ABSTRACT

Title of Dissertation:	MICROBIAL BIOFILMS ON MICROPLASTICS: A LOOK INTO THE ESTUARINE PLASTISPHERE OF THE CHESAPEAKE BAY
	Ana Paula Sosa Morfin, Doctor of Philosophy, 2021
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Microplastics are plastic particles that are smaller than 5 millimeters and are often found as pollution in our waterways. These polymer particles are globally distributed and are a direct result of human activity. Because of their rigidity and durability, microplastics are an ideal substrate for enhanced microbial growth and biofilm development. While microplastics have been studied in various contexts, only few studies have characterized the microbial communities on different types of plastic particles, but no study has been done in the estuarine water. In this study, we exposed three different types of plastics (polypropylene, polystyrene, and polylactic acid) to the water of Baltimore's Inner Harbor, along with a non-plastic glass control. We used both *in situ* and

in vitro incubations to understand the development of biofilm communities on microplastics. Microbial communities were analyzed based on the 16S rRNA gene sequences. We found that microbial composition on biofilm is distinct from that in the surrounding water, and different microplastic types have a minor impact on the composition of biofilm communities. The similarity between microbial communities on plastic and non-plastic particles suggests that surface supports rather than material types could be more critical for biofilm formation. Succession of microbial communities on the microplastics and interesting bacterial groups were described. Isolation and microscopic observations were also applied in this study. The presence of phototrophic organisms like filamentous cyanobacteria and *Auxenochlorella* on microplastic biofilms is interesting, and little is known about their contribution to carbon fixation in the ocean. Biofilms formed on microplastic surfaces could potentially affect the ecosystems via different mechanisms, including local nutrient cycling and the transportation of invasive or harmful species. As plastic production and mismanagement continues to be pervasive in our society, it is paramount that we include biofilm development into the framework of general ecology in order to truly understand the impact of plastic pollution and safeguard our ecosystems.

MICROBIAL BIOFILMS ON MICROPLASTICS: A LOOK INTO THE ESTUARINE PLASTISPHERE OF THE CHESAPEAKE BAY

by

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

To my parents and my brother, for always believing in me and loving me unconditionally.

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List of Abbreviations

Basic Local Alignment Search Tool (BLAST)

BioAnalytical Services Lab (BASLab)

Chlorophyll a (chl a)

Department of Natural Resources (DNR)

Dissolved organic carbon (DOC)

Dissolved organic matter (DOM)

Dissolved organic nitrogen (DON)

Dissolved organic phosphorus (DOP)

Dissolved organic sulfur (DOS)

Extracellular polymeric substances (EPS)

High molecular weight (HMW)

Low molecular weight (LMW)

Luria Bertani media (LB)

MUSCLE (Multiple Sequence Alignment)

National Center for Biotechnology Information (NCBI)

Next-generation sequencing (NGS)

Operational taxonomic units (OTU)

Particulate organic matter (POM)

Phosphate-buffered saline (PBS)

Polyethylene (PE)

Polyethylene therpalate (PET)

Polylactic acid (PLA)

Polymerase Chain Reaction (PCR)

Polypropylene (PP)

Polystyrene (PS)

Polyvinyl chloride (PVC)

Principal component analyses (PCoA)

Reasoner's 2A media (R2A)

Scanning electron microscopy (SEM)

Tryptic Soy Agar media (TSA)

Chapter 1 – Introduction

Organic matter in the ocean

Organic matter in the ocean is defined as any compound that can act as an electron and proton donor and thus a source of energy for organism growth (Morel & Price, 2003). Organic matter can be divided into two main categories, particulate organic matter (POM) and dissolved organic matter (DOM). POM is the organic matter retained on filters with 0.45-0.7 μm pore-size, while the organic matter in the filtrate is defined as DOM (Verdugo et al., 2004). Generally speaking, POM is present in sediments and suspended in the water column and DOM acts as a solute and can remain in the water column for long periods of time (Schneider et al., 2003). Because of the classification of organic matter being operational, some prokaryotes and viral particles can be included in the DOM pool; this pool is otherwise lifeless and most of the living biomass is part of the POM fraction (Carlson & Hansell, 2015). The POM pool is made up of a varying mixture of large proteins, lipids, carbohydrates, nucleic acids, among others (Fraga et al., 1998).

A large fraction of DOM is composed of dissolved organic carbon (DOC), dissolved organic nitrogen (DON), dissolved organic phosphorus (DOP) and dissolved organic sulfur (DOS) and some necessary metals for biological activity such as zinc and iron. DOC can be divided into air-sea exchangeable organic carbon, low molecular weight (LMW) and high molecular weight (HMW) forms (Dachs et al., 2005). Some DOM can pass through the classification filters but not be “truly dissolved” but can sometimes appear as its colloidal or gel-like form, formed by the aggregation of its molecules (Alldredge et al., 1993)

Both DOM and POM serve as an energy source because they are degraded by microbes and grazed by zooplankton in various pathways (Azam et al., 1983). Organic matter also absorbs UV and visible light and can influence phytoplankton growth and productivity in the ocean (West et al., 1999).

LMW DOC makes up around 75% of the DOC pool and most of it can be transported across the cell membrane of prokaryotes but HMW DOC requires pre-uptake extracellular digestion (Nagata, 2008). The metabolic processing of DOC is a vital part of the oceanic carbon cycle in which this nutrient is produced by phytoplankton in the surface waters, metabolized by microbes and stored in the deep layers of the water column (Wagner et al. 2020).

Organic pollution

Organic pollutants are ubiquitously present in waterways, particularly in regions where human activity contribute greatly to effluents and water treatment (Bedding et al 1982). Organic matter, both naturally occurring and synthetic, is introduced into bodies of water in the form of chemicals used as medications, cleaners, fertilizers, etc. (Kolpin et al., 2002). Plastics are synthetic organic polymers and can be considered a persistent form of organic pollution as they can accumulate in the environment and remain for extended periods of time (Worm et al., 2017). Microplastics that enter ecosystems can add to the existing POM pool and contribute to the sinking of organic matter into the water column and the sediments.

History of plastics

Polymers are defined as chemicals composed of large monomer units bound in chains that can form one-dimensional, two-dimensional and even three-dimensional molecules (IUPAC, 2014). Polymers can be found in nature and can play essential roles in biological processes; examples of this include rubber, cellulose, proteins and even nucleic acids.

Plastics are polymers that are man-made and have been invented and developed by material scientists for over a century. The first fully synthetic plastic was created in 1907 by Leo Baekeland as he combined phenol and formaldehyde and named the product Bakelite (Baekeland, 1909). Synthetic plastics are composed of a chain of repeated identical molecules, or polymers, usually made up of carbon and hydrogen. These materials are highly flexible and extremely durable, making them an excellent choice in manufacturing.

Pure plastics, however, often need added chemicals to have the desired properties for specific products. Additives can make these materials more flexible and malleable, as well as give them a different color or a softer finish. Virtually all modern plastics contain at least one chemical additive (Lithner et al., 2011).

Currently, hundreds of millions of tons of plastics are produced each year globally, and their uses range from packaging and insulation to manufacturing of equipment, furniture and vehicles (Andrady & Neal, 2009). The initial production purpose of plastic was to make goods that were easy to manipulate, cheap to manufacture, and would last a long time. Today, most plastic goods are made to be used one time and then be disposable. Well-known examples

of this are plastic cutlery, take-out food containers, grocery bags, drink bottles, and straws, among many others (Andrady & Neal, 2009).

The most common polymers produced are polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET), all of which are primarily petroleum-based substances (Geyer, 2020). In general, these materials are not considered biodegradable as the degradation of their hydrocarbon composition happens in a timescale that can be deemed irrelevant for their waste management (Andrady, 2015). Plastics that are produced as deliberately biodegradable are characterized as bio-based and can be metabolized by microbes in ideal growth conditions (Andrady, 2015).

Plastic pollution

Nearly 400 million tons of plastic are produced world-wide every year (Geyer et al., 2017) and 8 million tons of plastic waste makes its way to the ocean through stormwater runoff, wind and illegal dumping (Jambeck et al., 2015). Millions of tons of plastic enter the marine ecosystem every year, with 80% originating from litter (Li et al., 2016). Sources for the other 20% include commercial fishing gear and waste products or effluent from wastewater treatment plants (Ramírez-Álvarez et al., 2020).

The majority of plastic waste comes from post-consumer sources but there is a portion of waste that is generated during the manufacturing process (Geyer, 2020). Global plastic production has superseded that of any other material and PE and PP account for approximately 45% of this production (Plat, 2018). Only

about 8% of all-time plastic manufacturing has been that of recycled materials and the other 92% has been produced from fossil-fuel sources (Geyer et al., 2017).

Life-cycle analysis of plastic materials shows a lack of consideration for waste management in most products. Plastic litter finds its way into all waterways throughout the chain of disposal and even through wastewater after it has gone through treatment plants (Jambeck et al., 2015). Unfortunately, plastic debris is now considered as ubiquitous and potentially harmful to all members of marine ecosystems (Lusher, 2015).

As plastic enters the freshwater and marine systems, it is transported by currents and can be suspended in the water column or sink to sediments, depending on the density of the material (Galgani et al., 2015). Denser plastics like PET tend to sink and less dense plastics like PS will float unless aggregated with dense materials.

Marine plastic debris research has been prevalent in the last few decades, revealing deep impacts to human health and safety, as well as threats to the wildlife and natural processes of an ecosystem (Sheavly & Register, 2007). Animals that live in or near the water are in risk of entangling in or consuming plastic debris and suffering lethal consequences (Laist, 1997). Additionally, harmful or invasive species can grow on pieces of debris and be transported through large distances (Winston et al., 1997). In contrast, freshwater debris research is only now starting to become more extensive (Blettler & Wantzen, 2019). There is a large knowledge gap in freshwater and estuary plastic

pollution research and future work should be encouraged and considered in the creation of environmental policy in regard to this problem.

Plastic pollution at the Baltimore Inner Harbor

Baltimore's Inner Harbor, located in the Patapsco River region of the Chesapeake Bay, is exposed to increased plastic pollution. Heavy rains bring large amount of plastic trash into the Inner Harbor. The pollution in this harbor, as in many other urban waterfronts, is mostly composed of single-use consumer plastics such as plastic bags, Styrofoam containers, cigarette butts, plastic bottles and even sports balls (The Waterfront Partnership of Baltimore, 2021). Much of this pollution is introduced into the Inner Harbor in the form of public littering combined with stormwater runoff. Additionally, the Jones Falls tributary is the largest source of water in the Harbor and it can be an important source of debris (Kormann, 2019). The number of plastic debris pieces entering the Harbor has not yet been quantified exactly but it can be assumed that it is a larger amount than what is being filtered by the trash wheels, as only big plastics can be trapped by their mechanism and smaller pieces, such as micro and nanoplastics, can easily flow through.

Baltimore's urban waterfront currently has four anthropomorphic solar-powered trash collecting wheels (Fig. 1.1). The first one that was installed, Mr. Trash Wheel sits only a few hundred feet away from the sampling site of my thesis study. This trash wheel is an attempt to make the plastic pollution problem personal to the citizens of Baltimore City and the collective trash wheels have

filtered out 1608 tons of trash to this day (The Waterfront Partnership of Baltimore, 2021).

Figure 1.1. The anthropomorphic solar-powered trash collector in Baltimore's Inner Harbor Mr. Trash Wheel.

Microplastics

Microplastics are polymer fragments that are smaller than 5 millimeters, and they can be classified as primary or secondary microplastics (Cole et al., 2011). Primary microplastics are released into the environment directly from industrial processes, cosmetics, toothpaste, and textiles (Costa et al., 2010; Zitko & Hanlon, 1991). These beads and microfibers from synthetic clothing are the most common type of waste. Secondary microplastics are those that break

down from larger pieces of plastic debris; this fragmentation is usually caused by physical stress such as wave action or exposure to light (Andrady, 2011; 2017). These microplastic particles are ubiquitous, abundant and long-lasting in the aquatic environment.

It is hypothesized that the concentration of microplastics in aquatic systems greatly varies across different geographical locations, as well as for different zones in bodies of water. Their quantification has represented a challenge because of the lack of standardized protocols (Cole et al., 2011). Variations in microplastic quantification are also a result of variable sizes, shapes, densities and chemical composition of microplastics (Ryan et al., 2009). Some polymers will float in surface waters and some others will sink down the water column and sometimes reach the bottom and accumulate in sediments (Cózar et al., 2014; Erni-Cassola et al., 2019). Concentrations of microplastics have been reported ranging from 3 to 4,500 mg/L in marine environments (Green et al., 2017).

In 2014, Yonkos et al. trawled four estuarine tributaries (Patapsco, Rhode, Magothy and Corsica) of the Chesapeake Bay and found significant concentrations of microplastic particles in a range of three orders of magnitude (<1.0 to >560 g/km^2) when calculating the concentration per unit of surface area (Yonkos et al., 2014). They concluded that the locations that had the higher concentration of microplastic particles correlated to locations with higher human population densities.

Due to the wide presence of microplastics in water column, bacterioplankton, phytoplankton and zooplankton and other marine life forms are exposed to microplastic particles. We have just begun to study the interactions between microplastics and marine living organisms.

Microplastics and the aquatic food chain

While large pieces of plastic debris represent a threat for living organisms through the potential for entanglement and ingestion, the ecological impact of microplastics are harder to identify and quantify. Physiological effects in animals can present as an inflammatory response, hindered growth and reproduction and decreased eating, among others (von Moos et al., 2012; Cole et al., 2015). These effects can be caused by the polymers themselves or by the chemical additives. These chemicals can include plasticizers, flame retardants or any sorbed organic pollutants from the surrounding environment (Andrady, 2011).

The accumulation of microplastic particles in the tissues of organisms has been documented, especially in those of aquatic invertebrates (Fernández & Albentosa, 2019) and fish (Ding et al., 2018) and microplastics were found in commercial aquaculture species (Feng et al., 2019; Wu et al., 2020). Polymers have been found in the guts and digestive tracks of mussels, oysters, crabs, sea urchin, sea bass and clams (Geyer, 2020).

Understanding the ecological impact of microplastics is a challenge because of all the varying sizes, shapes, chemical composition and environmental concentrations of the particles and the lack of standard methods for this type

of research. Plastic debris is widely present in seabirds (Cadée, 2002), fish (Carpenter et al., 1972; Foekema et al., 2013) and shellfish (van Cauwenberghe & Janssen, 2014; Vroom et al., 2017). Marine organisms can mistake microplastics as natural foods. Algae and bacteria grown on plastic surface can make the microplastics smell more like real food and prone to be grazed by marine organisms (Vroom et al., 2017). However, we know very little about what kind of microorganisms can form the biofilms on microplastics. Even less is known about colonization of microbes on different types of plastics. Marine and estuarine environments are affected by microplastics at every level. At the base of the trophic pyramid, microorganisms settle on plastic particles, creating a complex biofilm, making them easily available to zooplankton, crustaceans and bivalves.

Bacteria and certain types of eukaryotes (like diatoms and hydroids) are able to colonize microplastic particles and generate complex biofilms that allow them to use polymers as a persistent growth surface and a transportation vector; these attached biofilms have been proved to be distinct from the free-living communities existing in the surrounding water and to impact said communities (Oberbeckmann et al., 2014; Zettler et al., 2013). When the concentration of microplastics is high enough, nutrients in the surrounding water could potentially be impacted by the abundant microbes growing on microplastics, thus significantly affecting the biogeochemical cycling. If microbial communities change, the composition of phytoplankton could also change. Direct grazing of polymer particles by marine species is another potential concern when

concentrations of microplastics are high. Evidence shows that many marine organisms can confuse particles for food if they are in the same size range, same environment. Marine zooplankton are particularly vulnerable to this effect, especially invertebrates and fish. Filter-feeders like copepod and euphasiid (Desforges et al., 2015) species have been found to ingest microplastics and suffer negative effects to their health (reproductive problems, deformities, and mortality). Because these filter feeders serve as a food source for larger zooplankton, they can contribute to the transfer of particles up to higher trophic levels in a phenomenon known as bioaugmentation. Some copepod species have been found to selectively feed on polymers and to decrease their consumption of regular food after ingesting them (Cole et al., 2015). Other filter-feeders like bivalves respond differently when exposed to microplastics. With the presence of microplastics, bivalves increased water filtration rates in some cases, but decreased in some other cases. Bivalves can also be very efficient at egesting the ingested particles and purging after a few hours. It is clear that most of these effects are species-dependent (Green et al., 2017). It is important to understand that the interaction with microplastics is highly organism and species-dependent, and it is also affected by the state of the particles.

Another factor to consider is the effect that the ingestion of microplastic particles can alter the intestinal microbiome of animal species (Lu et al., 2019). Changes in microbiota composition can lead to decreased digestive rates and

absorption of nutrients but can also significantly impact the microbial community as a whole (Wang et al., 2021).

The plastisphere

Biofilms are formed by microorganisms on a surface as a form of protection, and as a way to improve their probability of survival by increasing their nutrient availability and their competitive advantages (Fig. 1.2); it is composed of microbial cells and extracellular polymeric substances that help them attach to surfaces (Costerton et al., 1995).

Biofilm formation on plastic is no exception, and microplastics provide further advantages for microbes as these particles can subsist for decades in aquatic environments (Zettler et al., 2013). Fish and other organisms consume plastics and move them up the food chain, potentially reaching humans. These particles can also act as transportation for harmful organisms (Masó et al., 2003a) and chemical pollutants (Hirai et al., 2011), helping them move and persist in the oceans.

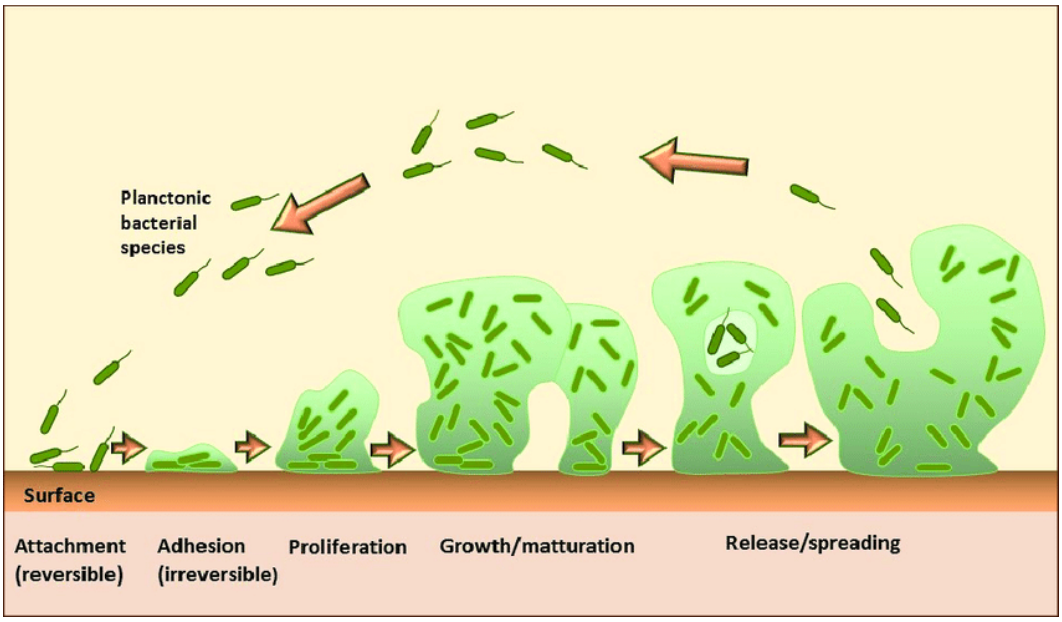

Figure 1.2. Schematic drawing of biofilm formation from Rukavina et al., 2016.

The term “plastisphere” (Zettler et al., 2013) was created and has been adapted to describe the interaction of any organism with microplastic particles, but for the purpose of this work, it will be used to describe the microorganisms that exist on the surface of polymer particles.

Factors that define the composition of the plastisphere

Polymers that have been in the water for some time can accumulate organisms on their surface in the form of biofilms and even favor their aggregation with other organic particles like marine snow (Jahnke et al., 2017). Known as aged microplastics, these particles can be weathered, fragmented, and when in big aggregates, can sink in the water column and settle in sediments. These aggregates can also absorb pollutants and potentially contain pathogenic species that are being transported in ways that they normally would not be (Bryant et al., 2016; Zettler et al., 2013).

It is crucial to understand the different microorganisms that attach to and live on plastic debris in aquatic systems and their metabolic capacity. This will help us assess their potential roles in global nutrient cycles, microbial species dynamics and even their potential to be utilized as bioremediation for plastic pollution.

Studies into the successive colonization of marine microplastics have shown that the initial colonization stage happens rapidly, and it is dominated by Alphaproteobacteria and Gammaproteobacteria (Oberbeckmann, Löder, Labrenz, et al., 2015), followed by the Bacteroidetes (Lee et al., 2008), and from that point, the physical and chemical properties of the attachment surface determine the composition of the biofilm in the early stages (Pompilio et al., 2008).

Community composition of plastisphere can be affected by many factors, just like that of the surrounding water. The 16S rRNA gene-based analysis has been used to study the microbial composition of biofilms on microplastics (and their perspective controls i.e., wood, glass) and the roles of factors in the determination of community composition (Oberbeckmann & Labrenz, 2020). Their study found that geographical region is a determining factor in biofilm composition, and particle chemical composition plays a small role as well. They also found that alpha and beta diversity on microplastic particles is significantly different than those on naturally occurring particles. Despite this dissimilarity, the ability of microplastics to transfer potentially pathogenic species like *Vibrio* has not been proven to be significantly greater than that of natural particles like

wood. It is suggested that the unnaturally long durability of plastic makes it a much better candidate as a long-term vector for pathogens and other harmful species (Oberbeckmann & Labrenz, 2020).

Early research focused on the plastisphere was limited by what could be observed by microscopy or cultured in a laboratory setting (Lobelle & Cunliffe, 2011a). The advancement of next-generation sequencing (NGS) has allowed for a more detailed description of the taxonomic diversity present in these biofilms (Zettler et al., 2013). High-throughput DNA sequencing is an invaluable tool for the study of the microorganisms that attach to polymer particles in any environment, but especially in the aquatic environment, where the culture and isolation of microbial species can be a special challenge relative to that of terrestrial species.

The ecological role of the plastisphere

Microplastic particles can cause the entire aquatic food chain to be affected and can even affect ecosystems by serving as vectors for pathogenic species, harmful algae and other invasive species. Harmful diatoms and bloom-causing dinoflagellates have been identified on plastic particles (Masó et al., 2003a, 2016). The presence and concentration of microplastics in aquatic environments, and the fact that all their exposed surfaces are colonized by microbes in a short period of time, results in a notable increase of biomass in the ecosystem. This in turn, inevitably affects the way in which microbial metabolism mediates the cycling of nutrients at a local scale.

At a global scale, however, the role that the plastisphere plays in biogeochemical cycling is not clear. It is possible that the inherent metabolism of the dominating microbial species could generate substrates that can influence global carbon cycling. It has been reported that as much as 23,600 metric tons of DOC is being released annually into the ocean reservoirs in the form of microplastic debris leaching (Romera-Castillo et al., 2018). Some prokaryotes have been found to have the capacity to transform polymer particles into DOC, increasing its concentration in areas with high incidence of microplastics (Huang et al., 2021). Furthermore, plastic particles that float in surface waters and are exposed to solar radiation have been found to release methane and ethylene gases, intermediate products that can act as electron donors for microbial metabolism (Royer et al., 2018).

The impacts of microplastic particles on the nitrogen cycle have been studied extensively. For example, polymer particles can alter both nitrification and denitrification in ecosystems (Seeley et al., 2020). PE particles have been proven to increase the incidence of denitrification and anammox genes in freshwater environments (Huang et al., 2021). Several studies have found contradicting results when utilizing various plastic substrates and measuring total nitrogen and ammonia concentrations (L. Li et al., 2020). Nitrogen cycling is mediated by microbial activity, and it would be hard to conceive that the plastisphere's metabolism is not closely related to the cycling of nitrogen.

In the case of phosphorus cycling, the presence of microplastics has been shown to lead to an increase in total phosphorus concentration in surrounding water as a result of the metabolism of the biofilms (Chen et al., 2020).

Plastics chosen for this study

Three different plastics were selected for the incubation studies of this dissertation. The plastic polystyrene was chosen as a substrate that is commonly found floating in surface waters, as it is less dense than water when in its expanded form (Wunsch, 2000). Polystyrene is a long chain hydrocarbon in which there is an aromatic phenyl group attached to alternating carbons (Fig. 1.3). This polymer is highly flexible and elastic, which allows it to be extruded and transformed into a foam, commonly known as Styrofoam (Scheirs, 2003).

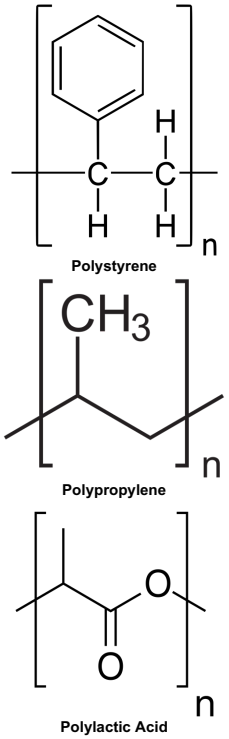

Figure 1.3. Molecular structure of synthetic polymers polystyrene, polypropylene and polylactic acid. Images obtained from Wikimedia Commons and are public domain.

Polypropylene is a synthetic polymer composed of a hydrocarbon chain that contains a methyl group on alternating carbons (Fig. 1.3). This methyl group confers the polymer great strength and thermal resistance (Gahleitner & Paulik, 2014).

Polylactic acid (PLA) is synthesized by fermenting plant starch and condensing lactic acid monomers (Södergård & Stolt, 2010). PLA is degradable by composting in industrial conditions which causes it to decompose into water and CO_2 (Iovino et al., 2008).

Scope of this dissertation

Previous studies on microplastic biofilm mainly focused on marine and freshwater environments. Little has been explored for the formation of biofilm on plastisphere in the estuarine water. My dissertation aims at understanding the development of plastisphere in the estuarine environment. Both *in vitro* and *in situ* incubations were performed in Baltimore's Inner Harbor or using the Inner Harbor water, and the microbial communities were analyzed by high throughput sequencing of 16S rRNA gene. In addition, cultivation methods were used to isolate bacteria and cyanobacteria grown on microplastics. The specific questions I would like to address are:

1. Are bacterial communities attached to microplastic particles in the Chesapeake Bay significantly different from the free-living bacterial communities in the surrounding water?
2. Are the bacterial communities different when they attach to the microplastics made from chemically different polymers?

3. What kind of biofilm is formed on non-plastic particles like glass beads?
4. Are photosynthetic cyanobacteria part of these biofilms? If so, what kind of cyanobacteria are they?

In correspondence to the above questions, four main hypotheses will be tested in this study.

Hypotheses:

1. Microbial biofilm communities developed on microplastics are different from free-living microbial communities in the surrounding water.
2. The chemical composition and physical properties of the substances making up the microparticles will be a determining factor for the bacterial community composition attached. A wide range of species will colonize the microplastic particles, including big players in biogeochemical cycling: primary producers, nitrogen fixers and methanogens.
3. The biofilms forming on the non-plastic inert control of glass microbeads will be significantly different to those attached to synthetic polymers.
4. Filamentous cyanobacteria are dominant cyanobacteria on plastisphere.

My whole thesis includes five chapters. Each chapter is briefly described as follows:

Chapter 1

The key concepts relevant to my thesis are introduced in Chapter 1, which include: plastic pollution in the Chesapeake Bay and Baltimore's Inner Harbor, microplastics and their impacts to aquatic ecosystems, the formation of biofilms on microplastics and what we know so far about their ecological characteristics.

Chapter 2

An *in vitro* experiment was conducted to address questions 1, 2 and 3 in Chapter 2. Bacterial communities developed on three different types of synthetic polymers were monitored over a period of 28 days. These biofilm communities were then visualized utilizing scanning electron microscopy (SEM). We also isolated and characterized 34 strains of bacteria grown on microplastics. The microbial communities of particle-associated samples were distinctly different to those from the water control, but microbial biofilm communities on different type of microplastics contained similar major bacterial populations. However, the communities on the non-plastic inert control of glass beads were significantly different than those on polymers. Interesting bacteria were found in the plastispheres of this study including filamentous cyanobacteria, Desuflobacterota and Planctomycetes.

Chapter 3

The main goal of chapter 3 is to address question 1, 2, and 3 in an *in situ* experiment. Microbial community analysis was performed on biofilm samples from three different types of microplastic beads placed in Baltimore's Inner Harbor to be colonized. We found similar trends to the results of chapter 2, in which microbial communities of water were significantly different from those on microplastic beads, but particle-associated communities were similar to each other. The biofilm communities on microplastics were similar to those on the glass beads (non-plastic control). The *in situ* study also suggests that microbial biofilm development is not affected by the different types of microplastics, and

surface matrix support leads to the formation of biofilm microbial communities which are different from free-living microbial communities.

Chapter 4.

Chapter 4 aims to answer question 4. The microbead samples from the *in vitro* experiment (Chapter 2) were used to isolate cyanobacteria. Five strains of filamentous cyanobacteria were isolated and morphologically characterized. The phylogenetic relationship of these filamentous cyanobacteria was investigated based their 16S rRNA gene sequences. The potential impact of phototrophic cyanobacteria on microplastics were speculated in terms of their carbon cycling and potentially harmful effects.

Chapter 5.

Chapter 5 discusses the main findings of these experiments and their implications. The limitations of the study are explored, and the potential future directions of this research are considered.

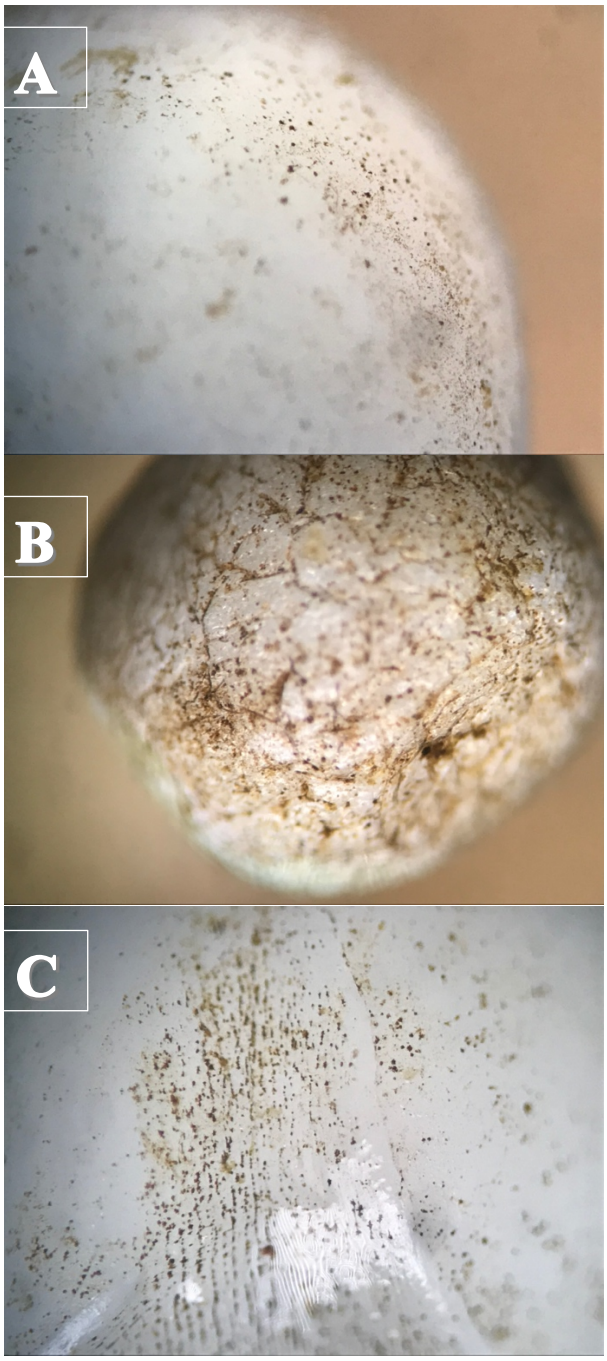

Figure 1.4. Microbial attachment onto polymer microbeads of the materials a. polylactic acid b. polystyrene and c. polypropylene. These beads were incubated in Inner Harbor water for 14 days prior to taking these photographs using a dissection microscope.

Chapter 2. Estuarine microbes on plastisphere*: in vitro* studies

Abstract

Plastic debris is present in virtually every environment, and there is an increasing concern on the impact of plastic pollution on ecosystem health. Microorganisms are known to colonize and form biofilms on plastic surface. However, the interactions of microbes and synthetic polymers in the estuarine environment have not been well studied. This study is aimed to investigate how the estuarine bacterial communities colonize on microplastic debris. Three different types of synthetic polymers were exposed to the estuarine water and incubated *in vitro*. The changes on microbial community structure and potential function on biofilms during two *in vitro* incubations were analyzed based on the 16S rRNA gene sequences. Using scanning electron microscopy (SEM), we observed development of interesting microbes such as bacteria, filamentous cyanobacteria, diatoms, etc on the surface of microbeads. A total of 34 bacterial strains were isolated from microbeads and water samples. Two *in vitro* incubation experiments show that the biofilm communities on plastic beads differ greatly from those in the surrounding water. The biofilms that formed on the glass control during the second incubation were also significantly different to the ones formed on synthetic polymers. Fluctuations in the community structure between the two incubation experiments suggest that the initial community in the incubation can affect the composition of the biofilms greatly. The *in vitro* incubation provides a relatively stable condition for biofilm development, but it does not reflect *in situ* environmental changes. Future work is needed to understand the formation of biofilms on microplastics in the real estuarine water.

Introduction

Complex biofilms that form on plastic particles have been dubbed the "plastisphere" (Zettler et al., 2013) and the microorganisms from these biofilms have not been studied extensively enough. While the plastisphere has been thoroughly documented in various marine environments (Jacquin et al., 2019; Oberbeckmann et al., 2015, 2016; Oberbeckmann & Labrenz, 2020; Quero & Luna, 2017), little is known about the formation of plastispheres on microplastics in the estuarine water. Estuaries are a unique ecosystem where freshwater meets seawater. The estuarine watershed like the Baltimore Inner Harbor contains the bacterioplankton populations from both freshwater and marine origin, and the microbial community is greatly affected by temperature and salinity (Kan et al. 2006).

Microplastic particles are an emerging concern as they exist ubiquitously in marine and freshwater systems and provide a substrate with a considerably large cumulative surface area for microorganism attachment and growth. Microplastics are any synthetic solid particle of size ranging from 1 μm to 5 millimeters and can come from a variety of sources (Frias & Nash, 2019). Primary microplastics have been manufactured to be that size and are often found as microbeads or plastic nurdles. Secondary microplastics are those that have broken off a large piece of plastic debris and become a smaller particle.

Studies have shown that sampled coastal waterways and ecosystems close to urban areas contain much higher densities of microplastics than open ocean environments (Ballent et al., 2016; McCormick et al., 2016; Yonkos et al.,

2014). Urban freshwater rivers have been identified as delivery systems for microplastics to the marine environment (Auta et al., 2017; Blettler et al., 2017; Boucher & Friot, 2017; Browne, 2015; Campanale et al., 2020; Mora-Teddy & Matthaei, 2020; Said & Heard, 2020; Simon-Sánchez et al., 2019), and the highest concentrations of microplastics have been found in nearshore areas that are more developed and industrial in nature (Ballent et al., 2016; Burns & Boxall, 2018; Cable et al., 2017; Corcoran et al., 2015; Deng et al., 2020; Ramírez-Álvarez et al., 2020).

Plastics can persist in bodies of water from a couple of decades to several hundred years and travel across freshwater and marine ecosystems (Cable et al., 2017; Xu et al., 2020). Denser plastics like polyethylene terephthalate can sink and settle into the sediment (Mistri et al., 2020), accumulating in large quantities over time. Less dense plastics like polypropylene, which makes up 80 - 90% of floating marine debris, have the potential to be transported long distances in these ecosystems, since they are often suspended in the water column and are moved by currents and winds (Corcoran et al., 2015; Engler, 2012).

Taking all of these factors into consideration, it is easy to understand why the attachment of microbial communities has the potential to vary greatly based on the type of polymer that is being colonized, the existing bacterial communities in the surrounding water, the environmental conditions of the site and the interaction with other organisms present in the ecosystem. The bacterial biofilms that colonize plastics in estuarine waters have been studied over the

last few years in selected estuaries around the world (Guo et al., 2020; Laverty et al., 2020; Li et al., 2019) but there remains a knowledge gap as to what type of bacteria colonize different types of microplastics of different polymers and what their metabolic potential may be.

In this study, we intended to investigate the succession of microbial communities on three different microplastics using *in vitro* experiments. In the laboratory, different plastic beads were introduced into waters collected from the Baltimore Inner Harbor and incubated at room temperature. Two *in vitro* experiments were conducted with water samples collected in different seasons. Microbial community structure and potential function will be explored based on 16S rRNA gene sequences. In addition, we also observed the biofilm structure using SEM, and culture bacteria from biofilms.

Materials and Methods

Selection of microplastics

Polypropylene (PP) and Polyethylene (PE) make up the largest portion of the plastic debris in the Chesapeake Bay water (Bikker et al., 2020). PP was chosen as a solid polymer that is less dense than the estuary's water and would float on the surface, polystyrene (PS) was chosen as an expanded plastic with a large surface area that would also float on the surface, and polylactic acid (PLA) was chosen as a biodegradable polymer option that does not float.

Two *in vitro* incubation experiments were run in this study. The first one took place in May 2019, and the second one in September 2020.

First *in vitro* incubation experiment

The Inner Harbor water was collected on May 17th, 2019. The location of sampling site is shown in Fig. 2.1. Water quality data for the collected water was downloaded from the Department of Natural Resources' (DNR) Eyes on the Bay project (http://eyesonthebay.dnr.maryland.gov/) which has a monitoring station on the east side of the National Aquarium and within 200 meters of the sampling site. At this station, pH, temperature, dissolved oxygen, salinity, turbidity, and chlorophyll concentration were recorded every 15 min. At the time of collection, the monitoring station indicated the salinity of the nearby water was 2.9 ppt, the pH was 7.5, and the temperature was 18.2 °C. Dissolved oxygen on this date was 5.8 mg/L. Microplastic beads of three different polymers (polypropylene (PollyPlastics™) polystyrene (PolyFil®), and polylactic acid (3DXTech©) were placed in glass bottles with 1.5 L of the harbor water. Microplastic beads were sterilized prior to being introduced into the water by soaking them in 70% v/v ethanol overnight and subsequently rinsed with sterile deionized water. A bottle with the inner harbor water and no beads was included for control.

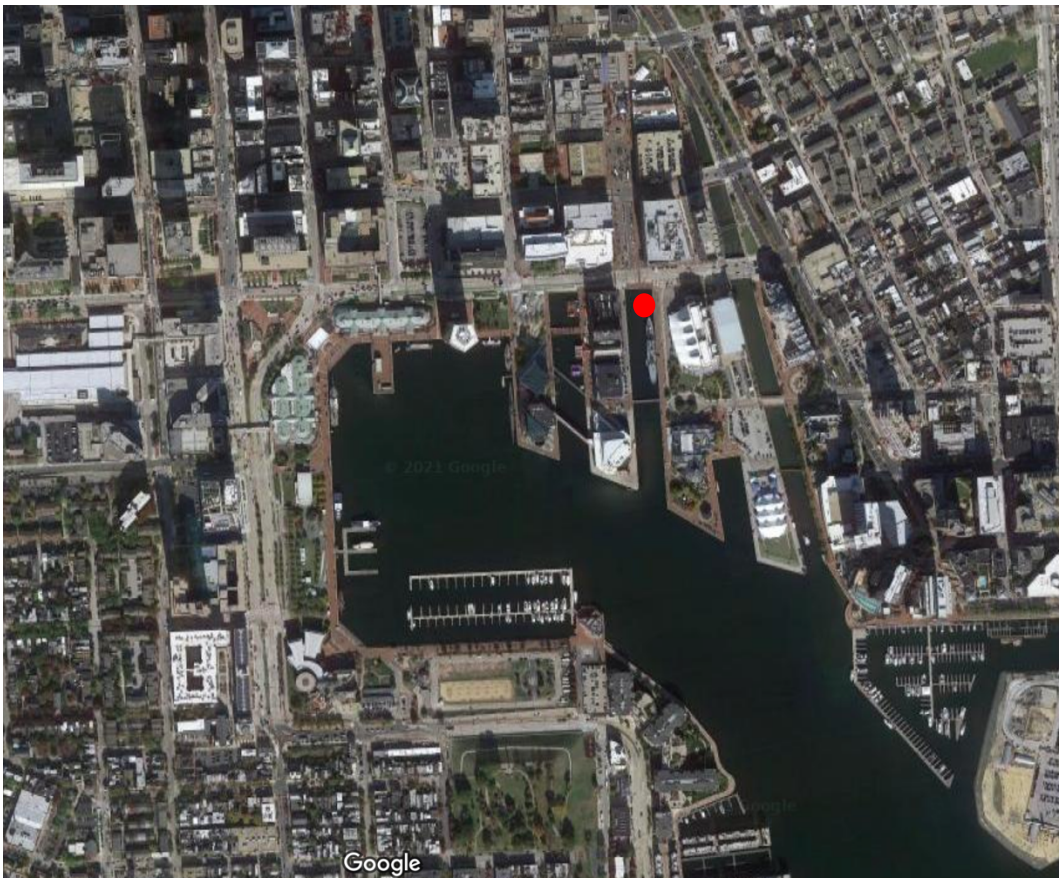

Figure 2.1. Satellite map of downtown Baltimore, Maryland, USA. The red circle marks the site where the water sample was collected.

All the bottles were set on the lab bench at room temperature (20-22 °C) and shaken by hand three times a week. Bottles were covered with a filter that allowed air flow but kept the bottles sterile (Fig. 2.2). The bottles were affected by the daylight from window and ceiling light in the lab, daylight light incidence for all bottles ranged from 6 to 8 μmol/m^2/s.

Figure 2.2. *In vitro* incubation of three different plastic microbeads and the water control on the benchtop. From left to right are PS, PP, PLA and the Harbor water, respectively.

Subsampling for bacterial community analysis

Microplastic beads were collected using a sterile metal scooping spoon and subsequently placed in 2 ml microcentrifuge tubes using sterile forceps for two timepoints: day 7 and day 28 of incubation. For the 16S rRNA gene community analysis, 50 beads were recovered at each time point from each material and rinsed with phosphate-buffered saline (PBS) buffer prior to being placed in microcentrifuge tubes. 100ml of water sample was collected from the control and filtered through a reusable filter tower (ThermoScientific™, Massachusetts, USA) using a .2 µm membrane filter. Filters were placed into a 2 ml microcentrifuge tube.

Subsampling for bacterial isolation and scanning electron microscopy work

To isolate bacteria, 10 beads of each material were recovered and rinsed with PBS buffer before being collected in a 2 ml microcentrifuge tube with 500 µl of sterile PBS buffer. Culture isolation was performed at the 10-month mark to attempt the growth of microbial strains enriched by the plastic material. 20 beads of each material were also collected for SEM observations (see below) and stored in 4% paraformaldehyde at -20°C until the time of sputter coating for SEM observations. Time points for SEM visualization were selected based on the end of the experimental incubation for the 28-day timepoint and the sample taken for culture isolation at the 10-month timepoint.

Bacterial isolation and identification

The beads submerged in PBS were shaken in a vortex for 1 minute to detach bacteria from the biofilm. 50 µl from each tube of PBS were spread onto agar plates of Reasoner's 2A (R2A) media (BD™ Difco™), Tryptic Soy Agar (TSA) media (Millipore®) and Luria Bertani (LB) Agar (Sigma-Aldrich®). Three different media types were selected for the growth and isolation of bacterial strains, R2A as a low-nutrient medium in which slow-growing bacteria can get the opportunity to grow without being overtaken by fast-growing species, such as enterococci. LB media was chosen as a universal media in which most bacteria that had the capability to grow would do so. Agar plates were kept at room temperature on a benchtop (20-22 °C) and laboratory lighting for 96 hours. Colonies from each of the three polymer times and from the control water bottle were picked based on different colors, sizes and morphologies to

streak in a new R2A, TSA and LB agar plates. Each different colony was attempted to be isolated. A total of 42 colonies were streaked and only 34 strains were able to be re-streaked after 96 hours of cultivation in the same conditions as the initial plates. Strains were further streaked two times for isolation. Isolated strains were characterized and photographed; a single colony from each strain was used to run colony PCR to amplify the 16S ribosomal RNA fragment with the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR products were purified with the GeneJet PCR Purification Kit (Thermo Fisher Scientific) and submitted for sequencing at the Insitute of Marine and Environmental Technology's BioAnalytical Services Lab (BASLab). Both forward and reverse primers were used for sequencing and two FASTA files were obtained for each sample. Obtained sequences were run on the Basic Local Alignment Search Tool (BLAST) to be compared to the National Center for Biotechnology Information (NCBI) database.

Scanning electron microscopy

SEM photographs were obtained at the Electron Microscopy Core Imaging Facility at the University of Maryland School of Dentistry in Baltimore, Maryland. The samples were processed and prepared by the facility prior to visualization by dehydration and sputter coating. The microscope used was the FEI Quanta 200 in the High Vacuum mode. Microbead samples from the *in vitro* experiment

were visualized at the time stamps of 28 days and 10 months for each respective polymer type.

Analysis of microbial community

DNA from biofilms of both incubation experiments was extracted using the IBI Soil DNA Extraction Kit (IBI Scientific©), water samples were processed using the PowerWater DNA Isolation Kit (QIAGEN©). Samples were sent in for MiSeq sequencing (Illumina) of the 16S ribosomal RNA subunit at the BioAnalytical Services Laboratory at the Institute of Marine and Environmental Technology. Obtained reads were paired and trimmed for quality using CLC Genomic Workbench 8 and put through the QIIME 2 bioinformatic pipeline (Bolyen et al., 2019) for bacterial community analysis utilizing the SILVA 132 taxonomy database (Quast et al., 2013). QIIME 2 was used to filter the raw reads for sequencing quality, denoise with the dada2 plug-in and to pick operational taxonomic units (OTU) with the standard settings of the software. OTUs were filtered by a minimum sequence number of 10 and subsequently classified by taxonomy using the Naive Bayes classifier trained for the V4 region of the 16S rRNA genes (515F/806R primers) and organized by abundance.

Diversity measures and PERMANOVA

An alpha rarefaction curve was generated using QIIME 2 based on the observed features (OTU) and the sequencing depth. Curves were generated by material type. Phylogenetic metrics and statistical analysis using PERMANOVA was performed in the form of pairwise tests to determine group significance. Principal component analyses (PCoA) using the weighted and

unweighted unifrac diversity measures were run to determine community dissimilarity in beta diversity. These tests were run on a python environment by using the pertinent QIIME 2 pipeline commands (Bolyen et al., 2019). Beta diversity represented by PCoA charts using unweighted unifrac (Fig. 2.12) and weighted unifrac (Fig. 2.13) distance show the water samples being isolated as a group in all instances. Unweighted unifrac distance analysis does not consider abundance of the OTUs and is a phylogeny-including taxonomic measure. Weighted unifrac distance, on the other hand, considers the abundance of taxonomic units. This provides two perspectives, one that gives weight to the most abundant taxas and one that gives equal weight to abundant and rare taxas.

Ecological Interpretation of 16S marker data

OTU tables with the corresponding taxonomy assigned were then compared to the FAPROTAX (Louca et al., 2016) database. This database will map the known OTUs to ecologically relevant metabolic functions of known and cultured species that are closely related. The collapse.py command in Python will collapse the OTU tables generated by QIIME 2 and convert them into a profile describing the functional group and its abundance for each known taxonomic unit in the database. This will result in an analysis of the metabolic or relevant ecological functions of the described bacterial communities that result from the 16S rRNA gene amplicon characterization using a reference database based on the literature of cultured bacterial representatives of genera or species.

Second *in vitro* incubation experiment

A second *in vitro* study was conducted on September 4, 2020 by including a non-plastic particle (glass beads) as a control. The first *in vitro* experiment was conducted without glass bead control using the Harbor water collected in spring (May), 2019. It should be noted that these two *in vitro* experiments were carried out with the Harbor water collected in two different seasons. The harbor water for the second *in vitro* experiment had higher salinity (8.7 ppt), temperature (27.2 °C), pH (7.7), and dissolved oxygen (6 mg/L) compared to the water used for the first *in vitro* experiment.

The incubation setup was similar to the first experiment except that a bottle with glass beads (BioSpec) was added as an inert control. Only microbial community analysis was performed for the second experiment. No culture isolation and SEM observations were conducted in the second experiment. Three more subsampling points (day 3, 14 and 21) were added for the second *in vitro* experiment. DNA extraction, sequencing and sequence analysis, statistical analysis, and metabolic prediction followed the same procedures described for the first *in vitro* experiment.

Due to the timing of these experiments being interrupted by the lockdowns, closures and material shortages of the COVID-19 pandemic, the SEM imaging and traditional culturing and isolation of microbial strains were not performed for the second *in vitro* incubation experiments and were only focused on the third.

Results

Bacterial isolation and identification in the first *in vitro* incubation

After 96 hours of incubation on the benchtop, agar plates of the three media showed varying amounts of colonies and bacterial growth (Fig. 2.3). The TSA agar plates from the water and PP samples showed no growth of any colonies, the plate from the PS sample showed 12 colonies with variable sizes, and the plate from the PLA sample showed 5 colonies of equal size and color. The LB plate from the water sample had 6 small white colonies, the plate from the PS sample showed similar growth of small white colonies. The LB plates from the PP and PLA samples showed many colonies of different sizes and morphologies, and an overgrowth film on the agar (Fig. 2.3).

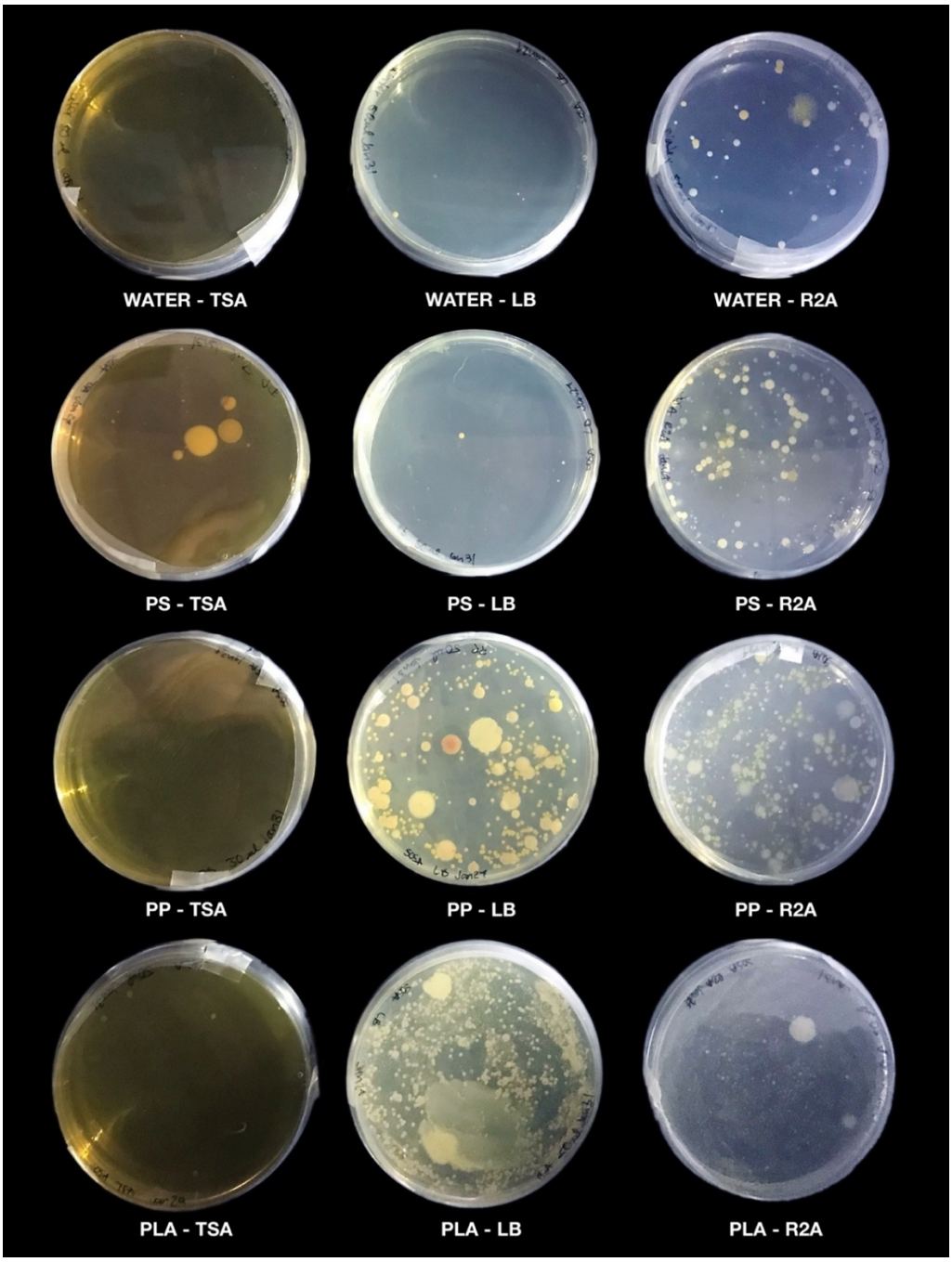

Figure 2.3. Agar plates of all materials: water, polystyrene (PS), polypropylene (PP) and polylactic acid (PLA). Samples were plated on three types of media: tryptic soy agar (TSA), Luria-Bertani Agar (LB) and Reasoner's 2A (R2A).

The plates of R2A media showed the most colonies for the water and PS samples, with 42 and over 100 colonies respectively. The R2A plates for PP

and PLA samples had too many colonies to count, and a film of growth all over the plates. Colonies of all plates showed a range of sizes, colors and textures. Some colorful strains were shown in Fig. 2.4.

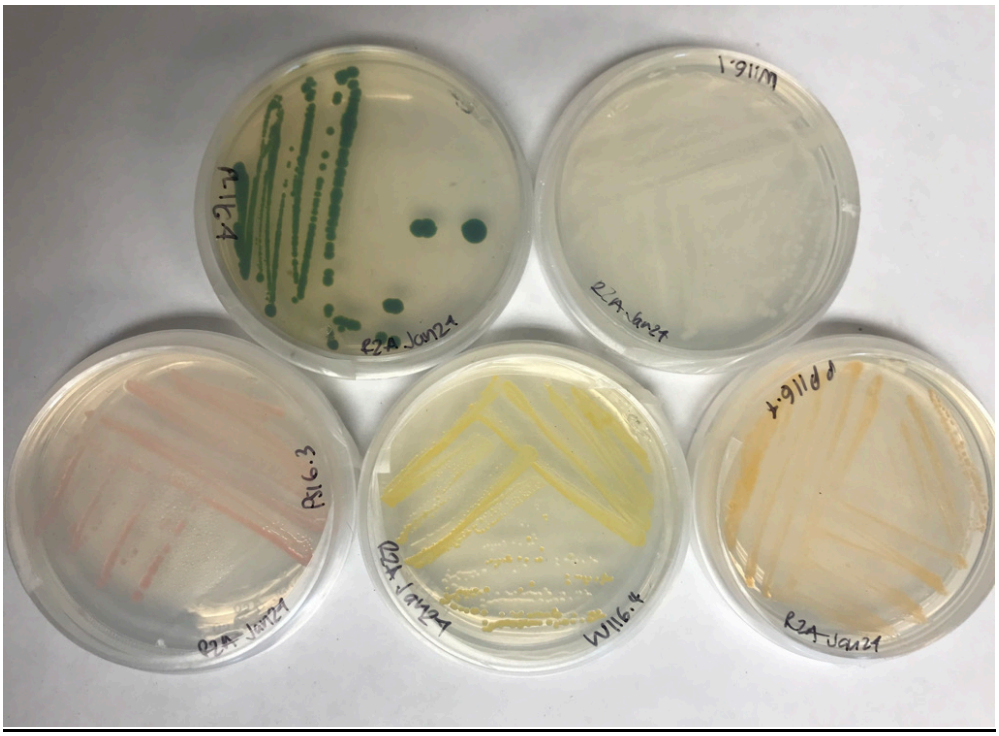

Figure 2.4. Five of the isolated strains grown on R2A media, demonstrating the pigmentation profile of the microorganisms cultured from the samples. From left to right, top to bottom: strain 12, 10, 4, 6 and 5.

A total of 34 bacterial strains from different microbeads and the surrounding water were isolated, purified and identified. Among them, 38.2, 26.6, 23.5 and 11.7% was isolated from PLA, PP, PS, and the water sample, respectively. Nearly full length of 16S rRNA gene of these 34 strains was sequenced and 23% of the strains belong to unknown phylum and classes based on the BLAST search of the NCBI database (Table 2.1). The query cover of each sequence put through this analysis ranged from 50 to 99%, and the sequence identity

ranged from 92.3 to 99.2%. Only three of the 34 strains were isolated from the TSA plates, and two of them (strains 19 and 20) matched with the sequences from chloroplasts in eukaryote *Auxenochlorella* (Table 2.1). Interesting genera of bacteria found through the BLAST analysis of the sequenced 16S genes included *Roseomonas*, *Sphingomonadaceae, Sphyngopixis, Erythtrobacter, Niveillspirilum, Microbacterium, Micrococcus, and Bacillus* (Table 2.1).

The taxonomic breakdown of these isolated strains resulted in 44% of the strains belonging to the Alphaproteobacteria class, 23% belonging to Unknown classes, 9% belonging to Bacilli, 9% to the eukaryote Trebouxiophyceae, 6% belonging to Betaproteobacteria, 6% to Actinobacteria and 3% belonging to Gammaproteobacteria (Fig. 2.5).

Table 2.1. Top BLAST hits for the 16S RNA gene sequences obtained for each isolated strain with percentages for query cover and identity and their taxonomic classification in the phyla and class levels. Each strain is represented by a sample number and their description includes colony color, source of isolation, and culture medium**.**

Strain number	Color	Isolated from	Media type	Top BLAST hit F	%Query Cover	% Identity
1	Pink	Polypropylene	R2A	Roseomonas sp. LB1-9 16S ribosomal RNA gene, partial sequence	99	96.92
2	Orange	Water	R2A	Uncultured bacterium clone 5'-80 16S ribosomal RNA gene, partial sequence	96	94.13
3	White	Polypropylene	R2A	Zhizhongheella caldifontis strain R2-2-5 16S ribosomal RNA gene, partial sequence	50	92.32
4	Pink	Polystyrene	R2A	Roseomonas sp. strain BU-1 16S ribosomal RNA gene, partial sequence	97	97.63
5	Orange	Polypropylene	R2A	Sphingomonadaceae bacterium NAMAF005 gene for 16S ribosomal RNA, partial sequence	96	96.03
7	White	Water	R2A	Uncultured bacterium clone A96 16S ribosomal RNA gene, partial sequence	96	97.87
8	White	Polystyrene	R2A	Sphingopyxis ummariensis strain 227-LR38 16S ribosomal RNA gene, partial sequence	95	96.89
9	Yellow	Polystyrene	R2A	Erythrobacter flavus strain 21-3 16S ribosomal RNA gene, partial sequence	99	99.2
10	White	Water	R2A	Uncultured bacterium clone ncd2067g07c1 16S ribosomal RNA gene, partial sequence	87	97.18
11	Yellow	Polypropylene	R2A	Microbacterium sp. CW-a23 16S ribosomal RNA gene, partial sequence	96	98.65
12	Green	Polylactic acid	R2A	Auxenochlorella protothecoides chloroplast, complete genome	88	96.53
13	Yellow	Polylactic acid	R2A	Uncultured Massilia sp. clone b5 16S ribosomal RNA gene, partial sequence	95	96.65
14	Yellow	Polylactic acid	R2A	Sphingomonas sp. DW649 16S ribosomal RNA gene, partial sequence	96	97.83
15	White	Polylactic acid	R2A	Uncultured organism clone ELU0037-T187-S-NIPCRAMgANb_000459 small subunit ribosomal RNA gene, partial sequence	92	98.12
16	Translucent	Polylactic acid	R2A	Uncultured organism clone ELU0177-T472-S-NIPCRAMgANa_000056 small subunit ribosomal RNA gene, partial sequence	99	98.01
17	Tan	Polystyrene	R2A	Bacillus tequilensis strain HEP15A3 16S ribosomal RNA gene, partial sequence	90	97.07
19	Dark green	Polypropylene	TSA	Auxenochlorella protothecoides strain UTEX 2341 chloroplast, complete genome	52	96.83
20	Dark green	Polylactic acid	TSA	Auxenochlorella protothecoides strain UTEX 2341 chloroplast, complete genome	47	93.23
22	White	Polylactic acid	TSA	Micrococcus yunnanensis strain JKR76 16S ribosomal RNA gene, partial sequence	96	98.11
23	Clear	Polystyrene	R2A	Nevskia sp. KNF004 gene for 16S ribosomal RNA, partial sequence	97	97.8
24	Clear	Polylactic acid	R2A	Niveispirillum cyanobacteriorum strain TH16 chromosome eg_2, complete sequence	97	95.72
26	White	Polylactic acid	R2A	Bacillus subtilis strain PEBS07032522 16S ribosomal RNA gene, partial sequence	68	91.02
27	Light brown	Polystyrene	R2A	Azospirillum sp. taihu16 16S ribosomal RNA gene, partial sequence	99	94.89
29	Yellow	Polypropylene	R2A	Sphingopyxis sp. DR 4-10 16S ribosomal RNA gene, partial sequence	98	97.36
30	Yellow	Polypropylene	R2A	Uncultured bacterium clone 290 16S ribosomal RNA gene, partial sequence	95	97.51
31	Yellow	Polypropylene	LB	Erythrobacter sp. strain CDJ15-CA06 16S ribosomal RNA gene, partial sequence	98	97.79
32	Yellow	Polystyrene	LB	Erythrobacter sp. strain JLT24 16S ribosomal RNA gene, partial sequence	99	97.38
33	Yellow-white	Polystyrene	LB	Mesorhizobium sp. strain AIY36S 16S ribosomal RNA gene, partial sequence	93	97.48
35	Dark brown	Water	LB	Uncultured alpha proteobacterium clone B-5-34 16S ribosomal RNA gene, partial sequence	99	97.21
36	White	Polylactic acid	LB	Bacillus altitudinis strain MGB1056 16S ribosomal RNA gene, partial sequence	98	97.52
37	Brown	Polylactic acid	LB	Niveispirillum cyanobacteriorum strain TH16 chromosome eg_2, complete sequence	88	95.93
38	White	Polypropylene	LB	Uncultured bacterium clone SEV1AH041 16S ribosomal RNA gene, partial sequence	96	95.43
41	Tan	Polylactic acid	R2A	Niveispirillum cyanobacteriorum strain TH16 chromosome eg_2, complete sequence	99	96.28
42	Clear	Polylactic acid	R2A	Uncultured bacterium clone D53 9 16S ribosomal RNA gene, partial sequence	91	96.23

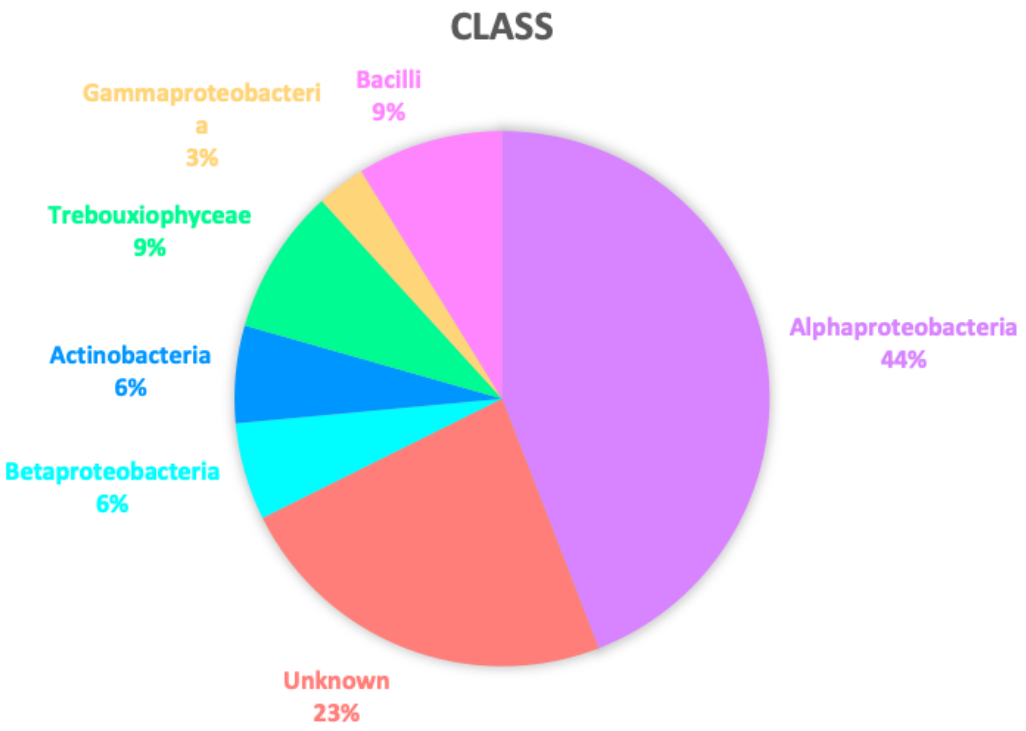

Figure 2.5. Taxonomic breakdown of the 34 isolated strains in the first *in vitro* incubation experiment by class.

Observation of plastic biofilms using SEM

To visualize the surface structure and biofilm formation on microbeads, samples from the first *in vitro* experiment were examined using a scanning electron microscope. Polystyrene beads have a very noticeable texture and big pores that could represent a higher surface area for biofilm growth (Fig. 2.6). It appears that thick cyanobacterial biofilms were formed on the surface of polystyrene beads, and pennate and centric diatoms with different sizes and shapes once settled on the biofilm surface (only diatom shell prints were evident in Fig. 2.6). Small cells with coccoid or rod shape (<1-2 μm) that resemble bacteria or picocyanobacteria covered the surface of polystyrene beads (see blue arrows in Fig. 2.6). In the case of polypropylene, there are

visible ridges where cells can accumulate and gain a competitive advantage over others (Fig. 2.7). Many pennate diatoms can be seen on the surface of polypropylene beads, filaments resembling filamentous cyanobacteria are also visible (Fig. 2.7). Polylactic acid appears to have the smoothest surface with a small number of ridges (Fig. 2.8). Massive development of filamentous cyanobacteria on PLA from day 28 to month 10 was evident (Fig. 2.8). In general, a variety of filamentous organisms that resemble cyanobacteria were present in all microplastic beads after 28 days and 10 months (Fig. 2.6, 2.7 and 2.8), but the matrix of filamentous cyanobacteria appeared to be better developed on PLA than PP and PS. Interestingly, eukaryotes such as pennate diatoms and dinoflagellates were visible on the surface of most of the microbead samples, prompting questions how they interact with heterotrophic and autotrophic bacteria on the plastisphere.

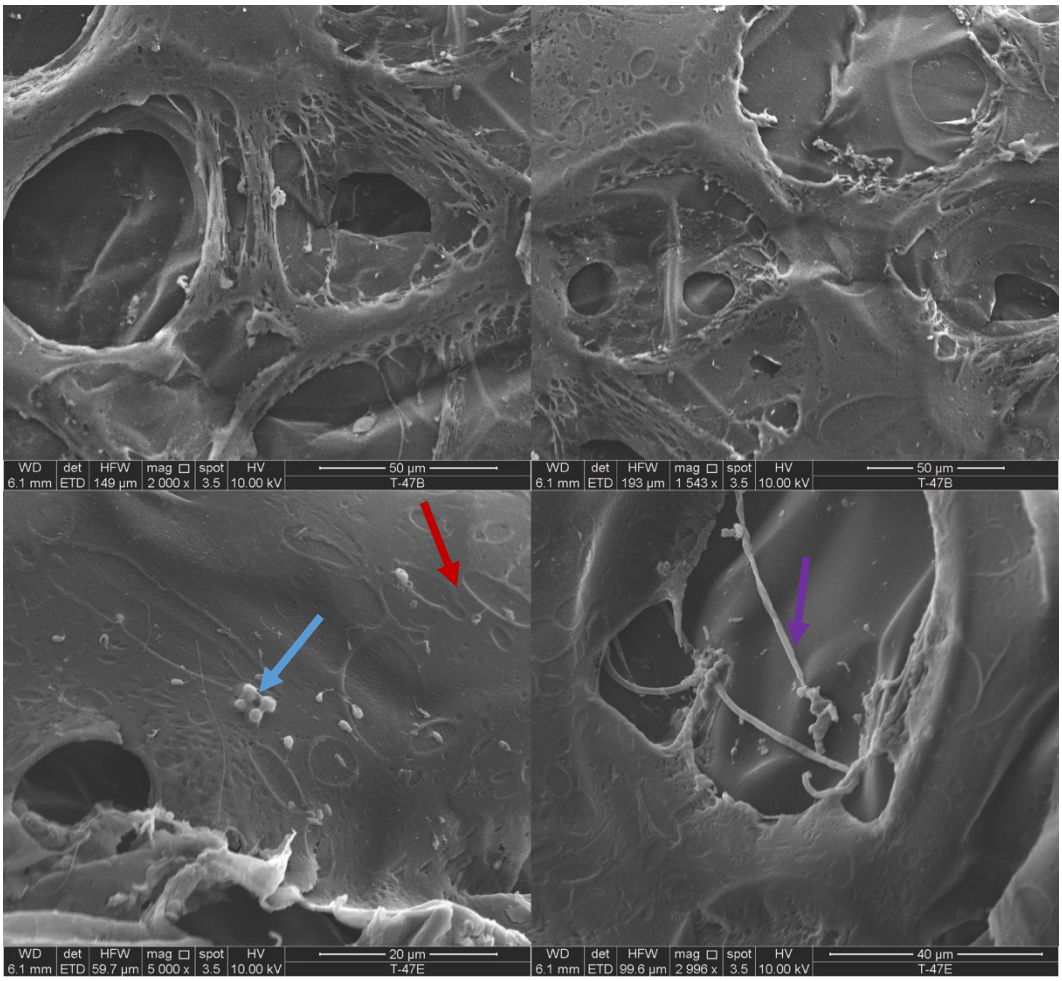

Figure 2.6. Scanning electron microscopy photographs showing the surface of polystyrene beads under different magnifications (magnifications and scale bars are shown on the micrographs). The upper two images were taken from the 28-day sample, and the lower two images were from the 10-month sample. Blue arrows point to bacteria with different sizes and shapes. Red arrow points to the diatom prints on the biofilm (tracks for both pennate and centric diatoms are visible) the purple arrow is pointing to a filamentous cyanobacterium.

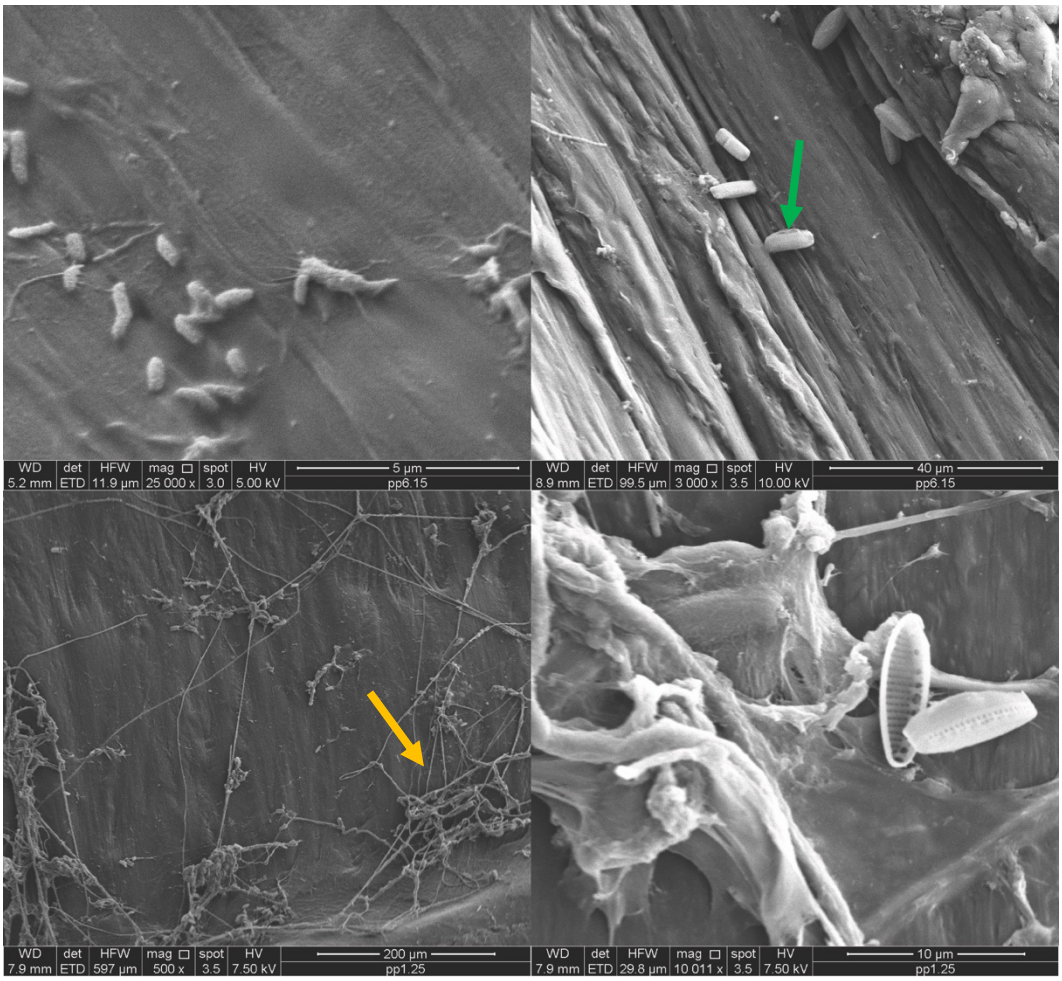

Figure 2.7. Scanning electron microscopy photographs showing the surface of polypropylene beads under different magnifications (magnifications are shown on the micrographs). The upper two images were taken from the 28-day sample, and the lower two images were from the 10-month sample. The green arrow is pointing to a pennate diatom, the orange arrow is pointing to a cluster of filamentous organisms.

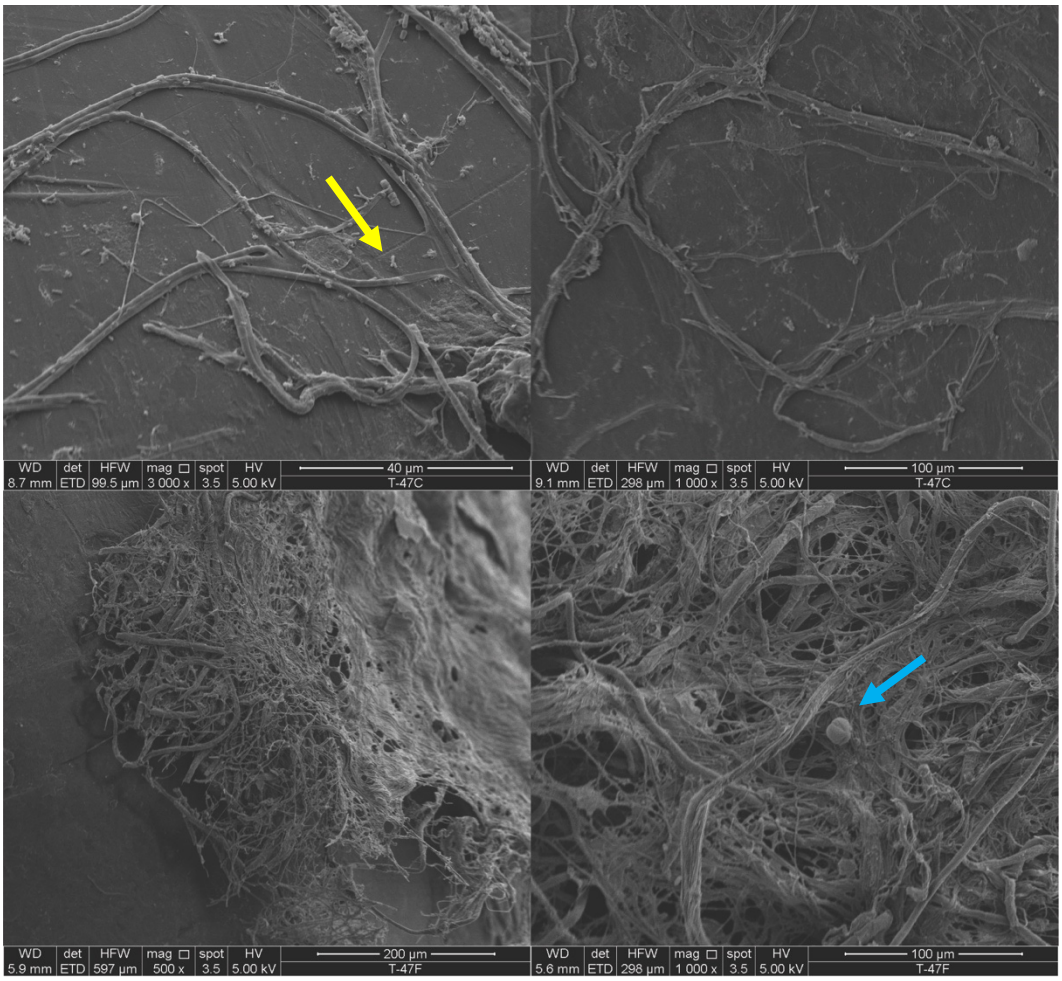

Figure 2.8. Scanning electron microscopy photographs showing the surface of polylactic acid beads under different magnifications (magnifications are shown on the micrographs). The upper two images were taken from the 28-day sample, and the lower two images were from the 10-month sample. The yellow arrow is pointing to bacterial cells, the blue arrow is pointing to a dinoflagellate-like organism attached to a large cluster of cyanobacteria.

Community structure and metabolism of the first *in vitro* incubation

Table 2.2 shows the number of raw reads that were sequenced for every sample, and the number of OTUs that were generated with the QIIME 2 pipeline. The number of raw reads range from 269,101 to 124,694 and the number of OTUs ranged from 15,115 to 5,327. The number of reads was higher

for all materials in the 28-day sampling time than it was in the 7-day sampling time.

Table 2.2. Number of raw reads, number of reads after filtration and total number of OTUs per sample, material of the sample and the date of subsampling for the first *in vitro* incubation.

Sample name	Raw reads	Filtered reads	Total OTU	Material	Date
W615	269101	108996	15115	Water	June 15, 2019
PP615	215883	80774	12119	Polypropylene	June 15, 2019
PP524	186130	73496	7461	Polypropylene	May 24, 2019
PLA524	183849	74857	10798	Polylactic Acid	May 24, 2019
PS615	180832	73594	12731	Polystyrene	June 15, 2019
PLA615	168551	69552	11996	Polylactic Acid	June 15, 2019
W524	125862	46402	13448	Water	May 24, 2019
PS524	124694	50809	5327	Polystyrene	May 24, 2019

The 16S rRNA gene sequencing and community analysis revealed that the community structure of water samples appears to be different to that of the polymer beads. Water samples have a higher abundance of Actinobacteriota and Verrucomicrobiota and appear to have the lowest abundance of Proteobacteria in the day 28 sample (25%). Unassigned bacteria also seem to be significantly more abundant in water samples than in particle-associated samples. Cyanobacterial abundance increased greatly from day 7 to 28 in all samples, with PLA showing the highest abundance of cyanobacteria on day 28 (Fig. 2.9). The phylum Planctomycetota also showed a significant increase in all samples after 28 days of incubation but especially in those from PP.

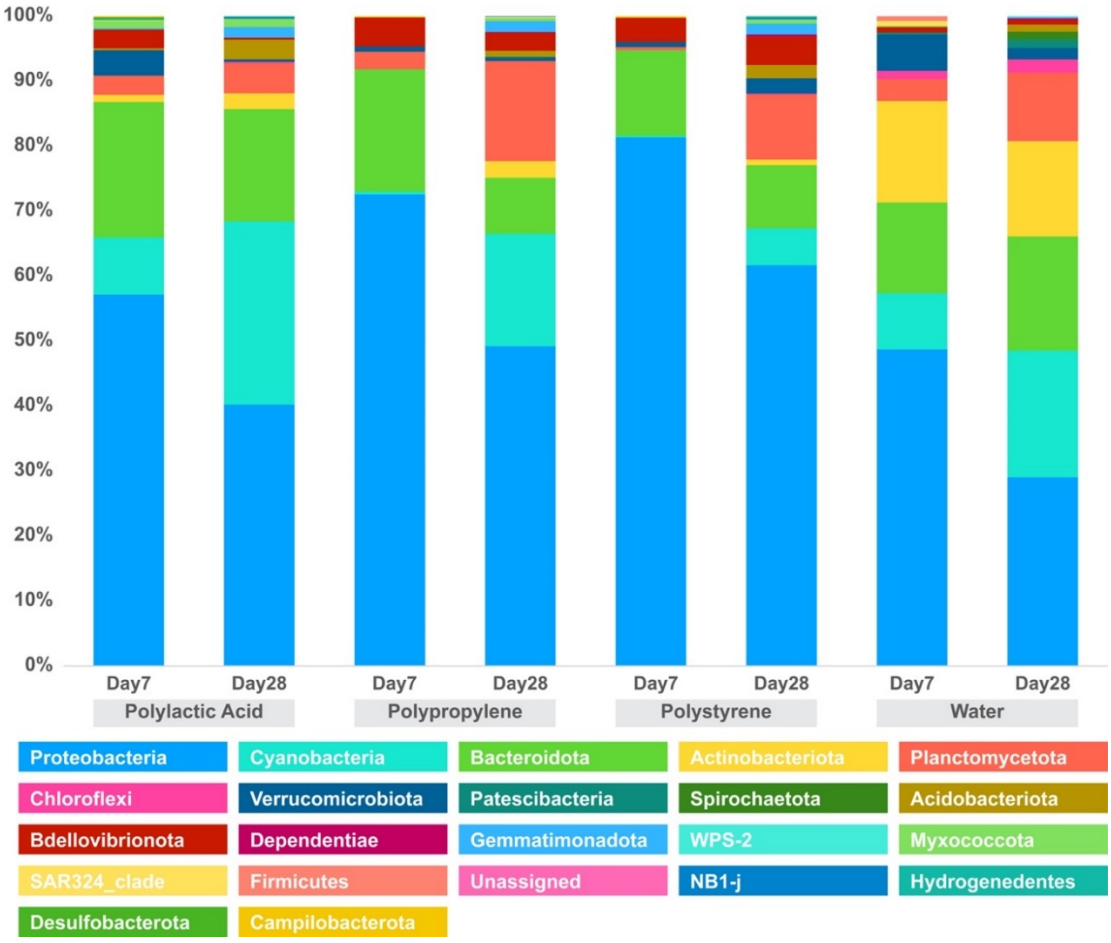

Figure 2.9. Relative abundance of major microbial phyla on day 7 and 28 during the first *in vitro* incubation.

The ecological interpretation of the 16S rRNA gene analysis using the FAPROTAX database, which shows a higher abundance of photosynthetic cyanobacteria in the PLA day 28 sample and to a lesser extent in the other plastic samples on day 28 (Fig. 2.10) is consistent with the Phylum based community analysis of Figure 2.9. This analysis of 16S rRNA gene data using the FAPROTAX database shows that in all material types there was an increase in OTUs related to the ecological functional phenotypes of oxygenic photoautotrophy, photoautotrophy, photosynthetic cyanobacteria, and phototrophy. All samples from polymer biofilms show a higher abundance of

OTUs related to nitrogen metabolism (nitrate and nitrogen respiration, nitrate reduction, denitrification) (Fig. 2.10). A look into the species of Cyanobacteria of the first *in vitro* incubation reveals that the most abundant genera on all samples are filamentous cyanobacteria, such as *Pseudanabaena*, *Nodosilinea*, *Leptolyngbya* and *Phormidium*, among others but this only represents the growth withing the 28-day incubation time and does not represent what the community looked like at the 10 month timepoint. The highest abundances of these filamentous cyanobacterial species are observed on the PLA sample of day 28.

Diversity measures present grouping of the water samples in the weighted and unweighted unifrac analysis when generating the PCoA charts (Fig. 2.11, 2.12). The rest of the materials appear to group separately, especially in the unweighted unifrac measure (Fig. 2.12).

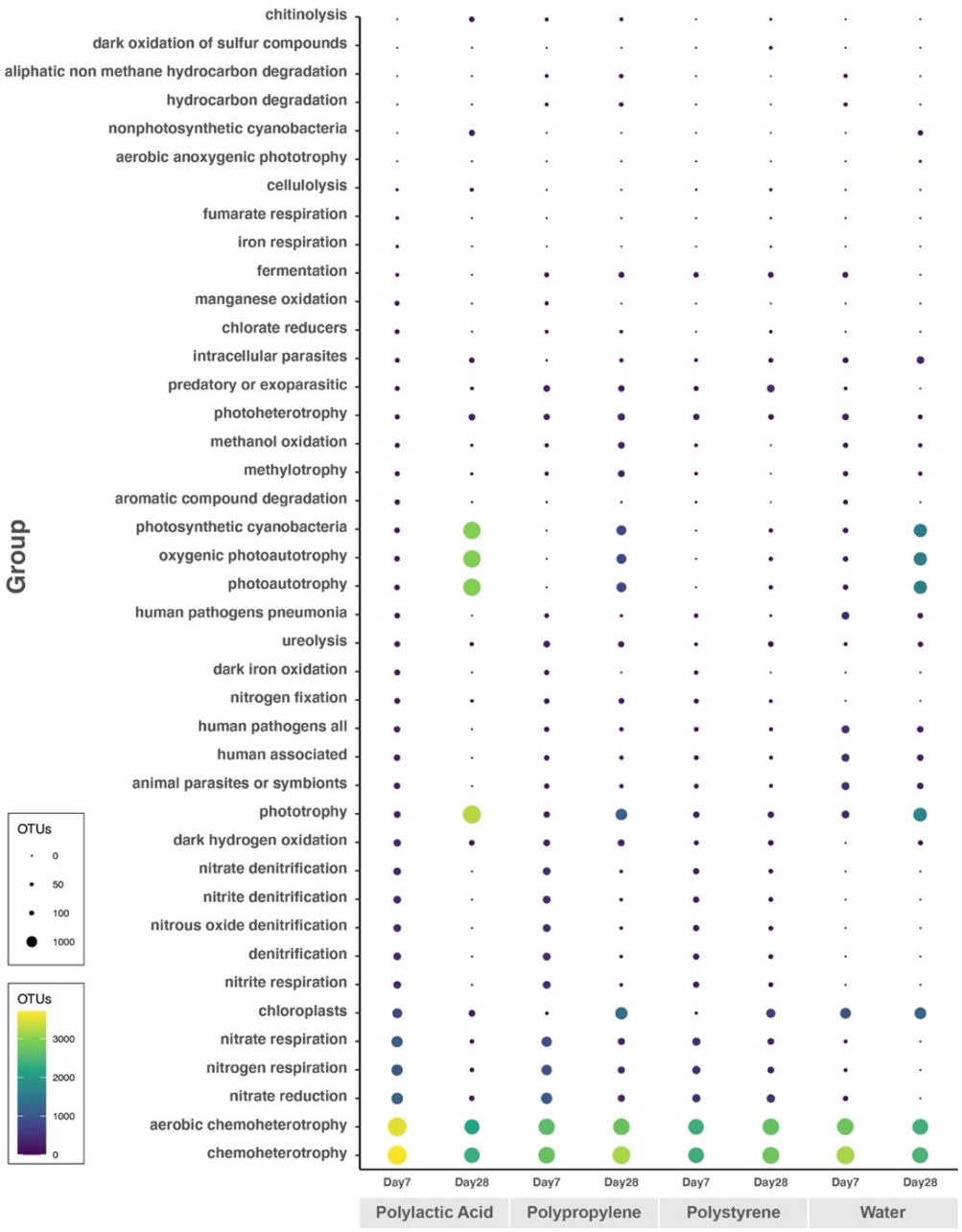

Figure 2.10. Ecologically relevant function abundance per sample for the first iteration of the laboratory incubation samples. Bubbles represent the number of OTUs that have been classified by FAPROTAX as a functional group in each sample.

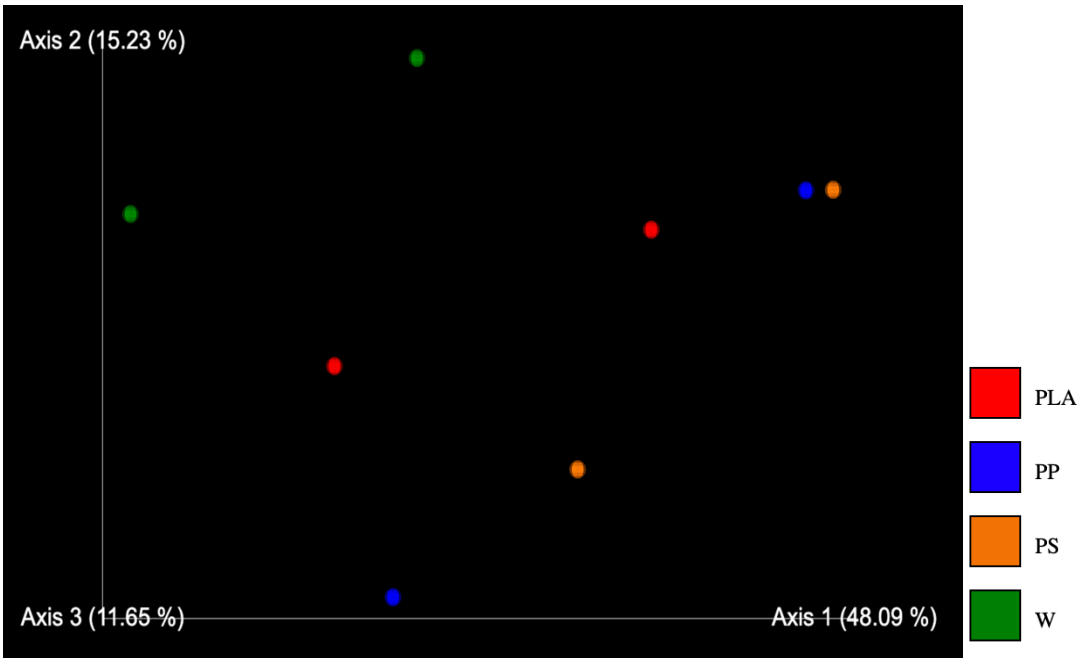

Figure 2.11. PCoA charts showing the dissimilarity of samples from the first *in vitro* experiment grouped by material they were extracted from. Beta diversity measures show the weighted unifrac distance.

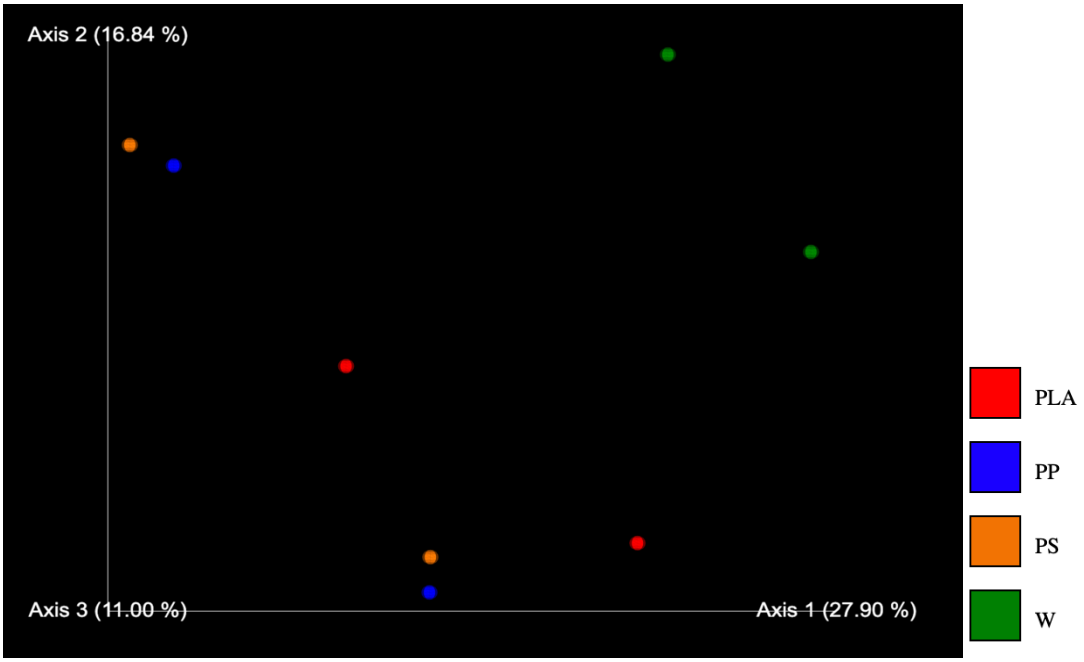

Figure 2.12. PCoA charts showing the dissimilarity of samples from the first *in vitro* experiment grouped by material they were extracted from. Beta diversity measures show the unweighted unifrac distance.

The second *in vitro* incubation experiment

In the second *in vitro* experiment, more subsampling points (day 3, 7, 14 21 and 28) were included. A total of 25 subsamples were collected throughout the experiment (Table 2.3). The number of raw reads in all samples ranged from 6,419 to 61, 426 and the number of final OTUs picked for each sample ranged from 4,490 to 27,270. The samples with the lowest number of both raw reads and OTUs were from the biofilms attached to glass microbeads but there was no other noticeable trend between read number and materials. The number of raw reads and OTUs appeared to be generally proportional in all the samples (Table 2.4).

Table 2.3. Sample labeling system including sample name, material of the sample and the date of subsampling (all in year 2020) for the second *in vitro* incubation.

Sample Name	Material	Date
BW97	Water	September 7
BW911	Water	September 11
BW918	Water	September 18
BW925	Water	September 25
BW102	Water	October 2
BG97	Glass	September 7
BG911	Glass	September 11
BG918	Glass	September 18
BG925	Glass	September 25
BG102	Glass	October 2
BPLA97	PLA	September 7
BPLA911	PLA	September 11
BPLA918	PLA	September 18
BPLA925	PLA	September 25
BPLA102	PLA	October 2
BPP97	PP	September 7
BPP911	PP	September 11
BPP918	PP	September 18
BPP925	PP	September 25
BPP102	PP	October 2
BPS97	PS	September 7
BPS911	PS	September 11
BPS918	PS	September 18
BPS925	PS	September 25
BPS102	PS	October 2

Table 2.4. Number of raw reads, number of reads after filtration and total number of OTUs per sample of the second *in vitro* incubation.

Sample name	Raw reads	Filtered reads	Total OTU
BW97	61,426	60,287	27,270
BPLA97	54,778	53,723	31,843
BPP918	50,269	49,618	31,598
BW102	49,783	48,918	37,459
BPS911	49,258	48,351	22,747
BPP97	46,700	45,909	26,457
BPS97	42,466	41,574	19,410
BW918	41,778	40,882	30,024
BPS925	40,110	39,319	25,941
BPLA918	38,995	38,425	28,539
BPP102	37,769	37,276	22,201
BPS918	36,834	36,129	36,834
BPS102	35,472	34,907	17,202
BPP925	28,878	28,446	19,900
BPLA925	27,981	27,562	19,480
BPP911	27,970	27,510	17,891
BW911	27,055	26,496	19,973
BPLA911	26,657	26,243	18,336
BW925	26,133	25,634	19,190
BPLA102	23,908	23,531	16,337
BG102	12,488	12,308	7,484
BG918	9,795	9,666	7,680
BG97	7,233	7,107	4,644
BG911	6,634	6,535	4,856
BG925	6,419	6,290	4,490

The 16S rRNA gene community analysis of the second *in vitro* experiment shows a different composition in water samples in contrast to the particle-attached samples of PP, PLA, PS and the glass bead control (Fig. 2.13). The most visible difference between these two groups is the high abundance of Verrucomicrobiota and Planctomycetes phyla in the water samples that does not appear in the biofilm samples. The relative abundance of the Desulfobacterota phyla in plastic and glass particles was higher than that in the

water samples. The water samples contained low or undetectable Desulfobacterota at all timepoints.

The community structures, however, were pretty stable from day 3 to day 28 in most of the samples. The community structure was pretty stable within the samples of same polymer type and the most different structures were present in the samples from the surrounding water (Fig. 2.13). In the water samples, there was a steady increase in the phylum Planctomycetes and Acidobacteria through the sampling timepoints, and a steady decrease of the phylum Verrucomicrobiota. The abundance of Proteobacteria in all samples appeared to decrease after the day 3 sampling but appeared to increase again on day 28 for all polymer types and glass. For the water communities, Proteobacteria gradually decreased from 73% to 57% from day 3 to day 14 and remained to be stable (~55%) for the remaining incubation time (Fig. 2.13).

In all the particle-associated samples, there was an increase in the phylum Desulfobacterota after day 7, with fluctuating levels through the rest of the incubation time, particularly for the glass samples. This phylum does not show a visible increase in the water samples. All of the samples from particles of PP, PS, PLA and glass appeared to have similar community structures, with the biggest difference being the low abundance of the phylum Firmicutes in the PS samples. Firmicutes was not a dominating phylum but it was consistently present among the polymer samples from both incubation experiments (Fig. 2.9, Fig. 2.13).

In the second *in vitro* incubation experiment, it is clear that the dominating phylum in all samples is Proteobacteria. The next most abundant phylum is Bacteroidota, consistent with all the samples from the first incubation experiment (Fig 2.9, Fig. 2.10). Cyanobacteria were also present in some of the second incubation samples, but in a much lesser abundance than in the first *in vitro* incubation experiment. The first *in vitro* experiment showed an increase of the phylum Planctomycetes across all the samples, but in the second incubation experiment this increase was limited to the water samples. Another important difference between both experiments, is the high abundance of Desulfobacterota in the second incubation experiment, while this phylum was barely detected in the first incubation experiment (Fig.2.9).

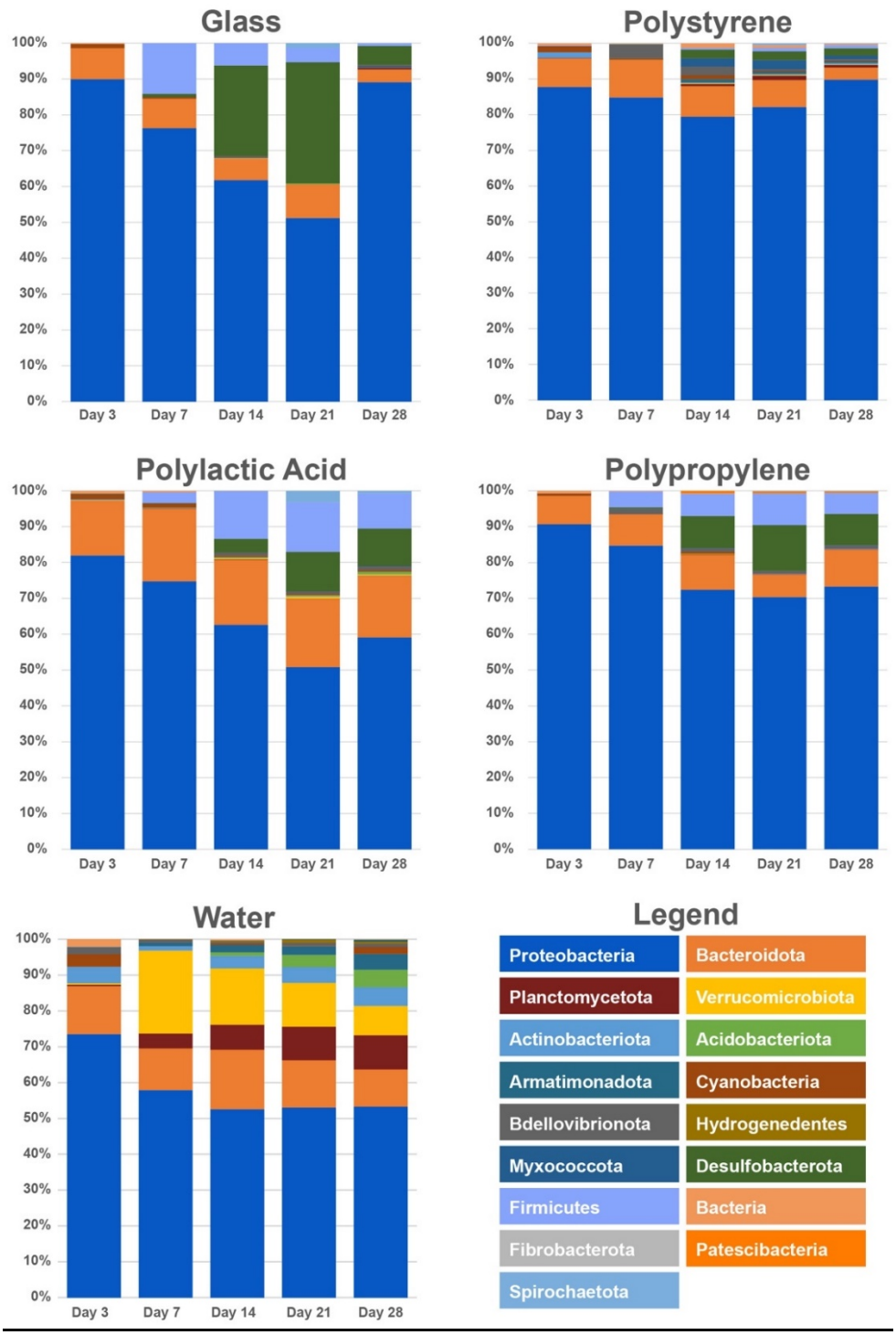

Figure 2.13. Relative abundance of major bacterial phyla in the second *in vitro* incubation at timepoints of 3, 7, 14, 21 and 28 days of each polymer, glass control and the surrounding water sample. The phyla labeled “Bacteria” represents unassigned bacteria.

The alpha rarefaction curves (Fig. 2.14) generated with QIIME 2 of all the particle types showed feature count plateauing at around 2000 sequencing depth, with the exception of the glass samples. This suggests the diversity of the communities from this experiment were captured at this depth and based on this the p sampling depth was selected as 2000; this number determines how many sequences will be randomly subsampled for the following diversity measures.

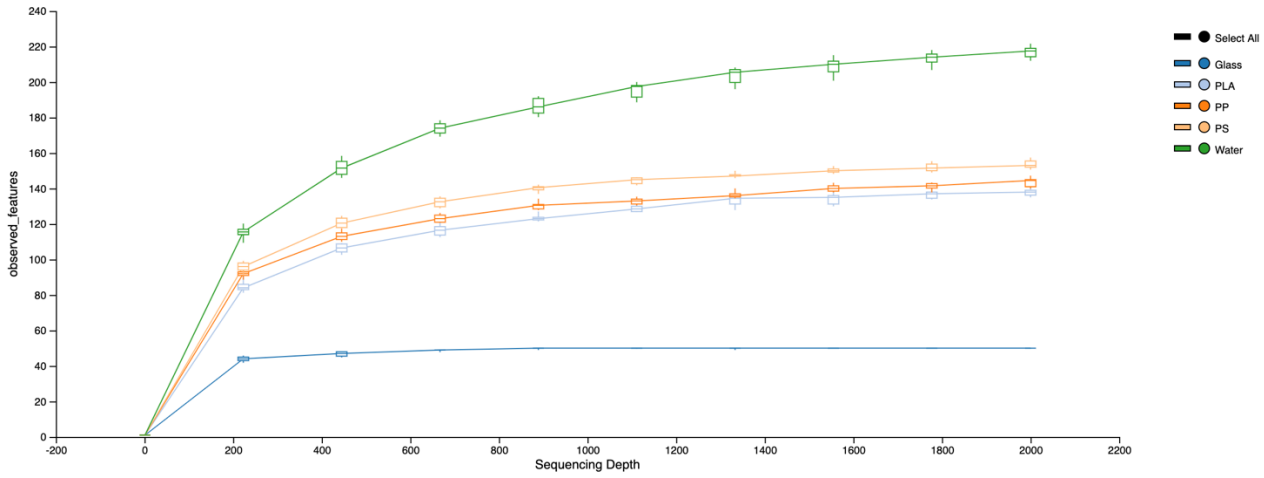

Figure 2.14. Alpha rarefaction curves based on observed features by sequencing depth in the second *in vitro* incubation.

The PERMANOVA analysis showed that the only communities attached to plastic types that were significantly different to each other were those from PLA and PS (Table 2.5). On the other hand, water control communities proved to be significantly different to all other communities (p-values ranging from 0.007 to 0.013). The same is true for the communities attached to glass microbeads, as it appears that these communities are statistically different to all other samples (p-values ranging from 0.007 to 0.024).

Beta diversity represented by PCoA charts using unweighted unifrac (Fig. 2.15) and weighted unifrac (Fig. 2.16) distance show the water samples being isolated as a group in all instances. Unifrac distances take into account the phylogeny in the communities and can either be a weighted or unweighted analysis. Weighted unifrac distance (Fig. 2.15), considers the abundance of taxonomic units, while unweighted unifrac distance gives equal weight to abundant and rare taxa (Fig. 2.16).

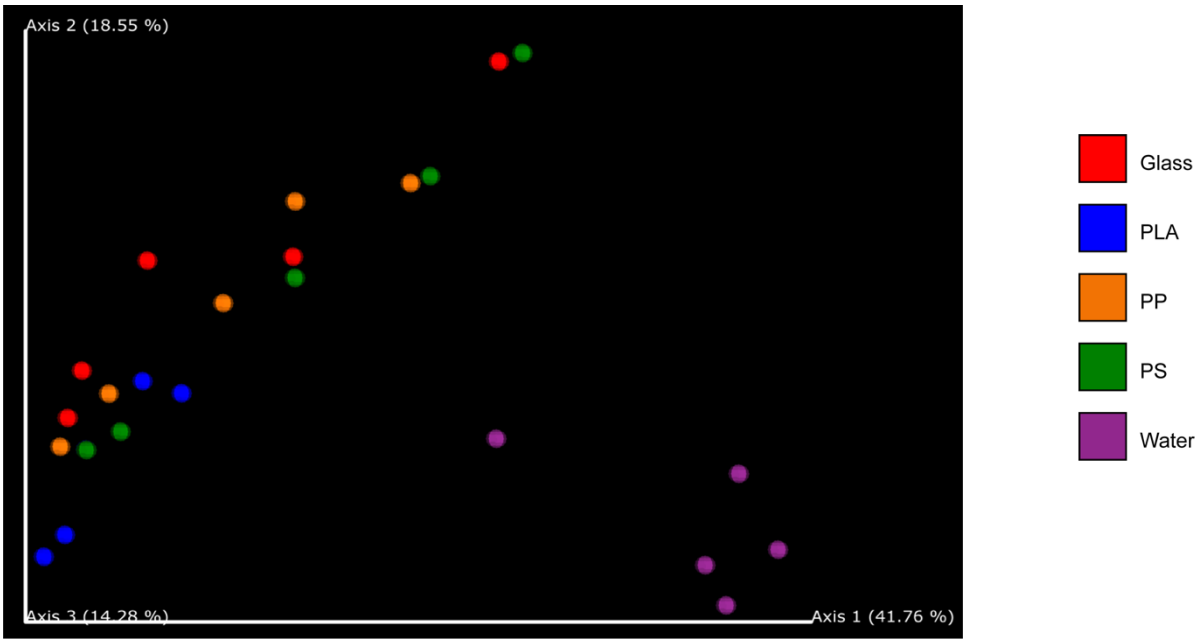

Figure 2.15. PCoA charts showing the dissimilarity of samples grouped by material they were extracted from. Beta diversity measures show the weighted unifrac distance of the second *in vitro* incubation.

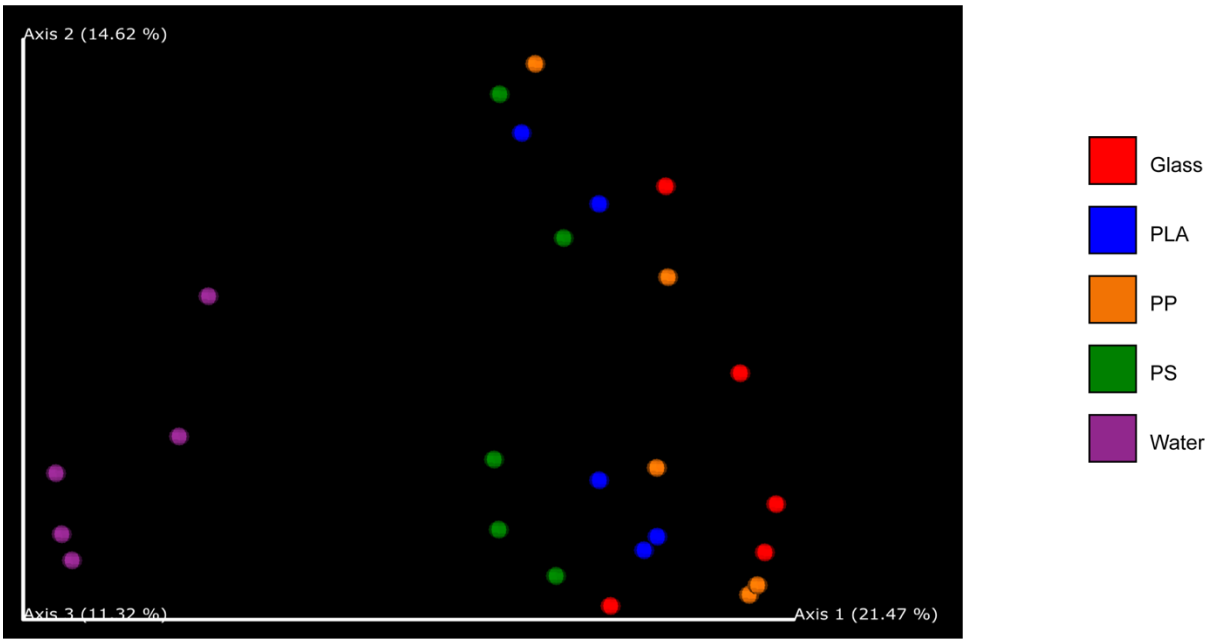

Figure 2.16. PCoA charts showing the dissimilarity of samples grouped by material they were extracted from. Beta diversity measures show the unweighted unifrac distance of the second *in vitro* incubation.

Table 2.5. PERMANOVA analysis to compare sampling groups for similarity in diversity of the second *in vitro* incubation.

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
Glass	PLA	10	999	2.845686704	0.01	0.025
Glass	PP	10	999	2.178097441	0.024	0.034285714
Glass	PS	10	999	3.155466224	0.016	0.026666667
Glass	Water	10	999	5.530987759	0.007	0.025
PLA	PP	10	999	1.792537307	0.078	0.078
PLA	PS	10	999	2.170659025	0.046	0.0575
PLA	Water	10	999	4.774843211	0.007	0.025
PP	PS	10	999	1.951270489	0.069	0.076666667
PP	Water	10	999	5.114787189	0.013	0.026
PS	Water	10	999	4.123208299	0.009	0.025

In Figure 2.17, it is apparent that little to none OTUs were identified by FAPROTAX as families known for plastic degradation. There is also some evidence of alternatives to oxygen metabolism being available to these biofilms, as many of their OTUs are being classified with ability to perform a wide range of nitrogen metabolism functions, such as nitrite and nitrate denitrification, nitrite respiration, nitrogen respiration, denitrification, as well as hydrogen metabolism such as dark hydrogen oxidation. All of these functional families appear to be more abundant in the particle-associated communities (Fig. 2.17) of glass, PLA, and especially PS and PP.

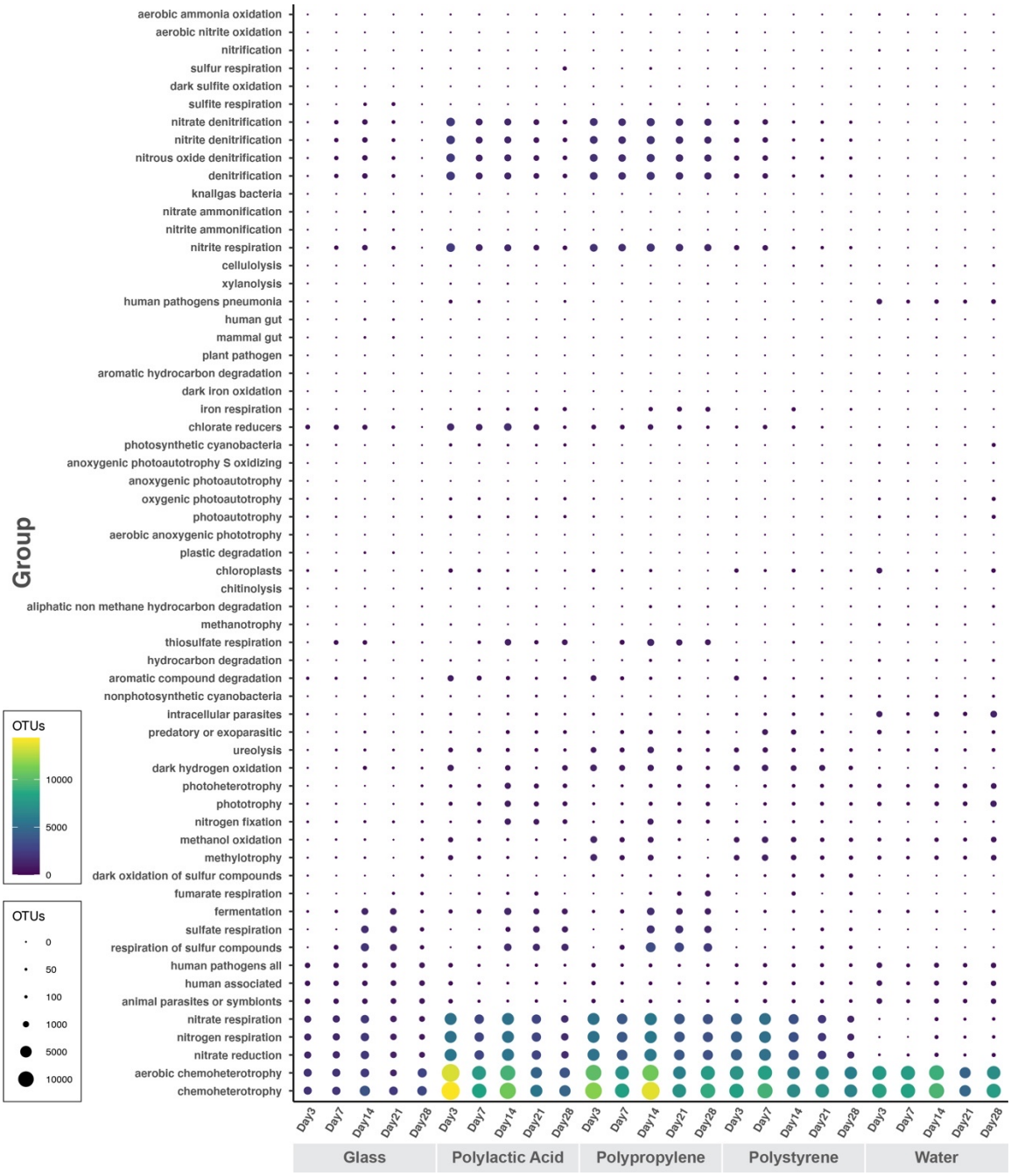

Figure 2.17. Ecologically relevant function abundance per sample for the second *in vitro* incubation samples. Bubbles represent the number of OTUs that have been classified by FAPROTAX as a functional group in each sample.

Discussion

The R2A culture medium has high recovery of bacterial cultures

The nutrient level of LB agar is considered a medium richness; its salinity (15%), however, may have proven to be prohibitive for the growth of estuarine bacteria as only a few colonies from the water and PS samples were able to grow, and the salinity of the sampled water was only 2.9 ppt. The TSA medium was selected as a high-nutrient medium with high concentrations of carbohydrates (glucose) and protein (casein) and nitrogenous substances from soybean, and only presented 12 colonies in all the plates (Fig. 2.3). A total of 70% of the isolated strains were successfully grown in R2A media, 20% in LB media and only 10% in TSA, suggesting that bacteria of the Inner Harbor plastisphere prefer a low-salinity low-nutrient environment as the salinity of R2A media is 0 ppt and the salinity of TSA media is 5 ppt.

The two strains isolated in the TSA medium were both eukaryotic microalgae identified as *Auxenochlorella protothecoides.* This is an interesting occurrence, as this strain behaved like a bacterial strain in every step of the culture and isolation work. This eukaryotic strain was also able to grow in the low-nutrient medium R2A as well, indicating that it may be resilient to a wide range of conditions and does not need a specific nutrient availability to grow rapidly and form large, dark green colonies (Fig. 2.4, strain 12). Mixotrophic microalgae are able to perform heterotrophic metabolism due to their ability to transport and activate carbohydrates like glucose, lactase, glycerol and acetate; the enzymes responsible for these actions are called hexokinases and have been previously found in Chlorella (Morales-Sánchez et al., 2015). TSA medium and R2A

medium both contain glucose, but LB media only contains a small amount of carbohydrates. *Auxenochlorella protothecoides* has been found to grow rapidly when in a glucose-rich medium and to produce lipids in high concentrations with a profile similar to lipids used as biofuel (Patel et al., 2018).

Fifty-three percent of isolated strains belonged to Proteobacteria, followed closely by the strains who could not be classified and were deemed Unknown (23%), and 9% of the strains were identified as eukaryotes of the Chlorophyta phylum (Figure 2.2).

Profiles of the isolated genera

The sequencing of the 16S rRNA gene in the isolated strains showed similarity with many interesting bacterial genera including *Roseomonas*, *Sphingomonas, Sphyngopixis, Niveillspirilum.* The genus *Roseomonas* is known for having pink pigmentation and being gram-negative with an oxidative metabolism. They have been frequently found in human infections (Rihs et al., 1993) and in environmental water samples in which they were able to form biofilms (Furuhata et al., 2008). The genera *Sphingomonas* and *Sphyngopixys* are part of the Sphingomonadaceae family, a gram-negative and in most cases, a yellow pigmentation (Glaeser & Kämpfer, 2014). *Sphingomonas* have been found to produce acidic polysaccharides to form biofilms on plastics in aquatic environments (Czieborowski et al., 2020).

Niveispirillum is a genus of Alphaproteobacteria that presents as nitrogen-fixing, gram-negative bacteria in cyanobacterial aggregates in eutrophic systems (Cai et al., 2015; Lin et al., 2014).

Presence of filamentous cyanobacteria and diatoms on the surface of microplastic beads

Similar results of diatoms, dinoflagellates and filamentous cyanobacteria were found in SEM images of the marine plastisphere of plastics recuperated from the North Atlantic Subtropical Gyre (Zettler et al., 2013). It is clear that all the colonization on the visualized microbeads is different for each plastic type, PLA showed a striking growth of filamentous cyanobacteria and unicellular small bacteria were less apparently abundant (Fig. 2.8). The community analysis revealed consistent results in which after the first 28-day incubation period, there was an increase in the abundance of the Cyanobacteria phyla, especially in the PLA biofilm samples. This increase in cyanobacteria could be due to the initial concentration of cyanobacteria in the water when it was collected in the month of May and with the light incidence on the benchtop being able to reach the transparent water bottles uniformly (Fig. 2.2). The proliferation of primary producers and photosynthetic organisms on microbeads can significantly increase the concentration of chlorophyll a (*Chl a*) in aquatic environments with one estimate stating that one 5 mm microbead can have equal concentration of *Chl a* as 700 milliliters of seawater (Zettler et al., 2013).

When looking into the details of the cyanobacterial species in the samples from the first *in vitro* incubation, some of the most abundant genera are filamentous cyanobacteria such as *Pseudanabaena*, *Nodosilinea* and *Leptolynbya*. A deeper look into these filamentous cyanobacterial genera is described in Chapter 5, including the cultivation of the cyanobacteria of these beads.

Some species of filamentous cyanobacteria have been identified in biofilms growing on polymer particles in the marine and estuarine areas of the Baltic Sea (Kaiser et al., 2017), as well as on the northern Mediterranean (Masó et al., 2016) and in Australian waters (Reisser et al., 2014). In marine environments, microplastics have presented a higher abundance of filamentous cyanobacteria than the surrounding water where unicellular cyanobacteria dominate (Zettler et al., 2013). Most of the bacteria that have been found to be part of the plastisphere, have been found to be heterotrophic bacteria, however, cyanobacteria are consistently found to be present, leading to the theory that primary producers can play an important role in the formation of biofilms that form on microplastics (Yokota et al., 2017). Studies into the ecological roles and metabolism of filamentous cyanobacteria have discovered that there are some hydrocarbon-degrading taxa, such as *Phormidium*, that could be hydrolyzing plastic in the north Atlantic Ocean (Zettler et al., 2013) and the North Sea (Oberbeckmann et al., 2014).

In vitro microbial colonization on the plastic beads

According to both the 16S marker community analysis of the second incubation and the diversity measures it generated, the communities that exist in the free-living environment of the water samples are significantly different to all those in the particle-associated samples (Table 2.5). Similar grouping was visible in the PCoA analysis of the weighted and unweighted unifrac distances of the first incubation experiment (Fig. 2.11, Fig. 2.12).

Communities from the PLA and PS samples in the second *in vitro* experiment did not show a significant difference, representing the only plastic communities that showed dissimilarity in the *in vitro* and *in situ* (see Chapter 3) studies of this document (Table 2.5). Water control communities proved to be significantly different to all other communities. Surprisingly, the communities from glass microbeads appear to be statistically different to all other samples, directly contradicting the results from the *in situ* study (Chapter 3). In the meta-analysis that included multiple marine plastisphere, it was found that the type of plastic does not play a big selective role in the formation of plastisphere, not even in naturally-occurring non-plastic particles (Oberbeckmann & Labrenz, 2020). It is unclear the difference between the *in vitro* and *in situ* study can be caused by the bottle effect or different incubation conditions. On the other hand, the dissimilarity of the water communities that are free-living to those communities from particle-associated samples actually support the finding of the previously mentioned meta-analysis, which concluded that surrounding water microbial communities are statistically different to those on plastispheres. In both *in vitro* incubation experiments, the community structure analysis showed that the initial colonization stages of the plastisphere is dominated by Proteobacteria and Bacteroidetes and they remained the most abundant two phyla throughout all sampling points. This is consistent with other colonization experiments that have shown that Proteobacteria and Bacteroidetes are the first ones to form biofilms on plastic particles and consistently dominate the community as it fluctuates (Schlundt et al., 2020).

In the first incubation experiment, bacteria from the phylum Firmicutes, increased in abundance in all samples after both 28-day incubation periods (Fig. 2.9), supporting the theory that they are able to survive in many environmental conditions through sporulation (Onyenwoke et al., 2004) and that potential pathogens may be attaching to the surface of polymer microbeads and other pieces of debris (Kirstein et al., 2016). Unassigned bacteria represent almost a negligible portion of the amplified community, but that can be due to the fact that any OTU with less than 10 sequences is filtered out in the QIIME 2 pipeline. Additionally, the cultivation results showed a higher portion of unassigned bacterial strains, which could be due to a large portion of the bacterial strains that are detected by the 16S community analysis are not able to be cultured in a laboratory setting.

In the second *in vitro* incubation experiment, such trend is not seen, as the abundance of Cyanobacteria was low in all the samples over the 28-day incubation period. This could be due to the difference in the water samples in different seasons. The water sample for the first *in vitro* study was collected in May, while the water sample for the second *in vitro* study was obtained in September. The water for the second *in vitro* study has higher salinity (8.7 ppt) compared to the first *in vitro* study (2.9 ppt). It is known that filamentous cyanobacteria prefer low salinity environment (Olofsson et al. 2020). It is possible that lower water salinity in the first *in vitro* experiment nurtured more filamentous cyanobacteria than the second *in vitro* experiment. Cyanobacterial populations tended to increase in the control water sample. These

cyanobacteria are likely dominated by picocyanobacteria,; the 16S community analysis reveals that the most abundant species in the water samples belongs to the Prochlorales order. Another noticeable difference between the two *in vitro* incubation experiments is the high abundance of the phyla Desulfobacterota in the polymer samples in the second experiment (Fig 2.13) which was not seen in the first experiment. It is possible that the water sample from this experiment was taken during a time in which sulfur reducing bacteria were widespread in the water and sediments of Baltimore's Inner Harbor. Since Desulfobacteria are usually found in sediments, this could explain their propensity to attach to a surface, such as glass or plastic, and form biofilms (Bak & Widdel, 1986). In the second incubation experiment there was a clear domination of Proteobacteria and Bacteroidetes that matches the communities from the first incubation experiment, as well as those from the *in situ* studies (See Chapter 3). Additionally, this experiment showed an incidence of Verrucomicrobiota in high abundance in the water samples but low to none in the plastic samples (Fig. 2.13). Not much is known about the phyla Verrucomicrobiota other than its strict need for oxygen for their metabolism and survival (Schlesner et al., 2006). This quality could potentially limit Verrucomicrobiota from thriving in the layers of the biofilms of the plastisphere. These findings suggest that the community structure in the collected water at the beginning time of the incubation can greatly influence the colonization of the plastic and microbeads in the experiment, as well as the shifts in the community in the water control. This initial community can greatly vary

depending on the season of the sampling and the environmental conditions at the time (Kan et al. 2006).

Differences in PLA and PS communities

The PLA and PS communities of the second incubation experiment showed dissimilarity in the PERMANOVA analysis (Table 2.5). In the relative abundance plots of PLA and PS in Fig.13, some of the most striking differences are the high abundance of Firmicutes in the PLA samples, and very low incidence of this phylum in the PS samples. Another difference that is evident in this analysis, is the domination of Proteobacteria in the PS samples being somewhat stable and staying within 80 to 90% relative abundance. In the PLA samples however, the Proteobacteria phylum fluctuated from a high of 80% in day 3, to a low of 50% in day 21. This difference in abundance was compensated with Bacteroidota abundance in the PLA samples, in which their abundance is constantly 20-25%. During these *in vitro* experiments, it was evident that plastic microbeads do not all behave in the same way in the surrounding water. The polypropylene and polystyrene beads floated on the surface of the sampled water and did not sink for the duration of the experiment. It has been demonstrated that some low-density plastics, including polypropylene, are the most common microplastics found in both sediment and surface water due to their ability to adsorb and interact with other substances that result in their becoming denser and sinking out of the water column (Frère et al., 2017). The polylactic acid beads, on the other hand, are denser than the estuarine water of the Inner Harbor, and they stayed on the bottom of bottle

throughout the entire incubation time. The varying surface of the materials and their porosity and roughness is evident in the photographs (Fig. 2.6, 2.7, 2.8) and can potentially play a role in the development of the communities and how their members interact with the interface (Oberbeckmann & Labrenz, 2020).

The potential metabolism of these biofilms

In the ecological interpretation of the 16S community analysis, there was a high abundance of OTUs that have been previously related to nitrogen metabolism, suggesting that these biofilms have the metabolic capability to survive a wide range of nutrient availability conditions, even though nitrogen metabolism in a biofilm usually depends on the nutrients present, and not entirely in genetic capability (Li et al., 2019). This could suggest that the biofilms that are forming on these microbeads able to perform metabolisms alternative to oxygen metabolism and surviving even in the deepest layer of the biofilms where conditions can be stressful and anoxic (He et al., 2018). Another example of this, is the high incidence of OTUs that were grouped into functional families that are capable of dark hydrogen oxidation and sulfate respiration, especially in those polymers that are able to float (PS, PP) in the *in vitro* incubation (Fig. 2.17).

Potentially harmful species on microplastics

It has been hypothesized that pathogens could be easily transported by microplastic particles in aquatic environments and pathogenic vibrio species have been found in high concentrations in estuarine plastispheres (Laverty et al., 2020). In this study, we identified species that have the potential to be

pathogenic or harmful. The genus *Roseomonas* has been known to cause infections in humans (Rihs et al., 1993) and cause bacteremia. Another genus that is known for having many pathogenic species is *Microbacterium*, as a study has revealed 18 different species of the genus in human infections (Gneiding et al., 2008). The incidence of pathogenic species is of great concern because microplastics can be transported by organisms such as algae and fish, and aquatic and marine food chains can be affected by microplastics at every level (Yu et al., 2018). Another concern is the high incidence of filamentous cyanobacteria, as they can produce blooms and mats, and they can secrete cytotoxins that can affect humans and other animals (Codd et al., 2005). Some of the identified cyanobacterial species in the 16S community analysis are known for forming blooms and for being able to cause harmful effects. The genus *Nodularia* can produce hepatotoxins known as Nodularins that can affect membrane integrity and can even be carcinogenic. The genus *Phormidium* can secrete anatoxin-a, a neurological blocker; and the genus *Lyngbya* can produce saxitoxins, another neurotoxin, that can block sodium channels (Codd et al., 2005). Further discussion of filamentous cyanobacteria attaching to these microbeads can be found in Chapter 5.

Bacteria and some types of eukaryotes (such as diatoms and hydroids) are able to settle on and colonize microplastic particles and generate complex biofilms that allow them to use polymers as a persistent growth surface and a transportation vector (Oberbeckmann et al., 2014; Zettler et al., 2013).

Conclusion

In this study, we demonstrated that the development of microbial biofilms microplastics by incubating estuarine water *in vitro* with three different types of microplastics: polypropylene, polystyrene, and polylactic acid. The two *in vitro* experiments conducted using water samples collected in spring and fall all show the highest relative abundance belonging to the Proteobacteria and Bacteroidetes phyla. Some microbial populations, however, were different between these two *in vitro* studies. For example, filamentous cyanobacteria increased in abundance in all the samples of the first incubation, especially in the PLA polymer. Desulfobacterota increased in abundance in all polymer samples of the second incubation but were barely detectable in the first incubation. These results imply that the formation of biofilms on microplastics is affected by the composition of original microbial community and environmental conditions. Unfortunately, no environmental parameters such as, nutrients, pH and dissolved oxygen were measured during the *in vitro* studies. The availability of these environmental factors can help us better interpret the succession of biofilm microbial community on microplastics. In the following *in situ* studies (Chapter 3), more environmental data will be available. Additionally, diversity analysis of the *in vitro* incubations suggests that the communities attaching to the particles in this study are significantly different from those in the surrounding water, opening the door to further study of the impact of these polluting biofilms on the ecosystem.

Chapter 3. An *in situ* study to understand community structure and potential function of estuarine microbes on plastisphere

Abstract

Microbial communities that settle on microplastic particles can potentially lead to the transport of pathogenic and harmful bloom-forming species, as well as have an impact on global biogeochemical cycles. However, little is known about the acclimation of microbes to different types of microplastic in the estuarine environment. In chapter 2, an *in vitro* incubation approach was used to investigate the microbial formation on microplastics in the Baltimore harbor water. In this study, we investigated the succession of microbial communities of biofilms developed on the three different types of microplastic beads placed in Baltimore's Inner Harbor (an *in situ* approach). Bacterial communities associated with microplastic particles and glass bead control were monitored throughout the 28-day incubation time. A significant taxonomic composition dissimilarity was observed between particles-associated and free-living communities, suggesting a unique microbial adaptation to these biofilms. The polymer types, however, did not significantly influence the bacterial community composition. Some interesting bacterial phyla were identified in the plastisphere samples, including Cyanobacteria, Planctomycetes, Desulfobacteriota, and Firmicutes, leading into speculation of their ecological responses and metabolic roles in the estuarine environment. It is crucial to understand the microorganisms that inhabit plastic debris in aquatic systems and their potential metabolic capacity in order to assess their roles in global nutrient cycles and if they have ability to be utilized in bioremediation for plastic pollution.

Introduction

In today's world, plastic pollution is a ubiquitous problem. Plastic debris can be found in virtually every environment: from glaciers to sediments and in all bodies of water as well (Barnes et al., 2009). The physical characteristics that make synthetic polymers ideal for manufacturing, like durability and solidity, also makes them the perfect growth surface for a wide array of organisms. Debris of all sizes can be found in aquatic ecosystems but there is a particular concern for particles that are smaller than 5 millimeters, known as microplastics (Betts, 2008). These particles can be classified as primary or secondary microplastics, with the first being those that were manufactured to be in this size range and the second being those that break down from large pieces of plastic (Cooper & Corcoran, 2010; Fendall & Sewell, 2009). Microplastics can be extremely difficult to quantify, isolate and characterize and they can be virtually impossible to remove from ecosystems without disturbing them. Research into the environmental impact of microplastics has been an emergent topic in the last decade, especially in sediments and marine systems (Eriksen et al., 2014a), but insight into their effects in estuaries is not yet sufficient or extensive. An in situ study in which microplastic particles are colonized could provide insight into how these pieces of debris develop biofilms in the fluctuating environmental conditions of an estuary.

The Chesapeake Bay is the largest estuary in the United States, and includes four major rivers: the Susquehanna, the Potomac, the Patapsco and the Patuxent. In 2011, a study trawled four estuarine tributaries (Patapsco, Rhode, Magothy and Corsica) of the Chesapeake Bay and found significant

concentrations of microplastic particles in a wide range of concentrations and concluded that the locations that had the higher concentration of particles correlated to locations with higher population densities (Yonkos et al., 2014a). Studies on microplastics in the surface waters of the Chesapeake Bay have found that polymer particles are present in concentrations from 0.007 to 1.245 particles per m^3, and that polypropylene (PP) and polyethylene (PE) are the most common types (Bikker et al., 2020).

Microorganisms colonize surfaces and form biofilms in order to protect themselves from grazing, mitigate the competition between species, facilitate horizontal gene transfer, exchange nutrients and to overall increase their probability of survival (Costerton et al., 1987). Biofilms can be complex and be conformed of multiple layers of prokaryotes and eukaryotes that make up a constantly adapting community that can interact and become more and more diverse. Biofilms on plastic particles are no exception, and microplastics provide further advantages for microbes as they can subsist for decades in aquatic environments and the roughness and enhanced half-life of these particles contribute to making them the ideal candidates for colonization. In 2013, a study named the biofilms of microplastic particles the “plastisphere” and found heterotrophic bacteria, filamentous cyanobacteria and some eukaryotes such as diatoms as members of the microbial communities (Zettler et al., 2013). The plastisphere has a greater evenness compared to the communities of the water around them that included many rare and undescribed species (Zettler et al., 2013). Physical factors such as water flow

and UV light exposure can significantly increase the roughness of particles by creating cracks and holes and/or oxidation of their hydrophobic surface (Zettler et al., 2013). Microcosm studies have shown that the biofilm formation on microplastic particles is fast and total colonization can take anywhere from a couple of days to a couple of weeks (Harrison et al., 2014). Some of the factors that determine the rate of colonization and the species composition of the biofilms are the chemical and morphological characteristics of the plastic particles, seasonality, geographical location (Eerkes-Medrano et al., 2015) and other environmental conditions such as salinity, temperature, hydrology and oxygen availability (Zettler et al., 2013; Bryant et al., 2016).

Recently, the total biomass of the oceanic plastisphere have been approximated to represent 0.01 to 0.2% of the total microbial biomass of the ocean (Mincer et al., 2016). The formation of biofilms can cause microplastics that would normally float on the water's surface to become biofouled and sink. They can be accumulated in sediments or even be ingested and incorporated into the food web as part of the microbial loop (Lobelle & Cunliffe, 2011b). Studying the role of microplastics in the cycling of the elements will be crucial to understanding the effects of plastic debris (Amaral-Zettler et al., 2020) especially considering the fact that growth limiting elements like nitrogen and phosphorus can become more available to microbes growing on microplastic surfaces than those in oligotrophic waters (Mincer et al., 2016).

Studies into biofilm formation in controlled environmental conditions have demonstrated that bacteria can grow selectively on plastic substrates, due to

their hydrophobicity, and the biofilms can be different to those that form on other naturally occurring particles like glass and cellulose (Ogonowski et al., 2018).

In this study, three different types of polymers (PP, PLA and PS) were introduced to the water of Baltimore Inner Harbor inside of mesh containers made of stainless steel, alongside an inert control of glass microbeads. Subsamples were collected from each containers and surrounding water at different time points over a total of 28-day incubation period. Microbial community structure was analyzed by sequencing the 16S ribosomal RNA gene. The objective is to explore if biofilm microbial community on microplastic particles differ from the microbial community of the surrounding waters, and if there are any significant differences in microbial colonization of chemically different polymers and a non-plastic control (glass).

Materials and methods

Selection of microplastics

Three types of synthetic polymers were selected: polypropylene, polystyrene and polylactic acid. In the second incubation experiment, a glass microbead control was used as an inert material. The selection of microplastics for this incubation experiment is described in the methods section of Chapter 2.

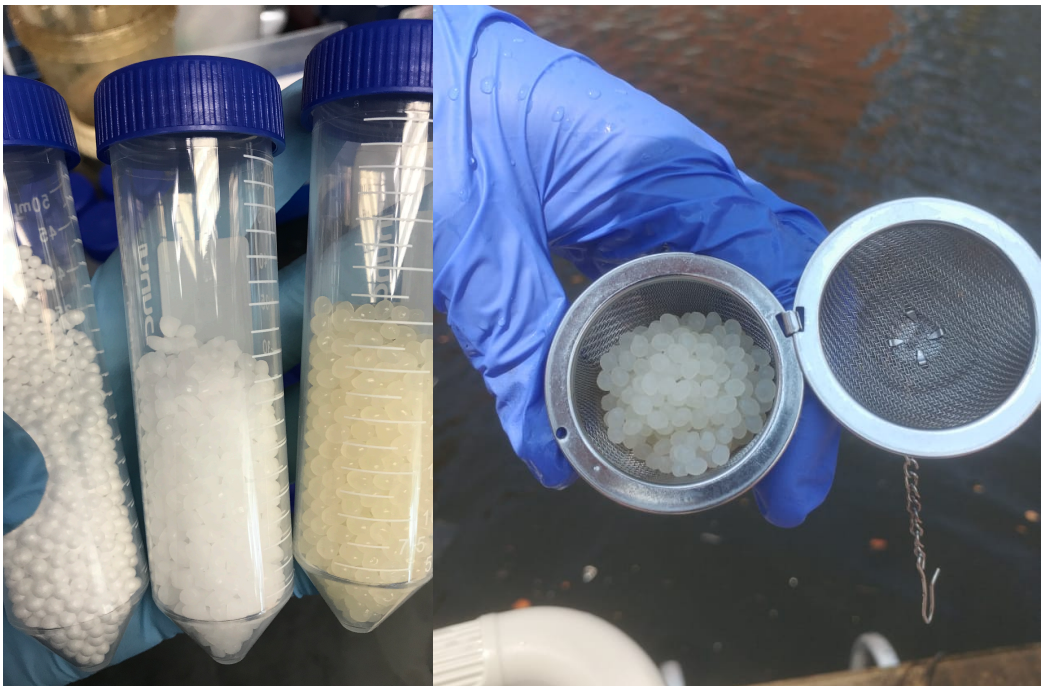

Figure 3.1. Microplastic beads composed of polystyrene, polypropylene and polylactic acid respectively (left panel). Stainless steel mesh container with polylactic acid microbeads (right panel).

Preparation and sterilization of beads

Three different microplastic spheres composed of pure PP (PollyPlastics^{TM,} Michigan, USA), PS (Fairfield®, Connecticut, USA), and PLA (3DXTech©, Michigan, USA) were used for this experiment. Glass spheres with a 4mm diameter (BioSpec, Oklahoma, USA) were used as a no-plastic control. The sterilization and preparation of the beads is described in Chapter 2. Plastic and glass beads were placed in stainless steel mesh containers which were soaked in 70% v/v ethanol for an hour and subsequently rinsed with sterile deionized water prior to the use. Stainless steel mesh containers were chosen because they allow free water flow.

First *in situ* incubation

Stainless steel mesh containers with microbeads (2-4mm) were introduced into the Baltimore's Inner Harbor sampling site on June 19th, 2019 and allowed to be colonized for 28 days. Subsamples were taken on day 7 and day 28.

Second *in situ* incubation

Stainless steel mesh containers with microbeads (2-4mm) were introduced into the Baltimore's Inner Harbor sampling site on September 5th, 2020 and allowed to be colonized for 28 days. Subsamples were taken on day 3, 7, 14, 21 and 28.

Subsampling for bacterial community analysis

Approximately 30 microbeads were taken per sample, with sterile forceps and in duplicate, one for processing and one for storage and archiving. Microbeads were rinsed using Phosphate-Buffered Saline (PBS) buffer and stored in sterile 2 milliliter microcentrifuge tubes. Water samples were taken using a sterile 1-liter glass bottle, subsequently 100 milliliters of the sample were filtered on a reusable filter tower (ThermoScientific™, Massachusetts, USA) with a filter with a 0.2 µm pore size for the retention of microorganisms.

16S sequencing and bioinformatic pipeline

DNA from biofilms was extracted using the IBI Soil DNA Extraction Kit (IBI Scientific, Iowa, USA) and the DNA in the water samples was extracted using the PowerWater DNA Isolation Kit (QIAGEN, Maryland, USA). Obtained reads were paired and trimmed for quality using CLC Genomic Workbench 8 and analyzed using the QIIME 2 bioinformatic pipeline for bacterial community

analysis and the SILVA 132 taxonomy database (de.NBI). Details of this informatic pipeline and the protocols are included in Chapter 2.

Diversity measures and PERMANOVA

QIIME 2 was used to generate an alpha rarefaction curve of the OTUs and the sequencing depth. Phylogenetic metrics and statistical analysis using a PERMANOVA assay was performed in the form of pairwise tests to determine group significance and determine similarity between material types. Principal component analyses (PCoA) using the weighted and unweighted unifrac diversity measures were used to determine community dissimilarity based on beta diversity.

Hydrological data

Water quality data was downloaded from the Department of Natural Resources' (DNR) Eyes on the Bay project (http://eyesonthebay.dnr.maryland.gov/) which has a monitoring station on the east side of the National Aquarium and within 200 meters of the sampling site. This station takes a snapshot of water quality every 15 minutes continuously and measures pH, temperature, dissolved oxygen, salinity, turbidity, and chlorophyll concentration. Data for the pertinent dates (Fig. 3.2) for June and July 2019 and September and early October 2020 were accessed on the DNR website and analyzed to obtain the average conditions for the sampling days.

Ecological interpretation of 16S marker data

The taxonomy tables generated by QIIME 2 were used to compare to the FAPROTAX (Louca et al., 2016) database. This database will map the known OTUs to ecologically relevant metabolic functions from published literature of known species that are closely related. This pipeline generated a profile describing the functional group and its abundance for each known taxonomic unit in the database. Full details on this pipeline can be found in Chapter 2.

Comparison of pooled data from *in situ* and *in vitro* incubations

The sequenced reads for both experiments from 2020 were combined (both *in vitro* from Chapter 2 and *in situ*) and compared based on the principle that they were conducted on the same dates and the initial communities were identical. Alpha and beta diversity measures were calculated using the QIIME 2 bioinformatic pipeline in the manner that was previously described in Chapter 2. Additionally, all OTUs from the 2020 experiment group were used to generate a Venn diagram using RStudio (PBC©) and the packages ggVennDiagram and tidiverse.

Results

First *in situ* incubation

A total of 8 subsamples were collected in the first 28-day *in situ* incubation, all samples from the 28-day timepoint had a higher number of raw reads after sequencing than those from the 7-day timepoints (Table 3.1).

Table 3.1. Number of raw reads, number of reads after filtration and total number of OTUs per sample, material of the sample and the date it was recovered for the first *in situ* incubation.

Sample name	Raw reads	Filtered reads	Total OTU	Material	Date
PS717	621011	94577	10451	Polystyrene	July 17, 2019
W717	591941	90894	12913	Water	July 17, 2019
PP717	426212	60731	6616	Polypropylene	July 17, 2019
PLA717	394220	59089	7909	Polylactic Acid	July 17, 2019
PP626	178959	84792	3966	Polypropylene	June 16, 2019
PLA626	174336	84015	7883	Polylactic Acid	June 16, 2019
PS626	171482	81946	6089	Polystyrene	June 16, 2019
W626	149375	66135	4087	Water	June 16, 2019

During the first *in situ* incubation period, salinity fluctuated between 2.6 and 5.4 ppt, and temperature increased from 24.4°C on day 7 to 28.4°C on day 28. Chlorophyll a concentration had a maximum of 20.9 µg/L on day 7 and a minimum of 7.9 µg/L on day 21, and a concentration of 11.9 on sampling day 28. Levels of dissolved oxygen in the water fluctuated between 4.4 to 7.1 mg/L throughout the 28-day incubation period (Fig. 3.2).

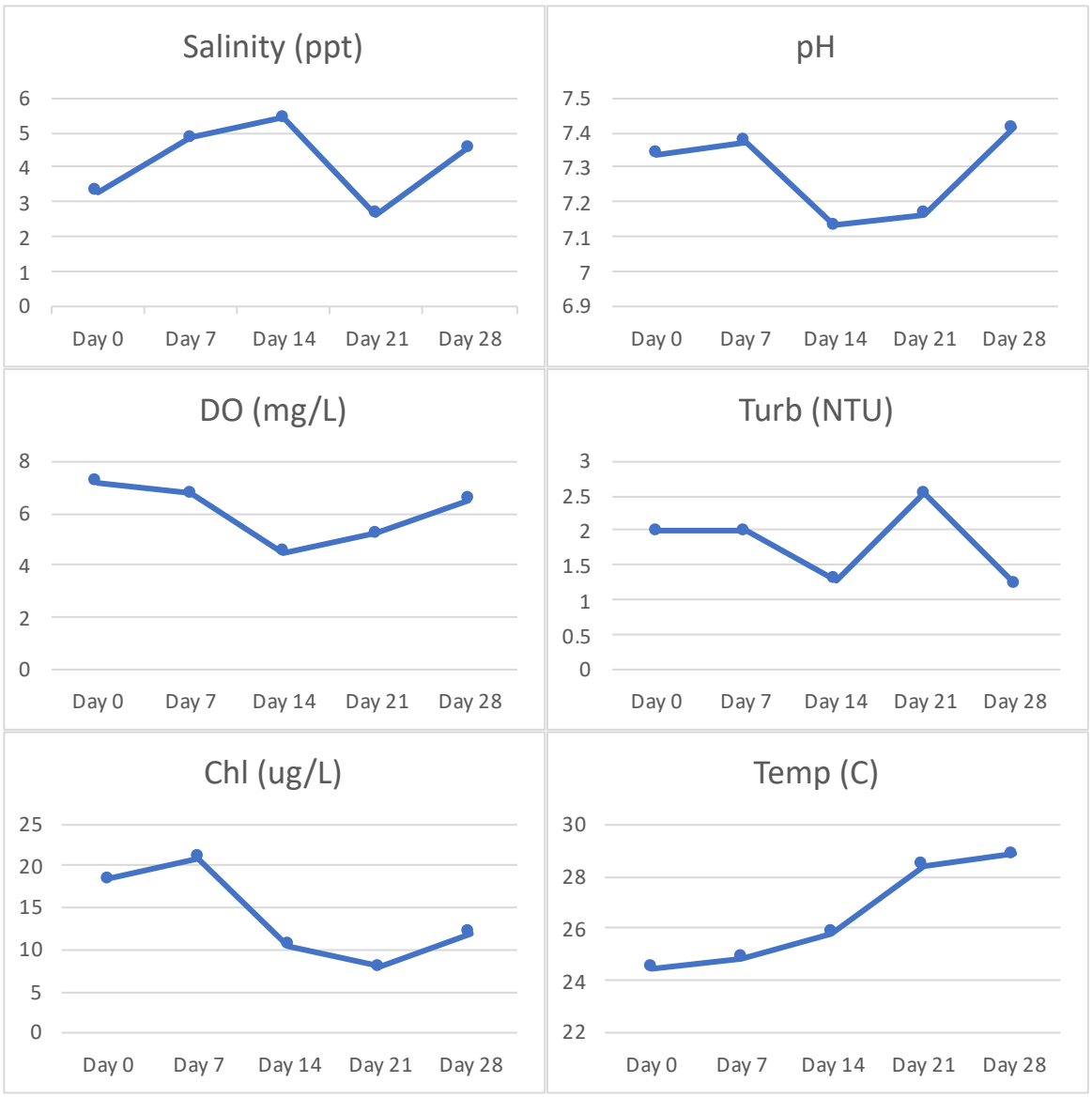

Figure 3.2. The hydrological data obtained from the Inner Harbor monitoring site of DNR. The site is located at the National Aquarium, about 200m from the study site. Each bullet represents the average of all that day's measurements during the first *in situ* incubation.

Through the 28 day incubation, the relative abundance of phyla Firmicutes, Actinobacteria and Patescibacteria increased in all samples (Fig. 3.3). Conversely, the abundance of Proteobacteria decreased in all samples from day 7 to day 28.

In all particle-associated samples there is a visible increase in abundance for the phyla Planctomycetes and Acidobacteria (Fig. 3.3). Cyanobacteria was less abundant after 28 days for PP, PLA and water samples but more abundant in the PS samples after the incubation.

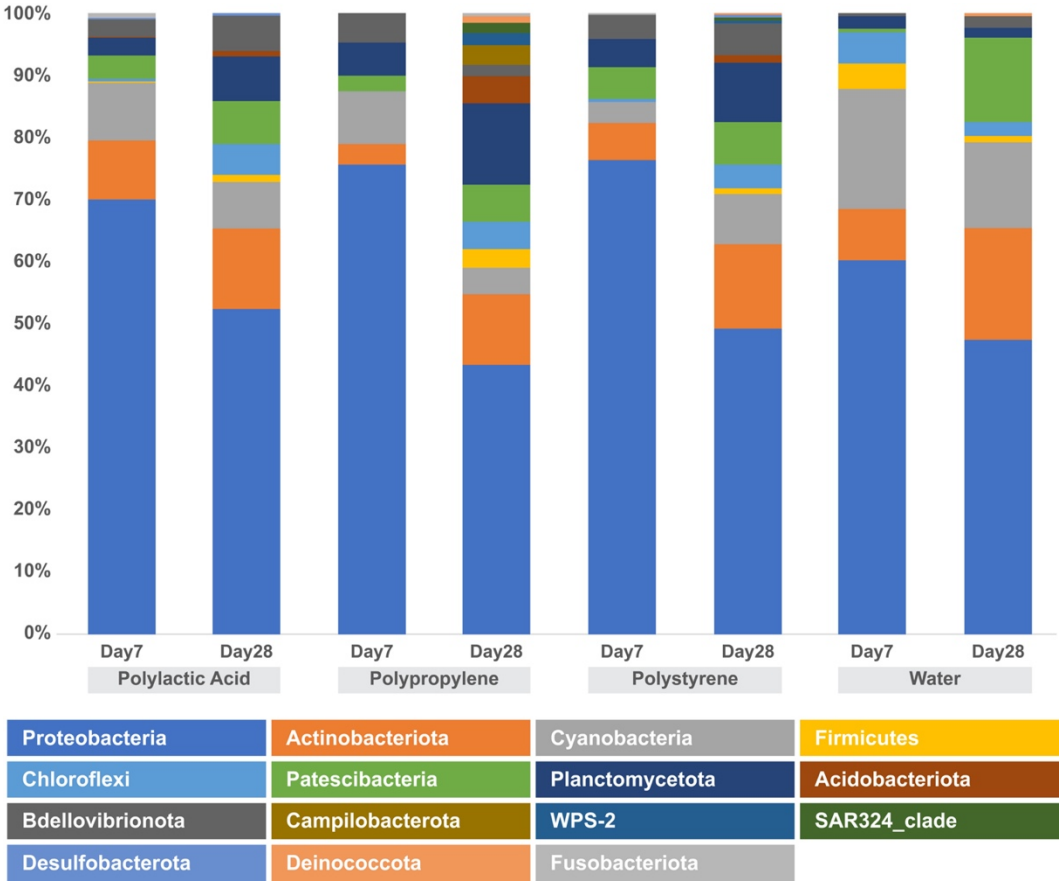

Figure 3.3. Relative abundance of major microbial phyla on day 7 and 28 during the first *in situ* incubation.

Diversity measures present grouping of the water samples in the weighted and unweighted unifrac analysis when generating the PCoA charts (Fig. 3.4, 3.5). This grouping effect is particularly noticeable in the weighted unifrac distance measure (Fig. 3.4). The other materials (PLA, PP and PS) appear to group together in two distinct clusters for both diversity measures.

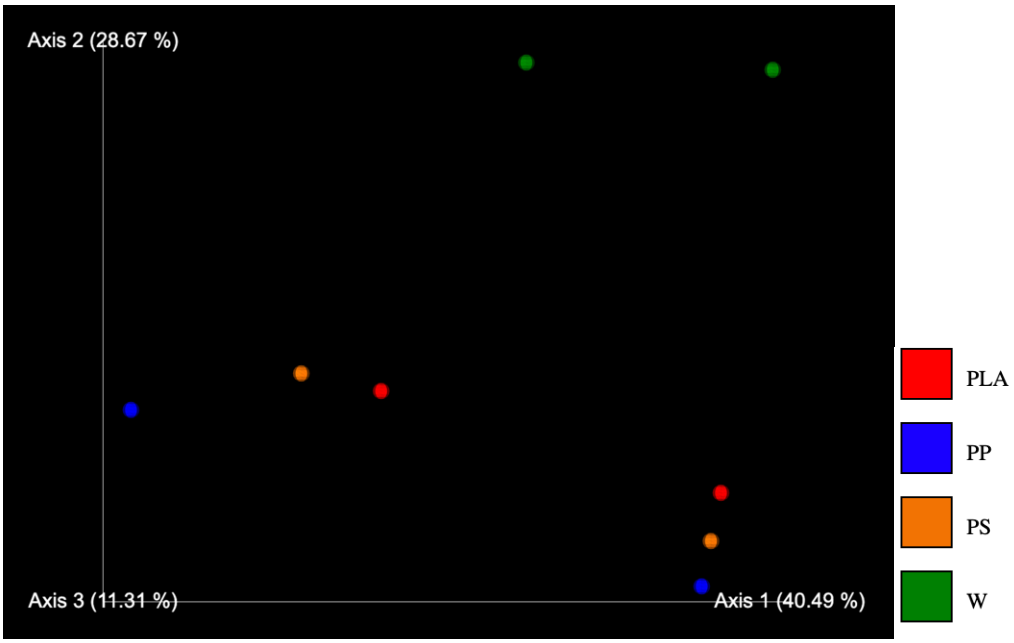

Figure 3.4. PCoA charts showing the dissimilarity of samples from the first *in situ* experiment grouped by material they were extracted from. Beta diversity measures show the weighted unifrac distance.

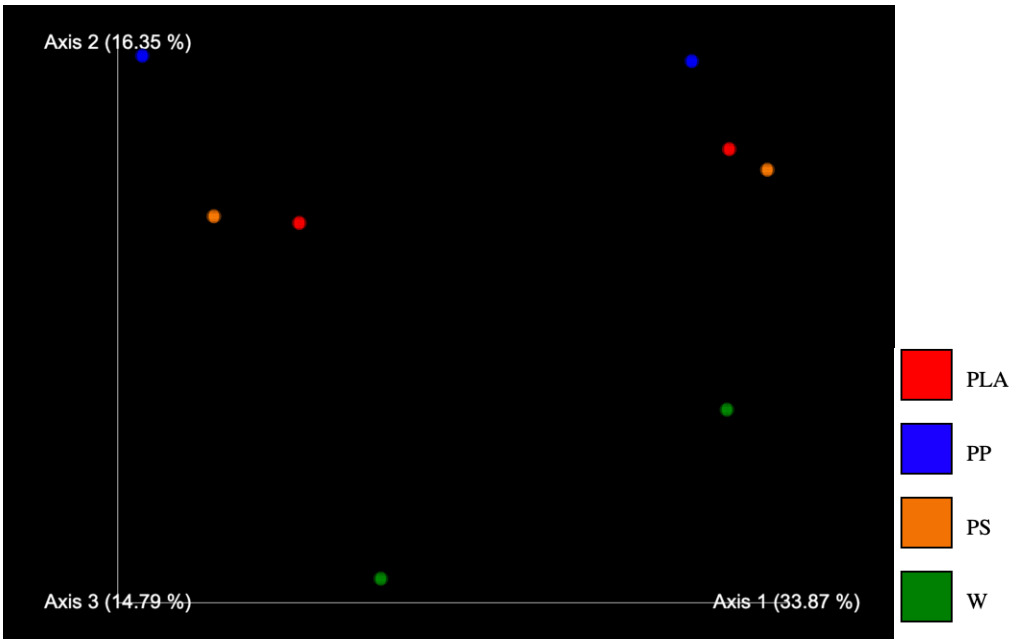

Figure 3.5. PCoA charts showing the dissimilarity of samples from the first *in situ* experiment grouped by material they were extracted from. Beta diversity measures show the unweighted unifrac distance.

Second *in situ* incubation

A total of 25 subsamples were collected during the second *in situ* incubation period (Table 3.2). Five subsamples were taken at each time point from the treatments and controls.

Table 3.2. Sample labeling system including sample name, material of the sample and the date it was recovered during the second *in situ* incubation.

Sample Name	Material	Date
IHW97	Water	September 7
IHW911	Water	September 11
IHW918	Water	September 18
IHW925	Water	September 25
IHW102	Water	October 2
TG97	Glass	September 7
TG911	Glass	September 11
TG918	Glass	September 18
TG925	Glass	September 25
TG102	Glass	October 2
TPLA97	PLA	September 7
TPLA911	PLA	September 11
TPLA918	PLA	September 18
TPLA925	PLA	September 25
TPLA102	PLA	October 2
TPP97	PP	September 7
TPP911	PP	September 11
TPP918	PP	September 18
TPP925	PP	September 25
TPP102	PP	October 2
TPS97	PS	September 7
TPS911	PS	September 11
TPS918	PS	September 18
TPS925	PS	September 25
TPS102	PS	October 2

Hydrological data

During the second *in situ* incubation period, salinity fluctuated between 9.3 and 12.7 ppt, and temperature decreased gradually from 27°C on day 7 to 22.5°C on day 28 (Fig. 3.3). The concentration of chlorophyll a decreased dramatically from 15.7 to 0.72 µg/L, and the level of dissolved oxygen also decreased markedly (from 5.89 to 0.23 mg/L). It is noticeable that the Inner Harbor water was in the hypoxic condition during the *in situ* study period and was almost anoxic on day 21. In addition, pH also fell during this period (September 5 to October 2, 2020) (Fig. 3.3).

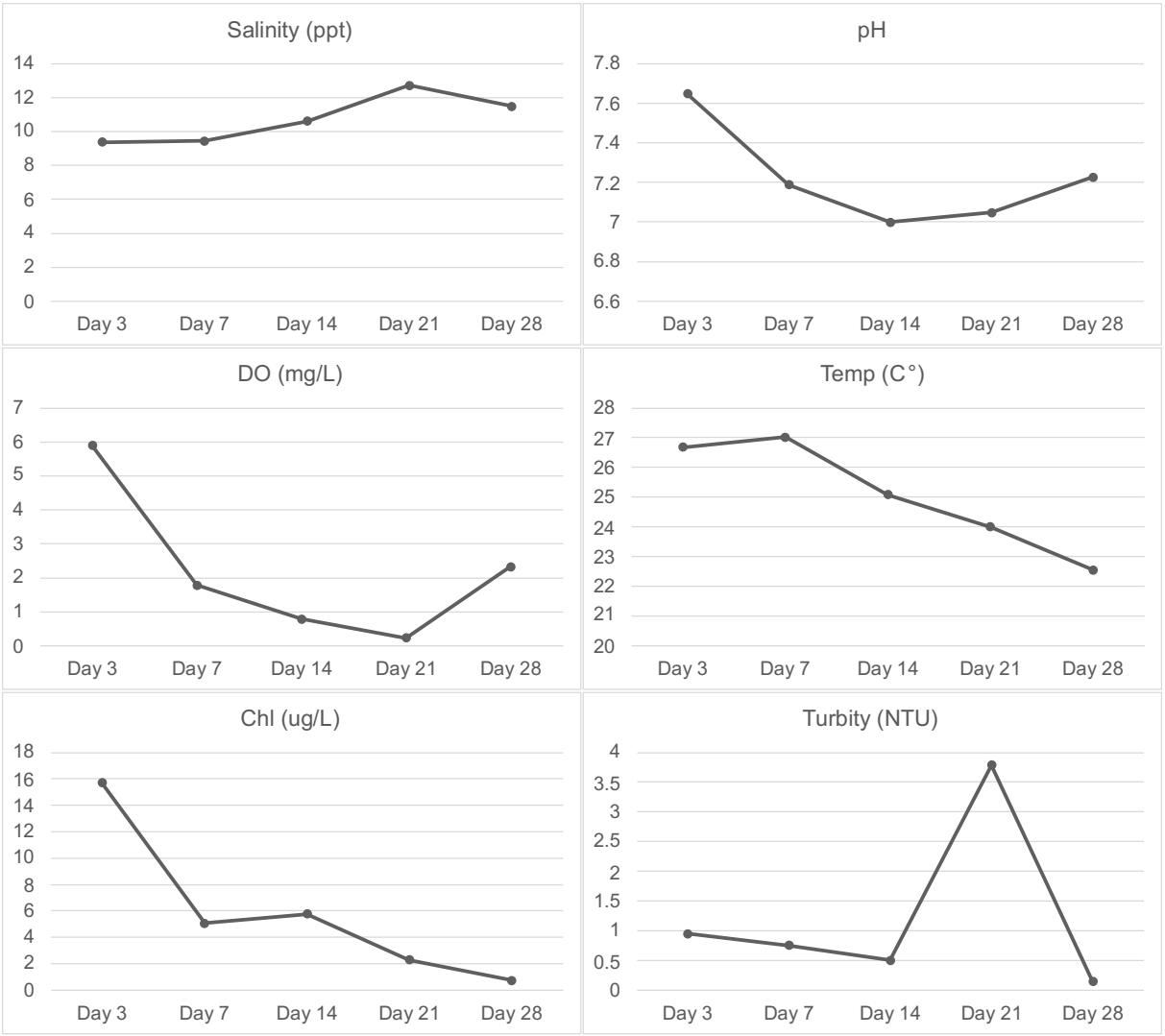

Figure 3.6. The hydrological data obtained from the Inner Harbor monitoring site of DNR. The site is located at the National Aquarium, about 200m from the study site. Each bullet represents the average of all that day's measurements during the second *in situ* incubation.

DNA sequencing and bioinformatic pipeline

DNA extracted from the 25 subsamples (Table 3.2) were sequenced. A summary of the resulting raw reads from the Illumina sequencing of samples is shown in Table 3.3. Reads were filtered using QIIME2 and the OTUs were clustered and enumerated through the same pipeline. Raw reads were in a

range of 16,525 to 89,012 and the number of OTUs in a range of 12,750 to 51,068. Generally, it appears that samples from PP, PS, and PLA have the largest number of raw reads and OTUs, with a few exceptions like the TPS102 sample. The relationship between number of raw reads and final picked OTUs is overall proportional.

Table 3.3. Number of raw reads, number of reads after filtration and total number of OTUs per sample for the second *in situ* incubation.

Sample name	Raw reads	Filtered reads	Total OTUs
TPP918	89,012	87,180	51,068
TPS97	86,722	84,806	52,429
TPP911	81,232	79,914	45,136
TPLA102	81,198	79,646	49,963
TPLA97	79,838	78,042	20,296
TPLA911	71,672	70,183	34,545
TPP925	71,366	70,247	44,202
TPP97	70,822	69,493	22,443
TPP102	70,623	69,284	45,136
TPS911	66,111	64,789	44,677
TPS918	58,716	57,441	44,557
TPLA925	58,648	57,554	35,586
TG97	56,307	55,149	34,308
TPLA918	56,272	55,293	29,932
TG925	56,230	55,255	44,396
TPS925	55,547	54,522	42,135
IHW925	53,544	52,547	27,485
TG918	48,332	47,495	36,619
TG102	43,913	43,030	31,958
IHW102	42,485	41,687	33,393
TPS102	37,252	36,368	28,003
TG911	31,910	31,349	24,459
IHW911	30,615	29,882	20,690
IHW97	20,689	20,241	16,192
IHW918	16,525	16,251	12,750

Microbial community analysis

The change of microbial community at the phylum level in different microplastics, glass beads, and surrounding water during the 28-day incubation period was shown in Fig. 3.7. The composition of microbial communities on

microplastics and glass beads appeared to be similar to each other from day 3 to day 28 (Fig. 3.7). In contrast, microbial community in the surrounding water maintained its own populations distinguishable from the biofilm microbial communities (Fig. 3.7). For the biofilm community, phylum Planctomycetes increased from a few percent on day 3 to ca. 10-20% for the rest of incubation time (Fig. 3.7). Such a trend was not seen in the surrounding water (Fig. 3.7). It appears that more Proteobacteria colonized on microplastics on day 3 compared to glass beads (Fig. 3.7). On day 3, Proteobacteria made up ca. 82, 66, and 66% of bacterial communities of PLA, PP, and PS, respectively, while glass beads contained ca. 50% of Proteobacteria. From day 3 to day 28, the relative abundance of Proteobacteria decreased gradually in all biofilm communities (PP, PS, PLA and glass beads), but they were still the most abundant bacterial group (>40% for all microplastics) at the end of incubation (Fig. 3.7). Bacteriodota appeared to be relatively stable (15-20% in most cases) throughout the incubation period. The abundance of phylum Cyanobacteria either stayed stable (Glass, Polystyrene) or slightly increased (PLA, PP) in all particle-associated particles, whereas it showed a decrease in water communities on day 14 and 21 (Fig. 3.7). Interestingly, the relative abundance of Desulfobacterota increased on all biofilms (PP, PS, PLA and glass beads) on day 21, and also increased somewhat in the water sample on day 21 (Fig. 3.7). Water communities showed a significantly higher abundance of the Actinobacteriota phylum in all time points, as well as a higher abundance of the phylum Crenarchaeota.

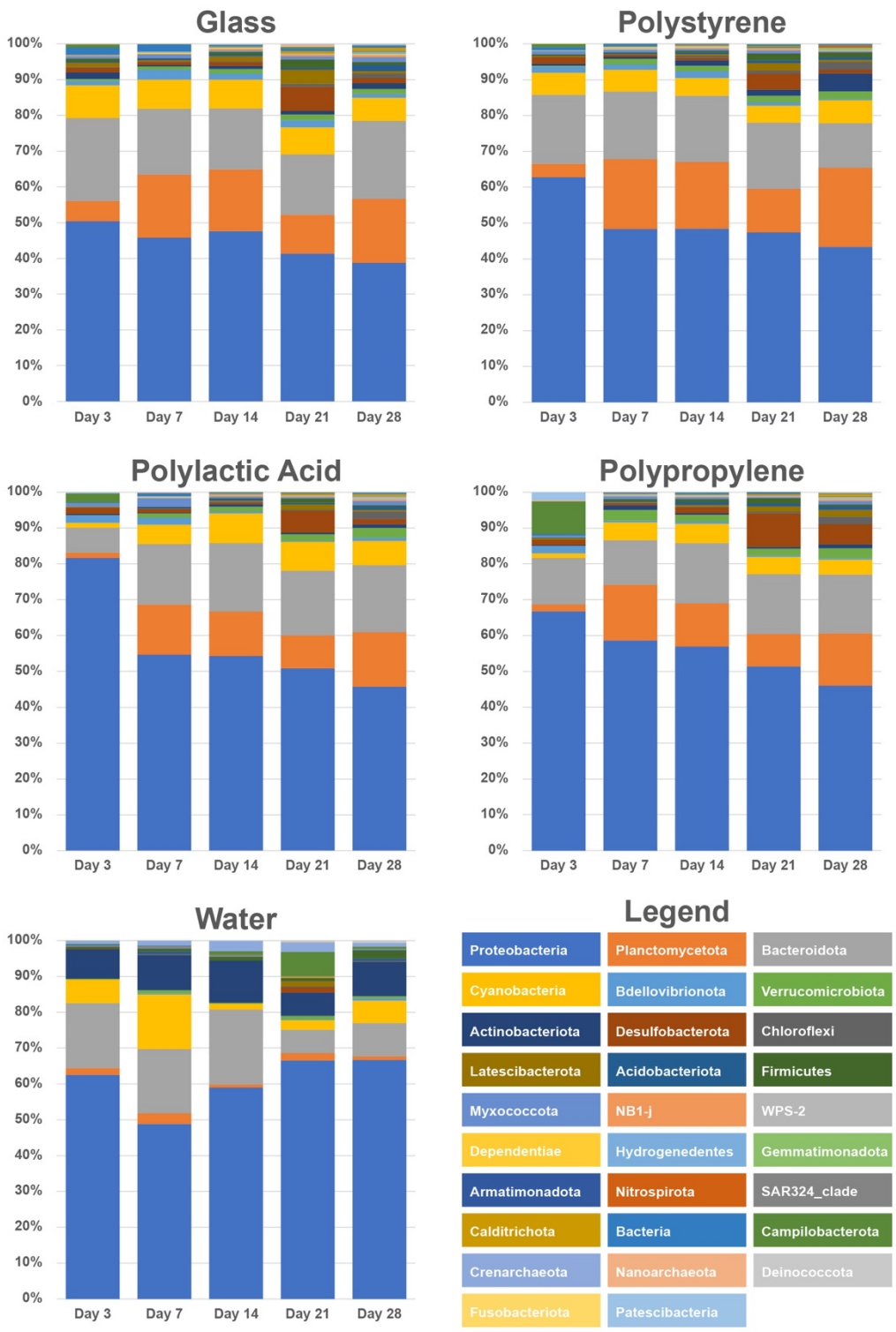

Figure 3.7. Relative abundance of major bacterial and archaeal lineages at the phylum level for bacterial communities. The relative abundance was analyzed based on the 16S ribosomal RNA gene sequences obtained on day 3, 7, 14, 21 and 28 for the second *in situ* incubation.

Diversity measures and PERMANOVA

Alpha rarefaction curves (Fig. 3.8) of all material types showed feature count plateauing at around 2000 sequencing depth, suggesting the diversity of the communities are captured at this depth and based on this the p sampling depth was selected as 2000 to determine how many sequences will be randomly subsampled for the following diversity measures.

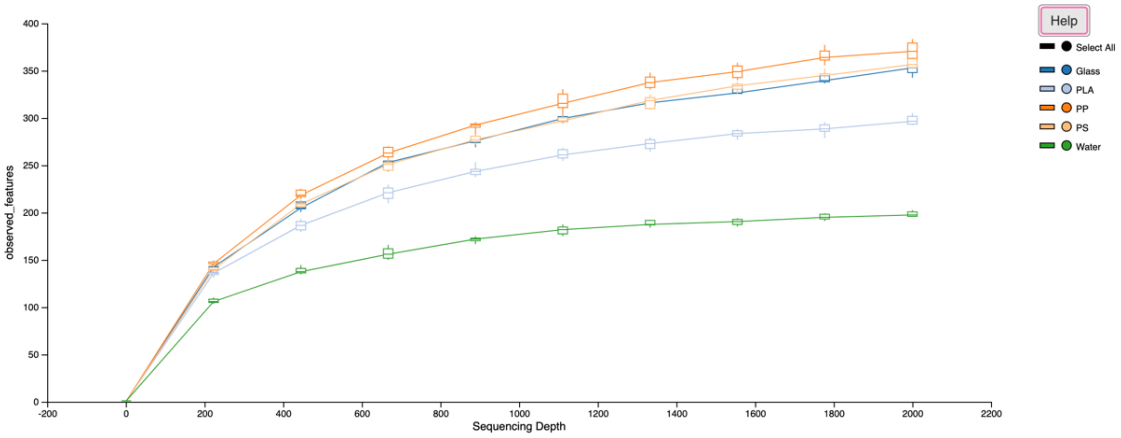

Figure 3.8. Alpha rarefaction curves based on observed features by sequencing depth for the second *in situ* incubation.

Statistical analysis using pairwise PERMANOVA tests is positive for dissimilarity if p-value is <.05 (Table 3.4). Beta diversity represented by PCA charts using unweighted unifrac (Fig. 3.9) and weighted unifrac (Fig. 3.10) distance show the water samples being isolated as a group in all instances. Unweighted unifrac distance analysis does not consider abundance of the OTUs and is a phylogeny-including taxonomic measure. Weighted unifrac distance, on the other hand, considers the abundance of taxonomic units. This provides two perspectives, one that gives weight to the most abundant taxa and one that gives equal weight to abundant and rare taxa.

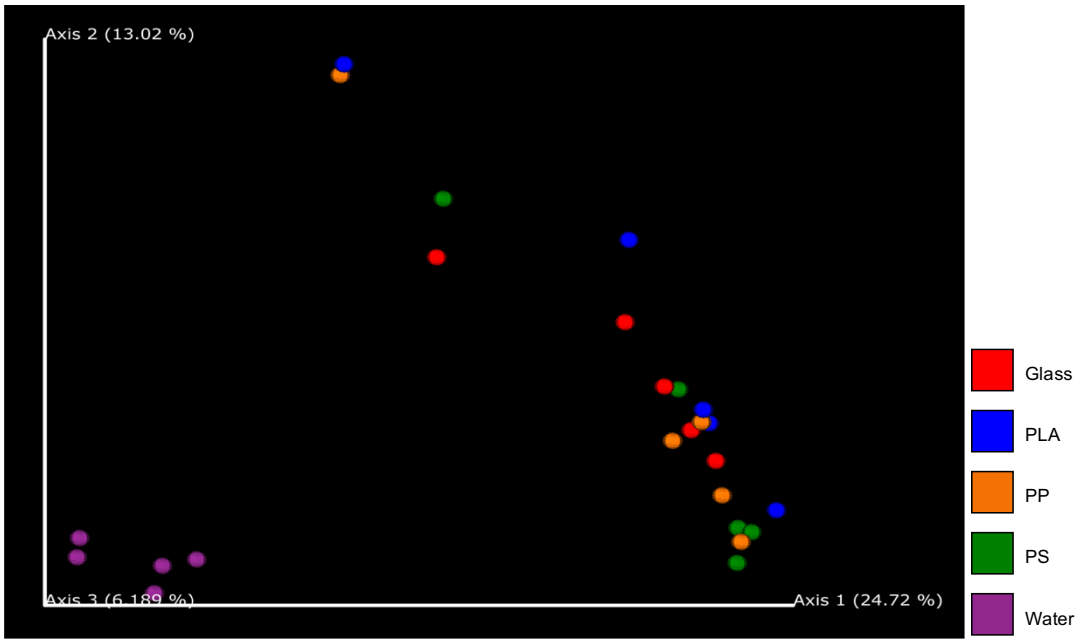

Figure 3.9. PCoA charts showing the dissimilarity of samples grouped by material they were extracted from in the second *in situ* incubation. Beta diversity measures show the unweighted unifrac distance.

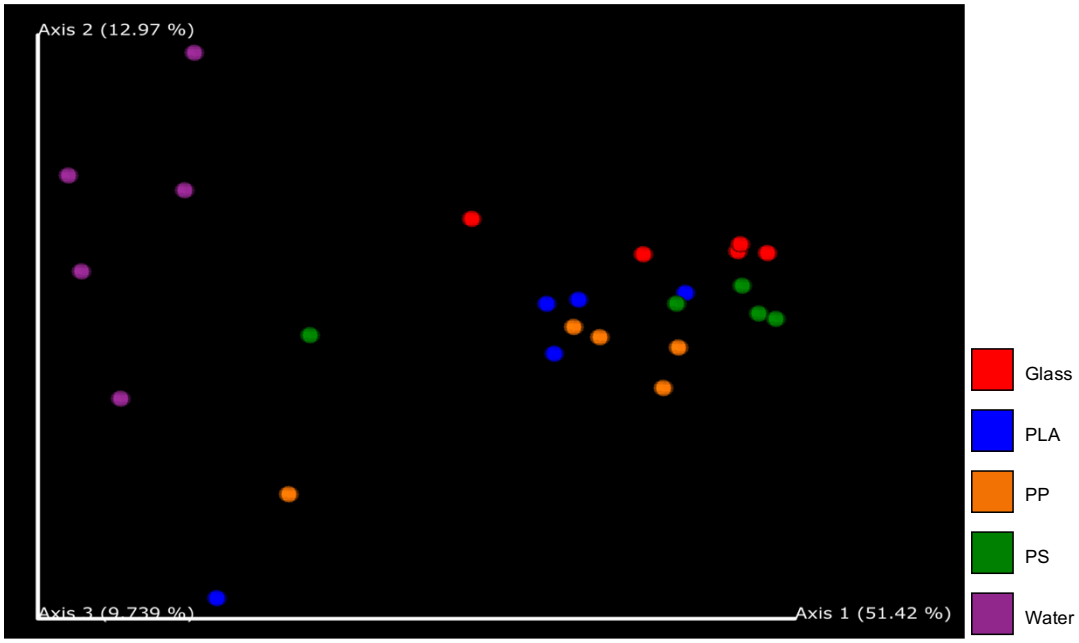

Figure 3.10. PCoA charts showing the dissimilarity of samples grouped by material they were extracted from in the second *in situ* incubation. Beta diversity measures show the weighted unifrac distance.

In both weighted and unweighted analyses, water groups together and separately from all the other particle-associated communities. There is no noticeable clustering of plastic materials separately from the glass inert control in either of the diversity measures.

PERMANOVA analysis confirmed the statistical significance of this dissimilarity. The p-values for the comparison between sampling groups showed dissimilarity exclusively when particle-associated communities were compared to water communities (glass-water=0.01, PLA-water=0.01, PP-water=0.006, PS-water=0.009). Every other p-value in the PERMANOVA analysis was larger than the 0.05 cutoff for dissimilarity (Table 3.4).

Table 3.4. Group significance PERMANOVA in a pairwise analysis for the second *in situ* incubation.

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
Glass	PLA	10	999	1.3036273	0.138	0.18125
Glass	PP	10	999	1.4050173	0.088	0.176
Glass	PS	10	999	1.4156289	0.115	0.18125
Glass	Water	10	999	4.6538861	0.01	0.025
PLA	PP	10	999	0.8238911	0.492	0.492
PLA	PS	10	999	1.3589694	0.145	0.18125
PLA	Water	10	999	4.5207674	0.01	0.025
PP	PS	10	999	1.0297202	0.448	0.492
PP	Water	10	999	4.1069582	0.006	0.025
PS	Water	10	999	4.7291459	0.009	0.025

Ecological interpretation of 16S marker data

The most abundant potential metabolic phenotype in all OTUs from all samples is chemoheterotrophy (Fig. 3.11). These two are closely followed by phenotypes that are related to phototrophy, photoautotrophy and lastly by photosynthetic cyanobacteria, with a similar abundance in all samples but slightly higher for all water samples. However, all particle-associated samples

contain more chloroplast-containing phenotypes than the surrounding water. All nitrogen metabolism phenotypes are highly abundant in all biofilm samples, especially for PP (Fig. 3.11). This is especially noticeable for nitrate and nitrite denitrification, nitrite respiration, nitrogen respiration and denitrification. Methylotrophy and methanol oxidation are more abundant in PLA compared to the rest samples (Fig. 3.11). Dark hydrogen oxidation in PP is more abundant compared to the other samples.

There is no significant increase in OTUs that have been related to plastic degradation in any of the samples, but there is however, a uniform abundance of hydrocarbon degradation potential metabolism OTUs. Additionally, OTUs related to human pathogenesis (general, pneumonia) seem to be abundant in communities from PLA samples and in those from water samples (Fig. 3.11). Potentially predatory, intracellular parasitic or exoparasitic OTUs are more abundant in all particle-associated communities but animal parasites or symbionts are equally abundant among all samples.

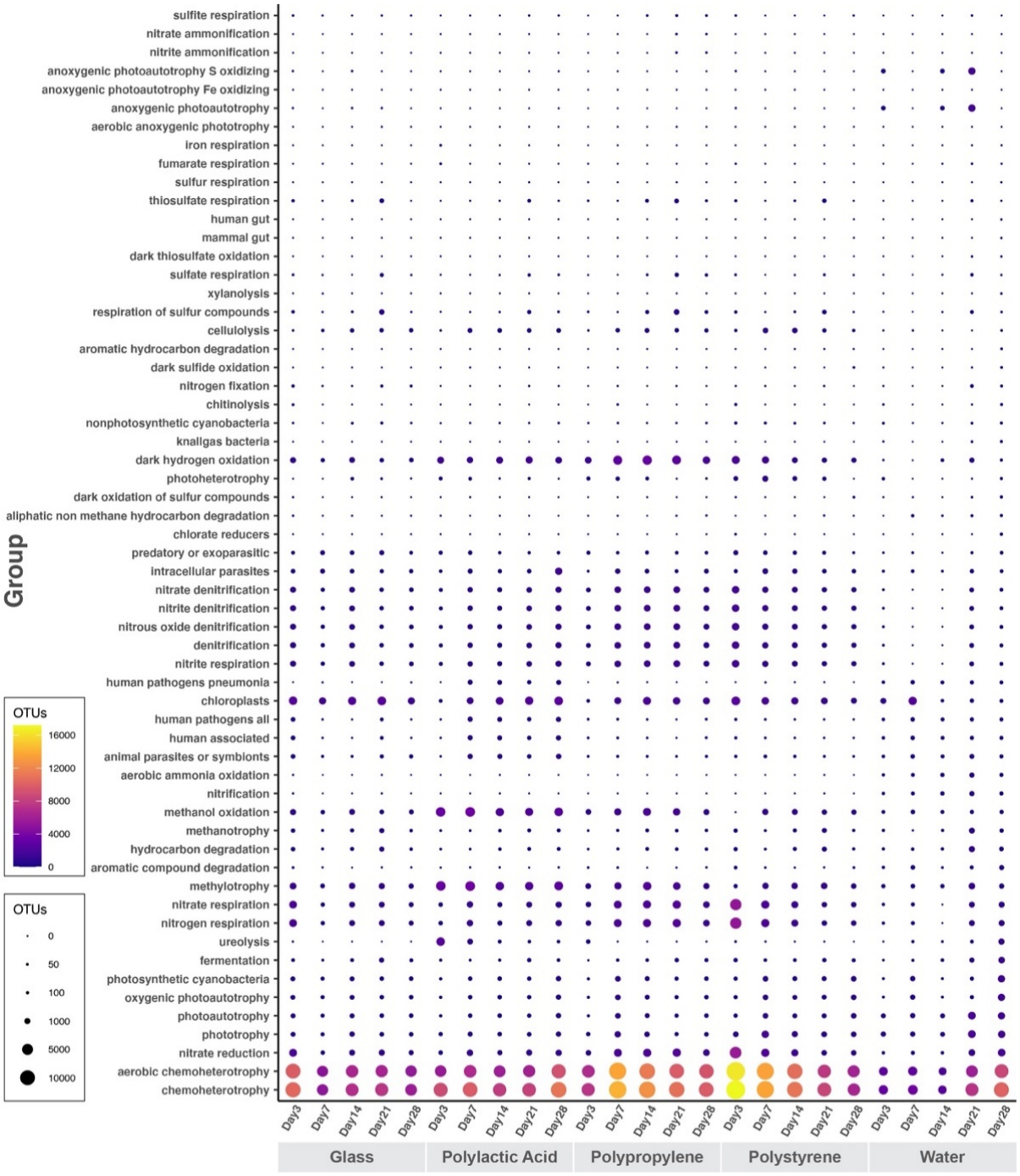

Figure 3.11. Ecologically relevant function abundance per sample. Bubbles represent the number of OTUs that have been classified by FAPROTAX as a functional group in each sample.

Comparison of pooled data from *in situ* and *in vitro* incubations

After pooling all samples from both experiments that were incubated starting on September 3rd, 2020, the diversity measures showed that the *in vitro* experiments described in Chapter 2 showed a lower alpha diversity than the *in situ* experiments described in this chapter (Fig. 3.14). Additionally, the beta diversity measures showed distinct grouping of each experiment's samples in the PCoA analysis (Fig. 3.12, Fig.3.13). All the water samples in both experiments appear to group closely together in the unweighted unifrac analysis (Fig. 3.12).

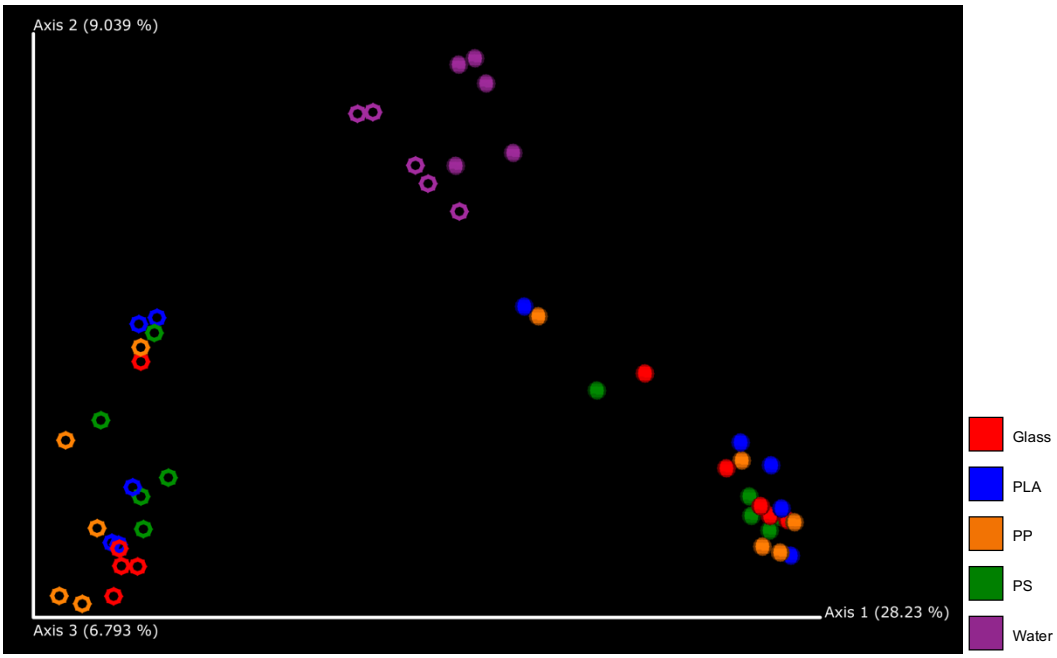

Figure 3.12. PCoA charts showing the dissimilarity of samples grouped by material they were extracted from in both 2020 incubations. Beta diversity measures show the unweighted unifrac distance. Rings represent the samples from the *in vitro* experiments and the spheres represent the samples from the *in situ* experiments.

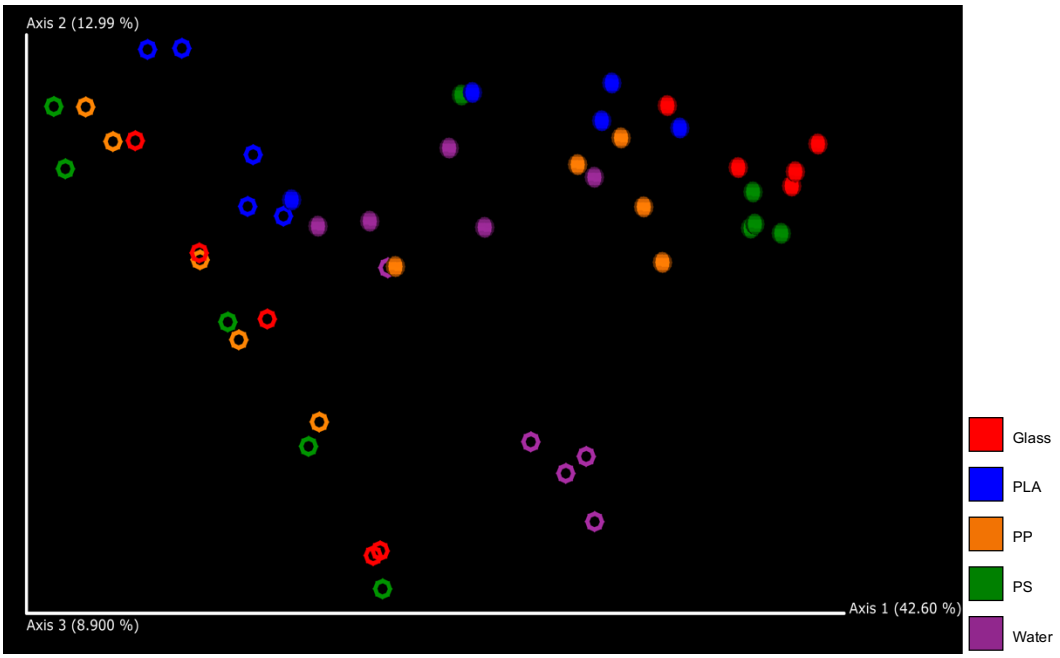

Figure 3.13. PCoA charts showing the dissimilarity of samples grouped by material they were extracted from in both 2020 incubations. Beta diversity measures show the weighted unifrac distance. Rings represent the samples from the *in vitro* experiments and the spheres represent the samples from the *in situ* experiments.

Alpha diversity in the samples from the PS microbeads presented the highest number of observed features, followed by PLA, then water, then PP and finally glass (Fig. 3.14). The PERMANOVA statistical analysis of pairwise group significance showed that the only significantly dissimilar samples are those from water (p-value=0.001) (Table 3.5). There was no significant difference between any of the microbead types.

The Venn diagram reveals that only 1% of all OTUs from the 2020 experiments is shared among all sample types, and that water and polystyrene each have the most unique OTUs to the sample type (3782 and 2326 respectively). Glass, PLA and PP only have approximately 1700 tom 1900 unique OTUs each. Only 0-2% of OTUs overlap for all varying sample type combinations (Fig. 3.15).

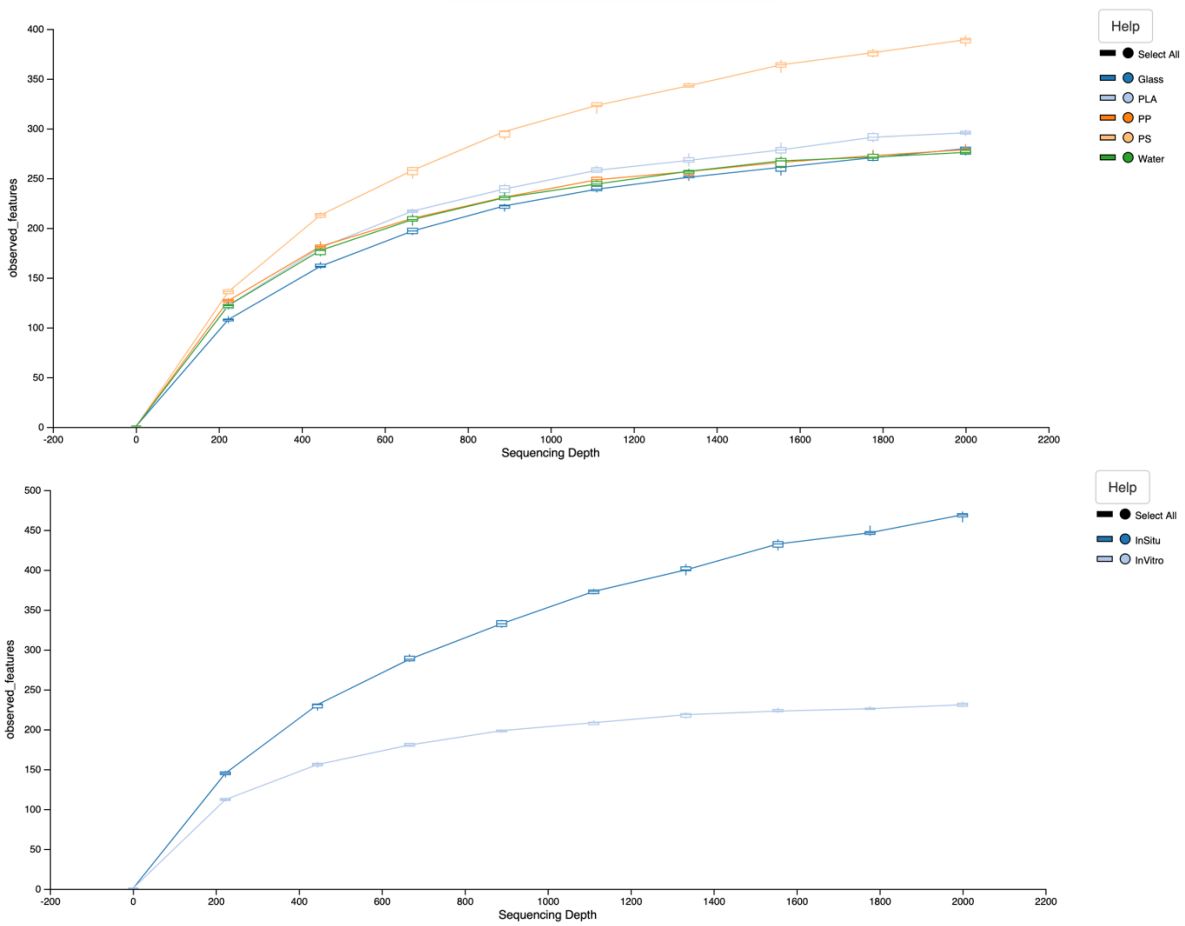

Figure 3.14. Alpha rarefaction curves based on observed features by sequencing depth for the pooled data for both 2020 experiments. Top graph represents alpha diversity by material type and bottom graph represents alpha diversity by experiment type (*in situ* vs. *in vitro*).

Table 3.5. Group significance PERMANOVA in a pairwise analysis for both 2020 experiments.

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
Glass	PLA	20	999	0.999538	0.344	0.43
Glass	PP	20	999	0.912766	0.398	0.442222
Glass	PS	20	999	1.118299	0.259	0.43
Glass	Water	20	999	3.359699	0.001	0.0025
PLA	PP	20	999	0.728485	0.691	0.691
PLA	PS	20	999	1.143047	0.251	0.43
PLA	Water	20	999	2.951865	0.001	0.0025
PP	PS	20	999	0.991455	0.343	0.43
PP	Water	20	999	3.269329	0.001	0.0025
PS	Water	20	999	3.173964	0.001	0.0025

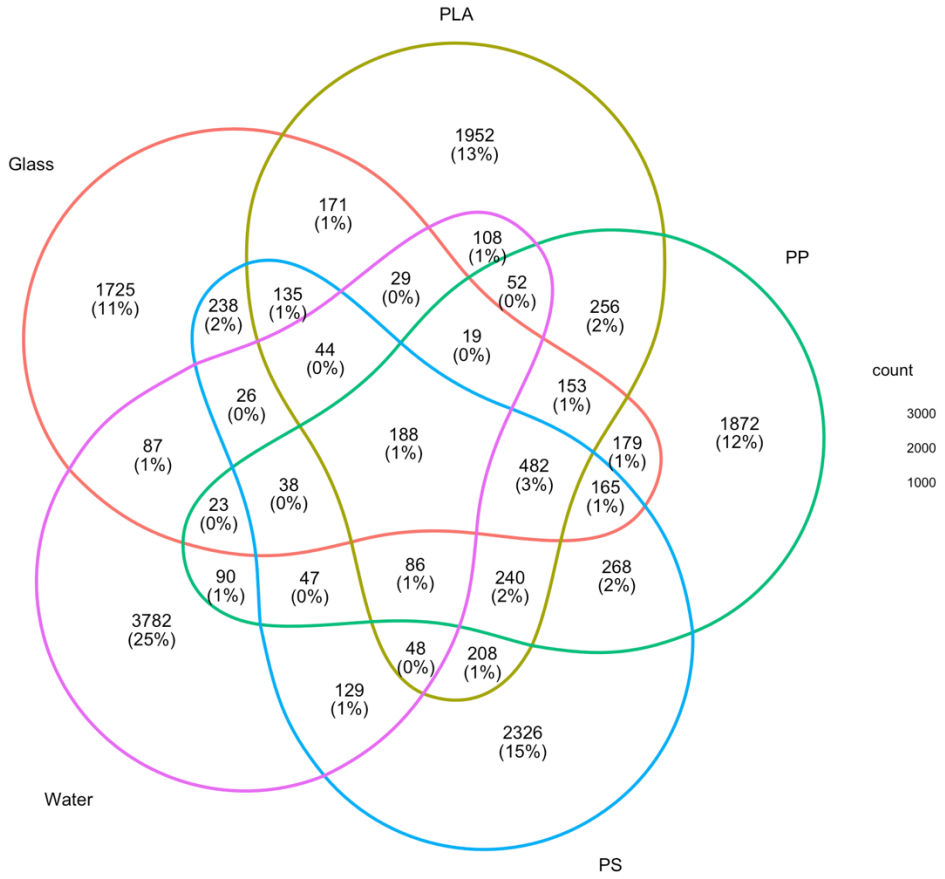

Figure 3.15. Venn diagram of OTU overlap between sample types including all the samples from the *in vitro* and *in situ* incubations from the 2020 experiments.

Discussion

Changes in community structure of the first incubation period

During the first incubation period in June-July of 2019, there was an increase in the abundance of the Firmicutes phyla. While Actinobacteria showed an increase in abundance in all samples (Fig. 3.3), it is more abundant in the water samples from both timepoints. Actinobacteria are considered to be favored by higher temperatures as well, as their higher GC content (higher than 55%) (Schaal et al., 2006) confers them an advantage (Shivlata & Satyanarayana,

2015). *Patescibacteria* is a phylum of very small bacterial cells that have small genomes of approximately 1 Mbp due to their reduced non-essential functions and their simplified cellular structure (Tian et al., 2020). Their abundance increased in all samples after 28 days. This phylum has been found to be abundant in plastisphere samples from Alpine and Artic soils (Rüthi et al., 2020). Cyanobacterial abundance decreased in all samples, except for the PS sample after 28 days of incubation, in which there was a slight increase. This decrease is consistent with a slight decrease in the chlorophyll a level in the water of the sampling site (Fig. 3.2).

The phyla that increased in all polymer communities were Planctomycetes and Acidobacteria (Fig. 3.3). This increase in Planctomycete abundance is consistent with the results of the second incubation experiment, in which Planctomycetes showed a sharp increase in all polymer samples, as well as in the non-plastic glass control (Fig. 3.7).

Low dissolved oxygen in the Inner Harbor during the second incubation period

The hydrological data showed that the water's environmental conditions changed rapidly during the *in situ* incubation study. Although we do not know how bacterial and algal abundance changed during this period, we speculate that the rapid decrease of chlorophyll a was caused by biomass reduction or die off of phytoplankton. Colder temperatures in the later period of incubation might result in the decrease of phytoplankton abundance. When phytoplankton die, they release a large quantity of organic matter which support massive growth of heterotrophic bacteria. Respiration of organic matter by bacteria

consumes oxygen and quickly lowers dissolved oxygen in the harbor. It is likely that the hypoxic/anoxic status was formed due to massive consumption of phytoplankton-released organic matter by bacteria. Meanwhile, bacterial respiration also releases CO_2, which may contribute to the lower pH observed in the later stage of incubation. It is unclear why the water turbidity increased on day 21. Understanding the change of water quality during the incubation time is an important step towards comprehensive analysis of bacterial community on plastisphere.

Planctomycetes thrived on biofilms

It is noticeable that Planctomycetes became more abundant after day 7 on all microplastic particles and glass beads (Fig. 3.7) and remained stable until the final timepoint of the second *in situ* incubation. Planctomycetes is one of the most commonly reported phyla in marine plastics in a wide range of locations (Oberbeckmann & Labrenz, 2020). In this study, Planctomycetes made up ca. 15-20% of microbial communities from day 7 to the end of experiment (day 28). For the most of incubation time, the local water experienced the hypoxic and anoxic condition (Fig. 3.6), and the low oxygen condition is preferable to Planctomycetes. Fuerst et al. (2011) reported that Planctomycetes generally thrive in an attached lifestyle, especially in low oxygen conditions, as they are able to oxidize ammonia to dinitrogen without oxygen. Planctomycetes have been previously found in sediments from Baltimore's Inner Harbor and their ammonia aerobic oxidation (anammox) activity has been characterized in an effort to understand how an increase of nitrogen due to urban activity and the

use of fertilizers has affected the nitrogen cycle in the Chesapeake Bay water (Tal et al., 2005).

A similar phenomenon was evident in the first *in situ* incubation, in which there was a clear increase in all biofilm samples of the phylum Planctomycetes (Fig. 3.3), and there was a decrease in dissolved oxygen (Fig. 3.2) in the water during the time of incubation (from 7.18 mg/L in day 0, to 4.4 mg/L in day 14 and back up to 6.5 mg/L in day 28).

In the *in vitro* studies of chapter 2, Planctomycetes showed a consistent increase in abundance throughout the 28-day incubation in the biofilm samples for the first incubation and in the water samples for the second incubation.

Desulfobacteriota became abundant on day 21

The abundance of Desulfobacteriota increased in all samples on day 21 of the second incubation experiment and then decreased to the previous levels for the day 28 sampling timepoint (Fig. 3.7). Hydrological data show a decrease in dissolved oxygen for the 21 day timepoint with a drop to 0.23 mg/ml (Fig. 3.6). Metagenomic studies have found a high abundance of mercury methylation genes in Desulfobacteria species that are commonly found in low-oxygen waters of the Baltic Sea and found that these genes were found in higher relative quantities in marine particle-associated communities than those in free-living communities (Capo et al., 2020). These bacteria have been found in oxygen deficient zones, including both anoxic and hypoxic waters.

Succession of biofilm communities

In the second incubation experiment, we found that initial colonization could be proven from the day 3 samples and that the number of days of incubation was not proportional to the number of raw reads or classified OTUs obtained (Table 3.3), as some of the samples with the highest number of reads are from the earliest sampling timepoints (i.e., TPS97). In all particle-associated samples, there was a decrease in proteobacteria after the initial colonization of about 5 to 25% from day 3 to 7 and it was followed by further decrease throughout the 28-day incubation. The water samples showed the same initial decrease from day to day 7 but slowly recovered during the rest of the timepoints to an even higher abundance on day 28 than on day 3. This suggests that after the initial colonization period in the first few days where Proteobacteria usually dominate, the rest of the members of the particle-associated community has a chance to increase in abundance and this is consistent with the findings from the first *in situ* incubation as well. The initial colonization patterns described by studies looking into the early stages of marine microbial plastic biofilms is consistent with the dominating classes of the particle-associated communities being Gammaproteobacteria and Alphaproteobacteria (Oberbeckmann & Labrenz, 2020) in the first two sampling days. After these initial stages, the composition of Proteobacteria in all plastic and glass samples seemed to adjust (Fig. 3.7). Studies into the successive colonization of plastic pollution in marine ecosystems have shown that once microbes colonize a surface and the biofilm becomes mature, the community stabilizes and less changes in composition are perceivable (Dussud et al., 2018). Bacterial colonization of any surface can

happen extremely rapidly in most environments (Costerton et al., 1999) and biofilm formation on polymers has been reported to happen as quickly as in a few hours to a few days (Harrison et al., 2014). Differences in community structure between day 3 and 7 could be explained by the different environmental conditions they were exposed to, as these conditions started changing within this time period. There was a decrease in DO, pH, turbidity and *chl a* concentration and a slight increase in temperature and salinity (Fig. 3.6). Samples from polymers in all dates of this *in situ* experiment show a higher percentage of cyanobacterial species, indicating that photosynthetic species are attaching and persisting on these particles. In the water and glass communities, however, there was a decrease in the abundance of Cyanobacteria (Fig. 3.7) consistent the overall decrease of chlorophyll a in the sampling site (Fig. 3.6). This could potentially affect nutrients available in the water and general biogeochemical cycles.

Several studies have found photosynthetic species in the plastisphere, including prokaryotes dominated by cyanobacteria (Bryant et al., 2016; Zettler et al., 2013).

Interestingly, the abundance of Actinobacteria was 10-15% higher in water communities than in particle-associated communities, directly contradicting studies in which Actinobacteria thrive in the plastisphere and increase in abundance after incubation on polymers (Rüthi et al., 2020). Actinobacteria are known to play a part in the decomposition of organic materials like cellulose and chitin and thus in the carbon cycle (Anandan et al., 2016), but marine

Actinomycetes are also considered mainly free-living bacteria (Barka et al., 2016). The abundance of Crenarchaeota in water samples being so high (2-5%) relative to that (0-2%) of the polymer samples is consistent with studies of the marine plastisphere in the Mediterranean Sea, in which all types of archaea were found in low abundances in all plastic related samples (Gutierrez et al., 2021). It is possible that the availability of sulfur in the plastisphere is too low for archaeal species to grow and develop and this may be the limiting factor for Crenarchaeota in these biofilms (Kletzin, 2007; Leigh & Whitman, 2013).

Additionally, there is a higher number of present phyla in the *in situ* incubation samples, compared to those in the *in vitro* experiment of Chapter 2 and this may be because of the constant water flow of the mesh containers. The fluctuating environmental conditions including salinity, temperature and dissolved oxygen available to the *in situ* samples could have been one of the factors that contributed to the very diverse communities, with a relatively higher number of OTUs across all samples compared to the *in vitro* study samples (see Chapter 2).

Diversity measures and PERMANOVA

The PERMANOVA analysis showed that there was no significant difference between all particle-associated groups. This result is consistent with a previous study where chemical composition of the surface was a minor factor in determining the composition of the biofilm (Oberbeckmann et al., 2020). Interestingly, there were no significant differences between the microplastic microbead samples and the inert glass beads control, and this directly

contradicts previous findings in which naturally occurring substances were colonized selectively by microorganisms (Ogonowski et al., 2018), but supports the previously mentioned meta-analysis results regarding naturally occurring particles (wood, cellulose, glass) and their communities being statistically similar to those on polymers (Oberbeckmann & Labrenz, 2020). These statistical results support our observations of the community structure based on relative abundance of phyla (Fig. 3.7) in which water communities appear to be distinguishable from all the particle-associated communities, which are similar across timepoint samples (Fig. 3.7).

Ecological interpretation of 16S marker data

The bubble plot generated with the FAPROTAX database shows that the most abundant function in all OTUs of all samples is chemoheterotrophy (Fig. 3.11), suggesting that there is an abundance of bacteria that are able to use metabolic products as nutrient sources and perform nutrient regeneration in the communities (Bharti et al., 2017). Functional families related to prototrophy and photoheterotrophy show high abundance in all samples including water, but photosynthetic cyanobacteria appear more abundant in water communities (Fig. 3.11). This directly contradicts our findings from the 16S rRNA gene community analysis, in which cyanobacteria proved more abundant in polymer and glass samples over surrounding water samples (Fig. 3.7). This discrepancy may be due to FAPROTAX being a uniquely predictive tool and not an actual metabolic descriptor.

As in the communities found in Chapter 2, there is also some evidence of metabolisms that confer bacteria advantages in low oxygen or anoxic conditions, as many of identified functions are related to nitrogen metabolism, methane metabolism and even dark hydrogen metabolism (Fig. 3.11). All of these functional families appear to be more abundant in the particle-associated communities (Fig 3.11) of glass, PLA, PS and PP. This suggests that the plastispheres attaching and forming on these particles are able to adapt to a low-oxygen or anoxic environment and survive even in the deepest layer of the biofilms where oxygen availability may be low to none (He et al., 2018).

Bacterial OTUs with potentially pathogenic functions appear to be the highest in communities from PLA biofilms and in water, contradicting the theory that pathogens can accumulate in large quantities on plastic biofilms and increase in abundance relative to their abundance in the surrounding water of aquatic environments (Barnes & Milner, 2005; Zettler et al., 2013). The higher abundance of exoparasites and intracellular parasites in all samples of particle-associated communities, however, supports this theory.

Comparison of pooled data from *in situ* and *in vitro* incubations

The fact that both second incubations from the *in situ* and *in vitro* studies in this dissertation were started on the same day and their colonization started from the same initial microbial community, allows for the pooling of the sequencing reads for a comparative analysis. The alpha diversity curves show that the *in situ e*xperiments presented a higher diversity, suggesting that the constant

water flow and open colonization of surrounding water could be more promotive of a diverse community composition.

The material type with the highest alpha diversity is polystyrene. This is interesting because polystyrene consists of big pores and ridges that form the foam structure. These pores and ridges confer polystyrene a higher surface area, which could potentially allow for a higher diversity of microorganisms. Although all other sample types seem to cluster together in the alpha diversity curves (Fig. 3.14), glass is the material that appear to have the lowest alpha diversity of all. Glass was chosen as an inert non-plastic control, and it's physical properties could contribute to the diversity of these biofilms being lower.

The PERMANOVA analysis further confirms the findings of this chapter, in which we found that water samples are all statistically different to particle-attached samples, and there is no significant difference between plastic types or even glass (Table 3.5).

Conclusion

In this study, development of biofilm communities on different microplastic beads and a glass bead control was monitored. Different types of microbeads were placed in stainless steel tea mesh containers. Two 28-day *in situ* incubation experiments took place in the Baltimore Inner Harbor in two different years. The first incubation took place in the summer of 2019 and the second incubation in the fall of 2020. We found that formation of biofilms on particles happened rapidly and was overall dominated by the phyla Proteobacteria. After

the initial colonization period, other phyla in the community were able to grow and increased in abundance. The phylum Planctomycetes was found to increase in abundance when attached to plastic and glass particles, especially in low-oxygen conditions. The second *in vitro* incubation had a low-oxygen event in which there was a dramatic increase in Desulfobacterota, especially in the particle-associated communities. Additionally, the microbial communities of the biofilms in this *in situ* study were found to be significantly different to those in the surrounding water. Understanding how chemically different polymers are colonized in estuarine environments is crucial to elucidating the environmental impacts of plastic pollution. The pollution of our waterways is ubiquitous; future work will have to focus on the interactions of these pollutants with all organisms in the ecosystem and it will have to define the extent of its impact on nutrient cycling, transportation of harmful species, and primary production.

Chapter 4. Isolation and characterization of filamentous cyanobacteria from microplastic beads in the *in vitro* study.

Abstract

Microbial biofilms formed on microplastics contain a wide range of microorganisms. Blue-green color was developed on the microplastic biofilms during the *in vitro* study, and microbial matrix resembled filamentous cyanobacteria were abundant on the surface of microplastics based on SEM observations (see Chapter 2). It is interesting to know if they are filamentous cyanobacteria, and if so, which species they belong to. This study focuses on the isolation of the filamentous cyanobacteria attached to three different types of microbeads incubated with Baltimore's Inner Harbor water *in vitro*. A total of five filamentous strains with blue-green color were isolated. Colony and cell morphology of these isolates were described. They were further identified based on their 16S rRNA gene sequences. The phylogenetic relationship between these isolates and other filamentous cyanobacteria was constructed. Our isolates were most closely related to the members in genera *Jaaginema*, *Tildeniella* and *Nodisinilea*, but also related to the species in *Leptolymbya* and *Phormidium*. These genera can all form blooms, and some of them are potentially harmful. Colonization of massive filamentous cyanobacteria on microplastics suggests can have multifaceted effects on the aquatic ecosystem such as increased activities of carbon and nitrogen fixation, increased oxygen level on the biofilm surface, and potential carbon sinking into sediments.

Introduction

Cyanobacteria are present in most marine and freshwater environments (Curren & Leong, 2020), and they play an important role in global primary production and can account for up to 10^{15} grams of wet biomass (Garcia-Pichel, 2009). They are phototrophic organisms and contribute greatly to the richness and abundance of benthic communities in freshwater ecosystems (Wang et al., 2018).

Cyanobacteria have many ecological roles, including but not limited to primary production, bloom formation, nitrogen fixation and serving as a food source for phytoplankton. Cyanobacterial bloom formation is necessary for the natural management processes of freshwater systems (Garcia-Pichel, 2009) but harmful cyanobacterial blooms (CyanoHABs) have been increasing in number in the last decade, including *Anabaena*, *Microcystis* and *Nodularia* (Carey et al., 2012). Despite this rise, little is known about the dispersal mechanism of cyanobacteria in aquatic environments, but it is known that CyanoHABs can happen in marine, freshwater and brackish systems (Curren & Leong, 2020). Since cyanobacteria are able to withstand a wide range of environmental conditions and can survive high and low temperatures (Castenholz, 1981), nutrient availabilities and even UV exposure (Sinha et al., 2008). Benthic genera like *Oscillatoria* and *Phormidum* can settle onto plastic debris and use these surfaces as transport vectors through normally unfavorable settings (Palińska et al., 2012).

Plastics account for approximately 60 to 80 percent of all debris in marine waters (Gregory & Ryan, 1997) and it is estimated that around 250,000 tons of

plastic are currently floating in the world's oceans (Eriksen et al., 2014). The most abundant plastic pollution form is microplastics. Cyanobacterial species have been found to attach to plastic debris, both in macro and micro sizes (Masó et al., 2003; Zettler et al., 2013). Filamentous cyanobacteria have been identified in biofilms attached to polystyrene particles in the marine and estuarine areas of the Baltic Sea (Kaiser et al., 2017), as well as on the northern Mediterranean (Masó et al., 2016) and in Australian waters (Reisser et al., 2014). In marine microplastics composed of polyprolene (PP) and Polyethylene (PE) filamentous cyanobacteria are present in a significantly higher abundance than in the surrounding seawater, in which unicellular cyanobacteria seem to dominate (Zettler et al., 2013). Some of these cyanobacterial species are potentially pathogenic or form harmful blooms, increasing the concern for the durability of microplastics and their potential to transport microorganisms across long distances and through bodies of water and making them invasive species.

During the *in vitro* study, development of blue-green color on the bead surfaces and the visualization of filamentous cyanobacteria like microbes on the bead surfaces using scanning electron microscopy (SEM) (see Chapter 2) suggest that filamentous cyanobacteria are likely an important player on the biofilm formation on the plastics. This also prompted the question of what species of filamentous cyanobacteria were attaching to these plastics during the *in vitro* incubation.

In this study, we attempt to isolate filamentous cyanobacteria from microplastic particles that were incubated *in vitro* with water collected from Baltimore's Inner Harbor. Upon successful isolation, we will further character them phenotypically and genotypically.

Materials and Methods

Isolation of cyanobacteria from microplastics

In this study, we intended to isolate cyanobacteria grown on three different microplastic beads (polypropylene, polystyrene, and polylactic acid), the full incubation protocols can be found in Chapter 2. SEM photographs were obtained at the Electron Microscopy Core Imaging Facility at the University of Maryland School of Dentistry in Baltimore, Maryland. The microscope used was the FEI Quanta 200 in the High Vacuum mode. Microbead samples from the *in vitro* experiment were visualized at the time stamps of 28 days and 10 months for each respective polymer type, full sample preparation details can be found in Chapter 2.

Following the first *in vitro* incubation experiment, 3 beads of each polymer type were taken from the glass bottles 10 month later, after a period of incubation and enrichment. The beads were rinsed with phosphate-buffered saline (PBS), and subsequently streaked across solid SN15 medium (Waterbury et al., 1986; Xu et al., 2015) using sterile forceps. SN is a common medium used for isolating cyanobacteria in aquatic environments, and SN15 is the same SN medium with adjusted salinity (15ppt). The water sample of the control was also plated by spreading 100 µl of control sample with a disposable plastic spreader

(Corning™, NY, USA). Inoculated SN15 agar plates were kept at room temperature (20-22°C) on the laboratory bench for 90 days. No specific light intensity was set, the plates received light from windows and fluorescent light from the ceiling with a daytime incidence of 6-8 µmol/m2/s and dark at nighttime. After 90 days, filamentous cyanobacteria were recovered from all three types of microbeads. Filamentous cyanobacteria were visible under a light compound microscope (AMSCOPE) in the lab. Filaments were randomly picked to streak on new SN15 agar plates and cultivated in the same conditions as the initial plates. To observe cyanobacteria under the epifluorescence microscope, a few filaments were taken from the culture plate using forceps and placed onto a glass slide. One drop of sterile deionized water was added on top of the filaments, and the glass slide was covered with a glass cover slip. The slides were examined using a fluorescent microscope model Axioplan (Zeiss, West Germany). Photographs were taken with the 100X magnification lens and using immersion oil. Microscopic images were captured by the AxioCam MR3 camera operated through an image analysis software Zen Lite.

Strain identification based on 16S rRNA gene sequences

Filaments from each strain were picked with a sterile plastic loop and transferred into 25 milliliters of liquid SN15 medium in sterile tissue culture flasks with vented cap and allowed to grow at room temperature (20-22 °C) and with laboratory lighting conditions, with a daytime incidence of 6-8 µmol/m^2/s and light daily shaking by hand for 4 weeks. Liquid cultures were used for DNA extraction, using 4 ml of liquid culture to centrifugate and collect a pellet of cells.

The DNA from cultures was extracted using the UltraClean Microbial DNA Isolation Kit (MOBIO, CA, USA) following the manufacturer's protocol. Extracted DNA was recovered in 50 µl of the included elution buffer.

Extracted DNA was used to amplify the V3-V4 region of the 16S rRNA gene of each sample with the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Products were purified using the GeneJET PCR Purification Kit (ThermoScientific™, Massachusetts, USA) submitted for sequencing at the Institute of Marine and Environmental Technology's BioAnalytical Services Lab (BASLab) Both forward and reverse primers were used for sequencing and two FASTA files were obtained for each sample. Obtained sequences were assembled to obtain one single sequence per strain using the Merge tool by EMBOSS (Williams, 1999) and run on the Basic Local Alignment Search Tool (BLAST) to be compared to the National Center for Biotechnology Information (NCBI) database.

Phylogenetic reconstruction

The tree was generated by taking the generated sequences and aligning them against a few representative strains of cyanobacteria on the BLAST NCBI database. These strains included filamentous cyanobacterial species, as well as a strain of unicellular cyanobacteria to serve as a root to the tree. The tree was built using the MEGA 11 software (Kumar et al., 2018). Phylogenetic relationship of four cyanobacterial isolates (PLA1, PP1, PP2, and PS1) with known cyanobacterial representatives based on 16S rRNA gene sequences

(758 nt aligned sequences). Neighbor-Joining tree was constructed with the Jukes-Cantor model and 1000 bootstrap replications. The scale bar represents the number of substitutions per site (Fig. 4.5).

Results

Scanning Electron Microscopy

Filamentous organisms attached to the plastic surfaces were visible in all samples of all polymer types (Fig. 4.1), but much more concentrated and clustered in the PLA beads. In the PP sample, it was possible to see a single filament with segmentation of cells. In the PS sample, filamentous organisms were spread out and settled in some of the pores of the material.

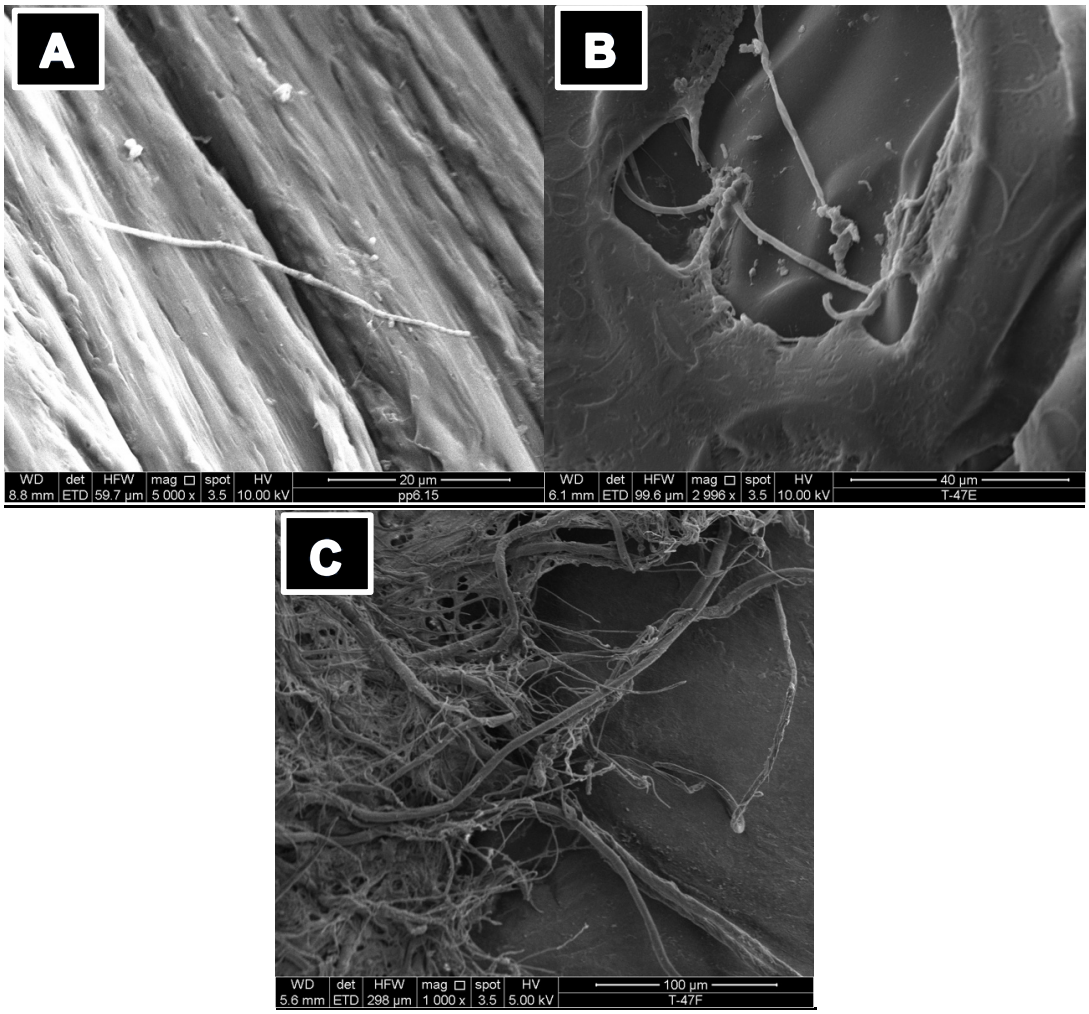

Figure 4.1. SEM images showing the colonization of filamentous organisms on the three different plastic microbeads at the 10 months of *in vitro* incubation. The scale bar and magnification are shown on the images.

Isolation of cyanobacteria from microbeads

After 3 months incubation, blue-green color filamentous microorganisms were grown on the plates inoculated from all three types of microbeads (PS, PP, and PLA) (Fig. 4.2). In addition, microorganisms with yellow-brown color were also present, especially on the PLA plate (Fig. 4.2). The number of microbes growing on the SN15 plates increased from PS, PP to PLA, based on eye

observation. Under the dissecting microscope, filamentous cyanobacteria (blue-green color) and pennate diatoms (yellow-brown color) are the dominant forms of microbes grown the plates. The original time of incubation was planned to be four weeks, but this experiment was started shortly before the COVID-19 pandemic restrictions and could not be accessed for three months. The SN15 agar plates had their surfaces completely covered in dark green, yellow and brown filaments for the plastic microbead samples. The water control sample plate, however, had no growth at all and showed no filaments or colonies of any kind (Fig. 4.2). A total of 7 strains were isolated originally, but only 5 strains were available after multiple transfers for purification.

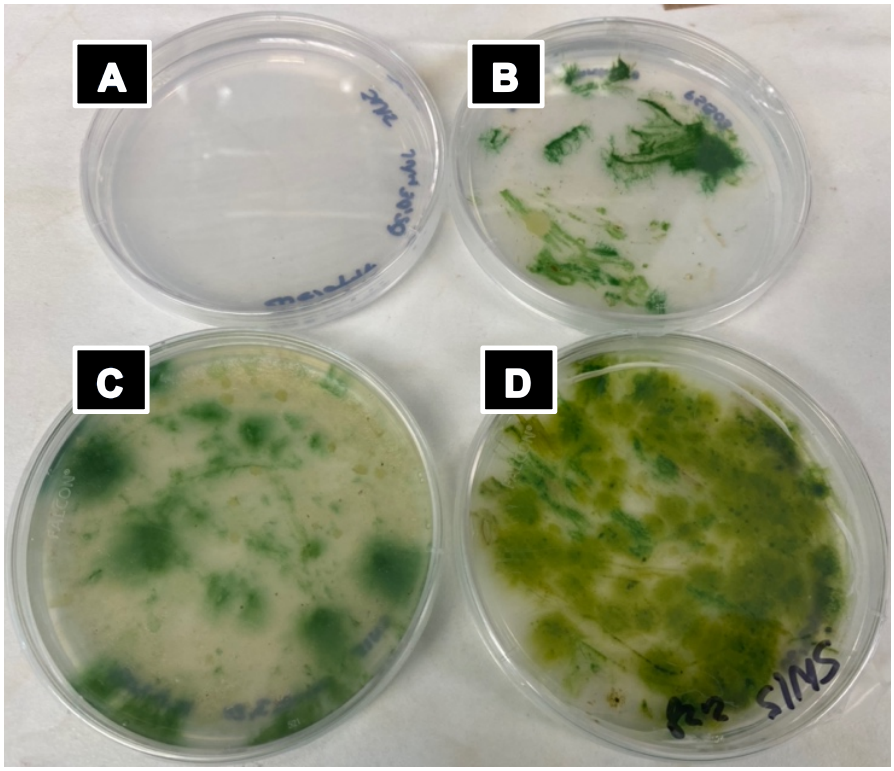

Figure 4.2. Growth of microalgae on the culture plates streaked from three different microplastic beads. A. Water control, B. Polystyrene, C. Polypropylene, D. Polylactic Acid.

After multiple transfer, pure cultures of filamentous cyanobacteria were obtained. Different forms of filamentous matrix can be visualized using a dissecting microscope (Fig. 4.3). Two strains were purified from the PP sample, two strains were purified from the PLA sample and one strain was purified from the PS sample. Strains were named PP-1, PP-2, PLA-1, PLA-2 and PS-2 (Fig. 4.3). The colors of the filaments varied from bright blue green to intensely dark blue green. When transferred to liquid media, all strains grew in clusters and formed aggregates that sunk to the bottom of the culture flasks.

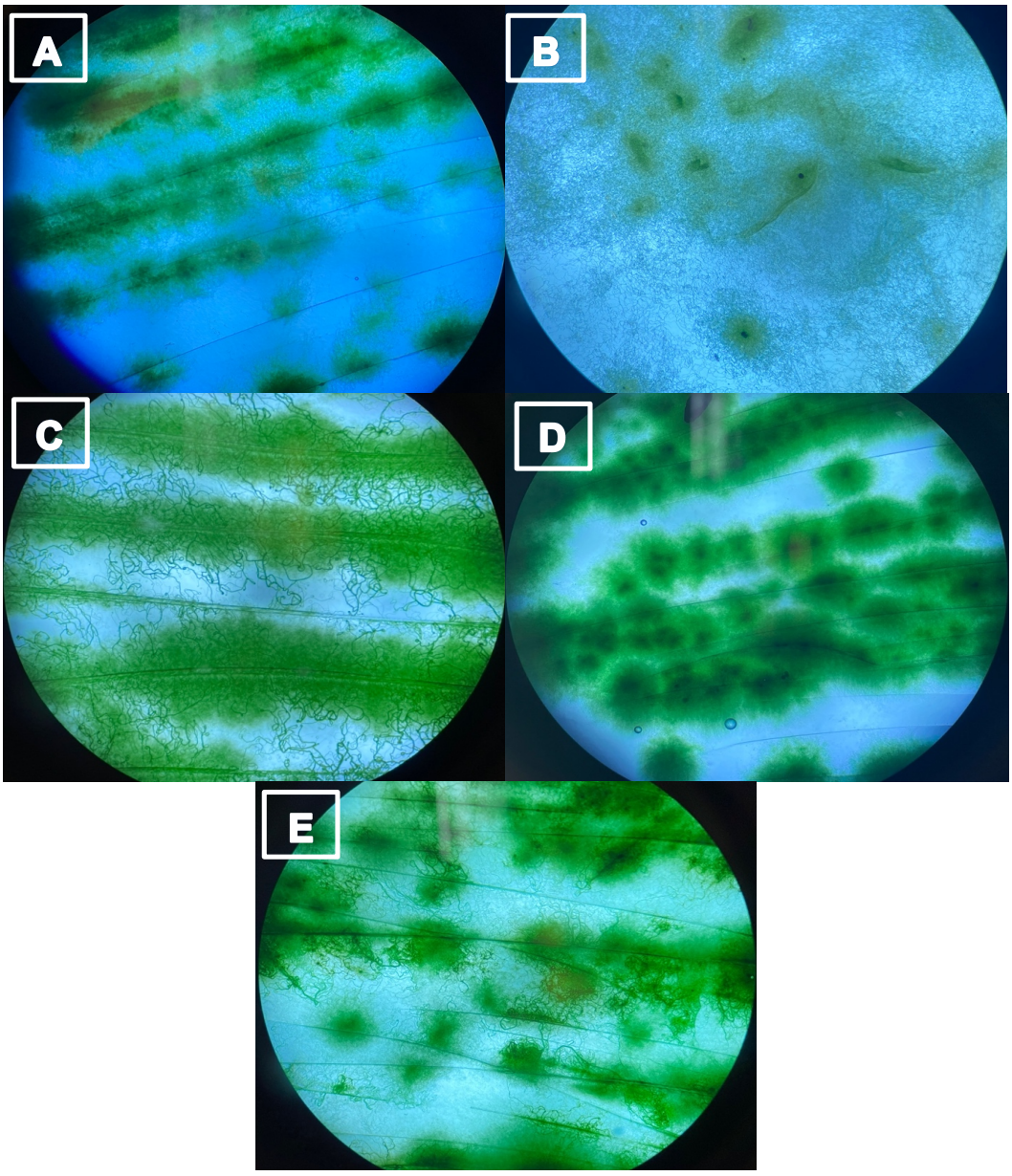

Figure 4.3. Different matrix forms of filamentous cyanobacteria observed under a dissecting microscope. A. PP-1 B. PP-2, C. PS-2, D. PLA-1, E. PLA-2. Photos were taken using a mobile phone pointing at the eye piece.

Identification of filamentous strains

Utilizing the top hits of the search, the 5 strains that grew were characterized, and their potential identity was determined (Table 4.1). After running the assembled 16S rRNA gene sequences through the BLAST alignment tool, strains were identified using their top hit as 3 different types of previously described cyanobacterial species. The query cover percentages ranged from 71 to 100% and the identities ranged from 87.2 to 99.01%. Strans PS-2 and PP-2 both had the best hit with the same strain of *Jaaginema germinatum* 16S RNA gene. Strains PP-1 and PLA-1 both had 100% query cover and over 99% identity with the same strain of *Tildeniella torsiva* UHER 1998/13D. Strain PLA-2 had only an 87.2% identity with cyanobacterium *Nodosilinea signiensis*.

Table 4.1. The best hits of five filamentous strains in GenBank.

Strain	Top BLAST hit	Query cover	%identity
PP-1	Tildeniella torsiva strain UHER1998/13D clone 3	100	99.7
PP-2	Jaaginema geminatum SAG 1459-8 16S ribosomal RNA gene, partial sequence	100	99.76
PS-2	Jaaginema geminatum SAG 1459-8 16S ribosomal RNA gene, partial sequence	100	99.01
PLA-1	Tildeniella torsiva strain UHER1998/13D 16S ribisomal RNA gene, partial sequence	100	99.7
PLA-2	Nodosilinea signiensis USMFM 16S ribosomal RNA gene, partial sequence	71	87.2

Microscopic observations

Five filamentous strains were visualized under a fluorescent microscope (Fig. 4.4). Red fluorescence emitted from pigments under green light enabled viewing cell segments. All filaments appear to have cells of varying lengths, the

longest appearing in PP-2 and the shortest in PLA-2. PLA-1 appears to have cells in different sizes within the same trichomes. PS-1 appears to have cells that have leached out their pigments and no longer fluoresce. Filaments also vary in thickness, with those from PS-2 being the thickest and those from PP-2 and PLA-2 being the thinnest. PLA-2 also presents an interesting formation denominated a rotating disk-like cluster (Yamamoto et al., 2021). All of the visible filaments in the micrographs present a thin mucilaginous sheath that covers the trichomes, but this especially visible in strain PS-2.

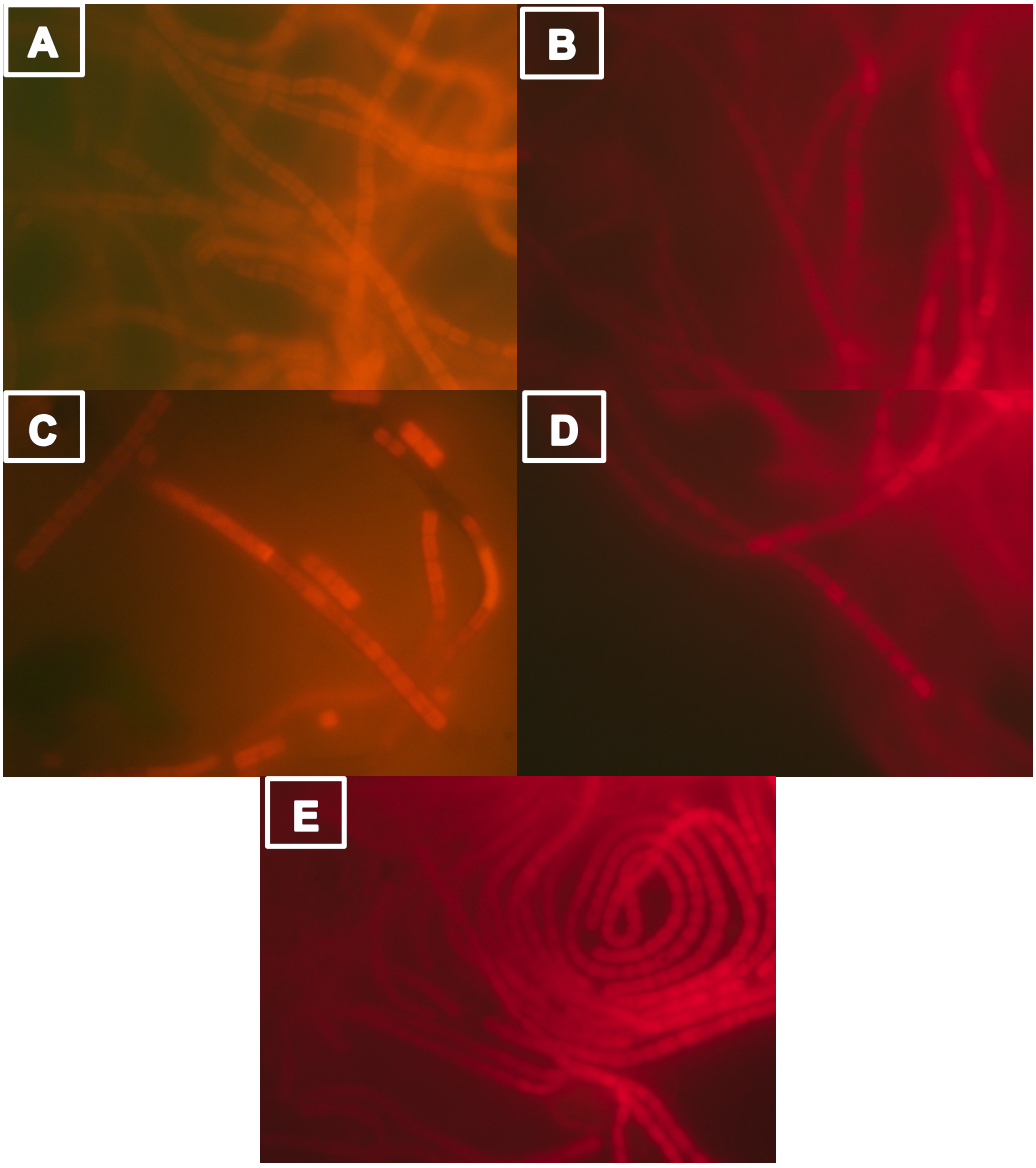

Figure 4.4. Micrographs of the filamentous cyanobacterial strains under the epifluoresence microscope with green light excitation. A. PP-1 B. PP-2, C. PS-2, D. PLA-1, E. PLA-2. Amplification is 100X for all samples.

Phylogenetic analysis of isolated filamentous cyanobacteria

Based on the 16S rRNA gene phylogeny, PLA1, PP1 and PP2 are closely related, and appear to cluster with *Jaaginema* spp. and *Tildeniella* spp. PS-2 also appears to be affiliated with the *Jaaginema-Tildeniella* cluster, but it does

not cluster closely with PLA-1, PP-1 and PP-2 (Fig. 4.5). *Synechococcus elongatus* was included as a unicellular cyanobacterial species to serve as a root for the tree. PLA-2 presented sequences of low quality that were affecting the alignment and the construction of the tree, so they were removed until further purification and future resequencing.

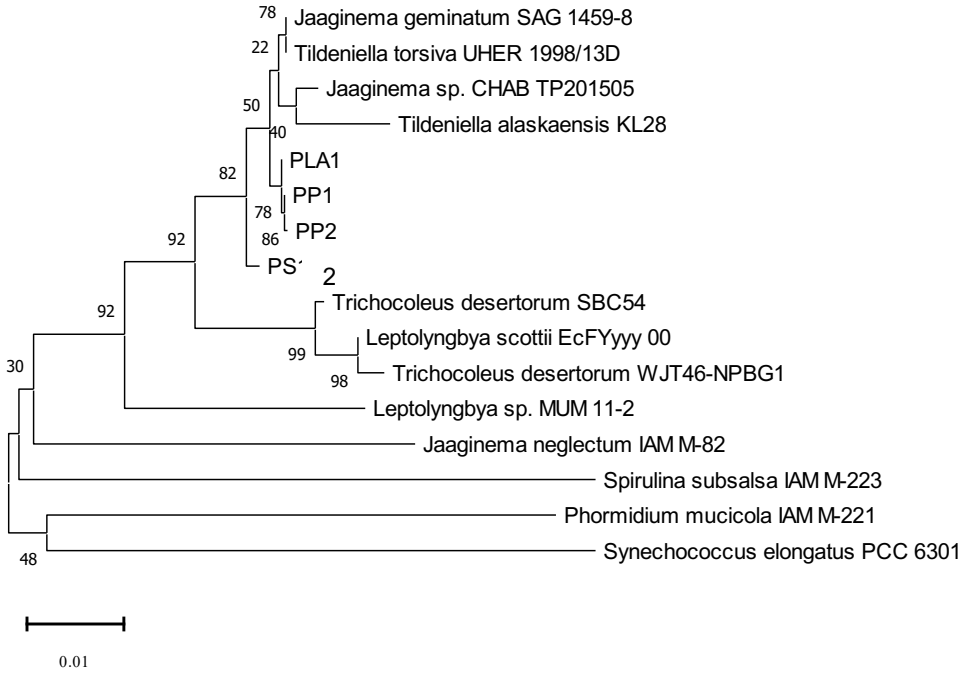

Figure 4.5. Phylogenetic reconstruction of the cultivated strains. Numbers represent the bootstrap values.

Discussion

Isolation of filamentous cyanobacteria

During the *in vitro* incubation of plastic microbeads with the water from Baltimore's Inner Harbor, there was a constant presence of cyanobacteria in the biofilms throughout the experiment. The SEM observations found the development of filamentous cyanobacteria on all the microbeads, and the 16S

rRNA gene community analysis also confirmed the presence of filamentous cyanobacteria on microplastic beads (Chapter 2). These findings suggest that filamentous cyanobacteria can play an important role on the formation of biofilm. To further characterize the cyanobacteria on the plastic surface, five strains of filamentous cyanobacteria were isolated in this study. The full length of 16S rRNA gene of these five strains were sequenced, and their colony and cell morphology were examined under the light and epifluorescent microscope. The phylogenetic analysis shows that these filamentous cyanobacteria are closely related to *Nodosilinea, Jaaginema, Tildeniella, Phormidium* and *Leptolyngbya*. The purification of cyanobacterial species attaching to plastic particles will allow for future characterization of these strains, including genome sequencing and screening for toxicity. Their isolation could also allow for interaction assays between these filamentous species and different organisms, such as heterotrophic bacteria or eukaryotic microbes like diatoms.

The isolated strains were obtained from polymer samples, two from PP, two from PLA and one from PS. Interestingly, there was no cyanobacterial growth originating from the water control sample. This could be potentially explained by a much lower abundance of filamentous cyanobacteria in the surrounding water compared to plastic biofilm. An earlier study showed a similar result, in which abundant filamentous cyanobacteria were found on plastics but not in the surrounding water (Zettler et al., 2013). The isolation of filamentous cyanobacteria from the plastic bead samples further confirms the presence of

filamentous cyanobacteria observed by SEM. The samples for the cultivation work and the TEM observations were collected at the same timepoint.

Identification of isolated cyanobacteria

Strain PLA-2 is taxonomically close to the species *Nodosilinea signiensis* (query cover of 71%, percent identity of 87%) (Table 4.1), and although the sequence quality of this strain was not enough for certain identification, further purification and future resequencing of PLA-1 could complete this task. The cell morphology matches that of *Nodosilinea* species, which usually present thin sheaths covering a single trichome (Komárek & Johansen, 2015). *Nodosilinea* is a genus from the Synechoccales family as well and can sometimes be morphologically similar and easily confused with *Leptolyngya*, and its name comes from the fact that it has the unique ability to form nodules in its trichomes, especially when exposed to low-light conditions for an extended period of time (Li & Brand, 2007; Radzi et al., 2019). *Nodosilinea signiesis* is a species that has been described as being genetically distinct from other species in the *Nodosilinea* genus and was first isolated from terrestrial mats in Anarctica (Radzi et al., 2019). Additionally, it has been reported that the genus *Nodosilinea* is capable of nitrogen fixation and has been found to attach to rocky surfaces like walls and stone monuments (Radzi et al., 2019). The strain PLA-2 had the top hit as *Nodosilinea* but the next few results on BLAST were closely related to the genus *Phormidium*. The filamentous freshwater cyanobacteria *Phormidium foveolarum* has been proven to degrade hydrocarbons (X. M. Deng et al., 1982) and other hydrocarbon-degrading

members of the genus *Phormidium* have been theorized to be hydrolyzing plastic collected from the north Atlantic (Zettler et al., 2013) and Polyethylene Terphalate (PET) bottles incubated in the North Sea (Oberbeckmann et al., 2014).

Strains PS-2 and PP-2 had an over 99% identity with the same sequence corresponding to *Jaaginema germinatum* SAG 1459-8 16S ribosomal RNA gene on the NCBI database. *Jaaginema* is a genus of cyanobacteria that belongs to the Synechoccocales family and it is known for being a filamentous benthic genus (Mai et al., 2018). According to AlgaeBase (National University of Ireland), the species *Jaaginema geminatum* was previously known as *Oscillatoria geminate* (Guiry, 2021) but has since then been reclassified by taxonomy. The cell morphology of *Jaagineema* usually presents as rounded cells, sometimes conical, and usually without sheaths but occasionally presenting very thin mucilaginous sheaths (Komárek & Johansen, 2015), matching the morphology of the observed cells for both strains (Fig. 4.4).

The sequence of *Tildeniella torsiva* that strains PP-1 and PLA-1 have proximity with is one isolated from a limestone wall in a park in Slovakia (Mai et al., 2018) (Fig. 4.5). These filamentous species could be highly adapted to living sessile lives attached to a wide range of surfaces, including those naturally occurring and even synthetic plastics. The morphology of *Tildeniella* usually presents as bright blue green filaments that are often entangled and form irregular clumps on solid media, as well as a thin colorless sheath and rounded cell ends (Mai et al., 2018).

It is important to note that in the generation of the microbial communities from the *in vitro* incubation described in Chapter 2, there was evidence of filamentous cyanobacteria being abundant in plastic particles, especially in the PLA microbeads. The most abundant cyanobacterial genera described in this community analysis were *Pseudanabaena* and *Nodosilinea* but *Leptolyngbya* and *Oscillatoria* were also present. Cultivation and molecular methods produced consistent results in which, these genera were the most abundant in the DNA sequencing and were also able to be sequenced, suggesting that the cultivation recovery of the filamentous cyanobacteria of the plastisphere is easier than that of the heterotrophic bacterial members.

Interestingly, after the cyanobacterial strains were transferred to the liquid media the cultures formed clusters and aggregated into visible filamentous granules. Similar formations have been previously described for filamentous cyanobacteria as a strategy for survival in unfavorable conditions and have been found to rapidly sink and settle in their environments (Milferstedt et al., 2017). These aggregates could potentially interact with microplastic particles that are floating on the water's surface and cause them to sink into the water column and even settle onto sediments. In previous studies, *Microcystis* cyanobacteria incubated with plastic particles (PE) formed aggregates when enriched with calcium (Ca2+) and promoted the sinking of a portion of the microplastics (Leiser et al., 2021).

Potential impacts of microplastic cyanobacteria on biogeochemical cycle

The isolation work, community analysis and SEM observations all seem to suggest that filamentous cyanobacteria can quickly colonize on microplastics. Despite the first settlers of the plastisphere being bacteria, fungi, and microalgae, photosynthetic organisms like cyanobacteria and diatoms are important for the biofilm formation and metabolism and play a role in the colonization process (Bryant et al., 2016; Yokota et al., 2017; Zettler et al., 2013). While heterotrophic bacteria seem to dominate the plastisphere, cyanobacteria are consistently identified as present, so it has been theorized that primary producers play an important role in the formation of plastic-associated biofilms (Yokota et al., 2017). The presence of highly buoyant microorganism, such as cyanobacteria that contain gas vacuoles for floating purposes, can temporarily increase the buoyancy of plastic particles (Barnes et al., 2009). On the other hand, cyanobacterial strains that do not contain vacuoles can create aggregations and clumps and contribute to the sinking and settling of microplastic particles that otherwise would have been buoyant, and this sinking can help cyanobacteria to survive otherwise fatal environmental conditions in the surface waters (Lee, 2008). This sinking of microplastic beads covered in biofilms and aggregating with other organic matter can increase the amount of carbon that is sinking to the sea floor (Cole et al., 2016; Kvale et al., 2020). Understanding the role that primary producers like cyanobacteria and diatoms play in the complex biofilms that form on microplastics will be an important step in understanding the impact of microplastic pollution on the

nutrient cycles of an ecosystem. Formation of filamentous cyanobacteria and diatoms on microplastics could potentially increase the amount of chlorophyll *a* and carbon fixation in an ecosystem, as well as increase nitrogen fixation (Zettler et al., 2013).

Potential harmful impacts of cyanobacteria

While none of the specific identified cyanobacterial species have been classified as forming harmful blooms, other species of *Jaaginema* (sp. TAU-MAC 0210) have been found to have antibacterial properties against *Staphylococcus* and have some cytotoxic effects on HuH-7 cells in cultures (Gkelis et al., 2019). Additionally, filamentous genera *Leptolyngbya*, *Oscillatoria* and *Phormidium* have been found to be toxic and cause mortality in brine shrimp in the Portuguese coast (Frazão et al., 2010). The genus *Leptolynbya* is considered to be highly toxic to organisms, as it produces proteins known as saxitoxins, which can block sodium channels in neural cells (Codd et al., 2005; Wiegand & Pflugmacher, 2005). Some large blooms of *Leptolynbya* have been reported in marine environments in Hawaii (Smith et al., 2008). On the other hand, diatoms like *Nitzchia* can form toxic blooms and be harmful to many species of fish and invertebrates by producing toxins (Parrish et al., 1991). Because of the durable nature of synthetic plastics, their colonization by filamentous cyanobacteria can potentially lead to their transportation and subsistence through the waterways and cause the introduction of harmful invasive species. It is possible that the presence of filamentous cyanobacteria on polymers can even lead to harmful blooms that

can affect the organisms that exist in the ecosystem and affect the communities that rely on it.

Conclusion

In this study, five filamentous cyanobacteria were grown from three different microplastics. They are related to *Nodosilinea*, *Jaaginema*, *Leptolyngba*, *Phormidium, Pseudanabaena* and *Tildeniella*, based on the phylogenetic analysis of full 16S rRNA gene sequences. A close examination of partial 16S rRNA gene sequences of cyanobacteria in the *in vitro* microbial community analysis (Chapter 2) suggests that isolated cyanobacteria and cyanobacteria in the *in vitro* community all belong to filamentous cyanobacteria. They are affiliated with genera *Nodosilinea*, *Leptolyngba*, *Phormidium and Pseudanabaena*. These results further support the SEM observations of filamentous cyanobacteria colonized on the surface of microplastics. Availability of these cyanobacterial cultures allowed us to characterize their colony and cell morphology, obtain their full 16S rRNA gene sequences, and construct the phylogenetic analysis. In the future, these cultures will enable to conduct further biological and physiological study after further purification of the strains. For example, genome sequencing, toxicity test, salt tolerance test, etc., all benefit from the availability of cultured cyanobacteria. The impacts of cyanobacterial biofilm on microplastics on the ecosystem are multifaceted. They can affect biogeochemical cycling by increasing carbon and nitrogen fixation in surface water and sinking more carbon to the deep ocean. These cyanobacterial strains can also be toxic and be transported throughout bodies

of water, as well as up and down the water column. We also do not know how these estuarine filamentous cyanobacteria adapt to the higher salinity water when they are transported to the offshore water.

Chapter 5. Conclusions and future directions

This dissertation focuses on the biofilm formation on three different types of microplastic when introduced to Baltimore's Inner Harbor. Two main types of studies were performed: *in vitro* incubation in the lab and *in situ* incubation at a sampling site. Three types of microplastic were chosen based on their chemical and physical properties: polypropylene as one of the most common plastics in all aquatic environments, polystyrene as a surface with a large number of pores and ridges, and polylactic acid as a "biodegradable" plastic made out of plant material. After an initial run of incubation experiments, it was decided that a second set of *in vitro* and *in situ* experiments were to be performed with an inert non-plastic control added. Samples were taken at different timepoints and DNA was extracted for community analysis using 16S rRNA gene amplification and the QIIME 2 bioinformatic pipeline. Biofilms were also visualized with scanning electron microscopy and photographs of interesting organisms attached to the surface of the plastic beads were captured. After 10 months of incubation and enrichment of the cultures, biofilms were detached by shaking with a vortex and inoculated onto three different types of solid culture media. Bacterial strains that were incubated on the plates picked based on morphology and were isolated for characterization. A similar protocol was used for the cultivation of cyanobacterial strains from microplastic particles after finding a high abundance of filamentous cyanobacteria on the SEM images and the microbial community analysis from the incubation experiments.

Our main findings

Free-living and particle-associated communities are different

The described microbial communities attached to particles are significantly different to those in the surrounding water in all experiments, supporting the first hypothesis described in Chapter 1 which states that the polymer-associated communities would show dissimilarity with those from the free-living communities of the water. The type of plastic was found to be less of a differentiating factor, as there was no difference between plastic types or glass in the *in situ* study. In the *in vitro* study, however, there was dissimilarity between the plastic type PLA and PS, as well as dissimilarity between glass communities and all other communities. This finding supports the second hypothesis put forth by this dissertation which states that polymer type affects the composition of the plastisphere and leads to speculation about the extent in which chemical composition can affect colonization in *in vitro* incubations. The effect of chemical composition of polymers could potentially have very important policy implications and could affect the way in which different types of waste are managed in the future.

Contradicting findings *in situ* and *in vitro*

The fact that polymer type did not prove to be a big determining factor in the *in situ* study but showed dissimilarity in the *in vitro* study can be explained by the variations in conditions in both experiments: the environmental conditions in the *in situ* experiment were not controlled and were fluctuating with local weather and water quality. In the laboratory incubation, however, the conditions were stable throughout the incubation time, with the light incidence being

uniform during the daytime and the temperature being controlled inside the lab. Environmental conditions like temperature, dissolved oxygen and salinity were fluctuating throughout the incubation times. The water conditions in the *in vitro* study remained controlled but the water community was limited to what was collected on the initial sampling date. Our third hypothesis states that the communities attached to glass particles were to be different than those in synthetic polymers, and the findings in these studies contradicted each other as there was dissimilarity in the glass control in the *in vitro* incubation but no statistical difference in the colonization of glass in the *in situ* incubation. These results could be explained by the fact that the microbeads of all different polymers were allowed to float or sink according to their properties in the *in vitro* experiments, where in the *in situ* incubation they were all suspended at the same depth and exposed to the same lighting and nutrient conditions. The pooled analysis in chapter 3 that includes all of the samples from 2020 confirms that material type does not dictate community composition as no particle samples were statistically dissimilar.

The colonization of synthetic polymers being significantly different to the colonization of naturally-occurring materials has presented conflicting results in a variety of studies in the last decade. More research and the standardization of protocols could help elucidate this issue in future work.

The plastisphere and the seasons

The experiments in this study were all performed in different seasons: the first *in vitro* incubation was performed in Spring of 2019, the fist *in situ* incubation

was performer in the Summer of 2019 and the second incubations for both experiments were run in the Fall of 2020.Communities across samples and experiments showed different compositions at the phylum level, which supports the idea that the seasonal changes in the water's bacterial communities can significantly alter the composition of the plastisphere that attaches to synthetic polymers and potentially to other non-plastic particles of the same size, like glass or wood. An interesting phenomenon we witnessed in these experiments was that of succession in the colonization of the major bacterial groups. Proteobacteria was the dominating phyla across all samples in all experiments but the levels of abundance fluctuated even within the same material in the same experiment, with a difference of only a few days. The abundance of Proteobacteria generally decreased after the first 7 days of colonization and in some cases the decrease was of considerable size. Proteobacteria, however, remained the most dominant group through the incubations.

Planctomycetes and Desulfobacterota in low-oxygen environments

The abundance of Planctomycetes showed a considerable increase in the biofilm samples of both the *in situ* incubation and the first *in vitro* incubation. Particularly in the *in situ* incubation samples, there was a noticeable increase in the abundance of the Planctomycetes phylum in all of the biofilm samples that coincided with a decrease in dissolved oxygen in the water of the sampling site. Planctomycetes generally thrive in an attached lifestyle, especially in low oxygen conditions, as they are able to oxidize ammonia to dinitrogen without oxygen. Although some species of Planctomycetes are commonly found in

sediments, they are also able to have a free-living lifestyle and thrive in low-oxygen water conditions. Another interesting finding was that the abundance of the Desulfobacterota phylum increased on day 21 of the second *in situ* incubation (Fall, 2020) and dissolved oxygen in the Inner Harbor was nearly depleted at this time. Desulfobacteria have been found to thrive in low and no oxygen environments and to form biofilms on polymer surfaces.

Cyanobacteria in the plastisphere

During the first iteration of the *in vitro* incubation experiment, there was a sharp increase in Cyanobacteria species in all polymer samples but particularly in those from PLA. This striking growth of Cyanobacteria was also able to be seen using SEM and captured in micrographs. This increase in abundance in the Cyanobacteria phylum was not consistent in the second *in vitro* experiment. This is surprising but suggests that the initial community in the collected water on the day of the incubation start and its environmental condition can strongly influence the composition of the colonizing plastisphere.

In chapter 4, five strains of filamentous Cyanobacteria were grown and characterized in terms of their morphology. The results in this chapter suggest that further cultivation work and purification may be necessary for the characterization of one or more of the strains. They were visualized under epifluorescence microscopy and small differences in their morphology were observed. A phylogenetic reconstruction revealed that these five strains are closely related to the genera *Jaaginema*, *Nodosilinea*, *Tildeniella* and *Phormidium*. The cyanobacterial isolations were done after 10 months of

incubating the microbeads in estuarine water and it was evident that after this time, filamentous cyanobacteria dominated the biofilms and picocyanobacterial were potentially outcompeted by filamentous species after long incubation. This supports the fourth hypothesis stated in Chapter 1, which asserts that the cyanobacteria on the plastisphere are dominated by filamentous species. The colonization of filamentous cyanobacteria on polymer samples can lead to the formation of aggregates and clusters that include the biofilm and the polymer particles. These aggregates can increase the primary production of surface water and when they sink, they can increase the rate of carbon sinking onto the ocean floor. Additionally, filamentous cyanobacteria can form mats and bloom, as well as produce toxins that can be harmful to humans and other animals. Many of the microbeads in these experiments were covered in massive filamentous cyanobacterial growth, which could contribute to changes in their buoyancy and their transportation into other bodies of water. Some of these cyanobacterial species may be toxic and may form invasive blooms in ecosystems that they otherwise would not have been able to reach.

Limitations and future directions

This study is limited by the choice of the three types of plastics: there's many other types of plastics that could be included in future studies, as well as mixed polymers and polymers that contain additives in their formulations. All of these chemical characteristics could alter the biofilm's microbial composition. The glass control provides a good reference to inert materials, but a natural material such as wood or cellulose could add a layer of complexity for a non-plastic

perspective and include naturally-occurring particles that are present in large amounts in the waterways. Another limitation of this study is the lack of analysis of the day 0 water community in both *in vitro* and *in situ* incubation as it could have answered some questions related to the difference in colonization in all experiments, such as the difference in Cyanobacteria abundance between both *in vitro* incubations. Future studies should include an initial sampling point in which the composition of the community in the sampling site is described to investigate how the initial community can impact the colonization and how the different seasons of these experiments could have affected the community composition throughout the incubations. Additionally, it would be advantageous to look at the initial parts of the biofilm formation and consider what the conditioning layer looks like and what type of macromolecules are attaching to the surfaces of the plastics to allow for microbial colonization.

A consideration in further experiments is the timepoints in which community analysis and bacterial isolation are done. In this study, there was a 9 month difference between the incubation and the traditional culture methods of identification, as well as the observation of the surfaces using SEM. In the future, all methods should be employed simultaneously to obtain corresponding datasets that can be compared with each other in a better manner. In the visualization of the SEM surface, there was a lot less microbial cells visible than generally expected, and this could be due to the manner of sample preparation that could have caused stripping of some of the biofilm from the microbeads. Future work should be done in an imaging laboratory that is specialized for

environmental biofilm visualization. There should also be some image capturing of the virgin materials prior to any incubation to look at the features of the surface and compare them to the colonized and prepared samples.

In a future iteration of these incubation experiments, it would be beneficial to measure nutrient levels in the water to understand how they may be shifting with the communities' shifts. The results of these experiments lead me to believe that the best perspective of bacterial colonization on plastic particles can be obtained through *in situ* incubations in which there is a more natural exposure of these plastics to the ecosystems fluctuating conditions. Close monitoring of the water's environmental conditions can provide detailed relationships between measurements (nutrient concentrations, temperature, dissolved oxygen, etc.) and shifts in the community. Additionally, transcriptomic analysis could help understand the metabolism of main microbial players in the community and look into their primary production, nitrogen fixation and their responses to hypoxic and anoxic conditions. The potential release of organic matter from the biofilms could be measured to elucidate the contribution of each polymer's plastisphere to the nutrient levels in the water.

I suggest that any further cultivation experiments from similar biofilms are done in low-nutrient media, such as R2A, as it yielded the highest culture recovery in this study. The eukaryotic strain that was isolated in this experiment and classified as *Auxenochlorella* would also provide an interesting insight into the eukaryotic microalgae on the biofilm community. This eukaryotic strain behaved like bacteria in all respects when being traditionally cultured and

presented rapid growth in solid R2A medium, demonstrating potential for biotechnological applications such as biofuel production. Further research into its characteristics and functions could prove useful for technology development. *Auxenochlorella* has been found to grow efficiently in media with glucose as a carbon source and to produce high concentrations of lipids in these conditions. These lipids have a profile that closely resembles the vegetable oils often used as biofuel (Patel et al., 2018). This rapidly growing strain could provide an efficiently-growing organism that can produce a large amount of biofuel and thrive in a scaled up industrial cultivation environment.

Other interesting isolated species, such as those from the *Sphingomonas* and *Sphingopyxys* genera, could be analyzed with genome sequencing and important genes could be searched for, including genes for motility, biofilm formation, antibiotic resistance, pathogenesis, plastic degradation, etcetera.

The strains of cyanobacteria isolated could also be further described by genomic analysis and growth assays. Their ability to form biofilms as well as the manner in which they interact with other members of the plastisphere could bring light to the issue of aggregation of cyanobacterial mats and plastic particles, their further sinking and the impact this may have in the carbon cycle. Closely related species of cyanobacteria that have been previously described could serve as a reference and their genomes could be searched for interesting ecological and taxonomic features, such as biofilm formation and responses in stressful conditions. The potential for some of these cyanobacterial strains to be toxic and/or harmful bloom forming should also be considered in this

genomic survey as members of the *Leptolyngbya*, *Phormidium* and *Jaaginema* genera have been found to produce cytotoxins that can be harmful to organisms. Another interesting experiment would be the measurement of the biomass forming on the microbeads and assess the surface area of each bead to estimate the thickness and coverage of the cyanobacterial mats. Another consideration when it comes to these thick biomass mats is their potential contribution to the fixing and sinking of carbon in aquatic systems. The estimation of the biomass could be a good way to estimate how much carbon will be fixed by the biofilm, but because the biofilm is composed of more than primary producers, there could be an experimental design that could better measure the primary production the plastisphere of these microbeads forming biomass aggregates. The addition of radiolabeled C^{14} carbon could measure the assimilation of inorganic carbon by the microorganisms of the plastisphere and thus estimate primary production. An experiment to measure the sinking of microbeads clustering with biomass could be designed to look at the sinking rates in a laboratory setting utilizing a transparent column and estuarine water. These combined experiments could shed light into the carbon fixation and sinking potential of the estuarine plastisphere.

This study was able to investigate the biofilms formed on microplastic particles in one of the Chesapeake Bay's harbors located in downtown Baltimore. The results of these experiments provide a good starting point in which new questions can be developed for future investigations. It was clear that in these incubations, the free-living water communities were significantly different to

those that attach to the surface of the microbeads and form complex biofilms. It was also clear that the seasonality of the water communities played a bigger role in the composition of the biofilms than the material of which each microbead is composed, including the non-plastic control. A successive colonization of these particles was visible and a broad understanding of the main phyla that exists in these biofilms was able to be achieved. Additionally, it was found that the alpha diversity of the *in situ* experiments was higher than that of the *in vitro* experiments in the second incubation, possibly indicating that constant water flow can contribute to a higher diversity. The alpha diversity of all glass samples from all experiments was the lowest out of all the surface types, suggesting that an inert surface can generate biofilms with less diversity than synthetic polymers. There were, however, some questions left unanswered. Some of these questions include what impact these biofilms may have in the nutrient cycling of the body of water and how this may impact other organisms in these ecosystems. Another unanswered question is how naturally occurring organic particles, such as wood or cellulose, could be colonized in similar incubations and if these biofilms would be significantly different to those from microplastics. This is important because these particles are commonly found in all aquatic systems but tend to be degraded by physical and biological factors in a much faster manner than synthetic polymers are. Another unanswered question from this study is how the plastic type affects the attachment of microbes and if it's possible that the polymers may be leaching their monomers or other potentially harmful chemicals, such as phthalates or

bisphenol A and if these chemicals could potentially be affecting the colonization profiles by promoting the growth of certain microorganisms over others.

Future studies into the plastisphere of the Chesapeake Bay should include multiple sampling sites in different parts of the Bay with distinct environmental conditions and distinct local microbial communities, as well as sampling timepoints that include changes in seasonality. The environmental gradients and sampling throughout different seasons can provide an interesting perspective into the colonization of plastic polymers and how they may be interacting with the other organisms native to the Chesapeake Bay estuary to understand their true impacts in the ecosystem.

Appendices

Appendix Chapter 2

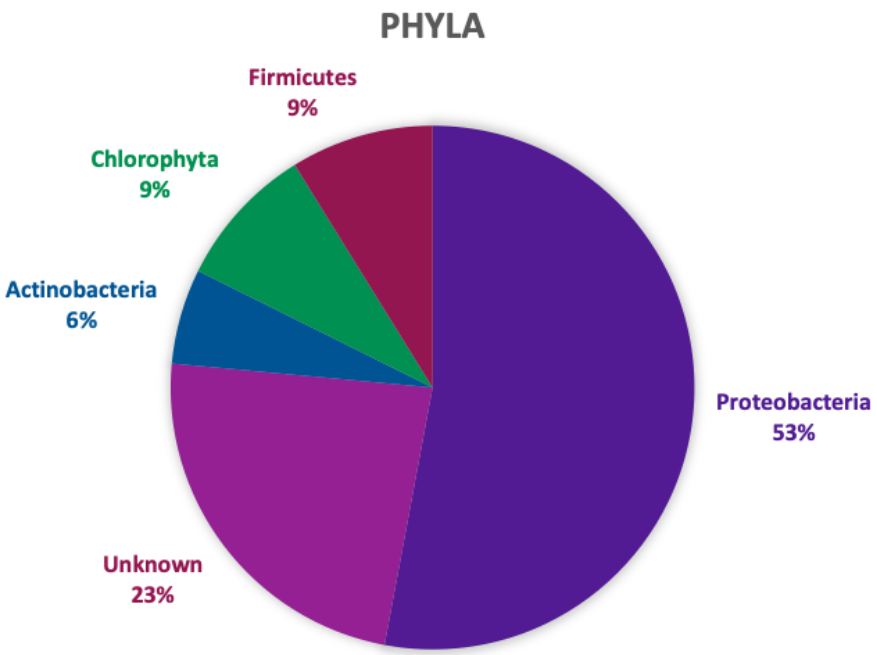

Figure A.2.1. Taxonomic breakdown of the 34 isolated strains in the first *in vitro* incubation experiment by phyla.

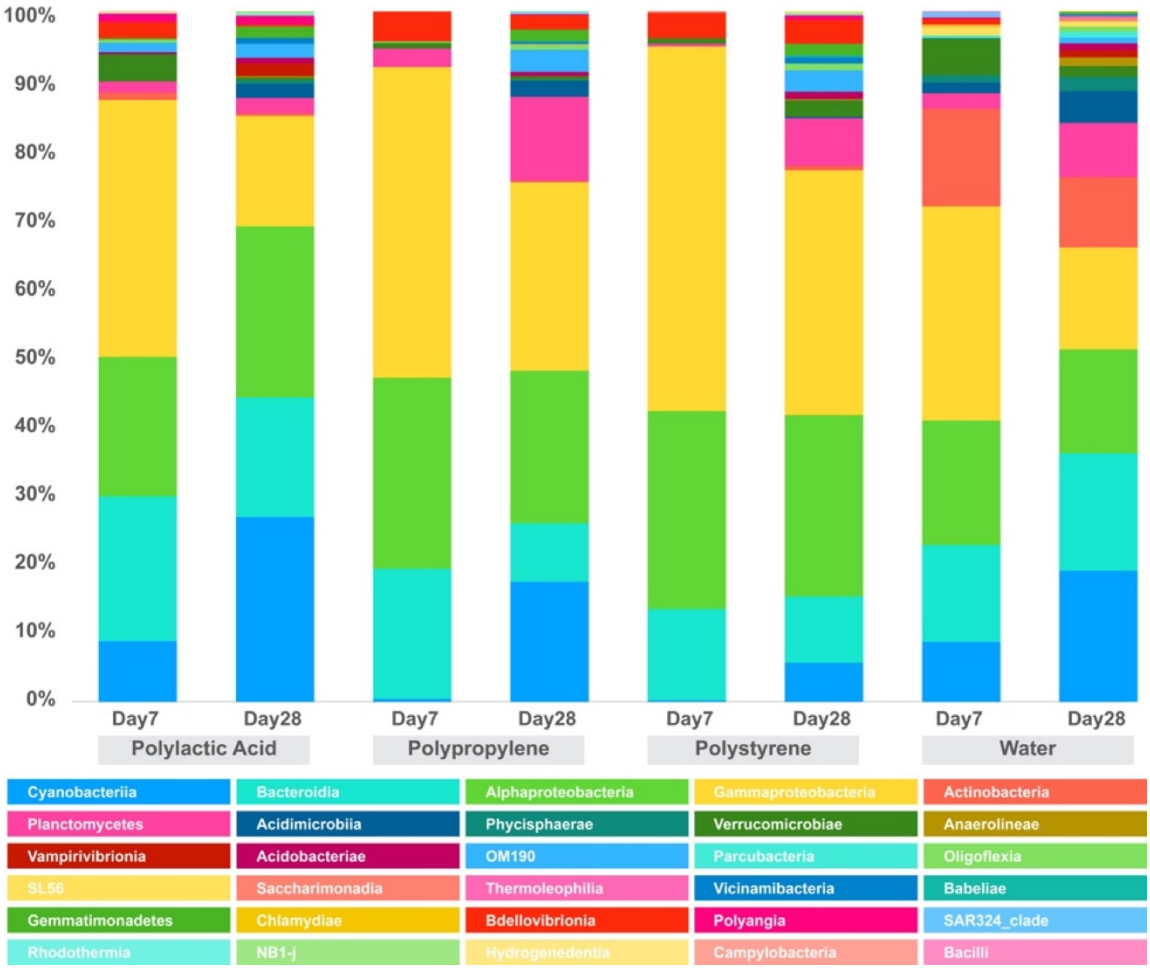

Figure A.2.2. Relative abundance of major microbial classes on day 7 and 28 during the first *in vitro* incubation.

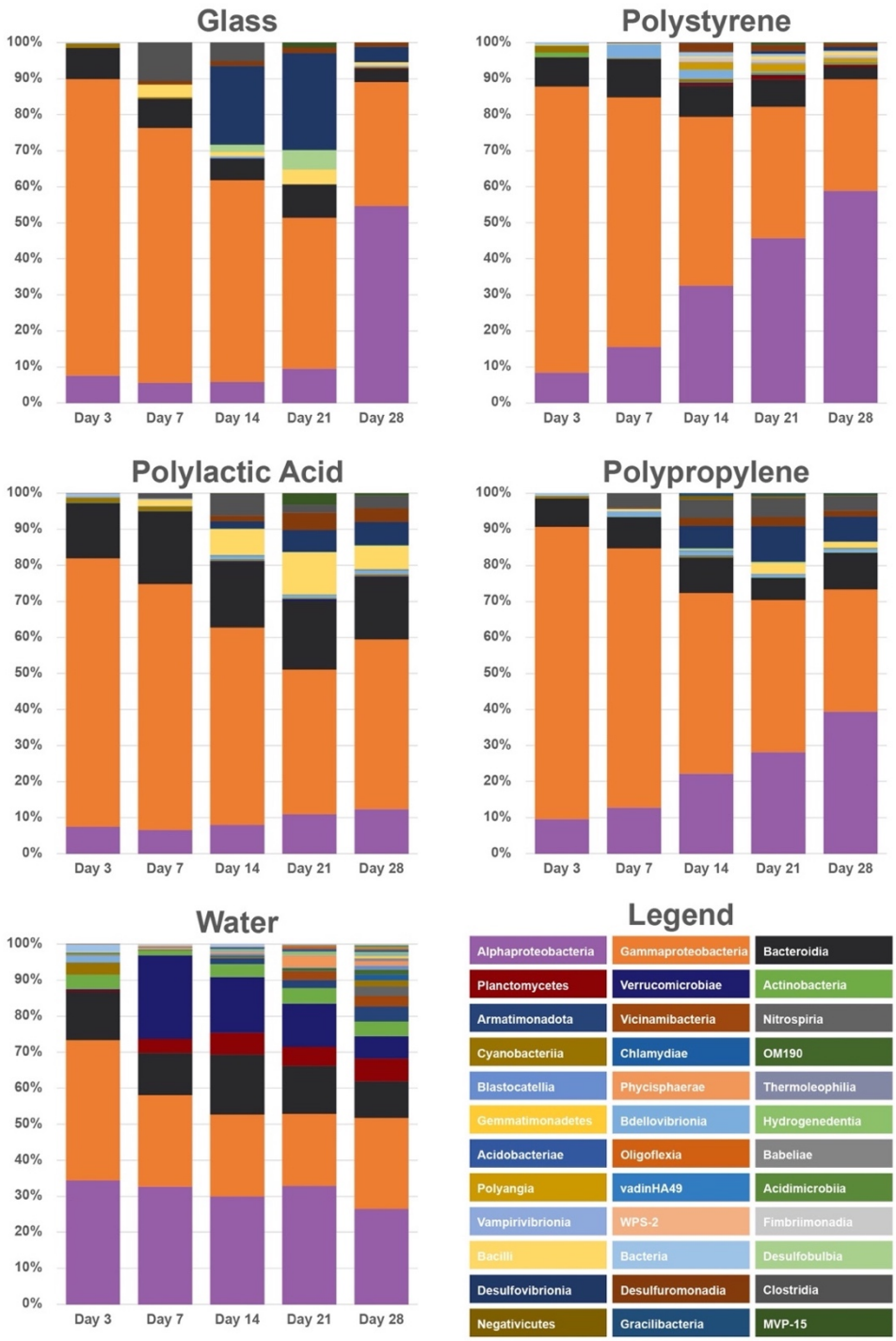

Figure A.2.3. Relative abundance of major bacterial classes in the second *in vitro* incubation in timepoints of 3, 7, 14, 21 and 28 days of each polymer, glass control and the surrounding water sample,. The class labeled “Bacteria” represents unassigned bacteria.

Appendix Chapter 3

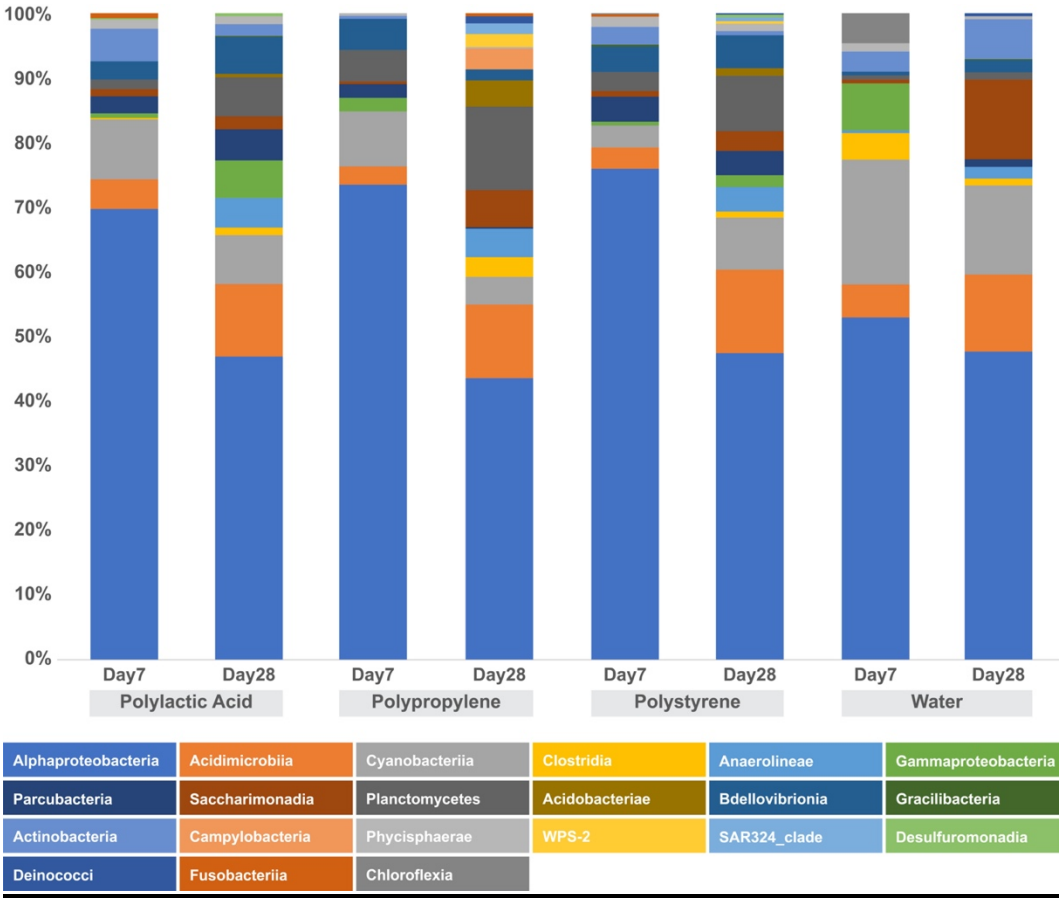

Figure A.3.1. Relative abundance of major microbial classes on day 7 and 28 during the first *in situ* incubation.

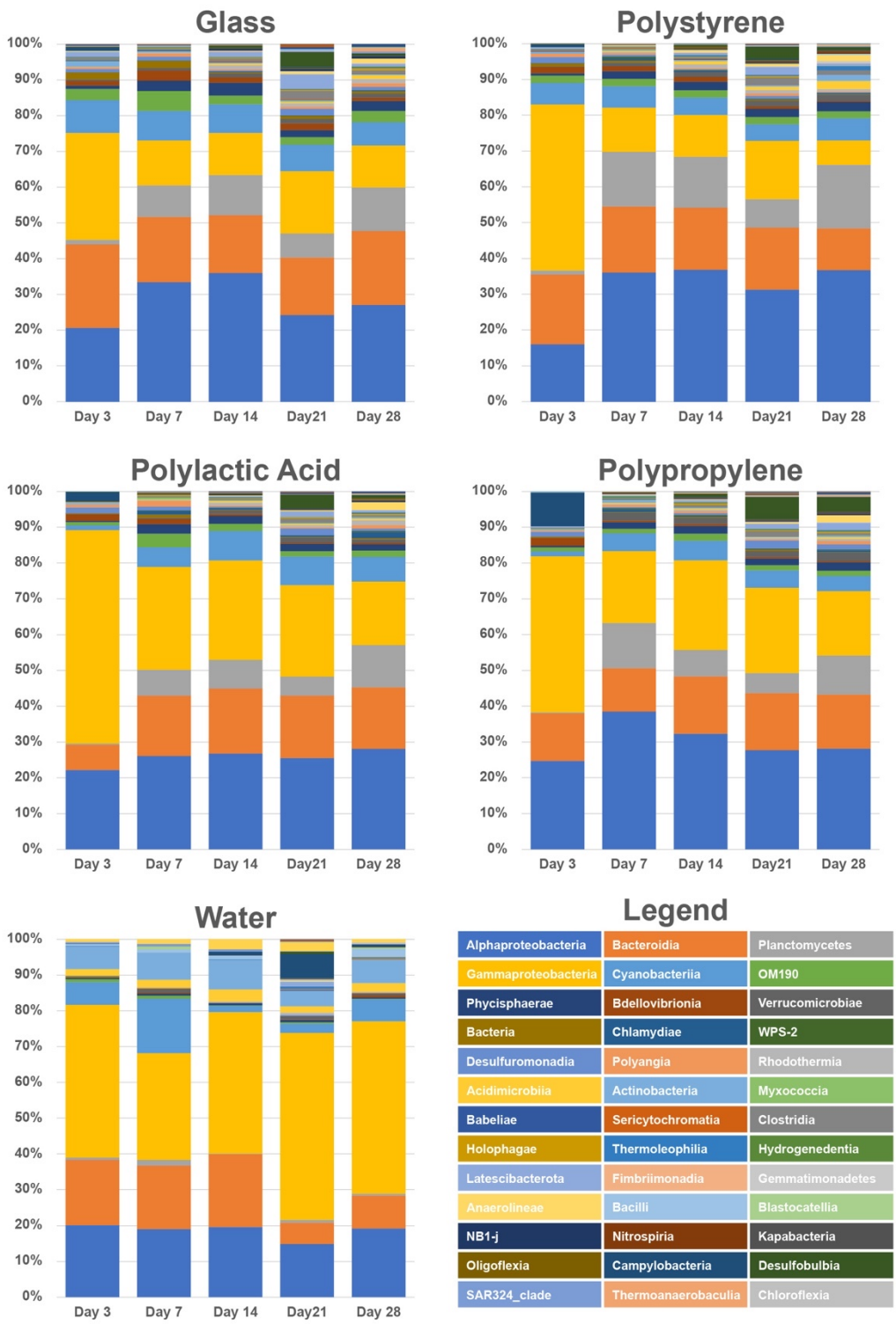

Figure A.3.2. Relative abundance of major bacterial and archaeal lineages at the class level in the second *in situ* incubation. The relative abundance was analyzed based on the 16S ribosomal RNA gene sequences obtained on day 3, 7, 14, 21 and 28.

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