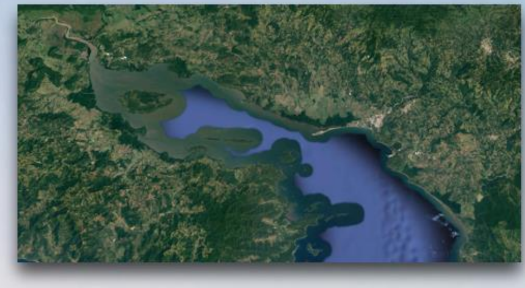
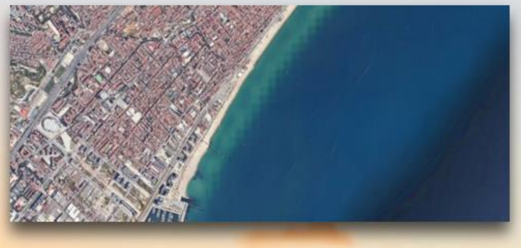
Patterns of connectivity in marine microbial communities





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Ecology of Marine Microbes

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COSTA RICA

Patterns of connectivity in marine microbial communities

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Abril 2023

Tesi presentada per a obtenir el títol de Doctora per la Universitat Politècnica de Catalunya
Programa de doctorat en Ciències del Mar

A Samuel

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General Introduction

Chapters

Summary

Marine microorganisms are fundamental for life on Earth. For example, they have produced most of the oxygen existing in our planet and are essential in biogeochemical cycles and energy flow to different trophic levels. One of the critical aspects of microbial ecology is the distribution of microorganisms at spatial and temporal scales since it allows us to understand the effect of environmental factors on the structure of microbial communities. In this thesis, the aim was to determine the structure of microbial communities at three spatial scales: In the first chapter, a study was conducted on the spatiotemporal dynamics of bacterioplankton communities in a tropical estuary strongly impacted by the discharge of the Tempisque River during the rainy season. The results show high spatial heterogeneity in the communities, and represent the first report on the spatial and temporal patterns in which the bacterioplankton of the Gulf of Nicoya is structured. The second chapter determined the effect of environmental gradients on the horizontal distribution of bacterioplankton in the northwestern Mediterranean. The samples were taken along cross-shore transects so that the gradients would presumably be due to the change in environmental parameters along the transect and a dilution of the anthropogenic and terrestrial effect. Community structure was observed to vary depending on the sites, but a large fraction of the microorganisms were shared in all sampled areas, reflecting a relatively high connectivity of the communities. The third chapter presents the vertical distribution of prokaryotic and eukaryotic microorganisms within the Deep Chlorophyll Maxima (DCM) in the NW Mediterranean sea, as determined by high-resolution sampling and separating two microbial size fractions (0.2-3 and 2-20 µm). The results show that the DCM acts as a transition zone (ecotone), but despite the drastic changes observed in the relative abundance of taxa in this layer both prokaryotic and eukaryotic communities below the DCM were dominated (~70% of the sequences) by taxa already detected at the ocean surface.

Resumen

Los microorganismos marinos son fundamentales para la vida en la Tierra. Por ejemplo, han producido la mayor parte del oxígeno de nuestro planeta y son esenciales en los ciclos biogeoquímicos y en el flujo de energía a los diferentes niveles tróficos. Uno de los aspectos críticos en la ecología microbiana es la distribución de los microorganismos a distintas escalas espaciales y temporales, ya que permite comprender el efecto de los factores ambientales sobre la estructura de las comunidades microbianas. El objetivo de esta tesis fue determinar la estructura de las comunidades microbianas en tres escalas espaciales: En el primer capítulo, se realizó un estudio sobre la dinámica espaciotemporal de las comunidades de bacterioplancton en un estuario tropical fuertemente impactado por la descarga del río Tempisque durante la temporada de lluvias. Los resultados muestran una alta heterogeneidad espacial en las comunidades, y representan el primer estudio sobre los patrones espaciales y temporales en los que se estructura el bacterioplancton del Golfo de Nicoya. En el segundo capítulo determinamos el efecto de varios gradientes ambientales sobre la distribución horizontal del bacterioplancton en el Mediterráneo noroccidental. Las muestras se tomaron desde la costa hasta mar abierto por lo que los gradientes son resultado del cambio de condiciones ambientales a lo largo del transecto y a una dilución del efecto antropogénico y terrestre. Se observó que la estructura de la comunidad variaba según los áreas de muestreo, pero se encontró que la gran mayoría de los microorganismos aparecían en todas las áreas muestreadas, lo que refleja una conectividad relativamente alta de las comunidades. El tercer capítulo presenta la distribución vertical de los microorganismos procariotas y eucariotas a través del máximo profundo de clorofila (DCM) en el NW del mar Mediterráneo, mediante un muestreo de alta resolución y separando dos fracciones de tamaño (0.2-3 y 2-20 µm). Los resultados muestran que el DCM actúa como una zona de transición (ecotono), pero que a pesar de los cambios drásticos en la abundancia relativa de los microorganismos en esta capa, el 70% de las comunidades de procariotas y eucariotas debajo del DCM están constituidas por taxones ya detectados en la superficie.

Resum

Els microorganismes marins són fonamentals per a la vida a la Terra. Per exemple, han produït la major part de l'oxigen del nostre planeta i són essencials en els cicles biogeoquímics i en el flux d'energia cap als diferents nivells tròfics. Un dels aspectes crítics en l'ecologia microbiana és la distribució dels microorganismes en escales espacials i temporals, ja que permet comprendre l'efecte dels factors ambientals sobre l'estructura de les comunitats microbianes. L'objectiu d'aquesta tesi ha estat determinar l'estructura de les comunitats microbianes en tres escales espacials. Al primer capítol, es va realitzar un estudi sobre la dinàmica espaciotemporal de les comunitats de bacterioplancton en un estuari tropical fortament impactat per la descàrrega del riu Tempisque durant la temporada de pluges. Els resultats mostren una alta heterogeneïtat espacial a les comunitats, i representen el primer estudi sobre els patrons espacials i temporals en què s'estructura el bacterioplàncton del Golf de Nicoya. Al segon capítol determinem l'efecte de diversos gradients ambientals sobre la distribució horitzontal del bacterioplàncton a la Mediterrània nord-occidental. Les mostres es van prendre des de la costa fins a mar obert, per la qual cosa els gradients es deuen al canvi de paràmetres ambientals al llarg del transecte i a una dilució de l'efecte antròpic i terrestre. Es va observar que l'estructura de la comunitat variava segons els llocs, però es van trobar els mateixos microorganismes a totes les àrees mostrejades, cosa que reflecteix una connectivitat relativament alta de les comunitats. El tercer capítol presenta la distribució vertical dels microorganismes procariòtics i eucariòtics a través del màxim profund de clorofil·la (DCM) al mar Mediterrani Nordoccidental, mitjançant un mostreig d'alta resolució i separant dues fraccions de mida (0.2-3 i 2-20 µm). Els resultats mostren que el DCM actua com una zona de transició (ecoton), però que a pesar dels canvis dràstics en l'abundància relativa dels microorganismes en aquesta capa, el 70% de les comunitats de procariotes i eucariotes sota el DCM estan constituïdes per taxons detectats a la superfície.

General Introduction

Three-quarters of the planet is covered by water, with the oceans representing 97% of total water on Earth. It is therefore the largest ecosystem in our planet, encompassing diverse ecosystems, from the poles to the great coral reefs, submarine trenches, and coasts worldwide. In addition, half of the primary production on the planet occurs in the ocean (Field et al., 1998), and 90% is done by microorganisms (Duarte and Cebrián, 1996). Moreover, the ca. 10^{29} microbial cells in the oceans (Whitman et al., 1998) account for the largest share of the total respiration (Del Giorgio and Duarte, 2002; Robinson, 2008).

The importance of microbes became evident after the postulation of the microbial loop concept (Pomeroy, 1974), which unveiled that prokaryotes play a central role in the remineralization of the large oceanic dissolved organic carbon (DOC) pool and also transfer a large proportion of this carbon to higher trophic levels, mainly by predation by small sized eukaryotic plankton. Besides the carbon pool, microorganisms are responsible for the transformation of nutrients and other elements throughout the water column. Despite the irrefutable relevance of microbial species in the oceans, their population dynamics and biogeography, biogeochemical impact, metabolic complexity and synergistic interactions remain still largely unknown.

Over the years, marine microbiology has consolidated as a discipline on its own. It has advanced greatly thanks to the development of molecular techniques (Fig. 1), allowing a better understanding of the diversity, distribution, ecology, physiology, and evolution of marine microorganisms (Brochier-Armanet et al., 2011; del Giorgio and Bouvier, 2002; Fuhrman et al., 2002; Galand et al., 2009; Massana and Pedrós-Alió, 2008; Pedrós-Alió, 2006; Salazar and Sunagawa, 2017). The advent of high-throughput sequencing techniques has also unveiled a high diversity of very low abundance microorganisms called the *rare* biosphere (Pedrós-Alió, 2011; Sogin et al., 2006). These microorganisms are part of the seed bank (Gibbons et al., 2013; Lennon and Jones, 2011), which is defined as 'a reservoir of dormant individuals that can potentially resuscitate in the future under different environmental conditions'. The seed bank could affect the composition of the communities and could help explain biogeography patterns

(Lennon and Jones, 2011). This is especially important since understanding distribution patterns will allow us to understand the structure and function of marine ecosystems. The seed bank may play a relevant role in the connectivity of marine ecosystems, i.e. the presence of organisms across ecosystem boundaries, organisms that might disperse from one community to the next, traveling e.g. with marine currents (Gibbons et al., 2013) or sinking particles (Mestre et al., 2018). A full understanding of life in the ocean requires the knowledge of biogeographical patterns of marine prokaryotes as they vary across environmental, spatial, and temporal gradients. Both at a large scale (e.g. ocean basins) or at the small scale (e.g. within a single bay). There is still much to learn about the vertical and spatial scales of bacterioplankton community composition in the ocean.

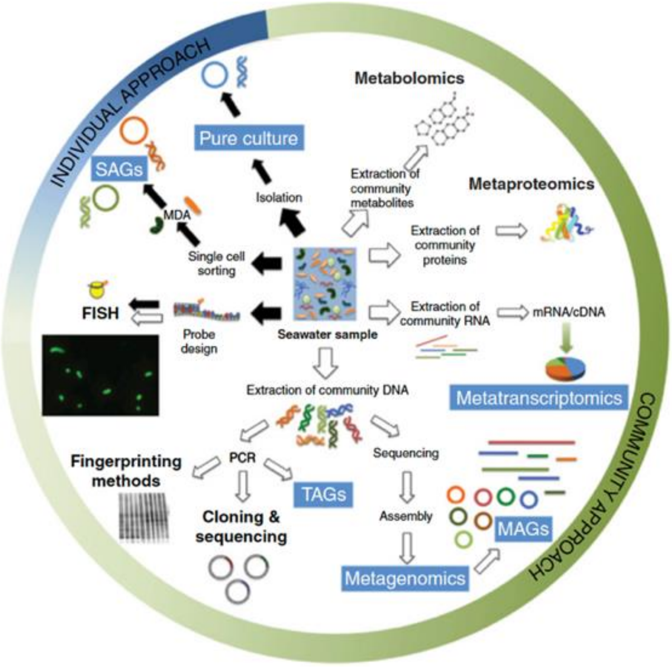

Fig. 1. Range of approaches available to microbial ecologists. Some allow examination of single cells (solid arrows), whereas others provide information at the community level (empty arrows). Several of these approaches have experienced a revolution thanks to development of HTS (white letters on dark background), and other approaches rely on different technologies. From Gasol and Kirchman (2018). The TAGs approach was used in this thesis.

Initial efforts at describing the ocean's microbial diversity were centered in the researchers' backyards, which were placed in coastal sites of the Northern Hemisphere. A few rather global collaborative initiatives such as the JGOFS (e.g.

Ducklow, 1999) or the AMT cruises (Robinson et al., 2009) allowed synthesis of the role of microbes in carbon and nutrient fluxes in the ocean, yet these studies did cover the global ocean only fragmentarily. The advent and democratization of gene sequencing technologies has facilitated global efforts at understanding the identity (ICoMM, Amaral-Zettler et al., 2010; OSD, Kopf et al., 2015) and the genomic characteristics of prokaryotes in the oceanic habitat (GOS, Rusch et al., 2007; Tara Oceans, Sunagawa et al., 2015). These studies with large geographic coverage are combined with high time coverage (time-series stations) sites such as the HOT (Karl and Church, 2014), BATS (Giovannoni et al., 2014), English Channel L4 (Gilbert et al., 2012), Helgoland (Teeling et al., 2012), San Pedro time series (Chow et al., 2014), Delaware estuary (Campbell et al., 2011), or the Blanes Bay Microbial Observatory (Alonso-Sáez et al., 2007) to facilitate an spectacular change of perspective in how we understand the ecology of the microbes inhabiting the ocean.

Biogeography of marine microbes: patterns of connectivity

The vertical and horizontal movement of microorganisms varies with the ocean currents (Richter et al., 2022). Dispersal processes and the resulting connectivity (or lack of) contribute to define the microbial biogeography and determine microbial diversity. However, some factors limit dispersal, e.g., environmental gradients and oceanographic features, which might act as barriers to exchanging microorganisms from one area to another (Villarino et al., 2018).

The advent of high-throughput sequencing techniques have allowed the first studies of microbial biogeography (Ruiz-González et al., 2019; Sebastián et al., 2021; Sul et al., 2013). While marine large-scale samplings provide fundamental insights into global patterns of biodiversity of marine microbes, small scale studies across highly structured ocean spatial features help to understand the niche-partitioning of marine microbes. In fact, the connectivity through these structures and the demonstration of the presence (or absence) of spatial barriers and ecotones is a need of aquatic microbial ecology to develop a science capable of making predictions.

The biophysically-coupled marine realm is the ideal example to describe the interconnectedness of diverse marine ecosystems on a planetary scale (Mestre and Höfer, 2021; Sunagawa et al., 2020). The rapid changes and reorganization of modern ocean ecosystems driven by climate change (ocean warming and acidification), biomass depletion, eutrophication and pollution (Duarte et al., 2020; Elahi et al., 2015; Henson et al., 2021) needs a clearer understanding of spatial biodiversity patterns across environmental gradients. At the base of the marine foodweb, Bacteria and Archaea are essential for ecosystem functioning (Falkowski, 1997), and alterations in their diversity and distribution could lead to changes in ocean biogeochemistry (Cavicchioli et al., 2019; Horner-Devine et al., 2003; Hutchins et al., 2007).

The connectivity between terrestrial and marine ecosystems (horizontal connectivity) is also another underexplored aspect. The ocean receives considerable amounts of water from river or groundwater discharges, especially important in coastal areas, bays, and estuaries, and these waters carry on microbes that may have an impact in marine biodiversity.

Untangling patterns in bacterial and archaeal diversity across small scale spatial gradients is thus essential for understanding, modelling and predicting microbially-driven ecosystem functions (Hatosy et al., 2013; Louca et al., 2016; Vallina et al., 2014).

Microbial distribution across environmental gradients

Dispersed microbes could either thrive in the environment reached if they encounter conditions that are favorable for their growth or they could become part of the seedbank, waiting for favorable conditions to arrive. Factors like latitude, depth, light intensity, salinity, temperature, nutrient availability, organic resources and anthropogenic effect, among others (Barton et al., 2013), regulate microbial communities structure. Thus, determining the effect of environmental barriers on microbial distribution will contribute to our understanding of how microbial communities adapt to a changing oceanic environment (Jönsson and Watson, 2016; York, 2018).

Seasonality has arisen as the main factor structuring microbial communities in temperate latitudes (Auladell et al., 2022; Fuhrman et al., 2006), as it causes temperature and light intensity fluctuations around the year, as well as oscillations in the mixed layer depth and in nutrient availability. In tropical areas, temperatures and light intensity are constant throughout the year (Lee et al., 2015), but the overall climatic variability is determined by the marked seasonality in rainfall. Along the vertical dimension, light intensity decreases with depth whereas nutrient concentration increases, and these gradients allow microorganisms to locate vertically according to their photosensitivity and nutrient requirements. Some microbes show ecotypes associated with zones of different light intensity, such as the case of *Prochlorococcus*, a picocyanobacterium whose ecotypes are distributed at different depths, according to the light regime they are adapted to (Johnson et al., 2006; Kashtan et al., 2014).

The ocean is thus a heterogeneous environment, and microbial communities have to adapt to the different environmental conditions encountered. Besides, temperature and nutrients, salinity is another factor that influences the distribution of microbial communities. In the open ocean, salinity is relatively constant, but as we get closer to the coast, natural salinity gradients are formed in areas affected by runoff, groundwater or riverine discharge (Canuel and Hardison, 2016). Several studies have shown marked changes in abundance and diversity of estuarine bacteria along salinity gradients (Bouvier et al., 2002; Crump et al., 2004; Herlemann et al., 2011), with different bacterial groups dominating in the fresh and marine sections of the estuaries. However, most of these studies have targeted temperate estuaries. In the tropics, drastic salinity changes do occur near the coast during the rainy season, when freshwater inputs due to rainfall and river runoff increase considerably. Areas affected by freshwater inputs from rivers, runoff, or precipitation such as the monsoons are known to alter the structure of microbial communities (Lee et al., 2015; Mitbavkar et al., 2021; Rajaneesh et al., 2017). It is thus likely that bacterioplankton communities in tropical estuaries experience dramatic changes between the rainy and the dry seasons, but due to the scarcity of studies in tropical estuaries almost nothing is known about this. This has been the focus of chapter 1, in which the distribution of bacterial taxa within a large tropical estuary in Costa Rica, and how it changes among seasons, were explored.

In addition, several studies have shown that bacterioplankton communities can be influenced by both large-scale environmental factors, such as water mass boundaries and ocean currents, and small-scale factors, such as hydrodynamic mixing and microbial interactions (Grossart et al., 2019). One important gradient that can structure the ocean is that created by anthropogenic impact, which decreases as we separate from nearshore coastal waters and get into the open ocean. For example, the increase in sediment resuspension, the entry of nutrients and pollutants from agricultural and industrial activities, the presence of plastics, and the creation of ports, among other factors, have affected not only the natural landscape but have also modified the associated microbial communities, which in turn, might affect the functioning of ecosystems (Ager et al., 2010; Nogales et al., 2011; Thanigaivel et al., 2023). Thus, the study of changes in bacterioplankton community composition in nearshore-open ocean gradients with different levels of anthropogenic impact provide information on the distribution of microbial taxa across these gradients and is crucial to evaluate changes in microbial diversity in the context of global change. This issue has been the subject of chapter 2, in which the prokaryotic distribution along coastal-open ocean transects in areas of differential anthropogenic and natural impact was investigated.

Changing from the horizontal to the vertical scale, despite studies carried out on the last two decades have unveiled that the vertical distribution of microbes is largely driven by the change in environmental conditions along the water column (Pommier et al., 2010; Sunagawa et al., 2015; Walsh et al., 2016), these analyses have been done basically ignoring the sharp environmental transition that occurs within the most relevant oceanographic structure defining the ecology of the surface ocean: the deep chlorophyll maxima (DCM). DCM is the water layer with the maximum concentration of chlorophyll-a. It originates in stratified oceans and freshwater ecosystems, at the depth where there is adequate light for photosynthesis and sufficient nutrient supply from below to sustain phytoplankton biomass (Estrada et al., 1993; Latasa et al., 2017). In highly stratified waters, between 50-60% of the water column primary productivity takes place within the DCM (Lorenzo et al., 2004; Weston et al., 2005), highlighting the importance of this oceanographic feature. The DCM is usually sampled with a resolution of tens of meters, but the change in conditions may occur at shorter spatial scales. Moreover, there is sometimes an uncoupling between the depth at which chlorophyll is maximal and the depth where maximal primary production rates occur (Marañón et al., 2021). This defines different layers within the DCM structure, but hardly nothing is known about the distribution of bacteria, protists and phytoplankton (but see Cabello et al., 2016; Dolan and Marrasé, 1995; Haro-Moreno et al., 2018, as exceptional studies) at high spatial resolution along this feature. Chapter three will assess the vertical distribution of microbial communities across the vertical gradients of light, nutrients and phytoplankton distribution that define the DCM.

In summary, how bacterial communities respond to the three types of gradients across small or relatively large distances, and the degree of connectivity between these communities is the subject of this PhD thesis,

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Aims and outlines of the thesis

Aims of the thesis

General: To explore microbial community structure across gradients at the local to regional spatial and seasonal scale in different marine environments, by using 16S rRNA gene metabarcoding to determine prokaryotic community structure and the connectivity between communities.

In particular, we aim at exploring with particular attention three situations that correspond to clear knowledge gaps: i) shifts in community composition between rainy and dry seasons in a tropical estuary strongly affected by riverine discharge (chapter 1), ii) changes in community structure along inshore-offshore gradients in coastal areas with different anthropogenic and natural impact (Chapter 2) and ii) shifts in community structure along the DCM structure in the temperate ocean (Chapter 3).

Chapter 1. Shifts in bacterioplankton community structure between dry and wet seasons in a tropical estuary strongly affected by riverine discharges . This chapter aims at exploring spatial (different sampling sites) and temporal (dry and wet seasons) changes in the structure of the bacterioplankton community in the Gulf of Nicoya (Costa Rica). This tropical estuary is strongly affected by riverine discharges, particularly during the rainy season. This area was chosen because of the scarcity of studies in tropical estuaries and the unique dynamism that this type of ecosystem presents.

In this chapter, the specific objectives were:
- To evaluate the spatiotemporal dynamics of the microbial community of the Gulf of Nicoya.
- To establish the degree of spatial connectivity between the microbial communities in different parts of the Gulf with more or less riverine inputs.

Chapter 2. Environmental gradients that shape the structure of the bacterioplankton communities in the Northwestern Mediterranean Sea.

Chapter 2 explored changes in the structure of bacterial communities in nearshore-open ocean gradients in areas with differential land and human impact.

The specific objectives were:

- To explore the microbial community structure in the NW Mediterranean in gradients inshore-offshore in areas with little or null human impacts, areas with natural riverine-derived impacts and areas with a stronger anthropogenic impact.
- To look at the connectivity within each one of the sides of each gradient and across the transects at different latitudes in the NW Mediterranean.

Chapter 3. Fine-scale structuring of microbial populations across the Deep Chlorophyll Maxima layer in the Northwestern Mediterranean Sea.

This chapter covers the vertical distribution of eukaryotic and prokaryotic microbial communities across one of the most relevant ocean layers, the DCM. A high-resolution sampling was performed to obtain a broad picture of the microbial distribution within the different layers of the DCM. The analyses were performed by separating the 0.2-3 µm and 3-20 µm size fractions, which represent free-living and particle-associated prokaryotes and pico and nano-eukaryotic plankton.

The specific objectives of this chapter were:

- To determine the vertical distribution of microbial communities within the deep chlorophyll maximum, with a high spatial resolution.
- To establish the degree of spatial and vertical connectivity between the different microbial communities.

Chapter 1

Shifts in bacterioplankton community structure between dry and wet seasons in a tropical estuary strongly affected by riverine discharge.

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Submitted to Science of the Total Environment

The Gulf of Nicoya is one of the most productive estuaries in the world.

Gocke et al, 2001

ABSTRACT

Estuaries are among the most productive ecosystems in the world and are highly dynamic due to the interaction of freshwater and seawater, which results in strong spatial gradients in physico-chemical conditions. Bacterioplankton play a central role in these systems, driving the fluxes of carbon and energy, and being central for contaminant removal in human-impacted areas. Most studies on bacterioplankton dynamics have been carried out in temperate estuaries, and they show that salinity is a major factor driving bacterioplankton distribution. Tropical estuaries, although largely understudied, experience drastic variations in river discharge between the dry and the rainy seasons, influencing the spatial distribution of the salinity gradient and thus likely impacting bacterioplankton communities. Using Illumina sequencing of the 16S rRNA gene, here we studied bacterial communities from the Nicoya’s gulf (Costa Rica), a large tropical estuary characterized by high riverine discharge during the rainy season, to explore seasonal changes in the spatial distribution and connectivity of these communities along the Gulf. Our results show pronounced differences in bacterial diversity and community structure between seasons and zones within the estuary (the shallow upper Gulf, the middle zone and the lower zone, located in the marine end of the estuary). Various Enterobacteriales genera and Cyanobacteria dominated the bacterial communities in the dry season, whereas Alphaproteobacteria dominated the communities in the rainy season. These seasonal patterns were similar to the seasonal variations observed in bacterial assemblages during almost a year within a single station in the upper region of the Gulf. We conclude that the Gulf is highly dynamic in both the spatial and temporal scale and that bacterioplankton communities are strongly influenced by the riverine and tidal inputs during both seasons.

1. Introduction

Estuaries are transition zones where seawater mixes with freshwater, generating large salinity gradients. These dynamic and complex aquatic ecosystems are among the most productive ecosystems in the world. They play a major role in erosion control and carbon sequestration, provide shelter, habitat, and food resources for many marine organisms, and are fish breeding and recruitment areas, representing important ecosystems for the economic development of human populations that depend on fishing (Vasconcelos, Reis-Santos and Cabral, 2011; Barbier *et al*., 2011). Estuaries occur from the poles to the tropics, and are widely studied because of their role as buffer ecosystems and recruitment areas for marine life (Barbier et al., 2011; Vasconcelos et al., 2011; Canuel and Hardison, 2016).

Bacterioplankton are essential players within estuaries, as they control the energy and nutrient cycles, organic matter decomposition, and contaminant removal in aquatic systems (Li et al., 2017; Newton and McLellan, 2015; Wang et al., 2017). In estuaries, bacterial communities from the inflowing rivers are mixed with seawater communities, and several studies have shown marked changes in abundance and diversity of estuarine bacteria along the salinity gradients and also between seasons (Revilla et al., 2000; Troussellier et al., 2002; Bouvier and Del Giorgio, 2002; Selje and Simon, 2003; Crump et al., 2004; Herlemann et al., 2011), with different bacterial groups dominating in the fresh and marine sections of the estuaries. However, most of these studies have targeted temperate estuaries, whereas estuaries in tropical regions have been largely understudied (but see Li et al., 2017).

In the tropics, although day-length and temperature are fairly constant, the overall climatic variability is determined by the marked seasonality in rainfall, which controls the fluxes of sediments, nutrients and organic matter from land to the sea. Consequently, tropical estuaries are highly influenced by the riverine discharge during the rainy season (Palter et al., 2006), which not only changes the physico-chemical environment but can also modulate the circulation patterns within the estuary (Zhang et al., 2022). In turn, rivers can transport large numbers of allochthonous bacterial taxa of terrestrial origin (Ruiz-González et al., 2015), especially in situations of high precipitation and runoff

(Stadler and del Giorgio, 2021). It is possible that the spatial structuring of bacterioplankton communities in tropical estuaries changes dramatically between the rainy and the dry seasons, but due to the scarcity of studies in tropical estuaries almost nothing is known about this.

Here we evaluated the spatio-temporal changes in bacterioplankton communities from the Gulf of Nicoya, a tropical estuary subjected to high riverine discharge during the rainy season, and explored the distribution patterns of typical marine and freshwater taxa within the estuary. The Gulf of Nicoya is located in the Pacific coast of Costa Rica and represents one of the largest estuaries (1530 km^2 surface area) of Central America and the most important fishing ground of Costa Rica. Two large rivers, the Tempisque and Grande de Tárcoles deliver most of the freshwater coming into the gulf (Wolff et al., 1998), affecting differentially the shallow upper zone (influenced by the Tempisque river) and the much deeper lower zone (influenced by the Grande de Tárcoles river). The discharge of these two rivers has large impacts on the circulation patterns and salinity gradients within the gulf: the Tempisque River generates a southward surface flow that carries fresh- to brackish water towards the lower gulf, which is compensated by a northward flow of saline water into the upper gulf along the bottom of the estuary (Peterson, 1960; Voorhis et al., 1983). In turn, the discharge from the Grande de Tárcoles river along the eastern shore generates a marked east-west asymmetric circulation in the lower gulf (Voorhis et al., 1983; Brenes et al., 2001; Kress et al., 2002). The seasonal variations in riverine discharge cause the circulation within the gulf to be faster during the rainy season (May-November), whereas during the dry season (December-April) the circulation becomes slower due to the low freshwater input (Lizano and Vargas, 1996). This has consequences in the water residence time within the Gulf, and therefore will have an impact on the distribution and dispersion of planktonic organisms. However, besides a few studies on the occurrence of harmful algal blooms (Viquez and Hargraves, 1995; Vargas-montero and Freer, 2004; Vargas-Montero et al., 2006; Calvo Vargas et al., 2016) and microphytoplankton abundance dynamics (Seguro et al., 2015), little is known about bacterioplankton communities in the Gulf of Nicoya. Here, we characterized the bacterial communities from six sites located throughout the estuary in two different occasions representing the rainy and dry seasons and explored variations in their abundance and diversity in relation to their proximity to the two main rivers. In addition, we performed 7 sampling over almost a year in a single station to explore the seasonal variations at a given site. We expected to find less spatial heterogeneity in communities during the rainy season due to homogenization of environmental conditions caused by riverine discharge, as well as a larger contribution of freshwater or terrestrial taxa.

2. Materials and methods

2.1. Study area and sample collection

The Gulf of Nicoya extends ca. 80 km north from the Pacific to the mouth of the Tempisque river. Due to its bathymetry, the gulf is divided into two main regions, the upper zone fringed by mangrove swamps and with less than 20 m in depth, and the lower zone, with depths exceeding 200 m at the mouth of the gulf, characterized by rocky shores and sandy beaches (Voorhis et al., 1983; Wolff et al., 1998) (Fig. 1). The gulf has two climatological seasons, a rainy period from May to November with rainfalls above 600 mm month^{-1} and a dry season from December to April with a precipitation of less than 50 mm month^{-1} (Wolff et al., 1998; Kress et al., 2002). The Tempisque (upper zone) and Grande de Tárcoles (lower zone) are the rivers that contribute the most to the freshwater coming into the gulf (Wolff et al., 1998).

We selected six sampling sites according to the characteristics of the gulf, two near the mouth of the Tempisque River (3 and 4), two in the middle of the upper zone (2 and 5), and two in the lower gulf (1 and 6) (Fig 1). Samples were collected both during the dry (December 2013) and the rainy (September 2014) seasons, after a scenario of moderate El Niño (i.e., drier than usual). In addition, we performed 7 samplings (between November 2012 and October 2013) at site 4, in order to better understand the temporal dynamics in an area strongly influenced by riverine discharges. Water samples were taken from the surface (1 m depth) using a Niskin water sampler. Physical parameters such as temperature and salinity were measured in situ with a YSI multiprobe model 566. About 2 L of seawater per sample were filtered through a 0.2 μm sterivex filter (Millipore) using a peristaltic pump and the sterivex filters were kept at -80ºC until subsequent extraction.

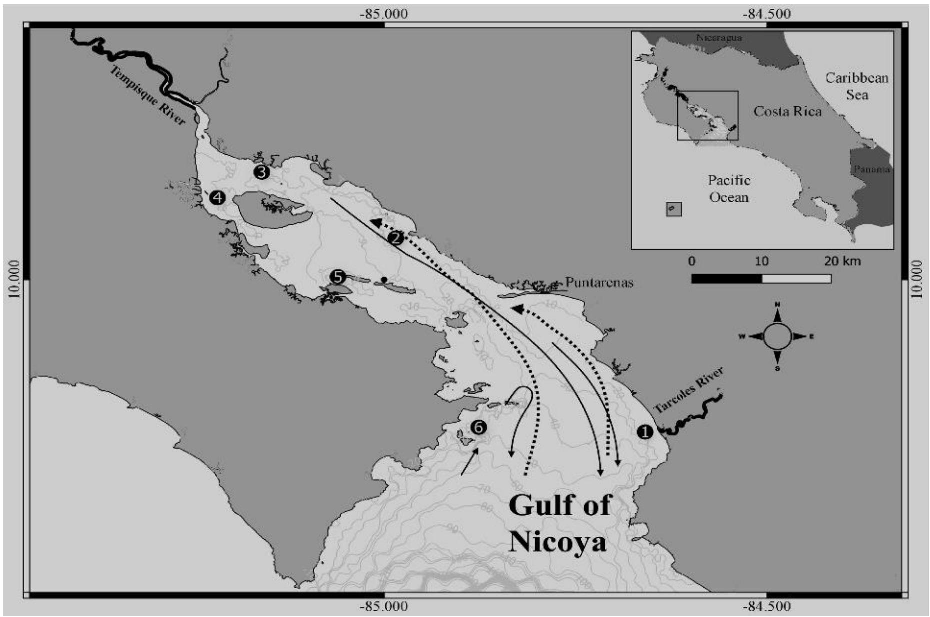

Fig. 1. Sampling area and location of the sampling sites. The upper zone (sites from 2 to 5) extends from Puntarenas to the Tempisque river and the lower zone (sites 1 and 6) covers the area between Puntarenas and the gulf mouth (including the Tárcoles river mouth). Arrows represents the tidally averaged circulation during the rainy season in the upper 0-15 m, solid lines) and lower (15 m-bottom, dotted lines) layers (Modified from Vargas et al., 2010). Depth contours in meters.

2.2. Bacterial and picophytoplankton abundance

Duplicate samples (1.8 mL) were preserved adding 10% P+G solution (1% paraformaldehyde + 0.05% glutaraldehyde final conc.). Bacterial abundances were determined after staining with SybrGreen I (Molecular Probes) in the dark for a few minutes and using a BD FACSCalibur cytometer with a laser emitting at 488 nm (following Gasol and Morán, 2015). High and Low nucleic acid content bacteria (HNA, LNA) were discriminated in plots of side scatter (SSC) vs green SyberGreen fluorescence.

2.3. DNA extraction and sequencing

The sterivex filters were filled with 1.8 ml of Lysis buffer (40 mM EDTA; 50 mM Tris pH=8.3; 0.75 M Sucrose), treated with lysozyme, proteinase K and sodium duodecyl sulfate, and the DNA was later extracted with phenol-chloroform, as described previously (Massana et al., 1997). Primers 28F (5’- GAGTTTGATCNTGGCTCAG-3’) and 519R (5’- GTNTTACNGCGGCKGCTG-3’) were used to generate amplicons spanning the V1 to V3 regions of the bacterial 16S rRNA gene and sequencing was performed in an Illumina MiSeq sequencer (2 × 250 bp, Research and Testing Laboratory; http://rtlgenomics.com/). The amplicons were processed with UPARSE (Edgar, 2013). The reads were merged with PEAR (Zhang et al., 2014), and those with >100 nucleotides were selected. Quality check, dereplication, OTU clustering (99%), and chimera filtering (using SILVA v.123 database) were processed with USEARCH (Edgar, 2010). A total of 60.024 sequences were obtained by illumina sequencing and grouped into 6.560 OTUs (at the 99% cutoff). Singletons were discarded as well as those affiliated with eukaryotes, chloroplasts and mitochondria. The OTU abundance table was then randomly subsampled to the lowest number of reads per sample (5002) using the *rrarefy* function in the *vegan* R package (Oksanen et al., 2015). Raw data have been deposited at the European Nucleotide Archive (Acc. No. PRJEB59522).

To determine which OTUs showed the largest changes in abundance from the upper region of the Gulf to the most oceanic station (st. 6), we calculated the average Euclidean distance of the relative abundance of each OTU between all samples, using the script presented in Appendix S1 of Ruiz-González et al., (2015). Those OTUs showing a mean distance >10 were considered ‘shifting’ OTUs. This value represented an average change of 238 sequences between two samples (range 24-1936). Those shifting OTUs displaying higher abundances in the upper Gulf where categorized as ‘upper gulf’ shifters, whereas the shifting OTUs displaying higher abundances in st. 6 were categorized as ‘marine’.

2.4. Statistical analyses

The data were grouped by location (inner and outer estuary) and season (dry and rainy) for statistical analyses. Shannon diversity calculations (H index), non-metric multidimensional scaling (nMDS) and cluster plots were performed and plotted in R (*stats* package). We used one-way analyses of variance (ANOVAs) to test for significant variations in microbial diversity or abundances across sites or seasons. All analyses were performed with R (3.6 version) and Rstudio software (1.2.1335 version).

3. Results and discussion

3.1. Environmental setting

Salinity showed marked changes between the different zones of the gulf during the rainy season (Table 1), with lower values in the upper region due to the Tempisque river discharge. In contrast, salinity values in the dry season were more homogenous along the gulf, in agreement with previous observations indicating the estuary is fully mixed during summer (Brenes et al., 2001; Kress et al., 2002). Water temperatures followed the pattern of a tropical estuary: quite constant throughout the year, with small increases in the middle and lower zones during the dry season due to the increase in solar radiation over a shallow water column (Lizano, 1998; Brenes et al., 2001; Kress et al., 2002).

Picophytoplankton abundance remained relatively constant along the estuary (Table 1), with slightly higher values in the dry season ($p<0.05$, ANOVA test). This may be explained by the fact that in the dry season turbidity is lower, allowing a deeper penetration of sunlight (Rochelle-Newall et al., 2011; Seguro et al., 2015, Panhwar and Mairaj, 2022), and that water residence time is longer. In contrast, bacteria were more abundant in the rainy season (ANOVA test, $p<0.01$), especially in the most saline areas of the estuary (Table 1 and Supplementary figure S1), suggesting that the entry of fresh water might affect the distribution of bacteria throughout the gulf. Bacterial diversity was also higher in the rainy season than in the dry season (ANOVA test, $p<0.01$), perhaps related to a higher niche availability for prokaryotes due to the high particle load associated to riverine discharge (Kress et al., 2002). Alternative, this could be due to the transport of freshwater or terrestrial bacterial taxa into the estuary, as rivers are known to carry a large diversity of soil-derived taxa (Ruiz-González et al., 2015; Hauptmann et al., 2016; Niño-García et al., 2016; Stadler and del Giorgio, 2021). During the dry season, diversity was higher in the upper and middle sections of the gulf ($p>0.05$, ANOVA test, Table 1). This may be a consequence of several factors: the Tempisque river, which is the largest river discharging into the Gulf (Kress et al., 2002), may act as an important source of bacterial taxa in the upper region. Also, most tidal mixing takes place in the middle section of the Gulf (Whelan et al., 1989), and tidal currents will likely have a higher influence in the dynamics of the Gulf circulation during the dry season than in the rainy season, when circulation is driven by the riverine discharge. Thus, tides may also act as sources of taxa, leading to an increase in bacterial diversity of seawater origin.

Table 1. Physical and biological parameters measured across all sampling sites during the dry and rainy seasons.

	Site ID	Zone	Sampling date	Temp ºC	Salinity	H index	Bacterial abundance Cells mL^{-1}	Picophytoplankton abundance Cells mL^{-1}	Precipitation * mm^{3} month^{-1}
DRY	**1**	Lower	December 2012	28.56	30.20	3.07	7.24E+05	1.36E+05	65.6
	6	Lower		28.22	31.22	3.78	5.17E+05	1.04E+05	
	2	Middle		28.99	30.82	2.96	1.09E+06	1.18E+05	
	5	Middle		28.80	30.13	4.78	1.01E+06	1.32E+05	
	3	Upper		28.88	29.07	4.01	7.07E+05	1.01E+05	
	4	Upper		29.20	27.52	4.43	1.18E+06	1.35E+05	
RAINY	**1**	Lower	September 2013	28.25	30.04	4.94	2.16E+06	1.21E+05	242.8
	6	Lower		27.84	31.37	5.10	2.09E+06	2.83E+04	
	2	Middle		28.60	28.87	5.10	1.98E+06	1.07E+05	
	5	Middle		29.15	28.01	5.17	2.09E+06	1.29E+05	
	3	Upper		30.05	22.46	5.32	1.28E+06	7.15E+04	
	4	Upper		30.05	23.33	5.41	1.42E+06	1.05E+05	

Headers: Temp (temperature), *Total monthly precipitation in Puntarenas observatory station (Instituto Metereológico Nacional).

3.2. Bacterial community composition

The studied bacterial communities showed both temporal and spatial segregation (Fig. 2), as samples were clustered by season and by zone of the Gulf. Communities changed gradually from those in the lower zone towards the upper zone, with those from the middle zone falling in between the two. However, the samples from the lower zone were more clearly separated from the rest in both seasons (ANOSIM, P<0.01). Samples from the upper and middle zones were more similar among them in the rainy season than in the dry season, and communities from the lower zone were the ones showing the largest seasonal differences (Fig. 2).

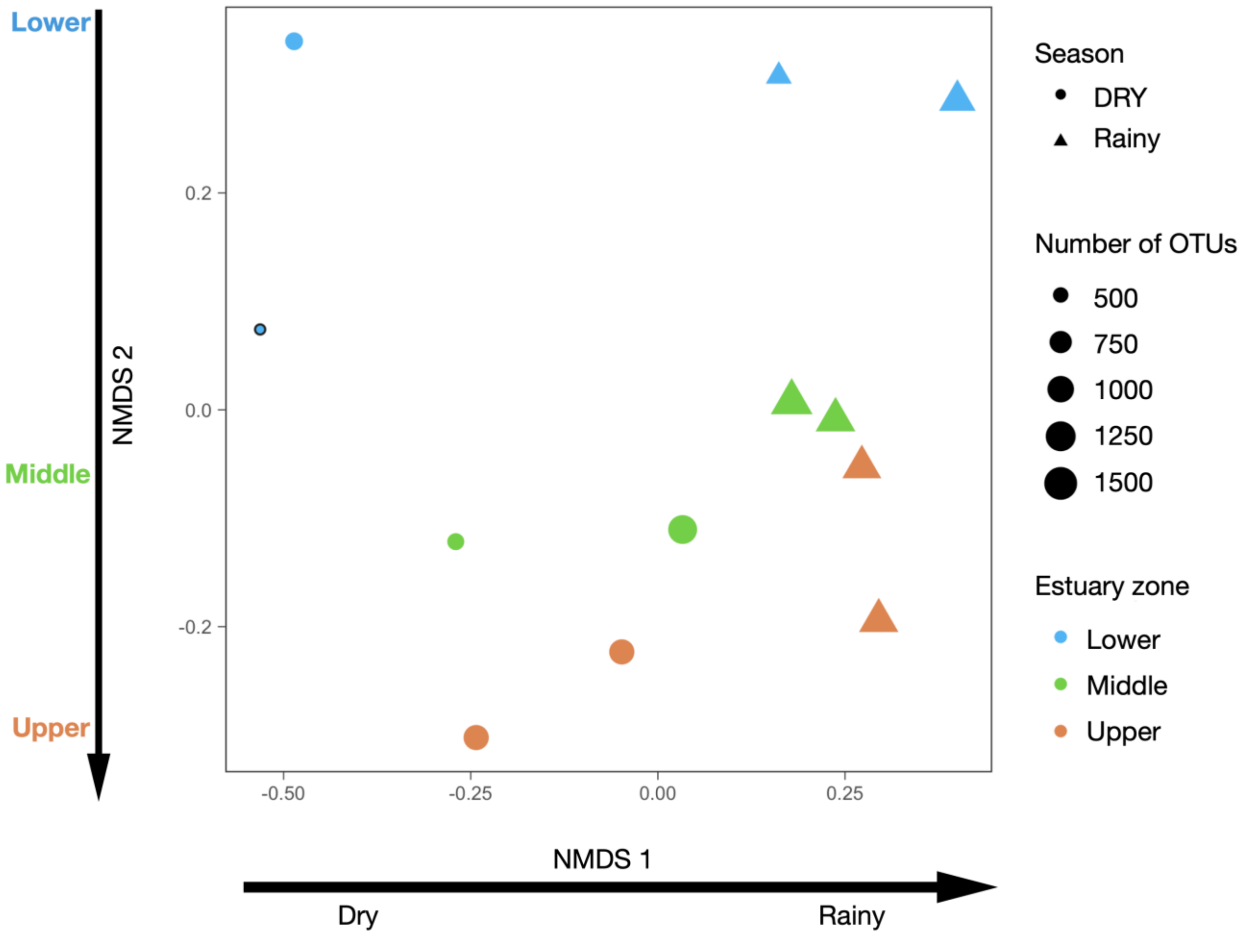

Fig. 2. Non-metric multidimensional scaling (NMDS) analysis based on Bray–Curtis distances between the studied bacterioplankton communities. Dot size is proportional to the richness (number of OTUs after rarification).

The taxonomic composition of the bacterial communities differed largely between seasons. During the dry season, Cyanobacteria and Enterobacteriales dominated the communities throughout the estuary (Fig. 3). Among the Enterobacteriales, distinct genera that are considered opportunistic pathogens or epibionts (Cooney et al., 2014) were abundant along the estuary, representing between 23% and 70% of local community sequences during the dry season. The genus *Pantoea*, which includes some species that are usually associated with plants (Delétoile et al., 2009), dominated in stations 1, 3 and 5 (Fig. 4) although different species were abundant in the different stations (Fig. S2). Genus *Enterobacter* was mostly restricted to station 4, affected by the Tempisque discharge, where it represented more than half of the Enterobacteriales reads. The genus *Serratia* accounted for the other half of the reads in this station, and dominated the Enterobacteriales population at station 6. In contrast, the genus *Tatumella* dominated the Enterobacteriales sequences at station 2 (Fig. 4).

Alphaproteobacteria such as Rhodobacterales, Rhodospirillales and Rickettsiales, were restricted to the medium and upper region of the gulf (Fig. 3A). SAR11 bacteria, which usually dominates surface ocean bacterial communities (Morris et al., 2002) represented a notable fraction of the community at station 5 (~25% Fig. 3A), but their contribution to the rest of communities sampled during the dry season was low.

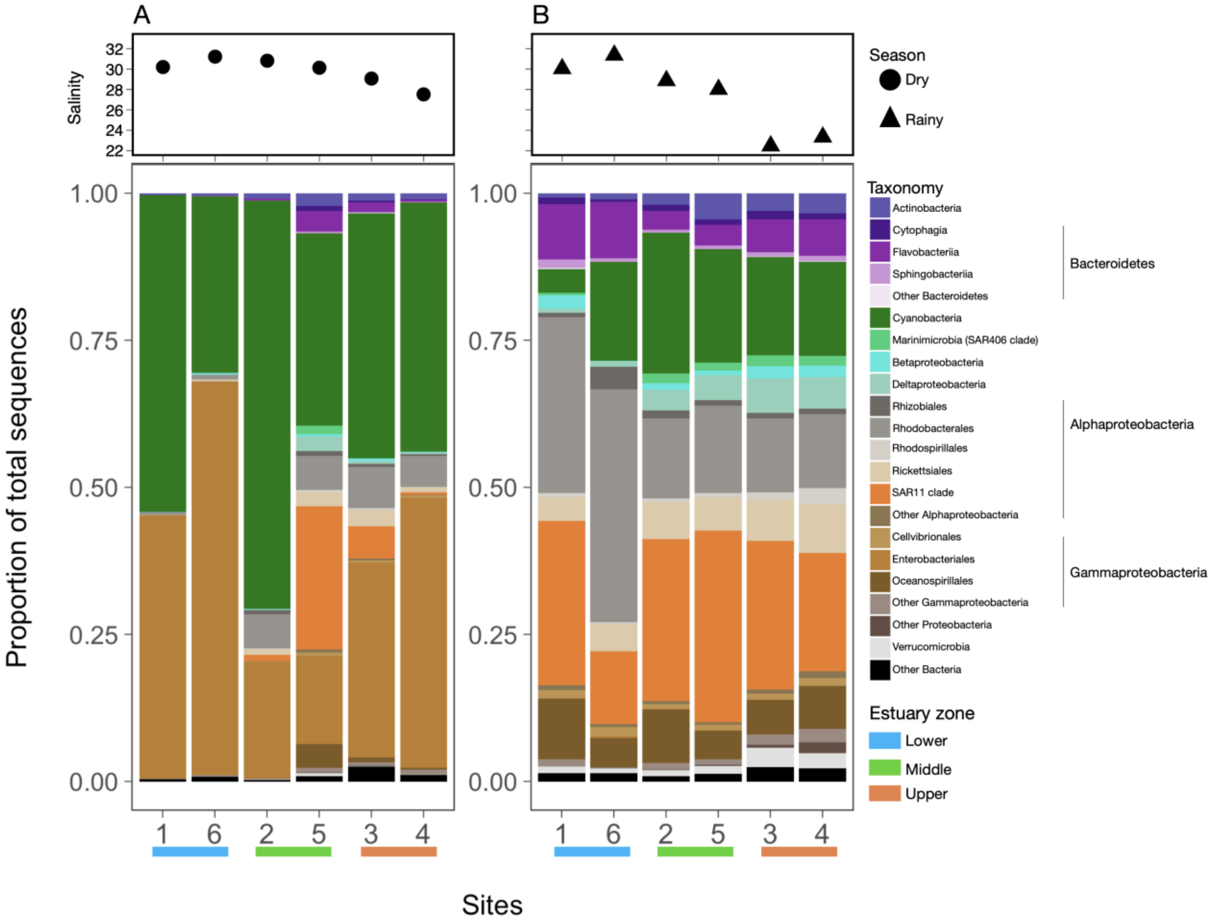

Fig. 3. Patterns of salinity (upper panels) and bacterial community composition (lower panels) along the estuary during the dry (A) and rainy (B) seasons. Bacterioplankton taxonomy is shown at the level of phylum except for Bacteroidetes, which is at the class level and Alpha- and Gammaproteobacteria that are at the order level. Taxonomic groups with relative abundances >1% are represented, whereas the remaining sequences were classified as “other bacteria”.

During the rainy season, the communities were more diverse taxonomically (Fig.3B, Table 1). The input of freshwater, the decrease in salinity, the putative contribution of nutrients due to the river discharge and the high hydrographic dynamism during this period likely explain the observed changes in the composition and the spatial distribution of bacteria. Alphaproteobacteria (Rhodobacterales, Rickettsiales and SAR11) dominated bacterial communities, whereas the contribution of Cyanobacteria remarkably decreased, and Enterobacterales were not detected (Fig. 3B, Fig. S3). The lower abundance of Cyanobacteria during the rainy season is likely related to the increase in water turbidity due to river discharge and the associated sediment loads. Groups such as Actinobacteria and Betaproteobacteria, which are typically found in the freshwater end of estuaries (Crump et al., 1999; Cottrell and Kirchman, 2003; Herlemann et al., 2011) were present. Actinobacteria were more abundant and diverse in the upper and middle zones of the Gulf, in the region affected by the Tempisque discharge (Fig. S3), whereas Betaproteobacteria displayed similar abundances in all the stations affected by freshwater inputs (i.e. stations 1, 3 and 4, Fig. S3). Deltaproteobacteria, and Rickettsiales (Alphaproteobacteria), exhibited a gradient with a maximum in the upper estuary, and decreased towards the middle and lower estuary. In contrast, Rhodobacterales and Flavobacteriia were more abundant in the lower gulf and decreased towards the upper estuary (Fig.3B, Fig. S3).

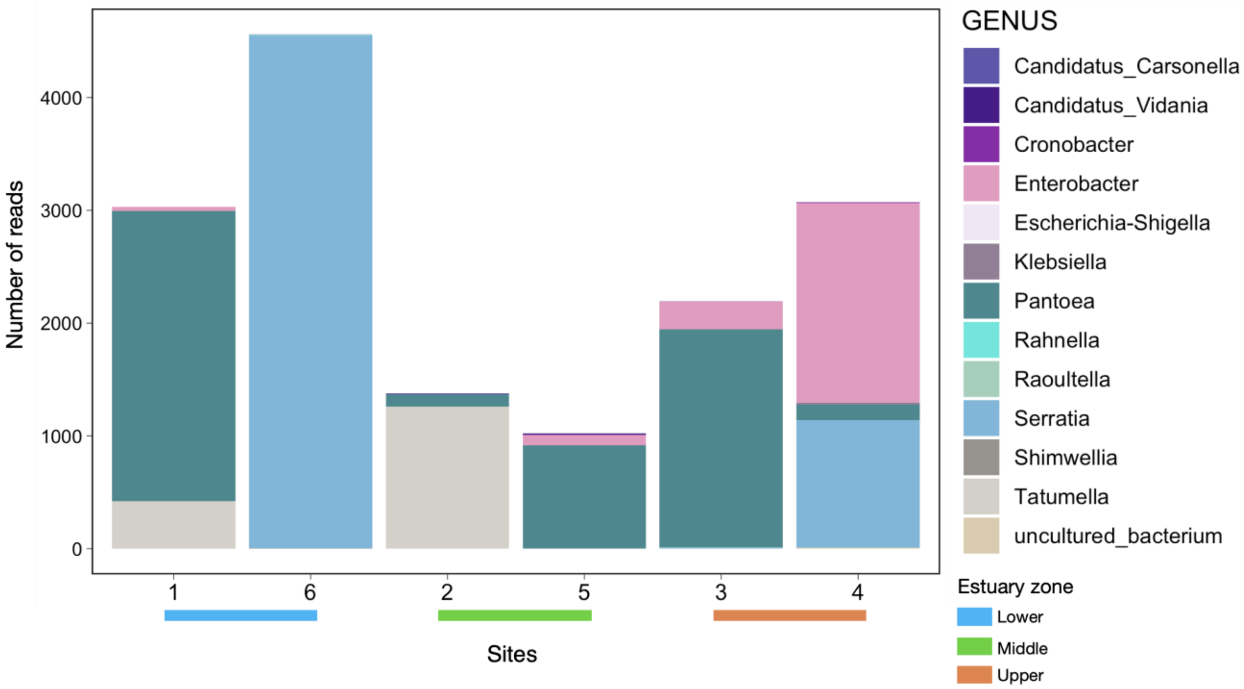

Fig. 4. Taxonomic affiliation of the Enterobacteriales sequences (after rarification) at the Genus level during the dry season. The bars in the X-axis indicate the region of the estuary.

We next explored those OTUs that displayed the largest spatial changes in abundance (hereafter referred as ‘shifting OTUs’) from the shallow stations in the upper gulf, strongly affected by the Tempisque river runoff, to st. 6, which was the most oceanic station. Shifting OTUs were then categorized as ‘marine’ if they presented higher abundances at st. 6 than in the upper section of the Gulf (st. 3 and 4), whereas they were considered ‘upper gulf’ OTUs when they showed the opposite abundance pattern. This categorization was done pooling together samples from the two seasons. Looking at the contribution of these shifting OTUs to the communities throughout the Gulf we observed that ‘marine’ shifters accounted for 60% and 40% of the community at stations 6 and 1, respectively, whereas their contribution at stations 3, 4 and 5 was lower than 20% (Fig. 5). The ‘upper gulf’ shifters displayed the opposite trend, representing between 50 and 60% of the community at stations 3, 4 and 5 and between 10-20% of the community in the lower Gulf. The trend was similar in both seasons, suggesting a clear segregation at the level of individual bacterial taxa along the Gulf regardless the hydrodynamic conditions.

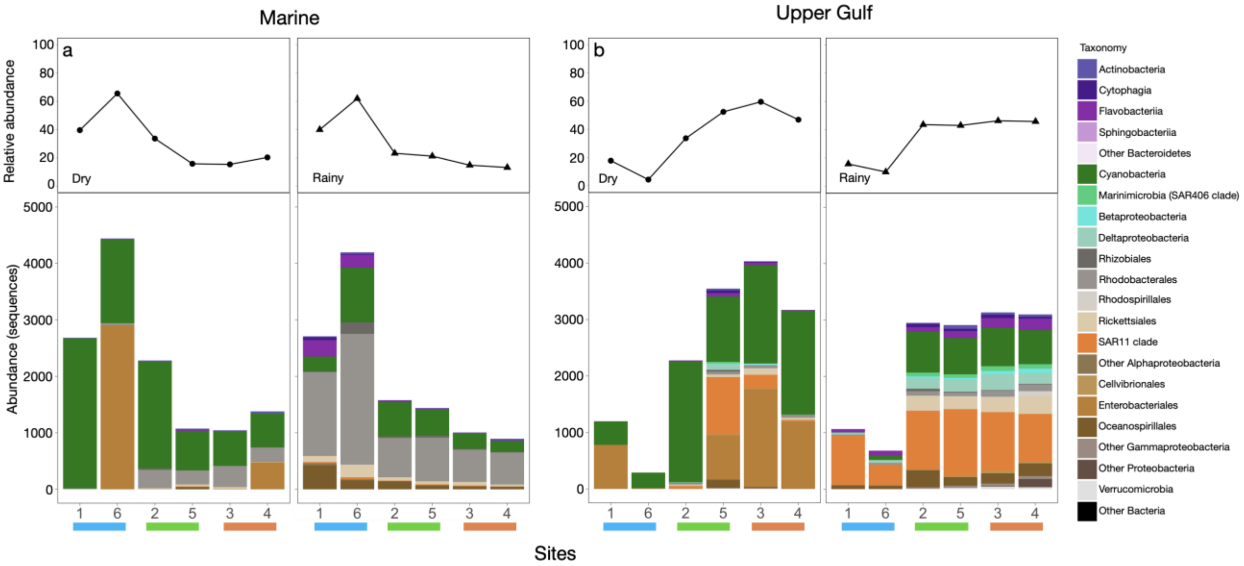

Fig. 5. Contribution of a) ‘marine’ and b) ‘upper gulf’ shifting OTUs (see main text) to each prokaryotic community during the dry (left) and the rainy (right) season (upper panels), and the taxonomic affiliation of the sequences (lower panels). The bars in the X-axis indicate the region of the estuary (blue: lower, green: middle and orange: upper zone). Bacterioplankton taxonomy is shown at the level of phyla except for Bacteroidetes, which is at the class level and Alpha- and Gammaproteobacteria that are presented at the order level. Taxonomic groups with relative abundances >1% are represented, whereas the remaining sequences were classified as “other bacteria”.

During the dry season, Cyanobacteria largely dominated the taxonomic affiliation of the ‘marine’ shifters (Fig. 5A), with one *Prochlorococcus* OTU representing more than half of the reads (Figs. S4, S5), whereas the ‘upper gulf’ cyanobacterial shifters were composed only by *Synechococcus* (Fig. S4) with two OTUs dominating the *Synechococcus* population (Fig. S5). During the rainy season, the ‘marine’ shifters pool was dominated by Rhodobacterales, and there was also some presence of Flavobacteriia, Oceanospirillales and Rickettsiales in the lower zone of the Gulf. In turn, the 'upper gulf' shifters comprised some cyanobacterial and enterobacteriales OTUs in the dry season, whereas in the rainy season there was a notable contribution of SAR11 sequences (Fig. 5B). This likely reflects the complexity of the Gulf of Nicoya hydrodynamics: during the rainy season there is a seaward surface flow induced by the Tempisque river discharge, which is compensated by a northward subsurface flow (Gocke et al., 2001) that due to the abrupt change in the Gulf bathymetry (Brenes et al., 2001, see Fig. 1) reaches the surface in the middle Gulf and distributes through the shallow upper region. During the dry season water circulation within the Gulf becomes slower due to the low riverine discharge (Lizano and Vargas, 1996). This change in water circulation leads to longer residence times, which may allow the blooming of species displaying fast growth rates (Crump et al., 2004), such as bacteria belonging to the Enterobacteriales (Fig. 3). This may be one of the reasons for the practically absence of SAR11 bacteria during the dry season, which have likely been outcompeted given their known slow growth rates (Lankiewicz et al., 2015; Giovanonni, 2017). The Puntarenas estuary located in the eastern side of the Gulf? (Fig. 1), which is one of the most important fishing and commercial ports that flow into the Gulf of Nicoya, and the Tárcoles river, are known to be highly contaminated with wastewater (Acuña et al., 1998; Herre et al., 1999; Nielsen and Quesada, 2006; Pérez-Gómez, 2021), and therefore may contribute to the presence of Enterobacteriales. However, some Enterobacteriales taxa have also been shown to be associated to late phases of algal blooms (Morrison et al., 2017; Zhou et al., 2019), which are frequent in the Gulf of Nicoya during the dry season (Gocke et al., 2001), and therefore the presence of Enterobacteriales may not be related exclusively to anthropogenic pollution. Differences in the taxonomic composition of the communities in the lower Gulf (stations 1 and 6) during the dry season are likely driven by the east-west asymmetry in water circulation due to the runoff of the Barranca and Tárcoles rivers along the eastern shore of the Gulf (Voorhis et al., 1983).

3.3. Temporal dynamics in bacterial community structure

To further understand the complexity of the temporal dynamics that communities display within the Gulf, we conducted 7 additional samplings at site 4 (Fig.1), comprising the dry (Dec-12, Feb-13, and Mar-13) and rainy (Sept-13 and Oct-13) seasons, as well as two transition periods (Nov-12 for the rainy to dry transition and Apr-13 from dry to rainy transition). According to the National Meteorological Institute, the Costa Rican Pacific was in 2012 under the regional influence of the El Niño Southern Oscillation, which, together with the drought that the country was experiencing, left a substantial rainfall deficit (K. Calderón, personal communication). Thus, the sampling carried out in November 2012, which occurred just after the El Niño phenomenon dissipated, does not reflect a typical month of transition from rainy to dry season due to the lack of rain in 2012.

Our results show a high temporal dynamism in bacterial community structure at site 4, located in the upper Gulf, accompanied by subtle variations in water temperature but pronounced decreases in salinity from March to October 2013 (Fig. 6). This region encompasses large mud flat areas surrounded by mangrove swamps (Gocke et al., 2001). Site 4 is a very shallow station, with the water column at the time of sampling ranging from 2 to 8 m depth, depending on the tidal cycle phase. Despite the shallow depth, the temporal trends reflected the same patterns observed in the dry and rainy seasons throughout the Gulf. The contribution of Enterobacteriales to total bacterial community composition was high at the beginning of the dry season and, in agreement with our previous results, the dominant genera were Pantoea, Serratia and Enterobacter (Fig. 6).

The presence of Enterobacteriales coincided with relatively low water-temperatures and a slight decrease in salinity (Fig. 6), suggesting a potential riverine input. Enterobacteriales may be just passively transported into the Gulf by the rivers, and it has been shown they can survive for very long periods of time in seawater (Grimes et al., 1986) although they usually become nonculturable with traditional methods. DNA analyses cannot differentiate whether the cells are dead, dormant or active, so the detection of Enterobacteriales does not imply that these cells are alive. However, their high abundances throughout the Gulf suggest they likely undergo transient blooms over the year. In agreement with this idea, some Enterobacteriales are salinity tolerant and some are natural components of the bacterioplankton communities (Morrison et al., 2017; Zhou et al., 2019). Nevertheless, as they could pose a health risk due to their potential pathogenicity, their origin and dynamics should be the focus of further investigations.

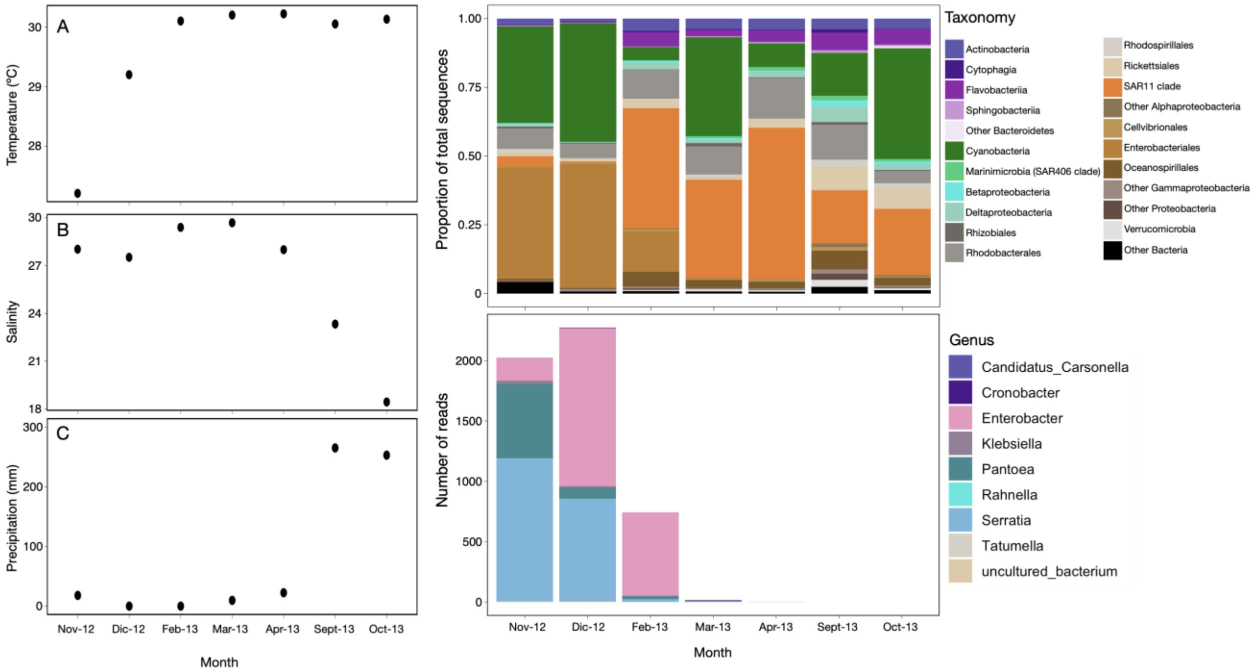

Fig. 6. Temporal dynamics of water temperature, salinity, precipitation (A, B and C) and bacterial community composition (right panels) in site 4 from November 2012 to October 2013. The right-upper plot corresponds to bacterial taxonomy at the phylum level except for Bacteroidetes, which is shown at the class level, and Alpha- and Gammaproteobacteria that are presented at the Order level. Only the taxonomic groups with >1% are represented, and the remaining sequences are classified as "other bacteria". The right-lower plot corresponds to sequences belonging to Enterobacteriales with their corresponding taxonomic affiliation at the Genus level.

SAR11 was nearly absent during November and December 2012, but became an important contributor to the bacterial communities from February 2013 onwards coincident with the increases in water temperature and salinity (Fig. 6), and decreased slightly in the period of maximal precipitation. This is consistent with the fact that we did not practically observe SAR11 bacteria in our spatial sampling during the dry season (Fig. 3), performed in December 2012. Overall, our results highlight a highly complex spatio-temporal structuring of bacterial communities within the Gulf of Nicoya, which likely reflects the large environmental heterogeneity caused by riverine discharge, tides and currents. Further studies with higher temporal and spatial resolution will be required to fully comprehend the dynamics of this complex estuarine system.

4. Conclusions

Here, we present the first report on the seasonal and spatial patterns of bacterial communities structure from the dry to the rainy season through the Gulf of Nicoya in a period after El Niño Southern Oscillation phenomena (a particularly dry period). Our results show a high spatial heterogeneity in the communities, which are dominated by seasonally changing proportions of Cyanobacteria, Enterobacteriales, and Alphaproteobacteria. The analysis of the OTUs that change more in abundance between the upper Gulf and the most oceanic station indicate that the gulf is very dynamic and the spatial distribution of bacterial taxa is highly influenced by riverine and tidal (marine) inputs, which appear to deliver or favor the establishment of different bacterial groups. Our work constitutes the first contribution to the knowledge of bacterioplankton composition and spatial and temporal dynamics in the Gulf of Nicoya, and adds to the body of information about the ecology of microbial communities in tropical estuaries, which have been poorly studied despite representing some of the most productive ecosystems in the world.

Acknowledgements

We thank Elman Calvo and Orlando Torres for helping to obtain the samples and Rebeca Quesada Céspedes for map elaboration. The author was supported by a PhD grant from Universidad Nacional (Costa Rica), and the ICM authors were supported by a Severo Ochoa Excellence Award (Ministerio de Ciencia e Innovación) CEX2019-000928-S.

Supplementary information

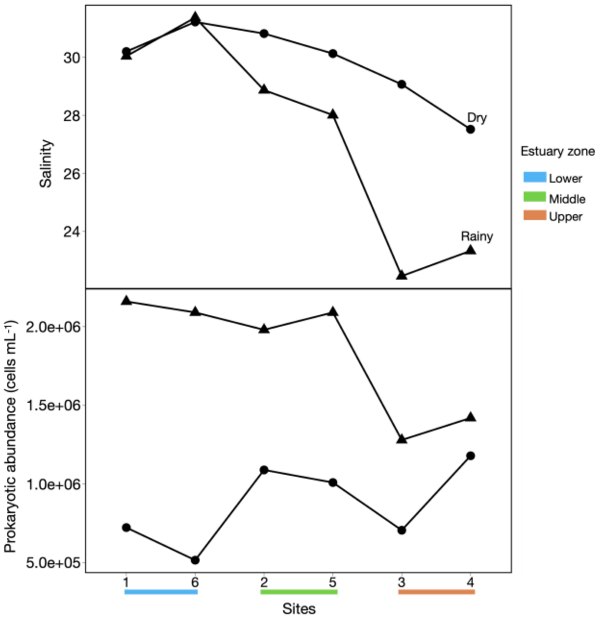

Fig. S1**.** Bacterial abundance by flow cytometry during dry and rainy seasons.

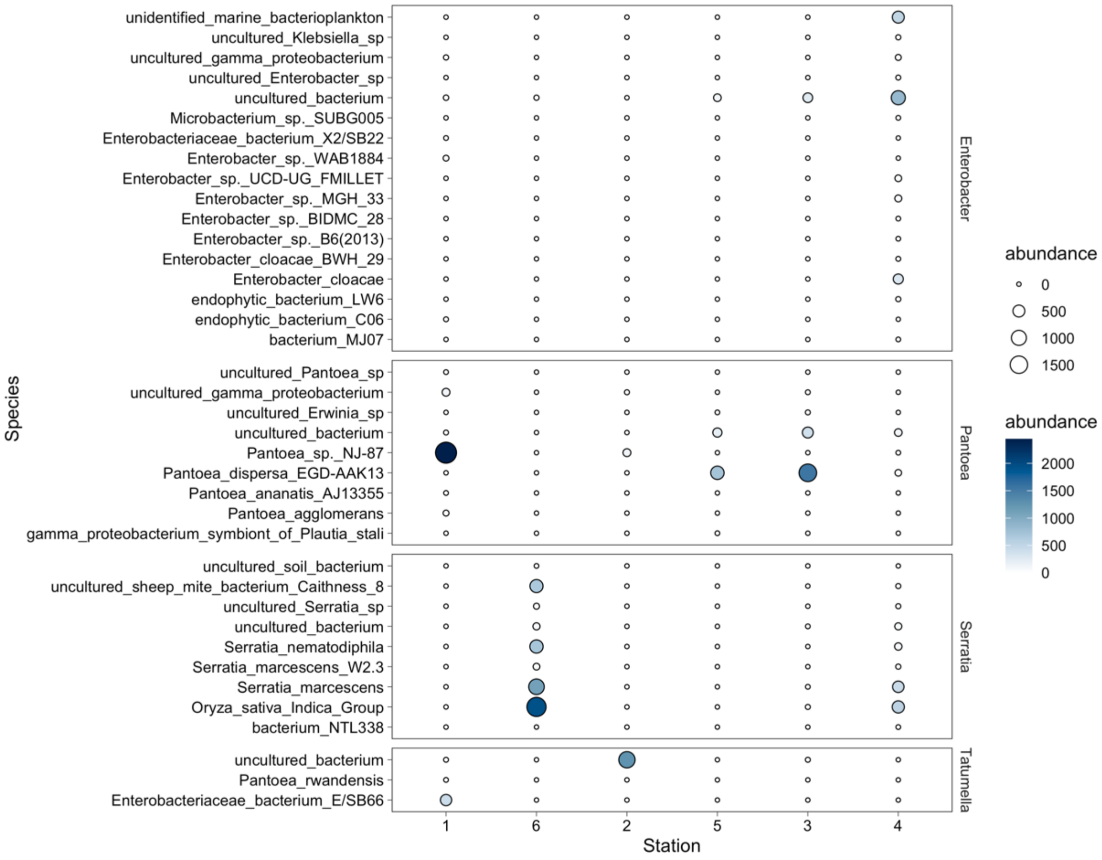

Fig. S2. Abundance of different species of Enterobacteriales in the Gulf of Nicoya.

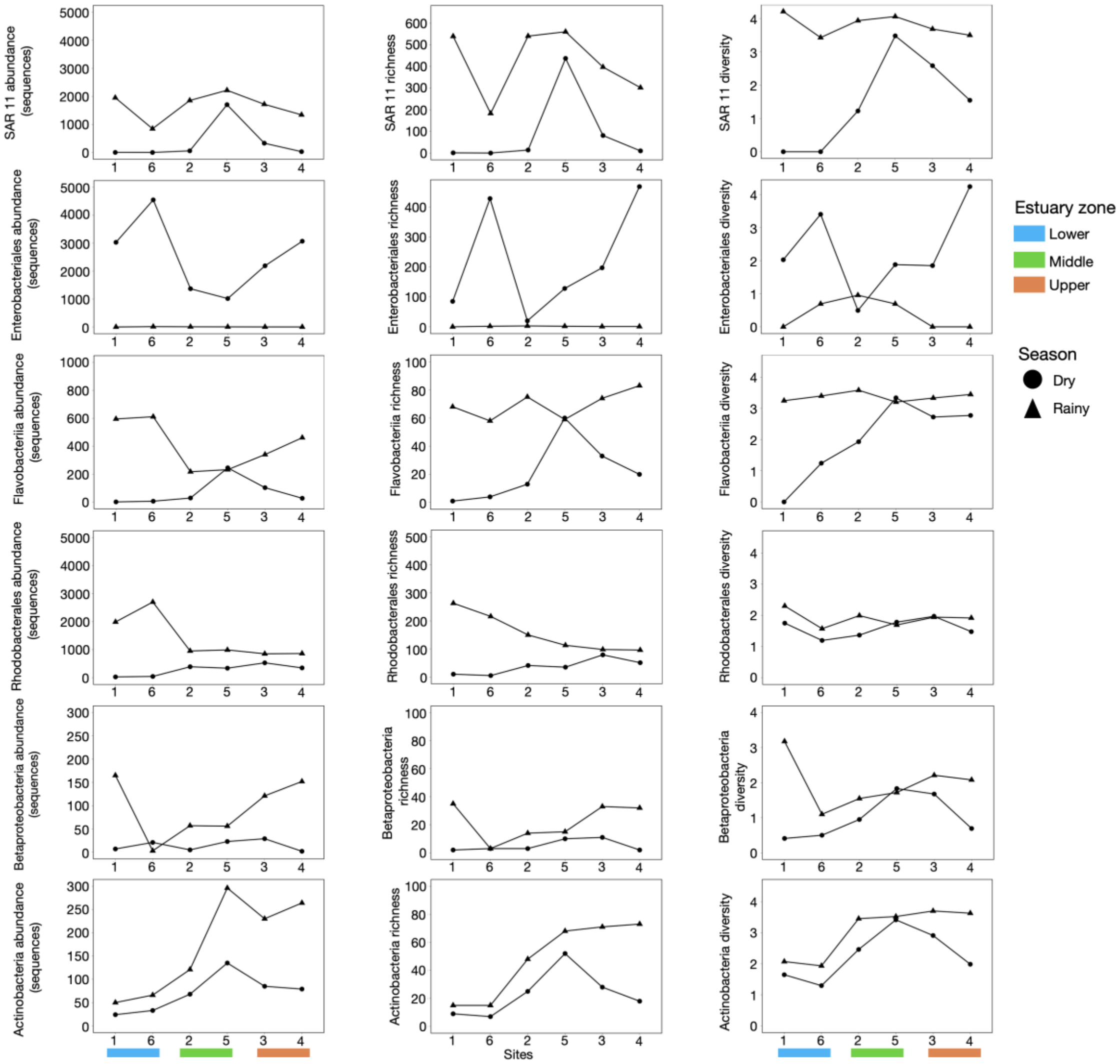

Fig. S3. Bacterial abundance (in terms of number of reads), richness and diversity (Shannon index) during the dry and rainy season for the most abundant taxonomic groups. Note different scale in y-axis.

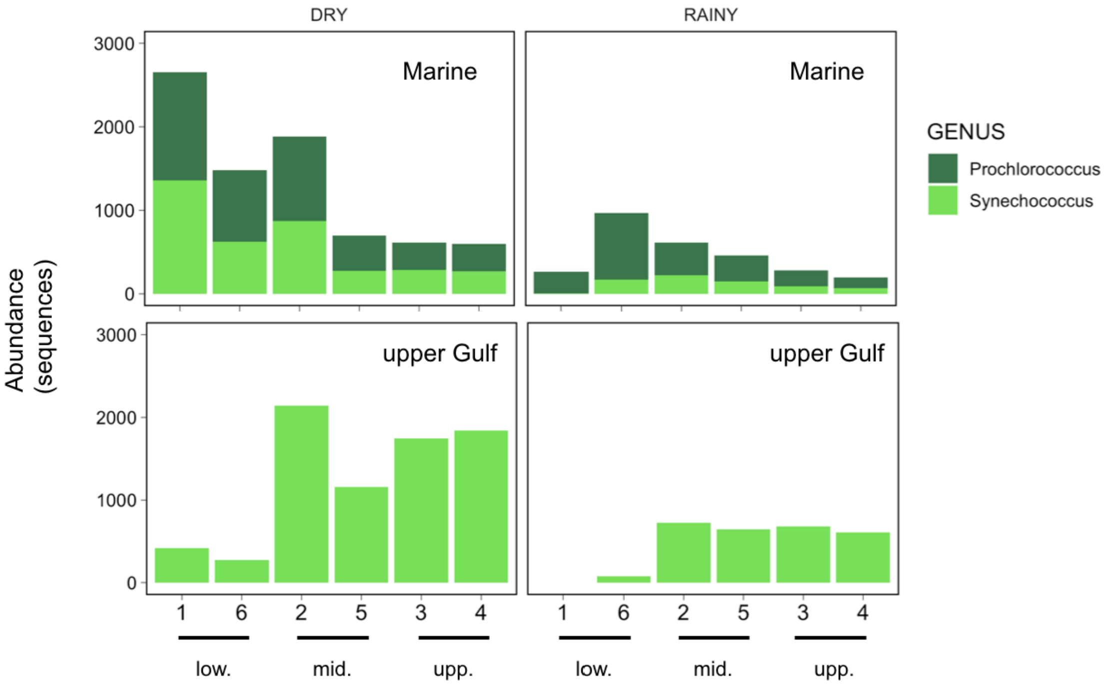

Fig. S4. Contribution of *Prochlorococcus* and *Synechococcus* to cyanobacterial ‘marine’ (upper panels) and ‘upper Gulf’ (lower panels) shifters (see main text for details) during the dry and the rainy season in the lower, middle and upper zones of the Gulf of Nicoya.

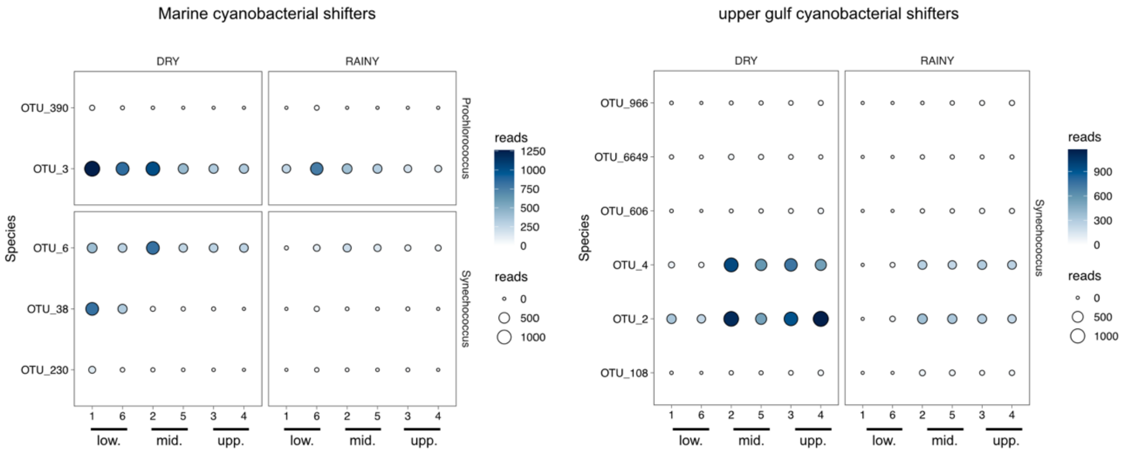

Fig. S5. Abundance of different OTUs of *Prochlorococcus* and *Synechococcus* belonging to the ‘marine’ shifters (left panel) and ‘upper gulf’ shifters (right panel) during the dry and the rainy season in the Gulf of Nicoya.

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Chapter 2 Cross-shore distribution of bacterioplankton communities in different areas of the Western Mediterranean.

The Mediterranean Sea is the largest
semi-enclosed basin on the planet

ABSTRACT

Coastal zones are productive ecosystems that are exposed to changing environmental conditions as well as to the impact of human activity, and are extremely sensitive to global change. Despite the important role that bacterioplankton communities play in biogeochemical cycles and as sentinels of changes in environmental conditions, very little research has been done on the impact of differential land-uses and anthropogenic pressures on the community structure of bacterioplankton along cross-shore gradients. In this work, we sampled four coastal-offshore transects in the northwestern Mediterranean in areas with different population densities and levels of agriculture and riverine impact to determine the continental effect on the free-living and particle-attached fractions of bacterioplankton communities. Our results show no obvious common changes in diversity as we move towards open sea but more community differences at the most coastal sites than in the more oceanic ones, a pattern more evident in the particle-attached communities. We also inspected the connectivity between communities both within the gradients and among them. The free-living open ocean communities contained ca 80% of the ASVs of the more coastal communities, except for the Girona transect that had an unusual coastal sample. In the particle-attached communities the values ranged from 50-70%. Among transects, and taking as a reference the Barcelona transect, the free-living communities consisted in ca. 75% of the community in the Barcelona more coastal station after 1500 m from the coast. This value was lower (50%) and much more variable for the particle-attached communities that were more distinct between sites. Despite the degree of shared species across areas was very high the community structure was different enough and characteristic across environmental gradients and across areas.

1. Introduction

Coastal zones are highly productive sites and breeding, nursery, and recruitment areas for many marine organisms (Barbier et al., 2011; Vasconcelos et al., 2011). They have great socioeconomic value supporting industry, trade, and growing urban populations, in addition to the provision od important ecosystem services such as nutrient cycling and food (Costanza et al., 1997). These ecosystems are highly impacted by anthropogenic activities such as fishing, tourism, and commercial and recreational shipping, as well as by the input of rivers and groundwater, and are the first marine environments to be impacted by global change (Nogales et al., 2011; Vasconcelos et al., 2011).

In order to carry out integrated coastal management, it is necessary to understand the functioning of these ecosystems (Flo et al., 2011; Leach et al., 2018), and a fundamental part is the structure and composition of microbial communities. Microorganisms fix carbon and nitrogen and are responsible for the remineralization of organic matter, forming the basis of marine food webs ,driving global biogeochemical cycles, and can be modulated by natural or anthropogenic perturbations, acting as sentinels of ocean's health (Stewart et al., 2008).

Environmental gradients such as temperature, salinity, and nutrient concentration influence the diversity and abundance of bacterioplankton (Fortunato et al., 2012; Fuhrman et al., 2008; Lozupone and Knight, 2007), which, in turn, influences the functioning of ecosystems (Lambert et al., 2021). Diverse studies have been conducted on the effect of environmental gradients on bacterioplankton distribution in systems such as estuaries, coastal transitions and marine basins (Doherty et al., 2017; Fortunato and Crump, 2011; Ghosh and Bhadury, 2019; Hu et al., 2020; Sebastián et al., 2021). However, more studies are needed in different environments to understand how differential land or human impact may alter bacterioplankton community structure distribution.

Describing the distribution patterns of microbial communities in areas transitioning diverse environmental gradients will contribute to our understanding of the implications of global change and the anthropogenic effect on coastal ecosystems (Basterretxea et al., 2018). In addition, we were interested in describing how well connected were communities along transects from the coast to the open ocean, and how well connected were they among each other, i.e. how many bacterial “species” appeared both at the coast and in the open ocean, and how many of them co-occurred in transects separated by a few hundred km or even, across the Catalano-Balearic Basin of the NW Mediterranean Sea. We set to assess the effect of environmental gradients on the bacterioplankton community structure in four areas of the northwestern Mediterranean Sea, covering different degrees of land and anthropogenic impact, and separated by varying distances.

2. Materials and methods

2.1. *Study area and sample collection*

Samples were obtained in July 2014 in four regions in the Balearic and Catalan coasts, encompassing varying land uses from agricultural to urban. In the four regions, sampling was performed at one-meter depth, following a transect from the shore to 4 km offshore. The northernmost transect was located near the town of L’Estartit (labelled as Girona In Fig. 1), with a low population density, agricultural activity, and the influence of a river with high nutrient loads (Liquete et al., 2009). The second transect was done near Barcelona, a highly populated urban area with high commercial and recreational shipping transit activity (Basterretxea et al., 2018). The third transect was performed south of L’Hospitalet de l’Infant (labelled as Tarragona), in an area with low population density, but affected by agricultural activity, and potentially by groundwater discharge. Finally, the four transect was performed close to Palma in the Mallorca island, a fairly densely populated island with intense agricultural activity and the effect of nutrient-rich groundwater (Basterretxea et al., 2010; Rodellas et al., 2014; Tovar-Sánchez et al., 2014).

2.2. *Bacterial abundance*

Samples (1.6 mL) were fixed using a solution of 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration) deep frozen in liquid nitrogen and stored at −80 °C until analyzed. Subsamples for prokaryotes were stained with SybrGreen I (Molecular Probes, final concentration 1000× dilution of the commercial product) for some minutes in the dark. For picophytoplankton analyses, subsamples were unstained. The cell counting was performed by using a BD FACSCalibur cytometer with a laser emitting at 488 nm and following Gasol and Morán (2015).

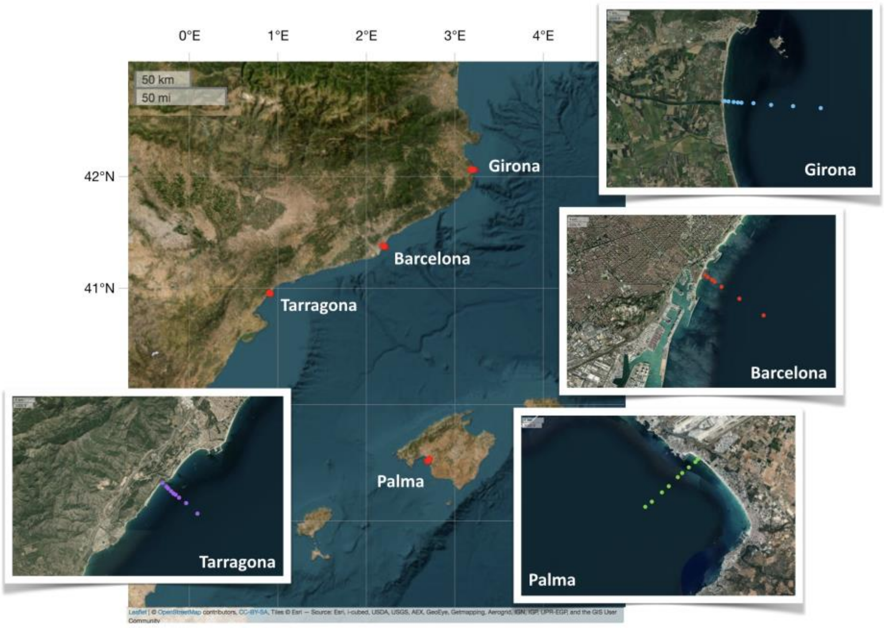

Fig. 1. Sampling area and location of the sampling sites. Maps created in R with package *leaflet* and the ESRI World Imagery background.

2.3. DNA extraction and sequencing

Water samples (10L) were sequentially filtered through a 20 µm mesh onto 3 µm and then 0.2 µm polycarbonate filters and frozen in liquid nitrogen; DNA was later extracted with the phenol-chloroform protocol following (Massana et al., 1997). The 357wF and 806bR (Apprill et al., 2015) primers were used to generate amplicons spanning the V4 region of the bacterial 16S rRNA gene.

Primers and spurious sequences were trimmed using cutadapt (Martin, 2011). DADA2 v1.8 was used to differentiate exact sequence variants (ASVs, Callahan et al., 2016). This software resolves ASVs by modelling the errors in Illumina-sequenced amplicon reads.

2.4. Statistical analysis

The data were grouped by location (Girona, Barcelona, Tarragona and Palma) and size fraction (free-living and particle-attached) for statistical analyses. Diversity index calculations (Shannon H´ and Chao1), non-metric multidimensional scaling (nMDS) and taxonomy plots were performed and plotted in R (*stats* package). We used one-way analyses of variance (ANOVAs) to test for significant variations in microbial diversity and PERMANOVA for differences in abundances of microbial communities. All analyses were performed with R (3.6 version) and Rstudio software (1.2.1335 version).

3. Results and discussion

3.1. Environmental setting

Salinity was constant along the Barcelona and Palma transects, while in Girona and Tarragona, abrupt decreases in salinity were observed, likely caused by the influence of the river and groundwater, respectively (Fig. 2). These fresh water inputs caused an increase in chlorophyll, and in the abundance of heterotrophic bacteria. Palma showed decreasing values of chlorophyll towards offshore waters, whereas in Barcelona there was a peak in the middle of the transect, around 1000m from the coast. Heterotrophic prokaryotes showed decreasing values from the coast towards the open ocean in Palma and Barcelona, but in the later the values remained high in offshore waters (Fig. 2). The temperature was roughly constant in each of the transects, with values decreasing in this order: Palma>Tarragona> Barcelona> Girona, and coincided with typical summer temperatures in the northwestern Mediterranean (Fig. 2). Inorganic nutrients showed a considerable increase in Girona, probably caused by the effect of the river. Palma showed an elevated peak in the near-shore zone, possibly related to submarine groundwater discharge. Ammonium fluctuated along the four transects (Fig. S1).

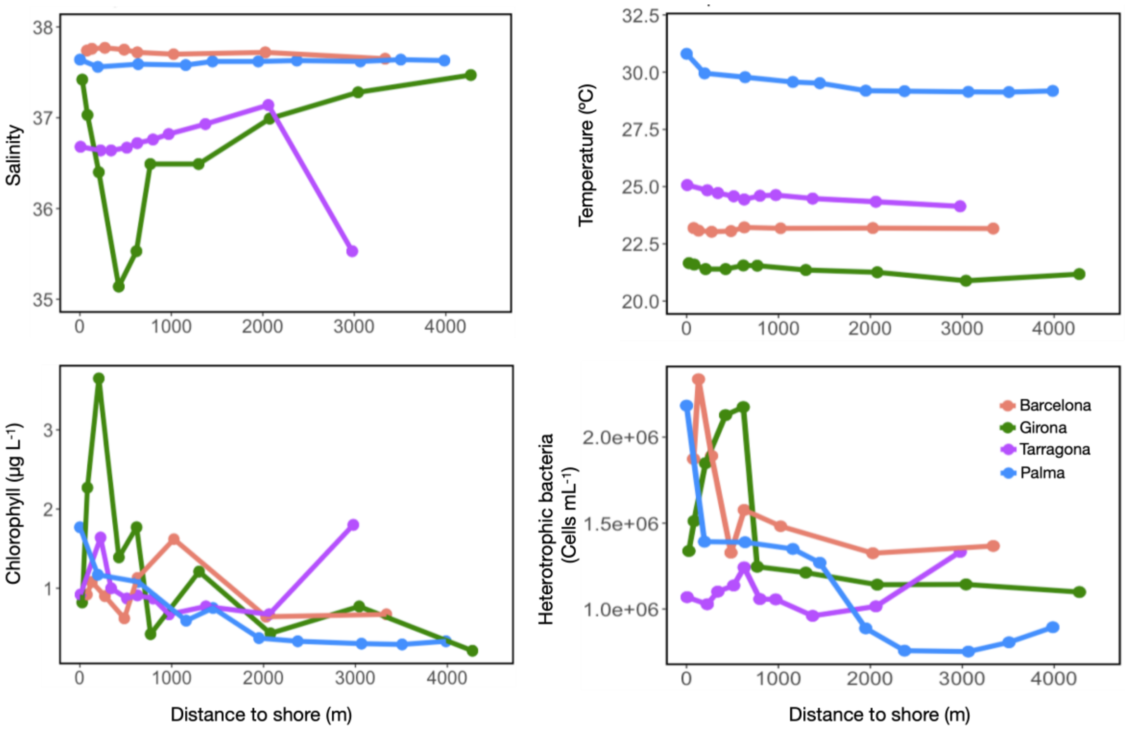

Fig. 2. Environmental parameters along the four gradients studied: Barcelona, Girona, Tarragona ad Palma.

3.2. Changes in alpha and beta diversity along the transects

Bacterial communities showed overall the highest richness and Shannon diversity values in the Barcelona and Girona transects (Fig. S2). Richness in these two transects was higher in the particle-associated than in the free-living bacterial communities, whereas in Palma and Tarragona the opposite pattern was observed (Fig. S2).

The diversity indexes (Shannon and richness) were generally quite variable along the transects, except for the Barcelona transect, where values were more uniform in both size fractions (Fig. 3, Fig. S3). The effect of salinity on alpha diversity could also be observed, as decreases in salinity generally coincided with important increases in the indexes in the Girona and Tarragona transects (Fig. 2, 3, S3).

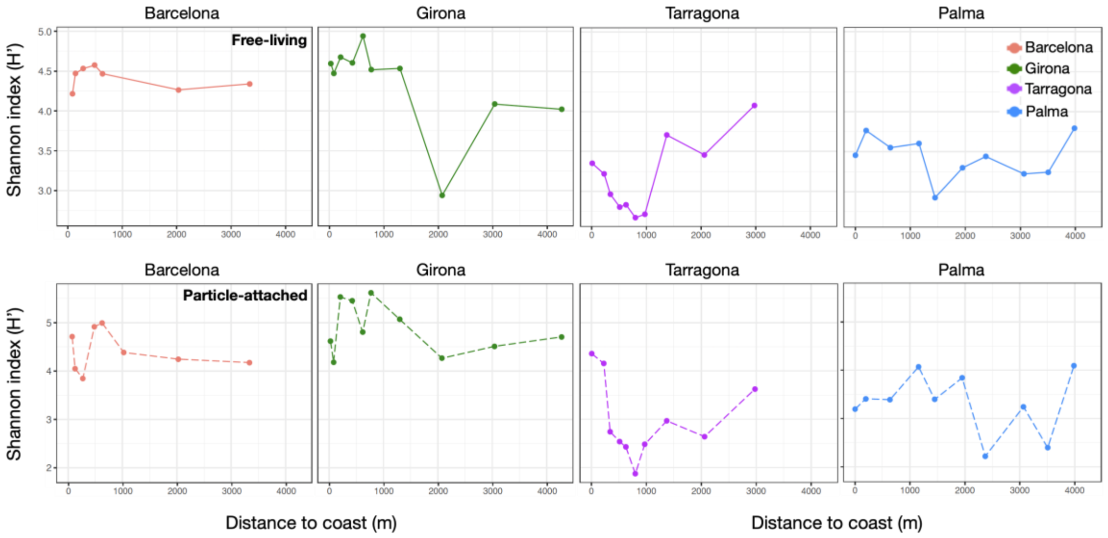

Fig. 3. Bacterial diversity estimates (Shannon index) by location, along the four different gradients.

Regarding the structure of the prokaryotic communities, an evident clustering was observed according to the different regions and also according to distance to shore (PERMANOVA $p<0.001$; Fig. 4A-D) and, to a lower degree due to salinity (PERMANOVA $p<0.05$ and 0.01 for free-living and particle-attached, respectively; Fig. 4E, F). Community structures were more similar (closer ordination in the nMDS) in the free-living fraction than in the particle associated one. Salinity had a strong effect in the community structure of particle associated communities of Girona and Tarragona (Fig. 4F). Also, the offshore free-living communities had more similar compositions than the communities closer to shore (Fig. 4C). Previous studies have shown that distance to shore (Luna, 2015; Mo et al., 2018) as well as salinity gradients influence the structuring of bacterioplankton communities, particularly in areas with riverine influence (Bouvier and del Giorgio, 2002; Campbell and Kirchman, 2013; Telesh and Khlebovich, 2010). The fact that offshore communities were more similar among the transects than the coastal ones suggests that continental pressure was different across gradients, yet limited to the areas closer to shore.

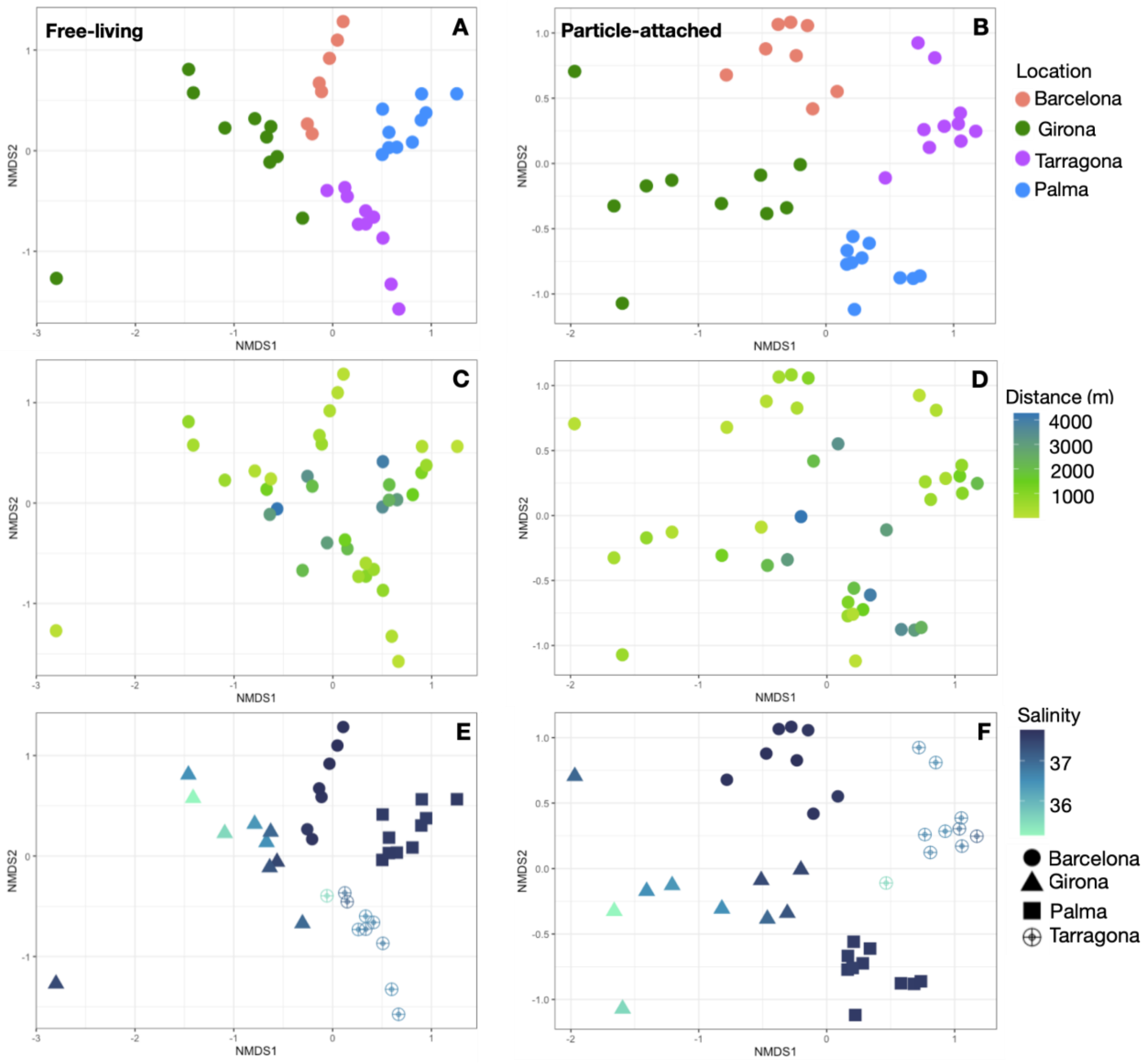

Fig. 4. Nonmetric multidimensional scaling (nMDS) ordination plots showing clustering of prokaryotic communities according to area (A, B), distance to shore (C, D) and salinity (E, F).

3.3. Changes in community composition along the different transects

To further understand the variations observed in community structure we investigated the taxonomic composition of the bacterioplankton communities along the four transects (Fig. 5).

In the Barcelona transect, the taxonomic composition of the free-living communities fluctuated in the first 500 m from shore, being this zone the only one where Actinobacteria were found. From this point towards offshore waters, community composition was rather uniform (Fig. 5). Alteromonadales, Oceanospirillales, and Rhodobacterales decreased towards offshore waters, and the contribution of Synechococcales and SAR11 increased. In the particle-attached fraction, there was higher heterogeneity along the transect than in the free-living communities, but not as evident as in the rest of the areas (Fig. 5). Flavobacterales and Rhodobacterales presented abundance peaks in the near-shore zone and then decreased. From 1000 m towards offshore waters, community composition in the particulate fraction was also rather uniform, similar to that of the free-living communities.

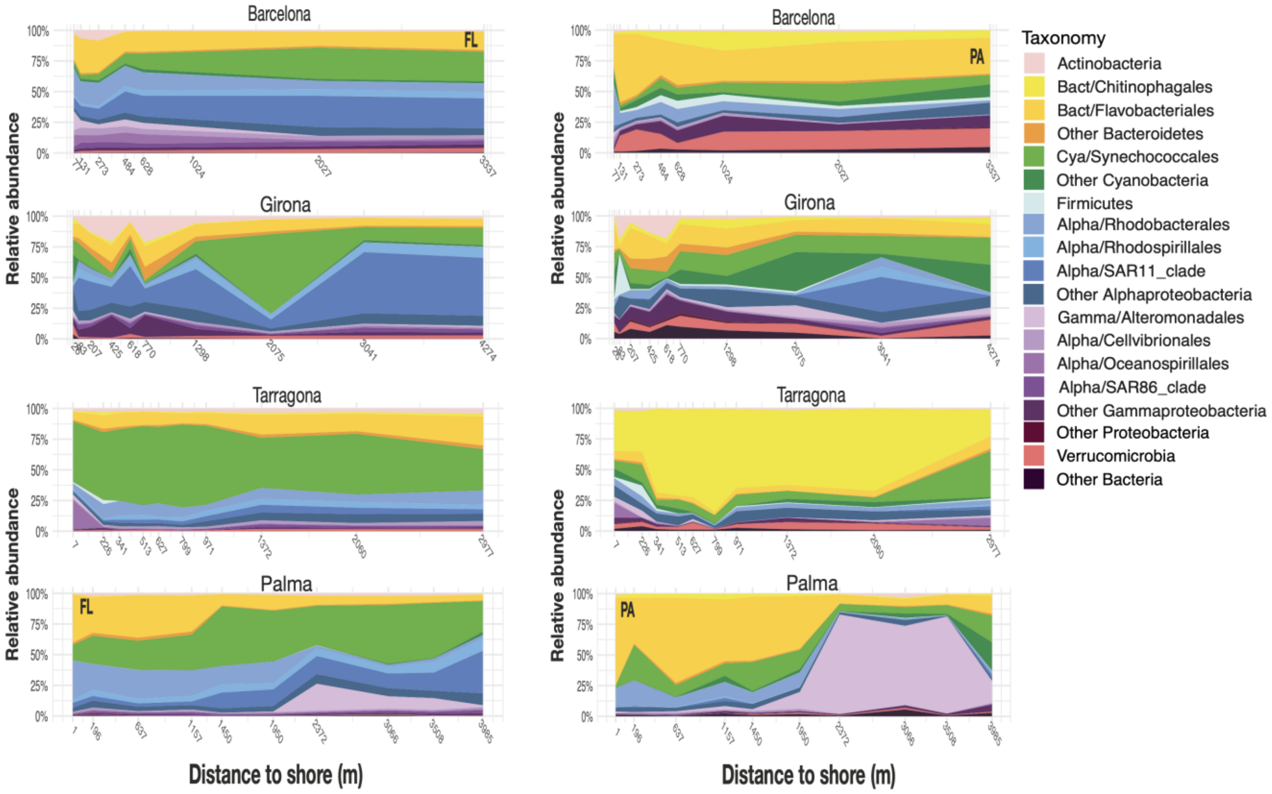

Fig. 5. Taxonomic composition along the four transects in both size-fractions. Bacterioplankton taxonomy is shown at the level of phylum except for Bacteroidetes, Cyanobacteria, Alpha- and Gammaproteobacteria that are presented at the order level. Only taxonomic groups with relative abundances >1% are represented, whereas the remaining sequences were pooled as “other bacteria”. PA: Particle-attached, FL: Free-living.

In the Girona transect, even if relatively more similar to the Barcelona transect, we observed the most remarkable variation in community composition, most likely associated to the riverine influence. In the middle section of the transect (at 2075 m) the relative contribution of SAR11 to the free-living communities decreased strongly, coincident with a maximum in Synechococcales. This change in taxonomic composition led to a drop in the diversity indexes at this site

(Fig. 3), and coincided with a larger contribution of coastal ASVs to the free-living communities (see later Fig. 6). There seems to be special conditions for this to occur in this area, as it does not happen in other parts of the transect. In the particle-attached fraction, Actinobacteria and Firmicutes represented a large fraction of the community in the freshwater-influenced area, but they decreased considerably afterwards. Around 3000 m from shore (where salinity increased again), there was a notable change in community structure, with an increase in the contribution of SAR11.

In the Tarragona transect the composition of the free-living bacterial community was rather homogeneous, with a dominance of Flavobacteriales and Synechococcales (Fig. 5). In near-shore waters there was a larger contribution of Firmicutes and Oceanospirillales that disappeared towards offshore waters (Fig. 5). In the particle-attached community, Chitinophagales dominated throughout the transect, representing ca. 75% of de community from 341 to 2060 m from shore. At the end of the transect, particle-associated communities had a larger representation of Synechococcales, coincident with the decrease in salinity potentially associated with groundwater discharge (Fig. 1). In the Palma transect, we observed in the free-living size fraction a shift in community composition at >1000 m from shore, where the contribution of Flavobacteriales decreased near 1500 m, coincident with an increase in Synechococcales. Also, around >2000 m, the relative abundance of Rhodobacterales decreased and instead Alteromonadales increased notably in abundance (Fig. 5). In the particle-associated size-fraction the shift in community composition was much more dramatic: Flavobacteriales, which represented around 50% of the communities in the first 2000 m were replaced afterwards by Alteromonadales, which accounted for ~80% of the communities from 2300 m to 3500 m, and then decreased slightly at the most offshore station.

To further explore the continental effect on the bacterioplankton communities in each of the transects, the ASVs present in the first (most coastal) site of each transect were selected, and the contribution of these ASVs to total read abundance and total number of ASVs along each transect were computed (Fig. 6).

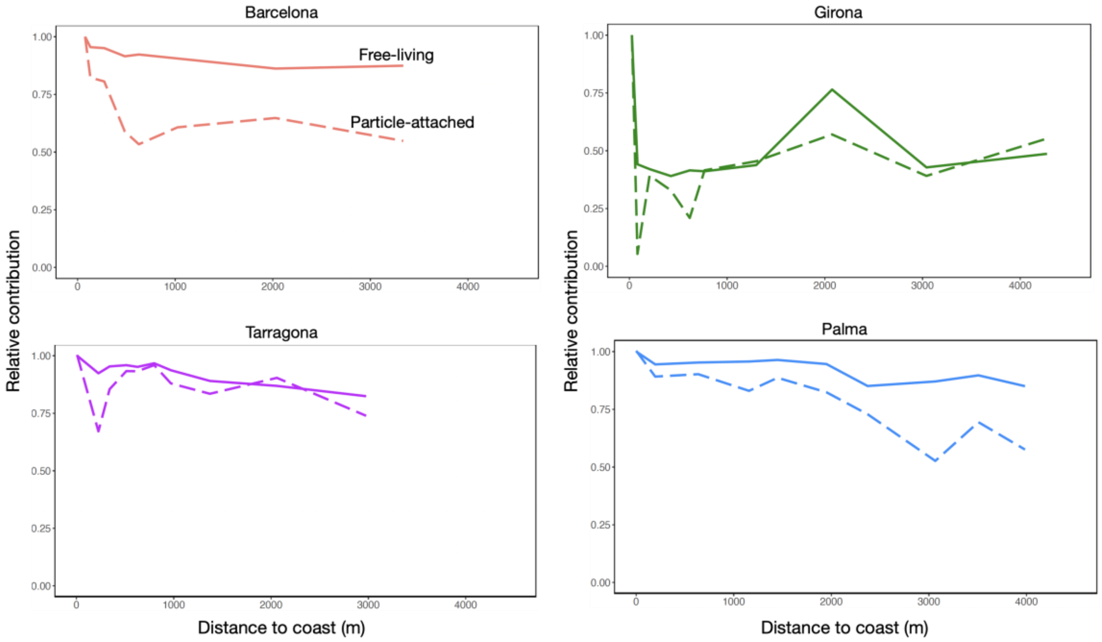

Fig. 6. Relative abundance of coastal ASVs along the four transects and in both size-fractions. Those ASVs that were present in the most coastal sample were selected and their relative contribution to total abundance along each transect was plotted.

In the free-living fraction, the contribution of coastal ASVs along the transects decreased slightly towards offshore waters, but represented between 80 and 90% of the sequences in all transects except for the Girona one, where a marked decreased was observed within the first 1300 m of the transect (Fig. 6, solid line). In this area, influenced by riverine discharges, coastal ASVs represented only 45% of the communities at the end of the transect (Fig. 2 and 6).

In the particle-attached fraction, the contribution of coastal ASVs decreased more rapidly (Fig. 6, dashed line). In Barcelona, the relative abundance of the coastal ASVs decreased down to 50% at around 600 m from shore; the same occurred in Palma but farther from shore, at around 3000 m. In Girona, the contribution of coastal bacterial ASVs fluctuated along the transect, similarly to the free-living size fraction, but with more remarkable variability in the zone of riverine influence, where they almost disappeared. The contribution of coastal ASVs to the particle-associated fraction fluctuated throughout the Tarragona transect, but was generally above 75% (Fig. 6). Prokaryotes in this size fraction represent cells that are associated to abiotic (i.e. mineral) and biotic particles, which may be formed by either living phytoplankton or detritus. Rivers transport inorganic and organic particles towards the coastal margin (Beusen et al., 2005), which may explain the low proportion of coastal ASVs in the area of the Girona transect affected by riverine discharge (Fig. 6). Shifts in particle-associated bacterial composition are known to occur following shifts in phytoplankton composition (Teeling et al., 2012), and a previous study in the same transects observed changes in the abundance of different large phytoplankton from the coastal margin to offshore waters (Basterretxea et al., 2018). This could be the reason for the generally lower contribution of coastal ASVs to particle-associated communities towards offshore waters, particularly in the Barcelona and Palma transects

The combined observations of Figs 5 and 6 indicate that although coastal ASVs represented in most cases a large fraction of offshore communities (Fig. 6), there were considerable changes in the relative abundance of these coastal ASVs along the transects in both size fractions. This is in agreement with other studies where they also compared the coastal zone with more oceanic samples (Fortunato et al., 2012; Garate et al., 2022; He et al., 2018; Wang et al., 2019).

3.4. Connectivity of prokaryotic communities between transects

We next evaluated the degree of connectivity (i.e. the pool of taxa shared) between the different areas by analyzing the distribution of Barcelona coastal ASVs as they appeared in the other transects (Fig. 7).

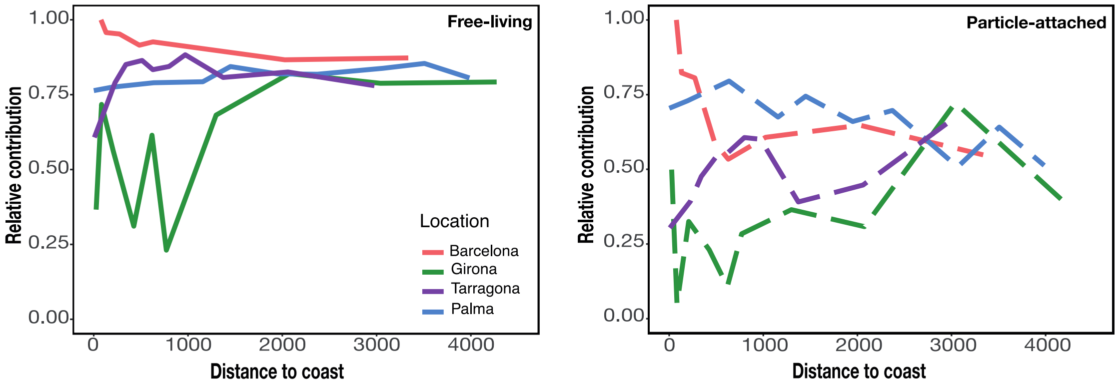

Fig. 7. Total relative contribution (in terms of reads) of Barcelona's coastal ASVs along the 4 transects in both size-fractions.

The contribution of Barcelona's coastal ASVs to the free-living communities was above 75% from the middle section of the transects (~2000 m) to offshore waters even in places as distinct as Palma (Fig 2) and separated >200 km, but was lower in near-shore waters (Fig. 7), highlighting the imprint of the different continental pressures. Barcelona's coastal ASVs represented around 60% of the free-living communities in coastal waters of Tarragona, and variable proportions, between 25% and 70%, of the communities in the areas affected by riverine discharge in the Girona transect. In the particle-attached fraction, the contribution of Barcelona's coastal ASVs was more heterogeneous, but generally lower than in the free-living realm (Fig. 7). Even in the sites where Barcelona's coastal ASVs represented 75% of the communities, there were large variations in the relative abundances of the different taxonomic groups (Fig. S3). This indicates that despite the degree of shared species across areas was very high (i.e. there was a high connectivity between the different marine systems), the community structure was different enough and characteristic across environmental gradients and across areas.

Dispersal by ocean currents is a key mechanism connecting distant bacterial communities across systems. The biogeography of bacterial communities thus results from the balance between two opposing forces: dispersal and selection by local environmental conditions (Ward et al., 2021). Communities from the Balearic and Catalan coasts seemed to be largely interconnected, but environmental conditions drove the changes in community structure, particularly nearshore. The larger divergence generally observed in the near-shore waters was likely a consequence of the different land uses and natural and anthropogenic impact among the four areas. In contrast, at distances from the coast >1.5-2 km the free-living communities were quite similar, indicating that the anthropogenic impacts were likely diluted at these distances.

Few studies have sampled bacterial community composition at the short scales we sampled, that comprised the coast and the continental platform, and in some cases (i.e. Palma) within a bay. Wang et al. (2019) studied an 87 km transect, well beyond the continental slope. They found distance from shore and temperature to drive differences in community composition and they postulated that the continental platform communities were composed by some members from the offshore waters and some from the very coastal stations. Across a similar distance in the German Bight at the mouth of the Elbe river estuary, Lucas et al. (2016) found bacterial communities from the river mouth and the open sea to be strongly segregated, something compatible with the well-known effect of low salinity river plumes on community composition (Fortunato et al., 2013; Hewson and Fuhrman, 2004; Satinsky et al., 2017) and basically the effect of salinity in determining the global bacterioplankton community structure (Lozupone and Knight, 2007). They also observed that this effect was stronger in the particle attached communities. These results concur with our observations of the effect of decreased salinity on community composition (Figs 2A and Fig 5) and the stronger variability of the particle-attached communities (Fig 3). Finally, Haber et al. (2022) in the Eastern Mediterranean also looked at the differences in community composition along an inshore-offshore transect. Their first station, however, was at 16 km from the coast. It was distinct from the stations further away across the continental slope, yet this spatial scale was rather different than the one we sampled. In all these studies, as well as in ours, it is quite common to observe that the coastal-more stations are different from the offshore sites. Yet, in our case, and while a difference in community structure exists (Fig 4), we show that in some of the studied gradients (e.g. Barcelona or Tarragona, where no freshwater inputs are obvious) the communities offshore (i.e. > 3 km from the coast) are quite similar (>75% ASVs, Fig 6) to those of the more coastal station.

Finally, our study was done in a relatively short time interval (July 2014) yet we observed 9°C differences between the Palma and the Girona transects. The temporal evolution of bacterioplankton community structure has been seen to strongly influence the gradients in community composition (Haber et al., 2022; Lucas et al., 2016; Wang et al., 2019), and this might have contributed to the observed variability in community composition at the different sites (Fig 4) and to the relatively low connectivity between Barcelona coastal communities and those at the coast in the other sites. Yet, in spite of these temperature differences, the communities at the offshore sites had a large number of common ASVs (Fig 7), indicating that a relatively reduced pool of bacterial species contribute to bacterioplankton community structure in areas within at least 200 km, and even across the Catalano-Balearic Sea.

4. Conclusions

We compared the effect of cross-shore gradients on bacterioplankton communities along four inshore-offshore transects in the northwestern Mediterranean Sea, covering areas with different degrees of land use and anthropogenic impact. The prokaryotic taxa present in the four areas were largely shared, but their relative abundances fluctuated between transects and distances from shore, likely reflecting changes in environmental conditions. Communities were more similar among the four areas in offshore waters, indicating that the continental effect was limited to near-shore waters. Riverine discharge and freshwater intrusions through groundwater discharge caused drastic changes in community composition. These results contribute to the knowledge about bacterial biogeography in coastal ecosystems, and how different taxonomic groups vary along environmental gradients. This information is particularly important to evolve to a predictive understanding of how the ocean is going to evolve in the context of global change.

Acknowledgements

I thank Isabel Ferrera, Adrià Auladell, Albert Reñé, Elisabet Alacid, Gotzon Basterretxea and Esther Garcés for their help in the sampling, in the lab and with the data analysis. The help of I. Esteve and E. Garcés was particularly important. This study was financed by project DEVOTES, grant FP7-ENV-2012-308392 from 7th Framework Program of the UE to E. Garcés. The author was supported by a PhD grant from Universidad Nacional (Costa Rica), and the ICM authors were supported by a Severo Ochoa Excellence Award (Ministerio de Ciencia e Innovación) CEX2019-000928-S.

Supplementary Information

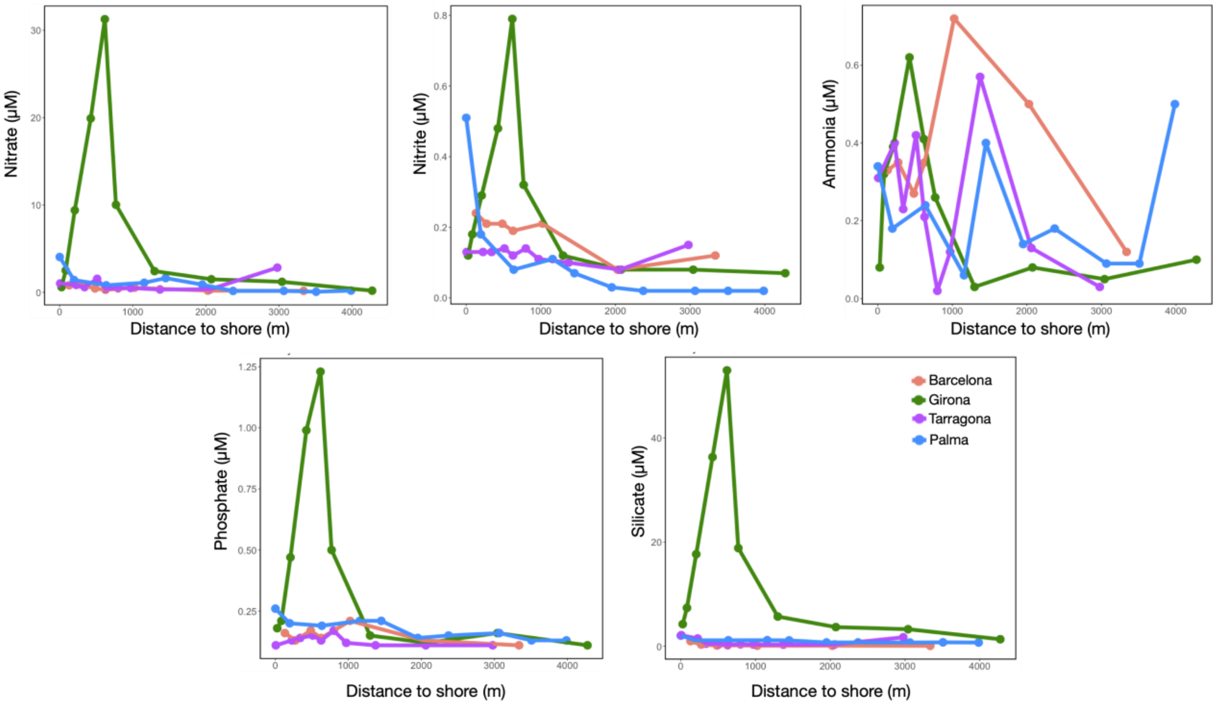

Fig. S1. Inorganic nutrients along four locations in the Northwestern Mediterranean Sea.

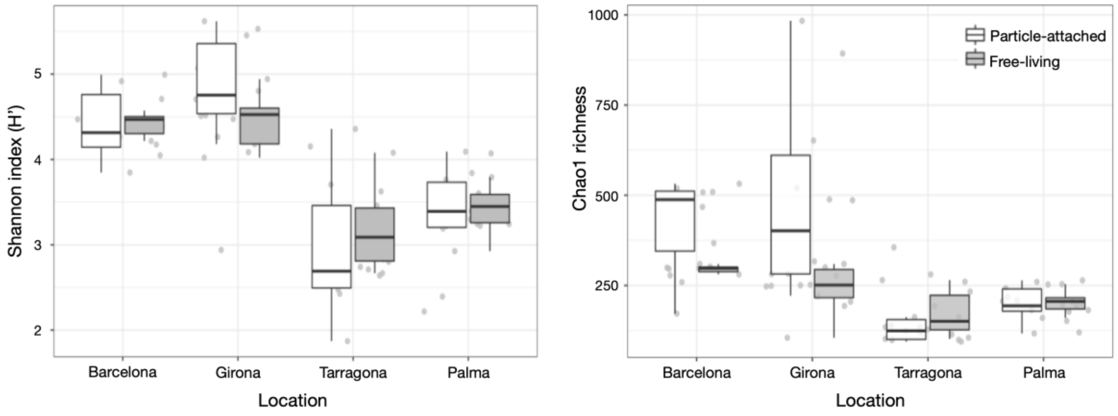

Fig. S2. Shannon diversity (H') and richness (Chao1) indexes of bacterioplankton communities at the four sites in the northwestern Mediterranean Sea. White boxes represent the particle-attached and gray boxes the free-living fraction. The points indicate the samples.

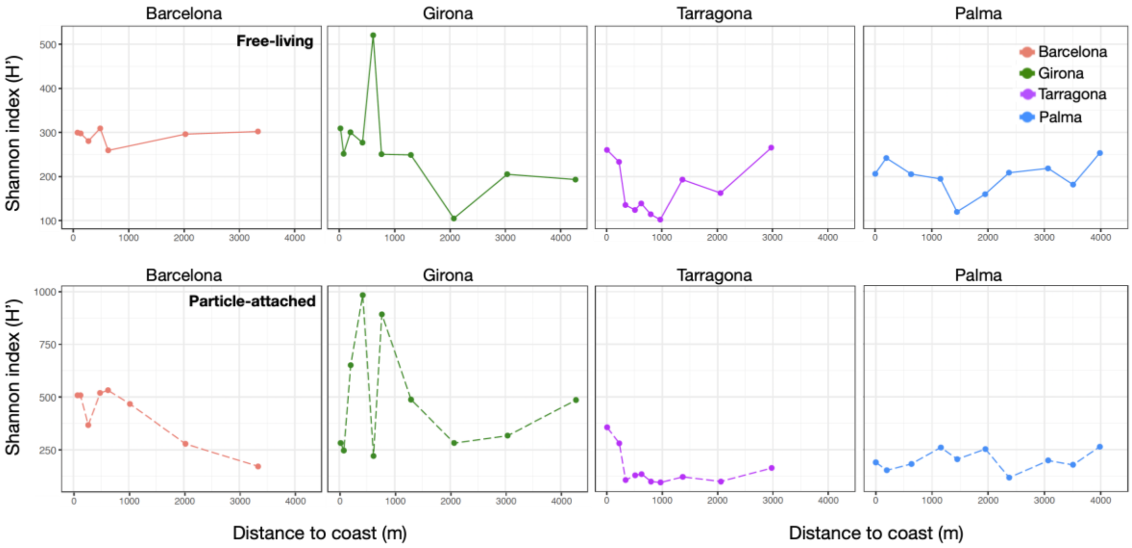

Fig. S3. Richness index (Chao1) of bacterioplankton communities along transects at four sites in the NW Mediterranean Sea. The continuous lines represent the free-living fraction and the dashed lines the particle-attached fraction.

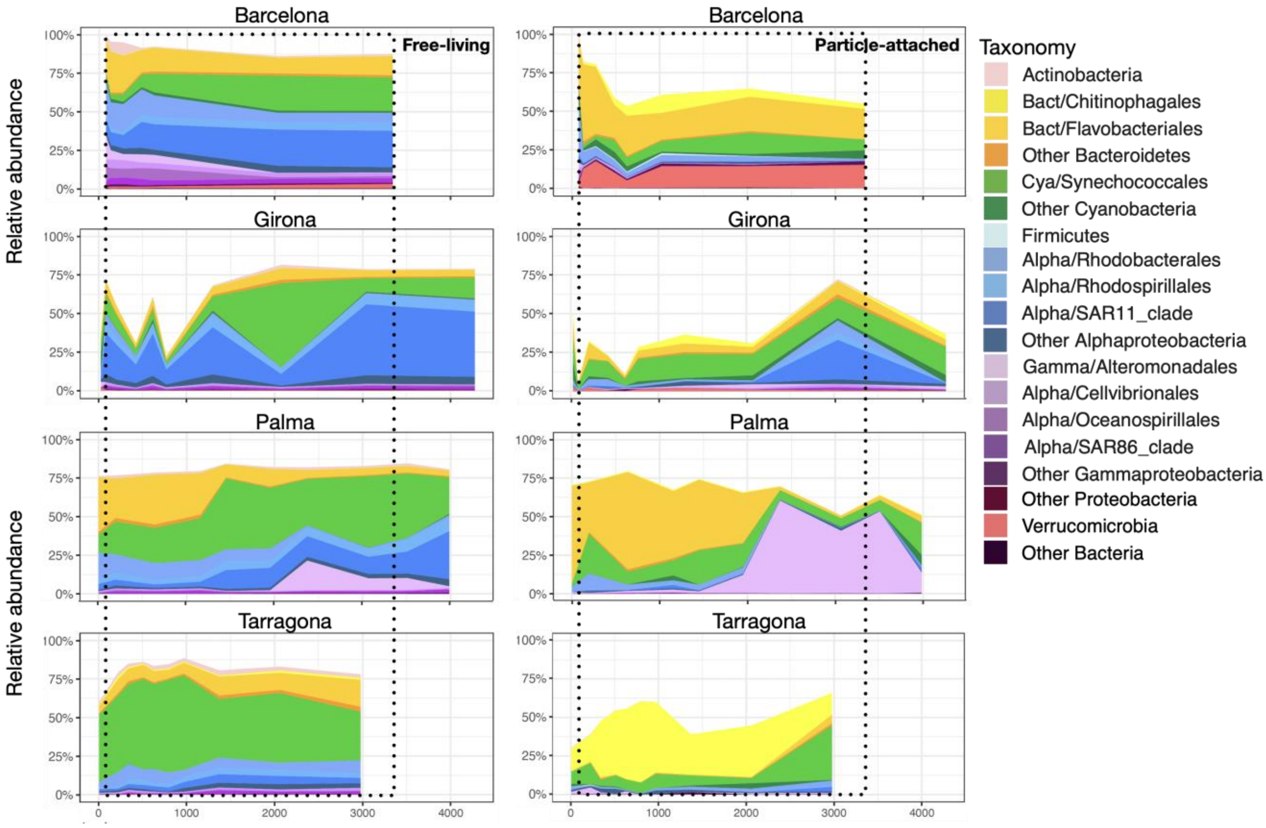

Fig. S4. Taxonomic affiliation of Barcelona's coastal ASVs as they appear along the four transects. Bacterioplankton taxonomy is shown at the level of phylum except for Bacteroidetes, Cyanobacteria, Alpha- and Gammaproteobacteria that are at the order level. Taxonomic groups with relative abundances >1% are represented, whereas the remaining sequences are pooled under "other bacteria". PA: Particle-attached, FL: Free-living. Pointed line delimits common transect distances.

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Chapter 3

Fine-scale structuring of microbial populations across the Deep Chlorophyll maxima layers in the NorthwesternMediterranean Sea

Si hay magia en este planeta,
está contenida en una gota de agua.
Loren Eiseley

ABSTRACT

The deep chlorophyll maxima (DCM) is likely the most important feature organizing the epipelagic environment. At the DCM, contrasting gradients of light and nutrients determine highly stratified marine habitats. While some knowledge exists about the distribution of phytoplankton along the DCM, almost nothing is known of the changes in bacterial community structure through this feature. We investigated the vertical distribution of prokaryotes and small eukaryotes in two size fractions (0.2 -3 µm and 3 – 20 µm) through 4 samplings at the same station, 2 during the day and 2 during the night, to also inspect potential changes related to the daily cell cycle of phytoplankton. Prokaryotic richness increased with depth, while that of small eukaryotes had a maximum at the depth of maximal chlorophyll. Larger eukaryotes had a rather uniform change in richness with depth. Eukaryotic communities changed remarkably at the depth of maximal chlorophyll while prokaryotic communities changed more gradually. We distinguished 6 groups of prokaryotes and 4 groups of eukaryotes based on their distribution patterns with depth: organisms present in the upper layer, others in both the upper layer and upper DCM, others increasing below the DCM, and others with maximal abundance at the DCM peak, and these groups had specific taxonomic distributions. In spite of these differential distributions and the sharp change in environmental conditions, both prokaryotic and eukaryotic communities below the DCM were dominated (~70% of the sequences) by taxa already detected at the ocean surface.

1. Introduction

The Deep Chlorophyll Maximum (DCM) is the water layer with the maximum concentration of chlorophyll *a*. It appears in stratified oceans and freshwater ecosystems, at the depth where there is adequate light for photosynthesis and sufficient nutrient supply from below (by upwelling and diffusion) to sustain phytoplankton biomass (Cullen, 1982; Estrada et al., 1993; Latasa et al., 2017). The presence of the DCM requires stability and in the ocean is thus typical of tropical and subtropical zones, where they can persist over weeks to months, whereas in template zones are seasonal, depending on how stratified the water column is (Cornec et al., 2021).

A significant fraction of the water column primary productivity is concentrated in the DCM, representing between 50-60% in highly stratified waters (Lorenzo et al., 2004; Weston et al., 2005). This fact, and the proximity of the DCM to export depth thresholds (Guidi et al., 2016; Tilstone et al., 2017), make this feature a relevant contributor to the biological carbon pump.

DCM's centered studies have mostly focused on addressing the different mechanisms that are involved in their formation and maintenance (Cornec et al., 2021; Cullen, 1982; Moeller et al., 2019) or the vertical distribution of major phytoplankton groups using pigment-markers, flow cytometry or fluorescent *in situ* hybridization (Cabello et al., 2016; Latasa et al., 2017, 1992; Zubkov et al., 2000). The distribution of other members of microbial communities, such as prokaryotes and non-pigmented eukaryotes have only been generally assessed considering a single depth or using a rough resolution of tens of meters (Beman et al., 2011; Giner et al., 2020; Medina-Silva et al., 2018; Mestre et al., 2018; Sunagawa et al., 2015; Walsh et al., 2015). Yet, DCM's are located in a transition where strong vertical environmental gradients occur, and this sampling approach misses most of the spatial heterogeneity of this important oceanographic feature. Indeed, the few high-resolution samplings performed to date have demonstrated that the distribution of both prokaryotes (Haro-Moreno et al., 2018) and eukaryotes (Cabello et al., 2016; Dolan and Marrasé, 1995; Latasa et al., 2017) along the DCM layer is complex and dynamic.

A well-developed deep chlorophyll maximum is a prominent feature of the Northwestern Mediterranean sea during most of the year (Estrada et al., 1993). Knowledge on spatial changes of the marine microbiome along this structure is limited but a previous study on a single location with sampling at 15 m depth intervals showed that significant changes in prokaryotic communities occur within the stratified photic layer (Haro-Moreno et al., 2018). Mechanisms underlying DCM formation such as phytoplankton photoacclimation (Steele, 1962), as well as nutrient availability and grazing (Moeller et al., 2019) change over the diel cycle, following shifts in the light regime or circadian cycles of biological activity, and thus, the vertical distribution of microbes along the DCM may also change. Picophytoplankton are known to divide following diel cycles that vary with depth from the surface to below the DCM (e.g. Vaulot et al., 1995) and bacteria follow at least in terms of activity these diel cycles (Ruiz-González et al., 2012), suggesting that strong changes in bacterial diversity should occur along the DCM.

Here we performed fine scale (every 5 to 10 m) sampling along a DCM structure at a single station in the Northwestern Mediterranean Sea over two consecutive days at 10 h GMT and 20 h GMT. We aimed at investigating spatial patterns in the distribution of the prokaryotes and eukaryotes in two size fractions (0.2-3.0 µm and 3.0-20 µm), as well as their level of connectivity along the DCM, and additionally whether there were potential changes in their depth distribution over the diel cycle.

2. Materials and methods

2.1. Study area and sample collection

Sampling was performed from on board the RV García del Cid from September 27th to September 29th, 2017 at 40º49 N 3º05 E, in the Western Mediterranean sea between the Barcelona coast and the island of Mallorca. Four vertical casts were performed with a CTD profiler on September 27th at 20h, on September 28th at 10h, and on September 29th at 10h and 20h (GMT). Water samples were collected with a 12-bottle Niskin rosette. High-resolution samplings were carried out in all the profiles every 5-10 m within the DCM structure, and additionally samples were collected from the surface and below the DCM (around 160-175 m) in each of the casts. The rosette was retrieved at low speed, and the bottles were closed without stopping the rosette retrieval to avoid disturbing the layered physical and biological structure.

2.2 Chlorophyll a

Chlorophyll a (Chl a) measurements were performed by filtering 200 mL of water through a Whatman GF/F filter stored frozen on board until analysis. The pigment was extracted in cold acetone (90% v/v) for 24 h and analyzed with a 10 AU Turner Designs bench fluorometer, previously calibrated with pure Chl *a* (Sigma Aldrich) (Holm-Hansen et al., 1965).

2.3 Inorganic nutrients

Samples for nitrate, nitrite, ammonium, phosphorus and were collected in 10 mL polyethylene bottles and kept in the dark at 4°C until analysis. Their concentrations were determined using a CFA Bran + Luebbe autoanalyser following the methods described by Hansen and Koroleff, (2007).

2.4. Heterotrophic bacteria and picophytoplankton abundance

Samples (1.6 mL) were fixed using a solution of 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), deep frozen in liquid nitrogen and stored at −80 °C until analyzed. Subsamples for prokaryotes were stained with SybrGreen I (Molecular Probes, final concentration 1000× dilution of the commercial product) for some minutes in the dark. For picophytoplankton analyses, subsamples were unstained. The cell counting was performed by using the BD FACSCalibur cytometer with a 488 nm laser emission as explained in Gasol and Morán (2015).

2.5. DNA extraction and sequencing

Water samples (2L) were sequentially filtered through a 20 µm mesh onto 3 µm and then 0.2 µm polycarbonate filters and frozen in liquid nitrogen; DNA was later extracted with the phenol-chloroform protocol following (Massana et al., 1997). . The 515F-Y and 926R primers (Parada et al., 2016) and the V4_Balzano primer (Balzano et al., 2015) were used to generate amplicons targeting the V4 region of the prokaryotic 16S rRNA gene and the eukaryotic 18S rRNA gene respectively.

Primers and spurious sequences were trimmed using cutadapt (Martin, 2011). DADA2 v1.8 was used to differentiate exact sequence variants (Callahan et al., 2016) DADA2 resolves ASVs by modelling the errors in Illumina-sequenced amplicon reads. In the case of eukaryotes, we built a consensus taxonomy for ASVs at the group level (in general formal Class) using blast (Altschul et al., 1990) against the eukaryotes V4 (Obiol et al., 2020) and PR2 (Guillou et al., 2013) databases. After their classification, we removed the ASVs assigned to Metazoa, land plants, and nucleomorphs. The samples were randomly subsampled to the lowest number of reads for prokaryotes (4.795 and 5.694 reads for the 0.2-3 µm and 3-20 µm size fractions, respectively) and eukaryotes (5.094 and 4.954 reads for the 0.2-3 µm and 3-20 µm size fractions, respectively), using the rarefy function in the vegan R package (Oksanen et al., 2015).

2.6 Data analysis

Data treatment was performed using R (3.6 version) and Rstudio (1.2.1335 version). Prokaryotic richness (the number of ASV per sample) and Sample evenness (using the Pielou index ($J = H/\ln(\text{nASV})$, where H is the Shannon index and nASV is the richness in every sample)), were calculated using the *vegan* (Oksanen et al., 2019) package. A K-means algorithm (*vegan* package) was used to classify the ASVs based on their depth distribution patterns. Vertical connectivity (from the surface to the deepest sample) was estimated by categorizing each ASV at the first depth (from ocean surface to DCM bottom) in which they were detected considering 3 different layers: upslope, DCM and downslope.

3. Results and discussion

3.1. Environmental properties of DCMs

Fluorescence and oxygen concentration followed a similar pattern, increasing below the mixed layer and slowly decreasing between 80 and 100 m depth (Fig. 1). The first profile (Fig. 1A) showed a fairly broad DCM, with two chlorophyll peaks at 60 m (0.371 mg m^{-3}) and 85 m (0.367 mg m^{-3}, Fig. 2A, Table S1). In the second profile the DCM was more defined (Fig. 1B), and showed maximum values of Chl a at 90 m (0.380 mg m^{-3}, Fig. 2A, Table S1). In the third sampling the fluorescence profile showed a broad DCM (Fig. 1C), but the maximum chlorophyll concentration was measured at 56 m depth (0.357 mg m^{-3}, Fig. 2A, table S1), and around 50 m there was a maximum in the oxygen profile and a minimum in the salinity distribution (Fig. 1C). The fourth profile showed the narrowest DCM, with the highest chlorophyll concentrations measured between 45 and 82 m and a maximum peak at 58 m (0.345 mg m^{-3}, Fig. 2A, Table S1). The fact that in all profiles the oxygen maxima coincided with the fluorescence maxima, suggests an increase in primary productivity (Estrada and Salat, 1989) at that depth. The presence of the DCMs between 50-80 m is consistent with what is expected in the northwestern Mediterranean, especially during late summer and early fall (Lavigne et al., 2015).

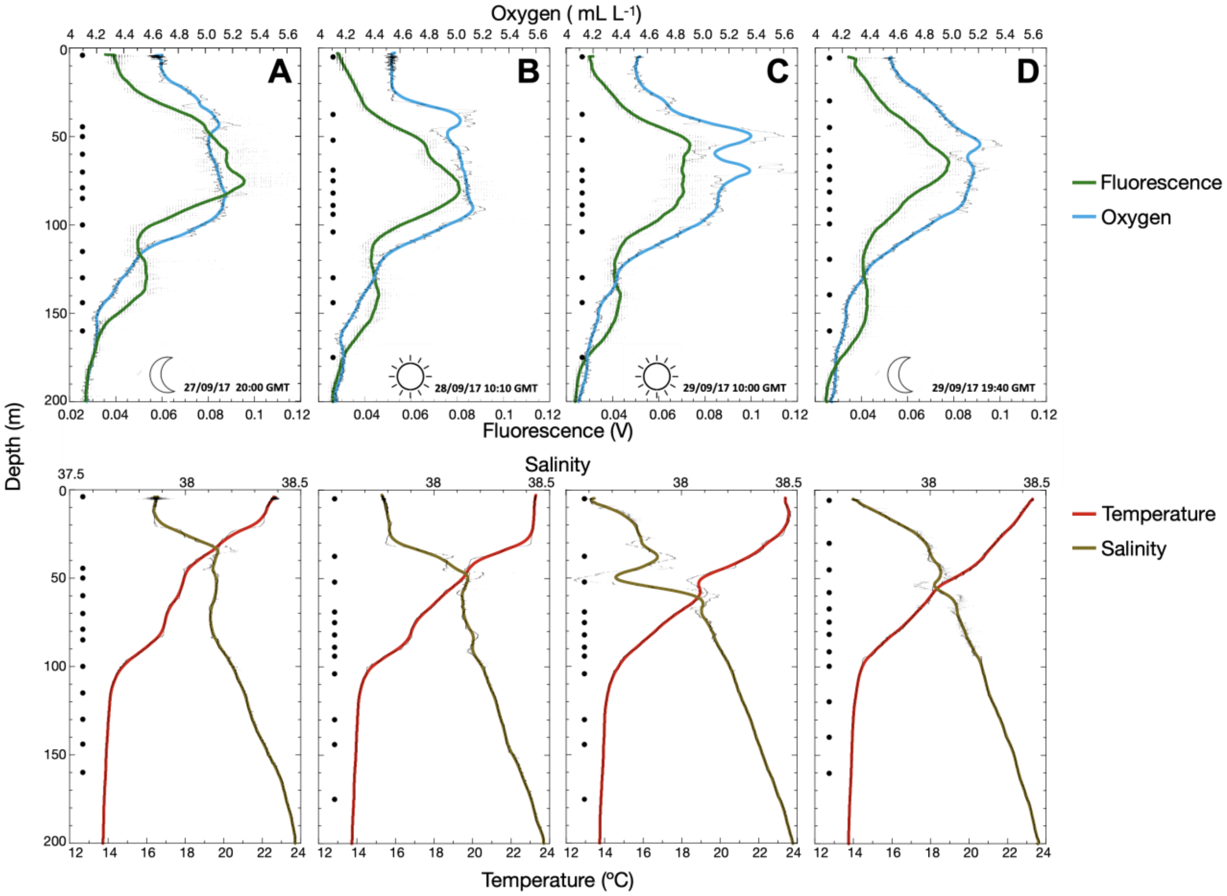

Fig. 1. CTD vertical profile (Upward cast) of Oxygen (mL L^{-1}), fluorescence (V), temperature (ºC) and salinity along four DCMs in a single station in the Northwestern Mediterranean Sea. The sampling periods (day/night) are shown on each plot.

The base of the DCM coincided with the presence of the nutricline, as depicted by the nitrate, silicate and phosphate profiles (Fig. 2, Table S1). Ammonium fluctuated throughout the water column, with comparatively higher concentrations at the depths where chlorophyll peaks occurred. Nitrite remained low in the first few meters and, just below the DCM, rose to their maximum peak to drop again abruptly (Fig. 2), as tipically observed throughout the ocean (Zakem et al., 2018).

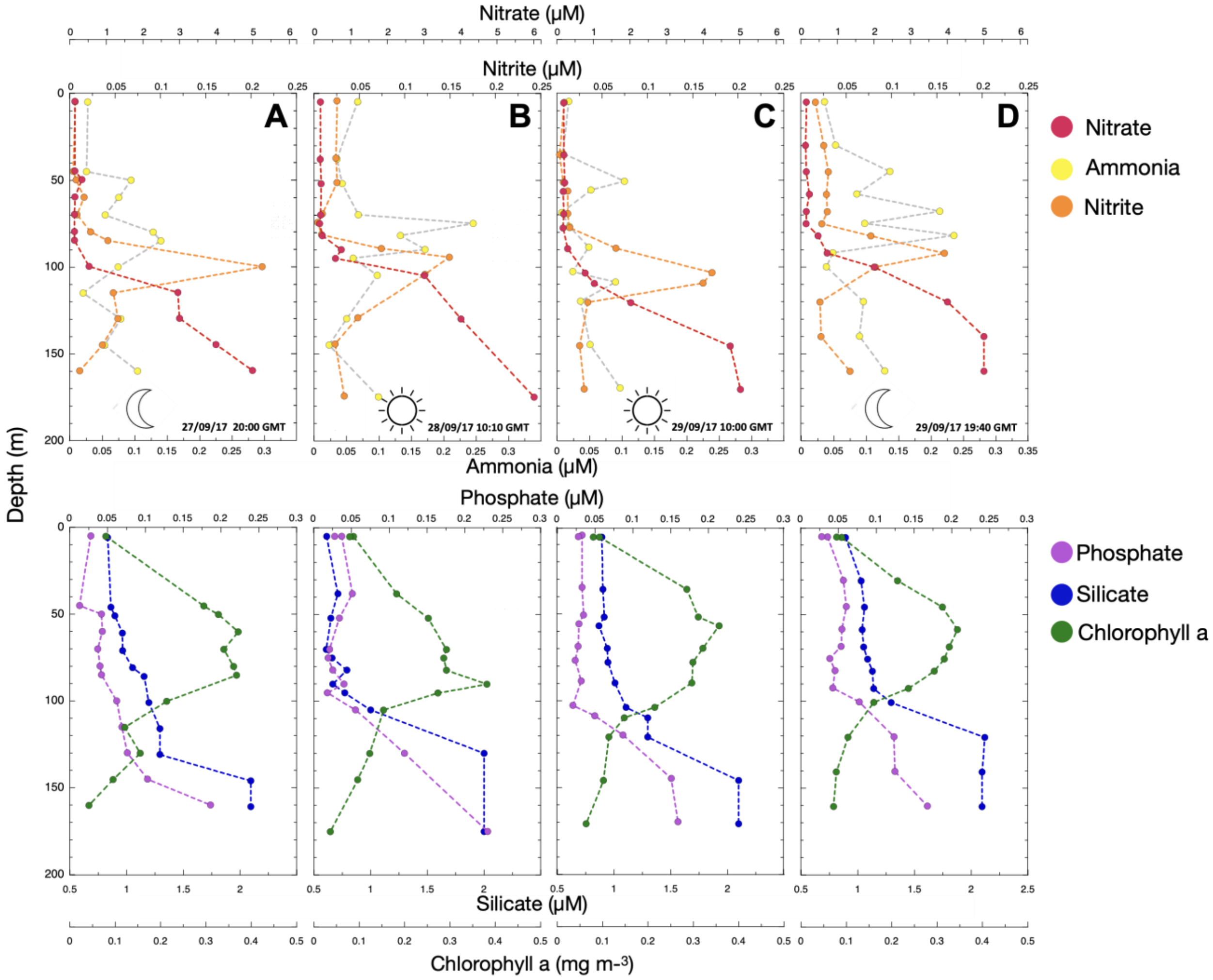

Fig. 2. Vertical profiles of inorganic nutrients and Chla along four DCMs in a single station in the Northwestern Mediterranean Sea. The sampling periods (day/night) are shown in each plot.

Regarding microbial abundances obtained by flow cytometry, heterotrophic bacteria presented increased values in the upper 90 m, and, in agreement with previous observations, the maximum values were found within the the DCM (Acinas et al., 1997; Haro-Moreno et al., 2018; Pedrós-Alió et al., 1999). *Prochlorococcus* and *Synechococcus* showed parallel abundance distributions, with higher abundances of *Prochlorococcus* along the DCM structure (Fig. 3).

Picoeukaryotes showed lower abundance than the *Prochlorococcus* values (except in DCM1) but also displayed similar distributions along the DCM.

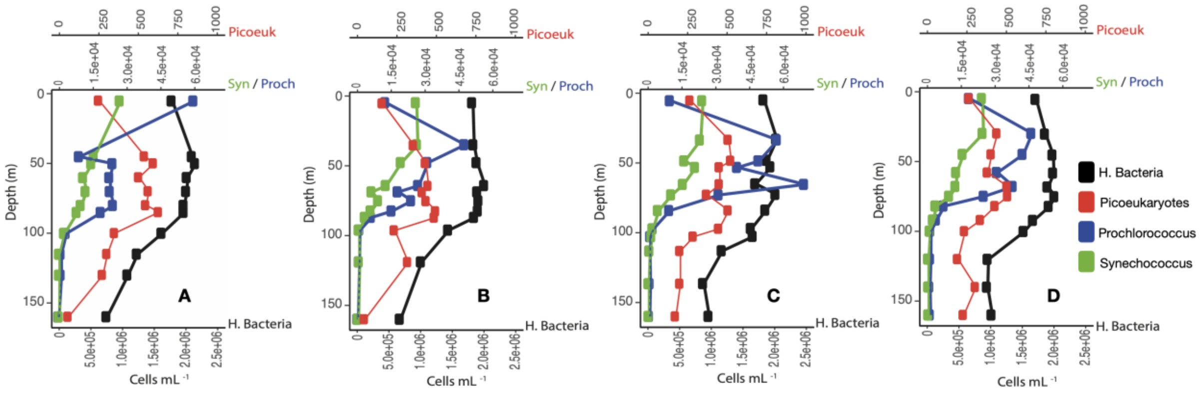

Fig. 3. Cellular abundance (Cells mL^{-1}) of heterotrophic bacteria (H. Bacteria), *Synechococcus* (Syn), *Prochlorococcus* (Proch) and picoeukaryotes (Picoeuk), along four vertical profiles of DCMs (A, B, C and D) in a single station in the Northwestern Mediterranean Sea. DCMs B/C were sampled during the day and A/D during the night. Nanoeukaryote abundances were very low to be plotted alongside the other groups (>100 cells mL-1).

3.2. Changes in alpha diversity indexes along the DCMs

In the 0.2-3 µm size fraction, the richness of prokaryotic communities showed little variation among the four DCMs, with a clear increase from the surface to 150 m depth. This agrees with previous observations showing increased richness towards deep waters (Haro-Moreno et al., 2018; Sebastián et al., 2021). Likewise, the richness patterns of eukaryotic communities in this size fraction was consistent among the 4 DCMs profiles, with maximum values between 50-100 m depth, where maximum values of Chl *a* occurred (Fig. 4). In contrast, in the 3-20 µm size fraction the richness of prokaryotic communities showed a minimum in the 50-100 m depth layer, whereas eukaryotic communities showed rather constant values with depth (Fig. 4).

In terms of evenness, free-living prokaryotic communities showed higher values (i.e. less dominance) at deeper waters, whereas the particle associated counterparts showed variable values along the DCM structure (Fig. 4). In the case of eukaryotes, picoeukaryotic (0.2-3 µm) communities showed maximum evenness between 50-100 m depth, whereas in the nanoeukaryotic (3 – 20 µm) communities evenness experienced a slight increase with depth. Communities with high richness and evenness indices are considered highly stable (Nogales et al., 2011; Wittebolle et al., 2009).

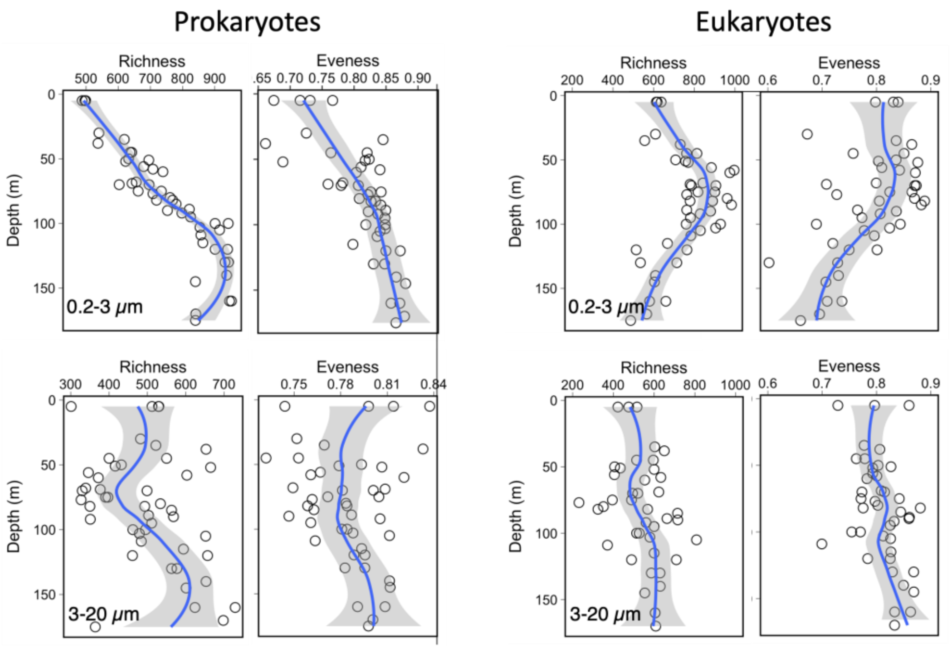

Fig. 4. Alpha diversity, represented by the richness (number of ASVs per sample) and evenness indices, in both size-fractions of the prokaryotic and eukaryotic communities along the DCMs (considering the pooled four casts). Blue lines represent the best fit smooth curves through the center of the data calculated using weighted least squares to highlight the relationship between richness and evenness with depth. Grey shading represents the confidence intervals around the smooths.

3.3. Shifts *in prokaryotic and eukaryotic community structure along the DCM*

Free-living and particle attached prokaryotes.

The taxonomic composition of free-living prokaryotic communities (0.2-3 µm size fraction) was notably consistent among the four samplings. Communities above 75 m depth were largely dominated by SAR11, Cyanobacteria and Bacteroidetes, whereas communities deeper than 100 m were dominated by SAR11, Thaumarchaeota and various contributions of other less abundant groups (Fig. 5 upper panel). The major changes in taxonomic composition occurred in just a few meters at the base of the DCM, where Cyanobacteria disappeared and the contribution of Thaumarchaeota increased. This increase in Thaumarchaeota coincided with the nitrite peak, in agreement with the involvement of this phylum in the oxidation of ammonia to nitrite (Könneke et al., 2005).

The vertical changes in the community composition of particle associated prokaryotes (3-20 µm size fraction) were likewise rather consistent among the four profiles (Fig. 5 lower panel), although more differences than in the free-living counterparts could be seen. Communities in the upper 50 m were dominated by Cyanobacteria, Bacteroidetes and Planctomycetes, but DCM1 and DCM2 had a lower representation of Planctomycetes, and instead the gammaproteobacteria Pseudomonadales in the DCM1 and the alphaproteobacteria Sphingomonadales in DCM2 showed increased abundances (Fig. 5 lower panel). The largest shifts in community composition of the particle associated prokaryotes occurred in general between 50 and 100 m depth, coincident with the higher values in Chl *a*. Communities below 100 m showed a high contribution of planctomycetes (Planctomycetes classes that were not present above) and Cyanobacteria. DCM 2 showed also a large contribution of Sphingomonadales in the deepest samples (Fig. 5).

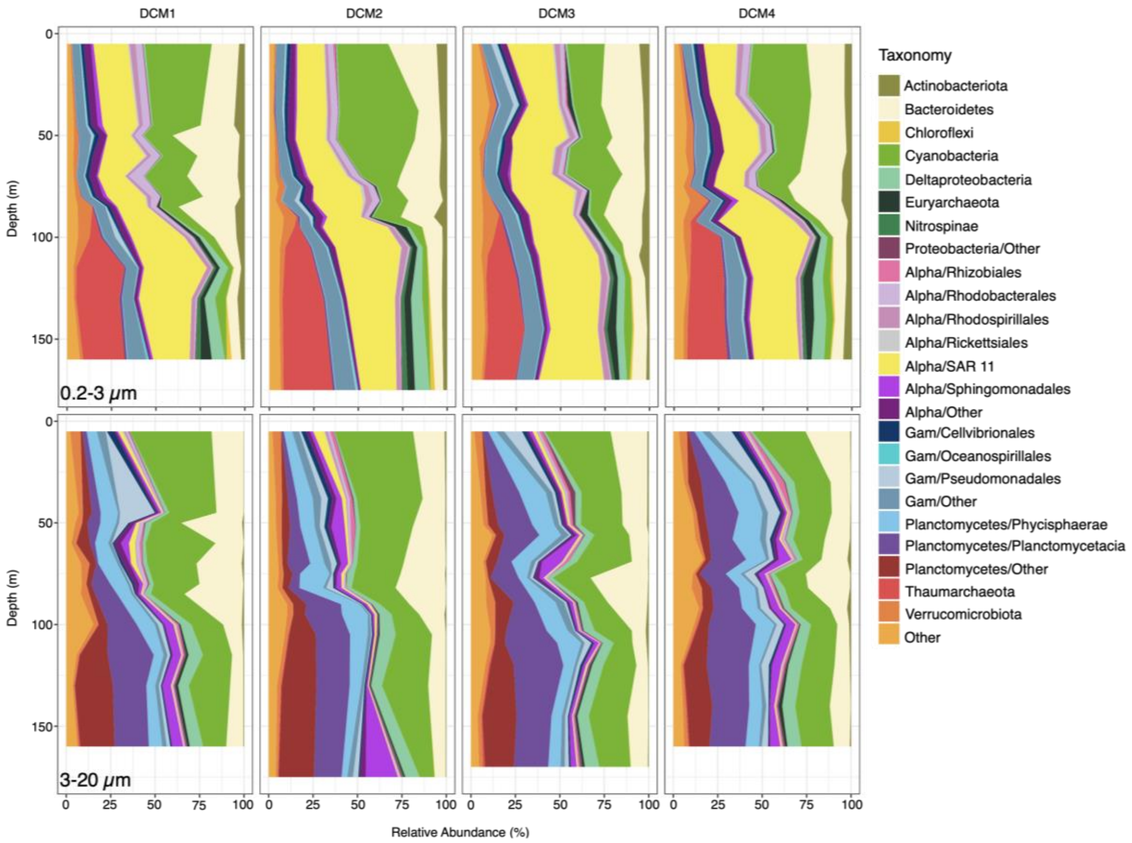

Fig. 5. Taxonomic composition of prokaryotic communities, in the four vertical profiles (DCM1-DCM4), shown at the phylum level, except for Alpha and Gammaproteobacteria which are at the order level and Planctomycetes at the class level. Taxonomic groups >1% relative abundances are represented while the rest of organisms are pooled as "other bacteria".

Pico and nanoeukaryotes.

Within the picoeukaryotes (0.2-3 µm size fraction), MALV-I and MALV-II were dominant between 0 and ~75 m depth (25-55% and 20-25%, respectively) and decreased towards the deepest samples to values close to 5% and 12%, respectively (Fig. 6). MALV-I were also abundant in the nanoeukaryotes size fraction (3-20 µm) but did not show such a decrease with depth. Rhizaria increased in abundance at the base of the DCM in both size fractions, with Acantharia and Polycistinea dominating picoeukaryotic communities and RAD-A having a notable representation in the nanoeukaryotic communities. Finally, Dinoflagellata was the group with the highest abundance within the largest size fraction being found at all depths (Fig. 6), but representing between 50-75% in the upper 75 m.

The distribution patterns of the different eukaryotic groups are consistent with reported vertical distributions in the global ocean at much lower resolution (Giner et al., 2020).

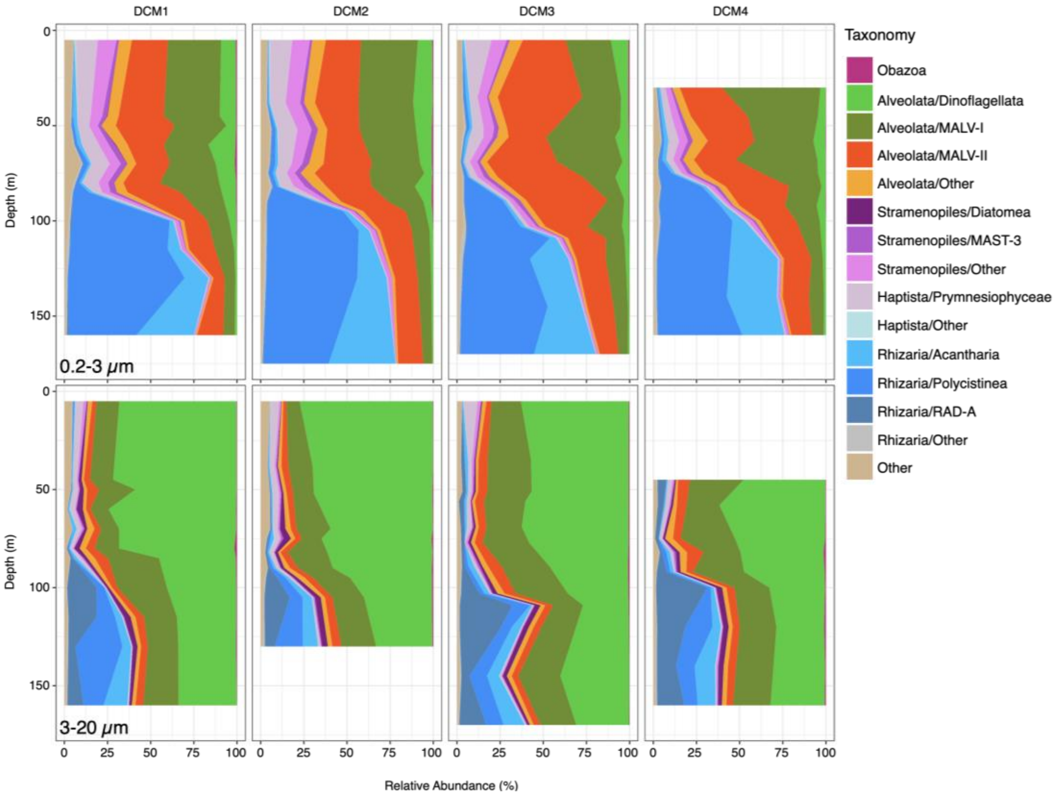

Fig.6. Taxonomic composition of pico (0-2-3 µm) and nanoeukaryotes (3-20 µm), in the four vertical profiles (DCM1-DCM4), shown at the group and supergroup level. Only the taxonomic groups >1% in relative abundances are represented, the others are pooled as "Other".

We next used K-means clustering to group ASVs according to their depth distributions. We obtained 6 clusters in the case of prokaryotes and 4 clusters in the case of eukaryotes. In the free-living size fraction, cluster 1 was entirely dominated by cyanobacteria and showed maximum values in the surface and a decreasing trend in the upper 100 m, being practically absent below (Fig. 7A). Cluster 2 likewise decreased with depth but displayed higher values at depth than cluster 1 and a small maximum at around 70 m —particularly in DCM3. This cluster 2 comprised members of the cyanobacteria, the Alphaproteobacteria Rhodobacterales and SAR11. Cluster 3 showed a peak in their vertical distribution at around 90 m and then decreased rapidly afterwards, and was largely dominated by Bacteroidetes and various other taxa. Cluster 4 showed a slight increasing trend down to the basis of the DCM and then increased drastically in abundance from 90 m onwards, and was represented by a diverse group of prokaryotic taxa. This distribution of cluster 4 correlated very positively with the concentration of nitrate along the water column (Fig. 7B). Cluster 5 was nearly absent in the upper 90 m but showed a peak at around 120 m depth, and then slightly decreased in the deepest samples, and was largely dominated by Thaumarchaeota (~60% of the ASVs) and some members of the SAR11 and deltaproteobacteria. Cluster 6 represented a large fraction of the prokaryotic communities in terms of reads, and included ASVs from diverse groups that decreased in abundance from the surface to the deepest samples (Fig. 7A).

In the fraction 3-20 µm, clusters 1 and 3 showed similar distributions, with cluster 3 displaying slightly higher values in the upper 80 m, but both showing low contributions below 100 m. Both had a large representation of cyanobacteria and Bacteroidetes, and cluster 1 comprised a notable contribution of the Planctomycetes Phycisphaerae (Fig. 7A lower panels). Cluster 2 showed minimum values in the surface and displayed a maximum between 50 and 100 m, with a large contribution of Planctomycetacia. Cluster 4 displayed relatively low values in the upper 90 m and then a maximum around 100 m depth, with also a large representation of Planctomycetacia (~40% of the ASVs). Cluster 5 comprised ASVs that were mostly absent in the upper 100 m and then increased notably in abundance around the nitracline (Fig. 2 and 7B), and encompassed Cyanobacteria and various Planctomycetes ASVs. Cluster 6 comprised a cluster of ASVs from diverse groups that represented a large fraction of the communities in terms of reads and displayed a minimum in abundance at around 70 m depth and higher values in both the surface and deepest samples (Fig. 7A).

In the case of eukaryotes, the differences in the clusters of both fractions show a different structuring between pico and nano eukaryotes, which was also evident in Figure 6. The picoeukaryotes showed 4 types of distributions. In cluster 1, minimum abundances occurred in surface, and then presented a maximum at around 50-70 m depth and decreased drastically in abundance below 100 m depth, comprising ASVs that belonged to MALV-I and MALV-II. Cluster 2 displayed high abundances in the upper 90 m and then decreased at the base of the DCM, and also comprised MALV-I and MALV-II and other alveolate. Cluster 3 was characterized by ASVs increasing below the DCM with a large representation of Acantharia and Polycistinea, and cluster 4, which dominated communities in terms of reads, showed a slightly diffuse behavior decreasing with depth, and encompassed several eukaryotic groups. In the nanoeukaryotes (3-20 µm size fraction), two clusters (1 and 2) comprised ASVs from Dinoflagellata and MALV-I whose abundances decreased from the surface towards the deep layers. Cluster 3 showed very low values in the surface and then increased below 100 m and comprised only RAD-A ASVs. This cluster had a nitrate-like distribution (Fig. 8A and B). Finally, cluster 4, which represented a large fraction of the eukaryotic communities in terms of reads, showed lower abundance values along the DCM structure, and higher abundances at depth, comprising eukaryotes from diverse taxonomic groups (Fig. 8A).

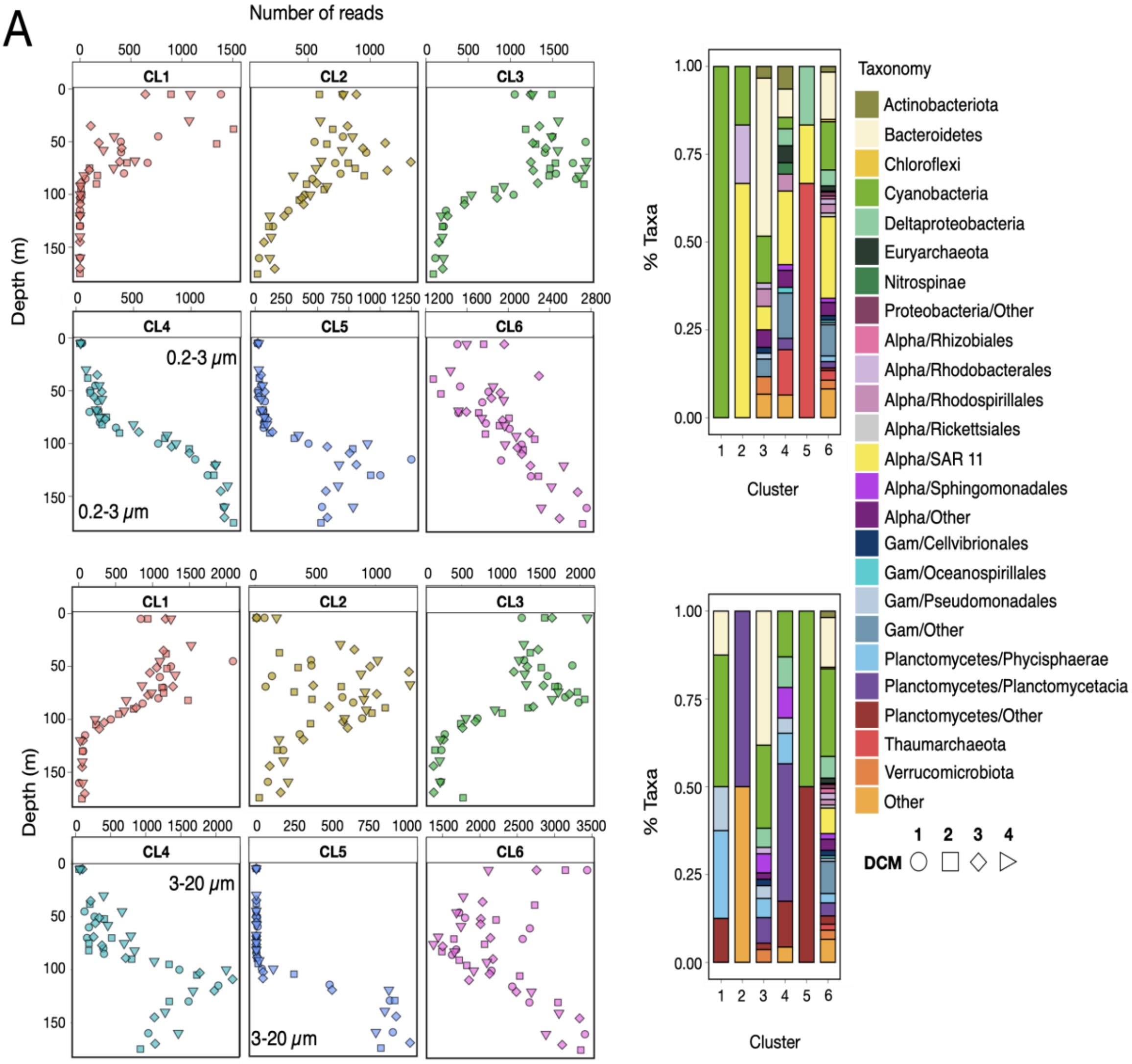

Fig. 7A. K-means clustering of vertical distribution patterns of prokaryotic ASVs using data from the four vertical profiles (**A**) (each symbol represents a different cast). Upper panels represent the 0.2-3 µm size fraction, and lower panels the 3-20 µm size fraction. Numbers above each panel represent the cluster assignation. Barplots on the right represent the taxonomic composition of the ASVs belonging to each of the clusters identified by the K-means analyses.

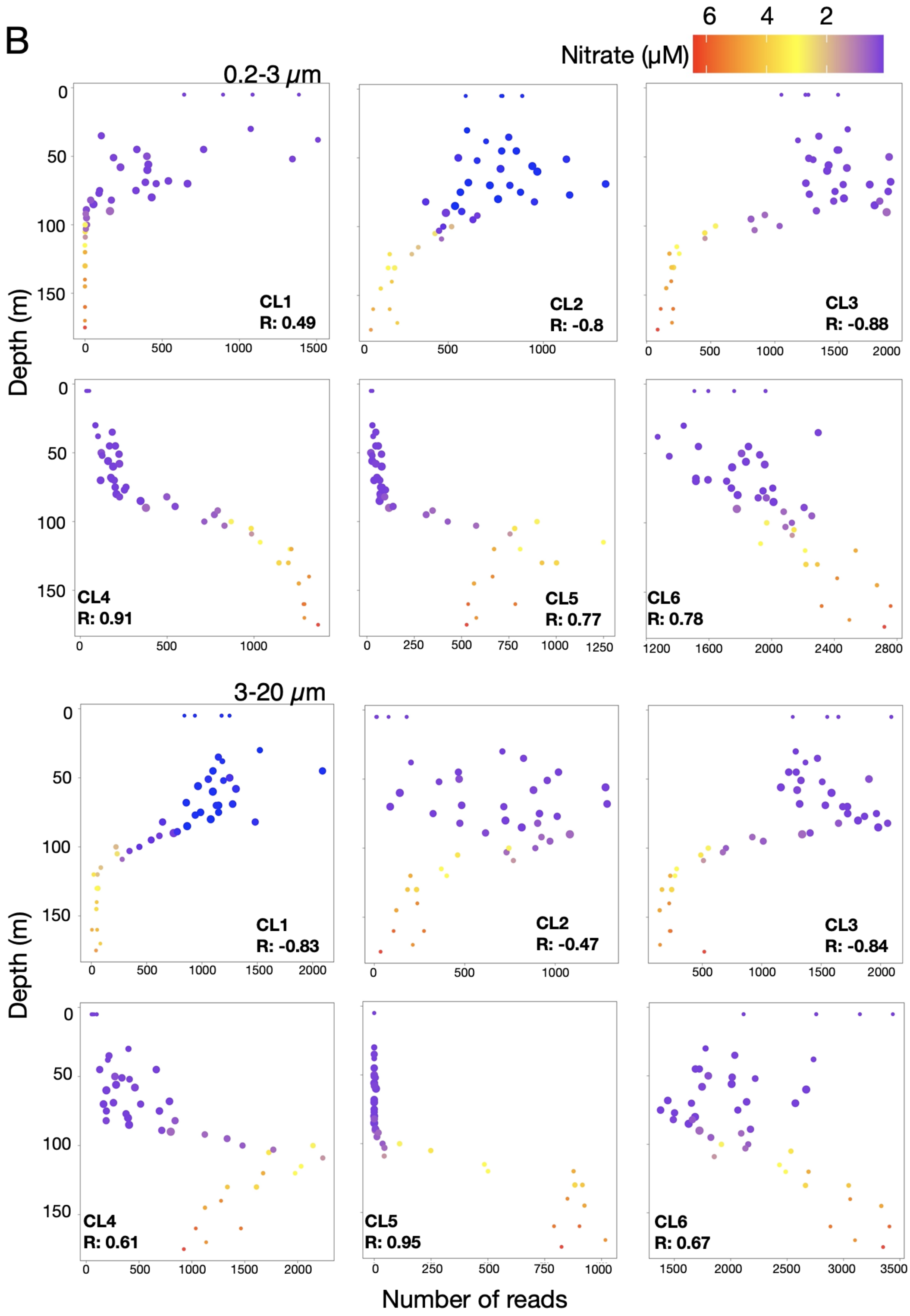

Fig. 7B. K-means clustering of vertical distribution patterns of prokaryotic ASVs using data from the four vertical profiles **B** represents the distribution of nitrate concentration for each sample in each prokaryote community cluster (CL). Spearman´s correlation test (R) was done relating the sum of reads with nitrate concentration.

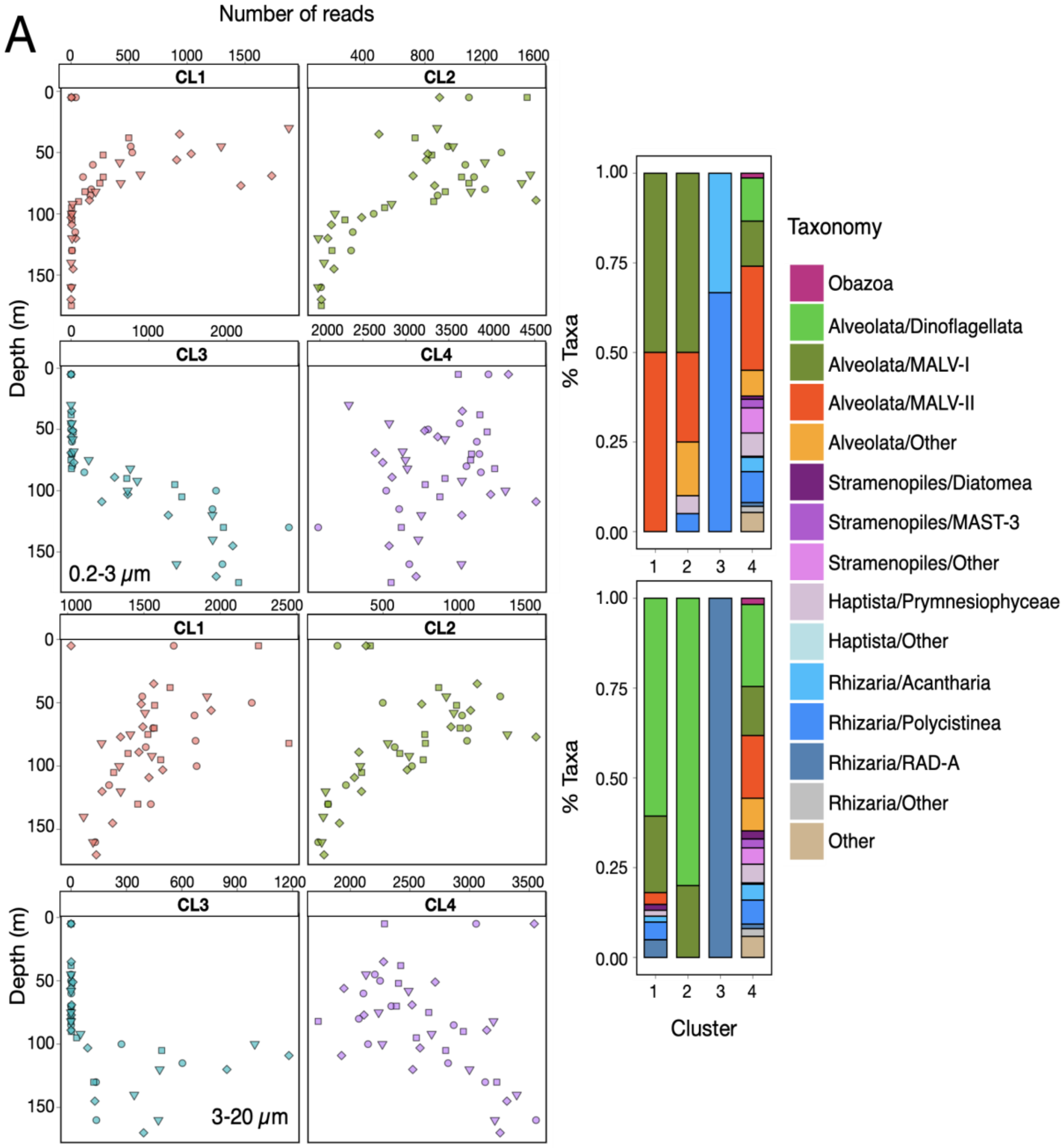

Fig. 8A. K-means clustering of vertical distribution patterns of eukaryotic ASVs using data from the four vertical profiles **(A)** (each symbol represents a different cast). Upper panels represent the 0.2-3 µm size fraction, and lower panels the 3-20 µm size fraction. Numbers above each panel represent the cluster assignation. Barplots on the right represent the taxonomic composition of the ASVs belonging to each of the clusters identified by the K-means analyses.

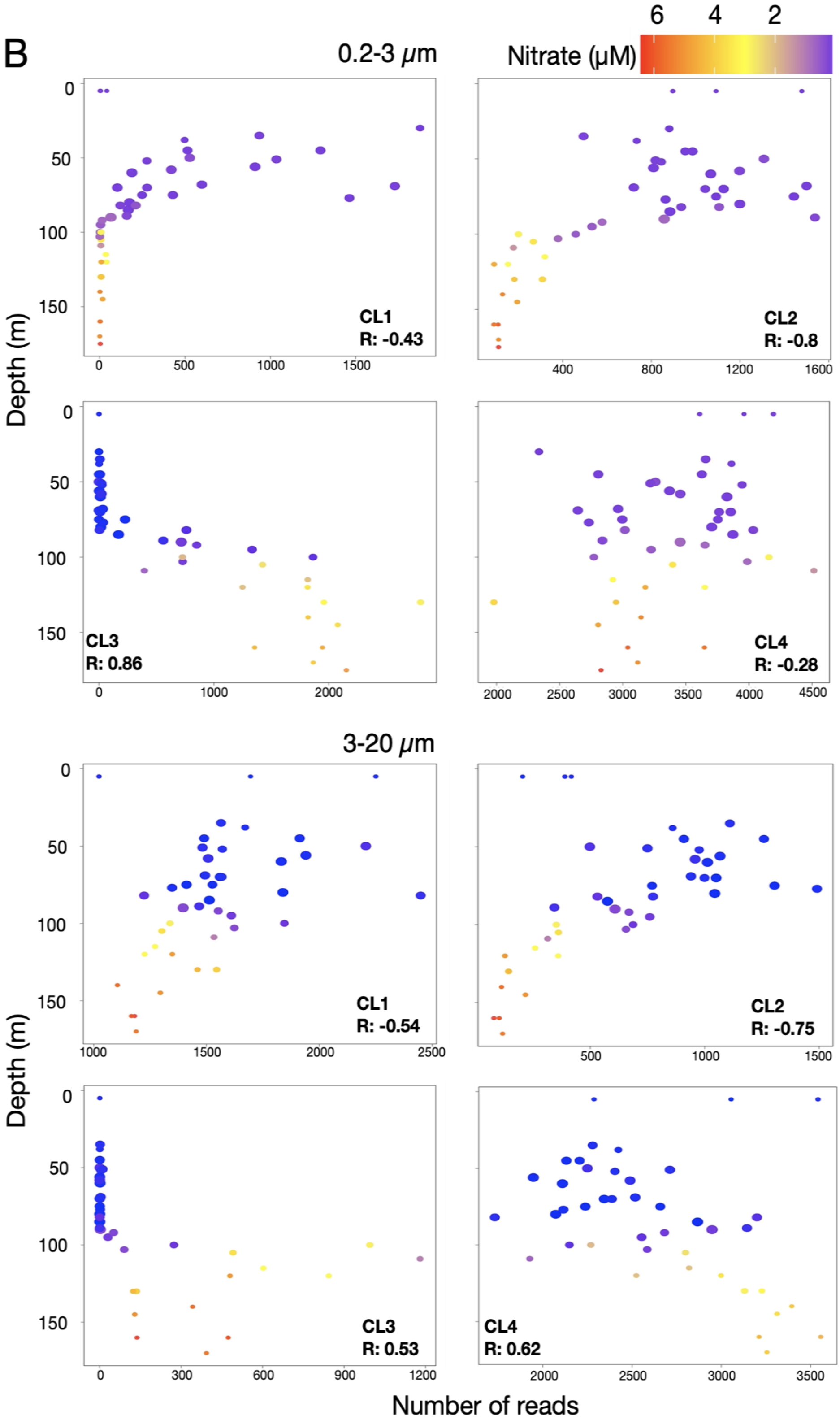

Fig. 8B. K-means clustering of vertical distribution patterns of eukaryotic ASVs using data from the four vertical profiles **B** represents the distribution of nitrate concentration for each sample in each prokaryote community cluster (CL). Spearman´s correlation test (R) was done relating the sum of reads with nitrate concentration.

Our results unveil marked differential vertical distribution patterns at the ASV level in both prokaryotic and eukaryotic communities, which may have a strong impact in the way community functioning is partitioned by depth.

3.4. *Vertical connectivity among communities*

Next, we assessed the vertical connectivity of taxa along the DCM structure to determine if the changes observed in the taxonomic composition were due to changes in the relative abundance of ASVs that were present in all the samples or in the recruitment of different ASVs with depth. ASVs were thus categorized based on the following criteria: those ASVs that were found at depths prior to the DCM peak were termed "upslope," those at the DCM peak that were not present in upslope were termed "DCM," and those below the DCM peak but not detected at previous depths were termed "downslope" (Fig. 9 and 10, Table S2). In the free-living prokaryotic communities, there was some recruitment of new taxa at the DCM, which represented around 20% of the ASVs but only accounted for a minor fraction of the total community sequences (~5%). Likewise, despite the recruitment of new ASVs was remarkable in the particle-associated (3-20 µm) size fraction, representing more than a third of the ASVs, they only accounted for ~5% of the community sequences. In contrast, the recruited ASVs at the 'downslope' level accounted for ~50% of the total ASVs detected and represented around 25% of the sequences in the free-living and particle-associated communities (Fig. 9).

Regarding the eukaryotic communities, recruitments were surprisingly similar in both the picoplankton and the nanoplankton communities (Fig. 10), despite the major changes observed in chlorophyll and the marked vertical stratification in phytoplanktonic groups that has been reported through pigment analyses (Latasa et al., 2017) and fluorescence in situ hybridization (Cabello et al., 2016). This means that the changes observed in eukaryotic communities are mostly driven by changes in the abundance of ASVs that are present throughout the photic zone, and not by a marked recruitment of novel taxa with depth.

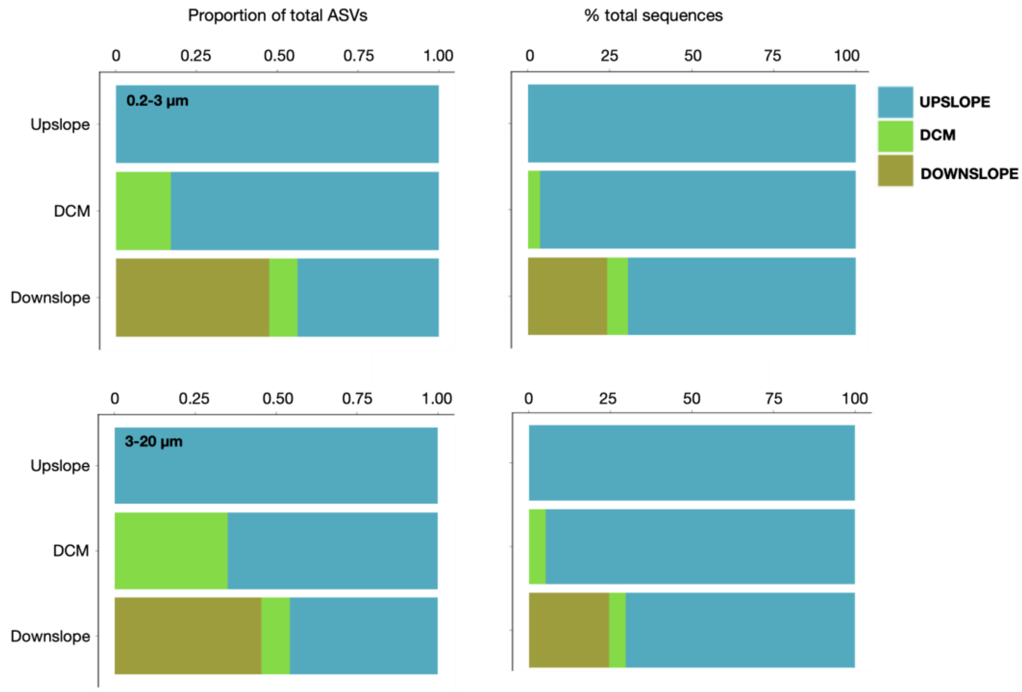

Fig. 9. Proportion of ASVs that are recruited in the different layers considering directionality from the surface to the deepest layer, and their corresponding proportion of the prokaryotic communities. The upper panels represent the 0.2-3 µm size fraction and the lower panels the 3-20 µm size fraction.

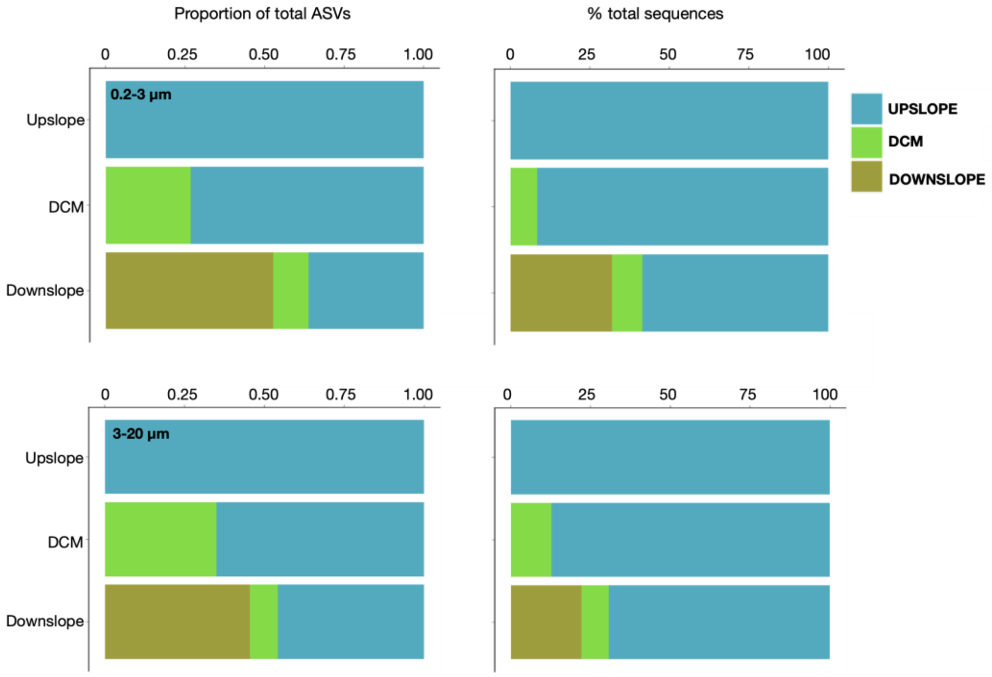

Fig. 10. Proportion of ASVs of eukaryotes that are recruited in the different layers considering directionality from the surface to the deepest layer, and their corresponding proportion in the eukaryotic communities. The upper panels represent the 0.2-3 µm size fraction and the lower panels the 3-20 µm size fraction.

The effects of depth on the distribution of prokaryotes and eukaryotes in the ocean water column have been widely discussed (Acinas et al., 1999; Cabello et al., 2016; Giner et al., 2020; Latasa et al., 2017; Lee' et al., 1991; Salazar et al., 2016; Sunagawa et al., 2015), indicating that the drastic environmental changes that occur with depth are a major driver of changes in prokaryotic and eukaryotic community composition. Our results show that these changes occur across very short spatial scales (gradients in the scale of a few meters). Therefore, the DCM represents a transition zone or ecotone, where these abrupt changes in the nutrient and light landscape model the structure of the microbial communities.

4. Conclusions

The DCM plays a crucial role in the carbon cycle of the ocean, as it is the primary site for primary production. Here, we evaluated microbial community structure (prokaryotes and eukaryotes) in two fraction sizes (0.2-3 and 2-20 µm) and with high spatial definition sampling within four DCM profiles in the Western Mediterranean sea. We find that the DCM acts as a transition zone or ecotone, encompassing dynamic environmental conditions that are generated above, within, and below these layers, and that, in turn, influence how microorganisms are distributed. The study of short spatial scales is essential for understanding microbial niche partitioning in the ocean and the potential impact on biogeochemical processes. Furthermore, in the context of climate change, ocean warming could affect the structure of the DCM, especially the depth at which it forms (Richon et al., 2019), and this would lead to shifts in microbial community structure and, expectedly, in their ecosystem functions.

Acknowledgements

I thank everybody onboard García del Cid for their help, and particularly Isabel Ferrera that acted as chief scientist. Special thanks go to the ship crew and the UTM technitian for smooth operation of the CTD. I thank Clara Cardelús, Vanessa Balagué and Olga Sánchez for their help on board and in the lab, and Aleix Obiol and Ramon Massana for the help with the eukaryote taxonomical assignation. This study was financed by grants CTM2015-70340-R and RTI2018-101025-B-I00 from the Spanish Ministry of Science to J.M. Gasol and O. Sánchez. The author was supported by a PhD grant from Universidad Nacional de Costa Rica, and the ICM authors were supported by a Severo Ochoa Excellence Award (Ministerio de Ciencia e Innovación) CEX2019-000928-S.

Supplementary Information

Table S1. Environmental parameters measured in samples of the four profiles carried out in the Northwestern Mediterranean Sea. Cell abundances obtained by flow cytometry. HB: heterotrophic bacteria and archaea. Peuk: picoeukaryotes, Proc: Prochlorococcus, Syn: Synechococcus

DCM	**Depth (m)**	**Sal**	**Temp (ºC)**	**Nitrate (μM)**	**Nitrite (μM)**	**Ammonia (μM)**	**Silicate (μM)**	**Phosphate (μM)**	**Chlorophyll a (mg m^{-3})**	**HB (cells mL^{-1})**	**Syn (cells mL^{-1})**	**Proc (cells mL^{-1})**	**Peuk (cells mL^{-1})**
1	160	38.4	13.88	5.872	0.011	0.104	2.484	0.186	0.042	7.58E+05	233.37	44.26	64.608
	130	38.29	14.01	3.767	0.053	0.078	1.902	0.076	0.155	1.09E+06	337.99	1066.28	120.951
	115	38.26	14.1	2.960	0.048	0.020	1.695	0.069	0.121	1.24E+06	333.97	1038.11	185.29
	100	38.21	14.64	0.532	0.212	0.074	0.878	0.062	0.213	1.63E+06	2526.88	3279.31	169.275
	85	38.13	16.4	0.130	0.042	0.140	0.824	0.042	0.367	1.98E+06	8143.96	18798.71	612.009
	80	38.11	16.89	0.132	0.023	0.128	0.698	0.040	0.361	1.98E+06	9946.58	24150.23	676.381
	70	38.11	16.99	0.133	0.008	0.054	0.587	0.037	0.339	2.02E+06	12131.45	22826.43	813.156
	60	38.13	17.7	0.144	0.016	0.075	0.586	0.043	0.371	2.03E+06	10980.67	22605.13	660.294
	50	38.14	17.93	0.334	0.007	0.094	0.504	0.042	0.327	2.16E+06	14638.21	24005.38	748.769
	45	38.11	17.94	0.144	0.004	0.025	0.460	0.013	0.295	2.11E+06	15700.47	9149.89	515.348
	5	37.89	22.75	0.141	0.006	0.027	0.424	0.028	0.079	1.79E+06	27224.34	59836.40	80.587
2	175	38.42	13.83	6.437	0.033	0.098	2.734	0.230	0.035	6.86E+05	394.32	225.33	64.645
	130	38.29	14.03	4.122	0.048	0.049	2.071	0.120	0.122	1.02E+06	893.26	840.95	137.042
	105	38.22	14.48	3.485	0.122	0.096	1.303	0.055	0.152	1.45E+06	776.57	1058.23	120.927
	95	38.17	15.53	0.567	0.149	0.059	0.772	0.018	0.272	1.86E+06	3508.66	6220.64	177.33
	90	38.16	16.36	0.727	0.074	0.170	0.665	0.040	0.380	1.92E+06	6087.85	15402.71	298.18
	82	38.19	16.83	0.214	0.008	0.132	0.791	0.025	0.291	1.93E+06	9395.33	24138.16	555.596
	75	38.14	16.92	0.138	0.004	0.244	0.659	0.019	0.285	1.91E+06	6486.20	18054.33	491.204
	70	38.13	17.33	0.163	0.009	0.067	0.609	0.021	0.291	2.02E+06	12799.38	27151.91	378.532
	52	38.15	19.36	0.178	0.025	0.042	0.650	0.034	0.251	1.90E+06	19454.58	31320.46	306.085

	38	38.07	20.21	0.154	0.024	0.034	0.712	0.051	0.181	1.85E+06	26697.23	47664.72	128.992
	5	37.78	23.24	0.158	0.025	0.066	0.613	0.037	0.086	1.83E+06	26218.41	12561.98	104.743
3	170	38.41	13.83	5.076	0.030	0.096	2.391	0.160	0.064	9.71E+05	297.75	547.22	48.504
	145	38.34	13.94	4.720	0.025	0.050	2.149	0.151	0.102	8.85E+05	281.66	828.88	56.593
	120	38.27	14.07	2.941	0.034	0.035	1.773	0.087	0.114	1.18E+06	704.15	696.10	80.681
	109	38.23	14.31	1.395	0.161	0.089	1.015	0.050	0.148	1.67E+06	1573.27	1062.26	193.338
	103	38.21	14.69	0.753	0.171	0.023	0.760	0.021	0.215	1.64E+06	2084.28	2180.84	241.656
	89	38.17	15.79	0.272	0.065	0.048	0.639	0.032	0.297	1.87E+06	4498.49	9516.04	362.469
	77	38.13	16.82	0.150	0.014	0.018	0.561	0.024	0.299	2.03E+06	10377.12	31497.50	386.603
	69	38.06	17.9	0.164	0.012	0.006	0.553	0.028	0.321	1.71E+06	15559.64	69505.35	475.149
	56	38.06	19.21	0.151	0.012	0.051	0.463	0.029	0.357	1.95E+06	20987.60	39709.87	418.846
	51	37.51	18.28	0.194	0.007	0.103	0.520	0.035	0.311	1.89E+06	16247.69	49213.84	499.286
	35	37.89	22.09	0.169	0.003	0.008	0.505	0.033	0.286	2.04E+06	23152.35	57357.80	394.639
	5	37.61	23.4	0.164	0.008	0.017	0.494	0.033	0.093	1.84E+06	24190.47	9813.80	80.606
4	160	38.38	13.89	5.508	0.054	0.129	2.318	0.167	0.072	1.02E+06	273.61	1001.90	120.959
	140	38.33	13.94	5.208	0.022	0.090	2.182	0.124	0.078	9.47E+05	245.45	752.43	128.962
	120	38.27	14.07	4.581	0.021	0.096	2.030	0.123	0.104	9.58E+05	181.07	1106.52	120.947
	100	38.22	14.43	2.805	0.082	0.039	1.449	0.077	0.161	1.53E+06	897.28	1178.94	128.998
	92	38.19	15.13	0.712	0.158	0.049	0.806	0.042	0.237	1.68E+06	2353.86	3725.94	201.459
	82	38.16	15.89	0.462	0.077	0.236	0.794	0.045	0.294	1.93E+06	3460.38	7105.85	265.899
	75	38.14	16.68	0.134	0.023	0.098	0.740	0.038	0.316	2.02E+06	9584.45	25015.33	644.154
	68	38.11	17.37	0.145	0.029	0.214	0.695	0.053	0.327	1.91E+06	12405.06	37867.01	555.66
	58	37.12	18.15	0.229	0.028	0.086	0.681	0.054	0.345	2.00E+06	12718.91	30777.26	467.139
	45	38.05	20.08	0.143	0.030	0.137	0.706	0.060	0.312	1.99E+06	15624.02	42305.15	338.372
	30	37.98	21.34	0.119	0.025	0.053	0.669	0.056	0.213	1.87E+06	24480.17	46179.97	273.889
	5	37.67	23.27	0.143	0.016	0.036	0.497	0.035	0.090	1.72E+06	24218.63	18424.51	112.785

Table S2. Percentage of surface ASVs and sequences recruited in layers below the surface.

Depth	Size fraction	Prokaryotes		Eukaryotes	
		ASVs %	Sequences %	ASVs %	Sequences %
DCM	0.2-3 µm	17	4	26.7	8.6
	3-20 µm	35	6.4	38.9	13.3
DCM + Downslope	0.2-3 µm	56.72	30.27	63.7	42.1
	3-20 µm	54.3	29.6	54.2	30.7

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General Discussion

Synthesis and contributions of the PhD thesis

In this thesis I used small subunit rRNA gene amplicon sequencing to explore several questions related with the variability at (relatively) short spatial scales of bacterial community structure in plankton environments. This resulted in three different studies in a gradient of spatial scales:

Chapter 1: The spatiotemporal distribution of bacterioplankton community structure in a tropical ecosystem strongly affected by riverine discharge and with a high level of anthropogenic impact.

Chapter 2: The horizontal distribution of bacterioplankton across inshore-offshore environmental gradients in temperate ecosystems with different degrees of river and anthropogenic impact

Chapter 3: The fine-scale vertical distribution of bacterioplankton and small protists across the major environmental gradient in the photic zone of a temperate ecosystem, the DCM.

The main results of each chapter are summarized below.

Chapter 1 explored the structure of the bacterial communities from the Gulf of Nicoya, a large tropical estuary located in Costa Rica (Central America) and characterized by high riverine discharges during the rainy season. The results indicated high heterogeneity in diversity and community structure due to seasonality and estuarine zone (upper, middle, and lower zone of the gulf), but a relatively high degree of connectivity between the zones. This study represents the first report on the spatiotemporal distribution of bacterioplankton communities in Costa Rican coastal areas, as well as a significant contribution to the ecology of microbial communities in tropical estuaries, which have been scarcely studied.

Chapter 2 studied the effect of environmental gradients on the structure of bacterioplankton communities along four coastal-offshore transects in the Northwestern Mediterranean Sea, covering areas with different anthropogenic and natural impacts. The results indicated that the four systems shared a large pool of bacterial taxa, but their relative abundances fluctuated between the four areas studied and also along the cross-shore transects, reflecting the underlying environmental gradients. Likewise, significant heterogeneity was observed in the areas closer to the coast, probably due to differential continental pressures.

Chapter 3 evaluated microbial community structure (prokaryotes and small eukaryotes) along detailed vertical gradients by sampling small-scale layers within the deep chlorophyll maximum (DCM) of the NW Mediterranean Sea. We found that changes in community composition occurred at very short spatial scales. Therefore, the DCM acts as a transition zone with highly dynamic environmental conditions that influence the vertical distribution of microorganisms.

Since each chapter has its own discussion, this section will address the most critical findings in the context of marine microbial communities distribution patterns. In particular, we put particular emphasis in the connectivity of bacterial communities across the gradients identified in the different ecosystems. In the first chapter by looking at the number and amount of bacterial taxa identified in the riverine mouth and appearing in several of the estuary sites (data recalculated for this discussion), in the second chapter by looking at the coastal taxa appearing in offshore sites in each of the gradients, and by looking at the taxa identified in one of the transects that could be observed also in the rest of the transects; and in the third chapter by looking at the bacterial and protists connectivity across the DCM, i.e. taxa identified at the surface that were also present below the DCM.

Patterns of microbial community structure in marine environments

The field of marine microbial ecology has sought to understand the role of microbes in the functioning of the marine environment. The patterns that define or model the biogeography of microorganisms have been extensively studied at global, regional, and local scales. We now know that dispersal mechanisms explain why the same taxon can be found in different environments but in different relative abundances (Gibbons et al., 2013). Some of these dispersal mechanisms are water mass circulation (Frank et al., 2016; Salazar et al., 2016), currents (Villarino et al., 2018), wind deposition (Cáliz et al., 2018; Mayol et al., 2017) or particle sinking (Mestre et al., 2018).

A very little studied dispersal mechanism is that which occurs between the terrestrial and marine environment, where there is a significant input of terrigenous material from runoff (Lawes et al., 2016), riverine discharge (Doherty et al., 2017; Fortunato and Crump, 2011; Raymond and Bauer, 2001) and groundwater (Basterretxea et al., 2010; Tovar-Sánchez et al., 2014). This material input is of particular importance in coastal areas (Chapter 1) and semi-enclosed, nutrient-poor seas (Chapter 2), such as the Mediterranean Sea (Ruiz-González, 2020), where allochthonous carbon from the continent has been shown to be essential for planktonic metabolism (Duarte et al., 2004). The continental water also transport microorganisms, and some of these dispersed microorganisms could end up thriving in the marine system (Ruiz-González et al., 2015).

The riverine contribution to the marine environment is so important that it represents about 95% of the sediments that reach the ocean (Syvitski, 2003). It is essential in areas with high rainfall, such as the tropics. Chapter 1 is framed in a tropical estuarine environment strongly influenced by the effect of a river whose discharge can reach up to 300 m^3 s^{-1} during the rainy season. In this chapter, we observed that the salinity gradient produced by the fluvial input contributed to the bacterioplankton diversity patterns and community structure, resulting in high spatial heterogeneity. OTUs that were dominant (50-60% of the community) in the area most impacted by riverine influence represented only 10-20% of the sequences in the most oceanic stations, and vice versa. This means that the spatial distribution of bacterioplankton taxa was strongly influenced by the fluvial and tidal inputs, but also by local environmental conditions which seem to favor the establishment of different bacterial groups. The information generated in this chapter adds to the scarce information on bacterioplankton communities in tropical estuaries. It is necessary to highlight the need to make greater efforts to study this type of tropical ecosystems with a larger spatial and temporal scale since they are under constant environmental pressure due to natural events such as El Niño Southern Oscillation/La Niña, hurricanes, tropical storms, as well as anthropogenic effects due to terrigenous input from agricultural and industrial activities. According to our findings, it is to be expected that changes in bacterial communities will occur in the face of these events. Remarkably, we observed a dominance of Enterobacterales in some stations, some of which could be pathogens. A high-density sampling at different seasons taking into account river flow as well as land use seasonality would be desirable if we want a good understanding of the factors that control bacterioplankton community structure as well as the presence of undesirable microbes in water that might be used for recreation and fish exploitation.

In addition to the above, tropical estuaries and, particularly, those from Central or South America have been hardly (or ever) been studied. A recent synthesis effort by a collective of South American researchers about microbial community composition in south American freshwater ecosystems has identified a clear lack of effort in comparison with European, north American, and southern Asia sites (Metz et al., 2022). As these authors did, we searched MGnify (www.ebi.ac.uk/metagenomics) with the word “estuary”. The database returned 132 studies and 1475 samples. Of these, 84% were located in North America, 8% in Europe, 5% in Asia, and 2% in Australia or South America. Not a single study of Central America existed in this database, which highlights the relevance of studies such as the one compiled in chapter 1.

The diversity of bacterioplankton communities in temperate ecosystems have been better studied those of tropical estuaries, but little is known about the degree of connectivity among communities separated a few hundreds of kilometers, and how land-use and anthropogenic impact may affect the distribution of taxa. The Mediterranean Sea, the largest semi-enclosed basin on the planet (Sebastián et al., 2021), is an oligotrophic environment considered one of the most heavily impacted areas by human activities in the world (Liquete et al., 2009), and has been identified as one of the most responsive regions to global change (Kim et al., 2019). In the second chapter, we focused on studying bacterioplankton distribution along cross-shore transects in four areas subjected to different anthropogenic activities and natural impacts. Of these four areas, one was under the effect of a river, one was affected by submarine water discharge, one was highly urbanized and densely populated, and the last one had a low population density and agricultural activities. Our objective was to evaluate the effect of continental pressure on the free-living and particle-attached fractions of the coastal bacterioplankton community. We observed that the four areas were highly connected (i.e. a high dispersal) among them, with a large share of common taxa accounting for ~75% of the bacterioplankton communities, except in the area strongly affected by riverine or groundwaters discharge. However, the relative abundance of the common taxa differed, indicating that community distribution patterns changed according to the environmental gradients found in the cross-shore transects (Basterretxea et al., 2018). The contribution of this thesis to the knowledge of bacterial dynamics in coastal systems is significant because coastal zones are considered among the most important and dynamic in the world, covering about 440 000 km in length, and are constantly under the effect of natural phenomena such as waves, wind, tides, and precipitation, as well as extraordinary events such as storms (Escudero Castillo et al., 2012). All this, in turn, has an impact on the bacterial communities, so it is important to emphasize that it is necessary to increase studies of microbial ecology in this type of environments, and do so at short spatial scales. Our data also indicate that the coastal effects are diluted at distances of 1-2 km from shore, even in this area with a relatively large continental slope.

Concerning the vertical distribution of microbial communities, it is known that environmental gradients shape how microorganisms are structured along the water column (Dobal-Amador et al., 2016; Massana et al., 1997; Sebastián et al., 2021; Sunagawa et al., 2015). Yet, most of the studies so far have low resolution in the vertical dimension, usually around tens of meters. One of the sharpest environmental gradients occurs in the layer of the deep chlorophyll maximum. This feature is formed in stratified water-columns at a depth where both light irradiance and nutrient supply are adequate for photosynthesis and phytoplankton metabolism (Cullen, 1982; Estrada et al., 1993), and sustains a large fraction of water-column productivity. Phytoplankton cells position themselves within this feature based on their light and nutrient requirements, leading to marked vertical distribution patterns across taxonomic groups (Cabello et al., 2016; Latasa et al., 2017). Given that the quantity and quality of dissolved organic matter compounds may change following the phytoplankton distribution we hypothesized that strong changes in prokaryotic diversity would also occur within the DCM. Likewise, as pico- and nano-eukaryotes comprise both phytoplankton species and potential prokaryotic grazers, we analyzed the vertical distribution of eukaryotes. A high-resolution sampling (every 5-10 m depth) was performed to detect environmental and/or community changes that cannot be detected at scales of tens of meters. Our findings indicate that the DCM is a transition zone where changes in nutrients and light availability occur in a very narrow depth layer, shaping community structure. This indicates that any situation that influences the shape, duration, and amplitude of the DCM will affect the structure of microbial communities. Some previous work on microorganisms in the DCM already showed high spatial heterogeneity (Cabello et al., 2016; Dolan and Marrasé, 1995; Haro-Moreno et al., 2018; Latasa et al., 2017; Mignot et al., 2014; Walsh et al., 2015; Zaccone et al., 2004) but this is the first study that considers both prokaryotes and eukaryotes, in two size-fractions, with high spatial resolution. This has allowed us to look in greater detail at what occurs within the most relevant oceanographic structure that defines the ecology of the surface ocean. Despite the sharp gradients encountered above and below the DCM, surface-derived taxa represented ~70% of the communities below the DCM, unveiling once again the high degree of connectivity occurring throughout the ocean.

Table 1 below synthesizes the various results observed taking into account the spatial scale and whether the gradient was vertically or horizontally structured. There are several insights that appear clear with this table:

First, the less connected environment was the Gulf of Nicoya, probably reflecting the high hydrographic dynamism, and the strong sorting effect of environmental constraints particularly in the areas affected by riverine discharge. The most connected environments were the Mediterranean Sea transects, although in this case, the particle-attached communities were less connected than the free-living ones. However, in the DCM analysis, we could not observe that difference, but we could see that protists were less connected than prokaryotes, something that fits with previous results comparing beta-similarities according to body size (Villarino et al., 2022, 2018) or, with the study of Logares et al. (2020), that found small eukaryotes to be more dispersal-limited than prokaryotes. Interestingly, in the coastal NW Mediterranean coastal study we observed that organisms were only marginally more connected through the transects (spatial scale of 4-5 km) than between the transects (spatial scale >100 km).

Table 1. Degree of connectivity of the microbial communities sampled during this thesis. Type H (horizontal dimension), V (vertical dimension)

Study site	Spatial scale	Type	% connected[$]
Gulf of Nicoya[#]	20 – 60 km	H	~30
Coastal Med Sea (within transects)	5 km	H	
Free-living			45-85
Particle-attached			50-75
Coastal Med Sea (between transects)	200 km	H	
Free-living			30*-80
Particle-attached			10*-50
Open Ocean Med Sea	0.1 km	V	
Prokaryotes free-living			70
Prokaryotes particle-attached			70
Eukaryotes small			60
Eukaryotes large			70

[#] Recalculated with data of Chapter 1

[$] Percentage of the community sequences that belong to shared taxa in the system considered

* Corresponds to the Girona gradient which had an inshore sample highly affected by freshwater. Not considering this point as an end member, the values would be ca 100%.

Beyond this thesis

In the three sections of this thesis, we have seen how communities respond to environmental or anthropogenic gradients over small (chapter 3) and relatively large (chapters 1 and 2) scales. In addition of having described bacterial (and small eukaryote) community structures, with the dominance of different organisms in different environments, we have also found that communities are more or less connected in each environment, which is demonstrated by the large fraction of taxa shared among the different communities. A step beyond this thesis would be to study bacterial communities as members of a metacommunity (Leibold et al., 2004; Wilson, 1992). The metacommunity paradigm provides an ecological framework to go beyond what was studied here. Under this approach, communities are spatially connected in a network by dispersal, and the processes that determine their structure are the result of the interplay between local factors (ecological factors selecting for certain organisms) and regional dynamics (such as mechanisms of dispersion, currents etc.). Vellend (2016) proposed a conceptual framework in which the structure of metacommunities could be explained by four processes: selection, dispersal, ecological drift, and diversification (this working at scales not relevant here). These processes seem to be universally present across ecological communities, but the strength in which they rule the assembly and the connectivity of communities is likely to vary across different communities and environmental heterogeneity. This approach has been recently applied to the surface ocean (Lindh et al., 2016; Logares et al., 2020), to different depths in the ocean (Junger et al., 2023) or to lakes and inland aquatic environments (Huber et al., 2020). Although this kind of analysis was not performed in this thesis, our results suggest that selection is likely to be driving community assembly across the DCM, while dispersion and selection are likely to be strong in Nicoya and dispersion in the sampled coastal sites in the NW Mediterranean

Therefore, this thesis contributes to the knowledge of the structure of microbial communities, their patterns of distribution at different scales and their connectivity, elements that are necessary to understand the functioning of marine ecosystems.

References (general discussion)

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General Conclusions

1. Our results show pronounced differences in bacterial diversity and community structure between seasons and estuarine zones within the Gulf of Nicoya

2. The Gulf is highly dynamic in both the spatial and temporal scale and bacterioplankton communities are strongly influenced by the riverine and tidal inputs during both seasons.

3. Enterobacteriales genera and Cyanobacteria dominated the bacterial communities of the Gulf of Nicoya in the dry season, whereas Alphaproteobacteria dominated the communities in the rainy season

4. The four areas sampled in the NW Mediterranean were highly connected (i.e. implying a high dispersal), with a large share of common taxa accounting for ~75% of the bacterioplankton communities, except in the area strongly affected by riverine or groundwaters discharges.

5. The relative abundance of the most common taxa differed, indicating that community distribution patterns changed according to the environmental gradients affecting each of the cross-shore transects studied.

6. At the depth of the chlorophyll maxima (DCM) in the NW Mediterranean, eukaryotic communities changed remarkably, while prokaryotic communities changed more gradually.

7. Prokaryotic richness increased with depth through the DCM, while the richness of small eukaryotes had a maximum at the depth of maximal chlorophyll. Larger eukaryotes had rather uniform richness values with depth.

8. We could differentiate 6 prokaryotic and 4 eukaryotic responses to the vertical gradient, responding differently to the vertical gradient in environmental conditions. Each group had a particular and distinct community structure.

9. Of the studied sites, the less connected environment was the Gulf of Nicoya, probably reflecting the high hydrographic dynamism, and the strong sorting effect of environmental constraints particularly in the areas affected by riverine discharge.

10. The most connected environments were the Mediterranean Sea transects, although in this case, the particle-attached communities were less connected than the free-living ones

Acknowledgements/Agradecimientos

Comienzo esta larga lista de agradecimientos con Marta y Pep. Ustedes saben que siempre los he admirado y respetado muchísimo y sin su ayuda nunca hubiera terminado esta tesis. A lo largo de este tiempo me han animado a terminar y muchas veces eso fue lo que me motivó a seguir, uds no lo saben pero los mails que me enviaban para saber cómo iba o si necesitaba ayuda, me sacaron mucha veces del “agujero” emocional en el que me encontraba. No tengo palabras para expresarles lo que han significado, no sólo en este proceso, sino en mi vida y estoy sumamente agradecida de haberlos conocido.

Isabel Ferrera, una persona increíble con quien compartí dos de mis mayores experiencias, las campañas REMEI y POSEIDON. Clara RG, gracias por la ayuda con R y con los comentarios al artículo pero gracias también por ese positivismo y esa alegría que siempre me contagió. Sigo siendo una gran admiradora de su música!!!! Gracias también a Andrea B. el año pasado en Barcelona fue increíble gracias a usted.

Gracias Olga porque junto con Pep han conseguido los proyectos que financian esta tesis, también por todos los ratos que compartimos en REMEI y en el García del Cid.

Gracias a la tripulación del García del Cid y Sarmiento de Gamboa, las campañas REMEI y POSEIDON fueron un sueño hecho realidad. Gracias al equipo científico de POSEIDON: Txetxu, Eugenio, Mar, Alba, Zoraida, Chie, Daniel y Bárbara.

Gracias Ramon por ser parte del capítulo del DCM y por ayudarme con los análisis de los eucariotas.

Vane y Clara C, uds son unas chicas que hacen magia, hacen que todo parezca muy fácil, ha sido fantástico aprender a hacer tantas cosas en el laboratorio, además de lo que compartimos en las campañas.

Al grupo de apoyo de la playa/guingueta: Mariri, Marta M, Yaiza, Caterina, Néstor, Dorle, Mireia, Estella, Ramiro y Xavi. Esas conversaciones me hacían perder el estrés. Gracias Aleix por la ayuda con los datos de eucariotas y por todo lo bueno que compartimos en Chile y en el Sarmiento.

A los que pasaron por el despacho P39 mientras estuve en el ICM, gracias!!!!! Gracias también Marta R, Isa S y Adrià, me encantó ir con uds de congreso y los ratos que conversamos en el ICM. Gracias también a Pablo por la ayuda con el clúster, todo lo hacía parecer muy fácil.

Gracias a los revisores externos, Hugo y Fernando y a los coautores de los capítulos, sin ustedes tampoco habría tesis.
Gracias también a Dolors, Cèlia, Cesc, Rafael, Silvia y Montse el departamento es increíble por la visión de personas como uds. Gracias también a Paula, Irene y a Eli Sa, siempre dispuestas a colaborar.

Gracias a todo el personal del ICM, este instituto es maravilloso por lo que hacen y por las personas que lo hacen.

Gracias Genoveva, Agustín y Vicente de la UPC por hacer que las gestiones administrativas fueran más fáciles y depositar a tiempo.

También le agradezco a Carmelo, Silvia, María, Alejandra, Jordi, P. Joaquim, JuanJo y Aracely por recibirnos en su comunidad, siempre nos sentimos como en casa.

De Barcelona paso a Costa Rica:

Gracias a la Junta de Becas de la Universidad Nacional por toda la gestión de mi beca, especialmente a María Teresa y a D. Randall. Gracias a Fran, Cuca y Tato por confiar en mi y ser mis garantes de la beca.

Luis Sierra, Luigi y Richard, uds tres impulsaron mi beca y siempre me dieron su apoyo, para mi eso es muy valioso. Gracias por creer en mi!!!! Gracias también Adri y Tania no sólo por apoyarme durante su gestión, sino también por darme ánimos para terminar.

Mi gran amigo Luis, mi marido laboral, gracias por el apoyo durante todo este tiempo. Gracias Alexiño y Ale, por su amistad y por darme ánimos tantas veces. También le quiero agradecer a Kali por escucharme, principalmente en estos últimos meses.

Gracias a las chicas de la ECB por ayudarme con todas las gestiones de mi beca, siempre con una gran sonrisa y la mejor disposición. Le agradezco también a mis compañeros de la EBM, el capítulo 1 es para uds, ya sabemos algo más de nuestro querido Golfo de Nicoya.

Un especial agradecimiento a mi querida comu, uds siempre estuvieron al lado de mi familia. D. Ana, Laurita, Xinita, Anita, D. María, D. Mary, Kathya, Ali, Marlon, David, Abi, Karo y al resto de esta gran familia.

Por último, pero no menos importante, a quienes siempre han estado conmigo. Gracias a mis padres y a mi hermana porque en nuestra familia el éxito de uno es el de todos, esto también es de uds.

A Carlos y Samuel por irse en esta gran aventura conmigo. Gracias Carlos por ser el mayor apoyo que cualquiera puede tener, por adoptar mi sueño de ir a sacar un doctorado y por animarme siempre en terminar la tesis. Gracias Sam por siempre decirme: ya casi mamá y por pedirme que te dedicara la tesis, no podía ser para nadie más. Ahora si, mamá tendrá más tiempo para que hagamos cosas juntos.

Puede que se me olvide alguien pero será porque mi mente ya está un poco agotada. Gracias a todos lo que han tenido que ver con esta tesis y con mi paso por Barcelona.

This thesis has been funded by the Universidad Nacional, Costa Rica, through a PhD fellowship to Carolina Marín Vindas.