Plastisphere of marine environment -a meta-analysis

Msc. Civil Engineering and Management

Bozhen Chen (Student ID: 2490325)

Supervisor: Dr. Umer Zeeshan Ijaz

Co-supervisor: Ciara Keating

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Abstract

The main research direction of this project is the impact of microplastics on the ocean on the environment and the distribution and composition of microbial communities attached to the surface of microplastics. It mainly studies whether plastic material, water temperature, salinity, ph and sea area will affect the distribution and composition of microbial communities. This project used second-hand data collection and used DADA2 to preprocess the data, and then used R studio to perform image analysis on the data. Finally, it was found that the impact of microplastics on the marine environment and ecological environment is serious. Plastic material, water temperature, salinity, pH and the composition and distribution of the microbial community in the sea all have a certain impact.

1.0 Introduction

1.1 Background

With the development of chemical industry, the global total industrial output of plastic products has increased year by year due to the demand of light, durable, cheap and easy to be made into disposable products. By 2018, the world's total production of plastics has reached 360 million tons, and Europe's plastic production has reached the peak value of 62 million tons (Plastics Europe, 2018). Plastic materials have been widely used in a variety of applications in the 1970s. Due to the imperfection of waste plastics treatment schemes in the world, the plastic wastes produced are transported by wind, transported by rivers and discharged by sewage treatment plants into the marine environment, which leads to a more serious situation of plastic waste pollution in the marine environment. According to statistics, the total amount of plastic waste floating in the marine environment is as high as 250000 million tons (Eriksen, et al, 2013), which makes people realize that plastic garbage in the ocean is already a serious environmental problem.

In 1972, when a large number of tiny plastic particles were found on the surface of the Sargasso Sea, the world first realized that microplastics existed in the marine environment (Carpenter & Smith, 1972). According to Goldberg's statistics, the total amount of marine plastic waste produced worldwide in 1975 was only about 6.4 million tons (Goldberg, 1975). After entering the 21st century, in 2010, the total amount of plastic waste entering the ocean reached 12.7 million tons (Jambeck et al, 2015). According to the current production rate of marine plastic waste, the amount of plastic waste entering the ocean will reach 32 million tons by 2050 (Neufeld et al, 2016). In the marine environment, the effects of marine plastic waste pollution on ocean and coastal ecosystem have been widely studied (Eriksen et al, 2014). These include ingestion by marine organisms as bait, entanglement of marine organisms, suffocation

and death of entangled organisms, release of plastic additives and adsorption of persistent organic pollutants in the environment. As a floating substrate in the marine environment, it can spread invasive species or pathogenic microorganisms, which will cause serious harm to the marine ecosystem (Oberbeckmann, et al, 2014). In the current global environmental problems, in addition to the familiar global climate change, ozone depletion and ocean acidification, marine plastic pollution is also included (Galloway & Lewis, 2016). At the same time, due to the stable chemical properties of plastics and degradation time as long as hundreds of years, the plastic waste entering the marine environment did not degrade because of the passage of time. Under the action of physical, chemical and biological processes in the natural environment, large pieces of plastic waste gradually become old and form more and more microplastic waste fragments disperse into the world ocean with the ocean current movement every corner.

1.2 Microplastics

Microplastics is a kind of high molecular compound, which has strong hydrophobicity and anti-biodegradation ability. It has variable density, various colors and different shapes. In 2004, British scientist Thompson put forward the concept of microplastics in science magazine (Thompson, 2004), so that microplastics came into people's view. According to the EU Maritime Strategic Framework Directive, the size of large-scale plastics is larger than 25mm, the size of medium-sized plastics is between 5mm and 25mm, and the size of micro plastics is less than 5mm. When the size of plastic fragments is as small as 1nm ~ 100nm, it is called nano micro plastics (Galgani et al, 2013). Microplastics generally refer to tiny plastic particles or fragments with radius less than 5mm, which are too small to be found by naked eyes. Compared with particulate matter 2.5 (PM2.5 refers to particulate matter with a diameter less than or equal to 2.5 microns in the bai atmosphere) in the ocean, the amount of microplastics in the ocean can reach 35400 tons (Rochman et al, 2014). Microplastics are ubiquitous in the environment, including toothpaste and facial scrub cream. About 280 million tons

of plastics are disposed of as waste every year. In the natural environment of fresh water, ocean and land, the accumulation of micro plastics has become increasingly prominent. Most plastic wastes will also be degraded by light sources, oxygen, and organisms, or be affected by physical effects such as waves to form broken small objects. And under long-term chemical action, it degrades into smaller fibers, fragments, films and small balls to form microplastics (Cózar, et al, 2014). At present, high concentrations of micro plastic waste have been found in the ocean, remote fresh water lakes, saltwater lakes and other waters, and even in the remote places such as the north and south poles. (Koelmans et al, 2014). From the origin of microplastics, it can be divided into two categories: consumer goods and industrial raw materials production debris. According to the erosion level, the microplastics can be divided into novel, UN weathered and initial transformed ones. According to their composition, they are polyethylene microplastics (PE), polystyrene (PS) and polypropylene microplastics (PP) (Brunner, et al, 2015). The primary source of microplastics in the ocean is the input from land, and the discarded plastic products are dispersed into the ocean through the current. The wastes piled up near the coastline, factories and domestic sewage discharge will enter the water environment through the sewage treatment system. In the ocean, plankton feed on microplastics, which are wrapped in feces. Other organisms can ingest microplastics indirectly by eating fecal balls, and then enter the ocean (Arthur, et al, 2009).

1.3 The Harm of Microplastics

At present, microplastics mainly affect marine organisms, followed by freshwater organisms and soil organisms.

First of all, it causes physical harm to marine organisms. When the microplastics are ingested by marine organisms, it may inhibit the digestive tract function, and block the digestive tract of marine organisms, causing feeding disorders. This effect is universal and extensive (Xu, 2020). Secondly, microplastics will cause chemical hazards to

marine organisms. For example, plastic products will be produced by adding different chemical reagents according to different ways of use, such as stabilizers, plasticizers, plasticizers, foaming agents, colorants and lubricants (Koelmans, et al, 2013). In addition, when microplastics enter the ocean, they are easy to absorb the chemical pollutants in the water. When the microplastics are ingested by organisms, these toxic chemical additives will be released in the organisms, causing chemical toxicity to the feeding organisms (Punyauppa-path, et al, 2020). Finally, the floating plastic waste has more stable physical and chemical properties, and can be used as a continuous substrate to provide habitat for marine organisms, which may bring new organisms to the sea area and cause biological damage to the local marine environment (Horton, 2020). So far, statistics and forecasting of microplastic waste around the world is a difficult task. However, this new environmental problem has aroused the research of the majority of academic enthusiasts and scientists.

1.4 Plastisphere

According to the results of study, there is a significant difference between the microbial community attached to the surface of plastic waste floating in fresh water and that in sea water. In the study, the combined environment of micro plastics and attached microorganisms is named " Plastisphere" (Zettler, et al, 2013). At the same time, it is found that there are potential pathogens in the community of plastic attached organisms. When the ingestion of this type of microplastics by the feeding organisms, the risk of disease may occur.

The concept of Plasticsphere makes people more deeply realize the severity and complexity of the impact of marine plastic pollution on marine ecosystem. First of all, microorganisms inhabiting on the plastic will change the adsorption state of pollutants on the surface of plastics and affect the decomposition rate of plastics in seawater (Diaz,et al, 2013). In some biological communities, autotrophic bacteria produce oxygen during photosynthesis, which accelerates the oxidative decomposition of

microplastics. Some fungi have been proved to have the function of decomposing plastics (Bond, 2020). On the other hand, to assist foreign species and community spread and diffusion, micro plastic as attached microbial habitats, its adherent microbes can do it with the micro plastic in the ocean vertical and horizontal distance to travel, so may lead to the spread of invasive species and diffusion problem, Especially when the micro-plastic attached microorganisms contain microorganisms related to aquatic animal diseases, the impact on the marine ecological environment is more significant (Onda, et al, 2020).

1.5 Microplastics on Other Researcher

Since microplastics are common pollutants in marine sediments and seawater, the environmental hazards of microplastics have been questioned. Marine microplastics pollution has gradually become the focus of scientific research.

The latest study found that the amount of microplastics less than 5mm in the ocean is about 90% less than the predicted amount. This may be due to the fact that some microplastics have been decomposed or broken into smaller plastic particles by microorganisms. In addition, these microplastics have been swallowed by marine organisms or have settled into deep-sea sediments (Cordova, 2020). Due to the characteristics of small particles, large specific surface area and strong hydrophobicity, microplastics are easy to adsorb persistent organic pollutants (POPs) and heavy metals. In addition, some clay particles and organic debris may be accumulated on the surface of microplastics, and the proportion of microplastics will increase, which will eventually lead to the accumulation of microplastics in the deep sea (Cordova, 2020). Therefore, microplastics can carry POPs and other pollutants from the surface water to the seabed sediments, which increases the exposure risk of marine benthos to POPs (Mato, et al, 2001). The toxic additives contained in plastics, such as plasticizers, flame retardants, pigments and other additives, can be released into the sea water after entering the marine environment. A large amount of hexabromocyclododecane

(HBCDs) was detected in expanded polystyrene (EPS) fragments from Korea and the Coast of Asia Pacific. EPS fragments may be the source of HBCDs in marine environment and marine food web (Jang, et al, 2017). Therefore, microplastics in marine environment are not only the source of pollutants, but also the carrier of toxic substances transmission, which has obvious transport effect on the environmental migration of toxic substances (Batel, et al, 2018). The heterogeneity of microplastics in polymer type, particle size, shape and density lead to different ecological effects on marine environment. The unique characteristics of different types of microplastics affect their interaction with chemical pollutants (Naji, et al, 2017). For example, microplastics made from non-polar monomers such as polyethylene (PE), polypropylene (PP), and polystyrene (PS) adsorb POPs from the surrounding Marine environment and concentrate them at concentrations up to a million times that of the environment (Hirai, et al, 2011). Based on floating buoys and physical ocean models, it is found that plastic floating on the ocean surface can migrate from the east coast of the United States to the interior of the North Atlantic subtropical circulation in less than 60 days (Zettler, et al, 2013). Therefore, the pollutants carried by microplastics also migrate. Plastic particles in the marine environment provide carriers for the migration of seaweed, seaweed, microorganisms and even some invasive species. Aggressive alien invaders can drift into different ecological environments with these vectors, causing ecological disasters. Marine microplastics double the chance of biological species migrating to other latitudes (Barnes, 2002). Microplastics have increased the opportunity for migration and spread. For example, the Membranipora tuberculate found on plastic particles in New Zealand originated in Australia (Gregory, 2009).

With the continuous migration of microplastics, it may promote the rapid spread of pathogenic bacteria attached to its surface, leading to large-scale infections and even endangering human health. In addition, microplastics can also provide a stable habitat for plankton, allowing them to obtain rich nutrition, thereby attracting lower trophic fishery organisms to eat microplastics, and at the same time may aggravate the toxic

effects caused by ingestion. Relevant studies have found that benthic diatoms, dinoflagellate and harmful dinoflagellate are attached to microplastics on the northwest coast of the Mediterranean Sea (Masó, et al, 2003). Once these microplastics migrate to sea areas suitable for the growth of harmful dinoflagellates, they may rapidly multiply and diffuse, leading to the release of large amounts of toxins that affect the quality and safety of aquatic products, and ultimately cause potential harm to human health.

In the marine environment, microplastics are not only easily treated as plankton by predators, but also ingested by marine organisms into the food chain (Moore, 2008). Moreover, microplastics are also easily adsorbed on the surface of marine life, and enter the food chain as they are ingested. Gutow (Gutow, et al, 2016) found that the surface of fucales was easy to adsorb microplastic particles in laboratory experiments. The presence of microplastics was found in the stomach and intestines of conch after feeding on the algae polluted by microplastics.

As a new biological habitat in the marine environment, marine microplastics are significantly different from the surrounding environment. Zettler used high-throughput sequencing technology for the first time to study the microorganisms attached to Marine microplastics. By comparing and analyzing the microbial communities in polypropylene (PP), polyethylene (PE) and seawater samples, he found that the microbial communities on plastics were significantly different from those in the surrounding environment. Some types of microbes were found in polypropylene (PP) and polyethylene (PE) plastic materials, but not in environmental water samples. At the same time, more than 1000 OTUs (OTU refers to the general term of taxonomic units as objects in quantitative taxonomy. There are species, varieties and individuals, such as a specific genus, a specific family, and a specific order) were detected on each surface of the microplastics, and a high abundance of potential pathogenic bacteria *Vibrio* was found on the surface of some of the microplastics samples. Therefore, the researchers named the structure of microplastics and attached microbial communities as

"plastisphere" (Zettler, et al, 2013).

Microplastics are also the transmission media of many harmful microorganisms in the marine environment. The physical and chemical properties of plastic materials are more stable than the natural floating objects in the natural environment. According to Lyons's research results, compared with the free distribution of microorganisms in the water environment, when the microbial communities gather together, the biological metabolism efficiency in the polymerized microbial community will be significantly improved, and the abundance of culturable Vibrio and fecal indicator bacteria in the aggregation community will increase (Lyons et al, 2010). When conducting experiments on the aggregation of the animal infectious parasite Toxoplasma gondii, Shapiro discovered that the plastic debris floating in the ocean has a spreading effect on harmful microorganisms (Shapiro, et al, 2014). However, in different regions and environments, the abundance and species of pathogenic microorganisms attached to marine plastic waste are quite different. Goldstein found halofoliculina corallasia on the surface of plastic waste in the North Pacific Ocean. This ciliate is the pathogen causing coral bone erosion (Goldstein, et al, 2014). Additionally, found that a harmful dinoflagellate was present in the bacterial community attached to the microplastics while studying the marine microplastics in the Australian offshore waters (Reisser, et al, 2014).

Plastic is a kind of artificial polymer, which is mainly composed of hydrocarbon. It can be used as carbon source by heterotrophic microorganisms in the environment (Shah, 2008). After immersion in the bay for 6 months, the weight of bio attached olefin polymers is reduced, the surface cracking occurred, and the mechanical properties and chemical functional groups were changed (Sudhakar, et al, 2007). Artham and Doble research pointed out that microorganisms attached to plastics can not only obtain energy through degradation, but also use extracellular polymers secreted by other attached organisms as carbon sources (Artham & Doble, 2009). Finally, Microorganisms attached to microplastics also play an important role in the adsorption of persistent

organic pollutants (POPs), In an experiment to evaluate the adsorption of microplastics to environmental chemicals, Gouin found that microorganisms attached to microplastics can significantly reduce the absorption rate of POPs by microplastics (Gouin, et al, 2011). To sum up, The impact of microplastics on the environment is extensive and complex, and the microorganisms in microplastics are still not well understood. This project will research and explain plastic microorganisms in the next few chapters.

1.6 Aims and Objectives

The purpose of this project is to study the biological communities associated with microplastics in the Northern Corsica, North Sea, Adriatic Sea and coast of Italy. In order to systematically understand the biodiversity and function of microbial communities on the surface of micro plastics, this project will try to explore the essential factors of marine pollution and plastic pollution. Dada2 was used to preprocess the collected data, and then r studio was used to analyze the pretreatment data to study the microbial community and environmental impact of different plastic materials in different pH, salinity, temperature and marine environment.

2.0 Methods

All data processing models for this project were built with the assistance by Dr Umer Zeeshan Ijaz and Ciara Keating of Glasgow University, and provided relevant training to help me independently complete the relevant data processing and analysis in the later stage.

2.1 Data Collection

The main research direction of the project is to process and analyze the metadata of microorganisms adsorbed on microplastics, so all the data of the project will be second-hand data sources, which means that other researchers and related scholars have discovered in this field And the relevant data can be used at any time, which will save a lot of the collection and processing of the original data, and improve the efficiency of the project progress.

In order to make the project data closer to the research theme, the data mainly collected academic articles related to the V3-V4, microplastics and microbes, and finally decided to use the data in the following four academic articles as the data basis for the project research for processing and analysis:

- The plastisphere in marine ecosystem hosts potential specific microbial degraders including Alcanivorax borkumensis as a key player for the low-density polyethylene degradation (Delacuvellerie, et al, 2019).
- Major Role of Surrounding Environment in Shaping Biofilm Community Composition on Marine Plastic Debris (Basili, et al, 2020).
- Bacterial Community Profiling of Plastic Litter in the Belgian Part of the North Sea (De Tender, et al, 2015).
- The composition of bacterial communities associated with plastic biofilms differs between different polymers and stages of biofilm succession (Pinto, et al, 2019).

According to the research content of each academic article and extract the relevant data codes are as follows:

- PRJNA495136
- PRJNA558771
- PRJNA272679
- PRJNA515271

Using the above project data codes, the relevant research data can be downloaded from https://www.ncbi.nlm.nih.gov/. The specific data download will be elaborated in part of 2.2 model's organization.

2.2 16S rRNA

All data of this project are from 16S rRNA. 16S rRNA is an important part of the ribosome. Together with 22 proteins, it forms the 30S subunit on the ribosome (Schluenzen, et al, 2000). Because the functions performed are very basic and important, the structure of 16S rRNA is very conserved during evolution. The length of 16S rRNA is about 1500 bp. In addition to the typical GC and AU base complementation, its secondary structure is also full of atypical base pair linkages such as GU and GA, forming more than 50 helical structures, and Single chain ring structure between spiral structures (Woese, et al, 1983). Due to the influence of secondary structure, the evolution rate of different nucleic acid sites on 16S rRNA is not the same. The part of single strand loop structure does not need complementary pairing, so the evolution speed is faster, while the spiral part needs to complete complementary pairing by gyric structure, which is very conservative. The evolution rate between the fastest evolving site and the slowest evolving site can differ by as much as 1000 times. According to the speed of evolution, the full length of 16S rRNA is usually divided into 9 highly variable regions (V1-V9) And the relatively conserved regions in between (Van, et al, 1996). For this project research, all data will use the highly variable region of V3-V4 for final

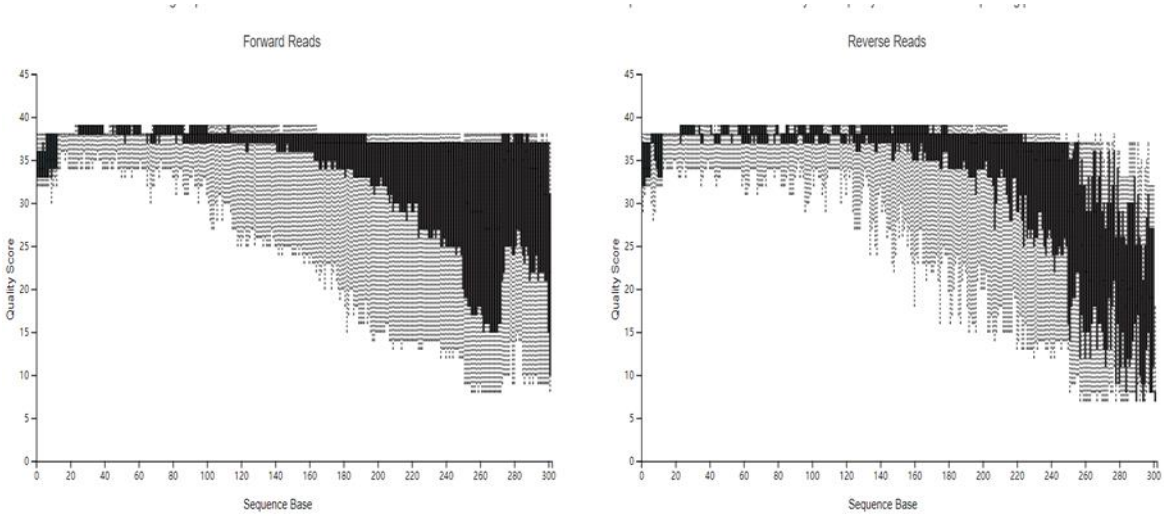
research and analysis. Carl Woese first proposed that rRNA has good clock characteristics and can be used to trace the evolutionary relationship of organisms, thus becoming a pioneer in rRNA research (Woese, 1987). On the basis of Carl's work, Norman initiated a molecular biological method for microbial diversity research, which was represented by rRNA gene sequencing. They directly sequenced 16S rRNA gene in environmental samples and directly captured the evolution and diversity information of environmental microorganisms by bypassing the cultivation step (Pace, 1997). With the application of molecular biology technology represented by 16S rRNA research, researchers have gradually realized that compared with a few microorganisms that can be cultured, most of the microorganisms that cannot be cultured have higher diversity and more important ecological functions, such as alpha diversity, beta-diversity, taxa bars, environmental filtering.

2.3 Illumina Miseq

Benefiting from the development of sequencing technology, the throughput of sequencing platforms is getting higher and higher. If only one microbial community sample is sequenced in each sequencing batch, it is obviously a big waste. At this time, you need to use multiplexed sequencing, barcoded sequencing and indexed sequencing technology (Goodrich, et al, 2014). The core of this method is that when the target fragment of 16S rRNA gene is amplified by PCR, a characteristic barcode of oligonucleotide segment is added to the primer, and the PCR product will also carry the same sample specific barcode. The barcode carried by the PCR primers of each sample is different. After the sequencing is completed, the barcode sequence can be detected to know which sample each sequence belongs to. In this way, the DNA template of hundreds or even thousands of samples can be mixed in the same sequencing batch, which greatly improves the detection efficiency and utilization rate.

The Illumina MiSeq platform pioneered the use of external barcode design. The barcode sequence is not directly connected to the PCR primers used to amplify the target

fragment. There is an extension adapter in the middle. The actual sequencing starts from the end of the extension adapter. The final sequencing sequence only contains the PCR primer and the target fragment. After the target fragment is sequenced, the barcode regions at both ends are separately sequenced to obtain the sample information of the sequence (Illumina, 2013). The advantage of this design is that through high-quality short fragment sequencing, the barcode sequence detection will be more accurate; and the barcode area does not occupy the sequencing read length of the target fragment, and the utilization rate is higher. At the same time, Illumina Miseq platform sequenced and identified barcode regions automatically during the sequencing process. The final sequencing results have been separated according to the samples, and no additional bioinformatics analysis is required.



2.4 DADA2 Analysis

With the development of high-throughput PCR sequencing, it is of great significance to the study of environmental microbial community. In amplification sequencing, a specific gene sequence is amplified from the DNA extracted from the target population and sequenced on the next generation sequencing platform. This technology avoids the necessity of microbial cultivation and detection, and effectively provides in-depth investigation of microbial communities (Callahan, et al, 2016). In the process of

amplicon sequencing, some incorrect sequencing will be introduced into the data, which makes the results of serious deviations and complicates interpretation. DADA2 brings a brand-new algorithm, which can model the errors introduced in the amplification process and use the error model to infer the real sample composition. (Callahan, et al, 2015).

The starting point of the DADA2 pipeline is a set of demultiplexed fastq files corresponding to the samples sequenced by amplicons. In other words, DADA2 expects that each sample will have two separate fastq files, one forward and one reverse. Like the DADA2 features introduced before, the ASV table records each sequence and its quantity information to obtain microbial classification information with higher resolution than the OTU table obtained by traditional clustering (Bolyen, et al, 2019). The appendix provides a brief description of how to generate virtual barcodes and explains how to combine forward and reverse barcodes together (see the appendix figure 2 and figure 3). After the analysis and processing of DADA2, the project finally obtained 11566 ASVs for the final data analysis (see the appendix figure 8).

2.5 Biological Diversity

In order to study the microbial diversity of microplastics and plastisphere in the ocean, the statistical analysis methods of alpha diversity and beta diversity will be used in this project. Alpha diversity refers to the diversity of microorganism in a specific area or ecosystem, and it is a comprehensive indicator reflecting abundance and uniformity. Alpha diversity is mainly related to two factors, one is the number of species, which is richness, the other is diversity, the uniformity of individual distribution in the community. Community richness index mainly includes Chao index and Ace index. Community diversity index, including Shannon index and Simpson index (Reese & Dunn, 2018).

- Simpson Index. It is an index commonly used in ecology. It reflects the status and

role of dominant species in the community. If a community has more dominant species, the proportion of other non-dominant species will decrease. Then Simpson index value is larger, which indicates that the community diversity is low, and this index is negatively correlated with other diversity indexes.

- Shannon index. One of the indexes used to estimate the diversity of microorganisms in the sample. Both it and the Simpson diversity index are commonly used indexes of alpha diversity. The larger the Shannon value, the higher the community diversity.

The term beta-diversity was proposed by Whitaker (Whittaker, 1960). It is defined as the degree of community composition change, or the degree of community differentiation, which is related to the complex gradient of the environment or the pattern of the environment (Legendre, 2014).

- Bray–Curtis dissimilarity. Difference in microbial abundance between two samples. 0 means that the two samples have the same species richness. 1 is the species richness of two samples that are completely different
- Jaccard distance.0 indicates that two samples have the same species, and 1 indicates that the two samples have no common species.
- UniFrac. sequence distances (phylogenetic tree), unweighted UniFrac is based on sequence distance, but does not consider abundance information. weighted UniFrac considers abundance information and sequence distance.

2.6 R Studio

In this project, R studio will be used to analyze the pre-processing data, and box plot, two-dimensional distribution chart and histogram will be used to give the results in alpha diversity, beta diversity, environmental filtering and taxa bars. Based on the standard of plastic type, the effects of different conditions (such as water temperature, salinity, pH and sea area) on microbial community composition were analyzed.

3.0 Results

	Northern Corsica	North Sea	Adriatic Sea	coast of Italy
ph	8.5	8	8.2	8.2
Temperature	20	8	21	18.6
Salinity g/L	38	33.5	38.3	38
Plastic type	PP, PE, PVE, LDPE	PE	LDPE, HDPE, PVE-DEHP, PVE-DIHP, PVE-DINP	PE, PP

Table 1: Basic Data

As showed in Table 1, The table summarizes the specific values of the relevant analysis collected in this project.

3.1 Alpha Diversity

This chapter will show 4 graphs, which use pH, salinity, temperature and sea area as variables to analyze the microbial communities on different plastic materials. Each graph will analyze each index in order to more clearly reflect the impact of each variable on the microbial community.

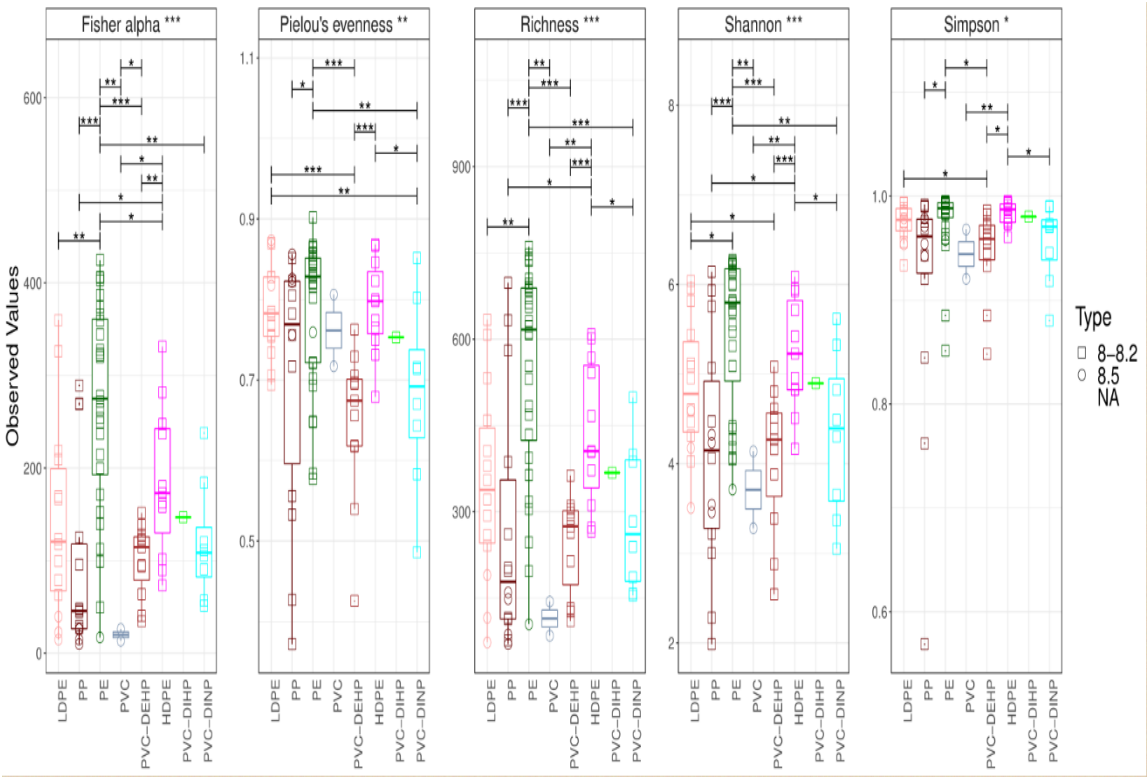


Figure 2: Plastic Type and ph in Alpha Diversity

As showed in Figure 2, the influence of pH value on the diversity of microbial community on different types of plastic materials. ph value would be divided into two variables of 8.5 and 8-8.2 to show the distribution of microbial diversity on different types of plastics. Under the Simpson index, according to the P value ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***.), it can be concluded that the difference in microbial diversity on the sample groups of different materials of plastic is weakly significant. In a single sample group, the sample distribution is relatively Similar , so the difference between individual samples is small. The Simpson index value of nearly 1 reflects the low microbial diversity. By comparing LDPE and PVC-DEHP, the P value between the two groups is $P < 0.05$*. The distribution between the two groups of samples is relatively similar, which indicates that the difference in the microbial community becomes a weaker display.

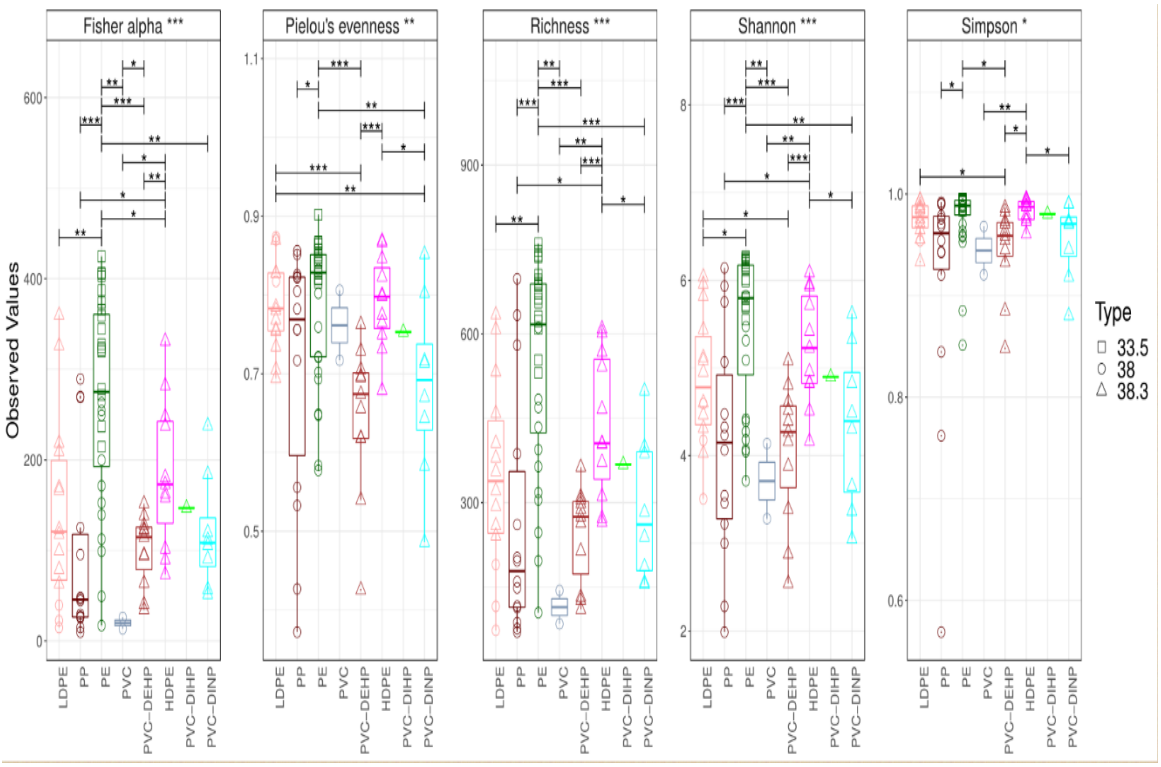


Figure 3: Plastic Type and Salinity in Alpha Diversity

As showed in Figure 3, salinity was divided into three variables: 38 g/L, 33.5 g/L and 38.3 g/L to show the diversity of the microbial community on the different plastic types (PE, PP, PVE, LDPE, PVC-DEHP, PVC-DIHP,PVC-DINP, HDPE) and the impact salinity has on this. Three salinity levels were compared (38 g/L, 33.5 g/L and 38.3 g/L). According to richness index, P value ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***.) can be concluded that the difference of microbial diversity among different materials of plastic samples is significant. By comparing PE and PVC-DINP, the P value between the two groups of samples is shown as $P < 0.001$***, the distribution of samples is not concentrated, so the difference between individual samples is great. It can be seen that the diversity of the samples is different due to the rich microbial species. In the samples of PE and PVC-DINP, when the salinity is 33.5g/L, the richness index is close to 6. When the temperature is 38g/L, the richness is directly close to 4. Therefore, when the salinity is lower, the types of microorganisms would be rich.

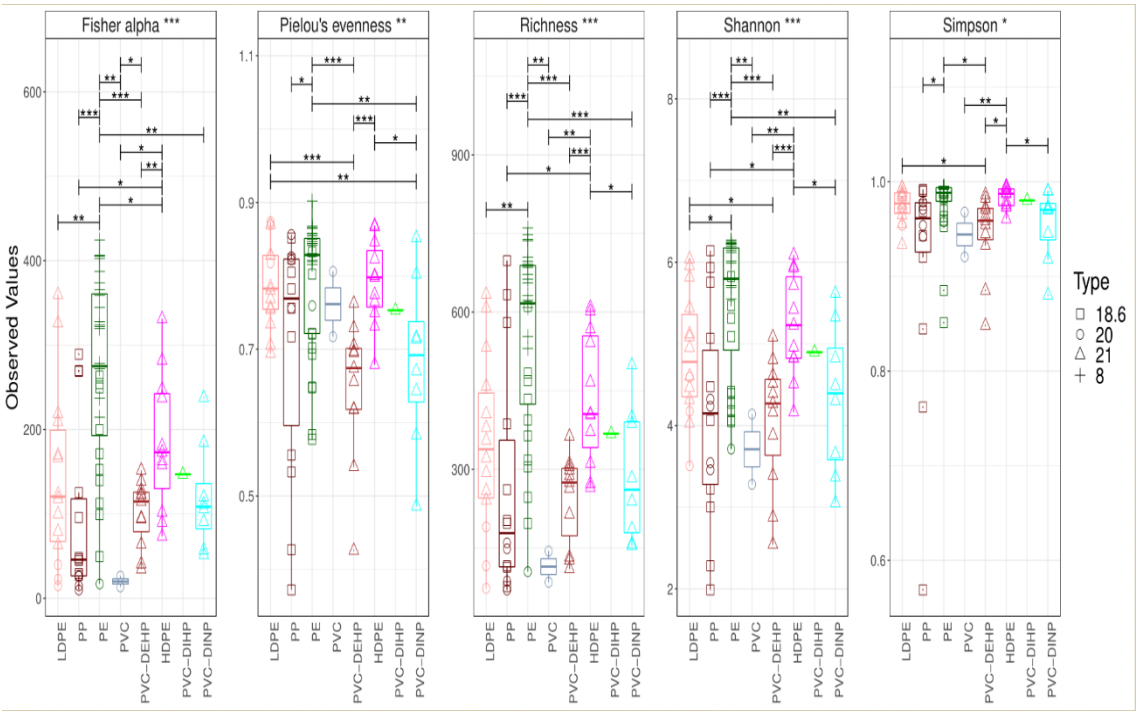


Figure 4: Plastic Type and Temperature in Alpha Diversity

As showed in Figure 4, the effect of water temperature on the diversity of microbial communities on different types of plastic materials. The measured water temperature is divided into four variables of 20℃, 21℃, 8℃ and 18.6℃ to illustrate the distribution of microbial diversity on different types of plastics (20℃, 21℃, 8℃ and 18.6℃). Evenness data pointed out that the small P value leads to significant differences in the microbial diversity of the sample groups on different plastic materials. By comparing HDPE and PVC-DEHP, the P value between the two groups of samples is shown as P < 0.001***, the sample distribution is not concentrated, and the comparison between samples also shows the diversity, which the difference is significant. Therefore, the large number of microorganisms in the sample leads to difference in diversity. In the samples of PE and PVC-DEHP, when the temperature is 8°C, the evenness index is close to 0.9. When the temperature is 21°C, the evenness is directly close to 0.7. Therefore, when the temperature is lower, the number of microorganisms would increase.

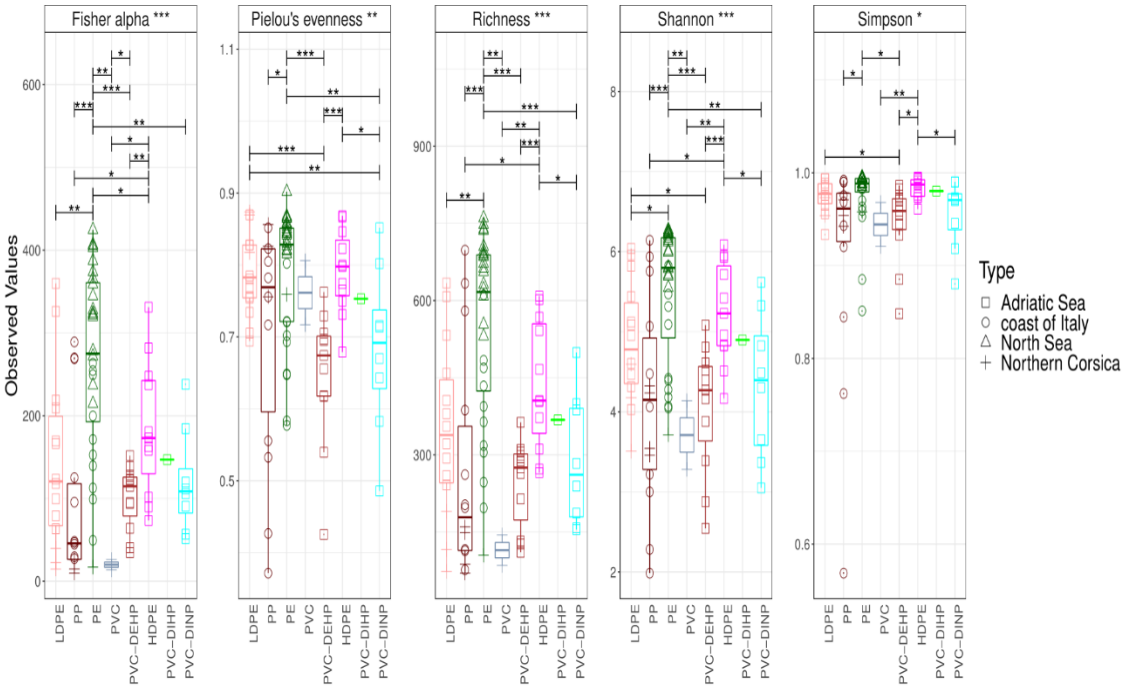


Figure 5: Sea Area-Plastic Type of Alpha Diversity

As showed in Figure 5, this project collected plastics of the same material from four different regions of the sea to analyze the distribution of microbial diversity. According to the observation of the Shannon index, the P value is significantly smaller than the P value in the Simpson index. The difference in microbial community diversity displayed under this index is significant. In a single sample group, the sample distribution is not concentrated, But the comparison between the sample and the sample shows that there are six groups (PVC-DEHP and PE, PVC-DEHP and HDPE, PVC-DINP and PE, PVC and HDPE, PP and PE, PE and PVC) with significant differences in microbial diversity and four groups (LDPE and PE, LDPE and PVC-DEHP, PP and HDPE, HDPE and PVC-DINP) with weakly significant differences.

According to the analysis of the above four graphs, the differences in the diversity of the microbial communities shown in the project data are significant, and the uniformity and richness are also relatively large. Because the content displayed by each graphic is not much different. Therefore, the influence of external environmental factors on the microbial community on different plastic materials is not significant.

3.2 Environmental Filtering

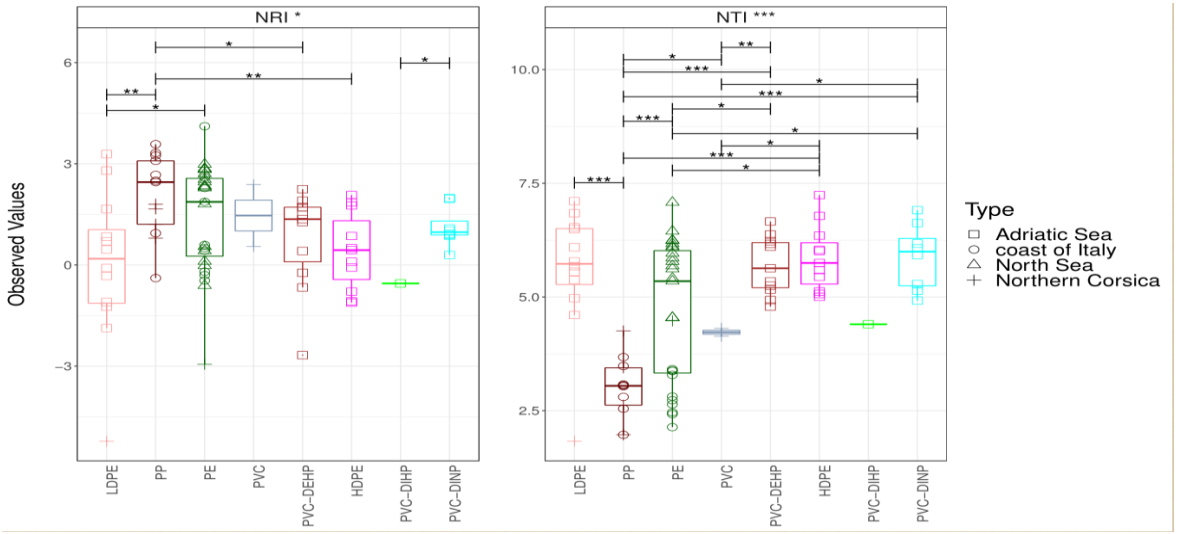
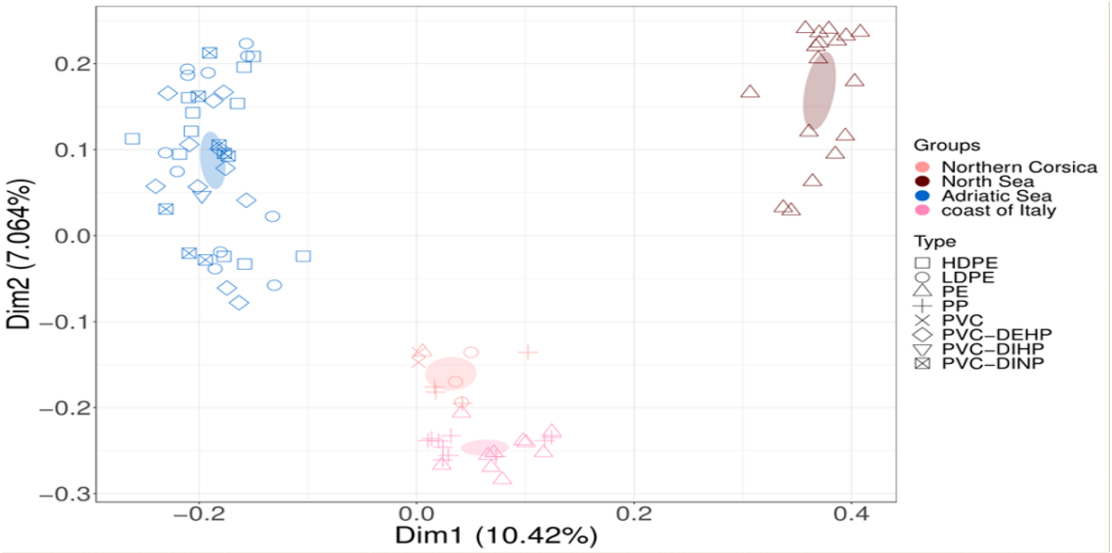


Figure 6: Sea Area-Plastic Type of Environmental Filtering

As showed in Figure 6, Analyzed ecological driving factors to determine whether the composition of the microbial on the different plastic types with respect to geographical location is related to any environmental pressure. When the ordinate value is greater than 2, NRI and NTI have significant clusters in the phylogenetic tree, which indicates a clustered phylogeny where coexisting taxa are more related to each other than expected by chance – therefore environmental pressure . When it is less than -2, this indicates members of these communities are less related to each other than expected by chance (i.e. phylogenetically over dispersed). – therefore, stochastic no environmental pressure.. When there are many clusters of phylogenetic trees, this is enough to indicate that environmental factors or other related parameters are involved in data analysis. According to Figure 6, the value of NRI is between 0-3, while the value of NTI is basically between 5-7, which is enough to show that the impact of environmental factors on the microbial community is significant. Since the marine environment is an infinitely open external environment, there will be more factors involved in it and affect the composition of the microbial community. Therefore, under the NTI index, the impact of different sea areas on the microbial community is increasing.

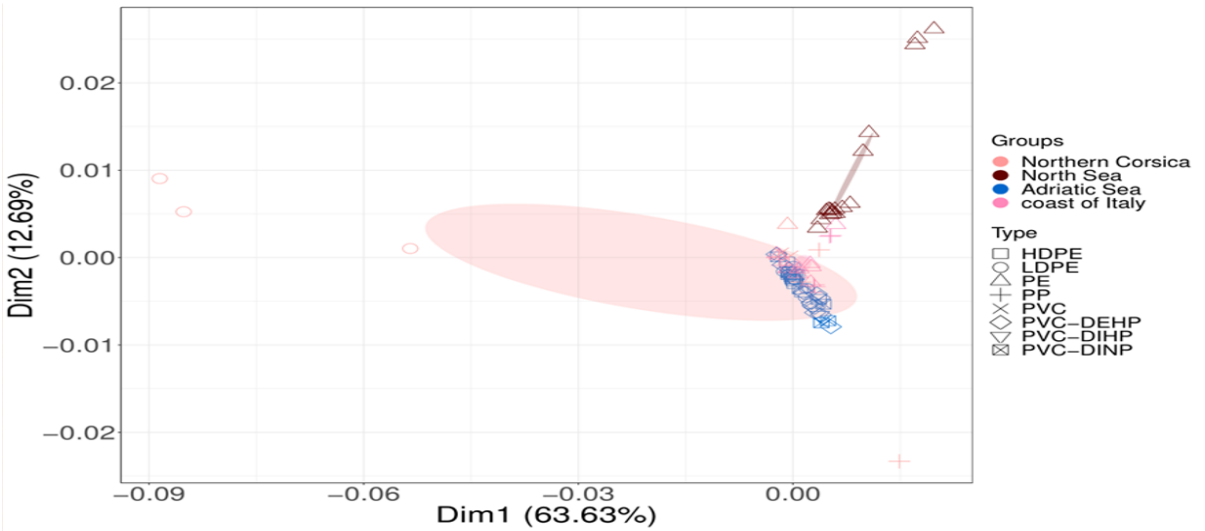
3.3 Beta Diversity



name	R square	P value
sea_Location	0.19884	0.001 ***
PLastic_Type	0.08012	0.004 **

Figure 7: Sea Area and Plastic Type in Unifrac Bate Diversity

As showed in figure 7, Using unifrac to analyze Beta diversity, unifrac is phylogenetic distance, which considers whether the sequence appears in the community, and does not consider the abundance of the sequence. The p value of sea location is 0.001, which is lower than p value of plastic type. This indicates that the difference in the microbial community on the sea location is a weaker indication. Through the observation of R-squared, the microbial community on the sea location (19%) is more than the plastic type (8%) Comparing the distance between the Adriatic Sea area and the North Sea area, it can be seen that there is a large difference, which is sufficient to show that the difference in the microbial communities in these two areas is obvious, and the distribution of microbial communities is relatively wide. Comparing the distance between the Northern Corsica area and the coast of Italy area, it is found that they are very close to each other, which means that the microbial community difference between the two areas is not significant and the similarity is high.



name	R square	P value
sea_Location	0.28160	0.001 ***
PLastic_Type	0.42746	0.001 **

Figure 8: Sea Area-Plastic Type of Wunifrac Bate Diversity

As showed in figure 8, Using wunifrac to analyze Beta diversity, On the basis of uniFrac, weighted unifrac is phylogenetic distance but considers species abundance., it can distinguish the difference in species abundance. The p value of sea location is 0.001, which is same as p value of plastic type. This indicates that the difference of microbial community in plastic type and sea location is the same. Through the observation of R-squared, the microbial community on the sea location (28%) is lower than the plastic type (42%). Adriatic Sea area, Northern Corsica area and coast of Italy area. These three areas overlap completely, and the distribution of microbial communities is relatively concentrated. This is enough to show that the composition of microbial communities in these three areas is very similar and the differences are weakly significant. The distance between the North Sea area and the above three areas is also very close, and the distribution of microbial communities in this area is also very concentrated. Therefore, regardless of sequence abundance, the difference in the composition of microbial communities is weakly significant. Unweighted unifrac can detect the presence of variation between samples, while weighted unifrac can further quantify the variation that occurs in different lineages between samples.

3.4 Composition of the microbial communities on microplastics

The analysis of taxa bars will use the top 25 most abundant genera to reflect the distribution of different microbial species on different plastic materials.

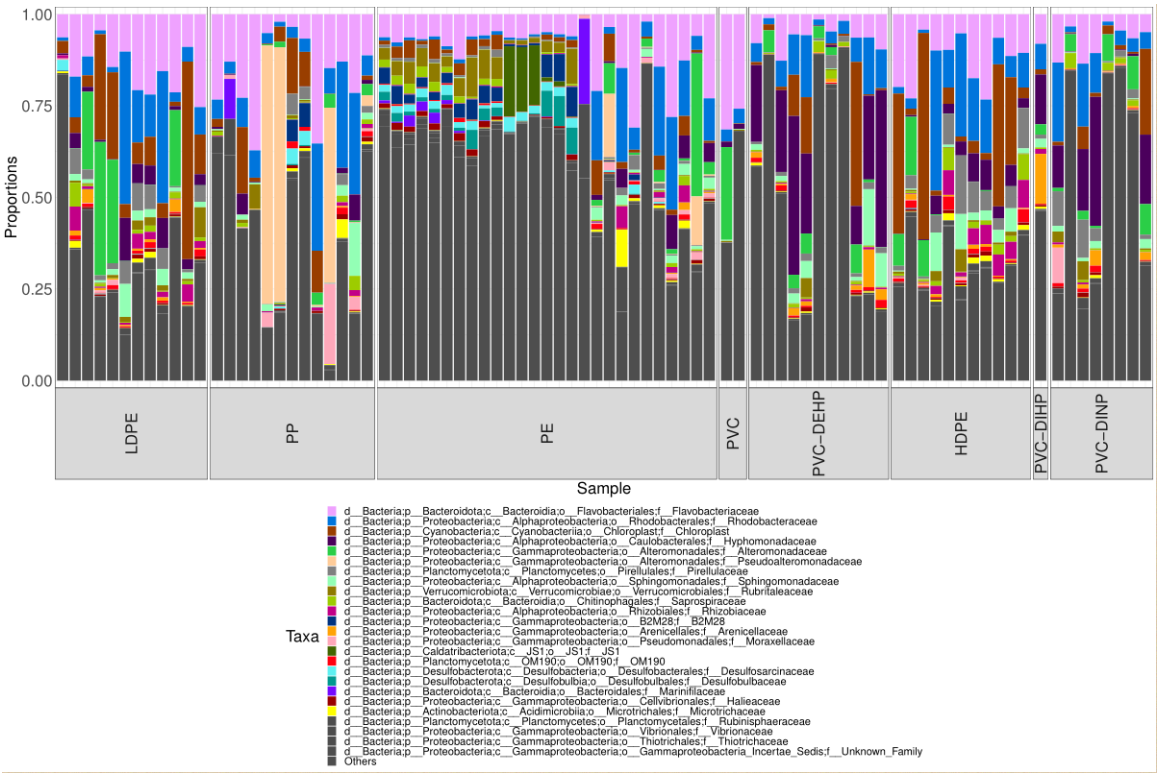


Figure 9: Plastic Type of Family Taxa Bars

As showed in figure 9, in the family level, Bacteroidota, Bacteroidia, Flavobacteriales and Flavobacteriaceae account for the highest proportion of all plastics of different materials. Proteobacteria, Alphaproteobacteria, Rhodobacterales and Rhodobacteraceae have the second highest proportion of bacteria. The third highest percentage (about 30%) of bacteria are Cyanobacteria, Cyanobacteria, Chloroplast and Chloroplast. These three types of bacteria account for the largest proportion of all plastic materials, and the distribution of other bacteria is relatively uniform. However, Proteobacteria, Gammaproteobacteria, Alteromonadales and Pseudoalteromonadaceae are distributed (about 70%) in PP more than other plastic types.

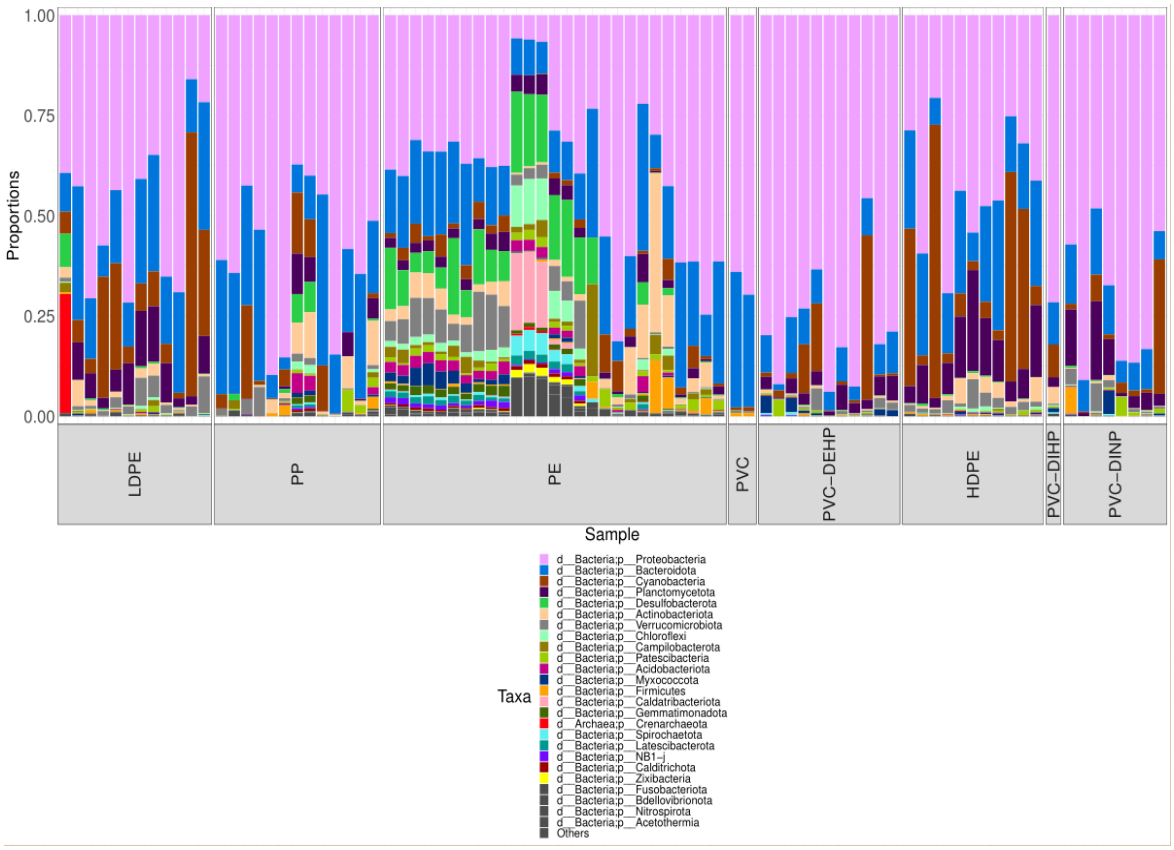


Figure 10: Plastic Type of Phylum Taxa Bars

As showed in figure 10, in the phylum level, the proportion of Proteobacteria (about 50%) is the highest among all plastics of different materials. Secondly, Bacteroidetes (about 15%) has the second highest proportion. The proportion of Cyanobacteria (about 10%) is the third highest. These three bacteria are the three types with the largest proportion of microorganisms in all plastic materials, and the distribution of other bacteria is relatively even. PE contains the most types of microorganisms in all plastics, which also shows that PE provides a good environment for the formation of microbial communities.

4.0 Discussion

In this research i aimed to environmental impact of microplastics and the impact of microbial communities on microplastics. It was found that according to the analysis results in Chapter 3, the change of location increases the impact of the environment on the microbial community, but environmental factors (pH, seawater temperature and salinity) have no significant impact on the composition and impact of the microbial community. Under extreme environmental conditions (Antarctic, Arctic, and submarine volcanoes), it can have a significant impact on the formation and composition of microbial communities (Li, et al, 2014). However, according to the data analysis in Chapter 3, it can be seen that the samples used are from ordinary marine environments, such as sand and gravel sediments and microplastics floating in shallow waters, which are not extreme environments under such environmental conditions. Therefore, this chapter will focus on the impact of microplastics and microbes on the marine and ecological environment.

4.1 Microplastics and Microbes

After entering the marine environment, microplastics are easy to absorb excrement, organic matter and inorganic nutrients, and then attract microorganisms and phytoplankton to adhere to the surface. according to the data analysis in Chapter 3, bacteria such as *Bacteroidetes*, *Proteobacteria* and *cyanobacteria* were attached on the surface of plastic particles collected from the North Sea. At the same time, a study in the North Pacific Circulation Zone showed that the microorganisms gathered on the surface of the microplastics mainly include Bacillus, Coccus, and pinnate diatoms (Carson, et al, 2013). In this project, high-throughput sequencing technology was used to analyze the bacterial community on the micro plastic samples. It was found that the main bacteria were *Alphaproteobacteria*, *Gammaproteobacteria*, *Flavobacterium*, *acidobacteria* and *cyanobacteria*. According to the information in Fig. 9 in Chapter 3,

Vibrio pathogenic was detected on the surface of the micro plastic (Kirstein, et al, 2016).

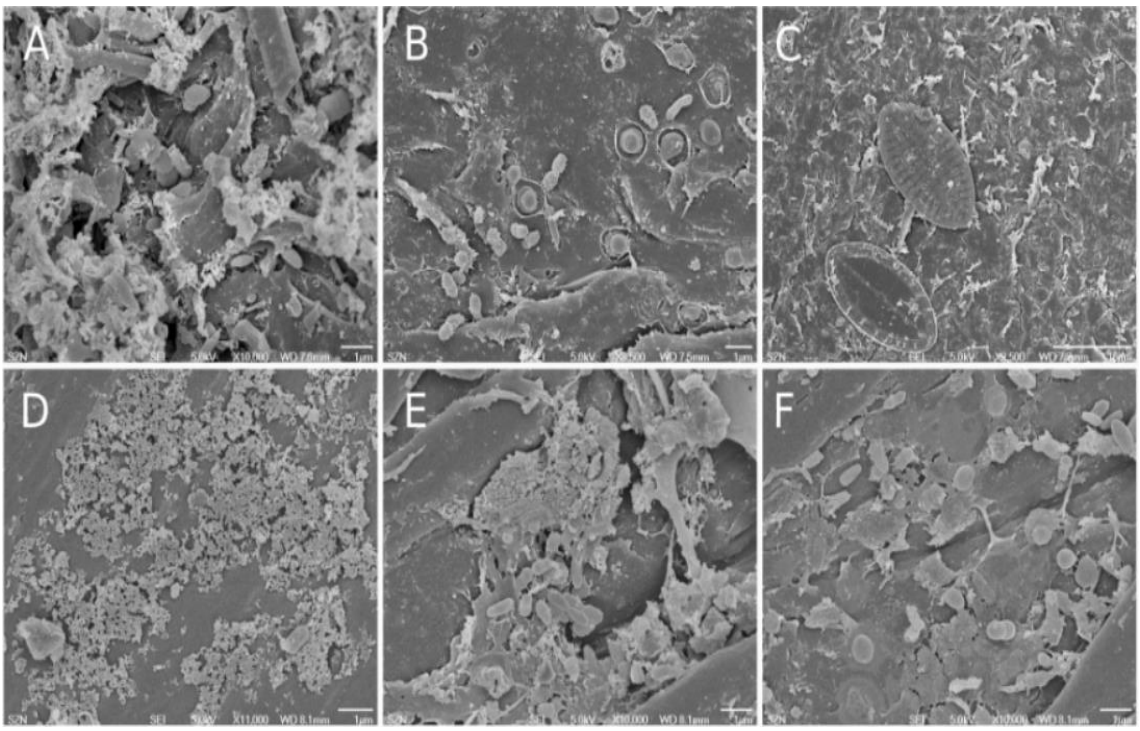


Figure 11: SEM Images of Microbial Biofilm on PE and PP (Basili, et al, 2020)

As showed in figure, Images A, B and C show microorganisms on the surface of PP, and images D, C and E show microorganisms on the surface of PE. Microplastic surfaces exposed to seawater are prone to form visible microbial communities on their surfaces (De Tender, 2015). A few studies have shown that the formation of microbial communities will affect the physical and chemical properties of microplastics to a certain extent. This includes: increasing the weight of microplastics, enhancing the ability of microplastics to resist ultraviolet radiation, reducing the hydrophobicity of the microplastics surface, and changing the buoyancy and density of microplastics (Weinstein, et al, 2016). From the analysis of Phylum level in chapter, the early and middle stages of biofilm formation on the surface of PE and PP are mainly *Proteobacteria* and *Bacteroidetes*. The late stages are mainly *Proteobacteria*, *Bacteroidetes* and *Verrucomicrobia*. Studies have shown that *Proteobacteria* is the primary colonizing organism for the formation of marine bacterial biofilm, and

Actinobacteria is the secondary colonizing organism for the formation of biofilm. These two types of bacteria play an important role in the formation of marine biofilm (Elifantz, et al, 2013).

4.2 Microplastic Degradation

The strains bacillus and *Pseudoalteromonadaceae* is one that helps degrade microplastics PE and PP. According to the enrichment of PE and PP plastic degrading bacteria by Indian scientists, a strain with obvious degradation ability was isolated from the water body and identified as *Pseudoalteromonadaceae* (Sudhakar, et al, 2007). In addition, Indian scientists have also isolated different types of PE degrading bacteria from the coastal waters of the Arabian Sea, including *Kocuria palustris*, Bacillus *pumilus* and Bacillus subtilis (Harshvardhan, et al, 2013). At the same time, Indian scientists used the isolation of a strain of Bacillus to treat plastics for 90 days. By means of weight determination, microbial metabolic activity, and atomic force microscope observation, they proved that the bacteria can degrade PE and PVC plastics. In addition, Arthrobacter and *pseudoalteromonadaceae*, which can degrade HDPE, were isolated by Indian scientists, which could reduce the crystallinity of HDPE within 30 days (Balasubramanian, 2010). Indian scientists also tried to carry out plastic degradation experiments and initially found that *Pseudoalteromonadaceae* from marine sources can partially degrade PC and release products such as Bisphenol A and BPA (Artham & Doble, 2012).

The degradation of plastic by marine microorganisms first comes into contact with the plastic and forms a biofilm on the surface of the plastic. In order to understand the degradation process of plastic by marine microorganisms, *Oberbeckmann* used PET plastic bottles as attachment substrates and enriched in the water environment of different stations in the North Sea for 35 to 42 days. The analysis found that the main group of microorganisms on the surface of plastic bottles was *Bacteroidetes*, which specifically included In order to efficiently degrade complex organic carbon, bacterial groups, such as *Flavobacteriaceae*, *Cryomorphace-ae* and *Saprospiraceae* (Oberbeckmann, 2016). This indicates that the separation and purification of plastic-degrading bacteria from microplastics and their use for plastic waste treatment are forward-looking. However,

whether these bacteria only degrade plastics or use the chemical components in plastics to participate in the metabolism is still unknown, and which factors will affect the degradation process remains to be studied.

4.3 Influence in Marine Environment

The buoyancy and stability of microplastics can support the survival and long-term drifting of surface microorganisms. Keswani's research has shown that floating plastic particles diffuse with ocean currents and waves, which can bring microbial communities into new habitats (Keswani, et al, 2016). Special attention should be paid to the presence of toxic or pathogenic bacteria on microplastics. With the drift of microplastics, there may be a large number of alien species that invade new habitats, and multiply due to suitable conditions, which will change the ecological risk of the community structure in the area. Studies have shown that Vibrio alginolyticus can adhere to PS, PE and PVC in a large amount, and it can easily spread with rivers. (Snoussi, et al, 2009). Several coral pathogens such as Halofolliculina have been detected in 95 plastics and debris in the Eastern Pacific Ocean, which are considered to be potential invaders that migrate with plastics (Goldstein, et al, 2014). The high abundance of Vibrio, a potential human pathogen, was frequently detected on plastic particles in the North Sea, Baltic Sea and North Atlantic Ocean, which further confirmed Zettler's inference.

According to the research results of this project, the pathogenic bacteria *Vibrio* was also found on the plastic materials of PE and PP. This makes people have to notice that microplastics not only affect the environment, but also the bacteria attached to the surface will also affect the environment. Human health poses a threat.

The biofilm attached to the microplastics is extremely complex and contains many bacteria, which may lead to gene exchange between biofilm communities or between biofilm communities and surrounding environmental communities (Stewart, 2013).

Stewart pointed out that in oligotrophic salt seas, there are a variety of bacteria attached to organic particles. Due to the strong variability of bacteria, community communication occurs through the horizontal transfer of bacteria, such as the absorption and utilization of DNA in the environment, cell transformation, and transduction through phage. Therefore, organic particulate matter may become a hotspot for bacterial communication, resulting in new bacteria. Since microplastics are organic particulate matter discharged from human activities, the pathogenic microorganisms attached to it are rich in pathogenic bacteria, which spread through the water flow, leading to the outbreak of pathogenic bacteria in the transmission path, and thus triggering large-scale infections. Therefore, in addition to being a carrier for the diffusion and migration of microorganisms, microplastic particles are also carriers for the exchange and transformation of various pathogenic bacteria, such as *Vibrio*. In this project, plastics made of PP and PE were found in these three sea areas (Northern Corsica, North Sea and coast of Italy). Through research, it was also found that the surfaces of these plastics were all adsorbed by pathogenic bacteria *Vibrio.*

In the marine environment, due to its small particle size and large specific surface area, microplastics are easy to adsorb organic pollutants in the environment, and thus produce compound toxic effects on marine organisms. Commonly adsorbed organic pollutants include PCB, PAH and PBBs, etc (Scopetani, et al, 2018). in addition to some heavy metals such as zinc, copper, lead, chromium and cadmium (Ashton, et al,2010). This will become a carrier of pollutants and cause compound pollution in the marine environment, and its compound toxicity is much higher than that of a single toxicity. In addition, the surface of microplastics will also adsorb some microorganisms, such as bacteria and viruses. When these microorganisms enter the organism, they will cause the bioaccumulation of microorganisms at various trophic levels (Gregory, 2009). In addition, compared with a single microplastic, the microplastics after the aggregation of microorganisms can produce a stronger compound toxicity effect, which can cause biological infections, which in turn poses a serious threat to the survival of marine life

and human health (Wagner, et al, 2014).

4.4 Limitations

At present, at the level of science and technology, there are still many problems and deficiencies in the global response to marine plastic waste and micro plastic pollution.

In terms of microplastic analysis methodology, as a new type of marine pollutant, international research on marine microplastics still lacks unified technical standards for monitoring, analysis and evaluation. The quantitative and qualitative analysis methodologies of microplastics still need to be further explored and improved (Shim, et al, 2017). Especially in the collection methods, characterization methods and spectral analysis of microplastic samples from different environmental media, further investigation is needed.

Marine micro plastic pollution control measures. Marine plastic waste and micro plastic pollution are global environmental problems. So far, there is a lack of global joint research in the world. The efficient collection and removal technology of plastic waste and microplastics in terrestrial and marine environments also needs to be developed. Governance in rivers is internationally recognized as an effective way to reduce the entry of plastic waste into the ocean, but so far there has not been a significant and efficient technology or project to treat river plastics globally (Auta, 2017).

Evaluation of potential ecological risks of microplastics. In the actual environment, there is no direct evidence that marine microplastics have affected ecosystems. The toxic effects and mechanisms of marine plastics and microplastics at ambient concentration levels are still not clear (Peng, et al, 2018). In summary, microplastics have become a new type of pollutant, which has an important impact on the marine ecological environment and freshwater ecological environment.

5.0 Conclusion

Microplastics have become a new type of pollutant and have an important impact on the marine ecological environment. Due to the biological adhesion effect, microplastics can become the growth carrier of microorganisms and algae, and carry them for long-distance migration, which brings the potential risk of biological invasion. The compound of microplastics and other pollutants can produce toxic effects and accumulate in the human body through the food chain effect, which poses a potential threat to human life and health. In addition, the risks to the ecological environment caused by the accumulation of microplastics on the seafloor are issues that urgently require more attention and research.

According to the meta-analysis of microorganisms on the surface of microplastics in this project, it can be seen that ph, salinity, temperature, sea area and plastic materials will all affect the composition of the microbial community. It can be known from the analysis that different bacteria have an effect on the degradation of microplastics. At the same time, the bacteria Vibrio found on the surface of microplastics is a kind of disease-curing bacteria, which will cause certain harm to human health.

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Appendix

DATA2 Organization

In order to realize the function and flexibility of FASTQ files, all the settings will be established in the Linux environment:

mkdir cbz # this command is to create my own project folder

cd cbz # this command is to go to my own project folder

mkdir sequences # this command is to create my own project data folder

After the sequences folder is created, the next step is to download the required data into the folder. For the specific operation steps (see, fig 1).

gunzip -r /home/eng/MScBioinf/Caroline/cbz/sequences # this command is to extract all downloaded data into the current folder

mkdir qiime2_tutorial # this command is to create a folder for qiime2 to process the data.

Step 1:

```
export PATH=/home/opt/sratoolkit.2.9.0-centos_linux64/bin:$PATH
export PATH=/home/opt/edirect:$PATH
```

Step 2: Get all the SRR numbers associated with a bioproject PRJNA*

```
esearch -db sra -query PRJNA495136 | efetch --format runinfo |cut -d "," -f 1 > SRR.numbers
```

Step 3: Retain only the SRR numbers in the files

```
awk '/SRR/' SRR.numbers > SRR.numbers.filtered
```

Step 4: of you can use the command in a for loop

```
for i in $(cat SRR.numbers.filtered); do echo Processing $i; fastq-dump --split-files --origfmt
--gzip $i ; done
```

Figure 1: Download of Data

In sequences folder:

```
[MScBioinf@becker ~/umer/sequences]$ for i in $(awk -F"_" '{print $1}'
<(ls *.fastq) | sort | uniq); do mkdir $i; mkdir $i/Raw; mv $i*.fastq
$i/Raw/.; done

[MScBioinf@becker ~/umer]$ cd qiime2_tutorial
[MScBioinf@becker ~/umer/qiime2_tutorial]$
```

There are different ways in which we can import data to qiime2:
https://docs.qiime2.org/2020.2/tutorials/importing/

```
[MScBioinf@becker
~/umer/sequences]$ d="/home/eng/MScBioinf/Caroline/Mu/sequences/";

[MScBioinf@becker ~/umer/sequences]$ cd ../qiime2_tutorial
```

Next step is to generate fictitious barcodes required to import data in Earth Microbiome Project (EMP) format (consult http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/oneliners.html#PERLOL on how I have written one-liner in perl to generate fictitious barcodes):

```
[MScBioinf@becker ~/umer/qiime2_tutorial]$ t=$(ls $d | wc -l);
[MScBioinf@becker ~/umer/qiime2_tutorial]$ paste <(ls $d) <(perl -le
'sub p{my $l=pop @_;unless(@_){return map [$_],@$l;}return map { my
$ll=$_; map [@$ll,$_],@$l} p(@_);} @a=[A,C,G,T]; print join("", @$_) for
p(@a,@a,@a,@a,@a,@a,@a,@a);' | awk -v k=$t 'NR<=k{print}') | awk
'BEGIN{print "sample-id\tbarcode-sequence\n#q2:types\tcategorical"}1' >
sample_metadata.tsv
```

This command shows you the barcodes on your samples:

```
[MScBioinf@becker ~/umer/qiime2_tutorial]$ cat sample_metadata.tsv
sample-id       barcode-sequence
#q2:types       categorical
```

Step 2: Generate barcodes for each read using the file as above

```
[MScBioinf@becker ~/umer/qiime2_tutorial]$ (for i in $(ls $d); do
bc=$(awk -v k=$i '$1==k{print $2}' sample_metadata.tsv); bioawk -cfastx
-v k=$bc '{print "@"$1" "$4"\n"k"\n+";for(i=0;i< length(k);i++){printf
"#"};printf "\n"}' $d/$i/Raw/*_1.fastq ; done) > barcodes.fastq
```

Essentially, we are extracting the read headers from all the forward FASTQ files, and we assign the barcodes generated from sample_metadata.tsv file to those headers

```
[MScBioinf@becker ~/umer/qiime2_tutorial]$ head barcodes.fastq
@M01359:18:000000000-A5HVT:1:1101:15648:3435 1:N:0:109
```

Figure 2

```
AAAAAAAA
+
########
@M01359:18:000000000-A5HVT:1:1101:15642:3453 1:N:0:109
AAAAAAAA
+
########
@M01359:18:000000000-A5HVT:1:1101:22693:3963 1:N:0:109
AAAAAAAA
[MScBioinf@becker ~/umer/qiime2_tutorial1$ tail barcodes.fastq
+
########
@M01359:18:000000000-A5HVT:1:2114:15029:28668 1:N:0:68
AAAAACCG
+
########
@M01359:18:000000000-A5HVT:1:1106:13378:23600 1:N:0:7
AAAAACCT
+
########
```

Step 3: Collate all the forward reads from all the folders together in a single forward.fastq file

```
[MScBioinf@becker ~/umer/qiime2_tutorial1$ (for i in $(ls $d); do cat
$d/$i/Raw/*_1.fastq ; done) > forward.fastq
```

Step 4: Assemble all the reverse reads from all the folders together in a single reverse.fastq file

```
[MScBioinf@becker ~/umer/qiime2_tutorial1$ (for i in $(ls $d); do cat
$d/$i/Raw/*_2.fastq ; done) > reverse.fastq
```

See if the numbers match

```
[MScBioinf@becker ~/umer/qiime2_tutorial1$ ls
barcodes.fastq  forward.fastq  reverse.fastq  sample_metadata.tsv
[MScBioinf@becker ~/umer/qiime2_tutorial1$ bioawk -cfastx 'END{print
NR}' forward.fastq
8889688
[MScBioinf@becker ~/umer/qiime2_tutorial1$ bioawk -cfastx 'END{print
NR}' reverse.fastq
8889688
[MScBioinf@becker ~/umer/qiime2_tutorial1$ bioawk -cfastx 'END{print
NR}' barcodes.fastq
8889688
[MScBioinf@becker ~/umer/qiime2_tutorial1$
```

Figure 3

```
Step 5 (in your qiime2 tutorial folder): Zip all the FASTQ files and
move them to emp-paired-end-sequences folder

[MScBioinf@becker ~/umer/qiime2_tutorial]$ gzip *.fastq

[MScBioinf@becker ~/umer/qiime2_tutorial]$ mkdir emp-paired-end-
sequences; mv *.gz emp-paired-end-sequences/.

[MScBioinf@becker ~/umer/qiime2_tutorial]$ ls

emp-paired-end-sequences  sample_metadata.tsv

[MScBioinf@becker ~/umer/qiime2_tutorial]$

Next, we enable Qiime2 on the Orion cluster

[MScBioinf@becker ~/umer/qiime2_tutorial]$ export
PATH=/home/opt/miniconda2/bin:$PATH

[MScBioinf@becker ~/umer/qiime2_tutorial]$ source activate qiime2-2019.7

Step 6: Import the sequences to Qiime2

[MScBioinf@becker ~/umer/qiime2_tutorial]$ qiime tools import --type
EMPPairedEndSequences --input-path emp-paired-end-sequences --output-
path emp-paired-end-sequences.qza

Step 7: Demultiplex the sequences in Qiime2

(qiime2-2019.7) [MScBioinf@becker ~/umer/qiime2_tutorial]$ qiime demux
emp-paired --p-no-golay-error-correction --i-seqs emp-paired-end-
sequences.qza --m-barcodes-file sample_metadata.tsv --m-barcodes-column
barcode-sequence --o-per-sample-sequences demux.qza --o-error-
correction-details demux-details.qza

Step 8: Depends on the quality of your run, we want to fine tune Dada2
algorithm by specifying the thresholds

(qiime2-2019.7) [MScBioinf@becker ~/umer/qiime2_tutorial]$ qiime demux
summarize --i-data ./demux.qza  --o-visualization ./demux.qzv

(qiime2-2019.7) [MScBioinf@becker ~/umer/qiime2_tutorial]$ qiime tools
export --input-path demux.qzv --output-path output
```

Figure 4

As showed in figure 1, 2 and 3, next download the file to your local computer by double clicking ‘demux.qzv’ file in Cyberduck. File should go to your Downloads folder on your laptop.

Next drag and drop the file on the Qiime2 viewer https://view.qiime2.org and manually figure out the thresholds, i.e., where the quality drops down significantly.

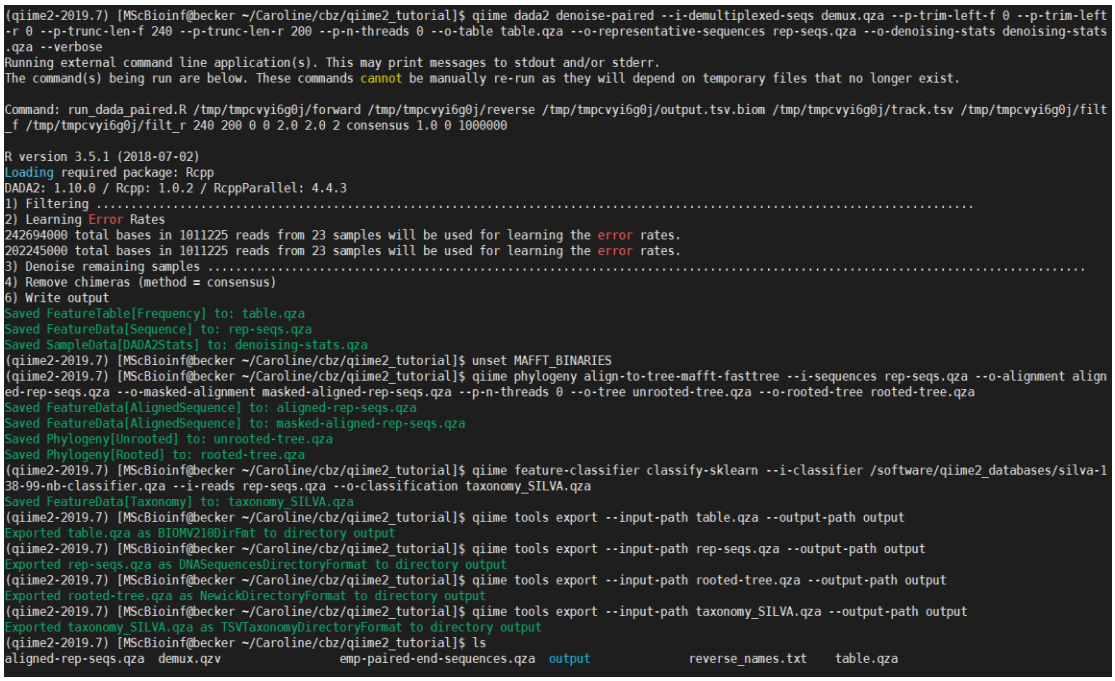

Figure 5

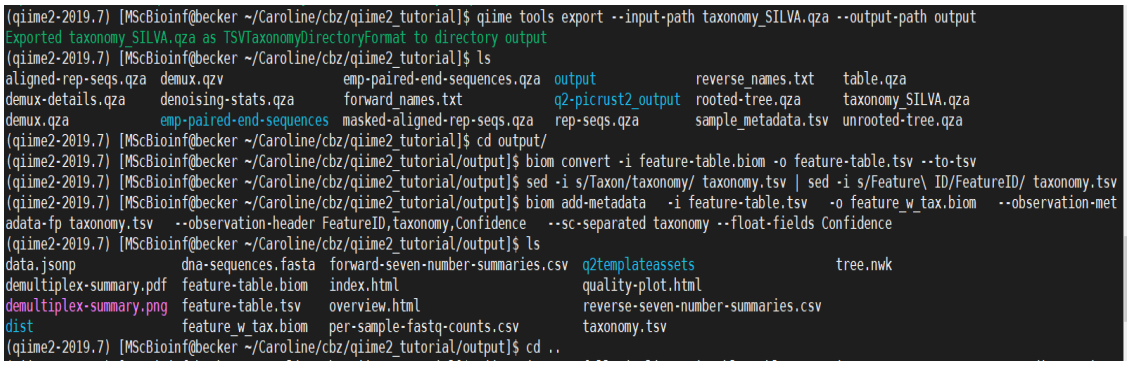

Figure 6

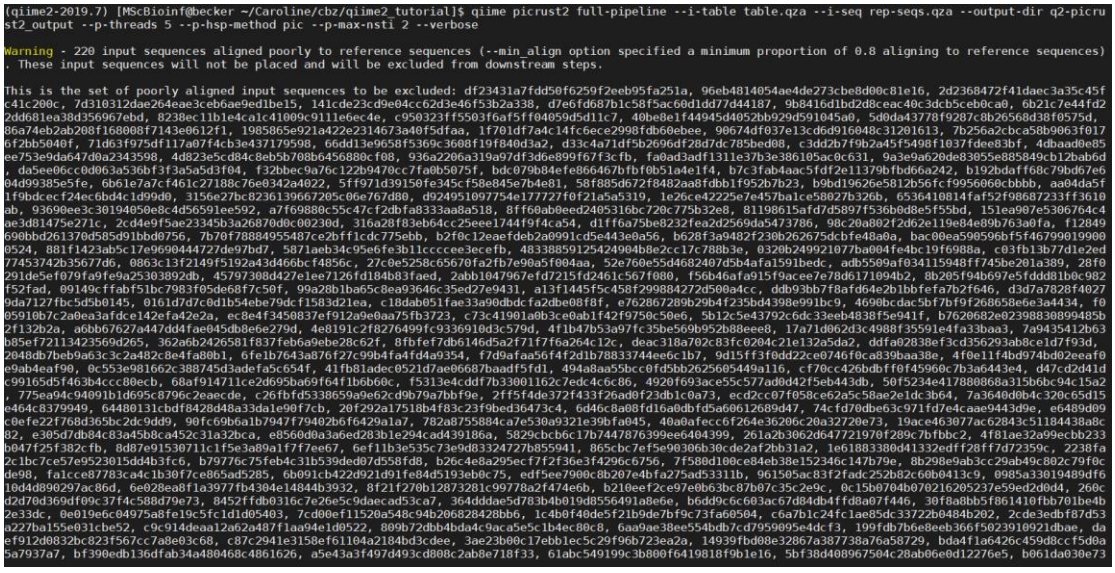

Figure 7

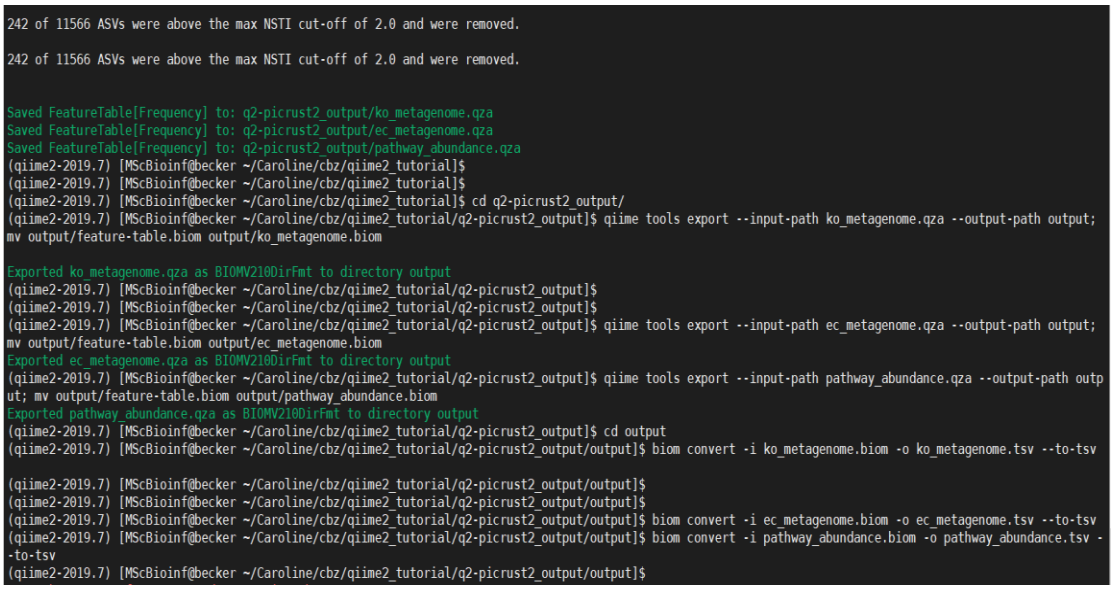

Figure 8

As showed in figure 1,2,3,4,5,6 and 7, it will be going to organize our data in such a manner that for every sample we have the folder name extracted from the paired-end files, and we are going to dump the raw sequences in a “Raw” folder.

- **Step 9**: Re-Run Dada2 algorithm
- **Step 10**: Generate the phylogenetic tree for the ASVs
- **Step 11**: Generate taxonomy for these ASVs (If you are not getting a very good taxonomy profile, use BLCA approach at the end of this Qiime2 tutorial)
- **Step 12**: Export all the files that Qiime2 generated
- **Step 13**: Attach the abundance table of ASVs with their corresponding taxonomy to generate the biom file that you will use in the downstream statistical analysis. For making Biome file compatible with R and phyloseq, please check https://github.com/joey711/phyloseq/issues/821. Go inside the "output" folder generated in the previous step and write these commands
- **Step 14**: Use Picrust2 to do the functional analysis https://github.com/picrust/picrust2/wiki
- **Step 15**: Export q2_picrust2_output files as biom files and then as TSV files