis proposed by Walsh et al. (2021).

Our study provides evidence that plastics represent a habitat that selects, to a certain extent, the bacteria that are attached to them. We found a slightly lower diversity in the plastics compared to their surrounding environments, although this was not statistically significant. In agreement with our observations, different studies have shown that when compared to a different environment such as landfill or aquatic ecosystems, lower α-diversity is observed on plastics (McCormick et al., 2014, Puglisi et al., 2019).

β-diversity analysis shows that the bacterial community attached to plastics evolves as the greenhouse plastics move towards the sea, except for G1 and CG2 plastics because these locations are similar biotopes. This confirms the findings of previous studies that location is the most important factor affecting the variation of plastic-associated bacterial communities. (Amaral-Zettler et al., 2020, Wright et al., 2021, Martínez-Campos et al., 2021). In this study, we also address the influence of environmental factors in modifying the microbial community adhered onto plastic, resulting in pH being the most relevant factor. pH was described previously as an important factor in the bacterial community developed in plastics located in soil (Li et al., 2021c). This can explain the difference between G1 and CG2 plastics and R3 plastics. Furthermore, nitrogen concentration was the second environmental factor with a key impact on the changes in the bacterial community diversity. The concentration of nitrogen-related ions was confirmed previously as an environmental factor that significantly affects the plastisphere in freshwater and seawater ecosystems (Li et al., 2021b). Still, this study confirms its influence on dry environments. The high concentration

of nitrates in R4 freshwater can explain the main difference of this plastic compared to the rest. The high concentration of nitrate in the R4 may be due to the intense fertiliser application in all the peripheral crops. Moreover, the river water was largely stagnant in the dry season, without any water renewal. The effect of fertilisers in increasing nitrogen concentration in rivers has been previously demonstrated (Lassaletta et al., 2009). Phosphorus was considered another important factor affecting the community attached to the plastisphere (Amaral-Zettler et al., 2020), and this study corroborates this hypothesis. Furthermore, our study confirmed the potential role of salinity in affecting the plastisphere's community composition, as previous studies denoted in different marine ecosystems (Oberbeckmann and Labrenz, 2020). Our study, which includes dry sampling locations (G1, CG2, R3) and freshwater (R4), denotes that the effect of this factor was not only limited to marine ecosystems but also influenced terrestrial and freshwater environments.

The significantly more abundant genera identified by Lefse in the microbial communities of the plastics at each location played different roles in the maturation of the biofilm. They adapt to their environment, confirming an evolution of the LDPE-associated microbial community as greenhouse plastic are transferred between sampling locations.

On G1 plastics, where the biofilm is in the first stage, more abundant genera were associated with this process. The high abundance genus *Methylobacterium*, usually implicated in the phyllosphere (Green and Ardley, 2018), can be explained because it was found as the primary coloniser in the plastisphere in aquatic ecosystems (Purohit et al., 2020). Furthermore, the presence of the genera *Sphingomonas* (Bereschenko et al., 2010, Martínez-Campos et al., 2018) and *Jeotgalicoccus* (Arti et al., 2020) are associated with the first stages of the formation of the biofilm under high salinity conditions, producing the EPS, which facilitates the adhesion and colonisation of other microorganisms over the plastic. The presence of primary producers, such as *Calothrix_KVSF5* and *Chamaesiphon_PCC_7430*, can stimulate biofilm growth and develop complex bacterial communities (Yokota et al., 2017). Also, the genus *Calothrix* produces microcystin (Shardlow, 2021), which could be toxic when the plastic arrives in the aquatic environment. Lastly, the presence of *Corynebacterium* can indicate the initiation of LDPE biodegradation as previous studies suggested the potential of this genus to biodegrade the polymer in marine conditions (Sudhakar et al., 2008).

On the CG2 plastics, the high abundance of *Hymenobacter,* previously detected in biodegradable plastic mulching (Bandopadhyay et al., 2020), can implicate the importance of this genus in the formation of the biofilm attached to plastics in soil ecosystems (Bandopadhyay et al., 2020). The elimination of the superficial layers on plastic in this

sampling location can be explained by the significant abundance of the genera *Arthrobacter* and *Kocuria*, microorganisms with the capacity to biodegrade the LDPE in natural conditions (Bolo et al., 2015, Han et al., 2020). The high abundance of *Modestobacter*, involved in the nitrate reduction (Song et al., 2018), indicates major function activities in the microbial community attached to the plastic. R3 plastics had a significant layer of biofilm (detected using SEM). For this reason, the high abundance of the genus *Pseudomonas* is not a surprise since this genus is known for its importance in the development of biofilms (Chien et al., 2013) and its potential to degrade polymers (Abdullah et al., 2021, Sivan et al., 2006). Other genera that could be involved in the biodegradation of the LDPE are *Rhodococcus*, which have some species that only used LDPE as a carbon source (Gilan and Sivan, 2013), *Devosia*, found previously in marine plastic debris (Zettler et al., 2013) and known by its capacity of biodegrading a high number of substrates, including hydrocarbons compounds (Talwar et al., 2020) and *Nocardiopsis*, that can biodegrade LDPE and may favour the biodegradation for the rest of the microorganisms producing biosurfactant (Priyadarshini et al., 2018). Also, the high abundance of the genus *Crinalium*, a cyanobacterium common in terrestrial sandy areas with a high desiccation-resistance (Wickham et al., 2019), indicates the importance of the primary producers in the community attached to the plastic. Furthermore, the high abundance of genera that can be opportunist pathogens, such as *Roseomonas* (Rihs et al., 1993), indicates the plastic's potential to carry pathogens, even on the ground.

On R4 plastics, some of the more abundant genera were previously associated with biofilms that grow in different freshwater ecosystems, such as *Porphyrobacter* (Di Pippo et al., 2020), *Tabrizicola* (Murphy et al., 2020), *Gemmobacter* (Nguyen et al., 2021), and *Pseudorhodobacter* (Di Pippo et al., 2020). Specifically, *Porphyrobacter* is an aerobic bacterium that participates in biogeochemical cycles in aquatic environments (Liu et al., 2017); *Rhodopirellula* and *Rubribacterium* have been reported as hydrocarbon-degrading bacteria (de Araujo et al., 2021, Urbance et al., 2001); *Algoriphagus* has been associated with polypropylene in a freshwater lake, whose development indicates significant algae growth on plastic (Szabó et al., 2021). *Ketogulonicigenium* is a facultatively anaerobic chemoheterotroph (Urbance et al., 2001) although its role in the plastisphere has not been defined and *Sandaracinobacter* is mainly found in freshwater environments (Lee et al., 2020). *Sandarakinorhabdus*, *Nodosilinea,* and *Rhodobacter* are primary producers playing a role in biofilm formation as previously documented (Yokota et al., 2017). Furthermore, some species of *Roseomonas*, are known to be opportunistic bacteria for humans (Rihs et al., 1993); and *Legionella*, a well-known biofilm participant is usually denoted as a pathogen as well (Edelstein and Lück, 2015). The specific

eutrophic conditions in this location, along with the presence of R4 weathered plastics, providing an extra carbon source and a surface in which nutrients can adhere are probably the main drivers of the significant increase of genera and their respective abundances in R4 plastics.

On S5 plastics, the plastic-associated communities agreed with the genera found in other studies in marine habitats such as *Lewinella* (Roager and Sonnenschein, 2019), *Dokdonia* (Basili et al., 2020), *Loktanella* (Delacuvellerie et al., 2019, Pinto et al., 2019), *Pseudahrensia* (Zhang et al., 2021), *Erythrobacter* (Kirstein et al., 2019) and *Parvularcula* (Kirstein et al., 2019). The repetitive detection of these genera suggested having an essential role in the marine plastic biofilm, suggesting that future studies can discover their function in these communities. Furthermore, the abundance of autotrophs at all sampling locations and their changes according to the different sampling locations (on S5 plastics, the more abundant genera were *Pleurocapsa* and *Schizothrix*) shows their importance in the plastisphere, independently of the environment. Most of the studies that address the plastisphere are based mainly on heterotrophic bacteria (Yokota et al., 2017), but determining the presence of photosynthetic bacteria can contribute information about the different relationships established in the bacterial communities associated with the plastisphere.

Many studies suggest that plastics and their smaller fractions (microplastics and even nano plastics) can be vectors of microorganisms between different habitats (Meng et al., 2021, Shen et al., 2019). The negative effect on ecosystems is not entirely clear, but some studies propose that plastics can introduce invasive species (Carter et al., 2010), pathogens (Goldstein et al., 2014, Kirstein et al., 2016), or increase the gene exchange between attached biofilm communities and the surrounding environments (Arias-Andres et al., 2018). Other studies indicate the potential of these microorganisms to use plastics as a carbon source (Bornscheuer, 2016). Our study confirms that greenhouse plastics can effectively function as vectors of bacteria, showing six genera (i.e., *Flavobacterium, Georgfuchsia, Acinetobacter, Pleurocapsa, Altererythrobacter* and *Rhodococcus*) preserved on the plastics independently of the sampling location and their surrounding environment (soil, freshwater or seawater). The genus *Flavobacterium* can be found generally in soil and freshwater (Bernardet and Bowman, 2006); it is a potential pathogen for some fish species (Bernardet and Bowman, 2006, Nematollahi et al., 2003). *Georgfuchsia* has been described previously as capable of biodegrading aromatic hydrocarbons (Staats et al., 2011). The genus *Acinetobacter* was reported for its implication in some human infections (Joly-Guillou, 2005) and its capacity for its resistance to multiple antibiotics (Manchanda et al., 2010), as it also happens with the

genus *Pleurocapsa* (Li et al., 2021a). Some species of the genus *Altererythrobacter* were reported as PHA/PHB degraders in previous studies (Vannini et al., 2021), and lastly, the genus *Rhodococcus*, can degrade LDPE under laboratory conditions (Abdullah et al., 2021, Sivan et al., 2006). Nonetheless, the highest number of these common taxa were detected in G1, CG2, R3, and R4 plastics suggesting that the arrival of the plastics to the sea may limit the capacity of the plastics to act as a vector of microorganisms. The presence of bacteria involved in the development of biofilms such as *Sphingomonas* (Bereschenko et, 2010; Martínez-Campos et al., 2018); others capable of biodegrading plastics such as *Pseudomonas* (Kyaw et al., 2012) and *Arthrobacter* (Han et al., 2020); potential pathogens such as *Brevundimonas* (Ryan and Pembroke, 2018) and *Roseomonas* (Rihs et al., 1993) urges not to underestimate the impact that plastics and associated plastisphere can have in each environment along their life cycle.

5. Conclusions

This study analyses for the first time the evolution of the bacterial community adhered to plastics across different environments from their point of use to their final destination in the sea. Greenhouse plastics were chosen for this study because their mismanagement facilitates their debris reaching the ocean. Bacterial communities detected on greenhouse plastics change with increasing distance from the point of use. Additionally, changes were caused by their surrounding environments, especially for plastics arriving in freshwater and the sea. Furthermore, the statistical analysis revealed that the pH, salinity, and concentration of nutrients (nitrogen and phosphorus) had an essential role in the successive changes produced in the bacterial community attached to the plastics.

The presence of six common genera independently of the sampling location (*Flavobacterium, Altererythrobacter, Acinetobacter, Pleurocapsa, Georgfuchsia and Rhodococcus*) confirmed that plastics could act as vectors of microorganisms between different environments along their life cycle. The potential of these bacteria to act as human and animal pathogens, invasive species, or to carry antibiotic resistance genes could be an important concern for human health and the environment. Nevertheless, the demonstrated implication of these genera in the degradation of different types of plastics provides insights into the possible future elimination of these plastics in the environment. Future studies should perform complete sequencing metagenomics to evaluate the real impact of the plastisphere on the ecosystems of the planet. Lastly, studies should focus on verifying which of these microorganisms may pose a real risk to the environment or the importance of isolating degrading microorganisms to discover better mechanisms for eliminating plastic

waste. This work provides information about the way by which greenhouse plastics act as vectors of microorganisms posing an added risk to receiving environments. Finally, appropriate waste management techniques such as centralised collection systems and targeted waste management education seminars can be proposed.

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7. SUPPLEMENTARY MATERIAL 1

CONTENTS:

Figure S1 Sampling sites, their location and images of each sampling site: a) Cyprus map showing in general and in detail the location of sampling sites, b) Images of each sampling site. Legend: G1: greenhouse sampling site; CG2: sampling site close to the greenhouses; R3: dry riverbed; R4: river delta; S5: sea, near the shoreline.

Figure S2 Direct visualization of fragments of the plastics collected in each sampling site.

Figure S3 Genera abundance clustering heat map using ASVs. The left clustering tree was based on Bray Curtis matrix. Sample name is given at the right. The abundance heat map based on ASVs is in the middle, showing genera in each sample with a significant higher abundance.

Figure S4 Venn diagrams obtained using the taxonomy assignment at genus level. The figure is the analysis chart of the G1 plastic in comparison with the soil.

Figure S5 Venn diagrams obtained using the taxonomy assignment at genus level. The figure is the analysis chart of the CG2 plastic in comparison with the soil.

Figure S6 Venn diagrams obtained using the taxonomy assignment at genus level. The figure is the analysis chart of the R3 plastic in comparison with the soil.

Figure S7 Venn diagrams obtained using the taxonomy assignment at genus level. The figure is the analysis chart of the R4 plastic in comparison with the freshwater.

Figure S8 Venn diagrams obtained using the taxonomy assignment at genus level. The figure is the analysis chart of the S5 plastic in comparison with the sea.

Table S1
Summary about the sampling sites, their location and the type of sample collected in each sampling site.

	Coordinates	Name	Type of sample
G1	34º 44′ 42.9′′ N; 33º 21′ 38.4′′ E	Greenhouse sampling site	Plastic and soil
CG2	34º 44′ 42.5′′ N; 33º 21′ 39.1′′ E	Sampling site close to the greenhouses	Plastic and soil
R3	34º 43′ 58.4′′ N; 33º 21′ 58.8′′ E	Dry riverbed, 1 km from greenhouses	Plastic and soil
R4	34º 42′ 48.2′′ N; 33º 09′ 52.3′′ E	River delta	Plastic and freshwater
S5	34º 42′ 44.0′′ N; 33º 09′ 51.8′′ E	Sea, near the shoreline, 2 m depth	Plastic and seawater

Table S2
Summary of the results obtained using the ATR-FTIR spectra.

Location of plastic collected	Plastic identification	Matching for identification	Database	Carbonyl Index	Hydroxyl Index	Carbon-Oxygen Index	Summatory of Index
G1	Poly(ethylene) low density	88.1%	Aldrich Condensed Phase Sample Library	0.08	0.05	0.37	0.50
CG2	Poly(ethylene) low density	80.1%	Aldrich Condensed Phase Sample Library	0.14	0.13	1.7	1.97
R3	Poly(ethylene) low density	85.2%	Aldrich Condensed Phase Sample Library	0.10	0.07	0.49	0.66
R4	Poly(ethylene) low density	86.6%	Aldrich Condensed Phase Sample Library	0.08	0.07	0.64	0.79
S5	Poly(ethylene) low density	88.1%	Aldrich Condensed Phase Sample Library	0.07	0.06	0.43	0.56

Table S3
Pairwise ANOVA comparison between the different samples based on Shannon Index

Comparison of samples	Shannon Index		
	Mean Difference	95% Confidence Interval of Difference	Significant?
Plastic *vs.* Environment	-0.48	-----	No
G1 *vs.* CG2	-0.06	-2.02 to 1.90	No
G1 *vs.* R3	0.65	-1.32 to 2.61	No
G1 *vs.* R4	-0.76	-2.72 to 1.20	No
G1 *vs.* S5	-0.46	-2.42 to 1.50	No
G1 *vs.* Soil	-0.69	-2.65 to 1.27	No
G1 *vs.* Freshwater	-1.22	-3.18 to 0.74	No
G1 *vs.* Seawater	-0.01	-2.20 to 2.18	No
GC2 *vs.* R3	0.71	-1.25 to 2.66	No
GC2 *vs.* R4	-0.70	-2.66 to 1.26	No
GC2 *vs.* S5	-0.40	-2.36 to 1.56	No
GC2 *vs.* Soil	-0.63	-2.59 to 1.33	No
GC2 *vs.* Freshwater	-1.16	-3.12 to 0.80	No
GC2 *vs.* Seawater	0.05	-2.14 to 2.24	No
R3 *vs.* R4	-1.41	-3.36 to 0.55	No
R3 *vs.* S5	-1.11	-3.06 to 0.85	No
R3 *vs.* Soil	-1.34	-3.29 to 0.62	No
R3 *vs.* Freshwater	-1.86	-3.82 to 0.10	No
R3 *vs.* Seawater	-0.66	-2.85 to 1.53	No
R4 *vs.* S5	0.30	-1.66 to 2.26	No
R4 *vs.* Soil	0.07	-1.89 to 2.03	No
R4 *vs.* Freshwater	-0.46	-2.41 to 1.50	No
R4 *vs.* Seawater	0.75	-1.44 to 2.94	No
S5 *vs.* Soil	-0.23	-2.18 to 1.73	No
S5 *vs.* Freshwater	-0.76	-2.71 to 1.20	No
S5 *vs.* Seawater	0.45	-1.74 to 2.64	No
Soil *vs.* Freshwater	-0.53	-2.49 to 1.43	No
Soil *vs.* Seawater	0.68	-1.51 to 2.87	No
Freshwater *vs.* Seawater	1.21	-0.98 to 3.39	No

Table S4
Pairwise ANOVA comparison between the different samples based on Gini Index

Comparison of samples	Gini test		
	Mean Difference	95% Confidence Interval of Difference	Significant?
Plastic *vs.* Environment	0.01	-----	No
G1 *vs.* CG2	0.00	-0.02 to 0.02	No
G1 *vs.* R3	0.00	-0.02 to 0.02	No
G1 *vs.* R4	0.01	-0.01 to 0.03	No
G1 *vs.* S5	0.00	-0.02 to 0.02	No
G1 *vs.* Soil	0.01	-0.01 to 0.03	No
G1 *vs.* Freshwater	0.03	0.01 to 0.05	Yes
G1 *vs.* Seawater	0.00	-0.03 to 0.02	No
GC2 *vs.* R3	0.00	-0.02 to 0.03	No
GC2 *vs.* R4	0.01	-0.01 to 0.03	No
GC2 *vs.* S5	0.00	-0.02 to 0.03	No
GC2 *vs.* Soil	0.01	-0.01 to 0.03	No
GC2 *vs.* Freshwater	0.03	0.01 to 0.05	Yes
GC2 *vs.* Seawater	0.00	-0.02 to 0.02	No
R3 *vs.* R4	0.01	-0.01 to 0.03	No
R3 *vs.* S5	0.00	-0.02 to 0.02	No
R3 *vs.* Soil	0.01	-0.02 to 0.03	No
R3 *vs.* Freshwater	0.03	0.01 to 0.05	Yes
R3 *vs.* Seawater	0.00	-0.03 to 0.02	No
R4 *vs.* S5	-0.01	-0.03 to 0.02	No
R4 *vs.* Soil	0.00	-0.02 to 0.02	No
R4 *vs.* Freshwater	0.03	0.003 to 0.05	Yes
R4 *vs.* Seawater	-0.01	-0.03 to 0.02	No
S5 *vs.* Soil	0.01	-0.02 to 0.03	No
S5 *vs.* Freshwater	0.03	0.009 to 0.05	Yes
S5 *vs.* Seawater	0.00	-0.03 to 0.02	No
Soil *vs.* Freshwater	0.03	0.003 to 0.05	Yes
Soil *vs.* Seawater	-0.01	-0.03 to 0.01	No
Freshwater *vs.* Seawater	-0.03	-0.06 to -0.01	Yes

Table S5
Global and Pairwise PERMANOVA comparison between the different samples based in Bray-Curtis similarity matrix.

Groups comparison	PERMANOVA test			
	t	P. (perm)	Unique permutations	*p*–value (Monte-Carlo)
Global	5.84	0.00	996	0.00
Plastic *vs.* Environment	6.62	0.00	996	0.00
G1 *vs.* CG2	1.70	0.11	10	0.08
G1 *vs.* R3	1.90	0.09	10	0.05
G1 *vs.* R4	2.78	0.10	10	0.01
G1 *vs.* S5	3.09	0.11	10	0.01
G1 *vs.* Soil	1.50	0.12	10	0.09
G1 *vs.* Freshwater	3.29	0.09	10	0.01
G1 *vs.* Seawater	2.45	0.10	7	0.02
GC2 *vs.* R3	2.04	0.11	10	0.03
GC2 *vs.* R4	2.96	0.13	10	0.01
GC2 *vs.* S5	3.31	0.11	10	0.00
GC2 *vs.* Soil	1.57	0.08	10	0.08
GC2 *vs.* Freshwater	3.55	0.10	10	0.01
GC2 *vs.* Seawater	2.63	0.11	10	0.03
R3 *vs.* R4	2.65	0.10	10	0.01
R3 *vs.* S5	2.94	0.11	10	0.02
R3 *vs.* Soil	1.45	0.09	10	0.15
R3 *vs.* Freshwater	3.12	0.10	10	0.00
R3 *vs.* Seawater	2.32	0.10	7	0.04
R4 *vs.* S5	3.92	0.10	10	0.00
R4 *vs.* Soil	1.71	0.10	10	0.08
R4 *vs.* Freshwater	4.09	0.13	10	0.00
R4 *vs.* Seawater	3.19	0.10	10	0.02
S5 *vs.* Soil	1.78	0.09	10	0.05
S5 *vs.* Freshwater	5.29	0.12	10	0.00
S5 *vs.* Seawater	3.80	0.11	10	0.01
Soil *vs.* Freshwater	1.85	0.08	10	0.05
Soil *vs.* Seawater	1.41	0.10	10	0.18
Freshwater *vs.* Seawater	4.39	0.08	10	0.01

Table S6.
Physicochemical parameters measured in soil (G1, CG2 and R3 sampling stations)

Sampling site	Bulk density (g/cm^3)	Salinity (S)	pH	Nitrogen (mg/g)	Phosphorus (mg/g)	Total Organic Carbon (mg/g)	% Sand	% Silt	% Clay
G1	1.09 ± 0.01	609 ± 1	7.7 ± 0.1	0.12 ± 0.01	0.023 ± 0.001	7.07 ± 0.01	25 ± 1	25 ± 1	50 ± 1
CG2	0.73 ± 0.01	891 ± 1	8.4 ± 0.1	0.24 ± 0.01	0.009 ± 0.001	7.07 ± 0.01	41 ± 1	25 ± 1	34 ± 1
R3	1.19 ± 0.01	105 ± 1	8.8 ±0.1	0.03 ± 0.01	0.030 ± 0.001	7.07 ± 0.01	68 ± 1	7 ± 1	25 ± 1

Table S7. Physicochemical parameters measured in soil (R4 and S5 stations)

Sampling site	Temperature (°C)	Conductivity (mS)	TDS (g/L)	pH	Oxygen (%)	Oxygen (mg/L)	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	NT (mg/L)	PO_4^{3-} (mg/L)
R4	23.2 ± 0.1	5.0 ± 0.1	3.6 ± 0.1	6.9 ± 0.1	13.3 ± 0.1	1.11 ± 0.1	0.34 ± 0.01	0.470 ± 0.001	44.4 ± 0.1	45.2 ± 0.1	0.24 ± 0.01
S5	28.8 ± 0.1	73.7 ± 0.1	49.6 ± 0.1	7.9 ± 0.1	94.7 ± 0.1	7.35 ± 0.1	0.07 ± 0.01	0.004 ± 0.001	3.0 ± 0.1	3.1 ± 0.1	0.1 ± 0.01

Table S8
Differential bacterial genera abundance comparing the plastics collected in each sampling site using linear discriminant analysis (LEfSe).

Sampling site plastic	Taxa	LDA Score
G1	*Methylobacterium*	5.20
	Sphingomonas	4.90
	Chamaesiphon_PCC_7430	4.70
	Frigobacterium	3.66
	Ruminococcus_2	3.60
	Pantoea	3.53
	Calothrix_KVSF5	3.47
	Weisella	3.42
	Corynebacterium	3.32
	Bacillus	3.29
	Turicibacter	3.24
	Curtobacterium	3.10
	Clostridium_sensu_stricto_1	3.06
	Pseudoalteromonas	2.94
	Romboutsia	2.85
	Sphingobacterium	2.77
	Fructobacillus	2.69
	Jeotgalicoccus	2.65
	Atopostipes	2.58
CG2	*Hymenobacter*	4.92
	Arthrobacter	4.73
	Massilia	4.55
	Kocuria	4.40
	Paracoccus	4.38
	Planomicrobium	4.22
	Modestobacter	3.95
	Kineococcus	3.88
	Kineosporia	3.53
	Rhizorhapis	2.67
R3	*Geodermatophilus*	4.95
	Nocardiopsis	4.59
	Marmoricola	4.38
	Quadrisphaera	4.27
	Roseomonas	4.20
	Crinalium_SAG_22_89	4.11
	Blastococcus	4.00
	Rhodococcus	3.87

(Continued)

Sampling site plastic	Taxa	LDA Score
R3	*Skermanella*	3.85
	Tepidisphaera	3.82
	Pseudomonas	3.66
	Actinomycetospora	3.64
	Noviherbaspirillum	3.38
	Adhaeribacter	3.37
	Aureimonas	3.32
	Dyadobacter	3.28
	Devosia	3.25
	Cellulomonas	3.22
	Friedmanniella	2.97
	Patulibacter	2.93
	Nostoc_PCC_73102	2.81
	Aquipuribacter	2.80
	Tumebacillus	2.66
R4	*Porphyrobacter*	4.83
	Rhodopirellula	4.63
	Tabrizicola	4.45
	Rubribacterium	4.39
	Ketogulonicigenium	4.32
	Luteolibacter	4.31
	Sandaracinobacter	4.19
	Sandarakinorhabdus	4.10
	Nodosilinea_PCC_7104	4.05
	Pir4_lineage	4.05
	Gemmobacter	4.04
	Terrimicrobium	4.04
	Rhodobacter	3.99
	Chryseobacterium	3.99
	Pseudorhodobacter	3.90
	Hydrogenophaga	3.89
	Rubellimicrobium	3.85
	Cloacibacterium	3.79
	Leptolyngbya_ANT_L52_3	3.79
	Salinarimonas	3.30
	Dysgonomonas	3.29
	Silanimonas	3.27
	Mesorhizobium	3.16
	Synechocystis_PCC_6803	3.12

(Continued)

Sampling site plastic	Taxa	LDA Score
R4	*Palleronia*	3.10
	Phreatobacter	3.04
	Chitinibacter	3.02
	Aquimonas	2.99
	Bryobacter	2.94
	Filomicrobium	2.94
	Blastomonas	2.93
	Robiginitalea	2.92
	Acidibacter	2.92
	Bosea	2.91
	Legionella	2.88
	Cytophaga	2.81
	Rubinisphaera	2.78
	Runella	2.74
	Fodinicola	2.72
	Polymorphobacter	2.72
	Cellvibrio	2.69
	Aeromonas	2.68
	IMCC26207	2.65
	SM1A02	2.63
	CL500_29_marine_group	2.58
	Seohaeicola	2.54
	Candidatus_Bealeia	2.53
S5	*Phormidesmis_ANT_LACV5_1*	5.22
	Rubrivirga	4.83
	Maribius	4.72
	Loktanella	4.72
	Pleurocapsa_PCC_7319	4.38
	Lewinella	4.35
	Pseudahrensia	4.26
	Schizothrix_LEGE_07164	4.20
	Parvularcula	4.11
	Erythrobacter	4.10
	Chroococcidiopsis_PCC_6712	3.89
	Algimonas	3.80
	Truepera	3.70
	Granulosicoccus	3.69
	Nonlabens	3.68
	Jannaschia	3.64

(Continued)

Sampling site plastic	Taxa	LDA Score
	Muriicola	3.64
	Aquimarina	3.59
	Dokdonia	3.42
	Roseobacter	3.40
	Pontivivens	3.34
	Hellea	3.26
S5	*Rubidimonas*	3.15
	Ahrensia	3.13
	Rubripirellula	3.05
	Aureicoccus	2.95
	Sphingomicrobium	2.95
	Muricauda	2.94
	Pseudomonas	3.66

Table S9
Differential bacterial taxa abundance comparing G1 plastic associated assemblages and soil sample bacterial communities by linear discriminant analyses (using LEfSe).

Sampling site plastic	Taxa	LDA Score
	Rhizorhapis	2.58
	Jeotgalicoccus	2.63
	Fructobacillus	2.79
	Romboutsia	2.83
	Clostridium_sensu_stricto_1	3.02
	Aureimonas	3.16
	Ruminococcus_2	3.23
	Turicibacter	3.27
	Emticicia	3.30
	Rhodococcus	3.32
G1	*UKL13_1*	3.36
	Kineosporia	3.47
	Modestobacter	3.50
	Pantoea	3.57
	Frigobacterium	3.63
	Kineococcus	3.81
	Arthrobacter	3.89
	Geodermatophilus	4.05
	Sphingomonas	4.78
	Hymenobacter	4.83
	Methylobacterium	5.20

(Continued)

Sampling site plastic	Taxa	LDA Score
Soil	*Acinetobacter*	4.32
	Micrococcus	4.17
	Delftia	4.06
	Brachybacterium	3.46
	Acidibacter	3.23

Table S10
Differential bacterial taxa abundance comparing CG2 plastic associated assemblages and soil sample bacterial communities by linear discriminant analyses (using LEfSe).

Sampling site plastic	Taxa	LDA Score
CG2	*Hymenobacter*	4.88
	Arthrobacter	4.73
	Methylobacterium	4.71
	Planococcus	4.63
	Sphingomonas	4.50
	Planomicrobium	4.26
	Roseomonas	4.04
	Modestobacter	3.98
	Kineococcus	3.84
	Geodermatophilus	3.83
	Marmoricola	3.78
	Rathayibacter	3.68
	Deinococcus	3.68
	Frigoribacterium	3.66
	Rhodococcus	3.62
	Fibrella	3.59
	Pantoea	3.56
	Rhodobacter	3.56
	Sphingorhbdus	3.55
	Kineosporia	3.54
	Quadrisphaera	3.54
	Skermanella	3.47
	Variovorax	3.44
	Rhizorhapis	3.43
	Blastococcus	3.42
	Aminobacter	3.38
	Aureimonas	3.33
	Spirosoma	3.08
	Friedmanniella	2.99

(Continued)

Sampling site plastic	Taxa	LDA Score
Soil	*Acinetobacter*	4.35
	Micrococcus	4.20
	Cutibacterium	4.12
	Delftia	4.11
	Staphylococcus	3.99
	Pseudomonas	3.93
	Georgfuchsia	3.87
	Nocardiopsis	3.77
	Acidibacter	3.59

Table S11
Differential bacterial taxa abundance comparing R3 plastic associated assemblages and soil sample bacterial communities by linear discriminant analyses (using LEfSe).

Sampling site plastic	Taxa	LDA Score
R3	*Geodermatophilus*	4.92
	Methylobacterium	4.69
	Nocardiopsis	4.53
	Marmoricola	4.40
	Hymenobacter	4.38
	Crinalium_SAG_22_89	4.25
	Roseomonas	4.20
	Friedmanniella	4.11
	Arthrobacter	4.09
	Aquipuribacter	4.04
	Blastococcus	4.03
	Rhodococcus	4.01
	Tumebacillus	4.00
	Skermanella	3.92
	Rubellimicrobium	3.89
	Tepidisphaera	3.88
	Patulibacter	3.88
	Nostoc_PCC_73102	3.78
	Kineococcus	3.77
Soil	*Actinomycetospora*	3.72
	Aureimonas	3.64
	Noviherbaspirillum	3.61
	Modestobacter	3.59
	Acinetobacter	4.40

Table S12
Differential bacterial taxa abundance comparing R4 plastic associated assemblages and freshwater sample bacterial communities by linear discriminant analyses (using LEfSe).

Sampling site plastic	Taxa	LDA Score
R4	*Porphyrobacter*	4.75
	Rhodopirellula	4.62
	Tabrizicola	4.46
	Rubribacterium	4.39
	Algoriphagus	4.35
	Ketogulonicigenium	4.32
	Luteolibacter	4.21
	Sandaracinobacter	4.18
	Roseomonas	4.17
	Sandarakinorhabdus	4.09
	Pir4_lineage	4.04
	Chryseobacterium	4.03
	Terrimicrobium	4.02
	Nodosilinea_PCC_7104	3.99
	Gemmobacter	3.99
	Rubellimicrobium	3.90
	Rhodobacter	3.89
	Leptolyngbya_ANT_L52_3	3.81
	Erythrobacter	3.79
	Fimbriiglobus	3.72
	Tropicimonas	3.71
	Chroococcidiopsis_PCC_6712	3.70
	Microcystis_PCC_7915	3.68
	Hyphomonas	3.58
	Emticicia	3.48
	Cyanobium_PCC_6307	3.47
	Leptolyngbya_PCC_6306	3.42
	Gleocapsa	3.41
	Blastopirellula	3.34
	Pedomicrobium	3.32
	Ilumatobacter	3.31
	Blastomonas	3.21
	Mesorhizobium	3.21
	Marivita	3.18
	Palleronia	3.16
	Lewinella	3.15
	Truepera	3.13
	Phreatobacter	3.04
	Filomicrobium	3.02
	Proteiniclasticum	3.01

(Continued)

Sampling site plastic	Taxa	LDA Score
Freshwater	*Candidatus_Aquiluna*	4.67
	Fluviicola	4.59
	Sediminibacterium	4.54
	Limnobacter	4.51
	Hydrogenophaga	4.48
	Rheinheimera	4.46
	Arcobacter	4.27
	Perlucidibaca	4.24
	Vogesella	4.21
	Flavobacterium	4.20
	Marivivens	4.18
	Vibrio	4.13
	Limnohabitans	4.09
	Ferritrophicum	3.98
	Pseudomonas	3.79
	Cellvibrio	3.76
	Shewanella	3.57
	Sulfurimonas	3.52
	Bacillus	3.51
	Haematospirillum	3.50
	Candidatus_Omnitrophus	3.50
	Sphingorhabdus	3.49
	Bacteriovorax	3.46
	Novispirillum	3.44
	Leptothrix	3.44
	Dechloromonas	3.41
	Georgfuchsia	3.40
	Rhodoferax	3.40
	Rhodoluna	3.40
	Lutispora	3.39
	Imperialibacter	3.38
	Pelagicoccus	3.36
	Arenimonas	3.35
	Halobacteriovorax	3.34
	Peredibacter	3.34
	Lacunisphaera	3.28
	Pannonibacter	3.25
	Paludibacter	3.21
	Flaviramulus	3.17
	Aeromonas	3.16
	Azospirillum	3.15
	Roseinatronobacter	3.12
	Aquicella	3.10
	Thiobacillus	3.05

Table S13
Differential bacterial taxa abundance comparing S4 plastic associated assemblages and sea sample bacterial communities by linear discriminant analyses (using LEfSe).

Sampling site plastic	Taxa	LDA Score
S5	*Phormidesmis_ANT_LACV5_1*	5.22
	Rubrivirga	4.87
	Maribius	4.74
	Loktanella	4.72
	Pleurocapsa_PCC_7319	4.41
	Lewinella	4.33
	Pseudahrensia	4.28
	Schizothrix_LEGE_07164	4.21
	Parvularcula	4.11
	Erythrobacter	4.10
	Chroococcidipsis_PCC_6712	3.95
	Aquimarina	3.74
	Algimonas	3.72
	Nonlabens	3.70
	Granulosicoccus	3.69
	Truepera	3.66
	Jannaschia	3.61
	Altererythrobacter	3.55
	Roseobacter	3.52
	Ilumatobacter	3.48
	Dokdonia	3.45
	Tepidisphaera	3.44
	Rhodopirellula	3.43
	Hyphomonas	3.41
	Hellea	3.39
	Pontivivens	3.38
	Rubripirellula	3.35
	Pir4_leage	3.34
	Muriicola	3.31
	Muricauda	3.28
	Ahrensia	3.26
	Rubidimonas	3.22
	Marivita	3.20
	Aureicoccus	3.14
	Sphingomicrobium	3.11
	Sulfitobacter	3.02

(Continued)

Sampling site plastic	Taxa	LDA Score
Freshwater	*Vibrio*	4.92
	Synechococcus_CC9902	4.74
	NS5_marine_group	4.72
	NS4_marine_Group	4.72
	Arcobacter	4.71
	HIMB11	4.63
	Glaciecola	4.57
	Formosa	4.18
	Catenococcus	4.11
	Nereida	4.03
	Shimia	3.96
	Phaeodactylibacter	3.93
	Marinomonas	3.85
	Reichenbachiella	3.76
	NS2b_marine_group	3.72
	Fluviicola	3.62
	Balneola	3.61
	Candidatus_Alysiosphaera	3.6
	Sulfurimonas	3.53
	Litoricola	3.48
	Neptuniibacter	3.47
	Ascidiaceihabitants	3.47
	Thalassococcus	3.45
	Candidatus_tenderia	3.45
	Candidatus_Puniceispirillum	3.39
	Thalassobius	3.29
	Terasakiella	3.29
	Marinibacterium	3.27
	Cyanobium_PCC_6307	3.21
	Temperatibacter	3.19
	Pseudoalteromonas	3.18
	Marinibacterium	3.27
	Polaribacter_4	3.17
	Aurantivirga	3.14
	Ponticoccus	3.12
	Neptunomonas	3.09

Table S14
Shared genera found in greenhouse plastics according to the different sampling sites based on the results obtained from Venn Diagram.

Clusters	Genera
G1 - CG2	*Lysobacter*
	Myxococcus
	Arcicella
	Citricoccus
	Brucella
	Lawsonella
	Inhella
	Clavibacter
	Ruminococcaceae_UCG-005
	Falsirhodobacter
G1 - R3	*Reyranella*
	Leuconostoc
	Thermoactinomyces
	Adhaeribacter
	Brevibacterium
	Janibacter
	Actinoplanes
	Prevotella
	Oceanobacillus
	Faecalibacterium
	Patulibacter
	Phormidium_CYN64
	Limnobacter
	Qipengyuania
	Amaricoccus
	Paenibacillus
	Streptomyces
	Paeniclostridium
	Flaviaesturariibacter
	Pajaroellobacter
	Sphingobacterium
	Delftia
	Rubrobacter
	Tumebacillus
G1 - R4	*Ochrobactrum*
	Rheinheimera
	SM1A02
	Silanimonas
	Rubribacterium
	Proteiniclasticum
	Escherichia/Shigella
	Fimbriiglobus
	Lacihabitans
	Bacteroides

(Continued)

Clusters	Genera
G1 - R4	*Vibrio*
	Hyphomicrobium
	Providencia
	Halomonas
	Terrimonas
	Bosea
	Salinimicrobium
G1 - S5	*Rubripirellula*
	Fuerstia
	Woeseia
CG2 - R3	*Tychonema_CCAP_1459-11B*
	Geminicoccus
	Cellulomonas
	Pigmentiphaga
	Rufibacter
	Aquipuribacter
	Noviherbaspirillum
	Flavisolibacter
	Rhodoferax
	Gemmatirosa
	Nakamurella
	Belnapia
	Planomicrobium
	Verticia
	Cellulosimicrobium
CG2 - R4	*Synechocystis_PCC-6803*
	Ketogulonicigenium
	CL500-29_marine_group
	Aeromonas
	Hydrogenophaga
	Herpetosiphon
	Rhizobacter
	Leptothrix
	OM60(NOR5)_clade
R3 - R4	*Fluviicola*
	Caulobacter
	Oligoflexus
	Xanthomonas
	Peredibacter
	Kushneria
	Crinalium_SAG_22.89
	Psychroglaciecola
	Cnuella
	Leptolyngbya_PCC-6306
	Prosthecobacter
	Cellvibrio

(Continued)

Clusters	Genera
R4 - S5	*Ilumatobacter*
	Robiginitalea
	Marivita
	Lewinella
	Rubinisphaera
	Loktanella
	Maribacter
	Chroococcidiopsis_PCC-6712
	Sva0996_marine_group
G1 - CG2 - R3	*Kineococcus*
	Cutibacterium
	Fibrella
	Kineosporia
	Blastocatella
	Novosphingobium
	Aminobacter
	Sanguibacter
	Rhodocytophaga
	Dyadobacter
	Aureimonas
	Solirubrobacter
	Quadrisphaera
	Rathayibacter
	Pseudoclavibacter
	Pantoea
	Streptococcus
	Friedmanniella
	Staphylococcus
	Frigoribacterium
	Stenotrophomonas
	Bacillus
	Conexibacter
	Aeromicrobium
	Rhizorhapis
	Chthoniobacter
	Variovorax
	Lactobacillus
	Salana
G1 - CG2 - R4	*Sphingorhabdus*
	Exiguobacterium
	Clostridium_sensu_stricto_1
	Salegentibacter
	Arenimonas
	Rhodobacter
	Psychrobacter
	Thermomonas
	Tabrizicola
G1 - CG2 - S5	*Phormidesmis_ANT.LACV5.1*

(Continued)

Clusters	Genera
G1 - R3 - R4	*Emticicia*
	Curtobacterium
	Haloferula
G1 - R4 - S5	*Blastopirellula*
	Rhodopirellula
	Erythrobacter
	Schizothrix_LEGE_07164
CG2 - R3 - R4	*Pirellula*
	Sphingobium
	Luteolibacter
	Planococcus
	Mycobacterium
	Ellin6055
R3 - R4 - S5	*Truepera*
	Tepidisphaera
G1 - CG2 - R3 - R4	*Brevundimonas*
	Pedomicrobium
	Lamia
	Marmoricola
	UKL13-1
	Chryseobacterium
	Nocardiopsis
	Calothrix_KVSF5
	Roseomonas
	Pseudomonas
	Blastococcus
	Spirosoma
	Luteimonas
	Sphingomonas
	Geodermatophilus
	Deinococcus
	Kocuria
	Paracoccus
	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
	Modestobacter
	Pedobacter
	Microvirga
	Massilia
	Arthrobacter
	Rubellimicrobium
	Pseudorhodobacter
	Methylotenera
	Skermanella
	Methylobacterium
	Hymenobacter
	Devosia
	Chamaesiphon_PCC-7430

(Continued)

Clusters	Genera
G1 - CG2 - R3 - S5	*Nitrospira*
	Brachybacterium
G1 - R3 - R4 - S5	*Pir4_lineage*
	Algoriphagus
	Porphyrobacter
	Hyphomonas
	OM27_clade
CG2 - R3 - R4 - S5	*SH-PL14*
	Bdellovibrio
	Rubrivirga
G1 - CG2 - R3 - R4 - S5	*Flavobacterium*
	Georgfuchsia
	Acinetobacter
	Pleurocapsa_PCC-7319
	Altererythrobacter
	Rhodococcus

Table S15
Genera in common between each plastic and their corresponding surrounding environment.

Clusters	Genera
G1 - Soil	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
	Brevundimonas
	Georgfuchsia
	Micrococcus
	Limnobacter
	Psychrobacter
	Ralstonia
	Acinetobacter
	Pedobacter
	Iamia
	Brachybacterium
	Marinobacterium
	Enhydrobacter
	Quadrisphaera
	Lysobacter
	Leuconostoc
	Qipengyuania
	Amaricoccus
	Pleurocapsa_PCC-7319
	Cutibacterium
	Exiguobacterium
	Microvirga
	Adhaeribacter
	Brevibacterium
	Lechevalieria
	Curtobacterium
	Thermomonas
	Fimbriiglobus

(Continued)

Clusters	Genera
G1 - Soil	*Massilia*
	Fusicatenibacter
	Acetobacter
	Luteitalea
	Methylophilus
	Chryseobacterium
	Janibacter
	Rubellimicrobium
	Rhodopirellula
	Actinoplanes
	Sandaracinus
	Nocardiopsis
	Prevotella
	Pseudorhodobacter
	Weissella
	Dongia
	Phormidesmis_ANT.LACV5.1
	Vibrio
	Pseudoalteromonas
	Calothrix_KVSF5
	Pajaroellobacter
	Rheinheimera
	Streptococcus
	SM1A02
	Lawsonella
	Roseomonas
	Pseudomonas
	Altererythrobacter
	Blastococcus
	Corynebacterium
	Sphingobacterium
	Staphylococcus
	Delftia
	Rubrobacter
	Woeseia
	Halomonas
	Stenotrophomonas
	Dietzia
	Bradyrhizobium
	Blastocatella
	Arenimonas
	Porphyrobacter
	Bacillus
	Luteimonas
	Bifidobacterium
	Nannocystis
	Flavobacterium
	Novosphingobium

(Continued)

Clusters	Genera
G1 - Soil	*Aeromicrobium*
	Sphingomonas
	Methylobacterium
	Chthoniobacter
	Phormidium_CYN64
	Hymenobacter
	Neisseria
	Erythrobacter
	Deinococcus
	Schizothrix_LEGE_07164
	Hyphomonas
	Kocuria
	Rhodocytophaga
	Paracoccus
	OM27_clade
	Devosia
	Dyadobacter
	Chamaesiphon_PCC-7430
	Lactobacillus
CG2 - Soil	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
	Brevundimonas
	Georgfuchsia
	Synechocystis_PCC-6803
	Psychrobacter
	Pirellula
	Acinetobacter
	Pedobacter
	Iamia
	Brachybacterium
	Quadrisphaera
	Sphingobium
	Lysobacter
	Luteolibacter
	Pleurocapsa_PCC-7319
	Cutibacterium
	Exiguobacterium
	Microvirga
	Thermomonas
	Massilia
	Gaiella
	Chryseobacterium
	Rubellimicrobium
	Pontibacter
	Nocardiopsis
	Geminicoccus
	Pseudorhodobacter
	Flavisolibacter

(Continued)

Clusters	Genera
	Phormidesmis_ANT.LACV5.1
	Calothrix_KVSF5
	Cellulomonas
	Streptococcus
	Lawsonella
	Roseomonas
	Pseudomonas
	Ketogulonicigenium
	Hydrogenophaga
	Altererythrobacter
	Blastococcus
	Herpetosiphon
	Mycobacterium
	Staphylococcus
	Rhodoferax
	Gemmatirosa
	Saccharibacillus
	SH-PL14
	Rhizobacter
	Stenotrophomonas
	Blastocatella
	Arenimonas
CG2 - Soil	*Bacillus*
	Luteimonas
	Flavobacterium
	Novosphingobium
	Bdellovibrio
	Aeromicrobium
	Rubrivirga
	Sphingomonas
	Methylobacterium
	Chthoniobacter
	Actinomyces
	Hymenobacter
	Deinococcus
	Kocuria
	Rhodocytophaga
	Cellulosimicrobium
	Paracoccus
	Christensenellaceae_R-7_group
	Ellin6055
	Devosia
	Dyadobacter
	Chamaesiphon_PCC-7430
	Lactobacillus

(Continued)

Clusters	Genera
	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
	Oxalicibacterium
	Brevundimonas
	Georgfuchsia
	Limnobacter
	Lacunisphaera
	Pirellula
	Lachnospiraceae_NK4A136_group
	Acinetobacter
	Pedobacter
	Iamia
	Opitutus
	Brachybacterium
	Quadrisphaera
	Sphingobium
	Luteolibacter
	Leuconostoc
	Lautropia
	Qipengyuania
	Amaricoccus
	Pleurocapsa_PCC-7319
	Cutibacterium
	Fluviicola
R3 - Soil	*Microvirga*
	Adhaeribacter
	Brevibacterium
	Curtobacterium
	Caulobacter
	Massilia
	Sphingoaurantiacus
	Pseudonocardia
	Chryseobacterium
	Janibacter
	Rubellimicrobium
	Acidovorax
	Actinoplanes
	Pseudarthrobacter
	Taibaiella
	Nocardiopsis
	Geminicoccus
	Prevotella
	Pseudorhodobacter
	Flavisolibacter
	Calothrix_KVSF5
	Cellulomonas
	Pajaroellobacter
	Streptococcus
	Xanthomonas

(Continued)

Clusters	Genera
R3 - Soil	*Prosthecobacter*
	Haliangium
	Roseomonas
	Pseudomonas
	Altererythrobacter
	Blastococcus
	Moheibacter
	Mycobacterium
	Sphingobacterium
	Staphylococcus
	Delftia
	Rhodoferax
	Gemmatirosa
	Rubrobacter
	Duganella
	SH-PL14
	Peredibacter
	Stenotrophomonas
	Blastocatella
	Porphyrobacter
	Bacillus
	Luteimonas
	Truepera
	Flavobacterium
	Novosphingobium
	Bdellovibrio
	Aeromicrobium
	Rubrivirga
	Sphingomonas
	Segetibacter
	Methylobacterium
	Chthoniobacter
	Phormidium_CYN64
	Cellvibrio
	Hymenobacter
	Deinococcus
	Peptoniphilus
	Hyphomonas
	Kocuria
	Rhodocytophaga
	Williamsia
	Cellulosimicrobium
	Paracoccus
	OM27_clade
	Ellin6055
	Laceyella

(Continued)

Clusters	Genera
R3 - Soil	*Devosia*
	Dyadobacter
	Glutamicibacter
	Chamaesiphon_PCC-7430
	Lactobacillus
R4 - Freshwater	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
	Brevundimonas
	Synechocystis_PCC-6803
	Georgfuchsia
	Pirellula
	Acinetobacter
	CL500-3
	Sphingorhabdus
	Ilumatobacter
	UKL13-1
	Rubrivivax
	Luteolibacter
	Chitinibacter
	Algoriphagus
	Pleurocapsa_PCC-7319
	Marivita
	Fluviicola
	Fimbriiglobus
	Caulobacter
	Massilia
	Anaerosporobacter
	Leptolyngbya_LEGE-06070
	Rahnella
	Chryseobacterium
	Roseinatronobacter
	Rubellimicrobium
	Rhodopirellula
	Candidatus_Soleaferrea
	Seohaeicola
	Acetoanaerobium
	Mucinivorans
	Bacteriovorax
	Bacteroides
	Vogesella
	Salinarimonas
	Pseudorhodobacter
	Acidibacter
	Aeromonas
	Vibrio
	Alistipes
	Phenylobacterium
	Robiginitalea
	Calothrix_KVSF5

(Continued)

Clusters	Genera
R4 - Freshwater	*Legionella*
	Hyphomicrobium
	Rheinheimera
	Pannonibacter
	Empedobacter
	Oligoflexus
	Phreatobacter
	SM1A02
	Terrimicrobium
	Azoarcus
	Prosthecobacter
	Roseomonas
	Blastopirellula
	Pseudomonas
	Ketogulonicigenium
	Hydrogenophaga
	Herpetosiphon
	Rs-D38_termite_group
	Cytophaga
	Roseivivax
	Providencia
	Enterococcus
	Methylotenera
	Mesorhizobium
	Silanimonas
	Pseudoxanthomonas
	Tepidisphaera
	Ignatzschineria
	Arcobacter
	Peredibacter
	CL500-29_marine_group
	Sandarakinorhabdus
	Arenimonas
	Porphyrobacter
	Bosea
	Leptothrix
	Rhodobacter
	Flavobacterium
	Pir4_lineage
	Bdellovibrio
	Tabrizicola
	Sphingomonas
	Cellvibrio
	Aquabacterium
	Chitinimonas
	Erythrobacter
	Nodosilinea_PCC-7104
	Aquimonas

(Continued)

Clusters	Genera
R4 - Freshwater	*Hyphomonas*
	Dechloromonas
	Intestinimonas
	Parabacteroides
	Dysgonomonas
	OM27_clade
	Asticcacaulis
	Azospira
	Tyzzerella
	IMCC26207
	Psychroglaciecola
	Devosia
	Rubrimonas
	Cloacibacterium
	Sediminibacterium
	Chamaesiphon_PCC-7430
	Desulfovibrio
	Gemmobacter
	Ochrobactrum
	Escherichia/Shigella
	Planctopirus
	Thermostilla
	Roseovarius
	Leptolyngbya_ANT.L52.3
	Pedomicrobium
	Thiothrix
	Thiocystis
	Psychrobacter
	Paludibaculum
	Modestobacter
	Pedobacter
	Marmoricola
	Iamia
	Polymorphobacter
	Pygmaiobacter
	Sphingobium
	Elev-16S-1166
	Ferruginibacter
	Moellerella
	Cyanobium_PCC-6307
	Anderseniella
	Filomicrobium
	Exiguobacterium
	Microvirga
	Mariniflexile
	Azovibrio
	Curtobacterium
	Thermomonas

(Continued)

Clusters	Genera
R4 - Freshwater	*B48*
	Haloferula
	Arthrobacter
	Lacihabitans
	Leucobacter
	Blastomonas
	Prosthecomicrobium
	Rhodovastum
	NS4_marine_group
	Nocardiopsis
	Fodinicola
	Lacibacter
	Clostridium_sensu_stricto_1
	Cnuella
	Candidatus_Bealeia
	Plesiomonas
	Emticicia
	Chthonobacter
	Microcystis_PCC-7915
	Flavihumibacter
	Lewinella
	Aquiflexum
	Leptolyngbya_PCC-6306
	Paraclostridium
	Parablastomonas
	Planococcus
	Xanthomonas
	Luteimicrobium
	Sebaldella
	Roseococcus
	Gleocapsa
	Tropicimonas
	Palleronia
	Salegentibacter
	Altererythrobacter
	Blastococcus
	Oxalobacter
	Mycobacterium
	Rubinisphaera
	Isobaculum
	Geminocystis_PCC-6309
	GCA-900066226
	Chloronema
	SH-PL14
	Halomonas
	Leeuwenhoekiella
	Rhizobacter
	Skermanella

(Continued)

Clusters	Genera
	Flavimarina
	Loktanella
	Terrimonas
	Spirosoma
	Rickettsia
	Luteimonas
	Salinimicrobium
	Truepera
	Candidatus_Alysiosphaera
	Bryobacter
	Rubrivirga
	Methylobacterium
	Ruminiclostridium_5
	Kushneria
	Hymenobacter
	Geodermatophilus
	OM60(NOR5)_clade
	Maribacter
	Haliscomenobacter
	Sedimentibacter
	Ornithinibacter
R4 - Freshwater	*Proteiniclasticum*
	Deinococcus
	Schizothrix_LEGE_07164
	Rubribacterium
	Crinalium_SAG_22.89
	Roseobacter_clade_CHAB-I-5_lineage
	Roseimaritima
	Kocuria
	Chroococcidiopsis_PCC-6712
	Rhodococcus
	Paracoccus
	Runella
	Sandaracinobacter
	Elioraea
	Ellin6055
	Enterobacter
	Sva0996_marine_group
	Hirschia
	Jeotgalibacillus
	Pseudanabaena_PCC-7429
	Lysinimicrobium
	Roseicyclus

(Continued)

Clusters	Genera
S5 – Seawater	*Georgfuchsia*
	Jannaschia
	BD1-7_clade
	Granulosicoccus
	Aureicoccus
	Aurantivirga
	Porphyrobacter
	Nonlabens
	Truepera
	Pir4_lineage
	Bdellovibrio
	Portibacter
	Parvularcula
	Algimonas
	Aquimarina
	OM27_clade
	Polaribacter_4
	Bythopirellula
	Sphingomicrobium
	Muriicola
	Pontivivens
	Acrophormium_PCC-7375
	Bernardetia
	Blastopirellula
	Altererythrobacter
	Planctomicrobium
	Acinetobacter
	Pseudobacteriovorax
	Brachybacterium
	Ilumatobacter
	Rivularia_PCC-7116
	Dokdonia
	Rubinisphaera
	Muricauda
	Silicimonas
	Roseobacter
	Algoriphagus
	Fuerstia
	Sulfitobacter
	Tepidisphaera
	Pleurocapsa_PCC-7319
	Woeseia
	Zeaxanthinibacter
	Marivita
	Hellea
	SH-PL14
	Ahrensia
	Loktanella

(Continued)

Clusters	Genera
S5 - Seawater	*Gilvibacter*
	Pseudahrensia
	Flavobacterium
	Rubrivirga
	Maribius
	Rhodopirellula
	Litorimonas
	Aquibacter
	Erythrobacter
	Rubripirellula
	Maribacter
	Schizothrix_LEGE_07164
	Hyphomonas
	Chroococcidiopsis_PCC-6712
	Rhodococcus
	Nitrospira
	Rubidimonas
	Phormidesmis_ANT.LACV5.1
	Robiginitalea
	Lewinella
	Sva0996_marine_group

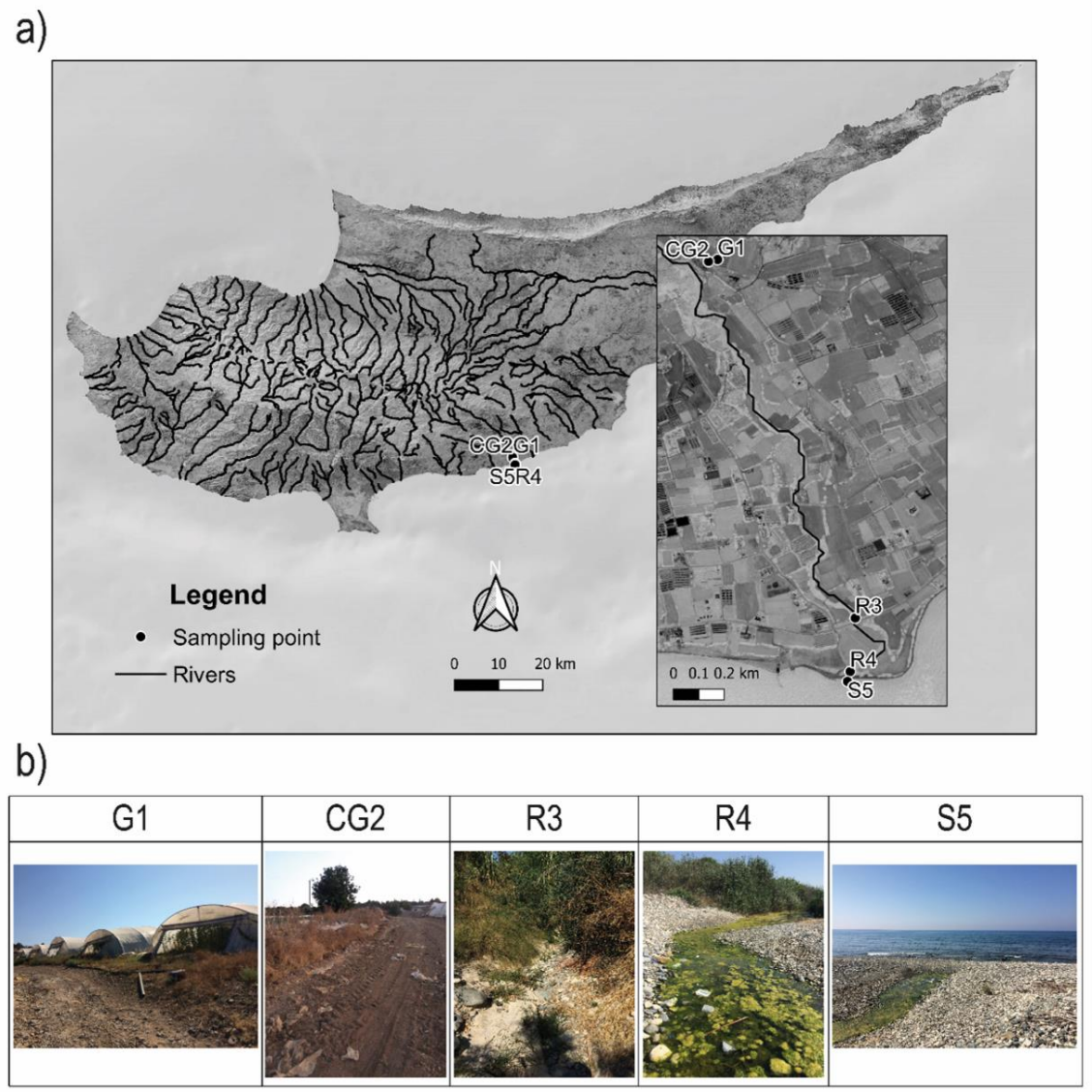


Figure S1. Sampling sites, their location and images of each sampling site: a) Cyprus map showing in general and in detail the location of sampling sites, b) Images of each sampling site. Legend: G1: greenhouse sampling site; CG2: sampling site close to the greenhouses; R3: dry riverbed; R4: river delta; S5: sea, near the shoreline.

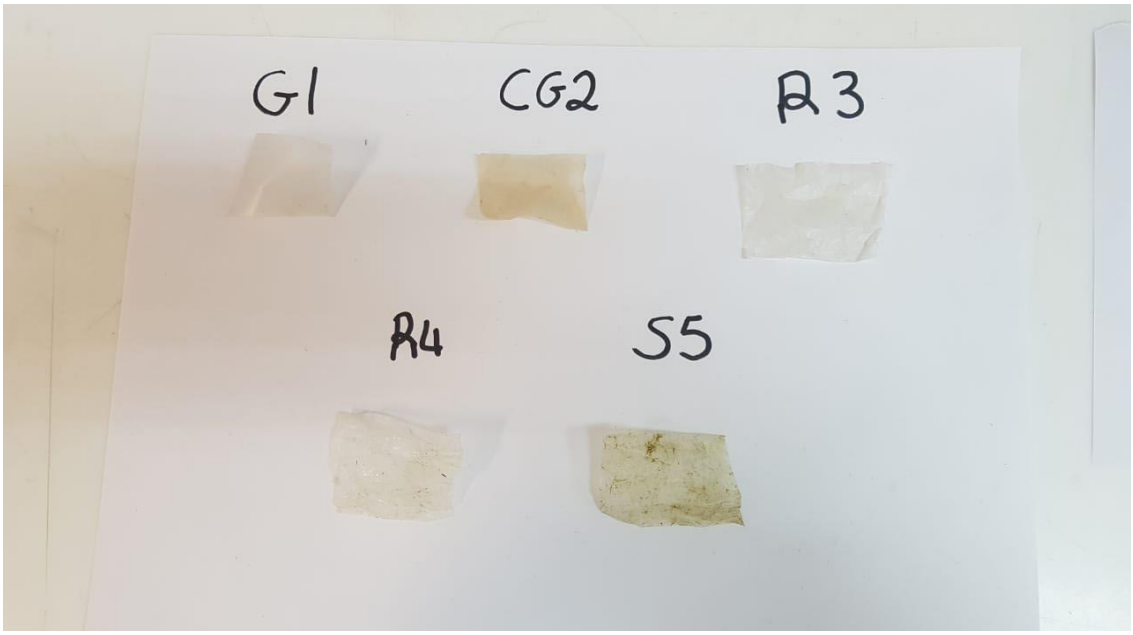


Figure S2. Direct visualization of fragments of the plastics collected in each sampling site

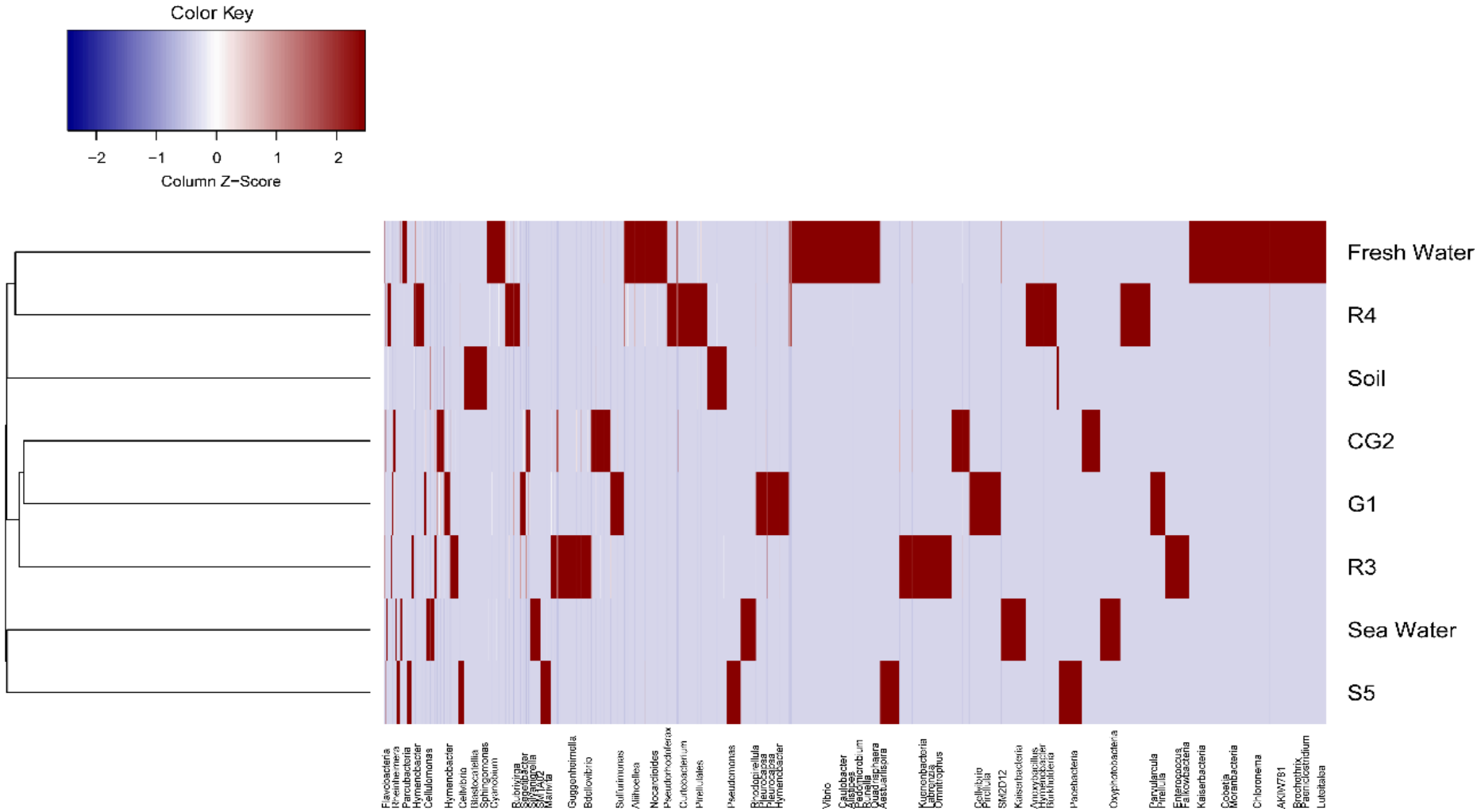


Figure S3. Genera abundance clustering heat map using ASVs. The left clustering tree was based on the Bray-Curtis matrix. Sample name is given at the right. The abundance heat map based on ASVs is in the middle, showing genera in each sample with a significantly higher abundance.

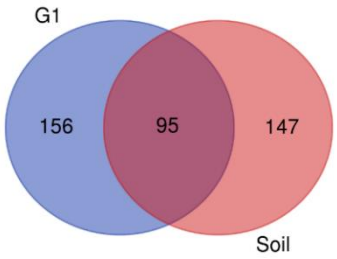


Figure S4. Venn diagrams were obtained using the taxonomy assignment at the genus level. The figure is the analysis chart of the G1 plastic in comparison with the soil.

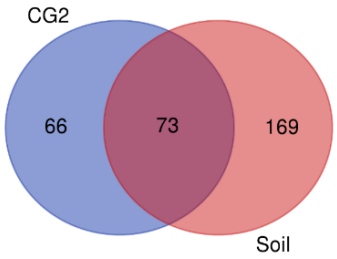


Figure S5. Venn diagrams were obtained using the taxonomy assignment at the genus level. The figure is the analysis chart of the CG2 plastic in comparison with the soil.

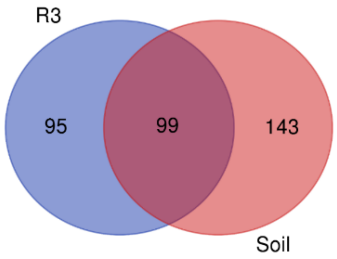


Figure S6. Venn diagrams were obtained using the taxonomy assignment at the genus level. The figure is the analysis chart of the R3 plastic in comparison with the soil.

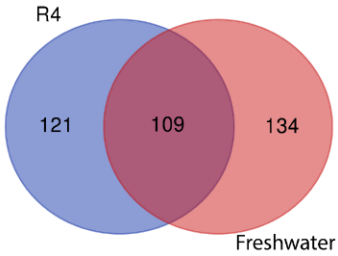


Figure S7. Venn diagrams were obtained using the taxonomy assignment at the genus level. The figure is the analysis chart of the R4 plastic in comparison with the freshwater.

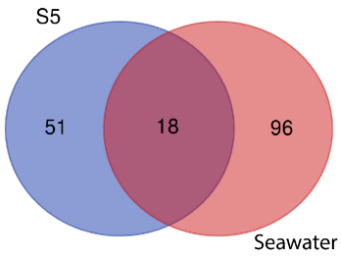


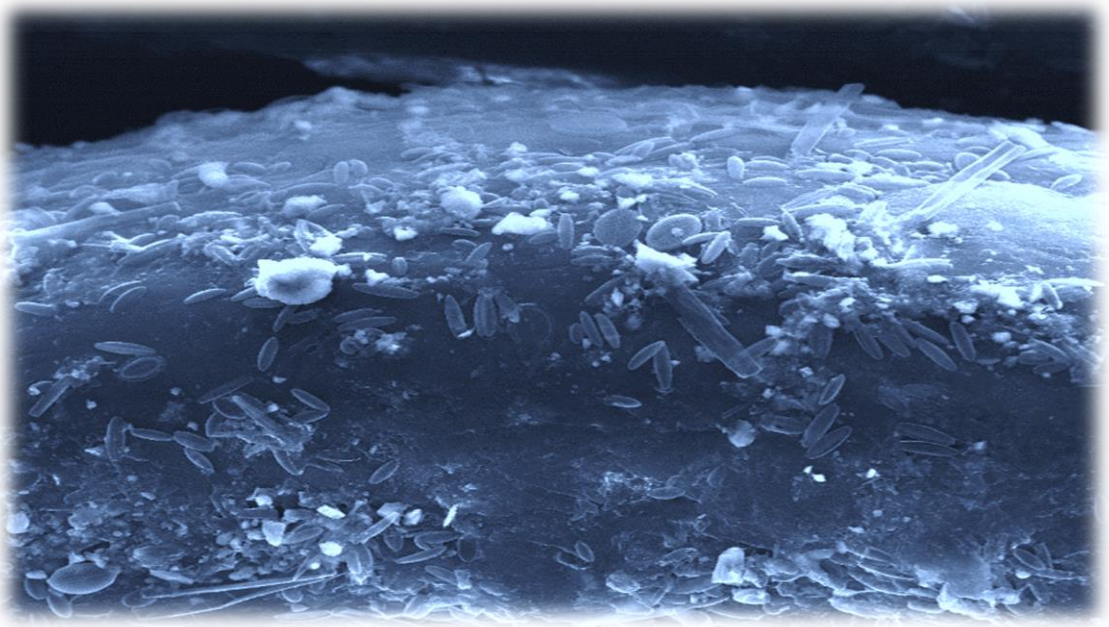
Figure S8. Venn diagrams were obtained using the taxonomy assignment at the genus level. The figure is the analysis chart of the S5 plastic in comparison with the sea.

8. SUPPLEMENTARY MATERIAL 2

The following supplementary material accompanies which details the taxonomic classification of all samples obtained from the sequencing of the gene region 16S rRNA can be downloaded from https://zenodo.org/record/6562784#.YoXtfKjP1D8

CHAPTER 6

GENERAL DISCUSSION



The main aim of this Ph. D Thesis was to increase the knowledge about the microbial community colonizing plastic debris in freshwater ecosystems, usually referred to as "plastisphere", as well as to verify the potential role of plastics as reservoirs and vectors of antibiotic resistance genes (ARGs). Therefore, this Doctoral Thesis focuses on: (i) Determining the main prokaryotic taxa developed on different plastics in rivers, including macroplastics (MaPs), microplastics (MPs), and end-of-life plastic products, such as greenhouse plastics or reverse osmosis (RO) membranes. Additionally, the main eukaryotic and fungal taxa developed on specific plastic substrates were also identified. (ii) Comparing the diversity of organisms in each environment, including plastics, non-plastic samples (glass or rocks), and the surrounding environment (water or soil). (iii) Evaluating the factors influencing the formation of biofilms such as sampling site, type of substrate, intrinsic properties of the plastic, environmental conditions, and concentrations of the antibiotics in the water samples. (iv) Defining the capacity of plastics to behave as vectors of microorganisms that may be pathogenic or harmful for other organisms including humans once abandoned in the environment and transported through different environments. (v) Evaluating the capacity of plastics to act as reservoirs and vectors of ARGs, whether exposed to the influence of wastewater treatment plant (WWTP) effluents or under other environmental conditions. Furthermore, the effect of water antibiotic concentration on the abundance of ARGs will be evaluated.

In order to meet the first objective, the number of eukaryotic and prokaryotic taxa found in different plastic substrates were determined. The prokaryotic community was extensively studied along this Doctoral Thesis, so that common taxa could be detected, especially for the higher taxonomic levels. Although some sequences were taxonomically assigned as Archaea, they did not present in any of the cases relative abundances higher than 0.1 %. In contrast, bacteria dominated the prokaryotic communities according to the results obtained by metabarcoding.

Concerning higher taxonomic levels, the phyla Proteobacteria, Bacteroidetes, Cyanobacteria and Actinobacteria were mostly identified. At the class level, Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, Oxyphotobacteria and Actinobacteria were clearly predominant. All these taxa had been previously identified in colonized plastics and were generally dominant regardless of sampling or incubation site (Amaral-Zettler et al., 2020; Li et al., 2021a).

At the order and family level, some taxa are closely related to the nature of plastic substrates. The order Sphingomonadales (mostly represented by the families

Sphingomonadaceae and Erythrobacteraceae) is characterized by a large number of genera capable of generating extracellular polymeric substances (EPS) that facilitate the adhesion and colonization of new bacteria (Bereschenko et al., 2010; Di Gregorio et al., 2017). The order Rhodobacterales (family Rhodobacteraceae) can produce quorum-sensing signals, involved in several microbial processes such as biofilm development (Jiang et al., 2018). The order Burkholderiales (family Burkholderiaceae) is characterized by taxa with the ability to develop in oligotrophic environments and have the capacity to use different compounds as carbon sources (Balkwill et al., 2006). Other orders that appear consistently in the plastisphere are Rhizobiales (mostly represented by the family Rhizobiaceae) and Cytophagales (represented by the family Hymenobacteraceae). At lower taxonomic levels (genus), taxa differ since other factors such as sampling site influence the bacterial taxa found.

The more abundant eukaryotic organisms in colonized plastics were analysed in **Chapter 4**. At phylum level, the phyla Ochrophyta (specifically the class Diatom), Platyhelminthes (specifically the class Gastropoda), Bryozoa (class Phylactolaemata) and Annelida (class Clitellata) were the most prominent. At the order and family levels, high variability depending on colonization time was observed in the eukaryotic community, but the orders Tricladida (family Planariidae), Caenogastropoda (family Caecidae), Ulvales (family Monostromataceae) Tectophilosida, Plumatellida, Haplotaxida, Triplonchida and Diptera were prevalent in all plastics. The complexity of the plastisphere is evidenced by the fact that many of the organisms identified were multi-cellular organisms, showing that plastic substrates are not only used for the development of a unique biofilm, but can also serve as a refuge for multiple organisms or as a food source (De-la-Torre et al., 2021; Gallitelli et al., 2021). Also, the abundance of diatoms, which were easily observed by scanning electron microscopy (SEM), has been previously detected in the plastisphere, reflecting the importance of these organisms as primary producers (Yokota et al., 2017).

The fungal community was examined in **Chapter 2** by ITS sequencing and, by 18S rRNA sequencing in **Chapter 4**. The results showed that they were scarcely abundant in most samples. In **Chapter 2**, most of the fungal organisms identified belonged to the phyla Ascomycota and Basidiomycota.

The second objective of this Dissertation which concerned the comparison of the diversity among plastics, non-plastics, and the surrounding environment, was studied in **Chapter 3**, **Chapter 4** and **Chapter 5**. In **Chapter 3**, it was found that, at early colonization times, the diversity identified on MPs was higher than that of the surrounding water and similar to that on other artificial substrates such as borosilicate (BS) glass. On the contrary,

in **Chapters 4** and **Chapter 5**, a generally lower diversity was observed on plastics compared to the environment, although similar to the other non-plastic substrates as indicated in **Chapter 4** for BS glass and rocks. The changes in diversity could be explained by the fact that **Chapter 3** studies the earliest stage of plastic colonization, in which the pioneer organisms allow the adhesion of other microorganisms to the plastic. This stage is not particularly specific or selective, so diversity might be higher than that in the surrounding water (Peng et al., 2018). This fact has already been reported in the early stages of biofilm in plastics in contact with WWTP effluents (Amaral-Zettler et al., 2020; Peng et al., 2018). In **Chapter 4**, which studies a long-term colonization experiment, the diversity decline could be related to the maturation of the microbial community, which tends to reduce the plastisphere diversity (Amaral-Zettler et al., 2020; Peng et al., 2018).

The results obtained from the beta diversity and PERMANOVA tests indicated that the community attached to the plastics was significantly different from that of the rest of the artificial substrates and that of the surrounding environment. Subsequent LeFSe analyses identified specific genera which were in higher abundance in specific plastics when compared with other samples (including all other plastics, artificial substrates, or the surrounding environment), which were referred to as core microbiome or core biome. Within these taxa, some have potential harmful effects the biota and even for human health. This is the case of the genus *Pseudomonas*, which includes opportunistic pathogens, such as *Pseudomonas aeruginosa*, a multidrug-resistant bacterium to several types of antibiotics (McCormick et al., 2014; Slekovec et al., 2012). *Arcobacter* is another frequent genus that appears associated with MPs as shown in **Chapter 3**, as well as with the greenhouse plastics immersed in freshwater as described in **Chapter 5**. This genus has been associated with gastrointestinal diseases in humans (Harrison et al., 2014). The genus *Vibrio*, which has also been reported as pathogenic in numerous studies of colonized plastics (Kirstein et al., 2016; Laverty et al., 2020; Oberbeckmann and Labrenz, 2020), also appeared in the biodegradable MPs studied in **Chapter 3** as well as in greenhouse plastic sampled at sea as shown in **Chapter 5**. **Chapter 4** also reports the presence of potentially pathogenic organisms within the core microbiome such as the eukaryotic *Candida* (Gkoutselis et al., 2021) in polystyrene (PS) dish and *Aeromonas*, a fish pathogen (Zettler et al., 2013) in low density polyethylene (LDPE) bag that was also identified in the work covered in **Chapter 3**.

The third objective was to assess the factors that could affect, to a higher degree, the formation and composition of the communities colonizing plastic substrates, conforming, thus, the plastisphere. This objective is extensively discussed along this Doctoral Thesis

(**Chapter 2**, **Chapter 3**, **Chapter 4** and **Chapter 5**), each chapter focusing on different factors. The results were mainly drawn from beta diversity data analyses.

In all cases, the location of the colonized plastics (sampling site) was considered as a critical factor influencing the constitution of the biofilm, with major influence on the community composition. **Chapter 2** analyzes three RO membranes from two different sites, showing major differences in the fungal and prokaryotic community composition between the membranes that belong to different desalination plants. In the study described in **Chapter 3**, the major differences in the bacterial communities attached to the tested MPs were explained by the different characteristics of the two WWTP where the MPs were deployed for the colonization experiments. In **Chapter 4**, which details the evolution of the plastisphere in everyday plastic items deployed in two sampling sites with different anthropogenic influence during a year, the location of the sampling site was what most conditioned plastisphere development. In **Chapter 5**, which examines the evolution of the bacterial community in greenhouse plastics during their life cycle from the greenhouse to the sea (soil, river, and sea), it could be observed that the most significant changes in bacterial communities in the greenhouse plastic were explained by the site where colonization took place. These results agreed with previous research found in the literature showing that sampling site is the main variable affecting the communities attached to the plastics (Kettner et al., 2019; Oberbeckmann et al., 2018, 2014; Rummel et al., 2017). In this context, Wright et al. (2021) analysed data from 50 metagenomics assays performed on different plastics. One of their most important conclusions is that site, followed by salinity, were the main drivers influencing the constitution of the plastisphere.

In close connection which site, different environmental conditions including both physical-chemical parameters and the presence of co-occurring contaminants, such as antibiotics. These factors are discussed in **Chapter 4** and **Chapter 5** of this Dissertation. **Chapter 4** uses Monte Carlos tests to assess the influence of the concentration of sulfamide, erythromycin, quinolones and trimethoprim antibiotics in water in the formation of bacterial biofilms on plastics. In line with these results, Xue et al., (2020) showed that, although the microorganisms attached to the plastics were relatively tolerant to anthropogenic pollutants, including antibiotics, the changes in their concentration, accounted for a major proportion of the changes in the bacterial community observed for different sections of the same river. Regarding the rest of the physical-chemical variables considered in **Chapter 5**, pH was the factor that better explained the changes in the microbial community attached to plastics. pH has been previously described as one of the most important factors affecting the bacterial

communities on soil plastics (Li et al., 2021b) and could explain the variability between terrestrial habitats and aquatic environments. The changes in nutrients (particularly nitrogen and phosphorus concentrations) could also be significant factors, as previously evidenced in reports on the plastisphere developed in freshwater and seawater (Amaral-Zettler et al., 2020; Li et al., 2021b).

The type of substrate, and therefore if the substrate is plastic or not, was also a crucial factor and allowed to understand whether the plastisphere is a unique ecosystem or whether it is similar to the biofilms forming in other substrates artificial such as borosilicate (BS) glass or natural such as rocks. This analysis appears in **Chapter 3** and **Chapter 4**. In this context, **Chapter 3** is the study that tested a higher number of MPs. The beta diversity results confirmed that there was a significant difference in the bacterial community attached to the different plastics, although the difference was most significant when compared with the bacterial community developing in BS glass. These results were confirmed in **Chapter 4**, in which an even stronger difference between the biological communities in plastics and those in the natural substrate (rocks) was observed. Several previous studies suggest (De Tender et al., 2015; Ogonowski et al., 2018) that there were differences in the bacterial and eukaryotic community depending on the type of substrate. **Chapter 3** studies two of the plastic intrinsic surface properties that commonly affect colonization: hydrophobicity and roughness. The results obtained by redundance analysis (RDA) showed that only hydrophobicity plays a significant role in early bacterial colonization. This is because the pioneering organisms that attach during the first hours contribute to reduce surface hydrophobicity, thereby allowing the further adhesion of other microorganisms that constitute the mature plastisphere (Lobelle and Cunliffe, 2011).

Incubation time is another important factor that was considered in **Chapter 4**. In this chapter, it was shown that, although time was the factor that had the least influence on the eukaryotic and bacterial communities in the plastisphere, it was still significant according to the results obtained by Monte Carlo tests. In this sense, a series of temporal stages were identified in which the microbial community evolved and changed towards maturity of the plastisphere: an early stage (up to 1 month of colonization), an intermediate stage (up to 3 month of incubation), and a late stage corresponding to the last colonization phase, which would encompass the time between 6 and 12 months of incubation, in which the plastisphere becomes mature without further significant variation. The most recent studies suggested that the earliest stages of plastic colonization, involving the adhesion of pioneer organisms and further development of plastisphere, lasted from only a few hours to days or even weeks

(Erni-Cassola et al., 2020; Quero and Luna, 2017; Tu et al., 2020). During the first phase, pioneer microorganisms produce EPS that is further used by secondary microorganisms to be attached to the initial biofilm. These organisms may play different functions that support the community attached to plastics, such as using the secondary products of other organisms as a source of carbon and energy. (Amaral-Zettler et al., 2020; Lorite et al., 2011). In the study, after 6 months, a final maturation state is reached and the microbial community was established with fewer changes until up to the 1 year of incubation (Wright et al., 2020).

The ability of plastics to act as vectors of microorganisms after the end of their useful life is mostly discussed in **Chapters 2** and **Chapter 5**. In these chapters, the analyses focus on plastic materials that had already completed their useful life, particularly in two very specific cases, RO membranes and greenhouse plastics. Most studies focus on evaluating the colonization of plastics once they have been discarded into the environment (Bryant et al., 2016), or virgin plastics that have been incubated to evaluate how colonization is initiated and how it evolves under different environmental factors (González-Pleiter et al., 2021; Muthukrishnan et al., 2019; Oberbeckmann et al., 2016) or, even, artificially aged plastics (Bao et al., 2022). Most of these studies consequently exclude other factors, such as the previous life of the plastic, during which it can be colonized by different types of microorganisms found in the surrounding environment or as a direct consequence of their use such as the plastic life cycles studies involving RO membranes and greenhouse plastics studied in the present Doctoral Thesis.

In both chapters, the biofilm developed on the materials at the end of their useful life was monitored by SEM. SEM analysis revealed a complex structure, where bacteria embedded in EPS could be easily observed, as well as a significant presence of inorganic fouling. In **Chapter 2**, dealing with RO membranes, the presence of a thin layer of crystals established on the biofilm was observed which is a usual outcome on the polyamide layer during desalination procedures. The biofouling developed in the RO membrane depended on different factors such as feed water, pre-treatment, and the chemical structure of the membrane (Al Ashhab et al., 2017, 2014; Bereschenko et al., 2011). In addition, the application of the Filmtracer™ LIVE/DEAD® biofilm viability kit and Filmtracer ™ SYPRO® Ruby biofilm matrix Stain demonstrated not only the extensive production of EPS over the entire membrane, which provides stability to the biofilm (Flemming and Wingender, 2010) but also that a certain number of cells remained alive and viable long after the RO membranes are no longer in use.

In **Chapter 5**, the potential of greenhouse plastics to act as a vector of different microorganisms along its life cycle in the environment is dealt with. 6 taxa were identified, at the genus level, that are present in the different habitats through which the plastic moves, including soil, river, and sea. These identified genera also had the potential for adverse effects to the environment and human health. The genus *Flavobacterium* is considered a potential pathogen for some fish species (Bernardet and Bowman, 2006; Nematollahi et al., 2003). Some species of the genus *Acinetobacter* are implicated in human infections (Joly-Guillou, 2005) whose treatment can be difficult because they often show resistance to multiple antibiotics (Manchanda et al., 2010). The genus *Pleurocapsa* is a type of nitrogen-fixing cyanobacteria capable of forming calcareous structures. (Bergman et al., 1997). Some of the identified genera could also be involved in organic pollutants/plastic degradation: The genus *Georgfuchsia* has the capacity to biodegrade aromatic hydrocarbons (Staats et al., 2011). Species of the genera *Altererythrobacter* and *Rhodococcus* are capable of degrading different types of plastics, polyhydroxyalkanoates (PHA) and polyhydroxybutyrate (PHB) (Vannini et al., 2021) in the case of the first one and low-density polyethylene (LDPE) in the case of the second one (Abdullah et al., 2021; Sivan et al., 2006).

The potential role of the plastics to act as reservoirs/vectors of ARGs was explored in **Chapter 3** and **Chapter 4**. In **Chapter 3**, the relative abundance of *sul1* and *tetM* genes in 7 types of microplastics was analysed and compared with their relative abundance in the effluent itself and in the biofilms of BS glass. The interest of this experiment lies on the fact that WWTPs are known hotspot for microplastics and antibiotic resistant bacteria (ARBs) harbouring cognate ARGs, which are constantly discharged into the environment (Edo et al., 2020; Hendriksen et al., 2019). The MPs of this study were deployed immediately downstream a WWTP, which avoids any dilution of the ARGs as the effluent mixes with river water. The selection of both genes (*sul1* conferring sulfamide resistance and tetM conferring tetracycline resistance) was based on previous reports of their presence in WWTP effluents (Garner et al., 2018; Hendriksen et al., 2019). The results obtained showed that the *sul1* gene increased its relative abundance in the MPs in comparison with the surrounding water. The *tetM* gene, although detected in the plastics, was more abundant in water. A comparable result was obtained by González-Pleiter et al. (2021), who performed a similar colonization experiment in an Arctic freshwater lake, finding that MPs accumulated higher concentrations of the *sul1* gene in comparison with the *tetM* gene. Therefore, the results obtained in this chapter confirmed that MPs act as reservoirs of ARGs, but in a selective

way. Furthermore, there are also differences between the ARGs present in each MPs, so the type of polymer affects the ability of a plastic to act as a reservoir for ARGs.

In **Chapter 4**, two completely different sites, one located in a natural area and the other influenced by an upstream WWTP, were compared to further assess the role of WWTP effluents as a determining factor for plastics to act as reservoirs of ARGs. Furthermore, the potential correlation between the relative abundance of ARGs in the plastics and the relative concentration of antibiotics in the surrounding water was evaluated. For this purpose, four types of ARGs were selected, *sul1*, *ermB* conferring (sulfamide resistance), *dfrA* (providing trimethoprim tolerance), and *qnrSrtF11A* (quinolone resistance) widely found in WWTP effluent outflow from all over Europe (Pärnänen et al., 2019). The results generally showed that although these ARG were found in the tested plastics there was no enrichment of ARGs in the plastics compared to the surrounding water. In contrast, the influence of the nearby WWTP was shown to affect the ability of plastics to concentrate ARGs. There was very limited enrichment of ARGs in plastics deployed at the natural sampling site, whereas they were abundant in the plastics downstream the WWTP. It was previously recognized that a high concentration of antibiotics in the environment was a decisive factor for the development of ARGs in aquatic environments (Zhao et al., 2020), but it was not fully clarified how this factor could influence the plastisphere resistome (Syranidou and Kalogerakis, 2022). This result confirms that there is a positive correlation between the concentration of antibiotics in the environment and the relative abundance of ARGs in plastics.

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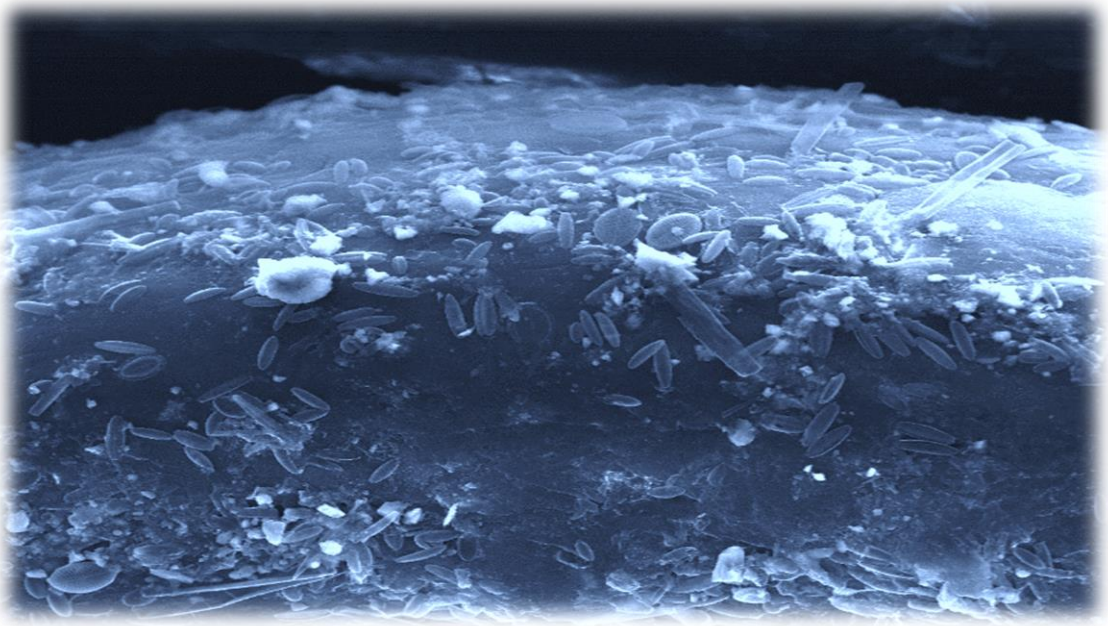
GENERAL CONCLUSSIONS



1. Plastics provide a unique habitat for a myriad of organisms. The nature of the polymer might select the attachment of specific organisms as core microbiomes/biomes could be identified in each plastic, which were clearly different to the organisms attached to other artificial and natural substrates as well as to the organisms present in the water column or soil. Key bacterial, eukaryotic and fungal taxa were identified in these core microbiomes/biomes. The presence of certain bacteria and eukaryotes could suggest the possibility of complex interactions in the plastisphere such as food webs or the involvement of plastics in biogeochemical cycles.
2. The formation of biofilms depends mainly on site-specific factors, including physical-chemical variables and the concentration of other pollutants. In addition, intrinsic polymer properties such as hydrophobicity play a crucial role in the formation of a specific plastisphere. Colonization time is another crucial factor in shaping the communities forming the plastisphere with a clear differentiation between early colonizers, which are pioneer microorganisms that facilitate further attachment of intermediate and late colonizers, which conform the mature plastisphere.
3. During their life cycle, plastics can act as vectors of microorganisms as they move between environmental compartments. Taxa that may act as human and animal pathogens o as invasive species have been identified in the plastics. Furthermore, the fact that some of these taxa are found in biodegradable plastics suggests that the capacity of the plastic to act as vector of potentially pathogenic taxa may be facilitated by their biodegradability. Interestingly, other identified taxa could be potential plastic degraders.
4. Plastics can act as reservoirs of Antibiotic Resistance Genes (ARGs) which may have serious implications in human health. A positive correlation was observed between the concentrations of selected antibiotic in water and the relative abundance of certain ARGs on plastics emphasizing a potential role of plastics in the spreading of antibiotic resistance.

ABBREVIATIONS

ARB ---------- Antibiotic Resistance Bacteria
ARGs ---------- Antibiotic Resistance Genes
ASVs ---------- Amplicon Sequence Variants
ATR-FTIR ---------- Attenuated Total Reflection Fourier Transform Infrared Spectroscopy
BS ---------- Borosilicate
CLSM ---------- Confocal Laser Scanning Microscopy
Ct ---------- Cycle Threshold
dbRDA ---------- Distance-based Redundancy Analysis
DDTs ---------- Dichlorodiphenyltrichloroethane
DO ---------- Dissolved Oxygen
EPS ---------- Extracellular Polymeric Substances
EU ---------- Europe
HDPE ---------- High-density Polyethylene
LDA ---------- Linear Discriminant Analysis
LDPE ---------- Low-density Polyethylene
LEfSe ---------- Linear Discriminant Analysis Effect Size Method
LLDPE ---------- Linear Low-density Polyethylene
MaPs ---------- Macroplastic
MBRs ---------- Membrane Bioreactors
MPs ---------- Microplastic
Mt ---------- Million Tons
NH_4^+ ---------- Ammonium
NO_2^- ---------- Nitrite
NO_3^- ---------- Nitrate
NOAA's ---------- National Oceanic and Atmospheric Administration
Nps ---------- Nanoplastic
OTU ---------- Operational Taxonomic Unit
PA ---------- Polyamide
PAHs ---------- Polycyclic Aromatic Hydrocarbons
PBDEs ---------- Polybrominated Diphenyl Ethers
PBS ---------- Poly(butylene succinate)
PC ---------- Polycarbonate
PCBs ---------- Polychlorinated biphenyl
PCL ---------- Polycaprolactone
PCoA ---------- Principal Coordinates Analysis
PCR ---------- Polymerase Chain Reaction
PERMANOVA ----- Permutational Multivariate Analysis of Variance
PET ---------- Polyethylene Terephthalate
PHA ---------- Polyhydroxyalkanoates
PHB ---------- Poly-3-hydroxybutyrate
PLA ---------- Polylactic Acid
PO_4^{3-} ---------- Orthophosphate
POM ---------- Polyoxymethylene

PP ---------- Polypropylene
PS ---------- Polystyrene
PU---------- Polyurethane
PVC---------- Polyvinyl Chloride
QIIME ---------- Quantitative Insights into Microbial Ecology
QIIME2---------- Quantitative Insights into Microbial Ecology 2
qPCR ---------- Quantitative PCR
RDA ---------- Redundance Analysis
RO ---------- Reverse Osmosis
Sdr ---------- Developed Interfacial Area Ratio
SEM ---------- Scanning Electron Microscopy
Sku ---------- Kurtosis Value
SRA ---------- Sequence Read Archive
TKN ---------- Total Kjeldahl Nitrogen
TN ---------- Total Nitrogen
TOC ---------- Total Organic Carbon
TP ---------- Total Phosphorus
UPGMA ---------- Unweighted-pair Group Method With Arithmetic Mean
vP ---------- Very Persistent
WWTP ---------- Wastewater Treatment Plant

PUBLICATIONS

Martínez-Campos, S., González-Pleiter, M., Fernández-Piñas, F., Rosal, R., Leganés, F., 2021. Early and differential bacterial colonization on microplastics deployed into the effluents of wastewater treatment plants. Sci. Total Environ. 757, 143832. https://doi.org/10.1016/j.scitotenv.2020.143832

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