UNIVERSITY OF ALGARVE

MASTER THESIS

Disentangling Microbial Community Variations across Three European Coastal Lagoons

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A thesis submitted in fulfillment of the requirements for the degree of Master in Applied Ecohydrology

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Supervisor Statement

DECLARATION

From the supervisor(s):

I hereby declare that I have thoroughly reviewed the content of the proposed thesis for the Erasmus mundus Master course in applied ecohydrology intitled "Disentangling Microbial Community Variations across Three European Coastal Lagoons" by Helena GALYS, and that the thesis report is complete and prepared for submission. The research, analysis, and documentation presented therein meet the required standards for examination and evaluation.

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I, Helena GALYS, declare I am the author of this work, titled "Disentangling Microbial Community Variations across Three European Coastal Lagoons" which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references. I further confirm that:

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UNIVERSITY OF ALGARVE

Abstract

Faculty of Science and Technology
Marine and Environmental Research Centre

Master in Applied Ecohydrology

Disentangling Microbial Community Variations across Three European Coastal Lagoons

by Helena GALYS

Coastal lagoon ecosystems are vital and dynamic habitats, yet their microbial community structures remain poorly understood. This thesis presents a comprehensive investigation of microbial communities in the three Southern European Coastal Lagoons Butrinti Lagoon (Albania), Sabaudia (Italy), and Ria Formosa (Portugal) aiming to identify key taxa and potential interactions that may offer generalizable insights into lagoon microbial ecology. A multi-faceted approach was employed, combining elemental composition analysis and high-throughput DNA sequencing to characterize both the quantitative and qualitative aspects of microbial communities. The results provide in-depth descriptions of microbial composition and diversity in each lagoon, highlighting unique taxonomic signatures. Subsequently, a correlative analysis suggested the potential of key bacteria (e.g. *Flacobacteriaceae* and *Desulfobulbaceae*) as potential indicator species for anthropogenic impacts, such as heavy metal pollution. Further network inference revealed distinct genera (*Haliglobus*, *Woeseia*, *Actibacter*, *Sandaracinaceae* and an unknown species of *Gammaproteobacteria*) as shared key taxa across the lagoons. Notably, the findings suggest potential general patterns in microbial community composition within the three European coastal lagoons. However, given the exploratory nature of this study, further research is encouraged to confirm and expand upon these preliminary insights.

Keywords: European Coastal Lagoons, Microbial Communities, 16S rRNA gene amplicon sequencing, Comparative Analysis, Community Patterns

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Chapter 1

Introduction

Coastal lagoons are unique and ecologically significant environments, acting as vital links between terrestrial and marine ecosystems governed by complex ecohydrological processes. These dynamic habitats provide essential ecosystem services, supporting a diverse array of flora and fauna while serving as buffers against environmental fluctuations. Central to the ecological functioning and resilience of coastal lagoons are microbial communities, representing a foundational component of coastal lagoon ecosystems that orchestrate essential ecological processes. Through nutrient cycling and biogeochemical transformations, microbial communities drive the availability and turnover of vital elements, shaping the productivity and biodiversity within these habitats. Moreover, their role in the degradation of organic matter and the bioremediation of pollutants underscores their crucial contributions to the maintenance of environmental quality and ecosystem resilience. Said natural functions of microbial communities elevate them to key players in defining ecohydrological interventions capable of enhancing the natural carrying capacity of the system and improving ecosystem health. Yet, a profound knowledge on microbial functioning in coastal lagoons is crucial to fully harvest their potential.

While scientific research on microbial communities in coastal lagoons has amassed considerable knowledge, the majority of studies have predominantly focused on analyzing microbial community structures and functioning within individual lagoons. However, due to the great diversity of coastal lagoons, the analysis of inidvidual lagoons cannot distinguish between local characteristics and overarching patterns and trends. Consequently, insights into broader trends and comparative assessments across multiple coastal lagoons have remained scarce. Multi-lagoon studies would thus be of particular interest to help close knowledge gaps on interactions and synergies between microbial communities and the abiotic factors governing these ecosystems. Additionally, they could provide a fertile ground to investigate potential impacts of anthropogenic disturbances, including climate change, pollution, and habitat alteration, on microbial communities and their functional roles in coastal lagoons.

This master thesis thus aims to contribute to our understanding of microbial processes in coastal lagoons by unravelling the variations and commonalities in microbial communities across three European coastal lagoons - the Butrinti Lagoon in Albania, Sabaudia Lagoon in Italy, and the Ria Formosa Lagoon in Portugal. For this purpose, the study first provides an in-depth analysis of the taxonomic composition, environmental drivers, and functional potential of each lagoon separately, founded on the integration of advanced molecular techniques such as Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (CARD-FISH) and Illumina-Sequencing of the 16S rRNA gene as well as environmental analyses through Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), CHN-Analysis, and multimeter water quality measurements. Subsequently, an attempt was made to derive trends and variations through a comparative assessment of all three lagoons. Thus, by embracing the complexity of these systems, this research attempts to unlock valuable insights into the general principles governing microbial community dynamics in coastal lagoons and assessing potential consequences of anthropogenic disturbances. The findings enhance our knowledge of coastal lagoon ecosystems, providing valuable insights for conservation and sustainable management practices in the face of environmental challenges.

The following sections of this chapter will introduce the reader to the coastal lagoon ecosystem and the importance of microbial communities therein. Chapter 2 will elaborate on the materials and methods employed in sample collection, laboratory analyses, and data processing. Chapter 3 will present the results of the physicochemical characterisation of the lagoon samples as well as the qualitative, and quantitative characterization of the lagoon microbiome, including the outcomes of the CARD-FISH and Illumina sequencing analyses. This chapter also comprises a comparison of all three lagoons. Subsequently, Chapter 4 will offer a comprehensive discussion, interpretation, and integration of the results, culminating in a holistic understanding of the microbial community variations across Butrinti Lagoon, Sabaudia Lagoon, and Ria Formosa. Finally, a short conclusion will be drawn in chapter 6.

1.1 Coastal Lagoons

Coastal lagoons are incredibly rich ecosystems that harbor a wealth of species and provide vital ecosystem services to millions of people (Kjerfve, 1994). Defined as "an inland water body, usually oriented parallel to the coast, separated from the ocean by a barrier, connected to the ocean by one or more restricted inlets, and having depths which seldom exceed a couple of meters" Kjerfve, 1994, p. 2, coastal lagoons usually formed as a consequence of rising sea levels and the natural formation of coastal

barriers through marine processes (Kjerfve, 1994). Accordingly, coastal lagoons are widely distributed in regions of past relative sea level rise, as opposed to their rare presence in regions where isostatic uplift [1] exceeds eustatic sea level [2] rise (Martin and Landim Dominguez, 1994). Today, coastal lagoons make up 13% of the global coastline and 5% of the European coastline, accounting for 6400 km^2 of coastal area in the Mediterranean region alone (Barnes, 1980; General Fisheries Commission for the Mediterranean, 2015).

In light of their ever-changing nature, coastal lagoons are frequently referred to as ephemeral ecosystems (Chapman, 2012; Duck and Da Silva, 2012; Ponti et al., 2009) that are part of the natural continuum of coastal environments (Duck and Da Silva, 2012). They are considered to be transitional ecosystems on the interface of epicontinental aquatic ecosystems, transitional waters, and coastal marine ecosystems characterised by strong physico-chemical gradients, including salinity, marine water renewal, nutrients, turbidity, and sediment structure (Pérez-Ruzafa et al., 2019; Snickars et al., 2009; Tagliapietra et al., 2009). As one of the most productive ecosystems worldwide, coastal lagoons support and foster a complex mosaic of valuable habitats like wetlands, mangroves, seagrass meadows, and salt marshes, to name just a few, and their rich biodiversity (Anthony et al., 2009; Inácio et al., 2023; Kennish and Paerl, 2010; Newton et al., 2018). The diverse sedimentological, biological, and hydrological gradients present a great heterogeneity that provides refuge to many endangered species and serves as a nursery for a number of coastal fish species (General Fisheries Commission for the Mediterranean, 2015; Müller et al., 2023).

1.1.1 Evolution of Perception and Ongoing Challenges for Coastal Lagoons

Yet, the high ecological value of coastal lagoons has not always been appreciated. Instead, the establishment of human settlements and the human-driven utilization of coastal lagoons have led to significant transformations and ecological degradation: within the terrestrial areas, dunes were lost due to the disruption of sediment dynamics and land transformation (Morales, 2022) and wetlands were land-filled for agricultural purposes (Doody, 2008) or drained to combat Malaria (Gilroy et al., 1948; Roberts, 2010; Sousa et al., 2014; Strickland, 1938). In the water, mangroves were converted into intensive aquaculture farming ponds (Newton et al., 2020) and aquatic habitats were

[1] The rise of land masses that were depressed by the huge weight of ice sheets during the last ice age (National Snow and Ice Data Center, 2023)

[2] The distance from the center of the Earth to the sea surface (Patzkowsky and Holland, 2012)

replaced by anthropogenic structures like ports and marinas (Manent et al., 2020). Consequently, a significant portion of the original coastal lagoon area has been irreversibly destroyed (Ruiz-Luna and Berlanga-Robles, 2003).

The perception of coastal lagoons began to change during a surge of scientific interest in lagoon ecosystems in the 1970s and 1980s. During this time, fundamental research was conducted, leading to the recognition of their value, and pioneering international frameworks for their protection were adopted. Notably, the Convention on Wetlands (Ramsar Convention) in 1971 (Gardner and Davidson, 2011) and the Convention on Biological Diversity (CBD) in 1992 (United Nations, 1992) played crucial roles in acknowledging the importance of coastal lagoons and establishing guidelines for their conservation.

Yet, in spite of the increased recognition of their ecological values, coastal lagoons are still facing numerous threats from anthropogenic and climate impacts, such as habitat destruction, pollution, water withdrawal, overexploitation, and invasive species (Miththapala, 2013; Pérez-Ruzafa et al., 2019). This strongly modifies the structure and functioning of lagoons (General Fisheries Commission for the Mediterranean, 2015) and impacts all levels of complexity of life within, including microbial communities.

In the European Union, coastal lagoons are declared as a habitat of priority in the EU Habitats Directive, highlighting their status as "habitats threatened by extinction" (European Union, 1992). Despite this designation, numerous coastal lagoons still exist throughout Europe. In Southern Europe, most of these coastal lagoons originated from river delta formations, such as the Rhone, Danube, and Po rivers, while others formed due to coastal morphology processes (Soria et al., 2022). The majority of lagoons located in the Mediterranean Sea have a depth that does not exceed 10 meters and exhibit microtidal conditions with a tidal range of 0-2 meters (Wit et al., 2001). A detailed review of the available literature suggests that the ecosystem health status of the Mediterranean lagoons is rather poor and that most lagoons are either eutrophic or hypertrophic (Soria et al., 2022).

1.1.2 Classification of Coastal Lagoons

Three main types of coastal lagoons are commonly distinguished according to their water exchange with the coastal ocean: choked lagoons, restricted lagoons, and leaky lagoons (Kjerfve, 1986).

Chocked lagoons: Choked lagoons consist of a series of interconnected elliptical cells, typically oriented parallel or perpendicular to the shoreline, and are connected to the

ocean through a narrow entrance channel. These lagoons are often found in coastal areas with high wave energy and significant littoral drift [3] (Kjerfve, 1994). The narrow entrance channel often limits tidal currents and water level fluctuations to less than 5% as compared to the bordering coastal tides. Consequently, water exchange remains limited and water and sediment flushing times are long, thus making wind forcing the dominant environmental influence. In choked lagoons, intense solar radiation and runoff frequently cause intermittent stratification and, in semi-arid and arid regions in particular, facilitate permanently or temporarily hypersaline conditions.

Restricted lagoons: are large water bodies that are oriented parallel to the shoreline and have two or more entrance channels or inlets, allowing for good tidal circulation. Along with wind influence, this facilitates effective vertical mixing and a wide range of salinity concentrations, ranging from near-limnetic (lake-like) to ocean-like conditions. Flushing rates in restricted lagoons are generally shorter compared to choked lagoons.

Leaky lagoons: Leaky lagoons are also oriented parallel to the shoreline but are characterized by multiple ocean entrance channels. They are typically located in areas with strong tidal currents that can overcome environmental forces, e.g. wave action and littoral drift, that would close the channel entrance. With their many inlets or entrance channels, leaky lagoons exhibit opposite characteristics to choked lagoons. Strong tidal currents and relatively unrestricted water exchange with the ocean lead to salinity levels similar to those of the adjacent ocean.

While other classifications of coastal lagoons have been proposed, the aforementioned categorization is commonly used due to its incorporation of information on dominant environmental influences and hydrological variability time scales (Mahapatro et al., 2016; Pérez-Ruzafa et al., 2007).

1.2 Microbial Communities in Coastal Lagoons

Microbial communities are crucial to the functioning of coastal lagoon ecosystem. While several studies have been conducted to characterise microbial communities in coastal lagoons, an overarching analysis has so far not been performed and a comprehensive database on microbes in coastal lagoons is not yet in place. Regardless, the available results already indicate that microbial communities differ greatly among and even within lagoons, depending on specific environmental conditions, geographical location, and other factors, thus making it difficult to give general statements about their

[3] A geological process that consists of the transportation of sediments (sand, shingle, pebbles) along a coast parallel to the shoreline, which is dependent on the angle incoming wave direction (Coastal Partners, 2023)

composition (Ghai et al., 2012). Nonetheless, a tentative trend can be derived from the performed studies, suggesting that certain phyla are more common than others and could potentially be associated with coastal lagoon ecosystems. In the domain of bacteria, these phyla include *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Actinobacteria*, and *Firmicutes*, while *Euryarchaeota* and *Thaumarchaeota* are the most commonly found phyla within the Archaea kingdom (Aldeguer-Riquelme et al., 2022; Ghai et al., 2012; Gómez-Acata et al., 2023; Trombetta et al., 2022).

It is important to note that the relative abundance and specific taxa within these bacterial and archaeal groups can vary significantly across different coastal lagoons and their respective environmental conditions. Following the recent advancements in molecular techniques, more detailed studies on microbial diversity and community structures in coastal lagoons were enabled, slowly bringing to light the vast diversity of microbes in this environment and the key functional roles they play. Thus, even though further research on the detailed composition of microbial communities in coastal lagoons remains necessary, their crucial role in environmental processes is undisputed (Kayranli et al., 2010). These microbial processes and functions shall further be introduced in the following paragraphs.

1.2.1 Microbial Processes in Coastal Lagoons

Microbial communities wield influence over their surrounding environment through their metabolic activities, while simultaneously being shaped by the prevailing environmental conditions. This dynamic interplay forms a complex and intricate relationship between microbial communities and their surroundings, especially in coastal lagoons. The scientific literature has extensively detailed numerous mutual interactions between microbial communities and the local environment, encompassing roles in essential cycles like carbon and nitrogen, as well as participation in both aerobic and anaerobic processes.

Microbial activities play a pivotal role in driving carbon cycling within coastal lagoons, showcasing their multifaceted impacts. Integral to this role is their involvement in the degradation of organic matter, encompassing processes like microbial respiration, decomposition, and mineralization (Gocke et al., 2003; Suhett et al., 2013; Viaroli et al., 2008). The versatility of microbial communities extends to their contribution to both short-term and long-term carbon storage within these ecosystems. While microbial biomass, though perishable, serves as a repository for substantial carbon content in the benthic zone, where organic matter collects in the form of decaying microbes

(Kayranli et al., 2010), microbial communities also facilitate longer-term carbon storage through mineralization processes. These include their participation in organic matter dynamics, converting dissolved organic matter (DOM) through microbial oxidation into inorganic substances (Kayranli et al., 2010).

Beyond carbon storage, microbial activities significantly influence the transfer of organic carbon to higher trophic levels (Rodrigues-Filho et al., 2023; Whalen, 2005). The presence of DOM has far-reaching effects on the composition and dynamics of microbial communities in lagoons. Variations in the origins and composition of DOM exert diverse impacts on microbial metabolism, affecting community dynamics and influencing rates of carbon processing (Boadella et al., 2021; Judd et al., 2006). Concurrently, soil oxidants such as carbon dioxide and oxygen, along with their respective availabilities, substantially shape soil microbial processes by serving as electron acceptors for organic matter degradation (Kayranli et al., 2010). This positioning bacteria as primary agents in organic matter diagenesis (Deming and Baross, 1993). Furthermore, the presence of organic carbon creates a connection to microbial influences on the nitrogen cycle, as DOM acts as a crucial energy source for bacterial denitrification processes.

Microbial communities in coastal lagoons encompass a diverse array of microbial taxa with specialized functions, collectively orchestrating the conversion of nitrogen compounds between different forms. Nitrogen-fixing bacteria and archaea, for instance, facilitate the transformation of atmospheric nitrogen into biologically accessible forms (Woitchik et al., 1997), while ammonia-oxidizing bacteria and archaea contribute to the conversion of ammonia into nitrite and nitrate. Nitrite-oxidizing bacteria further mediate the conversion of nitrite into nitrate, completing the nitrification process. On the other hand, denitrifying bacteria, archaea, and protists drive denitrification, a process that removes nitrogen from the ecosystem by converting nitrate and nitrite into nitrogen gas, thereby closing the nitrogen cycle (Herbert, 1999; Purvaja et al., 2008). Microbial activities and the intricate interplay of microbial communities hence not only shapes the availability of essential nutrients within coastal lagoons, collectively contributing to the conversion of nitrogen between different chemical forms, but also exerts profound influences on the ecosystem's overall biogeochemical dynamics.

Beyond their involvement in nutrient cycling, microbial communities also wield significant influence over the oxygen dynamics of the environment, a relationship that operates reciprocally (Fontes and Abreu, 2009). Notably, the interplay of oxygen production and consumption is intricately governed by the influence of microbial processes alongside the contributions of primary producers. One example of microbial impacts on oxygen dynamics lies in the potency of microbial degradation, capable of generating pronounced oxygen deficits (Zaldívar, 2003). This phenomenon, evident

in instances such as the decomposition of macroalgae, effectively paves the way for the onset of anoxic conditions (Eyre and Ferguson, 2002). The intricate network of microbial interactions is further compounded by the accumulation of phytoplankton biomass and epiphytic slime onto the benthic substrate, amplyfying the rates of oxygen consumption through microbial-driven decomposition pathways (Viaroli et al., 2013). Consequently, these processes serve as catalysts for the development of anoxic settings within coastal lagoons. Subsequent anaerobic processes that release dissolved sulfide can then determine the final shift of the system from one state to another (Viaroli et al., 2013).

Under anoxic conditions, sulfate-reducing bacteria are dominant. In the absence of nitrification, anaerobic nitrogen fixation may additionally be supported by such sulfate-reducing bacteria, thus enhancing the internal nitrogen loading of the lagoon as well as the nitrogen recycling and availability within the system (Viaroli et al., 2013). In anoxic sediments, these sulfate-reducing bacteria moreover facilitate the production of hydrogen sulfide and the mineralisation of organic compounds (Caumette, 1992).)

In summary, the interactions of the microbiome with coastal lagoons are manifold and should not be neglected when studying this type of environment, as they build the backbone to lagoon functioning (Boadella et al., 2021). Understanding microbial community composition is pivotal in understanding the entirety of the system and its reaction to anthropogenic impacts, such as trophic changes (Caruso et al., 2016).

1.2.2 Anthropogenic Impacts on Coastal Lagoon Microbial Communities

The manifold anthropogenic impacts straining coastal lagoons worldwide also effect microbial communities. Although for a long time understudied, the introduction of pollutants, nutrients, and contaminants into lagoon environments disrupts microbial diversity, composition, and interactions. Consequences of increased nutrient levels, organic chemical and heavy metal pollution, as well as the introduction of antibiotics and pathogens into the environment all affect both water column and sediments and are thus reflected in microbial community composition and abundance. Yet, identifying microbial community reactions to single anthropogenic stressors can be difficult as multiple stressors often simultaneously excert impacts on the same region (Crain et al., 2008).

Regardless, studies have shown that the introduction of sewage and wastewater as a consequence of urbanisation alters nutrient levels, in turn promoting the growth of certain bacteria, alongside notable shifts in community composition, greater diversity, and temporal fluctuations (Nogales et al., 2011). Similarly, agricultural runoff contributes excess nutrients like nitrogen and phosphorus to the lagoon environment and stipulate microbial communities to increase availability of nitrogen and phosphorus, resulting in eutrophication and subsequent harmful algal blooms that again cause shifts in microbial community structure (Aires et al., 2018; Berry et al., 2017; Michalak et al., 2013; Murray et al., 2015; Turner et al., 2015). According to Kodama et al., 2006 microbes are capable of regulating harmful algal blooms, both positively and negatively, linking them tightly to this environmental phenomenon.

Nutrient loads can also be introduced via aquaculture activities. These have a particularly high impact on sediment communities below cages. The increase in organic load raises the oxygen demand, thus altering the biogeochemistry of the sediments. In turn, bacterial abundances increase and the organic load facilitates microbial anaerobic respiration processes, e.g. sulphate, iron and manganese reduction, denitrification, methanogenesis and fermentation (Bissett et al., 2006; Christensen et al., 2000; Holmer et al., 2003; Nogales et al., 2011).

Reactions of microbial communities have also been observed as a consequence of chemical pollution, such as hydrocarbons and heavy metals, usually originating from insutrial discharge. Hydrocarbon pollution in particular was shown to cause lasting shifts in microbial community composition, as community composition was found to not return to the state prior to contamination (Allison and Martiny, 2008). Notably, the shift does not imply an improved degradation or removal of hydrocarbons (Head et al., 2006), but does usually involve an acute reduction in biodiversity as an immediate response to pollution (Head et al., 2006; Yakimov et al., 2007). Heavy metal contamination, as opposed to nutrient input or hydrocarbon pollution, is frequently associated with a decrease in total prokaryotic cell number, particularly under the presence of cadmium, copper, zinc, and lead (Gillan et al., 2005; Gillan and Pernet, 2007). Heavy metals were shown to inhibit microbial growth and impair cell viability (Sengör et al., 2009). They moreover interfere with essential metabolic pathways, disrupt enzyme activities, and cause oxidative stress (Gnanamani et al., 2010; Jaiswal and Pandey, 2018). This can lead to reduced microbial populations and altered community structures. Conversely, certain taxa appear to thrive in heavy metal-polluted sediments (Obi et al., 2016), fostering their recommendation as indicator taxa for heavy metal pollution.

These changes in microbial communities have cascading effects on lagoon ecosystems. Shifts in microbial functions impact nutrient cycling, organic matter degradation, and overall ecosystem stability. Harmful changes in microbial assemblages can lead

to water quality deterioration, reduced habitat quality for aquatic life, and decreased overall ecosystem health (Dang et al., 2021).

1.3 Methods for Microbial Community Analysis

In order to study the microbial communities and their involvement in environmental processes, in-depth studies of microbial community composition and functioning are needed. The technologies and methods needed to conduct such in-depth studies nowadays rely on advances in DNA sequencing and microscopic techniques achieved over the last two decades. In this study, a combination of quantitative analysis using CARD-FISH and qualitative characterization through Next-Generation Sequencing (NGS) was employed as this integrated approach allowed for both quantitative assessment and detailed characterization of the microbial communities. The basic principles of DNA sequencing as well as the CARD-FISH protocol will be summarised shortly in this chapter.

1.3.1 DNA Sequencing

Shortly after unveiling the three-dimensional structure of DNA, a significant advancement in microbial ecology emerged with Carl Woese's introduction of 16S rRNA sequencing in 1977 (Woese and Fox, 1977). Woese proposed sequencing ribosomal ribonucleic acid (rRNA) for prokaryotes due to its ubiquity, enabling the measurement of phylogenetic differences across diverse organisms. Among the available options, the 16S rRNA gene stood out as the ideal candidate for sequencing due to its distinctive attributes: it displays consistency across various domains of life, is adequately sized for direct sequencing unlike other cellular components, and contains both highly conserved regions for primer design and nine hypervariable regions (V1-9) for pinpointing microbial phylogenetic traits (Woese and Fox, 1977; Yang et al., 2016). The 16S gene spans 1,500 base pairs and comprises both 9 conserved and 9 hypervariable regions (Tringe and Hugenholtz, 2008; Wang and Qian, 2009). Given the expense associated with sequencing the entire gene, researchers commonly target one of the nine hypervariable regions based on their study's focus and research question (Rajeev et al., 2020; Teng et al., 2018).

Since its first description and propelled by advancements in sequencing technology, 16S rRNA sequencing has become the gold standard for determining the taxonomic composition and phylogenetic diversity of prokaryotes in environmental samples with high degrees of accuracy (Langille et al., 2013; Rausch et al., 2019; Yang et al., 2016). Most studies utilise a short-read NGS approach, in most cases on an Illumina platform,

in which short, clonally amplified DNA molecules are sequenced in parallel (Tucker et al., 2009). Generally, NGS pipelines encompass three main steps: library preparation, sequencing, and data analysis (Hu et al., 2021).

To prepare a sequencing library, DNA is fragmented into a platform-specific size range and the fragment-ends are repaired for adaptor ligation. Then, adaptors (e.g. platform-specific sequences) consisting of platform-specific sequences, are attached to the fragments to facilitate their binding to flow cells, which are necessary for amplification. The library is finalised by selecting a defined size range to enrich DNA and remove contaminants (Hu et al., 2021).

DNA is then clonally amplified to generate detectable signals. For this purpose, the DNA fragments previously attached to the flow cell are bridge-amplified to produce millions of single fragments which can subsequently be sequenced. After successful sequencing, a bioinformatics pipeline is employed for quality control of raw data, sequence alignment against a reference database, and data interpretation through taxonomic assignment, filtering, and prioritization (Dillies et al., 2013; Pereira et al., 2020).

The transition from characterizing microorganisms through plate cultivation to sequencing entire genomes at a fraction of the cost and time has been groundbreaking, revolutionizing the study of microbial communities and their role in the environment.

1.3.2 CARD-FISH

Mentioned new sequencing technologies have revolutionized the field of microbial ecology. However, they still fall short in providing quantitative assessments of microbial communities in samples. As a result, microscopic techniques remain necessary to accurately determine the abundance of microbial communities (Piwosz et al., 2021). Among these techniques, the CARD-FISH treatment of polycarbonate filters stands out as the most effective visualization method for microbes. It enables phylogenetic identification, enumeration, and direct spatial visualization of microorganisms by targeting the rRNA of bacterial and archaeal cells using the enzyme horseradish peroxidase (HRP)(Thompson et al., 2018). It is thus an advancement of the standard FISH protocol with signals up to several orders of magnitude stronger.

Similar to the FISH protocol, the CARD-FISH protocol is based on the binding of rRNA-specific HRP-labelled oligonucleotide molecular probes to complementary segments of the genetic makeup. Typically, these probes consist of 15-30 base pairs and carry the HRP label, normally on the 5'-end of the probe (Behrens et al., 2008; Hoshino et al., 2008; Moter and Göbel, 2000; Pernthaler et al., 2002). Oligonucleotide probes can

be designed to target DNA of individual organisms as well as entire domains, such as Bacteria or Archaea (Haroon et al., 2013), thus offering great versatility and flexibility in study design.

In principle, a fluorescently-labeled tyramide is deposited in situ on a target nucleic acid sequence through HRP catalysation under the presence of hydrogen peroxide. The HRP creates a radical intermediate of the tyramide able to bind to tyrosine residues near the HRP, generating a high-density tyramide labeling that is well detectable by fluorescence microscopy (Kubota, 2013). In this manner, CARD-FISH poses a more efficient FISH approach throught the inclusion of the catalysed reporter deposition.

The abundance of each bacterial or archaeal strain (or the entire domain, depending on the chosen probe) can afterward be determined through microscopic counting techniques and is usually expressed as a percentage of the total abundance. To facilitate this analysis, the prepared filters are commonly stained with 4',6-diamidino-2-phenylindole (DAPI) (Eickhorst and Tippkötter, 2008), a DNA-specific fluorescent dye that attaches to AT-rich sequences of DNA, forming a detectable fluorescent complex (Kapuscinski, 1995).

Chapter 2

Materials and Methods

In this chapter, the three designated study sites will be introduced (section 2.1), and the employed sampling strategy will be described (section 2.2). The chosen methodology for the physical and chemical characterization of the samples will be introduced in section 2.3. Lastly, the methodologies for microbiome characterization, namely DNA sequencing and microscopic visualization, will be elaborated on in sections 2.4 and 2.5.

2.1 Site Description

The thesis analysed three different Southern European coastal lagoons, all of which are located in a temperate climate with hot, dry, summers according to the Köppen-Geiger classification Csa (Kottek et al., 2006). The three selected study sites were the Ria Formosa Lagoon in Portugal, the Sabaudia Lagoon in Italy, and the Butrinti Lagoon in Albania, all of which are impacted by differing anthropogenic pressures. The lagoons were thus chosen to ensure a sufficient geographic coverage throughout Southern Europe while analysing a broad variety of anthropogenic impacts. In the following, the three lagoons are briefly introduced, categorised, and anthropogenic pressures are described.

2.1.1 Butrinti Lagoon, Albania

Located in the Eastern Mediterranean region of Southern Albania, Butrinti lagoon is a typical coastal lagoon of tectonic origin with a microtidal [1], restricted character (Ariztegui et al., 2010; Moisiu et al., 2016). The lagoon is located in the heart of the National Park of Butrinti and is classified as a UNESCO World Heritage and Ramsar Wetland Site of international importance (Ramsar, 2003), and serves as a habitat for a large number of rare or endangered plant and animal species. It is additionally an important spawning ground, food source, and migration path for fish (Ramsar, 2003).

[1] Applied to coastal areas in which the tidal range is less than 2 m (Dipper, 2022)

In the region surrounding Butrnit lagoon, annual temperatures range from 13°C in January to 27°C in July with a yearly precipitation of 1200 to 1300 mm (Topi et al., 2013). Surrounded by the Vurgu Plain in the North, the Mil Mountain in the East, the Vrina plein in the South, and the mountainous Ksamil peninsula in the West, the lagoon is connected to the Ionian Sea only through the 1 km long natural Vivari channel in the South of the lagoon (Moisiu et al., 2016). Although the channel, with its width of up to 120 m and depth of 6 m, allows for some exchange with the Ionian Sea, considerable sea water inflow remains scarce and mostly occurs during storm surges or very high tides (Ariztegui et al., 2010). Freshwater inflow, on the other hand, is maintained from a catchment area of 128 km^2, characterised mostly by the Bistrica River in the North and the Pavllo River in the South (Bego et al., 2012). Yet, some studies have suggested that the major freshwater inflow of Butrinti lagoon is fed from Bufi Lake in the South and the irrigation networks in use on Vurgu and Vrina plains, providing an accumulated freshwater feed of 10 m^3/s (Osmani and Peja, 2010). It is furthermore noteworthy that, according to **Kilotari.2013**, annual rainfall is offset by an annual water loss of roughly 1280 mm/y.

The lagoon itself is characterised by an average depth of 14 m and a maximum of 21.3 m. Overall, circulation within the lagoon remains weak, causing a stratification into an epilimnion, metalimnion, and a pronounced hypolimnion where anoxic conditions prevail below a depth of 8 m (**Moisiu.2013**; Bacu and Zaho, 2022). Most aquatic life in Butrinti Lagoon is present in the epilimnion as it provides the most favourable habitat, as opposed to the hypolimnion which is mostly dominated by sulfate-reducing bacteria. Water levels are maintained at a constant 9 cm a.s.l. and a slightly basic pH of above 8 (**Kilotari.2013**; Topi et al., 2013).

Apart from its outstanding ecological importance, Butrinti lagoon provides livelihoods to many locals that live off of fishing, mussel farming, stock raising, and cultural tourism (Ramsar, 2003). Naturally, such activities pose anthropogenic pressure on the lagoon and its natural ecosystem. Especially agricultural activities in the region, as well as tourism and its concomitant urban development, are increasingly impacting the lagoon (Bani et al., 2013). As a consequence, heavy metal pollution has been detected in soil, sediment, water, and mussel samples (Topi et al., 2013). Heavy metal pollution can further be ascribed to pedo-geological processes in the region and the release of household wastewater into the lagoon, causing an increasing contamination (Malltezi et al., 2010). As a consequence, elevated concentrations have repeatedly been reported for Chromium and Lead, especially around the urban area of Ksamil (Topi et al., 2012).

2.1.2 Sabaudia Lagoon, Italy

Sabaudia Lagoon, also known as Lago di Paola, is a restricted coastal lagoon located in the Lazio region in Central Italy (Zoppini and Amalfitano, 2011). The lagoon is incorporated into the Circeo National Park and is the largest in a group of four coastal lakes (Zoppini and Amalfitano, 2011). In 1998 and 2000 the area was recognised as a Ramsar and Natura 2000 site with high ecological value (Ramsar, 1998a; Ramsar, 1998b). The lagoon comprises an area of 390 ha with an average depth of 4 m and a maximum depth of 10 m. In total, the lagoon has a volume of 14 million m^3 (Minervini and Bianchini, 1990). Separated from the Thyrrenian Sea by coastal dunes and sandy beaches, Sabaudia Lagoon stretches parallel to the coast for a length of 7 km. It displays a straight shore on the western side and characteristic inlets penetrating the dunes in the East (Zucchetta et al., 2021). Due to limited water exchange with the Tyrrhenian Sea, the lagoon can be classified as microtidal; tidal fluxes usually remain in a range of 20 cm +- and can only pass through a small channel in the far north, called Caterattino, and the bigger Torre Paola channel in the far South of the lagoon (Zucchetta et al., 2021). In combination with frequent freshwater abstractions, marsh reclamations, and limited freshwater inflow from three superficial tributaries, the opening and embankment of the Torre Paola channel by the Romans is the most probable cause for the increasing salinisation of the Sabaudia lagoon.

Sabaudia Lagoon experienced severe anthropogenic pressure as a consequence of an increase in population throughout the 20^{th} century. Wastewater input into the lagoon, agricultural activities on the adjacent land, and large-scale pig farming lead to the gradual eutrophication of the lagoon and caused several anoxic crises (Vollenweider et al., 1996; Zoppini and Amalfitano, 2011). Today, anoxic conditions still prevail below a depth of 3m, despite the reduction of anthropogenic impacts through the inclusion of the lagoon in the Circeo National Park (Ramsar, 1998a). The recent detection of feacal pollution in Sabaudia Lagoon highlights, however, that anthropogenic pollution is still ongoing (Zoppini and Amalfitano, 2011).

2.1.3 Ria Formosa Lagoon, Portugal

The Ria Formosa Lagoon is a mesotidal [2] lagoon located on the Southern coast of Portugal and is part of the Ria Formosa national park (Anibal et al., 2019). With a total size of 170 km^2, the Ria Formosa belongs to the biggest coasta lagoon national parks in Europe, though the lagoon itself comprises only 80 km^2. The lagoon is separated from the Atlantic Ocean by five barrier islands and two sand pits that remain connected to the mainland (Sousa et al., 2020), thus connecting the lagoon to the Atlantic Ocean via

[2] Tidal range of 2-4 metres (Dipper, 2022)

seven inlets that allow a complex exchange between the coastal zone and the lagoon (Fabião et al., 2016). From the mainland, the Ria Formosa is fed by one permanently flowing river, the Gilão, as well as several ephemeral rivers and streams, all of which mostly originate in the Caldeirão Mountains (National Research Institute for Fisheries and Sea and Fernando Pensao University, 2003)

In contrast to microtidal Mediterranean lagoons, the mesotidal Ria Formosa shows a semi-diurnal tidal regime (Anibal et al., 2019; Newton and Mudge, 2003). Each tide replaces approximately 50 to 70% of the lagoon's water which, due to its shallow depth of 3.5 m on average (Brotas et al., 1990), prevents stratification and results in good vertical mixing. Regardless, the conditions in the lagoon are not fully homogenous as temperature and salinity differ significantly between the inlet channels and the inner lagoon.

As a Ramsar and Natura 2000 site, the Ria Formosa is a national park of international importance that provides the region with valuable ecosystem services and ensures the livelihood of local communities (Newton and Mudge, 2003). Of particularly high ecological value are its extensive salt marshes and seagrass meadows that pose important spawning grounds and nurseries for aquatic species in the shallow waters of the lagoon (Arnaud-Fassetta et al., 2006; Cabaço et al., 2009; Sousa et al., 2020).

Yet, the entirety of the national park is highly impacted by several anthropogenic pressures: Apart from the three main towns Faro, Olhão, and Tavira, and several smaller urban areas, the Ria Formosa experiences great pressure from annual tourism activities. The influx of tourists increases the population in the area severalfold and causes the overwhelming of infrastructure initially designed for the local population only. Wastewater treatment plants (WWTP) in particular are often unable to deal with the increased water consumption throughout the summer months (Cravo et al., 2022; Verissimo et al., 2019). Via its catchment, the Ria Formosa receives effluent from 28 domestic and industrial WWTPs in addition to untreated household wastewater, of which two plants discharge their water directly into the lagoon (Ferreira et al., 2003; Santos et al., 2004). Naturally, this impacts the lagoon's water quality by e.g. increasing the inorganic nutrient load or causing E. coli contamination of the water (Bettencourt et al., 2013; Malta et al., 2017).

The Ria Formosa moreover counts as the most important bivalve production area of Portugal where over 2500 tons of bivalves are produced annually in approximately 1200 aquaculture sites (Bettencourt et al., 2013; Ferreira et al., 2012). According to Chicharo et al., 2001 these bivalve production activities are associated with an overall decrease in macrofauna diversity, although a contradictive positive impact on the nutrient balance of the lagoon is similarly possible (Guyondet et al., 2022). The lagoon is

further impacted by agricultural activities in the catchment area and runoff from adjacent golf courses, both of which further increase the anthropogenic nutriet input. The development of a commercial port in Olhão fueled shipping and port activities and salt and sand extractions, threatening the ecological integrity of the lagoon (Newton et al., 2014). Further pressure is imposed by the international airport located within the Ria Formosa Lagoon (Sousa et al., 2020).

2.2 Sampling Strategy

The sampling campaign for the three study sites was conducted in the period from October 2022 to April 2023 by collaborating teams from the University of Algarve, the University of Tirana, and the project coordinators of the Water Research Institute in Rome.
In October 2022, the first samples were taken in the Butrinti Lagoon guided by the local representatives of the University of Tirana. The sampling points were strategically chosen to reflect a gradient of increasing urbanisation and thus environmental impact from the remote Northern part of the lagoon to the tourist town Ksamil, located in the South. Therefore, a total of seven sediment samples and 11 water samples were taken from seven sampling points on two different transect lines: Three samples were taken on a transect along the Western shore and another four points were sampled along a transect in the middle of the lagoon, including its deepest points and thus an active targeting of its anoxic bottom layer. In this manner, full coverage of the lagoon and its different environments could be guaranteed. Along the shore, surface water and sediment samples were taken whereas along the second gradient in the centre of the lagoon sediment, surface water, and deep water were sampled. An overview of the sampling points in Butrinti can be derived from Fig. 2.1. Further graphical visualisation of the sampling depth along the central lagoon transect is provided in Fig. 2.2

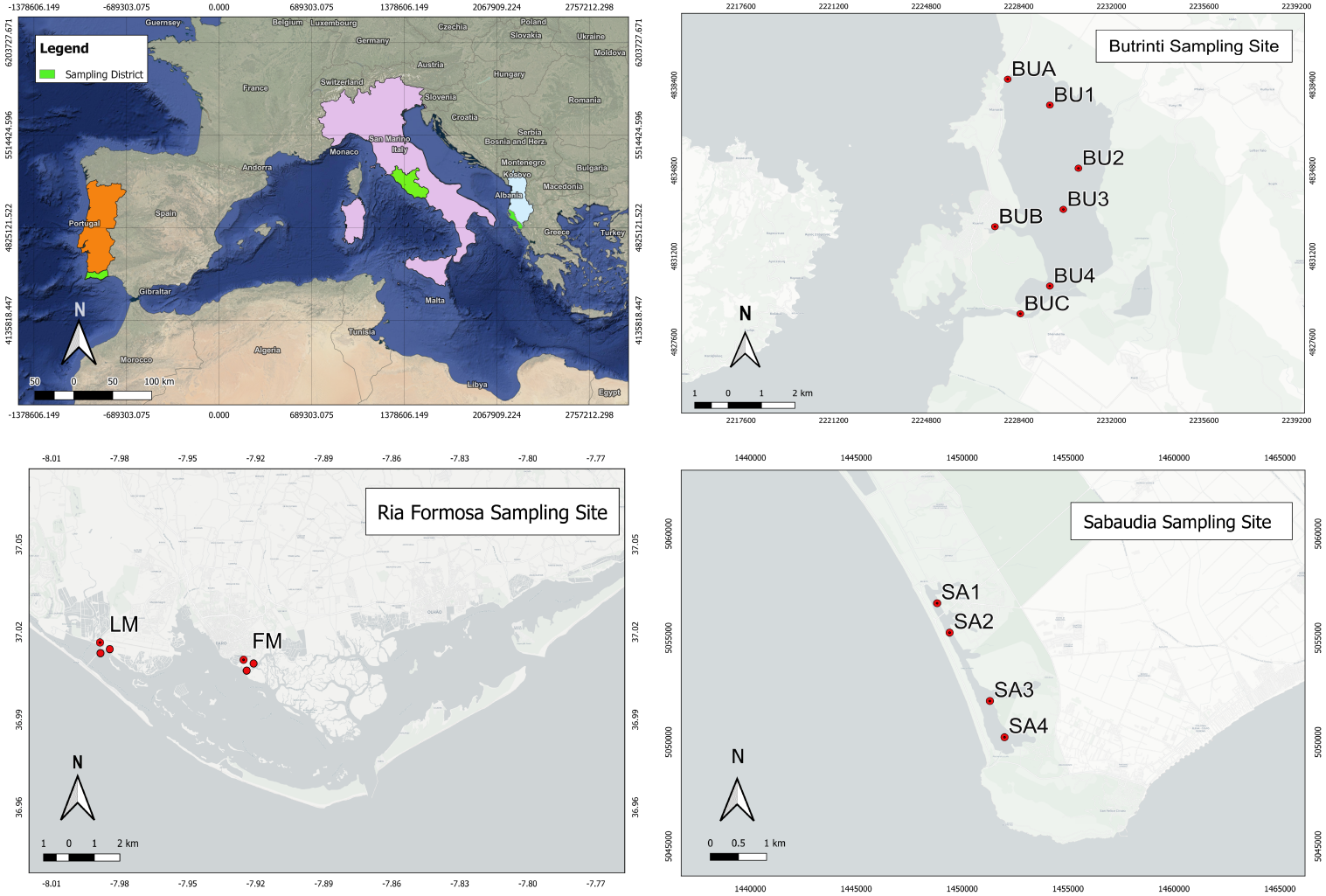

FIGURE 2.1: Map of the sampled lagoons and the sampling points

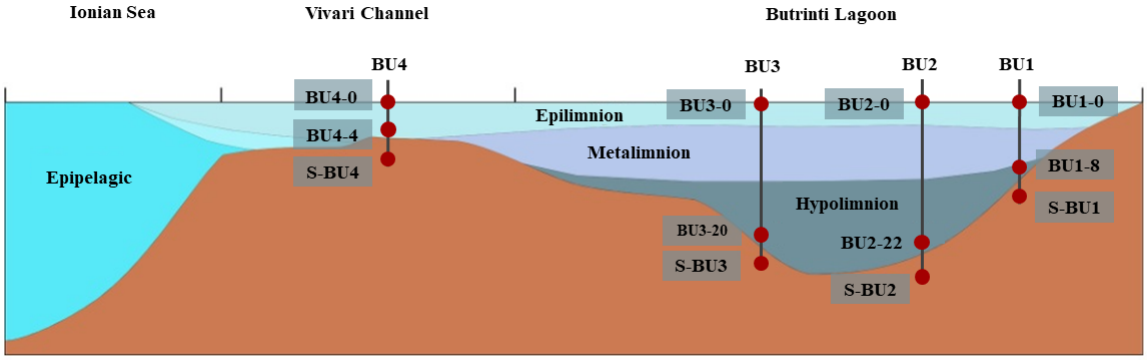

FIGURE 2.2: Lake sampling points in Butrinti Lagoon. Figure of Butrinti Lagoon profile was adopted from Moisiu et al., 2016

At the sampling site of Ria Formosa, six points were sampled for sediments by the collaborating research team of the University of Algarve on 08.02.2023 at low tide. Two sampling regions were chosen within the Ria Formosa, both of which are heavily impacted by anthropogenic impacts, and three sediment samples were taken per region at a distance of 10 to 20 m from each other. One sampling region was chosen close to the city of Faro at the outflow of the main WWTP; the second region was located further within the Ria Formosa in direct proximity to the international airport. Thereby, two different anthropogenic impacts could be assessed.
The campaign was finalised with the sampling of the Sabaudia lagoon in Italy on 17.04.2023. In line with the gradient approach chosen for the Butrinti lagoon, four points were sampled along a North-South transect spanning the entire length of the lagoon. At each point, surface water and sediment were collected for further analysis. The transect covers a salinity gradient and encompasses the impact of the mussel farm.

Comparability of the results was ensured via strict coherence in sampling methodology. Sediment samples were always taken from the surface with a Van Veen grab sampler. Obtained from the sampler, up to 50 ml of sediment were stored in a 50 ml Falcon tube and immediately placed on cooling elements in a cooling box until proper storage in the laboratory could be ensured. In order to take samples from the deep waters, a Niskin bottle was utilised from which the sampled water was transferred to a 500 ml sterile sampling bottle that was kept cool on the cooling packs. Finally, the surface water samples were taken manually with a 500 ml sterile sampling bottle and likewise cooled immediately after retrieval. At each sampling point, additional water quality parameters were taken with a multimeter probe. Despite the measured parameters varying slightly between sampling sites due to differences in the available multimeter probe, the basic parameters temperature, salinity, dissolved oxygen, and pH were measured at each site.

2.3 Sample Characterisation

Upon arrival of all samples in the laboratory, further analyses were conducted to chemically and physically characterise the samples. For this purpose, several sediment aliquots were taken from the 50 ml Falcon tube. First, aliquots of 5 ml were taken from each sample and stored as a sediment slury in 96% ethanol at -20°C to guarantee cell preservation for further processing. Additionally, an aliquot of 1 g was extracted for subsequent DNA extraction. Finally, further sediment aliquots were sampled for organic matter (OM) content, metal content, and chemical analysis. In addition to the subsequently elaborated analysis steps that have been conducted at the research laboratory of the Istituto di Ricerca sulle Aqua at the Consiglio Nazionale della Ricerche in Italy, individual analyses of sediment samples have been conducted by the Albanian and Portuguese project partners. In some cases, the partners conducted analyses that could not be performed for all other samples at the coordinating laboratory in Italy. For all data and insights that have been adopted from the collaborating partners to broaden the spectrum of analysed parameters, the respective internationally recognised methodology will be listed in the following two subchapters.

2.3.1 Physical Characterisation

All obtained sediment samples were first characterised for their OM content with the Loss on Ignition (LOI) technique as described by Heiri et al., 2001. To determine the weight percent of OM in the sediments, the samples were oven dried and heated in a muffle furnace. For this purpose, two approximately 5 g sediment aliquots were placed in a small aluminium container of known weight. At a temperature of 105°C, all samples were dried overnight to remove hygroscopic water adsorbed onto the sediment particles (Magdoff et al., 1996). The samples were then cooled in a desiccator and their weight was again determined at a precision of 0.01 g on an analytical balance (Gibertini E42, Italy). The difference between the initial, wet weight, and dry weight constitutes the water content of the sample (Veres, 2002). In a second step, the dried samples were transferred to a pre-weighed evaporation dish and placed in the muffle oven at 500°C for 3 hours, causing the release of volatile compounds from the samples. After combustion, all samples were again cooled in the desiccator and weighed. Via subtraction of the final ash weight from the dry weight, conclusions were drawn on the OM content of the sediments (Walter E. Dean JR., 1974).

2.3.2 Chemical Analysis

Major insight into the chemical composition of all sediment samples was acquired through two conducted approaches: first, a CHN elemental analysis was conducted for the determination of total nitrogen (TN) and total organic carbon (TOC) (Phillips et al., 2011). Additionally, metal digestion according to EPA 3051A with subsequent inductively-coupled plasma optical emission spectrometry (ICP-OES) analysis allowed to obtain insights on the elemental composition of the samples (Thermo Fisher Scientific, 2023a).

CHN Analysis

In preparation for the CHN analysis, one gram of sediment per sample was air-dried for 96 hours prior to its grinding in a mortar. For the TN analysis, 15-20 mg of ground sediments were weighed in a tin capsule and combined with an oxidiser (Vanadium pentoxide [V2O5]). The capsule is then combusted in an elemental analyser (FLASH EA 1112 CHN Analyzer, Thermo Scientific, USA) at 1000°C. Both the sample and the container melt upon which the tin promotes a violent reaction called flash combustion (Analytical Methods Committee, 2008). In the oxygen-enriched atmosphere, carbon dioxide, water, and nitrogen oxides are formed which are carried by the carrier gas helium over heated high-purity copper to ensure the removal of unconsumed oxygen and convert nitrogen oxides to nitrogen gas (Farina et al., 1991). After separation of the components by gas chromatography, CO_2, N_2, H_2O, and SO_2 were quantified via thermal conductivity detection (Analytical Methods Committee, 2008). Indirect TOC determination required an additional preparatory step in which 15-20 mg of the sample were instead weighed in a silver capsule and acidified with 10% HCl solution, removing carbonates and bicarbonates present in the sample. Otherwise, the analysis was conducted as described above.

ICP-OES Elemental Analysis

Generally acknowledged as one of the most powerful analytical tools for trace element determination, the ICP-OES analysis was chosen for its high accuracy, wide range, and most importantly, its high matrix tolerance (Hou et al., 2006; Thermo Fisher Scientific, 2023a). To enable the analysis of the samples with the ICP-OES, a preparatory microwave-assisted acid digestion of sediments had to be performed, here according to EPA guideline 3051A (United States Environmental Protection Agency, 2007). The approach allows for a near-total to total recovery and shows a higher efficiency in comparison to other methods (Hassan et al., 2007; Da Silva et al., 2014). For this purpose, the formerly dried sediments (105°C overnight) were homogenised with a mortar, and

approximately 0.3 g of sediment was weighed on an analytical balance (XS BL 224, Italy) and transferred into a digestion vessel. The weight was noted down to a precision of 0.001 g. Then, $9 \pm 0.1ml$ of 65% concentrated nitric acid (HNO_3, Carlo Erba, Italy) and $3 \pm 0.1ml$ of 37% concentrated hydrochloric acid (HCl, Carlo Erba, Italy) were added to the vessel under a fume ventilation system. This step was repeated for each sample. Additionally, 5 blank vessels without sediment, only containing the acids, were included for control purposes. All vessels were then properly sealed and placed into the microwave system, before the temperature and pressure sensors were connected (Milestone Ethos Touch Control - Advanced Microwave Labstation, Italy). The digestion was operated under a pre-set programme.

After the finalisation of the microwave programme, the vessels were left to cool down to a maximum temperature of 45°C and uncapped under the fume ventilation system. The vessel content was transferred to a 50 ml Falcon tube and diluted to a volume of 50 ml with Milli-Q water before the tube was centrifuged for 5 min at a speed of 9000 rpm. This step enhanced the precipitation of remaining particles in the sample and prevents clogging of the ICP-OES nebuliser. As a next step, the supernatant was transferred to acid-washed bottles which served as the stock for the final analyte. The analyte was subsequently prepared by bringing 1 ml of stock solution to a total volume of 10 ml via dilution with Milli-Q water and transferred to 14 ml polystyrene test tubes for the ICP-OES autosampler. Finally, each sample was autosampled (Autosampler Agilent SPS4 Autosampler, USA) and analysed by the ICP-OES.

Major and minor elemental analysis with the ICP-OES is based on the injection of liquid samples into a radiofrequency-induced argon plasma where the sample is quickly dried and vaporised. With an inherent temperature of 10.000 K, the high temperatures in the plasma energise the sample through collisional excitation, causing an atomic emission that emanates from the plasma. The different wavelengths of the emission are measured and allow for conclusions on the elemental composition of each sample (Hou et al., 2006). In this way, the ICP-OES provides accurate concentration values for a range of chosen elements. The final output, given in ppm or ppb for each element, was then converted to mg/kg to allow for comparison with literature values.

2.4 DNA Extraction, Library Preparation, and Sequencing

To sequence the 16S rRNA of the samples, DNA was extracted and sequenced on an Illumina platform.

2.4.1 DNA Extraction

As a prerequisite for DNA sequencing, the DNA has to first be extracted from the water and the sediment samples. A range of DNA extraction kits is commercially available and tailored for both water and sediment samples. In this case, the Qiagen DNeasy®PowerSoil®Pro Kit was utilised to extract DNA from ∼1g of sample and the provided protocol was strictly followed. The detailed protocol can be derived from Appendix A. Per sample, small aliquots of around 50 μl were extracted and stored at -20°C.

After the finalisation of the DNA extraction protocol, the concentration of extracted DNA was quantified for each sample with the Thermo Scientific ™NanoDrop™2000c Spectrophotometer (Thermo Fischer Scientific, USA). Designed specifically for the quantification of DNA, RNA, and protein content in samples, the NanoDrop measures variations in absorbance using micro volumes of 1-2 μl at very high precision (García-Alegría et al., 2020; Thermo Fisher Scientific, 2023b). In this manner, 2 μl of sample were transferred onto the NanoDrop and analysed to confirm a sufficiently high DNA concentration for subsequent sequencing.

Finally, the extracted DNA was sent to the DNA-sequencing laboratory DNASense ApS (Aalborg, Denmark) for the qualitative analysis of the microbial diversity in the samples. The sequencing laboratory conducted a high-throughput Illumina sequencing of the highly preserved 16S rRNA gene's hypervariable V4 region.

2.4.2 Library Preparation and Sequencing

A customised procedure based on an Illumina standard protocol was used for library preparation (Illumina, 2015). The V4 region of the 16S rRNA gene was amplified with the primer pair 515FB [GTGYCAGCMGCCGCGGTAA] and 806RB [GGACTAC-NVGGGTWTCTAAT], in alignment with the standards of the Earth Microbiome Project (Greg Caporaso et al., 2018). The resulting amplicon libraries were purified with CleanNGS SPRI beads (CleanNA, NL) according to the recommended methodology, with a bead-to-sample ratio of 4:5. From the purified amplicon libraries the final sequencing libraries were prepared via a second PCR and again purified according to the aforementioned protocol. Libraries were pair-end sequenced on a MiSeq (Illumina, USA) utilising a MiSeq Reagent kit v3 (Illumina, USA) under recommended guidelines. The reads were trimmed, clustered (97%similarity), taxonomically classified against the Silva 132 database, and demultiplexed in RStudio IDE (2022.2.3.492) running R version 4.2.2 Patched (2022-11-10 r83330) with R packages ampvis (2.7.27), tidyverse (1.3.1), seqinr (4.2.16), ShortRead (a.54.0) and iNext (2.0.20) (Charif and Lobry, 2007; Hsieh et al., 2016; Morgan et al., 2009).

2.5 Microscopic Visualisation and Abundance

In opposition to the majority of publications on microbial community characterisation, this study relies on a two-method approach for both qualitative and quantitative characterisation of the community. While the qualitative characterisation is achieved by the conducted sequencing, quantitative insights were in this study acquired through epifluorescence microscopy of prepared filters according to the CARD-FISH procedure. Microscopic analysis was conducted only for sediment samples.

2.5.1 Filter Preparation

In order to visualise cells via the CARD-FISH method, 1 g aliquots of fixed sediment slurry were filtered onto 47 mm Merck Isopore™ Polycarbonate Membrane Filters, 0.2 μm (Fisher Scientific, USA). To first detach and purify cells from the sediment slurry, physical and chemical pretreatment following a protocol proposed by Amalfitano and Fazi, 2008 was performed: in a first step, 10 ml of a release solution containing Milli-Q water, PBS, pyrophosphate tetrasodium (0.1 M), formalin (pH7, 2%), and Tween 80 (0.5%) were filtered through a syringe with millex filter and added to the sediment aliquot in a 15 ml conical test tube (Kemp, 1993). Formaldehyde has been shown to strengthen bacteria in samples but required additional filtering due to aggregate formation upon long-term storage (Thavarajah et al., 2012). Samples were then incubated under agitation for 15 mins at 720 rpm using an orbital shaker (IKA®KS 130 B, Staufen, Germany)and sonicated on ice for 1 min at 20W (Microson XL2000 ultrasonic liquid processor with 1.6 mm diameter micro-tip probe, Misonix, New York, USA), detaching the cells while avoiding cell shrinkage.

Subsequent improvement of visual quality via elimination of background noise in form of sediment particles was achieved through high-speed density gradient centrifugation, utilising Nycodenz (Nycomed, Oslo, Norway; density $1.310 \pm 0.002 g ml^{-1}$ as a density gradient medium. Therefore, 1 ml of Nycodenz was introduced employing a syringe at the bottom of a 2 ml Eppendorf tube, beneath 1 ml of treated sediment sample. The tubes were then centrifuged (Eppendorf Centrifuge 5810 R, Hamburg, Germany) for 90 min at 14000 rcf and 4°C to separate the contained cells from the sediment particles. The detached and purified cells are now contained in the supernatant which was transferred to a separate test tube.

Adequate filter preparation targets a suitable cell concentration on the filters. Flow cytometry (Apogee A-50micro, Apogee Flow System, London, UK) was thus used to preliminarily determine the cell abundance in the supernatant and choose the correct volume for filtration. Operational settings for the flow cytometer analyses were adopted from Manti et al., 2011 and Amalfitano and Fazi, 2008. While the accuracy of

flow cytometrical determination of microbial abundance in sediment samples is limited in comparison to less complex matrices (Gruden et al., 2004), the analyses still gave a sufficiently accurate indication on the required sample volume. As determined from the flow cytometry analyses results (Appendix B), an amount of 1 to 2 ml of pretreated sample were filtered onto the polycarbonate membrane filters mounted on a glass holder connected to a vacuum pump (Millipore Sigma WP6122050, USA 0.3 bar). All filters were then DAPI-stained (1 μg ml^{-1} and checked under the fluorescence microscope (Leica, DM LB 30, Wetzlar, Germany) for adequate cell abundance and visual quality. If both were sufficiently achieved, filters were stored in Petri dishes at -20°C for further analyses.

Total prokaryotic cell abundance was analysed through microscopic counting. For this purpose, small sections of the prepared filters were stained with DAPI solution through immersion in Vectashield oil (Vectashield Antifade Mounting Medium with DAPI, LS-J1033, Vector Laboratories Inc., California, USA) and mounted on microscope slides. A sterile cover slip was placed on the filters and the slides were incubated for 10 min in the dark prior to microscopic counting. The number of cells on each slide was counted using the epifluorescence microscope (magnification 1000x with 100x objective, $\lambda_{Ex} = 488nm, \lambda_{Em} = 500 - 530nm$) (Salehiziri et al., 2020). Per section, a minimum of 300 cells was counted (in at least 10 randomly selected fields) (Sheahan et al., 2005) to calculate overall cell abundance, taking into account the dillution from the slurry preparation

2.5.2 CARD-FISH

CARD-FISH was performed after the protocol proposed by Eickhorst and Tippkötter, 2008 with slight alterations according to Fazi et al., 2007. Sections of the previously prepared filters were mounted onto glass slides and dipped into 0.2% low melting-point agarose to limit cell loss in subsequent treatment steps and incubated at 37°C until all agarose was fully dried. After washing in 96% EtOH and air-drying, the filter sections were enzymatically treated with lysozyme solution (62970 Fluka Lysozime, 20 mg $ml^{-}1$, dissolved in 400 μl 0.05 M EDTA pH 8, and 200 μl 1 M Tris–HCl pH 7.4) and proteinase K working solution (18 μl proteinase K standard solution (P2308 34 U/mg) in 6 ml Tris-EDTA buffer (0.1 U^{ml-1})) to permeabilise the cell walls and enable the intrusion of the oligonucleotide probes inside the cells. Incubation was performed after both steps at 37°C for 60 min and 25 min respectively. After washing in Milli-Q water, a subsequent incubation in 0.01M HCl was performed to deactivate the endogenous peroxidase and prevent probe-unspecific signal emitation. Again, filters

were washed with Milli-Q water, dehydrated in 96% ethanol and air-dried to prepare for *in situ* hydridisation.

For the hybridisation of the filter sections, different HRP-labelled oligonucleotide probes and hybridisation buffers were needed to target the Bacteria and Archaea domains. Bacteria were targeted through mixing of a 200 μl hybridisation buffer with 30% formamide concentration (v/v) and 2 μl of oligonucleotide probes EUB338 I-III (50 ng / μl at 4°C) whereas Archaea were targeted with the same quantities of a 55% hybridisation buffer and the ARCH915 probe (50 ng / μl at 4°C); all probes were HRP-labelled at the 5'-end according to the sequences given in table 2.1. The hybridisation buffer was prepared as followed: 1800 μl of 5 M NaCl were mixed with 200 μl 1M Tris/HCl, 1 gr of Dextran sulfate, 1000 μl of 10% blocking reagent (ROCHE, cat n. 1096176, Roche Diagnosis GmbH dissolved in Maleic Acid, and 0.05% Triton X100). Depending on the desired formamide concentration, 5500 μl of 55% formamide and 1500 μl of Milli-Q water or 3000 μl of 55% formamide and 4000 μl of Milli-Q water were added.

TABLE 2.1: HRP-labelled oligonucleotide probes utilised for the CARD-FISH protocol

Probe	Target Domain	Sequence (5'-3')	rRNA location
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	16S (915-934)
EUB338 - I	Bacteria	GCTGCTCCCGTAGGAGT	16S (338-355)
EUB338 - II	Bacteria	GCAGCCACCCGTAGGTGT	16S (338-355)
EUB338 - III	Bacteria	GCTGCCACCCGTAGGTGT	16S (338-355)

Under constant rotation, the sections were incubated in the prepared probe-buffer-mix at 35°C overnight. On the subsequent day, the filter slices were moved to a washing buffer to eliminate formamide and enhance the binding of the probes to the target sites. Composed of 30 μl 5 M NaCL, 1000 μl 1M Tris/HCl, 500 μl 0.5 M EDTA, 50 μl 10% Sodium dodecyl sulfate filled with Milli-Q water to 50 ml, the washing buffer was preheated to 37°C and filter slices were again incubated at 37°C for 30 min. After incubation, the excess liquid was removed and filters were washed in phosphate-buffered saline solution (PBS) under mid-agitation for 15 min. Again removing excess liquid, a final incubation in a dye-tyramide substrate mix composed of 600 μl amplification buffer [3], 0.03 μl 30% H_2O_2 and 6 μl PBS was performed at 37°C on a rotisserie for 10 min, followed by consecutive washing in PBS (25 min under mid-agitation), Milli-Q water (1 min), and 96% Ethanol (1 min). To avoid bleaching of the tyramide, all steps following the final incubation were conducted in the dark.

[3]Mixture of 5 ml 10XPBS, 20 ml 5M NaCl, 0.5 ml Blocking reagent, and 5 g Dextran Sulfate filled up to 50 ml with Milli-Q water

After successful finalisation of the CARD-FISH protocol, all filter sections were counter-stained with 1.5 $\mu\ gml^{-1}$ DAPI solution and analysed under the microscope. A typical sight of the prepared filters under the microscope can be found in Fig. 2.3.

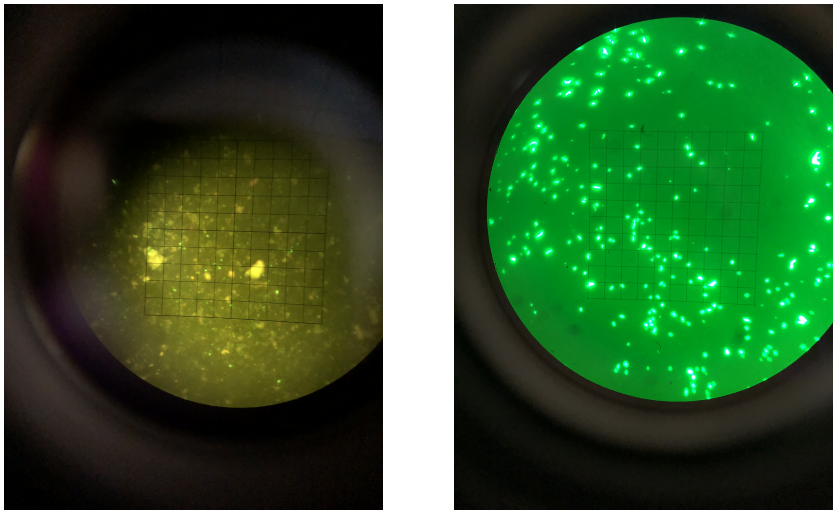

FIGURE 2.3: Typical view of the prepared filters through the epifluorescence microscope

2.6 Statistical Analysis and Graphical Visualisation

Statistical analysis of DNA-sequencing results was performed in R version 4.3.1. as well as in the R Shiny application Namco, version 1.0.1. (Dietrich et al., 2022). If not indicated differently, R was used for analysis and visualisation. The sequencing results of each site were normalised to a total sum of 10,000 reads (Total Sum Normalisation) in Namco and alpha and beta diversity were analysed and plotted. To gain further insights into microbial community composition of each lagoon, both phylum and order level were further inspected to provide a holistic description of the system balanced with a more in-depth analysis. Barplots of the most abundant taxa (>1%) were produced in Namco for both taxonomic levels. To deepen the insights into the fine-scale taxonomic structure of the microbial communities lagoon sediments, a heatmap analysis at the family level was performed using the heatmap() package in R. Distances were calculated according to the Bray-Curtis dissimilarity (vegan() package) and hirarchical clustering was based on average linkage. Again, taxa were prefiltered to an abundance of 1%. For Butrinti lagoon, a Bubble Plot was created to better represent potential compositional differences between samples and environments. Again, sequencing results were prefiltered to visualise only abundant taxa at the highest possible taxonomic resolution. Additionally, a differential network was calculated in R with the NetCoMi-package (Peschel et al., 2021), comparing the community network between the water

and sediment samples. For this purpose, counts were normalised using a total sum scaling normalisation and the network calculated based on pearson correlations. Differential associations were determined with the fisher test. The visualised network was chosen to represent the top 100 edges with the highest absolute difference in association.
All environmental variables were further processed in R using its base functions. A depth profile of Butrinti lagoon was produced based on water quality parameters taken at sampling point BU3 in a depth interval of 2 m. Furthermore, the impact of heavy metals on sampling sites was assessed via a PCA plot. For this purpose, heavy metal contamination was converted into a contamination factor to account for varying backrgound values. Base values were therefore derived from the European Soil Data Center (Toth, 2018)

To investigate environmental impacts on community composition, all measured or analysed environmental parameters were correlated to the presence of taxa found at all three sampling sites at family level using a pairwise comparison of the complete observations. The analysis was performed with the corrplot() package, following a prefiltering step (abundance > 0.25%), and finally visually presented as a correlogram. Analysis of abundant taxa (>0.25%) was conducted at class, order, and family level to identify and solidify correlations between taxa and environmental variables, with a focus on heavy metals in the subsequent analysis.

Additionally, a pariwise Permanova was performed on family level to test for statistical significance (p-value = <0.05) in difference between the three sampled lagoons. Distances were calculated as Bray-Curtis dissimilarities and p-values adjusted after the bonferri correction utilising the vegan () package. Finally, a network analysis was conducted in Namco to identify key taxa and potentially generalisable trends in community composition. Again, only taxa present at all three sites with an overall abundance of 1% in each sample were considered. The network was constructed on OTU level with underlying parameters similar to the dissimilarity network of Butrinti, although 120 nodes and 1000 edges were graphically represented. Additionally, clusters were identified using the fast greedy modularity optimization algorithm for finding community structures.
Measured concentrations for the heavy metals (arsenic, cadmium, chromium, copper, lead, manganese, and nickel)were further expressed in contamination factors C_f^i

$$C_f^i = C^i / C_n^i \tag{2.1}$$

where C^i is the measured concentration of heavy metals in sediment and C_n^i. The method was chosen after (Rahman et al., 2022) to account for varying background

values between sampling sites. Background levels were retrieved from Toth, 2018.

Chapter 3

Results

Over the course of this chapter, the results of the physicochemical characterisation and the qualitative and quantiative analysis of the microbial communities will be presented for each lagoon individually. Then, comparative statistical and network analyses will be presented to derive community structures from the data of all three lagoons.

3.1 Butrinti Lagoon, Albania

With a total of 16 samples, originating from both sediment and water, Butrinti Lagoon had the highest sample density among all three investigated lagoons. It was the only lagoon in this study were an in-depth description of the water and sediment environment could be attempted via the integration of insights into microbial community composition of different water depths and sediment samples. The results presented in this section reflect the prepared data of the conducted elemental characterisation - based on the elemental analysis, CHN-analysis, and water quality parameter measurements - coupled with the qualitative and quantitative insights into the microbial community composition origination from the DNA-sequencing and microscopic analysis.

3.1.1 Environmental Characterisation

In the investigated lagoon, a detailed assessment of key water quality parameters - namely conductivity, dissolved oxygen (DO), salinity, and temperature - in a depth interval of 2 m revealed distinctive patterns that underscore the lagoon's habitat conditions. The depth profiles of all variables measured at BU3 are depicted in Fig. 3.1.

The conductivity and salinity demonstrated an upward trend, steadily increasing from 35000 μS/cm and 26 PSU respectively over the first 8 meters of the water column. Beyond this depth, salinity levels reached a plateau, remaining constant at 34 to 35 PSU throughout the subsequent layers. Concurrently, conductivity peaked at 8 meters and 50,000 μS/cm and gradually decreased thereafter, finally reaching a level of 44,000

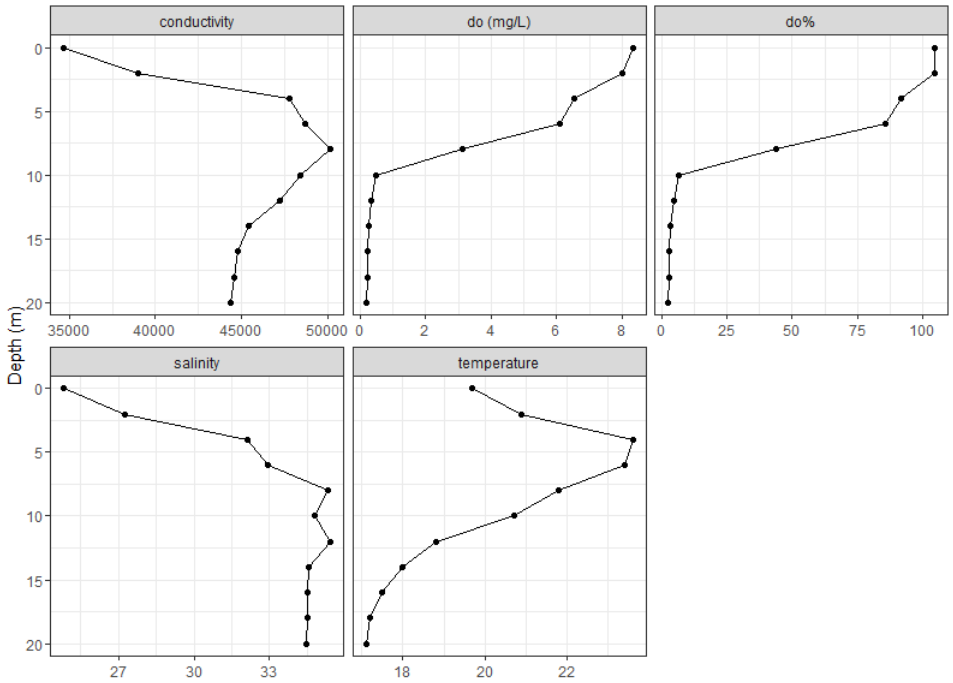

FIGURE 3.1: Depth profiles of (A) conductivity, (B) dissolved oxygen (mg/L), (C) dissolved oxygen (%), (D) salinity (PSI), and (E) temperature (°C) at BU3

μS/cm at the lagoon bottom. Such spatial variations in salinity and conductivity indicate the influence of external factors, such as tidal influences and freshwater input, shaping the lagoon's stratification.

Further insights emerged from the DO content, highlighting the presence of distinct oxygen zones within the lagoon's water column. The uppermost layer (0 to 2 m) exhibited a well-defined oxic zone, where dissolved oxygen levels were at over 100%, supporting aerobic processes. However, an abrupt transition occurred below 2 m, leading to a profound decline in oxygen concentrations. From 2 m down to a depth of 10 m, the lagoon's water became strongly anoxic, with dissolved oxygen reaching a minimal value of 6%. The subsequent anoxic layer persisted uniformly until the bottom of the lagoon, mounting into an oxygen content of 2%, which clearly indicates limited oxygen availability in these deeper strata.

Furthermore, the temperature distribution within the lagoon resembled that of a stratified lake. The uppermost layer experienced a gradual increase in temperature from 20 to 23.5°C within the first 5 meters, showing an atypical incline in temperature in the epilimnion. Below this depth, the water temperature drastically declined, reaching 18°C at a depth of 14 m. This strata thus shows the typical characteristics of the thermocline. Below a depth of 14 m, water temperatures stagnate and stabilise around 17°C towards the bottom of the lagoon, forming a clear hypolimnion. This stratified temperature profile accentuates the compartmentalization of the water column and reflects the complex interplay of heat absorption and mixing processes within

the lagoon.

Water quality parameters were additionally measured in the surface waters following the sampling transects in order to assess potential longitudinal variations (Appendix C). Surprisingly, no clear gradient could be observed in salinity or DO from North to South, neither along the coastal, nor along the central transect. For both parameters, levels peak at S-BU3 and showed a slight decrease at S-BU4 along the central transect. Temperature, on the other hand, increased steadily along this central transect. Along the coast, the lowest levels for salinity, DO, and temperature were all observed at S-BUB.

The results of the LOI method and CHN analysis revealed the OM, TN and TOC content (all in %) of the taken sediment samples. Significant fluctuations were only detected in the OM content of the sediment samples, were values ranged from 5.2 % in S-BUC to 13.8 % in S-BU3, thereby showing no clear gradient along the transects or correlation between station and lagoon samples. Both TN and TOC content exhibited only minimal differences among all samples and revolved around a content of 1% TN and 2% TOC. These findings are in lign with the concentrations of NO3, NH4, and NO in the water samples provided by the partners in Albania. Their analyses showed that concentrations for all water samples, along the transect as well as along the depth gradient, were negligible as concentrations were mostly below the detection limit. The same observation applies to the phosphorus content of the water samples. For further insights the reader is referred to Appendix D, were TN, TOC, and OM contents are presented.

Sediments of Butrinti Lagoon all exhibited critical concentrations exceeding the normal background values of the region. Sediments from the central transect across the lagoon (S-BU1 to S-BU4) were more contaminated than sediments from the coastal transect (S-BUA to S-BUC). The highest pollution was found in the deep sediments, where S-BU2 exceeded natural concentrations in all assessed heavy metals. Similarly, S-BU3 was found to be contaminated by six out of the seven assessed heavy metals, with only Lead concentrations lying within the natural background values. Sediment samles S-BU1 and S-BU4 still showed elevated concentrations for five and four heavy metals respectively. In contrast to the central transect, coastal sediment samples S-BUA and S-BUC remained uncontaminated apart from slightly elevated copper levels. However, heavy metal concentrations factors at sample S-BUB - located in immediate proximity to the town of Ksamil - again indicated pollution with Arsenic, Cadmium, Chromium, Copper, and Lead, suggesting strong anthropogenic impacts.

3.1.2 Microbial Community Composition

Quantitative analysis of prokaryotic cell abundance in the sediment samples of Butrinti Lagoon revealed a mean cell abundance of 1.44E+08 per gram of sediment, ranging from 3.87E+07 in S-BU3 to 2.59E+08 in S-BU4. Overall, cell abundance increased along the sampling transect until prokaryotic cell abundance collapsed in S-BU4. Among these, 80.45% of cell were on average identified as bacterial cell, constituting an abundance of 1.27E+08 cells per gram of sediment, whereas 2.57%(3.26E+08) could be assigned to the domain of archaea. All results of the DAPI and CARD-FISH counts are visually represented in Fig. 3.14 and 3.15 respectively. Despite accurate analysis, roughly 17% of all DAPI-stained cells remained unaccounted for.

Sequencing results of the 16S rRNA gene revealed a complex and distinct community composition in each of the sequenced samples. A total of 1287144 high-quality reads were obtained from 16 samples, comprising an average of 80,447 reads per sample. Two samples from point BU1 had to be excluded due to insufficient quality of extracted DNA.

From the total reads, 1118773 reads were identified as bacteria and 97695 reads assigned to the domain of Archaea. The bacterial domain within Butrinti Lagoon is comprised of 61 phyla, 121 distinct classes, and 283 identified orders. Reads could moreover be assigned to 388 different families. Less diverse is the archaeal domain of Butrinti lagoon, encompassing 17 different classes from 10 phyla. On a lower taxonomic level, reads from 24 different orders were identified, hosting a diversity of 20 distinct families. When compared to the SILVA database, 4.5% of reads remained unassigned, highlighting the need to further explore undiscovered taxa in coastal lagoons.

The analysis of community composition of the samples taken from Butrinti Lagoon reveals a clear dominance of *Proteobacteria* in all sequenced samples, accounting for 39.4% in total. Yet, the remaining composition of samples differs notably between water and sediment samples. These variations are graphically presented in Fig. 3.2.

Among water samples and superficial sediment samples, *Bacteroidetes* are equally dominant, accounting for as much as 30% in some samples. Moreover, it can be derived that community composition of deep water samples (BU2-22 and BU3-20) shows a greater similarity to the composition of sediment samples taken from shallow depths, namely S-BUA to S-BUC as well as S-BU4. This observation is in lign with the moderate presence of *Cyanobacteria* in water samples from high depths and sediment samples from low depths. In superficial water samples, *Cyanobacteria* present the third most abundant phylum with around 10%, whereas their share falls below 1% in sediment samples from greater depths. Overall, on the phylum level, coastal sediment samples S-BUA to S-BUC revealed similarities to the deep water samples of BU2-22 and BU3-20.

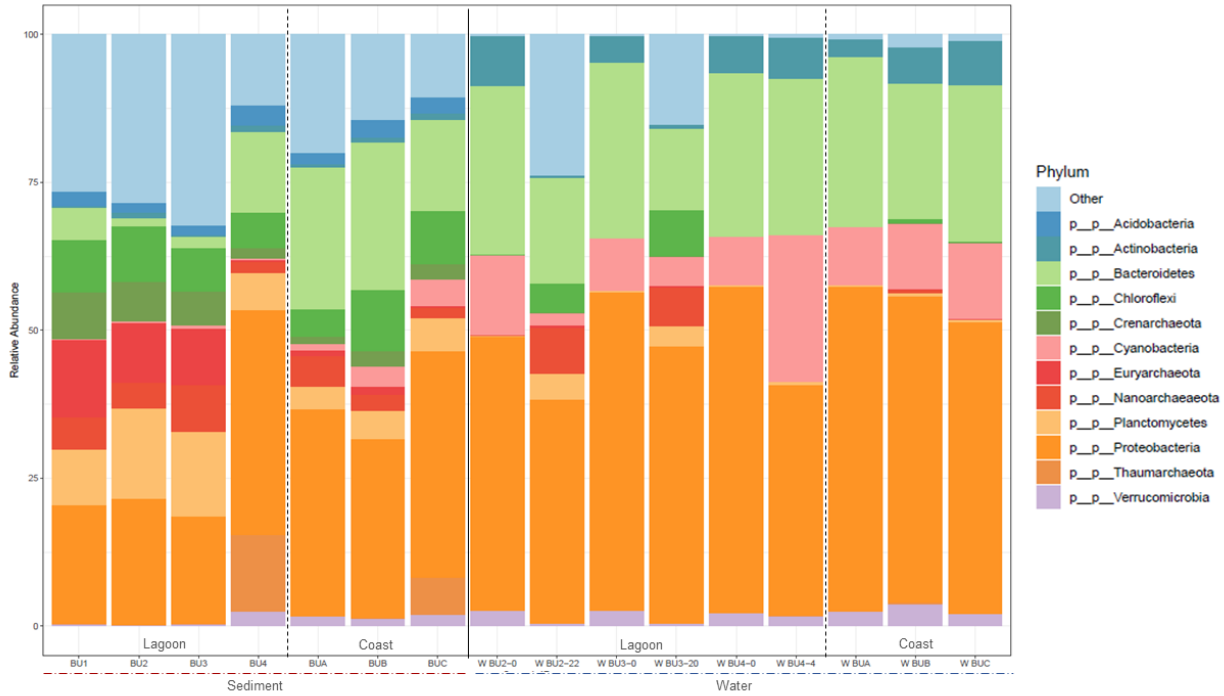

FIGURE 3.2: Taxonomy bar plots of Butrinti Lagoon samples at phylum level. Less abundant taxa (<1% abundance) are not displayed. Sediment samples are displayed on the left, water samples of the right side.

To further support these trends, a bubble plot depicting the most abundant taxa (>1%) at the highest taxonomic resolution was plotted (Fig. 3.3. Opposing the findings of the barplot, a difference in community composition between water and sediment samples is clearly evident. The plot further suggests significant distinctions in composition at location BU4 at 0 and 4 m depth as compared to all other water samples, thus isolating this sampling point from the others with regard to its composition. The observed high abundance of *Desulfatiglans* remains unique among the examined samples. Consequently, the analysis at a finer taxonomic resolution does not seem to support the similarities found in composition between deep water samples and sediment samples - regardless of their depth. Instead, the dendrogram indicates clustering of sediment samples based on the transects, exhibiting analogies between the central samples S-BU1 to S-BU4 in one cluster, and the coastal samples S-BUA, B, and C in a second cluster.

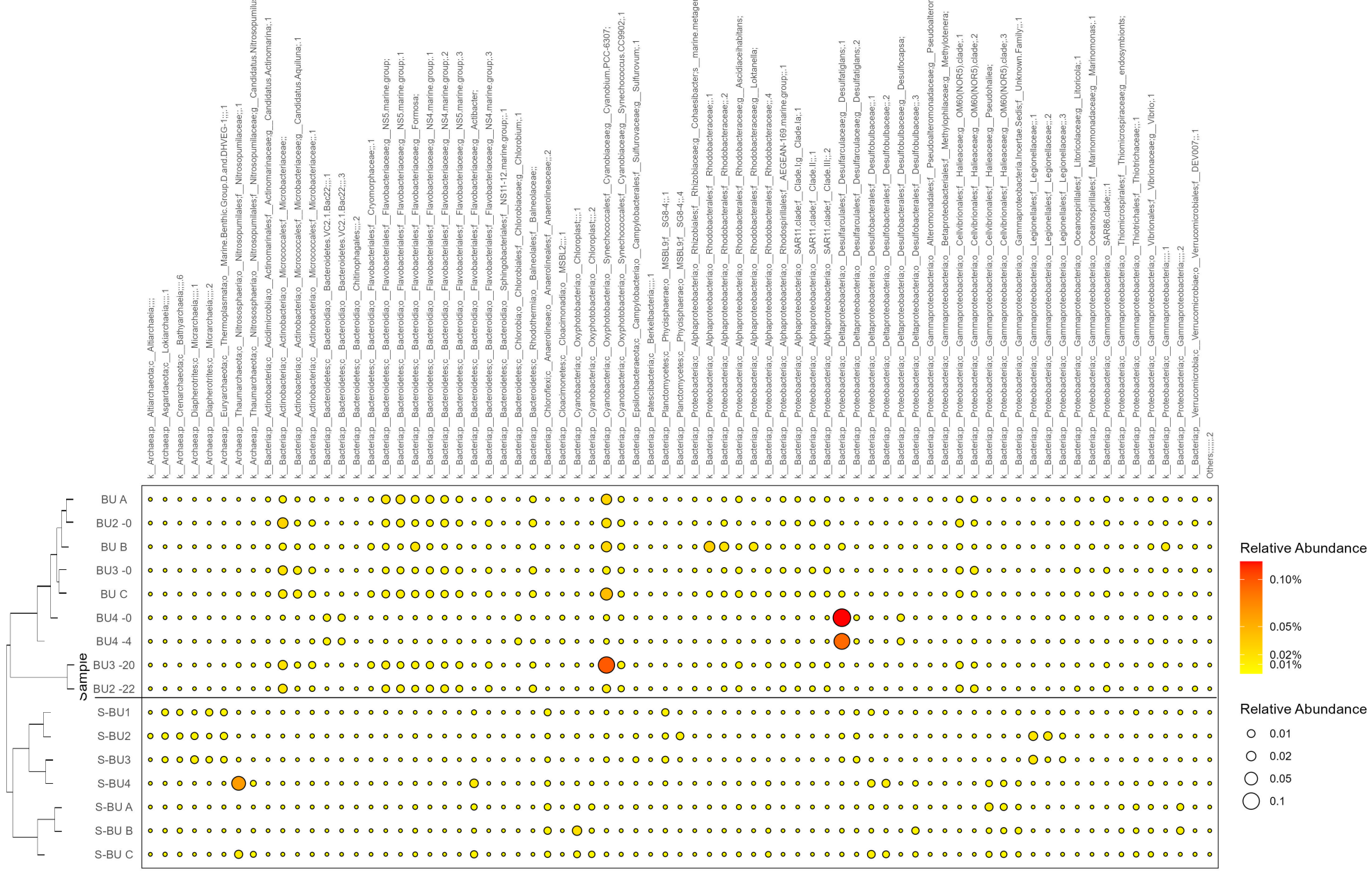

FIGURE 3.3: Bubble plot depicting the relative abundance of the most abundant retrieved taxa (relative abundance >1%) per sediment sample from Butrinti Lagoon. Samples are ordered in increasing depth

In order to derive insights about potential key microbial players within Butrinti Lagoon and draw conclusions on potential environmental interactions, a differential network analysis was performed, comparing the associations between the two environments sediment and water within the lagoon. The differential network analysis allows to detect significant changes in the co-occurrence patterns of microbial species present in both environments. As can be derived from Fig. 3.4, associations among several taxa vary between the water and sediment environments, thus indicating potential differences in functional roles or interactions of taxa.

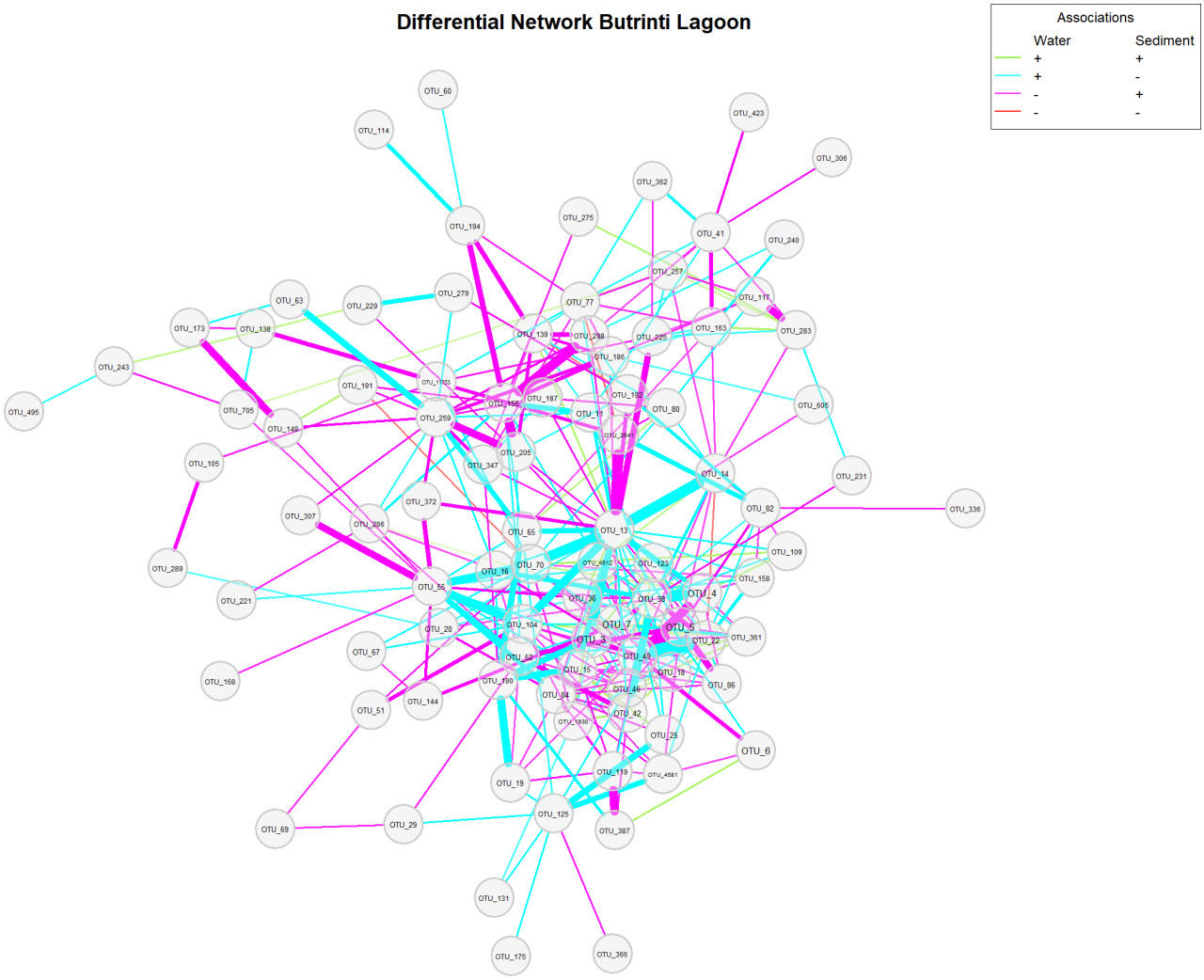

FIGURE 3.4: Differential network analysis comparing associations between sediment and water samples. Nodes represent microbial taxa, while edges signify significant connections.

Upon closer analysis, the Jaccard index - measuring the similarity between the sets of most central nodes - revealed complete dissimilarity of both networks with regard

to betweenness centrality and hub taxa, as their index value was 0. Eigenvector centrality and degree values are equally low with 0.163. This finding was supported by an Adjusted Rand Index, used to assess the similarity between clusterings, of 0.203, as would be seen in a network with limited agreement between clusterings.

While OTU_41 (*f_Legionellaceae*) and OTU_11 (*g_Cyanobium PCC-6307*) displayed eigenvector centrality in water, the microbial network in sediments was impacted strongest by OTUs 3 (*g_NS4 marine group*), 49 (*f_Clade III*), and 18 (*g_OM43 clade*). Of special interest for this study are taxa with varying associations in the water network and the sediment network, as these could potentially indicate different roles in the two environments. In the presented network, the five nodes with the highest absolute difference in association are OTUs 13 (*g_Desulfatiglans*), 38 (*f_Anaerolineaceae*), 49 (*f_SAR11 clade III*, 55 (*o_Bacteroidetes VC2.1 Bac22*), and 259 (*s_archaeon GW2011_AR13*). A visual analysis for taxa with differing positive associations in water and sediment draws attention to OTUs 14 (*g_Balneola*), 77 (*c_Bathyarchaeia*), OTUs 82 (*g_Coraliomargarita*), and 194 (*o_Chloroplast*.

3.2 Sabaudia Lagoon, Italy

Sabaudia Lagoon presents the smallest of the three studies lagoons and was thus only described via four samples. This section comprises an overview of the environmental characteristics of the lagoon, the taxonomic composition obtained through DNA sequencing, and the prokaryotic cell abundance to gain insights into the microbial diversity and ecosystem dynamics.

3.2.1 Environmental Characterisation

The determined water quality parameters at Sabaudia Lagoon showed a gradual increase in salinity from 33.7 PSU at SA1 to 36.1 PSU at SA4. Conversely, the measured temperature decreased along the sampled transect from 18.5°C to 16.7°C at SA4. A different trend emerged for the taken DO (mg/L) and organic matter (%) measurements: here, both levels increased gradually from SA1 (10.67 mg/L DO and 13.67% OM) to SA3 (11.39 mg/L DO and 25.85% OM), before levels dropped to 9.81 mg/L DO and 8.34% OM at SA4. All water quality parameter measurements are listed in Appendix C.

The CHN analysis of Sabaudia Lagoon sediments revealed constant levels of total nitrogen (%) along the sampled gradient as well as slightly lower TOC (%) values in SA1 as opposed to the remaining samples. All results from of the CHN analysis, LOI method, as well as the elemental concentrations determined via metal digestion and

subsequent ICP-OES analysis are presented in Appendix D.

The further processing of the elemental analysis results (presented in Appendix E) revealed elevated heavy metal concentrations in all four examined samples. Concentration factors of all seven examined heavy metals (arsenic, cadmium, chromium, copper, lead, manganese, and nickel) indicated a moderate to strong contamination of both SA2 and SA3, with factors as high as 9.12 for chromium in SA2 and 12.62 for copper in SA3. SA1 is characterised by elevated concentrations of arsenic, chromium, copper, lead, and nickel but could be categorised as unpolluted by cadmium and manganese. In contrast to these significant contamination profiles, SA4 exhibited normal concentrations of heavy metals and only showed slightly elevated concentrations for manganese.

3.2.2 Microbial Community Composition

Prokaryotic cell abundance in Sabaudia Lagoon sediments was investigated using DAPI staining and CARD-FISH analysis. DAPI staining revealed a moderate range of cell densities across the four sediment samples, ranging from 2.1e+08 cells per gram of sediment in SA4 to 3.6e+08 cells per gram of sediment in SA1. However, prokaryoitic cell abundance did not follow a clear gradient along the transect as abundance in SA3 exceeded abundance in SA2. Subsequent CARD-FISH analysis revealed a strong dominance of the bacteria domain in all sediment samples (on average 71.98% of total DAPI-stained cells) with an average cell abundance of 2.06e+08 cells per gram of sediment. With on average 1.90e+07 cells per gram of sediment, roughly 6.7% of total DAPI-stained cells could be assigned to the archaeal domain. All results of the DAPI and CARD-FISH counts are visually represented in Fig. 3.14 and 3.15 respectively.

Analysis of the 16S rRNA gene sequencing data revealed the taxonomic composition and diversity of the microbial communities inhabiting the sediments of Sabaudia Lagoon. A total of four sediment samples was analyzed for their microbial abundance and composition. After quality filtering and data processing, a total of 399963 high-quality reads were obtained, with an average of 99991 reads per sample.

Among the high-quality reads obtained from the 16S rRNA gene sequencing of Sabaudia Lagoon sediments, a total of 274858 reads were classified as bacteria, while 95825 reads were identified as archaea. This indicates a substantial representation of both prokaryotic domains within the microbial community. The taxonomic analysis of these reads revealed a diverse assemblage of microorganisms at various taxonomic

levels. At the phylum level, a total of 59 bacterial phyla and 9 archaeal phyla was identified. Within the bacterial phyla, further examination revealed 103 distinct classes, 218 bacterial orders, and 279 families. Within the archaeal phyla, 23 orders from 16 classes were detected, of which reads could be assigned to 17 different families. It is worth noting that despite rigorous taxonomic classification efforts, a portion of the high-quality reads remained unclassified, representing 7.32% of the total reads. The presence of unclassified reads underscores the richness and complexity of the microbial community within Sabaudia Lagoon sediments, leaving room for further exploration of novel and potentially undiscovered taxa in this unique ecosystem.

Within Sabaudia Lagoon, the most abundant phyla observed in the sediment samples were *Chloroflexi*, *Crenarchaeota*, *Proteobacteria*, and *Planktomycetes* constituting 19.99%, 14.41%, 9.53%, and 9.1% of the total microbial community, respectively. The relative abundance of each phylum in the sediment samples after an appropriate filtering step is presented in Fig.3.5. For a higher taxonomic resolution, the order level was investigated (bar plot depicted in Appendix F). The top five orders identified in the samples were *SBR1031*, *Anaerolineales*, *Marine Benthic Group D and DHVEG-1*, *Desulfobacterales*, and *MSBL9*, accounting for 7.18%, 7.13%, 4.96%, 3.89%, and 3.48% total microbial community, respectively.

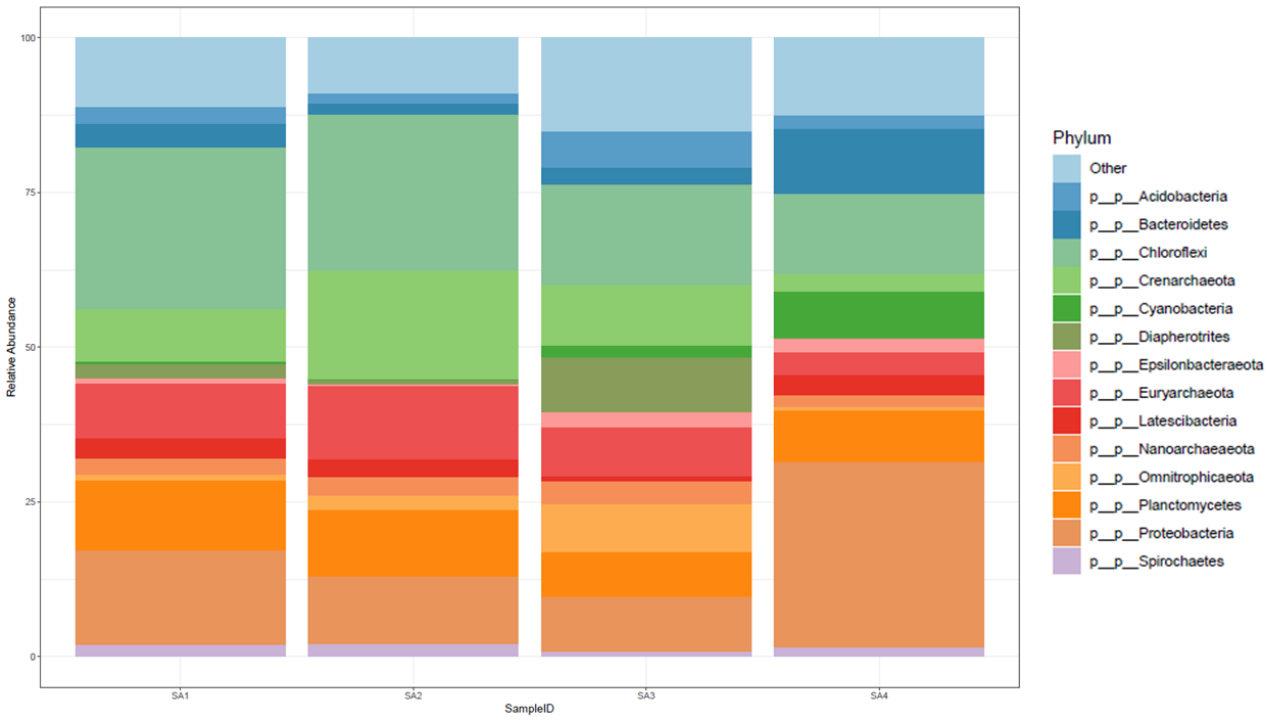

FIGURE 3.5: Taxonomy bar plot of Sabaudia Lagoon samples at phylum level

With *Desulfaculales* and *Desulfobacterales* both being abundant on order level in all

four sediment samples, the presence of sulfate-reducing bacteria becomes evident, indicating the cycling of sulfur within the sediments.

The finer taxonomic resolution was visualised in form of a heatmap. The heatmap, presented in Fig. 3.6, illustrates the relative abundance of the most abundant families across all 4 sediment samples. Each column in the heatmap represents a different family, while each row corresponds to a specific sediment sample. The color scale depicts the normalized relative abundance, with red indicating higher abundance and yellow indicating lower abundance.

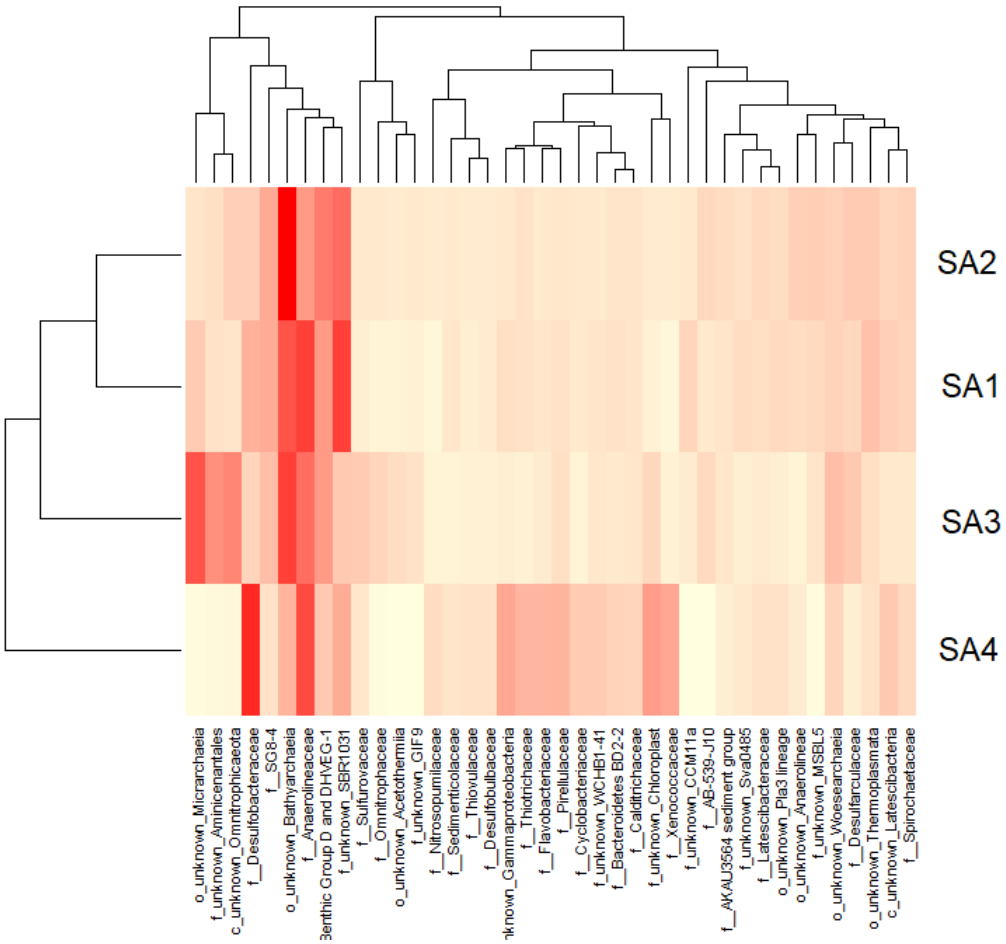

FIGURE 3.6: Heatmap and hierarchical clustering of family-level microial community composition profiles per sample at Sabaudia Lagoon

The heatmap revealed distinct patterns of family-level composition per sample. Notably, families such as *Anaerolineaceae*, *Desulfobacteraceae*, and *SG8-4* were consistently prevalent in most samples. Interestingly, a small shift can be noted in the relative abundace of *Desulfobacteraceae* which prevailed in SU4 and is of lower relative abundance in SA1 to 3. Conversely, families of the order *SBR1031* and class *Bathyarchaeia*

show a higher abundance in SA1 and 2 than in the remaining samples.

3.3 Ria Formosa Lagoon, Portugal

In parallel to section one and two of this chapter, section three will cover the results of the environmental characterisation, the taxonomic composition obtained through DNA sequencing, and the assessment of prokaryotic cell abundance to gain insights into the microbial diversity and ecosystem dynamics of Ria Formosa Lagoon.

3.3.1 Environmental Characterisation

As indicated in section 2.2, water quality parameters were measured for FM1-3 individually, but were taken for all samples of the LM group collectively. The measurements are shown in Appendix C. Within the FM group, salinity (PSU) and DO (mg/L) increased gradually from FM1 to FM3, rising from 0.91 PSU to 2.75 PSU and 1.73 mg/L to 2.24 mg/L. Contradictory to this, temperature (°C) as well as OM (%) fall from 16.45°C to 15.16°C and 9.65% to 0.56%. In the LM group, salinity is, with 16.6 PSU, significantly higher than in the FM group. DO lies at 2.5 mg/L, whereas temperature was determined at 14.85°C.

All further environmental variables were measures at the laboratory in Italy and are thus available for all six samples individually. The OM-content was found to spike drastically in LM2 and LM3, but TN and TOC, as determined by the CHN analysis, remain fairly constant for all samples (around 0.9% TN and 2.3% TOC) (See Appendix D).

Contamination factors for the assessed heavy metals showed no contamination of the LM1 side. Conversely, LM2 and LM3 are notably contaminated, with Arsenic, Cadmium, Chromium, Copper, and Lead contamination factors ranging from 1.5 (Lead) to 3.9 (Chromium). Sediment quality in the FM sample group remains good in most cases, apart from a Copper and Lead contamination of FM1, a Chromium contamination of FM2, and a Copper contamination of FM3.

Similar patterns are visible within the overall elemental composition of all samples. Measured concentrations show great similarities between LM2 and LM3, particularly for Boron, Calcium, Iron, Potassium, Magnesium, and Sulphate concentrations. Besides this, no clear patterns are detectable. All determined elemental contentrations can be derived from Appendix D.

3.3.2 Microbial Community Composition

Prokaryotic cell abundances of Ria Formosa lagoon are, in lign with the results presented for Butrinti Lagoon and Sabaudia Lagoon, visually presented in Fig. 3.14 and 3.15. Microscopic analysis of DAPI-stained cells revealed an average cell abundance of 1.17E+08 cells per gram of sediment with no clear trends within samples. The lowest abundance was observed in LM1 with an average prokaryotic cell abundance of 5.46E+07 cells per gram of sediment. The highest abundance, on the other hand, was observed in FM1 (1.82E+08 cells / gram of sediment). Prokaryotic cells could to 76.6% be assigned to the domain of bacteria and to 4.46% to the domain of archaea. The respective average cell abundance was determined at 9.38E+07 cells per gram of sediment and 5.12E+07 cells per gram of sediment. Notably, the high share of 11.15% of archaeal cells found in FM2 strikes out when compared to the remaining samples.

The analysis of 16S rRNA gene sequencing data provided comprehensive insights into the taxonomic composition and diversity of microbial communities in Ria Formosa Lagoon sediments. Six sediment samples were examined, yielding a total of 646,173 high-quality reads, with an average of 107,606 reads per sample.

The results indicated significant representation of both bacterial and archaeal domains within the microbial community. Specifically, 597,237 reads were classified as bacteria, and 41,528 reads were identified as archaea. The taxonomic analysis unveiled a diverse microbial assemblage at various taxonomic levels, including 55 bacterial phyla and 8 archaeal phyla. Within the bacterial domain, 106 classes, 220 orders, and 292 families could be identified. For archaea, 18 orders from 16 classes were detected, along with 15 families. Despite rigorous classification efforts, 1.15% of the high-quality reads remained unclassified, reflecting the complexity of the microbial community in Ria Formosa Lagoon sediments.

As for Sabaudia Lagoon, the phylum and order level were selected for further analysis, guaranteeing a balanced insight into the microbial community composition of the Ria Formosa Lagoon.

The taxonomic classification was first examined at phylum level, showing the relative abundances in Fig. 3.7. *Proteobacteria*, *Firmicutes*, *Chloroflexi*, and *Epsilonbacteria* posed the most abundant phyla, accounting for 37.23%, 23.83%, 6.75%, and 5.92% of the total microbial community, respectively.

At order level, the top five orders identified in the samples were *Shewanellaceae*, *Moraxellaceae*, *Clostridiaceae 1*, *Peptostreptococcaceae*, and *Anaerolineaceae*, constituting 9.72%, 7.56%, 6.24%, 5.14%, and 4.81% of the total microbial community. The community composition at order level is additionally depicted in Appendix F.

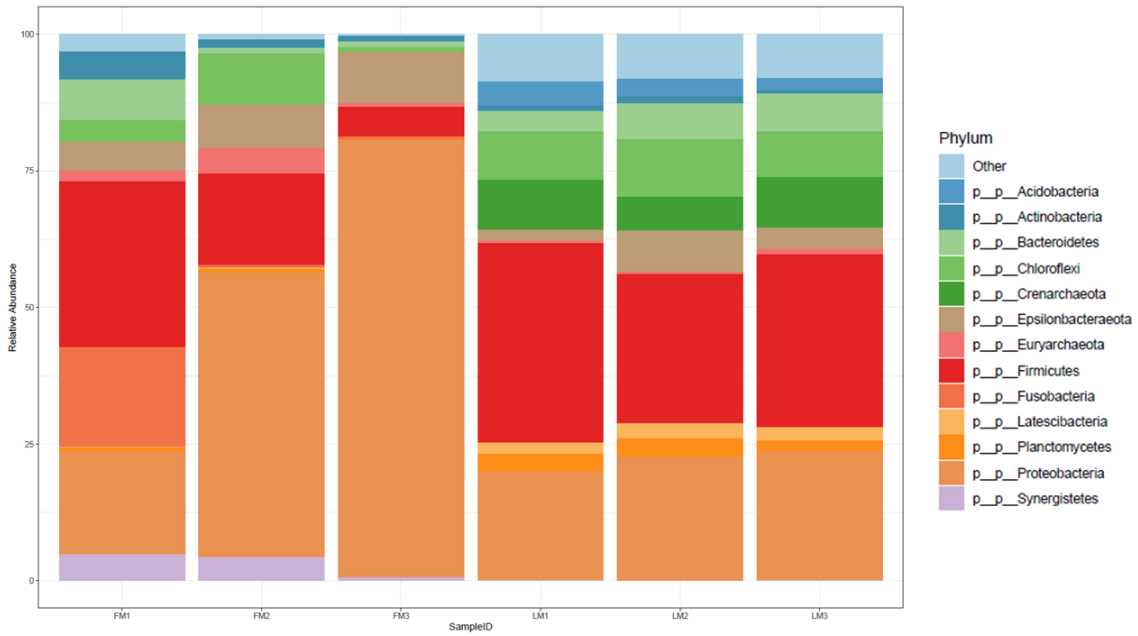

FIGURE 3.7: Taxonomy bar plot of Ria Formosa Lagoon samples at phylum level

To deepen the insights into the fine-scale taxonomic structure of the microbial communities in Ria Formosa Lagoon sediments, a heatmap analysis at the family level was performed. The heatmap, presented in Fig. 3.8, illustrates the relative abundance of the most abundant families across all 6 sediment samples. Each column in the heatmap represents a different family, while each row corresponds to a specific sediment sample. The color scale depicts the normalized relative abundance, with red indicating higher abundance and yellow indicating lower abundance.

The heatmap revealed the consistently high abundance of families such as *Anaerolineaceae*, and *Clostridiaceae 1*. Unsurprisingly, the microbial composition within the sampling groups only varies slightly. Notable differences in sample composition within the FM group lie in the higher abundance of *Fusobacteriaceae*, *Cranobacterieaceae*, *Lachnospiraceae*, and *Clostridiaceae 1* in FM1. FM2 and FM3, on the other hand, are characterised by a stronger community of *Shewanellaceae*, *Enterobacterieaceae*, and *Moraxellaceae*. Within the LM group, community composition appears more homogenic. Evident are only higher abundances of Vibrionaceae in LM3 and Clostridiaceae 3 in LM1 respectively.

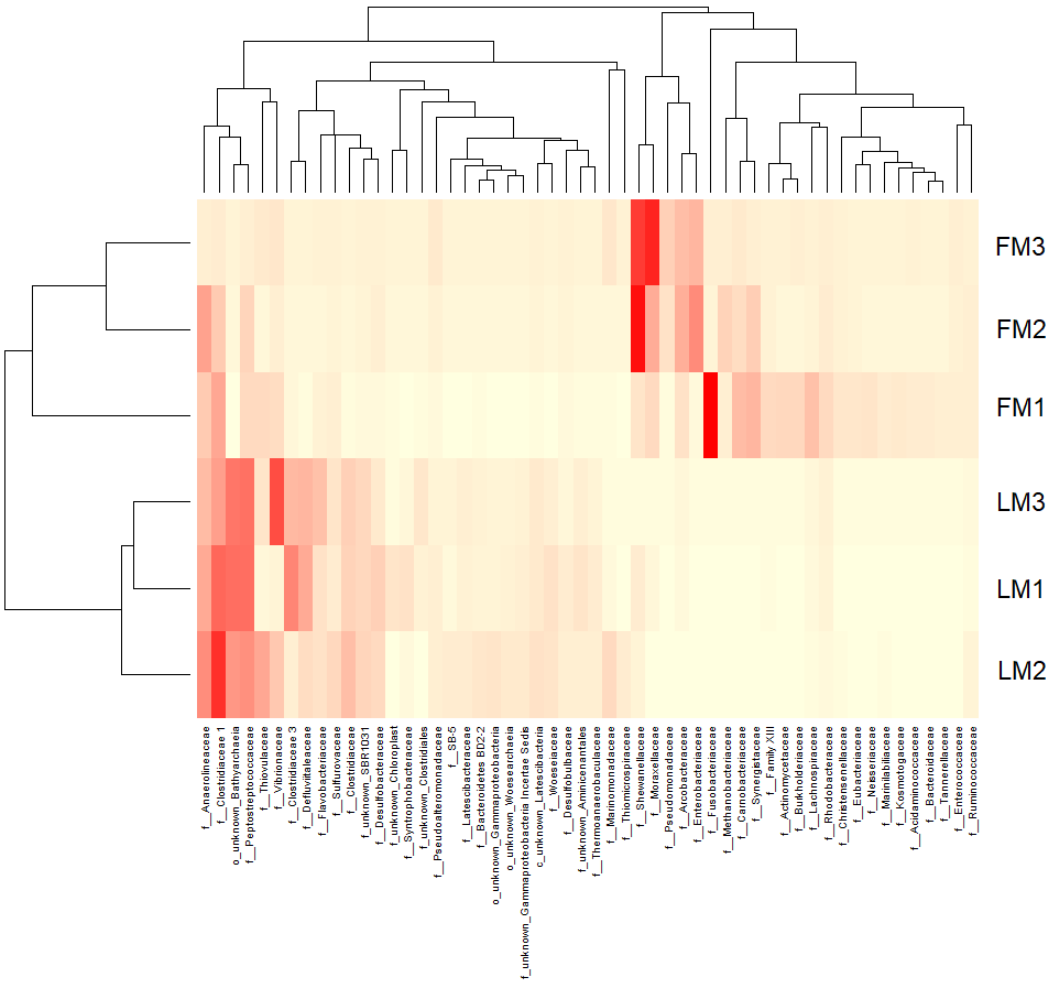

FIGURE 3.8: Heatmap and hierarchical clustering of family-level microial community composition profiles per sample at Ria Formosa Lagoon

3.4 Comparison

Finally, the comparison of all investigated sample sites was conducted based on the presented sediment samples. The results of the statistical analysis and the direct comparison of prokaryotic cell abundance will be presented.

3.4.1 Environmental Characterisation

The assessment of environmental variables in the three lagoons had to be limited to the elemental and heavy metal concentrations determined at the laboratory. A principle component analysis (3.9) revealed that heavy metal concentrations correlate the most to the samples taken in Sabaudia Lagoon, in particular correlating woth Lead and Copper, and indicates a close association. On the other hand, Arsenic, Cadmium, Iron, and Aluminium contribut strongest to the variation in data in Butrinti Lagoon. The lowest correlation was found between heavy metal concentration and sediment samples of the Ria Formosa, with the exception of LM2 and LM3.

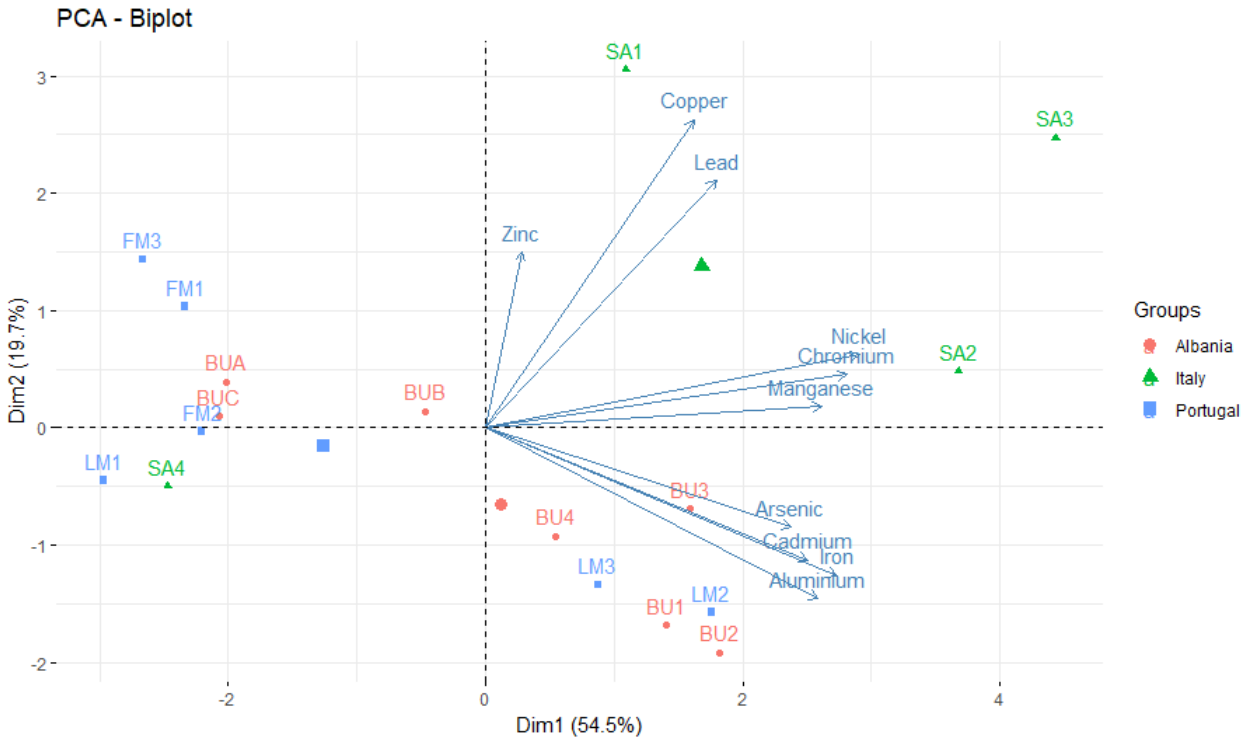

FIGURE 3.9: Principle component analysis biplot showing scores (microbial diversity in each sample analysed in the study as well as per study site) and heavy metal vectors

While these trends remain true when assessing the sampling site as a whole, variations must be considered within sampling sites. Yet, explaining the possible reasons

for these within-sampling site variations lies outside the scope of this study.

Attention shall otherwise be drawn to the notable difference in salinity of the sampled lagoons. The three lagoons are characterised by different degrees of water exchange with the adjacent sea and thus by varying salinity contents. Salinity was highest in Sabaudia Lagoon, followed by Butrinti Lagoon and finally by the Ria Formosa, which showed the strongest freshwater impact.

3.4.2 Microbial Community Composition

The performed pairwise Permanova showed highly significant differences in community composition between Ria Formosa Lagoon and Butrinti Lagoo (adjusted p-value = 0.009). Differences were even starker between Butrinti Lagoon and Sabaudia Lagoon, as indicated by the adjusted p-value of 0.003. Ria Formosa Lagoon and Sabaudia Lagoon were found to be significantly different with an adjusted p-value of 0.03. These findings are graphically represented in a non-metric multidimensional scaling plot shown in Fig. 3.10. Overlap in OTUs between the study sites is depicted in Fig. 3.11.

The correlation analysis depicted in Fig. 3.12 disclosed significant negative correlations at class level between *Alpha-* and *Gammaproteobacteria* and analysed elements, including the heavy metals manganese and nickel. Here, a deeper investigation at order level proved that negative correlations between elemental concentration and *Gammaproteobacteria* can be traced back to the order *Alteromonadales*. Contrastingly, several classes were significantly positivly correlated to heavy metal presence, in particular an unspecified class of the phylum bacteria and the classes *Parcubacteria*, *Micrarchaeia*, *Altiarchaeia*, *MD2902-B12*, and *LD1-PA32*,

of which positive correlations with at least five out of the seven investigated heavy metals were calculated.

In the scope of this study, it was not possible to further specify significant negative correlations between heavy metals and abundant taxa when analysed at a family level. Yet, as shown in Fig. 3.12, significant positive correlations were present. Namely, *Flavobacteriaceae* were found to be positively correlated to all seven heavy metals, with significant positive correlations to chromium and nickel. *Flavobacteriaceae* was furthermore significantly positively correlated to copper and lead, as was the family *Desulfobulbaceae*. Finally, the family *Sedimenticolaceae* boasted similar positive relations to the presence of Manganese.

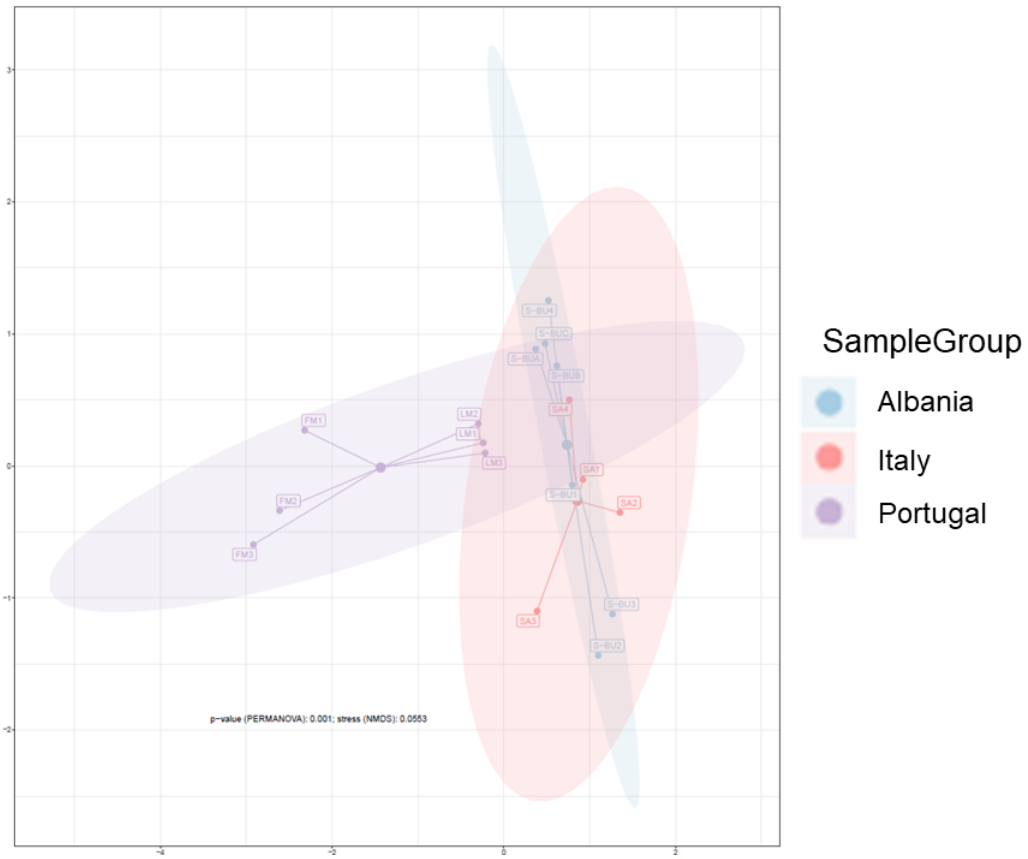

FIGURE 3.10: Bray-Curtis distance NMDS plot of microbial communities in the sediment samples

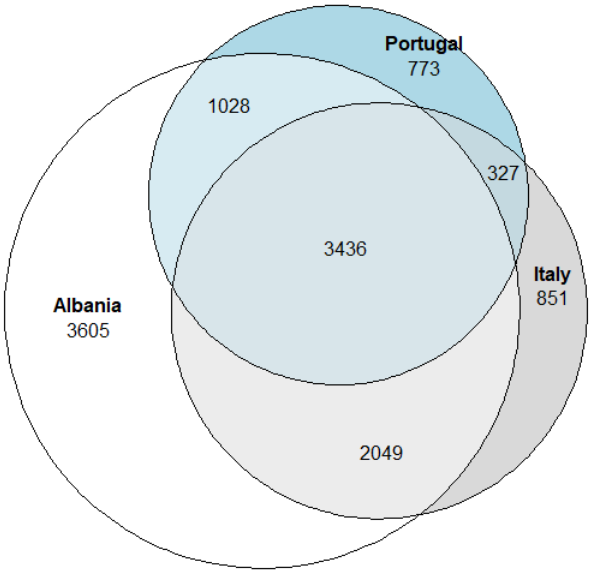

FIGURE 3.11: Euler diagram of OTU overlap between the three studied lagoons

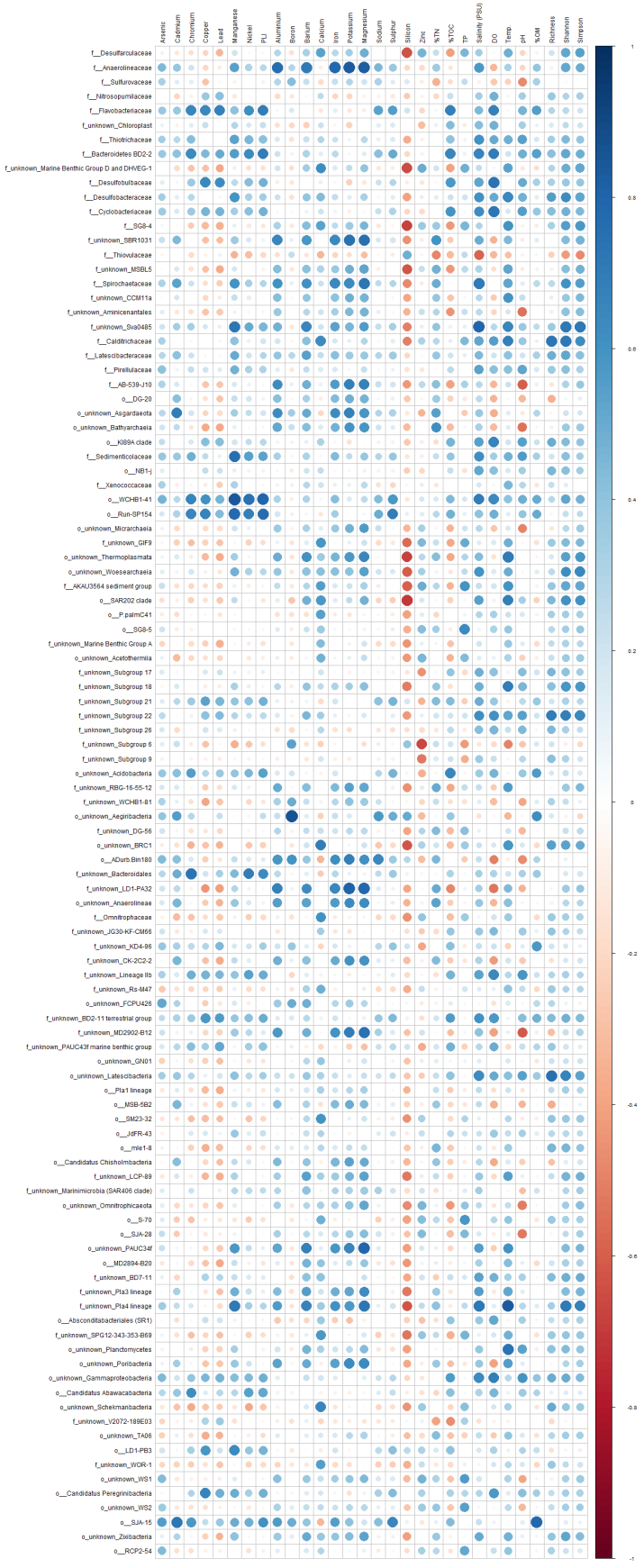

FIGURE 3.12: Pearson correlogram depicting the correlations between environmental variables and the abundance of the 30 most abundant taxa at family level. Positive correlations are displayed in blue and negative in red. Color intensity and the size of the circle are proportional to the correlation coefficients.

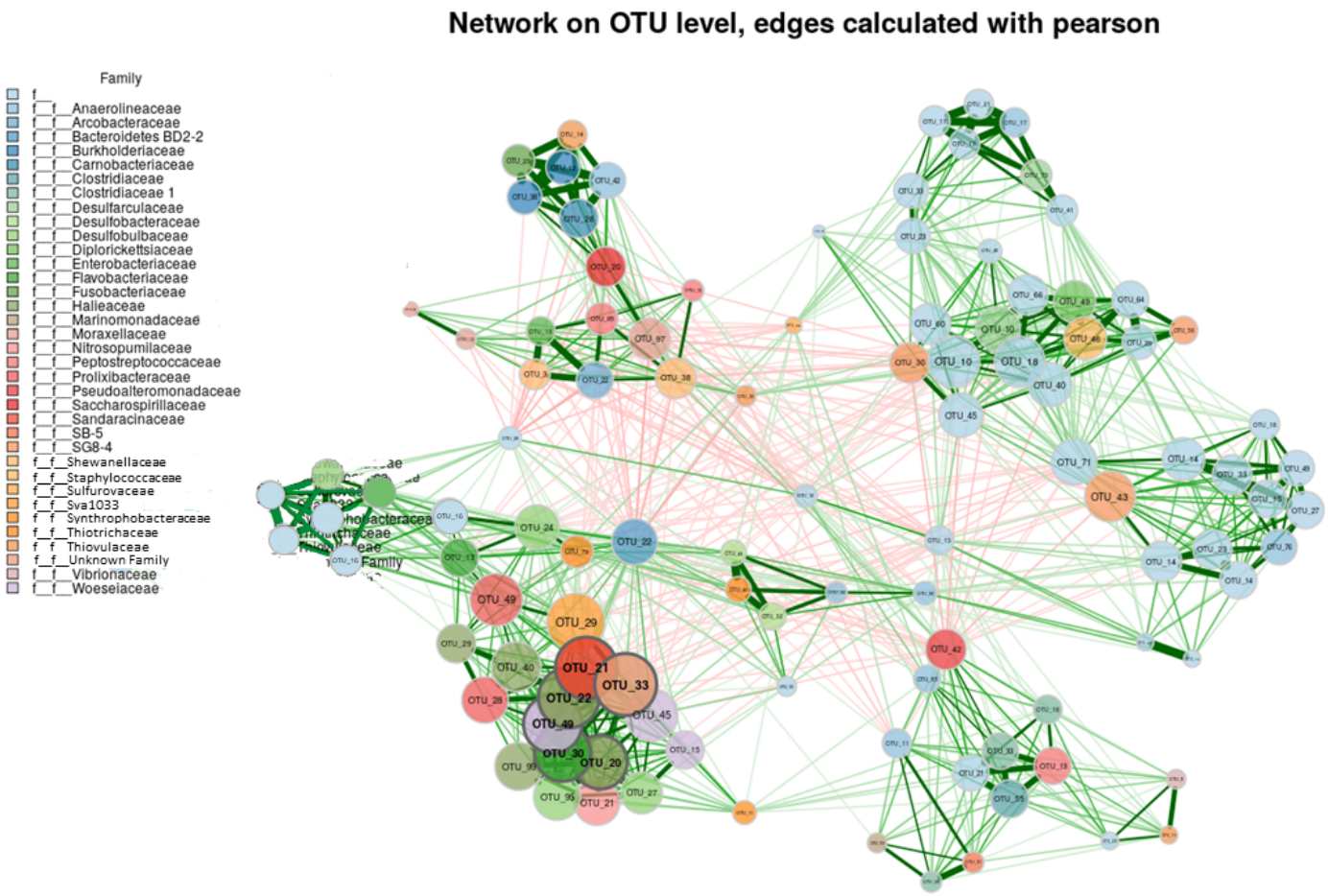

FIGURE 3.13: Microbial community network of taxa shared between all three lagoons based on pearson correlation analysis. Represented are the 120 nodes with the highest degree centrality and the 1000 edges with the highest edge weight. Node size represents the weighted degree centrality. Nodes are coloured by family. Hubs are highlighted by bold text and borders

To analyse trends within community composition, the network presented in Fig. 3.13 was examined. The network analysis revealed a single connected component, consisting of 107 nodes. Seven distinct clusters of taxa were identified, shedding light on potential functional modules in the microbial communities. Moreover, the analysis highlighted specific taxa that serve as hubs, exhibiting higher centrality measures such as degree, betweenness, closeness, and eigenvector centrality. These hub taxa are two OTUs of the genus *Haliglobus*, as well as one OTU per genera *Sandarinaceae*, *Actibacter*, and *Woeseia*, and of the order *Gammaproteobacteria Incertae Sedis*.

3.4.3 Prokaryotic Cell Abundance

Finally, the comparison of prokaryotic cell abundance revealed fairly homogenous prokaryotic cell abundaces throughout the three lagoons of around 2e+08 cells per gram of dry weight. As is depicted in Fig. 3.14 and Fig. 3.15, prokaryotic cell abundance and percentage of archaea is overall slightly higher in Sabaudia Lagoon. However, these differences are minimal when assessed on a logarithmic scale. In summary, around 85% of all prokaryotic cells, as determined via DAPI-staining, could be accounted for with CARD-FISH.

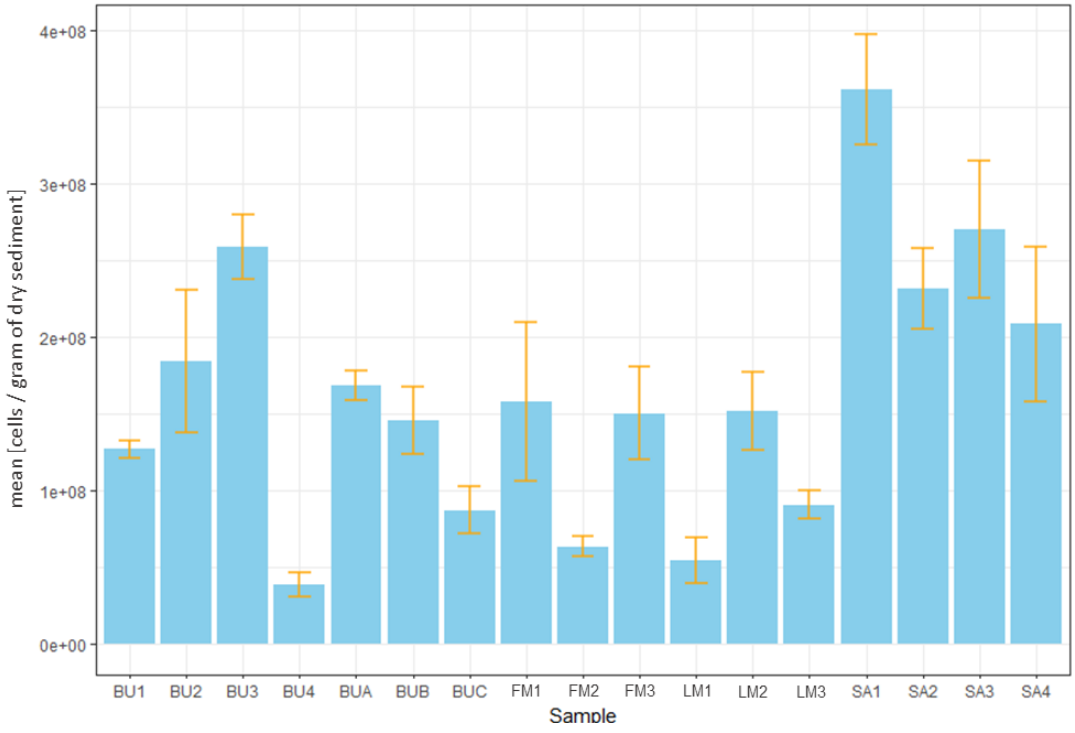

FIGURE 3.14: Mean cell count established by DAPI in the sediment samples. Data are expressed as number of cells per gram of dry weight (DW). Error bars represent the standard deviation. B-samples were taken at Butrinti Lagoon. FM and LM-samples were sampled at Ria Formosa Lagoon. S-samples were taken from Sabaudia Lagoon.

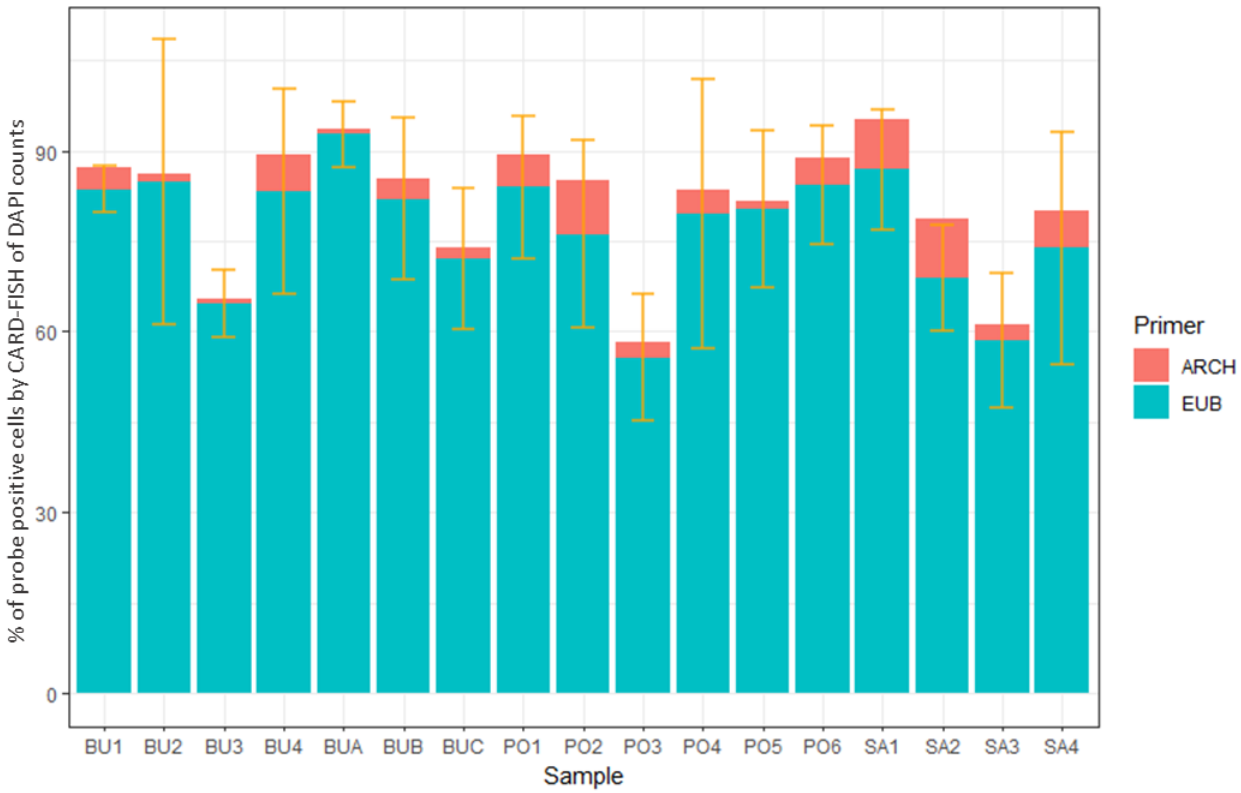

FIGURE 3.15: Bacteria and Archaea abundance estimated by CARD-FISH in the sediment samples. Data are expressed as number of cells per gram of dry weight (DW). Error bars represent the standard deviation. Arch and EUB indicate primers targeting Archaea and Bacteria cells respectively.

Chapter 4

Discussion

Through the analysis of three significantly different lagoons, deeper insights into the microbial community composition of Southern European coastal lagoons can be discussed. Based on the individual desciption of the three lagoons, presented over the course of the first sections of this chapter, an overarching analysis is finally attempted in section 4.

4.1 Butrinti Lagoon, Albania

Butrinti Lagoon has been subject of several previous studies, characterising, among others, its bathymetry, hydrology, water quality, and heavy metal content (Moisiu et al., 2016; Topi et al., 2013), giving a detailed reference for the observations made in this study. While the presented concentrations of heavy metals in lagoon sediments are within the range previously reported by Topi et al., 2012, the mean concentrations detected in this study increased notably, causing the exceedance of natural background values. Urban development and increased tourism fluxes were already pointed out as a source of heavy metal pollution in Butrinti Lagoon (Bani et al., 2013), coinciding with the elevated concentrations found at S-BUB. in light of the rapid urban development and increase in tourism around Butrinti Lagoon over the past decade (Kosova and Sinaj, 2021), changes could point towards the accumulation of heavy metals in sediments of Butrinti Lagoon, particularly in the deep anoxic sediments. Literature findings have highlighted the potential of acid-volatile sulfides in anoxic sediments to reduce the solubility and toxicity of heavy metals (Zhang et al., 2014), fostering their deposition and accumulation, and could be one possible explanation for the severe contamination of deep sediment.

Depth profiles, nutrient concentrations, and water quality parameters fully coindice with literature findings reported by Moisiu et al., 2016, indicating consistent environmental conditions in Butrinti Lagoon. This convergence of results provides robust validation and enhances the reliability of our measurements. The results thus underscore the intense stratification of the lagoon and give reason to adopt the finding of anoxic,

hypoxic, and oxic sediment sectors from Moisiu et al., 2016 for further discussion of this study's results.

Upon integration of the proposed sediment sectors, the taken samples can be categorised into samples from oxic, hypoxic, and anoxic sediment sectors to help disentangle the found community structures. Butrinti Lagoon samples BUA to BUC were all take from oxic sectors, whereas BU4 is located at the interface between the oxic and hypoxic zone. BU 1, 2 and 3, originating from greater depths, are thus the three samples taken from anoxic sediments. These conditions are clearly reflected in community composition at phylum level, where S-BU1 to S-BU3 and water samples BU2 -22 and BU3 -20 from the anoxic zone show great similarities in composition within the respective environments. However, oxygen availability appears to be surpassed as the driving factor for community composition in the remaining sediments, since samples from BUC and BU4 - both located in close proximity to the Vivari channel - differ significantly from S-BUA and S-BUB. As salinity can be ruled out, investigating potential other marine impacts on these sampling points could prove as crucial to understanding differences in community structure among these sites.

Water exchange with the adjacent sea might also serve as a mitigating factor for cyanobacterial growth in waters from BU4, as was found on the finest taxonomic level via the bubble plot. The increased abundances of *Cyanobium. PCC-6307* and *Synechococcus.CC9902* in water samples from BU1 to BU3 could indicate elevated nutrient concentrations within the lagoon (Partensky et al., 1999) that are dilluted at the channel entrance. Especially the presence of Synechococcus should be closely monitored in light of their ability to alter fish behaviour (Hamilton et al., 2014). The dominant influence of marine factors on BU4 samples is also highlighted by the increased abundance of *Desulfatiglans*, one of the most abundant lineages of *Deltaproteobacteria* predominant in marine sediments (Jochum et al., 2018), and *Bacteroidetes CV2.1 Bacc22*, a frequent inhabitant of marine environments (Leng et al., 2022). Their presence moreover indicates anoxic conditions and increased sulfur cycling at this location (Jochum et al., 2018; Leng et al., 2022). Conversely, all remaining water samples exhibited abundances of *NS5 marine group*, a genus of *Flavobacteriaceae* with an aerobic heterotrophic metabolism (Priest et al., 2022), throughout the entire water column, including the anoxic zone. A study by Priest et al., 2022 found the mechanisms for nitrogen and phosphorus metabolism to be genetically preserved in *NS5* and concluded that all members of the genus could be capable of ammonium transport and nitrogen response regulation, as well as building and hydrolysing long chain polyphosphates (Priest et al., 2022). Identifying environmental factors that facilitate the abundance of *NS5* as well as their metabolic functioning could thus have interesting implications for conservation measures as well as for insights into lagoon functioning. Equally abundant

was the closely related genus *NS4* marine group, but less knowledge is available on their function and metabolism.

The two clusters of sediment samples determined based on the finer taxonomic resolution are characterised by an increased abundance in Archaea in central lagoon samples. Since Archaea remain vastly understudied and are only recently receiving more attention (Vázquez-Campos et al., 2021), their functional role within Butrinti Lagoon remains unknown.

The coastal cluster, comprised of S-BUA, B, and C is characterised by several genera of the family *Haliaceae*, namely *Pseudohaliae* and *NOR5/OM60*, frequently referred to as a cosmopolitan (meaning ubiquitiou) branch of *Gammaproteobacteria*. With these two taxa, the sediments were found to have notable abundances of stricly aerobic as well as aerobic anoxigenic phototrophic bacteria (Spring et al., 2013; Yan et al., 2009), supporting the initially discussed sediment sectors and the proposed classification of samples. The presence of *NOR5/OM60* as aerobic anoxigenic phototrophic bacteria in lower depths coincides with the described metabolism which relies on bacteriochlorophylls to capture light energy during photosynthesis (Spring et al., 2013). Posessing the unique ability to carry out photosynthesis in the presence of oxygen without producing oxygen as a byproduct, *NOR5/OM60* are suspected to play an important role in carbon cycling (Ritchie and Johnson, 2012). During anoxygenic photosynthesis, they use light energy to assimilate carbon dioxide (CO2) and convert it into organic carbon compounds (Li et al., 2017). This process could contributes to carbon sequestration and affect the carbon balance in aquatic ecosystems (Tang et al., 2021).

A connective link between the two clusters is posed by the ubiquiously high abundance of Anaerolineaceae in all sediment samples, highlighting the important role anaerobic processes play in sediments of Butrinti Lagoon (Liang et al., 2015).

Like many other taxa, *Anaerolineaceae* were detected in both water and sediment samples, but their functional roles and community interactions could differ depending on the environment. Indications on this functional plasticity or variations in interactions with other taxa were assessed via the differential network. Indeed, *Anaerolineaceae* as well as *Desulfatiglans*, *SAR11 clade III*, *Bacteroidetes VC2.1 Bac22*, and *archaeon GW2011_AR13* all exhibited high absolute differences in associations. Indeed, all species have been reported in water and sediment samples before and comprise some of the most abundant taxa in the marine environment (see e.g. (Bolaños et al., 2022; Jochum et al., 2018; Kraiselburd et al., 2019; Lee et al., 2017; Walker et al., 2021)). These species constitute widely distributed and frequently reported taxa, hence potentially pointing towards a potential dominance of generalists with high functional plasticity (Székely et al., 2013). Yet, the results should be treated with caution as positive associations do not automatically imply real-life interactions or functional links.

The findings should be regarded as suggestions and starting points for future research to fully understand the microbial processes in coastal lagoons and the potential of their microbial communities.

Based on the presented findings, Butrinti Lagoon can be described as a stratified lagoon with ecologically highly complex microbial communities, performed processes and microbial interactions. By studying the microbial community of Butrinti Lagoon it was possible to gain insights into the hidden world of the anoxic bottom waters of the lagoon to decipher the crucial roles microbial communities play in this harsh environment.

4.2 Sabaudia Lagoon, Italy

The presented environmental variables characterising Sabaudia Lagoon coincide with the surrounding environment as well as with the available literature. High heavy metal concentrations could stem from the vulcanic origin of the region and the resulting high background values (Pippo et al., 2000). Although this should be accounted for via the calculation of the contamination factor, the methodology might not be equipped to deal with such unique conditions.

Gradients in salinity and temperature are in line with the increasing marine impact along the sampled transect. OM content, on the other hand, did not exhibit such a gradient, bearing in mind the elevated content in SA3. SA3 was sampled from the deepest point of the lagoon and is thus the only sample in this study that is exposed to permanently anoxic conditions. In combination with high amounts of OM- and nutrient-rich agricultural runoff at the location of SA3, feeding from the biggest lateral branch of the lagoon, this interplay of anthropogenic impacts and environmental factors could cause the high OM content. Former studies underlined that a large fraction of OM is only degradable under oxic conditions (Bastviken et al., 2004), supporting the role of anoxia in the observed OM content. A potential factor in OM-dynamics within Sabaudia Lagoon could also be posed by mussel farming activities. Mussel farming and particularly the activity called mussel fattening has been associated with strong nutrient enrichment, eventually causing hypoxic conditions (Pérez-Ruzafa and Marcos, 2012). Consequently, the prevailing anoxic conditions created through anthropogenic impacts in the 20^{th} century might further be excacerbated. Conversely, the high levels of DO in the surface waters of the sampling point could originate from an increased freshwater inflow at the supplying arm and indicate a profound stratification of the lagoon.

The anoxic conditions at the bottom of Sabaudia Lagoon reported in the literature are reflected in the sediment communities identified as abundant in this study. Both *Anaerolineales* and *Desulfobacterales* are frequently observed in anaerobic environments. The presence of *SBR1031*, *MSBLD9*, and *Marine Benthic Group D* and *DHVEG-1*, all families of bacteria from the marine environment, correspond to the high salinity, almost equal to that of the adjacent sea. The most abundant families moreover indicate the importance of microbial communities for lagoon functioning and nutrient cycling: *Desulfobacterales* are known for their ability to reduce sulfate to sulfide, thereby contributing to the sulfur cycle (Miletto et al., 2011), while *Anaerolineales* contribute to the degradation of complex organic matter, thus fostering nutrient cycling (Liang et al., 2015).

While community composition varied only moderately between samples, the small shifts in relative abundance of certain families across the sediment samples could still indicate potential spatial variation in community structure within the lagoon. The localised enrichment in *Micrarcheia*, *Aminicenantales*, and *Omnitrophicaeota* in SA3 is noteworthy, as little knowledge is available on either of the three taxonomic groups and their potential roles in coastal lagoon ecosystems. *Micrarcheia* was recently described as a novel class in the phylum *Micrarchaeota* based on identified protein phylogenies. Found at a radioactive legacy site in Australia, the class remains so far uncultured and undescribed, leaving its metabolism, environmental role, and characteristics in the dark (Vázquez-Campos et al., 2021). It has since been reported only once in the environment, namely in lake sediments (Han et al., 2020).

The phylum *Omnitrophicaeota* has been observed repeatedly in globally distributed water and sediment samples (Perez-Molphe-Montoya et al., 2022; Rinke et al., 2013; Seymour et al., 2022). Due to their frequent observation in oligotrophic systems, a high importance in ecosystem functioning is assumed (Perez-Molphe-Montoya et al., 2022). Encompassing two class-level and seven order-level clades with different metabolic pathways, assumptions on the role of *Omnitrophicaeota* in Sabaudia Lagoon remain pointless without further taxonomic analysis, although a role in the carbon cycle appears most likely (Seymour et al., 2022).

Finally, the class *Aminicenantales* is a member of the phylum *Aminicenantes*, known for its abundance in hydrocarbon-impacted environments and marine habitats (Farag et al., 2014). A study presented by Wang et al., 2022 testing the effect of hydrothermal pretreatment on anaerobic digestion of erythromycin fermentation dregs proposed the performance of hydrolysis and acidogenesis of *Aminicenantale*, potentially in cooperation with other hydrolysis and acidogenesis bacteria, and determined a crucial role in methane production.

Further investigating environmental factors distinguishing SA3 from the other samples could thus lead to conclusions on the habitat and methabolic pathways of these newly described taxa.

In the presented study, Sabaudia Lagoon was characterised as an anoxic coastal lagoon with high concentrations of heavy metals, thus suggesting distinct habital conditions. These are reflected in the discovered diverse microbial communities derived from the sediment samples. The key taxa identified could point towards increased rates of sulfur cycling in the surface sediments of Sabaudia Lagoon.

4.3 Ria Formosa Lagoon, Portugal

Ria Formosa was identified as a lagoon with an aplitude of anthropogenic impacts, of which two were directly investigated in this study: the impact of an international airport on the sampling region LM and the influence of wastewater effluent on the microbial community of the lagoon on the region FM.

Assessment of water quality parameters indicated a low oxygen content throughout the lagoon and a minor mixing of sea water with freshwater at FM. These findings are surprising, as the Ria Formosa has been classified as a mesotidal lagoon with large tidal exchange (Newton and Mudge, 2003) and rare hypoxic events (DO <5mg/L) (Cravo et al., 2020). But measurements are supported by the detection of several species prevailing in conditions of low oxygen availability.

The large variation in environmental variables between the two sampling regions, on the other hand, as well as the measured water temperatures appear to correspond to previous findings (Newton and Mudge, 2003). Although heavy metal contamination, nutrient content, and organic matter in sediments of Ria Formosa Lagoon have been investigated, the results were not published and can thus not be compared with this study (Brito et al., 2010; Da Moreira Silva et al., 2015). Several studies have, however, shown that airports frequently cause heavy metal contamination of adjacent soils (Özkan et al., 2017; Ray et al., 2012). Consequently, the elevated concentrations of Arsenic, Cadmium, Chromium, Copper, and Lead in LM2 and LM3 are likely to be a result of airport runoff and atmospheric depositions. Such elevated concentrations in heavy metals have previously been associated with increased abundances of Desulfobacterales, Clostridium kluyveri (Liao et al., 2019) and generally with bacterial communities involved in the sulphur cycle (Hatam et al., 2019), also found in the presented study. Heavy metal concentrations do, however, not appear to be the primary shaping factor, considering the lack of compositional variation between LM1, a supposedly

uncontaminated sample, and LM2 and LM3, which were found to be heavily contaminated.

Links between anthropogenic impacts and microbial community composition could also be observed in sampling region FM. The region was deliberately chosen to be exposed to the effluent of the local wastewater treatment plant in order to assess potential impacts and compare with literature findings. Indeed, all examined alpha-diversity indices (Richness, Shannon, and Simpson Index) were considerably lower in the FM samples than in the LM group, a finding formerly presented by Drury et al., 2013. Yet, the indicator species *Nitrospirae* and *Sphingobacteriales* identified in several studies (Drury et al., 2013; Lu and Lu, 2014) were not abundant in the analysed samples.

Between sample differences were best assessed via the presented heatmap. The increased abundance of *Fusobacteriaceae*, *Lachnospiraceae*, and *Clostridiaceae 1* in FM1 indicates a greater importance of anaerobic bacteria in this sample. Their increased abundance could be presupposed by the lower concentration of DO found in that sample, although the correlation would have to be further investigated. Yet, the higher abundance of *Shewanellaceae* and *Enterobacteriaceae*, two facultatively anaerobic bacteria families, and *Moraxellaceae*, an aerobic familiy, in FM2 and FM3 indicates impactful changes in oxygen content. It could thus be of interest for future studies to investigate microbial communities and changes in bacterial metabolism around an oxygen content of 2 mg/L.

Despite the slightly higher oxygen concentration in samples of the LM region, their microbial communities are equally dominated by anaerobic bacteria families like *Anaerolineaceae* and *Peptostreptococcaceae*. The high abundance of *Bathyarchaeia* shall further be pointed out, posing the only archaeal family found to be dominant in this study. Frequently reported from methane-rich sites (Romano et al., 2021; Qi et al., 2021), the presence of *Bathyarchaeia* could indicate an intricate web of microbial interactions with methane-producing bacteria. Both *Anaerolineaceae* and *Peptostreptococcaceae* are known to be associated with methane production in anaerobic environments, giving cause for further research on co-dependencies between these taxa.

4.4 Disentangling Microbial Community Structures

Among all coastal lagoons, many abundant phyla were in line with previously performed studies. Phyla like *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi* were consistently present in all three sampled lagoon, indicating their importance within coastal lagoon ecosystems. Yet, the three lagoons were found to be statistically significantly different systems at family level, as was determined via the pairwise Permanova. This gives the

study a sufficiently broad spectrum do disentangle overarching trends in community structure or impacts of environmental parameters on the same.

In light of their high overall productivity, prokaryotic cell abundance poses a valuable measure to assess coastal lagoon productivity on a microbial level. This study presents the first assessment of prokaryotic cell abundance in all three investigated lagoons. Cell abundances determined in this study proved to be generally lower than abundances presented for other lagoon sediments (Ferrara-Guerrero and Garza-Mouriño, 2007; Gomes and Mendonca-Hagler, 2004), with abundances being up to one order of magnitude lower than formerly published (Gomes and Mendonca-Hagler, 2004; Oliveira et al., 2012). In general, the reported abundances are closer to those of marine sediments (Aldeguer-Riquelme et al., 2022), suggesting a strong influence of marine factors on the lagoons.

The performed correlative analysis can be regarded as ecologically highly interesting, as microbial communities are increasingly proposed as potential indicators for ecosystem health (Astudillo-García et al., 2019; Ribas et al., 2023; Sims et al., 2013). Significantly positive or negative correlations could thus point towards taxa with potential suitability as ecosystem health indicators. For this purpose, the fine-scaled taxonomic resolution presented in this study could serve as crucial ground work for further investigations to strengthen or refute the potential of taxa as indicators for single environmental variables. Interestingly, the positive correlations between *Flavobacteriaceae* and Chromium and Nickel has already been reported by Senthil Kumar et al., 2023 in a study on rhizosphere microbiome community structures in serpentine geoecosystems. Thanks to a metabarcoding sequencing approach, the correlation could be followed down to the genus level, indicating the positive relationship between Nickel and Chromium and the genus *Flavobacterium*. As a matter of fact, even the negative correlation between *Flavobacteriaceae* and Calcium / Magnesium, although insignificant in this setting, is supported by the findings of the study, highlighting the robustness of the utilised methodology and presented findings.

Similarly, the identified trends between presence of *Desulfobulbaceae* and heavy metals, particularly Copper and Lead, align with already published findings (Dell'Anno et al., 2021). The class *Desulfobulbia* has previously been described as abundant in marine environments rich in hydrocarbons and/or heavy metals (Dell'Anno et al., 2021), pointing to a potential importance both quanitatively and functionally in contaminated coastal lagoons and marine environments in general. Yet, as also negative correlations between *Desulfobulbaceae* abundance and heavy metal presence have been found (Wu et al., 2019; Yi et al., 2021), further analysis is needed to assess its suitability as a potential bioindicator as well as its role in contaminated coastal lagoons.

Furthermore, the identified positive correlation of *Sedimenticolaceae* and Manganese

was so far not described in the scientific literature. But, interestingly enough, *Sedimenticolaceae*, formerly reported in marine and coastal benthic communities, were abundantly found on Arctic Fe-Mn deposits (Shulga et al., 2022). This finding could be regarded as a supporting indicator of the positive correlation found in this study. *Sedimenticolaceae* have moreover been reported to occur in Manganese-rich marine sediments, yet without assessment of a possible correlation (Vavourakis et al., 2019).

While *Proteobacteria* generally count as one of the most abundant phyla in coastal lagoon ecosystems, their resistance to heavy metal presence was previously described as low (Yin et al., 2015). This observation is supported by the results presented in this study. With further correspondance in correlations identified in this study and published results, e.g. between *SJA-15* and Cadmium (Liu et al., 2020), confidence in the presented is once again underscored.

Bearing these results in mind, the discussed taxa can be seen as of highest interest for further analyses investigating potential indicator taxa for heavy metal contamination in the coastal environment.

Insights on potentially generalisable structures in microbial communities of coastal lagoons could mainly be derived from the presented network. In this context, the identified hub taxa may play critical roles in shaping the structure and dynamics of the microbial communities in these lagoons and shall thus be further analysed.

Of the 5 identified key taxa, *Haliglobus*, *Actibacter*, *Sandaracinaceae*, and *Woeseia* have so far been cultured and described. The genus *Haliglobus* (phylum *Proteobacteria*) was isolated from seawater and classified as gram-negative, obligately aerobic, heterotrophic, and catalase negative (Park et al., 2012). Their optimal temperature range lies between 20 and 25°C but can range from 10-30°C. The genus has been identified to be positive in esterase, esterase lipase, and lipase, among others, potentially suggesting the genus' involvement in the degradation of organic matter. In the environment, the genus *Haliglobus* has so far been reported in two estuarine microbial communities (Curtis-Harper, 2017; Erazo and Bowman, 2021).

The genus *Woeseia* has repeatedly and reliably been identified in coastal lagoon sediments (Aldeguer-Riquelme et al., 2022). It has been cultivated and determined as a facultatively anaerobic chemoheterotroph with ideal growth conditions in a pH range of 7 to 8 (Du et al., 2016). Regardless, their role in coastal lagoon communities is not well researched. It shall here be pointed out that their functional role in aquatic ecosystems appears diverse, considering their recent identification in coral colonies (Krishnaswamy et al., 2023), potentially indicating a tendency for interactions with other species. Potential hypotheses for further studies could, however, be derived from their ability to produce catalase enzymes, thus suggesting a role in OM composition,

redox balancing, or oxidative stress protection.

Actibacter, a genus of the phylum *Bacteroidetes*, has previously been reported in aquatic environments, particularly sediments, (Bourhane et al., 2022; Zhang et al., 2022), but remains without mention in coastal lagoons. The genus has been isolated from tidal flat sediments and found to be gram-negative, aerobic, chemoheterotrophic, and mesophilic. They were furthermore classified as oxidase and catalase positive (Kim et al., 2008). Analyses of the genus of *Actibacter* in coastal lagoons and their potential function have not been performed. The oxidase and catalase positivity could hint towards an important role in the oxygen balance of coastal lagoons, as the combination provides the bacterium with an interesting metabolic niche, allowing it to occupy specific habitats within the lagoon where oxygen availability and oxidative stress conditions may vary. This versatility could also allow the genus to thrive under diverse environmental conditions.

Similarly, the family *Sandaracinaceae* (phylum *Proteobacteria*) encompasses gram-negative, strictly aerobic, mesophilic, and chemoheterotrophic bacteria (Mohr et al., 2012). Isolated from a soil sample, they have been found to be oxidase- and catalase-positive. Up to this point, the family *Sandarinaceae* was, to the best of the authors knowledge, never mentioned in the aquatic environment, including coastal lagoons. Thus, a substantial need for further research on this family, its functional role and occurence patterns is present.

Knowledge gaps are also highlighted by the identification of *Gammaproteobacteria Incertae Sedis*. *Incertae Sedis*, generally used to indicate the insecure placement of a taxon within the biological systematics, holding a spot as hub in the calculated network, indicates the importance of *Gammaproteobacteria* within microbial communities of coastal lagoons, while stretching the lack of knowledge in current microbial ecology even on vastly abundant and described taxa. Due to the diversity, various ecological roles, and presence in a wide range of environments, further statements about the potential function of *Gammaproteobacteria* in coastal lagoons are not sensible, despite the profound knowledge available on this class.

With these taxa structuring the microbial communities of the analysed coastal lagoons, the network indicated a highly interconnected community. The presence of shared key taxa and potential interactions across the lagoons hints at the possibility of general patterns in microbial community composition across European coastal lagoons. However, further research is warranted to confirm and expand upon the findings, considering the complex and dynamic nature of these ecosystems.

It is further noteworthy that the network analysis identified seven distinct clusters comprised of six to 29 taxa with mostly negative associations between and strongly

positive associations within clusters, possibly indicating a division of the microbial community in coastal lagoons after up to here unknown factors. Such factors could be ecological niches, functional groups, environmental gradients, or habitat segregation. It shall also be mentioned that clusters associated families and OTUs with diverse metabolisms and characteristics with each other. OTUs of the families *Anaerolineaceae* and *Clostridiaceae*, commonly known as anaerobic bacteria are clustered together with members of the families *Vibrionaceae* and *Pseudoalteromonadaceae* that are known for their ecological significance in marine environments. With *Thiovulaceae* and *Nitrosopumilaceae* families also present in the cluster, it covers families associated with nitrogen and sulfur cycling in aerobic and anaerobic environments. Further investigating potential links between these families could be of interest to deepen the understanding of delicate interactions between different environments and nutrient cycles in coastal lagoons and for understanding clusterings within microbial communities.

Due to the dominance of OTUs in the network that remained unassigned at family level, it is difficult to derive further conclusions from proposed clusters. Again, research needs are highlighted to improve taxonomic databases and further identify and characterise unknown members of microbial communities. Constructing additional networks in future studies will, however, be crucial to analyse the potential implications and meanings that computed clusters could have.

Independently of the overarching trends in community composition presented in this study, some trends might also be easier to assess through greater similarity between the studied systems. One example lies in the identification of an increased abundance of bacteria related to the sulphur cycle in both Sabaudia Lagoon and Butrinti Lagoon, the two restricted lagoons investigated in this study. This phenomenon might be limited to the special characteristics of this lagoon type and could thus not be identified in Ria Formosa Lagoon, the only leaky lagoon investigated in this study. Increasing the number of sampling sites and a greater coverage of leaky lagoons in future studies would be crucial to verifying this observation. Regardless, this hypothesis simply highlights how little knowledge is currently available on overarching trends in microbial community composition of coastal lagoons and how much further research is still needed.

Chapter 5

Conclusion

In the presented study, the microbial community structures of three previously unexplored Southern European Coastal Lagoons - Butrinti Lagoon, Sabaudia Lagoon, and Ria Formosa Lagoon - were investigated, thereby unravelling their environmental characteristics and microbial diversity. Each lagoon's unique microbial community was characterised, providing valuable insights into the ecological distinctiveness and biodiversity of these pristine environments. The presence of key taxa was examined in each lagoon, enabling the identification of potential ecosystem health indicators and functioning, as well as their ecological roles within these complex ecosystems.

Building on the individual lagoon analyses, a comparative analysis showed possible similarities and differences among sedimental microbial communities. The performed correlation analysis identified taxa like *Flavobacteriaceae* and *Desulfobulbaceae* as taxa of potential interest for ecosystem health analyses within coastal lagoons, thereby providing the groundwork for future studies and contributing to the growing knowledge on microbial communities as indicators of ecosystem health. Insights into trends in community composition were derived from a co-occurence network, enabling the identification of core taxa like *Haliglobus*, *Woeseia*, *Actibacter*, *Sandaracinaceae* and an unknown species of *Gammaproteobacteria* present across all three lagoons, thus signifying their potential importance as fundamental players in the ecological dynamics of European coastal lagoons. The identification of these key taxa opens the door to future research, where their roles and functions can be further explored, adding depth to our understanding of microbial community dynamics in these unique ecosystems.

The performed analysis and comparison of microbial community composition across three European Coastal lagoons poses, to the best of the author's knowledge, the first comprehensive analysis presenting an attempt to identify potential generalisable trends in the microbial community composition of European Coastal Lagoon ecosystems and the factors shaping them. By exploring how microbial diversity responds to stressors, insights could be gained into the broader implications for ecosystem health,

nutrient cycling, and water quality. The findings emphasize the critical role of microbial communities as indicators of environmental changes, reflecting the ecohydrological balance within these complex systems. However, it shall be emphasised that these findings are preliminary and serve as a foundational exploration of microbial community structures in Southern European Coastal Lagoons. In light of the inherent complexity of coastal lagoon ecosystems, future studies will be needed to build upon this work, validate observations and increase our knowledge on relationships between microbial communities, anthropogenic impacts, and ecohydrological dynamics in order to enable more informed protection and management strategies.

Overall, this study contributes novel insights into the microbial diversity of unexplored coastal lagoon habitats, expanding our understanding of the intricate relationships between microbial communities and their surrounding environments. By laying the groundwork for future investigations, we hope to inspire and support further research endeavors that will unlock the hidden secrets of these captivating ecosystems, ultimately advancing our knowledge of microbial ecology and enhancing conservation efforts in coastal lagoon environments.

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Appendix A

Qiagen DNeasy ®PowerSoil ®Kit

Quick Start Protocol

Notes before starting

- Perform all centrifugation steps at room temperature (15-25°C)
- Of Solution C1 has precipitated, heat at 60°C until precipitate dissolves
- 2 ml collection tubes are provided

Procedure:

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
2. Add 60 μl of Solution C1 and invert several times or vortex briefly.
   Note: Solution C1 may be added to the PowerBead tube before adding soil sample.
3. Secure PowerBead Tubes horizontally using a Vortex Adapter tube holder (cat. no. 13000-V1-24).
4. Vortex at maximum speed for 10 min.
   Note: If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 min.
5. Centrifuge tubes at 10,000 x g for 30 s.
   Note: Expect between 400-500 μl of supernatant. Supernatant may still contain some soil particles.
6. Add 250 μl of Solution C2 and vortex for 5 s. Incubate at 4°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the DNeasy PowerSoil extraction with this incubation we recommend you retain the step.
7. Centrifuge the tubes for 1 min at 10,00 x g.

8. Avoiding the pellet, transfer up to 600 μl of supernatant to a clean 2 ml collection tube.
9. Add 200 μl of Solution C3 and vortex briefly. Incubate at 4°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with this incubation we recommend you retain the step.
10. Centrifuge the tubes for 1 min at 10,000 x *g*.
11. Avoiding the pellet, transfer up to 750 μl of supernatant to a clean 2 ml collection tube.
12. Shake to mix Solution C4 and add 1200 μl to the supernatant. Vortex for 5 s.
13. Load 675 μl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow through.
14. Repeat step 14 twice, until all of the sample has been processed.
15. Add 500 μl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
16. Discard the flow through. Centrifuge again for 1 min at 10,000 x *g*.
17. Carefully place the MB SPin Column into a clean 2 ml collection tube. Avoide splashing any Solution C5 onto the column.
18. Add 100 μl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-Free PCR Grade Water for this step (cat. no 17000-10).
19. Centrifuge at room temperature for 30 s at 10,000 x *g*. Discard the MB Spin Column. The DNA is now ready for downstream applications.
    Note: Solution C6 is 10 mM Tris-HCL, pH 8.5. We recommend storing DNA frozen (-20°C to -80°C) as solution C6 does not contain EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

Appendix B

Results Flow Cytometry

TABLE B.1: Flow cytometry cell counts of Butrinti and Ria Formosa Lagoon. Shown are total cell count (TCC), percentage of high nucleic acid cells (HNA cells), and percentage of low nucleic acid cells (LNA cells).

	TCC	**HNA cells**	**LNA cells**
Unit	cells/ml	% of TCC	% of TCC
BU1	1.34E+07	1.1	99.0
BU1	5.50E+06	0.4	99.6
BU3	5.29E+06	0.3	99.7
BU4	9.20E+06	8.7	91.3
BUA	1.69E+07	1.7	98.3
BUB	4.36E+07	1.1	98.9
BUC	9.28E+06	25.6	74.5
FM1	3.86E+07	61.0	39.0
FM2	1.16E+07	63.6	36.4
FM3	1.55E+07	80.1	19.9
LM1	4.29E+06	1.9	98.1
LM2	4.28E+07	26.6	73.4
LM3	3.14E+07	31.7	68.3

Appendix C

Water Quality Parameters

TABLE C.1: Water quality parameters measured at the investigated sampling locations

SampleID	Salinity	DO	Temp.	pH
Unit	PSU	mg/L	°C	
S-BU1	34.73	0.25	22.7	8.5
S-BU2	34.47	0.17	17.1	7
S-BU3	34.47	0.18	17.1	6.5
S-BU4	32.66	5.99	22.2	8.5
S-BU ST A	25.59	7.75	19.8	8.2
S-BU ST B	19.45	8.95	20.1	7.7
S-BU ST C	26.65	7.58	20	8.2
BU2 -0	24.25	8.29	19.6	8.5
BU2 -22	34.47	0.17	17.1	7
BU3 -0	24.83	8.32	19.7	8.5
BU3 -20	34.47	0.18	17.1	6.5
BU4 -0	24.03	8.19	19.9	8.5
BU4 -4	32.66	5.99	22.2	8.5
BU ST A	25.49	7.92	19.9	8.2
BU ST B	20.28	7.5	18.6	7.7
BU ST C	26.65	7.58	20	8.2
SA1	33.7	10.67	18.5	8.4
SA2	35.1	10.83	18.3	8.4
SA3	35.8	11.39	17	8.35
SA4	36.5	9.81	16.7	8.22
FM1	0.91	1.73	16.45	7.93
FM2	0.95	1.86	15.18	7.88
FM3	2.75	2.24	15.16	7.59
LM1	16.6	2.5	14.85	7.4
LM2	16.6	2.5	14.85	7.4
LM3	16.6	2.5	14.85	7.4

Appendix D

Elemental Composition of Sediment Samples

TABLE D.1: Results of the CHN and LOI methods. Total Nitrogen (TN), Total Organic Carbon (OC), and Organic Matter (OM) are expressed in percent of gramm of dry sediment

Sample	%TN	%OC	%OM
S-BU1	1	1.8	11.91
S-BU2	1	1.9	11.94
S-BU3	0.9	1.8	13.83
S-BU4	0.9	2.3	6.71
S-BU ST A	0.9	2.4	5.78
S-BU ST B	1	1.9	10.52
S-BU ST C	0.9	1.9	5.2
SA1	0.9	2.5	13.67
SA2	0.9	3.2	19.75
SA3	1	3.4	25.85
SA4	0.9	3.1	8.34
FM1	0.8	1	9.65
FM2	0.9	2.3	1.04
FM3	0.9	2.8	0.56
LM1	1	1.9	1.31
LM2	0.9	2.9	27.83
LM3	1	3	16.9

TABLE D.2: Elemental composition of sediment samples as determined by metal digestion and ICP-OES analysis. All values are given in mg/Kg

SampleID	Al	As	B	Ba	Ca	Cd	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	Pb	S	Si	Zn
S-BU1	39571.09	93	95.13	111.82	57094.46	196	316	13	34270.69	14178.91	21483.64	153	18134.18	148	612.48	78	34506.01	1275.03	143.08
S-BU2	40116.55	117	93.24	104.90	55184.82	281	333	144	35845.82	14144.19	21858.14	152	21403.60	166	569.43	1	43586.41	1801.53	162.35
S-BU3	40181.12	166	108.01	104.69	64416.75	27	352	178	36193.09	14774.01	24675.97	151	37778.33	171	845.80	95	45569.96	2442.67	257.66
S-BU4	24282.88	223	56.31	107.65	204711.83	52	225	128	22369.99	8116.93	18357.07	191	7880.09	98	730.37	7	7328.59	2729.38	220.84
S-BU ST A	9274.33	0	39.85	94.65	311979.41	0	73	119	7741.61	3268.02	9340.75	87	8327.80	29	966.46	56	13092.00	1464.63	254.23
S-BU ST B	24339.65	206	84.83	73.19	225919.83	17	116	268	15199.60	5439.12	9577.51	41	10014.97	46	1417.17	131	33893.88	1621.76	293.70
S-BU ST C	8007.05	36	31.90	40.30	339754.87	0	75	115	7860.98	2577.23	9565.14	69	4435.86	35	779.05	5	5285.43	1113.16	242.86
SA1	16813.06	119	53.03	94.46	84426.58	48	343	1052	12252.24	3776.93	6920.78	75	15884.99	165	773.95	434	29744.78	2422.94	239.20
SA2	26683.91	190	78.44	66.76	18978.64	299	912	469	22333.11	5035.05	8150.87	165	26887.52	307	717.62	271	58501.34	2595.13	154.02
SA3	25921.95	157	93.33	56.56	18552.04	189	782	1262	30856.90	5356.33	9541.86	296	48195.70	295	828.62	329	109544.68	5008.48	229.45
SA4	3643.38	73	45.33	23.51	325095.70	0	57	83	3109.47	1131.63	9971.46	15	13678.64	35	636.33	39	12978.51	1359.97	51.41
FM1	4241.50	0	35.71	44.22	22690.48	0	76	312	3158.16	850.34	2059.52	7	6437.07	17	1244.90	171	9537.41	4862.24	184.00
FM2	1711.71	7	6.44	8.04	29673.42	0	333	98	2331.08	287.97	707.85	11	653.15	74	310.49	34	1266.09	5048.26	74.91
FM3	852.71	0	5.06	8.43	21407.15	0	10	376	776.88	188.74	569.60	3	288.17	20	299.97	78	1012.81	4260.20	322.21
LM1	3453.37	0	16.59	13.28	10079.65	0	40	49	2484.23	964.16	930.97	4	2102.56	7	144.37	39	3347.16	5240.62	48.50
LM2	39114.93	251	554.82	107.33	20721.60	300	349	168	28517.17	11396.96	11420.08	58	27440.55	75	713.34	210	62138.38	4029.06	117.84
LM3	25736.36	210	553.91	60.82	16623.93	240	393	144	22771.20	8379.36	12102.24	45	50775.81	92	506.25	135	59386.92	6443.13	90.60

Appendix E

Heavy Metal Concentration Factor

TABLE E.1: Contamination factor of heavy metals in sediment samples

Sample	Arsenic	Cadmium	Chromium	Copper	Lead	Manganese	Nickel	PLI
S-BU1	0.93	1.96	3.16	1.3	0.78	1.53	1.48	1.54
S-BU2	1.17	2.81	3.33	1.44	1.0	1.52	1.66	1.85
S-BU3	1.66	2.70	3.52	1.78	0.95	1.51	1.71	2.02
S-BU4	2.23	0.52	2.25	1.28	0.70	1.91	0.98	1.28
S-BU ST A	0.0	0.0	0.73	1.19	0.56	0.87	0.29	0.70
S-BU ST B	2.06	1.70	1.16	2.68	1.31	0.41	0.46	1.18
S-BU ST C	0.36	0.0	0.75	1.15	0.5	0.69	0.35	0.69
SA1	1.19	0.48	3.43	10.52	4.34	0.75	1.65	2.19
SA2	1.9	2.99	9.12	4.69	2.71	1.65	3.07	3.86
SA3	1.57	1.89	7.82	12.62	3.29	2.96	2.95	4.51
SA4	0.73	0.0	0.57	0.83	0.39	1.5	0.35	0.64
FM1	0	0	0.76	3.12	1.71	0.07	0.17	0.60
FM2	0.07	0	3.33	0.98	0.34	0.11	0.74	0.43
FM3	0	0	0.1	3.76	0.78	0.03	0.20	0.34
LM1	0	0	0.4	0.49	0.39	0.04	0.07	0.25
LM2	2.51	3	3.49	1.68	2.1	0.58	0.75	1.85
LM3	2.1	2.4	3.93	1.44	1.35	0.45	0.92	1.59

Appendix F

Appendix F

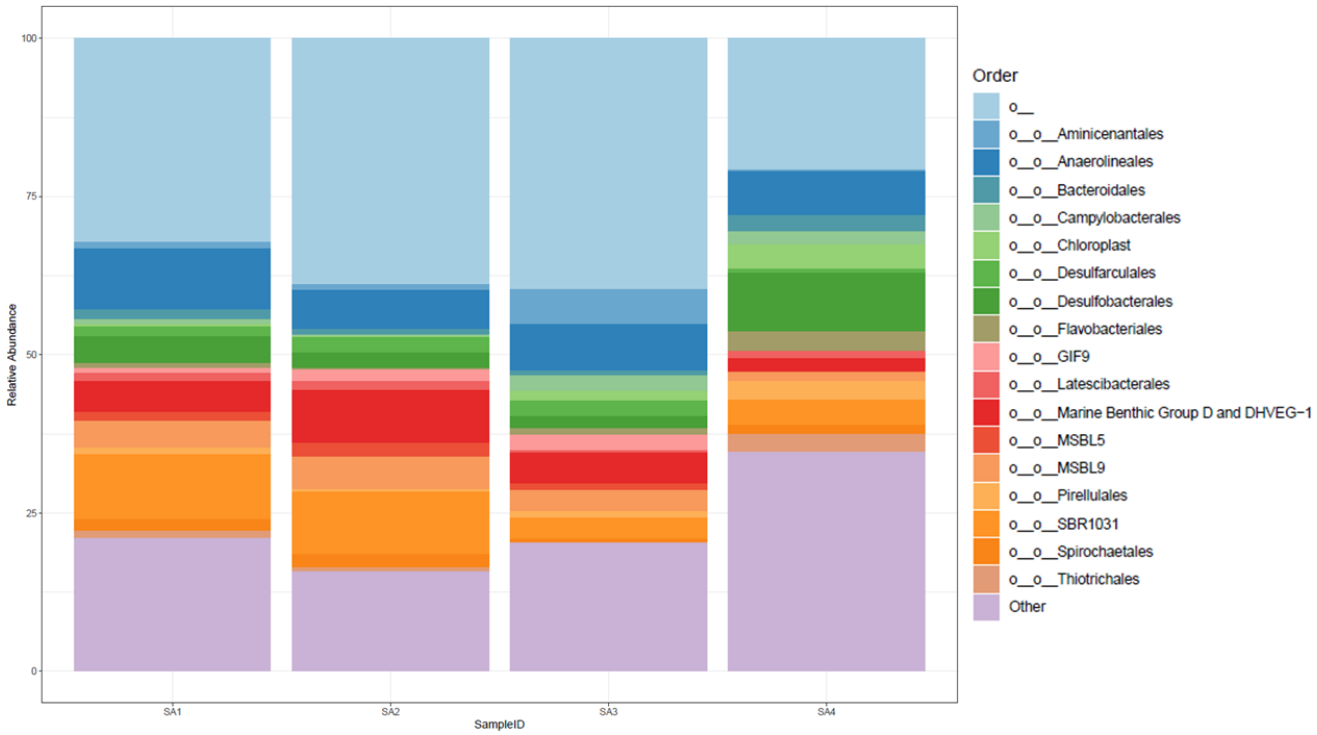

FIGURE F.1: Taxonomy bar plot of Sabaudia Lagoon samples at order level

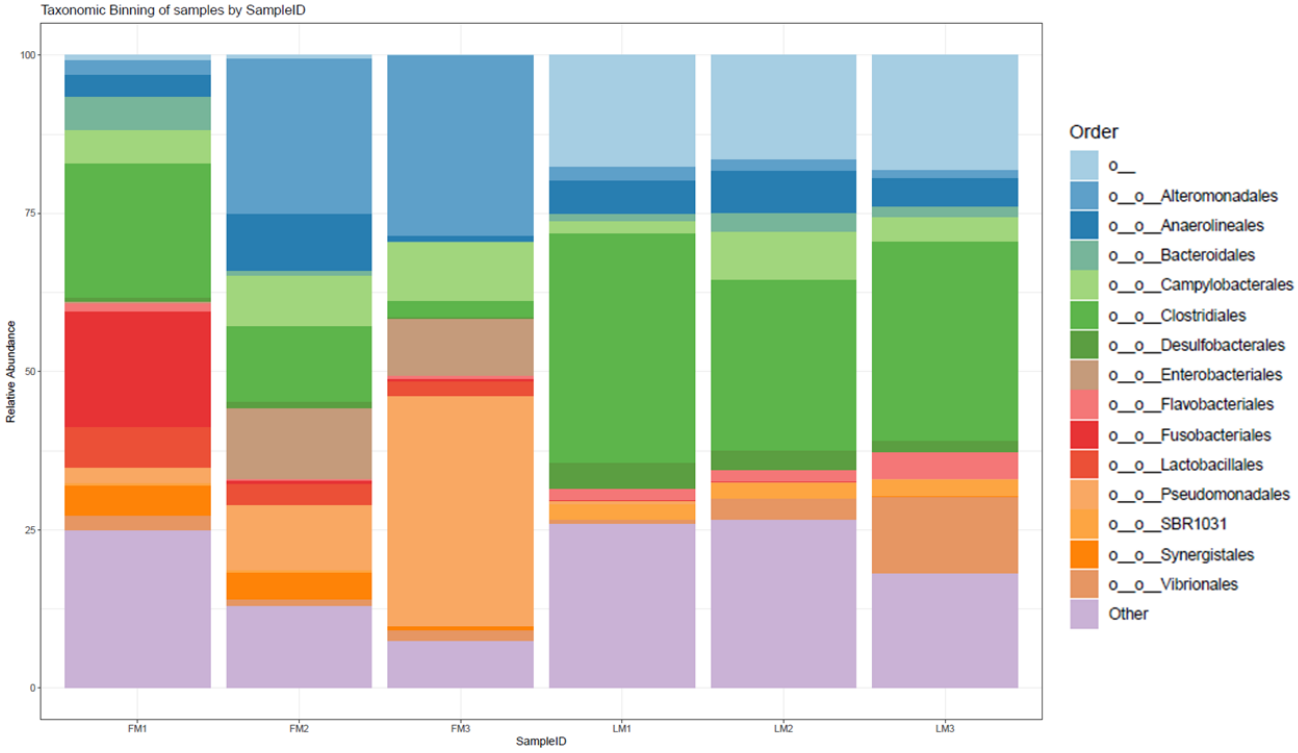

FIGURE F.2: Taxonomy bar plot of Ria Formosa Lagoon samples at order level