UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Comparative Biomedicine and Food Science

School of Agricultural Sciences and Veterinary Medicine

Second Cycle Degree (MSc) in
Biotechnologies for Food Science

Manila Clams Microbiome Biodiversity and Its Association With Seasonal and Environmental Variables

Supervisor:

Prof. Andrea Quagliariello

Submitted by:
Aline Karaki (Student ID: 2070782)

ACADEMIC YEAR
2023/2024

Abstract

The vital role of host microbiota in shaping the development and resilience of organisms is now widely recognized. With rising global populations placing a growing demand on food security, aquaculture is increasingly crucial for sustainable food production. To further explore this, we investigated the influence of the North Adriatic Sea's unique and challenging environment on the tissue-specific microbiota of clams. This fragile ecosystem faces mounting environmental pressures, making it crucial to understand how these factors influence the clams-microbiota relationship, in order to optimize clam health and resilience within aquaculture practices. To address this knowledge gap, we employed a bioinformatics approach to analyze the composition and function of microbiota across clam tissues (gills and digestive gland) in five sites throughout summer and winter 2019. This approach allowed for a detailed examination of the complex bacterial communities within the clams. Our findings revealed significant variations in the microbiota composition across both tissues and seasons. The digestive gland, in particular, exhibited the most diverse and balanced bacterial communities, also displaying the clearest seasonal shift between summer and winter. Additionally, differential abundance analysis revealed that sites affected by pollution harbored distinct microbiota compared to cleaner areas. Notably, the presence of the potential pathogenic Vibrio was observed in polluted areas during summer, while Rickettsiella was found to be abundant in the digestive gland during winter. This study provides valuable insights into the interplay between environmental factors, tissue specificity, and the clam-microbiota relationship within the Venice Lagoon's challenging environment. This knowledge can be instrumental in developing sustainable aquaculture practices that promote healthy and resilient clam populations, ultimately contributing to global food security.

Table of Contents

List of figures

List of Tables

1. Introduction

1.1. Aquaculture

With our planet's population on a non-stop rise, the need for sustainable, nutritious food sources becomes pressing. In order to meet the population's needs, new sustainable ways need to be implemented. Traditional land-based agriculture faces well-documented limitations, including high costs, inadequate macronutrient profiles, and reliance on fertilizers and pesticides (Carvajal-Muñoz & Carmona-Garcia, 2012). These practices can negatively impact plant growth and development, raising concerns about long-term sustainability. For this reason, aquaculture, the cultivation of aquatic organisms like fish, shellfish and algae, has emerged as a crucial solution in ensuring global food security. It plays a vital role in solving hunger and malnutrition by providing readily available sources of protein, essential fatty acids, vitamins, and minerals (Subasinghe et al., 2009; Kawarazuka, 2010). In addition, studies suggest that incorporating more seafood into our diets offers a multitude of health benefits including a reduced risk of chronic diseases, weight management and even childhood cognitive development (Lund, 2013). Aquaculture offers a distinct advantage over traditional land agriculture in terms of seasonal availability. Unlike terrestrial crops that are subject to the limitations of growing seasons, aquaculture allows for the year-round cultivation of aquatic species. This leads to a consistent and reliable supply of diverse seafood products, irrespective of the season (Islam, 2007). This consistency benefits both consumers who can access a wider range of fish throughout the year and businesses involved in the seafood industry who can maintain stable production cycles. Moreover, it is considered more sustainable than traditional agriculture, as we utilize areas that are often unsuitable for farming (Jayanthi et al., 2019; Subasinghe et al., 2009). This in turn reduces the use of land

and leaves a smaller environmental footprint compared to land-based animal farming (Jiang et al., 2022). Notably, certain aquaculture practices, like shellfish farming, actually improve water quality and clarity, contributing to a healthier ecosystem, due to the shellfish's characteristics of being filter-feeders, thus acting as natural biofilters, facilitating the removal of nitrogen and other nutrients from coastal waters (Rice, 2001; Shumway et al., 2003). By embracing sustainable aquaculture practices, a steady supply of nutritious seafood can be secured, while safeguarding natural resources for future generations.

1.2. Manila Clams

1.2.1. History and aquaculture

Considered a rising star in the world of sustainable aquaculture, the Manila clam (*Ruditapes philippinarum)* stands as the second most important bivalve species in fisheries and aquaculture (Cordero et al., 2017; Yoon et al., 2013). This is due to many reasons: i) they have minimal resource requirements to be cultivated; ii) they demonstrate remarkable adaptability to diverse coastal environments, and even thrive in some previously unsuitable aquatic areas (Dang et al., 2010); ii) in addition, they provide a source of protein, vitamins, minerals, and omega-3 fatty acids (Venugopal & Gopakumar, 2017). Finally, clams play a vital role in maintaining clean and healthy water ecosystems. Indeed, nutrient levels in aquatic environments can lead to excessive growth of microalgae. This is known as algal blooms, and it can disrupt the ecological balance and lead to oxygen depletion on the seafloor (Frascari et al., 1988). Since clams act as natural biofilters, they efficiently remove excess nutrients from the water column through filter feeding. By consuming phytoplankton, clams

help to regulate nutrient levels and prevent algal blooms, therefore contributing to a cleaner and healthier aquatic environment. All of these characteristics make them a highly suitable species for aquaculture, providing a healthy source of nutrients but also a more sustainable and environmentally conscious food system.

Manila clams originated from the Indo-Pacific region and quickly found their way to many countries for aquaculture purposes (Chiesa et al., 2011). In Europe, they were first introduced in France in the 1970s (Coelho et al., 2021), not reaching Italy until 1983, but despite that, Italy quickly became the leading European country for their production, accounting for 95 % of the total European yield, with an annual output of 33,500 tonnes of clams (Chiesa et al., 2011; FAO, 2020).

1.2.2. Manila clams and the North Adriatic Sea

Italy's shellfish farming industry thrives on two main bivalves: the Manila clam (*Ruditapes philippinarum)* and the Mediterranean mussel (*Mytilus galloprovincialis)* (Robert et al.,2013). These cultivated shellfish are perfect for the unique environmental conditions of the Northern Adriatic Sea. The heart of Italian clam farming lies within the highly productive coastal lagoons that border this sea (Bordignon et al., 2021; Sladonja et al., 2011). The key to the North Adriatic's success as a clam farming hotspot lies in the captivating complexity of the Venice Lagoon. This dynamic ecosystem presents a blend of complexity and uniqueness. Internal water movements and a diverse range of shapes and depths combine to create a constantly evolving ecosystem. Channels, shallow flats, and connections to the open sea all contribute to this dynamic characteristic. The lagoon is linked to the Adriatic Sea through a network of three narrow channels, ensuring a healthy exchange of water for the lagoon's

ecosystem (Bellafiore et al., 2008). This exchange of freshwater and saltwater brings a constant flow of nutrients, creating a rich feeding ground for clams, and making the north Adriatic Sea a hotspot for sustainable clam aquaculture.

However, this region faces a number of complex challenges. The North Adriatic Sea and, in particular, the Venice Lagoon, have faced significant pollution challenges for decades (Frascari et al., 1988), and these challenges remain a concern today (Pizzini et al., 2024; Basili et al., 2022). While the high sediment load carried by the Po River can dilute some pollutants (Riminucci et al., 2022), the primary source of the problem lies with the continuously growing human population around the lagoon. Waste from homes and industries, rich in nutrients like nitrogen and phosphorus, flows into the lagoon from surrounding cities (Çevirgen et al., 2020). Consequently, intensified agricultural practices to feed this population further deepens the problem by adding even more nutrients to the aquatic ecosystem.

The complex ecosystem is witnessing the effect of centuries of human activity (Bellucci et al., 2002). The industrial area of Porto Marghera stands out as a major source of contaminants, with heavy metals and persistent organic pollutants like PCDDs/Fs, PCBs, and HCB accumulating in the lagoon's sediments (Ravera, 2000; Bellucci et al., 2002; Frignani et al., 2004, 2005). Industrial activity in Porto Marghera began in the early 20th century, following World War I (Bellucci et al., 2002), coinciding with the reported increase in lagoon sediment contamination (Pavoni et al., 1992). While clam fishing practices may have contributed by redistributing some of these contaminants within the lagoon (Bellucci et al., 2002), the industrial activity remains the primary culprit. Mercury released during this period has also left a lasting impact (Rosati et al., 2020), leading to highly toxic sediments with documented DNA damage in marine organisms (Losso & Ghirardini, 2010).

The challenges that the Venice Lagoon is facing are due to profound human influence for the longest time (Ravera, 2000). The Venetian Republic itself significantly altered the lagoon's landscape through various modifications like expanding the canals, or making the city more attractive to tourists (Ravera, 2000), and today, the lagoon stands as a global example of the impact humans can have on their surrounding environment (Gieskes et al., 2015). The diverse industrial activities concentrated around Porto Marghera, ranging from chemical production to oil refining, has contaminated the air, soil, and water for decades, and it continues to challenge the health of the lagoon and its inhabitants (Pavoni et al., 1992; Bellucci et al., 2002; Guarino & Sciarrillo, 2017).

With the presence of these various contaminants in the habitat of Italy's valuable Manila clams, investigating the impact of these pollutants on clam health and potential human health risks becomes of great importance.

1.3. Microbiome

1.3.1. Definition

In order to maintain the health of clams and ensure the sustainability of clam aquaculture, it is crucial to understand the composition and function of their microbiome.

Although the idea of microbial communities existing within an organisms has been studied for centuries, the term “microbiome” did not appear until 2001, after Joshua Lederberg, microbiologist and Nobel Laureate, first used it to describe “an ecological system of commensal, symbiotic, and perhaps pathogenic microorganisms that reside in the human body” (Lederberg & McCray, 2001). Today, it is widely known that the microbiome goes

beyond just the human body, and the term is used to describe the microscopic organisms inhabiting a particular environment and the organisms themselves, typically including bacteria, fungi, and archaea (Liu, 2016). This community of microbes, referred to as "microbiota", forms a relationship with the host it inhabits, along a spectrum that ranges from mutualism, where both the bacteria and the host benefit, to pathogenicity, where the bacteria harm the host. Under the broader category of mutualism, we can define symbiosis and commensalism, where both parties gain some advantage or neither is harmed (Hooper & Gordon, 2001). These relationships can be observed across all organisms in the animal kingdom, some examples include on one end of the spectrum, the mutualistic symbiosis between squids and the bioluminescent bacteria *Vibrio fischeri*, where these bacteria provide the squid with light for anti-predation tactics, while the squid presents the bacteria with nutrients (Visick & McFall-Ngai, 2000). And on the other end of the spectrum, the relationship between *Xylella fastidiosa* and plants, that can range from commensalism, where the bacteria colonize the plant without causing harm, to pathogenicity, where the bacteria cause devastating plant diseases (Roper et al., 2019).

Several previous reserarch helped identify the numerous functions that microbiota plays within animals' physiology. These micro-organisms contribute significantly to their host's metabolism and fitness (Moran & Baumann, 2000), acting as nutritional supplements by synthesizing essential vitamins and amino acids (Wu et al., 2006). This partnership also extends to defense mechanisms, with the microbiome aiding the host in adapting to changing environmental conditions and even resisting disease (O'briend et al., 2019; Zilber-Rosenberg & Rosenberg, 2008). Research has even linked variations in gut microbiota to size and weight development in certain marine invertebrates (Sha et al. 2016).

An important feature of the microbiome is that is not static but rather dynamic. It is

constantly evolving throughout an organism's life in response to various factors, including maturation, dietary changes, environment, illnesses, and medical treatments (van Oppen & Blackall, 2019; Gerber, 2014). Microbial colonization is known to begin at birth and is shaped by a multitude of influences, establishing a foundation for the complex and vital functions it performs (van Oppen & Blackall, 2019; Zhou et al., 2017; Lema et al., 2014). A significant disruption in this composition, termed dysbiosis, can manifest as a loss of beneficial bacteria, reduced overall diversity, or an increase in potentially harmful pathogens within the microbiome (Walker, 2017), often leading to disease expression (Petersen & Round, 2014).

The following sections will delve deeper into the factors that can influence this delicate balance within the microbiome.

1.3.2. Microbiome of the Manila Clam

Similar to other organisms, the clam's microbiota plays a crucial role in their health and well-being, forming a mutually beneficial relationship. Studies have revealed that Proteobacteria are the dominant phylum within the clam microbiome, typically accounting for over 80% of the bacterial community (Leite et al., 2017). Other phyla commonly found include Firmicutes, Bacteroidetes, and Chlamydiae (Milan et al., 2018). At the genus level, Mycoplasma is often the most abundant, with Arcobacter and representatives from families such as Rhodobacteraceae and Endozoicimonaceae also frequently identified (Milan et al., 2018). Research also suggests that the composition of the microbiota can vary significantly between different clam tissues (Meisterhans et al., 2016). This variation likely reflects the specific functions performed by each tissue, with the microbiota composition adapting to support these functions.

1.3.3. The effect of the environment and the diet on microbiome biodiversity

1.3.3.1. Environmental Impact

The environment plays a crucial role in shaping animal microbiomes, with various stressors significantly affecting these microbial communities. For instance, during the warmer months, rising temperatures can increase the concentration of inorganic nitrogen compounds due to the rapid decomposition of organic matter, leading to decreased dissolved oxygen and pH levels in water. Such changes were found to impact bacterial communities in aquatic systems (Li et al., 2017). Moreover, pollution has a marked influence on intestinal bacterial communities. In heavily polluted environments, such as Jakarta Bay, there is a dominance of Vibrionales in wild shrimp, while cleaner environments and aquaculture facilities show a higher abundance of Alteromonadales, along with an overall higher alpha diversity in comparison with the polluted site (Oetama et al., 2016).

Environmental stressors can lead to bacterial dysbiosis, where the normal microbial balance is disrupted, potentially resulting in disease. For example, high mortality rates in aquatic animals have been linked to concurrent heat stress and changes in bacterial community structure, favoring opportunistic pathogens like *Arcobacter* and *Vibrio* (Green et al., 2019). This shift is often accompanied by a decrease in beneficial bacteria, allowing opportunistic microbes to become infectious (Boutin et al., 2013). The microbiome's role in an organism's adaptation to changing environments is increasingly recognized, with evidence suggesting that a stable host-microbiome association is vital for adaptation (Alberdi et al., 2016). Overall, both field and experimental studies indicate that environmental stressors can induce bacterial dysbiosis, leading to health issues in animals (Infante-Villamil et al., 2021).

1.3.3.2. Dietary Influence

Diet also has a profound effect on the microbiome composition and, as a consequence, on the host's health. For instance, in the shrimp *Penaeus vannamei*, partial replacement of fish meal with dehulled oil-extracted soybean meal negatively affected growth, feed efficiency, and innate immunity, while also leading to the development of an unfavorable gut microbiome (Hu et al., 2019). Similarly, in the Chinese mitten crab *Eriocheir sinensis*, the anti-nutritional factor glycinin found in soybean disrupted gut microbiome balance and impeded growth (Han et al., 2019, 2020). Researches also agree that the levels of proteins, lipids, and carbohydrates in an animal's diet can significantly impact both the microbiome and overall animal performance (Qiao et al., 2017; Sun et al., 2018).

Diet-induced dysbiosis is a notable concern. Diets high in soybean oil have been associated with decreased bacterial alpha-diversity and an increased abundance of potentially pathogenic bacteria like Mycoplasma and Vibrio, while reducing beneficial bacteria such as Bacillus and Lactococcus, and conversely, diets rich in long-chain fatty acids have a beneficial effect, promoting the growth of these beneficial bacteria in the carnivorous marine fish golden pompano (*Trachinotus ovatus*), (You et al., 2019). Additionally, dietary copper has been shown to increase the abundance of potential pathogens like Vibrio in the gut of shrimp, even if it does not affect the shrimp's performance directly (Zhou et al., 2017).

1.3.3.2. Effect of environment and diet on Manila Clams microbiome

The Manila clam exemplifies how environmental factors and diet interact to influence the microbiome. It has been known for decades, that the growth of Manila clams is primarily determined by water temperature and food availability (Chew, 1989; Shpigel & Fridman, 1990). Laboratory studies have shown that meat growth in clams is highest at 12°C and

decreases at higher temperatures, with no significant differences noted in shell growth (Mann & Glomb, 1978; Mann, 1979). More recent studies also agreed that seasonal changes affect the microbiome, with higher bacterial diversity observed in winter compared to summer (Milan et al., 2018; Meisterhans et al., 2016).

Environmental pollutants also have a significant impact on the clam microbiome. Clams from contaminated sites exhibit distinct transcriptomic signatures related to drug metabolism, detoxification processes, and immune response (Milan et al., 2013). This pollution can also open the doors to several opportunistic pathogens to infect the clams. Changes in the microbiome composition have been noted from many studies that investigated clams in polluted zones, and the most concerning finding was the abundance of Vibrio is several of the samples collected (Zhu et al., 2023; Zampieri et al., 2020). Moreover, the microbiota in clams' digestive glands can be modified by environmental fluctuations and direct acquisition of microbial species from the surroundings (Beleneva & Zhukova, 2009; Dubilier et al., 2008). Geographic variations also significantly influence microbial composition, with distinct bacterial communities observed in different habitats (Meisterhans et al., 2016).
Overall, the interaction of seasonality and exposure to toxicants substantially affects clam microbiota, mirroring the host's response to environmental changes (Milan et al., 2018). This complex interplay between environment and diet reveals how crucial they are for maintaining a healthy and balanced microbiome in Manila clams.

1.3.4. Effect of disease on the microbiome

The impact of health status on the microbiome has been extensively documented, highlighting that disease states often correlate with reduced microbial diversity. A decrease in alpha diversity is commonly associated with diseased conditions (Infante-Villamil et al.,

2021). Also, the overall microbiome diversity can be affected by health status (Wang et al., 2019). For instance, in the Pacific oyster *Crassostrea gigas*, heat stress alone did not cause mortality, but when combined with infection by the pathogen Vibrio, it triggered infections by opportunistic pathogens such as Arcobacter. In these cases, diseased individuals showed a high abundance of Arcobacter and reduced bacterial diversity, whereas infected survivors and control groups did not exhibit such a dramatic decrease in diversity (Lokmer & Wegner, 2015). Similarly, in the sea cucumber *Apostichopus japonicus*, animals suffering from Skin Ulceration Syndrome exhibited dysbiosis characterized by decreased alpha diversity as well as changes in beta diversity (Zhang et al., 2018). Interestingly, it was found that bacterial diversity did not decline at the onset of disease but often decreases as the disease progresses (Xiong et al., 2017).

For clams, the health condition significantly affects microbiome composition, especially in polluted environments. Being bivalves, Manila clams are sedentary, filter-feeding organisms that tend to accumulate metals and other pollutants in their tissues, particularly the gills and digestive gland (Milan et al., 2013). This accumulation can lead to significant health impacts, including the upregulation of genes involved in xenobiotic metabolism, as observed in clams from Porto Marghera, a highly polluted area (Iannello et al., 2021; Matozzo et al., 2010; Apitz et al., 2007). The interaction between toxicant exposure and microbiota changes in clams often mirrors the host's response to environmental variations (Milan et al., 2018).

In the Venice Lagoon, Porto Marghera's sediments were found to have some of the highest contaminant levels among the sites studied (Apitz et al., 2007). Such extreme levels of pollution significantly affect the microbiota associated with clams, with notable differences in the hepatopancreas-associated microbiota between clams from Porto Marghera and those from less polluted areas (Iannello et al., 2021). The exposure to these contaminants not only

impacts microbial diversity but also drives the upregulation of genes involved in detoxification processes, highlighting a complex interplay between the clams' health and their microbiome composition (Iannello et al., 2021).

1.4. Aim of the thesis

This thesis aims to investigate the impact of environmental factors, including pollution, on the Manila clam microbiome in the North Adriatic Sea. Utilizing a bioinformatics approach, we will analyze the composition of bacterial communities within both the digestive gland and gills of clams collected from five sites during summer and winter of 2019. The research focuses on comparing alpha and beta diversities, alongside differential abundance analyses, to understand how spatiotemporal variations influence the health and balance of the clam microbiome in relation to several environmental variables (i.e., Oxygen, Temperature, Conductivity, Salinity and pH). Understanding these interactions can ultimately be used to develop strategies for promoting healthy clam populations and safeguarding the balance of marine ecosystems.

2. Materials and methods

2.1. Data Collection

This study investigated the bacterial communities from Manila clams (*Ruditapes philippinarum*) collected in different farming sites and one polluted area. Clams were collected across two seasons (summer 2019 and winter 2019) from five locations within the Venice Lagoon and surrounding areas (Figure 1). At each location, approximately 100 clams were collected from four different farming areas (Chioggia (CH), Colmata (CO), Marano Lagunare (MA) and Scardovari (SC)), and one polluted site (Porto Marghera (PM)), using a mechanical rake, adhering to regulations for commercial bivalve harvesting. After collection, the clams were placed in a depuration center for at least 16 hours. Here, they were kept in a flowing seawater system with mechanical, biological, and ultraviolet (UV) filtration. Following depuration, the clams were transported to the laboratory where gill (GL) and digestive gland (DG) tissues were carefully dissected from each clam using sterilized scalpels. These tissue samples were immediately transferred to tubes containing 90% ethanol and refrigerated for further analysis.

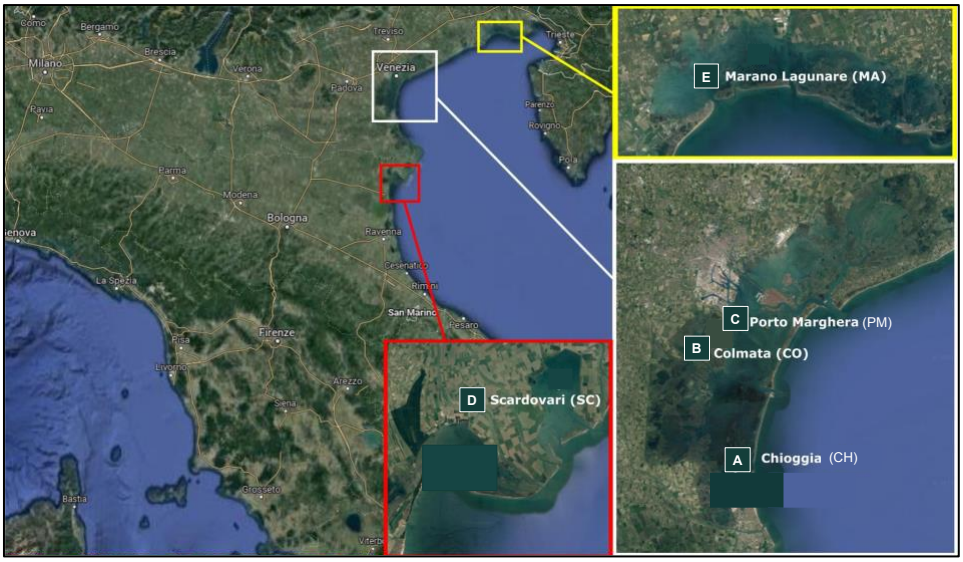

Figure 1. North Adriatic sea and Venice Lagoon: areas of study

2.2. DNA extraction and 16S gene Sequencing

For DNA analysis, tissues from 5 samples (each one composed of 10 individuals) within the same location and sampling period (either gill or digestive gland) were extracted and purified using a commercial kit (QIAGEN DNA Power Soil) with an additional proteinase K treatment to enhance cell disruption (Table 1). The quality and quantity of extracted DNA were assessed using gel electrophoresis and a NanoDrop 1000 instrument. Finally, DNA aliquots were sent to BMR Genomics (Padua, Italy) for sequencing. This sequencing process targeted a specific region of the 16S ribosomal RNA gene (V3-V4) using MiSeq technology, generating paired-end reads of 300 base pairs each.

	CH		CO		MA		PM		SC		Total
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	
DG	5	5	5	5	5	5	5	5	5	5	50
GL	5	5	5	5	5	5	5	5	5	5	50
SED	3	3	3	3	3	3	3	3	3	3	30
Total	13	13	13	13	13	13	13	13	13	13	130

Table 1. Number of samples analysed for each tissue type in each site and season

2.3. Environmental data

In order to investigate the possible association between clam microbiome and environmental conditions in 2019, we collected various environmental variables for the summer and winter months, which were available via the "Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto" (ARPAV; https://www.arpa.veneto.it/). The ARPAV pursues two closely related objectives: (i) protection, through environmental controls that protect the health of the population and the safety of the territory; (ii) prevention, through research, training, information, and environmental education. For the present study we considered the following variables: Temperature, conductivity, salinity, dissociated oxygen level and pH of the water.

2.4. Bioinformatics analyses

All subsequent analyses were performed in R (v4.3.3) using mainly the *phyloseq* package (v1.46.0) and other relevant packages mentioned below.

2.4.1. Analysis of environmental variables

Descriptive statistics were calculated, and preliminary graphs were generated to compare environmental parameters within and across sampling sites using *dplyr* and *ggplot* packages in R. Additionally, the Kruskal-Wallis and Wilcoxon tests were used to compare the different groups of interest: The Kruskal-Wallis test is a non-parametric alternative to ANOVA, to compare if medians of a variable differ significantly across multiple groups defined by another variable, (McKight & Najab, 2010), while the Wilcoxon test is used to compare a variable between all possible pairs of groups defined by another variable (Rosner et al., 2006). The obtained p-value would determine how significant the difference between the studied groups is. Both tests were performed using functions from the base R package stats: *Kruskal.test* and *Wilcox.test*.

2.4.2. Alpha diversity

In an ecological context, alpha diversity serves as an important measure of biodiversity within a habitat. It helps us identify the richness of species: the number of different species present (i.e., Observed index), but also its evenness, their relative abundance (i.e., Shannon index). A more specific index used to quantify alpha diversity is the Shannon index. It is based on the distribution of the individuals within a species and takes the proportion of each species into account, giving an accurate description of the diversity in an ecosystem (Konopiński, 2020). Hence, a higher Shannon value indicates greater diversity in terms of

species present and their relative abundances. Alpha diversities were calculated between seasons (summer, winter), between tissues (DG, GL, SED) and between sites for each tissue (CH, CO, MA, PM, SC). In this study, alpha diversity was calculated using the *phyloseq* function *estimate_richness*.

2.4.3. Beta diversity

On a broader scale, beta diversity becomes more useful, as it measures the relation among different habitats based on their microbiome composition, helping us identify how similar or dissimilar the different areas are. Metrics such as Bray-Curtis dissimilarity, Jaccard similarity are used to quantify the beta diversity. In this study, Bray-Curtis dissimilarity index was used. This index is used to quantify the compositional dissimilarity between two different sites or samples, based on the abundances of species (Bray & Curtis, 1957). Several visualization techniques are then used to visualize the results obtained from the previously mentioned metrics such as Principal Coordinates Analysis (PCoA). It projects the dissimilarity data onto orthogonal axes, with each axis representing a principal coordinate. Sites or samples with similar species composition will cluster together in the plot, while those with dissimilar composition will be farther apart (Xia, Y., & Sun, J., 2023). Beta diversity was calculated using the *phyloseq* functions *ordinate* and *distance*. Three-dimensional plot of beta diversity was generated using the *plotly* package (v4.10.4) (Sievert et al., 2021). Then a beta diversity plot was generated for each tissue type alone, with samples colored by site and shaped by season. Finally, Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) was calculated using *adonis2* from the vegan package (v2.6-4) (Oksanen et al., 2013).

2.4.4. Differential Abundance Analysis

Differential abundance analysis is a technique used to identify microorganisms that exhibit significant differences in abundance between different conditions within a dataset. It was performed in our study to determine which bacterial populations responded differently to seasonal changes across the three studied tissues. We used the R package *DESeq2* (v1.42.1) to statistically assess these differences while accounting for potential variations in sequencing depth. *DESeq2* is a package that helps scientists identify significant differences in gene expression levels. It does this using a special type of statistical model that accounts for variations in how genes are expressed (Love et al., 2014). This allows us to pinpoint key bacterial taxa that may contribute to the observed seasonal shifts within the digestive gland, gill, and sediment microbiomes.

2.4.5. Phylum and Genus composition

To quantify bacterial community composition within the tissues, the abundance of each genus and phylum was determined by summing the reads assigned to those taxonomic ranks. To do that, the data was first grouped by the taxonomic rank "Genus", the smallest taxonomic level we are interested in, using the *tax_glom* function from the *phyloseq* package. Normalization was then performed to account for variations in sequencing depth across samples. This involved calculating the relative abundance for each genus and phylum by dividing their read counts by the total number of reads per sample using another *phyloseq* function, *transform_sample_counts*. Finally, these normalized abundances were used to construct box plots, visualizing the distribution of each genus and phylum proportion by site and season within the digestive gland and gill tissues, and the sediment substrates. Several functions of the *dplyr* package were used (v1.1.4) to manipulate the data (*filter, group_by, mutate, select,*

summarise, ungroup) (Wickham et al., 2023).

2.4.6. Correspondence analysis

To understand how bacterial communities interact with their environment within the tissues, correspondence analysis (CA) was employed. This method is particularly useful because it can visualize relationships between different variables, which is exactly what we need (Greenacre, 2010). In this context, CA will create a biplot, where samples and bacterial genera are positioned based on their abundance patterns. By analyzing the relative positions of samples and genera in the biplot, we can identify potential correlations between specific environmental variables (season and site) and the observed bacterial communities. This allows us to explore which environmental factors are associated with the presence or absence of particular bacterial genera. For statistical comparisons and interactive visualizations, a list of R packages was used: for comparison and interactive visualization: *digest* (v0.6.35) and *ranacapa* (v0.1.0), for ecology and microbiome data analysis: *phyloseq* (v.1.46.0), *microbiome* (v1.24.0) and *vegan* (v2.6-4), (Eddelbuettel et al., 2024; Kandlikar et al., 2018; McMurdie & Holmes, 2013; Lahti & Shetty, 2018; Oksanen et al., 2013)

2.4.7. Data visualization

All graphs and visualizations were created using several functions from the *ggplot2* package (v3.5.0) in R, (Wickham et al., 2016).

3. Results

3.1. Analysis of environmental variables:

3.1.1. Analysis of environmental and seasonal differences during 2019.

Wilcoxon signed-rank tests revealed significant seasonal changes in several environmental parameters across all sites in 2019 (Table 2). Water temperature exhibited the most dramatic shift, with a highly significant difference (p-value < 2.22e-16) between summer (average: 21.36 °C) and winter (average: 11.81 °C). Similarly, dissolved oxygen levels displayed a significant decrease (p-value = 5.7e-16) from winter (average: 7.76 mg/L) to summer (average: 6.33 mg/L). Water pH also showed a significant rise (p-value < 2.22e-16), averaging 8.09 in winter and 8.44 in summer. Conversely, no significant seasonal differences were observed for conductivity (p-value = 0.12) and salinity (p-value = 0.25). In conclusion, the year 2019 witnessed significant seasonal variations in temperature, oxygen levels, and pH across all sites studied.

Year	Variable	Summer	Winter	p-value	Significance
2019	Temperature (°C)	21.36	11.81	p<2.22e-16	****
	Conductivity (µS/cm)	44	45.43	0.12	ns
	Salinity (psu)	28.35	30.01	0.25	ns
	Oxygen (mg/l)	6.33	7.76	5.70E-16	****
	pH	8.44	8.09	p<2.22e-16	****

Table 2. Averages and significance of the environmental variables between seasons in 2019

3.1.2. Analysis of environmental and seasonal differences for Chioggia (CH)

Similar to the overall trend across all sites, CH exhibited significant seasonal variations in several environmental parameters in 2019 (Table 3). Water temperature displayed the most dramatic change, with a highly significant difference (p-value = 8.7e-10) between winter (average: 10.85 °C) and summer (average: 20.08 °C), representing a near 10-degree increase. pH also showed a significant rise (p-value = 5.8e-10), averaging 8.14 in winter and 8.57 in summer. Interestingly, unlike the overall trend, conductivity and salinity displayed significant seasonal decreases at site CH. Conductivity dropped from an average of 54.63 µS/cm in winter to 47.37 µS/cm in summer (p-value = 2.4e-09), and salinity decreased from 33.79 to 30.50 (p-value = 6e-06). Finally, dissolved oxygen levels showed a slight but significant decrease (p-value = 0.04), averaging 7.36 mg/l in winter and 7.10 mg/l in summer. These observations suggest unique seasonal patterns in conductivity and salinity specifically at site CH compared to the overall trend across all sites.

Site	Variable	Summer	Winter	p-value	Significance
CH	Temperature (°C)	20.08	10.85	8.70E-10	****
	Conductivity (µS/cm)	47.37	54.63	2.40E-09	****
	Salinity (psu)	30.5	33.79	6.00E-06	****
	Oxygen (mg/l)	7.1	7.36	0.04	*
	pH	8.57	8.14	5.80E-10	****

Table 3. Averages and significance of the environmental variables between seasons in CH

3.1.3. Analysis of environmental and seasonal differences for Colmata (CO)

Out of the measured environmental variables in CO, only conductivity, oxygen levels, and pH exhibited significant changes between summer and winter (Table 4). While temperature showed a decrease from a summer average of 20.10°C to 13.33°C in winter, this difference was not statistically significant (p-value = 0.049). Similarly, salinity variations between seasons (winter average: 33.45 psu, summer average: 31.72 psu) were not significant (p-value = 0.45). In contrast, conductivity, oxygen levels, and pH all displayed highly significant seasonal differences. Conductivity was higher in winter (average: 52.41 µS/cm) compared to summer (average: 49.09 µS/cm) with a p-value of 6.2e-14. Likewise, oxygen levels were significantly higher in winter (average: 7.65 mg/l) than in summer (average: 6.43 mg/l) with a p-value of 5.4e-15. Finally, pH levels were also significantly higher in summer (average: 8.53) compared to winter (average: 7.87) with a p-value of 4.2e-15.

Site	Variable	Summer	Winter	p-value	Significance
CO	Temperature (°C)	20.1	13.43	0.049	*
	Conductivity (µS/cm)	49.09	52.41	6.30E-14	****
	Salinity (psu)	31.72	33.45	0.45	ns
	Oxygen (mg/l)	6.43	7.65	5.40E-15	****
	pH	8.53	7.87	4.30E-15	****

Table 4. Averages and significance of the environmental variables between seasons in CO

3.1.4. Analysis of environmental and seasonal differences for Marano Lagunare (MA)

In MA, temperature displayed the most significant seasonal change, with a p-value of 8.3e-07. The summer average of 23.24°C dropped considerably to an average of 11.62°C in

winter. Conductivity also exhibited a significant difference between seasons (p-value = 0.0018), decreasing from a summer average of 37.85 µS/cm to 29.92 µS/cm in winter, which were the lowest conductivity values across all sites. Similarly, winter witnessed a slight but significant increase in oxygen levels (p-value = 0.017), with the average rising from 5.23 mg/l in summer to 5.92 mg/l in winter. Interestingly, pH showed a significant decrease (p-value = 0.0095) from a summer average of 8.36 to 8.07 in winter. Unlike the other variables, salinity remained unchanged between seasons (p-value = 0.45), with averages being around 24.73 psu in summer and 25.56 psu in winter (Table 5).

Site	Variable	Summer	Winter	p-value	Significance
MA	Temperature (°C)	23.24	11.62	8.30E-07	****
	Conductivity (µS/cm)	37.85	29.92	0.0018	**
	Salinity (psu)	24.73	25.56	0.45	ns
	Oxygen (mg/l)	5.23	5.92	0.017	*
	pH	8.36	8.07	0.0095	**

Table 5. Averages and significance of the environmental variables between seasons in MA

3.1.5. Analysis of environmental and seasonal differences for Porto Marghera (PM)

In PM, all measured environmental variables except for pH exhibited significant seasonal changes (Table 6). Summer temperatures (average: 20.28°C) dropped significantly in winter (average: 12.65°C) with a p-value of 0.0058. Similarly, conductivity increased significantly from a summer average of 42.99 µS/cm to 49.68 µS/cm in winter (p-value = 5.8e-08). Salinity also displayed a significant rise between seasons (p-value = 5.6e-08), with winter values (average: 31.44 psu) being higher compared to summer (average: 27.43 psu).

Interestingly, unlike the other variables, pH remained relatively stable across seasons, with summer and winter averages at 8.30 and 8.25, respectively. Finally, dissolved oxygen levels followed the opposite trend, with a significant decrease (p-value = 4e-08) from a summer average of 8.43 mg/l to only 4.78 mg/l in winter, the lowest conductivity level across all sites.

Site	Variable	Summer	Winter	p-value	Significance
PM	Temperature (°C)	20.28	12.65	5.80E-03	**
	Conductivity (µS/cm)	42.99	49.68	5.80E-08	****
	Salinity (psu)	27.43	31.44	5.60E-08	****
	Oxygen (mg/l)	4.78	8.43	4.00E-08	****
	pH	8.3	8.25	1	ns

Table 6. Averages and significance of the environmental variables between seasons in PM

3.1.6. Analysis of environmental and seasonal differences for Scardovari (SC)

SC exhibited the most significant seasonal temperature change (p-value < 2.22e-16), with summer temperatures (average: 23.12°C) dropping considerably to winter averages (10.39°C). This represents the lowest summer temperature average across all sites. Unlike PM, pH in SC also showed a significant seasonal decline (p-value = 1.2e-09), going from a summer average of 8.48 to 8.18 in winter. Interestingly, winter witnessed a significant increase in oxygen levels (p-value = 5.8e-06) compared to summer. Oxygen levels rose from an average of 8.09 mg/l in summer to 9.47 mg/l in winter. In contrast to these significant changes, salinity and conductivity levels in SC remained relatively stable across seasons. Summer salinity (average: 27.39 psu) and conductivity (average: 42.69 µS/cm) did not differ

significantly from winter values (salinity: 25.81 psu, conductivity: 40.55 µS/cm), with p-values of 0.045 and 0.079, respectively (Table 7).

Site	Variable	Summer	Winter	p-value	Significance
SC	Temperature (°C)	23.12	10.49	p<2.22e-16	****
	Conductivity (µS/cm)	42.69	40.55	0.079	ns
	Salinity (psu)	27.39	25.81	0.045	*
	Oxygen (mg/l)	8.09	9.47	5.80E-06	****
	pH	8.48	8.15	1.20E-09	****

Table 7. Averages and significance of the environmental variables between seasons in SC

3.2. Alpha Diversity:

Alpha diversity was calculated for all the different tissues, seasons, and sites. We used two different indices: (i) the Observed richness, which reflects the total number of unique species identified, and (ii) the Shannon index, which incorporates both species richness and evenness.

3.2.1. Alpha diversity analysis by tissue:

Investigating alpha diversity across tissue types revealed pronounced patterns. Notably, significant differences were observed between clam tissues (DG and GL) and the surrounding sediments. Within the clam tissues, the digestive gland displayed a higher observed species richness, evident by its elevated mean value. This trend was mirrored by the Shannon index, showing both higher values and less variation in the DG compared to the gills. Interestingly, the alpha diversity of the sediments far exceeded that of both clam tissues. The observed species richness in the sediments presented a mean well above 1000, further supported by a high and tightly clustered Shannon index. These findings suggest that the digestive gland harbors a greater number of unique and more evenly distributed species compared to the gills. Furthermore, the sediment environment exhibited a remarkably higher richness and evenness of species compared to both clam tissues. To validate these observations, a Kruskal-Wallis test was employed, aligning with the initial visual assessment. The test yielded statistically significant p-values ($p < 0.05$) for all comparisons between tissues and sediment for both observed and Shannon indices. Notably, the p-values were particularly low ($p < 0.001$) when comparing the sediments to the clam tissues, highlighting the substantial difference in diversity between these environments (Figures 2 and 3).

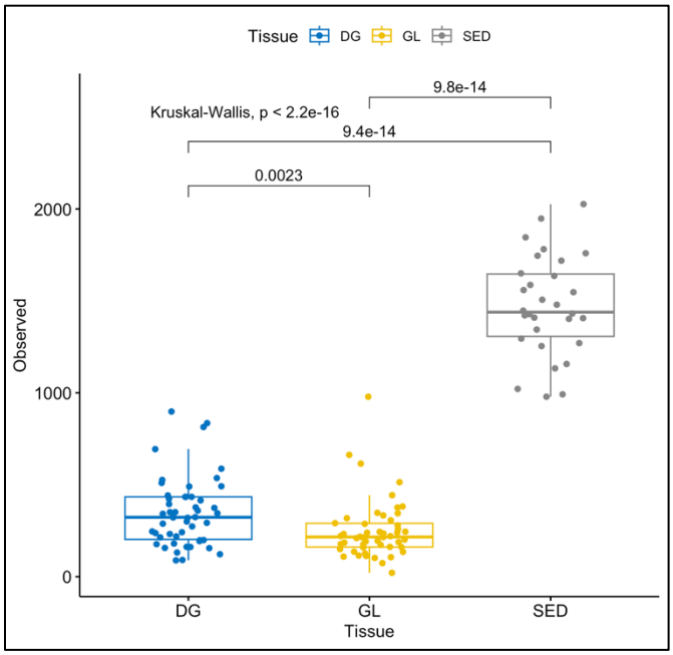

Figure 2. Kruskal-Wallis and Wilcoxon tests of alpha diversity by tissue - Observed

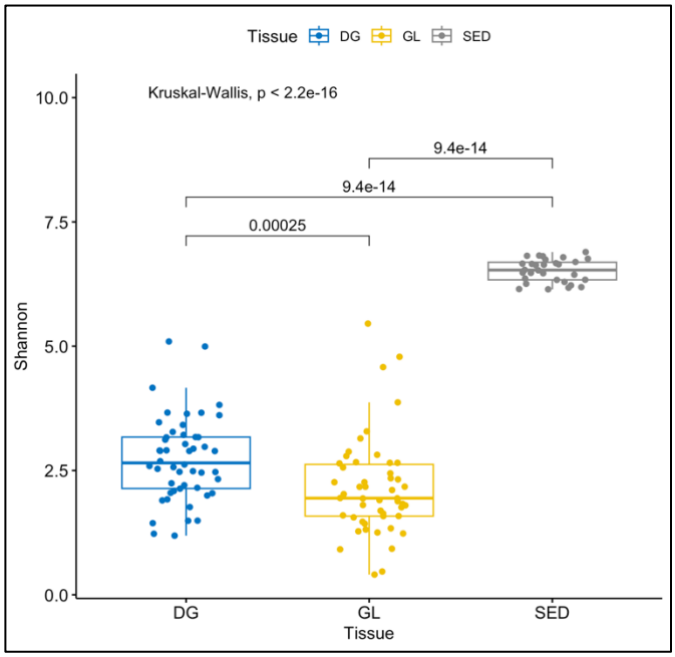

Figure 3. Kruskal-Wallis and Wilcoxon tests of alpha diversity by tissue - Shannon

3.2.2. Alpha diversity analysis by season:

Observed species richness displayed minimal variation between summer and winter (Figure 4). The mean values for both seasons were nearly identical, suggesting that the studied areas harbored a very similar number of unique species irrespective of the season. However, the Shannon diversity index, showed a slightly more pronounced seasonal difference, but it is still not statistically significant (Figure 5). While the mean Shannon index remained relatively close between summer and winter, summer exhibited a marginally higher value. This indicates that while the total number of species found might be similar across seasons, summer displayed a slightly greater evenness in species distribution compared to winter. To statistically verify these observations, a Wilcoxon signed-rank test was conducted. The test results agreed with our findings, demonstrating no significant differences in observed species richness (p-value = 0.79) and Shannon diversity index (p-value = 0.20) between summer and

winter. This suggests that the observed seasonal patterns may not be statistically robust.

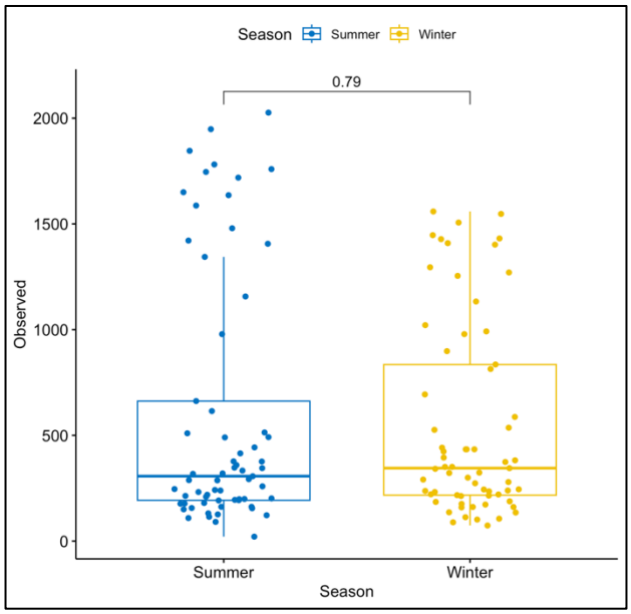

Figure 4. Wilcoxon test of the alpha diversity by season - Observed

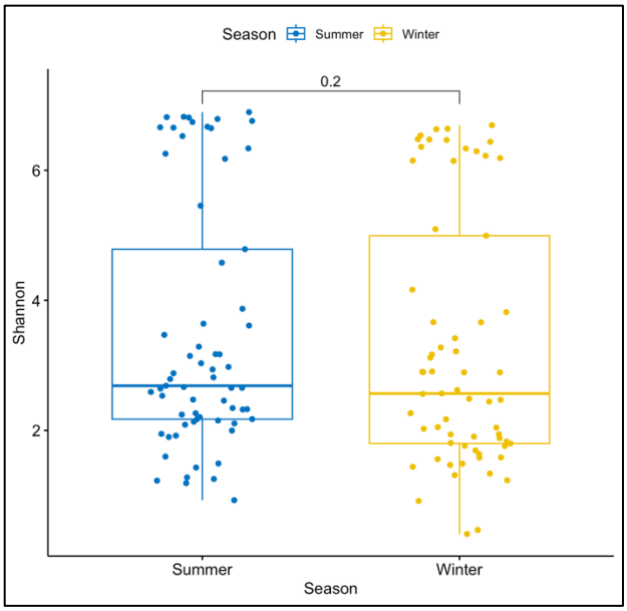

Figure 5. Wilcoxon test of the alpha diversity by season - Shannon

3.2.3. Alpha diversity analysis by site and tissue:

3.2.3.1. DG:

Analysis of alpha diversity within the DG revealed no significant variation between sampling sites (CH, CO, MA, PM, and SC). Both observed species richness (Figure 6) and the Shannon diversity index (Figure 7) displayed minimal fluctuations across locations. Notably, all sites exhibited similar species richness and evenness, with MA and PM showing a slight, but non-significant, tendency towards higher values. A Kruskal-Wallis test confirmed this observation, with all p-values exceeding 0.05, except for the comparison between CO and SC (p-value = 0.033). However, the overall significance level for both observed and Shannon indices remained non-significant (0.15 and 0.47, respectively).

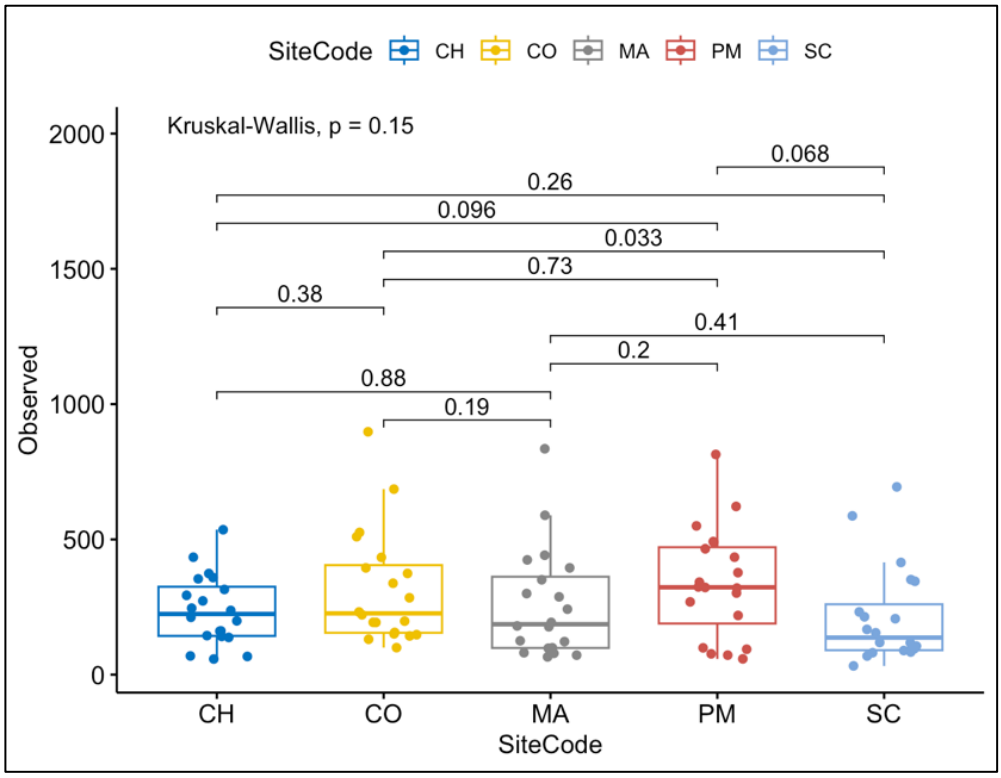

Figure 6. Kruskal-Wallis and Wilcoxon tests of alpha diversity by site (DG) – Observed

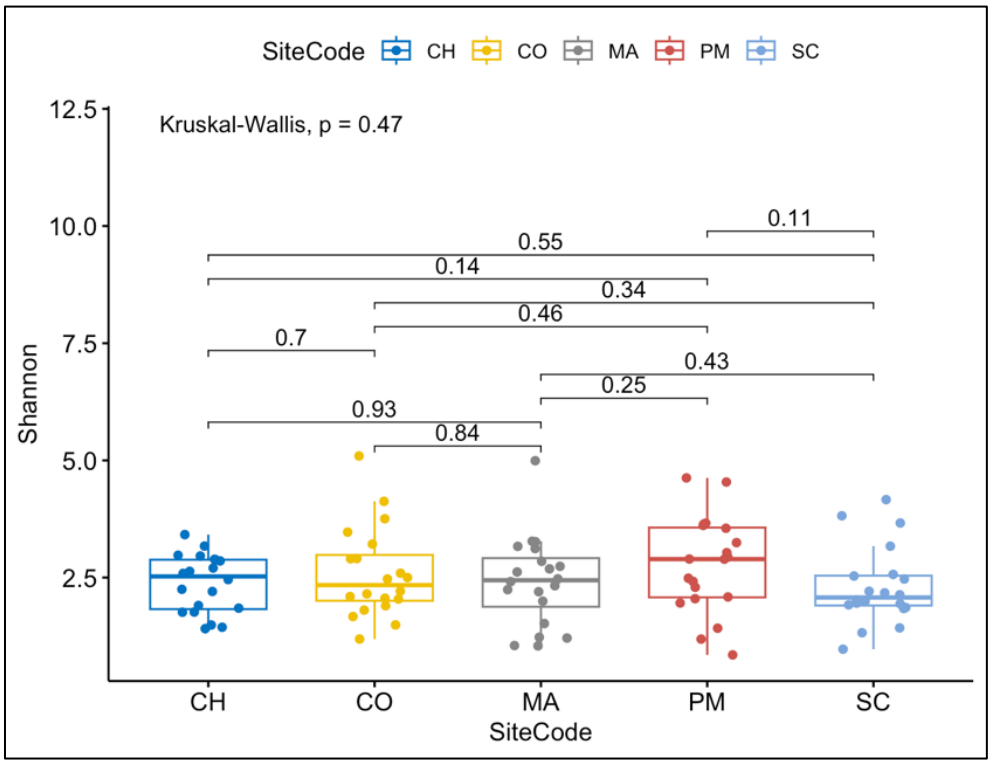

Figure 7. Kruskal-Wallis and Wilcoxon tests of alpha diversity by site (DG) – Shannon

3.2.3.2. GL:

In contrast, alpha diversity within the GL yielded more intriguing results. The Kruskal-Wallis test (Figure 8) revealed a significant overall difference between sites (p-value = 0.013). There appears to be significant differences specifically between SC and the remaining sites: PM: p-value = 0.0047, CO: p-value = 0.0073, and MA: p-value = 0.028. These findings suggest that SC exhibits the most distinct alpha diversity compared to other locations. The Shannon diversity index (Figure 9) presented even stronger statistical significance (p-value = 3.6e-06), with all pairwise comparisons between sites significant except for CH and SC, CO, and MA and finally PM and MA.

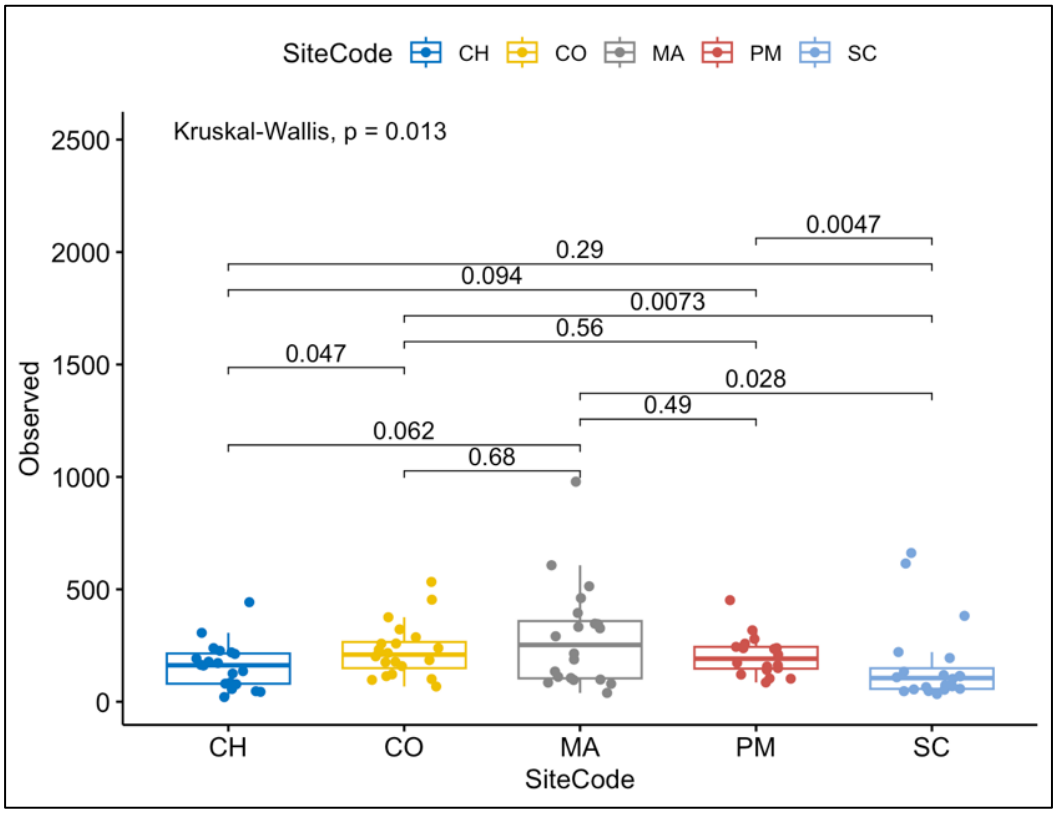

Figure 8. Kruskal-Wallis and Wilcoxon tests of alpha diversity by site (GL) - Observed

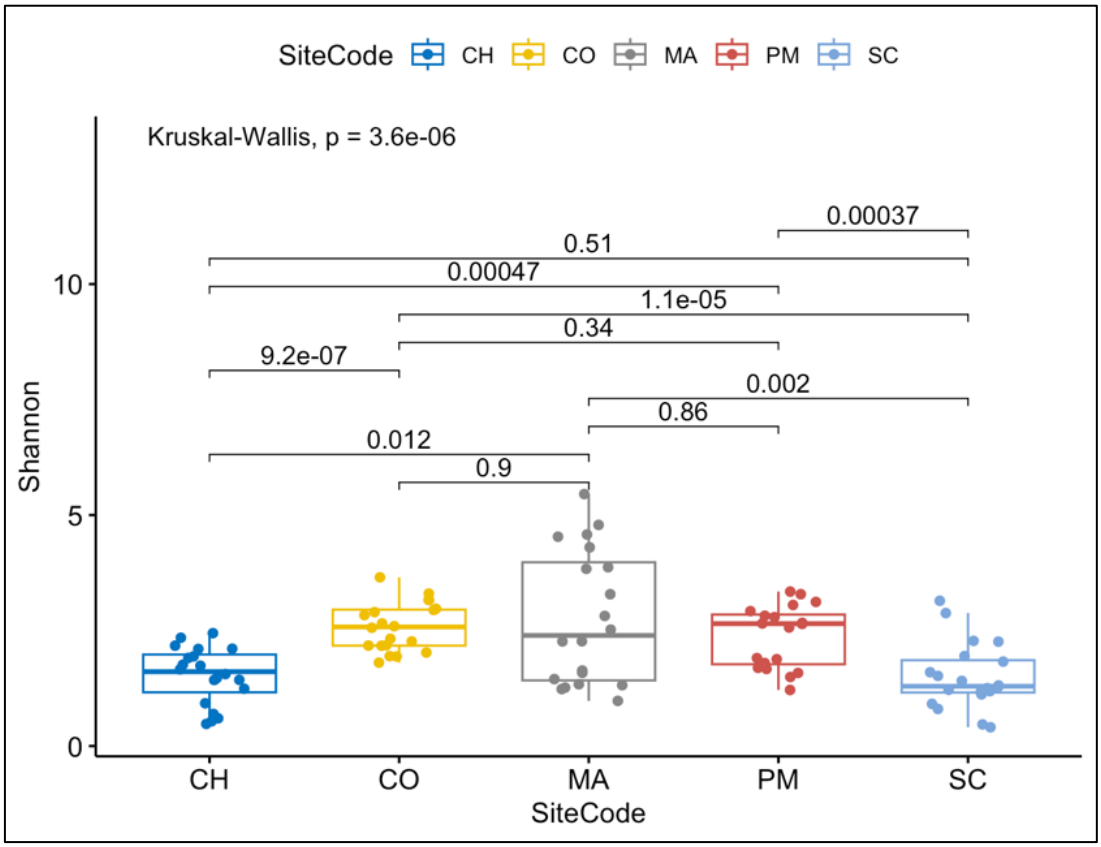

Figure 9. Kruskal-Wallis and Wilcoxon tests of alpha diversity by site (GL) - Shannon

3.3. Beta Diversity:

3.3.1. Beta-Diversity analysis on the entire dataset:

A Principal Coordinate Analysis (PCoA) plot revealed a clear separation of microbial communities across the three tissue types (DG, GL, and SED). The plot depicted three distinct clusters, with no overlap between groups, suggesting significant dissimilarity in species composition between the tissues (Figure 10). This observation was confirmed with a PERMANOVA test resulting in a significant p-value of 1e-04. However, to gain a more comprehensive understanding of the community structure, we employed a 3D PCoA plot for further analysis.

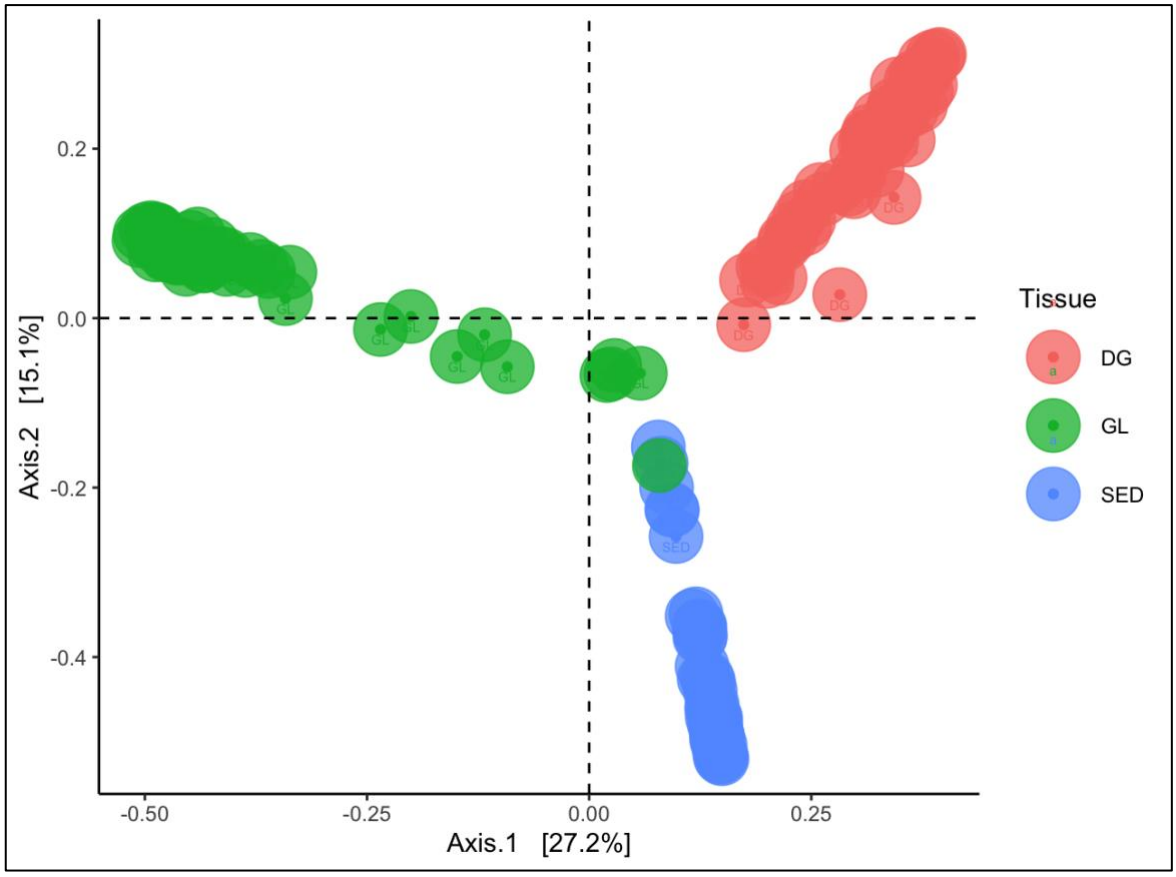

Figure 10. Bray-Curtis PCoA on the entire dataset

The 3D PCoA plot incorporating seasonality provided a more nuanced view of beta diversity within each tissue (Figure 11). For GL, winter samples exhibited tighter clustering compared to summer samples, indicating potentially more similar bacterial communities between samples in the winter. However, microbial communities in both seasons remained relatively close within the GL tissue. Conversely, SED samples displayed minimal separation by season. The most striking observation emerged from the DG analysis. Here, summer and winter samples formed distinct clusters, with summer exhibiting significantly higher dispersion compared to winter. This suggests a pronounced seasonal influence on the beta diversity of the DG microbial community. Interestingly, the 3D plot also revealed a revised perspective on overall beta diversity. Unlike the initial 2D plot, DG samples displayed the loosest clustering, indicating potentially the highest difference in microbial communities between summer and winter between the three tissues. Despite this, the distinct separation between all three tissue clusters remained evident. Notably, GL and SED samples formed a more uniform and tightly clustered structure, contrasting with the scattered distribution observed in DG. This reinforces the notion of distinct microbial communities within each tissue type.

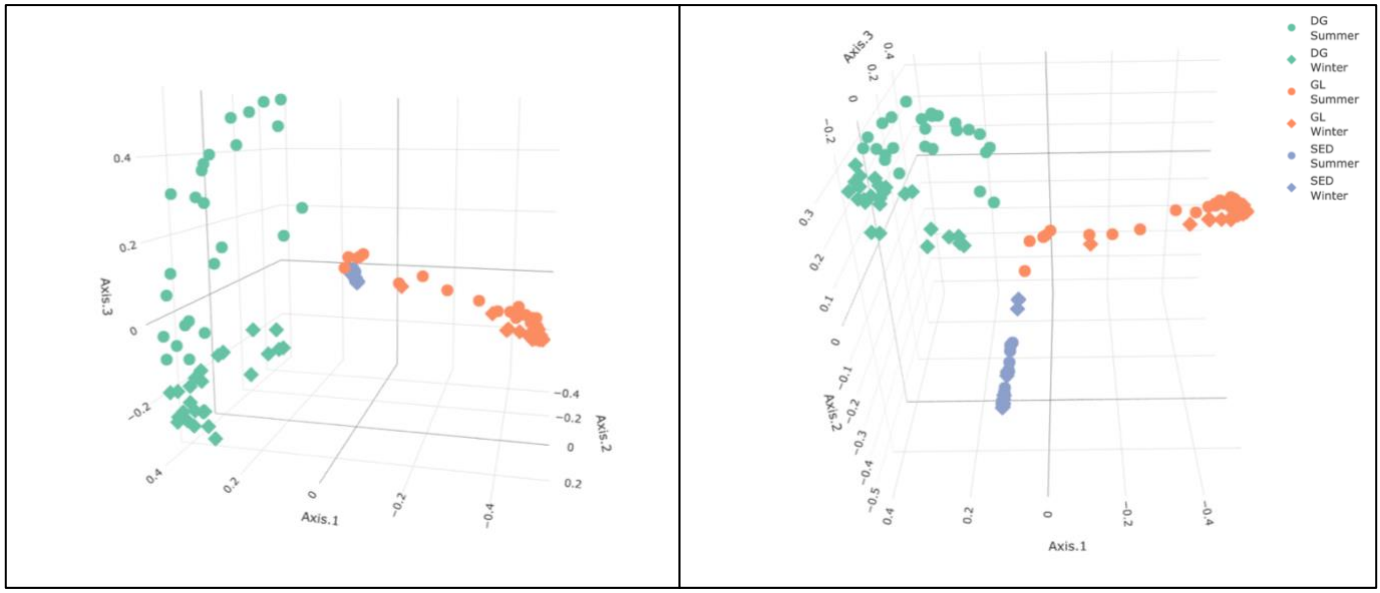

Figure 11. 3D Bray-Curtis PCoA on the entire dataset

3.3.2. Beta diversity by tissue:

After having a broad picture on bacterial communities in clams and sediments, we will now delve deeper into the beta diversity, to understand how these bacterial communities within each tissue respond to seasonal variations in each site.

3.3.2.1. DG:

It is possible to observe the existence of multiple summer and winter sub-clusters across every site among the DG samples (Figure 12). Notably, the distribution of samples within each cluster indicates similar microbial communities within seasons. Interestingly, sites from Venice (CH, PM, and CO) displayed comparable microbial communities with distinct but closely positioned summer and winter clusters. In contrast, MA and SC exhibited more pronounced seasonal differences, with summer samples showing greater dispersion compared to winter: Microbial communities in samples from MA and SC were the most different between summer and winter. Overall, PERMANOVA confirmed these observations, revealing significant seasonal and spatial effects (p-value = 0.001) on the DG microbial community composition (Table 8).

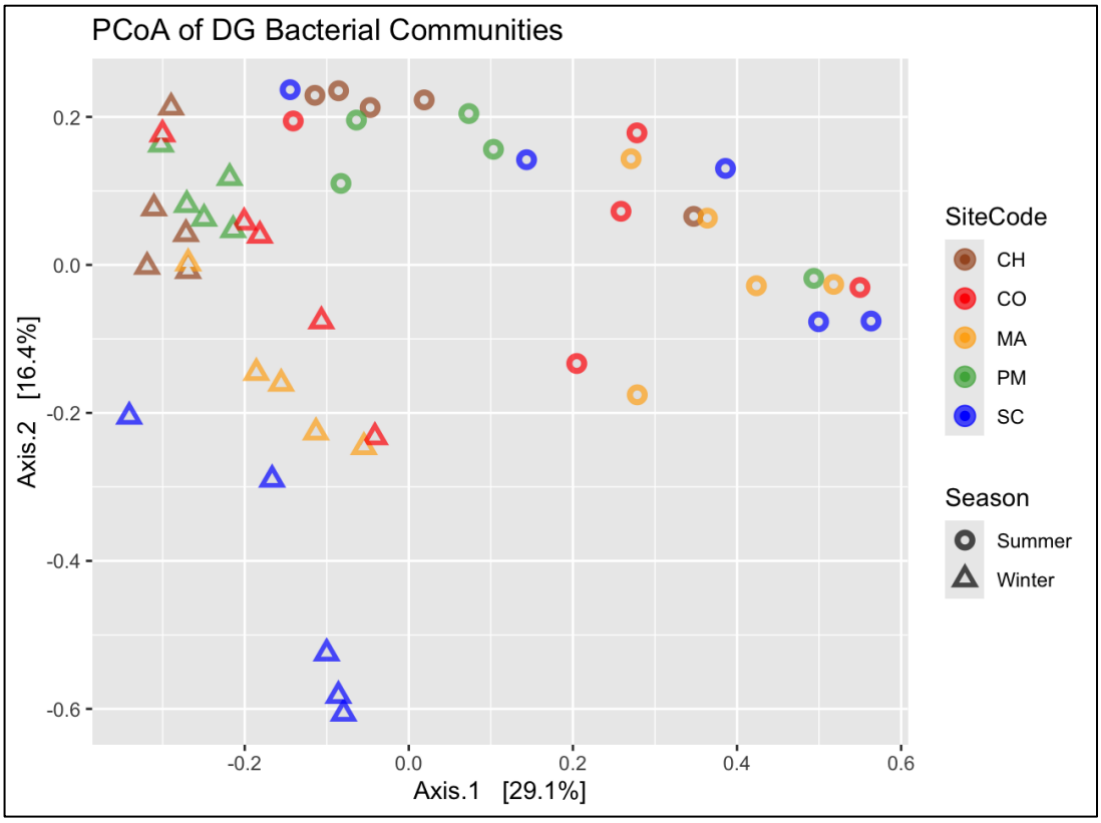

Figure 12. Bray-Curtis PCoA on the DG tissues

3.3.2.2. GL:

GL displayed a less pronounced seasonal pattern compared to DG (Figure 13). While some separation between summer and winter samples was observed, there were also overlaps, particularly in Venetian sites (CH, CO, and PM). These sites exhibited remarkably similar microbial communities across seasons, with CH showing the tightest clustering. Conversely, SC and MA displayed distinct patterns. Winter samples in SC clustered with winter samples from Venice, suggesting similar communities. However, summer samples from SC were highly scattered, distinct from all other sites. MA also showed a clear seasonal difference, with winter and summer samples forming separate clusters and both being distant from other sites: When it comes to gills, microbial communities in MA were the most different from

microbial communities in all other sites, but also the most different from each other between summer and winter. These observations were supported by a significant PERMANOVA test result (p-value = 0.001), indicating a strong influence of seasonality and geographic location on the gill microbial community structure (Table 8).

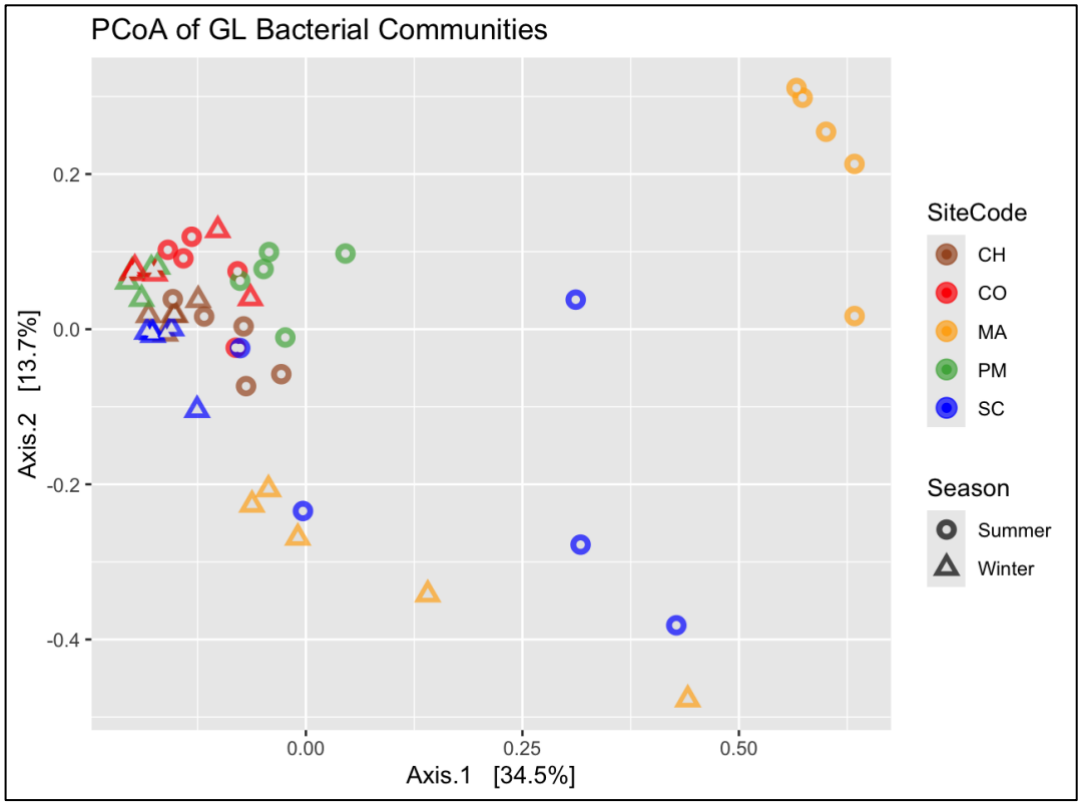

Figure 13. Bray-Curtis PCoA on the GL tissues

3.3.2.3. SED:

Sediment samples revealed yet another pattern (Figure 14). Here, CH, PM, and MA displayed closer clustering, suggesting more similar microbial communities compared to other sites. Seasonality also led to distinct groupings across all locations. Each site appeared to have its own cluster for summer and winter samples, although these clusters remained separate. This highlights the unique composition of sediment microbial communities at each site, further

confirmed by a significant PERMANOVA test result for both season (p-value = 0.001) and site (p-value = 0.001).

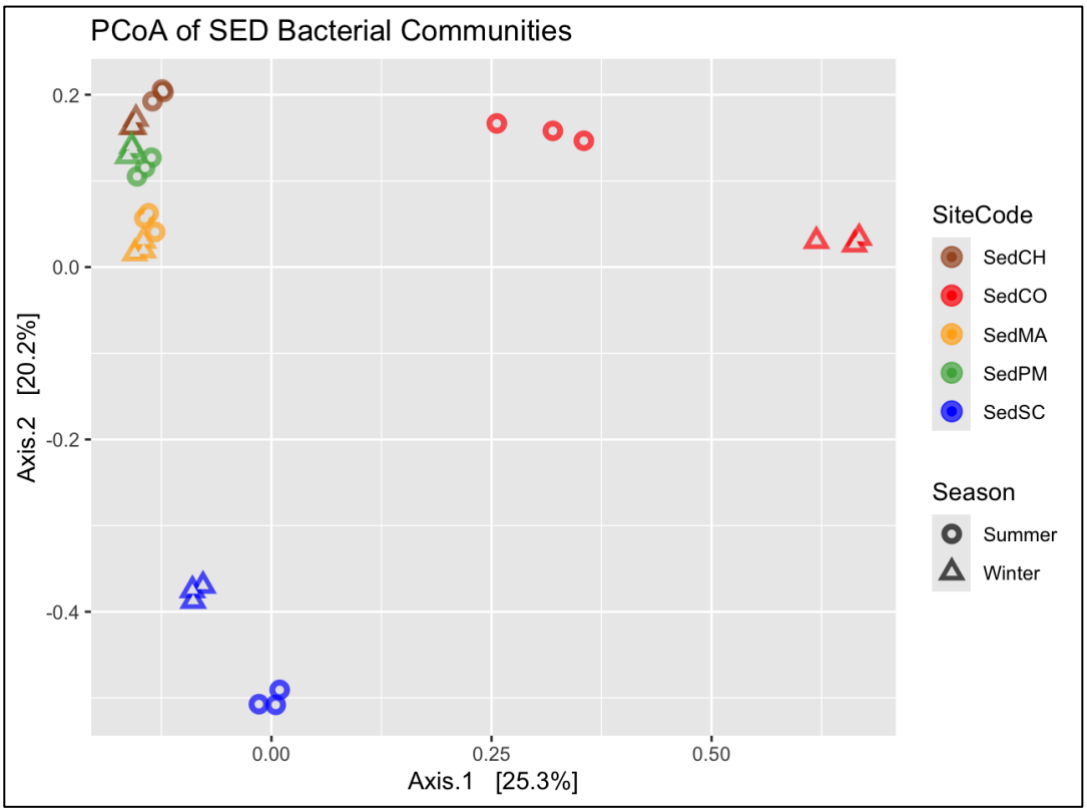

Figure 14. Bray-Curtis PCoA on the SED substrates

Overall, these findings highlight the distinct microbial communities within each tissue type (DG, GL, and SED). The influence of seasonality also varied across tissues, indicating that both variables studied, season and site, appear to have an influence on the microbial communities within the three tissues.

	DG	GL	SED
Season	0.001	0.001	0.001
Site	0.001	0.001	0.001

Table 8. PERMANOVA p-value results for each tissue

3.4. Microbiome composition at Phylum level:

3.4.1. Phylum composition in DG tissue:

DG displayed the most notable seasonal variation in bacterial composition (Figure 15). Summer samples were dominated by three phyla: Firmicutes, Proteobacteria, and Bacteroidota, with their abundance exhibiting minimal spatial variation across all sites. Notably, only SC displayed the emergence of Fusobacteriota during summer. Winter, however, revealed a shift in community composition. While Firmicutes and Proteobacteria remained dominant, the abundance of Bacteroidota declined. Additionally, new phyla emerged in winter, with Verrucomicrobiota and Desulfobacterota appearing in MA and Actinobacteriota in CO. These findings suggest that seasonal fluctuations have a stronger influence on the digestive gland microbiota compared to spatial factors. The full list of phyla can be found in Table 9.

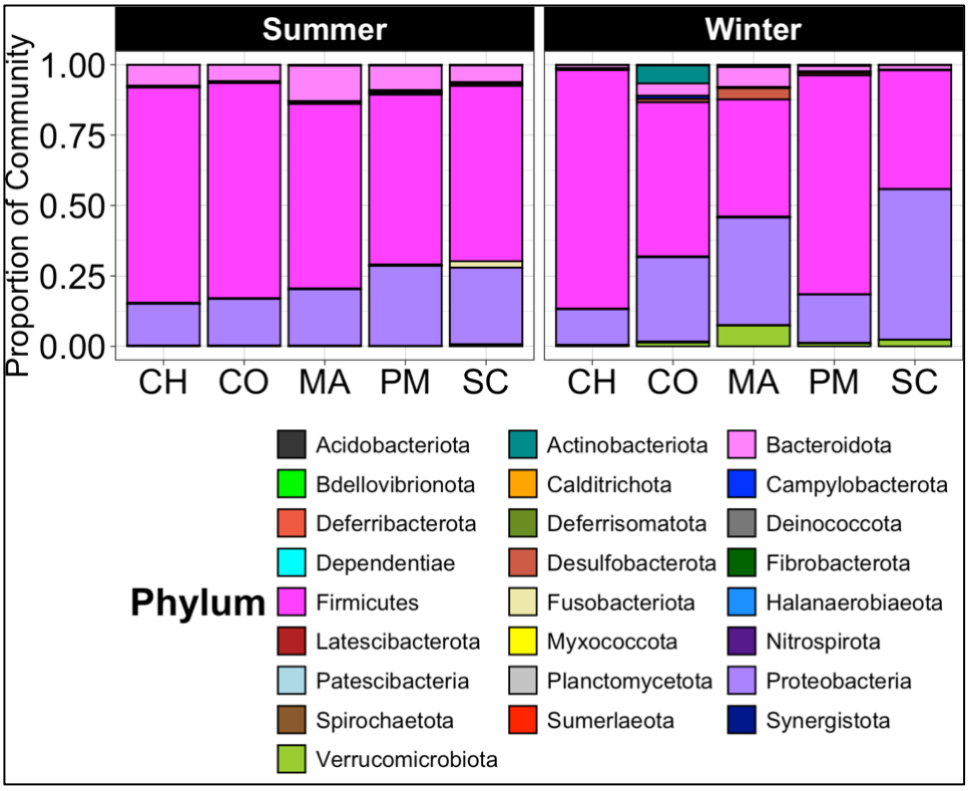

Figure 15. Phylum composition - DG

Summer		
Site	Phylum	Abundance
CH	Firmicutes	3.818849901
CH	Proteobacteria	0.740482446
CH	Bacteroidota	0.369550442
CH	Fusobacteriota	0.020891542
CH	Campylobacterota	0.015414637
CH	Verrucomicrobiota	0.012877281
CH	Desulfobacterota	0.010840298
CH	Bdellovibrionota	0.004747082
CH	Actinobacteriota	0.003395439
CH	Spirochaetota	0.00102861
CO	Firmicutes	3.81556547
CO	Proteobacteria	0.826105014
CO	Bacteroidota	0.290801529
CO	Desulfobacterota	0.016019039
CO	Fusobacteriota	0.015040995
CO	Verrucomicrobiota	0.012335942
CO	Campylobacterota	0.006378217
CO	Fibrobacterota	0.006297085
CO	Bdellovibrionota	0.00398502
CO	Spirochaetota	0.002792418
CO	Actinobacteriota	0.00279025
MA	Firmicutes	3.272443084
MA	Proteobacteria	0.998098976
MA	Bacteroidota	0.627695805
MA	Desulfobacterota	0.037184244
MA	Fusobacteriota	0.016768081
MA	Bdellovibrionota	0.01193012
MA	Spirochaetota	0.010015379
MA	Actinobacteriota	0.009830313
MA	Acidobacteriota	0.007690309
MA	Verrucomicrobiota	0.002931228
MA	Campylobacterota	0.002559756
MA	Nitrospirota	0.001338161
PM	Firmicutes	3.014881288
PM	Proteobacteria	1.419113895
PM	Bacteroidota	0.435872789
PM	Campylobacterota	0.033674017
PM	Desulfobacterota	0.030023439
PM	Fusobacteriota	0.018664166
PM	Actinobacteriota	0.012151794
PM	Fibrobacterota	0.01005442
PM	Bdellovibrionota	0.006996184
PM	Verrucomicrobiota	0.005136735
PM	Planctomycetota	0.004755589
PM	Spirochaetota	0.00423926
PM	Acidobacteriota	0.00354053
SC	Firmicutes	3.112551869
SC	Proteobacteria	1.357717176
SC	Bacteroidota	0.296543644
SC	Fusobacteriota	0.111776917
SC	Desulfobacterota	0.038919128
SC	Spirochaetota	0.01927743
SC	Verrucomicrobiota	0.018812744
SC	Bdellovibrionota	0.018704507
SC	Actinobacteriota	0.008916045
SC	Campylobacterota	0.008280583
SC	Acidobacteriota	0.003774857
SC	Myxococcota	0.001841683
SC	Fibrobacterota	0.001565341

Winter		
Site	Phylum	Abundance
CH	Firmicutes	4.232982094
CH	Proteobacteria	0.629177733
CH	Bacteroidota	0.051381293
CH	Desulfobacterota	0.025635638
CH	Verrucomicrobiota	0.018566954
CH	Campylobacterota	0.010197386
CH	Spirochaetota	0.00875951
CH	Planctomycetota	0.007435353
CH	Fusobacteriota	0.006206186
CH	Actinobacteriota	0.006045932
CH	Acidobacteriota	0.001940685
CO	Firmicutes	2.73678334
CO	Proteobacteria	1.4948231
CO	Actinobacteriota	0.319630075
CO	Bacteroidota	0.218225788
CO	Verrucomicrobiota	0.065094553
CO	Desulfobacterota	0.058608662
CO	Campylobacterota	0.05320304
CO	Spirochaetota	0.02137004
CO	Acidobacteriota	0.012921802
CO	Planctomycetota	0.007763999
CO	Fusobacteriota	0.005795602
CO	Bdellovibrionota	0.002285687
CO	Deferrisomatota	0.001160093
MA	Firmicutes	2.077009336
MA	Proteobacteria	1.906138556
MA	Verrucomicrobiota	0.368641
MA	Bacteroidota	0.353091747
MA	Desulfobacterota	0.199621709
MA	Acidobacteriota	0.024138459
MA	Actinobacteriota	0.019366857
MA	Campylobacterota	0.014658074
MA	Fusobacteriota	0.011938444
MA	Spirochaetota	0.009300456
MA	Planctomycetota	0.005849962
MA	Calditrichota	0.004482421
MA	Bdellovibrionota	0.002795359
MA	Myxococcota	0.00130992
PM	Firmicutes	3.882796495
PM	Proteobacteria	0.855416498
PM	Bacteroidota	0.094327391
PM	Verrucomicrobiota	0.052466279
PM	Desulfobacterota	0.04138152
PM	Campylobacterota	0.025638108
PM	Actinobacteriota	0.013157127
PM	Spirochaetota	0.010835134
PM	Acidobacteriota	0.009674234
PM	Planctomycetota	0.004013007
PM	Fusobacteriota	0.002527546
PM	Bdellovibrionota	0.002421063
PM	Calditrichota	0.002024338
SC	Proteobacteria	2.66745535
SC	Firmicutes	2.105742091
SC	Verrucomicrobiota	0.116372441
SC	Bacteroidota	0.081300969
SC	Actinobacteriota	0.007543338
SC	Fusobacteriota	0.005356007
SC	Desulfobacterota	0.004227787
SC	Campylobacterota	0.003667571
SC	Spirochaetota	0.002815124
SC	Planctomycetota	0.001444018
SC	Bdellovibrionota	0.001325082

Table 9. Most abundant phyla by site and season – DG.

3.4.2. Phylum composition in GL tissue:

GL displayed less evident seasonal differences at Phylum level (Figure 16). Proteobacteria consistently dominated across all sites and seasons, with a much higher abundance compared to DG. Bacteroidota displayed a site-specific seasonal pattern, with a notable presence only in summer for MA and a minimal presence in other sites and seasons. Verrucomicrobiota was scarce in GL, with slight detections in CH and CO during summer. The full list of phyla can be found in Table 10.

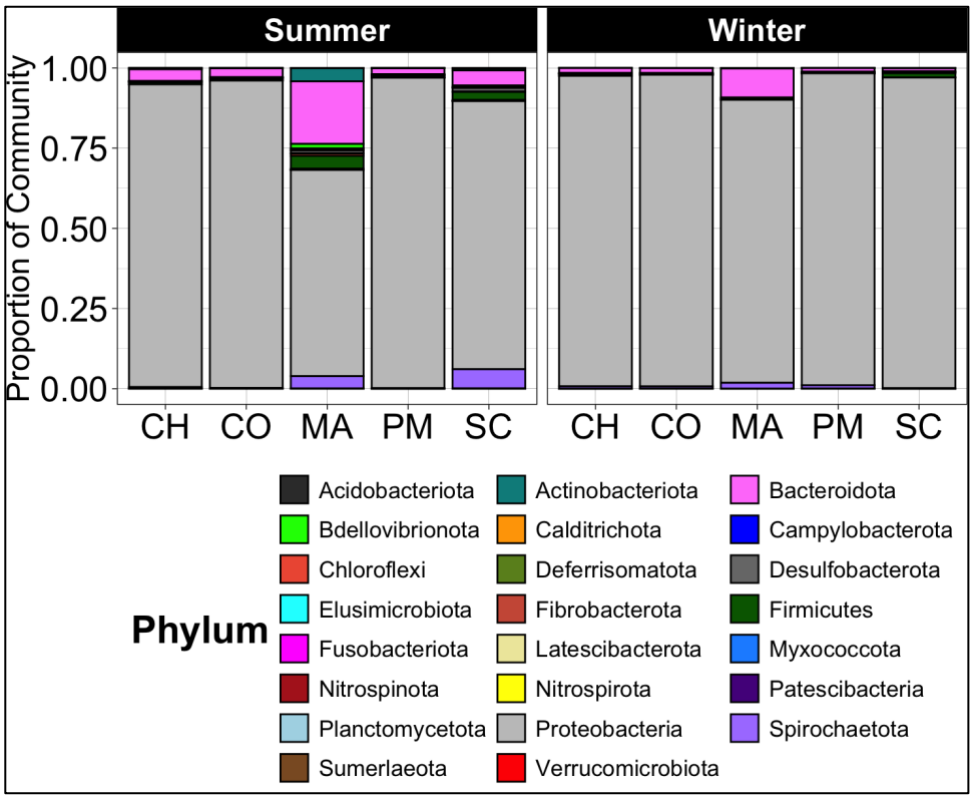

Figure 16. Phylum composition - GL

Summer		
Site	Phylum	Abundance
CH	Proteobacteria	4.717973616
CH	Bacteroidota	0.18344724
CH	Firmicutes	0.034932168
CH	Spirochaetota	0.021430926
CH	Actinobacteriota	0.020119839
CH	Campylobacterota	0.006931474
CH	Verrucomicrobiota	0.00483544
CH	Desulfobacterota	0.004138992
CH	Bdellovibrionota	0.003119062
CH	Fusobacteriota	0.001123808
CO	Proteobacteria	4.79156936
CO	Bacteroidota	0.137893158
CO	Firmicutes	0.018286191
CO	Fusobacteriota	0.011812839
CO	Fibrobacterota	0.00926304
CO	Spirochaetota	0.00916961
CO	Actinobacteriota	0.005742699
CO	Desulfobacterota	0.005639428
CO	Campylobacterota	0.005015647
CO	Bdellovibrionota	0.002780916
CO	Verrucomicrobiota	0.002494426
MA	Proteobacteria	3.214102835
MA	Bacteroidota	0.975441331
MA	Actinobacteriota	0.204614973
MA	Firmicutes	0.201442523
MA	Spirochaetota	0.191312818
MA	Bdellovibrionota	0.071848599
MA	Desulfobacterota	0.045859529
MA	Fibrobacterota	0.03695531
MA	Campylobacterota	0.028801654
MA	Fusobacteriota	0.014873718
MA	Verrucomicrobiota	0.004932832
MA	Acidobacteriota	0.003937742
MA	Myxococcota	0.002019025
MA	Calditrichota	0.001045227
PM	Proteobacteria	4.839513087
PM	Bacteroidota	0.09276707
PM	Firmicutes	0.025611341
PM	Campylobacterota	0.01336485
PM	Spirochaetota	0.008218097
PM	Fusobacteriota	0.006470668
PM	Actinobacteriota	0.004893012
PM	Fibrobacterota	0.004787556
PM	Bdellovibrionota	0.002060757
PM	Desulfobacterota	0.00128772
SC	Proteobacteria	4.178838618
SC	Spirochaetota	0.298246931
SC	Bacteroidota	0.235891618
SC	Firmicutes	0.125789981
SC	Desulfobacterota	0.05779439
SC	Actinobacteriota	0.031992753
SC	Bdellovibrionota	0.020041942
SC	Fusobacteriota	0.010119219
SC	Campylobacterota	0.008993972
SC	Verrucomicrobiota	0.006061925
SC	Acidobacteriota	0.005840931
SC	Fibrobacterota	0.005280715
SC	Calditrichota	0.004872345
SC	Myxococcota	0.004530483
SC	Deferrisomatota	0.002500093
SC	Latescibacterota	0.001016684

Winter		
Site	Phylum	Abundance
CH	Proteobacteria	4.83944097
CH	Bacteroidota	0.077732931
CH	Spirochaetota	0.037490639
CH	Fusobacteriota	0.028373665
CH	Campylobacterota	0.008550107
CH	Firmicutes	0.004398779
CH	Desulfobacterota	0.001901009
CH	Actinobacteriota	0.001154267
CO	Proteobacteria	4.856985252
CO	Bacteroidota	0.077653555
CO	Spirochaetota	0.034870492
CO	Campylobacterota	0.014211905
CO	Desulfobacterota	0.005858717
CO	Verrucomicrobiota	0.002224591
CO	Firmicutes	0.002035371
CO	Acidobacteriota	0.001945399
CO	Actinobacteriota	0.001346469
CO	Fusobacteriota	0.00123609
MA	Proteobacteria	4.4104948
MA	Bacteroidota	0.451929226
MA	Spirochaetota	0.091607409
MA	Desulfobacterota	0.016350996
MA	Firmicutes	0.008589493
MA	Campylobacterota	0.005754568
MA	Acidobacteriota	0.004773055
MA	Actinobacteriota	0.00368201
MA	Bdellovibrionota	0.002835312
MA	Verrucomicrobiota	0.001279158
MA	Fusobacteriota	0.001019875
PM	Proteobacteria	4.861525333
PM	Bacteroidota	0.058597827
PM	Spirochaetota	0.050318164
PM	Campylobacterota	0.013088342
PM	Desulfobacterota	0.006123066
PM	Verrucomicrobiota	0.004844414
PM	Fusobacteriota	0.00136743
PM	Acidobacteriota	0.001033595
SC	Proteobacteria	4.842135117
SC	Firmicutes	0.066718776
SC	Bacteroidota	0.047509275
SC	Desulfobacterota	0.015628195
SC	Campylobacterota	0.010238547
SC	Spirochaetota	0.007690353
SC	Bdellovibrionota	0.003152229
SC	Actinobacteriota	0.00207865
SC	Acidobacteriota	0.001602162
SC	Calditrichota	0.001274041

Table 10. Most abundant phyla by site and season – GL

3.4.3. Phylum composition in SED substrates:

SED samples exhibited the most diverse phylum composition and the least seasonal variation at Phylum level. Bacteroidota, Proteobacteria and Desulfobacterota, maintained a high abundance throughout both seasons at all sites (Figure 17). Additionally, phyla like Campylobacterota, and Acidobacteriota were also detected. Notably, CO displayed a unique presence of Spirochaetota in both seasons. Overall, the phylum composition in sediments remained remarkably consistent across seasons, contrasting significantly with the more dynamic patterns observed in the clam tissues. The full list of phyla can be found in Table 11.

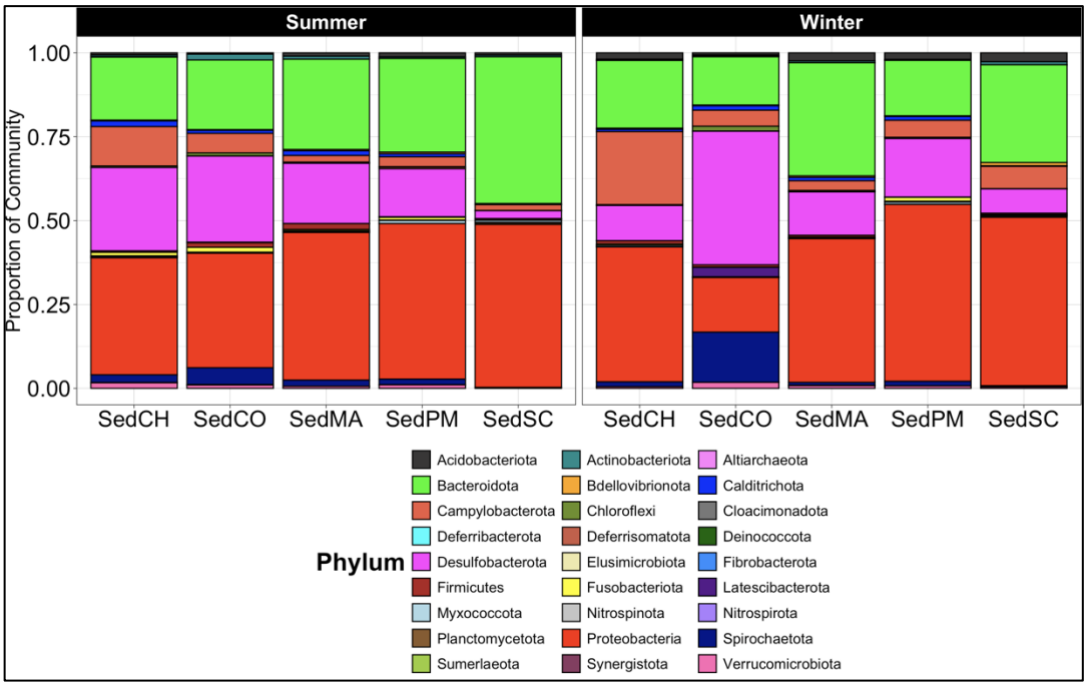

Figure 17. Phylum composition - SED

Overall, these findings highlight significant variations in phylum composition: seasonally, with DG exhibiting the most pronounced changes, spatially, with slight variations in dominance patterns between sites for the same tissue, and by tissue type, with sediments displaying the most diverse and seasonally stable community compared to the clam tissues.

Summer		
Site	Phylum	Abundance
SedCH	Proteobacteria	1.045684143
SedCH	Desulfobacterota	0.744685072
SedCH	Bacteroidota	0.563353872
SedCH	Campylobacterota	0.352595453
SedCH	Spirochaetota	0.06947919
SedCH	Calditrichota	0.05019254
SedCH	Verrucomicrobiota	0.049363588
SedCH	Fusobacteriota	0.031381581
SedCH	Acidobacteriota	0.019588015
SedCH	Actinobacteriota	0.015253639
SedCH	Chloroflexi	0.013006593
SedCH	Firmicutes	0.01281568
SedCH	Myxococcota	0.011049034
SedCH	Bdellovibrionota	0.007900474
SedCH	Latescibacterota	0.003553478
SedCH	Altiarchaeota	0.003305191
SedCH	Planctomycetota	0.002599558
SedCH	Sumerlaeota	0.002504089
SedCO	Proteobacteria	1.024478119
SedCO	Desulfobacterota	0.769743506
SedCO	Bacteroidota	0.622507937
SedCO	Campylobacterota	0.174001187
SedCO	Spirochaetota	0.149473502
SedCO	Actinobacteriota	0.052311546
SedCO	Fusobacteriota	0.044061398
SedCO	Firmicutes	0.041256025
SedCO	Verrucomicrobiota	0.030102916
SedCO	Chloroflexi	0.027221564
SedCO	Calditrichota	0.027150596
SedCO	Acidobacteriota	0.011409485
SedCO	Myxococcota	0.006870301
SedCO	Bdellovibrionota	0.006358493
SedCO	Sumerlaeota	0.003841342
SedCO	Fibrobacterota	0.0036278
SedCO	Planctomycetota	0.001432008
SedCO	Elusimicrobiota	0.001249958
SedCO	Deferrisomatota	0.001096799
SedMA	Proteobacteria	1.319778446
SedMA	Bacteroidota	0.806989542
SedMA	Desulfobacterota	0.538236794
SedMA	Campylobacterota	0.058481371
SedMA	Spirochaetota	0.054168932
SedMA	Firmicutes	0.052908883
SedMA	Calditrichota	0.04215269
SedMA	Acidobacteriota	0.029557748
SedMA	Actinobacteriota	0.026866124
SedMA	Verrucomicrobiota	0.018727217
SedMA	Fusobacteriota	0.012326507
SedMA	Bdellovibrionota	0.012125789
SedMA	Myxococcota	0.010815647
SedMA	Chloroflexi	0.008292551
SedMA	Deferrisomatota	0.002893326
SedMA	Planctomycetota	0.002227953
SedMA	Latescibacterota	0.001279431
SedPM	Proteobacteria	1.390894368
SedPM	Bacteroidota	0.837621438
SedPM	Desulfobacterota	0.428879737
SedPM	Campylobacterota	0.0876555
SedPM	Spirochaetota	0.049747161
SedPM	Acidobacteriota	0.036236166
SedPM	Verrucomicrobiota	0.031508597
SedPM	Myxococcota	0.027315679
SedPM	Calditrichota	0.026819142
SedPM	Fusobacteriota	0.02468494
SedPM	Bdellovibrionota	0.015291603
SedPM	Deferrisomatota	0.014700578
SedPM	Actinobacteriota	0.01328309
SedPM	Firmicutes	0.004479305
SedPM	Chloroflexi	0.004043604
SedPM	Latescibacterota	0.002671551
SedPM	Fibrobacterota	0.00122985
SedPM	Sumerlaeota	0.001100094
SedSC	Proteobacteria	1.456134513
SedSC	Bacteroidota	1.312360391
SedSC	Desulfobacterota	0.07121539
SedSC	Campylobacterota	0.052211626
SedSC	Acidobacteriota	0.020552279
SedSC	Myxococcota	0.018806325
SedSC	Actinobacteriota	0.014900084
SedSC	Planctomycetota	0.014324189
SedSC	Firmicutes	0.012144563
SedSC	Bdellovibrionota	0.009223862
SedSC	Verrucomicrobiota	0.006917103
SedSC	Nitrospirota	0.004650841
SedSC	Spirochaetota	0.002091686
SedSC	Deinococcota	0.001706938
SedSC	Fusobacteriota	0.001304348

Winter		
Site	Phylum	Abundance
SedCH	Proteobacteria	1.205659148
SedCH	Campylobacterota	0.653481342
SedCH	Bacteroidota	0.605935887
SedCH	Desulfobacterota	0.314390905
SedCH	Acidobacteriota	0.056714812
SedCH	Spirochaetota	0.0439981
SedCH	Firmicutes	0.029746923
SedCH	Calditrichota	0.020428428
SedCH	Verrucomicrobiota	0.014100783
SedCH	Myxococcota	0.0130224
SedCH	Fusobacteriota	0.011781555
SedCH	Actinobacteriota	0.009975526
SedCH	Bdellovibrionota	0.009823383
SedCH	Chloroflexi	0.005452236
SedCH	Deferrisomatota	0.001586582
SedCH	Altiarchaeota	0.001529608
SedCH	Latescibacterota	0.001177413
SedCO	Desulfobacterota	1.195033095
SedCO	Proteobacteria	0.488131147
SedCO	Spirochaetota	0.448242247
SedCO	Bacteroidota	0.431628893
SedCO	Campylobacterota	0.14289358
SedCO	Latescibacterota	0.084057061
SedCO	Verrucomicrobiota	0.053530166
SedCO	Chloroflexi	0.042449541
SedCO	Calditrichota	0.040067216
SedCO	Firmicutes	0.021342227
SedCO	Acidobacteriota	0.019128237
SedCO	Actinobacteriota	0.009665123
SedCO	Altiarchaeota	0.006684286
SedCO	Bdellovibrionota	0.006158097
SedCO	Myxococcota	0.003968693
SedCO	Planctomycetota	0.002139201
SedCO	Deferrisomatota	0.001814078
SedMA	Proteobacteria	1.284316252
SedMA	Bacteroidota	1.01150723
SedMA	Desulfobacterota	0.387525721
SedMA	Campylobacterota	0.086995462
SedMA	Acidobacteriota	0.071721994
SedMA	Calditrichota	0.030410103
SedMA	Spirochaetota	0.025857397
SedMA	Verrucomicrobiota	0.024561504
SedMA	Firmicutes	0.016919282
SedMA	Actinobacteriota	0.014701481
SedMA	Bdellovibrionota	0.01360625
SedMA	Chloroflexi	0.009574443
SedMA	Myxococcota	0.006161934
SedMA	Planctomycetota	0.003603977
SedMA	Sumerlaeota	0.003375053
SedMA	Deferrisomatota	0.002814495
SedMA	Altiarchaeota	0.002128681
SedMA	Latescibacterota	0.001845093
SedMA	Fusobacteriota	0.001424033
SedPM	Proteobacteria	1.58095575
SedPM	Desulfobacterota	0.523416329
SedPM	Bacteroidota	0.495504581
SedPM	Campylobacterota	0.151484731
SedPM	Acidobacteriota	0.059171884
SedPM	Spirochaetota	0.040386913
SedPM	Fusobacteriota	0.03470896
SedPM	Calditrichota	0.034035508
SedPM	Verrucomicrobiota	0.02183088
SedPM	Myxococcota	0.020637426
SedPM	Latescibacterota	0.007344755
SedPM	Deferrisomatota	0.007320559
SedPM	Actinobacteriota	0.007166408
SedPM	Bdellovibrionota	0.006324809
SedPM	Chloroflexi	0.004231678
SedPM	Firmicutes	0.00180041
SedPM	Altiarchaeota	0.001255589
SedSC	Proteobacteria	1.504719849
SedSC	Bacteroidota	0.874835042
SedSC	Desulfobacterota	0.217636987
SedSC	Campylobacterota	0.200295439
SedSC	Acidobacteriota	0.079880918
SedSC	Bdellovibrionota	0.028192099
SedSC	Actinobacteriota	0.026805613
SedSC	Planctomycetota	0.012737703
SedSC	Verrucomicrobiota	0.012303145
SedSC	Firmicutes	0.01199876
SedSC	Myxococcota	0.011158694
SedSC	Spirochaetota	0.010919422
SedSC	Calditrichota	0.005017805
SedSC	Chloroflexi	0.001226732

Table 11. Most abundant phyla by site and season – SED.

3.5. Microbiome composition at Genus level:

3.5.1. Genus composition in DG tissue:

DG displayed a seasonal shift in dominance (Figure 18). Mycoplasma dominated across all sites in both summer and winter, although winter also saw a significant presence of Rickettsiella, particularly in SC where it was more abundant than Mycoplasma. Summer samples from various sites showed additional genera in low abundances: Candidatus Endoecteinascidia (CO), Aurantivirga, Vibrio (mostly in PM, SC, CH, and CO) and Pseudoalteromonas (PM); Winter samples displayed a similar pattern with Mycoplasma and Rickettsiella, along with small relative abundance of Cutibacterium (CO) and Candidatus Rhabdochlamydia (MA). The full list of abundant genera can be found in Table 12.

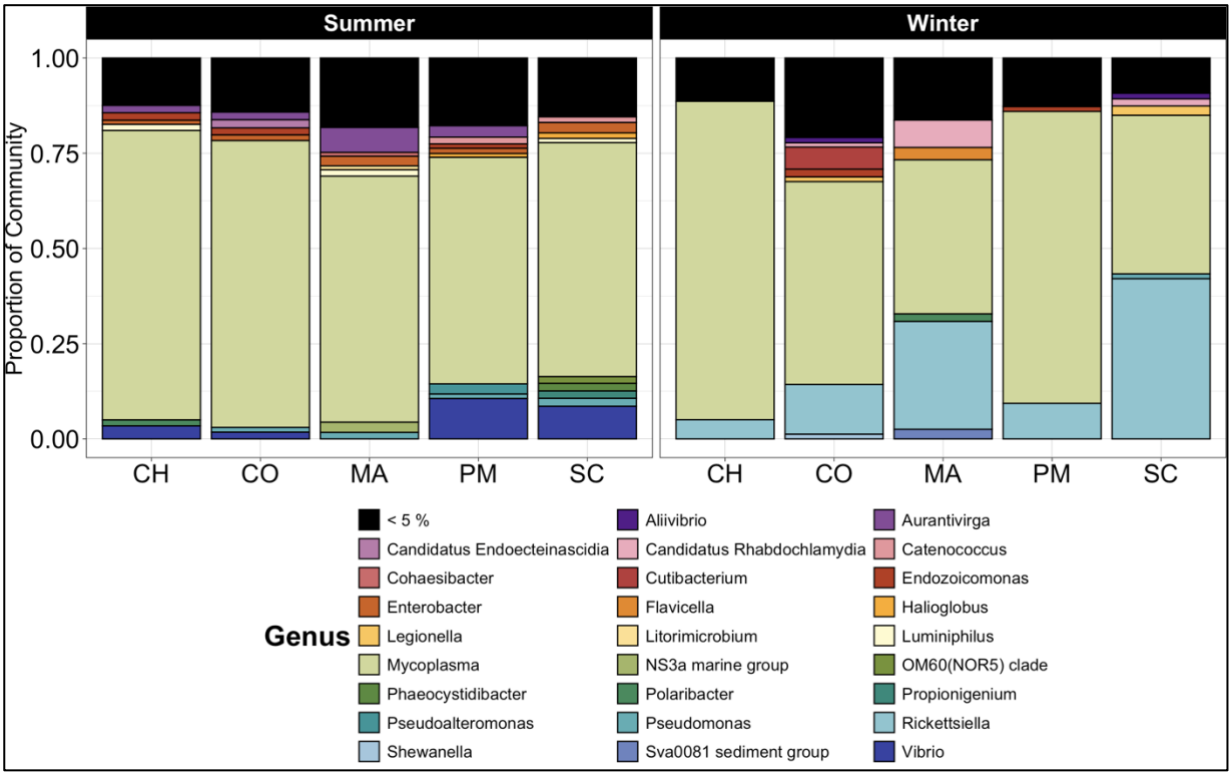

Figure 18. Genus composition - DG

Summer		
Site	Genus	Abundance
CH	Mycoplasma	3.798923697
CH	< 5 %	0.623177666
CH	Vibrio	0.171229849
CH	Endozoicomonas	0.098464273
CH	Aurantivirga	0.096263621
CH	Luminiphilus	0.080110669
CH	Polaribacter	0.078302863
CH	Enterobacter	0.053527362
CO	Mycoplasma	3.763483086
CO	< 5 %	0.710929316
CO	Candidatus Endoecteinascidia	0.10726437
CO	Aurantivirga	0.100197449
CO	Endozoicomonas	0.092347863
CO	Vibrio	0.088947698
CO	Enterobacter	0.073997615
CO	Pseudomonas	0.062832605
MA	Mycoplasma	3.228785682
MA	< 5 %	0.91258105
MA	Aurantivirga	0.32633784
MA	NS3a marine group	0.134291095
MA	Enterobacter	0.126482546
MA	Pseudomonas	0.085362172
MA	Luminiphilus	0.083251945
MA	Litorimicrobium	0.051870834
MA	Cohaesibacter	0.051036835
PM	Mycoplasma	2.973037517
PM	< 5 %	0.88988629
PM	Vibrio	0.530224157
PM	Aurantivirga	0.149059184
PM	Pseudoalteromonas	0.132741933
PM	Catenococcus	0.089456785
PM	Enterobacter	0.068260284
PM	Pseudomonas	0.059547407
PM	Endozoicomonas	0.057657991
PM	Halioglobus	0.050128452
SC	Mycoplasma	3.070229634
SC	< 5 %	0.773537325
SC	Vibrio	0.429108051
SC	Enterobacter	0.135505532
SC	Pseudomonas	0.104211131
SC	Phaeocystidibacter	0.099938464
SC	Propionigenium	0.095995977
SC	OM60(NOR5) clade	0.088354722
SC	Catenococcus	0.074158212
SC	Halioglobus	0.072925318
SC	Luminiphilus	0.056035632

Winter		
Site	Genus	Abundance
CH	Mycoplasma	4.178491308
CH	< 5 %	0.568936676
CH	Rickettsiella	0.252572017
CO	Mycoplasma	2.661899842
CO	< 5 %	1.043575647
CO	Rickettsiella	0.652458523
CO	Cutibacterium	0.289878475
CO	Endozoicomonas	0.102466634
CO	Aliivibrio	0.070384808
CO	Shewanella	0.062079956
CO	Legionella	0.061533307
CO	Candidatus Rhabdochlamydia	0.055722809
MA	Mycoplasma	2.022538649
MA	Rickettsiella	1.414463548
MA	< 5 %	0.81524189
MA	Candidatus Rhabdochlamydia	0.360021669
MA	Flavicella	0.161784652
MA	Sva0081 sediment group	0.12669182
MA	Polaribacter	0.099257772
PM	Mycoplasma	3.831906554
PM	< 5 %	0.638791905
PM	Rickettsiella	0.466547415
PM	Endozoicomonas	0.062754126
SC	Rickettsiella	2.102119816
SC	Mycoplasma	2.081245257
SC	< 5 %	0.462736852
SC	Legionella	0.122791238
SC	Candidatus Rhabdochlamydia	0.093193822
SC	Aliivibrio	0.074647681
SC	Pseudomonas	0.063265334

Table 12. Most abundant gerena by site and season – DG

3.5.2. Genus composition in GL tissue:

GL exhibited a more prominent genus as Endozoicomonas dominated in most sites and seasons (Figure 19). Summer samples from some sites displayed additional genera alongside Endozoicomonas, including Candidatus Endoecteinascidia (CO), Candidatus Aquiluna, Spirochaeta 2 (MA), Vibrio (MA and PM), and Spirochaeta 2 (SC). MA is particularly interesting, as it contains a very wide list of different abundant genera. Winter, however, showed a stronger dominance by Endozoicomonas across all sites except MA, where a minor presence of Colwellia and Cutibacterium was observed. Catenococcus also appears in the summer in CO only. The full list of genera can be found in Table 13.

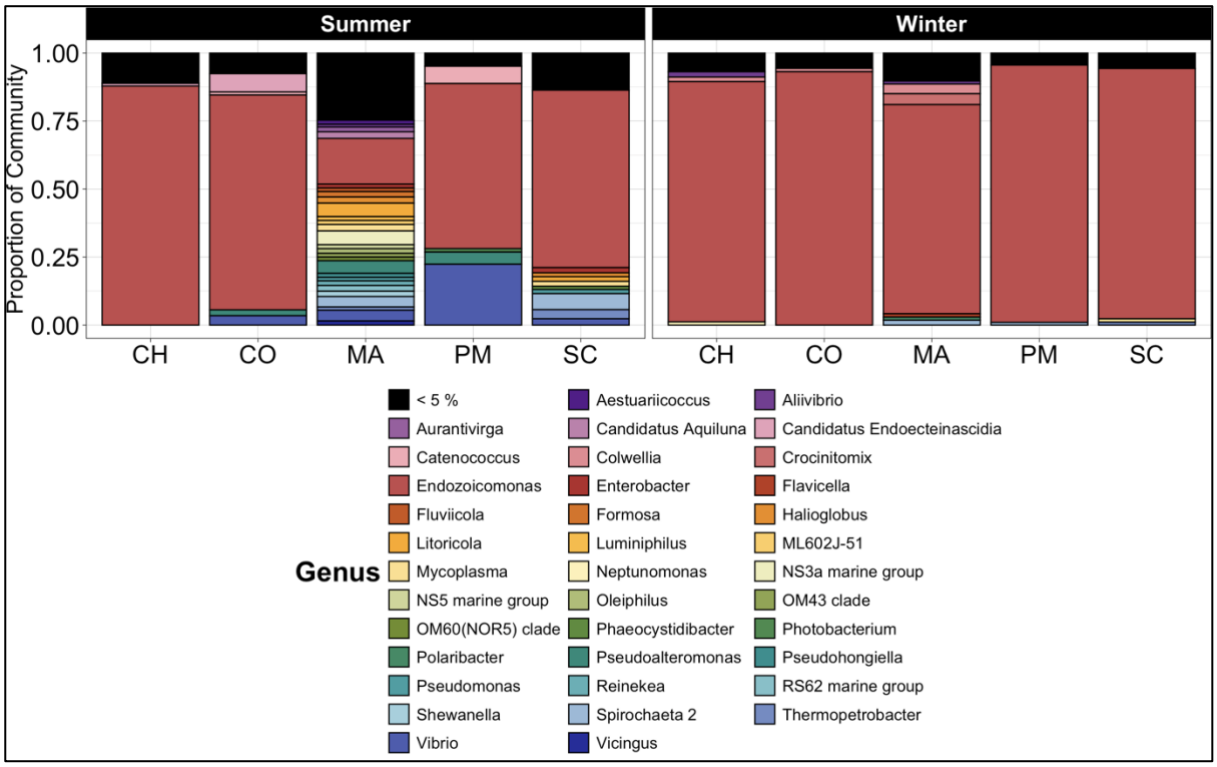

Figure 19. Genus composition - GL

Summer		
Site	Genus	Abundance
CH	Endozoicomonas	4.394581578
CH	< 5 %	0.554860291
CH	Candidatus Endoecteinascidia	0.050558131
CO	Endozoicomonas	3.950128105
CO	< 5 %	0.378175948
CO	Candidatus Endoecteinascidia	0.336638538
CO	Vibrio	0.172732467
CO	Pseudoalteromonas	0.106989022
CO	Catenococcus	0.055335921
MA	< 5 %	1.233399854
MA	Endozoicomonas	0.839747745
MA	NS3a marine group	0.252971616
MA	Litoricola	0.24836592
MA	Pseudoalteromonas	0.23199314
MA	Vibrio	0.197113511
MA	Spirochaeta 2	0.190225556
MA	Candidatus Aquiluna	0.121240193
MA	Mycoplasma	0.118739672
MA	Halioglobus	0.112576961
MA	RS62 marine group	0.106062817
MA	Formosa	0.101057934
MA	Shewanella	0.098148961
MA	Aurantivirga	0.086455265
MA	Reinekea	0.084496568
MA	Oleiphilus	0.083050131
MA	Vicingus	0.076637842
MA	Pseudohongiella	0.074692781
MA	Enterobacter	0.074145479
MA	ML602J-51	0.073215221
MA	Aestuariicoccus	0.073193698
MA	NS5 marine group	0.073137649
MA	OM43 clade	0.072049304
MA	Luminiphilus	0.070494809
MA	OM60(NOR5) clade	0.065825011
MA	Pseudomonas	0.063815563
MA	Fluviicola	0.0638151
MA	Thermopetrobacter	0.061116177
MA	Aliivibrio	0.052215522
PM	Endozoicomonas	3.035003083
PM	Vibrio	1.118630207
PM	Catenococcus	0.317139453
PM	< 5 %	0.241660873
PM	Pseudoalteromonas	0.224979848
PM	Photobacterium	0.062586537
SC	Endozoicomonas	3.261670804
SC	< 5 %	0.680925415
SC	Spirochaeta 2	0.294949371
SC	Thermopetrobacter	0.167142261
SC	Vibrio	0.117216591
SC	Enterobacter	0.096512956
SC	Mycoplasma	0.089212323
SC	Litoricola	0.084656807
SC	Pseudomonas	0.075671961
SC	Halioglobus	0.071348372
SC	Phaeocystidibacter	0.060693138

Winter		
Site	Genus	Abundance
CH	Endozoicomonas	4.415793153
CH	< 5 %	0.343495731
CH	Aliivibrio	0.096407583
CH	Colwellia	0.08436807
CH	Neptunomonas	0.059935463
CO	Endozoicomonas	4.657864399
CO	< 5 %	0.280050046
CO	Colwellia	0.062085554
MA	Endozoicomonas	3.844865645
MA	< 5 %	0.514481812
MA	Crocinitomix	0.198900179
MA	Colwellia	0.179413723
MA	Spirochaeta 2	0.091371072
MA	Flavicella	0.061310741
MA	Polaribacter	0.056505337
MA	Aliivibrio	0.053151492
PM	Endozoicomonas	4.730106861
PM	< 5 %	0.219671131
PM	Spirochaeta 2	0.050222008
SC	Endozoicomonas	4.601207682
SC	< 5 %	0.282287057
SC	Mycoplasma	0.061455854
SC	Thermopetrobacter	0.055049407

Table 13. Most abundant genera by site and season – GL

3.5.3. Genus composition in SED substrates:

SED samples displayed the most distinct community composition compared to the clam tissues and the most diverse as also observed by previous alfa diversity analysis. Unlike the clam tissues, a good proportion here was represented by rare genera that presented less than 5% of the total abundance (Figure 20). In addition, there was not only one genus that clearly dominated across all sites or seasons, instead there appears to be a wide list of genera at each site. The most abundant genera across samples were Woeseia, Halioglobus, and Sva0081 sediment group (Phylum: Desulfobacterota, Family: Desulfosarcinaceae, Class: Desulfobacteria). Additionally, Sulfurovum, Aquibacter and Actibacter appear in slightly lower abundances. A complete list of the different genera is found in Table 14.

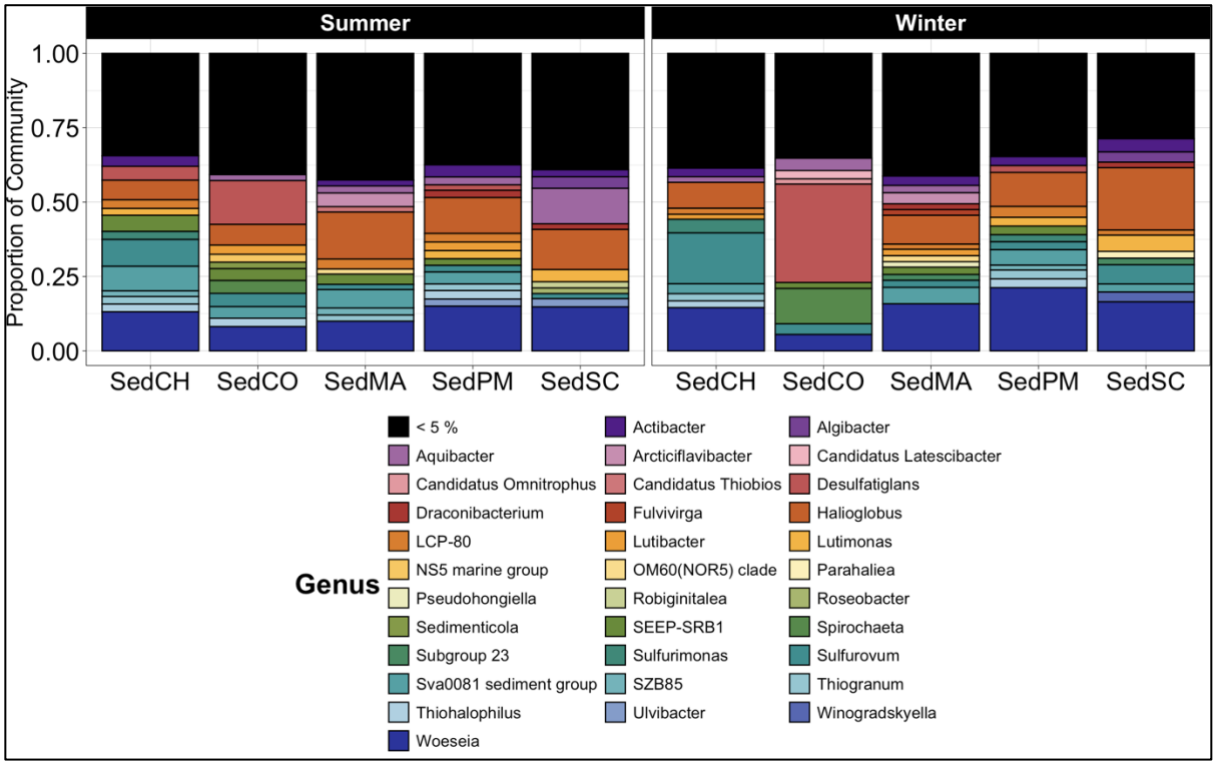

Figure 20. Genus composition - SED

Summer		
Site	Genus	Abundance
SedCH	< 5 %	1.032711352
SedCH	Woeseia	0.394550155
SedCH	Sulfurovum	0.270481334
SedCH	Sva0081 sediment group	0.247019173
SedCH	Halioglobus	0.197216326
SedCH	SEEP-SRB1	0.162797332
SedCH	Desulfatiglans	0.140040636
SedCH	Actibacter	0.106374256
SedCH	LCP-80	0.08782301
SedCH	Sulfurimonas	0.080646848
SedCH	Thiohalophilus	0.07794617
SedCH	Thiogranum	0.075685741
SedCH	Lutimonas	0.068399985
SedCH	SZB85	0.058307681
SedCO	< 5 %	1.22323398
SedCO	Desulfatiglans	0.440757108
SedCO	Woeseia	0.245314142
SedCO	Halioglobus	0.209050373
SedCO	Sulfurovum	0.134174622
SedCO	Spirochaeta	0.129250452
SedCO	SEEP-SRB1	0.120203881
SedCO	Sva0081 sediment group	0.116947125
SedCO	Lutibacter	0.092496463
SedCO	Thiohalophilus	0.084455441
SedCO	NS5 marine group	0.079815326
SedCO	Sedimenticola	0.064165783
SedCO	Aquibacter	0.060135305
SedMA	< 5 %	1.277302157
SedMA	Halioglobus	0.473072322
SedMA	Woeseia	0.299661646
SedMA	Sva0081 sediment group	0.185609692
SedMA	Arcticiflavibacter	0.137666388
SedMA	SEEP-SRB1	0.102578329
SedMA	LCP-80	0.101585236
SedMA	SZB85	0.071428741
SedMA	Aquibacter	0.071058064
SedMA	Thiogranum	0.061772617
SedMA	Actibacter	0.059865444
SedMA	Candidatus Thiobios	0.053906087
SedMA	Sulfurovum	0.052868044
SedMA	OM60(NOR5) clade	0.051625232
SedPM	< 5 %	1.124248003
SedPM	Woeseia	0.45116893
SedPM	Halioglobus	0.361850888
SedPM	Actibacter	0.12210831
SedPM	Sva0081 sediment group	0.121203049
SedPM	LCP-80	0.086651371
SedPM	Lutibacter	0.086392541
SedPM	Thiohalophilus	0.084918876
SedPM	Lutimonas	0.082099018
SedPM	Aquibacter	0.079319201
SedPM	Draconibacterium	0.073344444
SedPM	Ulvibacter	0.072588509
SedPM	Sulfurovum	0.066674169
SedPM	SEEP-SRB1	0.066491154
SedPM	Thiogranum	0.066276401
SedPM	Desulfatiglans	0.054665137
SedSC	< 5 %	1.173375694
SedSC	Woeseia	0.444089519
SedSC	Halioglobus	0.404836609
SedSC	Aquibacter	0.357384018
SedSC	Lutimonas	0.123062861
SedSC	Algibacter	0.116538444
SedSC	Ulvibacter	0.082651672
SedSC	Actibacter	0.070965356
SedSC	Robiginitalea	0.062217059
SedSC	Draconibacterium	0.057144372
SedSC	Roseobacter	0.056232479
SedSC	Sulfurovum	0.051501915

Winter		
Site	Genus	Abundance
SedCH	< 5 %	1.157197949
SedCH	Sulfurovum	0.512927182
SedCH	Woeseia	0.436046548
SedCH	Halioglobus	0.261667998
SedCH	Sulfurimonas	0.137490075
SedCH	Sva0081 sediment group	0.099736713
SedCH	Actibacter	0.086441827
SedCH	Thiogranum	0.072034086
SedCH	Thiohalophilus	0.069027074
SedCH	LCP-80	0.060280403
SedCH	Aquibacter	0.056756951
SedCH	Lutibacter	0.050393193
SedCO	< 5 %	1.057647599
SedCO	Desulfatiglans	0.992598879
SedCO	Spirochaeta	0.356217668
SedCO	Woeseia	0.165613283
SedCO	Aquibacter	0.123647
SedCO	Sulfurovum	0.107820919
SedCO	Candidatus Latescibacter	0.084057061
SedCO	SEEP-SRB1	0.059867241
SedCO	Candidatus Omnitrophus	0.05253035
SedMA	< 5 %	1.237268729
SedMA	Woeseia	0.474371417
SedMA	Halioglobus	0.290633963
SedMA	Sva0081 sediment group	0.166115056
SedMA	Arcticiflavibacter	0.111117972
SedMA	Actibacter	0.094636684
SedMA	Aquibacter	0.073777351
SedMA	SEEP-SRB1	0.07326228
SedMA	Sulfurovum	0.071213823
SedMA	Lutibacter	0.064800259
SedMA	OM60(NOR5) clade	0.060204043
SedMA	Subgroup 23	0.059098988
SedMA	Fulvivirga	0.057944619
SedMA	Draconibacterium	0.057818417
SedMA	Pseudohongiella	0.055044614
SedMA	LCP-80	0.052691786
SedPM	< 5 %	1.041388453
SedPM	Woeseia	0.637787103
SedPM	Halioglobus	0.343382134
SedPM	Sva0081 sediment group	0.154068313
SedPM	LCP-80	0.109375796
SedPM	Actibacter	0.090307994
SedPM	Thiohalophilus	0.08901758
SedPM	Thiogranum	0.088275501
SedPM	SEEP-SRB1	0.088067111
SedPM	Lutimonas	0.087831626
SedPM	Sulfurovum	0.079654404
SedPM	Sulfurimonas	0.070921589
SedPM	Desulfatiglans	0.068750896
SedPM	SZB85	0.051171499
SedSC	< 5 %	0.861173619
SedSC	Halioglobus	0.628458185
SedSC	Woeseia	0.494649541
SedSC	Sulfurovum	0.194057103
SedSC	Lutimonas	0.162595232
SedSC	Actibacter	0.13304293
SedSC	Algibacter	0.102952223
SedSC	Winogradskyella	0.099753163
SedSC	Sva0081 sediment group	0.080690163
SedSC	Parahaliea	0.069000163
SedSC	Subgroup 23	0.065581336
SedSC	Draconibacterium	0.055732939
SedSC	LCP-80	0.052313402

Table 14. Most abundant genera by site and season – SED

3.6. Differential abundance

To determine the influence of season on the microbial community, we used a negative binomial analysis (*DESeq2*) to test differential abundance at genus taxonomic level within every tissue. For GL and DG, we considered only genera with an adjusted p-value lower than 0.01, and for SED, due to the wide array of significant genera, only the ones with an adjusted p-value lower than 0.05 were considered.

3.6.1. Differential abundance analysis for DG tissue:

52 significant genera were obtained for this tissue, with the most significant adjusted p-value belonging to the genus Rickettsiella (padj=6.08e-71), which does not come as a surprise as this genus was abundantly present in the winter, but completely absent in the summer. Following Rickettsiella, the top 10 ASVs that were significantly different between summer and winter were: Phaeocystidibacter, Candidatus Rhabdochlamydia, Fabibacter, Erythrobacter, Litorimicrobium, Legionella, Colwellia, Luminiphilus, Coxiella and Aestuariicoccus (Figure 21).

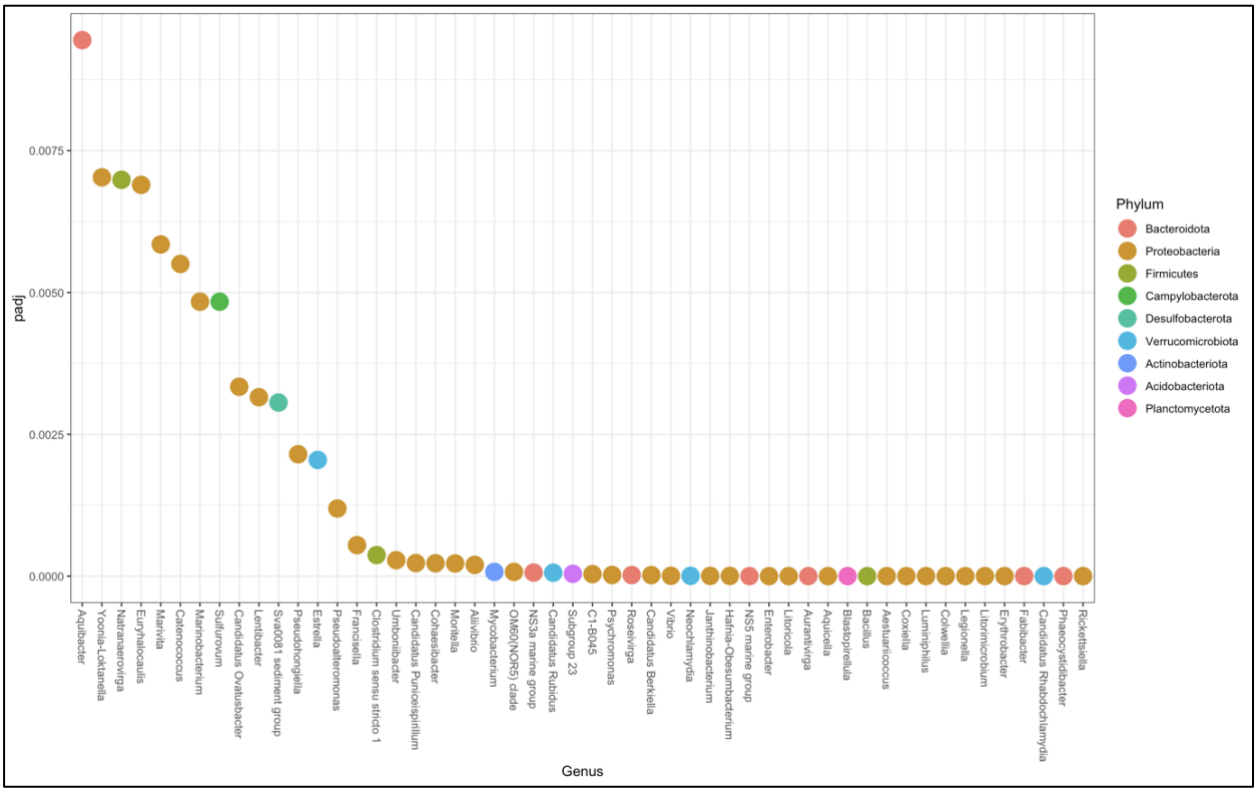

Figure 21. Significantly different genera across seasons as obtained by DESeq2 for DG

3.6.2. Differential abundance analysis for GL tissue:

For GL, 50 significant genera were obtained. The genus with the highest adjusted p-value was Catenococcus (padj=2.46e-109). The top 10 ASVs that were significantly different between summer and winter belonged to the genera: Malaciobacter, Phaeocystidibacter, Bacillus, MD3-55, Vibrio, Colwellia, Luminiphilus, Erythrobacter, Fabibacter and Litoricola (Figure 22).

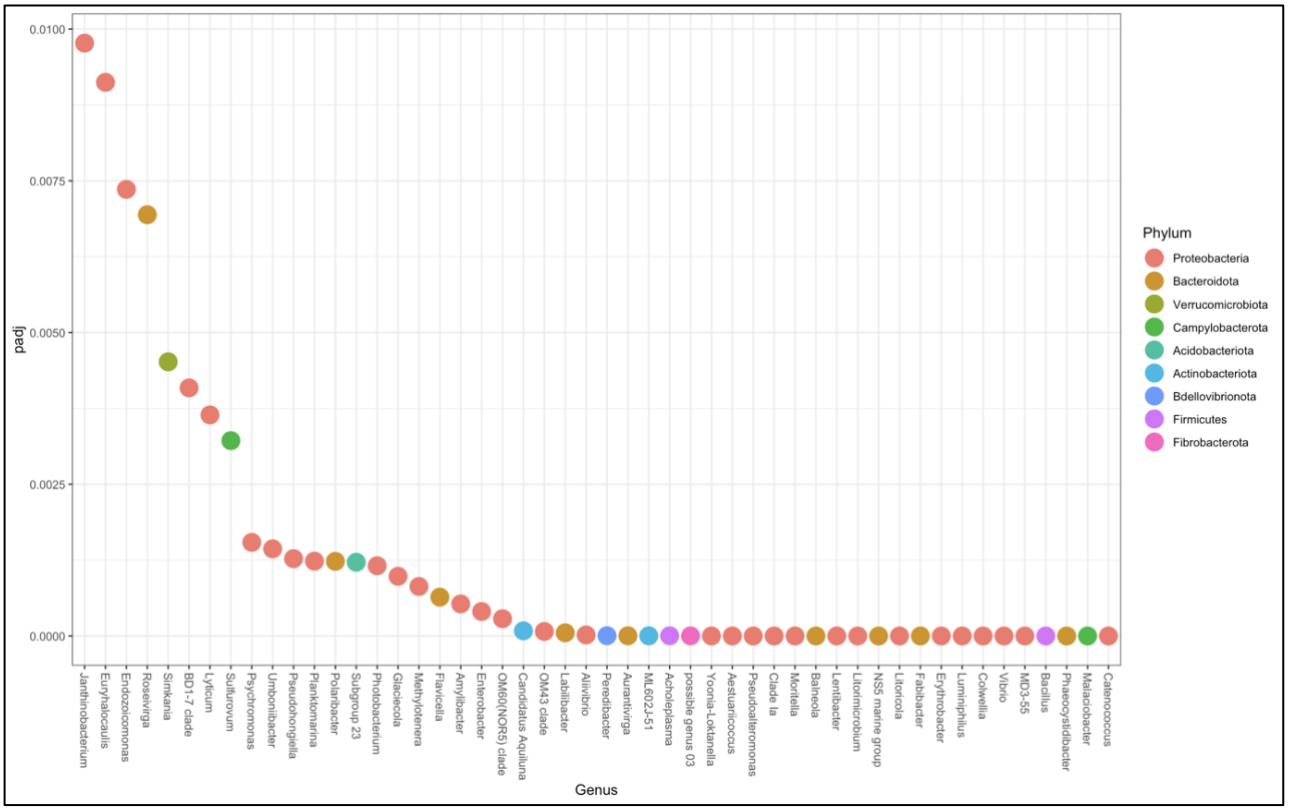

Figure 22. Significantly different genera across seasons as obtained by DESeq2 for GL

3.6.3. Differential abundance analysis for SED substrates:

17 significant genera were obtained for sediments, with the smallest adjusted p-value (9.6e-17) belonged to the genus Subgroup 23 sp. (Phylum: Acidobacteriota, Family: Thermoanaerobaculaceae, Class: Thermoanaerobaculia). Following that first genus, the top 10 ASVs that were significantly different across seasons belonged to the genera: Catenococcus, Endozoicomonas, Aliivibrio, Desulfocapsa, Hellea, Schleiferia, Ulvibacter, Photobacterium, Candidatus Latescibacter and Lewinella (Figure 23).

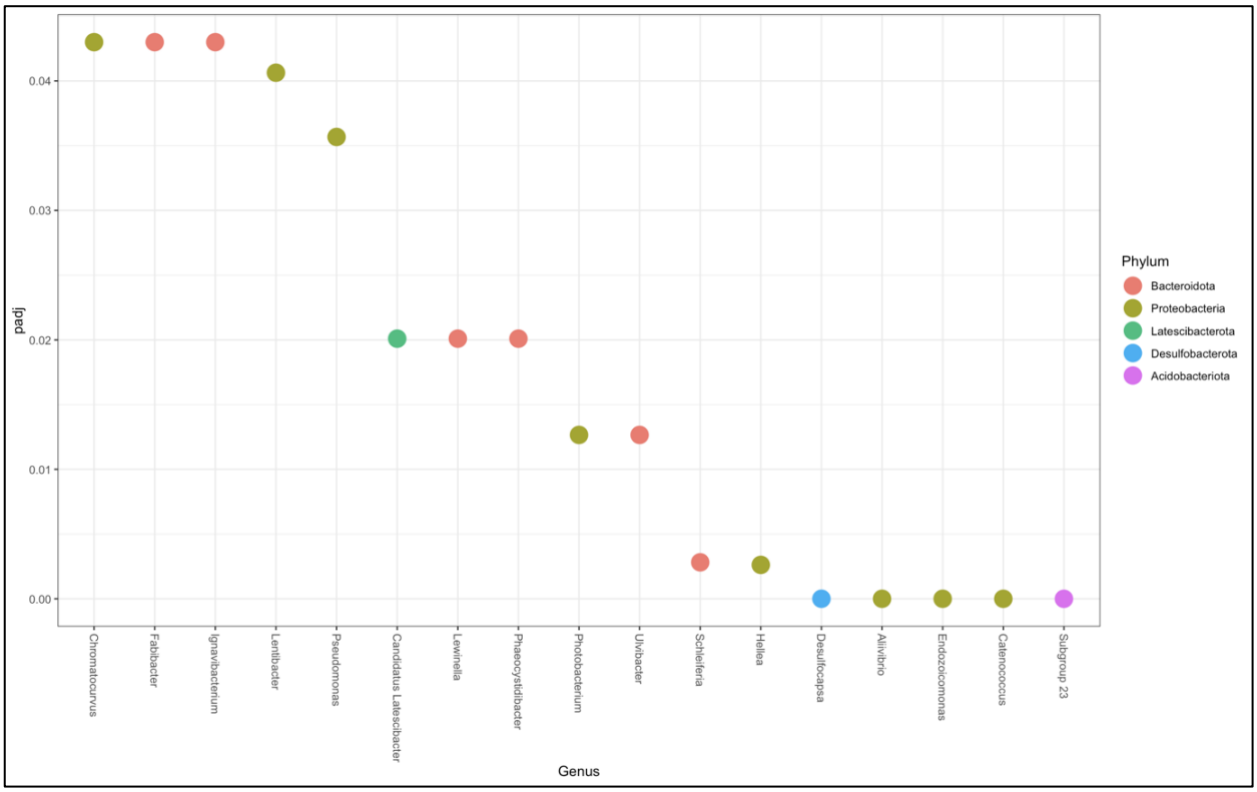

Figure 23. Significantly different genera across seasons as obtained by DESeq2 for SED

3.7. Seasonal and Spatial Distribution of the significant Genera

3.7.1. Seasonal and spatial distribution of the significant genera for DG tissue:

Our analysis of genus composition revealed five significant genera for DG: Mycoplasma, Vibrio, Rickettsiella, Aurantivirga, and Endozoicomonas. We examined more closely the presence and abundance of these genera across different sites and seasons. Mycoplasma displayed consistent presence across sites, with slightly higher relative abundance (RelAb) in summer (between 3% and 4%) compared to winter (around 2%), except for sites CH and PM which showed similar abundance year-round (Figure 24). Endozoicomonas, on the other hand, was primarily present in summer, except for CO and PM. This genus had the highest abundance in summer in CH and CO (RelAb = 0.098% and 0.092%) followed by PM (RelAb = 0.058%), MA, and SC (RelAb < 0.025%). In winter, it had a considerably high abundance in CO (RelAb = 0.1%) (Figure 25). The most striking seasonal variations occurred with Vibrio, Aurantivirga, and Rickettsiella. Vibrio dominated in the summer across all sites, reaching its peak abundances in MA and SC (RelAb = 0.53% and 0.43%). Conversely, this genus was nearly absent in winter, especially in PM and SC where it was almost non-existent (RelAb very close to 0), (Figure 26). Aurantivirga mirrored this pattern (Figure 27), being primarily a summer genus with minimal winter presence (RelAb < 0.025% and < 0.01% in CO and SC respectively). Rickettsiella displayed the opposite trend, being almost exclusively present in winter with all summer abundances being very close to 0. In winter, its highest abundance was found in SC (RelAb = 2.1%), followed by MA (RelAb = 1.41%) with lower abundances observed in the remaining sites (CO = 0.65%, PM = 0.47% and CH = 0.27%), (Figure 28).

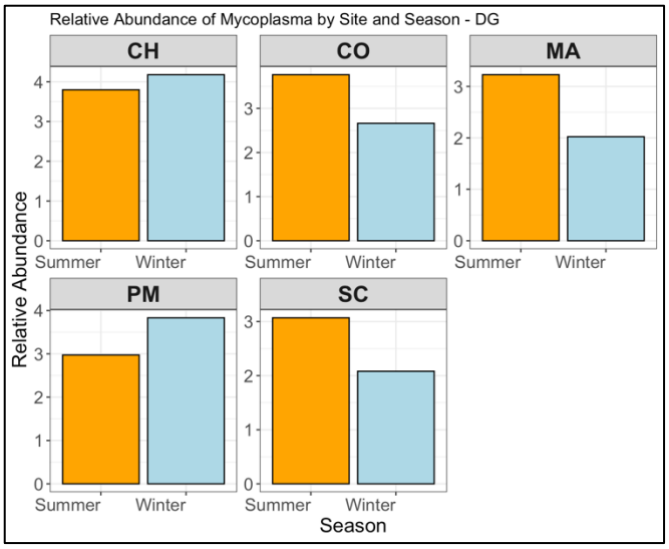

Figure 24. Relative abundance of Mycoplasma across sites and seasons - DG

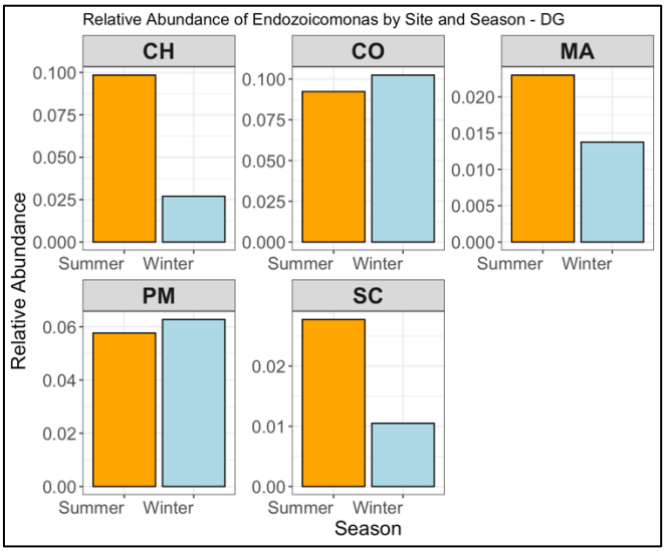

Figure 25. Relative abundance of Endozoicomonas across sites and seasons - DG

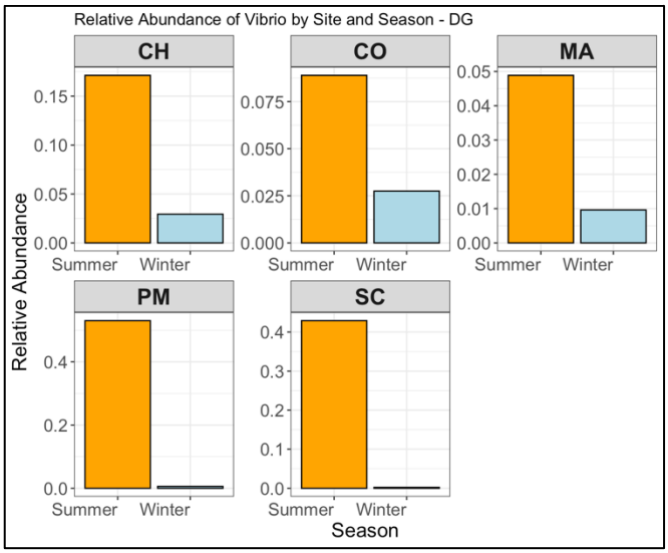

Figure 26. Relative abundance of Vibrio across sites and seasons - DG

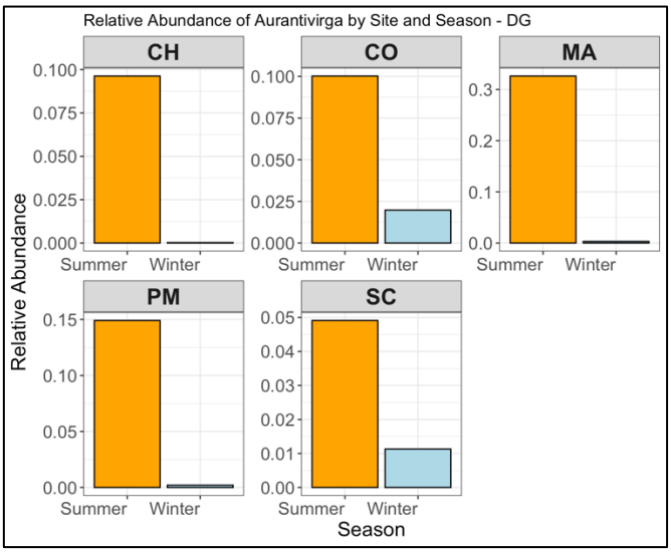

Figure 27. Relative abundance of Aurantivirga across sites and seasons - DG

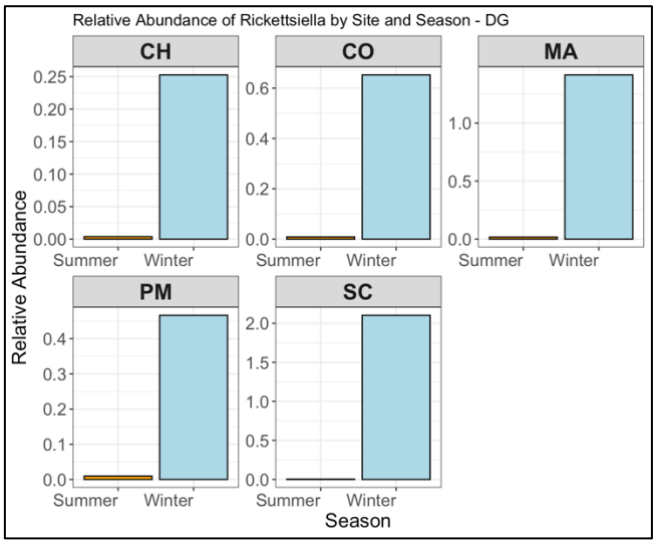

Figure 28. Relative abundance of Rickettsiella across sites and seasons - DG

3.7.2. Seasonal and spatial distribution of the significant genera for GL tissue:

Similar to DG, five genera emerged as the most abundant in GL tissues: Endozoicomonas, Vibrio, Spirochaeta 2, Catenococcus, and Candidatus Endoecteinascidia. Endozoicomonas dominated across all sites during both seasons, with a slight preference for winter months. Notably, its summer abundance in MA was the lowest (RelAb = 0.84%) compared to other sites that had relative abundances above 3.4% for this season. It constantly stayed the number one most abundant genus in winters win all sites with relative abundance above 3.84% (Figure 29). Vibrio mirrored its behavior in DG tissues, dominating summer across all sites, particularly in PM (RelAb = 1.12%), while being virtually absent in winter (Figure 30). Spirochaeta 2 displayed a more complex pattern, favoring summer in SC and MA but winter in CH, CO, and PM. Its highest abundance was observed in summer at SC (RelAb = 0.29%), where it was almost absent during winter (Figure 31). Catenococcus, the third most abundant genus in summer at PM (RelAb = 0.32%), followed a similar seasonal trend as Vibrio, being nearly undetectable in winter (Figure 32). Finally, Candidatus Endoecteinascidia, the third most abundant genus in both CO and CH during summer (RelAb = 0.34% and 0.05% respectively), exhibited lower abundances in the other sites. However, it appeared to favor winter months, particularly in SC and MA (Figure 33).

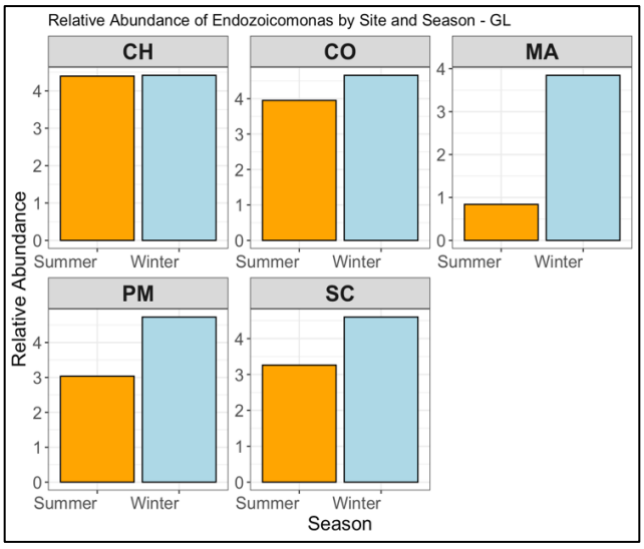

Figure 29. Relative abundance of Endozoicomonas across sites and seasons - GL

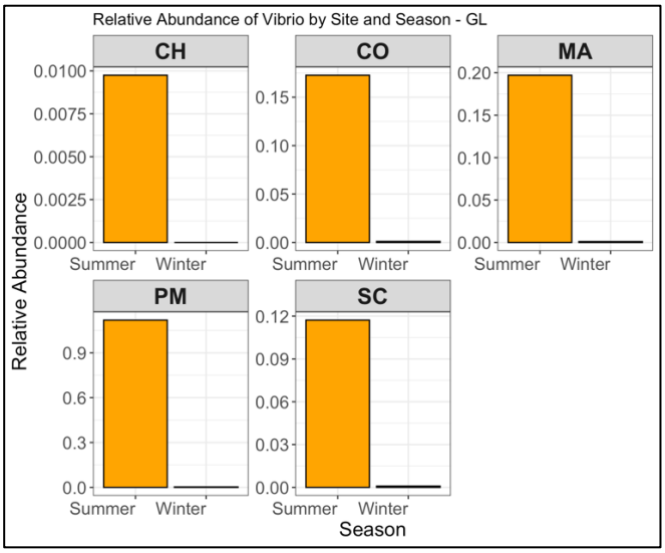

Figure 30. Relative abundance of Vibrio across sites and seasons - GL

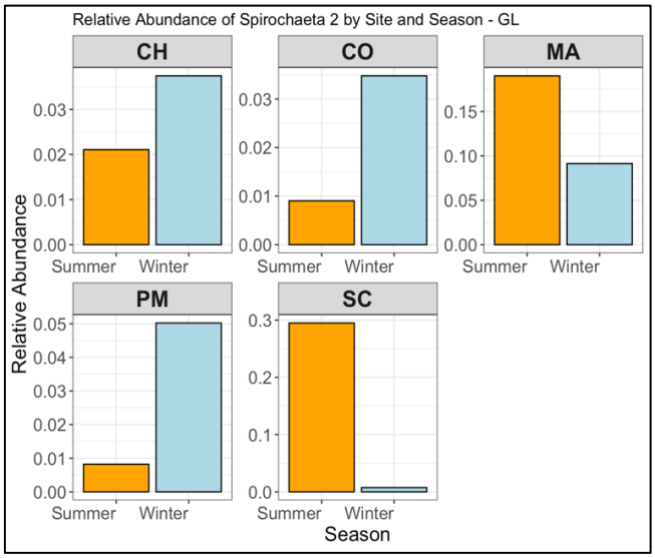

Figure 31. Relative abundance of Spirochaeta 2 across sites and seasons - GL

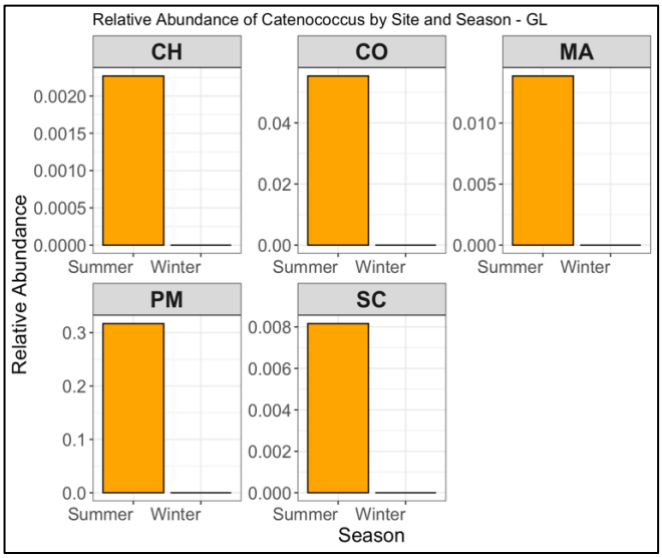

Figure 32. Relative abundance of Catenococcus across sites and seasons - GL

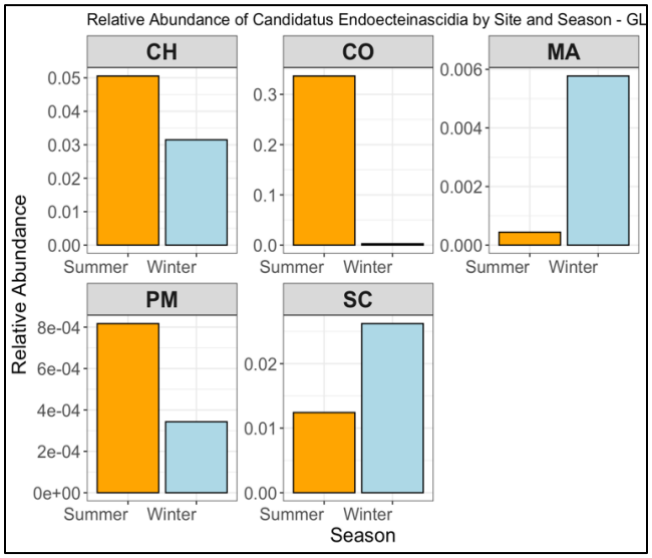

Figure 33. Relative abundance of Candidatus Endoecteinascidia across sites and seasons - GL

3.7.3. Seasonal and spatial distribution of the significant genera for SED substrates:

The analysis of sediment samples revealed a rich microbial community with six dominant genera: Woeseia, Halioglobus, Sulfurovum, Sediment group Sva0081, Actibacter, and Aquibacter. Woeseia stood out as the most prevalent genus, particularly during winter across all sites except CO, where its abundance was considerably lower (RelAb = 0.17%) compared to PM (RelAb = 0.64%) and SC (RelAb = 0.50%) (Figure 34). Halioglobus also displayed a seasonal preference for winter, reaching its peak abundance in SC (RelAb = 0.63%) but exhibiting higher summer abundance in MA (RelAb = 0.47%) (Figure 35). Sulfurovum, another highly abundant genus, showed a more complex seasonal pattern. Winter dominance was observed in all sites except CO, where summer abundance was higher. Its highest winter abundance was recorded in CH (RelAb = 0.51%), while winter abundances in MA and SC were remarkably low (around 0.05%) (Figure 36). Sva0081 (Phylum: Desulfobacterota) exhibited some seasonal variation, being more abundant in winter for PM and SC but favoring summer in CH, CO, and MA. Its highest abundance was observed in CH during winter (RelAb = 0.25%) (Figure 37). The remaining two genera, Actibacter and Aquibacter, while less abundant, were still significant. Their seasonal patterns were less clear. Actibacter displayed winter dominance in MA and SC but shifted to summer dominance in CH, CO, and PM. Its highest abundance was recorded in PM during summer with a relative abundance of 0.12%, while its lowest abundance was in CO during winter with a relative abundance of 0.02% (Figure 38). Aquibacter uniquely showed a strong preference for summer in SC (RelAb = 0.36%), with a dramatic decrease in winter abundance. Interestingly, only in SC and PM did Aquibacter exhibit higher winter abundance (Figure 39).

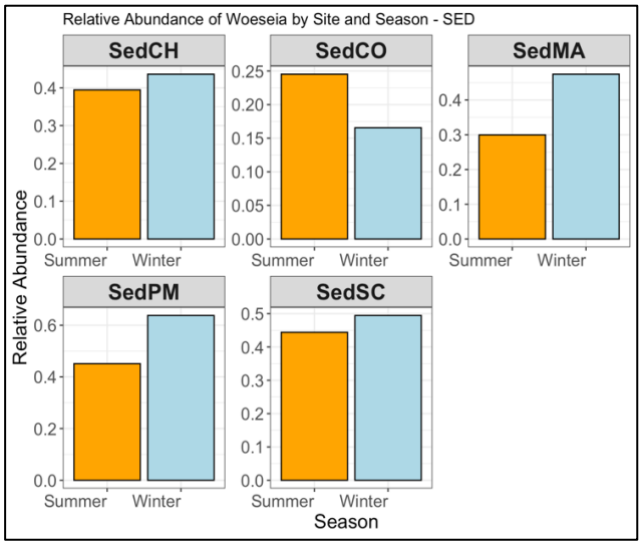

Figure 34. Relative abundance of Woeseia across sites and seasons - SED

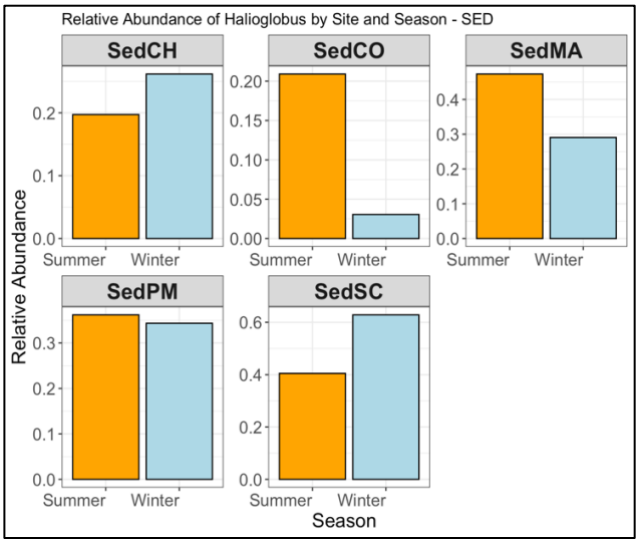

Figure 35. Relative abundance of Halioglobus across sites and seasons - SED

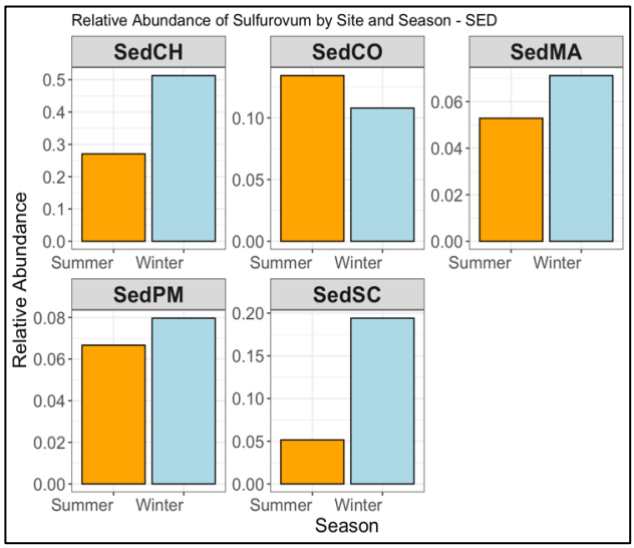

Figure 36. Relative abundance of Sulfurovum across sites and seasons - SED

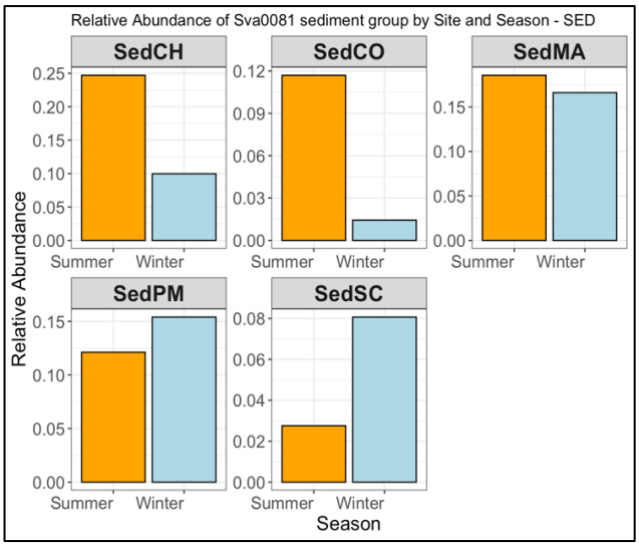

Figure 37. Relative abundance of Sva0081 across sites and seasons - SED

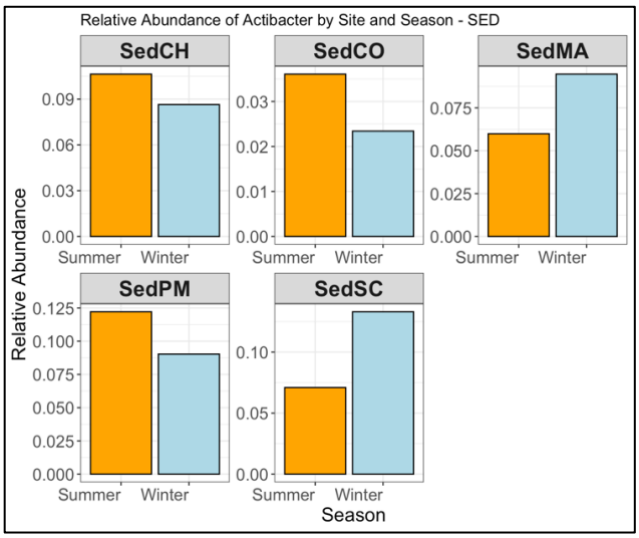

Figure 38. Relative abundance of Actibacter across sites and seasons - SED

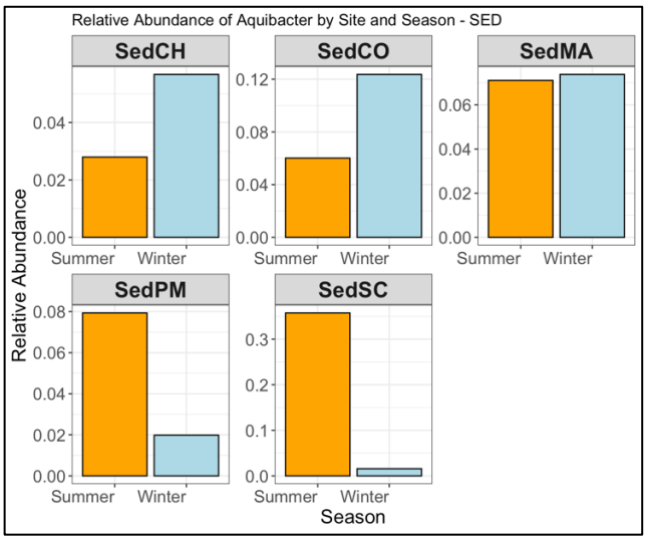

Figure 39. Relative abundance of Aquibacter across sites and seasons - SED

3.8. Correspondence analysis

In order to associate the environmental data with the microbiome composition in every site and during the two considered seasons, we perform a correspondence analysis for every tissue considered.

3.8.1. Correspondence analysis for the DG tissue:

Starting with DG, so far, it was clear that winter samples are less homogeneous than summer ones, and that we can distinguish between sites in this period more than in summer. Also, we observed that while the most abundant genera were Mycoplasma, Rickettsiella appears in the winter in good abundance, mostly in SC. We also saw that the difference between average oxygen values in summer and in winter was significant, with values being higher in winter. This is all evident from the correspondence analysis for the digestive gland (Figure 40). Ricketsiella, strongly associated with winter values, particularly at SC, emerges alongside the higher oxygen levels characteristic of winter. Mycoplasma, however, remains abundant throughout the year. Another important observation we can make, is the strong association of Vibrio with the temperature, and overall summer samples. Interestingly, the analysis reveals oxygen as the most influential factor on winter samples, followed by salinity and conductivity, while summer samples seem more influenced by pH and temperature.

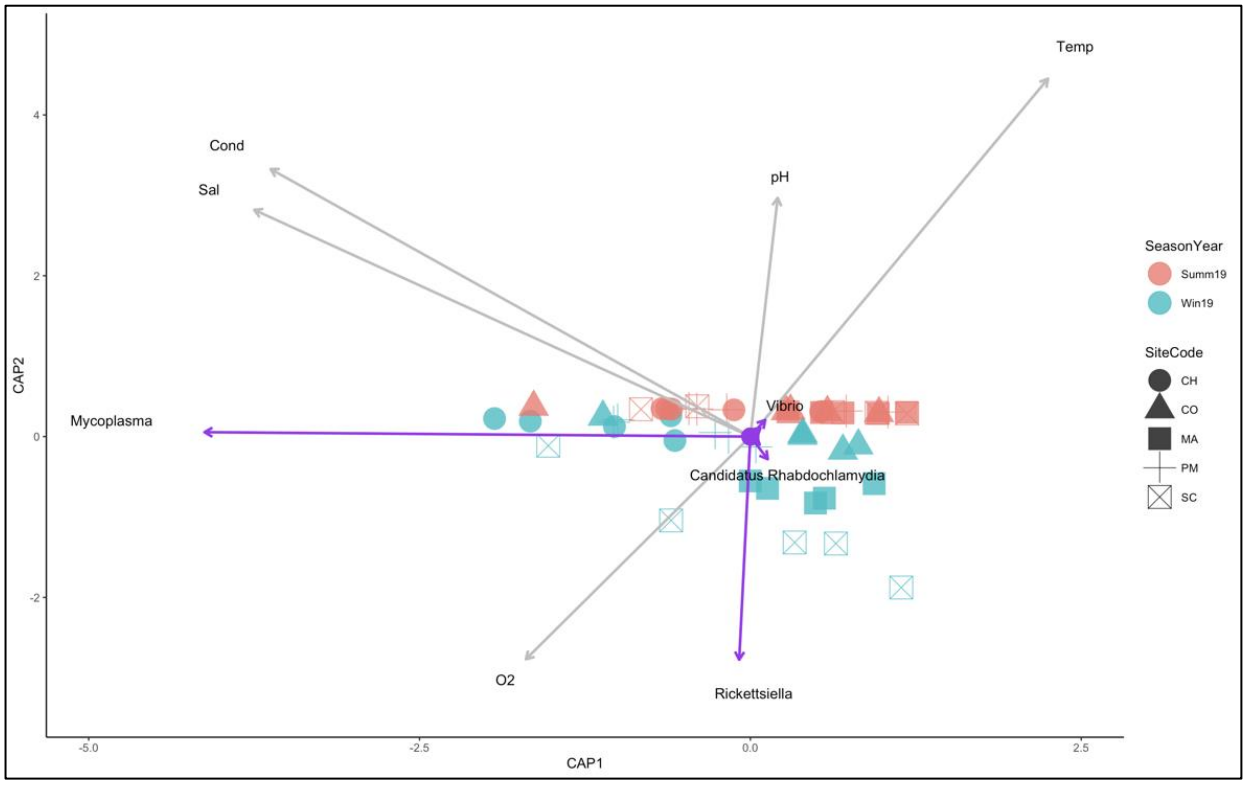

Figure 40. Correspondence analysis - DG

3.8.2. Correspondence analysis for the GL tissue:

Moving on to GL, we saw earlier that there is a clustering between samples from CH, PM and CO in both seasons, and that MA and SC have the most distinct microbiome, with MA having the most diverse microbiome between winter and summer. We also saw that Endozoicomonas dominates in both seasons, but in summer Vibrio has a high abundance as well in MA and PM.

This was also all evident in the correspondence analysis graph (Figure 41). Vibrio shows a strong association with summer samples, particularly at SC. With oxygen being the farthest away from Vibrio and conductivity, followed by salinity and temperature, it can be suggested that lower conductivity values, alongside the generally higher summer temperatures and

lower salinity, might be linked to Vibrio's presence in PM during summer. Overall, the analysis suggests that conductivity and salinity influence summer samples more, while Endozoicomonas associates with higher oxygen values, prevalent during winter 2019.

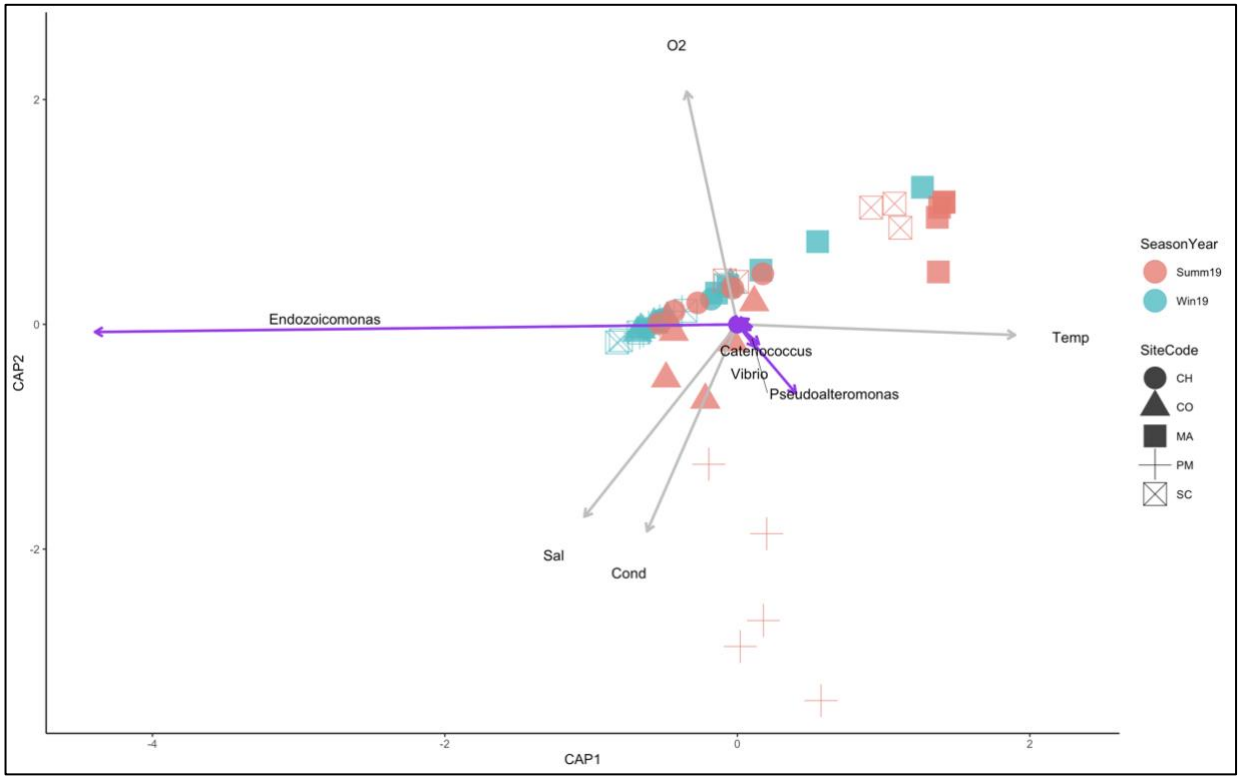

Figure 41. Correspondence analysis - GL

3.8.3. Correspondence analysis for the SED substrates:

Finally, for SED, we saw that there is a clear distinction between the sites, with a less evident difference between the seasons in CH, MA and PM, and that CO has the most distinct bacterial communities. It was also clear that many genera are abundant in sediment tissues, with Woeseia dominating, followed by Halioglobus. Other genera such as Aquibacter is abundant in summer in SC only, while Sulfurovum was abundant in winter in CH only. According to the correspondence analysis (Figure 42), Aquibacter correlates with summer values, particularly at site SC, aligning with the significantly higher summer temperatures (average 21.36°C, especially in SC with 23.12°C). Conversely, Sulfurovum and Woeseia associate with winter values at CH and display the closest link to conductivity, which was significantly higher in CH during winter 2019 (average 54.63 µS/cm). Halioglobus appears to be connected to winter SC samples, where it was previously found to have the highest abundance among genera.

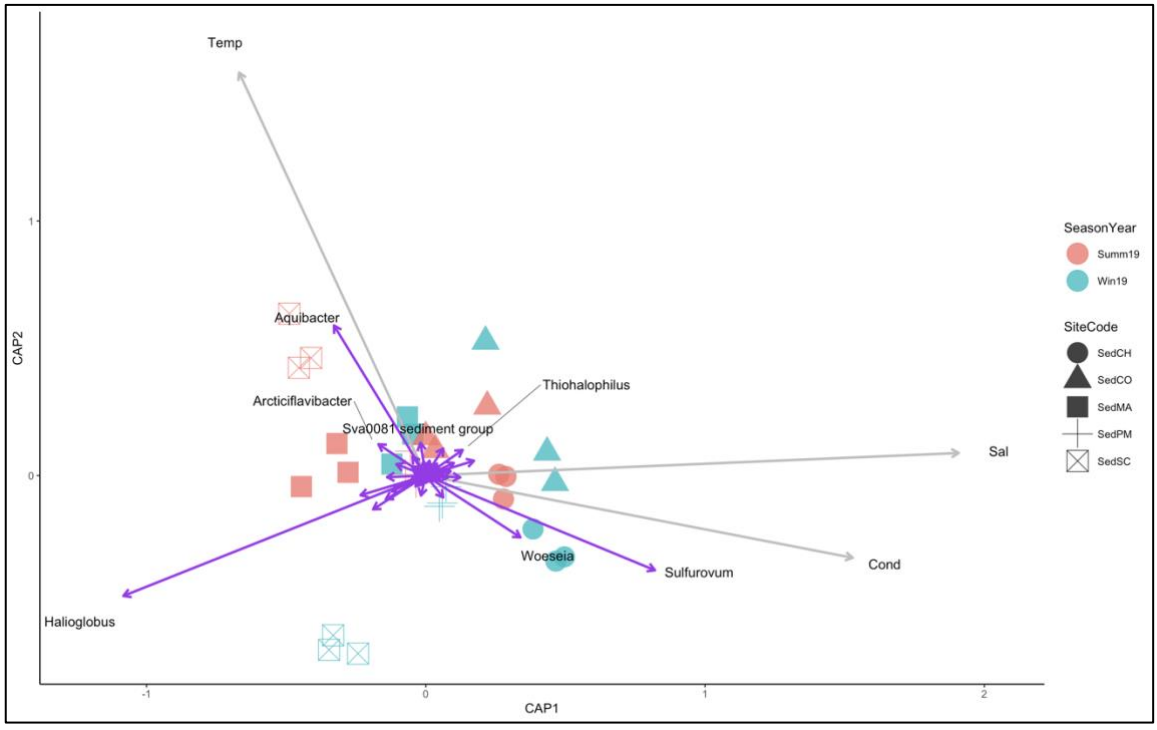

Figure 42. Correspondence analysis - SED

4. Discussion

This research investigated how seasonal and environmental variables influence the microbiome biodiversity of Manila clams (Ruditapes philippinarum) along the northern Adriatic coast. Five areas were chosen: Marano Lagunare (MA) in the Marano lagoon, Scardovari (SC) near the Po River delta, and three sites within the Venice Lagoon: Chioggia (CH), Colmata (CO), and the industrially polluted Porto Marghera (PM). This selection aimed to address the study's main objective by including four farming sites and one polluted site across diverse geographical locations and taking into consideration both the summer and the winter seasons of 2019. Extensive bioinformatics analysis began by examining how environmental conditions (temperature, conductivity, salinity, oxygen level, and pH of the water) varied across these sites and seasons. As expected, significant variations occurred both between sites and between summer and winter, creating an ideal setting to explore how the clams' microbiome responded to different conditions. Our study focused on the digestive gland and gills, as these tissues are known to be primary sites of bacterial bioaccumulation in filter-feeding organisms like clams (Zhu et al., 2023). This selection is further justified by their direct interface with the surrounding environment. The initial phase of the research investigated alpha and beta diversity within these tissues across different sites and seasons. This analysis served as a crucial foundation for subsequent, more in-depth investigations into the seasonal and spatial dynamics of the microbial communities within these two key tissues. General alpha diversity analysis across seasons revealed no significant difference in species richness between summer and winter (p-value = 0.79) when considering samples from all studied tissues and sites. This lack of seasonal distinction could be due to the combined analysis of potentially divergent microbial communities within the different tissues and sites. To address this, the data was further segregated by tissue type (GL and DG). Overall, the microbial communities within the clam tissues differed significantly from those found in the

sediments. The species richness in DG was significantly higher than the one in GL, and the one from SED was significantly higher than both clam tissues. Our findings so far are consistent with previous observations. Milan et al. (2018) reported higher microbial richness in winter for clams collected from the northern Adriatic Sea. This trend extends geographically, with studies in the Czech Republic (Kaevska et al., 2016) and China (Wang et al., 2019) demonstrating higher alpha diversity in colder seasons compared to warmer ones in aquatic environments. This analysis revealed a significantly higher species richness within DG during winter, while GL displayed greater seasonal stability. Zurel et al. (2011) observed a more seasonally stable microbiota in GL compared to DG of oysters in the eastern Mediterranean Sea, which aligns with our results. These observations can be explained biologically. The DG, directly involved in food processing, encounters a wider range of microorganisms through ingested particles (Milan et al., 2018; Zhu et al., 2023). This continuous influx, followed by physical and biochemical processing likely contributes to fluctuations in DG's microbiota. Conversely, GL primarily functions in respiration and filtration, resulting in a more stable environment, particularly across seasons where food availability fluctuates but respiratory demands remain constant. Notably, both clam tissues exhibit a degree of selectivity in filtering microbes compared to the surrounding sediments, which naturally harbor a highly diverse bacterial community (Sweet et al., 2011). When it came to observing the richness of the species in the tissues in each one of the sites, results were somehow surprising, since so far, it has been shown that GL are somewhat more stable than DG, however the results showed that the difference in alpha diversities in the different sites for GL was significant (p-value=0.013), and that for DG was not (p-value= 0.15).

To gain deeper insights into these trends, we turned to beta diversity analysis, allowing us to compare the bacterial communities across tissues. Notably, the overall beta diversity revealed distinct clustering based on tissue type. This is all expected since the tissues studied had

different physiological functions, but also since the gills act as a filter that selectively excludes some bacteria from reaching the gut resulting in different microbiota communities in gills versus the gut (Meisterhans et al., 2016).

However, the most interesting results emerged from the analysis of beta diversity in relation to tissue, site, and season. DG displayed significant seasonal variation across all sites, contrasting with the somewhat more stable bacterial communities observed in GL. This could most probably be due to the difference in food availability across the seasons, which would directly affect the feeding habits and microbial composition of the digestive gland (Yoon, Abe, & Kishi, 2013), but also due to the increased uptake of phytoplanktons that happens in response to higher demand of energy and nutrients when clams reach a complete maturation stage in the summer months (Meneghetti et al., 2004). Focusing first on this tissue, a striking observation was the distinctiveness of winter samples from Scardovari (SC). Geographically isolated from other sites, SC is situated near the Po River delta, Italy's largest wetland. This area has been significantly impacted by human activities including dam construction, river dredging, and coastal erosion (Simeoni & Corbau, 2009), resulting in altered environmental characteristics compared to the other study sites. Additionally, Scardovari does not receive direct freshwater inputs, and can thus be distinguished from the other lagoons (Casatta et al., 2016). Now to focus specifically on winter samples from SC, we observed the lowest temperatures values. Phytoplanktons thrive in higher temperatures, thus many species are significantly less abundant in winters (Ma & Mukai, 2009). This change in the diet could have an impact on the clams' microbiota. Notably, the impact of climate change on the Po River region has been documented, with studies reporting significant environmental alterations due to rising temperatures and sea levels (Brochier & Ramieri, 2001). Additionally, another study by Martinelli, Ruol, & Favaretto, (2021), reported severe weather events impacting the Po Delta from October 2018 to November 2019, coinciding with our

sample collection period. These events resulted in substantial coastal sandbank erosion and ecosystem disruption. Another study by Casatta et al. (2016) investigated the effects of sediment contamination in the Po River area on Manila clams, highlighting potential threats to aquatic life, and found that among the six areas studied, Scardovari exhibited the second worst environmental conditions and clam growth. The remaining sites displayed more similar beta diversity patterns, particularly the three sites within the Venetian Lagoon (Chioggia, Colmata, and Porto Marghera). This is unsurprising given their geographic proximity and likely shared environmental conditions and nutrient availability for the clams. Notably, the beta diversity analysis of DG revealed a clear distinction between the microbiota across these mentioned sites, but also between seasons.

The GL microbiotas exhibited less pronounced seasonal variations within the Venice lagoon. Notably, the Marano Lagoon displayed the most surprising difference in gill microbiotas between summer and winter. Samples from this lagoon also remained distinct from those collected at other sites throughout the study. In a previous study on the effect of the water flux in the Marano lagoon on clams, it was found that gills and siphons were the most influenced anatomical parts of the clams. With the water in Marano being contaminated by mercury, it was only normal for the gills of the Manila clams to harbor Hg-resistent strains from eight genera, including Enterobacter, Vibrio, Bacillus and Straphylococcus. (Baldi et al., 2013). Our samples from this site did indeed contain a significant abundance of both Enterobacter and Vibrio in gill tissues (0.1% and 0.2% respectively). It is important to note that so far clams in Marano are able to grow up healthy in this Hg-polluted area (Baldi et al., 2013). This distinction in the water condition could be a great explanation to the clear distinction in samples from MA in both seasons, from the rest of the sites studied.

As expected, the sediment microbial communities exhibited clear seasonal and geographic variations. Sediments directly reflect the surrounding environment, making them highly responsive to changes in environmental conditions (Meisterhans et al., 2016).

To further validate our previous observations, we examined the abundance of both phyla and genera across all tissues in relation to season and site. These findings further support our initial conclusions. First, distinct microbial communities were identified in DG, GL and SED. Second, GL microbiotas displayed greater seasonal stability compared to DG. Third, the digestive gland harbored a richer microbial community than the gills, with the sediments exhibiting the highest abundance. And fourth, the digestive gland displayed a clear seasonal shift in both phylum and genus composition. Proteobacteria and Bacteroidota, known members of common freshwater and marine microbial communities (Kaevska et al., 2016), were consistently abundant across all sites and seasons in both clam tissues. These findings align with previous studies reporting these two phyla as dominant contributors to the diversity and abundance of ASVs in marine organisms (Lokmer & Wegner, 2015; Zhu et al., 2023). Additionally, Firmicutes, well-established for their ability to process complex organic matter (Wang et al., 2019), emerged as the most abundant phylum in DG, reflecting its typical role within digestive systems. These three phyla constitute approximately 90% of the fish intestinal microbiota across various species (Ghanbari et al., 2015). At the genus level, the dominant presence of Mycoplasma in DG across all sites aligns with previous research demonstrating its dominance within the gut core microbiome (Milan et al., 2018; Aceves et al., 2018; Zhu et al., 2023). This dominance can be attributed to its parthenogenetic anaerobic properties, enabling it to ferment glucose and hydrolyze arginine to adapt to the gut environment (Zhu et al., 2023). In contrast, GL tissues were dominated by Endozoicomonas, another frequently encountered genus associated with diverse marine animal hosts, including bivalves (Schuett et al., 2007; Zurel et al., 2011; Neave et al., 2016; Zampieri et al., 2020).

Endozoicomonas possesses unusually flexible genomes and a vast array of metabolic pathways (Neave et al., 2016). Additional studies have linked this genus to aerobic heterotrophy, essential nutrient synthesis, and the secretion of depolymerizing enzymes within the gills (Jensen et al., 2021).

A particularly interesting finding was the elevated abundance of Vibrio spp. observed in summer, particularly within the gill tissues of clams from Porto Marghera, but also in the digestive gland. Vibrio are ubiquitous Gram-negative bacteria found in freshwater, and marine environments (Beleneva & Zhukova, 2009; Baker-Austin et al., 2018). Several species within this genus are known human and marine animal pathogens, with infections typically arising from contaminated water exposure or consumption of seafood harboring these bacteria. *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus,* and *Vibrio alginolyticus* are among the most common pathogenic Vibrio species (Siboni et al., 2016; Baker-Austin et al., 2018; Zampieri et al., 2020; Zampieri et al., 2021). While our analysis did not resolve Vibrio to the species level, previous research on Manila clams from the northern Adriatic Sea by Zampieri et al. (2020) and another research by Zhu et al. (2023) identified these pathogenic species within the clams. The seasonal presence of Vibrio spp. aligns with their well-documented preference for warmer, slightly saline waters (Beleneva et al., 2009; Siboni et al., 2016; Baker-Austin et al., 2018). This is further supported by our correspondence analysis, which revealed a positive correlation between Vibrio abundance, temperature, and summer samples, in both GL and DG in clams from Porto Marghera. Notably, this site is the most industrially polluted site among those studied, and it bioaccumulates a great number of organic pollutants deposited by the nearby factories (Bernardini et al.,2023). Patel & Koornhof (2004), previously demonstrated the ability of certain Vibrio species to thrive in industrially polluted environments, potentially contributing to their observed abundance in this location.

Another interesting observation was the striking abundance of Rickettsiella in the digestive tissue during the winter. It was abundant in all five sites, but it was surprisingly the most abundant genus in SC followed by MA. This bacterium appears to be completely absent in summer samples and in gill samples, which can suggest that Rickettsiella might have specific ecological or physiological interactions with the clam's digestive system that are influenced by winter conditions.The correspondence analysis agrees with these observations, where it is clear that Rickettsiella is mostly correlated with winter samples, particularly from SC and MA, but interestingly, there also appears to be a correlation with oxygen levels. When compared with the other sites, SC presented the highest average of oxygen values in winter 2019 (9.47 mg/l) but MA had the lowest average in winter (5.92 mg/l). Rickettsiella, a gram-negative bacteria, belonging to the phylum Pseudomonadota, is an intracellular parasite that lives inside other cells, and cannot survive on the outside. It is found within invertebrate hosts including bivalves. At the moment, it is still a relatively understudied genus, and the exact relationship with its host is still not widely known. While a previous study noted the presence of Rickettsiella among the genus composition of clams (Milan et al., 2018), its effect and importance were not investigated. No other study noted the striking presence of Rickettsiella among the genus composition of clams.

Despite the promising results, it is important to acknowledge certain limitations of our study. One major limitation was the short timeframe, the study only focused on samples from 2019, across 2 seasons, which is not enough time to get an idea of the long-term effects, or how the microbial communities could have changed across the years due to potential changes in environmental conditions across the years. This directly leads us to a second limitation,which was the sample size. Dealing with the high biodiversity observed within the microbial communities, it would be necessary to enlarge our sample size to better describe the

variability present within each site. Finally, other seasons could also be studied, to observe if some changes in the trends would arise.

To further build upon our findings, future research could investigate the abundance of Rickettsiella particularly in the digestive tissue during winter, as this bacterium was notably abundant only in the digestive gland and exclusively in winter, indicating a clear seasonal preference. Given the bacterium's very high abundance in the clam tissue and the current lack of knowledge about its role, it is essential to determine whether this association is beneficial, neutral, or harmful to the clams. Understanding the nature of this relationship could provide crucial insights into the health and biology of clams. Additionally, it would be useful to compare how the results from 2019 compare to results from different years when studying the same sites, in order to have a clearer picture of the long-term effects of the environmental conditions and pollution on clams.

5. Conclusion

This study unveiled the fascinating interplay between Manila clam tissues and their microbial communities. By looking deeper into the influence of season and environmental variables, bioinformatic analysis revealed distinct microbial profiles for each tissue. Sediments had the richest and most even bacterial communities, while the gill exhibited the lowest richness. Notably, beta diversity analysis painted a clear picture: each tissue formed a distinct microbial cluster, highlighting their unique compositions.

Further exploration into seasonal and spatial variations exposed more captivating results. The digestive gland, as well as the sediments, exhibited more pronounced seasonal shifts between summer and winter with respect to the gills.

While analysis revealed Proteobacteria and Bacteroidota as the predominant phyla across all samples, their presence varied in relative abundance across both site and season. At genus level, Mycoplasma, Vibrio, and Rickettsiella dominated in the digestive gland, particularly in polluted areas. In contrast, Endozoicomonas and Vibrio dominated the gill, while Woeseia, Halioglobus, and Sulfurovum were the sediment's most abundant genera. The abundance of these genera was affected by both season and site. Particularly, Vibrio, thrives in warmer weather and was linked to temperature, while Rickettsiella flourished in colder months and potentially responded to dissolved oxygen levels.

In conclusion, these findings painted a compelling picture: the site and its unique environment, the season, and the tissue type all play a crucial role in shaping the microbial communities within Manila clams. This knowledge is crutial for Manila clam aquaculture. By understanding the ideal microbial communities for healthy clams, we can select the best locations for farming. This not only ensures optimal growth but also helps us avoid areas with potentially harmful bacteria that can thrive in polluted environments.

This research opens doors for future exploration, acting as a stepping stone for developing methods for traceability studies to track where clams come from, helping to fight food fraud. Additionally, it shows promise in helping to identify clams harvested from areas restricted due to pollution concerns, ultimately ensuring that consumers have access to clams from clean and healthy waters.

6. REFERENCES

Aceves, A. K., Johnson, P., Bullard, S. A., Lafrentz, S., & Arias, C. R. (2018). Description and characterization of the digestive gland microbiome in the freshwater mussel Villosa nebulosa (Bivalvia: Unionidae). *Journal of Molluscan Studies, 84(3)*, 240-246.

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L., & Gilbert, M. T. P. (2016). Do vertebrate gut metagenomes confer rapid ecological adaptation?. *Trends in ecology & evolution, 31(9),* 689-699.

Apitz, S. E., Barbanti, A., Bocci, M., Carlin, A., Montobbio, L., & Bernstein, A. G. (2007). The sediments of the Venice Lagoon (Italy) evaluated in a screening risk assessment approach: Part II—Lagoon sediment quality compared to hot spots, regional, and international case studies. *Integrated Environmental Assessment and Management: An International Journal, 3(3)*, 415-438.

Baker-Austin, C., Oliver, J. D., Alam, M., Ali, A., Waldor, M. K., Qadri, F., & Martinez-Urtaza, J. (2018). Vibrio spp. infections. *Nature Reviews Disease Primers, 4(1),* 1-19.

Baldi, F., Gallo, M., Marchetto, D., Faleri, C., Maida, I., & Fani, R. (2013). Manila clams from Hg polluted sediments of Marano and Grado lagoons (Italy) harbor detoxifying Hg resistant bacteria in soft tissues. *Environmental research, 125*, 188-196.

Basili, M., Techtmann, S. M., Zaggia, L., Luna, G. M., & Quero, G. M. (2022). Partitioning and sources of microbial pollution in the Venice Lagoon. *Science of The Total Environment, 818*, 151755.

Beleneva, I. A., & Zhukova, N. V. (2009). Seasonal dynamics of cell numbers and biodiversity of marine heterotrophic bacteria inhabiting invertebrates and water ecosystems of the Peter the Great Bay, Sea of Japan. *Microbiology, 78*, 369-375.

Bellafiore, D., Umgiesser, G., & Cucco, A. (2008). Modeling the water exchanges between the Venice Lagoon and the Adriatic Sea. *Ocean Dynamics, 58*, 397-413.

Bellucci, L. G., Frignani, M., Paolucci, D., & Ravanelli, M. (2002). Distribution of heavy metals in sediments of the Venice Lagoon: the role of the industrial area. *Science of the total Environment, 295(1-3),* 35-49.

Bellucci, L. G., Mugnai, C., Giuliani, S., Romano, S., Albertazzi, S., & Frignani, M. (2013). PCDD/F contamination of the Venice Lagoon: A history of industrial activities and past management choices. *Aquatic ecosystem health & management, 16(1),* 62-69.

Bernardini, I., Quagliariello, A., Peruzza, L., Martino, M. E., Dalla Rovere, G., Iori, S., Asnicar, D., Ciscato, M., Fabrello, J., Corami, F., & Cecchetto, M. (2023). Contaminants from dredged sediments alter the transcriptome of Manila clam and induce shifts in microbiota composition. *BMC Biology, 21(1)*, 234.

Bordignon, F., Trocino, A., Rossetti, E., Zomeño, C., Pascual, A., Birolo, M., Llorens, S. M., & Xiccato, G. (2021). Effect of stocking density on growth and survival of juvenile

Manila clams (Ruditapes philippinarum) farmed in suspended lanterns in a North Italian lagoon. *Aquaculture Reports, 20*, 100719.

Boutin, S., Bernatchez, L., Audet, C., & Derôme, N. (2013). Network analysis highlights complex interactions between pathogen, host and commensal microbiota. *PloS one, 8(12),* e84772.

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs, 27(4)*, 326-349.

Brochier, F., & Ramieri, E. (2001). Climate change impacts on the Mediterranean coastal zones. *Available at SSRN 277549.*

Carvajal-Muñoz, J. S., & Carmona-Garcia, C. E. (2012). Benefits and limitations of biofertilization in agricultural practices. *Livestock Research for Rural Development, 24(3)*, 1-8.

Casatta, N., Stefani, F., Pozzoni, F., Guzzella, L., Marziali, L., Mascolo, G., & Viganò, L. (2016). Endocrine-disrupting chemicals in coastal lagoons of the Po River delta: sediment contamination, bioaccumulation and effects on Manila clams. *Environmental Science and Pollution Research, 23,* 10477-10493.

Çevirgen, S., Elwany, H., Pesce, M., & Zirino, A. (2020). Managing nutrient pollution in Venice Lagoon (Italy): a practical tool for assessment of water quality. *Sustainable Water Resources Management, 6*, 1-13.

CHEW, K. K. (1989). Manila clam biology and fishery development in western North America. *Clam Mariculture in North America*, 243-261.

Chiesa, S., Nonnis Marzano, F., Minervini, G., De Lucrezia, D., Baccarani, G., Bordignon, G., Poli, I., Ravagnan, G., & Argese, E. (2011). The invasive Manila clam Ruditapes philippinarum (Adams and Reeve, 1850) in Northern Adriatic Sea: Population genetics assessed by an integrated molecular approach. *Fisheries Research, 110(2)*, 259-267.

Coelho, P., Carvalho, F., Goulding, T., Chainho, P., & Guerreiro, J. (2021). Management models of the Manila clam (Ruditapes philippinarum) fisheries in invaded European coastal systems. *Frontiers in Marine Science, 8*, 685307.

Cordero, D., Delgado, M., Liu, B., Ruesink, J., & Saavedra, C. (2017). Population genetics of the Manila clam (Ruditapes philippinarum) introduced in North America and Europe. *Scientific Reports, 7(1)*, 39745.

Dubilier, N., Bergin, C., & Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology, 6(10)*, 725-740.

Eddelbuettel, D., Lucas, A., Tuszynski, J., Bengtsson, H., Urbanek, S., Frasca, M., Lewis, B., Stokely, M., Muehleisen, H., Murdoch, D., & Hester, J. (2024). Package 'digest'.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The gut microbiota of marine fish. *Frontiers in microbiology, 9,* 873.

Frascari, F., Frignani, M., Guerzoni, S., & Ravaioli, M. (1988). Sediments and Pollution in the Northern Adriatic Sea a. *Annals of the New York Academy of Sciences, 534(1),* 1000-1020.

Frignani, M., Bellucci, L. G., Carraro, C., & Favotto, M. (2004). Accumulation of polychlorinated biphenyls in sediments of the Venice Lagoon and the industrial area of Porto Marghera. *Chemosphere, 54(10)*, 1563-1572.

Frignani, M., Bellucci, L. G., Favotto, M., & Albertazzi, S. (2005). Pollution historical trends as recorded by sediments at selected sites of the Venice Lagoon. *Environment International, 31(7)*, 1011-1022.

Gerber, G. K. (2014). The dynamic microbiome. *FEBS letters, 588(22)*, 4131-4139.

Ghanbari, M., Kneifel, W., & Domig, K. J. (2015). A new view of the fish gut microbiome: advances from next-generation sequencing. *Aquaculture, 448*, 464-475.

Green, T. J., Siboni, N., King, W. L., Labbate, M., Seymour, J. R., & Raftos, D. (2019). Simulated marine heat wave alters abundance and structure of Vibrio populations associated with the Pacific Oyster resulting in a mass mortality event. *Microbial ecology, 77,* 736-747.

Greenacre, M. J. (2010). Correspondence analysis. *Wiley Interdisciplinary Reviews: Computational Statistics, 2(5)*, 613-619.

Guarino, C., & Sciarrillo, R. (2017). The effectiveness and efficiency of phytoremediation of a multicontaminated industrial site: Porto Marghera (Venice Lagoon, Italy). *Chemosphere, 183,* 371-379.

Han, F., Wang, X., Guo, J., Qi, C., Xu, C., Luo, Y., Li, E., Qin, J. G., & Chen, L. (2019). Effects of glycinin and β-conglycinin on growth performance and intestinal health in juvenile Chinese mitten crabs (Eriocheir sinensis). *Fish & Shellfish Immunology, 84*, 269-279.

Han, F., Xu, C., Qi, C., Lin, Z., Li, E., Wang, C., Wang, X., Qin, J. G., & Chen, L. (2020). Sodium butyrate can improve intestinal integrity and immunity in juvenile Chinese mitten crab (Eriocheir sinensis) fed glycinin. *Fish & Shellfish Immunology, 102*, 400-411.

Hooper, L. V., & Gordon, J. I. (2001). Commensal host-bacterial relationships in the gut. *Science, 292(5519)*, 1115-1118.

Hu, X., Yang, H.-L., Yan, Y.-Y., Zhang, C.-X., Ye, J., Lu, K.-L., Hu, L.-H., Zhang, J.-J., Ruan, L., & Sun, Y.-Z. (2019). Effects of fructooligosaccharide on growth, immunity and intestinal microbiota of shrimp (Litopenaeus vannamei) fed diets with fish meal partially replaced by soybean meal. *Aquaculture Nutrition, 25(1)*, 194-204.

Iannello, M., Mezzelani, M., Dalla Rovere, G., Smits, M., Patarnello, T., Ciofi, C., Carraro, L., Boffo, L., Ferraresso, S., Babbucci, M., Mazzariol, S., Centelleghe, C., Cardazzo, B., Carrer, C., Varagnolo, M., Nardi, A., Pittura, L., Benedetti, M., Fattorini, D., Regoli, F., Ghiselli, F., Gorbi, S., Bargelloni, L., & Milan, M. (2021). Long-lasting effects of chronic exposure to chemical pollution on the hologenome of the Manila clam. *Evolutionary Applications, 14(12)*, 2864-2880.

Infante-Villamil, S., Huerlimann, R., & Jerry, D. R. (2021). Microbiome diversity and dysbiosis in aquaculture. *Reviews in Aquaculture, 13(2),* 1077-1096.

Islam, F. U. A. (2007). Self-recruiting species (SRS) in aquaculture: their role in rural livelihoods in two areas of Bangladesh.

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D. N. (2015). Role of the normal gut microbiota. *World journal of gastroenterology: WJG, 21(29)*, 8787.

Jayanthi, M., Ravisankar, T., Nagaraj, G., Thirumurthy, S., Muralidhar, M., & Saraswathy, R. (2019). Is aquaculture abandonment a threat to sustainable coastal resource use?–a case study of Andhra Pradesh, India, with options for reuse. *Land use policy, 86,* 54-66.

Jensen, S., Frank, J. A., Arntzen, M. Ø., Duperron, S., Vaaje-Kolstad, G., & Hovland, M. (2021). Endozoicomonadaceae symbiont in gills of Acesta clam encodes genes for essential nutrients and polysaccharide degradation. *FEMS Microbiology Ecology, 97(6)*, fiab070.

Jiang, Q., Bhattarai, N., Pahlow, M., & Xu, Z. (2022). Environmental sustainability and footprints of global aquaculture. *Resources, Conservation and Recycling, 180,* 106183.

Kaevska, M., Videnska, P., Sedlar, K., & Slana, I. (2016). Seasonal changes in microbial community composition in river water studied using 454-pyrosequencing. *SpringerPlus, 5*, 1-8.

Kandlikar, G. S., Gold, Z. J., Cowen, M. C., Meyer, R. S., Freise, A. C., Kraft, N. J., Moberg-Parker, J., Sprague, J., Kushner, D. J., & Curd, E. E. (2018). ranacapa: An R package and Shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. *F1000Research, 7.*

Kawarazuka, N. (2010). The contribution of fish intake, aquaculture, and small-scale fisheries to improving nutrition: a literature review.

Konopiński, M. K. (2020). Shannon diversity index: a call to replace the original Shannon's formula with unbiased estimator in the population genetics studies. *PeerJ, 8, e9391.*

Lahti, L., & Shetty, S. (2018). Introduction to the microbiome R package. Preprint at https://microbiome.github.io/tutorials.

Lederberg, J., & McCray, A. T. (2001). Ome SweetOmics--A genealogical treasury of words. *The scientist, 15(7)*, 8-8.

Leite, L., Jude-Lemeilleur, F., Raymond, N., Henriques, I., Garabetian, F., & Alves, A. (2017). Phylogenetic diversity and functional characterization of the Manila clam microbiota: a culture-based approach. *Environmental Science and Pollution Research, 24*, 21721-21732.

Lema, K. A., Bourne, D. G., & Willis, B. L. (2014). Onset and establishment of diazotrophs and other bacterial associates in the early life history stages of the coral Acropora millepora. *Molecular ecology, 23(19)*, 4682-4695.

Li, T., Li, H., Gatesoupe, F.-J., She, R., Lin, Q., Yan, X., Li, J., & Li, X. (2017). Bacterial signatures of "red-operculum" disease in the gut of crucian carp (Carassius auratus). *Microbial Ecology, 74(3)*, 510-521.

Liu, X. (2016). Focus: Microbiome: Microbiome. *The Yale Journal of Biology and Medicine, 89(3)*, 275.

Lokmer, A., & Wegner, K. M. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *The ISME journal, 9(3)*, 670-682.

Losso, C., & Ghirardini, A. V. (2010). Overview of ecotoxicological studies performed in the Venice Lagoon (Italy). *Environment international, 36(1),* 92-121.

Love, M., Anders, S., & Huber, W. (2014). Differential analysis of count data–the DESeq2 package. *Genome Biol, 15(550)*, 10-1186.

Lund, E. K. (2013). Health benefits of seafood; is it just the fatty acids?. *Food chemistry, 140(3),* 413-420.

Ma, R. K., & Mukai, H. (2009). Food sources of the oyster (Crassostrea gigas) and the clam (Ruditapes philippinarum) in the Akkeshi-ko estuary. *Plankton and Benthos Research*, *4*(3), 104-114.

Mann, R. (1979). The effect of temperature on growth, physiology, and gametogenesis in the Manila clam Tapes philippinarum (Adams & Reeve, 1850). *Journal of Experimental Marine Biology and Ecology, 38(2),* 121-133.

Mann, R., & Glomb, S. J. (1978). The effect of temperature on growth and ammonia excretion of the Manila clam Tapes japonica. *Estuarine and Coastal Marine Science, 6(3)*, 335-339.

Martinelli, L., Ruol, P., & Favaretto, C. (2021, June). Analysis of overflow and wave overtopping of the Scardovari lagoon levees. In ISOPE International Ocean and Polar Engineering Conference (pp. ISOPE-I). ISOPE.

Matozzo, V., Binelli, A., Parolini, M., Locatello, L., & Marin, M. G. (2010). Biomarker responses and contamination levels in the clam Ruditapes philippinarum for biomonitoring the Lagoon of Venice (Italy). *Journal of Environmental Monitoring, 12(3)*, 776-786.

McKight, P. E., & Najab, J. (2010). Kruskal-wallis test. *The corsini encyclopedia of psychology,* 1-1.

McMurdie, P. J., & Holmes, S. (2013). Package 'phyloseq'. gan, 2(7).
Meisterhans, G., Raymond, N., Girault, E., Lambert, C., Bourrasseau, L., De Montaudouin, X., Garabetian, F., & Jude-Lemeilleur, F. (2016). Structure of Manila clam (Ruditapes philippinarum) microbiota at the organ scale in contrasting sets of individuals. *Microbial Ecology, 71,* 194-206.

Meneghetti, F., Moschino, V., & Da Ros, L. (2004). Gametogenic cycle and variations in oocyte size of Tapes philippinarum from the Lagoon of Venice. *Aquaculture, 240(1-4)*, 473-488.

Milan, M., Carraro, L., Fariselli, P., Martino, M. E., Cavalieri, D., Vitali, F., Boffo, L., Patarnello, T., Bargelloni, L., & Cardazzo, B. (2018). Microbiota and environmental stress: How pollution affects microbial communities in Manila clams. *Aquatic Toxicology, 194*, 195-207.

Milan, M., Ferraresso, S., Ciofi, C., Chelazzi, G., Carrer, C., Ferrari, G., Pavan, L., Patarnello, T., & Bargelloni, L. (2013). Exploring the effects of seasonality and chemical pollution on the hepatopancreas transcriptome of the Manila clam. *Molecular Ecology, 22(8)*, 2157-2172.

Moran, N. A., & Baumann, P. (2000). Bacterial endosymbionts in animals. *Current opinion in microbiology, 3(3)*, 270-275.

Neave, M. J., Apprill, A., Ferrier-Pagès, C., & Voolstra, C. R. (2016). Diversity and function of prevalent symbiotic marine bacteria in the genus Endozoicomonas. *Applied microbiology and biotechnology, 100*, 8315-8324.

O'Brien, P. A., Webster, N. S., Miller, D. J., & Bourne, D. G. (2019). Host-microbe coevolution: applying evidence from model systems to complex marine invertebrate holobionts. *MBio, 10(1),* 10-1128.

Oetama, V. S., Hennersdorf, P., Abdul-Aziz, M. A., Mrotzek, G., Haryanti, H., & Saluz, H. P. (2016). Microbiome analysis and detection of pathogenic bacteria of Penaeus monodon from Jakarta Bay and Bali. *Marine Pollution Bulletin, 110(2),* 718-725.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Wagner, H., & Oksanen, M. J. (2013). Package 'vegan': Community ecology package (Version 2.9) 1-295.

Patel, M. M., & Koornhof, H. J. (2004). Survival of Vibrio cholerae in industrially polluted water, with particular reference to iron concentrations. *Water Sa, 30(1),* 65-69.

Pavoni, B., Marcomini, A., Sfriso, A., Donazzolo, R., & Orio, A. A. (1992). Changes in an estuarine ecosystem: the lagoon of Venice as a case study.

Petersen, C., & Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cellular microbiology, 16(7),* 1024-1033.

Pizzini, S., Giubilato, E., Morabito, E., Barbaro, E., Bonetto, A., Calgaro, L., Feltracco, M., Semenzin, E., Vecchiato, M., Zangrando, R., & Gambaro, A. (2024). Contaminants of emerging concern in water and sediment of the Venice lagoon, Italy. *Environmental Research, 118401.*

Qiao, F., Liu, Y. K., Sun, Y. H., Wang, X. D., Chen, K., Li, T. Y., Li, E. C., & Zhang, M. L. (2017). Influence of different dietary carbohydrate sources on the growth and intestinal microbiota of Litopenaeus vannamei at low salinity. *Aquaculture Nutrition, 23(3),* 444-452.

Ravera, O. (2000). The Lagoon of Venice: the result of both natural factors and human influence. *Journal of Limnology, 59(1)*, 19-30.

Rice, M. A. (2001, January). Environmental impacts of shellfish aquaculture: filter feeding to control eutrophication. In Marine aquaculture and the environment: a meeting for stakeholders in the Northeast. *Cape Cod Press*, Falmouth, MA, USA (pp. 77-86).

Riminucci, F., Funari, V., Ravaioli, M., & Capotondi, L. (2022). Trace metals accumulation on modern sediments from Po river prodelta, North Adriatic Sea. *Marine Pollution Bulletin, 175*, 113399.

Robert, R., Sánchez, J. L., Pérez-Parallé, L., Ponis, E., Kamermans, P., & O'Mahoney, M. (2013). A glimpse on the mollusc industry in Europe. *Aquaculture Europe, 38(1)*, 5-11.

Roper, C., Castro, C., & Ingel, B. (2019). Xylella fastidiosa: bacterial parasitism with hallmarks of commensalism. *Current opinion in plant biology, 50,* 140-147.

Rosati, G., Solidoro, C., & Canu, D. (2020). Mercury dynamics in a changing coastal area over industrial and postindustrial phases: Lessons from the Venice Lagoon. *Science of the total environment, 743*, 140586.

Rosner, B., Glynn, R. J., & Lee, M. L. T. (2006). The Wilcoxon signed rank test for paired comparisons of clustered data. *Biometrics, 62(1),* 185-192.

Schuett, C., Doepke, H., Grathoff, A., & Gedde, M. (2007). Bacterial aggregates in the tentacles of the sea anemone Metridium senile. *Helgoland Marine Research, 61*, 211-216.

Sha, Y., Liu, M., Wang, B., Jiang, K., Sun, G., & Wang, L. (2016). Gut bacterial diversity of farmed sea cucumbers Apostichopus japonicus with different growth rates. *Microbiology, 85*, 109-115.

Shpigel, M., & Fridman, R. (1990). Propagation of the Manila clam (Tapes semidecussatus) in the effluent of fish aquaculture ponds in Eilat, Israel. *Aquaculture, 90(2)*, 113-122.

Shumway, S. E., Davis, C., Downey, R., Karney, R., Kraeuter, J., Parsons, J., Rheault, R., & Wikfors, G. (2003). Shellfish aquaculture–in praise of sustainable economies and

environments. *World Aquaculture, 34(4),* 8-10.

Siboni, N., Balaraju, V., Carney, R., Labbate, M., & Seymour, J. R. (2016). Spatiotemporal dynamics of Vibrio spp. within the Sydney Harbour Estuary. *Frontiers in Microbiology, 7,* 181053.
Sievert, C., Parmer, C., Hocking, T., Chamberlain, S., Ram, K., Corvellec, M., Despouy, P., & Sievert, M. C. (2021). Package 'plotly' (Version 4.10.1) [R package]. R Foundation for Statistical Computing.

Simeoni, U., & Corbau, C. (2009). A review of the Delta Po evolution (Italy) related to climatic changes and human impacts. *Geomorphology, 107(1-2),* 64-71.

Sladonja, B., Bettoso, N., Zentilin, A., Tamberlich, F., & Acquavita, A. (2011). Manila Clam (Tapes philippinarum Adams & Reeve, 1852) in the Lagoon of Marano and Grado (Northern Adriatic Sea, Italy): socio-economic and environmental pathway of a shell farm. *Aquaculture and the environment–A shared destiny, InTech Publisher*, 51-78.

Subasinghe, R., Soto, D., & Jia, J. (2009). Global aquaculture and its role in sustainable development. *Reviews in aquaculture, 1(1),* 2-9.

Sun, P., Jin, M., Ding, L., Lu, Y., Ma, H., Yuan, Y., & Zhou, Q. (2018). Dietary lipid levels could improve growth and intestinal microbiota of juvenile swimming crab, Portunus trituberculatus. *Aquaculture, 490*, 208-216.

Sweet, M. J., Croquer, A., & Bythell, J. C. (2011). Bacterial assemblages differ between compartments within the coral holobiont. *Coral Reefs, 30*, 39-52.

Thukral, A. K. (2017). A review on measurement of Alpha diversity in biology. *Agricultural Research Journal, 54(1).*

van Oppen, M. J., & Blackall, L. L. (2019). Coral microbiome dynamics, functions and design in a changing world. *Nature Reviews Microbiology*, *17*(9), 557-567.

Venugopal, V., & Gopakumar, K. (2017). Shellfish: nutritive value, health benefits, and consumer safety. *Comprehensive Reviews in Food Science and Food Safety, 16(6),* 1219-1242.

Visick, K. L., & McFall-Ngai, M. J. (2000). An exclusive contract: specificity in the Vibrio fischeri-Euprymna scolopes partnership. *Journal of Bacteriology, 182(7),* 1779-1787.

Walker, W. A. (2017). Dysbiosis. In *The microbiota in gastrointestinal pathophysiology* (pp. 227-232). Academic Press.

Wang, J., Huang, Y., Xu, K., Zhang, X., Sun, H., Fan, L., & Yan, M. (2019). White spot syndrome virus (WSSV) infection impacts intestinal microbiota composition and function in Litopenaeus vannamei. *Fish & shellfish immunology, 84*, 130-137.

Wang, Y., Liu, Y., Wang, J., Luo, T., Zhang, R., Sun, J., Zheng, Q., & Jiao, N. (2019). Seasonal dynamics of bacterial communities in the surface seawater around subtropical

Xiamen Island, China, as determined by 16S rRNA gene profiling. *Marine Pollution Bulletin, 142*, 135-144.

Wickham, H., Chang, W., & Wickham, M. H. (2016). Package 'ggplot2'. Create elegant data visualisations using the grammar of graphics. Version, 2(1), 1-189.

Wickham, H., François, R., Henry, L., Müller, K., & Vaughan, D. Posit Software PBC (2023). dplyr: A Grammar of Data Manipulation.

Wu, D., Daugherty, S. C., Van Aken, S. E., Pai, G. H., Watkins, K. L., Khouri, H., Tallon, L. J., Zaborsky, J. M., Dunbar, H. E., Tran, P. L., & Moran, N. A. (2006). Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. *PLoS Biology, 4(6),* e188.

Xia, Y., & Sun, J. (2023). Beta Diversity Metrics and Ordination. In *Bioinformatic and Statistical Analysis of Microbiome Data: From Raw Sequences to Advanced Modeling with QIIME 2 and R* (pp. 335-395). Cham: Springer International Publishing.

Xiong, J., Zhu, J., Dai, W., Dong, C., Qiu, Q., & Li, C. (2017). Integrating gut microbiota immaturity and disease-discriminatory taxa to diagnose the initiation and severity of shrimp disease. *Environmental microbiology, 19(4),* 1490-1501.

Yoon, S., Abe, H., & Kishi, M. J. (2013). Responses of Manila clam growth and its food sources to global warming in a subarctic lagoon in Japan. *Progress in oceanography, 119,* 48-58.

You, C., Chen, B., Wang, M., Wang, S., Zhang, M., Sun, Z., Aweya, J. J., Ma, H., & Li, Y. (2019). Effects of dietary lipid sources on the intestinal microbiome and health of golden pompano (Trachinotus ovatus). *Fish & Shellfish Immunology, 89,* 187-197.

Zampieri, A., Babbucci, M., Carraro, L., Milan, M., Fasolato, L., & Cardazzo, B. (2021). Combining Culture-dependent and culture-independent methods: New methodology insight on the Vibrio community of Ruditapes philippinarum. *Foods, 10(6),* 1271.

Zampieri, A., Carraro, L., Cardazzo, B., Milan, M., Babbucci, M., Smits, M., Boffo, L., & Fasolato, L. (2020). Depuration processes affect the Vibrio community in the microbiota of the Manila clam, *Ruditapes philippinarum*. *Environmental Microbiology*, 22(10), 4456-4472.

Zhang, Z., Xing, R., Lv, Z., Shao, Y., Zhang, W., Zhao, X., & Li, C. (2018). Analysis of gut microbiota revealed Lactococcus garviaeae could be an indicative of skin ulceration syndrome in farmed sea cucumber Apostichopus japonicus. *Fish & shellfish immunology, 80,* 148-154.

Zhou, G., Cai, L., Yuan, T., Tian, R., Tong, H., Zhang, W., Jiang, L., Guo, M., Liu, S., Qian, P. Y., & Huang, H. (2017). Microbiome dynamics in early life stages of the scleractinian coral *Acropora gemmifera* in response to elevated pCO2. *Environmental Microbiology*, 19(8), 3342-3352.

Zhou, Y., Zhang, D., Peatman, E., Rhodes, M. A., Liu, J., & Davis, D. A. (2017). Effects of various levels of dietary copper supplementation with copper sulfate and copper hydroxychloride on Pacific white shrimp Litopenaeus vannamei performance and microbial communities. *Aquaculture, 476*, 94-105.

Zhu, F., Zhang, X., Liu, J., Ma, X., Yang, J., Wang, Y., ... & Li, Y. (2023). Effects of Natural and Artificial Seawater on Digestive Gland and Gill Microorganisms of the Manila Clam (Ruditapes philippinarum) during Temporary Rearing without Feeding. *Aquaculture Research, 2023*.

Zhu, F., Zhang, X., Liu, J., Ma, X., Yang, J., Wang, Y., Lou, Y., & Li, Y. (2023). Effects of natural and artificial seawater on digestive gland and gill microorganisms of the Manila clam (Ruditapes philippinarum) during temporary rearing without feeding. *Aquaculture Research, 2023(1)*, 1042316.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS microbiology reviews, 32(5)*, 723-735.

Zurel, D., Benayahu, Y., Or, A., Kovacs, A., & Gophna, U. (2011). Composition and dynamics of the gill microbiota of an invasive Indo-Pacific oyster in the eastern Mediterranean Sea. *Environmental Microbiology, 13(6),* 1467-1476.