

Marine microbial community diversity and responses to environmental change

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Abstract

The ocean is the largest biome on Earth and hosts an immense diversity of marine microorganisms. These include prokaryotes (bacteria and archaea), protists, fungi, viruses and microzooplankton. They form the base of marine food webs and sustain higher trophic levels through their critical role in the ocean carbon cycle and many other biogeochemical processes. Microorganisms are found across a large variety of ecosystems and show both global and local biogeographical distribution patterns. Different types of microbial organisation, e.g. free-living and surface-attached, led to a multifarious lifestyle. Over time, the specialized nature of both environmental lifestyles resulted in the development of unique communities with different capabilities. In this thesis both lifestyles were studied, and a further distinction between biotic and abiotic surface associated communities was made.

Climate change related stressors in the ocean have been linked to multiple changes that threaten the marine environment, such as increased sea surface temperature, ocean acidification, and deoxygenation. These changes have already impacted the structure, composition, and function of microorganism communities in several marine habitats. However, due to the large diversity that exists within the prokaryotic and eukaryotic groups, a lot is still unknown. In order to detect and quantify short-term and long-term responses to environmental change, baseline studies are needed that show the natural spatial and temporal variability within and between communities. This was the main objective in this thesis, to characterize microbial communities in different unique ecosystems by applying 16S and 18S amplicon sequencing and thereby providing a framework for future studies on climate change and future work on interspecies and interkingdom interactions.

In Chapter 2 free-living protist communities were studied along the Munida transect, off the east coast of the South Island, New Zealand. The transect is defined by Neritic Water, Subtropical Water, the Southland Front and sub-Antarctic Surface Water. The five-year study of protist communities along the transect employed amplicon sequencing of the 18S rRNA gene and optical methods (flow cytometry and FlowCam) to reveal spatiotemporal patterns and size distributions of the protist assemblages. The Southland Front was found to be a diversity hotspot with strong temporal structuring. The different water masses were primarily dominated by pico- and nanophytoplankton throughout the year, but only supported distinct protist communities amongst all four water masses in spring. The strongest differentiation in protist communities was found between the subtropical and sub-Antarctic water mass and was not only limited to spring

but consistent all-year round. In addition, samples were taken during a marine heatwave (MHW) and allowed for the characterisation of potential heatwave induced community shifts. During the MHW, protist diversity was highly reduced across the Southland Front and the communities were dominated by a dinoflagellate parasitoid species (*Syndiniales*). A substantial increase was also found in the small green algae *Chloropicophyceae*. These results demonstrate direct evidence of the importance of long-term studies and the provision of baseline data to detect environmental changes.

In a temperate *Macrocystis pyrifera* dominated kelp forest (Karitane, New Zealand), biofilm community composition and development was characterised over a small spatial scale (~200 m) using settlement plates (Chapter 3). Biofilm community succession was compared between the interior and exterior kelp bed, for prokaryotic (16S rRNA) and eukaryotic (18S rRNA) communities. At high taxonomic level the prokaryotic communities did not reveal any patterns, while eukaryotes showed obvious successional shifts and structural differences across the kelp bed. Overall, a delay in succession was observed at the exterior kelp bed for both kingdoms, likely due to wave disruption. For prokaryotic communities, the delayed development caught up with the interior biofilms after 20 days, while eukaryotic communities remained completely distinct between the two locations for the duration of the study (32 days). Overall, this study suggests that during early biofilm development prokaryotic communities are more strongly structured by inter-species interactions rather than the environment. The opposite trend was observed for the eukaryotic communities.

The biofilm taxonomic compositions of both prokaryotic and eukaryotic communities were additionally used in Chapter 4 for a comparison with *M. pyrifera* blade-associated microbial assemblages. Kelp are increasingly being threatened by direct and indirect anthropogenic stressors and therefore characterising the diversity and composition of the kelp microbiome will aid in understanding how and to what extent the microbiome contributes to kelp health. Distinct communities were observed between the kelp blades, the settlement plates and seawater. However, some generalist colonising species were present on both the kelp surface and the settlement plates (e.g. *Microtrichaceae*, *E. siliculosus*, and *Trochilia* sp.), while others were considered to be kelp-specific (e.g. *Blastopirellula*, *Persicirhabdus*, *Granulosicoccus*, and *U. leptochaeta*). Epi-endophytic prokaryotic and eukaryotic communities developing on young (meristem) and old (apex) kelp blade tissue, proved to be distinct from one another, from seawater communities, and from biofilms on the settlement plates. The spatial structuring along the kelp blade was also associated with a higher diversity of microorganisms on the blade apex. Further, a higher similarity was found for the eukaryotic biofilms between the kelp blade meristem and the young biofilms on the settlement plates than for the prokaryotic assemblages

between those sample types. Overall, the kelp exerted a higher selection pressure on the prokaryotic communities compared to the eukaryotic epi-endobionts.

To look at the possible responses of tropical biofilm communities to future ocean acidification, settlement plates were deployed near a CO₂ vent system in Dominica, Lesser Antilles (Chapter 5). Unique biofilm communities were found at the high *p*CO₂ site (~575 µatm) with species replacement being the driving force for the change in biofilm composition compared to the control site. Successional shifts during biofilm development were observed sooner at the acidified site suggesting a faster biofilm development. A higher phytoplankton biomass was present at the high *p*CO₂ site which was indicated by elevated chlorophyll-*a* concentrations. Additionally, microeukaryotic diversity increased under elevated *p*CO₂ levels, which included an increase in relative abundance of the toxin producing dinoflagellate *Alexandrium* and a small shift towards chain-forming diatom species.

Overall, this thesis established an important baseline of protist community composition, diversity and seasonality, and of biofilm composition and development. Thereby providing a framework for future studies looking at changes in free-living protist communities in the western South Pacific, and biofilm community dynamics in coastal ecosystems, including *M. pyrifera* associated microbiome interactions. This thesis also provided clear evidence of changes in microbial community composition and diversity in response to a marine heatwave event and ocean acidification. These findings have a wider application as they can be used to predict microbial responses to future environmental interruptions. However, more research is needed to identify the nature of these changes and the impact they have on the whole ecosystem.

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List of Abbreviations

rRNA	ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
HDPE	High-density polyethylene
DIC	Dissolved Inorganic Carbon
POM	Particulate Organic Matter
DOM	Dissolved Organic Matter
CTAB	Cetyl Trimethylammonium Bromide
TE	Tris-EDTA (Ethylenediamine Tetraacetic Acid; buffered solution)
PCR	Polymerase Chain Reaction
BSA	Bovine Serum Albumin
ASV	Amplicon Sequence Variant
DADA	Divisive Amplicon Denoising Algorithm
PERMANOVA	Permutational Multivariate Analysis of Variance
nMDS	non-metric Multidimensional Distance Scaling
PERMDISP	Permutational analysis of multivariate dispersions
MANOVA	Multivariate Analysis of Variance
ANOVA	Analysis of Variance
dbRDA	distance-based Redundancy Analysis
DESeq	Differential expression analysis for sequence count data
Ppmv	parts per million volume
$p\text{CO}_2$	partial pressure of carbon dioxide
OA	Ocean Acidification
TA	Total Alkalinity

T	Temperature
S	Salinity
SST	Sea Surface Temperature
SSS	Sea Surface Salinity
UV-VIS	Ultraviolet–Visible Spectroscopy
dsDNA	double stranded Deoxyribonucleic Acid
NGS	Next-Generation Sequencing
BH	Benjamini-Hochberg
dNTP	Deoxyribonucleoside Triphosphate
EPS	Extracellular Polymeric Substances
CCM	Carbon Concentrating Mechanism
CCA	Crustose Coralline Algae
RV	Research Vessel
CTD	Conductivity, Temperature, and Depth
SSC	Side Scatter
IDT	Integrated DNA Technologies
NW	Neritic Water
STW	Subtropical Water
SASW	Sub-Antarctic Surface Water
HNLC	High-nutrient, low-chlorophyll
MAST	Marine Stramenopiles

Table of Contents

Abstract.....	ii
Acknowledgements.....	v
List of Abbreviations.....	vi
Table of Contents.....	viii

Chapter 1 - General Introduction..... 1

1.1 General introduction.....	1
1.1.1 Marine microbial communities	1
1.1.1.1 Free-living marine microorganisms.....	3
1.1.1.2 Surface attached marine microorganisms.....	4
1.1.2 Macroalgae as host organisms for epibiotic biofilms.....	5
1.1.3 Current threats shaping future ocean ecosystems.....	6
1.1.4 Microbiome sequence data analysis.....	9
1.1.5 Molecular community analysis.....	11
1.2 Thesis outline.....	12
1.3 Author contributions.....	14

Chapter 2 - Seasonal protist community dynamics and taxonomic composition across the Southland Front and during a marine heatwave event..... 16

2.1 Introduction.....	17
2.2 Materials & Methods	20
2.2.1 Site description	20
2.2.2 Sampling.....	21
2.2.3 Environmental measurements.....	21
2.2.4 Flow cytometry.....	22

2.2.5	FlowCam analysis	23
2.2.6	Molecular methods.....	24
2.2.6.1	DNA extraction	24
2.2.6.2	18S rRNA Library preparation	24
2.2.7	Statistical analyses.....	25
2.3	Results	27
2.3.1	Hydrographic parameters.....	27
2.3.2	Macronutrient concentrations.....	30
2.3.3	Chlorophyll- <i>a</i> concentrations	32
2.3.4	Alpha-diversity.....	32
2.3.5	Community structure	34
2.3.5.1	Community structuring across water masses.....	35
2.3.5.2	Phytoplankton seasonality in individual water masses	36
2.3.6	Size class structuring.....	36
2.3.7	Taxonomical community composition.....	38
2.3.7.1	Structuring at low taxonomic resolution - division and class level.....	38
2.3.7.2	Structuring at high taxonomic resolution – genus and ASV level	40
2.3.8	Taxonomical structuring during the heatwave event.....	42
2.4	Discussion	44
2.4.1	Distinct protist communities across the individual water masses.....	44
2.4.2	Phytoplankton biomass trends and size distribution.....	46
2.4.3	The Front as a diversity hotspot in summer.....	47
2.4.4	Taxonomic composition of protist communities	48
2.4.5	Protist community dynamics during the 2017/2018 MHW	52
2.5	Conclusion	54
 Chapter 3 - Prokaryotic and eukaryotic biofilm development and succession dynamics on settlement plates in a kelp forest.....		57

3.1	Introduction.....	58
3.2	Materials & Methods	59
3.2.1	Site description	59
3.2.2	Experimental set up and sampling of settlement slides	60
3.2.3	Seawater microbial communities.....	62
3.2.4	Environmental variables.....	62
3.2.4.1	<i>In situ</i> pH and temperature	62
3.2.4.2	Description of A _T and DIC analytical methods	62
3.2.4.3	Biofilm chlorophyll- <i>a</i> analysis	63
3.2.4.4	Inorganic macronutrient analysis	63
3.2.5	Molecular methods.....	64
3.2.5.1	DNA extraction	64
3.2.5.2	16S rRNA Library preparation	64
3.2.5.3	18S rRNA Library preparation	65
3.2.6	Sequence post-processing and statistical analyses.....	66
3.3	Results	68
3.3.1	Environmental variables.....	68
3.3.2	Microbial biofilms on settlement plates	69
3.3.2.1	Biofilm community abundances	69
3.3.2.2	Spatio-temporal biofilm community composition dynamics	70
3.3.2.3	Small spatial structuring across the kelp bed.....	70
3.3.2.4	Community structuring over time.....	71
3.3.2.5	Correlation between beta-diversity and abiotic variables.....	72
3.3.2.6	Biofilm taxonomical succession across a small spatial scale	73
3.3.2.6.1	Prokaryotic communities	73
3.3.2.6.2	Eukaryotic communities	75
3.3.3	Seawater microbial communities.....	77
3.3.3.1	Microbial community diversity	77
3.3.3.2	Spatio-temporal taxonomic variation.....	78

3.4	Discussion	81
3.4.1	Successional dynamics and small spatial structuring of prokaryotic biofilms.....	81
3.4.2	Successional dynamics and small spatial structuring of eukaryotic biofilms.....	83
3.4.3	Ecological processes behind multi-kingdom communities	85
3.5	Conclusion	87

Chapter 4 - Assessment of kelp associated microbiome diversity and taxonomic composition..... 88

4.1	Introduction.....	89
4.2	Materials & Methods	91
4.2.1	Site description and sampling	91
4.2.2	Molecular methods.....	92
4.2.2.1	DNA extraction and sequence library preparation	92
4.2.3	Sequence analysis and Statistical Analyses.....	93
4.3	Results	95
4.3.1	Kelp blade associated microbiome	95
4.3.1.1	Epiphytic microbial abundances.....	95
4.3.1.2	Epiphytic microbial community dynamics and taxonomy	96
4.3.1.2.1	Prokaryotic kelp microbiome	96
4.3.1.2.2	Eukaryotic kelp microbiome	96
4.3.1.3	Kelp blade tissue type associated biofilm communities	97
4.3.1.3.1	Prokaryotic kelp microbiome	97
4.3.1.3.2	Eukaryotic kelp microbiome	98
4.3.2	Comparison of the microbial community diversity of settlement plates, kelp blades and seawater	99
4.3.2.1	Alpha-diversity.....	99
4.3.2.2	Beta-diversity.....	100
4.4	Discussion	102
4.4.1	Microbial communities of kelp blades, settlement plates and seawater were distinct	102

4.4.1.1	Taxonomy of kelp-associated prokaryotic communities	104
4.4.1.2	Taxonomy of kelp-associated eukaryotic communities.....	108
4.4.1.2.1	Comparison of epi-endophytic pigmented algae (Chlorophytes and Phaeophytes)	108
4.4.1.2.2	Comparison of epi-endophytic eukaryotes	109
4.4.2	Fine scale spatial microbial variation along <i>M. pyrifera</i> blades	110
4.4.2.1	Distinct prokaryotic communities on kelp blade tissue.....	110
4.4.2.2	Distinct eukaryotic communities on kelp blade tissue.....	112
4.4.2.2.1	Epi-endophytic pigmented algae (Chlorophytes and Phaeophytes)	112
4.4.2.2.2	Epi-endophytic eukaryotes	112
4.4.2.3	Tissue age associated community abundances and structure	114
4.5	Conclusion	114

Chapter 5 - Responses of microbial benthic communities to elevated $p\text{CO}_2$ levels at a tropical CO_2 vent..... 116

5.1	Introduction.....	117
5.2	Materials & Methods	119
5.2.1	Site description and geological setting.....	119
5.2.2	Experimental design and field measurements	121
5.2.2.1	Environmental data collection.....	122
5.2.3	Chlorophyll- <i>a</i> analysis	123
5.2.4	DNA extraction and amplicon sequencing.....	123
5.2.4.1	Prokaryotes (16S rRNA) library preparation.....	124
5.2.4.2	Microeukaryotes (18S rRNA) library preparation	124
5.2.5	Sequence data processing	125
5.2.6	Statistical analyses.....	126
5.3	Results	127
5.3.1	Alpha diversity	127
5.3.2	Biofilm community composition	128
5.3.3	Taxonomic composition at the control and high $p\text{CO}_2$ site.....	131

5.3.3.1	Prokaryotic community composition and succession	131
5.3.3.2	Microeukaryotic community composition and succession.....	133
5.3.4	Chlorophyll-a trends.....	135
5.4	Discussion	136
5.4.1	Biofilm community alpha diversity	136
5.4.2	Biofilm development processes under high $p\text{CO}_2$ levels	137
5.4.3	Biofilm taxonomic composition and community succession.....	138
5.4.3.1	Prokaryotic communities	138
5.4.3.1.1	Oxygenic Phototrophic Prokaryotes.....	140
5.4.3.2	Photo- and heterotrophic eukaryotes	140
5.5	Conclusion	143
Chapter 6 - General Discussion.....		145
6.1	Discussion	146
6.1.1	Free-living protist community dynamics.....	147
6.1.2	Microbiome of coastal kelp ecosystems	149
6.1.3	Prokaryotic and eukaryotic biofilm development patterns.....	149
6.1.4	Future trends in prokaryotic and protist communities	150
6.2	Future directions	151
References.....		154
Supplements.....		191

Chapter 1 |

General Introduction

1.1 General introduction

1.1.1 Marine microbial communities

The marine environment hosts an immense diversity of microorganisms including prokaryotes (bacteria and archaea), protists, fungi, viruses and microzooplankton (Giovannoni & Stingl, 2005; Zinger et al., 2011). In the upper ocean layer alone (0 – 300 m), microbial cell concentrations typically reach about 10^5 cells mL⁻¹ and represent the most dominant organisms in the marine environment (Ducklow & Kirchman, 2000). Major protist groups underlying the microbial communities include autotrophic, mixotrophic and heterotrophic species (Crane & Grover, 2010; Worden et al., 2015). Photosynthetic autotrophs that live in the euphotic zone are referred to as phytoplankton. Approximately, one-half of the global net primary productivity is mediated by these marine photosynthetic organisms (Ducklow & Kirchman, 2000; Buchan et al., 2014). Phytoplankton include one major prokaryotic group (oxygenic *Cyanobacteria*) and several unicellular eukaryotic groups (e.g. diatoms, chlorophytes, and certain dinoflagellates), who cover an incredibly wide size range: picophytoplankton (<2 µm), nanophytoplankton (2 – 20 µm), and microphytoplankton (20 – 200 µm). Within picophytoplankton, the picocyanobacteria size group has only been relatively recently discovered (1970s–1980s) and its members are considered to be the most abundant organisms on Earth. This group includes *Prochlorococcus* at 0.5 – 0.7 µm in diameter (Chisholm et al., 1988) and *Synechococcus* 0.8 to 1.5 µm in diameter (Waterbury et al., 1979; Johnson & Sieburth, 1979). The greatest diversity of phytoplankton can be found within the diatom group (picophytoplankton to microphytoplankton) with roughly 200,000 different members (Kooistra et al., 2007).

Heterotrophic species such as bacteria, fungi, several dinoflagellates, and flagellates rely on the oxidation of organic matter to assimilate carbon for growth (Ducklow & Kirchman, 2000). Next to viruses, heterotrophic bacteria are the most abundant microorganisms in the ocean. With cell sizes typically between 0.2 µm and 0.6 µm in diameter, bacteria form the smallest size group of the marine microbes (Whitman et al., 1998). Their small size gives them the largest surface area-to-volume ratio, an advantage that they use to out-compete other microorganisms for dissolved organic matter (Azam et al., 1983). Mixotrophic organisms can use both photosynthesis and food ingestion to acquire energy from either an inorganic or organic carbon source, respectively (Stoecker et al., 1997). They are represented in several protist groups (Graham & Wilcox, 2000) and have been well documented in dinoflagellates where they have been found across all the taxonomic orders (Stoecker, 1999).

Phytoplankton and bacterioplankton form the base of the food chain where they intricately sustain higher trophic levels through oxygen production, nutrient remineralisation, degradation of organic matter and the subsequent provision of energy and critical ecosystem functions (Figure 1.1) (Azam et al., 1983; Falkowski et al., 2008). More specifically, marine phytoplankton are responsible for converting about 50 petagrams of inorganic carbon dioxide to dissolved organic matter (DOM) annually by means of photosynthesis (Field et al., 1998; Yamasaki, 2003). DOM enters the food web through the release of biogenic carbon exudates or by heterotrophic protists and microzooplankton grazing on phytoplankton (Azam et al., 1983; Fenchel, 2008; Kirchman & Gasol, 2018). Bacteria and archaea subsequently decompose DOM through consumption, remineralisation and/or respiration, and use DOM as a direct source of energy for growth. In turn, these prokaryotes, as well as autotrophic cyanobacteria, are the food source for heterotrophic protists or fall victim to virus infections and subsequent cell lysis, thereby releasing organic carbon back into the microbial loop. A fraction of POM/DOM escapes predation, consumption and remineralisation and sinks to the deep ocean where carbon is sequestered over ecological to geological time scales with an average age of approximately 5,000 years (Bauer et al., 1992; Figure 1.1). Overall, microorganisms are the primary drivers behind marine biogeochemical cycles involving essential elements such as carbon, nitrogen, phosphorus, sulphur and iron (Paerl & Pinckney, 1996; Suttle, 2007; Falkowski et al., 2008; Zehr & Kudela, 2011; Hawley et al., 2017).

Over the past decades the marine microbial ecology field has received increasing attention. This is partly due to major technological advances that have been made in molecular analysis (see

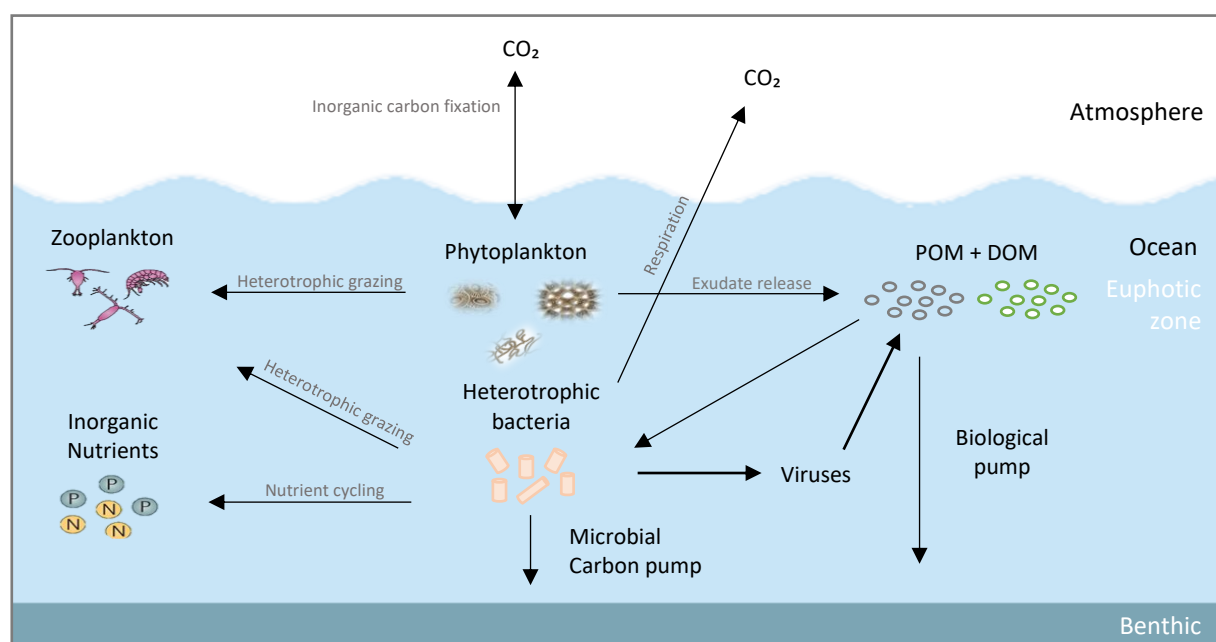


Figure 1.1: Simplified overview of microorganisms involvement in the carbon cycle. DOM: dissolved organic matter, POM: particulate organic matter, CO₂: carbon dioxide.

below: section 1.1.5), allowing a more comprehensive and in-depth assessment of microorganism diversity, composition, and spatiotemporal community dynamics. In order to study the ecology of marine microorganisms it is necessary to make a distinction between the different levels of microbial organisation. That is, microbial communities have multifarious lifestyles such as pelagic (free-living) and surface attached (e.g. on sediment, biotic and abiotic particles and surfaces). As a result of long-term evolution, a division between free-living and surface associated microbial communities occurred and led to the development of distinct capabilities in response to the different physical, chemical, and biological properties of their chosen environmental lifestyle (Amin et al., 2012; Luo et al., 2013; Dang & Lovell, 2016). Within this thesis the focus will be on (I) free-living protist communities in the ocean sunlit layer across distinct oceanographic water masses and (II) biofilm organised prokaryotic and eukaryotic microorganisms on biotic and abiotic surfaces in highly unique coastal ecosystems (coastal kelp forest and a hydrothermal vent).

1.1.1.1 Free-living marine microorganisms

The ocean represents the largest continuous biome on Earth with two thirds of its biomass accounted for by plankton communities (Bar-On et al., 2018). Overall, protist community composition and abundance are affected by seasonal variation as a result of changes in abiotic factors, e.g. temperature, light, and nutrient levels (Sherr et al., 2007; Vannier et al., 2016), and a change in biotic factors, e.g. prey availability, grazing pressure, and interspecies interactions including parasitism, symbiosis and mixotrophy (Stoecker et al., 1997; Gran-Stadniczeňko et al., 2019; Anderson & Harvey, 2020).

Diatoms (*Bacillariophyta*) are highly prevalent in the marine realm and account for ~40% of primary productivity in the ocean, making them major contributors to the carbon cycle (Nelson et al., 1995; Victor Smetacek, 1999). They increase in abundance during phytoplankton blooms when nutrient- (nitrate and silicate) and iron-rich conditions occur (Martin et al., 1991; Malviya et al., 2016). In general, diatoms tend to have a higher presence at high latitudes and in upwelling regions (Pierella Karlusich et al., 2020). Microzooplankton grazers, including ciliates and heterotrophic or mixotrophic dinoflagellates, have been suggested to be responsible for ~65% of grazing during diatom dominated blooms (Strom et al., 2001). Dinoflagellate taxa employ a variety of ecological strategies that define their abundance and distribution which is linked to the different trophic modes the members of this group belong to (e.g. autotrophy, heterotrophy and mixotrophy) (Stoecker et al., 2009; Worden et al., 2015). The presence of a mixotrophic lifestyle complicates the observation of general trends within dinoflagellates, and results in an underexplored ecology of this group. Chlorophytes can be divided into two main groups (*Mamiellophyceae* and *Chloropicophyceae*) with distinct distribution patterns as observed by the global *Tara* Oceans expedition and the Ocean Sampling Day initiative. *Mamiellophyceae* are

particularly abundant in coastal waters (Monier et al., 2016; Vannier et al., 2016), while *Chloropicophyceae* (formerly clade VII prasinophyte) dominate the offshore oceanic waters (Shi et al., 2009; Dos Santos et al., 2017).

The surface open ocean hosts a variety of unique ecosystems characterised by biogeochemical and physiological parameters, such as productive zones (upwelling and frontal systems), coastal adjacent regions and offshore open waters (Moore et al., 2001a). Higher nutrient levels are generally found in productive regions and offshore water masses due to coastal run-off, upwelling or increased mixing of deep nutrient-rich water (Boyd et al., 1999). In addition, high radiation levels and more shallow water depths in coastal water masses promote primary productivity and increased phytoplankton biomass (Trombetta et al., 2019). The opposite is true for open ocean offshore regions, such as the Southern Ocean, subarctic Northeast Pacific, and equatorial Pacific, which are known as high-nutrient low-chlorophyll zones characterised by exceedingly low iron availability (Moore, et al., 2001b). In the beginning of microbial ecology studies, all microorganisms were believed to have a pan-ocean distribution with local environmental factors being responsible for species selection and community structuring (i.e. ‘everything is everywhere, but the environment selects’; Baas-Becking, 1934). Due to the increased exploration of microorganism genetic diversity, the consensus about their unrestricted dispersal has been brought into question (Grosberg & Cunningham, 2000; Martiny et al., 2006) and multiple studies have now shown the presence of global biogeographic patterns for marine microorganisms across these diverse ecosystems (Foissner, 2006; Rusch et al., 2007; Pommier et al., 2007; Casteleyn et al., 2010; Sunagawa et al., 2015; Mousing et al., 2016; Vincent et al., 2022).

1.1.1.2 Surface attached marine microorganisms

Colonisation of any suitable marine surface by microbial assemblages is a well-regulated process that proceeds via sequential steps (Hall-Stoodley et al., 2004). The resulting surface community structures are referred to as biofilms and are defined as aggregates of mixed microorganisms including bacteria, archaea, protist, fungi and viruses (Costerton et al., 1978, 1995; Salta et al., 2013). The pioneer colonisers are usually free-living bacteria that adhere to substrata through initial interaction with the surfaces organic and inorganic particles (Dang & Lovell, 2000). Once bacterial cells have attached themselves to the substrate, they accumulate through growth and multiplication. The primary colonising bacteria also play a crucial role in modifying the surface physicochemical properties via the production of extracellular polymeric substances, thereby rendering the surface either suitable or unsuitable for subsequent colonisers including algae, cyanobacteria, and protists (Dang et al., 2008; Dang & Lovell, 2016). Extracellular polymeric substances (EPS) are a complex matrix of high-molecular weight polymers, such as polysaccharides and proteins, and are a characteristic and fundamental feature of marine biofilms

(Costerton et al., 1978, 1995; Flemming & Wingender, 2001, 2010; Steinberg & Kolodkin-Gal, 2015; Flemming, 2016). Further, a primary biofilm is established through interactions of this constructed monolayer (bacteria and EPS) with other free-living bacteria (Dang & Lovell, 2000). Finally, the primary biofilm community will develop into a mature three-dimensional biofilm through mutualistic and antagonistic interactions including cell-to-cell communication by employing chemical cues such as acylhomoserine lactone (quorum sensing; Gram et al., 2002), and the subsequent successive recruitment of additional unicellular and multicellular colonisers (Dang & Lovell, 2000, 2016; Davey & O'toole, 2000; Molino et al., 2009; Huggett et al., 2009).

The formation of these complex microbial colonies provides important advantages for all microorganisms included, such as i) protection from predation and environmental stressors, ii) protection against toxins and antibiotics, iii) increased availability of nutrients and metabolic cooperativity, iv) sustaining extracellular enzyme activities, and v) genetic trait exchange and acquisition (Dang & Lovell, 2000, 2016; Gram et al., 2002; Lehman & O'Connell, 2002). Further, biofilms are also vital in initiating settlement of invertebrate larvae and algal spores (Salta et al., 2013; Singh & Reddy, 2014; Kegler et al., 2017; Espinel-Velasco et al., 2021). Research has shown that no single bacterial species but rather a collaboration of several microorganism are needed to promote larval settlement (Qian et al., 2003; Chung et al., 2010). Changes to biofilm composition in light of climate change events could compromise the settlement process. This was shown in a study by Espinel-Velasco et al. (2021), where direct and indirect effects of ocean acidification led to changes in settlement selectivity. Interruptions at the early development stage could cascade effects on higher levels in the food chain and ultimately affect the whole ecosystem. This emphasizes the need to better understand the impacts of ocean acidification on biofilm community composition and development processes.

1.1.2 Macroalgae as host organisms for epibiotic biofilms

Seaweeds or marine macroalgae are multicellular protists that are important habitat forming organisms (foundational species). They thrive in temperate nutrient-rich cold-water coastal regions where they create highly productive three-dimensional underwater ecosystems that provide shelter, food, and nursery grounds for a myriad of biota including marine mammals, invertebrates, fishes, other algae, epibiota and birds (Mann, 1973; Duggins et al., 1989). In these coastal ecosystems, kelp and their associated epibionts contribute vastly to nutrient cycling and long-term carbon sequestration (Pessarrodona et al., 2018; Pfister et al., 2019). In a study of Wheeler & Druehl (1986), canopy-forming kelp in Canada were shown to fix up to 1.3 kg of carbon per m² each year. In addition to sustaining higher marine trophic levels, they further benefit local

coastal economies through providing commercially harvestable resources, tourism, shoreline protection (Schiel & Foster, 2015; Wernberg et al., 2019; Bayley et al., 2021).

Microbial communities have been found on the surfaces of different macroalgae anatomical structures and form symbiotic relationships with their host (Weigel & Pfister, 2019; Ihua et al., 2020; Lemay et al., 2021). The microbiome contributes to the kelp through nutrient provision, defence against pathogens and grazers, and supports overall development (e.g. spore release, growth, metamorphosis) (Weinberger, 2007; Goecke et al., 2010; Wahl et al., 2012; Hollants et al., 2013; Egan et al., 2013; James et al., 2020). These intimate interactions thereby are considered to be responsible for influencing kelp health, physiology, and ecology. Gaining a better understanding of the kelp microbiome diversity, composition and functioning is essential in determining how and to what extent these epibionts contribute to kelp health. An incubation experiment by Minich et al. (2018) revealed that increased temperature drives the dysbiosis of kelp-microbiome and leads to kelp degradation, thereby revealing a strong link between kelp health and the associated microbial communities. With the increasing anthropogenic impacts kelp forest are facing today this becomes a pressing research topic (Wernberg et al., 2011, 2013; Krumhansl et al., 2016; Thomsen et al., 2019).

1.1.3 Current threats shaping future ocean ecosystems

Climate change induced variations in ocean surface temperatures and ocean chemistry are impacting marine ecosystems on a large scale by altering the physical, chemical, and biological environment (Doney et al., 2012). The major climate change related stressors for the marine realm are an increase in sea surface temperature, ocean acidification, and deoxygenation (Doney et al., 2009; Keeling et al., 2010; Bopp et al., 2013). Atmospheric carbon dioxide (CO₂) to-date has reached levels higher than 410 ppm, which is almost 50% higher than preindustrial concentrations and continues to rise at a fast rate (Gingerich, 2019). This huge increase is attributed to excessive emissions of greenhouse gasses mainly sourced by anthropogenic processes such as fossil fuel burning, agricultural emissions, and deforestation (Caldeira & Wickett, 2005). Together with other greenhouse gasses, CO₂ causes global warming which also influences ocean temperatures. Over the past century, sea surface temperature (SST) has risen by about 0.7°C (Bindoff et al., 2007) and has been the cause of multiple additional changes that are threatening marine ecosystems, such as increased upper ocean stratification, rising sea level, decreasing ice cover, changing ocean currents and altered weather patterns (Doney et al., 2012; and references therein). Concurrent with continued gradual warming, marine heatwaves (MHW) have also been reported and are predicted to increase in frequency and duration (Oliver et al., 2018). MHWs are defined as anomalous warm water events that span a period of at least five days

with temperatures warmer than the 90th percentile, based on a 30-year baseline climatology (Hobday et al., 2016). On a regional scale, these events have been the cause for changes in ecosystem functioning and structuring by driving species range shifts, leading to widespread mortality and altering community compositions (Garrahou et al., 2009; Wernberg et al., 2013; Smale et al., 2013).

Ocean surface layers are slightly alkaline with a global average pH of about 8.1 and a variation of ± 0.3 units accounting for local, regional and temporal variability (Bindoff et al., 2007). Since pre-industrial times, sea surface pH has decreased by about 0.1 units and is predicted to further decline between 0.07 – 0.4 units, depending on the Shared Socioeconomic Pathway (SSP) scenario, by the end of 2100 (Figure 1.2) (IPCC, 2021). This process is referred to as ocean acidification and is a direct result of oceanic absorption of atmospheric CO₂ involving several chemical reactions (Doney et al., 2009). When atmospheric CO₂ is absorbed, it reacts with water and forms a weak acid (carbonic acid; H₂CO₃). Carbonic acid will release hydrogen ions (H⁺) and is thereby converted to bicarbonate (HCO₃⁻). The addition of hydrogen ions to the environment increases acidity and subsequently lowers seawater pH. Excess hydrogen ions in the ocean will lower the availability of carbonate ions (CO₃²⁻) in the water column by forming HCO₃⁻. Overall, ocean acidification results in an increase in CO₂(aq), HCO₃⁻ and H⁺, and a decrease in CO₃²⁻ and pH. Consequently, many marine organisms that use calcium carbonate (CaCO₃) to develop their shells and skeletons will be most vulnerable to ocean acidification.

Changes to the ocean chemistry and sea surface temperatures could influence metabolic rates, bacterial and protist community composition and growth, alter interactions between these

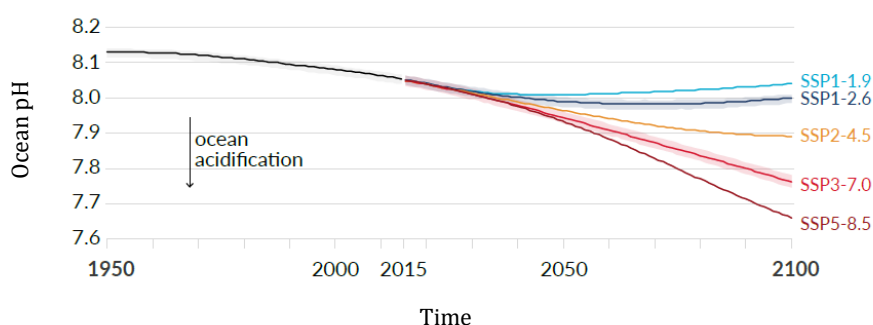


Figure 1.2: Global ocean surface pH projections for the end of the century (2100). The projections for each of the five-greenhouse gas (GHG) emission scenarios are shown in colour. The shades depict the uncertainty ranges for each scenario. The black curve represents the historical values. SSP: shared socioeconomic pathway. The five GHG emission scenarios: very low (SSP1-1.9), low (SSP1-2.6), intermediate (SSP2-4.5), high (SSP3-7.0), and very high (SSP5-8.5). All pH projections are climate models that are based on CMIP6 model simulations. CMIP6: coupled model intercomparison project phase 6. Image adapted from IPCC, 2021.

microorganisms, and ultimately impact ecosystem functioning (Thomas et al., 2012; Toseland et al., 2013; Nagelkerken & Connell, 2015; Ullah et al., 2018). For instance, global observations and predictions based on CO₂ emission modelling for the end of the 21st century (Bopp et al., 2013; IPCC et al., 2021) and metabarcoding data from a global plankton survey (Ibarbalz et al., 2019), both suggested that phytoplankton diversity will generally increase with the strongest trends at higher latitudes in the Northern Hemisphere (Vincent et al., 2022). This has also been suggested by Hutchins & Fu (2017) and further supports tropicalization of temperate marine ecosystems as shown for bacterioplankton (Morán et al., 2010, 2017), zooplankton (Beaugrand et al., 2015), fish (Cheung et al., 2013; Vergés et al., 2016), and kelp ecosystems (Wernberg et al., 2016).

Great effort has gone into researching the sensitivity and resilience of small marine organisms to climate change related impacts (Wernberg et al., 2012; Nagelkerken & Connell, 2015). However, the vast majority of earlier studies have focussed on experimental work under controlled conditions involving acute exposure to elevated *p*CO₂ levels, short-term mesocosm designs, and experiments using single species or simplified food webs with single stressors (Riebesell et al., 2010; Wernberg et al., 2012; González-Delgado & Hernández, 2018). Although those works have provided useful data, they do not account for the effects of chronic ocean acidification (incl. long-term acclimation and evolutionary adaptation), or the complexity of multi-species and multi-kingdom interactions which are influenced by a vast amount of biotic and abiotic variables associated with natural marine ecosystems (Riebesell et al., 2010). The limitations of those experiments were recognised and the need to study open ecosystems shifted the focus of the scientific community towards *in situ* observational studies and *in situ* perturbation experiments (Hall-Spencer et al., 2008; Kerfahi et al., 2014, 2020; Taylor et al., 2014; Allen et al., 2021).

Furthermore, baseline data including documentation of biological community compositions, natural variations in community ecological processes, spatiotemporal patterns, as well as identifying biotic and abiotic drivers structuring the marine systems are necessary to observe, predict and manage short-term and long-term responses to future ocean perturbations (Shade & Handelsman, 2012). A key example is the *Tara* Oceans expedition that took place from 2009 to 2013 and surveyed plankton community diversity and taxonomy on a global scale. The expedition generated a most comprehensive baseline dataset that has been the framework for a vast amount of plankton ecological studies and climate change prediction models (e.g. De Vargas et al., 2015; Lima-Mendez et al., 2015; Ibarbalz et al., 2019; Logares et al., 2020; Vincent et al., 2022). Nonetheless, a lot of local and regional marine ecosystems, especially around New Zealand are still lacking this type of baseline data.

1.1.4 Microbiome sequence data analysis

The first step in microbial community ecology to analyse the community composition and structure of microorganisms is describing the alpha diversity of amplicon sequence data. Alpha diversity refers to the mean species diversity within each sample, or within different sites on a local scale, and addresses both species richness and evenness. Species richness is a measurement of the number of taxonomic groups within each sample (Chao et al., 2016), while species evenness is a reflection of how evenly the abundances of those taxonomic groups are distributed. These alpha diversity indices can be measured separately (e.g. Chao1 richness and Pielou's evenness) or together as one metric (Shannon diversity). A detailed description of different alpha-diversity indices and their application in microbiome ecology research can be found in the work by Li et al. (2012) and Thukral (2017). Once the metrics have been calculated, a statistical comparison of the mean alpha diversity values is possible using modelling and hypothesis testing. If the assumptions of normality are met by the data, a parametric univariate analysis (ANOVA) is applied. However, when the data violates these assumptions, i.e. normal distribution and homogeneity of variances amongst the groups, or when the data is structured by an unbalanced design (unequal and/or small sample sizes), a non-parametric univariate analysis (Kruskal-Wallis) is used to compare the median alpha diversity values.

To study the diversity between samples, i.e. amongst microbial communities, rather than within each sample, beta diversity is measured. This involves creating a distance matrix which records the (dis)similarities between samples or between different sites. The distance matrix can be based on taxonomic abundance (Bray-Curtis dissimilarity), species presence or absence (Jaccard dissimilarity), or phylogeny (not used in this thesis). It follows that the Bray-Curtis index (Bray & Curtis, 1957) will determine the distances between the microbial communities quantitatively, while the Jaccard index (Gower, 1985) does this qualitatively. To graphically illustrate these differences, unconstrained ordination techniques are used that can project the multidimensional data into a low dimensional space and thereby reveal potential community structuring. The unconstrained ordination technique used throughout this thesis is non-metric multidimensional scaling (nMDS). This is a rank-based approach, which means that the original distance data is being replaced with ranks. Rank-based methods are generally considered as more robust techniques than the traditional ordination methods as they can tolerate data that lack an identifiable distribution. Specifically, nMDS can accept data with missing pairwise distances, it allows for any dissimilarity measure to be used in creating the distance matrix, and it can use different types of variables (quantitative, qualitative, or mixed). In the final nMDS plot, similar samples (microbial communities) will be plotted closer together, while dissimilar samples are placed further apart. The nMDS technique is explorative in nature and requires a non-parametric

multivariate analysis to follow up on any potentially community patterns that were observed. Permutational multivariate analysis of variance (PERMANOVA; Anderson, 2017) is the method of choice throughout this thesis. A PERMANOVA test analyses multivariate data in response to one or more variables or factors, based on any (dis)similarity matrix and using permutations to obtain *p*-values. The focus of a PERMANOVA is to detect 'location' effects, meaning the detection of differences in the centroids (or central locations) of the multivariate groups that are being assessed. However, PERMANOVA tests are sensitive to differences in dispersion amongst groups and cannot distinguish between true and false location effects due to heterogeneity of dispersions. It is therefore necessary to perform a separate analysis to investigate the homogeneity of multivariate dispersions, using PERMDISP analysis (Anderson et al., 2006). By running PERMDISP in addition to PERMANOVA and the nMDS ordination, it is possible to uncover the nature of the differences detected among the groups, i.e. location effects, dispersion effects or a combination of both.

In addition to unconstrained ordinations, it is also possible to use a set of explanatory variables (e.g. environmental parameters) to describe the variation among the microbial communities. This type of analysis is referred to as constrained ordination. Distance-based redundancy analysis (dbRDA) is a linear constrained ordination technique where dissimilarity data is regressed against explanatory variables (Legendre & Anderson, 1999). The results show the decomposition of the dissimilarities into variation (or inertia) related to environmental parameters (i.e. constrained axes) and not related to environmental parameters (i.e. unconstrained axes).

In 2010, Baselga facilitated the partitioning of beta diversity into its two separate components, spatial turnover and nestedness (Baselga, 2010). Turnover is the main cause for community structuring when the difference between the communities is explained by species replacement. Species replacement events are linked to environmental sorting or defined through spatial and historical limitations (Qian et al., 2005). Nestedness is the dominant driver behind community structuring when the community with the smallest amount of species numbers represents a subset of the richer community. This process reflects species loss and is the result of any parameter that promotes breaking up organism assemblages in an orderly manner (Gaston & Blackburn, 2000). Both components can be calculated from a beta diversity metric based on a species presence/absence distance matrix (e.g. Jaccard dissimilarity).

Once microbial community patterns have been investigated, the taxonomic composition of the microbial communities can be explored at both high and low resolution. A MANOVA test can be performed on the most abundant taxonomic groups to test for significant variations in their relative abundances across selected variables. A MANOVA is a multivariate parametric analysis

that allows to test multiple independent variables on multiple dependent variables, while correcting the p -value for multiple comparisons. Assumptions of normality and homogeneity of variances must be met. To look for differences between microbial communities at high resolution, differential abundance analysis can be performed using the DESeq test (Love et al., 2014). DESeq will identify the differentially abundant ASVs (amplicon sequence variant: a small taxonomic unit) along the chosen variable and based on a predefined significance level. This analysis could help identify which ASVs are driving the patterns observed by the beta-diversity analysis.

1.1.5 Molecular community analysis

Early studies on microbial communities applied enrichment and culture-based approaches developed by Pasteur, Koch, Beijerinck and Winogradsky (Beijerinck, 1895). In order for microorganisms to be successfully cultivated, knowledge about their physiological niche was required and needed to be experimentally mimicked. Needless to say, these techniques were time consuming and labour intensive, and it soon became evident that many species that were visible under light microscopy were unable to establish a culturable state (Amann et al., 1995). Even though microscopic identification of bacteria and protists, through the characterisation of their morphological features combined with species enumeration, resulted in great progress in the field of marine microbiology, these methods were deemed inadequate to study the true diversity and composition of microorganisms (Keeling & Campo, 2017). This led to the development of culture-independent techniques with molecular methods based on polymerase chain reaction (PCR) for DNA amplification being the predominant ones used today.

In the last two decades, high throughput sequencing (HTS) methods have made substantial advances in the field of marine microbial ecology. Dramatic improvements have been made in characterising prokaryotic and eukaryotic diversity and abundance (Flombaum et al., 2013; De Vargas et al., 2015; Massana et al., 2015; Le Bescot et al., 2016). New lineages of marine protists have been discovered (Moon-Van Der Staay et al., 2001; Lefranc et al., 2005) and previously under-appreciated small cell-groups, the pico- (0.2 – 2 μm) and nano- (2 – 20 μm) phytoplankton, have now been shown to be the most dominant groups in the ocean (Vaulot et al., 2008). Additionally, symbiotic and epibiotic relationships amongst microorganisms and marine plants and animals have generated new insight due to the discovery of the newly found diversity within the associated microbial communities (Apprill, 2017; Weigel & Pfister, 2019).

1.2 Thesis outline

The goal of this thesis is to characterise microbial taxonomic composition and community dynamics within local and regional ecosystems in New Zealand. Thereby providing baseline data, e.g. taxonomic composition, diversity, cell size groups, development processes, and spatiotemporal patterns, against which future studies can be compared to. Secondly, this thesis will investigate the responses of marine microorganism communities to extreme temperatures and ocean acidification. This will contribute to our understanding of how global, and perhaps future, climate change events impact marine microorganisms. The four data chapters of this thesis cover different lifestyles (free-living and surface attached) of marine microorganism communities in different unique ecosystems (Figure 1.3), and thus provide insight in different aspects of microbial ecology. Collectively, these data projects will provide a framework for future research focussing on revealing ecological network processes, and ultimately on a broader scale ecosystem-responses, to ongoing and future climate change events.

| Chapter 1

A review of the literature concerning marine microbial communities, their importance to ocean ecosystems and their different lifestyles is given. Current and future threats that impact these marine microorganisms are also discussed. Finally, an overview of the different methods that are being used throughout this thesis to analyse microbial amplicon sequence data, and thereby assess microbial community dynamics, is described in detail.

| Chapter 2

This chapter provides a comprehensive long-term analysis of protist community composition across four different water masses off the south-eastern coast of New Zealand. A five-year data set has been collected to describe protist diversity, composition, size distribution, and seasonal patterns across the Southland Front. A combination of amplicon sequencing (18S rRNA) and optical technologies, Flow cytometry and FlowCam, are used to analyse the data. Additionally, this chapter documents the impact of a summer marine heatwave on the protist taxonomical composition and community diversity across the Southland Front. The hypothesis put forward in this chapter are: (i) different water masses will host distinct protist communities all-year round, (ii) seasonal patterns are expected for the protist communities in each water mass, (iii) the frontal system acts as a diversity hotspot, and (iv) the marine heatwave will simplify protist community compositions across the Southland Front in terms of taxonomy and diversity.

| Chapter 3

In Chapter 3, biofilm communities across a small spatial scale within a local kelp ecosystem, New Zealand, are studied. The taxonomy and development of prokaryotic and eukaryotic biofilms on settlement plates are investigated at approximately one-week intervals for a month using amplicon sequencing. The objective of this study is to better understand biofilm development and the taxonomic composition of benthic biofilm communities within the kelp ecosystem. Microbial community structures are expected to differ across the kelp bed for both prokaryotic and eukaryotic communities. The ecological mechanism responsible for the differences across the kelp bed and over time is expected to be turnover. Further, the data analysed in this chapter is also used in Chapter 4.

| Chapter 4

Macrocystis pyrifera associated microbial communities (prokaryotic and eukaryotic) were sampled from a local kelp forest, New Zealand, to study their taxonomical composition and ultimately gain a better understanding of the functional role the microbiome plays in kelp health. To identify kelp-specific microbial taxa, a comparison between kelp associated microbial assemblages, biofilms on the settlement plates (Chapter 3), and seawater microbial communities is performed. Spatial structuring along the kelp blade was assessed by looking at the microbial communities on the young blade tissue (meristem) and comparing them to communities on the older blade tissue (apex). Further, the kelp blade microbiomes were sampled from two locations in the kelp forest to assess their variability across the kelp forest. Overall, (i) kelp microbial communities were expected to be distinct from the biofilms on the settlement plates and the surrounding seawater communities, (ii) spatial structuring was expected along the different kelp blade tissues with the apical tissue supporting higher alpha diversity for both prokaryotic and eukaryotic epi-endobionts, and the (ii) kelp-microbiome communities were expected to be similar across the kelp bed.

| Chapter 5

Chapter 5 studies the response of biofilm communities to elevated $p\text{CO}_2$ levels at an interval of five days for the duration of 20 days. This involved an *in situ* experiment near a coastal shallow CO_2 vent system in Dominica. The vent system was used as a natural analogue for predicting future ocean acidification impacts on marine microbial communities. Prokaryotic and eukaryotic biofilm

community composition and development were compared between a control site and the CO₂ vent, using amplicon sequencing. Several hypotheses were made: (i) elevated $p\text{CO}_2$ levels will alter the biofilm community composition, (ii) a higher degree of taxonomic homogenisation will be achieved near the high $p\text{CO}_2$ site, (iii) turnover is the main driver behind the distinct communities at the high $p\text{CO}_2$ site, and (iv) high $p\text{CO}_2$ will shift diatom communities towards larger species.

| Chapter 6

In this last chapter, the findings of all four data chapters are synthesised and their contribution to the current knowledge of microbial ecology is discussed. Finally, future directions for marine microbial community research are proposed.

1.3 Author contributions

Table 1.1: Summary of major author contributions to each chapter. HH = Hanneloor Heynderickx, LH = Linn J. Hoffmann, TS = Tina Summerfield, KC = Kim Currie, JD = Judith Murdoch, RA = Rohan Allen, SR = Sam Rastrick

	Writing	Editing	Experimental design	Data collection	Sequencing; preparation and post-processing	Statistical design and data analysis
Chapter 1	HH	HH, LH, TS	-	-	-	-
Chapter 2	HH	HH, LH, TS	HH, LH, TS, RA	HH, KC, JM, LH, RA	HH	HH
Chapter 3	HH	HH, LH, TS	HH	HH, TS	HH	HH
Chapter 4	HH	HH, LH, TS	HH	HH, TS	HH	HH
Chapter 5	HH	HH, LH, TS	LH, TS, RA	HH, SR, RA	HH	HH
Chapter 6	HH	HH, LH, TS	-	-	-	-

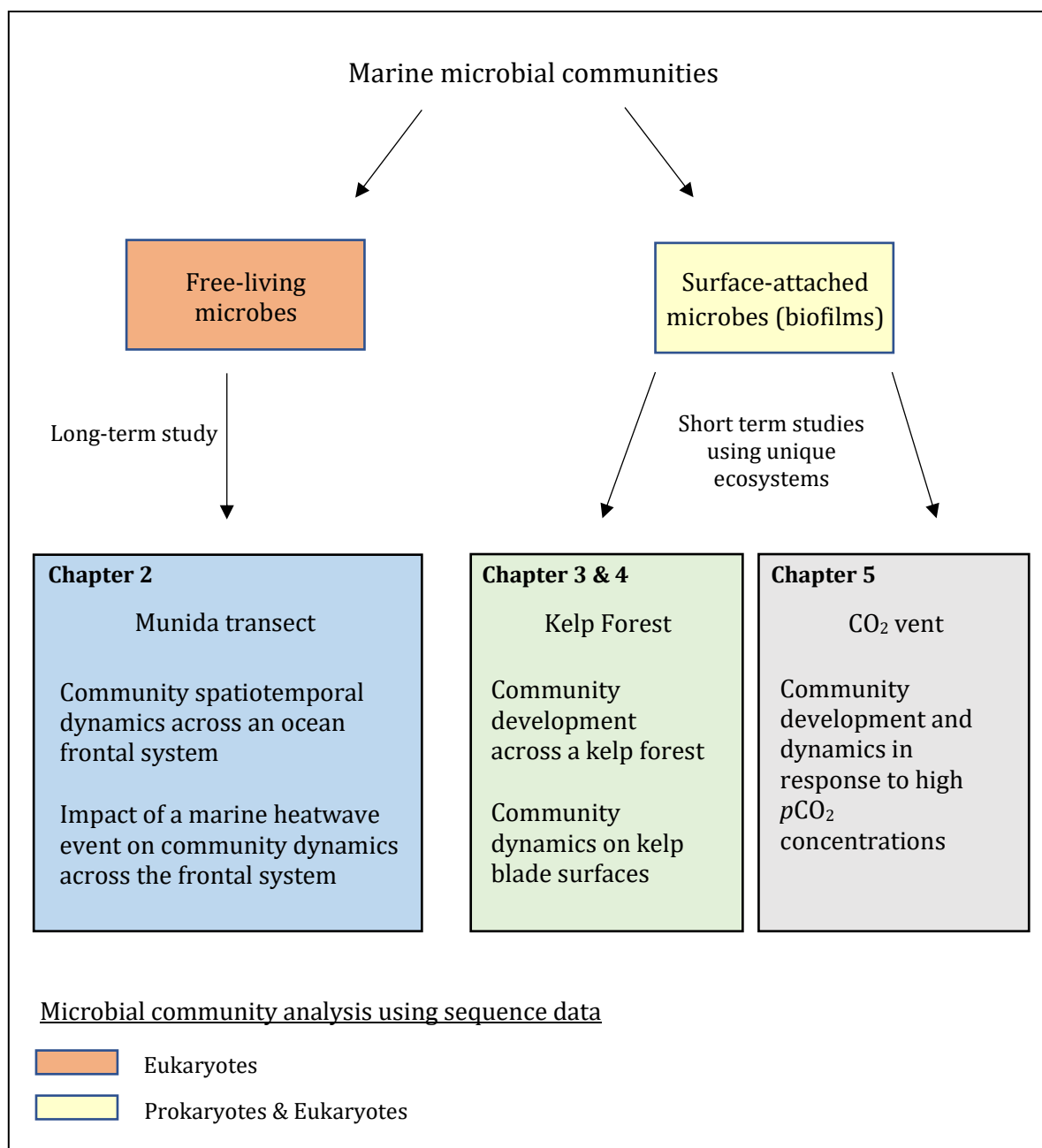


Figure 1.3: Schematic framework of the thesis structure.

Chapter 2|

Seasonal protist community dynamics and taxonomic composition across the Southland Front and during a marine heatwave event

Hanneloor Heynderickx, Linn Hoffmann, Tina Summerfield

2.1 Introduction

Protists are a diverse group of unicellular organisms, including cyanobacteria, diatoms, dinoflagellates, and chlorophytes. They dominate the sunlit layer of the ocean where they are largely responsible for the net primary productivity through their involvement in the carbon cycle and biogeochemical fluxes (Azam et al., 1983; Falkowski et al., 2008). Their high abundance in the ocean photic zone and their global distribution is attributed to the range of trophic modes that have been characterised within this group, e.g. autotrophy, heterotrophy, and mixotrophy (Crane & Grover, 2010; Worden et al., 2015). Unicellular protists cover different size groups with microphytoplankton (20-200 μm) representing the larger cells, mostly diatoms and dinoflagellates, and have received the most attention in the past (Pierella Karlusich et al., 2020). With the continuous improvement of optical and sequencing methods in the microbial field during the past two decades, the previously underexplored abundance and diversity of the smaller cell groups have gained increasing interest. A distinction was made between the picophytoplankton (0.2 – 2 μm) and nanophytoplankton (2 – 20 μm). The picocyanobacteria, *Prochlorococcus* and *Synechococcus* were relatively recent discovered (1970s – 1980s), yet they have been found to be the most abundant organisms in the upper ocean (Partensky et al., 1999).

The recent *Tara* Oceans expedition explored patterns of protist biodiversity on a global scale and provided the largest plankton survey to date (Vincent et al., 2022). The project confirmed global biogeographical trends of different phytoplankton size groups that have been characterised by multiple studies at local and regional scales over a century of research (Pierella Karlusich et al., 2020). The use of high throughput sequencing has been key in assessing the diversity of protist communities across a variety of ecosystems (Santoferrara et al., 2020). For instance, dinoflagellate abundance and diversity were shown to be much higher than had previously been reported with a specific increase in parasitic and mixotrophic species (De Vargas et al., 2015; Lima-Mendez et al., 2015). Overall, the project covered many different aspects of microbial ecology thereby hugely improving the current knowledge of protist community dynamics, distribution, and size range. Despite the global nature of the expedition, the western South Pacific Ocean received little attention. In addition, the survey was limited by studying single time points and thus did not characterise seasonal variability patterns. Long term monitoring is key in identifying gradual changes of community assemblages as a response to climate change. However, baseline data of natural community diversity and structuring patterns due to seasonal and inter-annual variability is needed in order to detect the true effects of climate change.

The Southland Front, located off the eastern coast of New Zealand, is a local manifestation of the circumpolar Subtropical Frontal Zone (STFZ) due to its close proximity to land and is subsequently influenced by seasonal coastal processes (Heath, 1972, 1981; Currie & Hunter, 1999; Van Hale & Frew, 2010; Hopkins et al., 2010; Currie et al., 2011). Like the STFZ, the Southland Front forms the boundary between warm, nutrient-poor and high salinity modified Subtropical Water (STW), and cold, nutrient-rich and low salinity sub-Antarctic Surface Water (SASW) (Heath, 1981; Hamilton, 2006). The frontal waters of the STFZ are advected northward along the south-eastern coastline of the South Island, New Zealand. They are confined along the Otago continental shelf, located about 40 – 50 km offshore (Figure 2.1) and thereby compacted to a narrow width of approximately 2 – 10 km (Heath, 1972; Sutton, 2010). In other oceanic regions the STFZ is characterised by a broad spatial zone (> 100 km) where the chemically different STW and SASW fully mix. Those frontal waters have been shown to support higher primary productivity and species diversity (Laubscher et al., 1993; Bradford-Grieve et al., 1997; Froneman et al., 1999; Murphy et al., 2001; Raes et al., 2018).

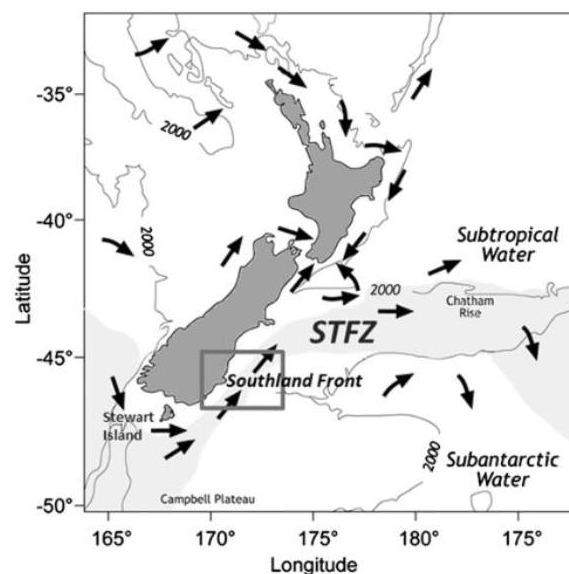


Figure 2.1: Map of New Zealand illustrating the Subtropical Frontal Zone and the near-coastal protrusion, the Southland Front. Boxed area represent region of the Munida Transect. Image adapted from Jones et al. (2013).

The SASW to the east of the Southland Front represents the characteristic high-nutrient low chlorophyll waters of the Southern Ocean (Boyd et al., 1999; Hopkins et al., 2010; Currie et al., 2011). The STW to the west of the Southland Front is modified through mixing with the coastal adjacent neritic water (NW). The physicochemical properties of the water masses across the Southland Front have been well characterised in previous research and generally follow a spatial

gradient. Macronutrient concentrations (nitrate and phosphate) increase offshore and are highest in SASW. However, due to river and land run-off, NW is also associated with slightly higher macronutrients (Hawke, 1989; Currie & Hunter, 1999; Jones et al., 2013). Iron and other trace element concentrations (e.g. zinc, copper, cadmium) decrease offshore (Croot & Hunter, 1998; Sander et al., 2015).

In Austral summer 2017/2018, New Zealand waters experienced a Marine Heatwave (MHW) that persisted for three months (December 2017 – February 2018) and was defined by an increase in sea surface temperature of 2 – 4°C relative to the 1981–2010 monthly average (NIWA, 2019). MHWs are identified as prolonged and discrete anomalously warm sea-surface water events (Hobday et al., 2016). More specifically, MHWs need to persist for at least five days with temperatures exceeding the 90th percentile based on a 30-year local baseline climatology (Hobday et al., 2016). They are caused by different localised (air-sea heat flux) and large-scale (e.g. El Niño Southern Oscillation) processes operating across multiple spatial and temporal ranges (Hobday et al., 2016). Their ecological impact has resulted in devastating changes to local ecosystems such as widespread mortality of foundational species (Wernberg et al., 2013; Smale & Wernberg, 2013) and benthic invertebrates (Garrahou et al., 2009), species distribution shifts and changes in species phenology, and altered valuable fishing practices (Mills et al., 2013). MHWs are predicted to increase in frequency and magnitude in the future as a result of climate change (IPCC, 2012). Further, the New Zealand Earth System Model projects that MHWs in the ocean around New Zealand will increase more intensely in STW compared to SASW with the strongest changes observed for the Subtropical Front (Behrens et al., 2022). The severity of these extreme events on biodiversity patterns and ecosystem structure and functioning urges more research to be conducted on their biological impact.

Patterns of photic-zone protist dynamics were assessed across four different water masses over a time series of five years. In general, this study provided a baseline of protist community composition across the Southland Front that can be further monitored to detect changes over a longer time scale in the context of a changing ocean. A combination of high-throughput 18S rRNA amplicon sequencing and size fractionated samples was used to describe the spatial distribution of protists taxonomic composition and size groups. This comprehensive dataset addressed the following main questions: (i) Do the four water masses host distinct communities all year round? (ii) Do the protist communities follow patterns of seasonal variation? (iii) Is the frontal system a diversity hotspot? (iv) Does the marine heatwave simplify the protist community composition in terms of taxonomy and diversity?

2.2 Materials & Methods

2.2.1 Site description

The 65-km long Munida time-series transect was established in 1998, based on the transect studied by Jillett (1969), and named after the RV Munida vessel that was used during the first sampling voyages. It is the longest record of oceanic CO₂ chemistry in the Southern Hemisphere. The transect extends off the east coast of Southern New Zealand covering four distinct water masses (Jillett, 1969; Heath, 1981). It runs from Taiaroa Head at the tip of the Otago Peninsula (45.770°S 170.720°E) across coastal neritic water (NW) into modified subtropical water (STW) of the Southland Current, followed by the Southland Front (Front) and ends at a sampling station in sub-Antarctic surface water (SASW; 45.83°S 171.50°E) (Figure 2.1). The Southland Front is a transition zone between the warm, nutrient-poor and salty water of STW (15 – 40 km offshore) and the cold, nutrient-rich and less saline sub-Antarctic surface water (SASW, >45 km offshore) (Heath, 1972, 1981; Currie & Hunter, 1999; Hopkins et al., 2010; Currie et al., 2011). Moreover, this front corresponds to the Subtropical Convergence but is seen as an independent system due to the interruption of the New-Zealand landmass (Jillett, 1976). It is a site of high variability and complexity regarding its strength, location, width, and nutrient concentrations (Heath, 1972; Hopkins et al., 2010; Jones et al., 2013). The hydrology and physical oceanography of these water masses were described in earlier research papers (Jillett, 1969; Hawke, 1989; Currie et al., 2011).

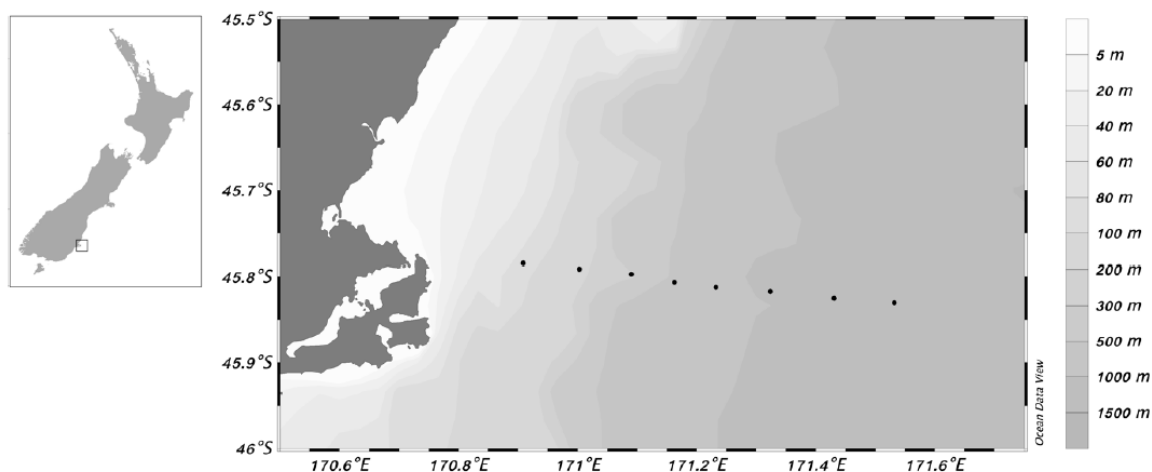


Figure 2.2: Map showing the region of the Munida transect (65 km) off the coast of the Otago Peninsula with eight sample stations. Bathymetry is shown by the colour scale. Image adapted from Meyers (2020).

2.2.2 Sampling

The phytoplankton time series along the Munida transect was initiated in early autumn 2016 and is still ongoing. This study includes the first 20 single-day cruises performed over five years from March 2016 until March 2020. The sampling trips took place every second month aboard the RV *Polaris II*. On each cruise, samples were collected at eight discrete stations along the Munida transect (Figure 2.2). These stations were chosen based on previous research (Currie et al., 2011; Jones et al., 2013; Baltar et al., 2015) and give a good representation of the different characteristic water masses present along the transect. Phytoplankton samples for flow cytometry, FlowCam and metagenomic analysis were all taken at 10 m depth using 12 L Niskin bottles mounted on a CTD Rosette (SBE 19plus V2 SEACAT Profiler CTD). For flow cytometry, triplicate seawater samples were collected in 1.5 mL microcentrifuge cryo-vials (National Scientific™), snap frozen and kept in liquid nitrogen on-board. All the samples were analysed within 12 h of collection. Seawater samples (200 mL) for FlowCam analysis were collected in acid-washed opaque bottles and immediately fixed with 2 mL Lugol's acid solution (2%). Metagenomic seawater samples (2.5 L) were collected in autoclaved transparent nalgene bottles (Thermo Scientific, USA), stored in a cooler, and filtered on-board onto 0.2 µm polyethersulfone membranes (47 mm diameter, Sterlitech) using an in-house designed closed filtration system. A pressure pump was used and kept at low vacuum (0.2 bar) to prevent rupture from compromising the filter membranes. After filtration, the filters were folded into 1.5 mL microcentrifuge cryo-vials (National Scientific™), snap frozen and stored in liquid nitrogen until they were transferred to -80°C on-shore awaiting extraction.

2.2.3 Environmental measurements

Continuous measurements of temperature and salinity were taken from surface waters (2 m depth) for the duration of each single day cruise. Sea-surface water temperature and conductivity were measured using a SBE21 thermosalinograph (Sea-Bird Electronics, Inc.) with an accuracy of $\pm 0.1^\circ\text{C}$ for sea-surface temperature. Additionally, conductivity, temperature and depth measurements were recorded during the CTD casts at each station from 10 m depth. Discrete seawater samples were collected at each sampling station ($n = 8$) for the measurement of macronutrients (nitrate, phosphate and silicate) and chlorophyll-*a* concentrations. Nutrient samples were filtered (Whatman GF/F) on board, collected in HDPE bottles (acid-washed) and colorimetrically analysed with an Astoria Pacific API 300 micro segmented flow analyser using a digital detector (EPA method 365.5). Water samples for chlorophyll-*a* concentrations were also filtered (Whatman GF/F) on board and frozen. Chlorophyll-*a* filters were extracted in 90% acetone for approximately 24 h and measured using a pre-calibrated Turner De- signs

Fluorometer as described in Jones et al. (2013). All environmental samples were analysed by NIWA / University of Otago Research Centre for Oceanography.

Continuous sea surface temperature (T) and salinity (S) measurements were visualised for each sample cruise (Figure S2.1 & Figure S2.2). Additionally, discrete temperature and salinity data points taken at 10 m depth were fitted onto the T/S graphs (Figure S2.1 & Figure S2.2). Following the seasonal threshold levels for temperature and salinity that were defined by Jillett (1969) (Table 2.1), the individual water types for each sample cruise were identified based on the patterns of the present T/S graphs.

Table 2.1: Seasonal salinity and temperature characteristic for neritic water, subtropical water and sub-Antarctic surface water. Modified from Jillett (1969).

		Neritic Water	Subtropical Water	Subantarctic Surface Water
Salinity (‰)	Winter	< 34.5	> 34.5	< 34.5
	Summer	< 34.6	> 34.6	< 34.6
Temperature (°C)	Winter	< 10.0	> 9.5	< 9.5
	Summer	< 12.0	> 12.0	< 12.0

2.2.4 Flow cytometry

Flow cytometry analysis was performed for each cruise, following Tarran & Bruun (2015), to quantify phytoplankton and to discriminate between major groups of these small protists (pico- and nano-phytoplankton). Samples were measured with a BD Accuri™ C6 flow cytometer fitted with a 488 nm (blue) and 640 nm (red) laser, two light scatter detectors, and four fluorescence detectors (3-blue/1-red), allowing for the detection of multiple natural phytoplankton

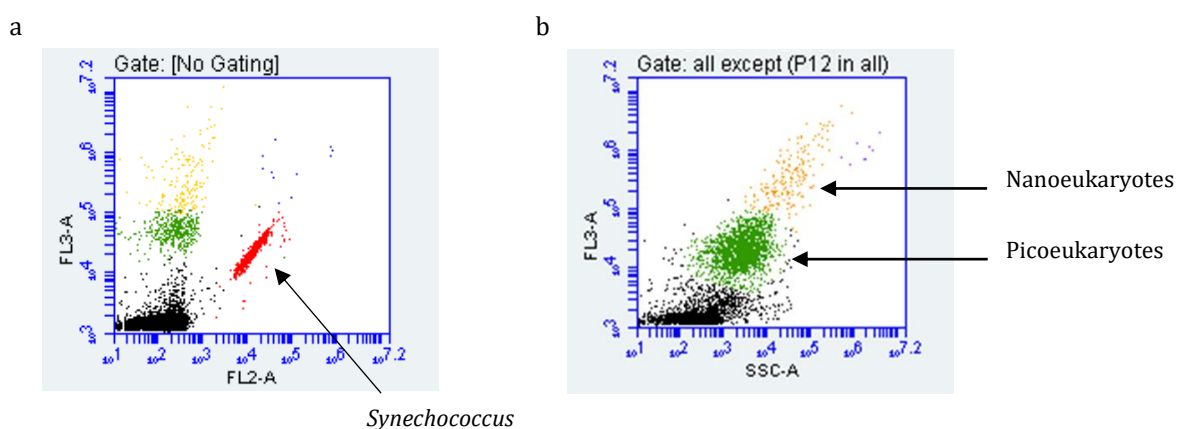


Figure 2.3: Cytogram profiles illustrating the different phytoplankton groups. (a) *Synechococcus* identified based on red (FL3) versus orange (FL2) fluorescence scatter plots. (b) Phytoplankton size groups, pico- (0.2 – 2 μm) and nano- (2 – 20 μm) eukaryotes, identified based on side scatter versus red (FL3) fluorescence scatter plots.

fluorochromes (e.g. chlorophylls and phycobilins). Deionized water ($0.22\ \mu\text{m}$) with bacteriostatic concentration solution was used as sheath fluid for all analyses. Phytoplankton populations were grouped according to their unique autofluorescence profiles in the red (FL3) versus orange (FL2) fluorescence bivariate scatter plots (Figure 2.3a). These groups were further identified based on their relative cell size depicted by the side scatter (SSC) versus FL3 fluorescence scatter plots (Figure 2.3b). Final phytoplankton cell counts (cells mL^{-1}) were computed from event counts in the gated size groups and the analysed volume.

2.2.5 FlowCam analysis

Phytoplankton samples (200 mL) were size fractionated using a $55\ \mu\text{m}$ mesh (Nitex) after mixing the sample by gently inverting it (3x). After filtering, the mesh membrane was immediately transferred into a beaker containing filtered seawater ($0.22\ \mu\text{m}$) to prevent the large size fractionated cells on top of the mesh from bursting. The membrane was thoroughly rinsed in the beaker, using a squirt bottle containing filtered seawater ($0.22\ \mu\text{m}$), before discarding it. Both size fractions were subsequently concentrated and topped up to a volume of 10 mL, using 1.2 mL polyethersulfone membranes (47 mm diameter, Sterlitech) to concentrate the cells and filtered seawater ($0.22\ \mu\text{m}$) to rinse and collect the cells into 15 mL falcon tubes. Samples were preserved with one drop of 2% Lugol's acid solution and stored in the dark. Phytoplankton abundance measurements and size group identifications were performed using the FlowCam (Yokogawa Fluid Imaging Technologies). The small size fractions ($< 55\ \mu\text{m}$) were examined using a $50\ \mu\text{m}$ glass flow cell with a 20x objective, 20x collimator, and a 0.5 mL syringe pump. For this size class, a volume of $300\ \mu\text{L}$ per sample was run at a flow rate of $0.020\ \text{mL min}^{-1}$. The large size fractions

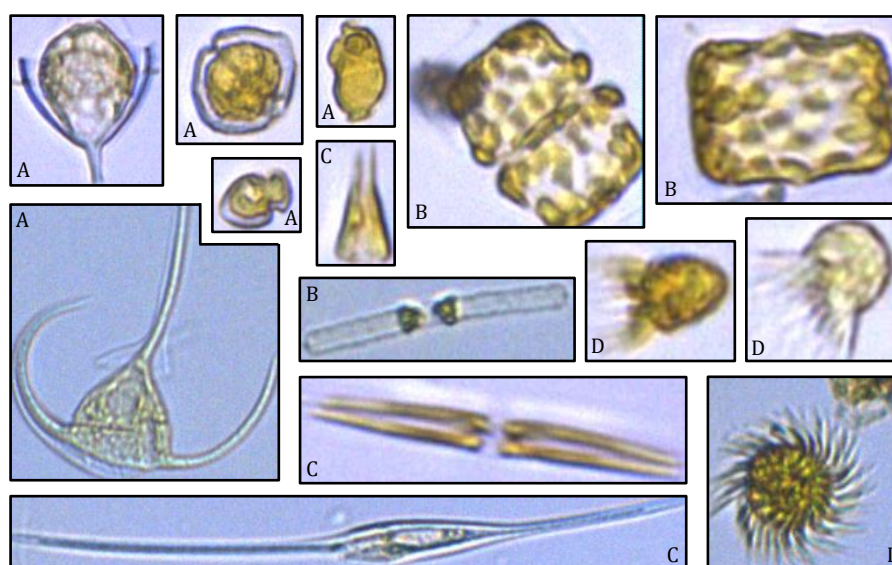


Figure 2.4: FlowCam images of the major protist taxonomical groups. (A) dinoflagellates, (B) centric and (C) pennate diatoms, and (D) ciliates

(> 55 μm) were measured using an 80 μm glass flow cell with a 10x objective, 10x collimator, and a 1 mL syringe pump. For each sample from this fraction, a volume of 1 mL was run at a rate of 0.1 mL min⁻¹. The sample volume and flow rate of both size-fractionated runs were calculated based on approximately a 30 % efficiency estimation to avoid multi-imaging of particles. Analyses were conducted in AutoImage mode. Particle images (Figure 2.4), counts and additional particle value calculations (mean, standard deviations, diameter etc.) were rendered by the Visual Spreadsheet software and processed therein. Particles were grouped, after deletion of abiotic fragments, according to size and taxonomy using the Classification Window. The different size groups were 0 – 5 μm , 5 – 20 μm , 20 – 50 μm , and 50 – 80 μm . The identified taxonomy groups were dinoflagellates, pennate diatoms, centric diatoms, ciliates, and marine flagellates (Figure 2.4). Some particles were classified as unidentified due to the limitations of image resolution.

2.2.6 Molecular methods

2.2.6.1 DNA extraction

For DNA extraction, the frozen filters were thawed and cut up in petri dishes (clean environment manufactured, Technoplas) with sterilized razor blades into small fragments of approximate 2 mm by 1 cm. The fragmented filters were collected in sterilized 15 mL falcon tubes (Greiner Bio-On, Kremsmünster, Austria). DNA was extracted from the polyethersulfone filters with 0.7 M NaCl, 1% Cetyl Trimethylammonium Bromide (CTAB) and 0.1 mm zirconia/silicabeads (dnature, BioSpec), following Countway et al. (2005). After the CTAB incubation, sample lysates were further extracted with 1:1 volume of phenol and chloroform. Samples were thoroughly shaken by hand for 30 s and centrifuged (16,000 x g) at room temperature for 5 min. The upper aqueous phase was transferred to a clean 2 mL micro-centrifuge tube and 1 x volume of chloroform was added. The samples were vortexed for 30 s and centrifuged (16,000 x g) at room temperature for 5 min. DNA was precipitated by adding 1/10 3M sodium acetate and 2.2 x volume of ice cold 95% ethanol. The solution was vortexed and incubated overnight at -20°C. After centrifugation (20 min at 10,000 x g) at 4°C, DNA precipitate was collected and washed in 250 μL of 70% ethanol followed by a spin (16,000 x g) of 2 min at 4°C to pellet the DNA and subsequently discard the ethanol (the wash step was repeated 2 to 3 times). DNA was resuspended in 30 μL of Tris-EDTA (TE; pH 8) and stored at -80°C.

2.2.6.2 18S rRNA Library preparation

Triplicate PCR mixtures were prepared using the KAPA HiFi HotStart DNA kit (Kapa Biosystems, Boston, MA, USA) for the amplification of the 18S rRNA V4 region (~548 bp, for this protocol). The PCR mixtures were constructed as follows: 5 x KAPA HiFi Buffer (2 mM MgCl₂), 0.3 mM KAPA

dNTP, 0.3 μ M Forward primer and 0.3 μ M Reverse primer, 0.5 U KAPA HiFi HotStart DNA Polymerase, and 0.05 mg ml⁻¹ Bovine Serum Albumin (BSA, Invitrogen, USA). The BSA protein was added to overcome PCR inhibition issues as a result of long-term storage and in case of high polysaccharide contamination. The forward primer TAREuk454FWD1 (5'-CCAGCASCYGCCGTAATTCC-3') and reverse primer TAREukREV3 (5'-ACTTTCGTTCTTGATYRA-3') suggested by Stoeck et al. (2010) were ordered from Integrated DNA Technologies Inc. (IDT), salt-free and purified. Both primers were modified with overhanging tags (forward, 5-ACGACGCTCTTCCGATCT-3'; reverse, 5-CGTGTGCTCTTCCGATC-3'; Griffith et al., 2017) to allow for first round (R1) amplicon target detection by the second-round primers during the library preparation process. First round amplification was performed using a two-step PCR program (adapted from Massana et al., 2015) to maximise specificity for the V4 region: an initial denaturation step at 95°C for 30 s; followed by step 1 – 10 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s; followed by step 2 – 15 cycles of 95°C for 30 s, 48°C for 30 s, and 75°C for 30 s; and a final extension period of 300 s at 72°C. Triplicate PCR products were pooled for each sample and visualised on a 2% agarose gel to confirm the expected amplicon length and rule out any contamination using the negative controls (non-template reactions). Clean-up of the PCR products was done using 1.8 x (vol:vol) magnetic AMPure XP beads (Beckman Coulter, Brea CA, USA) according to the Agencourt AMPure XP PCR Purification protocol. Purified products were quantified with a Qubit fluorometer using the Qubit™ dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and DNA concentrations were diluted accordingly to 1 ng μ l⁻¹. Samples with lower DNA concentrations were re-amplified using a higher amount of template DNA. After dilutions, a second round (R2) PCR was performed for the addition of Illumina sequencing adaptors and unique dual-index barcodes to the amplicon products of R1-PCR. Desalted and purified TruSeq primers, modified with the same overhanging tags as described earlier, were ordered from IDT. Thermocycling conditions were as followed: an initial denaturation step at 95 °C for 120 s; followed by 10 cycles of 98°C for 20 s, 60°C for 20 s, and 75°C for 20 s; and a final extension period of 60 s at 72°C. Products were visualised on a 2% agarose gel; clean-up and quantification was done as described for the R1-PCR. Finally, samples were pooled in equimolar concentrations and send off to the Otago Genomics Facility (Otago University, New Zealand) for Illumina paired-end sequencing on an MiSeq platform (Illumina, San Diego, CA, USA) using the V3 (2 x 300 bp) reagent kit.

2.2.7 Statistical analyses

The raw Illumina 18S rRNA sequences were returned demultiplexed with the linkers, adaptors and barcodes removed. Processing of the sequences prior to statistical analysis was done in R

(version 3.4.4; R Core Team, 2021) following the ‘DADA2’ pipeline (Callahan, Sankaran, et al., 2016). The mean quality score profiles were used to assess the need for additional trimming of the reads based on a Q30 score. Forward reads were truncated at position 270 while the reversed reads were truncated at position 230 due to the lower quality. Primers were removed and reads shorter than the selected threshold were discarded. Amplicon sequence variants (ASV) were resolved using the Divisive Amplicon Denoising Algorithm (Callahan, McMurdie, et al., 2016). To obtain full denoised sequences forward and reverse reads were merged (12,052 ASVs) based on a default overlap of 12 bp. Chimeric sequences accounted for 2 % of the merged reads and were subsequently removed. Taxonomy was assigned to the sequences with *assignTaxonomy* using the Protist Ribosomal Database (PR2 version 4.14; <https://github.com/pr2database/pr2database/releases>; Guillou et al., 2013). Sequence variants identified as ‘Metazoa’ were not of interest for this study and hence were removed from the dataset. Statistical analyses relied on the “phyloseq” package (McMurdie & Holmes, 2013) and all visualisations were done with *ggplot2* (Wickham, 2016), available in the “tidyverse” package (Wickham et al., 2019).

Protist alpha diversity (Chao1 Richness and Pielou’s evenness) was measured using *estimate_richness* (“phyloseq”) and tested for differences across water masses and seasonality with the non-parametric Kruskal-Wallis test and the pairwise Wilcoxon Rank Sum test. These non-parametric test were chosen because the data violated the assumptions of normality, homogeneity of variances and equal sample sizes. To analysis the protist community composition (beta diversity), distance matrices based on Bray-Curtis dissimilarities (reflects relative abundance) were calculated. The matrices were then used with a Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson, 2017) to assess the community composition variability across the four water masses, and for seasonal trends (*adonis* function; “vegan”). In order to evaluate the PERMANOVA results, multivariate homogeneity of the dispersions amongst the groups (water masses and seasonality) are also required to be tested. For this, the PERMDISP analysis was performed using the *Betadisper* function from the “vegan” package (Anderson et al., 2006). Community comparisons were visualised in an ordination plot using unconstrained non-metric multidimensional distance scaling (nMDS). All beta diversity analysis were performed with 10,000 permutations and a seed of 711 was set for reproducibility. The effect of environmental variables on protist community variability was tested with a constrained distance-based redundancy analysis (dbRDA; Legendre & Anderson, 1999) using the distance matrix based on Bray-Curtis dissimilarities. Response variables were tested for co-linearity by calculating their variance inflation factors (*vif.cca* function; scores >10 are removed). Next, the variables were fitted in the model through forward selection using the *ordiR2step* function (“vegan”). The significance of the overall model and the individual terms was assessed with an ANOVA test using

the *anova.cca* function. The prevalence of the most abundant taxonomic groups across the water masses and for seasonality was tested using the Multiple Analysis of Variance (MANOVA) test. Significant MANOVA results were further investigated using a univariate ANOVA test to determine which taxonomic groups were responsible for the differences between the groups. Finally, significant differences of nutrient levels in summer between ambient and heatwave conditions were tested with a parametric directional t-test.

2.3 Results

2.3.1 Hydrographic parameters

Sea surface temperature (SST) displayed a seasonal pattern across all water masses with the highest values in austral summer and the lowest values in winter (Figure 2.5a). The continuous surface temperatures (2 m depth) ranged from 8°C up to 18.4°C, while the temperatures at 10 m depth covered a slightly narrower range (7.8°C to 16.7°C). During the five-year study period, the Austral summer of 2017/2018 portrayed the warmest SSTs (Figure 2.5a). On land, this summer reflected the hottest temperatures New Zealand had encountered in the past 83 years, thereby surpassing the historical record of warmest summer of 1934/1935 (NIWA, 2019). Further, the 2017/2018 summer coincided with marine heatwave events in the Tasman Sea and New Zealand coastal waters. On the south-east coast of South Island New Zealand the MHW persisted for three months (December to February) characterised by an increase of 2°C to 4°C relative to the 1981-2010 average SST (NIWA, 2019) (Table 2.2). Sea surface temperature also varied spatially along the transect following a consistent pattern throughout the years with generally colder temperatures further offshore (SASW). Higher SST values were also found to extend further seawards during the MHW event.

Sea surface salinity (SSS) had a much less pronounced seasonal pattern compared to SST but showed a strong spatial structure. The highest salinity values were seen for STW (34.8‰, at 3 and 10 m), while the lowest measurements were found in near coastal waters (33.5‰, at 3 and 10 m). The offshore waters represented relative stable SSS all year-round (~34.3‰). During the cruise in January 2020, an extremely low salinity event was observed in NW (< 33.5‰) that extended 20 – 25 km offshore (Figure 2.5b). However, this salinity depression event did not extent to 10 m depth, indicating that diluted NW overlayed high-salinity STW at about 20 km off coast. Similar observations concerning overlying water masses were reported by Jillett (1969) with measurements between the surface and 25 m depth.

Table 2.2: Hydrographic parameters for summer months with and without a heatwave event. SST: Sea Surface Temperature ($^{\circ}\text{C}$) and SSS: Seas Surface Salinity (‰) values for the 2017/2018 summer heatwave event are compared to the average summer SST and SSS measurements of 2016, 2017, 2019 and 2020. Error estimates are standard deviations. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and sub-Antarctic Surface Water (SASW).

	Summer Heatwave Event				Summers without a Heatwave			
	Surface 3 m		Depth 10 m		Surface 3 m		Depth 10 m	
	SST	SSS	SST	SSS	SST	SSS	SST	SSS
NW	18.3 ± 0.1	34.7 ± 0.01	/	/	13.9 ± 0.9	33.9 ± 0.4	13.9 ± 1.2	34.4 ± 0.3
STW	17.7 ± 0.2	34.8 ± 0.02	16.3 ± 0.4	34.8 ± 0.03	14.1 ± 0.2	34.8 ± 0.0	13.2 ± 0.6	34.7 ± 0.1
Front	/	/	15.3 ± 0.6	34.7 ± 0.10	12.7 ± 0.4	34.6 ± 0.6	11.8 ± 0.6	34.5 ± 0.1
SASW	17.3 ± 0.2	34.3 ± 0.01	15.2 ± 0.2	34.4 ± 0.03	14.1 ± 0.6	11.6 ± 0.6	11.6 ± 0.6	34.3 ± 0.1
Min	17.1	34.3	14.7	34.3	11.3	33.3	10.9	34.2
Max	18.4	34.8	16.7	34.9	15.9	34.8	14.8	34.8
Range	1.3	0.6	2.0	0.52	4.6	1.5	3.9	0.7

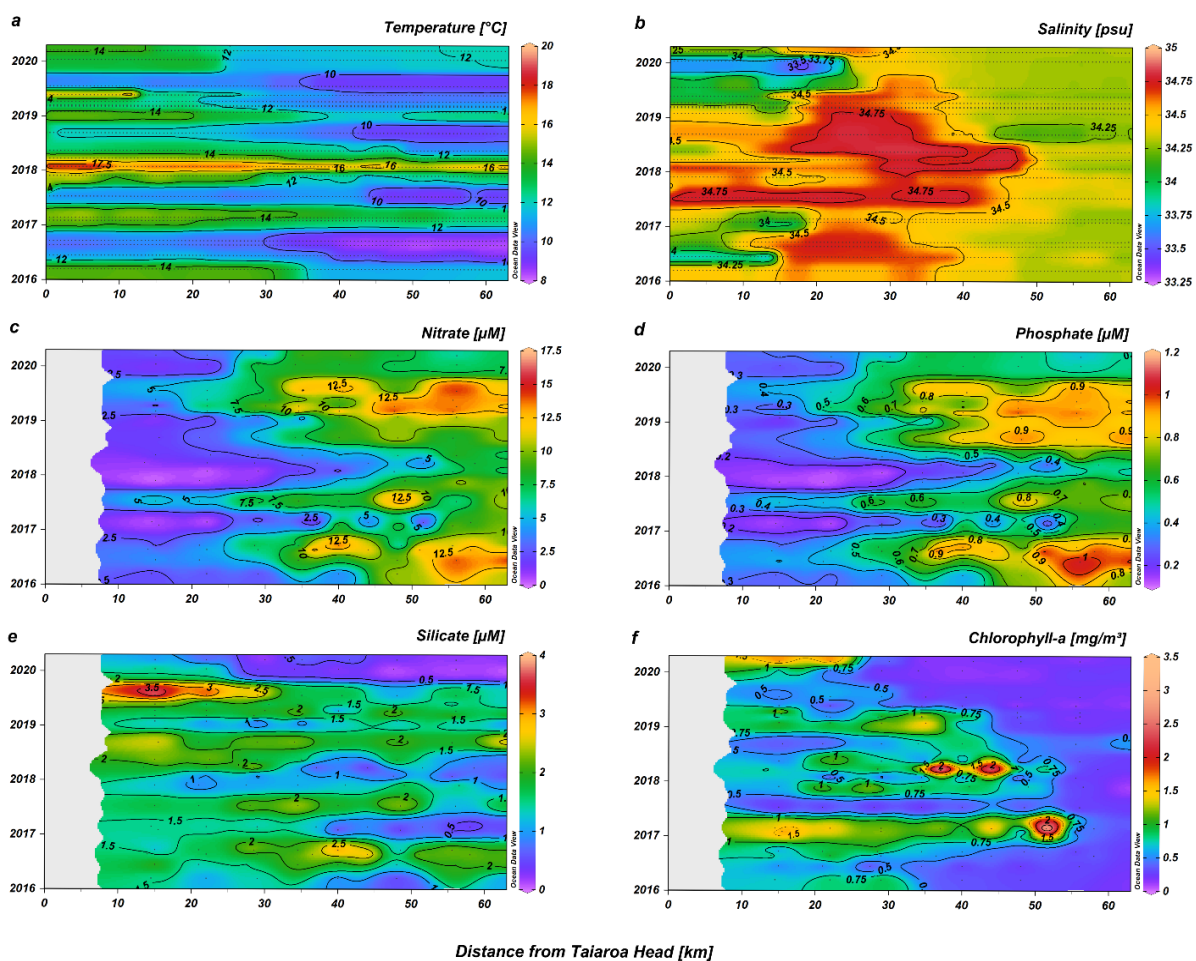


Figure 2.5: Surface seawater plots of environmental parameters measured along the Munida Time Series Transect from March 2016 to February 2021. Sea surface (a) Temperature (SST), (b) Salinity (SSS), (c) Nitrate, (d) Phosphate, (e) Silicate, and (f) Chlorophyll *a*. SST and SSS profiles were based on continuous measurements throughout each cruise. Macronutrient profiles and chlorophyll *a* data points were collected from eight stations (Figure 2.2) along the transect on each cruise. Plots were created using Ocean Data View software (Schlitzer, 2021).

The individual water masses identified at 10 m depth for each sample cruise are visualised in an area plot (Figure 2.6). The frontal zone was characterised as a dynamic system that oscillated

seasonally (Figure 2.6). It was generally found more inshore during the summer months and extended more offshore during winter. Noticeably, the higher temperatures of the MHW (2017/2018 summer) associated with the frontal zone were 3°C above average for the January cruises. Consequently, this resulted in the Front being established over a much wider area of approximately 13 km (Figure 2.6).

In February 2017, the near coastal water mass identified as neritic water expanded furthest seawards (± 30 km) compared to the other 19 sampling cruises (Figure 2.6). This also resulted in STW and the Front system to be further offshore (50 and 55 km, respectively) during that same sampling event. Noticeable, in May 2018 STW reached a maximum width of ± 31.5 km which almost covers twice as much area compared to an average of ± 17 km for STW during this five-year study. Lastly, the sampling cruise in January 2020 was associated with SASW protruding inshore as far as 27 km distance from Taiaroa Head.

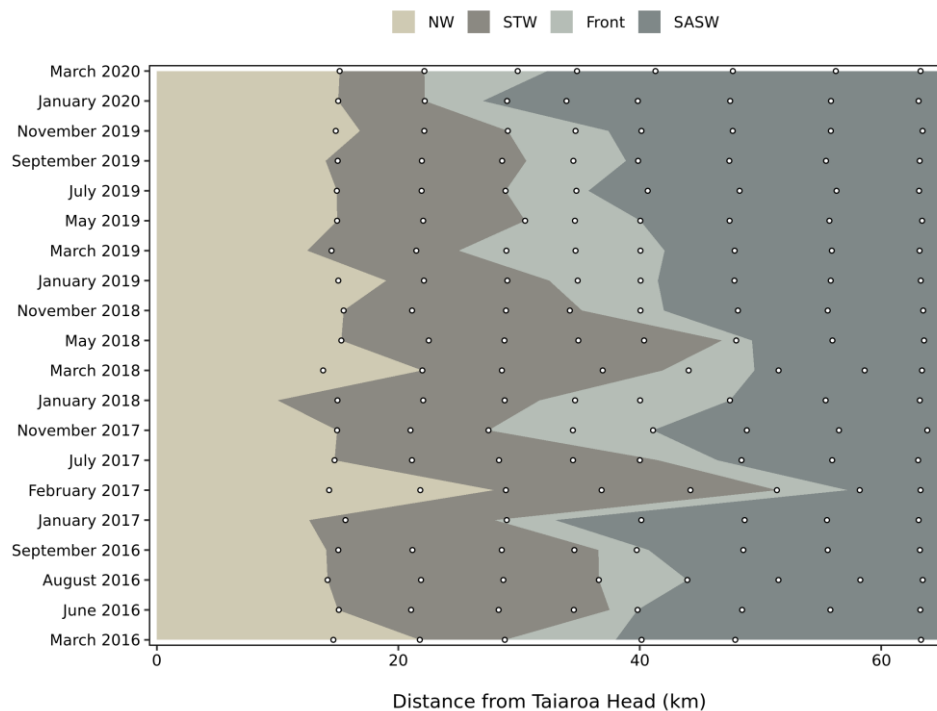


Figure 2.6: Area plot of the individual water masses for each sample cruise. Spatial distribution of the water masses was based primarily on temperatures and salinities at 10 m depth complemented with surface temperature and salinity measurements. White dots indicate sampling stations for each cruise. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and Subantarctic Surface Water (SASW).

The area plot was primarily guided by the discrete temperature and salinity measurements recorded at 10 m depth for each station. Continuous sea surface temperatures and salinities were used to add higher spatial resolution data to the plot (in-between the stations) only when temperature and salinity measurement at the surface and 10 m depth were characteristic for the

same water mass. Note, it is possible that for some cruises STW was found more inshore than depicted here, due to the first discrete sampling point at 10 m depth being located 15 km distance from offshore as shown in Figure 2.6 by the white dots. The area plot highlights the spatiotemporal dynamic nature of the Front along the Munida transect.

2.3.2 Macronutrient concentrations

Nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentrations show seasonal oscillations across all water masses with their highest values in Austral winter (NO_3^- : 4.7 – 12 μM & PO_4^{3-} : 0.4 – 0.9 μM) and their lowest throughout the summer months (NO_3^- : 0.4 – 8.8 μM & PO_4^{3-} : 0.1 – 0.6 μM) (Figure 2.5c,d; Table 2.3). During the 2017/2018 MHW, the macronutrient concentrations in the coastal adjacent water masses hit near depletion up to 35 – 40 km offshore (Table 2.3). In SASW, both nitrate and phosphate concentrations were significantly lower during the 2017/2018 heatwave (t-test; p-values < 0.001) compared to the other summers (January 2017, 2019 & 2020). Additionally, phosphate was also significantly lower in STW during the heatwave (t-test; p < 0.001). A similar pattern of nitrate and phosphate depressions was observed the year before as well (Austral summer of 2016/2017) with lower values extending up to 30 – 35 km offshore (Figure 2.5c,d). The spatial variability of these macronutrients displayed a consistent pattern with lower values in the water masses closest to shore (NW & STW) and peak concentrations for the offshore waters (STF & SASW) (Table 2.3). This is in agreement with previous studies along the Munida transect (Currie et al., 2011; Jones et al., 2013; Sander et al., 2015).

The seasonality of silicate is parallel to the patterns observed for nitrate and phosphate, with higher values found in Austral winter (Figure 2.5). In contrast, spatial differentiation of silicate along the transect was less pronounced. Grouping the values seasonally showed that silicate levels tended to be slightly higher inshore (Table 2.3). The two highest measurements were recorded during winter 2019 in NW (3.40 μM and 3.88 μM). No differences in silicate values were detected in any of the water masses during the 2017/2018 heatwave.

Table 2.3: Average seasonal macronutrient and chlorophyll-*a* concentrations for each individual water mass. Measurements for the 2017/2018 heatwave are shown separately (grey) and are associated with extreme reduced numbers. Mean chlorophyll-*a* levels including potential bloom events are highlighted in blue and seperatedly shown in Table 2.4. Mean values with standard deviations are depicted. March and May measurements depict a clear separation between early and late Autumn and therefore were split up in the table below. n.d. = no data available. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and sub-Antarctic Surface Water (SASW).

		NW	STW	Front	SASW
Chlorophyll- <i>a</i> (mg m ⁻³)	Spring	1.24 ± 0.89	0.68 ± 0.21	0.66 ± 0.13	0.31 ± 0.11
	Summer	1.02 ± 0.28	1.33 ± 0.28	2.31 ± 1.04	0.32 ± 0.11
	Early Autumn - March	0.75 ± 0.06	1.56 ± 0.68	1.97 ± 0.76	0.33 ± 0.12
	Late Autumn - May	0.48 ± 0.00	0.41 ± 0.21	0.15 ± 0.04	0.20 ± 0.13
	Winter	0.70 ± 0.31	0.48 ± 0.20	0.27 ± 0.13	0.27 ± 0.05
	Heatwave	n.d.	0.23 ± 0.08	0.25 ± 0.04	0.20 ± 0.04
Nitrate (μM)	Spring	0.39 ± 0.39	5.14 ± 1.93	10.75 ± 2.23	10.87 ± 1.79
	Summer	0.45 ± 0.35	1.04 ± 0.81	2.87 ± 2.80	8.82 ± 0.56
	Early Autumn - March	1.90 ± 0.50	2.62 ± 0.74	2.92 ± 0.92	7.47 ± 0.86
	Late Autumn - May	1.90 ± 0.00	4.84 ± 0.97	7.86 ± 1.32	10.18 ± 3.30
	Winter	4.72 ± 0.58	7.09 ± 2.53	10.84 ± 3.26	12.15 ± 2.18
	Heatwave	n.d.	0.28 ± 0.27	2.91 ± 2.31	6.51 ± 0.08
Phosphate (μM)	Spring	0.13 ± 0.00	0.45 ± 0.12	0.80 ± 0.12	0.84 ± 0.09
	Summer	0.17 ± 0.04	0.21 ± 0.18	0.31 ± 0.18	0.66 ± 0.05
	Early Autumn - March	0.24 ± 0.05	0.26 ± 0.05	0.29 ± 0.05	0.51 ± 0.10
	Late Autumn - May	0.28 ± 0.00	0.46 ± 0.06	0.62 ± 0.08	0.80 ± 0.11
	Winter	0.42 ± 0.01	0.59 ± 0.16	0.81 ± 0.19	0.90 ± 0.11
	Heatwave	n.d.	0.11 ± 0.01	0.26 ± 0.13	0.44 ± 0.01
Silicate (μM)	Spring	1.39 ± 0.20	1.80 ± 0.26	2.17 ± 0.37	1.87 ± 0.35
	Summer	1.38 ± 0.11	1.21 ± 0.29	0.95 ± 0.40	0.50 ± 0.16
	Early Autumn - March	1.29 ± 0.04	1.14 ± 0.77	0.86 ± 0.71	0.69 ± 0.22
	Late Autumn - May	2.35 ± 0.00	1.77 ± 0.41	1.59 ± 0.14	1.14 ± 0.33
	Winter	2.79 ± 1.54	2.09 ± 0.45	2.12 ± 0.66	1.83 ± 0.36
	Heatwave	n.d.	0.75 ± 0.05	0.93 ± 0.28	0.44 ± 0.01

Table 2.4: Potential bloom events identified across the water masses each year. Water masses with chlorophyll-*a* levels higher than 1.5 mg m⁻³ are shown and correspond to the trends seen in Figure 2.5f. Values shown are one-point measurements. n.d. = no data available. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and sub-Antarctic Surface Water (SASW).

Year	Month	Season	Distance offshore	Water mass	Chl- <i>a</i> (mg m ⁻³)	Nitrate (μM)	Phosphate (μM)	Silicate (μM)
2017	January	Summer	15.62	STW	1.88	0.84	0.18	1.51
2017	February	Summer	44.20	STW	1.58	3.19	0.32	0.9
2017	February	Summer	51.36	Front	3.45	0.63	0.16	0.74
2018	March	Autumn	36.93	STW	3.49	2.79	0.19	0.43
2018	March	Autumn	44.05	Front	2.84	3.57	0.25	0.36
2019	January	Summer	34.86	Front	1.69	1.65	0.28	0.44
2019	March	Autumn	28.95	Front	1.63	n.d.	n.d.	n.d.
2019	March	Autumn	34.68	Front	2.72	n.d.	n.d.	n.d.
2019	March	Autumn	40.06	Front	2.01	n.d.	n.d.	n.d.
2019	November	Spring	14.82	NW	1.87	0.66	0.13	1.53
2020	March	Autumn	15.14	STW	1.83	2.56	0.31	0.95
2020	March	Autumn	22.16	STW	1.89	3.57	0.36	0.59

2.3.3 Chlorophyll-*a* concentrations

Chlorophyll-*a* concentrations were measured as a proxy for phytoplankton biomass. Overall, surface chlorophyll-*a* covered a wide range from 0.08 to 3.49 mg m⁻³. Only a minor amount of all observed chl-*a* values exceeded bloom indicator thresholds (> 1.5 mg m⁻³) (Table 2.3 & Table 2.4). Compared to other analogous coastal systems, that are representative of typical highly productive coastal zones (Gibbs, 2000; Thomas et al., 2001; Evans et al., 2011), current chl-*a* concentrations are substantially lower. However, they are consistent with previous reports for this study area (Jones et al., 2013) as well as other oceanic areas defined by the presence of a Subtropical Frontal zone (Laubscher et al., 1993; Bradford-Grieve et al., 1997; Clementson et al., 1998; Froneman et al., 1999; Boyd & Abraham, 2001; Gall et al., 2010; Thomalla et al., 2011). Seasonality in the chl-*a* measurements was observed with peak values (2 – 3.5 mg m⁻³) in late Austral summer and early autumn (February – March). The increased chl-*a* values were associated with NW, STW and the Front (Table 2.4). Offshore SASW exhibited no seasonality and had low chl-*a* concentrations all year round (± 0.3 mg m⁻³; Table 2.3).

2.3.4 Alpha-diversity

On average all seasons showed a similar degree of richness per water mass, but the Chao1 index was overall most stable amongst the water masses during spring (Figure 2.7a). Note, in spring and winter, NW was represented by extremely low sample sizes ($n = 2$) and was therefore excluded from statistical analysis on protist alpha diversity within these seasons. Likewise, the Front in winter ($n = 2$) was limited by sample size and not included in the alpha diversity analysis for this

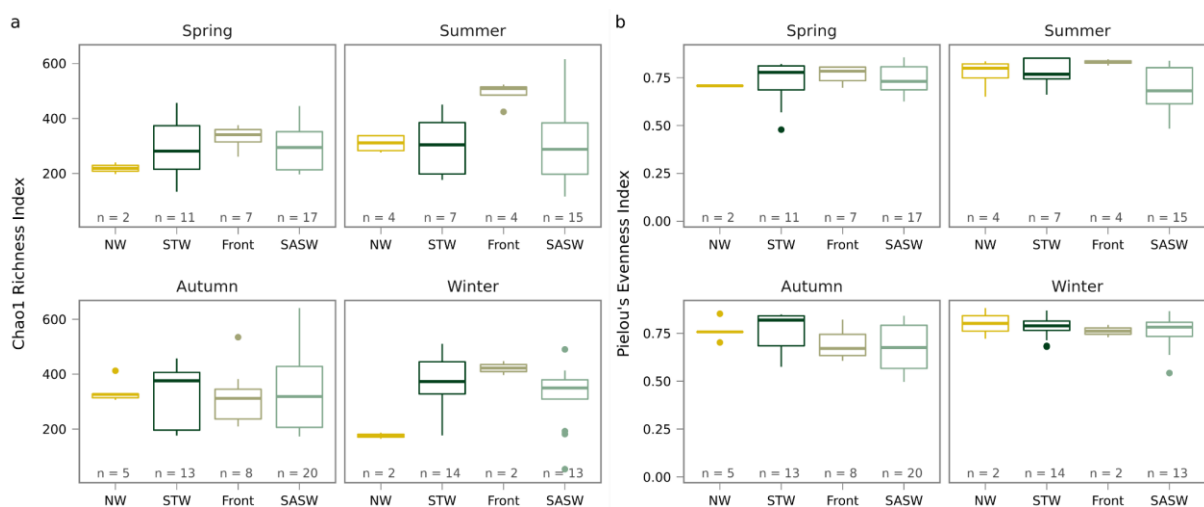


Figure 2.7: Protist alpha diversity metrics across seasons for each individual water mass. (a) Chao1 richness index and (b) Pielou's evenness index. Sample sizes (n) per group are also displayed. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and sub-Antarctic Surface Water (SASW). Marine heatwave samples collected in January 2018 are not included.

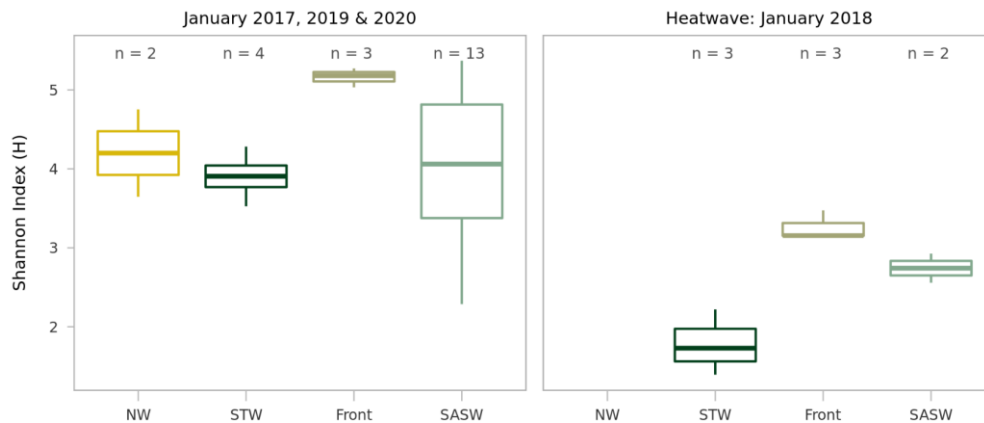


Figure 2.8: Comparison of protist alpha diversity measurements between ambient summer and heatwave conditions. Sample sizes (n) per group are displayed. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and sub-Antarctic Surface Water (SASW).

season. Only one significant difference in alpha diversity amongst the water masses was found. Namely, in summer the Front was associated with a significantly higher diversity compared to the neighbouring water masses (Shannon; $df = 3$, $X^2 = 8.67$, $p = 0.03$; Figure 2.7). This significant result was obtained after the heatwave sampling trip (January 2018) was removed from the analysis. Regarding interannual variability, no specific patterns in Chao1 measurements were found for the individual water masses. Protist alpha diversity in spring and winter were characterised by high and relatively similar Pielou measurements amongst the individual water masses, while protist communities in summer and autumn were more variable for each water mass (Figure 2.7b). The latter indicates that for some of the water masses in summer and autumn the protist assemblages are represented by a few high abundant species instead of a more diverse and equally represented community.

During the 2017/2018 heatwave, protist alpha diversity measures were significantly lower for STW (Shannon; $df = 1$, $X^2 = 4.5$, $p = 0.034$) and the Front (Shannon; $df = 1$, $X^2 = 3.9$, $p = 0.049$) compared to the mean diversity values of the other summer months (Figure 2.8). Analysis of Chao1 richness and Pielou's evenness indices showed that for the protist communities in STW the significantly lower Pielou value (Pielou; $df = 1$, $X^2 = 4.5$, $p = 0.034$) was the driver behind the difference in diversity. The reduced protist alpha diversity in the Front was a combination of variations in both richness and evenness (Chao1 and Pielou; $df = 1$, $X^2 = 3.9$, $p = 0.049$). No significant results were obtained for SASW communities. The lack of significance for SASW is likely the result of the reduced sample size for during the heatwave sampling trip paired with the high variability in diversity measures during the 2017/2019/2020 summers. Differences in alpha diversity between the water masses during the January 2018 trip (heatwave) were observed

between STW and the Front (Shannon; $df = 1$, $X^2 = 3.9$, $p = 0.049$) and resulted from a significant difference in both richness and evenness.

2.3.5 Community structure

PERMANOVA analysis showed that protist communities were significantly structured by water mass type ($df = 3$, pseudo- $F = 10.56$ and $p < 0.001$), seasonality ($df = 3$, pseudo- $F = 6.12$ and $p < 0.001$) and the interaction between both factors ($df = 9$, pseudo- $F = 2.01$ and $p < 0.001$) (Figure 2.9b). The assumption of homogeneity of variance amongst groups was violated for the community comparisons between all water masses except amongst NW and SASW (PERMDISP; $df = 3$, $F = 8.58$, $p = 0.001$). Despite the variance in dispersions, separate protist community clusters can be distinguished by looking at the nMDS ordination (Figure 2.9b), suggesting that the different community groupings are a result of both 'location' and 'dispersion' effects (see Chapter 1: section 1.1.4). Further, PERMANOVA pairwise comparisons showed that all water masses except NW – STW were significantly different from one another. The strongest distinction was observed between STW – SASW ($R^2 = 0.32$, $p < 0.001$) associated communities and the weakest between the Front – SASW ($R^2 = 0.08$, $p = 0.002$). For a more detailed understanding of protist dynamics along the Munida transect, protist assemblages across water masses were further

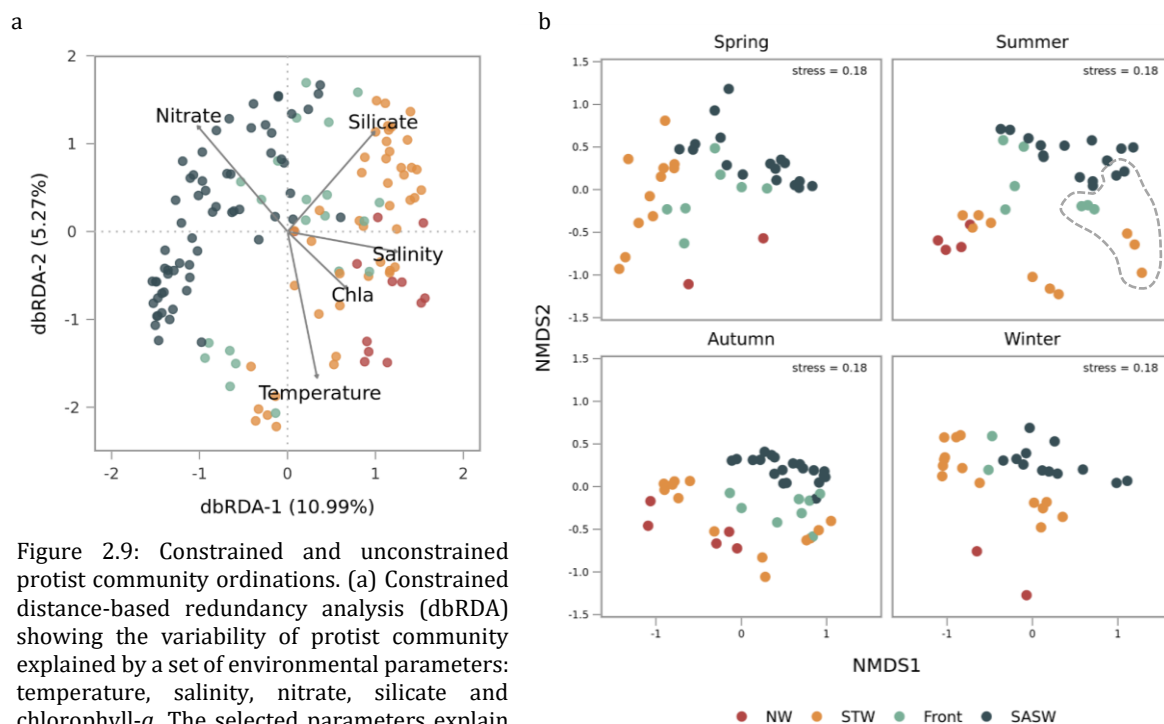


Figure 2.9: Constrained and unconstrained protist community ordinations. (a) Constrained distance-based redundancy analysis (dbRDA) showing the variability of protist community explained by a set of environmental parameters: temperature, salinity, nitrate, silicate and chlorophyll-*a*. The selected parameters explain 20% of the total variation amongst the protist communities. Note, phosphate was removed due to co-linearity with nitrate.

(b) Unconstrained non-metric multidimensional scaling (nMDS) for each season. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and sub-Antarctic Surface Water (SASW). Dashed line: heatwave samples.

analysed for each season individually, while seasonal variability was tested for each water mass separately.

Distance-based RDA (dbRDA) analysis was used to quantify the explanatory power of the environmental variables on the structure of the protist communities across the Southland Front system. The final minimal model that adequately represented the full set of environmental variables included temperature, chlorophyll *a*, salinity, nitrate, and silicate (Figure 2.9a). This model constrained 20% of the total variation measured in the community composition. The global dbRDA model and the individual explanatory variables were all significant ($p < 0.05$). To partially detrend the dataset, additional dbRDA models were calculated for the protist communities per season which resulted in a higher proportion of the protist community variation explained by the predictor variables (Table S2.1).

2.3.5.1 Community structuring across water masses

In spring and winter, the water masses were found to be significantly distinct (PERMANOVA; $df = 2$, pseudo- $F = 4.93$ & 5.60 respectively, and both p -values < 0.001) and the variance of dispersions amongst the groups was equal for both seasons. In spring, pairwise comparisons found differences between protist communities associated with STW vs. the Front (pseudo- $F = 5.66$ and $p = 0.005$), the Front vs. SASW (pseudo- $F = 3.24$ and $p = 0.04$) and STW vs. SASW (pseudo- $F = 15.18$ and $p = 0.003$). Pairwise assessments with communities from NW could not be performed due to low sample size ($n = 2$). In winter, significant differences were found between STW and SASW associated communities (pseudo- $F = 7.41$ and $p = 0.002$). NW and the Front (both $n = 2$) had insufficient sampling points which results in weak statistical power and consequently the inability to be tested for pairwise comparisons.

Summer and autumn protist communities also showed a significant difference amongst the water masses (PERMANOVA; $df = 3$, pseudo- $F = 3.93$ & 5.89 respectively and both p -values < 0.001). However, unlike the other seasons, summer and autumn associated communities exhibited heterogeneity of dispersions (PERMDISP, $df = 3$; $F = 4.47$ & 3.55 , and $p = 0.009$ & 0.02 , respectively) specifically between NW – STW and STW – SASW for both seasons, and also between STW – Front in summer. Despite the unequal variance of dispersion, STW and SASW associated protists still displayed distinct groupings for both seasons, visualised in the nMDS ordination (Figure 2.9b) and statistically shown with pairwise PERMANOVA tests (pseudo- $F = 10.108$ & 22.66 for summer and autumn, p -values $= 0.003$). Noticeably, a more stable protist community was observed for SASW compared to STW in both seasons based on community dissimilarities. In summer, additional pairwise differences were found between NW – Front (pseudo- $F = 6.52$ and $p = 0.02$), NW – SASW (pseudo- $F = 12.56$ and $p = 0.003$), and Front – SASW (pseudo- $F = 3.54$ and p

= 0.04). In autumn, the same distinctions were found, NW – Front (pseudo- F = 6.69 and p = 0.01), NW – SASW (pseudo- F = 15.93 and p = 0.003), and Front – SASW (pseudo- F = 5.5 and p = 0.006) but also additionally between STW – Front (pseudo- F = 4.88 and p = 0.025).

2.3.5.2 Phytoplankton seasonality in individual water masses

Seasonal variation in phytoplankton communities was observed for some but not all water masses. In neritic waters, a significant difference was detected between summer and autumn communities (PERMANOVA; df = 1, pseudo- F = 1.9 and p = 0.02) with the PERMDISP test confirming homogeneity of dispersions amongst the seasonal groups. Due to low sample sizes for neritic water in spring and winter, pairwise comparisons could not be performed amongst all the seasons.

Protist communities in STW and the Front both followed a seasonal pattern (PERMANOVA, df = 3; pseudo- F = 3.77 and 2.87 respectively, both p -values < 0.001) with equal variance of dispersions amongst the comparison groups. In STW, the strongest dissimilarities in protist assemblages were found between spring – summer (pseudo- F = 8.04 and adjusted p -value = 0.006) and summer – winter (pseudo- F = 8.16 and adj. p -value = 0.006). Additionally, smaller significant differences were also seen between spring – autumn (pseudo- F = 4.31 and adj. p = 0.04) and winter – spring (pseudo- F = 4.32 and adj. p = 0.02). For the Front, pairwise analysis revealed that the significant difference was observed between spring and autumn phytoplankton groups (pseudo- F = 7.93 and adj. p = 0.006). Noteworthy, there were only two data points collected in winter and therefore this season was removed from the pairwise comparisons.

The most offshore located water mass (SASW) exhibited an overall significant effect for seasonality (df = 3, pseudo- F = 3.44 and p < 0.001) combined with homogeneity of dispersion amongst the individual groups. However, despite the overall significance, pairwise comparisons did not reveal any differences between specific seasonal groups.

2.3.6 Size class structuring

Pico- (<2 μ m) and nano- (2 – 20 μ m) phytoplankton size classes were enumerated with flow cytometry while nano- and micro- (20 – 200 μ m) eukaryotic cells were counted and visualised with FlowCam analysis (Figure 2.10 & Figure 2.11). The distinct profile of *Synechococcus* in flow cytometry allowed for a separate characterisation of these picocyanobacteria. An increase in *Synechococcus* was observed in February across all water masses but was higher in NW (Figure 2.11). In the Front, *Synechococcus* numbers were generally higher in January. Overall, picophytoplankton was the most abundant size group (about 10,000 – 500,000 cells mL⁻¹)

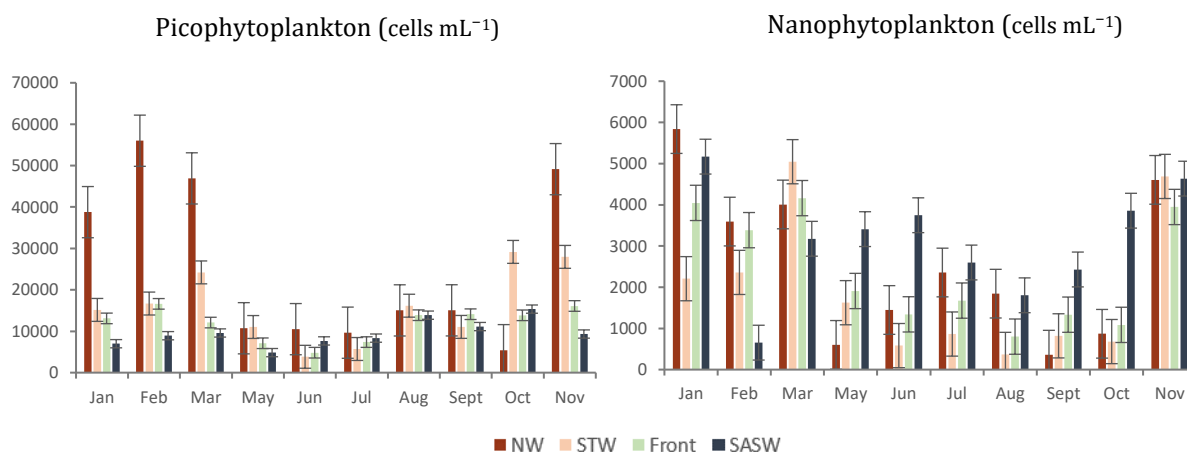


Figure 2.10: Pico- and nanophytoplankton temporal size class distribution across the four different water masses. Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and sub-Antarctic Surface Water (SASW). Pico- and nanophytoplankton numbers were quantified with flow cytometry. Error bars are standard error.

followed by nanophytoplankton (about 400 – 6,000 cells mL⁻¹) (Figure 2.10). Nanophytoplankton in NW and the Front showed the same pattern with increased numbers from November until March. In STW higher numbers were only observed in November and March. Throughout most of the year, similar levels of nanophytoplankton (3000 – 4000 cells mL⁻¹) were found in SASW with an increase in January (5000 cells mL⁻¹) and highly reduced numbers in February (600 cells mL⁻¹). FlowCam imaging and counts revealed that microeukaryotes (incl. diatom cells) were consistently observed across all water masses in March and November each year. FlowCam imaging also revealed that centric diatoms were more prevalent in the coastal adjacent water masses (NW and STW), while pennate diatoms were more abundant in the offshore water mass (SASW). However, both diatom groups were associated with the Front. Overall, micro-planktonic eukaryotes were low in abundance but showed peaks in March for NW and STW and in November for the Front and SASW.

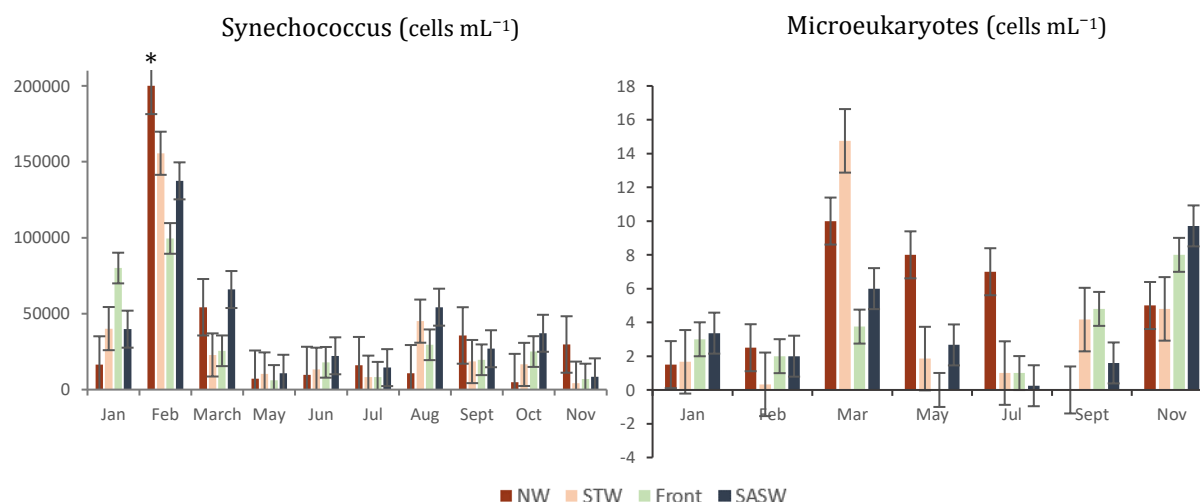


Figure 2.11: *Synechococcus* and microeukaryotic temporal size class distribution across the four different water masses. Neritic Water (NW), Subtropical Water (STW), Southland Front (Front) and Subantarctic Surface Water (SASW). Asterisk: value is cut-off, total amount of *Synechococcus* cells mL⁻¹ in NW (Feb) is about 490,400. *Synechococcus* was quantified by flow cytometry and microeukaryotes by FlowCam analysis. Error bars are standard

2.3.7 Taxonomical community composition

2.3.7.1 Structuring at low taxonomic resolution - division and class level

All four water masses were characterised by the same major phytoplankton groups, *Dinoflagellata* (autotroph and mixotroph), *Chlorophyta* (green algae) and *Ochrophyta* (incl. diatoms) (Figure 2.12). Overall, at division level the water masses showed similar relative abundances. However, at high taxonomic resolution (genus and ASV) and even from class level on differences in protist composition were observed. In addition to the dominant phytoplankton groups, other autotrophs with a substantial contribution to at least one water mass included *Haptophyta* and *Prasinodermophyta*. The heterotrophic component of the protist communities was covered by *Syndiniales* (dinoflagellates), *Cercozoa*, *Ciliophora*, *Sagenista* (Marine Stramenopiles or MASTs), and *Telonemia*. At division and class level, these heterotrophic taxonomical groups varied more across the water masses compared to the major autotrophic taxa. *Syndiniales* made up a substantial proportion of the communities in each water mass (20 – 30%) while the other heterotrophs were mostly detected in reduced amounts (< 5%).

Seasonal variability was also detected across the water masses for certain protist groups, such as *Dinophyceae* and *Syndiniales* (dinoflagellates), and *Mamiellophyceae* (*Chlorophyta*) (Figure 2.12). *Dinophyceae* show a clear trend with their highest levels in August and September in all water masses. After that, they show a decrease over November and stay consistent over the remaining months. The opposite pattern is observed for *Syndiniales* in all water masses except SASW, where this group showed similar relative abundances all year-round. Lastly, *Mamiellophyceae* shows different patterns across the water masses. In NW, this group has a higher prevalence during summer and early autumn, while in STW and the Front their presence is mostly observed in late autumn, winter and spring.

In NW, on average dinoflagellates (33%) and *Chlorophyta* (25%) dominated the protist communities together, while *Ochrophyta* accounted for 16%. In comparison, *Haptophyta* (mainly *Prymnesiophyceae*) made up a smaller proportion of the overall relative abundance (4%). Dinoflagellates were equally represented by *Dinophyceae* (15.5%) and the heterotrophic *Syndiniales* (17%). *Chlorophyta* members were mostly divided into three classes, primarily *Mamiellophyceae* (17%) but also *Pyramimonadophyceae* (3%), and *Trebouxiophyceae* and *Chloropicophyceae* (2.5% each). The *Ochrophyta* individuals were dominated by *Bacillariophyta* (diatoms, 12%) while *Pelagophyceae* only accounted for a small fraction (1.5%). Relative to the other water masses diatoms were significantly more affiliated with NW ($df = 3$, $F = 13.4$, $p\text{-adj} < 0.001$). Beside *Syndiniales*, the heterotrophic protists involved *Cercozoa* (5.5%), *Sagenista* (3%), *Ciliophora* (3%) and *Telonemia* (2%). *Cercozoa* and *Telonemia* affiliated taxa were significantly

more abundant in NW compared to STW, the Front and SASW ($df = 3, F = 16$, both $p\text{-adj} < 0.001$). Further, *Cercozoa* classes *Filosa-imbricatea* (2.5%) and *Filosa-Thecofilosea* (2%) were the most abundant, followed by *Spirotrichia* (2.4%, *Ciliophora*), *Telonemia_X* (2%) and minor contributions of the *Sagenista* classes MAST 4, MAST 7 and MAST 9 (each 1%).

STW was clearly dominated by dinoflagellates alone (52%), who were equally represented by *Dinophyceae* and *Syndiniales*. The second most abundant taxonomical group was *Chlorophyta* with a relative abundance of 16%. Similar to NW, *Mamiellophyceae* strongly dominated the *Chlorophyta* group (12.5%). Minor additions were observed from *Chloropicophyceae* (2%) and

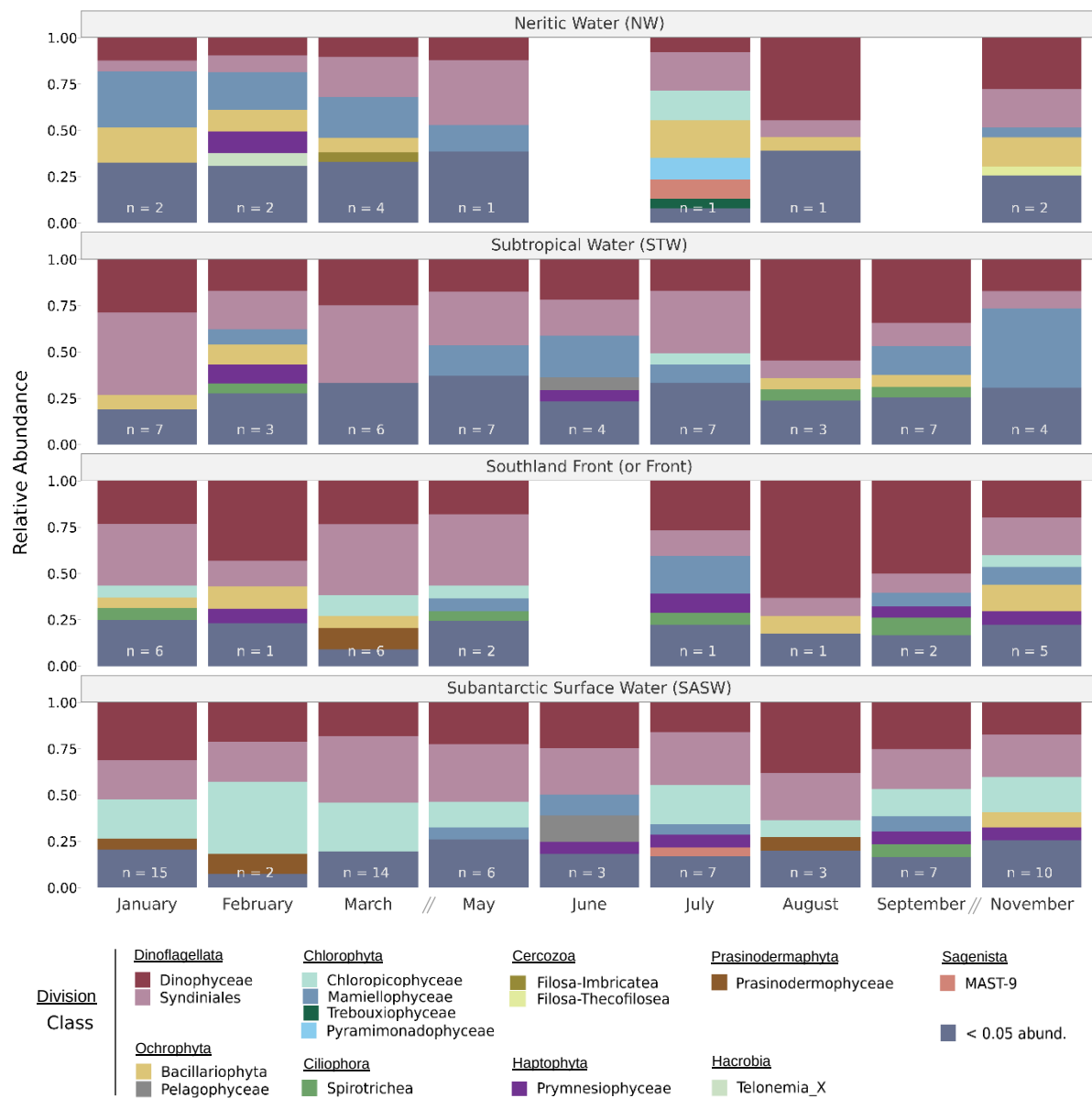


Figure 2.12: Class-level taxonomical composition of protist communities for each individual water mass. Mean relative abundances were averaged over the years and presented per month. No sampling cruises were conducted in April, October, or December. n = number of samples averaged per month. Heatwave summer month (January) included.

Pyramimonadophyceae (1.5%). *Ochrophyta* identified taxa in STW were reduced compared to NW but were still dominated by diatom species (5%), followed by *Pelagophyceae* (2%). *Haptophyta* remained a less abundant group (4%), while *Cercozoa* (1%) and *Telonemia* numbers (<1%) were drastically diminished compared to NW. In addition to *Sagenista* (2.5%), *Ciliophora* (5%) became more abundant amongst the heterotrophic group in STW with individuals primarily belonging to class *Spirotricha*.

The Front had the highest relative abundance of dinoflagellates (55%) amongst all water masses, followed by substantial proportions of *Chlorophyta* (13%) and *Ochrophyta* (10%). Dinoflagellates were still equally represented by *Dinophyceae* and *Syndiniales*. *Chlorophyta* taxa differed from NW and STW as they were dominated by both *Chloropicophyceae* (7%) and *Mamiellophyceae* (5.5%). Diatom affiliated reads increased slightly (7.5%) compared to STW while *Pelagophyceae* taxa dropped (1%). *Haptophyta* (5%) and *Ciliophora* numbers (5%) were comparable to STW while *Sagenista* taxa were largely diminished (1%). The Front was also associated with a relative high abundance of the autotrophic *Prasinodermophyta* (3.5%) group. *Cercozoa* and *Telonemia* remained near absent (<1%).

Like STW and the Front, SASW was also clearly dominated by dinoflagellates (50%, equally *Dinophyceae* and *Syndiniales*). Overall *Chlorophyta* relative abundance (24%) in SASW was more similar to NW, however at class level a clear shift was noticed. In SASW, *Chloropicophyceae* (20%) unambiguously dominated the *Chlorophyta* while *Mamiellophyceae* (4%) accounted for a less substantial proportion here. In addition, *Ochrophyta* (6.5%) depicted their lowest numbers in SASW and diatoms were no longer the most dominant group. Rather, diatoms and *Pelagophyceae* contributed equal proportions (2.5% each). Compared to NW and STW, *Haptophyta* (5.5%) showed a marginal increase. *Prasinodermophyta* were also present in SASW with a slightly higher fraction than in the Front (4%). *Ciliophora* (4%) remained the second most abundant heterotrophic group in SASW.

2.3.7.2 Structuring at high taxonomic resolution – genus and ASV level

In NW, the most abundant ASV was identified as a *Chlorophyta* (*Mamiellophyceae*) species, *Ostreococcus Lucimarinus* (7.4%), followed by two diatom species, *Minidiscus variabilis* (4%) and an unidentified *Polar-centric Mediophyceae* (3.9%), and lastly a dinoflagellate (*Syndiniales*) species belonging to Dino-Group-I-Clade-1_X (3.9%). Lower abundant *Mamiellophyceae* affiliated taxa were *Bathycoccus prasinos* (3%), *Micromonas commoda* A2 (2%) and *Micromonas bravo* B2 (1.5%). In addition to these, *Picochlorum*_sp. (class *Trebouxiophyceae*; 2.4%) and *Pycnococcaceae*_X_sp. (class *Pyramimonadophyceae*; 2%) were also relatively high abundant *Chlorophyta* species while *Chloroparvula pacifica* abundance was less substantial (class

Chloropicophyceae; 0.9%). The two most abundant *Dinophyceae* species in NW were *Gyrodinium fusiforme* (1.4%) and *Gyrodinium helveticum* (1%).

Compared to NW, the increase and decrease observed for dinoflagellates and diatoms in STW at division level, respectively, was also seen at lower taxonomic levels. The dinoflagellate (*Syndiniales*) species Dino-Group-I-Clade-1_X (8.8%) clearly dominated STW. This was followed by two *Chlorophyta* species *O. lucimarinus* (4.7%) and *B. prasinos* (3.8%), and an unidentified dinoflagellate species (*Gymnodiniaceae* sp.; 3%). Similar to NW, *Micromonas commode* A2 (2.5%) was also very abundant and *Chloroparvula pacifica* (1%) to a lesser extent. The other *Chlorophyta* species identified as relatively substantial in NW showed highly reduced abundances in STW. In addition to the highly prevalent *Gymnodiniaceae* sp., *G. fusiforme* (1.4%) and *G. helveticum* (1.8%) were also characteristic species for the dinoflagellate group. Finally, for the diatoms only one species had a relatively high abundance, *M. variabilis* (1.4%).

In the Front, the most abundant taxa was again the dinoflagellate (*Syndiniales*) species belonging to Dino-Group-I-Clade-1_X (10.5%). The second most abundant ASV was a *Chlorophyta* species *Chloroparvula pacifica* (5%), which defined a clear shift from NW and STW. The third most abundant ASV was a *Prasinodermophyta* species identified as *Prasinoderma singularis* (3.5%). For the *Chlorophyta*, *C. pacifica* was followed by *B. prasinos*, *O. lucimarinus* (both 1.8%) and *M. commode* A2 (1.3%). The dominating *Dinophyceae* species were the unidentified *Gymnodiniaceae* sp., *G. fusiforme* (both 2.2%) and an *Dinophyceae*_XXX_sp (1.5%). In addition to *M. variabilis* (1.3%) still being the most abundant diatom species, *Brockmanniella brockmannii* (1%) also contributed highly.

The most different water mass at lower taxonomical resolution was SASW. *Chloroparvula pacifica* (14.8%) was clearly the most abundant species in SASW, followed by a dinoflagellate (*Syndiniales*) species of genus Dino-Group-I-Clade-1_X (9.7%) and a second *C. pacifica* species (3.4%). Next was a *Prasinoderma singularis* (3.3%) and an unknown dinoflagellate *Peridinales* sp. (2.6%). The *Chlorophyta* group nearly in its entirety was accounted for by *C. pacifica* and *Bathycoccus prasinos* species. Next to *Peridinales* sp., the dinoflagellates were also represented by *G. fusiforme* (2.2%), an unidentified *Dinophyceae* sp. (1.6%) and *Karludinium* sp. (1.2%). The diatom group was composed out of low abundant species of which *Brockmanniella brockmannii* (0.8%) contributed the most.

The heterotrophic protists in NW were dominated by *Novel clade 2_X* sp. (*Cercozoa*) and *Telonemia-Group-2_X* sp. (each ~1%), followed by less abundant *MAST 9A_XX* and *4E_XX* sp. (*Sagenista*) and *Laboea strobila* (*Ciliophora*). *Sagenista* taxa *MAST 9A_XX* sp. were the most dominant heterotrophic protists in STW, the Front and SASW. Similar to NW, *Mast 4E_XX* sp. was

also relative abundant in STW. For the *Ciliophora*, two species were considered more prevalent *Strombidiidae M_X sp.* and *Strombidiida G_XX sp.* They showed relatively equally proportions in STW and SASW but were slightly more abundant in the Front.

2.3.8 Taxonomical structuring during the heatwave event

The changes from ambient to heatwave conditions were mainly defined by large reductions and increases in relative abundance for *Dinoflagellata*, followed by increases in *Chlorophyta* and decreases in diatoms (Figure 2.13). Additionally changes in less abundant groups were also noticed for *Haptophyta* and heterotrophs *Ciliophora*, *Radiolaria* and *Cercozoa*. The comparisons between ambient and heatwave communities are based on the average relative abundance of three years (= three summer cruises) for ambient conditions versus one year (= one cruise) under heatwave conditions.

In STW and NW, the dinoflagellate *Syndiniales* (mainly genus *Dino-Group-I-Clade-1_X*) increased dramatically under heatwave conditions (Figure 2.13). From 18% in both water masses under ambient conditions to 81% in STW and 48% in the Front during the heatwave. This shift coincided with a drastic reduction in the dinoflagellate class *Dinophyceae*. In STW, *Dinophyceae* decreased from 47% to 4.4% during the heatwave, while in the Front a decrease from 34% to 13% was observed. Overall, the shifts in dinoflagellates were most evident for the communities in STW.

In SASW, this dinoflagellate shift was not observed. Instead, a large increase in the oligotrophic green algae (*Chloropicophyceae*) took place (Figure 2.13). *Chlorophyta* taxa in STW and the Front also underwent changes. In STW, the overall *Chlorophyta* group only slightly increased with SST from 4% to 5.8%. This was associated with an increase in *Chloropicophyceae* (0.2% to 2%), an increase in *Trebouxiophyceae* (0.3% to 3%), and a decrease in *Mamiellophyceae* (1.5% to 0.5%). In the Front, *Chlorophyta* members belonging to *Chloropicophyceae* increased in relative abundance from 1.5% to 11% under heatwave conditions, while *Mamiellophyceae* members did not vary with the change in SST.

Diatoms (*Ochrophyta*) in STW and the Front also clearly underwent a reduction in relative abundance. In STW, a change from 13% to 0.2% was observed while in the Front the diatoms decreased from 10% to 1.8%. Conversely, the *Pelagophyceae* (*Ochrophyta*) in the Front were associated with a slight increase from 1.8% to 2.5% but remained unchanged in STW. *Haptophyta*, another phototrophic group, decreased in relative abundance in both STW (1.7% to 0.5%) and the Front (7.5% to 1.8%).

For the remaining heterotrophic protist groups, *Ciliophora* (*Spirotrichea*) decreased in both STW (5% to 1.8%) and the Front (8.5% to 5.5%), *Cercozoa* only reduced in relative abundance in STW (0.7% to 0.06%), while *Radiolaria* (*Acantharia*) increased in both STW (0.3% to 0.7%) and the Front (1.3% to 3.5%).

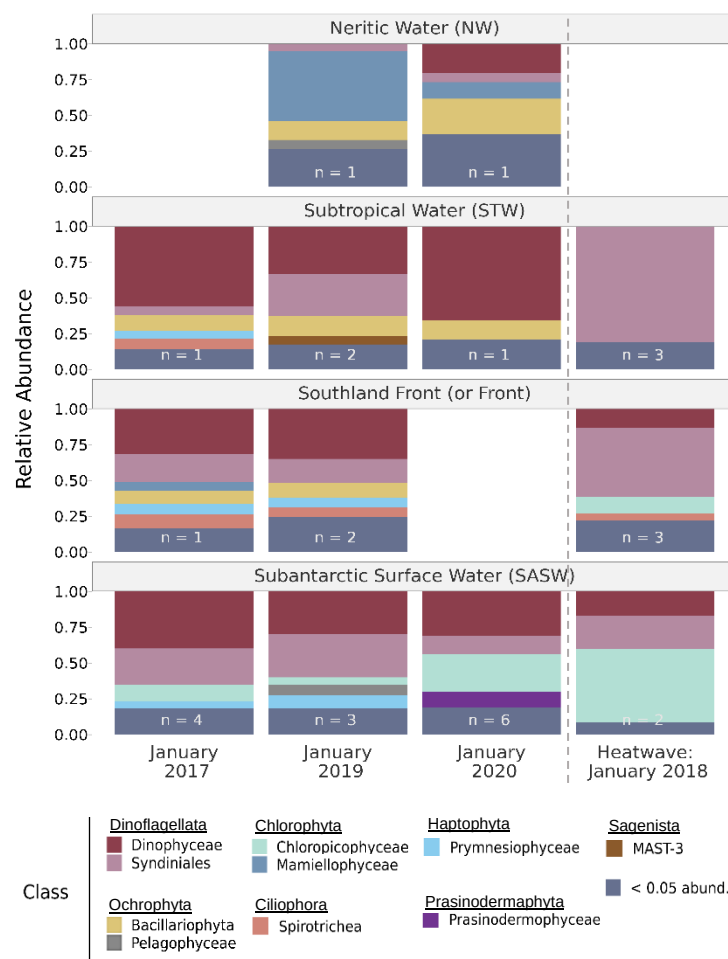


Figure 2.13: Class-level taxonomical composition of protist communities in January, including the heatwave in 2018. n = number of samples averaged.

2.4 Discussion

2.4.1 Distinct protist communities across the individual water masses

Each water mass across the Southland Front system is characterised by a unique set of hydrographical parameters and macronutrient concentrations (Jillett, 1969; Hopkins et al., 2010; Jones et al., 2013). Distinct protist communities were affiliated with each of these individual water masses. No significant difference was detected between protist communities of the coastal adjacent Neritic and Subtropical Waters (Table 2.5). This is in contrast to the high spatial resolution study by Allen et al. (2020) who observed that each of the four water masses hosted unique protist communities. However, the lack of a significant difference in the current study between NW and STW is likely due to the low statistical power associated with the seasonally small sample sizes for NW. Therefore, this result may suggest that neritic and subtropical protist assemblages are more similar to each other compared to the other water masses and that more intensive sampling is needed to accurately characterise the protist communities associated with the neritic water mass. The strongest differentiation in community composition was found between the nutrient-poor warm waters of STW and the nutrient-rich cold waters of SASW (Table 2.5). Similar water mass specificity for eukaryotic communities has been consistently reported across different oceanic regions (Hamilton et al., 2008; Clayton et al., 2014, 2017; Raes et al., 2018). The weakest community dissimilarity amongst STW, Front and SASW, was noticed between the Front and SASW. This suggests that the Front is more influenced by the offshore water mass ecology than the more coastal STW. This also implies that physical horizontal mixing is more intense between the Front and SASW. In the Northeast Pacific, a similar observation was tested by performing an iron-enrichment experiment on Southern Ocean associated phytoplankton communities (Ribalet et al., 2010). The results showed that the phytoplankton composition associated with the Front was more similar to the iron-enriched offshore community rather than the natural nitrate-enriched coastal community.

The protist communities in NW and STW showed some seasonal community structuring (Table 2.6). In contrast, SASW supported similar communities all year-round thereby not showing any seasonal trends (Banse, 1996). Due to low sample sizes the full extent of seasonality per water mass could not be tested. It was noticed that in NW a strong difference was observed between summer and autumn while in STW and the Front those seasons did not support distinct communities (Table 2.6), instead both water masses were characterised by bloom events during those months.

Table 2.5: Overview of the community composition differences between water masses for each season. ns: non-significant i.e. no differences in protist composition, sign: significant difference ($p < 0.05$) i.e. distinct protist communities, too low: sample size ≤ 2 samples. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front), sub-Antarctic Surface Water (SASW).

	NW-STW	STW-Front	Front-SASW	STW-SASW
Summer	ns	ns	ns	sign
Autumn	ns	ns	ns	sign
Winter	Too low	Too low	Too low	sign
Spring	Too low	Sign	Sign	sign

Table 2.6: Overview of the seasonal protist community differences for each water mass. ns: non-significant i.e. no differences in protist composition, sign: significant difference ($p < 0.05$) i.e. distinct protist communities, too low: sample size ≤ 2 samples. Water masses: Neritic Water (NW), Subtropical Water (STW), Southland Front (Front), sub-Antarctic Surface Water (SASW).

	Spring - Summer	Summer - Autumn	Autumn - Winter	Winter - Spring
NW	Too low	Sign	Too low	Too low
STW	Sign	ns	ns	sign
Front	ns	ns	Too low	Too low
SASW	ns	ns	ns	ns

The variability of the protist assemblages across the Southland Front system was explained by a combination of environmental variables (temperature, chlorophyll *a*, salinity, nitrate/phosphate and silicate) (dbRDA; 20%) with temperature being the strongest predictor. Despite all these variables, the overall explained variation was still considerably low. In comparison, Allen et al. (2020) accounted for the majority of the variability (dbRDA; 65.86%) in protist communities across the Southland Front, explained by temperature, phosphate, and ammonia. The comparison between dbRDA results from both studies suggested the high influence of seasonality and interannual variation on structuring the protist assemblages. Once the dataset was organised by season (spring, summer, autumn, and winter), a higher proportion of the community variability was explained (Table S2.1). This confirms that seasonal variability is one of the forcing factors behind protist compositional structuring across the Southland Front. Additionally, for each season a different set of environmental parameters explained the community structure indicating that different drivers dominate over time. The interaction between environmental variables and temporal variation in shaping protist assemblages across this small spatial scale highlights the importance of long-term surveys to understand the complex dynamics involved in protist community structuring. Additionally, previous studies have also shown that environmental factors alone cannot account for the complete community variability and that trophic interactions (grazing, pathogenicity, and parasitism) are also strong determinants of community structuring (Chow et al., 2013; Lima-Mendez et al., 2015).

2.4.2 Phytoplankton biomass trends and size distribution

Flow cytometry and FlowCam analysis revealed that the phytoplankton assemblages across the Southland Front were strictly dominated by smaller cell structures (pico- and nanophytoplankton). This is in agreement with the chlorophyll-*a* size fractionation analysis and net primary productivity results from Jones et al. (2013). The dominance of the pico- and nanophytoplankton were in line with the lower nutrient levels observed across the water masses that were in favour of smaller cells (Eppley et al., 1969). However, it cannot be excluded that the dominance of smaller phytoplankton reflect the interference of other variables, such as increased grazing pressure. Overall, phytoplankton blooms were observed in spring, summer, and early autumn in either NW, STW and/or the Front and coincided with small reductions in nitrate, phosphate and silicate (Figure 2.5 & Table 2.4). Only one spring bloom was detected which took place in NW. The highest phytoplankton biomass was found in STW and the Front with bloom levels being observed in summer and early autumn (January – March) for both water masses.

The summer bloom persisted across two water masses and was associated with low nutrient levels (STW and the Front; Figure 2.5 & Table 2.4). The higher biomass in the Front coincided with the lowest silicate levels compared to STW indicating that the Front potentially supports a small diatom bloom. This follows from the fact that diatoms cell walls (frustules) are constructed out of biogenic silica and therefore present major inventories of particulate silica in the ocean (Tréguer et al., 1995; Nelson et al., 1995; Ragueneau et al., 2000). Diatom biomass, cell size and morphology is therefore linked to silicate and nitrate concentrations in the environment, but in addition is also highly dependent on iron availability (Martin et al., 1991; Marchetti & Cassar, 2009). The increase in diatoms in the Front was confirmed by the metagenomic analysis and revealed the dominance of *Pseudo-nitzschia seriata*, *Thalassiosira delicatula* and a *Minidiscus* sp. In February, extremely high abundances of *Synechococcus* were observed in all water masses which could indicate a *Cyanobacteria* bloom (Figure 2.11). However, this data only reflected one cruise and could thus be an isolated event. Nonetheless, in January (over multiple cruises) high numbers of *Synechococcus* were also observed and primarily detected in the Front. Collectively, this suggest that during summer the Front is characterised by a bloom dominated by *Cyanobacteria* and diatoms.

In early autumn a second strong bloom event was establish in STW and the Front also coinciding with lower nutrient levels and a reduction in silicate, similar to the summer bloom. FlowCam analysis revealed that both water masses were particularly enriched in centric diatoms during that time period. Further, a dominance of picophytoplankton was accompanied by an increase in nanophytoplankton (particularly for STW) and a large drop in *Synechococcus* abundance in both

water masses compared to the summer bloom. This indicates a potential shift from pico- to nanophytoplankton during the transition from summer to early autumn. In STW this shift also coincided with an increase in microeukaryotes.

Jones et al. (2013) reported a spring bloom in STW and SASW that coincided with a shift to microphytoplankton and silicate depletion thereby suggesting a typical large cell-sized diatom dominated spring bloom. However, the only spring bloom observed in this study was associated with coastal NW and was dominated by pico- and nanophytoplankton. Potentially, the lack of a spring bloom event in the offshore water masses could be explained by a higher grazing pressure. Indeed, in SASW a higher number of nanophytoplankton and microeukaryotes were observed (Figure 2.10 & Figure 2.11). FlowCam imagery further revealed a high presence of pennate diatoms in the Front and SASW during spring. The higher prevalence of pennate diatoms in the offshore water masses is linked to the iron limiting conditions. To account for the iron limiting conditions in HNLC waters, raphid pennate diatoms can greatly reduce their iron requirements due to changes made to their photosynthetic apparatus giving them an advantage over centric diatom species in HNLC water masses (Sunda et al., 1991; Strzepek & Harrison, 2004; Kustka et al., 2007; Allen et al., 2008).

2.4.3 The Front as a diversity hotspot in summer

Previous research conducted across the Subtropical Front system has investigated, among other things, whether the Front between STW and SASW acts as a diversity hotspot for bacterioplankton and planktonic protists (Morales et al., 2018; Allen et al., 2020). These research questions were based on studies done on several oceanic front regions (Kuroshio Front, California Current System, Northeast Pacific Ocean) where the frontal systems support enriched protist biodiversity and productivity (Ribalet et al., 2010; Taylor et al., 2012; Clayton et al., 2017; Raes et al., 2018). Oceanic regions involving the Subtropical Frontal Zone can span a width of 10 up to hundreds of kilometres (Bradford-Grieve et al., 1997; Murphy et al., 2001; Thomalla et al., 2011; Raes et al., 2018). Unlike those frontal convergent zones, the Southland Front system along the Munida transect only spans a width of 2 to 10 km making it a very unique and dynamic system that might not support the same biodiversity trends as other oceanic regions (Jones et al., 2013).

Morales et al. (2018) performed a seasonal study (January 2014 – April 2015, excluding spring months) on bacterioplankton across the Southland Front system and showed that the Front is a clear transition zone between STW and SASW water masses with seasonally oscillating strength. However, no evidence was found of increased bacterioplankton diversity in the Front. In contrast, Allen et al. (2020) conducted a high spatial resolution survey across the Southland Front in Austral

spring (November 2017) and found that the Front did support an increase in diversity for bacterioplankton but not for protist communities. In the current study only protist assemblages were investigated, and no significant difference in the overall mean richness and evenness measures were found for each individual water mass. However, like the study of Morales et al. (2018) a seasonal dependency for protist communities was present as well. In Austral summer (January – February) the Front was associated with a higher mean alpha diversity compared to the other water masses (NW, STW & SASW). Note, only the comparison between the Front and STW was significantly different suggesting that the Front is more similar to SASW in terms of protist alpha diversity. This further supports the finding of more similar community compositions between the Front and SASW (see section 2.4.1).

The enhanced diversity in the Front coincided with increased phytoplankton biomass of the summer bloom. As stated earlier, this was complemented by low nitrate and phosphate levels and a reduction in silicate concentration. Collectively, these low values are an indication of increased nutrient uptake by protist communities, specifically silicic diatoms, thereby supporting an increase in species diversity and high phytoplankton productivity (Laubscher et al., 1993; Taylor et al., 2012). These results are in line with the observations from other oceanic front regions (Ribalet et al., 2010; Clayton et al., 2017; Raes et al., 2018). However, the establishment of the Front as a diversity hotspot was not repeated in any of the remaining seasons even though several more bloom events were discovered. In contrast, previous studies on frontal zones found enriched biodiversity throughout summer (Ribalet et al., 2010), autumn (Taylor et al., 2012; Clayton et al., 2017) and winter (Raes et al., 2018). The strong temporal variability of the Front as a diversity hotspot is likely reliant on a combined set of favourable abiotic and biotic environmental variables that are only achieved under the hydrology and physical oceanography processes that are characteristic for the Front in summer. These variables would involve sufficient nutrient levels and trace elements (e.g. iron, copper, zinc and vitamin B), light availability, thermal stratification, moderate grazing pressure and biological community interactions. Noteworthy, there is also the possibility that additional Front enriched biodiversity patterns in other months were missed due to low-resolution and inconsistent or rather weather dependent spatiotemporal sampling here. This highlights the importance of a combination of both long-termed surveys as well as short-term high-resolution studies.

2.4.4 Taxonomic composition of protist communities

While the physicochemical nature and spatiotemporal dynamics of the Southland Front system have been well documented (Jillett, 1969; Heath, 1972; Shaw & Vennell, 2001; Jones et al., 2013; Sander et al., 2015), an extensive overview of the taxonomical composition of protists including

autotrophic, heterotrophic and mixotrophic members has not been performed previously. Similar to the findings of De Vargas et al. (2015) in the global *Tara* Oceans project, a large unexpected abundance of heterotrophic protists was detected in this study, specifically involving parasitic dinoflagellates.

Dinophyceae

Dinoflagellata were the dominant protist group in each water mass and were equally represented by taxa from class *Dinophyceae* and from the parasitic class *Syndiniales* (Figure 2.12). Note that dinoflagellates possess variable 18S gene copy numbers and that the high abundance observed in this study could be slightly inflated (Lin, 2011; Wisecaver & Hackett, 2011; Lin et al., 2015; Krabberød et al., 2017). Nonetheless, their dominance across the Southland Front was strongly supported by the FlowCam imagery for the nanoplankton size groups. Dinoflagellates made up half of the relative abundance in STW, the Front and SASW while constituting a slightly lower proportion of the protist communities in NW. The dominance of this major phylogenetic group in NW and SASW was shared with *Chlorophyta* members, *Mamiellophyceae* and *Chloropicophyceae*, respectively. Despite being the most dominant group at high taxonomical level, at a fine resolution scale ASVs affiliated with *Chlorophyta* and diatoms were most abundant in NW, *Chlorophyta* and *Syndiniales* in STW, *Syndiniales* in the Front and *Chlorophyta* in SASW. This suggest that *Dinophyceae* across the Southland Front represent a more diverse group of lower abundant taxa compared to *Syndiniales* and the other major phylogenetic groups who are clearly more structured by a few highly abundant taxa. The most prevalent *Dinophyceae* ASVs belonged to genus *Gyrodinium* and were mainly identified as *G. fusiforme*, *G. helveticum* and *G. spirale*. In addition to *Gyrodinium* taxa, the *Peridinales* group was also relatively well represented in STW, the Front and SASW. The high relative abundance of the *Gyrodinium* species agrees with previous research along the Southland Front (Allen et al., 2020). Overall, *Gyrodinium* species have a global distribution and are potentially mixotrophic in nature (Chang, 1996; Gast et al., 2018). Their high abundance and mixotrophy feeding strategy highlights their importance as part of protist communities and the extent of their role in the carbon cycle. In each water mass *Dinophyceae* abundance was highly similar to *Syndiniales*, suggesting that these two dinoflagellate groups might have a positive relationship and perhaps even symbiotic or parasitic.

Syndiniales

The widespread distribution of *Syndiniales* was confirmed by the *Tara* Oceans exploration (De Vargas et al., 2015), and recent works have increasingly recognised these parasites as a major part of protist communities (Guillou et al., 2008; De Vargas et al., 2015; Cleary & Durbin, 2016; Clarke et al., 2019). Their high abundance and the fact that all known *Syndiniales* parasites obligately kill their host (Guillou et al., 2008) makes them important contributors to the marine carbon cycle.

Therefore it has been suggested that these parasitoids should be included in marine food web and biogeochemical models (Warren et al., 2010; Worden et al., 2015). *Syndiniales* affiliated taxa were highly prevalent in each water mass but showed the lowest association with neritic water. The most dominant ASVs were classified as Dino-Group I, followed by a smaller amount of Dino-Group II. In contrast, in a study by Guillou et al. (2008) the Dino-Group I taxa were found to mainly dominate anoxic and suboxic ecosystems (e.g. sediments and hydrothermal vents) in Atlantic and Mediterranean waters while Dino-Group II related taxa had a higher abundance throughout the water column. Likewise, Suter et al. (2022) observed a higher association of Dino-Group I ASVs with low-oxygen waters compared to the genetically more diverse Dino-Group II who dominated the oxycline. In general, Dino-Group II affiliated taxa tend to be the most dominant observed *Syndiniales* group throughout the water column (Guillou et al., 2008; Suter et al., 2022). This is in stark contrast to the current study where all samples were taken from the photic zone and none of the water masses involved have been recognised as oxygen low zones. Consequently, this is the first report of the high abundance and dominance of Dino-Group I over Dino-Group II in the photic zone for each of the sampled water masses (coastal, subtropical, front and subantarctic). Further, previous co-occurrence network studies have suggested that intra-community or rather intracellular biological interactions play a strong role in structuring the *Syndiniales* groups in protist assemblages (Lima-Mendez et al., 2015; Berdjeb et al., 2018; Anderson & Harvey, 2020; Käse et al., 2021), even more so than abiotic variables (e.g., temperature, salinity, nutrients, oxygen and light availability). This could help explain the high abundance of *Syndiniales* Dino-Group I through a potential strong interaction between this group and the equally dominant non-parasitic dinoflagellates, *Gyrodinium* (*Gymnodiniaceae*, *Dinophyceae*), in this study.

Bacillariophyta (= diatoms)

Diatom species represent an important component of the phytoplankton community in marine ecosystems and productive areas such as upwelling zones and frontal systems are often characterised by their seasonal blooms (Field et al., 1998; Armbrust, 2009). In accordance, diatoms here were primarily limited to coastal NW and the Front, however their overall abundance across the Southland Front system was very low. Further, while most global diatom bloom communities are predominantly represented by *Chaetoceros*, *Thalassiosira*, *Rhizosolenia* and *Pseudo-nitzschia* (Leblanc et al., 2012), the diatom assemblages in both the coastal adjacent and open ocean waters of the Southland Front were strongly dominated by nanoplanktonic species (2 – 20 µm), primarily *Minidiscus variabilis* and an unknown polar-centric *Mediophyceae* species. The high abundance of *Minidiscus* in the diatom community was also observed in a previous study across the Southland front (Allen et al., 2020). This genus contains the smallest known centric diatoms (2 – 5 µm; Hasle, 1973) in marine waters. Earlier studies have reported

the existence of quasi-single-species diatom blooms dominated by pico-nano-sized diatom species, but these events were seen as anomalies rather than a potential unexplored diversity within the diatom group (Gould & Wiesenburg, 1990; Buck et al., 2007; Dale et al., 2014; Daniels et al., 2015). Nonetheless, evidence from recent studies, including data from northwestern Mediterranean Sea sampled during the global scale *Tara* Oceans survey, has shown that these small diatoms (genus *Minidiscus*) can reach extremely high abundances and become major contributors to bloom events thereby questioning the common view of diatom bloom succession that involve larger species (Buck et al., 2007; Malviya et al., 2016; Leblanc et al., 2018). The dominance of pico-nanoplankton diatom species observed across the Southland Front in this study further supports these latest findings. The most likely driving force behind the observed diatom ecology is thought to be size-selective predation of zooplankton on the larger diatom cells (Leblanc et al., 2018). However, no concrete evidence has been given that supports this hypothesis and further research focussing on inter-species relationships is needed to unveil this potential trophic link.

Chlorophyta (=green algae)

Chloropicophyceae (formerly Prasinophytes Clade VII) are pico-eukaryotic green algae that are found in mesotrophic and moderately oligotrophic waters (Moon-Van Der Staay et al., 2001; Shi et al., 2009; Dos Santos et al., 2017). As mentioned before, their distribution across the Southland Front system opposes that of the picoplanktonic green algae *Mamiellophyceae*. Members of *Mamiellophyceae* (genera *Ostreococcus*, *Micromonas* and *Bathycoccus*) are typical of coastal waters (Monier et al., 2016; Vannier et al., 2016) which was confirmed in this study by their dominance in coastal neritic and subtropical water (Figure 2.12). *Chloropicophyceae* abundance increased with distance offshore and subsequently dominated the SASW. Although SASW is a HNLC water mass (Banse, 1996) and thus not macronutrient limited like the oceanic waters this group has previously been discovered in, it seems that *Chloropicophyceae* (here mainly *Chloroparvula pacifica*) can also thrive in these iron-limited HNLC areas. Interestingly, HNLC areas are not only characterised by limited iron (Fe) but trace metals such as zinc (Zn), cobalt (Co), and vitamin cobalamin (B₁₂) have also been shown to co-limit phytoplankton biomass in these waters (Croft & Hunter, 1998; Panzeca et al., 2006; Bertrand et al., 2007). Since *Mamiellophyceae* are vitamin auxotrophic for thiamine (B₁) and B₁₂, they need to acquire these vitamins from the surrounding environment for growth (Croft et al., 2005; Helliwell, 2017) which would help explain their lower abundances in SASW. Additionally, this suggest that *Chloropicophyceae* do not rely on exogenous vitamins but instead are able to synthesise thiamine (vitamin B₁) themselves. Although this ability has not been detected in genus *Chloroparvula*, which presents the most dominant *Chloropicophyceae* members in this study, it has been detected in the only other genus

(*Chloropicon*) belonging to the *Chloropicophyceae* lineage (Lemieux et al., 2019). More research into the genomic make-up of *Chloroparvula* species is needed to confirm this theory and help explain the distribution pattern of this genus in oceanic environments.

2.4.5 Protist community dynamics during the 2017/2018 MHW

An interesting opportunity presented itself when the sampling cruise of January 2018 coincided with a MHW that took place from December 2017 to February 2018 (NIWA, 2019). Subsequently, a comparison between the summer trips from 2017 to 2020 (strictly January cruises) was made to research the impact of increased sea surface temperature (approximately +2.5°C) on protist communities *in situ*, across three different water masses (STW, Front and SASW). Unfortunately, no NW was sampled during the MHW as STW extended further inshore. During the heatwave the Front was positively impacted and established a width of about 13 km. The widening of this transitional zone during a period of high sea surface temperatures is likely the result of enhanced ocean stratification and reduced vertical mixing thereby forcing the Front to expand horizontally to allow for the convergence between warm nutrient-poor STW and cold nutrient-rich SASW.

The protist alpha diversity during the MHW was drastically reduced in all three water masses (STW, Front and SASW) (Figure 2.8). Remarkably, the pattern of enhanced protist diversity associated with the Front during summer persisted under the heatwave conditions. The most abundant taxa that were present in the Front included dinoflagellate groups *Syndiniales* and *Dinophyceae*, *Chlorophyta* groups *Chloropicophyceae* and *Mamiellophyceae*, and to a smaller extent *Prasinodermophyta*, *Ciliophora* and *Radiolaria*. Strikingly, it was the heterotrophic unicellular parasitoid group *Syndiniales* (marine alveolates) who overwhelmingly dominated the Front and even more strongly the STW (Figure 2.13).

Despite still acting as a diversity hotspot, in relation to the neighbouring water masses, the Front under heatwave conditions was not accompanied by an increase in phytoplankton biomass. The low chlorophyll-*a* biomass was also true for the adjacent water masses and was associated with near depleted nitrate and phosphate levels. Silicate concentrations revealed minor reductions with the anomalous SSTs suggesting that silicic diatoms didn't positively correlate with the sharp increased temperatures. Likewise with the expansion of the Front, enhanced thermal stratification and shoaling of the mixed layer are likely responsible for significant reduction in species diversity by preventing the vertical nutrient exchange required to sustain high productivity and abundance in the euphotic zone (Sarmiento et al., 2004; Doney, 2006; Behrenfeld et al., 2006).

The change in community composition with the heatwave event was mainly defined by the shift in dominance from *Dinophyceae* (ambient conditions) to a high abundance of *Syndiniales* in both STW and the Front (Figure 2.13). The positive correlation between *Syndiniales* abundance and sea surface temperature has been previously observed in short- and long-term temporal studies on coastal waters, the open ocean and polar systems (Lima-Mendez et al., 2015; Berdjeb et al., 2018; Anderson & Harvey, 2020). Although temperature was the strongest factor responsible for the community variability in STW and the Front between ambient and heatwave conditions, together with salinity and pH these abiotic variables still only accounted for 50% of the overall community variation (dbRDA analysis). As mentioned before, other variables such as biotic interactions tend to be a major predictor in *Syndiniales* community structuring (Lima-Mendez et al., 2015; Anderson & Harvey, 2020). Consequently, it was noticed that the shift in dinoflagellates, beside resulting in a drop in *Dinophyceae* relative abundance, also coincided with a reduction in diatoms, ciliates and haptophytes, and an increase in *Chloropicophyceae* (Figure 2.13). The changes in these taxonomical groups took place in both STW and the Front but they occurred with different orders of magnitude. In STW, diatoms were drastically reduced during the heatwave and became virtually absent. Even though the green pico-algae *Chloropicophyceae* increased in STW, their overall relative abundance was still low partially due to extreme increase and resulting dominance of *Syndiniales* (81%). In the Front, the decrease in diatoms under the heatwave conditions was substantial but they did not completely disappear. The biggest change in composition in the Front (excluding *Syndiniales*) was defined by the sharp increase in *Chloropicophyceae*.

As previous and current research has shown, abiotic variables are not the major drivers behind *Syndiniales* structuring. Therefore, it is possible that the large increase of these parasitoids reflects a similar high enhancement of their host organisms. This means that the rapid short-term elevation of SSTs in STW and the Front would primarily have resulted in an increase in biomass of host protist groups. Based on the patterns of change in relative abundance between ambient and heatwave conditions it seems that *Dinophyceae*, diatoms, ciliates and haptophytes could have been potential hosts (Guillou et al., 2008). Although the reduction in some of these protist groups could also have been a consequence of unsuitable environmental conditions (e.g. high SSTs, low nutrient levels, iron limitation). For instance, it is unlikely that diatoms increased in abundance prior to the heatwave sampling day because that would have resulted in a change in silicate concentration (Jones et al., 2013; Sander et al., 2015). Even though *Syndiniales* are generalists and opportunists, previous reports on host-parasite interactions involving *Syndiniales* and ciliates have identified tintinnids as the preferred host organism (Guillou et al., 2008). Regardless, this taxonomically group was barely present in any of the water masses under both ambient and

heatwave settings. The most likely host group is *Dinophyceae* due to their more equal abundance to *Syndiniales* under ambient conditions. Nevertheless, these potential successional shifts and interactions in protist communities under heatwave conditions cannot be confirmed with this dataset as only one time point was sampled during the heatwave event. However, further analysis such as co-occurrence networks (Lima-Mendez et al., 2015; Anderson & Harvey, 2020; Käse et al., 2021) on the whole dataset could give a better understanding about the interspecies correlations involving *Syndiniales* across the Southland Front system and potentially discover new host-parasite interactions for *Syndiniales* Dino-Group I. Interestingly, very similar results were found in a subtropical estuary, where high temperatures (23 – 31°C) increased the relative abundance of *Syndiniales* while decreasing *Dinophyceae* and diatoms (Anderson et al., 2021). This might suggest a common response of protist communities in subtropical waters to rising temperatures.

The increased abundance of *Chloropicophyceae* was most evident in SASW and the Front. In SASW, *Chloropicophyceae* merely increased in abundance but in STW and the Front a shift in dominance was detected from *Mamiellophyceae* to *Chloropicophyceae*. Due to these compositional changes, a possible explanation could be an increased vitamin limitation in the coastal adjacent water masses under heatwave conditions (see section 2.4.4: *Chlorophyta*). As a result of thermal stratification, water masses closer to the shore would become increasingly limited by macronutrients as well as trace elements and vitamins (Fe and vitamins B)(Croot & Hunter, 1998; Bertrand et al., 2007; Sander et al., 2015). Additionally, the heatwave conditions could also have led to a significant change in grazing pressure. Overall, the huge impact of the heatwave conditions on these chlorophyte lineages urges more research to be done on the recent classified and still poorly studied *Chloropicophyceae* members.

2.5 Conclusion

This data provides a comprehensive overview of protist communities across the Southland Front offshore the east coast of South Island, New Zealand. A combination of technologies including metabarcoding and optical methods were used to analyse protist abundance, composition, diversity, and size range. Overall, distinct communities were found for Subtropical Water (STW), the Southland Front (or Front) and sub-Antarctic Surface Water (SASW), but not between Neritic Water (NW) and STW. Regarding seasonality, the protist communities only established distinct communities across the Southland Front in spring. It was clear that protist assemblages in SASW maintain a consistent community structure all year-round which was also evident from the lack of change in phytoplankton biomass (chl-*a*). No distinction was found between summer and

autumn communities in STW and the Front. Coincidentally, potential bloom events were mainly restricted to STW and the Front during those seasons. In summer the blooms were dominated by *Cyanobacteria* and diatoms which shifted towards diatoms in the nanophytoplankton size range and microeukaryotes during the early autumn bloom. In general, more sampling is needed for a full picture of seasonal trends in protist community structuring across the Southland Front. Due to the low temporal resolution of the ongoing Munida expeditions, a longer time-series dataset is needed to discuss spatiotemporal patterns of the protist communities more accurately.

The protist communities across all water masses were highly dominated by picophytoplankton followed by nanophytoplankton. Due to advances made in molecular and microscopic technologies in the 21st century it is now possible to start exploring the diversity and abundance of small-sized organisms that were previously missed or deemed unimportant. Heterotrophic and mixotrophic dinoflagellates (*Dinophyceae* and *Syndiniales*) dominated the protist assemblages across all water masses. Parasitic dinoflagellates (*Syndiniales*) belonging to Dino-Group-I were identified as important and abundant members in the photic zone and highlight the need for further research to better understand their involvement in trophic interactions. In contrast to most observations of diatoms in open ocean ecosystems, small-celled species belonging to the *Minidiscus* genus dominated the diatom communities in NW, STW and the Front. In SASW small green algae affiliated with *Chloropicophyceae* were the most abundant phototrophs.

The data confirmed that the Front acted as a diversity hotspot for protist communities, but this trend was limited to summer. Further, the Front was the only water mass associated with increased species richness. To definitively confirm that no other seasons support a higher diversity in the Front, continuous monitoring is needed because not all seasons were equally sampled, with spring having been covered the least.

Finally, the impact of the heatwave event had drastic effects on protist assemblages. Alpha diversity was highly reduced and both STW and the Front shifted toward *Syndiniales* dominated communities, while *Chloropicophyceae* became highly abundant in SASW. The Front however still supported an enriched diversity compared to STW and SASW. MHWs are predicted to increase in the future as a result of anthropogenic impacts and could greatly affect food webs through changes in biogeochemical cycles and species interactions. By studying the ecological interactions of species such as *Syndiniales* and *Chloropicophyceae*, who have only received little attention, a better understanding could be gained of what the consequences will be for marine food webs resulting from future disruptions.

Continuous monitoring of protist assemblages along the Munida transect will allow to observe long-term changes in protist communities in response to direct and indirect anthropogenic impacts such as increasing surface temperature, ocean acidification and marine heatwaves.

Chapter 3|

Prokaryotic and eukaryotic biofilm development and succession dynamics on settlement plates in a kelp forest

Hanneloor Heynderickx, Linn Hoffmann, Tina Summerfield

3.1 Introduction

Kelp forests are extremely productive temperate coastal ecosystems that create highly complex habitats for a variety of marine organism (Steneck et al., 2002; Teagle et al., 2017). These environments are characterised by highly variable pH conditions where daily seawater pH can increase up to 1 unit due to photosynthesis (Hofmann et al., 2011; Cornwall et al., 2013) and decrease again at night as a result of respiration (Hurd et al., 2011). These processes together with the high carbon fixation rate of kelp create a unique and selective ecosystem that governs the physiology and ecology of many organisms living in these near-shore habitats (Kavanaugh et al., 2009; Duarte, 2017; Pfister et al., 2019). Kelp forest ecosystems have been well studied in terms of hydrodynamics (Rosman et al., 2007; Huang et al., 2011; Stephens & Hepburn, 2014), population patterns (Schiel & Foster, 2006), productivity (Duggins et al., 1989), seawater chemistry (Hepburn et al., 2011; Pfister et al., 2019), community structures (Graham, 2004; Vásquez et al., 2006), and trophic and spatial dynamics (Ortiz, 2008, 2010). In the past decade there has also been an increase in research on kelp associated microorganisms (Bengtsson & Øvreås, 2010; Michelou et al., 2013; Lemay, Martone, Hind, et al., 2018; Weigel & Pfister, 2019). Yet little work has been done on characterising benthic biofilm development and successional processes within kelp forests.

Biofilm communities often benefit ecosystems primarily through their active involvement in biogeochemical cycles (Paerl & Pinckney, 1996), thereby providing organic materials and subsequent energy to higher trophic levels (Azam et al., 1983; Carvalho & Caramujo, 2012). Microbes further contribute to a healthy functioning ecosystem by mediating early development life stages of marine eukaryotes and invertebrates (Hadfield & Paul, 2001; Lau et al., 2005; Huggett et al., 2009), making them important members of many ecosystems. The biofilm matrix provides a suitable settlement surface and engages in spore and larval recruitment through chemical signalling (Hadfield & Paul, 2001; Hadfield, 2011). Further, in a study by Uribe et al. (2014), the organisation of benthic biofilm communities in kelp beds was compared to barren grounds. The authors showed that these microscopic assemblages were unique to each ecological state thereby highlighting the specificity of the biofilm communities to their immediate environment as well as the potential influence of the ecosystem onto the biofilm structures. Hence, it is important to identify the microbial biofilm composition, diversity and functioning in order to fully comprehend their involvement in biotic interactions with marine recruits and macroalgae, for each unique ecosystem. In light of the rapid changes the marine world is facing due to direct and indirect anthropogenic perturbations (Krumhansl et al., 2016; Smale, 2020) it is important to assess the

health of these highly productive ecosystems. For this, more research is needed to explore and understand kelp forest microbial ecology.

Marine biofilm development is controlled by several successional stages defined by shifts in major taxonomic groups, which have been well documented in several reviews (Dang & Lovell, 2000, 2016; Flemming et al., 2016). With the advances in sequencing technology, it is now also possible to study these successional changes at fine taxonomic level where the communities will more strongly reflect their local environment. In addition to determine if two communities are distinct in composition, it is also possible to look at the main ecological mechanisms responsible for the (dis)similarity, that is species replacement (Turnover) or species loss (Nestedness). These mechanisms are attained by the main ecological assembly processes, i.e. environmental selection, dispersal, ecological drift and speciation (Vellend, 2016).

To explore the extent to which benthic biofilm communities interact with their immediate environment and contribute to the complex dynamics of a kelp ecosystem, it is critical to understand the ecological mechanism and taxonomic composition of these assemblages. In this study, benthic microbial communities collected on settlement plates were examined to decipher the ecological mechanisms that are guiding microbial community composition across the kelp forest. For this, an in-depth analysis of their taxonomic composition and diversity was also described. Additionally, the variability of benthic biofilm communities was assessed across a small spatial scale within the kelp forest to gain a better understanding of how abiotic parameters, associated with this kelp ecosystem, influence these benthic ecological systems. Biofilm colonisation and development was studied at one-week intervals for an immersion period of 32 days. Next-generation sequencing was employed to characterise both prokaryotic and eukaryotic communities by targeting the 16S and 18S rRNA genes, respectively.

Overall, this study provides baseline data for the *Macrocystis pyrifera* dominated kelp forest in South Island, New Zealand which will aid in the further investigation of the overall kelp microbiome ecology (further explored in Chapter 4).

3.2 Materials & Methods

3.2.1 Site description

All field research was conducted in a temperate kelp forest located in Te Awa Mokihi/ Butterfly Bay (45°38'20.58''S, 170°40'19.38''E), Karitāne/Huriawa Peninsula, on the southeast coast of the

South Island, New Zealand (Figure 3.1). The bay is located 800 m south of where the Waikouaiti River debouches into the Pacific Ocean and approximately 50 km north of Dunedin, Otago. Due to its close proximity, the river effluent from the Waikouaiti River and the Water of Leith, which emerges into the Otago Harbour, strongly influence the study site. The macroalgal community in this wave-exposed coastal site is mainly characterised by the canopy forming giant kelp *Macrocystis pyrifera* and calcifying coralline species (Hepburn et al., 2011). A combination of bedrock and boulders interspaced with sand extends from the shallow (2 m) to the deep ($\pm 10 - 12$ m) subtidal and forms the substrate of this kelp forest. A detailed description of the macroalgal community and the light environment in this kelp forest was covered by Hepburn et al. (2011).

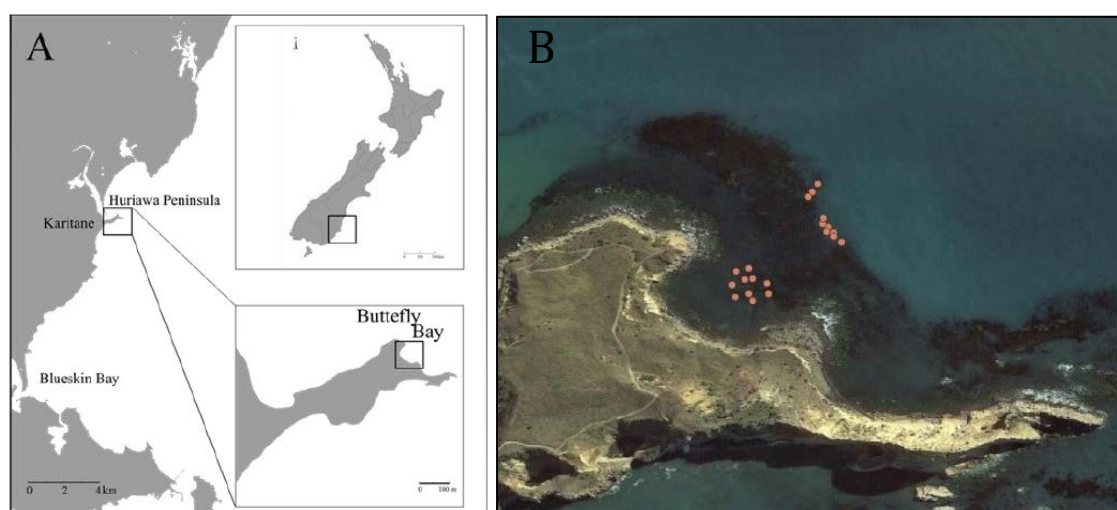


Figure 3.1: Map of the study site Te AWA Mokihi/Butterfly Bay at Huriawa Peninsula. (A) Image adapted from Kluibenschedl (2021). (B) Aerial image from Google Earth (earth.google.com). Dots show the location of the kelp individuals for the two sample locations ($n = 10$ per location).

3.2.2 Experimental set up and sampling of settlement slides

Two locations in the kelp forest were selected for sampling, namely the interior and exterior kelp bed. The interior bed was located inside the coastal bay while the exterior bed was located outside the bay, at the edge of the kelp forest (Figure 3.1). At both sites, settlement plates were attached to ten *M. pyrifera* kelp individuals that were randomly selected as independent replicates for this study. Acrylic plates cut up to the size of microscope slides (75 mm x 25 mm), served as settlement substrates for biofilm communities (Figure 3.2). Two holes were drilled one at each end of the slides (5 mm diameter) for attachment to the kelp stipe at the base of the air bladder structures (pneumatocyst). The slides were acid-washed and stored in sterile Ziplock bags prior to deployment. Attachment to the kelp stipe was done by snorkelling and with the use of flagging tape to minimise the environmental impact, and for ease of retrieval (Figure 3.2a,b). The ends of

the slides, as well as the flagging tape used for attachment, were both labelled. During low tide, five slides were affixed to each kelp host at a depth of approximately 1 m below surface. Extra flagging tape was added to each kelp individual to facilitate locating the slides throughout the experiment.

Sampling started in early February and ended early March 2021. At each time point, the following samples were collected through snorkelling: one settlement plate (biofilm slide) per kelp individual ($n=10$ per site), water samples for pelagic microbial community metagenomics ($n=4$ per site), water samples for inorganic nutrient analysis ($n=3$ per site), and physio-chemical water parameters (pH, temperature, salinity, and chlorophyll-*a*).

The settlement plates were planned to be recovered at seven-day intervals for a total of five time points. However, the time intervals varied depending on the weather and sea conditions. The first three sampling occasions took place at six-day intervals, followed by an eight-day interval and another six-day interval for the last two sampling days. Due to bad weather and sea conditions, visibility was not always favourable and limited the sampling efforts. An overview of the number of samples collected at each time point is shown in Table 3.1.

Table 3.1: Overview of the samples collected at each time point

Time points	Date	Interior Kelp Bed		Exterior Kelp Bed	
		Settlement plates	Seawater	Settlement plates	Seawater
Day 6	14.02.2021	9	4	9	4
Day 12	20.02.2021	10	4	9	4
Day 18	26.02.2021	8	4	7	4
Day 26	06.03.2021	6	4	8	4
Day 32	12.03.2021	5	4	7	4

The settlement plates were removed from the kelp by cutting the flagging tape with scissors and transferring the slides at the surface into 50 mL sterile conical tubes (Greiner Bio-One™, Germany), carefully avoiding any water from getting into the collection tubes. The tubes perfectly enclosed the slides, thereby restricting contact between the biofilm layer and the inside of the tubes. Next, the samples were placed in a Ziplock bag at the surface, to avoid any water contamination, and transported back to shore in a catch bag. The samples were kept in a cooler on shore until processing (within 1 hour of collection). The organic material on the artificial substrates was scraped off from half of each side of the slide with a sterile razor blade and transferred to a 1.5 mL microcentrifuge tube (Figure 3.2c). After collection, the microcentrifuge tubes were put on dry ice in the field and during transport. Back in the lab the samples were frozen at -80°C until DNA extraction.



Figure 3.2: Settlement plate design for biofilm communities. (a) before deployment, (b) attached to the kelp individual, and (c) after retrieval.

3.2.3 Seawater microbial communities

Seawater samples ($n = 4$, 2 L) from the top surface layer were collected in acid-washed HDPE Nalgene bottles by snorkelling. The bottle caps were removed underwater at the site of sampling and the bottles were rinsed three times before sampling. The samples were kept cool and dark on shore and during transport. Upon arrival in the lab, the samples were immediately filtered using 0.2 μm polyethersulfone membranes (47 mm diameter, Sterlitech), an in-house built closed filtration system, and a pressure pump (Vacuubrand, John Morris Science) at low vacuum (0.2 bar). The filters were collected in a 1.5 mL microcentrifuge tube and stored at -80°C until DNA extraction.

3.2.4 Environmental variables

3.2.4.1 *In situ* pH and temperature

Sea surface *in situ* temperature and pH were measured using a Sea-Bird Scientific SeaFET™ Ocean pH Sensor. By means of snorkeling the SeaFET sensor was dragged three times back and forth parallel to the coastline at both sampling sites. The temperature sensor was calibrated with a one-point measurement.

3.2.4.2 Description of A_T and DIC analytical methods

To calibrate the SeaFET pH sensor, a water sample (1 L) for the analysis of total alkalinity (A_T) and total dissolved inorganic carbon (DIC) was taken at the start of each SeaFET deployment. On shore, within 10 min of collection, the samples were poisoned with 2 drops of saturated HgCl_2 solution (0.05 %) to prevent biological activity from altering the carbon chemistry of the bottled water. The samples were further analysed by NIWA / University of Otago Research Centre for Oceanography following the best practice guide for CO_2 measurements (Dickson et al., 2007).

Total alkalinity was determined using a closed cell potentiometric titration, according to the SOP 3a of Dickson et al. (2007). The accuracy of the method was established to be less than $\pm 2 \mu\text{mol kg}^{-1}$ based on Certified Reference Material provided by Andrew Dickson (Scripps Institution of Oceanography). Analysis of DIC was performed following SOP 2 (Dickson et al., 2007), using a UIC Model 5011 coulometer system with a SOMMA style CO_2 extraction system, whereby seawater was reacted with acetic acid while CO_2 was subsequently stripped from the sample. The accuracy of the data was estimated to be less than $\pm 1 \mu\text{mol kg}^{-1}$ based on Certified Reference Material provided by Andrew Dickson (Scripps Institution of Oceanography).

Total alkalinity, DIC, *in situ* temperature and salinity were collectively used to calculate total scale pH and the remaining carbonate parameters using CO2Sys (Pierrot et al., 2006) with the carbonic acid dissociation constants of Mehrbach et al. (1973) refitted by Dickson & Millero (1987).

3.2.4.3 Biofilm chlorophyll-*a* analysis

For chlorophyll-*a* analysis, organic matter covering half of the biofilm settlement plate on each side was scraped off using a sterile razor blade and collected in a 1.5 mL microcentrifuge tube. The samples were stored on dry ice in the field and transferred to -20°C in the lab for a duration of two months. Chlorophyll-*a* was extracted in 1 mL 96 % ethanol. Following an incubation period of 24 hours at -20°C , the chlorophyll extracts were centrifuged for five minutes at 8,000 rpm to remove cell debris. Using the spectrophotometric method described by Jeffrey & Humphrey (1975), chlorophyll-*a* concentrations were determined by measuring the absorbance values of the chlorophyll extracts at 750, 664, 647 and 630 nm with a SV1200 VIS Spectrophotometer.

3.2.4.4 Inorganic macronutrient analysis

For inorganic nutrient analysis (nitrate, ammonium, phosphate, and silicate) triplicate seawater samples from both kelp bed locations were collected in acid-washed Nalgene Bottles (250 mL). The bottles were uncapped underwater within ± 1 m of the water surface and rinsed three times before sampling. On shore, 200 mL of each sample was immediately filtered using 0.8-micron Cellulose Acetate Membrane Filters (Sterlitech, USA), sterile disposable syringes (Interlab, New Zealand) and acid washed polycarbonate filter holders (Millipore, USA). For each sample a new filter and holder set was used to avoid contamination. The filtered samples were kept cool and dark in the field and transferred to -20°C in the lab until further processing. Nutrient analysis was performed using an XYZ Autosampler with a QuickChem 8500 Series 2 Automated Ion Analyser (Lachat instruments, USA).

3.2.5 Molecular methods

3.2.5.1 DNA extraction

DNA extractions were performed on the pellets scraped from the settlement plates and the filters concentrated with seawater microbial communities. The filter membranes were thawed and cut up in small fragments of approximate 2 mm by 1 cm in petri dishes (clean environment manufactured, Techno Plas, Australia) using sterile razor blades. Subsequently, the fragmented filters were transferred to sterile 15 mL conical tubes (Greiner Bio-One™, Germany). DNA extraction was performed following a modified version of the protocol used by Needham & Fuhrman (2016). Briefly, the cells were lysed with 0.1 mm zirconia/silicabeads (dnature, BioSpec, New Zealand) and hot lysis buffer (100 mM Tris - pH 8, 40 mM EDTA - pH 8, 100 mM NaCl, 1% SDS) and by repeatedly (3x) vortexing the lysis solution for 30 s and incubating for 15 min at 70°C. A final concentration of 0.7 M NaCl and 1% Cetyl Trimethylammonium Bromide (CTAB) was added to the samples and a second incubation step of 10 min at 70 °C was performed. Sample lysates were transferred to new 2 mL tubes and were further extracted with a phenol/chloroform extraction protocol. A 1:1 volume of phenol:chloroform was added to the lysates. The samples were thoroughly shaken by hand for 30 s and centrifuged (16,000 x g) at room temperature for 5 min. The upper aqueous phase was transferred to a clean 2 mL micro-centrifuge tube and 1 x volume of chloroform was added. The samples were vortexed for 30 s and centrifuged (16,000 x g) at room temperature for 5 min. DNA was precipitated by adding 1/10 3 M sodium acetate and 2.2 x volume of ice cold 95% ethanol. The solution was vortexed and incubated overnight at -20°C. After centrifugation (20 min at 10,000 x g) at 4°C, DNA precipitate was collected and washed in 250 µl of 70% ethanol followed by a spin (16,000 x g) of 2 min at 4°C to pellet the DNA and subsequently discard the ethanol (the wash step was repeated 2 to 3 times). DNA was resuspended in 30 µl of Tris-EDTA (TE; pH 8) and stored at - 80°C.

3.2.5.2 16S rRNA Library preparation

For the amplicon sequencing analysis of prokaryotes, the V4 region of the 16S ribosomal RNA genes was amplified using the KAPA HiFi HotStart DNA kit (Kapa Biosystems, Boston, MA, USA) and the universal Earth Microbiome Project primer sets (Caporaso et al., 2011, 2012) modified by Apprill et al. (2015) and Parada et al. (2016) to reduce bias against Crenarchaeota, Thaumarchaeota, and the SAR11 clade: 515F (5' – GTGYCAGCMGCCGCGTTAA – 3') and 806R (5' – GGACTACNVGGGTWTCTAAT – 3'). Both primers were further modified with additional overhang sequences at the 5' – ends (forward, 5-ACGACGCTCTTCCGATCT-3'; reverse, 5-CCGTGTGCTCTTCCGATC-3'; Griffith et al., 2017). These overhangs were incorporated into the first round (R1) amplicon product and allowed for a second round (R2) PCR to take place to add

Illumina adapter sequences and indexes to the target amplicon. This step is necessary in order to multiplex the samples on one Illumina MiSeq flowcell. PCR mixtures for the R1-PCR contained 5 x KAPA HiFi Buffer, 0.3 mM KAPA dNTP, 0.3 μ M Forward primer and 0.3 μ M Reverse primer, 0.5 U KAPA HiFi HotStart DNA Polymerase, 5 mg mL⁻¹ Bovin Serum Albumin (BSA) and approximately 10 ng template DNA. Thermocycling conditions were: an initial denaturation at 95°C for 2 min, 25 cycles of 98°C for 20 s, 50°C for 30 s, 72°C for 30 s, and a final extension period at 72°C for 1 min. The amplicon products were visualised on a 1.5 % agarose gel and had an expected band size of ~300 – 350 bp. Amplicon products were cleaned up with 1.8 x (vol:vol) magnetic AMPure XP beads (Beckman Coulter) following the Agencourt AMPure XP PCR Purification protocol. During the final step of clean-up, the samples were eluted in 30 μ L Tris-EDTA (pH 8). Prior to R2-PCR, the quantity of purified DNA in each sample was measured on a Qubit fluorometer using the Qubit™ dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently diluted to 1 ng μ L⁻¹. For the R2-PCR, dual index (i5 and i7) TruSeq primers (Illumina, San Diego, CA, USA) were used and tailed with the same oligonucleotide sequences as the R1-primers. Each reaction (25 μ L) contained 5 x KAPA HiFi Buffer, 0.3 mM KAPA dNTP, 0.3 μ M Forward and Reverse primer, 0.5 U KAPA HiFi HotStart DNA Polymerase and 1 ng template DNA. PCR conditions were 95 °C for 2 min; 10 cycles of 98°C for 20 s, 60°C for 20 s, and 75°C for 20 s; and a final extension at 72°C for 1 min. Amplification products were visualised on a 1.5 % agarose gel. PCR clean-up and quantification were performed as described for the R1-PCR. All samples were pooled equimolarly (10 nM) into one library and run on the Agilent Bioanalyzer 2100 (Agilent Technologies, USA) by the Otago Genomics Facility (Otago University, New Zealand) to confirm the expected quality and quantity of the PCR amplicons. Finally, the library pool was loaded on a MiSeq platform (Illumina, San Diego, CA, USA) using the V2 (2 x 250 bp) reagent kit for Illumina paired-end sequencing by the Otago Genomics Facility (Otago University, New Zealand).

3.2.5.3 18S rRNA Library preparation

Determining eukaryotic diversity in our samples was done by targeting the V4 region of the 18S rRNA eukaryotic gene. The forward primer TAREuk454FWD1 (5'–CCAGCASCYGC GGTAATTCC–3') and reverse primer TAREukREV3 (5'–ACTTTCGTTCTTGATYRA–3') designed by Stoeck et al. (2010) were used and further modified with overhanging adapters (forward, 5–ACGACGCTCTTCCGATCT–3'; reverse, 5–CGTGTGCTCTTCCGATC–3'; Griffith et al. (2017)). In the R1-PCR the V4 target amplicon was selected for and subsequently amplified using the Kapa HiFi Hotstart PCR Kit. Each PCR reaction consisted out of: x1 Kapa Hifi buffer, 0.3 mM Kapa dNTP mix, 0.3 μ M forward and reverse primers, 0.5 U Kapa Hifi Hotstart DNA polymerase, 5 mg mL⁻¹ Bovin Serum Albumin (BSA), and approximately 10 ng of template DNA. Due to the difference in length

between the forward and the reverse primer, a two-step PCR program was set up (Stoeck et al., 2010): an initial denaturation step at 95°C for 30 s; step 1 – 10 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s; step 2 – 15 cycles of 95°C for 30 s, 48°C for 30 s, and 75°C for 30 s; and a final extension time at 72 °C for 5 min. Amplification products were visualised on a 1.5 % agarose gel and had an expected band size of ~400 – 450 bp. Next, the DNA products were purified in a PCR clean-up step using magnetic AMPure XP beads (Beckman Coulter) following the Agencourt AMPure XP PCR Purification protocol. Purified products were recovered in 30 µL elution buffer (Tris-EDTA, pH 8), quantified with the Qubit™ dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit fluorometer and concentrated to 1 ng µL⁻¹. The PCR mixtures and cycling conditions, clean-up and quantification steps for the R2PCR were the same as described earlier in the 16S rRNA library preparation. All samples were pooled in equimolar (10 nM) concentrations and sent off to the Otago Genomics Facility (Otago University, New Zealand). The library pool was quantified and qualified (~550 bp) on the Agilent Bioanalyzer 2100 (Agilent Technologies, USA) prior to being loaded on a MiSeq platform (Illumina, San Diego, CA, USA) using the V3 (2 x 300 bp) reagent kit for Illumina paired-end sequencing.

3.2.6 Sequence post-processing and statistical analyses

The data obtained from the Illumina Miseq platform were paired-end sequences in fastq file format, demultiplexed by sample, and with the adapters and indexes removed. These raw sequences were processed following to the DADA2-pipeline (Callahan, Sankaran, et al., 2016) in R (version 3.4.4; R Core Team, 2021). First, a quality score of 30 was applied to both forward and reversed reads, trimming the forward reads at 240 and 250 bp and the reverse reads at 240 and 200 bp for prokaryotic (16S) and eukaryotic (18S) rRNA sequences, respectively. The chosen cut-off lengths allowed for sufficient overlap to merge the reads during downstream computation. Amplicon sequence variants (ASVs) were inferred using the DADA2 algorithm by Callahan & McMurdie (2016). Divisive Amplicon Denoising Algorithm (DADA) is an error model-based approach that accurately renders high resolution ASVs that can differ by only one single nucleotide. Next, paired reads were merged and a sequence table was constructed containing 18,130 ASVs (16S) and 4,465 (18S) ASVs. All sequences approximately fell within the expected size range of our V4 target amplicon (16S: 253 bp and 18S: 379 bp). At the end of the workflow, *removeChimeraDenovo* was used to identify and subsequently remove chimeric ASVs. In both dataset chimeras only accounted for 2% of the merged reads. For the final step, taxonomy was assigned to the sequence variants down to species level via the DADA2 functions *assignTaxonomy* and *assignSpecies*. For the prokaryotic sequences, the SILVA taxa database (version 138.1 with species assignment; <https://benjjneb.github.io/dada2/training.html>) was used, while for the

eukaryotic sequences the Protist Ribosomal Database (PR2 version 4.14; <https://github.com/pr2database/pr2database/releases>; Guillou et al., 2013) was utilised. Both taxonomy classifications were done using the naive Bayesian classifier method. Only prokaryotic sequences affiliated with Bacteria and Archaea were retained for downstream analysis. This means sequences assigned to Eukaryota, Chloroplast and Mitochondria for the prokaryotic dataset were removed. For the Eukaryotic data, ASVs identified as ‘Metazoa’ were excluded, as they were not a focus of this study. Statistical analyses and visualisations were done using the “phyloseq” package (McMurdie & Holmes, 2013) and *ggplot2* (Wickham, 2016), which is available in the “tidyverse” package (Wickham et al., 2019).

Alpha diversity measures (Chao1 and Pielou index) were calculated with *estimate_richness* (“phyloseq”). Diversity differences were tested between the two kelp bed locations and across time (five discrete time points). All the data was checked for normality using the Shapiro test, while homogeneity of variance between the samples was tested with the Levene test. Since the data did not meet the parametric assumptions, the non-parametric Kruskal-Wallis test was used followed by a pairwise Wilcoxon Rank Sum test to test variability of alpha diversity between the two kelp locations and across time.

Assessment of the community composition variability (beta diversity) was performed for the location and time factor. The beta diversity analysis were performed using Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson, 2017) with the *adonis* function (“vegan”) and were visualised with an unconstrained non-metric multidimensional distance scaling (nMDS) ordination. Pairwise PERMANOVAs were done with *pairwise.adonis* (<https://github.com/pmartinezarbizu/pairwiseAdonis>). Both the Jaccard (species incidence) and the Bray-Curtis dissimilarity index (abundance data) were used to generate a distance matrix for the nMDS method and the PERMANOVA analysis, in case of the biofilm samples on the settlement plates. The seawater communities only relied on Bray-Curtis dissimilarities. Homogeneity of dispersions amongst all groups, for both sample types, was tested for each comparison (location and time) with PERMDISP (Anderson et al., 2006). This additional multivariate analysis allows to further investigate the results of the PERMANOVA analysis. For the biofilm samples, the Jaccard dissimilarity index was further partitioned into spatial turnover (species replacement) and nestedness (species loss) with the *beta.pair* function from the “betapart” package (Baselga & Orme, 2012). This partitioning is a first step in investigating the ecological processes that are responsible for biofilm compositional variability. All beta diversity test statistics were executed with 10,000 permutations and a seed was set at 711 for reproducibility. A MANOVA analysis was performed to test the significance of multiple dependent variables (phylogenetic groups) across the kelp bed at high taxonomical rank (phylum and class). Significant MANOVA results were

further analysed for each taxonomical group individually with univariate ANOVA tests. To identify which microorganisms, at ASV level, were specifically associated with a certain group of samples (location and time) an indicator species analysis (“DESeq2”) was used (Love et al., 2014).

Community composition and the correlation with abiotic factors was explored using distance-based Redundancy Analysis (dbRDA; Legendre & Anderson, 1999). The constrained dbRDA analysis was performed with a Bray-Curtis dissimilarity matrix. Nutrients (ammonia, nitrate, phosphate and silicate), chlorophyll-*a*, temperature and pH were used as explanatory variables and were standardized using *decostand* (“vegan”). These variables were tested for co-linearity prior to analysis by calculating their variance inflation factors (*vif.cca* function). Variables with VIF-values higher than 10 were removed from the model. The explanatory variables were further defined through forward selection using *ordiR2step* to include only those parameters that best explain the variability amongst the biofilm communities. The effect of the environmental variables on biofilm community composition and their significance was tested with ANOVA permutations (*anova.cca*).

3.3 Results

After rarefying each of the datasets to an even sequencing depth, the prokaryotic (16S) sequences were represented by 8,010 reads per sample ($n = 75$) and the eukaryotic (18S) sequences by 5,472 reads per sample ($n = 77$). The seawater microorganism communities had a higher number of reads per sample for prokaryotic and mainly eukaryotic communities (both $n = 40$; 16S: 8,597 and 18S: 10,056).

3.3.1 Environmental variables

Nitrate ($<0.5 \mu\text{M}$) and phosphate ($<0.1 \mu\text{M}$) concentrations were low throughout the study, while ammonia ($0.3 - 1.1 \mu\text{M}$) and silicate ($4 - 7.5 \mu\text{M}$) showed high levels (Table 3.2). The low concentrations of nitrate and phosphate were in line with results from earlier studies on the South East coast of southern New Zealand and are characteristic for coastal waters at the end of Summer (Hepburn et al., 2006; Stephens & Hepburn, 2014; Desmond et al., 2015). Ammonia, nitrate and silicate exhibited their lowest values around Day 12 and their highest values on Day 26. Consequently, strong correlations were observed for silicate and ammonia (Spearman; $p < 0.01$, $\rho = 0.81$), and silicate and nitrate (Spearman; $p < 0.01$, $\rho = 0.58$) over time. Ammonia and phosphate were significantly higher at the interior kelp bed (t-test; $df = 28$, $t = 2.7$ & 2.9 , both p -

values < 0.05), while nitrate and silicate concentrations stayed consistent across the kelp bed for each time point. Further, biofilm chlorophyll-*a* concentrations were low ($< 0.1 \mu\text{g m}^{-2}$), similar between the two kelp bed locations, and showed an increasing trend over time (Table 3.2).

Mean seawater temperature ranged between 15.09°C and 17°C with peak values on Day 12 (Table 3.2). This was consistent with the weather patterns observed during the study period (in field personal observations), where no rain fall was recorded in the weeks surrounding the first two sampling days (Day 6 and Day 12). The third day of sampling (Day 18) was preceded by heavy rain and resulted in reduced visibility (1 – 2 m). Medium rainfall and cloud cover were persistent throughout the remainder of the study with the visibility limited to one meter or less on the last two sampling days. The pH levels were on average slightly higher at the interior bed and were highest for both locations on the first sampling day (Table 3.2).

Table 3.2: Overview of environmental parameters. The days are labelled according to the immersion time of the settlement plates. IN = Interior kelp bed and OUT = Exterior kelp bed. Chlorophyll-*a* concentrations are from the biofilm communities. Mean values with standard deviations are shown. Note that the standard deviations refer to the variations amongst the replicates and that they do not represent the range of daily temporal variation.

Day	Site	Ammonia (μM)	Silicate (μM)	Nitrate (μM)	Phosphate (μM)	Chlorophyll- <i>a</i> ($\mu\text{g m}^{-2}$)	T ($^{\circ}\text{C}$)	pH
6	IN	0.77 ± 0.13	4.99 ± 0.40	0.25 ± 0.03	0.09 ± 0.01	0.001 ± 0.00	16.96 ± 0.11	8.17 ± 0.02
6	OUT	0.64 ± 0.02	4.02 ± 0.05	0.28 ± 0.01	0.05 ± 0.00	0.001 ± 0.00	16.50 ± 0.14	8.05 ± 0.02
12	IN	0.74 ± 0.05	4.20 ± 0.02	0.14 ± 0.00	0.05 ± 0.00	0.004 ± 0.00	17.02 ± 0.07	8.11 ± 0.02
12	OUT	0.39 ± 0.02	4.05 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.004 ± 0.00	17.00 ± 0.03	8.07 ± 0.04
18	IN	0.87 ± 0.07	5.33 ± 0.09	0.11 ± 0.00	0.05 ± 0.00	0.015 ± 0.01	16.25 ± 0.03	8.05 ± 0.04
18	OUT	0.79 ± 0.06	5.12 ± 0.14	0.09 ± 0.03	0.01 ± 0.01	0.003 ± 0.00	16.41 ± 0.03	8.05 ± 0.00
26	IN	1.10 ± 0.04	7.54 ± 0.31	0.38 ± 0.00	0.05 ± 0.00	0.088 ± 0.04	15.82 ± 0.08	8.08 ± 0.02
26	OUT	0.88 ± 0.14	7.26 ± 0.41	0.41 ± 0.02	0.05 ± 0.01	0.020 ± 0.01	16.53 ± 0.07	8.05 ± 0.03
32	IN	0.84 ± 0.03	5.47 ± 0.01	0.30 ± 0.00	0.02 ± 0.00	0.062 ± 0.04	15.01 ± 0.06	8.07 ± 0.02
32	OUT	0.72 ± 0.01	4.78 ± 0.18	0.24 ± 0.01	0.00 ± 0.00	0.037 ± 0.02	15.09 ± 0.02	8.05 ± 0.02

3.3.2 Microbial biofilms on settlement plates

3.3.2.1 Biofilm community abundances

For the prokaryotic biofilm communities, Chao1 richness did not show any significant differences between the two locations (interior and exterior kelp bed) or across the different time points (Figure 3.3a). In contrast, Pielou's evenness was significantly higher at the interior location and was overall significant for the time factor (KW; Site: $df = 1$, $H = 0.52$, $p = 0.025$; Day: $df = 4$, $H = 13.23$, $p = 0.01$; Figure 3.3b). Pairwise analysis revealed that species evenness at the interior bed was significantly higher for Day 18 (vs. Day 6) and Day 26 (vs. Day 6 and Day 12). In comparison, diversity measurements for the eukaryotic communities displayed equal variability amongst the two locations and over time (Figure 3.3b,c; KW, all p -values > 0.05). Overall, species richness and evenness was substantially higher for prokaryotic communities.

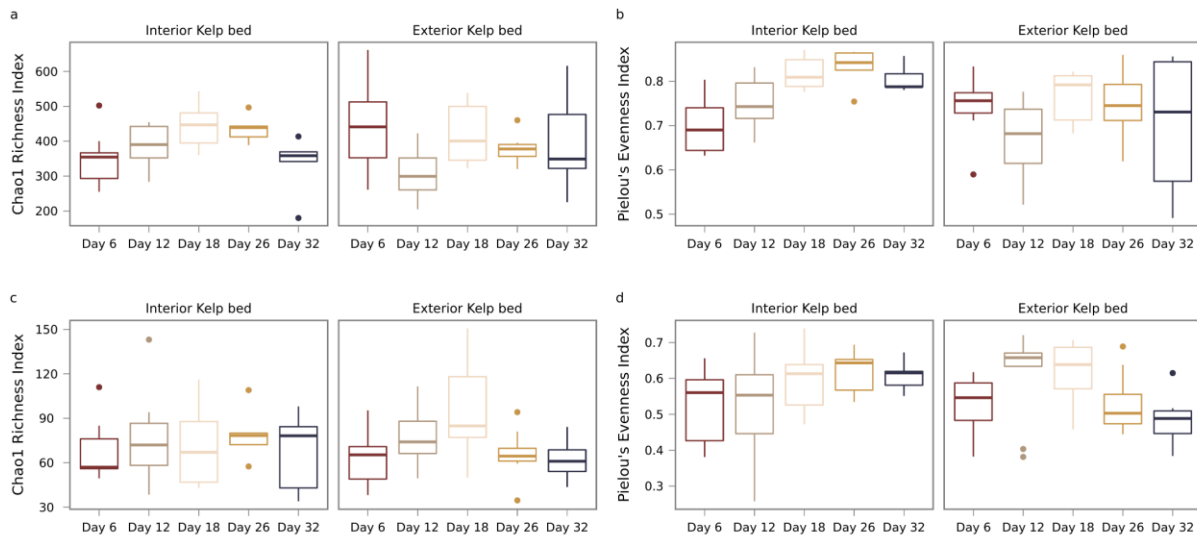


Figure 3.3: Alpha diversity measures for biofilm communities on the settlement plates across the kelp bed. (a – b) Prokaryotic communities and (c – d) eukaryotic communities. Colours represent the day of sampling and equals the age of the biofilms.

3.3.2.2 Spatio-temporal biofilm community composition dynamics

Community composition for prokaryotic biofilms was significantly different for location, time and the interaction factor, based on species incidence- (Jaccard) and relative abundance- (Bray-Curtis) dissimilarities (Table 3.3). For the eukaryotic biofilms, community composition was significantly different for location and time based on Bray-Curtis dissimilarities, and for location, time and the interaction factor based on Jaccard dissimilarities (Table 3.3). The Jaccard index was additionally partitioned into species replacement (Turnover) and species loss (Nestedness) to further investigate the processes behind the community structuring.

3.3.2.3 Small spatial structuring across the kelp bed

The prokaryotic biofilms at the interior and exterior kelp bed were significantly different from one another during the first 26 days of immersion, supported by both Jaccard and Bray-Curtis dissimilarities (Figure 3.4a & Table 3.3). The difference between the locations for Day 6, Day 12 and Day 18 was primarily explained by species Turnover with a contribution of Nestedness on the second day of sampling (Day 12). The difference across the kelp bed on Day 26 was entirely explained by Nestedness. PERMDISP analysis was not significant for any of the site comparisons based on either Jaccard or Bray-Curtis dissimilarities.

PERMANOVA for eukaryotic biofilms based on Jaccard distances revealed a significant difference in community composition between the locations at each time point, while Bray-Curtis dissimilarity only showed a significant difference between the locations for Day 6, Day 12 and Day 18 (Figure 3.4b,c & Table 3.3). Further, species Turnover was responsible for the community variability between the interior and exterior bed at each time point (Figure 3.4c & Table 3.3). The

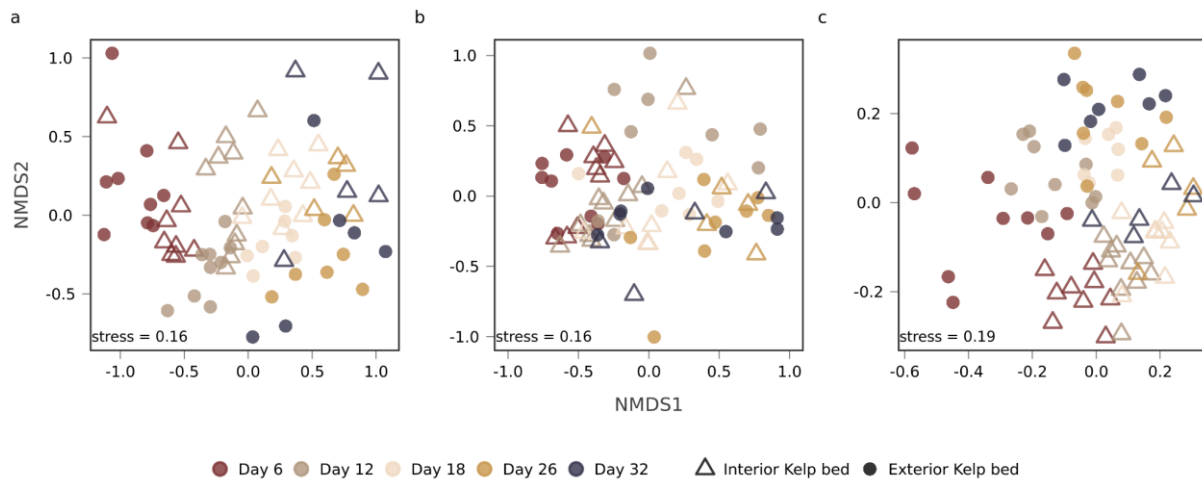


Figure 3.4: Community composition variability for biofilms on the settlement plates across the kelp forest. The ordinations were made using non-metric multidimensional scaling (nMDS). (a) prokaryotic community variability based on Bray-Curtis dissimilarity (abundance data), (b) eukaryotic community variability based on Bray-Curtis dissimilarity, and (c) eukaryotic community variability explained by the Turnover partition (species replacement) of Jaccard dissimilarity (presence/absence data). Colours represent the day of sampling and equals the age of the biofilms. Symbols represent the two sampled locations across the kelp forest, interior bed (triangles) and exterior bed (circles).

pairwise PERMDISP analysis based on Jaccard distances for eukaryotes revealed no heterogeneity of dispersions between the two locations, while PERMDISP with Bray-Curtis dissimilarities detected heterogeneity of dispersions between the locations on Day 12 (*betadisper*; $df = 2$, $F = 9$, $p = 0.008$).

3.3.2.4 Community structuring over time

Comparison of prokaryotic community compositions over time at the interior bed revealed significant differences between the first three consecutive time points (Day 6 – Day 12 and Day 12 – Day 18) regarding both species incidence as well as relative abundance (Figure 3.4a; Table 3.3). At the exterior location, prokaryotic composition varied significantly between the first four sampling days (Day 6 – Day 12, Day 12 – Day 18 and Day 18 – Day 26) based on both dissimilarity indices (Figure 3.4; Table 3.3).

The differences in composition over time at the interior bed were the result of species Turnover, while at the exterior bed a combination of both Turnover and Nestedness processes were driving the community differences (Table 3.3). The PERMDISP test did not reveal any heterogeneity in dispersions amongst the significantly different time points with regards to both Bray-Curtis and Jaccard indices. For eukaryotic communities, PERMANOVA comparisons based on both the Jaccard and Bray-Curtis distances revealed temporal variability between the first three sampling days (Day 6 - Day 12 and Day 12 – Day 18; Table 3.3), at both the interior and exterior bed. Additionally, Jaccard dissimilarities were also significantly different between Day 18 – Day 26 at the exterior kelp bed. Only the Turnover partition was responsible for the community variation

across time (Figure 3.4c). No significant PERMDISP results for eukaryotes were detected for the time factor.

Table 3.3: Beta-diversity for prokaryotic and eukaryotic communities. Both Bray-Curtis and Jaccard dissimilarities are shown for location and time. Jaccard dissimilarities are further partitioned into species replacement (Turnover) and species loss (Nestedness). Inner/IN = Interior kelp bed and Outer/OUT = Exterior Kelp bed. Significance: $p < 0.001$ (***) , $p < 0.01$ (**), $p < 0.05$ (*).

	Bray-Curtis distances			Jaccard distances			Turnover			Nestedness		
	Pseudo <i>F</i>	R ²	P	Pseudo <i>F</i>	R ²	P	Pseudo <i>F</i>	R ²	P	Pseudo <i>F</i>	R ²	P
Prokaryotes												
Location	3.67	0.04	***	2.36	0.03	***	2.71	0.03	***	-3.03	-0.05	1.00
Time	6.73	0.26	***	2.73	0.13	***	3.16	0.15	***	-2.71	-0.18	1.00
Location x Time	1.70	0.07	**	1.19	0.06	**	1.12	0.05	0.10	2.01	0.14	0.15
Inner - Outer (Day 6)	3.16	0.16	***	1.62	0.09	**	1.71	0.10	***	-0.08	0.00	0.86
Inner - Outer (Day 12)	3.28	0.16	***	1.91	0.10	**	1.75	0.09	**	5.47	0.24	*
Inner - Outer (Day 18)	1.79	0.12	**	1.39	0.10	**	1.53	0.11	**	-0.58	-0.05	0.95
Inner - Outer (Day 26)	1.53	0.13	*	1.18	0.11	*	1.05	0.10	0.27	8.83	0.47	0.02
Inner - Outer (Day 32)	1.07	0.11	0.39	1.04	0.10	0.27	1.17	0.12	0.13	-0.78	-0.10	0.98
Day 6 - Day 12 (IN)	8.23	0.33	**	2.25	0.12	***	2.34	0.12	***	1.95	0.10	0.19
Day 12 - Day 18 (IN)	7.04	0.31	**	1.31	0.08	*	1.28	0.07	*	3.32	0.17	0.10
Day 18 - Day 26 (IN)	1.29	0.11	0.27	0.96	0.08	0.62	1.01	0.08	0.43	-0.26	-0.02	0.83
Day 26 - Day 32 (IN)	1.21	0.13	0.31	0.90	0.10	0.89	0.74	0.08	0.98	5.37	0.40	0.02
Day 6 - Day 12 (OUT)	24.96	0.61	**	2.13	0.12	***	2.29	0.13	***	0.11	0.01	0.73
Day 12 - Day 18 (OUT)	10.61	0.43	**	1.56	0.10	**	1.27	0.08	*	6.87	0.33	*
Day 18 - Day 26 (OUT)	5.00	0.29	**	1.31	0.10	**	1.38	0.10	**	-0.08	-0.01	0.82
Day 26 - Day 32 (OUT)	1.13	0.09	0.34	1.00	0.08	0.41	0.99	0.08	0.48	0.43	0.04	0.57
Eukaryotes												
Location	6.39	0.07	***	4.60	0.06	***	6.42	0.08	***	-12.01	-0.21	1.00
Time	11.12	0.12	***	3.50	0.04	***	4.61	0.05	***	-4.51	-0.08	0.99
Location x Time	1.60	0.02	0.10	1.45	0.02	**	1.46	0.02	*	-0.40	-0.01	0.77
Inner - Outer (Day 6)	4.22	0.21	**	2.17	0.12	***	2.56	0.14	***	-2.42	-0.18	0.96
Inner - Outer (Day 12)	4.72	0.22	**	2.89	0.15	***	4.03	0.19	***	-4.47	-0.36	1.00
Inner - Outer (Day 18)	3.06	0.19	**	2.25	0.15	***	3.04	0.19	***	-1.07	-0.09	0.97
Inner - Outer (Day 26)	1.20	0.10	0.23	1.53	0.12	**	1.66	0.13	**	0.51	0.04	0.58
Inner - Outer (Day 32)	1.34	0.12	0.23	1.28	0.11	*	1.42	0.12	*	-1.01	-2.14	0.98
Day 6 - Day 12 (IN)	3.05	0.15	*	1.61	0.09	**	1.95	0.10	***	-1.93	-0.13	1.00
Day 12 - Day 18 (IN)	3.13	0.16	*	1.46	0.08	*	2.01	0.11	***	-0.88	-0.06	0.96
Day 18 - Day 26 (IN)	1.48	0.12	0.20	1.32	0.11	0.06	1.45	0.12	0.06	-0.39	-0.04	0.86
Day 26 - Day 32 (IN)	0.40	0.05	0.99	1.02	0.11	0.36	1.01	0.11	0.45	1.03	0.11	0.43
Day 6 - Day 12 (OUT)	5.72	0.26	**	2.26	0.12	***	2.65	0.14	***	-1.23	-0.08	0.89
Day 12 - Day 18 (OUT)	2.75	0.16	**	2.01	0.13	***	2.62	0.16	***	-2.44	-0.21	1.00
Day 18 - Day 26 (OUT)	1.74	0.12	0.12	1.40	0.10	**	1.26	0.09	0.13	4.19	0.24	0.10
Day 26 - Day 32 (OUT)	1.08	0.08	0.32	0.97	0.07	0.52	1.09	0.08	0.29	-0.31	-0.02	0.87

3.3.2.5 Correlation between beta-diversity and abiotic variables

The constrained dbRDA analysis identified five predictor variables (ammonia, phosphate, silicate, temperature and pH) that were significantly correlated with the prokaryotic biofilm community ($df = 4$, $F = 5.6$, $p < 0.001$). Collectively, they explained 24% of the total variance, while the majority of the observed variation (76%) was explained by the unconstrained axes.

For the eukaryotic biofilms, temperature was excluded due to co-linearity with pH and only five variables passed forward selection (chl-*a*, ammonia, phosphate, silicate and pH) explaining 31% of the total inertia ($df = 5$, $F = 5.78$, $p < 0.001$ based on 9,999 permutations), with each predictor variable significantly correlated to the response data. Similar to the bacterial biofilms, the largest part of the total variance was explained by the unconstrained axes. This indicates that both biofilm

communities might be structured by other strong abiotic and biotic variables that were not measured in this study.

3.3.2.6 Biofilm taxonomical succession across a small spatial scale

3.3.2.6.1 Prokaryotic communities

At high taxonomic levels (phylum and class), both locations displayed very similar compositions. The top prokaryotic phyla that accounted for approximately 92% of all bacterial and archaeal taxa, identified during the whole immersion period, were *Proteobacteria* (interior: 66.8% and exterior: 71.3%) and *Bacteroidota* (25.7% and 20.5%). The phylum *Proteobacteria* was predominantly represented by *Alphaproteobacteria* with a relative abundance of 48.8% at the interior and 56% at the exterior bed (Figure 3.5c). *Alphaproteobacteria* mainly consisted of members from the family *Rhodobacteraceae* and were consistently dominant across the kelp bed throughout the 32-day study. This was also observed at the lowest taxonomic level with the most abundant ASV classified as belonging to the genus *Yoonia* (*Rhodobacteriaceae*; interior: 10.2% and exterior: 14.7%). In comparison, *Gammaproteobacteria* accounted for a much lower proportion (interior: 17.8% and exterior: 15%) of ASVs (Figure 3.5) and comprised a more diverse group of taxa rather than being represented by a single dominant ASV. Sequences belonging to the second most dominant phylum *Bacteroidota* were primarily affiliated with two orders exhibiting relative equal abundances between the two locations, *Chitinophagales* (interior: 14.6% and exterior: 13%) and *Flavobacteriales* (10.1% and 6.8%). The remainder phyla that represented more than 1% of all taxa for at least one location were *Patescibacteria* (class *Gracilibacteria*), *Actinobacteria* (class *Acidimicrobiia*) and *Chloroflexi* (class *Anaerolineae*).

The succession of the most abundant taxa at high taxonomic level was analysed with a MANOVA test based on the PERMANOVA results for the time factor. As mentioned before, *Alphaproteobacteria* consistently dominated the whole 32-day immersion period but the decrease and/or increase of certain taxonomic classes marked several successional shifts. At the interior kelp bed, *Gammaproteobacteria* did not show any clear temporal patterns. Rather, the compositional difference between Day 6 and Day 12 was characterised by an increase in *Bacteroidota* (df = 4, F-value = 12.2, $p < 0.01$), while the difference between Day 12 and Day 18 was mainly explained by an increase in *Acidimicrobiia* (*Actinobacteria*; df = 4, F-value = 32.7, $p < 0.01$; Figure 3.5). In comparison, at the exterior kelp bed *Gammaproteobacteria* significantly decreased from Day 6 to Day 12 (df = 4, F-value = 4.7, $p < 0.01$), while *Bacteroidota* significantly increased from Day 12 to Day 18 (df = 4, F-value = 11.9, $p < 0.01$; Figure 3.5) and taxa affiliated with *Acidimicrobiia* had a significantly higher abundance after 32 days compared to the first 2.5 weeks of immersion (df = 4, F-value = 6.3, $p < 0.01$).

At a finer taxonomical level, DESeq analysis identified 19 differentially abundant ASVs ($p < 0.05$) between Day 6 and Day 12 at the interior kelp bed (Figure 3.5a). Only five ASVs had a higher relative abundance on Day 6, while the remaining 14 ASVs were characteristic for Day 12. The highest differentially abundant ASV between Day 6 and Day 12 was classified as *Rhodobacteraceae* (*Alphaproteobacteria*) and this was highly abundant on Day 6 (5.7%). The second highest differentially abundant ASV was characteristic for Day 12 (1.6%) and classified as *Saprospiraceae* (*Bacteroidota*). No ASVs were statistically different for the biofilm communities after 18 days compared to the 12-day old biofilms. This indicates that from day 12 on the biofilms at the interior kelp bed had a higher species evenness.

At the exterior kelp bed, DESeq analysis characterised 24 differentially abundant ASVs between Day 6 and Day 12 ($p < 0.05$), annotated as nine *Alphaproteobacteria*, nine *Gammaproteobacteria*, five *Bacteroidia* and one *Firmicutes* (Figure 3.5b). The highest differentially abundant ASV between Day 6 and Day 12 was identified as genus *Yoonia* and was the single most abundant ASV of Day 12 (29%). The following two most differentially abundant ASVs were classified as *Oleibacter* (*Gammaproteobacteria*; 4.1%) and *Litoreibacter* (*Alphaproteobacteria*; 4.5%) and were characteristic for Day 6. DESeq did not detect any significant differentially abundant ASVs

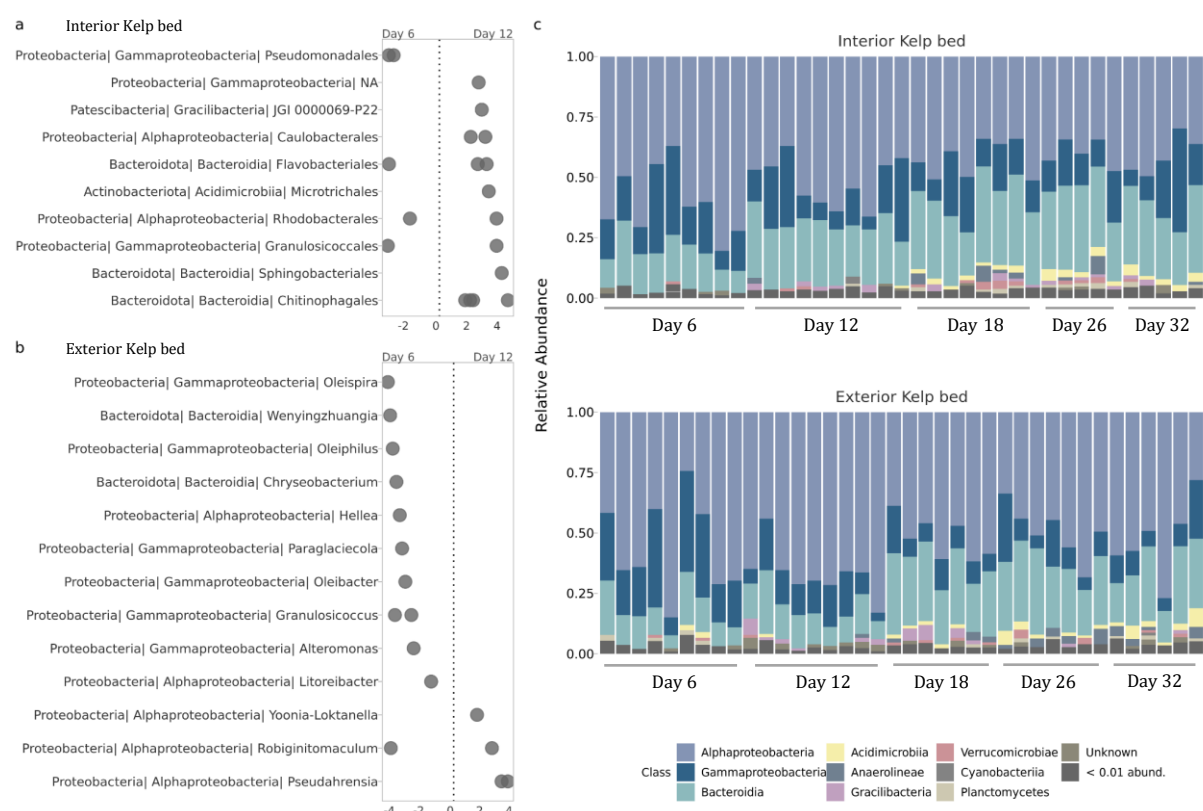


Figure 3.5: Taxonomical composition of prokaryotic biofilms. (a – b) DESeq analysis comparing two time points ($p < 0.05$), (a) Day 6 vs. Day 12 at the interior kelp bed with taxa labels including Phylum, Class and Order level. (b) Day 6 vs. Day 12 at the exterior kelp bed with taxa labels including Phylum, Class and Genus level. (c) Barplot showing the relative abundance of the prokaryotic taxa over time at class level for the interior and exterior location. Each individual replicate is shown.

between Day 12 – Day 18 and Day 18 – Day 26 which could be due to the high variability amongst the individual replicates.

Overall, the interior kelp bed had 2,009 unique ASVs, while the exterior kelp bed had 2,419 unique ASVs and they shared another 1,751 ASV. This indicates that the exterior location is characterised by a slightly higher species richness. Considering relative abundances, the shared taxa described approximately 95.5% of the total reads suggesting that the abundant ASVs in the prokaryotic biofilms are similar across both locations and that both sites contain unique characteristic ASVs with low relative abundance.

3.3.2.6.2 Eukaryotic communities

The eukaryotic biofilm communities were clearly dominated by *Ochrophyta* (~74% of reads) at both sites across the 32-day immersion period. Taxa affiliated with *Cercozoa* were also equally represented at both locations (~8% of reads). Large differences in relative abundance between the two locations were seen for *Rhodophyta* (interior: 10% and exterior: 1.4%) and *Ciliophora* (interior: 5% and exterior: 12%). These four divisions accounted for approximate 97% of reads at both locations. Within *Ochrophyta Phaeophyceae* (brown algae) consistently dominated the eukaryotic communities at every sampling day and with mean similar proportions across the kelp bed (interior: 59% and exterior: 55%) (Figure 3.6d). This was followed by a lower abundance of *Bacillariophyta* (diatoms) (15% and 19.5%), *Florideophyceae* (*Rhodophyta*, 10% and 1.4%), two *Ciliophora* classes *Phyllopharyngea* (4% and 9%) and *Spirotrichea* (5.8% and 2.8%), and *Endomyxa* (*Cercozoa*, both locations 5.8%) (Figure 3.6d).

Interior kelp bed

At fine taxonomic scale, the single brown filamentous algae *Ectocarpus siliculosus* dominated the communities at the interior bed for the first 2.5 weeks (30 – 50%). After 26 days, the diatom *Tabularia tabulate* (~20%) was the most abundant species but still closely followed by *E. siliculosus* (~18%). There were no significant changes in the abundance of a major taxonomical group between Day 6 and Day 12. DESeq analysis revealed that only one *Ciliophora* taxon, genus *Paracineta*, was identified as significantly differentially expressed ($p < 0.05$) between Day 6 and Day 12.

The first successional shift was defined by the significant decrease in *Ciliophora* after the first two weeks (MANOVA, Day 6 – Day 18; $df = 4$, $F = 3.72$, adjusted- $p = 0.025$). Along with the decrease of *Ciliophora* a significant increase in diatoms was observed (MANOVA, Day 6 – Day 18; $df = 4$, $F = 9.45$, adjusted- $p = 0.025$). Both successional changes happened over the duration of 2.5 weeks and

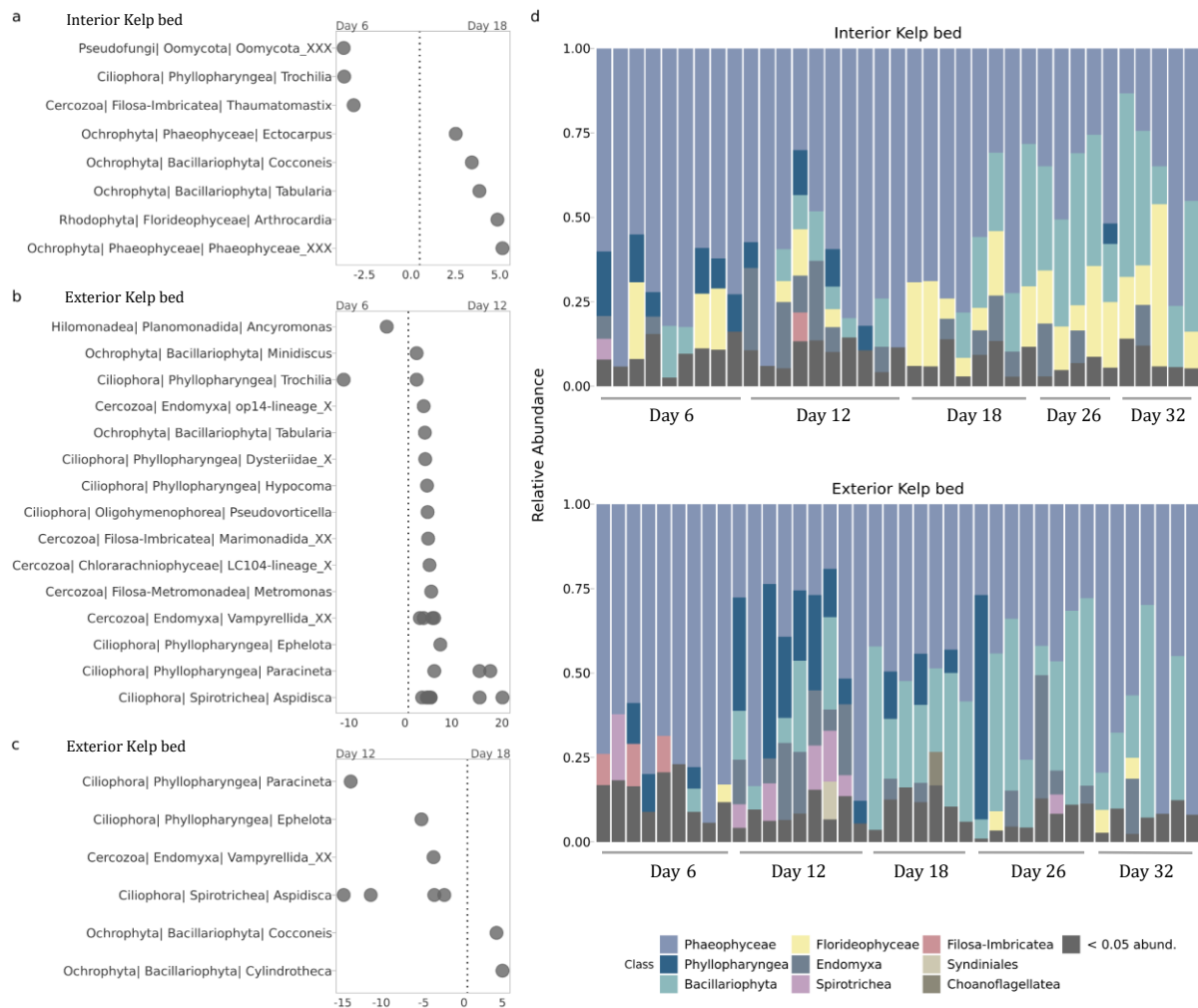


Figure 3.6: Taxonomical composition of eukaryotic biofilms. (a – c) DESeq analysis comparing two time points. (a) Day 6 vs. Day 18 at the interior kelp bed, (b) Day 6 vs. Day 12 at the exterior kelp bed and (c) Day 12 vs. Day 18 at the exterior Kelp bed. (d) Barplot showing the relative abundance of the eukaryotic taxa over time at class level. Each individual replicate is shown.

reached their strongest difference between Day 6 – Day 26. The DESeq analysis confirmed these findings by identifying eight differentially abundant ASVs between Day 6 and Day 18 (Figure 3.6a). Two of the eight ASVs were classified as *Ciliophora* and were characteristic for Day 6 while another two ASVs, *Cocconeis* and *Tabularia*, were identified as diatoms characteristic for Day 18. The most differentially expressed ASV was a *Corallinales* (*Rhodophyta*) species, *Arthrocardia carinata*, which accounted for 5.5% of all ASVs on Day 18. Although *Rhodophyta* did not significantly vary across time, the group was clearly more abundant during the later stages of biofilm development (Day 18 – Day 32). The final successional shift observed during this study was the significant decrease in *Phaeophyceae* after the first 26 days (Figure 3.6d). No significant changes in the major taxonomic groups were observed between the last two sampling days.

Exterior kelp bed

Similar to the interior bed, the brown algae *E. siliculosus* dominated the eukaryotic communities during the first 2.5 weeks at the exterior kelp site. However, the dominance of this single brown algae at the exterior bed was less drastic (19 – 40%). After 26 days the biofilm community was dominated by *T. tabulate* (~25%) but re-shifted to *E. siliculosus* (29.5%) after 32 days. In contrast to the interior bed, the first sampling days (Day 6 – Day 12) were marked by a significant decrease in *Phaeophyceae* ($df = 4$, $F\text{-value} = 5.7$, $p < 0.01$) and a significant increase in both *Phyllopharyngea* (*Ciliophora*; $df = 4$, $F\text{-value} = 2.9$, $p = 0.04$) and *Endomyxa* (*Cercozoa*; $df = 4$, $F\text{-value} = 3.4$, $p = 0.02$) (Figure 3.6d). DESeq analysis identified 37 differentially expressed ($p < 0.05$) ASVs of which 32 were representative for the 12-day-old biofilm and five for Day 6. The most differentially abundant ASVs were *Ciliophora* genera *Trochilia*, *Ephelota* and *Paracineta*, diatom species *T. tabulate* and ASVs identified as *Cercozoa* order *Vampyrellida*, all affiliated with Day 12 (Figure 3.6b). No characteristic *Phaeophyceae* ASVs were detected by the DESeq analysis for the comparison between Day 6 and Day 12. The transition from the 12-day-old to the 18-day-old biofilm was characterised by a clear increase in diatoms ($df = 4$, $F\text{-value} = 7.2$, $p < 0.01$; Figure 3.6c). Only nine ASVs were found to be differentially abundant between Day 12 and Day 18. Two of them were assigned to diatom genera *Cylindrotheca* and *Cocconeis* and were indicative for Day 18, while the remainder seven ASVs included six *Ciliophora* and one *Cercozoa* taxa which were representatives for Day 12. Diatoms relative abundance further increased for another week (Day 12 – Day 26) until reaching a steady level after 26 days of immersion. From Day 12 on, *Ciliophora* slowly decreased and reached significantly reduced number after 26 days (Day 12 – Day 26) until becoming nearly absent in the 32-day old biofilm.

Similar to the prokaryotic biofilm communities, the eukaryotic assemblages contained a higher total of unique ASVs at the exterior bed ($n = 444$) compared to the interior kelp site ($n = 361$). Both collections had another 310 ASVs in common that accounted for the most abundant ASVs.

3.3.3 Seawater microbial communities

3.3.3.1 Microbial community diversity

The seawater prokaryotic communities had higher species richness at the interior kelp bed (KW; $df = 1$, $H = 12.56$ & $p < 0.001$) while the species evenness was similar between the two locations. At the interior kelp bed, Chao1 richness did not vary across time while Pielou's evenness was significantly different for all the consecutive timepoints (KW; $df = 4$, $H = 16.99$ & all corrected $p\text{-values} = 0.041$). At the exterior location both diversity metrics differed over time (KW, $df = 4$; Chao1: $H = 10.27$ & $p = 0.036$; Pielou: $H = 13.89$ & $p = 0.008$). Nevertheless, pairwise analysis did not reveal any significant results between the time points. For the eukaryotic seawater

communities, Chao1 richness and Pielou's evenness did not show any distinction across the kelp bed. However, diversity metrics over time at the exterior bed were both significant (KW, $df = 4$; Chao1: $H = 27.61$ & $p < 0.001$; Pielou: $H = 27.88$ & $p < 0.001$). Pairwise comparisons further indicated that Chao1 richness was statistically different between the first four consecutive timepoints (Day 6 – Day 12, Day 12 – Day 18 and Day 18 – Day 26), but not between the last two (Day 26 – Day 32).

The composition of the microbial communities, prokaryotic and eukaryotic, in the seawater column significantly varied for location, time and the interaction factor (Figure 3.7). For the prokaryotic communities the PERMDISP test was significant for location on Day 6, Day 18 and Day 32. However, the unconstrained ordination (stress 0.16) showed a clear separation between the sites indicating that both a 'location' effect and 'dispersion' effect contributed to the significant variability between the interior and exterior seawater communities. PERMDISP was also significant for the communities at the interior bed over time.

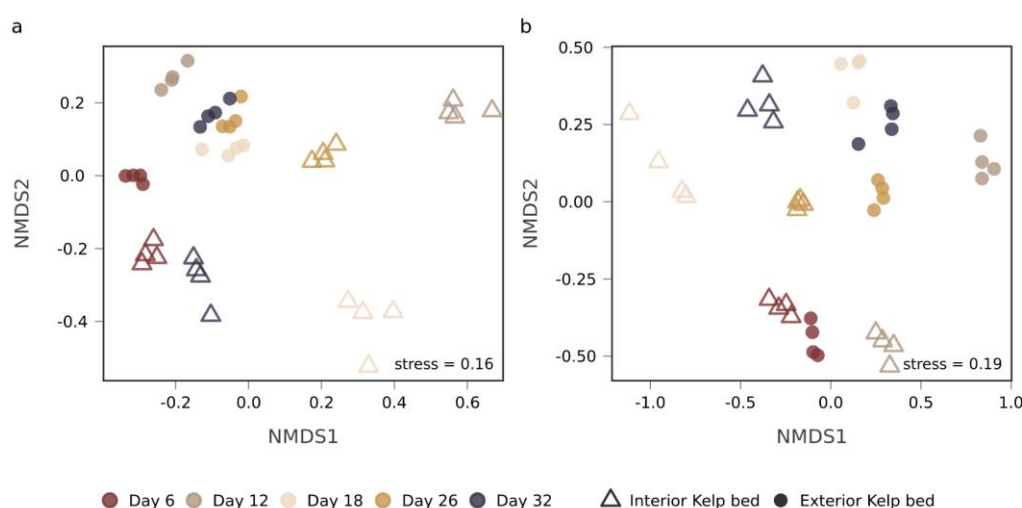


Figure 3.7: Beta diversity of seawater communities based on Bray-Curtis dissimilarity. Ordinations were made using non-metric multidimensional scaling (nMDS). (a) Prokaryotic communities and (b) Eukaryotic communities. Colours represent the day of sampling during this study. Symbols represent the two sampled locations across the kelp forest, interior bed (triangles) and exterior bed (circles).

3.3.3.2 Spatio-temporal taxonomic variation

At a high taxonomic level, the pelagic prokaryotic communities at the two sites were very similar (Figure 3.8a). Across all time points, the communities were dominated by *Proteobacteria* at both the interior (57%) and exterior (54%) kelp bed. The *Proteobacteria* were predominately represented by *Alphaproteobacteria* (interior: 33% and exterior: 38%) and to a lesser extent *Gammaproteobacteria* (24% and 16%). The second most dominant phylum was the *Bacteroidota* (mainly *Flavobacteria*), present at equal levels (21.7% and 21.3%) at both locations. These two main phyla were followed by *Cyanobacteria* (mainly *Synechococcus*; 6.3% and 9.6%),

Actinobacteriota (mainly *Accidomicrobia*; 3.5% and 4.7%), and the Archaea phylum *Thermoplasmotota* (2.3% and 3.9%). Overall, these major taxonomic groups at class level did not significantly vary across time at the interior bed (MANOVA; $p > 0.05$). At the exterior bed *Gammaproteobacteria* (lowest abundance; F-value = 10, $p < 0.05$) and *Bacteroidia* (peak values; F-value = 11.2, $p < 0.05$) were significantly different on Day 12 in relation to the other time points.

The four most abundant ASVs at both locations had similar relative abundances and belonged to: SAR 11 clade Ia (*Alphaproteobacteria*), *Nitrincolaceae* (*Gammaproteobacteria*), another SAR 11 clade Ia affiliated ASV, and *Synechococcus CC9902* (*Cyanobacteria*). The fifth most abundant ASV at the inner edge location belonged to the NS5 marine group genus (2.7%, *Flavobacteriales*), while at the outer edge location this was a *Rhodobacteraceae* species (*Alphaproteobacteria*) identified as *Nereida ignava* (2.3%)

The most abundant division at both locations was *Ochrophyta* (interior: 32%; interior site: 29.5%). *Ochrophyta* was equally represented by *Bacillariophyta* (14%) and *Phaeophyceae* (11%) at the interior bed (Figure 3.8b) but dominated by *Bacillariophyta* (15% vs. 5% for *Phaeophyceae*) at the exterior bed. In addition to *Ochrophyta*, taxa affiliated with *Chlorophyta* (23%, mainly

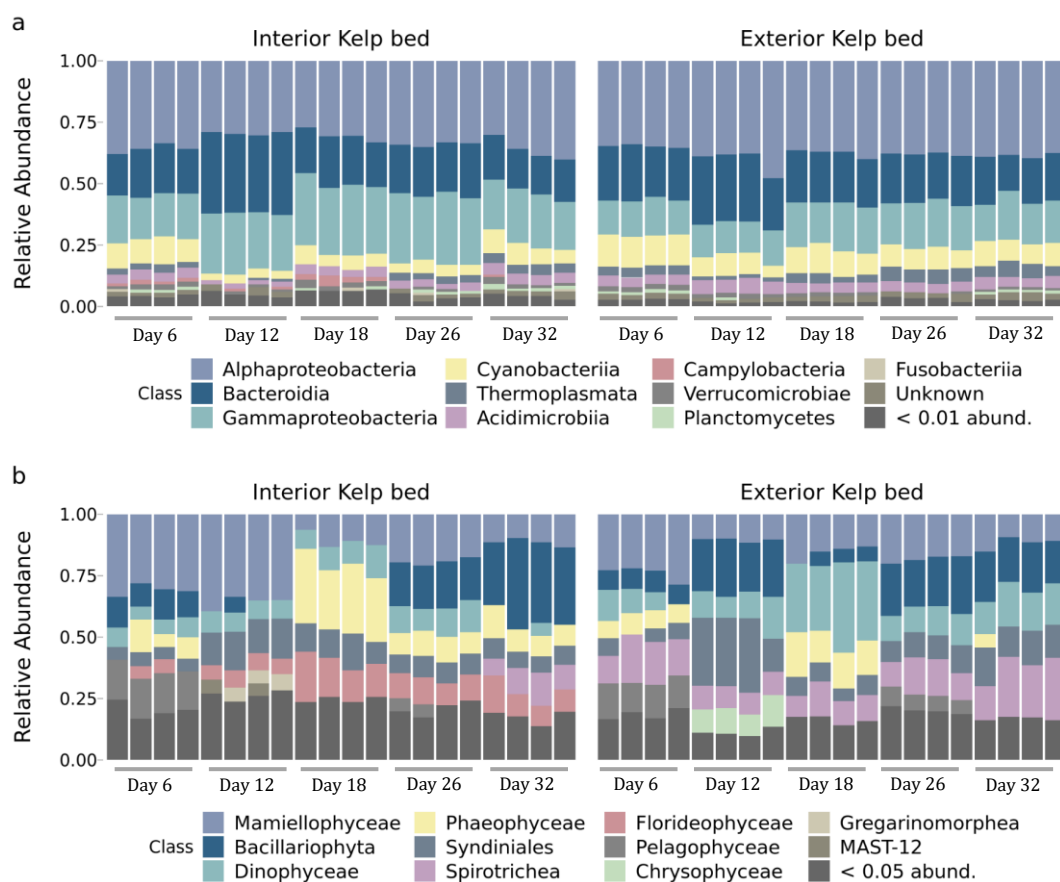


Figure 3.8: Taxonomic composition of seawater microbial communities across the kelp bed. (a) Prokaryotic communities and (b) eukaryotic communities. All the replicate samples are shown for each time point.

Mamiellophyceae), *Dinoflagellata* (17%, *Diniphyceae* and *Syndiniales*), *Rhodophyta* (9.5%, mainly *Florideophyceae: Corallinales*) and *Ciliophora* (5%, mainly *Spirotrichia*) represented 86.5% of the total reads at the interior kelp bed (Figure 3.8b). The top 90% of all the reads at the exterior bed were affiliated with only four divisions. *Ochrophyta* was followed by *Dinoflagellata* (28%; *Diniphyceae* and *Syndiniales*), *Chlorophyta* (17%, mainly *Mamiellophyceae*), and *Ciliophora* (15.5%, mainly *Spirotrichia*) (Figure 3.8b). *Rhodophyta (Florideophyceae)* at the exterior site only accounted for approximately 1%.

All these major taxonomic groups were significantly different over time at the interior bed (MANOVA; $df = 4$, $F\text{-value} = 17.6$, $p < 0.01$) and the exterior bed (MANOVA; $df = 4$, $F\text{-value} = 11.3$, $p < 0.01$). At the interior bed diatoms were significantly lower on Day 12 and Day 18 and reached increased level from Day 26 on. At the exterior bed diatoms were substantially present on Day 12, Day 26 and Day 32 but significantly lower on Day 18. *Phaeophyceae (Ochrophyta)* and *Florideophyceae (Rhodophyta)* were significantly higher on Day 18 at both locations, however both taxonomic groups did show a much higher relative abundance at the interior kelp bed. For the dinoflagellates, *Diniphyceae* was significantly lower on Day 32 at the interior bed compared to the other time points while the group was relatively abundant on all days at the exterior day with a substantial increase on Day 18. *Syndiniales* (dinoflagellates) was significantly lower on Day 6 at both locations and was additionally enriched on Day 12 at the exterior bed. Finally, *Spirotrichia* (ciliates) reached their highest relative abundance on Day 32 at both locations but was overall clearly more abundant at the exterior kelp bed.

The top five most abundant ASVs at the interior kelp site were *Ostreococcus lucimarinus* (9.7%, *Chlorophyta*), *Macrocystis pyrifera* (6.6%, *Ochrophyta*), *Bathycococcus prasinos* (5.6%, *Chlorophyta*), *Pelagomonadales sp.* (5.4%, *Ochrophyta*), and a dinoflagellate species belonging to the Dino group I clade 4 family (4%). At the exterior kelp bed, *Ostreococcus lucimarinus* (7%) was the most dominant ASV followed by the diatom *Chaetoceros tenuissimus* (6%), *Warnowia sp.* (5%, *Dinoflagellata*), *Macrocystis pyrifera* (5%), and a species belonging to the Dino-group I clade 4 family (5%).

3.4 Discussion

Strikingly at high taxonomic level (phylum & class), the prokaryotic biofilm communities did not show any major differences across space and time, while the eukaryotic communities were substantially different in both relative abundance as well as species presence/absence. The same pattern was observed for the seawater communities. Nonetheless, the alpha diversity trends of both biofilms and seawater communities, showed a much higher species richness for prokaryotes compared to the eukaryotes. This indicates that the prokaryotic communities contain a larger pool of lower abundance species that account for the community variability across time and space (Zhang et al., 2019). Thereby expressing the importance of exploring community composition at low taxonomic level to accurately assess prokaryotic community structuring.

3.4.1 Successional dynamics and small spatial structuring of prokaryotic biofilms

Marine biofilm settlement and subsequent development occurs within seconds of substrate exposure through the formation of a conditioning surface layer, surface sensing, cell-cell communication, and species and inter-kingdom interactions (Dang & Lovell, 2016). The major pioneer colonisers in temperate coastal marine waters have previously been identified as bacteria belonging to *Rhodobacteraceae* (*Alphaproteobacteria*) (Dang & Lovell, 2000, 2002; Jones et al., 2007; Dang et al., 2008). Consistent with these reports, the prokaryotic biofilms in this study were continuously dominated by members of *Rhodobacteraceae*. The success of *Rhodobacteraceae* for early colonisation has mainly been attributed to their efficiency for low level nutrient utilisation, associated with new surfaces, and their wide range of metabolic pathways (Buchan et al., 2005; Dang et al., 2008; Dang & Lovell, 2016). Taxa annotated as the *Rhodobacteraceae* genus *Yoonia* (previously *Loktanella*; Wirth & Whitman, 2018) dominated the biofilms for the whole 32-day period. Nevertheless, they clearly displayed a reduction in abundance over time emphasizing their importance as early colonisers across the kelp bed. Similarly, *Yoonia* was one of the major dominant genera identified on artificial substrates deployed in Sacheon harbor, Korean Peninsula (Lee et al., 2008) and has also previously been associated with growth on macroalgal species (Lachnit et al., 2011; Burke et al., 2011; Stratil et al., 2013; Mancuso et al., 2016). Furthermore, the affinity of *Yoonia*-related species for a variety of substrates (abiotic and biotic) suggests an opportunistic and generalist lifestyle.

Interestingly, the biofilms at the exterior kelp bed showed a significant decline in *Gammaproteobacteria* (predominantly the genus *Oleibacter*) during early biofilm development from Day 6 to Day 12. In 2008, Lee et al. was the first to study bacterial biofilm succession at a fine

temporal scale (0 - 36 h) and reported the predominance of *Gammaproteobacteria* during the initial hours of biofilm development. Similarly, Pollet et al. (2018) reported that members of genus *Oleibacter* (*Gammaproteobacteria*) dominated the pioneer biofilm up until 24 hours after immersion. *Oleibacter* affiliated sequences are well known to be hydrocarbonoclasts, mostly linking them to the degradation of petroleum (hydrocarbon rich) compounds (Teramoto et al., 2011). Hence, the presence of this genus could be indicative for the influence of anthropogenic activities (e.g., urban run-off and boating) in the environment that may have resulted in petroleum contamination in water column (Teramoto et al., 2013; Wu et al., 2021). Noteworthy, the settlement plates used in the previously mentioned studies (Lee et al., 2008; Pollet et al., 2018) as well as the current work, partially consist of petroleum derivative products and could be the main reason as to why *Oleibacter* is observed here. Nevertheless, the considerable decline of *Gammaproteobacteria* in the early biofilm at the exterior location could also support the hypothesis, put forward by Lee et al. (2008), that these bacteria might be the predominant species in pioneer biofilms and should therefore be recognised as the true primary colonisers. Unfortunately, in the present study no biofilms were collected prior to 6-days of immersion or from additional natural surfaces (e.g. rocks) to further assess and confirm the possible predominance of *Gammaproteobacteria*. Furthermore, the temporal shift (6 – 12 days) involving *Gammaproteobacteria*, observed in this study, indicates a substantial difference in time frame of biofilm development compared to the descriptions of Lee et al. (2008) (0 – 9 h) and Pollet et al. (2018) (0 – 24 h). Conversely, the biofilm communities at the interior kelp bed displayed no evidence of an early successional shift involving *Gammaproteobacteria*. Most likely the dissimilarity in temporal biofilm development amongst these aforementioned studies and between the interior and exterior kelp bed in the current study reflects the variability of environmental conditions in these different regions.

Differences in community composition at both locations revealed successional shifts in the early biofilm, defined by substantial increases in *Bacteroidia* (*Bacteroidota*) and *Acidimicrobiia* (*Actinobacteria*). The change in *Bacteroidia* abundance occurred on day 12 at the interior bed and day 18 at the exterior bed. This indicated a delay in time frame of approximately six days in biofilm development at the exterior bed, thereby also explaining the discrepancy in time for the late shift in *Gammaproteobacteria* (6 – 12 days), as discussed earlier. At both locations, *Bacteroidia* became the second most dominant class throughout the remainder of the 32-day period after their initial increase in relative abundance. Members of *Bacteroidia* have previously been recognised as successful secondary surface colonisers (Dang & Lovell, 2016; Pollet et al., 2018; Antunes et al., 2020). Unlike *Alphaproteobacteria*, *Bacteroidota* identified taxa require enriched levels of nutrients and organic substrates (POM and DOM) to facilitate and sustain surface colonisation,

which accounts for their secondary nature in early colonisation (Kirchman, 2002; Elifantz et al., 2013). Contrary to earlier reports (reviewed in Dang & Lovell, 2016), *Bacteroidia* in this study appeared to be dominated by members of the order *Chitinophagales* rather than *Flavobacteriales*, which could indicate a preference of *Chitinophagales* in *M. pyrifera* kelp beds. Nonetheless, the positive shift in *Bacteroidia* abundance was the result from an equal level of increase in the relative abundance of both orders. Taxa affiliated with the family *Saprospiraceae* predominately represented *Chitinophagales* and were characteristic for the successional change of *Bacteroidota* at both sites. Both *Saprospiraceae* and *Flavobacteriales* have been classified as macroalgal generalist bacterial families by Florez et al. (2017), who conducted a meta-analysis of macroalgae associated epiphytic bacteria. This emphasizes the complex nature and high connectivity of the kelp ecosystem. The successional shift in *Bacteroidota* was followed by a change in *Acidimicrobiia* (*Actinobacteria*), albeit at a lower magnitude. The increase in *Acidimicrobiia* was seen during the transition from Day 12 to Day 18 at the interior bed while the change in *Acidimicrobiia* at the exterior location was established over a longer time period (Day 18 – Day 32). This reflects the temporal lag in biofilm development across the kelp bed.

The three major successional shifts involving *Gammaproteobacteria*, *Bacteroidota* (*Chitinophagales* and *Flavobacteriales*) and *Acidimicrobiia*, together with the predominance of *Alphaproteobacteria* throughout the biofilm development period are in accordance with previous studies that have identified these bacterial groups, among others, as the primary early biofilm colonisers across a range of environmental conditions (Dang & Lovell, 2016; and references therein).

3.4.2 Successional dynamics and small spatial structuring of eukaryotic biofilms

The early eukaryotic biofilms were overwhelmingly dominated by the small brown filamentous algae *Ectocarpus siliculosus* (Dillwyn) Lyngb. This filamentous seaweed is the model organism for all brown algae and shares close evolutionary relatedness with the other brown kelps (Peters et al., 2004; Charrier et al., 2008; Cock et al., 2010). *E. siliculosus* has mainly been found in temperate and subtropical rocky coastlines and has been known to grow on abiotic (rocks, pebbles, artificial substrate) as well as biotic (macroalgae, seagrass) surfaces (Charrier et al., 2008). The dominance of this organism on the biofilm plates is a reflection of the coastal brown algae-rich environment. However, as the biofilm matured, brown algae (*Phaeophyceae*) overall decreased in relative abundance which coincided with the increase of diatoms (*Bacillariophyceae*) during mid-biofilm development. The latter is in line with a previous study focussed on eukaryotic biofilm assembly at a hypersaline lake (Washington State, USA) showing the dominance of diatoms at a later

development stage (Brislawn et al., 2019). The high abundance of brown algae and diatoms during early- and mid- biofilm, respectively, marks them as the dominant primary producers.

The temporal successional shifts involving brown algae and diatoms occurred approximately a week sooner at the exterior kelp bed. This temporal difference in biofilm development across the kelp bed was likely influenced by the seawater communities. At the interior bed, the same increase from Day 26 onwards for diatoms was observed in the water column. At the exterior kelp bed, the significant increase on Day 12 in the water column could have promoted the earlier colonisation of benthic diatoms onto the exterior settlement plates. Indeed, it has been previously shown that benthic biofilm constituents are recruited from seawater sample pools (Dang & Lovell, 2000, 2016; Jones et al., 2007; Lee et al., 2008). Note, the diatom species found in the water column were primarily centric species while the benthic diatoms consisted almost entirely out of pennate species. The reflection of the seawater communities in the eukaryotic assemblages was also apparent by looking at the distribution of *Corallinales* (*Rhodophyta*). Overall, members of the *Corallinales* group had a relative high abundance at the interior bed in the water column as well as on the settlement plates, while being near absent at the exterior site. Previous studies describing the *M. pyrifera* dominated kelp bed at Karitane reported high *Corallinales* cover across the kelp bed at 2 m depth in the interior and 10 m depth at the exterior location (Hepburn et al., 2011; Pritchard et al., 2013; Desmond et al., 2015). Due to the microorganisms being sampled from the surface ocean layer (0 – 1 m), the depth difference and subsequently the difference in proximity to *Corallinales* could have resulted in the pattern observed here.

Beside *Phaeophyceae* being pioneer colonisers, *Ciliophora* affiliated taxa also had their highest numbers in the early biofilm. The initial rapid colonisation by ciliates has been attributed to their motility and a high food supply (e.g., bacteria and algae) in biofilm structures (Coppellotti & Matarazzo, 2000; Risse-Buhl & Küsel, 2009). The dominant ciliate colonisers at both sites were known to be primarily bacterivorous and were mostly represented by a single ASV (genus *Trochilia*). Overall, biofilm ciliates were more abundant at the exterior kelp bed which was again a reflection of *Ciliophora* abundance in ambient seawater. However, on a temporal scale the settlement plates and seawater communities did not reflect one another. At the interior kelp, ciliates in the water column were most abundant at day 32, while consistently present throughout the study at the exterior bed. Further, a one-week delay in *Ciliophora* colonisation of the biofilm was observed at the exterior bed which supports the temporal lag seen in bacterial biofilm structuring across the kelp forest. Since the most dominant ciliates were bacterivorous predators, it is not that unexpected that they might be closely linked to the bacterial communities. Indeed, strong correlations between ciliates and bacteria abundance have been previously reported (e. g. Zingel & Ott, 2000). At the interior bed, from Day 6 on the ciliates slowly dropped to insignificant

numbers over the next 12 days. While at the exterior kelp bed a clear change in predator regime was noticed with the abundant bacterivorous species at Day 12 shifting towards mostly carnivorous species (order *Suctorina*) at Day 26. Similar ciliate colonisation dynamics were observed in previous reports of coastal waters (Zhang et al., 2012; Zhang et al., 2013). Following the transition in predator regime, ciliate numbers in the 32-day-old biofilm were drastically reduced. The successional shifts and different community structuring in the *Ciliophora* group across the small spatial gradient could be the result of varying resource availability, predation pressure or physical environmental disturbances (such as wave exposure, heavy rain event or riverine runoff).

3.4.3 Ecological processes behind multi-kingdom communities

Prokaryotic and eukaryotic biofilm communities, at the interior kelp bed, did not significantly change anymore in community composition after 2.5 weeks. It is assumed that at that time point they reached a stable biofilm community. At the exterior kelp bed, a stable community was established after 3.5 weeks for both prokaryotic and eukaryotic biofilms. This indicates that biofilm communities at the exterior bed have a temporal lag of one-week in their development process. The close association between prokaryotic and eukaryotic assemblages in terms of temporal community structuring is suggestive of an inter-kingdom relationship (Zancarini et al., 2017; Brislawn et al., 2019). For the seawater communities, distinction across the kelp bed and was observed for each sampling day. While the spatial community patterns in seawater across the kelp bed were relatively well reflected by the biofilm assemblages, the temporal structuring at both locations was not.

The later onset of biofilm development at the exterior kelp is most likely a reflection of the environmental variability between the two locations. However, despite the significance of the abiotic variables in explaining the variation of the benthic biofilm assemblages, they only accounted for a small amount of the community variability. This would suggest that other parameters are responsible for shaping the communities at this fine spatial scale. It should be noted though that the environmental variables measured during this study were discrete measurements rather than continuous data collections, and therefore do not reflect the full temporal variability of those environmental variables in the kelp ecosystem. In a study by Stephens & Hepburn (2014), a water mass-transfer gradient was identified in the same *M. pyrifera* dominated kelp forest, Karitane, New Zealand, with increased water motion at the exterior kelp bed. This could result in a physically interruption of biofilm settlement at the exterior kelp bed, leading to a delay in biofilm development.

Benthic prokaryotic assemblages differed significantly across the kelp bed during the first 3.5 weeks of development. Despite the clear differences in time scales of successional shifts, the communities between the two locations reached similar compositions after 32 days. The low community variability between the final biofilms is an indication of homogeneous selection over time for the prokaryotic communities (Stegen et al., 2012; Dini-Andreote et al., 2015; Zhou & Ning, 2017). A process which could have been induced by the heavy rainfall, subsequent water mixing and land and riverine runoff mid-way through the study. For instance, this was observed in a study on microbial communities in rock pools that underwent an environmental shift (intense rain events) which induced homogeneous selection across multiple rock pools (Vass et al., 2020). Furthermore, homogeneous selection was observed as the dominant process behind community structuring of particle associated prokaryotes as well as free-living communities in a variety of environments such as lakes, rivers, coastal settings and the open ocean (Brislawn et al., 2019; Wang et al., 2020; Logares et al., 2020; Allen, Hoffmann, et al., 2020; Wu et al., 2021). Nevertheless, it is not uncommon for bacterial biofilms to evolve towards similar community compositions and structure, independent of the environmental variability (Jones et al., 2007; Pollet et al., 2018; Antunes et al., 2020).

The outcome of the ecological processes responsible for the prokaryotic community structuring was primarily species Turnover yet there was a contribution of Nestedness on certain days. Hence, a combination of ecological processes took place to guide the prokaryotic communities toward similar one-month old biofilms across the kelp bed. Conversely, ecological processes behind eukaryotic biofilm development exclusively resulted in species replacement (Turnover) for the whole 32-day immersion period. The greater extent of species sorting that applies to eukaryotic assemblages was also observed for eukaryotic biofilms in a hypersaline lake (Washington State, USA) and in the East China Sea (Wu et al., 2017; Brislawn et al., 2019). Furthermore, this result is indicative of variable selection for eukaryotic communities and is also consistent with findings of previous studies (Brislawn et al., 2019; Vass et al., 2020). To correctly identify the ecological processes (deterministic versus stochastic) that are involved in biofilm development an ecological null model analysis is advised (Brislawn et al., 2019; Allen, Summerfield, et al., 2020; Allen, Hoffmann, et al., 2020; Vass et al., 2020). Overall, species recruitment and temporal succession operated on different time frames for each kingdom, most likely as a result of distinct assembly processes (selection, drift, dispersal and speciation).

Finally, compared to the swift dynamics of bacterial succession, eukaryotic community development occurred at a slower pace. It is possible that over a longer immersion period eukaryotic communities could also have reached equal biofilms across the kelp bed. However, the

different ecological mechanisms that shape these communities compared to prokaryotes more likely suggest against that outcome.

3.5 Conclusion

As expected, the biofilm communities were distinct from the free-living microorganisms. However, across the kelp bed compositional similarity between both sample types at high taxonomic levels was observed. Environmental variability (e.g. wave induced hydrodynamic patterns) across the kelp forest resulted in a one-week temporal lag for biofilm development at the exterior bed during the first 3.5 weeks of immersion. After a month, prokaryotic assemblages across the kelp bed did reach similar community compositions, suggesting that interspecies interactions and associated signalling mechanisms within the biofilm over time are a stronger force in structuring the community than abiotic influences. Prokaryotic communities that have been established over longer time periods across the kelp bed are thus expected to show similar assemblages. Eukaryotic biofilm communities from both sites were still distinct after one month and suggests that environmental variability across the kelp forest potentially plays a stronger role in structuring the eukaryotic biofilm communities compared to the prokaryotic biofilms. A longer experimental study is necessary to fully investigate the structuring of eukaryotic biofilm development. Additionally, biofilms on biotic surfaces could display different community dynamics, depending on to level of influence from the host organisms, and require further investigation. Furthermore, community differences across time and space for prokaryotic and eukaryotic constituents of the biofilm were observed at different taxonomic levels. Prokaryotic compositional differences were strictly present at fine taxonomical level, while eukaryotic members already showed strong differences at division and class level. Expanding more on the interkingdom interactions, within the biofilm, across the kelp would be the next step in gaining a better understanding of the microbial ecology in this highly variable ecosystem.

Chapter 4|

Assessment of kelp associated microbiome diversity and taxonomic composition

4.1 Introduction

Marine macroalgae contribute significantly to the productivity of temperate coastal ecosystems as important bioengineers (Teagle et al., 2017; Wernberg et al., 2019). They form complex habitat structures that support and maintain local biodiversity (Steneck et al., 2002; Teagle et al., 2017) thereby offering a large pool of resources for socioeconomic gains involving fisheries, shoreline protection and tourism (Schiel & Nelson, 1990; Madsen et al., 2001; Beaumont et al., 2007).

The giant bladder kelp, *Macrocystis pyrifera*, (Linnaeus) was first described by C.A. Agardh in 1820. This large macroalgae belongs to the order *Laminariales* of the *Phaeophyceae* (brown algae) and is renowned for building extensive underwater kelp forests along temperate coastlines in both the northern and southern hemisphere (Kain, 1982; Hay, 1990 & references therein). *M. pyrifera* is the sole form of *Macrocystis* that occurs in New Zealand and is one of the dominant canopy-forming macroalgae, supporting large kelp forests along the southern coastline of the North and South Island (Hay, 1990; Brown et al., 1997; Hepburn & Hurd, 2005; Hepburn et al., 2007). The highest biomass in the South Island is found on the East Otago coast where this perennial kelp species typically inhabits the low intertidal and subtidal zones through attachment to rocky substrata (Hay, 1990).

Due to their occurrence in the inter- and subtidal zone the surface floating kelp is exposed to changes in both air and ocean temperatures making them more susceptible to current climate related threats. For instance, the marine heatwave (MHW) of 2017/2018 led to reduced cover of *M. pyrifera* across multiple regions in New Zealand primarily where sea surface temperatures exceeded 18°C (Tait et al., 2021). Additionally, Minich et al. (2018) reported reduced growth of *M. pyrifera* (California, USA) and the disturbance of the kelp associated biofilms after exposure to increased temperature during a mesocosm experiment. Threats to kelp as a result of gradual ocean warming, more frequent and longer MHW, and ocean acidification have been reported for macroalgae across the world (Koch et al., 2013; Krumhansl et al., 2016; Noisette & Hurd, 2018; Smale et al., 2019; Smale, 2020). The magnitude of these events tend to be influenced by biogeography and not all ocean regions have similar responses to these climate related impacts (Krumhansl et al., 2016; Smale, 2020). However, during a heatwave in New Zealand (summer 2017/2018) a localized extinction event was reported for the canopy forming Bull Kelp (*Durvillaea* sp.) in the South Island (Thomsen et al., 2019). This highlights the need to investigate the ecology of temperate kelp forests at regional and local scales in New Zealand to better assess their vulnerability to current and future threats.

An important contributor to macroalgae ecology is the intimate interaction between kelp and marine microorganisms. Kelp provide ample settlement surfaces for microbial epibionts by producing polysaccharide rich matrices (Martin et al., 2015; Lin et al., 2018) and dissolved organic carbon (Abdullah & Fredriksen, 2004; Reed et al., 2015) across different anatomical structures (Ihua et al., 2020; Lemay et al., 2021). Several studies have investigated the functional role epiphytic bacteria play in modulating macroalgae health, growth, resilience and spore release (Weinberger, 2007; Joint et al., 2007; Goecke et al., 2010; Wahl et al., 2012; Hollants et al., 2013; Egan et al., 2013). Although an increase in research on kelp associated bacterial assemblages has been seen in the past decade (e.g. Lachnit et al., 2009; Wahl et al., 2012; Bengtsson et al., 2012; Hollants et al., 2013; Egan et al., 2013; Weigel & Pfister, 2019; Ihua et al., 2020; Lemay et al., 2021), there is still a lot unknown about the ecological processes that underpin these interactions on a regional scale. In addition, hardly any studies have directed their focus on exploring kelp affiliated epi-endophytic eukaryotes. So far, only Lemay et al. (2021) used high resolution sequencing to report the composition and diversity of kelp associated eukaryotic biofilm communities. Collectively, earlier work has shown that the microbial epiphytes found on kelp are host and tissue specific (Lachnit et al., 2009; Egan et al., 2013; Weigel & Pfister, 2019; Lemay et al., 2021), and are distinct from the microbial communities present in the surrounding waters (Bengtsson & Øvreås, 2010; Michelou et al., 2013; Mancuso et al., 2016; Weigel & Pfister, 2019). Along with host and tissue specificity, spatiotemporal variability (Mancuso et al., 2016; Weigel & Pfister, 2019; Korlević et al., 2021) and kelp condition (Marzinelli et al., 2015) have also been demonstrated to play a part in shaping the kelp microbiome. The consensus of these studies is that kelp prokaryotic microbiomes are mainly driven by kelp classification at high taxonomical level (division), followed by kelp-species, and then region; further indicating the importance of species- and region-specific studies of kelp-associated microbial communities.

Research on community composition and diversity of the *M. pyrifera* microbiome across oceans is still scarce and so far has been limited to studies in the Northern hemisphere (Michelou et al., 2013; Vollmers et al., 2017; Minich et al., 2018; Lin et al., 2018; Weigel & Pfister, 2019). *Gammaproteobacteria* (*Granulosicoccus* sp.) were shown to dominate the bacterial epibionts on *M. pyrifera* followed by *Alphaproteobacteria* (*Rhodobacteraceae*), *Bacteroidetes* (*Flavobacteriia* and *Saprospirae*) and to a lesser extent *Planctomycetes* and *Verrucomicrobiia* (Michelou et al., 2013; Vollmers et al., 2017; Weigel & Pfister, 2019). However, a detailed description of *M. pyrifera* associated microorganisms at a finer level of phylogenetic resolution is missing.

The continued increase of climate related threats stresses the importance of investigating and enhancing current knowledge on the close interactions between macroalgae and their microbial epibionts thereby unravelling the ecological role the microorganisms play in maintaining these

healthy complex coastal ecosystems. This study will focus on a local *M. pyrifera* kelp forest on the southern east coast of the South Island, New Zealand. The community composition and diversity of both prokaryotic and eukaryotic biofilm communities on the surface of the kelp will be investigated. Small scale spatial variation in microbial communities with tissue age and across the kelp forest will be described for both kingdoms. Finally, an in-depth comparison between the kelp microbiome, biofilm development on settlement plates (small transparent acrylic slides) and seawater microbial assemblages will assist in identifying kelp-specific communities.

4.2 Materials & Methods

4.2.1 Site description and sampling

The *in-situ* study was performed in a *Macrocystis pyrifera* dominated kelp forest located in Te Awa Mokihi/ Butterfly Bay (45°38'20.58''S, 170°40'19.38''E), Karitāne/Huriawa Peninsula (New Zealand) (Figure 3.1). A full description of the study region was given in Chapter 3. In late summer 2021 (February-March), kelp blades were sampled through snorkelling at two locations within the kelp bed. The interior site was located inside the coastal bay and the exterior site was at the seaward edge of the kelp forest. For the comparison of the kelp blade associated biofilms with communities on non-living surfaces and seawater microbial assemblages, additional samples were collected from settlement plates attached to the kelp individuals and from the surrounding seawater as described in Chapter 3 (Table 4.1). Water samples for the analysis of inorganic macronutrients (n=3 per site), and physio-chemical water parameters (pH, temperature, salinity, and chlorophyll-*a* concentration) were also collected on each sampling day (Chapter 3).

Table 4.1: Overview of the amount and type of samples collected. The corresponding sampling days from Chapter 3 are included in the table for comparison.

Corresponding Chapter 3 time points	Date	Interior Kelp Bed			Exterior Kelp Bed		
		Kelp blades	Settlement slides	Seawater	Kelp blades	Settlement slides	Seawater
Day 6	14.02.2021	9	9	4	9	9	4
Day 32	12.03.2021	5	5	4	7	7	4

The kelp blades were removed at the air bladder structure (pneumatocyst; Figure 4.1) and collected in a Ziplock bag at the surface, while keeping water from entering the plastic bag. The samples were placed in a catch bag and transported back to shore where they were kept in a cooler until processing (within 1 hour of collection). Extra flagging tape was added to each kelp

individual to facilitate locating the same selected macroalgae on the second sampling day. On shore, each kelp blade was photographed, measured, and rinsed for 10 s with sterile filtered (0.2 μm) seawater to remove any transient epiphytes present on the blade surface as a result from the sampling process. Three sections per kelp blade were subsequently sampled to examine the prokaryotic and eukaryotic community structure on different age blade tissue: the basal (meristem), medial and apical regions (Figure 4.1). Sampling from the blade was done by swabbing the tissue surface layer (10 cm^2) with a sterile cotton swab for 10 s. The swab from the basal region was taken approximately 2 cm from the air bladder structure. The swabs were collected in a 1.5 mL microcentrifuge tube and stored on dry ice. Back in the laboratory the samples were kept frozen at -80°C until DNA extraction. Only intact, healthy-looking blades were selected for downstream analysis of the kelp blade associated microbial communities. Additionally, the blade samples were classified into three categories based on their length: short (1 – 35 cm), medium (35 – 65 cm) and large (65 – 100 cm).

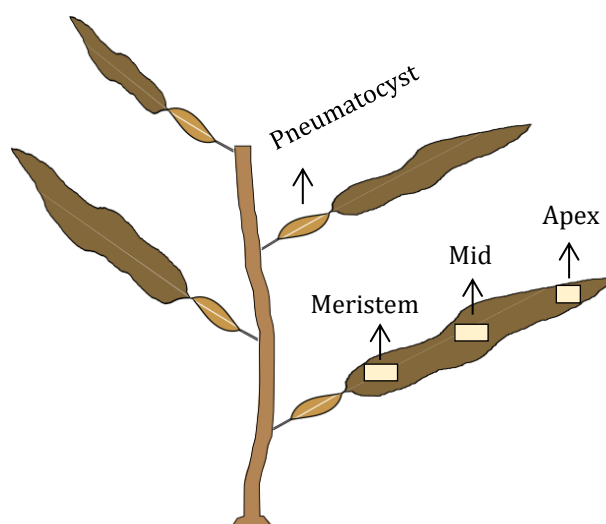


Figure 4.1: Schematic overview of the different blade tissue sections of *Macrocystis pyrifera*. Young blade tissue (meristem) and old blade tissue (apex). Pneumatocyst is the air bladder structure used by the kelp for buoyancy.

4.2.2 Molecular methods

4.2.2.1 DNA extraction and sequence library preparation

The cotton swabs containing kelp blade surface biofilms were cut off just above the cotton bud with sterile scissors and placed in a 2 mL microcentrifuge tube. DNA was subsequently extracted from the cotton buds using a modified version of the DNA extraction protocol described by Needham & Fuhrman (2016) and outlined in Chapter 3.

Analysis of bacteria and protist biofilm communities was performed by sequencing the V4 region of the 16S rRNA and 18S rRNA gene, respectively. For a detailed description of the library preparation see Chapter 3, however a brief summary will be provided here. Amplification of both V4 amplicons was performed using the KAPA HiFi HotStart DNA polymerase kit (Kapa Biosystems, Boston, MA, USA). The primers for the 16S rRNA gene were the updated universal Earth Microbiome Project primers (Caporaso et al., 2011, 2012; Apprill et al., 2015; Parada et al., 2016). For the 18S rRNA amplicons, the primers designed by Stoeck et al. (2010) were used. Both primer sets were additionally modified with overhanging sequences (Griffith et al., 2017) to enable the attachment of adaptors and indexes, during a second PCR round, necessary for downstream Illumina sequencing. The PCR mixtures and conditions, clean-up steps, and DNA quantification for both 16S and 18S amplicons were described in Chapter 3. A quick summary of the sequential steps involved is provided in Figure 4.2. Note, PCR inhibition was present for some samples due to the high presence of polysaccharides from the kelp. To successfully amplify these sample an extra 5 cycles for the R1-PCR were added.

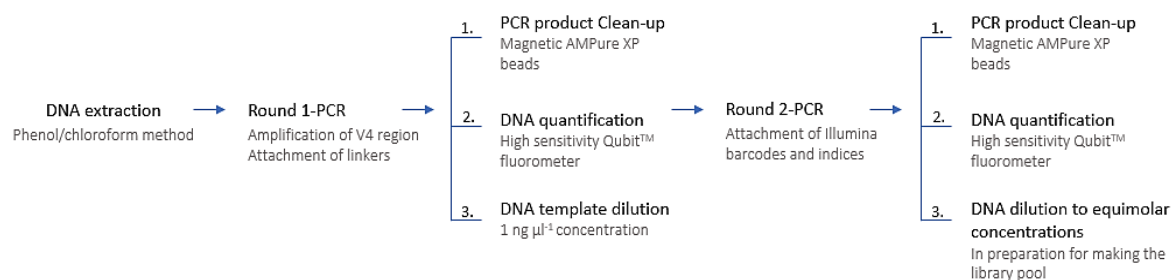


Figure 4.2: Flowchart of the molecular steps prior to DNA sequencing.

Finally, the samples were pooled into two libraries (16S and 18S), each with a concentration of 10 nM that was quantified with the Agilent Bioanalyzer 2100 (Agilent Technologies, USA) and subsequently sequenced by the Otago Genomics Facility (Otago University, New Zealand) on a MiSeq platform (Illumina, San Diego, CA, USA). For paired-end sequencing of the 16S rRNA amplicon a V2 (2 x 250 bp) run was performed, while the 18S rRNA gene required a V3 (2 x 300 bp) run due to its larger amplicon size.

4.2.3 Sequence analysis and Statistical Analyses

The raw sequences were run through the DADA-2 pipeline (Callahan, Sankaran, et al., 2016) in R (version 3.4.4; R Core Team, 2021) for post-processing thereby applying the DADA2 algorithm by Callahan & McMurdie (2016) to accurately identify high resolution amplicon sequence variants (ASVs). Only 2% of the total merged reads for both datasets were found to be chimeric and were

subsequently removed. Finally, taxonomy was assigned with *assignTaxonomy* and *assignSpecies* (DADA2 functions) using the SILVA taxa database (version 138.1 with species assignment; <https://benjjneb.github.io/dada2/training.html>) for the prokaryotes (16S) while the Protist Ribosomal Database (PR2 version 4.14; <https://github.com/pr2database/pr2database/releases>; Guillou et al., 2013) was used for the eukaryotes (18S). Sequences identified as Eukaryota, Chloroplast and Mitochondria were excluded from the prokaryotic kingdom and Metazoa affiliated sequences from the eukaryotes. Further, all downstream statistical analysis and graphical visualisations were performed using the R interface (version 3.4.4) mainly with the help of the “phyloseq” package (McMurdie & Holmes, 2013), the “vegan” package and *ggplot2* from the “tidyverse” package (Wickham et al., 2016 & 2019).

The normal distribution of the data was checked using the Shapiro test, while homogeneity of variance amongst the sample groups (location and age) was tested with the Levene test. Where the data met these assumptions, parametric tests (ANOVA or t-test) were used to define significance otherwise non-parametric analysis (Kruskal-Wallis or *Wilcoxon* Rank Sum test) were applied. Alpha diversity measures (Chao1 and Pielou index) were calculated with *estimate_richness* (“phyloseq”). A comparison of these indices between the two locations was analysed for the mid-section samples of the kelp blades. To test the variation in alpha diversity between the different kelp blade tissues (age comparison), samples from the base and the tip of the blade were used. This comparison was limited to medium and large sized blades (70 – 100 cm) of the exterior kelp bed to allow for a representation of healthy blades with enough individual replicates. Lastly, a comparison of diversity measures was made between all three sample types for both locations across the kelp bed.

The community composition (beta diversity) of the kelp blade associated biofilms was assessed for location and tissue age. Additionally, the community composition was also compared between the three sample types. This community variation was tested with Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson, 2017) using the *adonis* function (“vegan”) and visualised with an unconstrained non-metric multidimensional distance scaling (nMDS). A distance matrix was calculated for the PERMANOVA test using the Bray-Curtis dissimilarity index (focusses on species abundance data). Multivariate analyses were performed with 10,000 permutations and a seed of 711 for reproducibility. Variance of dispersions amongst groups was tested with the PERMDISP analysis (Anderson et al., 2006) to rule out a potential dispersion effect in case of a significant PERMANOVA result between the microbial communities. For the taxonomical composition, the most dominant bacterial and eukaryotic taxa groups were tested for a significant difference across the kelp bed and between the kelp blade tissues with a MANOVA test.

4.3 Results

Prior to analysing the metagenomic data, both datasets were rarefied to even sequence depths to validate comparisons of diversity measures amongst the samples. Note that this results in a loss of richness involving mainly low abundant ASVs. After rarefaction, the 16S sequences contained 7,552 paired reads (66 samples) and the 18S sequences 2,185 reads (68 samples). The macronutrient concentrations (nitrate, phosphate, ammonia and silica), chlorophyll-*a* levels and temperature range for the kelp forest study site were described in Chapter 3 (Table 3.2).

4.3.1 Kelp blade associated microbiome

4.3.1.1 Epiphytic microbial abundances

Analysis revealed that the blade-associated microbial communities (prokaryotic and eukaryotic biofilms) did not significantly differ in alpha diversity across the kelp bed (Figure 4.3). Additionally, no significant difference in alpha diversity was detected for prokaryotic communities along the different blade tissues (meristem and apex; Figure 4.3a,b). However, the number of unique ASVs differed as the meristem harboured fewer unique taxa (275 ASVs) compared to the tip of the blade (521 ASVs), suggesting a higher species richness for the apical tissue. Both tissue types had another 383 ASVs in common which mostly represented the high abundance ASVs. The difference in alpha diversity between the tissue types was stronger for the eukaryotic communities with significantly higher Chao1 richness (Wilcoxon test; $W = 32$, $p = 0.026$) for the apical tissue (103 unique ASVs) compared to the meristem (21 unique ASVs).

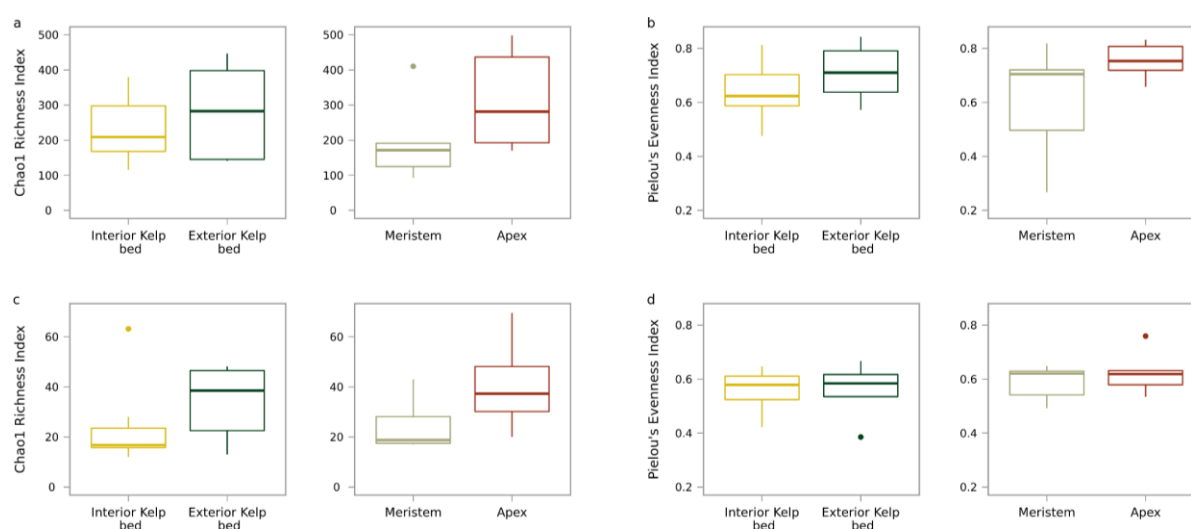


Figure 4.3: Alpha diversity measurements for kelp blade associated biofilms. (a – b) prokaryotic biofilm communities (16S) and (c – d) eukaryotic biofilm communities (18S). Species richness is depicted in (a) and (c), while species evenness is shown in (b) and (d). Yellow and green present the comparison between the two sampled locations while beige and red present the comparison between kelp tissues. Note, the different scales for prokaryotic and eukaryotic richness.

The meristem (young surface) associated assemblages shared a slightly higher fraction of their unique ASVs with the surrounding seawater communities (Chapter 3) compared to the apex affiliated biofilm communities. For the prokaryotic communities the young blade tissue shared 17% ASVs with seawater and the tip of the kelp blade shared 14.9%. A large difference in shared taxa was observed for the eukaryotic biofilms with the meristem sharing 30% of their unique ASVs with seawater and the apex 17%.

4.3.1.2 Epiphytic microbial community dynamics and taxonomy

PERMANOVA analysis revealed that the prokaryotic ($df = 1$, pseudo- $F = 1.67$, $p = 0.088$) and the eukaryotic ($df = 1$, pseudo- $F = 1.93$, $p = 0.058$) epiphytes were not significantly different between the two locations. PERMDISP analysis (p -values > 0.05) confirmed homogeneity of variances amongst the microbial groups. Noticeably, some variation between the replicates for both kingdoms was present (Figure 4.4a,c).

4.3.1.2.1 Prokaryotic kelp microbiome

In general, the kelp blade prokaryotic communities at both kelp locations were clearly dominated by *Proteobacteria* (62%) followed by *Bacteroidota* (15%), *Planctomycetota* (9%), *Verrucomicrobiota* (4.3%) and *Actinobacteriota* (4%) (Figure 4.4a). Collectively, these five dominant phyla accounted for approximately 94% of the total taxa. At class level, the epiphytic bacteria belonging to *Gammaproteobacteria* (44.5%; mainly *Granulosicoccaceae*) formed the most abundant group, followed by *Alphaproteobacteria* (16.5%; mainly *Hyphomonadaceae*). Taxa that were affiliated with the second most abundant phylum *Bacteroidota* were equally represented by two orders, *Chitinophagales* (8%; mainly *Saprospiraceae*) and *Flavobacteriales* (6%). *Planctomycetota* were mainly accounted for by one dominant genus, *Blastopirellula*. Likewise, *Verrucomicrobiota* and *Actinobacteriota* affiliated taxa belonged primarily to one taxonomical group *Rubritaleaceae* and *Microtrichaceae*, respectively. At the lowest taxonomical level, the most dominant ASVs ($n = 4$) accounted for 30% of all identified taxa. The top three ASVs were all grouped within the *Gammaproteobacteria* genus *Granulosicoccus* (approximately 7.5% each), while the fourth taxa was classified under the *Alphaproteobacterial* genus *Hellea* (7%).

4.3.1.2.2 Eukaryotic kelp microbiome

The four most abundant divisions representing the eukaryotic biofilms at both locations within the kelp bed were *Ochrophyta* (70.5%), *Chlorophyta* (19.7%), *Ciliophora* (7.6%) and *Psuedofungi* (1.2%) (Figure 4.4c). All taxa classified as *Ochrophyta* were affiliated with either *Phaeophyceae* (brown algae) or *Bacillariophyta* (diatoms). *Phaeophyceae* (66.5%) was the most dominant group, primarily represented by a single ASV *Ectocarpus siliculosus* (40%), followed by an unidentified brown algae (10.8%). Diatoms only accounted for 4% of all taxa. *Chlorophyta* (mainly

Ulvophyceae) affiliated taxa were predominately identified as *Acrochaete leptochaete*. *Ciliophora* (mainly *Phyllopharyngea*) were represented by two main dominant ASVs, the most abundant one was classified as genus *Trochilia* (3.7%; order *Cyrtophoria_8*) and the second highest was identified as genus *Acinetidae_1_X* (1.6%; order *Suctorina*). In contrast to the previously mentioned taxonomical groups, the relative abundance of *Pseudofungi* members was nearly absent from the interior bed (0.2%) while accounting for 2.3% of all ASVs at the exterior site. Neither of the four dominant divisions (MANOVA; $df = 1$, Pillai = 0.45, pseudo-F = 2.05 & $p = 0.162$) or the five classes (MANOVA; $df = 1$, Pillai = 0.55, pseudo-F = 2.2 & $p = 14$) dependently showed a significant difference between the two locations. However, independently (i.e. not taking correlations into account) the diatoms were significantly higher at the exterior bed ($p < 0.01$).

4.3.1.3 Kelp blade tissue type associated biofilm communities

The community compositions between blade tissue type (meristem and apex) were significantly different at the exterior bed for both bacterial ($df = 1$, pseudo-F = 2.02, $p = 0.038$) and eukaryotic epiphytes ($df = 1$, pseudo-F = 3.07, $p = 0.038$; Figure 4.4b,d). No group comparisons could be made at the interior bed due to the insufficient healthy representative samples. The homogeneity of dispersion was not violated for the tissue type factor.

4.3.1.3.1 Prokaryotic kelp microbiome

The biofilm communities of the meristem tissue were highly dominated by the *Gammaproteobacteria* genus *Granulosicoccus* (34.5%). The single most abundant ASV of all meristem associated sequences belonged to *Granulosicoccus* and accounted for 25% of all the taxa. In contrast, this ASV only accounted for 2.4% of all bacterial sequences on the apical tissue. As a result, *Gammaproteobacteria* represented a more diverse group of taxa on the tip of the blade with families *Granulosicoccaceae* (8.5%), *Arenicellaceae* (10%) and *Thiotrichaceae* (7%, mainly *Leucothrix*) in relatively equal proportions. Noticeably, the phylum *Bacteroidota* had a 2-fold higher relative abundance at the tip of the blade compared to the meristem. This resulted from an increase in both *Saprospiraceae* and *Flavobacteriaceae*. Note, on both blade tissues *Saprospiraceae* affiliated taxa were more abundant compared to *Flavobacteriaceae*. *Planctomycetota* sequences, mostly annotated to genus *Blastopirellula*, did not display drastic differences between the meristem and apical tissue, but nonetheless showed reduced numbers on the tip of the blade (meristem: 14%, apical: 9.5%). The remaining highly abundant taxonomical groups *Alphaproteobacteria* (20%), *Actinobacteria* (6%) and *Verrucomicrobiota* (4.5%) were very similar (± 1 -2%) between the two tissue types down to family level. At lower taxonomical level, the meristem was clearly dominated by a few single bacterial taxa, while the apical tissue of the kelp

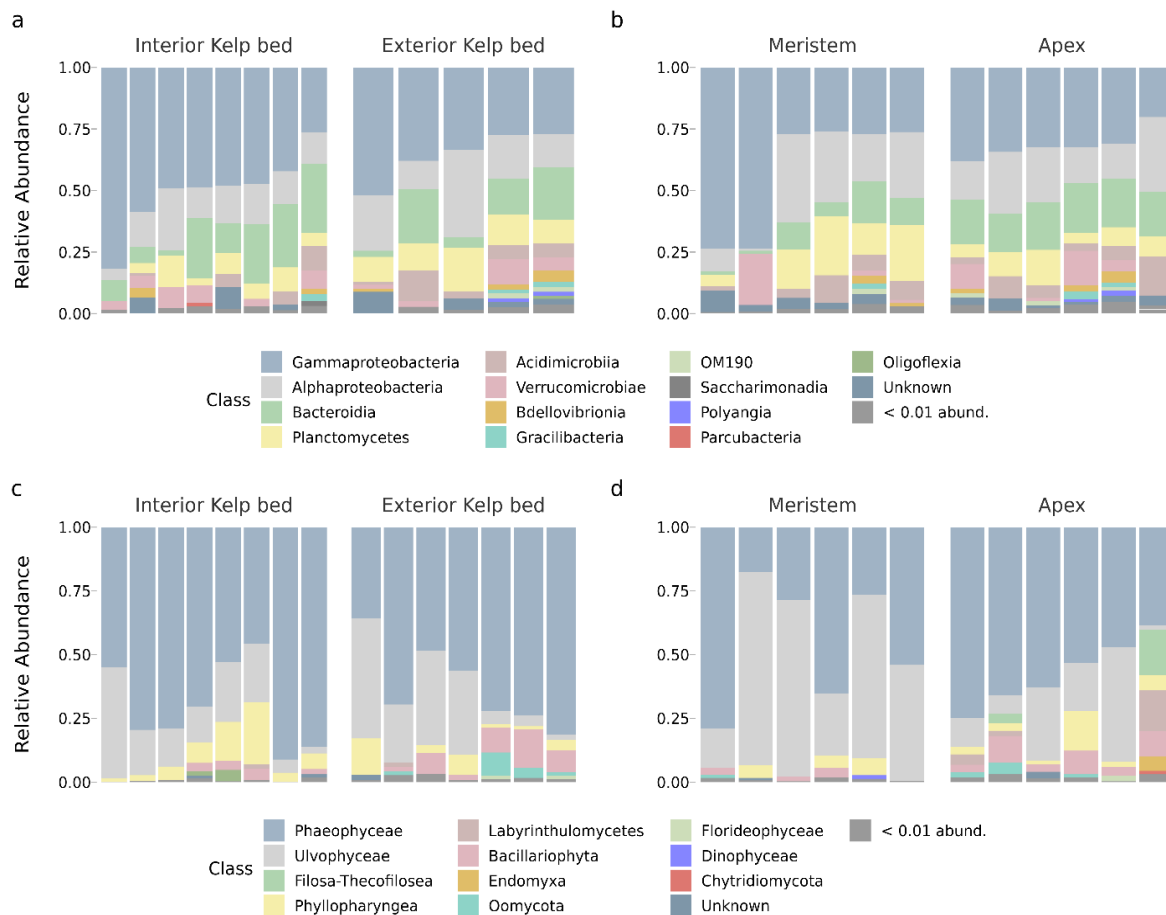


Figure 4.4: Taxonomical composition of kelp blade associated biofilms. (a – b) prokaryotic communities and (c – d) eukaryotic communities. (a,c) visualise the comparison between the two kelp bed locations for epibiotic communities on the medial region of the kelp blade and (b,d) show the comparison between kelp tissue age. Meristem = young surface, Apex = old surface.

blade hosted a greater diversity of bacterial individuals. The top eight most dominant ASVs on the young blade tissue accounted for 50% of all sequences, while the same proportion of relative abundance was accounted for by the top 21 most abundant ASVs on the older apical tissue. The biggest differences between the most dominant ASVs ($n = 10$) on both blade tissues were defined by two *Gammaproteobacteria* taxa, *Granulosicoccus* ASV (meristem: 25%, apex: 2.4%) and an *Arenicella* ASV (1.6% and 5%), and one *Verrucomicrobiota* taxa, a *Persicirhabdus* ASV (3.4% and 0.09%).

4.3.1.3.2 Eukaryotic kelp microbiome

The surfaces of the young blade tissue were dominated by *Ulvophyceae* (*Chlorophyta*) and *Phaeophyceae* (*Ochrophyta*; Figure 4.4d). Compared to the blade apex, the relative abundance of *Ulvophyceae* was more than twice as high on the meristem tissue (49% vs. 18.8%). *Phaeophyceae* however were more abundant at the tip of the blade (45.2% vs. 57%). These two families were each characterised by a few highly abundant ASVs. Two *Ulvella leptochaete* (*Ulvophyceae*) ASVs were in the top five most abundant ASVs at the meristem. One was the most dominant ASV in all

meristem assemblages with 25.8% and the other was ranked third highest with 19.3%. Both taxa were also part of the top five ASVs associated with the apical tissue but had a much lower relative abundance, 13.2% and 4.1%. *Ectocarpus siliculosus* was the most abundant ASV on the surface of the apical tissue with 30.3% while being the second most abundant ASV at the meristem with 24.3%. Diatoms (*Ochrophyta*) were overall not abundant on the surface of the kelp blade. At the meristem, they represented merely 1.5% of all ASVs compared to 6.2% at the apical tissue. *Ciliophora* were ranked as the third major taxonomical group for both blade tissues with 3.4% on the younger surfaces and 6.2% on the older surfaces. Interestingly, the blade tip showed *Cercozoa* (4.8%), *Sagenista* (3.7%) and *Pseudofungi* (1.5%) but these were almost completely absent at the meristem (all < 0.5%). Similar to the bacterial sequences, a greater diversity was witnessed on the surfaces of the older blade tissue. The top eight ASVs at the meristem collectively accounted for 86.3% of all taxa, while the top 16 ASVs were needed to roughly represent the same amount at the apex.

4.3.2 Comparison of the microbial community diversity of settlement plates, kelp blades and seawater

4.3.2.1 Alpha-diversity

The kelp blade associated prokaryotic biofilms were significantly lower in Chao1 richness compared to the settlement plates and seawater communities (KW; df = 2, H = 66.72 & corrected p-value < 0.001; Figure 4.5a). The prokaryotic communities on the plates and in the seawater did not significantly differ in species richness (pairwise tests; p > 0.05). Prokaryotic species richness was 385 ± 9.4 for the plate biofilms, 383 ± 10.4 for seawater, and 242 ± 7.3 for the kelp blade associated biofilms. All three sample types were significantly different from one another regarding the Pielou's evenness metric (KW; df = 2, H = 53 & corrected p-values < 0.05; Figure 4.5b). The highest evenness values were observed for the seawater prokaryotic communities (average = 0.82) while the lowest values were associated with the kelp blade prokaryotic biofilms (average = 0.69).

In agreement with the prokaryotic assemblages, the three eukaryotic communities were also significantly different from one another between the three sample types based on Chao1 richness (KW; df = 2, H = 129.07 & corrected p-values < 0.001; Figure 4.5c). Noticeably, the eukaryotic plate (average = 67.7 ± 5.9) and kelp biofilms (average = 37.6 ± 2.2) had a much lower species richness than the planktonic seawater communities (average = 209 ± 12.5). In terms of species evenness (Pielou index), the eukaryotic communities on the plates and the kelp blades were very similar and relatively low (average = 0.57), while seawater supported communities with a significantly higher species evenness (average = 0.77; KW, df = 2, H = 85.75 & corrected p-values < 0.001; Figure

4.5d). No differences in alpha diversity between the two locations (interior and exterior kelp bed) were detected for the eukaryotic communities. Overall, the prokaryotic assemblages revealed a higher magnitude of species diversity compared to the eukaryotes.

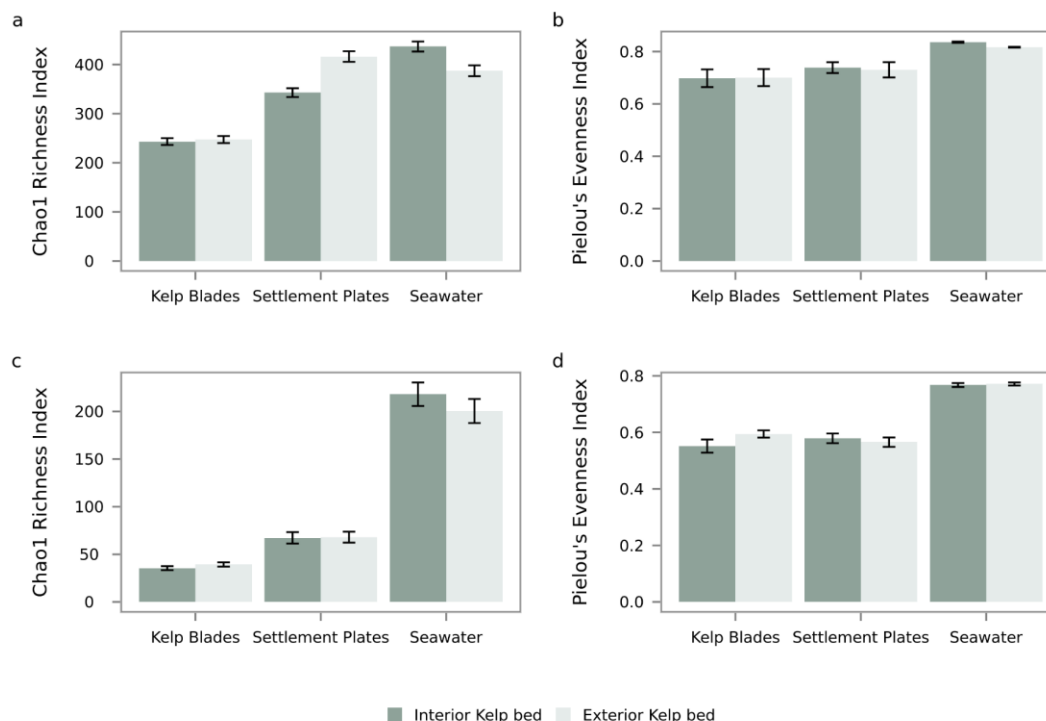


Figure 4.5: Comparison of alpha diversity measurements between the three sample types. (a – b) prokaryotic communities and (c – d) eukaryotic communities.

4.3.2.2 Beta-diversity

All three sample types displayed distinct prokaryotic and eukaryotic community compositions ($df = 2$; pseudo- $F = 64.20$ and 59.78 respectively, all corrected p -values = 0.001 ; Figure 4.6). However, the PERMDISP tests for both communities were also significant ($df = 2$; prokaryotes: $F = 137.66$ & eukaryotes: $F = 8.761$, both p -values < 0.001), indicating that the compositional variability might be the results of unequal dispersions amongst the sample groups. Pairwise analysis showed that for the prokaryotic communities the variance of dispersion of the seawater communities was heterogeneous compared to the plates and blades. Nevertheless, the unconstrained NMDS ordination had the confidence of a low stress value (0.11) and displayed a clear separation between the three sample types (Figure 4.6a). This indicates that 'location' was the primary effect responsible for the significant difference between the prokaryotic communities with a small contribution of dispersion effect. For the eukaryotic communities, pairwise analysis revealed that the kelp blade biofilms displayed unequal dispersions compared to the plates and the seawater communities. Here, the NMDS ordination (stress 0.13) provides further evidence that the

significant difference between the kelp blade and plate biofilms was mainly a result of the dispersion effect, while the difference between the kelp blade and seawater communities was mostly explained by a location effect (Figure 4.6b).

While all three sample types were distinct for the prokaryotic communities, they did have certain taxa in common. The kelp microbiome shared 34.8% of their taxa with the biofilm communities on the settlement plates and 17% with seawater microbes. The kelp eukaryotes however had 60% of their taxa in common with the biofilms on the settlement plates and 32.5% with seawater.

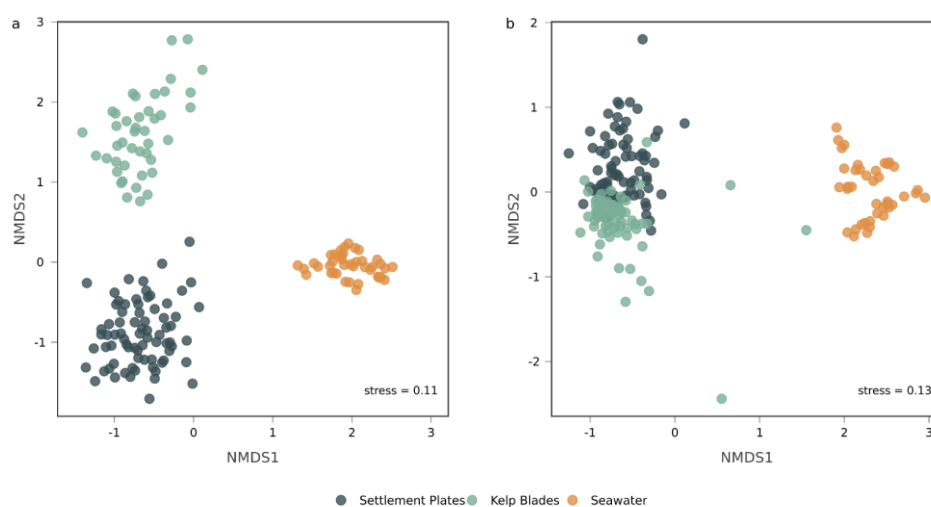


Figure 4.6: Community composition structuring based on Bray-Curtis dissimilarities amongst settlement plates, kelp blades and seawater. (a) prokaryotic communities and (b) eukaryotic communities.

Furthermore, the Bray-Curtis dissimilarities of the meristem associated communities for both kingdoms was compared with the biofilms on the settlement plates for each time point (Figure 4.7). For the prokaryotic communities no correlation was observed between living and non-living surface associated biofilms (Figure 4.7a) and each time point showed the same distance to the dissimilarity of the kelp meristem community. The eukaryotic communities however displayed differences between the time points. The young blade surface communities hosted more similar eukaryotic biofilms to the one-week-old biofilms versus the older biofilms on the settlement plates (Figure 4.7b). Additionally, the overall distance from the biofilms on the settlement plates to the dissimilarity of the meristem community was less strict compared to the prokaryotic communities.

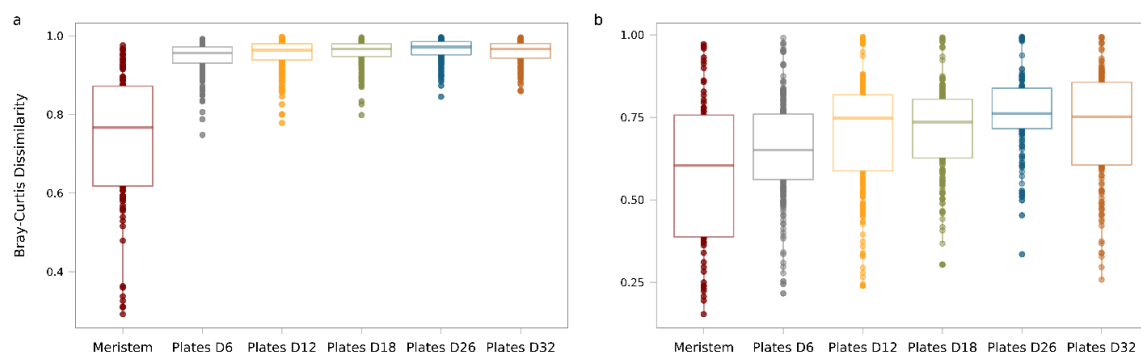


Figure 4.7: Bray-Curtis dissimilarity differences between biofilms associated with kelp blade meristem tissue and settlement plates over time. (a) Prokaryotic communities and (b) eukaryotic communities. Colours represent the different biofilm communities: kelp blade meristem (red), biofilms on the settlement plates (grey, yellow, green, blue, and orange). The biofilms on the settlement plates are shown for each sampling day: day 6 (D6), day 12 (D12), day 18 (D18), day 26 (D26), and day 32 (D32).

4.4 Discussion

4.4.1 Microbial communities of kelp blades, settlement plates and seawater were distinct

The bacterial communities associated with *Macrocystis pyrifera*, the settlement plates and ambient seawater were all distinct from one another (Figure 4.6 & Figure 4.8). The difference in community structure between free-living and surface attached prokaryotic communities was expected since it has been consistently reported for various kelp species (Staufenberger et al., 2008; Lachnit et al., 2009; Bengtsson & Øvreås, 2010; Burke et al., 2011; Mancuso et al., 2016; Weigel & Pfister, 2019; Lemay et al., 2021) and different types of abiotic surfaces (Jones et al., 2007; Lee et al., 2008; Zhang et al., 2014). Meanwhile, the dissimilarity in biofilm composition between the abiotic (plates) and biotic (kelp) surfaces strongly contributed to the consensus that macroalgae organisms exert selection pressure on the recruited microbial communities (Lachnit et al., 2009; Burke et al., 2011; Lemay, Martone, Hind, et al., 2018; Weigel & Pfister, 2021). This follows from the substrates being located in the same environment, had access to the same pool of microbial colonisers and were subjected to the same local biotic and abiotic variables. Hence, the current results support the finding that kelp hosts play a strong role in structuring the bacterial epibionts. Similar results were obtained by Lemay et al. (2018) and Weigel & Pfister (2021), who found distinct bacterial populations between kelp tissue surface and rocky or artificial substrates, respectively.

Eukaryotic communities did not reveal the same level of specificity with the kelp host as the bacterial assemblages. While biofilm communities on the plates and kelp were obviously distinct

from the seawater communities, they did not establish completely separate communities among each other (Figure 4.6). This suggests a higher similarity between the plate and the kelp blade associated eukaryotes compared to seawater. In comparison to the epiphytic bacterial populations, *M. pyrifer* exerted a lower degree of selection on eukaryotic taxa. This is in agreement with two other studies who reported that the relationship between kelp-host and associated eukaryotic communities is weaker compared to bacterial epibionts but still significant (Lemay, Martone, Hind, et al., 2018; Lemay et al., 2021). Furthermore, the same trend was demonstrated by species diversity. Kelp associated eukaryotic communities were more similar in diversity to the biofilms on the settlement plates, with both surface types supporting a community structured by a few very abundant species and an overall reduced species richness. The higher similarity between the surface associated eukaryotes, in comparison to the kelp-specific prokaryotic microbiome, suggests a stronger interaction of the kelp with the prokaryotic biofilm

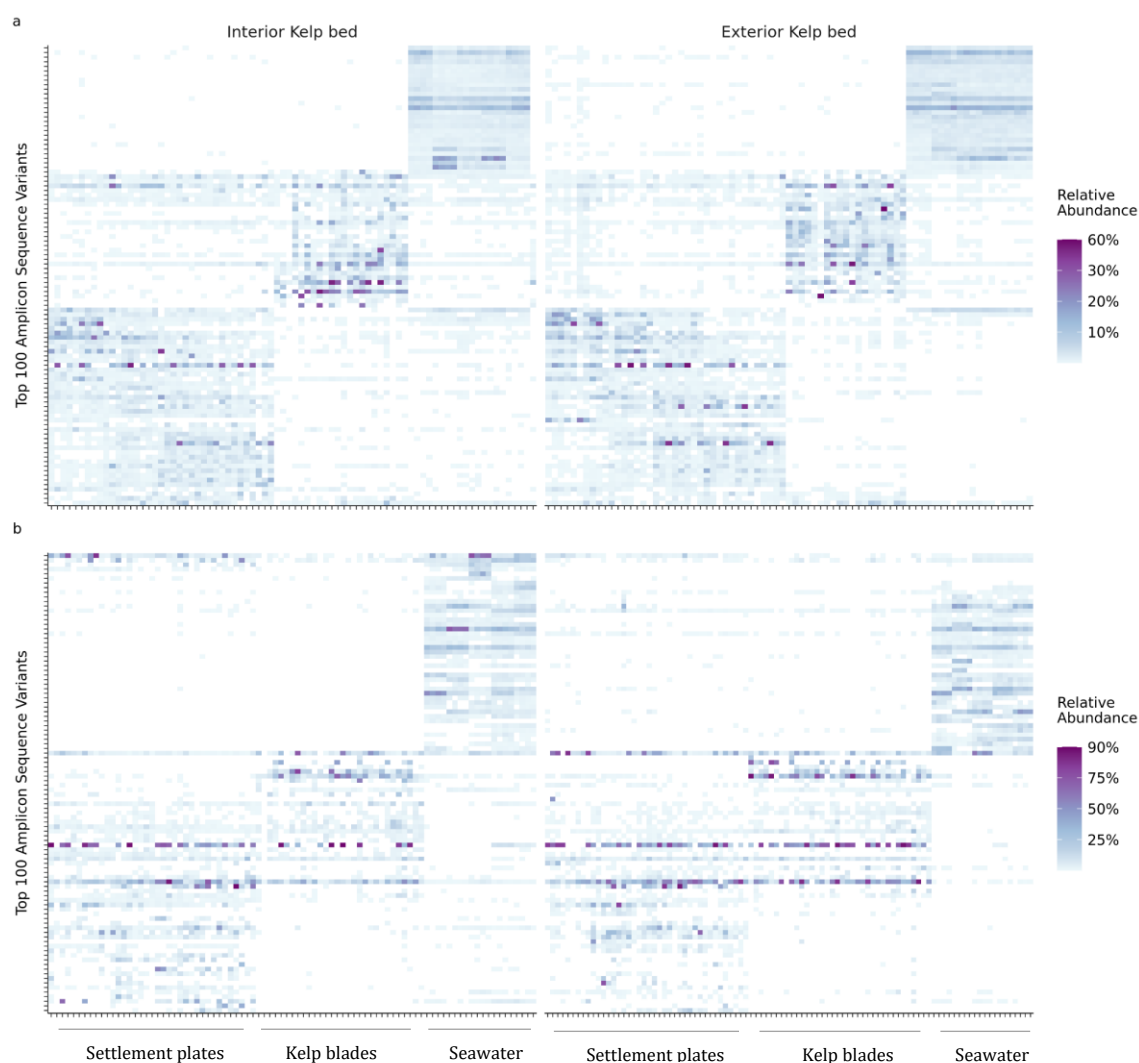


Figure 4.8: Heatmap of the top 100 most abundant amplicon sequence variants for the three sample types. (a) Prokaryotic communities, (b) eukaryotic communities. Note the different relative abundance scale bar for prokaryotic and eukaryotic communities.

e.g. through the release of dissolved organic matter. It also indicates that kelp associated eukaryotes are more region specific than kelp-host specific.

Spatial structuring for prokaryotic and eukaryotic assemblages across the kelp bed was found for the early biofilms on the settlement plates and the seawater microbial communities but not for the kelp microbiome. However, the statistical power was only marginally non-significant for the eukaryotes. Even though the lack of significance could be due to the small sample sizes and inter-sample variability, the homogenous community composition across the kelp bed could also be a reflection of the kelp selection pressure. It follows that the selection of the kelp on the microbiomes is stronger than the impact of the environmental parameters. This is in line with studies reporting host-phylum and host-species specificity of the microbial communities on a regional scale (Lachnit et al., 2011; Weigel & Pfister, 2019).

4.4.1.1 Taxonomy of kelp-associated prokaryotic communities

The same major prokaryotic phyla were found in all three sample pools, including predominant phyla *Proteobacteria* and *Bacteroidota*, and less abundant *Planctomycetota*, *Verrucomicrobia* and *Actinobacteria*. It is common to see little distinction in microorganisms at higher taxonomic level (Longford et al., 2007; Bengtsson & Øvreås, 2010; Mancuso et al., 2016; Weigel & Pfister, 2019) and these phyla have previously been shown to be abundant in both free-living and surface attached microbial communities (Rappé et al., 2000; Burke et al., 2011; Hollants et al., 2013; Dang & Lovell, 2016). Accounting for up to 80 – 90%, *Proteobacteria* and *Bacteroidota* clearly dominated all prokaryotic assemblages. While *Alphaproteobacteria* was the most abundant class for the bacterial communities associated with seawater and the settlement plates, *Gammaproteobacteria* made up the largest part the kelp blade microbiome. Both *Gammaproteobacteria* and *Bacteroidota* are two major groups that contain an extensive repertoire of polysaccharide degradative enzymes (Edwards et al., 2010) allowing them to rapidly and successfully colonise the complex polysaccharide-rich extracellular matrix of brown algae (Michel et al., 2010; Lin et al., 2018).

Bacteria belonging to *Planctomycetota* (mainly *Blastopirellula*) were more abundant (9-fold) on the kelp blades compared to the other sample types, consistent with prior work on *M. pyrifera* (Vollmers et al., 2017; Lin et al., 2018; Weigel & Pfister, 2019). For *Laminaria hyperborean* from Norway (Bengtsson & Øvreås, 2010) and *Fucus vesiculosus* from the Baltic Sea (Lachnit et al., 2011), *Planctomycetota* were the most abundant bacterial group. Similarly, *Verrucomicrobiota* (mainly *Persicirhabdus*) had a much higher abundance on the kelp blade surfaces compared to the other samples. Both genera (*Blastopirellula* and *Persicirhabdus*) appear to be selected for by *M. pyrifera* (Figure 4.9). Indeed, the enriched presence on kelp surfaces of both genera has been

increasingly reported across different kelp-species and has been attributed to their active degradation metabolism of sulphated polysaccharides produced by macroalgae (Lage & Bondoso, 2014; Vollmers et al., 2017; Lin et al., 2018). Finally, the presence of *Actinobacteria* on *M. pyrifera* has previously been linked to the agarolytic and carrageenanolytic properties of this group, and thus were assumed to be attracted to the kelp polysaccharides (Michelou et al., 2013). The slightly higher numbers of *Actinobacteria*-related organisms (mainly *Microtrichaceae*) on the kelp are in line with those findings. However, the same taxa were also observed on the settlement plates, suggesting that members of the family *Microtrichaceae* are more generalist biofilm colonisers.

Strikingly, *Cyanobacteria* (primarily *Synechococcus*) was the third most abundant phylum for the seawater communities but was near absent on both biofilm substrates. The low abundance of kelp-associated *Cyanobacteria* compared to ambient seawater was also observed for *M. pyrifera* in Calvert Island, Canada (Lin et al., 2018). *M. pyrifera* microbiome studies in Monterey Bay, CA (Michelou et al., 2013) and Puget Sound, Washington, US (Weigel & Pfister, 2019) did not mention *Cyanobacteria* as a dominant or low abundant bacterial phylum. Their near absence on *M. pyrifera* across different studies indicates that this could potentially be a general feature of *M. pyrifera*.

The most abundant taxon associated with the kelp blade biofilms was *Granulosicoccus* sp. (*Gammaproteobacteria*). The extremely high abundance of one single *Granulosicoccus* ASV was unique to the kelp tissue surfaces (Figure 4.9). Even at order level, members of this group were only observed at low relative abundance in seawater and on the settlement plates. This gives strong evidence that *Granulosicoccus*-related organisms were specifically selected by the *M. pyrifera* blade environment. Sequences annotated to the genus *Granulosicoccus* have been isolated from seagrass (Kurilenko et al., 2010), brown algae (Park et al., 2014), and from Antarctic seawater (Lee et al., 2007). They are obligate aerobes as well as chemoheterotrophs capable of nitrate reduction (Park et al., 2014; Baek et al., 2014). Overall, bacteria from the genus *Granulosicoccus* have been identified as important core epiphytic colonisers in brown macroalgae (Park et al., 2014), such as *F. vesiculosus* (Lachnit et al., 2011), *S. latissima* (Staufenberger et al., 2008), *N. luetkeana* and *M. pyrifera* (Weigel & Pfister, 2021), but also in the red alga *Delisea pulchra* and the green alga *U. australis* (Longford et al., 2007). The kelp-association with this genus across different kelp-phylum, kelp-species and geographical regions generalises that *Granulosicoccus* are actively selected for by macroalgae.

The seawater communities were dominated by the well-known SAR11 clade (*Alphaproteobacteria*), while *Yoonia* sp. (*Alphaproteobacteria*) was the most abundant ASV on the settlement plates (Figure 4.9). For the seawater samples, it is not surprising that the SAR11 clade had the highest prevalence since they have been documented as the most abundant

bacterioplankton in marine environments (Morris et al., 2002; Elifantz et al., 2013; Giovannoni, 2017). SAR11 bacteria are aerobic, strictly free-living, chemoheterotrophic microbes with an ancient evolutionary origin and have evolved the ability to efficiently harvest low-molecular-weight dissolved organic matter that are abundantly found in the water column (Elifantz et al., 2013; Giovannoni, 2017). In 2018, several *Loktonella* species were reclassified into the novel genus *Yoonia* (Wirth & Whitman, 2018). *Yoonia* sp. are characterised as members of the family *Rhodobacteraceae*, they are aerobic and lack the ability to perform nitrate reduction. While *Yoonia* sp. was the most abundant taxa on the settlement plates it was only present in very low numbers on kelp tissue and in seawater, suggesting that the kelp might deter this species. However, *Yoonia* sp. (formerly *Loktanella*) has previously been identified in several macroalgae species (incl. brown, green and red algae) ranging from low (Burke et al., 2011), mid (Lachnit et al., 2011; Miranda et al., 2013; Stratil et al., 2013) to even high relative abundances (Mancuso et al., 2016). Interestingly, these studies covered a variety of kelp at different taxonomic levels (division-level, species-level), geographic regions and season. Nevertheless, a similarity between these studies was observed at class level where *Yoonia* sp. appeared to be present only when *Alphaproteobacteria* overall exhibited much higher abundances than *Gammaproteobacteria*. This is also consistent with current results as *Alphaproteobacteria* dominated the biofilm communities on the settlement plates while *Gammaproteobacteria* were the most abundant symbionts on the kelp surfaces. While it is currently believed that *Yoonia* species tend to behave as generalists and opportunists, additional research into the functional metabolomics of this genus could shed more light on the clear association of this species with strictly high abundances of *Alphaproteobacteria* or specifically reduced abundances of *Gammaproteobacteria* on kelp surfaces.

Finally, the genus *Amylibacter* (formerly *NAC11-7 lineage*) was consistently present on the settlement plates and in the water column (Figure 4.9), yet it was almost entirely absent from kelp tissue. *Amylibacter* is a strictly aerobic alphaproteobacterial genus (Teramoto & Nishijima, 2014) a trait that should allow this genus to persist on the kelp surface from a metabolic point of view (Weigel & Pfister, 2021). Specifically, the most abundant *Amylibacter* species found on the plates and in seawater was *Amylibacter ulvae*, a species first isolated from a green macroalgae (Nedashkovskaya et al., 2016). Additionally, *Amylibacter* has also been detected on seagrass and in low amounts on green algae *Caulerpa cylindracea* (Korlević et al., 2021). It is possible that *M. pyrifera* deters species related to this genus or that these non-motile species lack the efficiency to rapidly colonise the kelp surface and compete for space. Another possibility may be that this genus is linked to diatom abundance. Since *Roseobacter* members and diatoms are known to have a strong relationship during planktonic lifestyle (Amin et al., 2012), this could also hold true for surface associated assemblages. Hence, the low observed diatom numbers on kelp (see later

section 4.4.1.2.2) could be responsible for the equally low occurrence of *Amylibacter* species. However, further research using network analysis would be required to validate or nullify this speculation.

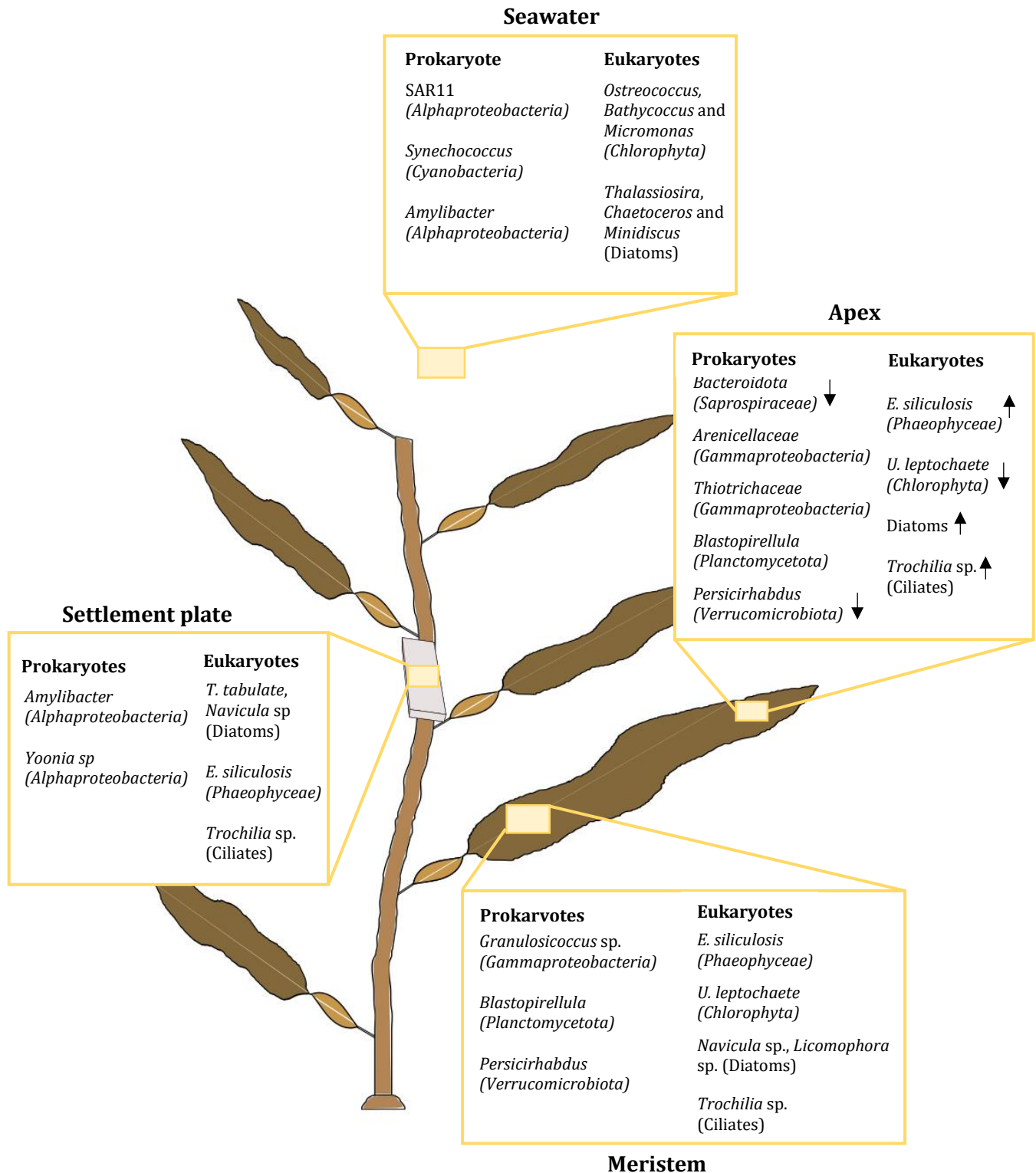


Figure 4.9: Comparison of the most dominant taxonomic groups between the three sample types. Kelp blades tissues: young tissue (Meristem) and older tissue (Apex). Arrows indicate an increase or decrease compared to the taxonomic groups on the Meristem.

Despite the clear separation of the three different sample types based on community composition, similarities in taxa were present as well (Figure 4.8). As expected, the surface attached communities shared the highest portion of taxa with each other (kelp shared 34.8% of their ASVs with plate biofilms), while they had a lower fraction of their microbes (approximately 17%) in common with the seawater samples. The amount of shared taxa between kelp associated microbiomes and seawater communities gathered from previous studies covers a wide range of proportions, including 2% for *M. pyrifera* (Michelou et al., 2013), 32% for both *N. luetkeana* and *M. pyrifera* (Weigel & Pfister, 2019), and 86% for sympatric kelp species (Lemay, Martone, Keeling, et al., 2018). This high variability is likely linked to the differences underlying the studies including geographic location, seawater retention time, seasonality, sampling method, and sequencing depth.

4.4.1.2 Taxonomy of kelp-associated eukaryotic communities

4.4.1.2.1 Comparison of epi-endophytic pigmented algae (Chlorophytes and Phaeophytes)

The biggest similarity between the kelp and plate associated communities was the overwhelming dominance of epi-endophytic brown algae, specifically *Ectocarpus siliculosus*. *E. siliculosus* together with *E. fasciculatus* make up the genus *Ectocarpus* and represent brown micro-filamentous algae that are well known fouling species (Morris & Russell, 1974). *E. siliculosus* has been identified as the main epi-endophytic algae on *Fucus vesiculosus* in the northern Baltic Proper (Kiirikki & Blomster, 1996). However, most reports of *Ectocarpus* sp. involved *E. fasciculatus* which has previously been isolated as an endophytic algae from *Laminaria saccharina*, *L. hyperborea* and *L. digitata* (Russell, 1983; Ellertsdottir & Peters, 1997). Here, the high prevalence of *E. siliculosus* on both settlement surfaces during late summer is consistent with previous studies that witnessed propagule release of *Ectocarpus* during summer after which the species stayed hidden during autumn and winter as a microthallus growing epi- or endophytically on other macroalgae (Russell, 1983; Kiirikki & Lehto, 2012). Further, the occurrence of this species, and brown filamentous algae more generally, on the settlement plates reveals that they are facultative colonisers who do not necessarily require specific kelp nutritional compounds.

In contrast to the high similarity of brown pigmented algae on the plates and kelp, both green and red epi-endophytic algae differed strongly between both substrates. For instance, green algae had an enriched presence on the kelp surfaces and in the water column but not on the settlement plates (Figure 4.9). Additionally, green algae on the kelp blade and in the surrounding seawater were represented by very different species. The high prevalence of the green algae *Ulvacella leptochaete* (previously *Acrochaete leptochaete*) (Rinkel et al., 2012; Nielsen et al., 2013) suggests an obligate relationship. *U. leptochaete* has previously been isolated as an endophytic species from

green algae *Chaetomorpha* in China (Deng et al., 2011, 2012), as well as from green algae *Cladophora glomerata* and red algae *Laurencia obtusa* in India (Bast et al., 2014). The green algae species identified in the seawater samples were dominated by three pico-eukaryotic genera of *Chlorophyta* (*Ostreococcus*, *Bathycoccus* and *Micromonas*; Moreau et al., 2012). Despite brown (*Phaeophyceae*) and green algae (*Chlorophyta*) being greatly enhanced on kelp, red algae (*Rhodophyta*) were barely detected. Red epi-endophytic algae were neither casual nor obligate colonisers on *M. pyrifera* tissue.

To date, no observations of epi-endophytic *E. siliculosus* and *U. leptochaete* on *M. pyrifera* have been reported. The use of next generation sequencing with universal 18S primers and the continuous improvement of online sequence databases could have greatly contributed to the in-depth identification of the true eukaryotic communities on the kelp blade. The high abundance of both chlorophytes and phaeophytes could also be linked to the temporal window explored in this study. There is a possibility that a shift took place during summer from eukaryotic symbionts to predominately epi-endophytic pigmented algae. This would then be linked to the increase in decay of *M. pyrifera* blades in late summer as the macroalgae host undergoes higher levels of stress that can be phenotypically observed through increased epiphytic growth (Marzinelli et al., 2015). This is mere speculation and cannot be confirmed with this dataset as samples were only taken during late summer, but it prompts the need for seasonal studies. Because no previous studies have focussed on kelp associated eukaryotic communities in New Zealand, the high abundance of epi-endophytic pigmented algae could also simply reflect the local or regional kelp ecosystem dynamics.

4.4.1.2.2 Comparison of epi-endophytic eukaryotes

Strikingly, diatom numbers on *M. pyrifera* were much lower compared to previous reports of several macroalgae host (Totti et al., 2009; Tiffany, 2011; Majewska et al., 2016; Mayombo et al., 2020) and also in comparison to the settlement plates and ambient seawater. However, it is possible that the true diatom diversity and abundance is being shrouded by the overwhelming numbers of the epi-endophytic pigmented algae. If the latter were excluded from biofilm communities, *Ciliophora* would become the most abundant taxa on the kelp blades followed by diatoms and other potentially abundant taxa which are underrepresented or undetected by the current sequencing efforts.

The diatom community found on the settlement plates was strongly dominated by *Tabularia tabulate* (Chapter 3) followed by the small motile *Navicula* sp., while the seawater communities were characterised by typical centric pico- and nanodiatoms (*Thalassiosira*, *Chaetoceros* and *Minidiscus*) (Figure 4.9). The diatom species on kelp tissue surfaces were similar to the plates and

mainly dominated by two small motile species, *Navicula* sp. and *Licomophora* sp. The latter is in contrast with previous studies on kelp epiphytic diatoms that have mostly found a strong dominance of adnate forms such as *Cocconeis* spp. and *Amphora* spp. (Totti et al., 2009; Tiffany, 2011; Majewska et al., 2016; Mayombo et al., 2019, 2020), which were entirely absent on *M. pyrifera* blades. It is noteworthy that most studies were performed during spring and early- to mid-summer whereas current work took place in late austral summer. This could explain the presence of the small diatom species observed for all three sample types as they are known to be more advantageous in post-bloom conditions (late summer), marked by lower nutrient concentrations, due to their high surface area to volume ratio (Durbin et al., 1975). Similarly, Majewska et al. (2016) observed a strong seasonal influence on epiphytic diatom communities and reported an increase of small motile diatom species towards the end of austral summer. Additionally, both Thomas & Jiang (1986) and Majewska et al. (2016) found evidence that macroalgal morphology also greatly impacted the associated epiphytic diatom community. Overall, macroalgae-specific and seasonally selected diatom epibionts could explain the contrasting findings in literature related to kelp tissue associated diatoms.

Similar to diatoms, both the kelp and the settlement plates were characterised by the same dominant ciliate species, i.e. *Trochilia* sp. In contrast, members of *Ciliophora* in seawater were more diverse and not dominated by a single taxa. The presence of *Trochilia* sp. on both living and non-living surfaces suggest that these ciliates are not obligate kelp coloniser but more likely generalist biofilm heterotrophs.

4.4.2 Fine scale spatial microbial variation along *M. pyrifera* blades

4.4.2.1 Distinct prokaryotic communities on kelp blade tissue

At high taxonomical level the same bacterial groups were present on the meristem and apex tissue of the blade (Figure 4.9). However, at a fine taxonomical scale (ASV level) and including bacterial relative abundances the apex and meristem tissue surfaces exhibited different communities. Dominance of a single *Granulosicoccus* classified ASV on the surface of young meristematic tissue is in line with previous studies of kelp meristem, such as *Saccharina japonica* (Balakirev et al., 2012), *Laminaria hyperborea* (Bengtsson et al., 2012), *Nereocystis luetkeana* and *M. pyrifera* (Weigel & Pfister, 2019), and *Laminaria setchellii* (Lemay et al., 2021). In these studies, members of the genus *Granulosicoccus* have been recognised as pioneer colonisers on young kelp surfaces and therefore play an important role in the early successional stages of epibiont biofilm development. This is also supported by the findings in the present study. On the apical tissue the dominance of genus *Granulosicoccus* was highly reduced while two other *Gammaproteobacteria* families, *Arenicellaceae* and *Thiotrichaceae* increased in abundance. Their presence was also

noticed on the settlement plates but at low relative abundance compared to the tip of the blade. *Arenicellaceae* sequences are chemo-organotrophic and strictly aerobic (Nedashkovskaya et al., 2013; Rua & Thompson, 2014). Although these bacteria have received little attention, they have been previously associated with the brown kelp *S. japonica* (Balakirev et al., 2012) and more specifically with the core bacteria group on the blade tip of *L. setchellii* (Lemay et al., 2021). *Thiotrichaceae* members are well known chemolithotrophic sulfur-oxidizing bacteria (Garritty et al., 2005). Similar to *Arenicellaceae*, they were also identified as part of the core group of apical tissue of *L. setchellii* (Lemay et al., 2021). This may suggest a higher production of inorganic sulphur on older kelp tissue and tissue-specific abundances of polysaccharides and kelp-produced carbon exudates. *Bacteroidota* (mainly *Saprospiraceae*) were found to be substantially more enriched at the tip of the blade. This is in agreement with the study of Lin et al. (2018) who discovered higher numbers of *Saprospiraceae*-related organisms on older *M. pyrifera* blade tissue occurring at lower depths in the water column. This may be explained by their ability to breakdown complex carbon polymers, which relates to increased polysaccharides and carbon exudates on older kelp tissue (McIlroy & Nielsen, 2014).

ASVs classified as *Persicirhabdus* (*Verrucomicrobiota*) and *Blastopirellula* (*Planctomycetota*) were reasonably abundant on both meristem and apical tissue and are considered to be obligate colonisers (see previous section 4.4.1.1). Despite their obvious role as primary colonisers (Chen & Parfrey, 2018; Lin et al., 2018; Pfister et al., 2019), they retained similar abundance levels at the apical tissue which indicates an additional secondary importance to the kelp. Noticeably, their presence on young meristem tissue is somewhat unexpected since both bacteria groups are known to be slow-growing and would likely be outcompeted by the more rapidly growing *Proteobacteria* and *Bacteroidota*. However, in a study by Vollmers et al. (2017) they found that both groups also contained a large fraction of encoded genes associated with secondary metabolism. They proposed that kelp-hosts would attract these bacteria by producing certain signalling carbohydrates and then use the antimicrobial secondary metabolites produced by *Verrucomicrobiota* and *Planctomycetota* as biofouling control against other undesired microbes. This would explain their unique but consistent presence on the kelp blade meristem and apex. Lastly, species classified as *Hyphomonadaceae* genera (*Hellea*, *Litorimonas*, and *Robiginitomaculum*) were also an important part of early colonisation on *M. pyrifera* blades but were not exclusively found on kelp meristem tissue. They were observed in equal abundances on the apical tissue and on the settlement plates hinting that *Hyphomonadaceae* species are generalist biofilm forming bacteria and thus facultative epiphytes on kelp. This is consistent with the literature on *Hyphomonas* bacteria, identifying them as primary colonisers on abiotic and biotic

surfaces as a result of the bacteria having a polar holdfast structure that facilitates surface attachment (Dang & Lovell, 2016 and references therein).

4.4.2.2 Distinct eukaryotic communities on kelp blade tissue

The most dominant eukaryotic groups on the surface of *M. pyrifera* young blade tissues were primarily epi-endophytic chlorophytes (green algae) and phaeophytes (brown algae), followed by *Baciliariophyta* (mainly pennate diatoms) and *Ciliophora*. Noticeably, on the tip of the blade three additional groups were also present, *Cercozoa*, *Sagenista* and *Pseudofungi*. The older apical tissue clearly represented a more diverse group of taxa compared to the meristem, which is in line with the patterns observed for the bacterial communities (Bengtsson et al., 2012; Weigel & Pfister, 2019; Lemay et al., 2021).

4.4.2.2.1 Epi-endophytic pigmented algae (Chlorophytes and Phaeophytes)

The green filamentous alga *U. leptochaetei* was the most abundant taxon on the meristem tissue accounting for half of the identified eukaryotic taxa. However, with tissue age a 2.5-fold reduction in green algae abundance, specifically *U. leptochaetei*, was observed. Nonetheless, the chlorophytes still made up the second most abundant group at the tip of the blade. Since the settlement of *U. leptochaetei* on kelp tissue was identified as obligate in this local ecosystem (see previous section 4.4.1.2.1), it is highly likely that this epi- or endophyte is strictly dependent on kelp-derived exudates. Their prominent presence on the young tissue could therefore be linked to the release of increased carbohydrates as a result of high meristem photosynthetic capacity (Arnold & Manley, 1985).

In contrast to green algae, *Phaeophyceae* abundance increased along the tissue age gradient, becoming the most dominant group at the tip of the blade. The high prevalence of the group was clearly driven by the single ASV *E. siliculosus*. Higher densities of epi-endophytes on older tissue surfaces could be the result of more colonizing events that took place over time. Since *E. siliculosus* was identified as a facultative epi-endophyte (see previous section 4.4.1.2.1), competition for available substrate with other eukaryotic taxa would most logically explain the increased abundance on the blade tip.

4.4.2.2.2 Epi-endophytic eukaryotes

In addition to the epi-endophytic pigmented algae, diatoms and ciliates were also identified as primary colonisers on the kelp blade meristem. Even though diatoms had a drastically reduced abundance on the *M. pyrifera* blades (see previous section 4.4.1.2.2) there was still a difference in numbers between the young and old tissue surface. Diatom abundance and diversity were both increased on the old apical tissue which was also observed for *Ecklonia maxima* and *Laminaria*

pallida in South Africa (Mayombo et al., 2019, 2020). However, the opposite pattern was reported for diatoms on *Laminaria setchellii* with higher numbers on younger tissues (stipe and meristem) compared to older tissues (holdfast and blade tip; Lemay et al., 2021). Both tissue surfaces were primarily associated with the small motile diatoms *Navicula* sp and *Licomophora* sp., indicative of late summer (see previous section 4.4.1.2.2). The preferential abundance of diatoms on the blade tip could be interpreted as a combination of factors. First, the meristem is affiliated with a higher production of antibacterial and grazing deterring compounds that limits the recruitment and colonisation of most epiphytic organisms (Mayombo et al., 2020). Secondly, the older tissues are generally more damaged and thereby release more dissolved organic matter that can be used as a nutrient source by heterotrophic bacteria and protists (Mayombo et al., 2020). Thirdly, the reduction in abundance of green algae with tissue age allows diatoms to compete more effectively for the higher availability of substrate and dissolved organic matter on the apical tissue. In addition to these factors, abiotic parameters such as seasonality could also have had a huge impact (Michelou et al., 2013).

Ciliates mirrored the same diversity pattern of the diatoms and bacterial epiphytes across tissue age. This is not surprising since ciliates are predators that feed on both bacteria as well as other eukaryotes (Armstrong et al., 2000). They were slightly more abundant on the meristem than the diatoms but showed equal relative abundance at the tip of the blade. Small ciliates affiliated with genus *Trochilia* were the most dominant taxa on the surface of both tissues. *Trochilia* taxa are bacterivorous, belong to order *Cyrtophoria* and are characterised by a podite structure that facilitates their attachment to substrates (Gismervik, 2004). The second most abundant taxa on the meristem was *Ephelota mammillata*, while on the apical tissue this was an *Acinetidae_1_X* sp. Both secondary taxa are associated with a carnivorous lifestyle and were followed by a diverse group representing both predation types equally. This suggests that there was no change in predation regime along the tissue age gradient. Similar to the diatoms, the dominance of these small ciliates in late summer indicates a connection to seasonality. The latter was reported by Gismervik (2004), who observed seasonal succession (spring to late summer) of ciliate taxa associated with macroalgae in a *Laminaria hyperborean* dominated kelp forest.

As mentioned before, it is likely that a more diverse group of diatoms and ciliates are associated with kelp tissues but were not detected due to the high dominance of the epi-endophytic pigmented algae. Furthermore, the clear influence of temporal succession suggests that more research is needed to explore the full diversity of the eukaryotic taxonomical groups that are associated with kelp tissues.

4.4.2.3 Tissue age associated community abundances and structure

The majority of the lamina were extensively degraded at the apical tissue which is in line with late summer deterioration of the perennial kelp (Brown et al., 1997). For the comparison of biofilm communities between the two tissue types, degraded blades were excluded from the dataset. This decision was based on the study from Marzinelli et al. (2015) which showed that once kelp entered into a stressed state (e.g. degraded or bleached) the relationship with their microbiome becomes disrupted and less conditioned by defining biotic and abiotic effects. The limited kelp dataset might also explain the lack of a significant result in species richness variation for prokaryotic communities along the tissue age, which was consistently found in literature (Bengtsson et al., 2012; Weigel & Pfister, 2019; Lemay et al., 2021). Although there was a clear pattern observed that younger meristematic tissue supported a lower ASV richness for both bacterial and eukaryotic communities. Despite the low statistical power, the trends put forward by this dataset are in concurrence with previous published work on kelp tissue-age associated communities (Bengtsson et al., 2012; Weigel & Pfister, 2019; Lemay et al., 2021). In addition to the differences in tissue-age related epibionts, the meristem had slightly (1-fold) more unique ASVs in common with the surrounding seawater than the apical tissue. A stronger difference between the sample types was found for *N. luetkeana* in a study by Weigel & Pfister (2019) where meristem microbial communities shared 3-times more ASVs with seawater than the blade tip communities.

4.5 Conclusion

Kelp forest are important biodiversity hotspots and create highly productive coastal ecosystems in temperate regions all around the world (Wheeler and Druehl, 1986). They sustain higher food webs and provide numerous goods and services to local communities. However, a large component of their ecology is still in the early stages of being explored, such as their close interaction with microbial epibionts. This study confirms that both prokaryotic and eukaryotic epibionts, affiliated with *M. pyrifera*, established different community structures from the surrounding seawater. Nevertheless, a clear difference in host-microbiome interaction between these kingdoms was witnessed. The results revealed that *M. pyrifera* has a stronger interaction with the epiphytic bacterial populations than the eukaryotic assemblages. Biofilm communities along kelp blade tissue confirmed findings from previous studies that the older blade surface (apex) supports a greater diversity of prokaryotic assemblages than the younger tissue (meristem). A structure that was shown here to be strongly mirrored by the protist biofilm

communities. Collectively, these outcomes could indicate that the relationship with the kelp prokaryotic assemblages are a greater determining factor in structuring the kelp eukaryotic communities than the kelp host itself.

In relation to *M. pyrifera* kelp forests in the northern hemisphere, the present kelp microbiome was found to support a highly similar taxonomical composition up to family level. Thereby confirming host-specificity across a wide geographical range. Note that a full comparison of the microbiomes was not possible on account of previous studies not exploring the phylogeny of the prokaryotic assemblages at the same high resolution that was used here. Furthermore, the eukaryotes associated with the kelp blade surface in this study suggested a relation with the seasonal time frame sampled here. This would indicate that temporal variation is also observed for kelp associated eukaryotes, as has been previously shown for *M. pyrifera* affiliated prokaryotes (Michelou et al., 2013). However, continued seasonal sampling is recommended to further investigate this.

Overall, this study provided great insight into the taxonomical composition of both prokaryotic and eukaryotic communities associated with *M. pyrifera* kelp blades. Thereby identifying preferred associations with species from both kingdoms, certain deterred taxonomical groups and taxa annotated as general colonizers. Although the kelp prokaryotic microbiome has been more extensively quantified (Hollants et al., 2013; Egan et al., 2013; Singh & Reddy, 2014), the eukaryotes have been poorly described so far. To verify if the current findings involving the kelp associated epi- and endophytic microeukaryotes are a generalised feature rather than region specific, more research is needed. Additional characterisation of the functional guilds of kelp microbiome associations as well as interkingdom interactions will further aid in gaining a better understanding of the kelp microbial ecology.

Chapter 5|

Responses of microbial benthic communities to elevated $p\text{CO}_2$ levels at a tropical CO_2 vent

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5.1 Introduction

Biofilms are important structures of marine coastal ecosystems that sustain food webs through their active involvement in fundamental microbial processes such as organic matter remineralization, contaminant degradation, biogeochemical cycling, primary productivity and the transfer of energy to higher trophic levels (Azam et al., 1983; Paerl & Pinckney, 1996; Carvalho & Caramujo, 2012; Hawley et al., 2017). Microorganisms can colonise available abiotic and biotic surfaces within seconds of exposure to the marine environment. When biofilms develop on biotic substrates such as corals, seagrasses, and kelp, they establish host-microbiome interactions that involve mutualistic benefits (Heyward & Negri, 1999; Apprill, 2017; Weigel & Pfister, 2019; Sanders-Smith et al., 2020). Furthermore, together with crustose coralline algae and certain filamentous algae, marine biofilms are also of vital importance for larval settlement induction and subsequent metamorphosis for a number of benthic invertebrate species (Hadfield & Paul, 2001; Huang & Hadfield, 2003).

Since the industrial revolution, the ocean has absorbed approximately 30% of atmospheric CO₂ and has thereby tempered the unprecedented rate at which climate change has been happening (IPCC, 2021). The increasing atmospheric CO₂ emissions as a result of burning fossil fuels, industrialisation, and deforestation are responsible for a substantial change in ocean carbonate chemistry, leading to a decrease in calcite and aragonite saturation (Doney et al., 2020; IPCC 2021). Uptake of atmospheric CO₂ by the ocean biome causes an increase in partial pressure of CO₂ in the upper ocean that further results in the formation of carbonic acid (weak acid) and thereby reduces seawater pH and carbonate ion concentrations (Caldeira & Wickett, 2005; Orr et al., 2005). This process is known as ocean acidification and has already decreased surface ocean water pH by 0.1 units over the past 200 years (Raven, Caldeira, Elderfield, & Hoegh-Guldberg, 2005). Further, it has been predicted to decrease pH by another 0.3 – 0.4 units if our current atmospheric levels (~415 ppmv) reach approximately 1000 ppmv by 2100 under the SSP5-8.5scenario (IPCC, 2021). These changes may profoundly impact several groups of marine organisms and their biotic and abiotic interactions, and inevitably leads to ecosystem diversity alterations and loss of related services (Doney et al., 2020). Moreover, ocean acidification could also potentially influence the architecture of marine biofilms by altering the community composition and subsequent functions, impact physiological homeostasis of the bacteria, and interrupt cell-cell signalling and community-level communication (Dang & Lovell, 2016; and references therein). These changes could further affect the settlement of invertebrate juveniles that is promoted by biofilm assemblages (Toupoint et al., 2012; Espinel-Velasco et al., 2018), drive

dysbiosis of essential macroalgae host-microbiomes (Minich et al., 2018) and directly and indirectly alter the productivity status of the higher trophic levels (Vizzini et al., 2017; Ullah et al., 2018). Collectively, these processes could result in major repercussions for the whole ecosystem.

Joint et al. (2010) predicted that marine microbes would possess the flexibility needed to accommodate the future ocean acidification induced pH change and that the marine biogeochemical processes driven by microorganisms would not be impacted. However, studies during the past decade have observed that under ocean acidification conditions primary productivity increases (Lidbury et al., 2012; Russell et al., 2013; Johnson et al., 2015; Harvey et al., 2019), microbial community composition and diversity are significantly altered (Witt et al., 2011; Kerfahi et al., 2014, 2020; Taylor et al., 2014), species replacement underpins community changes (Allen et al., 2021) and an overall shift from smaller diatoms to larger diatom species takes place (Tortell et al., 2008; Feng et al., 2009, 2010; Bach et al., 2017, 2019; Harvey et al., 2019). Some studies have also suggested that communities are expected to simplify in terms of taxonomy and functions with increasing $p\text{CO}_2$ (Kroeker et al., 2011; Vizzini et al., 2017; Agostini et al., 2018), this appears to be the case for larger organisms (Agostini et al., 2018; Hall-Spencer & Harvey, 2019) rather than microorganisms (Kerfahi et al., 2020).

Most studies looking at ocean acidification have focussed on short-term acute exposure to high $p\text{CO}_2$ and low pH, while coastal organisms can actually tolerate rapid perturbations in carbonate chemistry as a result of large natural variability (Wootton et al., 2008). Therefore, long term multi-species experiments and monitoring is necessary to further our understanding of ocean acidification impact on the whole ecosystem. In 2008, Hall-Spencer et al. described the use of CO_2 vents as natural analogues of global-scale ocean acidification to provide information about long-term ecological effects of increased $p\text{CO}_2$ and lowered pH on marine organisms. By using shallow submarine vent systems, natural ecological interactions amongst biota (e.g. production, competition and predation) and their adaptations to the surrounding ocean chemistry are retained within the experimental study. This gives valuable insight into the performance of organisms in a natural setting, which is difficult or impossible to achieve in laboratory experiments and mesocosm designs. Furthermore, it reduces the probability of observing and misinterpreting organismal stress responses as true reactions to CO_2 enrichment. However, *in situ* observational studies or perturbation experiments do not allow for the control of environmental parameters, making it hard to disentangle the effects of potential confounding factors (e.g. salinity, temperature, light and vent nutrients). Additionally, CO_2 vents are open systems where recruitment from outside is not hindered. This will impact species potential for genetic adaptation in response to the selective pressure of high $p\text{CO}_2$ (Sunday et al., 2014; Harvey et al., 2014; Agostini et al., 2018). It is therefore recommended that results and patterns from *in situ* studies be used in

conjunction with experimental approaches that allow for a more controlled setting, such as laboratory and mesocosms designs. This will greatly strengthen the understanding of ocean acidification studies and aid in the prediction and subsequent conservation and management of marine ecosystems in the context of future ocean dynamics. So far the majority of biofilm orientated studies in a natural environment have focussed on CO₂ vents in temperate climate regions such as Italy and Japan (Kerfahi et al., 2014, 2020; Taylor et al., 2014; Allen et al., 2021), while only a few studies have researched biofilm community dynamics at CO₂ vents in a tropical setting, e.g. Papua New Guinea (Hassenrück et al., 2017). Due to the limited spatial scale associated with venting sites, replication becomes difficult and comparisons between different venting sites (large pH variability) increases the potential of confounding factors. However, characterising the communities that can survive at these high *p*CO₂ environments provides important knowledge on its own regarding future changes to the marine ecosystem as a consequence of ocean acidification.

In the current study, high-throughput sequencing of the genomic V4 hypervariable regions from prokaryotic (16S rRNA gene) and eukaryotic (18S rRNA gene) organisms was used to gain a better understanding of marine biofilm compositions at a *p*CO₂ enriched area in a tropical setting. Specifically, marine biofilm colonisation and succession on settlement plates was explored *in situ* at a tropical hydrothermal CO₂ vent in Dominica, Lesser Antilles. To explore the variability of these benthic assemblages, alpha- and beta-diversity, chlorophyll-*a* (chl-*a*), and taxonomic composition was assessed. Additionally, an initial evaluation of the underlying ecological mechanisms that drive early biofilm succession and development was performed. For this, beta-diversity was partitioned into two components: Turnover or species replacement and Nestedness or species loss. The following hypotheses were tested: (i) elevated *p*CO₂ levels alter the biofilm community composition, (ii) a higher degree of taxonomic homogenisation is achieved near the high *p*CO₂ site, (iii) Turnover is the main driver behind community succession at the high *p*CO₂ site, (iv) and high *p*CO₂ shifts diatom communities towards larger species.

5.2 Materials & Methods

5.2.1 Site description and geological setting

Champagne Hot Springs (N 15.245846, W 61.373125) in the southwest of Dominica was chosen as the sampling site for this study (Figure 5.1). Dominica island is located along the eastern margin of the Caribbean Sea and is one of the younger constituent islands that comprise the Lesser Antilles archipelago. The Lesser Antilles is a two-parallel island-arc system that converges into a

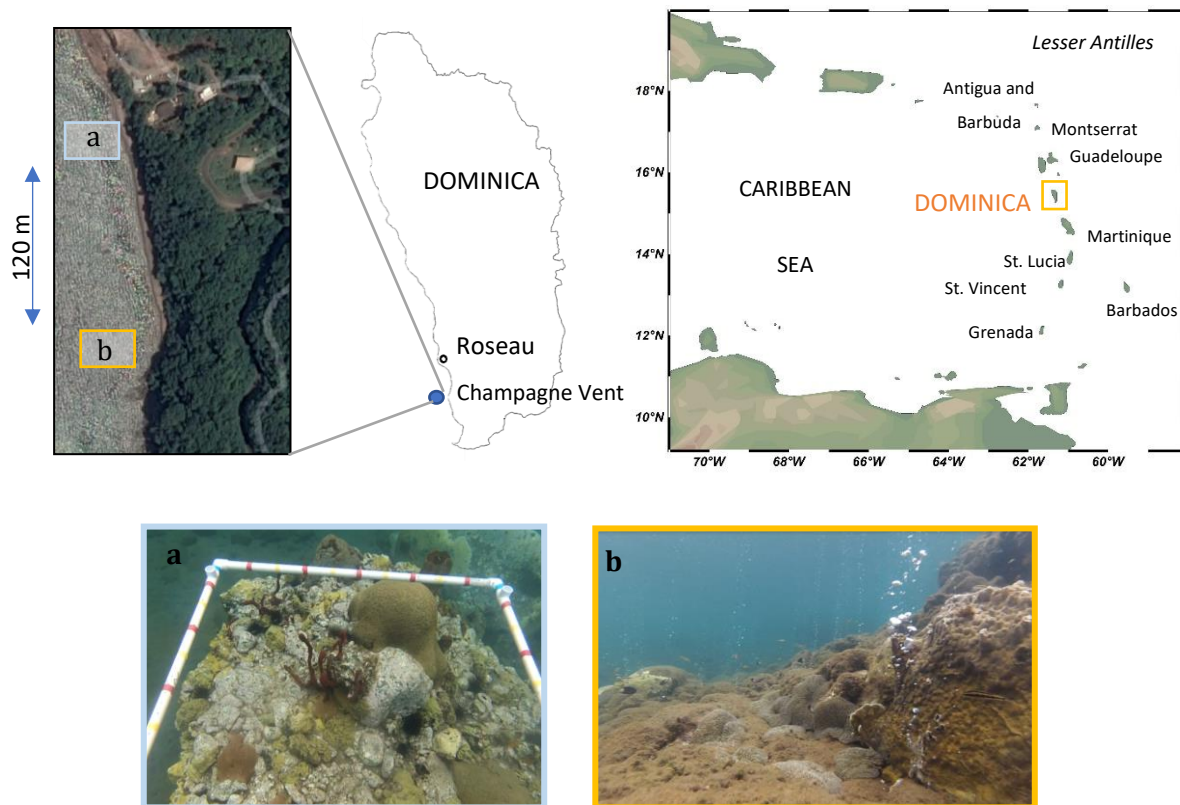


Figure 5.1: Map of Champagne Hot Springs CO₂ system, Dominica, Lesser Antilles. (A) The control site at 120 m away from the (b) high $p\text{CO}_2$ site.

single chain of oceanic islands to the southeast of Dominica (Martin-Kaye, 1969; Fink, 1972) and it is one of two active island arcs in the Atlantic Ocean. Dominica is volcanically the most extreme and hazardous nation with up to nine potentially active volcanic centres, both terrestrial and marine (Lindsay et al., 2003, 2005; Joseph et al., 2011). Champagne Hot Springs is a shallow submarine hydrothermal vent system (1 - 5 m water depth) that exhibits two types of fluid discharge: (i) focused discharge from discrete ports and (ii) dispersed or diffuse venting with streams of gas bubbles emerging through the sediment (McCarthy et al., 2005). The total venting site covers an area of 880 m², extends up to 40 m offshore and has a mean depth of 3 m. McCarthy et al. performed an initial study in 2005, analysing the geochemistry of the Champagne Hot Springs and found that the chemical compounds from the vent site were typical island-arc associated gasses (Table 5.1) with meteoric and magmatic signatures. The centre of the vent site was visibly colonised by brown non-calcifying algae and anemones and, less commonly, by small calcifying algae and brain corals that were noticeably eroded. The sampled area (high $p\text{CO}_2$ site) was 5 m away from the vent site where large assemblages of tube sponges (*Aplysina spp.*) dominated the field. In addition, a small cover of urchins and calcifying corals/alga were observed as well (pers. comm.). Approximately 100 m out from the vent fluid, the faunal assemblages were largely dominated by a number of corals, calcifying alga, and urchins (Figure 5.1).

Table 5.1: Composition of Champagne Hot Springs vent gas. Proportions represent the percentage of total analysed gas volume. Carbon dioxide is the major constituent. Data adapted from McCarthy et al. (2005).

Gas	Proportions
Carbon dioxide (CO ₂)	74-93%
Nitrogen (N ₂)	4-21%
Hydrogen Sulfide (H ₂ S)	1.6-3.9%
Methane (CH ₄)	0.15%
Hydrogen chloride (HCl)	0.73%
Oxygen (O ₂)	0.07%
Argon (Ar)	0.46%
Helium (He), hydrogen (H ₂) & cobalt (Co)	0.0-0.003%

5.2.2 Experimental design and field measurements

All field research was conducted from the 26th of June to the 15th of July in 2017 at Champagne Hot Springs in Dominica, Lesser Antilles, by means of snorkelling. Transparent acrylic slides were chosen as settlement plates for biofilm colonisation and development (Johnson et al., 2013, 2015; Allen et al., 2021). The slides (5 cm x 10 cm) were mounted on a secure underwater rig using cable ties. Five rigs with each four slides attached to, were deployed at both the control and the high $p\text{CO}_2$ site at a depth of 3 m. The rig design was not developed to prevent any form of grazing or predation from taking place. The 'high CO₂' rigs were placed at a distance of 5 m from the centre of the venting area to minimize the direct impact of metals and other chemical elements originating from the vent. The control site was located approximately 120 m up the shoreline behind a small, exposed rock where the slides were not impacted by hydrothermalism (Figure 5.1). The settlement plates were collected over a period of 20 days. Every five days, one slide per rig was retrieved and sampled for i) amplicon sequencing and ii) chl-*a* analysis. Sampling for amplicon sequencing was done by removing the organic matter covering 30 cm² of the plate with a sterile razor blade and transferring it to a 1.5 mL microcentrifuge tube. All samples were stored in *RNAlater* at ambient temperature for transportation to New Zealand. Upon arrival, the samples were transferred to -80°C and kept frozen until processing. *RNAlater* is a non-toxic preservative with a high salt content that stabilizes nucleic acid molecules. It can store cell samples safely for up to a week without the need to keep the samples frozen. Like all other preservatives, *RNAlater* has been shown to have a small impact on DNA-based community analysis (Gray et al., 2013; McCarthy et al., 2015). The biofilm material covering the remainder 20 cm² of the plate was sampled in an equal manner without the addition of a preservative. These samples were kept dark and cool at all times for the analysis of chl-*a*.

5.2.2.1 Environmental data collection

Temperature (T), salinity (S) and pH measurements (Figure 5.2) were collected daily at the control and high $p\text{CO}_2$ site, for the duration of the study (20 days), using a YSI-probe (Table 5.2). The YSI probe was calibrated every week, using a three point calibration. For total pH, between three to six measurements were taken every day (control, $n = 79$; high $p\text{CO}_2$, $n = 90$), while temperature and salinity were only measured once a day. Daily temporal variation between replicate measurements of total pH was lower at the control site (± 0.02) than the high $p\text{CO}_2$ site (± 0.06). Discrete water samples for total alkalinity (A_T) were taken each week and measured by hand titration (pers. comm). These parameters (T, S, pH & A_T) were collectively used to calculate the carbonate chemistry (Table 5.2) of the two sampling sites following the ocean acidification guidelines using CO2Sys (Pierrot et al., 2006).

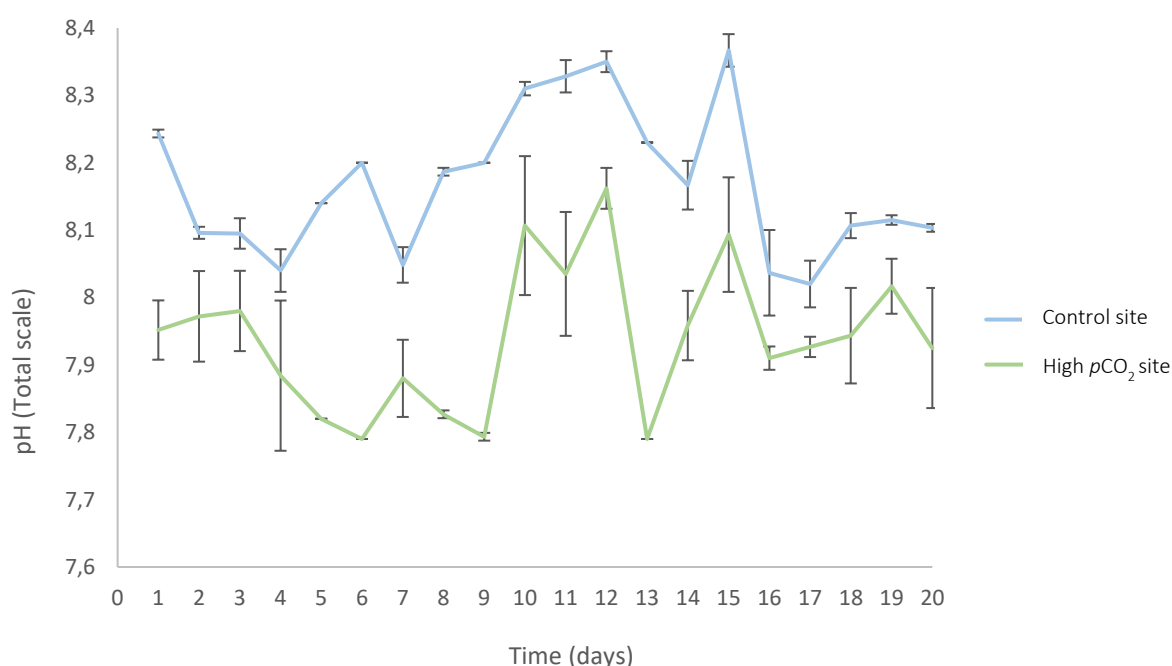


Figure 5.2: Mean pH_{Total} values for the duration of the study at the control (blue) and the high $p\text{CO}_2$ (green) site. Measurements were taken with an YSI probe. The study took place from the 26th of June (day one on the x-axis) to the 15th of July 2017 (day 20 on the x-axis) in Dominica, Lesser Antilles. Error bars represent standard deviation. The high $p\text{CO}_2$ site was located 5 m away from the centre of the CO_2 vent system, and the control site at 120 m.

Table 5.2: Carbonate chemistry parameters for the control and high $p\text{CO}_2$ site at Dominica, Lesser Antilles. pH total (control, $n = 79$; high $p\text{CO}_2$, $n = 90$), temperature (control, $n = 20$; high $p\text{CO}_2$, $n = 20$), salinity (control, $n = 20$; high $p\text{CO}_2$, $n = 20$) and total alkalinity (A_T ; control, $n = 6$; high $p\text{CO}_2$, $n = 6$) were measured in the field. The carbonate chemistry parameters were calculated using the CO2Sys software (Pierrot et al., 2006): hydrogen carbonate (HCO_3^-), carbonate (CO_3^{2-}), partial pressure of carbon dioxide ($p\text{CO}_2$), calcite (ΩCa) and aragonite (ΩAr). Mean values are shown $\pm$ standard deviation.

	Control	High $p\text{CO}_2$
pH total	8.17 ± 0.1	7.94 ± 0.1
Temperature ($^{\circ}\text{C}$)	29.7 ± 0.4	29.7 ± 0.3
Salinity (‰)	29.9 ± 0.3	29.9 ± 0.1
A_T ($\text{CaCO}_3 \text{ mg L}^{-1}$)	2334.63 ± 68.55	2334.63 ± 68.55
$p\text{CO}_2$ (μatm)	298.9 ± 89.76	574.65 ± 162.31
HCO_3 ($\mu\text{mol kg}^{-1} \text{ SW}$)	1650.27 ± 124.55	1874.94 ± 95.71
CO_3 ($\mu\text{mol kg}^{-1} \text{ SW}$)	284.55 ± 51.17	191.64 ± 39.68
ΩCa	7.23 ± 1.298	4.87 ± 1.01
ΩAr	4.77 ± 0.859	3.22 ± 0.67

5.2.3 Chlorophyll-*a* analysis

Chl-*a* was extracted by incubating the biofilm samples in 1 mL of 96 % ethanol for 24-hours at 20°C . After the extraction, the homogenates were centrifuged at $3,300 \times g$ for 5 minutes to pellet cellular debris. Chl-*a* concentration was then photometrically determined using a UV-1280 UV-VIS Spectrophotometer. The equation described by Jeffrey & Humphrey (1975) was used to calculate the chl-*a* concentration.

5.2.4 DNA extraction and amplicon sequencing

A total of 38 biofilm samples were thawed and spun down at $3,300 \times g$ for 3 min to remove the RNA later supernatant prior to DNA extraction. Genomic DNA was extracted using the Qiagen DNEasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The success of the DNA extraction was quantified on a Qubit fluorometer with the Qubit™ dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Two separate amplicon library types were prepared, one for the identification of prokaryotic (16S amplicons) communities and one to identify eukaryotic (18S amplicons) communities. Both types of library were created using a 2-step PCR method and the KAPA HiFi HotStart DNA kit (Kapa Biosystems, Boston, MA, USA). In the first round PCR (R1-PCR), the amplicons of interest were targeted and amplified using amplicon specific primers. To allow for a second round PCR (R2-PCR) to take place, the R1-PCR primers were adjusted by adding oligonucleotide linkers at the 5' – ends (forward, 5'–ACGACGCTCTTCCGATCT–3'; reverse, 5'–CGTGTGCTCTTCCGATC–3'; Griffith et al., 2017). Dual-index (i5 and i7) TruSeq primers were used for the R2-PCR and were modified

with the same linkers to enable the binding of second round primers to the R1-PCR amplicons. Furthermore, the R2-PCR primers attach a unique combination of Illumina adaptors and indices to each sample facilitating sample multiplexing on the MiSeq next-generation sequencing (NGS) platform.

5.2.4.1 Prokaryotes (16S rRNA) library preparation

To identify prokaryotic communities the V4 region of the 16S rRNA gene was amplified using the latest universal Earth Microbiome Project primers (modified as described above): forward primer 515F (5' - GTGYCAGCMGCCGCGTTAA - 3') and reverse primer 806R (5' - GGACTACNVGGGTWTCTAAT - 3') with reduced bias against *Crenarchaeota*/*Thaumarchaeota* and the Alphaproteobacterial clade SAR11 (Caporaso et al., 2011, 2012; Apprill et al., 2015; Parada et al., 2016). The R1-PCR reactions (25 µL) consisted of 1 µL DNA template of approximately 10 ng, 5 x KAPA HiFi Buffer, 0.3 mM KAPA dNTPs, 0.3 µM Forward primer and 0.3 µM Reverse primer, 0.5 U KAPA HiFi HotStart DNA Polymerase and 5 mg mL⁻¹ Bovin Serum Albumin (BSA). PCR cycling conditions were 95°C for 2 min, followed by 25 cycles of 98°C for 20 s, 50°C for 30 s, and 72°C for 30 s, and a final extension period for 1 min at 72°C. PCR products were visualised on a 1.5 % agarose gel and had an expected band size of ~300–350 bp. Next, the amplicons were purified with 1.8 x (vol:vol) magnetic AMPure XP beads (Beckman Coulter) following the Agencourt AMPure XP PCR Purification protocol. The resulting purified products were eluted with 30 µL of Tris-EDTA (pH 8) elution buffer, quantified on the Qubit fluorometer and diluted to 1 ng µL⁻¹. The R2-PCR reactions were made as follows: 5 x KAPA HiFi Buffer, 0.3 mM KAPA dNTP, 0.3 µM Forward and Reverse primer, 0.5 U KAPA HiFi HotStart DNA Polymerase and 1 ng template DNA. PCR conditions were 95 °C for 2 min; 10 cycles of 98°C for 20 s, 60°C for 20 s, and 75°C for 20 s; and a final extension at 72°C for 1 min. Amplicons were visualised on a 1.5 % gel (~400 – 450 bp), cleaned-up and quantified as described for the first round. Finally, all the samples were pooled into one amplicon library in equimolar amounts (10 nM) and run on the Agilent Bioanalyzer 2100 (Agilent Technologies, USA) by the Otago Genomics Facility (Otago University, New Zealand) to confirm the final quality and quantity of the amplicon library. The pool was then loaded on an Illumina MiSeq platform at the Otago Genomics Facility for paired-end sequencing using the V2 (2 x 250 bp) reagent kit.

5.2.4.2 Microeukaryotes (18S rRNA) library preparation

Microeukaryotes were identified by targeting the V4 region of the 18S rRNA gene using the TAREuk454FWD1 (5'-CCAGCASCYGC GGTAATTCC-3') and TAREukREV3 (5'-ACTTTCGTTCTTGATYRA-3') primers with linkers attached as described above (Stoeck et al., 2010). The following PCR mixtures (25 µL) were prepared for each sample: x1 Kapa Hifi buffer,

0.3 mM Kapa dNTP mix, 0.3 μ M forward and reverse primers, 0.5 U Kapa Hifi Hotstart DNA polymerase, and approximately 10 ng of DNA template. Stoeck et al. (2010) designed a 2-step PCR cycling program to account for the difference in primer length: an initial denaturation at 95°C for 30 s; step 1 – 10 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s; step 2 – 15 cycles of 95°C for 30 s, 48°C for 30 s, and 75°C for 30 s; and a final extension time for 5 min at 72 °C. PCR amplicons were verified on a 1.5 % agarose gel (~400 – 450 bp), followed by a clean-up step, quantification, and dilution to 1 ng μ L⁻¹, identical to the R1-PCR procedure for the 16S amplicons. The R2-PCR multiplexing workflow (PCR-step, purification, quantification and library pooling) was as followed for the 16S amplicon R2-PCR protocol, described earlier. After the samples were pooled in equimolar amounts (10 nM) the final library was validated on the Agilent Bioanalyzer 2100, by Otago Genomics Facility, for an expected band size of ~550 bp. The amplicon library was run on the MiSeq platform (Illumina, San Diego, CA, USA) using the V3 paired-end (2 x 300 bp) sequencing kit.

5.2.5 Sequence data processing

DNA sequence processing and all statistical tests were performed in R (version 3.4.4; R Core Team, 2021). Raw demultiplexed DNA sequence reads with barcodes already removed were obtained from Otago Genomics Facility and were processed following the DADA2-pipeline (Callahan, Sankaran, et al., 2016). In short, forward (F) and reverse (R) reads were quality trimmed based on their Q-scores (Q30 for all reads) at 240(F) – 230(R) bp and 250(F) – 240(R) bp for 16S and 18S rRNA sequences, respectively. Reads that were shorter than these cut-off thresholds were discarded. The difference in length between the forward and reverse reads is explained by the quality of the reads with the forward reads containing a higher quality. Samples were dereplicated and amplicon sequence variants (ASVs) were resolved using the DADA2 core denoising algorithm (Callahan, McMurdie, et al., 2016). After denoising, the reads were merged, and bimeric sequences (chimeras) were identified and subsequently removed. For both communities (16S and 18S), the chimera reads accounted for only 1 % of all merged sequence reads. Lastly, prokaryotic (16S) sequences were classified with the DADA2 functions *assignTaxonomy* and *assignSpecies* against the SILVA taxa database (version 138.1 with species assignment; <https://benjjneb.github.io/dada2/training.html>) using the naive Bayesian classifier method. Taxonomy for the microeukaryotes (18S) was assigned with the *assignTaxonomy* function via the Bayesian method and against the Protist Ribosomal Database (PR2 version 4.14; <https://github.com/pr2database/pr2database/releases>; Guillou et al., 2013). Only sequences classified as Bacteria and Archaea were selected for prokaryotic data analysis (Eukaryota,

Chloroplast and Mitochondria identified reads were removed). For the microeukaryotes, sequences classified as 'Metazoa' were removed.

5.2.6 Statistical analyses

Differences in alpha and beta diversity within and between the biofilm samples were measured in R with the “phyloseq” package (McMurdie & Holmes, 2013). Prior to computing diversity metrics, the 16S rRNA dataset was rarefied to an even sampling depth of 21,291 sequences, thereby removing two samples, one from the control site (C1D05) and one from the high $p\text{CO}_2$ site (A5D05). The 18S rRNA dataset was rarefied to an even sampling depth of 14,718 sequences, which excluded one sample from the control site (C2D15) that contained fewer reads. Rarefying thresholds were determined based on the rarefaction curves reaching saturation levels for all samples. This suggest that the sequencing depth per sample is an adequate representation of the whole community diversity for each biofilm sample. Outliers in the datasets were identified with the *grubbs.test* function from the “outliers” package (Komsta, 2011). Variation in biofilm alpha diversity, species richness (Chao1 index) and evenness (Pielou index), was statistically compared for all samples across the different time points (day 5, day 10, day 15 and day 20) and for the CO_2 condition (i.e. control vs. high $p\text{CO}_2$ site) using the non-parametric Kruskal-Wallis analysis. Significant results were further investigated using pairwise Wilcoxon Rank Sum tests with Benjamini-Hochberg (BH) corrections for multiple testing. These non-parametric tests were chosen based on the results from the Shapiro–Wilk normality test and Levene’s homogeneity of variance test, which showed that the data did not follow a normal distribution.

Biofilm community composition variation (beta diversity) was assessed for the time factor and CO_2 condition. For this, a Bray-Curtis dissimilarity matrix, based on species abundance, as well as a Jaccard dissimilarity matrix, based on species presence/absence, was measured. Additionally, the *beta.multi* and *beta.pair* function from the “betapart” package (Baselga, 2010; Baselga & Orme, 2012) were used to partition the overall Jaccard beta-diversity into a turnover component (species replacement) and a nestedness component (species loss). By quantifying the relative contribution of both components to the overall beta diversity, ecological processes that help shape biodiversity patterns can be revealed. The significance of observed compositional shifts over time and for CO_2 condition, based on both Bray-Curtis and Jaccard distance matrices, was tested with a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2017). This was done using the *adonis* function from the “vegan” package (2.5-7; Oksanen et al., 2020), based on 100,000 permutations. PERMANOVA results with significant outcomes were followed up with pairwise comparisons associated with BH corrections to account for multiple pairwise testing. Additionally, all significant PERMANOVA results were tested for heterogeneity of dispersion among the

predefined groups (time and CO₂ condition) using permutational multivariate analyses of dispersions (PERMDISP; Anderson et al., 2006). PERMDISP tests were performed using the *Betadisper* function implemented in the “vegan” package and followed up with pairwise comparisons using *permutest.betadisper* (vegan). Both tests were performed with 100,000 permutations. The resulting three beta diversity distance measures (beta.jtu, beta.jne & beta.jac) from *beta.pair*, as well as the Bray Curtis distances, were visualised with non-metric multidimensional scaling analysis (nMDS) using the *metaMDS* (vegan) function. A *set.seed* of 711 was set for all permutational tests to achieve reproducible results. To determine the structuring of the most dominant taxa groups over time and by CO₂ condition, a MANOVA test was performed.

Finally, the impact of time and CO₂ condition on chl-*a* concentrations of total biofilm communities was tested using a Kruskal-Wallis analysis, followed by pairwise Wilcoxon Rank Sum Tests with BH corrections. Barplots were created from relative abundance data of prokaryotic and eukaryotic taxa. All data visualisations were made in R with *ggplot2* (Wickham, 2016) available in the “tidyverse” package (Wickham et al., 2019).

5.3 Results

After rarefying, the prokaryotic dataset retained 18 samples for both *p*CO₂ sites, while the eukaryotic dataset retained 20 samples from the high *p*CO₂ site and 17 samples from the control site. A total of 21,291 reads per sample were used for the prokaryotes and 14,718 reads per sample for the eukaryotes.

5.3.1 Alpha diversity

Alpha diversity metrics including Chao1 richness and Pielou’s evenness were calculated based on the rarefied data. Overall, prokaryotic communities were substantially more diverse (higher Chao1 richness and Pielou’s evenness) than microeukaryotes (Figure 5.3).

For prokaryotic communities, Chao1 richness did not significantly differ between the two locations, although the first days (Day 5 and Day 10) did show higher richness values at the high *p*CO₂ site (Figure 5.3a). Prokaryotic evenness was significantly higher at the high *p*CO₂ site on Day 10 (Kruskal-Wallis; *df* = 1, *H* = 6.0, *p* = 0.01) (Figure 5.3b). Differences across time for prokaryotic communities were observed at both locations. At the control site, the prokaryotes had a significant drop in richness after Day 15 (Kruskal-Wallis; *df* = 3, *H* = 8.5, *p*_{adj} = 0.048), while at the high *p*CO₂

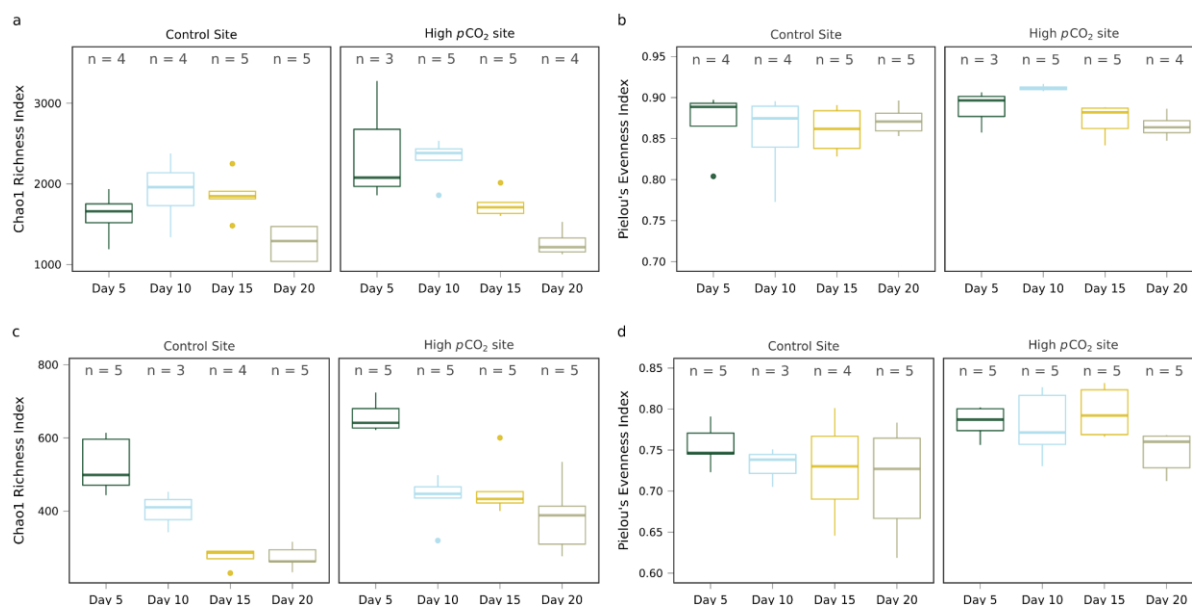


Figure 5.3: Alpha-diversity measures for biofilm communities between the control and high $p\text{CO}_2$ site. (a – b) Prokaryotic communities and (c – d) microeukaryotic communities. Sample size (n) per group are displayed. Colours represent the different days of sampling: green (day 5), blue (day 10), yellow (day 15), and beige (day 20). Note the different scales for prokaryotic and microeukaryotic diversity measures.

site a significant decrease in species richness and evenness was observed after Day 10 ($df = 3$, $H = 6.5$, $p_{adj} = 0.01$) (Figure 5.3a,b).

For eukaryotic communities, Chao1 richness was significantly higher at the high $p\text{CO}_2$ site on Day 5 ($df = 1$, $H = 6.8$, $p < 0.01$), Day 15 ($df = 1$, $H = 6.0$, $p = 0.01$) and marginally on Day 20 ($df = 1$, $H = 3.9$, $p = 0.05$). Similar to prokaryotic communities, temporal shifts in alpha diversity for microeukaryotes were observed at both locations. Species richness dropped after Day 5 at the control site but was only significantly different from Day 15 and Day 20 (Kruskal-Wallis; $df = 3$, $H = 12.9$, all p_{adj} – values < 0.05) (Figure 5.3c). At the high $p\text{CO}_2$ site species richness decreased after Day 5 and was significantly different from Day 10, Day 15, and Day 20 (Kruskal-Wallis; $df = 3$, $H = 12.9$, all p_{adj} – values = 0.02) (Figure 5.3c). No significant differences were observed for time and CO_2 condition based on Pielou's evenness.

5.3.2 Biofilm community composition

Prokaryotic and eukaryotic biofilm communities at the high $p\text{CO}_2$ site were distinct from the communities at the control site across all time points based on species incidence (Jaccard dissimilarity; Figure 5.4a,b & Table 5.3) as well as abundance (Bray-Curtis dissimilarity; Table 5.3 & Figure S5.1). For prokaryotic biofilms, the Turnover partition was significantly affected by CO_2 condition (control vs. high $p\text{CO}_2$ site), time, and the interaction term, while Nestedness was only

significant for time (Table 5.3). For microeukaryotes, Turnover and Nestedness were both significantly affected by CO₂ condition, time, and the interaction between these factors (Table 5.3).

Prokaryote biofilm differences between the CO₂ conditions at every time point were entirely driven by Turnover (Figure 5.4a & Table 5.3). For time, differences between the first two time points (Day 5 – 10 & Day 10 – Day 15) were structured by Turnover at both sites, while differences between the last time points (Day 15 – Day 20) was explained by Nestedness at both sites (Table 5.3). For eukaryotic communities, Turnover was also significant at each time point for CO₂ condition but with a contribution of Nestedness on Day 15 (Figure 5.4b & Table 5.3). Temporal variation for microeukaryotes at the control site was driven by Turnover between first (Day 5 – Day 10) and last time points (Day 15 – Day 20). Differences between Day 10 and Day 15 were explained by Nestedness (Table 5.3). At the high pCO₂ site, significant differences between all the consecutive time points for microeukaryotes was due to Turnover but with a contribution of Nestedness between Day 5 and Day 10 (Table 5.3).

Based on Jaccard dissimilarities, significant heterogeneity of multivariate dispersions for prokaryotes was detected with PERMDISP between the sites on Day 10 ($df = 1, F = 8.84, p < 0.05$), which means that there was unequal dispersions amongst the groups. The PERMDISP test also

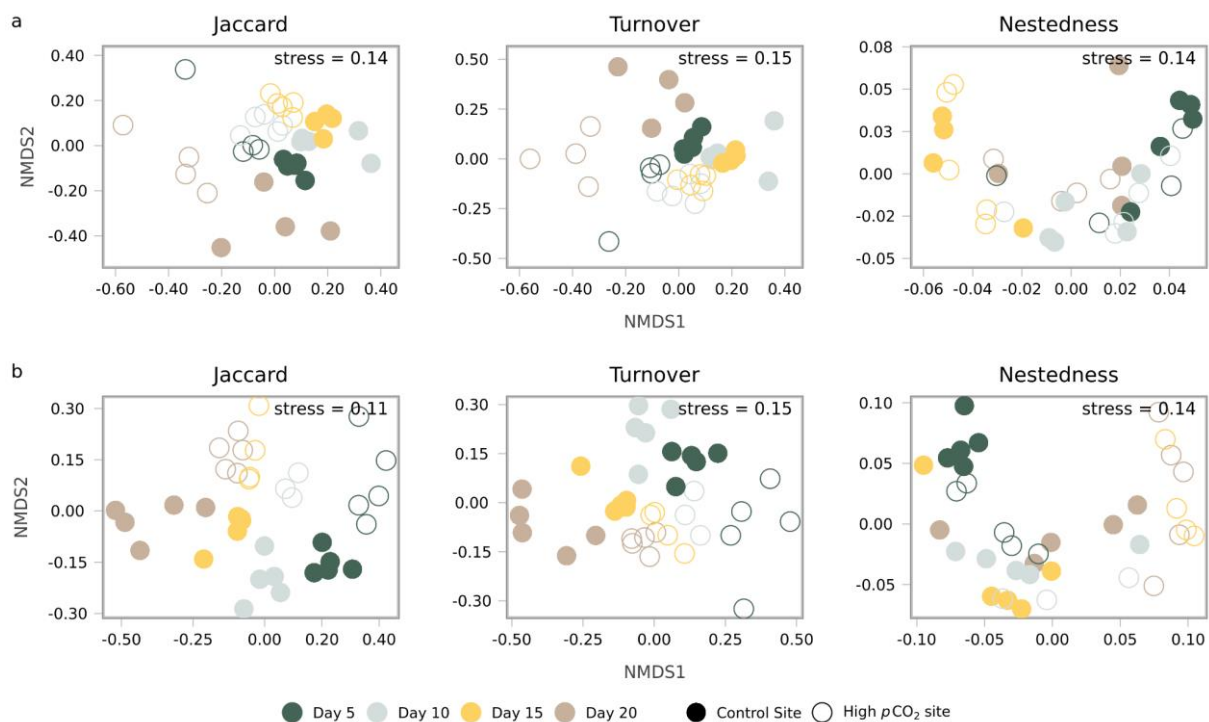


Figure 5.4: Non-metric multidimensional scaling for biofilm communities based on Jaccard dissimilarities. (a) prokaryotic and (b) microeukaryotic communities. Overall beta-diversity based on Jaccard dissimilarities (Jaccard) is partitioned in two components: species replacement (Turnover) and species loss (Nestedness). Stress value is indicated per ordination plot. Colour represent the different time points of sampling: green (day 5), blue (day 10), yellow (day 15), and beige (day 20). Symbols represent all the individual samples: closed circles (control site) and open circles (high pCO₂ site).

revealed a significant heterogeneity between the first two time points (Day 5 – Day 10; $df = 3$, $F = 5.12$, $p < 0.05$) at the high pCO_2 site. For eukaryotes, PERMDISP pairwise comparisons (Jaccard dissimilarities) resulted in significant differences for CO_2 condition on Day 5 ($df = 1$, $F = 16.29$, $p < 0.01$) and Day 20 ($df = 1$, $F = 21.69$, $p < 0.01$), and for time at the control site between the first two time points (Day 5 – Day 10; $df = 3$, $F = 3.17$, $p < 0.05$). Despite PERMDISP showing unequal dispersions of variability amongst these groups, for both prokaryotes and eukaryotes, the nMDS ordinations still showed a clear separation of the centroid positions for both factors (Figure 5.4a,b). This suggests that these significant differences, that were also detected by the PERMANOVA tests, are explained by a combination of both ‘location’ and ‘dispersion’ effects (see Chapter 1: section 1.1.4).

Table 5.3: PERMANOVA test results comparing Bray-Curtis and Jaccard dissimilarities among CO_2 conditions (control vs. high pCO_2 site) and time for prokaryotic and eukaryotic biofilm communities. Jaccard was partitioned in Turnover (species replacement) and Nestedness (species loss). All PERMANOVA tests were done with 100,000 permutations. Significance: $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$.*.

	Bray-Curtis			Jaccard			Turnover			Nestedness		
	Pseudo- <i>F</i>	R^2	<i>P</i>	Pseudo- <i>F</i>	R^2	<i>P</i>	Pseudo- <i>F</i>	R^2	<i>P</i>	Pseudo- <i>F</i>	R^2	<i>P</i>
Prokaryotes												
CO_2 condition	6,27	0,11	***	2,49	0,06	***	2,82	0,07	***	-8,84	-0,18	1,00
Time	5,04	0,28	***	2,00	0,15	***	1,82	0,14	***	8,40	0,51	**
CO_2 Condition x Time	1,77	0,10	*	1,24	0,09	**	1,26	0,09	**	1,57	0,10	0,26
Control - High pCO_2 (Day 5)	1,50	0,20	0,06	1,55	0,16	*	1,21	0,17	*	-0,67	-0,13	0,97
Control - High pCO_2 (Day 10)	3,43	0,33	**	1,69	0,20	**	1,76	0,20	**	-0,02	0,00	0,80
Control - High pCO_2 (Day 15)	2,82	0,26	**	1,47	0,16	**	1,53	0,16	**	-1,41	-0,21	1,00
Control - High pCO_2 (Day 20)	4,62	0,40	**	2,03	0,23	**	2,29	0,25	**	-2,05	-0,41	1,00
Day 5 - Day 10 (Control)	2,05	0,25	*	1,19	0,17	0,06	1,24	0,17	*	-0,27	-0,06	0,94
Day 10 - Day 15 (Control)	2,38	0,25	**	1,16	0,14	*	1,22	0,15	**	-0,09	-0,01	0,94
Day 15 - Day 20 (Control)	2,08	0,21	**	1,35	0,15	**	1,04	0,12	0,33	10,31	0,56	*
Day 5 - Day 10 (High pCO_2)	2,66	0,28	**	1,59	0,18	**	1,60	0,19	**	-0,05	-0,01	0,91
Day 10 - Day 15 (High pCO_2)	4,43	0,36	**	1,64	0,17	**	1,42	0,15	**	12,02	0,60	*
Day 15 - Day 20 (High pCO_2)	2,57	0,27	**	1,42	0,17	**	1,14	0,14	0,08	9,49	0,58	*
Eukaryotes												
CO_2 Condition	5,94	0,10	***	5,94	0,10	***	3,00	0,07	***	23,00	0,30	***
Time	6,71	0,33	***	6,71	0,33	***	2,68	0,18	***	4,20	0,16	*
CO_2 Condition x Time	1,97	0,10	***	1,97	0,10	**	1,41	0,10	**	4,03	0,16	*
Control - High pCO_2 (Day 5)	2,85	0,26	**	1,72	0,18	**	1,62	0,17	**	6,61	0,45	0,06
Control - High pCO_2 (Day 10)	2,16	0,26	*	1,55	0,21	*	1,65	0,22	*	-0,40	-0,07	0,88
Control - High pCO_2 (Day 15)	2,64	0,27	**	2,15	0,24	**	1,40	0,17	*	16,74	0,71	**
Control - High pCO_2 (Day 20)	4,11	0,34	**	2,52	0,24	**	2,55	0,24	**	7,58	0,49	0,06
Day 5 - Day 10 (Control)	4,01	0,40	*	1,46	0,20	*	1,36	0,19	*	6,24	0,51	0,07
Day 10 - Day 15 (Control)	2,02	0,29	0,07	1,30	0,21	*	0,92	0,16	0,63	22,96	0,82	*
Day 15 - Day 20 (Control)	0,96	0,12	0,50	1,29	0,16	*	1,37	0,16	*	-0,59	-0,09	0,94
Day 5 - Day 10 (High pCO_2)	4,34	0,35	*	2,12	0,21	**	1,68	0,17	**	16,18	0,67	**
Day 10 - Day 15 (High pCO_2)	2,83	0,26	*	1,91	0,19	**	2,12	0,21	**	-2,18	-0,37	1,00
Day 15 - Day 20 (High pCO_2)	3,66	0,31	*	2,13	0,21	*	2,39	0,23	**	-1,61	-0,25	1,00

Similar to the Jaccard indices, pairwise PERMANOVA tests based on Bray-Curtis dissimilarities for prokaryotic and eukaryotic communities were also significant for CO_2 condition, time and the interaction term. For CO_2 condition, the community composition of prokaryotic biofilms was

significantly different at each time point except on Day 5 (Table 5.3). Across time, the prokaryotic communities were significantly different between each consecutive time point at both the control and the high $p\text{CO}_2$ site. The eukaryotic communities were also distinct between the two sites at each time point and across every time point for the high $p\text{CO}_2$ site (Table 5.3). At the control site, only the first pair of time points (Day 5 – Day 10) were significantly different (Table 5.3). Furthermore, based on relative abundance (Bray-Curtis dissimilarities), multivariate dispersions (PERMDISP test) were more homogenous amongst all the groups compared to the Jaccard dissimilarities. PERMDISP pairwise analysis for prokaryotes found a significance between Day 5 – Day 10 and Day 5 – Day 20 at the high $p\text{CO}_2$ site ($df = 3$, $F = 3.43$, $p < 0.05$). For eukaryotes, only one significant PERMDISP difference was observed, namely between Day 5 – Day 10 ($df = 3$, $F = 2.74$, $p < 0.019$) at the high $p\text{CO}_2$ site (Figure S5.1).

5.3.3 Taxonomic composition at the control and high $p\text{CO}_2$ site

5.3.3.1 Prokaryotic community composition and succession

Overall, the relative abundance of the top bacterial phyla and classes identified at the control and the high $p\text{CO}_2$ site were very similar (Figure 5.5) but became more distinct at lower taxonomic levels (order, family, genus, and ASV). Within the Bacteria domain, *Proteobacteria* (*Alpha*- and *Gammaproteobacteria*) and *Bacteroidota* (*Bacteroidia*) dominated the biofilms across all times and at both sites (Figure 5.5). The Archaea domain was very rare comprising only 0.33% of all reads at the control site and 0.24% at the high $p\text{CO}_2$ site. Comparison of biofilm community composition between the two sites was based on the 3-week-old biofilms (Day 20).

The 3-week-old biofilms were dominated by *Proteobacteria* representing 49% at the control site and 61% at the high $p\text{CO}_2$ site. *Proteobacteria* were represented by *Alphaproteobacteria* (control: 29% and high $p\text{CO}_2$: 43.5%) and *Gammaproteobacteria* (20% and 18%). The *Alphaproteobacteria* at the high $p\text{CO}_2$ site showed an increased in two orders: *Rhizobiales* and *Rhodobacterales*. At both sites, a large proportion (50 – 60%) of *Alphaproteobacteria* was unidentified at genus level. Changes at order level were also observed for *Gammaproteobacteria* (*Pseudomonadales*, *Enterobacterales* and HOC36). *Pseudomonadales* and *Enterobacterales* were the most abundant groups at the control site (both 7%), while at the high $p\text{CO}_2$ site *Pseudomonadales* remained unchanged (7%), *Enterobacterales* (3%) showed a reduced prevalence and HOC36 (2%) increased. *Bacteroidota* was the second most dominant phylum in the 3-week-old biofilms, represented by orders *Flavobacteriales* and *Cytophagales*. Members belonging to *Bacteroidota* were more abundant at the control site (29.5%) than at the high $p\text{CO}_2$ site (17%), mainly due to a reduction (two-fold) of *Flavobacteriales*.

The third most abundant phylum in the 3-week-old biofilm was *Cyanobacteria* (control: 5.4% and high $p\text{CO}_2$: 3.5%) (*Cyanobacteriia*; Figure 5.5). At the control site members of the families *Nodosilineaceae* and *Phormidesmiaceae* were most abundant while the high $p\text{CO}_2$ site these families were reduced and there was an increase in *Synechococcus* ASVs. Next, *Verrucomicrobiota* changed from a community dominated by *Puniceicoccaceae* at the control site (4%) to *Rubritaleaceae* at the high $p\text{CO}_2$ site (5.7%). In contrast, *Planctomycetota* were slightly higher at the control site (4.5%) than at the high $p\text{CO}_2$ site (3.5%) but both CO_2 conditions (control and high $p\text{CO}_2$ site) were dominated by the OM190 class. The last taxonomic group with a relative high abundance ($>1.5\%$) at one of the sites was the *Actinobacteriota* class *Acidimicrobiia* (control: 1% and high $p\text{CO}_2$: 2.6%). At the control site, *Acidimicrobiia* members were mainly annotated as *Microtrichaceae* (family rank), while at the high $p\text{CO}_2$ site *Ilumatobacteraceae* species formed the most abundant group followed by *Microtrichaceae*.

Both sites were characterised by the same most dominant ASV, an *Alphaproteobacteria* belonging to the genus *Ruegeria*. The taxa following the *Reuveria* ASV were very different between the sites. At the control site, a diverse group of ASVs each with an individual relative abundance greater than 1% dominated the 3-week-old biofilm: *Flavobacteriaceae* ($n = 2$; *Bacteroidota*), *Nodosilineaceae* ($n = 1$; *Cyanobacteria*), and *Pseudomonadales* ($n = 1$; *Gammaproteobacteria*). At the high $p\text{CO}_2$ site, the most abundant taxa ($>1.5\%$) in addition to *Reuveria* included another three *Rhodobacterales* ASVs (*Alphaproteobacteria*) and one *Rubritaleaceae* (*Verrucomicrobiota*).

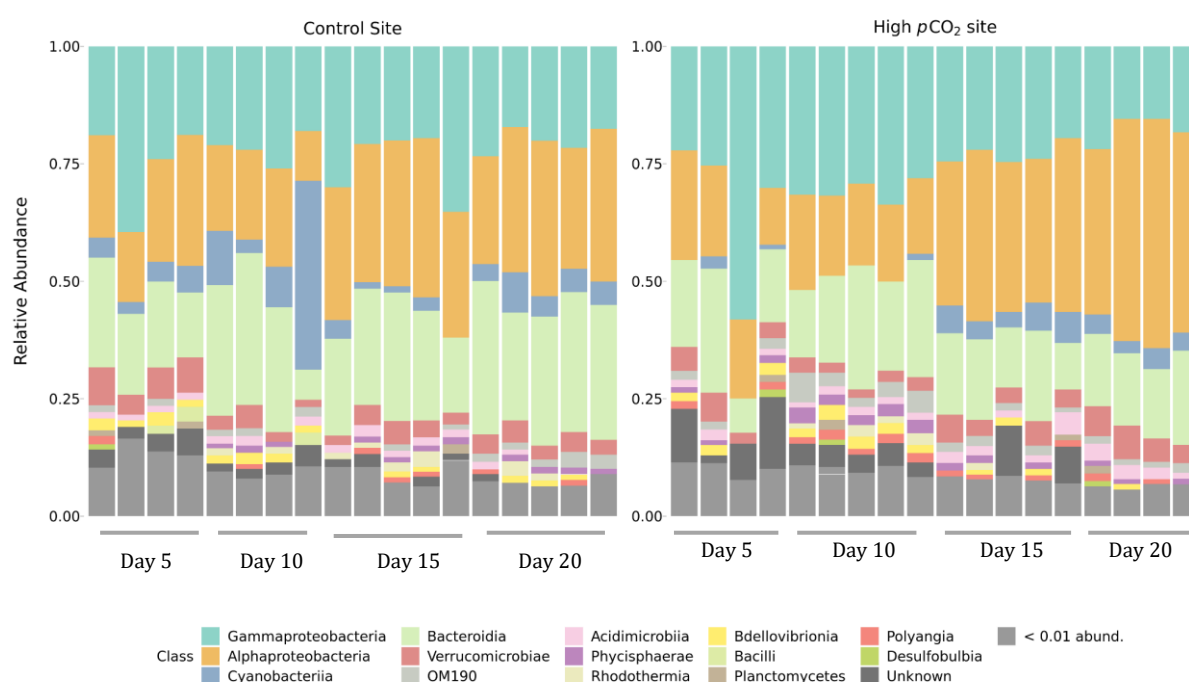


Figure 5.5: Class-level relative abundance of prokaryotic communities at the control and high $p\text{CO}_2$ site. All individual replicates are shown for each day of sampling (day 5, day 10, day 15 and day 20).

Prokaryotic biofilm development throughout the study period was characterised by several successional shifts at each site. At the control site, three major taxonomic groups (class rank: *Verrucomicrobiae*, *Alphaproteobacteria* and *Acidimicrobiia*) showed significant differences across time (MANOVA; $df = 3$, $F = 8.0$, $p < 0.01$) (Figure 5.5). *Verrucomicrobiae* reached their highest relative abundance on Day 5 followed by a fast significant decrease ($df = 3$, $F = 6.5$, $p\text{-adj} = 0.005$). The second shift was defined by an increase in *Alphaproteobacteria* after Day 10 ($df = 3$, $F = 8.8$, $p\text{-adj} < 0.01$). Finally, *Acidimicrobiia* (*Actinobacteriota*) underwent a significant decrease from Day 15 to Day 20 ($df = 3$, $F = 3.8$, $p\text{-adj} = 0.03$). At the high pCO_2 site, four successional shifts were observed for the prokaryotic biofilms (MANOVA; $df = 3$, $F = 3.6$, $p < 0.01$) (Figure 5.5). These shifts seemed to occur over longer time periods compared to the control site. *Gammaproteobacteria* slowly decreased during biofilm development (Day 5 – Day 20; $df = 3$, $F = 3.6$, $p\text{-adj} = 0.05$), reaching their lowest abundance in the 3-week-old biofilm. *Alphaproteobacteria* consistently increased in relative abundance from Day 10 to Day 15 to Day 20 ($df = 3$, $F = 42$, $p\text{-adj} < 0.01$). The third successional shift included a slow rise in *Verrucomicrobiae* from Day 10 to Day 20 ($df = 3$, $F = 4.8$, $p\text{-adj} = 0.01$). Finally, a clear increase in the class *Cyanobacteriia* occurred midway through the biofilm development from Day 10 to Day 15 ($df = 3$, $F = 20.7$, $p\text{-adj} < 0.01$).

5.3.3.2 Microeukaryotic community composition and succession

Microeukaryotic communities displayed greater heterogeneity and diversity in taxonomic composition between the two sites, at the higher taxonomic levels (Division and Class), compared to the prokaryotic communities (Figure 5.5 & Figure 5.6). Comparison of biofilm community composition between the two sites was based on the 3-week-old biofilms. The most abundant taxonomic divisions for the 3-week-old biofilms (20 days) were *Ochrophyta* (control: 70% and high pCO_2 : 77.5%) and *Rhodophyta* (22.5% and 7%). *Ochrophyta* were overwhelmingly comprised of *Bacillariophyta* (diatoms) at both sites, followed by *Phaeophyceae* to a lesser extent (Figure 5.6).

For the 3-week-old biofilms at the control site, the red algae group *Rhodophyta* (class *Florideophyceae*) was predominately represented by order *Ceramiales* (16.5%) and a smaller proportion of *Corallinales* (5.5%). At the high pCO_2 site, *Ceramiales* affiliated taxa were significantly reduced ($df = 1$, $F = 9.8$, $p = 0.003$) and together with *Corallinales* they each accounted for a relative abundance of 2%. Green algae (*Chlorophyta*, mainly *Ulvophyceae*) were equally present at both sites (approximately 2.5%). Dinoflagellates (mainly *Dinophyceae*) were significantly more prevalent at the high pCO_2 site (7%) compared to the control site (0.8%; $df = 1$, $F = 19.4$, $p < 0.001$). The *Dinophyceae* class at both sites was not dominated by a specific taxonomic group, rather similar proportions of taxa belonging to orders *Gonyaulacales* (genus *Alexandrium*), *Peridinales* and *Suessiales* were present. The heterotrophic ciliates were slightly higher at the high pCO_2 site (2.5%) compared to the control site (1.8%) and were mainly represented by CONTH_6,

followed by *Spirotrichea*. The remainder divisions with a relative abundance higher than 1% at either one or both sites were *Sagenista*, who were significantly more prevalent under elevated CO₂ levels (0.8% and 2.8%; mainly *Labyrinthulomycetes*), and *Apicomplexa* (0.02% and 1.4%).

At ASV level, both sites were dominated by the same pennate diatom species (11%), *Berkeleya hyalina*. The top five ASVs at the control site represented three diatom genera (*Berkeleya*, *Licmophora* and *Cylindrotheca*) and two *Rhodophyta* ASVs (genus *Ceramium*). at the high pCO₂ site, the top five ASVs were mainly diatoms (n = 4, including *Berkeleya*, *Mastogloian*, and *Melosira*) and one brown algae (*Phaeophyceae*).

Several successional shifts (class level) were present at each site throughout the study period (Figure 5.6). At the control site, *Bacillariophyta* (diatoms) showed a significant decrease after Day 5, displaying its lowest relative abundance on Day 10 (ANOVA; df = 3, F = 5.9, p-adj = 0.03). After Day 10 a slow increase occurred resulting in a significantly enriched diatom community after 20 days (p-adj < 0.01). *Ciliophora* class *Phyllopharyngea* reached their highest relative abundance in the early biofilm (Day 5) followed by a significant decrease (df = 3, F = 15.1, p-adj = 0.03). Another *Ciliophora* group, CONTH_6 significantly increased from Day 5 to Day 10 but decreased during further biofilm development (df = 3, F = 16.4, p-adj < 0.001). The last identified successional shift at the control site was a clear significant increase in *Florideophyceae* (*Rhodophyta*) between Day 5 and the older biofilm (Day 15 and Day 20; df = 3, F = 8.0, p-adj < 0.01). At the high pCO₂ site, the

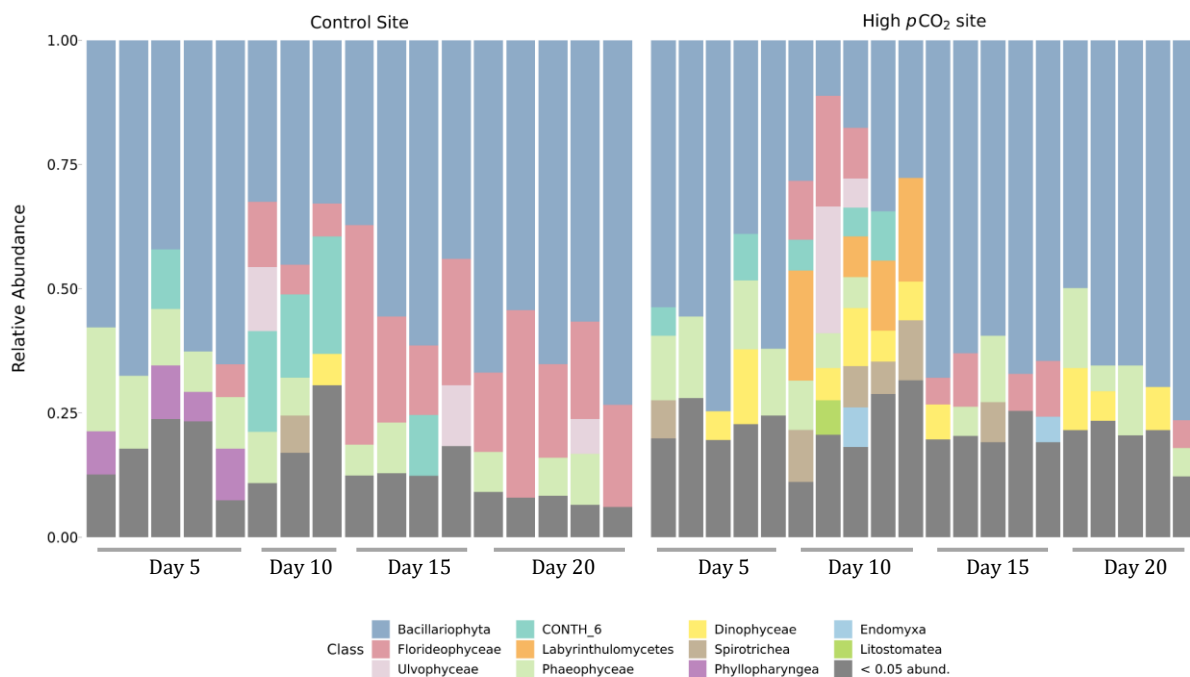


Figure 5.6: Class-level relative abundance of microeukaryotic communities at the control and high pCO₂ site. All individual replicate samples are shown for each day of sampling (day 5, day 10, day 15 and day 20).

successional shifts also involved a decrease in diatoms from Day 5 to Day 10, revealing their lowest numbers on Day 10, but was followed by a significant increase in the later biofilm (Day 20; $df = 3$, $F = 21.1$, $p\text{-adj} < 0.001$). The *Ciliophora* group that significantly changed over time at the high $p\text{CO}_2$ site was *Spirotrichea* (mainly *Hypotrichia*). This taxonomic group was significantly enriched on Day 10 and slowly decreased thereafter (Day 10 – Day 20; $df = 3$, $F = 6.7$, $p\text{-adj} < 0.01$). *Florideophyceae* marginally increased from Day 5 to Day 10 ($df = 3$, $F = 3.3$, $p\text{-adj} = 0.05$), while peak values for *Labyrinthomycetes* (*Sagenista*) were observed on Day 10 ($df = 3$, $F = 9.4$, $p\text{-adj} < 0.05$).

Diatoms were further analysed at genus level to reveal a more in-depth structure. At the high $p\text{CO}_2$ site, the diatom assemblages were more homogenous and there was less variability amongst replicates. In the early biofilms, diatom communities were dominated by *Cylindrotheca* at both sites. At the high $p\text{CO}_2$ site, the communities were enriched for *Nitzschia* throughout the 20-day study ($df = 1$, $F = 8.5$, $p = 0.006$) while *Mastogloia* and *Bacillaria* become more prevalent after Day 5 and Day 10, respectively ($df = 1$, $F = 5.3$, $p = 0.03$). Additionally, *Licmophora* sequences were highly reduced under elevated $p\text{CO}_2$ levels ($df = 1$, $F = 13.4$, $p < 0.001$).

5.3.4 Chlorophyll-a trends

Chl-*a* concentrations ($\mu\text{g cm}^{-2}$) were significantly affected by CO_2 condition (Kruskal-Wallis; $df = 1$; $H = 5.27$ and $P = 0.022$) and time point ($df = 3$, $H = 28.24$ and $P < 0.001$). At both locations, a temporal increase in chl-*a* was observed with a significant difference between each time point (Figure 5.7). The total chl-*a* concentrations ranged from 1.0 to 8.8 $\mu\text{g cm}^{-2}$ at the control site and from 2.3 to 12.1 at the high $p\text{CO}_2$ site.

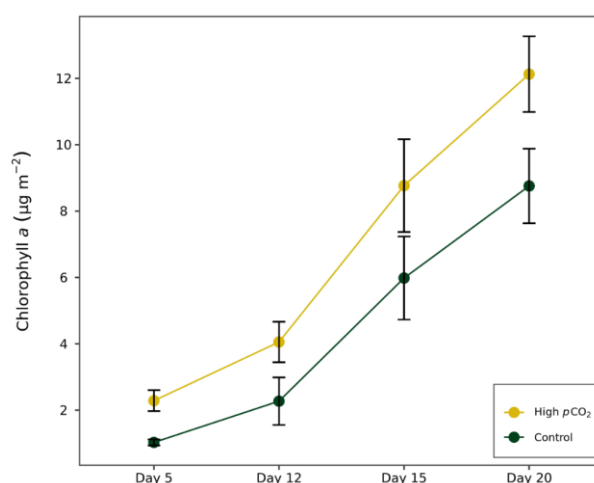


Figure 5.7: Chlorophyll-*a* concentrations at the control and high $p\text{CO}_2$ site over time. Error bars represent standard error.

5.4 Discussion

The mean pH_{Total} between the control and the high $p\text{CO}_2$ site differed significantly, while other potential confounding variables such as temperature, salinity and total alkalinity remained relatively constant between the two sites (Table 5.2). Light and macro-nutrients were not measured during this study, but due to the close proximity of the control site (~120 m) and no riverine discharge in the area, these parameters were assumed not to have significantly contributed to the variation in biofilm communities.

5.4.1 Biofilm community alpha diversity

High $p\text{CO}_2$ levels (~575 μatm) did not impact species richness in prokaryotic assemblages but did promote more diverse microeukaryotic communities. This suggests that microeukaryotic communities tend to be more resilient to elevated $p\text{CO}_2$ levels than prokaryotic assemblages. However, a change in grazing pressure (not measured in this study) due to the elevated $p\text{CO}_2$ levels could also have been responsible for a more diverse microeukaryotic community. Over time, reductions in species richness took place at both locations but occurred sooner at the high $p\text{CO}_2$ site for both prokaryotic and eukaryotic communities. This could indicate that biofilm structuring is happening at a faster rate under increased $p\text{CO}_2$ levels.

The lack of change in alpha diversity for prokaryotic biofilms was in agreement with previous studies in temperate (Allen et al., 2021) and tropical waters (Hassenrück et al., 2017). Notably, slightly higher richness values were observed under elevated $p\text{CO}_2$ levels for the early prokaryotic biofilms (Day 5 – Day 10) in the current study. The absence of a significant result could be a bias of the low sample sizes. Overall, contrasting findings have been reported in previous literature concerning prokaryotic alpha diversity under high $p\text{CO}_2$ levels. For instance, some studies showed greater diversity of prokaryotic biofilms at natural CO_2 -rich ecosystems (Lidbury et al., 2012; Kerfahi et al., 2014, 2020) and in a mesocosm system (Witt et al., 2011), while others showed reduced alpha diversity *in situ* (Taylor et al., 2014) as well as in a mesocosm experiment (Russell et al., 2013). The heterogeneous responses among all these studies suggest the interference of confounding variables. Differences in species interactions, nutrient limitation, light availability, study duration, climatic region, molecular methods, and substratum type, could all have contributed to the variability in alpha diversity and require further research.

The higher species richness of eukaryotic biofilms under high $p\text{CO}_2$ conditions was partly in line with the study of Allen et al. (2021), who reported a single significant increase under high $p\text{CO}_2$ levels after five days of substrate exposure. Both academic works, the study by Allen et al. (2021)

and the current study, were done by the same research group and used a similar experimental set-up, sampling process and sequencing flow, but the studies were performed in different climate zones. The consistent higher eukaryotic species richness under elevated $p\text{CO}_2$ levels observed at each time point in the current study was perhaps the result of elevated CO_2 in a tropical setting. However, other environmental parameters such as nutrient levels, metals, and reduced grazing pressure could also be responsible for an overall higher diversity at the high $p\text{CO}_2$ site. Further, increased eukaryotic richness under high $p\text{CO}_2$ was also observed by Lidbury et al. (2012) for biofilms under reduced light at a CO_2 vent in Italy (substrate was immersed for 16 days). Other CO_2 -enrichment experiments looking at eukaryotic biofilms have mainly focussed on primary producers (Johnson et al., 2013, 2015; Harvey et al., 2019). These studies all reported major increases in relative abundance of only a few taxa and mainly involved a shift towards larger diatoms. In terms of alpha diversity this was expressed as a decrease in species richness and evenness. In a review by Aiuppa et al. (2020), similar negative trends in diversity with high $p\text{CO}_2$ are described across different groups of organisms at tropical, sub-tropical and temperate vent sites and are said to result in more homogenous ecosystems.

5.4.2 Biofilm development processes under high $p\text{CO}_2$ levels

Prokaryotic and eukaryotic benthic communities established distinct biofilms at the CO_2 enriched site which is in agreement with previous studies (Witt et al., 2011; Lidbury et al., 2012; Johnson et al., 2013; Kerfahi et al., 2014, 2020; Taylor et al., 2014; Harvey et al., 2019, 2020; Allen et al., 2021). The difference for the biofilm communities observed between the two sites was a combination of varying relative abundance and species presence and absence. Previous research has attributed the modified biofilm assemblages under high $p\text{CO}_2$ levels to enhanced bacterial degradation of extracellular polymeric substances (EPS) as a result of increased extracellular enzyme activity in elevated CO_2 conditions (Piontek et al., 2010; Engel et al., 2014). In addition to enhanced EPS degradation, an increase in biofilm EPS production under high $p\text{CO}_2$ was also observed, allowing for species competition, the potential of cross-feeding and subsequent modification of niche availability (Engel, 2002).

The prokaryotic communities at the high $p\text{CO}_2$ site were consistently distinct from those at the control site as a result of species replacement (Turnover). Similarly, the distinct eukaryotic communities under high $p\text{CO}_2$ levels were also primarily driven by Turnover, but with a contribution of species loss (Nestedness) at Day 15 which was expressed as a significant reduction in species richness. The high dominance of Turnover for both kingdoms confirms that an environmental pressure, here CO_2 enrichment or vent nutrients, is the ecological driver behind the distinct community compositions between the two sites (Qian et al., 2005; Baselga, 2010). This

result was also in agreement with the findings of biofilm community structuring near a CO₂ vent in Japan (Allen et al., 2021).

Further, the processes involved in biofilm community development were also investigated at both locations. For prokaryotic communities, the first two weeks of biofilm development were defined by species replacement under both CO₂ conditions. This was strictly followed by species loss for the older biofilms (Day 15 – Day 20) at both sites. The only difference between the two sites was that species loss already contributed to the second week of biofilm development under high *p*CO₂ levels. A similar temporal anomaly in development processes was observed for the eukaryotic communities under high *p*CO₂. At the control site, the 3-week-old biofilms were structured by a sequence of Turnover (Day 5 – Day 10), Nestedness (Day 10 – Day 15) and Turnover (Day 15 – Day 20), while at the high *p*CO₂ site the sequence was Nestedness and Turnover, Turnover, and Turnover. The earlier appearance of species loss under high *p*CO₂ levels was in line with the results for biofilm alpha diversity, indicating that biofilms at the high *p*CO₂ site evolve faster towards a more homogeneous community.

5.4.3 Biofilm taxonomic composition and community succession

5.4.3.1 Prokaryotic communities

Although the prokaryotic communities at phylum and class level were very similar under both CO₂ conditions (control and high *p*CO₂ site), at lower taxonomic level the prokaryotic biofilms revealed shifts in relative abundance and dominance under high *p*CO₂ (Table 5.4). The prokaryotic assemblages at both sites were dominated by *Alphaproteobacteria* and showed an increase in relative abundance over time. The most abundant ASV for each CO₂ condition was a taxa belonging to genus *Ruegeria*. The prevalence of this genus is likely linked to the high temperature (29°C) of this study area. For instance, an increase in *Ruegeria* in the water column was associated with elevated temperature during an incubation experiment by Minich et al. (2018). Further, high *p*CO₂ levels did not seem to impact the relative abundance of *Ruegeria* in this study and thus might suggest that temperature in general has a stronger effect on this genus than CO₂.

Overall, *Alphaproteobacteria* were enriched at the high *p*CO₂ site as a result of increases in orders *Rhizobiales* and *Rhodobacterales*. An increase in *Rhizobiales* in the water column with elevated *p*CO₂ was observed in the study of Minich et al. (2018), while increases in *Rhodobacterales* with *p*CO₂ have previously been observed in sediments (Raulf et al., 2015). This could indicate a general positive response of these orders to elevated *p*CO₂ in a tropical setting. Changes in *Alphaproteobacteria* at lower taxonomic levels (genera) beside *Ruegeria* were not detected in this study due to the high number of unclassified taxa beyond the class rank at either site. This large

portion of unidentified bacteria highlights the unexplored diversity of this taxonomical group in biofilm communities in a tropical setting under both CO₂ conditions. The second most abundant group, *Bacteroidota*, showed a slight decrease under high *p*CO₂ levels but did not express any significant changes over time. Surprisingly, the biggest reduction was noticed for *Flavobacteriales* sequences. Previous studies have mainly reported a positive correlation between *p*CO₂ and *Flavobacteriales* in the water column, sediment and biofilms (Witt et al., 2011; Taylor et al., 2014; Raulf et al., 2015; Lin et al., 2018) due to their high role in carbon decomposition (Buchan et al., 2014). However, *Rhodobacteriales* are also major players in organic carbon remineralisation (Buchan et al., 2005) and although both bacterial groups benefit from elevated temperatures (Raulf et al., 2015; Arandia-Gorostidi et al., 2020), it appears that high *p*CO₂ levels and the tropical setting of this study favours *Rhodobacteriales* over *Flavobacteriales*.

Table 5.4 Changes in prokaryotic taxonomy at elevated *p*CO₂ levels.

Changes in relative abundance at the high <i>p</i> CO ₂ site			
Phylum	Class	Order	Change
Proteobacteria	Alphaproteobacteria	Rhizobiales	Increase
		Rhodobacterales	Increase
	Gammaproteobacteria	HOC36	Increase
Bacteroidota	Bacteroidia	Flavobacteriales	Decrease
Verrucomicrobiota	Verrucomicrobiae	Opitutales & Verrucomicrobiales	Shift
Actinobacteria	Acidimicrobiia	Microtrichales	Shift
Shift in dominance			
Phylum	Control <i>p</i> CO ₂ (Family)	Elevated <i>p</i> CO ₂ (Family)	
Cyanobacteria	Nodosilineaceae	Synechococcales; Synechococcus	
	Phormidiaceae		
Verrucomicrobiota	Puniceicoccaceae	Rubritaleaceae	
Actinobacteria	Microtrichaceae	Ilumatobacteraceae	

Acidimicrobiia had a low abundance at the control site which was unexpected as these bacteria grow well at high temperatures (Matsumoto et al., 2009, 2013). Here, we saw an increase in *Acidimicrobiia* and a shift at family level from *Microtrichaceae* to *Ilumatobacteriaceae* under high *p*CO₂ levels. *Ilumatobacteriaceae* is a relatively recent described family and includes aerobic non-motile bacteria identified from the sediment (Matsumoto et al., 2009, 2013). The present study suggests that elevated *p*CO₂ in a tropical setting benefits the growth of *Ilumatobacteriaceae* (genus *Ilumatobacter*). Similarly, a shift in *Verrucomicrobiota* at family level was observed under high *p*CO₂ from *Puniceicoccaceae* to *Rubritaleaceae*. In addition, *Verrucomicrobiota* were more abundant in the early biofilm at the control site, while they reached their highest values in the 3-week-old biofilm at the high *p*CO₂ site.

Archaea represented only a small portion (<0.3%) of the reads under both $p\text{CO}_2$ conditions. Information on archaea is still scarce owing to primer sets that are mostly biased against archaea. The primers used here were adapted to remove such biases (Parada et al., 2016), however detection remained low. This indicates that archaea are rare in these tropical marine biofilms. Similar numbers were reported by previous studies (Webster & Negri, 2006; Zhang et al., 2014; Pollet et al., 2018).

5.4.3.1.1 Oxygenic Phototrophic Prokaryotes

The mean relative abundance of *Cyanobacteria* was not impacted by $p\text{CO}_2$ enrichment, but a shift in dominant taxa was observed. Filamentous *Cyanobacteria* classified as *Nodosilineaceae* and *Phormidesmiaceae* dominated the 3-week-old biofilms at the control site, at the elevated $p\text{CO}_2$ site there was increased *Synechococcus* abundance. Previous studies that focussed on *Cyanobacteria* (incl. *Synechococcus*) under high $p\text{CO}_2$ reported an increase in growth (Morrow et al., 2015; Das & Mangwani, 2015). In an incubation experiment by Fu et al. (2007), increases in *Synechococcus* were observed under high $p\text{CO}_2$ but only when combined with elevated temperatures. The study showed that high temperature combined with high $p\text{CO}_2$ benefitted *Synechococcus* by increasing the maximum chl-*a* photosynthetic rates and raising the content of both phycobilin and chl-*a* (Fu et al., 2007). In line with that study, an increase in chl-*a* concentrations was also observed here and could potentially be linked to *Synechococcus* enrichment at the high $p\text{CO}_2$ site.

5.4.3.2 Photo- and heterotrophic eukaryotes

Measurements of chl-*a* concentrations can be used as a proxy to determine photosynthetic standing crop (Underwood, 1984). The significant increase of chl-*a* (x1.5) at the high $p\text{CO}_2$ site can therefore be interpreted as an increase in primary production under elevated $p\text{CO}_2$ levels at this tropical vent system. A similar result with a more substantial increase (fivefold) in periphyton biomass was observed for benthic assemblages on artificial acrylic substrates at a temperate CO_2 vent in Italy (Johnson et al., 2013). While a three- to fourfold increase was obtained for natural epilithic, epipellic and epipsammic communities at the same temperate vent system in Italy (Johnson et al., 2015). Despite chl-*a* displaying a significant increase at every time point, no variation in the overall mean relative abundance for the dominant primary producers (diatoms and *Cyanobacteria*) was found under high $p\text{CO}_2$ levels.

The photosynthetic component of the eukaryotes was overwhelmingly dominated by raphid pennate diatoms. This has been reported in earlier studies; benthic diatom communities, irrespectively of their substratum, are dominated by pennate diatoms, while phototrophic communities in the water column are primarily characterised by centric diatoms (Patil & Anil, 2007; and references therein). High $p\text{CO}_2$ levels in the tropical setting of this study did not impact

this structure. The 3-week-old established biofilms at both locations were equally dominated by diatom species *Berkeleya hyaline*. The high prevalence of genus *Berkeleya* coincided with the dominance of the *Alphaproteobacteria* genus *Ruegeria* (discussed earlier) under both CO₂ conditions and could therefore be the first evidence for a potential diatom – *Alphaproteobacteria* interaction (Amin et al., 2012) amongst these genera driven by the high temperature of this study.

Despite the similarity of the most abundant diatom species at the two sites, a clear community structural difference was also observed for diatoms as a result of species replacement. Diatom genera *Nitzschia*, *Mastogloia*, *Bacillaria* and chain forming diatoms of genus *Melosira* showed an obvious increase at the high pCO₂ site, while other genera such as *Licmophora* and *Cylindrotheca* became more scarce under high pCO₂ levels. Proliferation of some taxa and reduction of others revealed the genus specific responses to elevated pCO₂ levels (Tortell et al., 2008; Trimborn et al., 2009; Finkel et al., 2010; Torstensson et al., 2012). Carbon dioxide enrichment has been shown to select for large pelagic diatoms (Wu et al., 2014) and to favour large chain forming periphytic diatoms (Tortell et al., 2008; Johnson et al., 2013; Harvey et al., 2019). Phototrophic microorganisms have adjusted to ambient pCO₂ levels with Carbon Concentrating Mechanisms (CCMs) that allow them to actively improve the uptake of CO₂ (Giordano et al., 2005). Enriched seawater pCO₂ levels can benefit these phototrophs by increasing primary production and through the down regulation of the energy demanding CCMs (Beardall & Giordano, 2002; Rost et al., 2008; Trimborn et al., 2009). This reduction in CCM use and the associated carbon fixation cost permits resource allocation towards growth (Hopkinson et al., 2011). Further, the larger diatom species and increased primary production will likely enhance the transfer of energy to higher trophic levels e.g. marine animals by reducing the complexity of the food chain (Sommer et al., 2002).

The changes for the other eukaryotic communities beside diatoms were observed at high taxonomic level under high pCO₂ (Table 5.5). The impact of pCO₂ was most obvious for *Rhodophyta* affiliated taxa who showed a substantial decrease at the high pCO₂ site. The same adverse effect of high pCO₂ on red algae was found at tropical volcanic vents in Papua New Guinea (Fabricius et al., 2015) and temperate CO₂ vents in Japan (Agostini et al., 2018). These studies mention taxa belonging to the *Corallina* order, interestingly though, *Rhodophyta* in this study was predominantly represented by *Ceramiales* rather than the calcareous red algae. Here, a shift from *Ceramiales* dominated *Rhodophyta* communities to equal low numbers of *Ceramiales* and *Corallina* was observed under high pCO₂. The high relative abundance of *Ceramiales* is not surprising since they account for more than a third of *Rhodophyta* species diversity in general (Guiry & Guiry, 2019; Díaz-Tapia et al., 2019). The overall low numbers of crustose coralline algae (CCA) at the high pCO₂ site is expected, but the lower numbers at the control site are concerning. Coralline algae

play a key role in the development of coral reef habitats and provide a substrate for larvae settlement of many benthic organisms (Heyward & Negri, 1999; Perry & Hepburn, 2008). The average high temperature of this study area could have strongly impacted the CCA at both sites. In contrast to the observed decline in *Rhodophyta*, dinoflagellates appeared to benefit from elevated $p\text{CO}_2$ levels. Similar to diatoms and *Cyanobacteria*, the observed trend is likely related to their inorganic carbon uptake strategy (Reinfelder, 2011; Hepburn et al., 2011; Hyun et al., 2020). Specifically, dinoflagellates possess form II RubisCO which has a low affinity for CO_2 (Morse et al., 1995; Rost et al., 2006; Reinfelder, 2011) and therefore are assumed to benefit from a CO_2 enriched environment. Additionally, dinoflagellates have been recognised for their diverse lifestyles including not only autotrophy, heterotrophy but also mixotrophy (Stoecker et al., 1997, 2009; Gast et al., 2018). The ability to harvest energy and nutrients through both photosynthesis and food ingestion would give mixotrophic dinoflagellates a competitive advantage under elevated CO_2 conditions. Notably, an increase in *Alexandrium* genus was detected over the 3-week development period under high $p\text{CO}_2$. This genus is classified as a harmful algae species (Fu et al., 2012) as they can synthesize several paralytic shellfish toxins (Wang, 2008; Anderson et al., 2012). This observation is in line with previous research that has shown increased toxin production and growth of the dinoflagellate *Alexandrium* under high $p\text{CO}_2$ (Hattenrath-Lehmann et al., 2015), and thus supports the prediction of increased harmful algal blooms as a result of ocean acidification.

Table 5.5: Changes in eukaryotic taxonomy at elevated $p\text{CO}_2$ levels.

Changes in relative abundance at the high $p\text{CO}_2$ site			
Division	Class	Order	Change
Dinoflagellata	Dinophyceae	Gonyaulacales (genus <i>Alexandrium</i>), Peridinales and Suessiales	Increase
Sagenista	Labyrinthulomycetes	Labyrinthulida	Increase
Rhodophyta	Florideophyceae	Ceramiales	Decrease

Elevated $p\text{CO}_2$ levels also enriched *Labyrinthulomycetes* (*Sagenista*), which are heterotrophic fungus-like protists. These heterotrophic eukaryotes contribute to secondary production through mineralisation of organic matter and provide ω -3 polyunsaturated fatty acids to higher tropic levels (Raghukumar, 2002). These species stand to benefit indirectly from enriched $p\text{CO}_2$ levels through increased primary production.

Finally, the composition and relative abundance of *Ciliophora* in the 3-week-old biofilms was low and did not differ between the two CO_2 conditions. This is not surprising as ciliates are known to

be pioneer biofilm colonisers (Coppellotti & Matarazzo, 2000; Risse-Buhl & Küsel, 2009) and thus are expected to reach their highest numbers in the early biofilm. The early successional shifts that were observed for *Ciliophora* were characterised by different taxonomical groups between the two sites. The relative abundance of *Phyllophyrangia* and CONTH_6 that dominated the early biofilm at the control site were highly reduced under elevated $p\text{CO}_2$ levels, while *Spirotrichea* taxa clearly benefited from enriched $p\text{CO}_2$.

5.5 Conclusion

This study confirms the previous findings that an increase in $p\text{CO}_2$ results in unique biofilm communities and that this response is also present in a tropical setting. Distinct communities were observed for both prokaryotic and eukaryotic benthic biofilm assemblages. Several prokaryotic taxonomical groups benefitted from high $p\text{CO}_2$ levels (*Rhizobiales*, *Rhodobacterales*, *Ilumatobacteriaceae*, *Rubritaleaceae*, and *Synechococcus*) while others showed adverse effects (*Flavobacteriales*, *Microtrichaceae*, *Puniceicoccaceae*, *Nodosilineaceae*, and *Phormidesmiaceae*). Similarly, for eukaryotic microorganisms there were so-called ‘winners’ (*Nitzschia*, *Mastogloia*, *Bacillaria*, *Melosira*, *Labyrinthulomycetes*, and *Spirotrichea*) and ‘losers’ (*Licmophora*, *Cylindrotheca*, *Ceramiales*, *Phyllophyrangia*, and CONTH_6). Further, some genera were not affected by the $p\text{CO}_2$ enriched environment but were clearly selected for by the tropical temperatures and displayed a high prevalence at both sites (e.g. *Ruegeria* and *Berkeleya*).

The increase in chl-*a* under high $p\text{CO}_2$ that was previously observed in temperate climate zones was also confirmed in this tropical ecosystem and was linked to a shift towards larger chain forming diatom species and an increase in *Synechococcus* for the prokaryotic phototrophs (*Cyanobacteria*). While prokaryotic alpha diversity was not altered under high $p\text{CO}_2$, eukaryotic communities were found to support a higher species richness. However, due to the heterogeneity in literature surrounding biofilm alpha diversity no common response for prokaryotic and eukaryotic biofilms can be described to date. Nonetheless, the results in this study show that increased $p\text{CO}_2$ levels have a larger impact on benthic eukaryotic alpha diversity than on prokaryotes.

Turnover or species replacement was the dominant process that structured the biofilm communities under high $p\text{CO}_2$ compared to their analogues under ambient CO_2 conditions. For both kingdoms, increased $p\text{CO}_2$ directed biofilms earlier towards a more homogeneous community.

Even though distinct communities were observed under high $p\text{CO}_2$ levels, the functional composition of the biofilms could have remained unaltered. This was reported in a study by Kerfahi et al. (2020) who relied on predictions of the microbial functional guilds based on 16S amplicon sequence data. The next step would be to use full metagenomes to thoroughly investigate the functional composition of the biofilms and assess the biofilm communities in the tropical coastal ecosystem at seasonal scale to investigate the long-term exposure of biofilms.

Chapter 6 |

General Discussion

6.1 Discussion

The ocean is the largest biome on Earth covering over two-thirds of the planet's surface. It provides a wide variety of habitats and ecosystems that host an immense diversity of marine ecosystems and supports millions of people through ecosystem services (Beaumont et al., 2007). However, many marine organisms are currently being threatened by anthropogenic pollution, overfishing and climate change (Doney et al., 2012). In the marine realm climate change has caused sea surface temperatures to rise, created deoxygenation zones and continues to decrease ocean alkalinity (drop in pH), a process referred to as ocean acidification (Raven et al., 2005; Bopp et al., 2013; IPCC, 2021). The implications of the changes in ocean carbon chemistry as a result of ocean acidification (OA) are strongly linked to microorganisms due to their major involvement in ocean biogeochemical cycles and particularly the carbon cycle (Field et al., 1998; Falkowski et al., 2008). In order to predict holistic marine microorganism responses to future environmental changes, principally caused by the recent anthropogenic impacts, a better understanding of microbial ecology is required.

An important aspect of studying change in aquatic systems is the need for natural baseline data and continuous long-term observations. This was the main motivation behind the research work performed in this thesis. Chapter 2; Assessing long-term protist community dynamics across the Southland Front, New Zealand. This chapter describes the first comprehensive analysis of protist taxonomic composition with spatiotemporal variability patterns for this region. It documents the first five years of an ongoing long-term monitoring project (the Munida transect) comparing protist community diversity and dynamics across different water masses. Along the transect, continuous measurements of $p\text{CO}_2$ concentrations have monitored OA since 1998 (Currie & Hunter, 1999; Currie et al., 2011). By continuing the characterisation of protist communities alongside the $p\text{CO}_2$ measurements, these datasets collectively present a strong tool for the detection of OA impacts on microorganisms that underpin marine food webs. In Chapter 3, Bacterial and eukaryotic benthic biofilms were evaluated in terms of composition and structuring across a small spatial scale in a local temperate kelp forest. The objective of this experiment was to provide an overview of benthic biofilms communities as a comparison to further enhance our understanding of the macroalgae host-microbiome, which was covered in Chapter 4. These combined experiments illustrate the advantage of providing baseline data for an ecosystem. A comparison of the kelp microbiome, between young and old blade tissues, to the benthic biofilms reveals more in-depth knowledge about kelp specific microorganisms (Chapter 4). Additionally, by identifying both prokaryotic and eukaryotic biofilm communities a comprehensive description of the kelp microbiome is obtained. Finally, in a more direct approach to study microbial biofilm

responses to OA a shallow CO₂ vent system, as a natural analogue to OA, was used to gain insight into the potential composition of future biofilms. A comparison was made between benthic biofilms at a control site and biofilms near the CO₂ vent in a tropical coastal system (Chapter 5).

6.1.1 Free-living protist community dynamics

New Zealand is defined by one of the longest coastlines and largest marine regions in the world compared to its land surface area. The extremely diverse coastal and open ocean environments provide New Zealand with a wide variety of social and economic benefits ranging from regulatory ecosystem and provisioning services to non-consumptive opportunities (such as recreation and visual amenities) (MacDiarmid et al., 2013). Surprisingly, with this huge access to marine coastal and open ocean waters there is only limited information about phytoplankton taxonomic composition and distribution in the water masses east of the New Zealand coastline (DiTullio et al., 2003; Peloquin et al., 2011; Chiswell et al., 2019; Allen, Summerfield, et al., 2020). On a global scale, the *Tara* Oceans expedition from 2009 to 2013 undertook the largest plankton survey to date researching their distribution, abundance and diversity (e.g. De Vargas et al., 2015; Lima-Mendez et al., 2015; Vincent et al., 2022). The project provided a comprehensive overview of plankton community dynamics in the open ocean covering a wide variety of ecosystems such as upwelling regions, mesoscale eddies, and oxygen-poor zones (Vincent et al., 2022). The major limiting factor of this expedition were the single sampling efforts in time and space. This underlines the need for mapping microorganism dynamics on regional and local scales in addition to global surveys. The ongoing long-term monitoring of protist communities off the coast of New Zealand allows detection of protist spatiotemporal patterns and is a great addition to the existing phytoplankton long-term time-series located in the Northern Hemisphere (Bindoff et al., 2007).

Several findings from the global *Tara* Oceans project were highlighted including diversity, taxonomic compositions, biogeographical patterns of phytoplankton size groups and drivers behind community structuring. They found that heterotrophic protists comprise a larger diversity than had been previously reported with an emphasis on parasitic taxa (De Vargas et al., 2015; Lima-Mendez et al., 2015). Current results strongly confirm this observation and further extend this trend to New Zealand waters. The similar magnitude of abundance and co-occurrence across the different water masses that was seen for dinoflagellate species *Dinophyceae* and *Syndiniales* (parasitoids) in Chapter 2 suggests a larger trend in microbial ecology. Namely, that biotic interactions, e.g. heterotrophic/mixotrophic – parasites, are more important drivers for protist communities structuring (Lima-Mendez et al., 2015; Needham & Fuhrman, 2016; Berdjeb et al., 2018) rather than environmental resources and abiotic factors (Verity & Smetacek, 1996; V. Smetacek, 2001) which were shown to explain only a small fraction of marine protist variability

across the Southland Front. The trends seen in *Chlorophyta* with *Mamiellophyceae* being the dominant group in the coastal adjacent waters (NW and STW) and *Chloropicophyceae* in the offshore open ocean water (SASW) was also consistent with the global plankton patterns (Monier et al., 2016; Vannier et al., 2016; Dos Santos et al., 2017). The diatom species that were dominant across all water masses belonged to the pico-nanophytoplankton size range, specifically genus *Minidiscus*, and have been relatively unexplored. The dominance of these small-cell diatoms over larger species could be specific to this region but could also be a more general trend for temperate zones which are known to host multiple groups of nanophytoplankton. Overall, the water masses across the Southland Front were shown to be dominated by pico- and nanophytoplankton. Due to the advances made in sequencing technology combined with optical methods in the 20th century, smaller protist organisms belonging to pico- and nanophytoplankton are being increasingly discovered. A larger diversity than had so far been reported for these size groups is likely present in the ocean but has gone undetected partly due to their poor morphologic and physiologic characterisation. It has been argued that trophic models of the open ocean need to be updated to better include mixotrophic and parasitic protists as well as picophytoplankton on account of their high abundance and diversity as well as their involvement in the carbon cycle. The results from this study (Chapter 2) strongly support that idea.

The kelp forest ecosystem that was studied in Chapter 4 is located on the East Coast of the South Island in the coastal water mass adjacent to the water masses described in Chapter 2. The protist communities in the kelp forest seawater revealed a different community composition from the ones observed in coastal adjacent NW (Chapter 2). Although, similar taxonomic groups were present in both waters, including *Dinophyceae*, *Syndiniales*, diatoms, and *Chlorophyta* (*Mamiellophyceae*), their relative abundance was vastly different. Diatoms were more abundant across the kelp forest which reflects this highly productive coastal ecosystem (Steneck et al., 2002). A clear decline in heterotrophic and parasitic taxa (*Dinophyceae*, *Syndiniales* and ciliates) was observed from open ocean water towards the coastal kelp ecosystem and even at a finer scale from the exterior kelp bed towards the interior. Despite the decline of dinoflagellates, the co-occurrent abundance between *Dinophyceae* and *Syndiniales* stayed consistent, which presents further evidence of a potential interaction between these groups. Temperature was found to be the biggest driver behind the fine scale regional organisation for the overall dataset (Chapter 2) but only explained a small fraction of the community variability. Different drivers were found to structure the communities over time emphasizing underlying patterns as a result of natural seasonal variability. Evidence of seasonality was detected in NW, STW, and the Front but was only limited due to the disparity of the data. The continued monitoring of protist communities across the Southland Front will enable a better characterisation of the spatiotemporal patterns.

6.1.2 Microbiome of coastal kelp ecosystems

In Chapters 3 and 4, prokaryotic and eukaryotic biofilm community dynamics were investigated to gain a better understand of their interaction with macroalgae. Biofilm communities were first studied on settlement plates at the interior and exterior kelp bed to document their development dynamics and taxonomic composition within the kelp ecosystem. Overall, the kelp microbiome was found to be distinct from the plate biofilms and the surrounding environment. A higher selection pressure from the kelp on prokaryotic communities was observed compared to the eukaryotic epibionts. The latter seem to be more region-specific rather than kelp-species specific. The prokaryotic epibionts were largely similar to the microbiome that was found for *M. pyrifera* in California, USA (Weigel & Pfister, 2019), thereby confirming the overall consensus that prokaryotic assemblages are driven foremost by kelp-species rather than their environment (region-specific). By comparing the kelp microbiome to the plate biofilms and the surrounding seawater it was shown that certain taxa were selected for by *M. pyrifera* (e.g. *Granulosicoccus*, *Blastopirellula*, *Persicirhabdus*, *U. leptochaetei*) while others were deterred (e.g. *Amylibacter* and *Yoonia* sp.). The regional specificity of eukaryotic epibionts highlight the importance of studying kelp at regional and local scale. To determine how the eukaryotic communities benefit kelp and how important they are for the host kelp health future research should be done on the functions of these epibionts.

6.1.3 Prokaryotic and eukaryotic biofilm development patterns

Successional development processes were studied for the biofilm communities in two coastal ecosystems, the kelp forest (Chapter 3) and the CO₂ vent system (Chapter 5). The beta-diversity of the microbial communities was partitioned into Turnover and Nestedness to assess the driving mechanisms behind biofilm development. Biofilms (prokaryotic and eukaryotic) at both sites developed differently, in the kelp forest mature biofilms were formed after 18 – 26 days while at both sites for the CO₂ vent ecosystem biofilm composition was still changing after 20 days. Species replacement (Turnover) was the major driver for biofilm development in the kelp forest with changes in both species relative abundance and species presence/absence. In the CO₂ ecosystem biofilm communities were formed by a combination of both species replacement and species loss (Nestedness) under ambient and high *p*CO₂ conditions, supported by changes in species relative abundance and presence/absence. A third comparison can be made with the study from Allen et al. (2021) who performed the same experimental setup, sampling, processing, and analysis as was used here in Chapter 3 and 5. The biofilm development processes observed in the study by Allen et al. (2021) were also strictly guided by species replacement. Although a number of biotic and

abiotic variables are responsible for biofilm structuring, from these three studies it can be concluded that temperature is the main contributing factor for the differences observed.

However, similar patterns between Chapters 3 and 5 were observed when comparing prokaryotic vs. eukaryotic taxonomic structuring. Despite the different environmental selection pressures (high $p\text{CO}_2$ and hydrodynamics combined with wave disruption), prokaryotic communities at high taxonomic level showed only slight differences in each coastal ecosystem in response to the local stressors and became apparently more different at lower taxonomic level. For the eukaryotic biofilms clear patterns were already observed at the highest taxonomic ranks (division and class). This reflects the higher diversity that is associated with prokaryotic communities in general.

6.1.4 Future trends in prokaryotic and protist communities

Chapters 2 and 5 supplied direct evidence of microorganism responses to climate change induced environmental alterations or a proxy thereof (CO_2 vent). During the long-term measurements of protist communities across the Southland Front a summer marine heatwave (MHW) event was encountered. This offered unique insight into natural protist community reactions to short-term rapid temperature rises. Due to the long-term nature of the study a comparison with communities under ambient summer conditions was possible.

The community shifts observed with increasing sea surface temperatures (SST) were the result of substantial increases in abundance mainly for *Syndiniales* and *Chloropicophyceae*, rather than species replacement and are in line with previous reports on warming (Romaní et al., 2014; Smale et al., 2017). The trend of the previously described covariance of *Syndiniales* and *Dinophyceae* abundance was interrupted as *Dinophyceae* showed a massive decrease under heatwave conditions. This could indicate that a change in host-parasite interaction took place and that *Syndiniales* is killing off their host. Since it is not known if the *Syndiniales* numbers that were sequences were still associated with a host or free living in the water column this could be a likely scenario. Similar changes in host-parasite interactions with temperature have been reported for Arctic invertebrates (Gilg et al., 2012; and references therein).

Diatom abundance was also reduced under the heatwave conditions which is in contrast with previous studies that reported an increase in phytoplankton abundance and primary productivity with increasing SST (Hutchins & Fu, 2017; Ibarbalz et al., 2019). Most reports on increasing SST also reveal an increase in microorganism diversity, thereby supporting the trend of tropicalization of temperate plankton (Morán et al., 2010, 2017; Smale et al., 2017; Ibarbalz et al., 2019). However, the marine heatwave observed in this study drastically reduced the diversity of protist communities across STW, the Southland Front and SASW. These opposing results could be related

to the fact that MHWs are short-term rapid increases in temperature rather than a long-term continuous rise. Modelling predictions would be based on the gradual increase of SST whereas incubation experiments tend to include a condition period to avoid observing community stress responses. Additionally, temperature has also been predicted to shift the community towards organisms with a reduced body size (Sommer et al., 2017). These predictions, higher diversity and abundance of smaller organisms, would mean a reduced upward energy flow through to higher trophic levels (Beaugrand et al., 2008; Ullah et al., 2018). These contrasting findings emphasize the importance of studying climate related changes at different biogeographical scales, as it is clear here that not all ecosystems will respond in a similar manner. Further it also stresses the need for baseline data to understand the patterns of change that are observed.

To gain insight into the effect of OA on biofilm communities a CO₂ vent system was used as an analogue to ocean acidification. This provides the opportunity to study *in situ* community responses to high *p*CO₂ in an open systems, rather than closed laboratory settings that do not account for ecosystem interactions and include preconditioning periods which may confound the responses of the studied communities (González-Delgado & Hernández, 2018). High *p*CO₂ levels resulted in different prokaryotic and eukaryotic communities from the control site. These differences were driven by species replacement, a process that was also observed for the biofilm communities near a CO₂ vent in a temperate climate (Allen et al., 2021). Therefore, this finding is suggestive of a potential general response of biofilm assemblages to high *p*CO₂ levels. During biofilm development a decrease in species richness was observed which generally marks a successional shift towards a more mature biofilm. However, under high *p*CO₂ conditions this reduction in diversity occurred sooner for both prokaryotic and eukaryotic biofilms. This could indicate that under high *p*CO₂ levels biofilms develop at a faster rate.

6.2 Future directions

Long-term time-series provide important and powerful data to study community spatiotemporal patterns, and to serve as a framework for further investigations of future ocean changes (including modelling). The five-year long protist dataset that was investigated in Chapter 2, gave a good preliminary description of the regional marine ecosystem, off the east coast of Southern New Zealand, but it was also restricted by small sample sizes and limited temporal resolution. The ongoing monitoring of this transect will improve the statistical power of the data analyses by increasing the total number of samples. However, the dynamic nature of the Southland Front system makes it difficult to consistently sample all four water masses at every sampling

opportunity. In order to establish long-term observational studies, a trade-off is required between the cost and time of collecting and processing the samples, the availability and willingness of people to go on the sampling trips, and the spatial sampling resolution (including quantity and quality of the samples). Over time, a high spatial resolution sampling trip could be established in each season, whereby between 16 and 24 stations could be sampled instead of just eight. Doubling the amount of sampling stations along the transect would increase the spatial sampling resolution and the chance of covering each water mass during every sampling trip. To accurately describe seasonal and perhaps interannual patterns for each water mass a dataset of at least 10 years is needed. Continuing this time series and combining the current sampling approach with occasional high spatial resolution sampling events would allow for a more comprehensive assessment of these region in the future.

In terms of the kelp forest ecosystem, a stronger data framework can be built by including multiple local kelp forests in New Zealand. The kelp associated microbiome showed a high degree of kelp species specificity which could be expected to persist on a regional scale. Sampling additional *Macrocystis pyrifera* dominated kelp forests would greatly improve and expand the findings from this study. Additionally, different kelp species should also be targeted to further investigate the host-specificity of kelp associated prokaryotic and eukaryotic biofilm communities. Once a better baseline of kelp associated biofilms in New Zealand is established, a comparison (e.g. metadata analysis) with kelp species from the Northern hemisphere could be performed to study kelp microbiome patterns across large spatial scales, between the same kelp species and amongst different kelp species. Similarly for the high $p\text{CO}_2$ study, additional sites should be sampled to strengthen the statistical power of the findings. However, shallow vents usually only have a short spatial scale and do not allow for multiple locations within one study site. Repeated measurements across seasons or on an annual scale could help to verify the findings of this study.

The choice of sampling prokaryotic and eukaryotic communities together provides great opportunities for continued research looking at co-occurrence patterns (network analysis), thereby revealing interspecies and interkingdom interactions and greatly enhancing our understanding of microbial ecological processes. The data in this thesis suggested potential interactions between dinoflagellate species (*Syndiniales* – *Dinophyceae*), and between *Ruegeria* (*Alphaproteobacteria*) and *Berkeleya* (diatom). Network analyses are easily performed on sequence data and could provide further proof of the presence or absence of these potential interactions. Next, *in vitro* experiments involving culturing and small mesocosm designs would be needed for an accurate description of these interactions. Additionally, characterising the functional guilds of the prokaryotic groups would also give further insight into microbial

community structuring and network interactions. Major advances in bioinformatics have been essential in studying biological science, especially for environmental DNA analysis. Recently new pipelines and more comprehensive functional databases allow the use of amplicon sequence data to investigate the dominant functions certain taxonomic groups are involved with.

Finally, part of this thesis covered responses of microorganisms to climate change related impacts. The findings from these studies (Chapter 2 & 5) were based on *in situ* experimental work and thereby give strong evidence of how ecosystems are expected to change under future ocean conditions. The results from these studies could be used for designing models that could predict large-scale future ecosystem responses to climate change, which in turn could help inform mitigation and restoration scenarios.

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Supplements

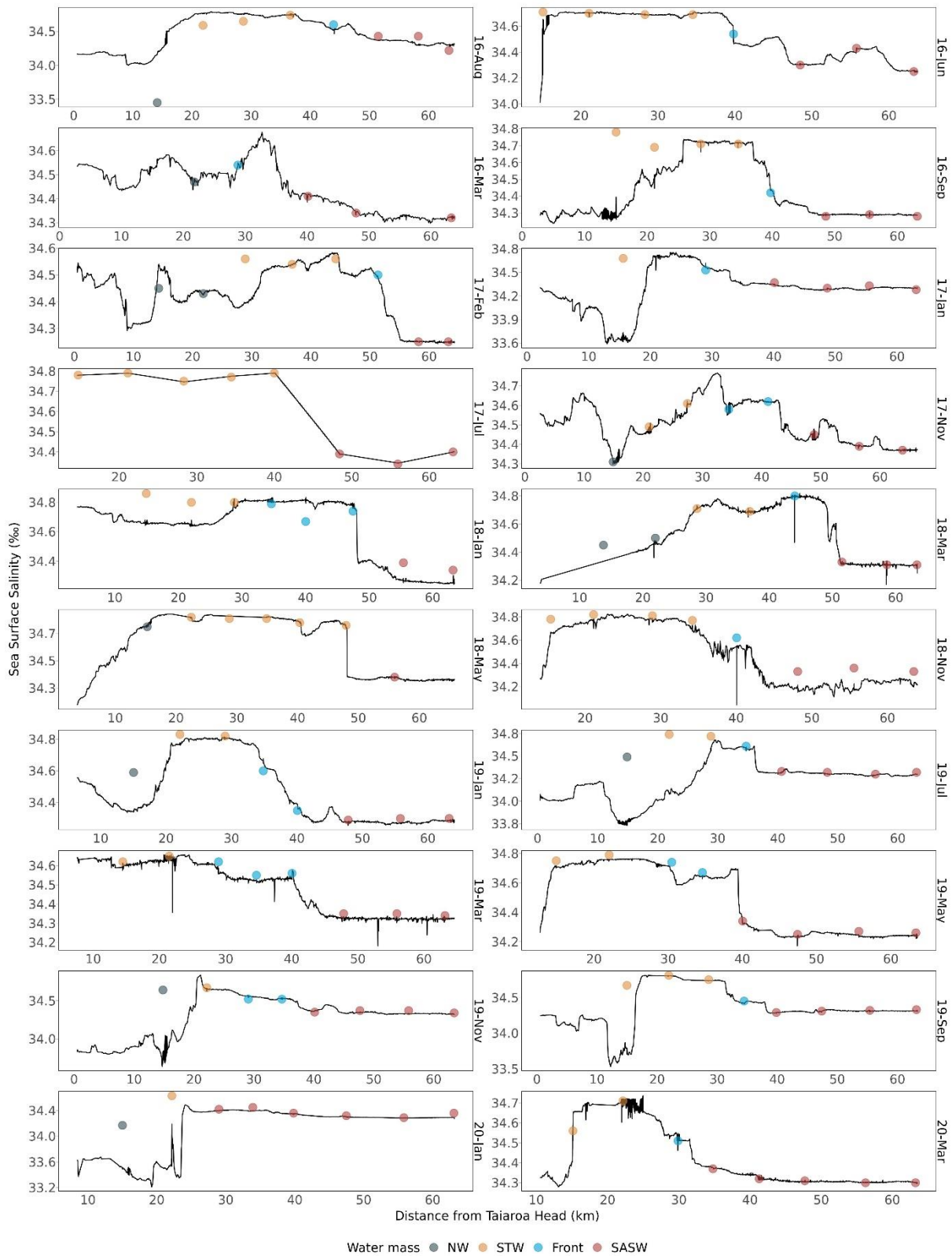


Figure S2.1: Continuous sea surface salinity profiles for each sample cruise. Sample station salinity values measured at 10 m depth are added and colored according to the characterised water mass. NW = Neritic Water, STW = Subtropical Water, Front = Southland Front, SASW = Subantarctic Surface Water.

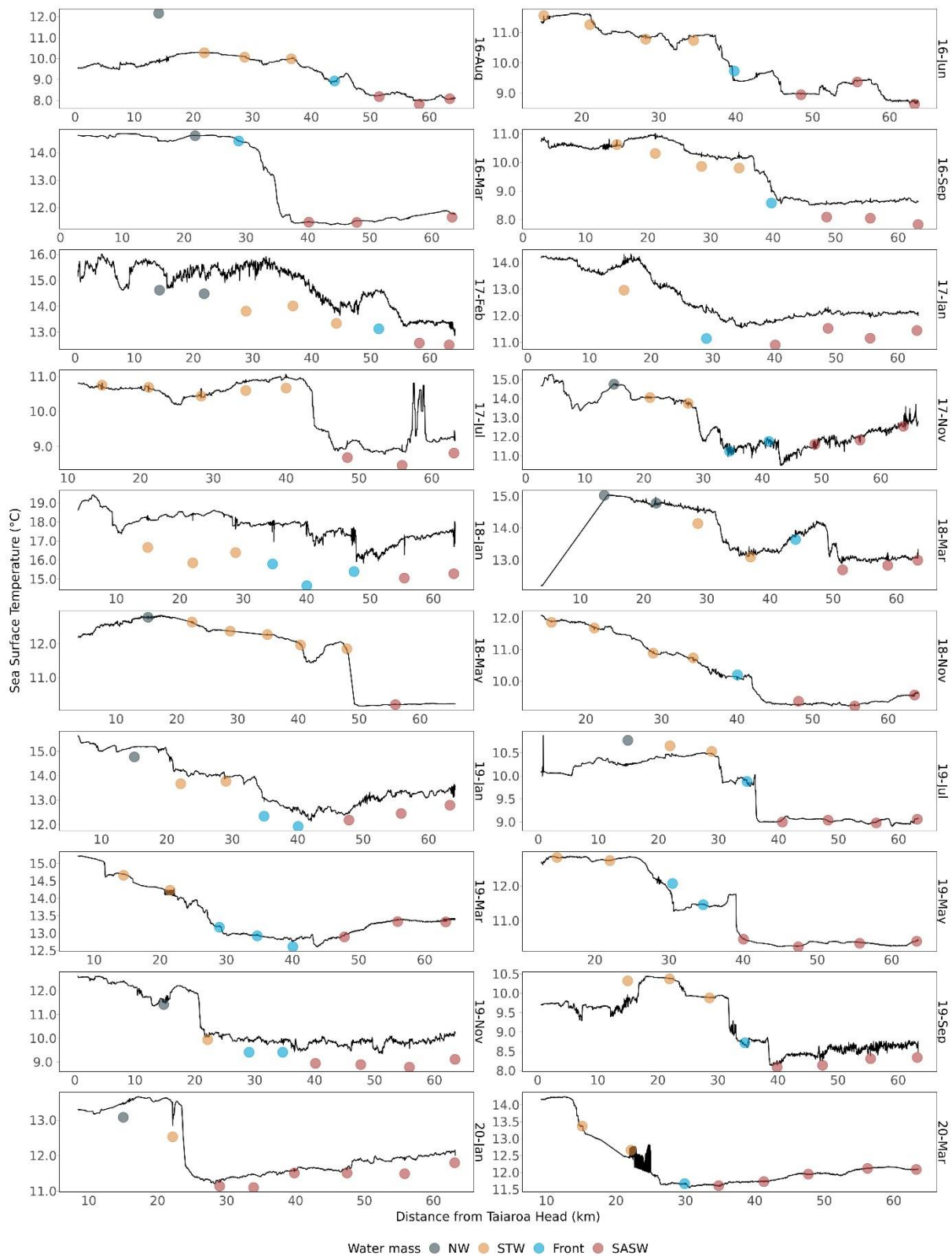


Figure S2.2: Continuous sea surface temperature profiles for each sample cruise. Sample station temperature values measured at 10 m depth are added and colored according to the characterised water mass. NW = Neritic Water, STW = Subtropical Water, Front = Southland Front, SASW = Subantarctic Surface Water.

Table S2.1: Constrained distance-based redundancy analysis (dbRDA) for spatial and temporal patterns. The main predictor value is shown in yellow. X = predictor value selected for the dbRDA model by forward selection., sign = significant, co-lin. = collinearity between phosphate and nitrate. NW = Neritic Water, STW = Subtropical Water, Front = Southland Front, SASW = sub-Antarctic Surface Water. There were insufficient samples for NW.

	NW	STW	Front	SASW
Temperature		x (sign)	x (sign)	x (sign)
Salinity		x (sign)		
Nitrate		co-lin.	x (NS)	x (sign)
Phosphate	/	x (sign)	co-lin.	x (sign)
Silicate				x (sign)
Chlorophyll a				x (sign)
pH		x (sign)		
p-value		<0.001	<0.001	<0.001
constrained		39%	28.00%	21%
unconstrained	/	59%	72.00%	79%
first axis		14.5	21.40%	11%

	Spring	Summer	Autumn	Winter
Temperature	x (sign)		x (sign)	x (sign)
Salinity	x (sign)	x (sign)	x (sign)	x (sign)
Nitrate		co-lin.	x (sign)	co-lin.
Phosphate		x (sign)	co-lin.	x (sign)
Silicate		x (sign)		
Chlorophyll a	x (sign)	x (sign)	x (sign)	
pH		x (NS)	x (sign)	
p-value	<0.001	<0.001	<0.001	<0.001
constrained	31%	43%	45%	40%
unconstrained	69%	57.00%	55%	60%
first axis	19%	20%	26%	22%

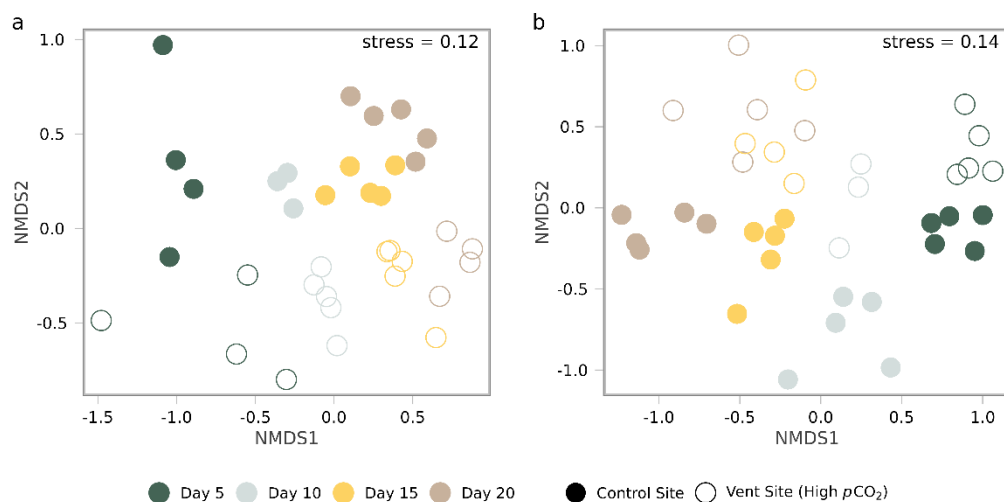


Figure S5.1: Non-metric multidimensional scaling (nMDS) of biofilm communities based on Bray-Curtis dissimilarities. (a) prokaryotic and (b) eukaryotic communities. Stress value is indicated per ordination plot. Colour represents the different time points of sampling: green (day 5), blue (day 10), yellow (day 15), and beige (day20). Symbols represent all the individual samples: closed circles (control site) and open circles (high $p\text{CO}_2$ site).