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# MAPPING THE 'PARTICLESCAPE': DIVERSITY AND ACTIVITY OF MICROBIAL COMMUNITIES ASSOCIATED WITH PARTICLES IN THE MARINE ENVIRONMENT.

by

**Cordelia Roberts** 

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

# DOCTOR OF PHILOSOPHY

School of Biological and Marine Sciences

May 2023

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# Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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## Mapping the 'particlescape': Diversity and activity of microbial communities associated with particles in the marine environment.

### **Cordelia Roberts**

#### Abstract

Particles are hotspots for marine microbial communities and impact ecosystem functioning, including the biological carbon pump. Although particle microbiomes differ across particle composition, type, size, source, and life history, this heterogeneity is often overlooked by using bulk-scale sampling approaches. In addition, knowledge of microbial eukaryotes in particle microbiomes is limited. I broadly set out to investigate how particle associated communities are structured at the macro and micro-scale which may have implications for wider food web interactions and biogeochemical cycling. In independent research chapters, I examined the diversity of bacteria (chapter 1) and activity of microbial eukaryotes associated with organic material (chapter 2 and 3) which play a role in marine carbon cycling and explored potential mechanisms structuring diversity dynamics observed. Here I discuss work done using both model and natural particles, including at the single particle level, combined with DNA and RNA amplicon sequencing of particle microbiomes in the North East Atlantic, Southern Ocean and Western English Channel. I show how particle source, life history and heterogeneity influences particle microbiomes across the whole water column (chapter 1) and reveal the diversity and activity of microbial eukaryotes (chapter 2) including fungi (chapter 3) associated with particles, the biological carbon pump and wider carbon cycling in the marine environment. This includes describing the identity and activity of microbial communities on particles across varying scales of complexity and incorporation of variability. I also add to the growing pool of knowledge of marine microbial communities in the understudied mesopelagic and bathypelagic. By exploring the particlescape, this work adds to the developing paradigm that particle microbiomes including microbial eukaryotes and particle-scale processes shaped by heterogeneity should be considered when understanding large-scale biogeochemical processes.

# **Table of Contents**

Abstracti
Table of Contentsii
List of Figuresv
List of Tables vii
Abbreviationsix
Chapter 1: Introduction1
1.1 Carbon cycling in the ocean2
1.1.1 The marine carbon cycle – a very brief summary2
1.1.2 The biological carbon pump (BCP)3
1.1.3 Biological influence on the BCP8
1.2 Particulate organic matter (POM) in the marine environment11
1.2.1 Defining particulate organic matter11
1.2.2 Particulate organic matter as hotspots for microbial activity15
1.3 Sampling microbial communities on particulate organic matter (POM)22
1.3.1 Collection of particles and associated microbial communities22
1.3.2 Molecular tools for characterisation of marine microbial communities27
1.4 Knowledge gaps31
1.4.1 Particle heterogeneity
1.4.2 Diversity and activity of particle associated microorganisms
1.4.3 Protists and fungi32
1.4.4. Impact of particle associated microorganism knowledge gaps on understanding the marine carbon cycle
1.5 Thesis Aims
1.6 Objectives
1.6 Thesis Outline
1.7 Author Contributions
Chapter 2: Depth-dependent diversity of particle associated bacteria on model chitin particles in the open ocean40
2.1 Abstract41
2.2 Introduction
2.3 Methods
2.3.1 Study site44
2.3.2Experimental set up45
2.3.3DNA extraction and metabarcoding46

2.3.4Data processing and statistical analysis	47
2.3.5Determining vertical microbial connectivity	48
2.4 Results	49
2.5 Discussion	
2.6 Conclusion	62
Chapter 3: DNA and RNA based assessment of the the Southern Ocean	e biological carbon pump in 64
3.1 Abstract	65
3.2 Introduction	67
3.3 Methods	
3.3.1 Sampling	
3.3.2 Bulk particulate material sampling	70
3.3.3 Particulate organic carbon measurements	71
3.3.4 DNA/RNA co-extraction, complementary DM metabarcoding	NA (cDNA) synthesis and72
3.3.5 Data processing and statistical analysis	
3.4 Results	
3.5 Discussion	
3.6 Conclusion	
Chapter 4: Mapping the 'particlescape': an assessn characteristics and fungal communities at the single	nent of particle e particle level in the coastal
4 1 Abstract	91
4.2 Introduction	Q3
4 3 Methods	96
4.3.1 Seawater sampling	
4.3.2 Environmental parameters	98
4.3.3 Single particle picking	
4.3.4 Particle characterisation and image analysis	s
4.3.5 DNA extractions for bulk community	
4.3.6 RNA extraction, complementary DNA (cDN, amplification of single particles.	A) and whole genome
4.3.3	Metabarcoding
4.3.4 Data proce	essing and statistical analysis
4.4 Results	
4.4.1 Particle characterisation	

4.4.1.1 Zooplankton-Derived105
4.4.1.2 Detritus-like
4.4.1.3 Aggregate-like105
4.4.2 Temporal and spatial resolution of particle characteristics
4.4.2.1 Particle abundance106
4.4.2.2 Particle Size106
4.4.2.3 Particle Complexity (RGB mean)107
4.4.3 Carbon stable isotope ratios and particulate organic carbon (POC) concentrations
4.4.4 Single particle eukaryote ASV richness against particle characteristics and environmental parameters
4.4.5 Single particle microbial community composition113
4.4.6 Single particle fungal community composition114
4.5 Discussion119
4.5 Conclusion127
Chapter 5: General Discussion128 5.1 Overview of
discussion
North East Atlantic Ocean
contribution to the biological carbon pump of the Southern Ocean
surrounding Plymouth sound
in coastal waters surrounding Plymouth Sound
Bibliography143
Appendix209

# **List of Figures**

Figure 1.1 Overview of the marine carbon cycle and its inorganic and organic carbon pumps. (MCP – microbial carbon pump)......4 Figure 1. 2 Attenuation of particulate organic carbon (POC) with depth as described by the power law function, the Martin Curve. Adapted from Martin et al, 1987. Ez (base of the euphotic zone)......6 Figure 1. 4 Varying size of POC and DOC/marine gels taken from Verdugo et al, (2004)......12 Figure 1.5 Sampling techniques used to collect POM in the marine environment. A) Water collection B) manual collection of particles using SCUBA C) size-fractionation filtration (adapted from Turner et al, 2014) D) sediment traps E) marine snow catchers F) in situ pumps, G) individual particle picking.23 Figure 2.1 A) Map of North East Atlantic ocean study, Porcupine Abyssal Plain Sustained Observatory (PAP-SO) indicated by yellow circle. B) Cruise transect across sampling sites within the PAP-SO area. Vertical profiles of C) salinity, D) temperature, E) oxygen and F) fluorescence as a measure of chlorophyll. Figure 2.2 Experimental incubation setup and sample processing pipeline.....46 Figure 2.3 Schematic figure of shared and unique amplicon sequence variants (ASVs). Green – epipelagic, Orange – mesopelagic, Blue – bathypelagic. ......48 Figure 2.4 A) Alpha diversity of bacterial community across all depths. Bars represent standard error and asterisks denote level of significance (\*\*\*p < 0.001). B) Non-metric multidimensional scaling (NMDS) visualisation of community structure across all treatments. Letters represent depths as follows E = epipelagic, M=mesopelagic and B = bathypelagic. Ellipses not statistically calculated but used to aid guidance of differences between depths and incubated chitin particle community......51 Figure 2.5 Order level relative abundance of bacterial and archaeal amplicon sequence variances (ASVs) across all treatments conducted across the three depths, epi-, meso- and bathypelagic for individual replicates grouped by Phyla. Figure 2.6 Order level relative abundance of bacterial amplicon sequence variants (ASVs) shared and unique found on particles incubated in epi-, mesoand bathypelagic water by individual replicates. Orders are grouped by Phyla. Relative abundances of less than 1% are grouped under 'other' categories... 56 Figure 3. 1 Three sampling stations OOI, TN and TS in the Southern Ocean where repeated transects took place......70 Figure 3. 2 Particulate Organic Carbon (POC) concentrations (top axis, bars) and fluorescence (bottom axis, points) measured at the three stations, OOI (red), TN (green), and TS (blue) across depth. POC Data measured by Sabena Blackbird and provided by Fred Le Moigne. Fluorescence data supplied from ship CTD and processed by Hugh Venables. .....74 Figure 3. 3 Non-metric multidimensional scaling (NMDS) plot A) microbial eukaryote and B) metazoan community structure based on Bray-Curtis dissimilarity. Colours represent occupations at stations, depths are labelled, triangles represent DNA samples and circles represent RNA. Hulls grouped by station for visual aid and not statisitcally calculated......75

Figure 4. 1 Map of study sites located in the Western English Channel. Figure 4.2 A) Representative images of particle major groups and particle types at L4 Station and Plymouth Sound combined over sampling period February-April 2021 (B) relative abundance, (C) surface area (mm2), (D) Mean Red Green Blue (RGB) of single p particles sampled. L4 station (circle) and Plymouth Sound (triangle). . "n=" represents number of particles in total found on each sampling date.(E) Key identifiable features of Aggregate-like group particles including (i) aggregate particles, (ii) dense aggregates and (iii) bloom particles. Arrows marked with 'p' highlight phytoplankton cells, 'c' highlight Chaetoceros sp. cells specifically and red circle and 'd' highlight dark, dense Figure 4.3 Bulk measurements of A) Particulate organic carbon (POC) and delta 13C ( $\delta$ 13C) for Station L4 (red) and Plymouth Sound (green) over Figure 4. 4. Relationship between total eukaryote species richness of 18S rRNA gene libraries on single particles (n = 9) and a) complexity (RGB mean), b) surface area, c) salinity d) rainfall, e) river flow, f) ammonia, g) nitrite, h) nitrate nitrite, i) silicate, j) phosphate, k) temperature l) POC, m) 13Carbon/12Carbon ratio. Blue lines represent linear regression, red = Station L4, green = Plymouth Figure 4. 5 Relative abundance of eukaryotes on single particles isolated from L4 station and Plymouth Sound. Pink = bloom particles, red = detritus, green = dense aggregates......114 Figure 4. 6 Relative abundance of fungal phyla found on single particles. Red -L4 Station. Green – Plymouth Sound. Numbers represent sampling event particle was isolated in......115 Figure 4.7 Bubble plot of fungal ASVs belonging to Ascomycota, Basidiomycota, Chytridiomycota and unassigned fungi with relative abundance greater than 1% abundance from all particle types. Red panels represent particles from L4 and green panels represent particles from Plymouth Sound. Colours on particle names represent particle type: pink - bloom aggregates, red  **Appendix Figure 1.1** POC concentration with depth across three stations **Appendix Figure 1.2** Images of single particles selected for RNA Appendix Figure 1.3 A) Longest length (mm) and B) Red Green Blue (RGB) standard deviation values of particles isolated from Plymouth Sound (triangle) and L4 Station (circle). Colours represent different particle **Appendix Figure 1.4** Phytoplankton cell counts at L4 Station performed by Clare Widdicombe (PML). Flagellates on left axis and other plankton groups on right **Appendix Figure 1.5** 18S rRNA gene community composition at phylum level for A) Station L4 and B) Plymouth Sound for each replicate over the sampling period. Grey shading represents phyla with less than 3% abundance......220 **Appendix Figure 1.6**Environmental variables taken from Western Channel Observatory (WCO) and shipboard measurements for Station L4 over sampling **Appendix Figure 1.7** Genus level fungal composition of single particles isolated from Station L4 and Plymouth Sound greater than 1% relative Appendix Figure 1.8 Ecological guilds of fungi identified on single particles as 

# List of Tables

**Table 2.3** Amplicon sequence variants (ASVs) shared between particles and in situ communities. Values were calculated by counting the total number of ASVs found in each replicate at each depth and then counting the number of ASVs that were also found in situ. This number was then divided by the total number of ASVs on the particle to identify the percentage of ASVs shared with the in situ community.....57

Appendix Table 1.1. Volume of SAPS water filtered and split size for individual

samples. Cells coloured green for euphotic and blue for mesopelagic samples......211

<b>Appendix Table 1.2</b> Taxonomic assignment of MArine STramenopile (MAST-3) amplicon sequence variance (ASV) using Tara Oceans eukaryote database (De Vargas et al
2015)
<b>Appendix Table 1.3</b> Counts of particle types collected from Plymouth Sound (PS) and L4 Station (L4) in the Western English
Channel
<b>Appendix Table 1.4</b> Leave-one-out cross validation of particle group according with the LDA performed on RGB mean
values219
<b>Appendix Table 1.5</b> Leave-one-out cross validation of particle Type according with the LDA performed on RGB mean values

# Abbreviations

ASV	Amplicon sequence variants
ВСР	Biological carbon pump
BGP	Biological gravitational pump
BLAST	Basic local alignment search tool
CaCO₃	Calcium carbonate
CARD-FISH	Catalysed reporter deposition fluorescent in-situ hybridisation
<b>CO</b> <sub>2</sub>	Carbon dioxide
CO3 <sup>2-</sup>	Carbonate ion
CSP	Coomassie stainable particles
CTD	Conductivity, temperature, depth
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EPS	Extracellular polymeric substances
FL	Free-living
L4	Station L4
MAST	Marine STramenopiles
МСР	Microbial carbon pump
MG II	Marine Group II Thermoplasmatota
MSC	Marine snow catcher
MUC	Molecularly uncharacterised components
ΟΤυ	Operational taxonomic unit
РА	Particle attached

PCR	Polymerase chain reaction
PIPs	Particle injection pumps
POC	Particulate organic carbon
РОМ	Particulate organic matter
PON	Particulate organic nitrogen
РОР	Particulate organic phosphorus
PR <sup>2</sup>	Protist ribosomal reference
PS	Plymouth Sound
Q-PCR	Quantitative polymerase chain reaction
rDOM	Recalcitrant dissolved organic matter
RDP	Ribosomal database project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCUBA	Self-contained underwater breathing apparatus
SSU	Small subunit
ТЕР	Transparent exopolymer polysaccharides
wco	Western channel observatory

# **Chapter 1: Introduction**

# **Chapter 1: Introduction**

#### 1.1 Carbon cycling in the ocean

1.1.1 The marine carbon cycle – a very brief summary

The global ocean is a net carbon sink, storing 2.5 Gt annually (Friedlingstein et al., 2019). As much as 50% of anthropogenic carbon dioxide (CO<sub>2</sub>) emissions are sequestered in the surface ocean (Sabine et al., 2004). The ocean's role within the global carbon cycle is mediated by a complex interaction of organic and inorganic carbon in physical and biological pumps (Boyd et al., 2019), including the solubility pump, the carbonate pump, the biological carbon pump (BCP) and the microbial carbon pump (MCP) (Figure 1.1).

The solubility pump drives the exchange of CO<sub>2</sub> in high latitude waters, in which CO<sub>2</sub> containing water sinks leading to deep water formation via thermohaline circulation and its subsequent upwelling and outgassing in low latitude equatorial regions (Volk and Hoffert, 1985). The carbonate pump relies on the dissolution of CO<sub>2</sub> in the surface waters and the production of bicarbonate and carbonate which can react with calcium to precipitate into calcium carbonate (CaCO<sub>3</sub>). The availability of carbonate ions (CO<sub>3</sub><sup>2</sup>-) supports the biological requirements for calcifying organisms as inorganic carbon, including coccolithophores, foraminifera and coral. In the BCP, production of dissolved organic matter (DOM) and particulate organic matter (POM) in the euphotic zone, supports the microbial loop (Pomeroy, 1974) and the MCP (Jiao et al., 2010), as well as, contributing to the mass flux of carbon from the surface waters to the deep sea via a multitude of physically and biologically mediated processes (Boyd et al., 2019). Conversion of CO<sub>2</sub> into carbon by plankton and its associated flux means the biological carbon pump is responsible for the

export of 5-12 Gt C year<sup>-1</sup> to the deep ocean (Henson et al., 2012). POM flux enables removal of carbon from the atmosphere and potential storage in the ocean from years to millennia.

1.1.2 The biological carbon pump (BCP)

Photosynthetic production of organic matter in the ocean, provides up to 50% of the global carbon fixation (Arrigo, 2005; Behrenfeld et al., 2006). The transfer of fixed carbon to the deep ocean via the BCP, largely as sinking detritus particles, forms a major component of the marine carbon cycle (Boyd and Trull, 2007).Carbon exported as POM into the deep sea, via the BCP can remain climatically inactive for >1000 years (Boyd et al., 2019) with the absence of this export, atmospheric  $CO_2$  would be nearly two-fold higher (Maier-Reimer et al., 1996; Parekh et al., 2006). Achieving a predictive understanding of the BCP is critical to understanding and mitigating the impacts of anthropogenic induced climate change (Siegel et al., 2022).

Chapter 1: Introduction



Figure 1. 1 Overview of the marine carbon cycle and its inorganic and organic carbon pumps. (MCP – microbial carbon pump).

Our current understanding of the BCP conveys a complex story of the fate of organic carbon produced in the surface, and its vertical transport and potential sequestration in the deep ocean. Organic matter is produced by primary production from dissolved inorganic carbon (DIC) uptake via photosynthesis. 'Living' and 'dead' organic carbon facilitates carbon cycling in two different ways. As described in traditional food webs, living organic carbon can be transferred via direct zooplanktonic grazing and subsequent consumption by fish and other large organisms (Ryther, 1969). 'Dead' organic carbon is created from the death of phytoplankton cells, due to limitation of cell requirements e.g. nutrients, light and inactivity, or viral lysis (C. Suttle, 2005). Cell death provides both dissolved and particulate forms of carbon available for wider carbon cycling. Leaching of cellular material via lysis contributes to the standing DOM pool helping to sustain microbial loop and microbial carbon pump. Larger dead material, or necromass, forms the foundation of the BCP as it creates a pool of particulate material along with other organic material within the water column. Organic material includes but is not limited to macro gels, microgels such as transparent exopolymer polysaccharides (TEP), microbial cells and zooplankton faecal pellets, moults and carcasses (Kharbush et al., 2020)

POM may remain suspended or sink via gravitational settling as part of the biological gravitational pump (BGP), resulting in the downward transport of material from the euphotic zone (<100 m) to the deep sea (>1000m), with very little reaching the sediments (Buesseler and Boyd, 2009). The BGP provides a key link between the euphotic and mesopelagic ocean (200-1000m) in terms of sustaining mesopelagic biota and carbon storage in the ocean interior. During Page | 5 the sedimentation of particles, material is exposed to remineralisation and degradation by microorganisms and planktonic filter feeders, resulting in the exponential attenuation of particle flux with depth (Figure 1.2 ,(Martin *et al.*, 1987)). Across oceanic regions, as little as 5-25% of primary production is exported below the euphotic zone (De La Rocha and Passow, 2007). It is estimated around 97% of primary production is remineralised, primarily occurring in the euphotic and upper mesopelagic ocean (Turner, 2015).





Material which is exported to depth and its subsequent recycling,

provides a feedback loop to the surface as it controls nutrient concentrations in

the deep sea which are eventually upwelled and responsible in part for controlling primary productivity in the euphotic zone (Buesseler et al., 2009). The interplay between the BGP and transformative processes (e.g. remineralisation, degradation, zooplankton grazing), oceanographic features (e.g. currents and turbulence) exerts a major influence on controlling the sequestration period of carbon within oceans (Briggs et al., 2020). The depth at which a particle reaches before it is remineralised is crucial, as this determines the length of time respired carbon remains within waters before it is upwelled again. Currently, geochemical tracer estimates suggest that the BGP associated flux alone is insufficient to close mesopelagic carbon budgets (Devries et al., 2012; Emerson, 2014) or meet carbon demands for mesopelagic biota (Boyd et al., 2019). This highlights the continued efforts needed to reassess pathways contributing to carbon storage in the ocean. Boyd et al. (2019) recently proposed that multi-faceted particle pumps drive carbon sequestration in ocean in coalition with the BGP. These include both physical (Bishop et al., 1986; Omand et al., 2015; Stukel et al., 2018) and biological (Davison et al., 2013; Jónasdóttir et al., 2015; Steinberg and Landry, 2017), particle injection pumps (PIPs) which work to export particles of all classes (e.g. sinking and suspended) and how efficiently the BCP sequesters carbon can be impacted by individual pumps (Boyd et al., 2019).

The efficiency of the BCP as a whole can be separated into two components: the magnitude of POC flux out of the surface e.g. strength and the fraction of sinking POC that is transported to depth e.g. transfer efficiency (Buesseler et al., 2020). The interaction between the magnitude and transfer efficiency, as particles undergo many transformative processes, creates a

gradient of POC within the water column resulting in a pronounced vertical attenuation with depth as described by the 'Martin Curve' (Figure 1.2, (Martin et al., 1987)). The Martin Curve is a power law function used to parameterize flux attenuation used widely within models to describe particle flux attenuation on regional and global scales. The exponential decline of POC with increasing depth, suggests that POC production is greatest in the surface. Considerable variability in the export of carbon exists across temporal and regional scales (Buesseler and Boyd, 2009; De La Rocha and Passow, 2007); therefore, the global applicability of this curve has been guestioned (Buesseler et al., 2007; Buesseler and Boyd, 2009). Variability in the exponential decline of POC is likely owing to both physical and biological processes including PIPs as described above. Undoubtedly, research into understanding these processes help to close the carbon budget (Boyd et al., 2019). However, other transformative processes including microbial action on particles still require more exploration to add to a more complete understanding of the ocean's dynamic role in carbon storage and to continue to close the carbon budget in the ocean.

#### 1.1.3 Biological influence on the BCP

The strength of the flux can be controlled by the community composition of the surface waters. Phytoplankton diversity in surface waters can influence POC production and carbon export efficiency. For example, mineral-ballasted phytoplankton such as diatoms and coccolithophores have enhanced sinking rates, reducing time for remineralisation action in the euphotic zone (Armstrong et al., 2001; Michaels and Silver, 1988). Whilst picophytoplankton like *Prochlorococcus* and *Synechococcus* are small and unballasted, are more likely

Page | 8

to be incorporated into larger particles through grazing and aggregation (Nissen and Vogt, 2021). The transfer efficiency is controlled by a complex interplay of processes (Figure 1.3) including grazing (and excretion), fragmentation, degradation and remineralisation, which influence the concentration of exported POC (e.g. Martin Curve) and the timescale(s) in which it is sequestered away from the surface ocean and atmosphere. Key candidates involved in these processes have been identified as heterotrophic communities including zooplankton and heterotrophic microbes (namely bacteria).





Traditionally, only bacterial and archaeal communities have been considered when referring to the microbial communities involved in the degradation and remineralisation of POM (Aristegui et al., 2009), including the dominance of heterotrophic osmotrophy which differentiates them from other organisms in the ocean (Jiao et al., 2010). Microbial respiration on POM can release DIC (as CO<sub>2</sub>) into the ocean (Raven and Falkowski, 1999) which depending on the depth of remineralisation can remain in the ocean on varying timescales. Degradation releases dissolved organics into the surrounding water column, due to inefficient uptake by attached bacteria (Smith et al., 1992). Through the microbial loop (Pomeroy, 1974) released DOM is taken up by bacteria and archaea, supporting the cycling of nutrients and dissolved organic carbon (DOC). These nutrients are made available for photosynthesis or through transfer of bacterial secondary production via food webs (Verdugo et al., 2004). As part of the MCP (Jiao et al., 2010), labile DOM is degraded by heterotrophic prokaryotes further into refractory or recalcitrant DOM (rDOM), resulting in a DOC reservoir of 662 Gt C (Jiao et al., 2010). As it is highly resistant to further microbial degradation, rDOC can persist in the ocean between 4000 and 6000 years (Hansell, 2013), highlighting the importance of the MCP in the oceanic carbon cycle and climate regulation (Zhang et al., 2018).

Migrating organisms such as zooplankton which consume carbon in the surface and deposit it at depth, as part of the 'active' biological pump, are most effective at injecting carbon deep into the ocean interior, as it by passes a large proportion of the remineralisation which would take place as a passively sinking particle (Boyd et al, 2019). The grazing of particles by micro- and meso-zooplankton is important in the creation of fast sinking faecal pellets reducing retention time in the surface (Gowing and Silver, 1985). Fragmentation of particles by zooplankton, in addition to physical fragmentation processes, may counter carbon export, by producing smaller slower sinking or suspended particles (Mayor et al., 2014). However, some zooplankton may purposefully fragment particles to encourage colonisation by microorganisms before

Page | 10

ingestion, known as 'microbial gardening' (Mayor et al, 2014). Microbial gardening allows for trophic upgrading and increased microbial biomass for detritivorous zooplankton and likely the wider particle grazing community (Cavan et al., 2021)and can increase POC turnover by 1.9 times (Cavan et al., 2021).

Biological processes across a range of trophic levels, in combination with abiotic processes, are responsible for current dynamics of export flux and carbon storage. However, microbial communities are integral for the dynamic changes between sinking and suspended POM and DOM pools, thereby shaping organic matter availability in the ocean. Exploration of microbial communities has been largely reduced to that of bacteria. Eukaryotic taxa such as phytoplankton contribute to sinking material, and other eukaryote groups including radiolarians, ciliates, MArine STramenopiles (MAST), fungi, and fungal-like organisms such as labyrinthulomycetes have been identified on and in association with exported POM (Bochdansky et al., 2017; Decelle et al., 2013; Gutierrez-Rodriguez et al., 2019). The diversity, activity and role of eukaryotes behind the BCP still requires further exploration.

#### 1.2 Particulate organic matter (POM) in the marine environment

#### 1.2.1 Defining particulate organic matter

Historically, sampling methods used to operationally define organic carbon as particulate or dissolved have spanned a range of filter pore sizes, namely 0.2-1 µm glass or quartz fibre filters (Kharbush et al., 2020). The literature therefore contains varying descriptions of particulate and dissolved material. The associated dynamics (biological and physical) of POM may not be truly

comparable to one another. For analytical purposes, the distribution of organic carbon between the dissolved and particulate fractions are broadly defined by any material retained on a 0.2 µm filter as POM, whilst DOM constitutes the resultant filtrate (Verdugo et al., 2004). Sampling methods across the literature are yet to be standardised. By consequence, defining POM and DOM solely by size is problematic as organic matter can span orders of magnitudes. For example, the presence of gels spans the 'traditional' size-based definition of both POC and DOC (Figure 1.4). Consequently, to understand the role POM and associated microbial communities play in the BCP and wider carbon cycling; origin, type and composition must also be considered.





Organic matter may be also delineated by its source as either allochthonous or autochthonous material. Allochthonous organic matter is nonmarine produced material that is transported into the ocean by either fluvial or atmospheric input. Allochthonous material can include leaf litter, wood, sediment and aerosols, which may also transport microbial communities including fungi (Delany et al., 1967; Folger, 1970). Autochthonous organic matter is produced in the marine environment via photosynthetic and zooplanktonic organisms adding to the DOC or POC pool. These pools encompass both living (plankton, macroalgae) and detrital fractions of organic matter, as described by the BCP.

Freshwater environments receive large inputs of allochthonous organic matter in both particulate leaf litter, wood and dissolved form (Solomon et al, 2015) from terrestrial surroundings. The diversity of microbial communities associated with freshwater POM, in particular spans both terrestrial and aquatic origin. Given the connectivity of aquatic ecosystems, for example, estuarine and brackish water ecosystems which exist at the interface fresh and saline environments, fluvial inputs of organic matter may shape the diversity and structure of POM associated microbial communities in marine, noticeably coastal environments, although this should be explored further.

The production of autochthonous particulate material can be generated from a number of sources of organic matter in the ocean. Broadly, particles in the ocean can be categorised into marine aggregates including marine snow and faecal pellets (>500  $\mu$ m), microparticles (500-1 $\mu$ m) and submicron particles (1-0.1 $\mu$ m) (Simon et al., 2002). Yet, the composition of the POC in these particles vary greatly. These can include but are not limited to the aggregation of living and non-living phytoplankton cells, microbial cells, larvacean houses, zooplankton faecal pellets, exuviae and moults, often bound in matrixes by extracellular polymeric substances (EPS) e.g. TEP (Engel, 2004; Passow,

Page | 13

2002) and protineaceous Coomassie stainable particles (CSP) (Long and Azam, 1996). More specifically, the POC of marine particles provide a variety of substrate ranges and potential niches for colonising microbial communities (Figure 1.4) These include lipids, amino acids and proteins and carbohydrates (Kharbush et al., 2020); references therein)). Marine particles also encompass allochthonous materials as previously described, including lignin and terrestrial plant matter, black carbon (light-absorbing carbonaceous aerosols), microplastics and dust particles (Iversen and Ploug, 2010; Kharbush et al., 2020); references therein). It is important to note that the POM can also constitute a much broader range of organic molecules than POC including particulate organic nitrogen (PON) and particulate organic phosphorus (POP), and likely includes many molecularly uncharacterised components (MUC) which likely span across POC, PON, and POP(Kharbush et al., 2020; Moran et al., 2016). MUC may remain uncharacterised as they are not identified by traditional molecular analyses (Kharbush et al., 2020). However, PON and POP are outside the scope of this research, so POC only will be considered moving forward.

POM can be divided based upon their physical properties including sinking rate (Riley et al., 2012). For example marine particles can be described as fast or slow sinking defined by their density and sinking speeds. Faecal pellets are described as fast sinking particles, with high density and enhanced sinking speeds which range from a rate of 5-2700 m day<sup>-1</sup> (Turner, 2002; Wilson et al., 2008). Marine snow particles are described as slow sinking with varying densities (Alldredge and Gotschalk, 1990) and speeds from 10-386 m day<sup>-1</sup> (Alldredge and Gotschalk, 1990; Nowald et al., 2009). Furthermore, many

Page | 14

marine particles do not sink and remain suspended in the water column, contributing to most of the particle mass and composing POC which is two orders of magnitude greater than that of sinking particles (Baker et al., 2017; Giering et al., 2014; Riley et al., 2012). Sinking velocity can be related to the size of a particle as settling velocity generally increases with size (Alldredge and Gotschalk, 1988; Iversen and Ploug, 2010); however, sinking velocity may depend on composition; shape and porosity (Iversen and Ploug, 2010). More recently, *in situ* observations also confirm that aggregate sinking velocity is not controlled by aggregate size, which may compromise previous estimates of particle fluxes (Iversen and Lampitt, 2020).

1.2.2 Particulate organic matter as hotspots for microbial activity POM is a hotspot for microbial activity (Fuhrman and Azam, 1982; Simon et al., 2002), likely owing to a higher density of organic carbon and nutrients (Alldredge and Silver, 1988). For example, particle associated carbon can exceed background levels by 2-4 orders of magnitude greater than surrounding seawater (Blackburn and Fenchel, 1999a; Grossart and Simon, 1998; Prgzelin and Alldredge, 1983).Enumeration of bacterial cells on particles has shown that in the mesopelagic zone especially, particles can be enriched > 1000-fold in comparison to the free-living (FL) community (Ploug et al., 1999; Thiele et al., 2015; Turley and Mackie, 1994). As the ability to attach to particles is a pivotal factor for colonising microbes (Grossart et al., 2006), particle attached (PA) microbes are phylogenetically (Salazar et al., 2015), morphologically (Caron et al., 1982; Malfatti et al., 2010) and metabolically distinct (Dang and Lovell, 2016) from those found FL in the water column. Particle communities are in taxonomically diverse microbial communities in comparison to the surrounding water column (Alldredge and Silver, 1988; Fenchel and Blackburn, 1999) including the presence of both bacteria (Alldredge et al., 1986), archaea (Crump and Baross, 2000; Jain and Krishnan, 2021; Orsi et al., 2015), microbial eukaryotes (Bochdansky et al., 2017; Duret et al., 2019a; Wurzbacher et al., 2014) and viruses (Bettarel et al., 2015).

Microbial communities associated with different particle types result in rapid and efficient turnover of POC (Simon et al., 2002). POM is typically associated with comparatively higher metabolic activity (Lyons and Dobbs, 2012) and supports metabolically different microbial communities from the surrounding water column; including the presence of ammonifying (Shanks and Trent, 1979) and nitrifying (Phillips et al., 1999) bacteria, as well as, methane producing archaea (Van Der Maarel et al., 1999). Differences in metabolic activity including rates and patterns of enzymatic hydrolysis can vary across oceanic scales (Arnosti et al., 2011) and depth (Hoarfrost and Arnosti, 2017), these can reflect variations in microbial community composition (Balmonte et al., 2018) and community succession, as well as the remineralisation and sequestration of carbon across these scales too. .

The life history of a particle plays a role in the colonisation and succession, and thereby wider carbon cycling. Homogeneous particles in laboratory (Datta et al., 2016) and *in situ* (Suominen et al., 2021) studies are known to undergo distinct community succession patterns over short (Datta et al., 2016; Roberts et al., 2020; Suominen et al., 2021) and long (Suominen et al., 2021) timescales. Community succession may mirror the change in a particles carbon quality and quantity as semi-labile and refractory components of particles increase (Bižic-Ionescu et al., 2018). As the POC content of the particle changes, communities may diverge from that of a particle-degrading one to a community reliant on secondary production (Datta et al., 2016; Roberts et al., 2020). Community succession on POM can also be impacted by the presence of zoosporic fungi, which may provide DOM as seen in laboratory studies with freshwater bacterial communities (Roberts et al., 2020).

Increasing evidence shows that microbial community composition and functionality differs across particles of different size and sinking rates (Duret et al., 2019a, 2019b) reflecting the inherent heterogeneity in their chemical composition. For example, PA communities associated with smaller particles display higher composition dissimilarity between sinking and suspended particles and are able to degrade and assimilate small carbohydrates and nitrogen compounds (Duret, 2018). However, PA communities of larger particles are more phylogenetically diverse and have complex carbohydrates and nitrogen compounds degrading abilities, with PA communities of larger suspended particles holding genes related to the degradation of refractory material (Duret, 2018). As suspended and sinking particles result in different fates for the long-term storage of carbon (Kwon et al., 2009) differences in PA community composition, degradation and subsequent remineralisation of POC should be explored further to better understand the role of a wide range of microbes in varying POC fluxes in the ocean. It has also been suggested that particles may provide seeding for deep sea environments by attachment and detachment (Mestre et al., 2018; Ruiz-González et al., 2020).
#### 1.2.2.1 Bacteria

Bacteria have been characterised on particles across a wide range of marine environments including coastal (Mestre et al., 2020), open ocean (Boeuf et al., 2019; Luo et al., 2022), polar ocean (Balmonte et al., 2018; Milici et al., 2017) and deep sea (Mislan et al., 2014; Pelve et al., 2017) using a range of tools (see section 1.3). Particle associated communities can differ reflecting particle composition (Datta et al., 2016; López-Pérez et al., 2016; Suominen et al., 2021), life history (Datta et al., 2016), particle size (Salazar et al., 2015) and environmental conditions and gradients such as seasonality (Wenley et al., 2021), oxygen (Fuchsman et al., 2011), depth (Mestre et al., 2018) and proximity to shore (Valencia et al., 2022).

Commonly attached bacterial groups include *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Gammaproteobacteria*, *Firmicutes* and *Planctomycetes*. Particle associated bacteria can overlap with free living bacteria (Ghiglione et al., 2007; Hollibaugh et al., 2000), therefore communities may reflect both generalist and specialist taxa, although community structure is additionally dependent on the life history and colonisation status of the particles (Datta et al., 2016). For example, early colonising taxa can be structured by particle attachment ability; whilst later taxa may be selected for owing to their substrate range or ability to degrade high molecular weight material which particles are often composed (e.g. Bacteroidetes (Puddu et al., 2003)). Given the evidence of different bacterial communities in association with different types of POM, it may not be appropriate to sample particles bulk at one time point in its life history in order to truly understand the contribution of bacterial taxa within POM processing and wider biogeochemical cycling.

#### 1.2.2.3 Archaea

Archaea are now recognised as ubiquitous members of the planktonic microbial community with a cosmopolitan distribution (Pereira et al., 2019). Most archaeal groups lack cultured representatives and knowledge on diversity of particle attached archaea is limited. Nevertheless, archaea including Marine Group II (MG II) are in high abundance in particle rich waters (Galand et al., 2008; Wells et al., 2006; Wells and Deming, 2003), and have been found in associated with particles (Crump and Baross, 2000; Jain and Krishnan, 2021; Orsi et al., 2015; Van Der Maarel et al., 1999). Archaea on particles exhibit higher diversity and different taxonomy to their respective free-living counterparts (Crump and Baross, 2000) and show clear habitat dependent patterns in their community composition (Galand et al., 2008). MG II and MG III also show functional potential for degradation of proteins, lipids and carbohydrates (Haro-Moreno et al., 2017; Orsi et al., 2015; Rinke et al., 2019), suggesting a role of POM processing for archaea although this requires further exploration.

1.2.2.4 Eukaryotes (protists, fungi and microbial metazoans) Eukaryotes in association with particulate material include both unicellular (protists) and multicellular (eukaryotes and metazoans) organisms. Eukaryotic taxa associated with POM exist across a spectrum of nutritional strategies including autotrophy, heterotrophy and mixotrophy (Caron et al., 2012; Stoecker et al., 2017) which can largely impact the production and resulting export flux of organic material (Worden et al., 2015). Photosynthetic protists including large phytoplankton such as diatoms, coccolithophores, as well as, pico- (<2 µm) and nanoplankton (2-10 µm) groups contribute heavily to sinking material as phytodetrital aggregates (Durkin et al., 2022; Gutierrez-Rodriguez et al., 2019; Richardson and Jackson, 2007; Silver and Gowing, 1991), as individual sinking cells (Durkin et al., 2015; Puigcorbé et al., 2020) and are incorporated into sinking faecal pellets (Durkin et al., 2022; Richardson and Jackson, 2007). Increasing evidence shows the contribution of additional eukaryote taxa to the BCP such as flagellates including dinoflagellates (Duret et al., 2019a), ciliates (Duret et al., 2019a; Gutierrez-Rodriguez et al., 2019a), ciliates (Duret et al., 2019a; Gutierrez-Rodriguez et al., 2019a; Gutierrez-Rodriguez et al., 2019), fungi and fungal-like organisms (Bochdansky et al., 2017; Duret et al., 2019a; Raghukumar, 2002).

The contribution of fungi to marine carbon cycling has received increasing attention in recent years (Amend et al., 2019; Grossart et al., 2019) as fungi are widespread members of microbial eukaryotes in the marine environment (Chrismas et al., 2023; Taylor and Cunliffe, 2016a) and are able to degrade phytoplankton (Cunliffe et al., 2017; Gutiérrez et al., 2011a) and macroalgae derived organic material (Pilgaard et al., 2019). Fungi are also shown to dominant biomass on particles in the open ocean (Bochdansky et al., 2017). Global assessments of marine fungi highlight the dominance of Ascomycota, Basidiomycota and Chytridiomycota (Hassett et al., 2019) and fungi in coastal marine ecosystems can include both marine and non-marine taxa suggesting land-sea exchange regularly takes place potentially via particles as a mechanism for their transport (Chrismas et al., 2023). The involvement of fungi in POM-associated processes is poorly understood which limits our understanding of their contribution to the carbon cycle, broader biogeochemical cycling and wider food web interactions.

There is a shifting paradigm revealing the diversity of eukaryotes and their connection to POM, the BCP and wider carbon cycling (Cordier et al., 2022; Duret et al., 2019). However, identifying eukaryote enrichments in particle fractions may reflect eukaryote contributions to the detrital component of particles, attachment to or association to particles. Evidence is needed to better understand microbial eukaryotic activity in relation to POC. For example, fungi undertake saprotrophic degradation of POC in coastal (Cunliffe et al., 2017; Gutiérrez et al., 2011) and open ocean (Baltar et al., 2021; Chrismas and Cunliffe, 2020) and fungal abundance increases with increasing POM concentrations (Taylor and Cunliffe, 2016b), yet a definitive contribution of fungi to the BCP is missing.

#### 1.2.2.5 Viruses

Viruses are a causative agent for marine bacterial, algal and protist mortality (Munn, 2006). The resulting production of cellular debris from viral lysis, highlights their contribution to both dissolved and organic matter pools (Shibata et al., 1997). The production of labile DOM as part of the viral shunt provides material available for remineralisation and subsequent nutrient cycling (Suttle, 2005) making them a driving force behind biogeochemical cycling in the ocean (Fuhrman, 1999; Suttle, 2005). Marine particles are sites of high viral accumulation (Mari et al., 2007; Weinbauer et al., 2009) with variations in abundance owing to the quality, age and size of aggregates (Bongiorni et al., 2007). As particles are hot spots for bacterial activity, viral activity may be

Page | 21

stimulated, as viruses rely on their host in order to replicate, meaning particles can act as viral factories (Bettarel et al., 2015; Weinbauer et al., 2009).

Despite ongoing work on particle communities, viruses on particles remain largely unexplored (Luo et al., 2022) and few taxa are named. Nevertheless, recent work has shown viral presence on particles throughout the water column, and positively correlated viral abundance of some novel viral taxa with particulate carbon flux (Luo et al., 2022). The interactions of viruses with particles and the major contribution of viruses to the BCP and wider carbon cycling requires much investigation.

#### 1.3 Sampling microbial communities on particulate organic matter (POM)

1.3.1 Collection of particles and associated microbial communities In general, sampling for POM to explore their microbial communities has commonly been explored in bulk using size-fractionation filtration of seawater. There are now however, a suite of tools to sample POM available for the research community to use (Figure 1.5) in combination with high-throughput sequencing to profile microbial communities, which have both benefits and costs associated with them depending on desired research outcomes.



Figure 1. 5 Sampling techniques used to collect POM in the marine environment. A) Water collection B) manual collection of particles using SCUBA C) size-fractionation filtration (adapted from Turner et al, 2014) D) sediment traps E) marine snow catchers F) in situ pumps, G) individual particle picking.

Size-fractionation filtration involves seawater, often collected in Niskin bottles on conductivity, temperature, and depth (CTD) rosettes (Figure 1.5A, C). During size-fractionation, PA and FL communities are separated from each other by passing seawater through a pre-filter of a larger pore size upstream of smaller pore sizes. Both filters retain microbial biomass with larger pore sizes retaining PA communities (typically 1.0-30  $\mu$ m) and the smallest pore size retaining FL communities (typically 0.1- 0.2  $\mu$ m).

There are several limitations to the size-fractionation method (Padilla et al., 2015). Size-fractionation sometimes includes a pre-filtration step using 200 µm mesh to remove larger eukaryotes such as diatom chains and mesozooplankton (Crump et al., 1999; Smith et al., 2013). A bias to the size, type of POM and thereby microbial community sampled may exist in the first instance. Variations exist in the pore sizes used to define PA and FL

communities making it difficult to draw comparisons between studies. Some studies define material retained on a 3.0  $\mu$ m filter as PA and the resulting filtrate retained on a 0.2  $\mu$ m as FL (Crump et al., 1999; D'Ambrosio et al., 2014; Smith et al., 2013). In addition to this, some studies include an additional filtration step between 3 and 0.2  $\mu$ m to further delineate smaller size classes of PA communities (Orsi et al., 2015). Other studies have used a much larger (30  $\mu$ m (Fuchsman et al., 2011) and broader range of pore sizes aimed at targeting a more complete view of particle sizes and communities (2.7-53  $\mu$ m (Cantarero et al., 2020).

The inaccuracy of the fractions is another limitation of size-fractionation. Biomass retained on the filter increases with increasing volumes filtered, which can result in an increased proportion of FL communities being retained on top of particulate material as pores become blocked (Lee et al., 1995; López-Pérez et al., 2016) which can significantly distort microbial diversity estimates (Padilla et al., 2015). Selection of filtration mechanism may also result in inaccuracies in PA and FL fractions, as intense vacuum filtration may break larger particles apart to sizes smaller than the pore-size meaning they are captured in the FL fraction.

Size-fractionation based filtration exist on a much larger scale with the use of *in situ* pumps (Figure 1.5F). *In situ* pumps sit in the water column, tethered to a research vessel for up to 4 hours filtering large volumes of water (hundreds of litres) typically through a 53 µm nylon mesh to retain particulate material (Bishop et al., 2012; McDonnell et al., 2015). Benefits of *in situ* pumps include keeping sampling communities at *in situ* conditions, which minimizes changes in pressure and temperature and potentially reduces the outcome of

Page | 24

altered PA communities (Puigcorbé et al., 2020). On comparison with Niskin bottle collected PA communities, *in situ* pumps recover a largely different but more diverse PA communities, likely due to increased volume of water and different particle types sampled (Puigcorbé et al., 2020). However, increased water volume may result in greater retention of FL communities on the particulate material retained on the mesh.

Other bulk based approaches include sediment traps (Figure1.5D), which collect sinking particles *in situ* and preserve them in formalin to prevent degradation (McDonnell et al., 2015). Moored sediment traps can also be used to collect time-series data too, as weekly sampling over a 6–12-month period, enabling fine scale resolution over seasonal periods including phytoplankton blooms for example (Honjo, 1976; LeCleir et al., 2014). Drifting traps also exist, however, sediment traps may exclude slow sinking or suspended particles. Marine snow catchers (MSC) can overcome the exclusion of particles of different sinking speeds. MSCs are giant water bottles which sample 100L of water and on-board settling procedures fractionate samples by settling velocity resulting in suspended, slow-sinking and fast-sinking particles (Figure 1.5E, (Riley et al., 2012)).

Sampling natural particles in bulk has been influential in providing an image of physical, chemical, and biological characteristics of particles including overall PA communities, in comparison to the surrounding water column. These tools provide little resolution over different particle compositions as the heterogeneous composition of particles, colonisation dynamics and life history of particles can make it complicated to understand the dynamics of microbial interactions with particles. Therefore, the use of short and long-term incubations

Page | 25

of model or homogenous laboratory made particles with seawater and microbial communities can help provide some insight into how particle composition impacts colonising taxa (Suominen et al., 2021), microbial interactions between taxa (Datta et al., 2016; Enke et al., 2019) and mechanisms behind degradation (Bunse et al., 2021)

Up until recently the manual selection of individual particles (Figure 1.5G) has only provided limited resolution on particle dynamics (DeLong et al., 1993). Early work on larger (>500 µm) marine snow particles involved the collection of water and individual particles via SCUBA (Figure 1B, (Alldredge, 1998)); however, these were combined to obtain enough material for analyses to understand physical and chemical properties (Alldredge, 1998). Manually picking particles provides a method to avoid disturbance of aggregate associated communities but can be labour intensive and using techniques such as SCUBA means it is restricted to the very upper ocean (McDonnell, 2015). Underwater vision profiler based studies have highlighted variations in particle size and concentration across temporal and spatial scales resulting in differing contributions towards carbon export (Bol et al., 2018; Lacour et al., 2023). The development of single particle approaches is therefore integral as differences in microscale functional states could translate to large scale variation in particle degradation rates (Szabo et al., 2022). More recently there have been efforts to utilise some of the above techniques (e.g. sediment traps) in combination with DNA-based assessments to sample individual particles and characterise eukaryote composition with success on a larger scale, showing differences in composition between individual particles (Durkin et al., 2022). Heterogeneity in particle associated bacterial communities on individual particles (<5 particles

pooled) has been noted in freshwater environments in comparison to bulk estimates (Bižic-Ionescu et al., 2018) and in few instances for individual marine particles including TEP (Zäncker et al., 2019) and marine snow particles (Lundgreen et al., 2019). Individually selected marine snow and faecal pellets in the North Atlantic also show distinct and variable lipidomes within and between particle types (Hunter et al., 2021). The variability of substrates within particles or the 'particlescape' (Bunse et al., 2021) can drive niche specialisation of bacteria colonising particles (Bunse et al, 2021).

1.3.2 Molecular tools for characterisation of marine microbial communities Investigations of marine microbial communities generally, has targeted bacterial communities using sequencing based approaches. The benefit of using sequenced based approaches for particles is two-fold. Firstly, some organisms are too difficult to visualise manually because of size, species crypticity or degradation. Secondly, particles are often comprised of multiple sources of organic matter, including fragments of organisms, which can be challenging to characterise the origin of.

only parts of organisms are also likely difficult to identify. Historically, bacterial studies have used Sanger sequencing of generated bacterial 16S ribosomal RNA (rRNA) gene clone libraries. However, Sanger sequencing is low throughput and unlikely to provide complete community diversity (Youssef et al., 2009). Eukaryote and protist-based investigations of marine microbial communities have always remained behind in comparison, perhaps due to their reliance on light-microscopy based taxonomy which is labour intensive requiring taxonomic specialists and difficultly in distinguishing between ecologically relevant protists (Edgcomb, 2016). High-throughput sequencing technology has

Page | 27

revolutionised the speed, ease and cost of characterising microbial communities, as well providing high depth and taxonomic resolution (Caporaso et al., 2011; Sogin et al., 2006).

Universal marker genes for bacteria, archaea and eukaryotes using the small subunit (SSU) ribsosomal RNA (rRNA) (bacteria and archaea: 16S rRNA gene, eukaryotes: 18S rRNA gene) have become a standard tool used in high-throughput amplicon sequencing. This is due to it being found across all taxa, lack of horizontal gene transfer and the presence of hypervariable regions flanked by highly conserved regions which act as reliable binding sites for universal primers (Head et al., 1998; Hugerth and Andersson, 2017). Briefly, polymerase chain reactions (PCR) amplify maker gene fragments from genomic DNA extracted from environmental samples. Multiple, well curated databases and tools exist for comparison of the resulting sequence data to assign taxonomic identity for bacteria, archaea and protists sequences generated (Table 1.1). Outputs of sequencing surveys can be impacted by DNA extraction efficiency, primer selection, library preparation, and sequencing based analysis (Soergel et al., 2012; Yarza et al., 2014).

Many community based studies, including particulate based work, have used an operational taxonomic unit (OTU) based approach to analyse sequence data, clustering sequences at a named threshold, typically a 97% similarity value (particle specific studies using OTU based approach:(Bižiclonescu et al., 2018; Duret et al., 2019a; LeCleir et al., 2014; López-Pérez et al., 2016; Mestre et al., 2020)). However, OTU approaches may lose some taxonomic resolution and biological variation that exists. Approaches such as amplicon sequence variants (ASV) build on traditional OTU-based approaches by providing improved taxonomic resolution by monitoring single nucleotide base changes within taxa and allow for reusability and reproducibility between studies (Callahan et al., 2017). ASVs do not eliminate the complexity of organisms, for example if a single genome has multiple copies of the gene marker, multiple ASVs can be presented within the dataset for the same organism (Callahan et al., 2017).

Database/tool	Marine	Bacteria	Archaea	Protists
	specific			
GreenGenes		Х	Х	
(DeSantis et al.,				
2006)				
SILVA (Quast et		Х	Х	Х
al., 2013)				
RDP (Ribosomal		Х	Х	Х
Database project)				
(Cole et al., 2014)				
BLAST (Zhang and		Х	Х	Х
Madden, 1997)				
PR <sup>2</sup> (Protist				Х
ribosomal				
reference) (Guillou				
et al., 2013)				
PR2 transitions				Х
(Jamy et al., 2022)				

Table 1. 1 Taxonomic databases for sequence data.

PhytoREF (Decelle	x		X (only major
et al., 2015)			photosynthetic
			eukaryote lineages
			considered)

High-throughput sequencing tools have been influential in the characterisation of marine microbial communities, with large global-scale surveys providing novel insights into the global distribution and diversity of bacteria, archaea (Santoro et al., 2019; Sunagawa et al., 2015) and protists and eukaryotes (Biard et al., 2016; De Vargas et al., 2015; Faure et al., 2019; Malviya et al., 2016). In addition to metabolically active cells, high-throughput sequencing of DNA (rRNA encoding genes) provides a picture of total diversity, capturing metabolically inactive cells, dead cells and cell free (Not et al., 2009; Stoeck et al., 2007). As POM contains dead cells which may contribute to particle composition, sequencing DNA can mask patterns of diversity for metabolically-active taxa (Bowsher et al., 2019)

High-throughput sequencing of complementary DNA produced from RNA (rRNA), enables a more resolved understanding of the metabolically-active community associated with particles involved in degradation, remineralisation and nutrient cycling (Blazewicz et al., 2013). The ratio between the two (rRNA: rRNA gene) can provide a proxy for activity (Giner et al., 2020), where those with low rRNA: rRNA gene ratios showing potential low activity, whilst those with higher rRNA: rRNA gene ratios indicating high activity which may suggest an important role in biogeochemical cycling for example. However, copy numbers for the rRNA gene can vary greatly across individual protist taxonomic

groups (Biard et al., 2018; Gong et al., 2013; Hu et al., 2016), relating to cell size (Godhe et al., 2008) and genome size (Godhe et al., 2008; Prokopowich et al., 2003) meaning it can be difficult to compare across taxonomic groups. Furthermore, variability rRNA copy numbers as a result of different life-history strategies can make it difficult to interpret within species (Blazewicz et al., 2013). Despite these caveats, using DNA/RNA approaches in particle studies provides a step-change needed to determine diversity and activity of microorganisms associated with particles. Previous studies have described the activity of microbial communities across the water column (Giner et al., 2020; Stoeck et al., 2007; Xu et al., 2017), but this has not yet been linked to particles or the biological carbon pump directly.

#### 1.4 Knowledge gaps

#### 1.4.1 Particle heterogeneity

Previous work investigating the particle associated microbial communities has heavily focussed on the bulk-scale collection of natural particles (Baumas & Bizic, 2023; *references therein*). However, bulk-scale collection of natural particles assumes each particle and its associated community is a replicate of each other, ignores particle life history and assumes all POC is represented the same within marine environments e.g. high molecular weight vs low molecular weight, when in fact it is much more complex. At present, our understanding of how particle heterogeneity impacts large scale processes and influences carbon turnover in the ocean is limited, meaning we cannot wholly interpret the true impact of marine microorganisms on marine carbon cycling. 1.4.2 Diversity and activity of particle associated microorganisms DNA sequencing of particles has captured the biological component of a particle's composition, dormant cells and the metabolically active community, but have not enabled a distinction between these components (Durkin et al., 2022). DNA based approaches have also allowed for an initial broad-scale understanding of the complexity of PA communities and how they differ from FL communities. Using a rRNA gene based approach likely masks patterns of diversity associated with metabolically active particle attached microbes which are underpinning wider ecosystem interactions and the biological carbon pump (Bowsher et al., 2019).

#### 1.4.3 Protists and fungi

Exploration of microbial communities on particles in question have been largely dominated by investigating bacterial communities with limited work on protists and fungi. Research has demonstrated protists contribution to the biological composition of POM (Amacher et al., 2009; Boeuf et al., 2019; Cordier et al., 2022; Durkin et al., 2022)and high fungal biomass on particles in the bathypelagic (Bochdansky et al., 2017). Yet, the inclusion of fungi in the canonical view of the marine carbon cycle is missing (Grossart et al., 2019). Limited knowledge on the eukaryotic role is likely due to the nonspecific nature of using rRNA genes as a tool to investigate POM which is largely composed of eukaryotic debris, resulting in a knowledge gap on the identity of metabolically active microbial eukaryotes associated with POM and their role in the biological carbon pump.

1.4.4. Impact of particle associated microorganism knowledge gaps on understanding the marine carbon cycle.

A 'bulk' based approach to examining POM, which only examines the diversity of organisms, and largely focuses on bacteria, hinders our progress in understanding how fixed atmospheric carbon is processed in the marine environment and how long carbon is sequestered in the marine environment. This is because it does not consider all pathways in which carbon may be respired or consumed, and transferred to higher trophic levels. Alternatively, acknowledging the heterogeneity of particles and what drives heterogeneity within the marine environment combined with a more holistic approach to surveying microbial communities will increase our ability to close carbon budgets. Closing these knowledge gaps will provide a better stance for a more complete understanding on marine carbon cycling and improved estimates for model parameterisation, which is needed in order to best predict the future of the oceans role in carbon sequestration under climate change.

#### 1.5 Thesis Aims

In this thesis, I set out to investigate how particle associated communities are structured at the macro (ocean basin) and micro-scale (single particles). As well as examining bacteria, I also wanted to explore the taxonomic identities and activity of microbial eukaryotes associated with marine particles, especially fungi, as there has been increasing evidence for their role in POC processing capabilities in marine environments (Baltar et al., 2021; Chrismas and Cunliffe, 2020; Cunliffe et al., 2017). In doing so, this thesis will contribute to the understanding of how particle communities are structured in the ocean, and

Page | 33

identify taxa which may contribute to cycling of carbon and wider food web ecology, to close carbon budgets and help us better predict the impact microorganisms play in biogeochemical cycling.

#### 1.6 Objectives

The general topic of this thesis was to investigate the diversity and activity of microbial communities across marine particles and drivers of particle associated microorganisms.

Specific Objectives:

### I. Investigate depth-dependent diversity of particle associated bacterial communities in the North East Atlantic Ocean.

I set out to investigate the connectivity and diversity of colonising bacterial communities on model chitin particles across the major depth zones of the North East Atlantic Ocean to gain insight on identities of general and depthspecific bacteria which are involved in wider ecosystem processes such as microbial gardening.

Explored in: 'Chapter 2: Depth-dependent diversity of particle associated bacteria on model chitin particles in the open ocean'.

## II. Determine diversity and activity of microbial eukaryotes in their contribution to the biological carbon pump of the Southern Ocean.

A major objective of this thesis was to determine the contribution of microbial eukaryotes to the BCP in terms of their activity, as historically surveys of

Page | 34

microbial eukaryotes identify only their contribution to composition of sinking and suspended particles. Using 18S rRNA:18S rRNA gene ratios, I aimed to highlight the diversity and activity of microbial eukaryotes to the BCP of the globally important Southern Ocean. I also aimed to identify if marine fungi were an active component of the eukaryotic community.

Explored in: 'Chapter 3: DNA and RNA based assessment of the biological carbon pump in the Southern Ocean'.

## III. Develop methods to describe physical characteristics and active microbial communities of individual naturally-occurring particles in coastal waters surrounding Plymouth Sound.

Particles in the marine environment exhibit enormous variety but this heterogeneity is often overlooked using bulk-scale sampling approaches. Firstly I wanted to develop a pipeline which would describe both the physical characteristics and active microbial communities of individual particles isolated from coastal waters surrounding Plymouth Sound. I then wanted to describe the heterogeneity of naturally occurring particles found and determine if this differs overs spatial and temporal scales and as a response to environmental variables including stable isotope ratios of carbon. To assess the impact of particle heterogeneity more broadly, I wanted to investigate if physical properties of particles influenced the active microbial eukaryote community.

Explored in 'Chapter 4: Mapping the 'particlescape': an assessment of particle characteristics and fungal communities at the single particle level in the coastal ocean.'

## IV. Conduct a pilot study on the drivers of active particle associated fungi in coastal waters surrounding Plymouth Sound.

Using the developed pipeline, I wanted to determine if fungi were an active component of particle attached eukaryote community and determine the drivers of active particle fungi diversity.

Explored in 'Chapter 4: Mapping the 'particlescape': an assessment of particle characteristics and fungal communities at the single particle level in the coastal ocean.'

#### **1.6 Thesis Outline**

This thesis has an introduction chapter, three research chapters and a general discussion chapter.

The introduction chapter (chapter 1) outlines the role the ocean's BCP plays in marine carbon cycling and the current understanding of microbial communities processing POM and its influence on the BCP. It details methodology and accompanying limitations used to sample POM and their associated communities. It highlights knowledge gaps in our understanding of the ecological processes underpinning PA community structure and limited knowledge on the contribution of microbial eukaryotes in the biological carbon pump and recognises the role expanding knowledge plays in understanding the future of the oceans role in climate change.

Chapter 2 describes the diversity of bacterial communities attached to model chitin particles incubated in water collected from three depth zones in the North East Atlantic ocean to help identify general and depth-specific candidates for microbial gardening. Mechanisms, including connectivity via sinking particles, are suggested for structuring particle attached communities and the implications of microbial particle colonisation for zooplankton grazers and wider ecosystem interactions and carbon cycling are suggested.

Chapter 3 investigates the diversity and activity of microbial eukaryotes behind the biological carbon pump in the globally significant Southern Ocean using high-volume *in situ* sampling of seawater. This chapter uses a DNA and RNA based assessment to explore microbial eukaryotes associated with POC attenuation. Using 18S rRNA: 18S rRNA gene ratios, the contribution of microbial taxa to production and degradation of POC in the surface and upper mesopelagic are suggested.

Chapter 4examines whether the heterogeneity of particles is reflected in the heterogeneity of 'active' microbial communities on single particles in the coastal ocean. This chapter uses a pipeline of isolation, imaging, and RNA sequencing of the 'active' microbial eukaryotic communities on individual particles collected over a winter and spring period across two sampling sites in the Western English Channel. Classification of particles into categories are accompanied by analysis of 'active' communities for individual particles in comparison to the bulk planktonic community. This chapter focuses especially on exploring the fungal diversity associated with different particle types, spatially and temporally and assesses the environmental mechanisms driving the contribution of fungi to the active communities on particles including using stable isotope composition of bulk POM.

The general discussion chapter (chapter 5) summarises the major contributions of the research to the knowledge gaps and aims outlined in the introduction chapter and in the broader context of the wider field. It also presents possible limitations of methodologies used in this thesis and suggest approaches to overcome these, in addition to identifying future avenues of research.

#### **1.7 Author Contributions**

The work presented in this thesis was carried out by the author except in the cases outlined in Table 1.2 and below:

Chapter 2

- Provision of logistical support for cruise sampling in Chapter 2 was provided by Sue Hartman and Kimberley Bird
- Illumina MiSeq sequencing was carried out by the IMR sequencing facility, Dalhousie, Canada

#### Chapter 3:

- Cruise sampling and samples for Chapter 3 were provided by Adrian
  Martin, Chelsey Baker, Katsia Pabortsava, Frederic LeMoigne
- Sabena Blackbird carried out elemental analysis of POC samples as part of the wider CUSTARD project.
- Illumina MiSeq sequencing was carried out by the NUOMICS, Northumbria, UK

#### Chapter 4:

- Water sampling was carried out by RV MBA Sepia crew
- Stable isotope analysis was conducted by OEA Labs, Okehampton.
- Processing of samples for eukaryote bulk dataset was conducted

alongside Laura Branscombe

- Illumina MiSeq sequencing of bulk eukaryote was carried out by the NUOMICS, Northumbria, UK
- Illumina MiSeq sequencing of single particles was carried out by the GENEWIZ, Leipzig, Germany
- Phytoplankton count data was provided by Claire Widdicombe at

Plymouth Marine Laboratory (PML)

- Nutrient data and environmental parameters were provided by the

Western Channel Observatory, PML, National River Flow Archive

(NRFA) and Bears by the Sea

Table 1.2 Summary of author contributions to each chapter (CR = CordeliaRoberts, MC = Michael Cunliffe, KH = Katherine Helliwell, KB = Kimberley Bird, NC= Nathan Chrismas, SH= Sue Hartman, HV =Hugh Venables, CB = ChelseyBaker, KP = Katsia Pabortsava, FLM = Fred Le Moigne, SB = SabenaBlackbird, LB= Laura Branscombe).

Chapter	Sample	Sample	Technic	Data	Writing	Editing
	Collection	Processin	al	Analysi		
		g	Support	S		
Chapter 1:	-	-	-	-	CR	CR,MC,
Introduction						КН
Chapter 2	CR,SH,	CR	NC	CR	CR	CR,MC,
	KB					КН
Chapter 3	HV,	HV,	NC	CR	CR	CR,MC,
	KP,FLM,C	SB,CR,KB				КН
	В					
Chapter 4	CR, LB	CR, LB	-	CR	CR	CR,MC

Chapter 5:	-	-	-	CR	CR,MC
General					
Discussion					

# Chapter 2: Depth-dependent diversity of particle associated bacteria on model chitin particles in the open ocean.

Co-authors of the manuscript: Kimberley Bird, Nathan Chrismas, Susan Hartman, Michael Cunliffe

# Chapter 2: Depth-dependent diversity of particle associated bacteria on model chitin particles in the open ocean.

#### 2.1 Abstract

Sinking particles transport carbon from the surface to the deep ocean. Microbial colonisation of sinking particles and remineralisation at depth plays an important role in the ecology and biogeochemistry of the deep ocean. Fragmentation of particles by zooplankton and the resulting colonisation by microorganisms before ingestion, known as 'microbial gardening', allows for trophic upgrading and increased microbial biomass for detritivorous zooplankton. Using model chitin particles incubated with seawater collected from surface, meso- and bathypelagic depths in the North East Atlantic Ocean, we determined particle-attaching bacterial communities to help identify general and depth-specific candidates for microbial gardening. Comparison of particleattached communities at the amplicon sequence variant (ASV) level showed that bacteria found on surface particles were also found attached in the bathypelagic, in line with sinking particles providing vertical connectivity through the water column. Bathypelagic particle-attached communities were most diverse. We propose that some microbial gardening candidates attach in the surface and sink out with the particle, whilst other candidates are depth-specific. This chapter suggests that candidates for microbial gardening differ with depth, which may be important when considering the implications of microbial particle

colonisation for wider carbon cycling and the role they play for zooplankton grazers and other ecological processes.

#### 2.2 Introduction

Sinking particles transport carbon from the surface to deep ocean and form a key component of the biological carbon pump (BCP), acting as a mode of organic carbon transport to meso- and bathypelagic ecosystems (Honjo et al., 2014). Particles are microbial hotspots because of higher density of organic carbon and nutrients (Alldredge and Silver, 1988). As attachment capability is a factor for colonising microbes (Grossart et al., 2006), particle-attached microbial communities are distinct from surrounding bacterioplankton (Alldredge and Silver, 1988; Fenchel and Blackburn, 1999).

Particles are grazed on by detritivorous zooplankton, such as some copepods (Jackson, 1993; Wilson et al., 2008b). In a process referred to as 'microbial gardening', grazing zooplankton fracture sinking particles to create new exposed surface areas, promoting microbial attachment and improving the trophic value of particles for subsequent grazers (Mayor et al., 2014). Microbial gardening allows zooplankton to utilise enzymatic repertoires of bacteria which degrade refractory compounds (e.g. cellulose), that they are unable to degrade themselves. Transformation of large detrital particles to smaller particles richer in microbial biomass provides an increased nutritional value available for metazoan growth (Mayor et al., 2014).

In temperate waters, zooplankton can fragment and ingest ~50% of sinking particles and stimulate microbial degradation (Giering et al., 2014). Microbial gardening is likely important in meso- and bathypelagic ecosystems where resources are scarce, however knowledge on ecological processes Page | 42 involved in processing organic material in the so-called 'twilight zone' is limited (Martin et al., 2020). At present, the taxa involved in microbial gardening (i.e. microbial taxa that attach to new particle surfaces) are poorly understood, with few studies characterising key members of the community involved (Kong et al., 2021) and with a limited focus on the mesopelagic and bathypelagic zones.

To address these knowledge gaps, we incubated model chitin particles in seawater collected from the epi-, meso- and bathypelagic depths at the Porcupine Abyssal Plain Sustained Observatory (PAP-SO) in the North East Atlantic Ocean. Chitin is one of the most abundant polysaccharides in the marine environment (Kirchner, 1995) and a structural component of many marine organisms and associated debris (e.g. carcasses, faecal pellets) (Durkin et al., 2009; Greco et al., 1990; Latgé, 2007; Weiner and Traub, 1984; Yoshikoshi and Kô, 1988). Observational and modelling based estimates, indicate that the North East Atlantic Ocean is the largest ocean sink for atmospheric CO<sub>2</sub> in the Northern Hemisphere, taking up  $\sim 0.7 \pm Pg C$  year<sup>-1</sup> (Gruber et al., 2002) The North East Atlantic Ocean is an area with a high-level of flux organic carbon (Billett and Rice, 2001; Lampitt et al., 2010), with chitinous sinking material estimated to comprise of 40% faecal pellets and 10% dead zooplankton (Lampitt et al., 2001, 1993). We aimed to explore the community composition on model chitin particles across the three major depth zones in the open ocean and identify cosmopolitan (i.e. throughout the water column) and depth-specific candidates for microbial gardening.

#### 2.3 Methods

#### 2.3.1 Study site

Sampling took place on board the *RRS Discovery* (cruise DY103) between 21 June to 9 July 2019 at the PAP-SO (49°N 16.5°W) (Figure 2.1A). Seawater properties (salinity, temperature, oxygen and fluorescence) throughout the water column were measured using a conductivity-temperature-depth mounted on a rosette sampler (CTD Seabird) (Figure 2.1C-F). Seawater was collected from epipelagic (termed "surface" (30 m), mesopelagic (600 m) and bathypelagic (3000 m) waters using Niskin bottles on the CTD rosette.



Figure 2. 1 A) Map of North East Atlantic ocean study, Porcupine Abyssal Plain Sustained Observatory (PAP-SO) indicated by yellow circle. B) Cruise transect across sampling sites within the PAP-SO area. Vertical profiles of C) salinity, D) temperature, E) oxygen and F) fluorescence as a measure of chlorophyll. Produced with Ocean Data View (Schlitzer, 2016).

#### 2.3.2 Experimental set up

To determine the microbial communities in the water column (termed "in situ"), seawater (1 L) was filtered through 0.2 µm cellulose filters. Magnetic chitin microbeads (New England Bio; Ø100 µm) were used for incubations using protocols adapted from (Datta et al., 2016; Roberts et al., 2020). Incubations were carried out in 1 L polycarbonate bottles (Nalgene) containing seawater only (i.e. no microbeads) and seawater plus chitin microbeads (1125 ±222 beads per mL) with 4 replicates for each treatment. (Figure 2.2). Bottles were kept at *in situ* temperatures (30m 15°C, 600m 10°C, 3000m 3°C) in the dark with twice daily inversion. After 48 hours, all bottles were harvested. Bottles containing seawater only were filtered on 0.2 µm cellulose nitrate filters (Cole-Parmer). For bottles containing microbeads, seawater was passed through a 40 µm sterile cell strainers (Corning) to collect the microbeads. The 40 µm mesh was inverted and washed with 0.2 µm filtered seawater from the respective depth into a sterile petri-dish (Sarstedt), from which the microbeads were harvested into a sterile centrifuge tube (Eppendorf). A magnet was used to collect the microbeads at the side of the tube and excess water was removed and discarded. The seawater that passed through the 40 µm mesh was filtered onto 0.2 µm cellulose nitrate filters. All filters and microbeads were preserved in DNA/RNA Shield (Zymo Research, USA) and stored at -80°C. Some replicates from the meso-and bathypelagic were lost during processing.

Chapter 2: Depth-dependent diversity of particle associated bacteria on model chitin particles in the open ocean.



Figure 2. 2 Experimental incubation setup and sample processing pipeline.

#### 2.3.3 DNA extraction and metabarcoding

DNA was extracted using the ZymoBIOMICS DNA/RNA Miniprep kit (Zymo Research, USA) following the manufacturer's instructions, including a blank extraction (e.g. no sample) to act as a 'kitome' to enable identification of potential contamination from reagents (Salter et al, 2014), which was also sequenced. Partial 16S rRNA gene (v4 region) were amplified using primers 515F (Parada) (Caporaso et al., 2011; Parada et al., 2016) -806R(Apprill) (Apprill et al., 2015) followed by sequencing on the Illumina MiSeq platform and processed using the DADA2 pipeline to determine amplicon sequence variants (ASVs) (Callahan et al., 2016) in R Studio (R Core Team, 2019). Demultiplexed reads were filtered and trimmed to remove primers and low-quality sequences. Paired end reads were merged to obtain full denoised sequences. Chimeric sequences were removed, before taxonomy was assigned using the SILVA database (release 128) (Quast et al., 2013). Chloroplast and mitochondria sequences were removed. ASV table, taxonomic assignment and metadata were combined into a phyloseq object using the *phyloseq* package (McMurdie and Holmes, 2013) and sequences were rarefied to 1614 reads before further analysis. Low rarefaction was required in order to retain enough replication of samples.

#### 2.3.4 Data processing and statistical analysis

Shannon's index (H) was used to calculate alpha diversity, and the effect of depth and treatment on alpha diversity was determined using two-way ANOVA with Tukey's HSD in R Studio (R Core Team, 2019). Differences in community structure between samples were calculated using a Bray-Curtis dissimilarity matrix and visualised through non-metric multidimensional scaling (NMDS) ordination. Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was used to test the effect of depth and treatment on differences in community structure using the Adonis function in the R package *vegan* (Oksanen et al., 2015).

#### 2.3.5 Determining vertical microbial connectivity

To determine vertical connectivity of microbial gardening candidates, the number of ASVs that were shared or unique on incubated particles between depths were compared. ASVs were categorised as shared or unique for each depth as follows (Figure 2.3).



Figure 2. 3 Schematic figure of shared and unique amplicon sequence variants (ASVs). Green – epipelagic, Orange – mesopelagic, Blue – bathypelagic.

ASVs that were detected only on the incubated particles and not detected in the respective *in situ* bacterioplankton (likely due to detection limits) were termed "unique to particle" ASVs. ASVs detected on both the incubated particles and in the *in situ* bacterioplankton for the respective depth were termed "shared *in situ*" ASVs. Assuming unidirectionality, i.e. the potential flow of ASVs is from surface to the bathypelagic only, ASVs detected on particles incubated in the meso- and bathypelagic water as well in the surface as were Page | 48 termed "shared with epipelagic incubated particles" and those not detected on surface incubated particles but detected on meso- and bathypelagic incubated particles were termed "shared with mesopelagic incubated particles". In the case where there was overlap between categories e.g. an ASV was "shared with epipelagic incubated particles" and "shared *in* situ" in the bathypelagic, the ASV was defined as "shared with epipelagic incubated particles" under the assumption of unidirectionality of ASVs transport in the water column. Individual replicates were compared against pooled replicates of bulk communities to overcome differences in replication.

#### 2.4 Results

Using the DNA concentration extracted from incubated chitin particles as a proxy for particle biomass and therefore colonisation, biomass across the particles seemed comparable, although this is difficult to interpret due to low replication (Table 2.1).

Table 2.1 DNA concentrations ( $\mu$ g/ $\mu$ L) extracted from chitin particle incubations across the three depth zones from NanoDrop spectrophotometer (ThermoFisher).

	DNA concentration (µg/µL)					
Depth	Replicate 1	Replicate 2	Replicate 3	Replicate 4		
Epipelagic	3.2	4.9	4.3	2.2		
Mesopelagic	-0.1	1.2	2.4	-		
Bathypelagic	3.0	2.6	-	-		

Diversity on incubated particles was reduced compared to the *in situ* 

water column, water only incubations and particle water communities across all

depths (Tukey's HSD p<0.05), with particle community diversity greatest in the bathypelagic incubations (Figure 2.4A; Tukey's HSD p<0.05). Community structure varied significantly between the depths and the incubated particle-attached and other treatments (water only and *in situ* community), with an overall significant interaction between these variables (Figure 2.4B; PERMANOVA p <0.001). Particle communities were also distinct with depth indicated by separation on the NMDS plot for chitin particles by depth, although this trend was not statistically significant (Figure 2.4B, Tukey's HSD p=0.1). This is likely because of loss of replication the meso-and bathypelagic incubations, resulting in low statistical power.

Chapter 2: Depth-dependent diversity of particle associated bacteria on model chitin particles in the open ocean.



Figure 2. 4 A) Alpha diversity of bacterial community across all depths. Bars represent standard error and asterisks denote level of significance (\*\*\*p < 0.001). B) Non-metric multidimensional scaling (NMDS) visualisation of community structure across all treatments. Letters represent depths as follows E = epipelagic, M=mesopelagic and B = bathypelagic. Ellipses not statistically calculated but used to aid guidance of differences between depths and incubated chitin particle community.
Matching the community structure, the phylogenetic composition of the *in situ*, water only and particle water treatment from each depth were broadly similar, dominated by Flavobacteriales (particularly in the surface), Oceanospirillales, Pelagibacterales (SAR11), SAR324 clade (Marine group B), Rhodobacterales, Planctomycetales and the Archaea order Thermoplasmatales (Figure 2.5).

Particles exposed to surface water were dominated by Cytophagales and a large proportion of unassigned ASV that the SILVA database could only assign to the kingdom of Bacteria (Figure 2.5). Particles incubated with mesopelagic water were dominated by Cellvibrionales and to a lesser extent also Cytophagales (Figure 2.5). Particles incubated with bathypelagic water were dominated by more major orders than the surface and mesopelagic, namely Flavobacteriales along with Alteromonadales, Cellvibrionales, Cytophagales and Sphingobacteriales (Figure 2.5). ASVs belonging to the Archaea were found attached to particles across the depth ranges, but these did not form greater than 0.12% of the relative abundance on particles (Table 2.2), so were not considered further.



Figure 2.5 Order level relative abundance of bacterial and archaeal amplicon sequence variances (ASVs) across all treatments conducted across the three depths, epi-, meso- and bathypelagic for individual replicates grouped by Phyla. Relative abundances of less than 3% are grouped under "other".

Table 2.2 Archaeal amplicon sequence variances (ASVs) found on particles across all depth ranges and replicates.

Depth	Replicate	Archaeal ASV (Order	Relative		
		level ID)	Abundance (%)		
	1	None detected	-		
Epipelagic (30 m)	2	Thermoplasmatales ASV	0.06		
		40			
	3	None detected	-		
	4	None detected	-		
Mesopelagic (600 m)	1	Halobacteriales ASV 2314	0.12		
	2	Thermoplasmatales ASV			
		16			
	3	Unknown Order	0.06		
		(Candidatus			
		nitropelagicus) ASV 87			
		Thermoplasmatales ASV	0.06		
		186			
Bathypelagic (3000 m)	1	Thermoplasmatales ASV	0.12		
		16			
		Thermoplasmatales ASV	0.3		
		45			
	2	Thermoplasmatales ASV	0.06		
		40			

Surface particle attached ASVs were also found attached on particles incubated with water from the meso- and bathypelagic, with increased abundances from orders dominating the bathypelagic incubated particles e.g. Flavobacteriales, Cytophagales, and Alteromonadales (Figure 2.6). Some bacterial ASVs were unique to specific depths (Figure 2.6), including all the mesopelagic Cellvibrionales and some bathypelagic Flavobacteriales. ASVs that were not found in the surface but found in the mesopelagic and bathypelagic treatments were also identified e.g. Rhodobacterales. In the bathypelagic, 19.18% ( $\pm$ 8.02 SD) of ASVs on particles were shared with ASVs found on particles in the surface, with a further 8.96% ( $\pm$ 0.89 SD) shared with particles from the mesopelagic (Table 2.3). In the mesopelagic, 29.60% ( $\pm$  6.78) of the of ASVs on particles were shared with particles in the surface trade to the surface (Table 2.3). In contrast, the number of ASVs shared between the particle attached and reciprocal *in situ* communities decreased with depth (Table 2.3 p<0.05). The surface particle-attached community shared a 51.20 % ( $\pm$ 4.43 SD) ASV similarity with the *in situ* community, whilst the meso- and bathypelagic particles shared a reduced ASV similarity with the reciprocal *in situ* communities at 13.91 % ( $\pm$ 8.96 SD) and 8.65 % ( $\pm$ 1.19 SD) respectively.



Figure 2.6 Order level relative abundance of bacterial amplicon sequence variants (ASVs) shared and unique found on particles incubated in epi-, meso- and bathypelagic water by individual replicates. Orders are grouped by Phyla. Relative abundances of less than 1% are grouped under 'other' categories.

Table 2.3 Amplicon sequence variants (ASVs) shared between particles and in situ communities. Values were calculated by counting the total number of ASVs found in each replicate at each depth and then counting the number of ASVs that were also found in situ. This number was then divided by the total number of ASVs on the particle to identify the percentage of ASVs shared with the in situ community.

Depth	Replicate	Total	Unique		Shared		Shared		Shared	
		ASVs on	to particle		in situ		epipelagic		mesopelagic	
		particles	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Epipelagic	1	61	28	45.90	33	54.09	-	-	-	-
(30 m)	2	74	32	43.24	42	56.75	-	-	-	-
	3	60	31	51.67	29	48.33	-	-	-	-
	4	57	31	54.39	26	45.61	-	-	-	-
Surface mean			48.80±4.49		51.2±4.43					
Mesopelagic	1	46	31	67.39	4	8.69	11	23.91	-	-
(600 m)	2	46	25	54.35	3	6.52	18	39.13	-	-
	3	132	63	47.73	35	26.52	34	25.76	-	-
Mesopelagic mean			56.49±8.17		13.91±8.96	-	29.60±6.78			
	1	161	93	62.11	12	7.45	43	26.70	13	8.07

Bathypelagic	2	122	60	49.18	12	9.84	13	10.66	12	9.84
(3000 m)										
Bathypelagic mean			55.65±6.47		8.65±1.195		19.18±8.02		8.96±0.885	

#### 2.5 Discussion

The diversity of particle-attached microbial communities was significantly reduced in comparison to the surrounding bacterioplankton communities across all depths, as has been previously reported of microbial communities colonising chitin particles in marine and freshwater (Datta et al., 2016; Roberts et al., 2020; Suominen et al., 2021). This suggests that there is selection for distinct particle associated communities (Datta et al., 2016), potentially as a result of r strategists responding rapidly to substrate enrichment on encountering a particle. While bacterioplankton alpha diversity remained relatively consistently high across the three depth zones, the alpha diversity of particle-attached communities was highest in the bathypelagic region, which has previously been reported at 2,000 m on chitin particles (Suominen et al., 2021). This suggests a mechanism for enrichment of bacterial ASVs at depth in the bathypelagic.

Evidence for identical bacterial ASVs found on chitin particles across the three depth zones also suggests in part this elevated diversity may be explained by particles promoting vertical connectivity in the open ocean microbiome (Mestre et al., 2018; Ruiz-González et al., 2020). Previous work has shown that initial diversity on chitin beads is high owing to nonspecific colonisation of particles, with changes in colonisation over time (Datta *et al*, 2016; Suominen *et al*, 2020). As differences in incubation temperatures representative of the *in situ* water temperatures likely impacts the rate at which growth may happen, patterns in the bathypelagic could be represented by differences in colonisation and growth. Although, DNA yields were comparable between depths suggesting colonisation and growth were similar, lack of

replication makes this difficult to interpret and this should be considered in future investigations.

Chitin particles incubated with surface water were dominated by an unassigned bacterial group that was detected at very low abundances at other depths (< 1%), suggesting a key candidate for microbial gardening of surface particles. BLAST searches of sequences against the NCBI nr database (Altschul et al., 1990) and the Microbe Atlas Project tool (Matias Rodrigues et al., 2017) showed hits with high similarity against previously recorded uncultured bacteria in the phylum Fibrobacteres (99% guery cover; >83.96 % identity cover) and the class Fibrobacteria (82% identity cover) from each database respectively. Fibrobacteres isolated from hypersaline soda lakes have been identified as chitin degraders (Rahman et al., 2016). Given the dominance of this taxon in the surface, further work should be done to uncover its identity and potential role as a candidate for microbial gardening. Our findings indicate that some members of particle-attached communities in surface waters are also found attached to particles in the meso- and bathypelagic region of the open ocean, while others are depth specific. As particles sink and conditions change, some bacteria may not be adapted to changes and detach (Mestre et al., 2018), potentially supporting the recorded higher diversity of the bacterioplankton around them and allowing new depthspecific microbial gardening candidates to colonise the particle under more optimal conditions. For example, the Cellvibrionales, as a group were not detected on surface particles but dominated on mesopelagic particles.

The same ASVs belonging to the orders Flavobacteriales, Cytophagales, Alteromonadales, and Sphingobacteriales were found on particles in the surface as well as the bathypelagic incubations, suggesting that they may be cosmopolitan candidates for microbial gardening. However, as these orders increased in their abundance in the bathypelagic, with the exception of Cytophagales, they may be of greater importance as microbial gardening candidates in the bathypelagic determined by their ability to withstand pressure and temperature changes below the photic zone or as dormant or slow growing ASVs in the surface that flourish as conditions become more favourable (Lennon and Jones, 2011; Mestre et al., 2018) in comparison to surface dominant ASVs (e.g., Cytophagales). These taxa were not at high abundances in the corresponding *in situ* data, and as niche partitioning within bathypelagic prokaryotes between free living and particle attached at a phylogenetic level has been previously reported (Salazar et al., 2015), this may also be responsible for the more pronounced enrichment at depth.

Zooplankton selectively graze on particles and their attached microbial communities to obtain a dietary supply of essential amino acids and fatty acids, which they are unable to synthesise (Anderson et al., 2017). Regulation of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) that are produced by Flavobacteriales (Shulse and Allen, 2011) and Cytophagales (Nichols and McMeekin, 2002) are important below the euphotic zone where high pressure and low temperature can influence the functioning of copepod cell membranes (Pond et al., 2014). EPA also has an important role in growth and fecundity of freshwater zooplankton (Muller-Navarra, 1995; Müller-Navarra D. C. et al., 2000). Therefore, diversity of particle colonising bacterial groups may be important when considering the role that they play in providing essential

nutrition for zooplankton communities throughout the water column, and potentially influence their migration dynamics, which should be explored further.

Microbial gardening on chitin-based particles may allow for the trophic upgrading of chitin carbon, which may otherwise be nutritionally devoid or energetically costly to undertake. Currently, the feeding dynamics of detritivorous zooplankton on chitin-based particulate organic matter (POM) is unknown. Further studies should attempt to unravel the feeding dynamics of detritivorous zooplankton on chitin-based POM, including the palatability of chitinous POM with and without associated bacterial communities and assess the trophic transfer capability of chitin carbon via microbial gardening.

#### 2.6 Conclusion

In this short-term experimental incubation, we show ASV level diversity of particle attached bacteria across the water column, with elevated bacterial diversity on particles in the bathypelagic region. We also demonstrate the vertical connectivity of microbial communities via particles through sharing of ASVs on particles found in the surface and bathypelagic as previously recorded in environmental sampling using an operational taxonomic unit (OTU) based diversity resolution (Mestre et al., 2018; Ruiz-González et al., 2020). We propose that high diversity on particles in the deep sea could be explained in part via sinking of particle-attached communities and colonisation from the surrounding bacterioplankton at depth. We suggest that taxa involved in microbial gardening at depth are likely to be a combination of those that have attached in the surface or mesopelagic and those attaching in the bathypelagic zone. This should be explored at a finer depth-resolution to determine the source of the remaining particle diversity of the bathypelagic zone. This study Page | 62

suggests that candidates for microbial gardening differ with depth which may be important when considering the wider implications of microbial colonisation on particles for carbon cycling and the role they play for zooplankton grazers.

These observations should also be investigated for other types of particle, such as microplastic particles, which may have further unknown implications for wider food web interactions.

# Chapter 3: DNA and RNA based assessment of the biological carbon pump in the Southern Ocean

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## Chapter 3: DNA and RNA based assessment of the biological carbon pump in the Southern Ocean.

#### 3.1 Abstract

The biological carbon pump drives the flux of particulate organic carbon (POC) in the ocean directly affecting atmospheric CO<sub>2</sub> concentrations. The Southern Ocean especially plays a disproportionate role within the biological carbon pump owing to both physical and biological processes. The interplay between production and gravitational sinking of material and transformative processes including microbial degradation exerts a major influence on controlling carbon sequestration in the ocean. The activity of microbial eukaryotes in contribution to production, and transformation of sinking material as part of the biological carbon pump is however limited. Here we show diversity and activity of microbial eukaryotes including microbial metazoans concomitant with the vertical attenuation of POC in the Southern Ocean. Using DNA/RNA ratios of microbial eukaryote amplicon sequence variants (ASVs) as an estimate for activity, we found many taxa including chain forming and pennate diatoms, polycystine radiolarians and MArine STramenopile group 3 (MAST-3) were active throughout the water column. We also identified ASVs that showed contrasting patterns of activity to their surface counterparts at depth, coupled with increasing dissimilarity between DNA and RNA species composition with depth including the potential decreasing activity of some diatom ASVs, dinoflagellates and copepods in the mesopelagic compared to the surface

rates. Our results suggest a more specific contribution of microbial eukaryote diversity and activity towards the vertical attenuation of POC in the Southern Ocean than previously identified. The distinction between total diversity and potential activity of microbial eukaryote taxa has potential to improve estimates for model parameterisations needed to close carbon budgets in the marine carbon cycle, and better predict the response of the ocean carbon sequestration under future climate scenarios.

#### 3.2 Introduction

Phytoplankton sustain ecosystems via primary production (Ducklow et al., 2012) by supporting zooplankton communities including copepods and krill (Shreeve et al., 2002), higher trophic levels and the microbial loop. Phytoplankton also contribute to the export of carbon to the deep sea as part of the biological carbon pump (BCP). As a result of surface productivity, a great variety of particulate organic matter (POM) is produced (Kharbush et al., 2020). POM includes, but is not limited to, phyto-detritus, zooplankton-based detritus (faecal pellets, carcasses, and moults) and gel-like particles. The subsequent sinking or removal of POM out of the euphotic zone via multi-faceted pumps (Boyd et al., 2019) drives the BCP, and thus removal of carbon to the deep ocean for potential long-term sequestration. Seasonality in biological processes governing particle formation result in orders of magnitude seasonal variations in POC flux (Buesseler et al., 2007; Manno et al., 2015).

The decreasing concentration of particulate organic carbon (POC) with depth, or attenuation, is generally described by the Martin curve (Martin et al., 1987) and captures the inefficient nature of the BCP. Flux attenuation of POC is generated by both physical processes including particle size and fragmentation, and biological processes such as heterotrophic consumption by microorganisms and metazoan taxa such as copepods which show clear variations in POC export across seasons and ocean basins (Buesseler et al., 2007). Degradation and remineralisation of POC by microorganisms play an important role in POC attenuation with depth and the depth that remineralisation occurs is tightly coupled to atmospheric levels of CO<sub>2</sub> (Kwon et al., 2009). Description of the flux-degradation relationship is crucial in understanding mechanisms behind

carbon flux efficiency and global carbon cycling (Lin et al., 2022), especially in the mesopelagic, as this is where many particles form and the most rapid loss of carbon occurs (Buesseler et al., 2009). Additionally, in some oceanic regions POC may only need to sink within the top few 100 meters in order to be stored for a climatically relevant sequestration period (100 years, (Robinson et al., 2014), whilst others require deeper penetration into the ocean interior (<1000 m) (Baker et al., 2022).

Many studies have correlated the attenuation of POC with zooplankton (metazoa) and bacterial respiration, although a lack of consensus still remains as to their relevant role (Belcher et al., 2016; Burd et al., 2010; Giering et al., 2014). Microbial eukaryotes are ubiquitous across the water column and display marked differences between surface and deep water eukaryotic assemblages (Duret et al., 2019a, 2015; Orsi et al., 2012). Despite this, the diversity and activity of microbial eukaryotes and metazoa, in carbon transfer pathways, carbon sequestration and modelling efforts is limited. Limited data on diversity and activity of microbial eukaryotes means we cannot close carbon budgets, or effectively predict the future climate.

High-throughput sequencing of DNA extracted from POM in the marine environment has identified the diversity of specific eukaryotic taxa associated with sinking material and POC export (Cordier et al., 2022; Duret et al., 2019a), including diatoms, dinoflagellates, fungi and radiolarians. DNA sequencing captures metabolically inactive cells and dead cells, in addition to metabolically active cells (Stoeck et al., 2007). Therefore, it is unlikely to provide an accurate reflection of the true activity of microbial eukaryotes in either the production or degradation and remineralisation of POC, but rather a picture of the total

diversity present, including dead organisms contributing to particle composition (e.g., production of POM) (Not et al., 2009; Stoeck et al., 2007). As an alternative, sampling RNA characterises metabolically active cells, and in concomitant with DNA can provide complimentary diversity information (Blazewicz et al., 2013). Furthermore, the ratio between rRNA (RNA) and rRNA gene (DNA) counts enable an estimate of protein syntheses as a potential for activity of individual taxa and has highlighted the mesopelagic as a hotspot for activity (Giner et al., 2020). Previous work has used RNA:DNA ratios as a proxy for activity for marine microbial eukaryotes (Giner et al., 2020; Hu et al., 2016; Logares et al., 2014; Massana et al., 2015; Not et al., 2009; Xu et al., 2017). Combined DNA and RNA based sequencing approaches enables the potential for a more accurate linked view of diversity and activity of microbial eukaryotes associated with the BCP and their contribution into biogeochemical models, which has been limited thus far.

The Southern Ocean is one of the largest carbon sinks in the global ocean (Frölicher et al., 2015; Khatiwala et al., 2013) and plays a disproportionate role in the global carbon cycling through its physical and biological properties(Hauck et al., 2015; Hauck and Völker, 2015). Knowledge on the Southern Ocean BCP is limited by its remote nature and high cloud cover (excludes extensive use of satellite ocean colour), and there is great spatial variation in the depth at which POC must reach in order to be sequestered for 100 years (DeVries and Weber, 2017).Collectively, this means *in situ* observations of diversity and activity of microbial eukaryotes over spatial scales in the Southern Ocean are key in providing a greater understanding of the BCP. Here we use high-volume water sampling using *in situ* pumps coupled

with a DNA and RNA-based metabarcoding approach to describe the diversity and activity of microbial eukaryotes behind measured POC vertical attenuation and specifically assess spatial trends in the upper limb of the Southern Ocean.

#### 3.3 Methods

#### 3.3.1 Sampling

Sampling took place on board the *RRS Discovery* (cruise DY111) during the austral summer (2<sup>nd</sup> December 2019 to 9<sup>th</sup> January 2020) in the Southern Ocean. Sampling consisted of repeated occupations of a transect across 3 stations along the 89<sup>th</sup> meridian west line (Station OOI (54 S 89 W), Station TN (57 S 89 W), Station TS (60 S, 89 W)) (Figure 3.1). Water column properties (i.e, salinity, temperature, oxygen, and fluorescence) were measured using a conductivity-temperature-depth rosette (CTD Seabird). Sampling depths ranged between 30-400 m. Depth zones were defined as follows epipelagic: 0-180m, mesopelagic 200-400m following literature definitions (Reygondeau et al., 2018).



135° W 180° 135° E Figure 3. 1 Three sampling stations OOI, TN and TS in the Southern Ocean where repeated transects took place.

3.3.2 Bulk particulate material sampling

Particles were collected using stand-alone pumps (SAPS). Briefly, SAPS were

deployed to pre-determined discrete depths (Appendix Table 1.1) at each Page | 70 station occupation and left to pump water for 60 minutes through a 53 µm Nytex mesh (PLASTOK Associates). Once recovered on board, 1L seawater was passed over the 53 µm Nytex mesh to concentrate and collect bulk particulate material and split between several fractions using a Folsom splitter (Wildco). One split (0.109 L) was used for analyses conducted here as sampling efforts were coordinated for different projects and required sharing between them during the cruise period. Volumes of water filtered by pumps ranged from 21-2000 L are detailed in Appendix Table 1.1. Bulk particulate material for DNA and RNA extractions was stored in DNA/RNA Shield (Zymo Research, USA) and at -80°C. For POC measurements 0.5- 1 L of seawater was filtered on to GF/F filters (Whatman) and stored at -20°C.

3.3.3 Particulate organic carbon measurements

Filters were freeze-dried, and quartered. For this study, two quarters were ran in duplicate and underwent vapour phase de-carbonation using <sup>12</sup>N Analar Grade hydrochloric acid (HCI) (Yamamuro and Kayanne, 1995). Briefly, filters were arranged on glass petri-dishes and left overnight in a desiccator with concentrated HCI in the bottom before drying in a low temperature oven. Filters were then analysed on a FlashSmart Organic Elemental Analyser (Thermo Scientific). Two point daily calibration using High Organic Sediment Standard OAS (Elemental Analysis Ltd, NIST certified) were then analysed twice as an 'unknown'. Results for the 'unknown' were within uncertainty limits of certified value (Carbon 7.7%  $\pm$  0.09%. Nitrogen 0.57%  $\pm$  0.02%).

3.3.4 DNA/RNA co-extraction, complementary DNA (cDNA) synthesis and metabarcoding

Particulate material was removed by scraping the particle mass off the mesh with a sterile scalpel and the remaining mesh was vortexed in 1.4 mL of DNA/RNA shield to dislodge as much trapped material as possible. Resulting mesh liquid and particulate material were combined before starting extractions. DNA and RNA was co-extracted from samples using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, USA) following manufacturer's instructions and quantified using a Qubit flurorometer (Invitrogen) with broad range and high sensitivity assays (Thermo-Fisher) as appropriate. Complementary DNA (cDNA) was synthesised using the SuperScript III (Invitrogen) reverse transcription protocol and random hexamer primers. The 18S V9 region was amplified using the PCR primers 1391F (Lane, Lane 1991)) and EukB (Medlin, (Medlin et al., 1988)) and sequenced on the Illumina MiSeq platform.

Sequences for DNA and cDNA (referred to as RNA from here onwards) were processed using the DADA2 pipeline to determine amplicon sequence variants (ASVs) collectively (Callahan et al., 2016)in R Studio (R Core Team, 2019). Demultiplexed reads were filtered and trimmed to remove primers and low-quality sequences. Paired end reads were merged to obtain full denoised sequences. Chimeric sequences were removed, before taxonomy was assigned using the PR2 database (release 4.140) (Guillou et al., 2013). Non-eukaryote, chloroplasts and mitochondria sequences were removed. ASV table, taxonomic assignment and environmental metadata were combined into a phyloseq object using the *phyloseq* package (McMurdie and Holmes, 2013) and sequences were rarefied to 13,821 reads before further analysis.

3.3.5 Data processing and statistical analysis

Differences in eukaryotic composition between samples (beta diversity) were calculated using a Bray-Curtis dissimilarity matrix and visualised through non-metric multidimensional scaling (NMDS) ordination. Permutational multivariate analysis of variance (PERMANOVA) was used to test the effect of station, depth, and nucleic acid type (DNA or RNA) on community structure using the *adonis* function in the R package *vegan* (Oksanen et al., 2015).

To explore the potential activity of microbial eukaryotes across the water column, we calculated RNA: DNA ratios at the ASV level for individual microbial eukaryote and microbial metazoan taxa separately. As a proxy for *in situ* potential activity 18S rRNA:18S rRNA gene ratios were calculated by dividing RNA read counts by corresponding DNA read count for ASVs. For each ASV, if the RNA read count was equal to or greater than 1, but the DNA read count was 0, 1 was added to the DNA read count prior to calculating (Allen et al., 2023).

To further examine the differences of activity throughout the water column, Bray Curtis dissimilarity was calculated between reciprocal DNA and RNA for a given sample and compared against depth within a linear regression model.

#### 3.4 Results

As expected, POC concentrations were highest in the surface and attenuated with depth to 400 m (Figure 3.2) and showed spatial and temporal variability. All samples showed a subsurface peak in concentrations between ~ 50 -70 m; yet Page | 73

Station TN showed an additional peak at ~ 130 m at TN 2 (Figure 3.2). POC concentrations at TN declined to similar concentrations found at station OOI and TS at deeper depths and also declined by later visits to TN. Fluorescence, as a proxy for chlorophyll a, mirrored the spatial and temporal dynamics of POC.



Figure 3. 2 Particulate Organic Carbon (POC) concentrations (top axis, bars) and fluorescence (bottom axis, points) measured at the three stations, OOI (red), TN (green), and TS (blue) across depth. POC Data measured by Sabena Blackbird and provided by Fred Le Moigne. Fluorescence data supplied from ship CTD and processed by Hugh Venables.

Comparison of composition of DNA and RNA ASVs by NMDS revealed distinct clustering for both microbial eukaryotes (Figure 3.3A) and metazoans (Figure 3.3B) by station (PERMANOVA p<0.05), except for the first occupation of the TN station (TN 1) where the microbial eukaryote ASVs clustered within the OOI station occupations. Depth was observed to be a strong variable structuring the ASV composition with ASV composition in the surface clustering



Figure 3. 3 Non-metric multidimensional scaling (NMDS) plot A) microbial eukaryote and B) metazoan community structure based on Bray-Curtis dissimilarity. Colours represent occupations at stations, depths are labelled, triangles represent DNA samples and circles represent RNA. Hulls grouped by station for visual aid and not statisitcally calculated.

together, and deeper ASV composition, particularly 400 m, clustering together across all stations (PERMANOVA p<0.05).

Overall, diatoms (Bacillariophyta) dominated TN and TS in terms of relative abundance of ASVs in comparison to OOI (Figure 3.4). Diatoms contributed to a large proportion of the diversity observed and were abundant throughout the water column at TS and to a lesser extent TN. Major ASVs contributing to diatom diversity included the chain forming diatom groups *Chaetoceros* and *Pseudo-nitzschia,* and the pennate diatom group *Fragilariopsis*. Dinoflagellates (Dinophyceae) were also an abundant taxa at station TN and TS, and were especially diverse in the surface waters at TN in comparison to TS (Figure 3.4). Dominant dinoflagellate ASVs included *Protoperidinium, Prorocentrum* and *Gyrodinium*. At TS, only *Gyrodinium* was observed throughout the water column (Figure 3.4).

For rhizarian taxa, radiolarians were the more dominant taxa in terms of relative abundance at OOI station occupations, and the first occupation of the TN station (TN 1). Specifically, a number of ASVs belonging to the colonial polycystine radiolarian *Collozoum amoeboides* were observed, but were dominated by the abundance of one ASV (*C. amoeboides ASV 1;* Figure 3.4) throughout the water column. At TS, additional rhizarian ASVs were observed including the acatharian radiolarian *Litholophus*, and the Phaeodarian *Protocystis.* MArine STramenopile group 3 (MAST-3) were also detected at OOI, across the water column. Fungi were not a major component of the eukaryotic diversity observed grouping to less than 0.01% abundance.

For metazoan taxa, copepod and largely unassigned Maxillopoda were the dominant taxa across all stations and throughout the water column.

Dominant copepod ASVs were assigned to *Neocalanus, Pseudocalanus* and *Oithona* (Figure 3.5). Additionally, at TS, *Oithona* ASV 2 and an unassigned *Metridia sp.* ASV (ASV 5) were dominant throughout the water column, which appeared to be replaced by *Subeucalanus subtenuis* at station OOI and to a lesser extent at TN (TN 1). Other metazoan groups including Cnidaria and Annelida showed patchier distributions across the water column and stations.

Analysis of relative activity at the ASV level using 18S rRNA: 18S rRNA ratios highlighted different relative activity patterns for taxonomic groups (Figure 3.4, 3.5). Diatoms were largely active and stable in their abundance throughout the water column across all stations, including *Fragilariopsis* and most *Chaetoceros* ASVs. However, one *Chaetoceros* ASV (*C. debilis* 2 ASV 7) showed reducing activity and increased abundance with depth at station TN and TS (Figure 3.4). At TN *Protoperidinium and Prorocentrum* ASVs were active in the surface and at TS, *Gyrodinium fusiforme* showed decreasing activity with increasing abundance, but decreased abundance at 400 m (Figure 3.4). *Collozoum amoeboides* ASV 1, showed increasing activity and decreasing abundance at depth at OOI and TN1, whilst at TS, *C. amoeboides* ASV 1 was detected only at 400 m and in low abundance (Figure 3.4). Although there were only a few MArine STramenopile (MAST) ASVs greater than 1% abundant detected, MAST-3 group ASVs (ASVs 16, 19 and 54) were very active in the surface, with ASV 16 active throughout the water column, at OOI (Figure 3.4).

Many Copepod and unassigned Maxillopoda ASVs were active in the surface, and showed greater activity at station TN, than the other stations. Many ASVs were stable in their abundance throughout the water column; however, at station TN and TS *Oithona sp.* ASV 2 showed decreasing activity and increased

abundance at depth, and at TS alone Metridia sp. ASV 5 showed decreasing

activity with increasing abundance to 200 m but a drop in abundance at 400 m.

## Chapter 3: DNA and RNA based assessment of the biological carbon pump in the Southern Ocean



Figure 3. 4 Relative activity of microbial eukaryote taxa by amplicon sequence variants (ASVs). Relative abundance for DNA represented by size of bubble, and 18S rRNA:18S rRNA gene represented by gradient colour. ASVs grouped at class (grey boxes) and phyla (colour) level.

## Chapter 3: DNA and RNA based assessment of the biological carbon pump in the Southern Ocean



Figure 3. 5 Relative activity of metazoan taxa by amplicon sequence variants (ASVs). Relative abundance for DNA represented by size of bubble, and 18S rRNA: 18S rRNA gene represented by gradient colour. ASVs grouped at class (grey boxes) and phyla (colour) level.

Comparison of the Bray-Curtis dissimilarity between reciprocal DNA and RNA based diversity of samples revealed increasing dissimilarity with increasing depth (Figure 3.4A, Figure 3.4B) and was significant for both microbial eukaryotes and metazoans (ANOVA P>0.05). At 400 m Bray-Curtis dissimilarity between reciprocal DNA and RNA reduced (Figure 3.4B), although this was not statistically significant.



## Chapter 3: DNA and RNA based assessment of the biological carbon pump in the Southern Ocean

Figure 3. 6 (A) Depth-averaged Bray-Curtis dissimilarity values between DNA and RNA samples. Line represents the median value and dot represents outliers. (B) Scatterplot showing the relationship between Bray-Curtis dissimilarity and depth across all stations. Dashed line represents where mesopelagic is defined in this study (180 m and below). Top panels – all, middle panels – microbial eukaryotes only, bottom panels – metazoans only. Blue line represents linear regression.

#### 3.5 Discussion

Previous studies have used DNA-based approaches to demonstrate microbial eukaryote diversity including its association with POC export (Cordier et al., 2022); however, they do not provide an accurate reflection of the activity of organisms identified in association with export. Additionally, our knowledge on microbial eukaryotes especially in the mesopelagic is limited (Caron, 2017; Edgcomb, 2016). This is at odds with the evidence that most POC flux attenuation occurs in the mesopelagic (Martin et al., 1987) and respiration of carbon in the 'dark ocean' contributes a third of biological production of CO<sub>2</sub> (Arístegui et al., 2005, 2003; Del Giorgio and Duarte, 2002). Using a DNA and RNA-based assessment of eukaryotic taxa across horizontal and vertical gradients of POC concentrations, we suggest a more linked contribution of microbial eukaryote activity to POC attenuation in the mesopelagic, which could be used to improve parameterisations within biogeochemical models to close carbon budgets.

RNA/DNA ratios have previously been used as a proxy for protein synthesis, highlighting the potential activity of microbial eukaryotes and metazoans spanning the water column (Giner et al., 2020; Hu et al., 2016; Massana et al., 2015; Xu et al., 2017). However, previous estimates have not interpreted data using an ASV level resolution, which may conceal underlying dynamics. The enriched and highest relative activity for many taxa was in the surface, consistent with the traditional view of the biological carbon pump (Buesseler, 1998). Although, species composition varied between stations including at a higher taxonomic resolution (species and ASV level), which likely influenced POC concentration dynamics reported here (Le Moigne et al., 2015) e.g. highest concentrations of POC associated with diatom heavy communities (Figure 3.2,3.3a). This included diatoms, dinoflagellates, and copepods but also additional taxa not yet part of the canonical view of the biological carbon pump, including MAST-3 group and colonial polycystine radiolarian, but previously recorded (Hu et al., 2016; Xu et al., 2017). This suggests here that DNA sampling was representative of the active community in the surface.

Entering into the mesopelagic, taxa began to display contrasting patterns of activities to their surface counterparts. Dinoflagellates and copepods showed decreased activity at depth. Heterotrophic dinoflagellates, including *Gyrodinium* have been found in suspended and sinking particle fractions (Duret et al., 2019a) and with decreasing activity with depth (Giner et al., 2020), here this might show their death contributing to sinking material. However, dinoflagellates have also shown increased activity deeper in the mesopelagic and bathypelagic (Xu et al., 2017) although this was not captured here. Copepods in the Southern Ocean undergo dormancy at depth (Atkinson, 1998) and copepods such as *Neocalanus*, detected in this study, undergo ontogenetic migration at depth, showing significantly reduced RNA:DNA ratios (Kobari et al., 2013). In this case, as average ratios are specific to life stage and species (Kobari et al., 2013) we cannot confidently assume the true activity of copepods here at depth.

Diatoms largely remained active in the mesopelagic as previously reported (Agusti et al., 2015; Agustí et al., 2020; Giner et al., 2020; Xu et al., 2017) suggesting their incorporation into fast sinking faecal pellets (Agusti et al., 2015), supported by the activity of copepods in the surface, which likely grazed on diatoms and deposited fast sinking faecal pellets (Dagg, 1993; Kobari et al.,

2010). Of the diatoms, ASVs within the genus *Chaetoceros* displayed different dynamics, suggesting the merit of the incorporation of finer resolution when considering the contribution of taxa, especially diatoms, towards the carbon flux (Tréguer et al., 2018). C. debilis 2 showed decreasing activity and increased abundance at depth, coupled with an increased POC concentration signature at ~ 130 m on the second and third occupation of TN (TN2,TN3) which disappeared at the next sampling point at TN (TN4) (Figure 3.2, Appendix Figure 1.1). This was coupled with an increased fluorescence signature (Figure 3.2) supporting photosynthetic organisms where responsible for the POC signature. Chaetoceros species have been previously identified as a key 'Csinker' diatom (Tréguer et al., 2018), through their bloom formation and mass mortality events that result in fast sinking aggregates (Assmy et al., 2013), which would likely be represented by DNA material or lower rRNA ratio (Blazewicz et al., 2013). Additionally, many Chaetoceros species can produce highly silicified fast sinking resting spores (Crosta et al., 1997), characterised by reduced rRNA synthesis (Pelusi et al., 2023), and can accumulate at depth. contributing substantially to carbon export (Rembauville et al., 2015). However, efforts to explore differences between rRNA ratio signatures for resting spores and dying cells, in addition to, living cells (including those possibly incorporated into faecal pellets), across a wider diversity of diatoms should be determined to unravel the dynamics displayed here further.

At station OOI, MAST-3 as a group were also active throughout the water column, and had the highest activity ratios (~10), which has been previously observed for MAST groups (Hu et al., 2016). MAST groups represent small heterotrophic flagellates, with the MAST-3 group or Solenicolida (Gómez et al., 2011) including the parasitic taxa *Solenicola setigera* (Buck and Bentham, 1998; Gómez et al., 2011). Yet, as an understudied group, the role of MAST groups in the BCP remains largely unknown. Alternative taxonomic assignment using the Tara Oceans Eukaryote V9 database (De Vargas et al., 2015) identified a large proportion of MAST-3 ASVs as S. *setigera* or the *Solenicola* genus (Appendix Table 1.2). These ASVs were previously identified only to 'Order level' taxonomy using the PR2 database, highlighting the unequal detail for different taxonomic groups (Kase et al., 2020). *Solenicola setigera*, is a known parasite of the diatom *Leptocylindricus mediterraneus* (Gómez et al., 2011) Although *L. mediterraneus* was not detected here, it is likely that as parasites can be broad range (Laundon et al., 2021), there is potential for the MAST-3 group here to play a role as a parasite on additional diatom taxa, or as a colonial nanoflagellate grazer (Gómez, 2007).

The polycystine collodarian radiolarian *Collozoum amoeboides* ASV 1, showed increasing abundance and activity with depth at OOI in comparison to the surface, which may demonstrate their role as mesopelagic grazers (Figure 3.4). Many radiolarians are mixotrophs (Decelle et al., 2012; Flynn et al., 2013), and are globally abundant in the mesopelagic (Biard et al., 2016). Collodarian radiolarians alone may represent a substantial fraction of the global carbon standing stock in the surface ocean (Biard et al., 2016; Guidi, 2016), and are major contributors to organic matter transfer due to their large cell size (Suzuki and Aita, 2011) and ballasting mineral exoskeletons (Biard et al., 2018). Other unassigned collodarian ASVs showed only some variation in activity and abundance but largely showed lower levels of activity, suggesting they may be contributing to sinking POM material. DNA sequencing surveys alone has

struggled to resolve whether radiolarians found in the mesopelagic are metabolically active or non-viable cellular material sinking from the euphotic (Biard et al., 2016; Preston et al., 2020). Here we provide additional evidence that some radiolarian taxa are active at depth (Hu et al., 2016), adding to the shifting paradigm that radiolarian play an important role in the BCP and wider geochemical cycling (Biard et al., 2016; Lampitt et al., 2009).

Further exploration of the DNA-RNA species composition relationship with depth, using Bray-Curtis dissimilarity, displayed the increasing dissimilarity of DNA-RNA communities with depth which echoed the attenuation of POC (Figure 3.2, Figure 3.6B: all panels). Therefore, we hypothesise that in the surface DNA provides a more accurate representation of the active community, achieved by congruence to RNA in the surface. At depth, we suggest dissimilarity occurs as RNA is representative of the active community, ASVs which are incorporated into fast sinking material or involved with biomass degradation and remineralisation. However, DNA accounts largely for metabolically inactive cells and debris, in addition to active cells, contributing to sinking biomass and facilitating carbon export to the deep sea. As DNA and RNA in the mid-mesopelagic (400 m) become more similar again, some taxa show reduced abundance and activity. Here we propose that declining POC concentrations which diminish with grazing, remineralisation and fragmentation, results in reduced activity of associated grazers, mirroring the constant reached in the attenuation of POC, as commonly described in the Martin Curve (Martin et al., 1987).

Examining the diversity and activity of microbial eukaryotes is crucial in understanding the mechanism and efficiency behind the BCP. However, many
microbial eukaryotes are still missing in detail from biogeochemical cycles, and for organisms which are included, knowledge on diversity, activity and functional type is lacking but should be included to better constrain current day carbon budgets (Tréguer et al., 2018). In addition, monitoring of diversity and activity may be of ecological significance when monitoring community changes, when combined with stoichiometric based assessments of taxa. This will help to improve model parameterisations, and enable us to predict how the BCP may respond with climate change in the Southern Ocean (Constable et al., 2014) and its subsequent impact on global carbon cycling and climate regulation.

### 3.6 Conclusion

Here we show that use of DNA-RNA based metabarcoding approach can provide a better linked view of the diversity and potential activity of microbial eukaryotes and metazoans associated with the sinking POC flux over spatial scales in the Southern Ocean. We highlight the activity of universally associated taxa to the sinking POC flux including diatoms, dinoflagellates and copepods, as well as, MAST-3 and polycystine radiolarians, groups which have only recently been suggested to play a role in carbon export. By calculating 18S rRNA ratios we have been able to provide additional evidence for the latter groups differing contribution to the sinking POC flux over spatial scales in the Southern Ocean. We show increasing dissimilarity of DNA and RNA eukaryotic assemblages across horizontal and vertical gradients of POC. Using RNA:DNA ratios we attribute this in part, to differences in diversity and activity of eukaryotic assemblages across POC gradients, which enables the identification of ecologically significant taxa involved with POC production and remineralisation in the understudied mesopelagic and BCP. Therefore,

Page | 88

consideration of opening the 'black box' of microbial eukaryotes within

biogeochemical modelling must be considered in order to best predict changes

in the Southern and wider ocean with climate change.

# Chapter 4: Mapping the 'particlescape': an assessment of particle characteristics and fungal communities at the single particle level in the coastal ocean.

## Chapter 4: Mapping the 'particlescape': an assessment of particle characteristics and fungal communities at the single particle level in the coastal ocean.

## 4.1 Abstract

The coastal ocean is a boundary between terrestrial and oceanic systems, is highly productive, and experiences great variability owing to the contribution of allochthonous and autochthonous particulate organic material which fluctuates on seasonal and temporal scales. Particulate organic matter is a hotspot for microbial activity. Despite the heterogeneity that exists within particle attached (PA) communities based on particle characteristics, particle life history, and environmental gradients, limited work has been conducted at the single particle level and concerning diversity and activity of PA eukaryotes including fungi. Here we developed a pipeline to isolate, image and characterise the 'particlescape' and PA eukaryote communities in the coastal environment at the single particle level. We show that particle source, type, size, complexity and PA eukaryotic microbial communities (e.g. the particlescape) are extremely variable across spatial and temporal scales. We hypothesise that variability is likely accounted for by the source, colonisation and life history of particles and demonstrates the importance of exploring the holistic particlescape especially in the coastal ocean. We also show that fungi are the dominant eukaryote group on single particles in the coastal environment and that species richness correlates with the mapped particlescape including particulate organic carbon and particle complexity. Fungi on particles include marine and non-marine fungi,

Page | 91

and we provide the first potential evidence of the activity of PA non-marine fungi into coastal environments which we propose are transported by allochthonous particles during high rainfall and river flow. Despite reported variability, many studies use bulk-based particle approaches to assess particle characteristics and associated communities against environmental parameters. Observations we report here suggest this likely has ramifications for how we interpret the dynamics of particulate organic carbon and its associated microbial activity in the marine environment, now and in the future.

### 4.2 Introduction

The coastal ocean is a boundary between terrestrial and oceanic systems including the transfer of externally supplied organic matter and nutrients, which, in part, shapes the high productivity of coastal environments (Säwström et al., 2016). The resulting impact of terrestrial-oceanic connectivity is the accumulation of a range of particulate organic matter (POM) composed of different sources of organic carbon making the coastal ocean a dynamic component of the global carbon cycle (Bauer et al., 2013; Smith and Hollibaugh, 1993). With increasing anthropogenic perturbation, the coastal ocean also contributes to the net annual growth of long term open ocean carbon storage (Regnier et al., 2013). POM in the coastal environment includes allochthonous, and marine derived autochthonous material (Watanabe and Kuwae, 2015). Coastal POM can include particulate leaf litter, wood (Solomon et al., 2015), pollen (Liénart et al., 2022), phytoplankton (Nakakuni et al., 2022), seagrass (Liénart et al., 2017), macroalgae (Krause-Jensen and Duarte, 2016), zooplankton carcasses, moults and faecal pellets (Stukel et al., 2013), and active, dormant and dead microbial cells bound in extracellular polymeric matrices (Alldredge and Silver, 1988; Crump et al., 1998; Sugimoto et al., 2021). The ratio between autochthonous and allochthonous POM in the coastal ocean can vary on seasonal and inter-annual scales based on physical and biological forcing (Auladell et al., 2023; Silori et al., 2022) which likely impacts resulting microbial activity (Poff et al., 2021).

Particles act as hotspots for microbial activity and organic remineralisation in aquatic ecosystems (Bižić-Ionescu et al., 2015). Microbial

colonisation and degradation plays a role in cycling carbon and other nutrients impacting ecosystem functioning (Alldredge and Silver, 1988; Azam and Long, 2001). Particle-attached (PA) microorganisms and functional diversity vary across different particles and may be structured by particle life history (Datta et al., 2016), and particle composition (Bunse et al., 2021; Datta et al., 2016; López-Pérez et al., 2016; Suominen et al., 2021) for model particles. Particle size (Lyons et al., 2010; Mestre et al., 2018; Orsi et al., 2015; Salazar et al., 2015), sinking speed (Duret et al., 2019b) and environmental gradients such as seasonality (Poff et al., 2021) and proximity to shore (Valencia et al., 2022) also influence community structure and function on natural particles. Furthermore, limited single particle based approaches have identified the variances between eukaryotic composition (Durkin et al., 2022; Lundgreen et al., 2019). PA bacterial community diversity of natural single particles of the same source, age and environment (Bižic-Ionescu et al., 2018; Zäncker et al., 2019) and PA functional diversity with particle size (Lyons et al., 2010). Despite reported variability, many studies use bulk-based particle approaches to assess associated communities and heterogeneity of particle characteristics have been largely ignored, which likely has ramifications for how we interpret microbial remineralisation and hinders our understanding of the carbon budget in the marine environment (Baumas and Bizic, 2023).

Fungi are widespread members of microbial eukaryotes in coastal environments (Chrismas et al., 2023; Taylor and Cunliffe, 2016a) and are able to degrade phytoplankton (Cunliffe et al., 2017; Gutiérrez et al., 2011a) and macroalgae derived organic material (Pilgaard et al., 2019). Global

assessments of marine fungi highlight the dominance of Ascomycota, Basidiomycota and Chytridiomycota (Hassett et al., 2019) and fungi in coastal marine ecosystems can include both marine and non-marine taxa suggesting land-sea exchange regularly takes place potentially via particles as a mechanism for their transport (Chrismas et al., 2023). Fungi are shown to dominate biomass on particles in the open ocean (Bochdansky et al., 2017), yet the contribution of fungi to POM-associated processes is poorly understood which limits our understanding of their contribution to the carbon cycle, broader biogeochemical cycling and wider food web interactions (Peng et al, *in review*).

To address limited knowledge on the variation of particle characteristics over space, time, and introspectively within particle types and how this influences the active particle attached eukaryote community we developed a pipeline to isolate, image and characterise the 'particlescape' in the coastal environment. Previous studies have used the term 'particlescape' to define natural variation in polysaccharide composition on model particles (Bunse et al., 2021) Here, we build on Bunse et al, (2021) by using the term 'particlescape' to describe both the macro and micro-scale characteristics driving particle variation within the landscape of the coastal ocean.

We collected natural single particles on a weekly basis, spanning winter and spring months from Plymouth Sound and at the long term sampling site L4 Station in the Western English Channel off Plymouth (UK) (Figure 4.1). Coastal environments (Beucher et al., 2004; Carstensen et al., 2015; Säwström et al., 2016)) and specifically at L4 Station (Smyth et al., 2010) exhibit varying degrees of productivity resulting in large phytoplankton blooms in the spring period. Since POC concentration generally mirrors productivity (Bol et al., 2018), sampling over the winter and spring period enabled us to capture this. Plymouth Sound sits within the Tamar Estuary catchment area and L4 station is periodically influenced by terrestrial and estuarine influences from surrounding rivers including the Tamar (Smyth et al., 2015, 2010; Uncles et al., 2015a). This allows the assessment of temporal and spatial variations in response to environmental parameters to understand potential drivers of active fungal diversity on particles. Additionally, we used stable isotope ratios ( $\delta$ 13C) of carbon ( $^{13}C/^{12}C$ ) to determine the contribution of terrestrial and marine POC to the coastal ecosystem as a potential driver of particle attached fungal diversity.

Using 18S rRNA sequencing from single particles we describe active fungal diversity across the particlescape of the coastal ecosystem in comparison to bulk sampling of microbial communities. Here our goal was to highlight a need to consider heterogeneity of the particlescape and evidence the active diversity of fungi on organic matter in coastal environments both knowledge gaps which likely influences our current and future understanding of the oceans role in carbon cycling. We also wanted to determine if non-marine fungi found in the coastal environment were active and if particles represent a means of transport from terrestrial environments.

### 4.3 Methods

### 4.3.1 Seawater sampling

Sampling took place on board the research vessel *MBA Sepia* between February and April 2021 (9 weeks) on a weekly basis in coastal waters surrounding Plymouth, UK. The exception to this was in February where there

Page | 96

were 2 sampling dates within the same week. To assess the variability of particles in the coastal ocean and their associated fungal communities, sampling was conducted in Plymouth Sound and at the long-term sampling site L4 station (Figure 4.1). 20 L of water was collected from 5 m depth from both sites using the *MBA Sepia*'s underway system and returned to the lab for processing. Water was also collected to assess bulk community assemblages, elemental analysis (particulate organic carbon (POC)) and bulk  $\delta^{13}$ C analysis. For bulk community assemblages, four replicates of 1 L of water from each of the sampling sites was filtered on a 0.2 µm cellulose filters membrane for DNA sampling, and four replicates of 1 L of water for elemental and bulk stable isotope analysis (combined) was filtered on one GF/F membrane filter. Filters were frozen at -20°C until analysis.



Figure 4. 1 Map of study sites located in the Western English Channel. Plymouth Sound in green, L4 station in red.

## 4.3.2 Environmental parameters

Temperature and salinity data were recorded from a Midas 606 CTD profiler (Valeport) immersed in water from the underway on *MBA Sepia*. Nutrient concentrations and phytoplankton count data was available for L4 Station only, at a weekly resolution and collected as part of the WCO sampling conducted by Plymouth Marine Laboratory (PML). Weekly measurements of nitrite, nitratenitrite, phosphate, silicate and ammonium concentrations for the sampling period was downloaded from the WCO website

(https://www.westernchannelobservatory.org.uk). Nutrient concentrations were provided in duplicate for 0 and 10 m from the WCO website and averaged to obtain an estimated value for 5 m.

Microscopic analysis of seawater sampled at 10 m depth provided count data for phytoplankton groups and biomass values were calculated using the Menden-Deuer and Lessard formula (Menden-Deuer and Lessard, 2000) at PML. Biomass values for phytoplankton groups were estimated by multiplying the biomass value assigned to the species within each phytoplankton group by the count of each species and summing the values. River flow data (Gauged Daily Flow) for the Tamar at Gunnislake was downloaded from the National River Flow Archive (NRFA) website (https://nrfa.ceh.ac.uk). Rainfall data was downloaded from the Plymouth Live Weather Station website (http://www.bearsbythesea.co.uk). For the above environmental parameters, where sampling dates did not match exactly, the nearest dates were found and used instead. Filters collected for elemental and stable isotope ratio ( $\delta^{13}$ C) analysis were freeze-dried for 72 hours and submitted to OEA Laboratories Ltd (Exeter, UK) for analysis. Samples were run on a continuous flow isotope ratio mass spectrometer interfaced with a configured elemental analyser. Carbon (C) isotopes ( $^{13}$ C/ $^{12}$ C) were calibrated to international reference standards and are presented using  $\delta$  notation (units per mil [ $^{0}$ / $_{00}$ ]).

4.3.3 Single particle picking

In order to concentrate particles that could be individually picked, 20 L of water was split between two 10 L carboys that were left to drain at a rate of 100 mL per minute through a 30  $\mu$ m nylon mesh (Millipore) which took ~2-2.5 hours. After the carboys had finished draining over the 30  $\mu$ m mesh, ~1 L of seawater remained that had concentrated particles below the tap line of the carboy. Two fractions of particles were collected, particles collected on the mesh and particles collected remaining in the bottle, to allow ease for picking. Meshes were removed from the filter holder and placed in sterile petri-dishes contained 0.2  $\mu$ m filtered seawater from the respective sampling site and the remaining seawater in the bottle was collected in autoclaved Nalgene bottles rinsed three times with 0.2  $\mu$ m filtered seawater. Both were kept at *in situ* temperature whilst waiting to be processed.

For the mesh fraction, meshes were left to soak in 0.2 µm filtered seawater in petri-dishes and then gently agitated to remove loose single particles. For both fractions, particles were identified by eye and light microscopy (Leica Microscope), and individually picked using a combination of a P1000 and a P10 pipette (PIPETMAN, Gilson) with filter tips (Biosphere). Single particles were then imaged on a light microscope (Leica Microscope), and individually preserved with 700  $\mu$ L DNA/RNA Shield (Zymo) in 1.5 mL tubes (Eppendorf) and stored at – 80 °C.

4.3.4 Particle characterisation and image analysis.

Particles were characterised into major groups using microscope images and further classified as particle types based on similarity in morphology and/or composition (see Appendix Key 1). Using ImageJ, particle metrics were measured for all single particles including longest length, surface area, Red, Green, Blue (RGB) mean value and RGB standard deviation value. Here we used RGB mean values as an indicator for particle complexity, which we hypothesise is a predictor of substrate complexity and therefore niche availability (Bunse et al., 2021).

4.3.5 DNA extractions for bulk community

For the bulk planktonic community DNA was extracted from filters using the ZymoBIOMICS DNA Miniprep kit (Zymo Research, USA) as per manufacturer's instructions with an additional filter cutting and mechanical bead beating step. The V9 region of the 18S rRNA gene was amplified using the PCR primers 1391F (Lane 1991) and EukB (Medlin et al, 1988), followed by sequencing on Illumina MiSeq Platform.

4.3.6 RNA extraction, complementary DNA (cDNA) and whole genome amplification of single particles.

9 particles were selected for RNA sequencing from both Plymouth Sound and L4 Station across the sampling period (Appendix Figure 1.1). Particles were thawed and centrifuged at x 12, 000 g to ensure the particle was at the bottom of the 1.5 mL tube. ~600  $\mu$ L of DNA/RNA Shield was removed to leave ~100  $\mu$ L Page | 100

of DNA/RNA Shield and the particle behind. An equal volume of RNA lysis buffer was added and the particle-lysis buffer mix was vortexed twice for 30 seconds to encourage mechanical lysis. RNA was then extracted using the ZymoBIOMICS Quick-RNA Fungal/Bacterial Microprep kit (Zymo Research, USA) according to manufacturer's instructions. Complementary DNA (cDNA) was produced from RNA using random hexamers and whole genome amplification was done to amplify synthesised cDNA using the QIAseq FX single cell RNA library kit (Qiagen) following the manufacturer's instructions. The V9 region of the 18S rRNA fragment was amplified using the PCR primers 1391F (Lane 1991) and EukB (Medlin et al, 1988), followed by sequencing on Illumina MiSeq Platform.

### 4.3.3 Metabarcoding

Sequences were processed using the DADA2 pipeline to determine amplicon sequence variants (ASVs) (Callahan et al., 2016) in R Studio (R Core Team, 2019) (separately for both bulk and single particle metabarcoding). Demultiplexed reads were filtered and trimmed to remove primers and low quality sequences. Paired ends were merged to obtain full denoised sequences. Chimeric sequences were removed before taxonomy was assigned the PR2 database (release 4.140) (Guillou et al., 2013). Non-eukaryote, chloroplast and mitochondria sequences were removed. ASV tables, taxonomic assignments and environmental metadata were combined into a phyloseq object using the *phyloseq* (McMurdie and Holmes, 2013) package for bulk and single particle metabarcoding independently. Bulk sequences were rarefied to 10,716 reads per sample before further analysis. Single particle sequences

were not rarefied to avoid the loss of diversity given the low biomass nature of the samples.

### 4.3.4 Data processing and statistical analysis

All statistical analyses were carried out in R studio (R Core Team, 2019). Particle surface area and RGB mean best showed the variation of particle characteristics and so are the only characteristics considered from this point onwards (longest length and RGB SD found in Appendix Figure 1.2). Particle surface area was log transformed to provide normally distributed data as confirmed by a general residual test (uniformity and dispersion of residuals against linear model) and a two-way analysis of variance (ANOVA) with Tukey's HSD was used to determine the effect of time (winter vs spring) and location on particle surface area at the particle group and particle type level.

For particle complexity (RGB mean), data was non-normally distributed and transformation did not improve distribution. Therefore, an ANOVA was used to determine if the linear model for particle complexity was significantly different to a null linear model. As this was significantly different (p<0.05), we chose to use an ANOVA to determine if particle complexity was different across particle groups and particle types, and exercise caution on interpreting results. Additionally, a linear discriminant analysis (LDA) with leave-one-out cross validation was used to evaluate the accuracy of the LDA model for predicting particle groups and particle types based on particle complexity values.

Species richness was calculated for single particles by determining the number of ASVs per single particle and Spearman's rank correlation coefficient was used to determine a correlation between particle characteristics (surface area and complexity) and environmental parameters (salinity, rainfall, river flow, ammonia, nitrite, nitrate-nitrite, silicate, phosphate, temperature, POC, stable isotope ratios). Species richness was used to limit the impact of PCR and WGA associated bias (Knight et al., 2018).

To investigate the occurrence of single particle fungal ASVs in the bulk eukaryote dataset a local BLAST database including only fungal ASVs from the bulk sequencing data was formed using Geneious prime (Biomatters Ltd) and single particle fungal sequences were BLAST against it. Percentage pairwise identity cut off was 100% and query coverage cut off was >95%. To assign trophic mode for abundant fungal taxa detected on single particles was predicted by searching either species or genus names against the FunGuild database (Nguyen et al., 2016).

## 4.4 Results

4.4.1 Particle characterisation

In total, 426 single particles were collected across the sampling period and two stations combined, with 261 and 165 collected from Plymouth Sound and L4 Station respectively (Appendix Table 1.3).

Visual classification of particles resulted in 3 major groups: "zooplanktonderived", "detritus-like" and "aggregate-like". These were further characterised into 8 particle types. For "zooplankton-derived" particles: zooplankton carcasses, zooplankton moults and zooplankton faecal pellets; for "detrituslike": fragmented detritus and detritus; and for "aggregate-like": aggregates detrital aggregates and bloom aggregates (Figure 4.2A)



Figure 4.2 A) Representative images of particle major groups and particle types at L4 Station and Plymouth Sound combined over sampling period February-April 2021 (B) relative abundance, (C) surface area (mm2), (D) Mean Red Green Blue (RGB) of single p particles sampled. L4 station (circle) and Plymouth Sound (triangle). . "n=" represents number of particles in total found on each sampling date.(E) Key identifiable features of Aggregate-like group particles including (i) aggregate particles, (ii) dense aggregates and (iii) bloom particles. Arrows marked with 'p' highlight phytoplankton cells, 'c' highlight Chaetoceros sp. cells specifically and red circle and 'd' highlight dark, dense sediment-like particles.

## 4.4.1.1 Zooplankton-Derived

Zooplankton-derived particles were defined by being particles that originated from zooplankton, based on visual observation (Figure 4.2A, top row). Zooplankton carcasses were defined as being dead zooplankton groups (e.g. copepods) and usually complete organisms. Zooplankton moults were defined by their transparent appearance, and whole shed of a zooplanktonic organism or appendages. Faecal pellets, were defined by being long cylindrical shapes, with smooth edges, often rounded ends.

4.4.1.2 Detritus-like

Detritus-like particles were mainly characterised by an amorphous or fragmented structure, as if they were previously part of large piece of material (Figure 4.2A middle row). Fragmented detritus particles had visibly jagged edges, pieces of larger organic matter. Detritus particles had no apparent visible phytoplankton or zooplankton present in the particle, and were amorphous.

## 4.4.1.3 Aggregate-like

Aggregate particles were characterised by clear aggregations of organic matter with phytoplankton cells (p) (Figure 4.2A bottom row; Figure 4.2E i). Dense aggregates were often dark in colour, densely packed aggregates with visible phytoplankton cells (p) and small dark particles (d) present within the structure (Figure 4.2E ii). Bloom aggregates were finer particles, with a gel like consistency characterised by small circular light particles (Figure 4.2E iii, red circles) and the major spring bloom forming diatom *Chaetocerous sp.* (Figure 4.2E iii).

### 4.4.2 Temporal and spatial resolution of particle characteristics

### 4.4.2.1 Particle abundance

Winter sampling (February and early March) was dominated by detritus-like particles including detritus and fragmented detritus at both sampling sites (Figure 4.2B). In March, as aggregate-like particles became a more common feature, differences in particle composition became more apparent. Plymouth Sound was dominated by dense aggregates, detritus-like particles and to a lesser extent aggregates, whilst L4 station was more variable in its particle composition. Initially in March, L4 station was dominated by aggregates and to a lesser extent dense aggregates and detritus particles, which was then succeeded by the appearance of bloom aggregates heading into April. Bloom aggregates did not appear until late April in Plymouth Sound. Zooplanktonderived particles were a minimal and irregular component of the particles collected over the sampling period, and carcasses were not found at the Plymouth Sound sampling station.

### 4.4.2.2 Particle Size

Collected particles exhibited a large variation in their surface area over time and space, however at the group and type level some patterns for particle surface area emerged. At the particle group level, there was no overall effect of site on surface area (Tukey's HSD p>0.05) but aggregate-like particles were the largest particle group (ANOVA p<0.05). Aggregate-like particles on the first sampling day in February were significantly smaller than for the rest of the sampling period. In contrast, aggregate-like particles sampled within the same week (3 days later), were on average the largest particles sampled and in comparison to

other aggregate-like particles sampled across the whole sampling period (ANOVA p<0.05), which showed no significant differences between them (Tukey's HSD p>0.05). Detritus-like particles showed no difference in surface area over time (Tukey's HSD p>0.05).

Within particle types, for aggregate-like particles, dense and bloom aggregates were significantly larger than aggregates (ANOVA p<0.05). Aggregates were larger overall in Plymouth Sound, whilst detritus and fragmented detritus particles were larger at L4 station (Tukey's HSD p<0.05). Dense aggregates were largest in winter (Tukey's HSD p<0.05) than in spring for Plymouth Sound, and although at L4 Station dense aggregates appeared more variable in their size in winter than in spring, there was no significant significance detected (Tukey's HSD p>0.05). For detritus-like particles, fragmented detritus particles were larger than detritus particles (ANOVA P p<0.05). Time was not an effect for the size of detritus particles (Tukey's HSD p>0.05), fragmented detritus (Tukey's HSD p>0.05), aggregates (Tukey's HSD p>0.05) and bloom aggregates (Tukey's HSD p>0.05). For zooplankton-derived particles there was no significant differences detected between the three types (Tukey's HSD p>0.05).

4.4.2.3 Particle Complexity (RGB mean)

Since we were more interested in comparing the complexity between particle types we only compared the complexity values between particle groups and particle types (Figure 4.2D). Particle complexity displayed clear observational differences across particle groups, although aggregate-like and zooplanktonderived particles were more complex than detritus-like particles (Tukey's HSD P<0.05). There was no difference in complexity between aggregate-like and zooplankton-derived (Tukey's HSD P>0.05) (Figure 4.2D). For detritus-like particles, fragmented detritus was less complex than detritus (Tukey's HSD P<0.05), whilst for aggregate-like particles, bloom aggregates were more complex than dense aggregates (Tukey's HSD P<0.05), but were both not significantly different from aggregate particle complexity (Tukey's HSD P>0.05).

LDA performed on RGB mean values extracted from the particle images confirmed that the three particle groups were distinct from each other (p<0.05). The first linear discriminant accounted for 88% of the variation and despite nearly a third of detritus-like particles overlapping with aggregate-like particles, the model clearly separated aggregate-like particles from detritus-like particles from each other but not from zooplankton-derived particles, which were generally misidentified by the model. Leave-one-out cross validation provided support for different groups and 80.5% of RGB mean values were correctly classified (Appendix Table 1.4).

At the particle type level, RGB mean values also showed that many particle types were distinct from each other (p<0.05), with the first linear discriminant describing 68.1% of the variation. Exceptions to this were aggregate-like particles, which were not distinct from each other within the group and within zooplankton-derived, zooplankton moults and carcasses were not distinct from each other and zooplankton carcasses were not distinct from faecal pellets (p>0.05). Leave-one-out cross classification correctly classified 52.1% of particle types using the RGB mean values (Appendix Table 1.5). 4.4.3 Carbon stable isotope ratios and particulate organic carbon (POC) concentrations.

To map the broader particlescape, carbon stable isotope ratios and POC concentrations were analysed from bulk water sampling across both stations. In the late winter (February-mid march), Plymouth Sound, was characterised by high POC concentrations and low  $\delta^{13}$ C values (-26‰) with a delayed effect observed at L4 Station which was succeeded by greater  $\delta^{13}$ C values (Figure 4.3). Moving into spring (late March-April), POC concentrations were lower initially but began to increase into April coinciding with increasing diatom cell abundance (Appendix Figure 1.3). Additionally,  $\delta^{13}$ C signatures were highly variable in Plymouth Sound (-26‰ to -15‰) and between stations but entering into April, Plymouth Sound  $\delta^{13}$ C signatures became more aligned with  $\delta^{13}$ C signatures at L4 Station.  $\Delta$ 13C signatures at L4 station were highly variable throughout the sampling period (Figure 4.3B)



Figure 4. 3 Bulk measurements of A) Particulate organic carbon (POC) and delta 13C ( $\delta^{13}$ C) for Station L4 (red) and Plymouth Sound (green) over sampling period.

4.4.4 Single particle eukaryote ASV richness against particle characteristics and environmental parameters.

There was a significant link between eukaryote species richness on single particles and complexity, with PA eukaryote diversity greatest at lowest complexity (n =9, rho = -0.79, p<0.05, Figure 4.4 a). There was an apparent decreasing species richness with increasing particle surface area although this was not significant (n=9, rho=-0.37, p>0.05, Figure 4.4 b). Increased eukaryote species richness on particles also coincided with increasing POC concentration (n=9, rho= 0.87, p<0.05, Figure 4.4 l). Eukaryote species richness on particles could not be significantly linked to any additional environmental parameters, although with increasing salinity, nitrite and nitrate-nitrite, eukaryotic species richness decreased (Figure 4.4 c, g, h), and with increasing river flow and temperature species richness increased (Figure 4.4 e, k). Particle species richness was also generally higher in Plymouth Sound.



Figure 4. 4. Relationship between total eukaryote species richness of 18S rRNA gene libraries on single particles (n =9) and a) complexity (RGB mean), b) surface area, c) salinity ,d) rainfall, e) river flow, f) ammonia, g) nitrite, h) nitrate nitrite, i) silicate, j) phosphate, k) temperature I) POC, m) <sup>13</sup>Carbon/<sup>12</sup>Carbon ratio. Blue lines represent linear regression, red = Station L4, green = Plymouth Sound.

4.4.5 Single particle microbial community composition.

Particles were enriched with active fungi across all particle types in comparison to other active eukaryotic groups which included metazoa, apicomplexa and ochrophyta (Figure 4.5). As fungal enrichment was greatest on single particles, we chose to consider fungal diversity and ecology as the focus for the single particle communities from here onwards. Single particles were also enriched in active fungi in comparison to the bulk eukaryote community (Appendix Figure 1.4) and more greatly enriched on particles isolated from Plymouth Sound. Furthermore, enrichment of fungi was greatest on a single particle (PS2-1) isolated from Plymouth Sound sampled during the second sampling event (Feb 2 - 25/02/2021) which coincided with high rainfall, river flow and reduced salinity (Figure 4.5, Appendix Figure 1.5 see 'Feb 2') and was also mirrored by an increased fungal abundance within the 18S bulk eukaryote community dataset (Appendix Figure 1.4, 25/02/2021).



Single Particle

Figure 4. 5 Relative abundance of eukaryotes on single particles isolated from L4 station and Plymouth Sound. Pink = bloom particles, red = detritus, green = dense aggregates.

4.4.6 Single particle fungal community composition

Fungal taxa on single particles were dominated largely by Ascomycota,

Basidiomycota, and a small number of Chytridiomycota and unassigned fungal

ASVS (Figure 4.6). At the order and family level, particle composition,

resembled that of phyla level, but at the genus level particles exhibited a great

degree of variation in their associated fungal communities. Variation was

observed between particles isolated from the same station, time and particle

type. For example, three bloom particles isolated from L4 station in the same

sampling event showed vastly different fungal community composition (Figure



4.6, Appendix Figure 1.6).

Of the 180 fungal ASVs identified from metabarcoding of the single particles, 21 ASVs (Ascomycota -13, Basidiomycota -6, Chytridiomycota -1, and Unassigned Fungi -1) were core fungal particle taxa meaning they were found at least twice on particles isolated from both L4 and Plymouth Sound to varying levels of abundance. Of the 21 ASVs, a number were universally dominant on particles ASVs including *Agariomyctetes ASV 2, ASV 7, Chytriomyces hyalinus* 

Figure 4. 6 Relative abundance of fungal phyla found on single particles. Red – L4 Station. Green – Plymouth Sound. Numbers represent sampling event particle was isolated in.

ASV 8, Candida wounanorum ASV 1, Davidiella ASV 6 and Pyrenophora triticirepentis ASV 5. Additional notable universal ASVs occurred at lower abundances included Aspergillus, Agaricomycetes, Boeremia, Candida, Davidiella, Lodderomyces, Malassezia, Metschnikowia, Sclerotinia, Wallemia and unassigned Fungal ASV (11) (Figure 4.7).

Ascomycota ASVs were mostly found across all particles or on a single particle, whilst Basidiomycota ASVs included a number of ASVs which were found largely only on particles isolated from Plymouth Sound (i.e found only on 1 L4 particle) (Figure 4.7). Basidiomycota ASVs found largely on Plymouth Sound single particles included *Agariomyctetes ASV 49, ASV 57, ASV 73, Guehomyces pullulans ASV 58, Meripilus giganteus ASV 108, and Wallemi sebi ASV 45* (Figure 4.7).

On comparison of active fungal ASV sequences from single particles with the bulk ASV fungal sequences, no single particle fungal ASV sequences matched with 100% query coverage (QC) of sequences for ASVs within the corresponding sampling time points in the bulk eukaryote dataset (Max QC: 96.97%). Overall there were 6 fungal ASVs found on single particles and in the bulk eukaryote dataset with greater than 95% QC of sequences including *Davidiella macrospora ASV 103, Pyrenophora tritici-repentis ASV 5, ASV 178, Cryptococcus vishiacii ASV 71* and *Rhizoclosmatium sp. ASV 101* (Figure 4.7). Many of the fungal genera and species identified on the single particles belonged to terrestrial ecological guilds including plant associated, animal pathogens, and saprotrophs (determined by FUNGuild, Appendix Figure 1.7).

However, a considerable number of guilds remained unknown likely owing to

the number of ASVs only identified to lower taxonomic resolution with PR2.



Figure 4. 7 Bubble plot of fungal ASVs belonging to Ascomycota, Basidiomycota, Chytridiomycota and unassigned fungi with relative abundance greater than 1% abundance from all particle types. Red panels represent particles from L4 and green panels represent particles from Plymouth Sound. Colours on particle names represent particle type: pink – bloom aggregates, red – detritus, green – dense aggregate

## 4.5 Discussion

The aim of this study, was to describe the natural variation of particles at the macro, (time and space) and micro (individual particle characteristics) scale, we term the 'particlescape'. We also aimed to develop a pipeline which captured the impact of this physical particlescape on 'active' particle-associated fungal communities at the single particle level. We chose fungal communities, since they show enriched biomass on particles in the open ocean (Bochdansky et al., 2017) but show an elevated abundance in the coastal ocean when measured in bulk (assumed free-living and particle attached) in comparison to the open ocean (Wang et al., 2014). Additionally, they can degrade phytoplankton derived material in the coastal ocean (Cunliffe et al., 2017), and display seasonal variations in their community structure (Banos et al., 2018; Duan et al., 2018; Priest et al., 2020) including at the long term sampling station L4 Station in response to environmental drivers (Chrismas et al., 2023; Taylor and Cunliffe, 2016a) suggesting they likely play an important role in the coastal marine carbon cycle. We demonstrate that there is seasonal variability in the coastal particlescape which includes the source, type, size and complexity of POM. Additionally, we show fungi are consistently isolated on single particles in the coastal ocean and their diversity is likely driven by the seasonality of the macroscale particlescape.

Elemental analysis of carbon and isotopic ratios have highlighted the contribution of terrestrial and marine POM to the spatial and temporal variation of POC composition in the coastal environment. This is driven by local scale hydrodynamic processes including river flow, sediment resuspension, and

geomorphology (Liénart et al., 2017, 2016; Uncles et al., 2015b). Across the sampling period and between stations (Plymouth Sound and L4 Station), we observed marked changes in both POC concentrations and <sup>13</sup>C/<sup>12</sup>C isotopic ratios in the bulk sampling, enabling us to discriminate terrestrial and marine contributions to the coastal particlescape. In winter, the landward station of Plymouth Sound was characterised with low  $\delta^{13}$ C values, indicative of terrestrial material (Savoye et al., 2003) and high POC concentrations, with a delayed effect of this observation seen at L4. This was likely due to physical advection of water masses from the coastline outward with tidal flows previously identified using hydrological data at L4 Station and within the Tamar catchment (Uncles et al., 2015b, 2015a). At the same time, stations were dominated by terrestrial looking detritus-like particles and large more complex dense aggregates which appeared to incorporate sediment like particulates within their structure which has been demonstrated in coastal particles previously (Liénart et al., 2017, 2016; Uncles et al., 2015b). This suggested an episodic pulse of terrestrial POM, driven by recently high rainfall and river flow as a mechanism of transporting allochthonous material (Hedges et al., 1997; Martineau et al., 2004; Raymond and Bauer, 2001).

The winter particlescape was quickly succeeded by a strengthened but variable autochthonous signature  $\delta^{13}$ C (-<24‰) (Savoye et al., 2003) coinciding with increasing temperature and depleting nutrients, combined with increasing diatom biomass, indicative of common but variable spring blooms recorded at L4 Station (Tait et al., 2015; Zhang et al., 2015). There was also the appearance of 'stereotypically' marine particles (i.e. bloom aggregates and

zooplankton derived) which propagated shoreward. Moving into spring, the  $\delta^{13}$ C signature seemed variable within stations, but began to stabilise and achieved congruence between the sampling stations, potentially showing the continual allochthonous contribution into the coastal system.

Temporal and spatial variability in the particlescape was observed at the microscale for PA eukaryote diversity across different particle characteristics and types. Despite previous evidence for increasing functional diversity, as a proxy for species richness, with increasing particle size (Lyons et al., 2010) which builds on island biogeography theory (MacArthur and Wilson, 1963,1967), we showed a trend of decreasing species richness with increasing size. However, given the low variation, skew of smaller surface area (0.019-0.241 mm) and number of sequenced particles (n=9), we are unlikely to have effectively captured any tangible relationship here.

We previously hypothesised increasing particle complexity could act as a proxy for increasing substrate complexity (e.g. polysaccharide components) and therefore niche availability. However, we show that species richness significantly decreases with increasing particle complexity, and earlier described overlaps between particle microbiomes on different polysaccharide composition supports our observations (Bunse et al., 2021). We could not account for the age of sampled particles in this study which may also influence the dynamics seen here (Datta et al., 2016; Enke et al., 2019; Hehemann et al., 2016). It is also likely that spatial variability produces a combined effect, as particles from Plymouth Sound generally showed higher species richness.

Previous studies in coastal environments have indicated long term seasonal observations in protist (Caracciolo et al., 2022; Giner et al., 2020) and fungal diversity (Chrismas et al., 2023; Duan et al., 2018; Taylor and Cunliffe, 2016a). Single particle eukaryote composition was variable but consistently dominated by fungi, previously only reported in the open ocean (Bochdansky et al., 2017). Total PA eukaryote richness was positively correlated with POC availability as previously identified for fungi (Gutiérrez et al., 2011a; Taylor and Cunliffe, 2016a), suggesting increased substrate availability. 18S rRNA sequencing provided ASV level resolution of fungal taxa that were active across single particles including both terrestrial (e.g. Pyrenophora and Boeremia) and marine (e.g. Metschnikowia, Malassezia) genus. Fungi show impressive carbohydrate active enzyme (CAZyme) repertoires including glycoside hydrolases (Chrismas and Cunliffe, 2020), and glycosyl transferases (Baltar et al., 2021) suggesting carbohydrate utilisation by fungi on particles as they are rich in carbohydrates (Amon and Benner, 2003; Pakulski and Benner, 1994). The contribution of different carbohydrates from terrestrial, freshwater and marine contributions to POC may drive differences in community composition across different particle types seen here (Paerl, 1975). Additionally, as lipids comprise a major component of particles in euphotic ocean (Wakeham et al., 1997; Wakeham and Canuel, 1988) and fungi are lipid rich (Gutiérrez et al., 2020; Thomas et al., 2022), reported variability in lipids(Hunter et al., 2021) may also drive fungal dynamics seen here.

Terrestrial ascomycetes *Pyrenophora tritici-repentis* and *Boeremia exigua* were dominant on single detritus particles isolated from Plymouth Sound

and to a lesser extent at particles from L4 Station and at greatest abundance following high rainfall and river flow. Both are globally distributed plant pathogens. P. tritici-repentis causing tan spot in wheat (Savary et al., 2019) and is a model necrotrophic parasite, meaning it acquires nutrients from dead and dying tissue (Aboukhaddour et al., 2022) utilising many CAZymes associated with plant wall degradation such as cellulases and endoglucanases (Gourlie et al., 2022). B. exigua is a facultative saprotroph and parasite of common ash (Fraxinus excelsior), a common species along the Tamar valley (Rouse and Edlin, 1958). *P. tritici-repentis* also shows great intragenomic which likely explains the high number of ASVs (22 ASVs) reported for the species here (Gourlie et al., 2022). Previous studies have highlighted the low abundance but seasonal winter and spring reoccurrence of terrestrial fungi at L4 Station from ITS sequencing of eDNA (Chrismas et al., 2023). The identified activity of terrestrial fungi in the coastal environment supports that salinity is not a constraint for survival of terrestrial fungi in the coastal ocean, likely owing to their chitin rich cell walls which can withstand osmotic shock on transition from fresh and brackish waters into seawater (Richards et al., 2012). In combination with increased inputs of terrestrial material, we provide the first potential evidence for the transport of active terrestrial taxa transported by rivers (Burgaud et al., 2013; Taylor and Cunliffe, 2016a) on allochthonous POM as previously suggested (Chrismas et al., 2023).

Fungal taxa previously detected in marine environments were also isolated across particles including the yeast *Metschnikowia*, (Chrismas et al., 2023; van Uden and Castelo-Branco, 1961), a known parasite of marine
copepods (Seki and Fulton, 1969) and freshwater zooplankton(Codreanu and Codreanu-Balcescu, 1981; Metschnikoff, 1884). The reason for potentially parasitic taxa detected in low abundances on particles in the coastal environmental remains to be determined. It is possibly a more beneficial lifehistory strategy, owing to higher nutrient concentrations than surrounding seawater (Blackburn and Fenchel, 1999b) or helps to increase parasitic transmission through suspension feeding by zooplankton as reported for parasitic transmission in bivalves-parasite systems(Ben-Horin et al., 2015).

Early diverging Chytridiomycota were detected on bloom particles and detritus including the facultative marine chytrid *Chytridiomycota hyalinus* (Booth, 1971). Many chytrids are parasitic, infecting phytoplankton (Kagami et al., 2007) and making large phytoplankton available to zooplankton through cell fragmentation and zoospore grazing as part of the mycoloop (Kagami et al., 2014) in freshwater environments, although evidence for a marine mycoloop is still missing (Grossart et al., 2019). Chytrids also undertake saprotrophic degradation of pollen particles(Kagami et al., 2017; Wurzbacher et al., 2014), chitin rich exuviae (Sparrow, 1937), zooplankton (Tang et al., 2009) and can promote increased bacterial diversity on model chitin particles (Roberts et al., 2020) highlighting complex ecological roles in biogeochemical cycling.

*Davidiella* was also isolated across single particles and had the greatest abundance on bloom particles but has not previously been recorded in marine environments. However, *Davidiella* is a known teleomorph of *Cladosporium* (Braun et al., 2003), a fungus regularly found in coastal environments (Richards et al., 2015), including L4 station (Chrismas et al., 2023; Cunliffe et al., 2017;

# Chapter 4: Mapping the 'particlescape': an assessment of microbial communities at the single particle level across different particle types in the coastal ocean.

Taylor and Cunliffe, 2016a). *Cladosporium* can assimilate phytoplanktonproduced organic matter (Cunliffe et al., 2017); therefore, the presence of *Davidiella* on bloom particles composed of the bloom forming diatom *Chaetoceros* may suggest its incorrect taxonomic assignment.

Despite the alliance of our single particle metabarcoding with previously reported patterns of bulk estimates of fungal diversity, many of the particle associated fungal ASVs were not found within our corresponding bulk eukaryote data set. As the bulk DNA should represent both particle attached and free living fungi the cause for missing ASV single particle fungal ASV sequences is uncertain but may potentially be due to biological and technical distortion from sampling and processing biases associated with methodologies (Alberdi et al., 2018) or simply the variation seen in the particlescape itself. Furthermore, additional fungal taxa found at L4 Station previously (Chrismas et al., 2023; Cunliffe et al., 2017; Taylor and Cunliffe, 2016a), were not found on our particles or within the bulk sampling. This may be a combined result of our study highlighting only active fungi and poor taxonomic resolution provided by 18S rRNA gene markers for below order level in comparison to the ITS region when sequencing fungi (Liu et al., 2015). Future efforts should determine fungal identity using ITS barcoding in addition to 18S marker genes to establish the best taxonomic resolution alongside its relative abundance to other important planktonic eukaryotes. Undoubtedly, increasing our currently limited knowledge of biology and ecology of fungi found in the marine environment will also help to better characterise fungal groups, which has resulted in their omission from central databases used for taxonomic identification.

Finally, variability of physical and microbial characteristics between single particles of the same type (e.g. bloom particles) and across single particles on temporal (e.g. winter vs spring) and spatial (e.g. Plymouth Sound vs L4 Station) scales has not been considered holistically before and heterogeneity is not generally included within the canonical view of marine carbon cycling. We hypothesise that variability is likely accounted for by the source, colonisation and life history of particles and demonstrates the importance of exploring the holistic particlescape especially in coastal oceans. Future work should focus efforts to translate the influence of the variability in physical and microbial particlescape on important processes within carbon cycling such as remineralisation (Omstedt et al., 2014), which likely has ramifications for how we interpret current estimates of carbon budgets in the coastal ocean (Baumas and Bizic, 2023). The impact of this should also be interpreted within the open ocean, as part of the biological carbon pump and wider marine carbon cycling. Looking forward, culture-independent (e.g. metatranscriptomics) and metabolic activity (e.g. remineralisation rates) assessments to determine and quantify how terrestrial and marine fungi contribute to coastal carbon cycling should be explored. This will also help address current knowledge gaps surrounding the evolution of fungi within aquatic environments (Grossart et al., 2019) and their role within the marine carbon cycle and boundless carbon cycle of the land-ocean aquatic continuum (Battin et al., 2009; Regnier et al., 2013).

Chapter 4: Mapping the 'particlescape': an assessment of microbial communities at the single particle level across different particle types in the coastal ocean.

### 4.5 Conclusion

We show that particle source, type, size, complexity and PA eukaryotic microbial communities (e.g. the particlescape) are extremely variable across spatial and temporal scales and hypothesise that variability in particle microbial communities is as a result of these variables. Using a pilot study of 9 particles we demonstrated fungi were dominant on particles and included marine and non-marine taxa. Furthermore, we provide the first potential evidence of the activity of PA non-marine fungi into coastal environments which we propose are transported by allochthonous particles during high rainfall and river flow. Despite reported variability, many studies use bulk-based particle approaches to assess particle characteristics and associated communities against environmental parameters. Observations we report here suggest this likely has ramifications for how we interpret the dynamics of particulate organic carbon and its associated microbial activity in the marine environment, now and in the future.

**General Discussion** 

### **General Discussion**

### 5.1 Overview of discussion

In this chapter, I will summarise the major contributions of the research to the knowledge gaps and aims outlined in the introduction chapter and in the broader context of the wider field. Furthermore, I will present limitations of methodologies used in thesis and suggest approaches to overcome these, in addition to identifying future avenues of research.

In this thesis, I broadly set out to investigate how particle associated communities are structured at the macro and micro-scale which may have implications for wider food web interactions and biogeochemical cycling. In independent research chapters, I examined the diversity of bacteria (chapter 1) and activity of microbial eukaryotes associated with organic material (chapter 2 and 3) which play a role in marine carbon cycling and explored potential mechanisms structuring diversity dynamics observed.

To do this, I used metabarcoding based approaches including sequencing of commonly used marker genes in DNA and associated rRNA, coupled with a variety of particle systems including model chitin particles, natural bulk material and single natural particles. By utilising increasing levels of particle heterogeneity (e.g. model bulk single substrate type, natural bulk material, single natural particle), spatial (North East Atlantic Ocean, Southern Ocean, Western English Channel, proximity to shore, coastal vs open ocean) and temporal (short-term incubations, repeated transect, seasonal sampling) complexity, I have developed methods (Chapter 3) and provided increasing resolution to understand mechanisms structuring microbial communities and its

Page | 129

uniformity over spatial and temporal scales. The environment, particles within it and particle associated microorganisms is a concept termed here, the 'particlescape'.

# I. Depth-dependent bacterial colonisation of model particles in the North East Atlantic Ocean.

In chapter 1, I investigated the depth-dependent diversity of particle associated bacterial communities in the North East Atlantic. Using model chitin particles incubated for 48 hours, combined with DNA metabarcoding across three distinct depth zones, enabled a 'simplistic' model to determine early colonizing bacteria on particles which may be representative of those involved in microbial gardening. Often understanding microbial dynamics on particles can be compounded by the variability exhibited in particle age, size, composition and life history which likely all interact in the natural environment. Using model (Datta et al., 2016) systems, such as the model chitin bead system used here can control for heterogeneity, whilst still maintaining high microbial diversity (Datta et al., 2016). Moreover, particles undergo micro-scale bacterial successions on model particles driven by ecological interactions within short time frames (Datta et al., 2016; Roberts et al., 2020, Appendix Paper 1) which can be difficult to capture on natural particles. Here I showed that despite composition of bacterial communities across the water column being largely similar, that early colonizing bacteria on chitin particles were different across the water column and to their respective in situ communities, identifying depthspecific potential candidates for microbial gardening. Comparison of taxa at the ASV level resolution found that bacteria on surface particles in low abundances were also found attached and at greater abundances in the bathypelagic which

I hypothesis that are likely general candidates for microbial gardening. As bacteria provide essential nutrition for zooplankton via amino and fatty acids (Anderson et al., 2017), work reported here may influence zooplankton grazing and migration dynamics and should be explored further. One example of future work could be to incubate zooplankton with particles colonised with different bacterial groups across different depths to ascertain dietary preferences and feeding ecology further.

This study has built on previous work at the OTU level (Mestre et al., 2018; Ruiz-González et al., 2020) by capturing exact sequence matches rather than grouping at a sequence nucleotide similarities over a 97% threshold. Trends observed here agree with previous findings that suggest sinking particles may already contain bacteria taxa which are dominant members at depths below (Mestre et al., 2018; Ruiz-González et al., 2020). Since there was no pre-filtration before incubating *in situ* seawater with chitin particles it is likely particles were present in the incubating seawater which were not accounted for, but may have contributed to dynamics seen here.

Initially, the experimental design included DNA and RNA metabarcoding allowing for the estimation of diversity and activity using RNA/DNA ratios as described in the following Chapter (Chapter 2). However, due to unsuccessful co-extraction of RNA without residual DNA contamination this was not conducted here. Despite this, future studies should endeavor to include RNA based assessment. As DNA sequencing captures both active, dormant, dead and cell free DNA (Stoeck et al., 2007) sampling RNA will enable a better estimate of potential activity (Blazewicz et al., 2013) of hypothesised dormant ASVs in the surface in comparison to the meso and bathypelagic. Finally, the initial experimental design also included 18S rRNA gene sequencing of the V4 region to determine the contribution of eukaryote taxa, specifically fungi to the early colonizing community on model chitin particles. Interactions between fungi and POM in the marine environment are limited and interactions between fungi and bacteria on marine POM further so. Even though fungi and bacteria co-exist in marine ecosystems, knowledge of fungal-bacteria interactions is limited to freshwater environments including niche-overlap (Baldy et al., 1995; Hieber and Gessner, 2002; Wurzbacher et al., 2014),promoted diversity (Roberts et al., 2020), synergistic (Romaní et al., 2006) and antagonistic (Mille-Lindblom et al., 2006) interactions. Following previous work showing chytrid fungi shaping freshwater bacterial communities on model chitin beads (Roberts et al., 2020), I hypothesised that fungi could play a role in structuring early colonizing bacterial communities on particles. Fungal sequences were not detected here in this study, the reasons for which may be three-fold.

Firstly, despite the previous description of V4 as a 'discriminative' variable region for fungi (Reich and Labes, 2017) and evidence for fungi on particles in the open ocean using the V4 region for PCR amplification (Duret et al., 2019) recent evidence suggests the choice of primer pairs makes a vast difference in recovered diversity of fungal diversity (Banos et al., 2018). Therefore, I suggest that the choice of the V4 region impacted the recovery of fungal diversity recorded here. However, Banos et al., (2018) informed the future primer selection for remaining work in this thesis.

Secondly, the "volume and bottle effect" may have influenced the sampling on both bacterial and fungal communities (Zobell and Anderson, 1936). Given the patchiness of microorganisms (Azam and Hodson, 1981;

Page | 132

Mitchell and Fuhrman, 1989) and unquantified distribution of fungal biomass (Grossart et al., 2019) in the open ocean, the likelihood of capturing fungal communities within the water column is small. This was also supported by the lack of detection of fungi within the *in situ* sampling and treatments. Additionally, communities could be subjected to the bottle effect, in which members of the community can be favored above others due to more optimal conditions or depletion of resources over time (Zobell and Anderson, 1936). However, comparison of bacterial communities sampled *in situ* and in the water only treatment showed congruence in their communities so it is likely that at 48 hours the bottle effect had not been at play here.

If indeed, fungi do dominate biomass on particles in the bathypelagic, as previously recorded in the North Atlantic Ocean (Bochdansky et al., 2017), attempts to understand the patchiness of fungi in the marine environment should be explored further to help determine dynamics reported here. Especially as particles are also patchy in their distribution (Azam and Long, 2001).

Thirdly, there is potential that fungi are not early colonisers on particles since particles were only incubated for 48 hours with seawater. Nonetheless, this chapter makes a substantial contribution to the identities of bacterial taxa and their structuring by depth on model particles in the open ocean, which may be important when considering the the wider implications of microbial colonisation on particles for carbon cycling and the role they play for zooplankton grazers.

# II. Determine diversity and activity of microbial eukaryotes in their contribution to the biological carbon pump of the Southern Ocean.

A major objective of this thesis was to determine the contribution of microbial eukaryotes to the BCP in terms of their activity, as historically surveys of microbial eukaryotes contribution to the BCP identify only their contribution to composition of sinking and suspended particles using microscopy and DNA based approaches. Using 18S rRNA: 18S rRNA gene ratios of microbial eukaryote ASVs as an estimate for activity, I found many taxa were active throughout the water column. I also identified ASVs that showed contrasting patterns of activity to their surface counterparts at depth, coupled with increasing dissimilarity between DNA and RNA species composition with depth. I hypothesise that in the surface DNA provides a more accurate representation of the active community, achieved by congruence to RNA in the surface. At depth, I suggest dissimilarity occurs as RNA is representative of the active community, ASVs which are incorporated into fast sinking material or involved with biomass degradation and remineralisation. Here I propose that declining POC concentrations which diminish with grazing, remineralisation and fragmentation, results in reduced activity of associated grazers, mirroring the constant reached in the attenuation of POC, as commonly described in the Martin Curve (Martin et al., 1987). Observed results suggest a more specific contribution of microbial eukaryote diversity and activity towards the vertical attenuation of POC in the Southern Ocean than previously identified.

Metatranscriptomics surveys provide a snapshot of genes transcribed by the metabolically active community to infer the functional profile enriched on particle communities (Boeuf et al., 2019; Zhao et al., 2020), and the role that active microorganisms play in particle degradation specifically. The use of DNA and RNA based tools together with traditional visualisation e.g., light microscopy, may help to disentangle the relationship between those organisms adding to bulk particle composition and those actively consuming and respiring POC as eluded to here. Collaborative uses of tools are useful e.g. microscopy can succeed where modern-day techniques fail such as the difficulty in capturing high taxonomic resolution within a single database such as the difficulties experienced here for the MArine STramenopile group 3 (MAST-3) using PR2.

Despite patchy sampling high-volume *in situ* sampling provided a diverse range of eukaryotes in association to the BCP, including typically associated and more recently identified taxa and still provided patterns of eukaryote association, which will only be strengthened by higher resolution observations. The benefits of using high-volume *in situ* sampling here was two-fold, as it allows for an increased reported diversity due to less sample manipulation and processing time in comparison to traditional niskin bottle sampling (Puigcorbé et al., 2020). It was the first use of this method for determining the diversity and activity of microbial eukaryotes. In situ pumps used here included sizefractionation at 53 µm. However, it is likely that the pumping of seawater captures not only particle associated material but also free-living and pelagic organisms as a result of filter clogging (Padilla et al., 2015) which may skew reported observations here. Additionally, differences within volume of water filtered through the SAPS could influence the recovery of taxa including rare taxa which may also skew reported observations (Brandt et al, 2021), especially since taxa which aren't very abundant could still be considered active and playing a role in the BCP. Furthermore, this is compounded by the lack of

replication within our reported observations and should be considered a potential limitation of the study.

Future studies, could undertake cross comparisons of different methodologies (e.g. sediment traps, *in situ* pumps, niskin bottles) to determine how efficient *in situ* pumps are at capturing particles and their associated eukaryotic communities.

Additionally, I also wanted to identify if marine fungi were an active component of the eukaryotic community. Since I chose a different primer set targeting the V9 region of the 18S rRNA gene fragment and increased the volume of water sampled to retain as much of the in situ diversity possible (Puigcorbé et al., 2020), I hoped I would be more successful in reporting fungal diversity and activity associated with organic material in the Southern Ocean. Yet again, I was unsuccessful in discovering a meaningful contribution of fungal ASVs to processed sequencing data. In this instance, targeting of the V9 region resulted in only 3 fungal ASVs being detected at low read counts at the most northern sampled station, OOI. Fungi have previously been reported in frozen Antarctic lakes (Gonçalves et al., 2012; Rojas-Jimenez et al., 2017) and in the Southern Ocean (Breyer et al., 2022), including on natural (Duret et al., 2019) and plastic (Lacerda et al., 2020) particles. However, as fungal abundance decreases with proximity from shore (Burgaud et al., 2013) and decreasing substrate availability for pelagic (Taylor and Cunliffe, 2016) and benthic (Orsi et al., 2013) taxa, this could provide a mechanism for lack of abundance of fungi reported here. Furthermore, mesh size, filter clogging and associated downstream sample processing and sequencing impacts may explain lack of detected fungi too. This may suggest that the use of *in situ* pumps is not the most appropriate to recover particle associated fungi from the marine

environment. At a bulk scale, alternatives such as use of sediment traps and niskin bottles may be more appropriate here.

# III. Develop methods to describe physical characteristics and active microbial communities of single naturally occurring particles in coastal waters surrounding Plymouth Sound.

Particles in the marine environment exhibit enormous variability but heterogeneity is often overlooked using bulk-scale approaches as previously outlined. In chapter 3, I developed a pipeline of isolation, imaging, and RNA sequencing of the 'active' microbial eukaryote communities on single particles collected across two sampling sites in the Western English Channel over the winter and spring period in 2021. This resulted in the classification of 8 particle types based on visual observations, which are not extensive list of particles found in the marine environment, given the variability in terms assigned to particles types (Durkin et al., 2021, 2022). Furthermore, some particles e.g., aggregate-like, characteristics likely exist on a continuum, based on visual observations, and fundamental to this study, likely vary based on environmental parameters and seasonality. Manual selection of particles was time consuming, roughly taking 7 hours from filtration to selection of all particles in a given time point. There is potential bias for the particles selected here owing to methodological implications e.g. mesh size, isolation of particles conducted by eye. Additionally, increased processing time and manipulation of the water on transfer from bottle to carboys may have altered particle characteristics and PA communities. However, this does not distract from the clear variability in particles characteristics observed.

Imaging of particles provides both manually and automated methods such as under water visual profiles combined with high throughput image sorting algorithms such as those used for plankton e.g., MorphoCluster (Schröder et al., 2020) can provide quantitative measurements of particle types across temporal and spatial scales (Giering et al., 2020; Giering et al., 2020). Here, relative abundance is only semi-quantitative due to manual selection but future efforts involving higher-throughput imaging of particle types could provide quantitative estimates. As morphology of particles plays a dynamic role in carbon export (Trudnowska et al., 2021) improving methods which quantify variance is required in order to capture the variability desperately needed to improve parameterisations within biogeochemical models.

In addition to single particle characterisation, I utilised environmental parameters collected at L4 Station Western Channel Observatory combined with stable isotope ratios of carbon to identify the source and potential mechanisms driving the dynamic influx of allochthonous material into the coastal system, alongside autochthonous contributions to POM and their associated communities. Since environmental parameters limited in their spatial resolution (e.g., WCO at L4 Station, Tamar river flow only, rainfall data for Plymouth), it is likely the variability within the system has been underestimated which may explain the poor correlations for some parameters which PA microbial eukaryote communities reported here. Nevertheless, there were clear trends associated to the PA microbial eukaryote communities I evidence is driven for by the seasonality of the macroscale particlescape including source, colonisation and life history of particles.

# IV. Conduct a pilot study on the drivers of active particle associated fungi in coastal waters surrounding Plymouth Sound.

Using the developed pipeline, Chapter 3 was successful in detecting particle associated fungi and was able to explore the fungal diversity associated with different particle types, spatially and temporally. In stark contrast to the other research chapters, fungi were the dominant eukaryote across particles, independent of particle type, site and season. Whilst it is important to note, the chapter only details the microbial diversity of 9 particles, the developed pipeline provides encouraging evidence for fungi's involvement within carbon cycling in the coastal boundary environment.

Current estimates of fungal abundance are greatest closer to the shore (Burgaud et al., 2013), with increasing substrate availability in coastal environments (Taylor and Cunliffe, 2016) and fungi dominate biomass on particles in the bathypelagic in the open ocean (Bochdansky et al., 2017). Combined with the improved recovery of fungi using of the V9 region (Banos et al., 2018) the single particle resolution and low biomass nature of this study, this likely explains the detection of fungi on single particles sampled here. Highthroughput sequencing data does well to provide compositional and relative abundance data for PA microbes but has been criticized for its lack of quantitative abilities and it can be difficult to disentangle the composition of the bulk particle itself and the active microbial community transforming it. To overcome lack of fungal abundance data, the use of tools such as Q-PCR (Datta et al., 2016; Zhang et al., 2022), epi-fluorescence microscopy (Kiørboe et al., 2003; Lochte and Turley, 1988; Turley and Mackie, 1995) and CARD-FISH (Bochdansky et al., 2017; Priest et al., 2020) could be used as a more quantitative based approach to count abundance and visualise PA fungi.

Page | 139

However, they are currently limited in their completeness by primer and probe selection bias.

I present the first evidence of PA non-marine fungi in coastal environments which I propose are transported on the increased allochthonous particles observed during high rainfall and river flow as previously suggested (Burgaud et al., 2013; Chrismas et al., 2023). The identified activity of terrestrial fungi in the coastal environment supports that salinity is not a constraint for survival of terrestrial fungi in the coastal ocean, likely owing to their chitin rich cell walls which can withstand osmotic shock on transition from fresh and brackish waters into seawater (Richards et al., 2012). Assessments of saltwater intrusion of soils show that soil associated fungal growth can be reduced (Rath and Rousk, 2015); however, fungi isolated from the marine environment exhibit uncoupled growth rates from final growth yield (Thomas et al., 2022). This could suggest investment in cellular resources as seen in the modification of macromolecular composition of the marine fungus Paradedryphiella salina in response to salinity (Paton and Jennings, 1988). Future studies should look to undertake both culture-dependent and independent assessments inferring the impact of salinity on PA terrestrial fungi in the coastal environment, especially given that substrate availability may influence the survival of a fungus. This will also help us to resolve pre-existing questions surrounding the evolution of fungi in aquatic environments.

Marine fungal taxa found at L4 Station previously (Chrismas et al., 2023; Cunliffe et al., 2017; Taylor and Cunliffe, 2016) were not found on ou particles or within the bulk sampling, and a number of fungal ASVs were only defined at the kingdom level. This may be a combined result of our study highlighting only active fungi and poor taxonomic resolution provided by 18S rRNA gene markers

Page | 140

for below order level in comparison to the ITS region when sequencing fungi (Liu et al., 2015). Future efforts should determine fungal identity using ITS barcoding in addition to 18S marker genes to establish the best taxonomic resolution alongside its relative abundance to other important planktonic eukaryotes. Undoubtedly, increasing our currently limited knowledge of biology and ecology of fungi found in the marine environment will also help to better characterise fungal groups, which has resulted in their omission from central databases used for taxonomic identification.

#### 5.2 Concluding statement:

Mapping the 'particlescape' at the macro and micro scale has helped to answer fundamental questions and points of future interest for the field of microbial ecology and biogeochemical cycling. This includes describing the identity and activity of microbial communities on particles across varying scales of complexity and incorporation of variability. I also add to the growing pool of knowledge of marine microbial communities in the understudied mesopelagic and bathypelagic (Aristegui et al., 2009). This thesis has also provided increased knowledge surrounding microbial eukaryotes, especially fungi, and in their contribution behind the biological carbon pump and wider marine carbon cycle. Prior to this thesis, knowledge surrounding the dominance and activity of marine and non-marine fungi on particles was missing. Work outlined in chapter 3, provides a step-change in knowledge surrounding the diversity and activity of fungi in marine environments and demonstrates a plethora of avenues to be explored to determine the true role of fungi in the marine carbon cycle.

Collectively, this thesis provides encouraging evidence needed to consider more than just a 'bulk' approach to examining particles and their associated communities in the marine environment. In doing so, studies mapping the particlescape in which diverse marine microbial communities exist, will not only accelerate our understanding of marine microorganism's role in carbon and wider biogeochemical cycling, but also allow progression in closing present day carbon budgets. Undertaking this is not a menial task, but is integral in predicting how marine carbon cycling may respond under future climate projections.

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Appendix Figures and Table for Chapter 3:

DNA and RNA based assessment of the biological carbon pump in the Southern Ocean

### Appendix 1. SAPS deployment and Calculation of Folsom Splitter split

The SAPs Mesh filters was rinsed with 1 L of filtered seawater and the resulting water particle mixture was split into several fractions for different analyses using a Folsom splitter (Wildco). Exact split sizes were calculated as a volume of seawater filtered. The volume of water that was filtered through the 53 µm mesh filter was known from the *in situ* pump flowmeter and the size of the whole filter was known. 1/8<sup>th</sup> of the sample was taken away for microplastics analyses and 7/8<sup>th</sup> was left remaining. The fraction of the sample for microplastics analyses can be calculated and subtracted from the remaining material which was then suspended in 1L of filtered seawater and split further with the Folsom splitter into several fractions including POC, PIC, BSi and this study. Exact split sizes are listed below.

Appendix	Table 1.1. Volume of SAPS water filtered and split size for individual
samples.	Cells coloured green for euphotic and blue for mesopelagic samples.

Sample No.	Cruise	Cruise	Dept	SAP	Actual
	Statio	Occupatio	h (m)	Volum	Volume (L)
	n	n		e (L)	
DY111_SAPS_00	001				
1		OOI 1	80	1534	167.7813
DY111_SAPS_00	001				
2		OOI 1	180	21	2.296875
DY111_SAPS_00	TS				
3		TS-1	80	1382	151.1563
DY111_SAPS_00	TS				
5		TS-1	400	1626	177.8438
DY111_SAPS_00	TN				
6		TN-1	80	1754	191.8438
DY111_SAPS_00	TN				
7		TN-1	180	1650	180.4688
DY111_SAPS_00	TN				
8		TN-1	400	1622	177.4063
DY111_SAPS_00	001				
9		001 2	80	1587	173.5781
DY111_SAPS_01	001				
1		OOI 2	400	1859	203.3281
DY111_SAPS_01	TS				
2		TS-2	400	1718	187.9063

DY111_SAPS_01	TS				
3		TS-2	200	1712	187.25
DY111_SAPS_01	TS				
4		TS-2	100	1640	179.375
DY111_SAPS_01	TN				
5		TN-2	30	1751	191.5156
DY111_SAPS_01	TN				
6		TN-2	130	1501	164.1719
DY111_SAPS_01	TN				
7		TN-2	400	1621	177.2969
DY111_SAPS_01	001				
8		OOI 3	400	1596	174.5625
DY111_SAPS_01	001				
9		OOI 3	150	1325	144.9219
DY111_SAPS_02	TS	TS3	40	1591	174.0156
2					
DY111_SAPS_02	TN				
4		TN3	40	1591	174.0156
DY111_SAPS_02	TN				
5		TN3	140	1626	177.8438
DY111_SAPS_02	TN				
6		TN3	400	1148	125.5625
DY111_SAPS_02	TS				
7		TS4	50	1033	112.9844

DY111_SAPS_02	TS				
8		TS4	150	2474	270.5938
DY111_SAPS_02	TS				
9		TS4	400	1686	184.4063
DY111_SAPS_03	001				
0		OOI4	80	806	88.15625
DY111_SAPS_03	001				
1		OOI4	280	1674	183.0938

# Appendix Table 1.2 Taxonomic assignment of MArine STramenopile (MAST-3) amplicon sequence variance (ASV) using Tara Oceans eukaryote database (De Vargas et al 2015).

ASV	Kingdom	Supergroup	Division	Phylum	Class	Order	Suborder	Family	taxa_ASV
asv_17	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+sp.	Solenicola+sp. asv_17
asv_227	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	NA	NA	MAST-3 asv_227
asv_49	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_49
asv_74	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_74
asv_46	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_46
asv_160	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_160
asv_115	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_115
asv_161	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_161
asv_214	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_214
asv_658	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	NA	NA	MAST-3 asv_658
asv_431	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_431
asv_538	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_538
asv_749	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	MAST-3D	MAST-3D+sp.	MAST-3D+sp. asv_749
asv_750	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Incisomonas	Incisomonas+sp.	Incisomonas+sp. asv_750
asv_293	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+sp.	Solenicola+sp. asv_293
asv_397	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_397
asv_395	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+sp.	Solenicola+sp. asv_395
asv_724	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_724
asv_620	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_620
asv_561	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+sp.	Solenicola+sp. asv_561
asv_1046	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	NA	NA	MAST-3 asv_1046
asv_510	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_510
asv_642	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_642
asv_1030	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+sp.	Solenicola+sp. asv_1030
asv_427	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_427
asv_641	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_641
asv_1139	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_1139
asv_1272	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	MAST-3D	MAST-3D+sp.	MAST-3D+sp. asv_1272
asv_911	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_911
asv_654	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	Solenicola+setigera	Solenicola+setigera asv_654
asv_984	Eukaryota	Stramenopiles	Stramenopiles_ X	MAST	MAST-3- 12	MAST-3	Solenicola	NA	Solenicola asv_984



Appendix Figure 1.1 POC concentration with depth across three stations (OOI, TN, TS) for 4 occupations sampled.

Appendix Figures and Tables for Chapter 4: Mapping the 'particlescape':

an assessment of particle characteristics and fungal communities at the single particle level in the coastal ocean.

## Particle characterisation key

- 1. Zooplankton derived or faecal pellet shaped go to 4
- Detritus an apparently single entity made of the same source material features include a distinct, sometimes entire, margin appears dense; no or few interstitial spaces – go to 5.
- 3. Aggregate a conglomerate of multiple particles. Can include detritus. features include a non-distinct, irregular, or undulate margin. May contain interstitial spaces *go to 6.*

-----

Carcass - Zooplankton Carcass (ZC)
 Carapace/moult - Zooplankton Moult (ZM)
 Faecal pellet – Zooplankton Faecal Pellet (ZF)

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Piece from a larger object - Fragmented Detritus (FD)
 Not obviously a piece from a larger object - Detritus (D)

\_\_\_\_\_

6. Contains detritus – Detrital Aggregate (DA)
 Contains prevailing bloom-forming phytoplankton – Bloom Aggregate (BA)
 Other aggregates – Aggregate (A)



Appendix Figure 1.2 Images of single particles selected for RNA sequencing.

Appendix Table 1.3 Counts of particle types collected from Plymouth Sound (PS) and L4 Station (L4) in the Western English Channel.

Particle Type	Site	Feb 3	Feb 4	March 1	March 2	March 3	March 4	April 1	April 2	April 3	April 4	total
				L								
				winter			spring					
Detritus	L4	7	3	1	1	9	2	3	8	0	10	44
	PS	13	6	3	9	7	11	5	10	4	6	75
Fragmented Detritus	L4	6	1	1	0	3	0	1	0	0	0	12
	PS	3	14	4	1	1	2	0	0	1	4	30
Aggregates	L4	3	3	7	5	3	0	0	10	2	0	33
	PS	0	0	12	5	5	7	0	0	0	1	30
Dense Aggregates	L4	0	11	4	3	0	1	0	5	0	2	26
	PS	0	13	3	19	13	14	14	0	0	3	78
Bloom aggregates	L4	0	0	0	0	0	18	14	4	1	1	38
	PS	0	0	0	0	0	0	0	20	16	6	42
Zooplankton	L4	0	2	0	1	1	0	0	0	1	0	5
Carcass	PS	0	0	0	0	0	0	0	0	0	0	0
Zooplankton Moults	L4	0	0	0	0	1	1	0	0	0	1	3
	PS	0	0	0	4	1	0	0	0	0	0	5
Zooplankton Faecal	L4	0	0	0	0	0	0	1	3	0	0	4
Pellets	PS	0	0	0	0	0	0	0	1	0	0	1
total		32	53	35	48	44	56	38	61	25	34	426



Appendix Figure 1.3 A) Longest length (mm) and B) Red Green Blue (RGB) standard deviation values of particles isolated from Plymouth Sound (triangle) and L4 Station (circle). Colours represent different particle types.

Appendix Table 1.4 Leave-one-out cross validation of particle group according with the LDA performed on RGB mean values.

	Actual							
Predicted	Aggregates	Detritus	Zooplankton					
Aggregates	236	53	11					
Detritus	10	106	6					
Zooplankton	2	1	1					

Appendix Table 1.5 Leave-one-out cross validation of particle Type according with the LDA performed on RGB mean values.

	Actual										
Predicted	А	BA	D	DA	FD	ZC	Z FP	ΖM			
А	0	0	0	0	0	0	0	0			
BA	22	49	14	4	4	2	0	7			
D	8	3	80	28	12	2	4	0			
DA	33	26	13	72	6	1	0	0			
FD	0	0	8	0	20	0	1	0			
ZC	0	0	0	0	0	0	0	0			
Z FP	0	0	0	0	0	0	0	0			
ZM	0	2	3	1	0	0	0	1			



Appendix Figure 1.4 Phytoplankton cell counts at L4 Station performed by Clare Widdicombe (PML). Flagellates on left axis and other plankton groups on right axis.



Appendix Figure 1.5 18S rRNA gene community composition at phylum level for A) Station L4 and B) Plymouth Sound for each replicate over the sampling period. Grey shading represents phyla with less than 3% abundance.



Appendix Figure 1.6 Environmental variables taken from Western Channel Observatory (WCO) and shipboard measurements for Station L4 over sampling period.



Appendix Figure 1.7 Genus level fungal composition of single particles isolated from Station L4 and Plymouth Sound greater than 1% relative abundance.



determined by FUNGuild.

Appendix Paper 1. Roberts et al, 2020 published paper providing context for methods used in first chapter.

# BIOLOGY royalsocietypublishing.org/journal/rsbl Research O Constraints G Constraints Cite this article: Roberts C, Allen R, Bird KE, Cunliffe M. 2020 Chytrid fungi shape bacterial communities on model particulate organic matter. *Biol. Lett.* 16: 20200368. http://dx.doi.org/10.1098/rsbl.2020.0368

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THE ROYAL SOCIETY

### **Community ecology**

# Chytrid fungi shape bacterial communities on model particulate organic matter

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Microbial colonization and degradation of particulate organic matter (POM) are important processes that influence the structure and function of aquatic ecosystems. Although POM is readily used by aquatic fungi and bacteria, there is a limited understanding of POM-associated interactions between these taxa, particularly for early-diverging fungal lineages. Using a model ecological system with the chitin-degrading freshwater chytrid fungus Rhizoclosmatium globosum and chitin microbeads, we assessed the impacts of chytrid fungi on POM-associated bacteria. We show that the presence of chytrids on POM alters concomitant bacterial community diversity and structure, including differing responses between chytrid life stages. We propose that chytrids can act as ecosystem facilitators through saprotrophic feeding by producing 'public goods' from POM degradation that modify bacterial POM communities. This study suggests that chytrid fungi have complex ecological roles in aquatic POM degradation not previously considered, including the regulation of bacterial colonization, community succession and subsequent biogeochemical potential.

### 1. Introduction

Particulate organic matter (POM) in aquatic ecosystems acts as 'hotspots' for bacteria [1–3] and fungi [4,5]. Microbial processing of POM has impacts on ecosystem functioning, including the biological carbon pump in the open ocean [6] and carbon transfer through freshwater food webs [7].

Bacteria–POM studies have characterized the microscale interactions between bacteria and particles, including the composition of colonizing communities [8–10], interactions between attached bacteria [11] and the dynamics of POM degradation [9–12]. Laboratory-based incubations with chitin microbeads as model POM have identified bacteria that colonize and degrade POM using extracellular enzymes, producing a pool of more freely available substrates, including dissolved organic matter (DOM), considered 'public goods' for other bacteria in the community to utilize [8].

Dikaryan fungi (Ascomycota and Basidiomycota) also attach to and degrade POM [13–18]. Given that POM-degrading fungi also use extracellular degradation mechanisms [19,20], it is likely that they produce 'public goods' for the wider community to exploit. Studies of freshwater leaf-degrading dikaryan fungi show that as bacteria lack key enzymes associated with plant polymer degradation [21], the production of low and intermediate weight DOM by fungi [22] may support enhanced bacterial growth on allochthonous leaf litter [21].

The roles of early-diverging saprotrophic fungal lineages, such as the Chytridiomycota (chytrids), in POM-associated processes are poorly understood.

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Page | 223



Figure 1. (a) Schematic summary of experimental design. Experimental treatments: Control = yellow, Zoospores = blue, Established = red. (b) Bacterial diversity measured as the Shannon's H index of treatments over time. Bars represent standard error and asterisks denote level of significance as follows: \*\*p < 0.01, \*\*\*p < 0.01. (c) NMDS plot of bacterial community structure based on weighted UniFrac dissimilarity between bacterial communities found with treatment over time.

Chytrids are widespread fungi that produce motile zoospores to search for substrates to colonize, including allochthonous and autochthonous POM such as pollen [4], chitin-rich exuviae [23] and zooplankton carcasses [24], as well as living substrates, such as amphibian epidermises [25] and phytoplankton [26]. Once attached, a zoospore loses its flagellum before developing a walled sporangium with a rhizoid network, which attaches to and penetrates the substrate [27]. Chytrids subsequently feed saprotrophically via the rhizoids, which secrete extracellular enzymes to degrade POM to low molecular weight substrates for uptake and assimilation [28].

Even though chytrids and bacteria coexist in aquatic ecosystems, knowledge of chytrid-bacteria POM interactions is limited to niche overlap [4] and infection-associated dynamics on amphibian epidermises [29,30]. To our understanding, there is no current research on the direct influence of chytrids on POM-attached bacterial diversity and community structure. To address these knowledge gaps, we used the chitinophilic Rhizoclosmatium globosum in an experimental study with chitin microbeads to assess the interactions between chytrids, bacteria and POM. Using chitin microbeads as a POM experimental system removes the complex heterogeneity of natural particles (e.g. age and composition) while retaining ecological relevance since chitin is an important POM component in aquatic ecosystems [8]. We aimed to explore how the different chytrid life history stages (i.e. attaching zoospores versus established sporangia with rhizoid networks) impact concomitant attaching bacterial diversity and community structure.

### 2. Material and methods

#### (a) Experimental set-up

R. globosum JEL800 was maintained on PmTG agar [31] as described previously [27]. To harvest zoospores, established plates were flooded with 4 ml distilled H<sub>2</sub>O and incubated at room temperature under laminar flow for 90 min. The zoospore suspension was passed through a 10 µm cell strainer and the concentration determined using a coulter Counter (Beckman Coulter, US).

Magnetic chitin microbeads (New England Bio) were used for the experiments using protocols adapted from [8]. Pond water containing a natural bacterial assemblage was collected from Efford Marsh pond (Plymouth, UK) and passed through a 40 µm mesh to remove detritus and large eukaryotes. Three experimental treatments were set up as follows: 'Control', 40 µm filtered pond water and chitin microbeads; 'Zoospores', *R. globusum* zoospores, 40 µm filtered pond water and chitin microbeads; and 'Established', *R. globusum* grown initially on chitin microbeads for 24 h in 0.2 µm filtered pond water before addition of experimental 40 µm filtered pond water (figure 1a). Lett

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For each treatment, 23.5 ml pond water was added to a 25 ml vented culture flask. Each treatment was conducted with three replicate flasks per timepoint that were sampled destructively. Chitin microbeads were added to the flask and inverted several times to ensure even distribution. For chytrid treatments, zoospores were added to a concentration of approximately  $3 \times 10^4$  cells ml<sup>-1</sup>. Flasks were incubated in the dark at 22°C with mixing at 50 rpm. After 2 h, a washing step took place in the chytrid treatments, with the water replaced to ensure that the experiments proceeded with microbead-attached chytrids only ('Established' 40 ± 14.9 chytrids microbead<sup>-1</sup>). After 2 (i.e. the wash step), 9, 24 and 34 h, all chitin microbeads from each flask were harvested using a magnet. The residual water was discarded, retaining the chitin microbeads, which were frozen in liquid nitrogen and stored at  $-80^\circ$ C.

### (b) DNA extraction, 16S rRNA gene sequencing and bioinformatics

DNA was extracted from the chitin microbeads using the Zymo Research Quick-DNA™ Fecal/Soil Microbe Microprep Kit (Zymo Research, USA) following the manufacturer's instructions. The V4-V5 region of the 16S rRNA gene was amplified using primers 515FB and 926R [32], and sequenced using the Illumina MiSeq platform. Sequences were processed in R [33] using the DADA2 pipeline [34]. Demultiplexed reads were filtered and trimmed to remove primers and low-quality sequences. The DADA2 algorithm was used to infer amplicon sequence variants (ASVs) [34]. Paired-end reads were merged to obtain full denoised sequences. Chimeric sequences were removed, before taxonomy was assigned using the SILVA database (release 128) [35]. ASVs assigned as chloroplasts and mitochondria were removed. A maximum likelihood phylogenetic tree was estimated using the phangorn package (v.2.5.5) [36] and combined with the ASV table, taxonomic assignment and experimental metadata into a phyloseq object using the phyloseq package [37]. Sequences were rarefied to 4955 reads before further analysis.

#### (c) Data processing and statistical analyses

Shannon's index (H) was used to calculate diversity, and the effect of treatment and time on diversity was assessed using a two-way



Figure 2. Bacterial community composition. Bacteria are grouped by the top 10 most abundant orders; orders outside this are grouped as 'other'.

ANOVA with Tukey's HSD. Differences in community structure between samples (beta diversity) were calculated using a weighted UniFrac [38] distance matrix and visualized through non-metric multidimensional scaling (NMDS) ordination. Permutational multivariate analysis of variance (PERMANOVA) [39] was used to test the effect of treatment and time on community structure using the 'adonis' function in the R package vegn [40].

### 3. Results

The diversity of the bacterial communities attached to chitin microbeads in all treatments followed the same pattern of decline in the first 9 h of the experiment (figure 1b). After 9 h, bacterial diversity in the control treatment continued to decrease for the remainder of the study. Conversely, bacterial diversity in the presence of chytrids increased after 9 h compared with the control treatment (Tukey's HSD p < 0.05). Attached bacterial diversity was greater in the zoospore treatment compared with the established chytrid treatment, although this trend was not statistically significant (Tukey's HSD p = 0.08).

Bacterial community composition varied significantly between treatments and timepoints, with a significant interaction between these variables (PERMANOVA, all p < 0.001). After 2 h, the bacterial communities attached to the chitin microbeads in the established treatment were distinct from the other treatments (figure 1c), dominated by Burkholderiales, Chromatiales and to a lesser extent Neisseriales and Pseudomonadales (figure 2). Conversely, bacterial communities in the zoospore and control treatments were similar (figure 1c) and dominated by Burkholderiales (figure 2).

As the experiment progressed, the structure of the bacterial communities in the zoospore and established chytrid treatments converged, while bacterial communities in the control treatment remained distinct and with relatively limited variation through time (figure 1c). At 24 h, Cellvibrionales were also dominant in both the zoospore and established treatments, while Burkholderiales remained dominant in the control treatment (figure 2). Together, these results suggest that the presence of chytrids on chitin particles impacts both initial bacterial colonization and community succession, with the effects specific to the chytrid life stage.

#### 4. Discussion

Understanding microbial–POM interactions has been largely dominated by bacteria-focused studies. Limited work has been conducted on fungi–bacteria–POM interactions, and the roles of early-diverging fungal lineages in these interactions remains unresolved. Here we show that chytrid fungi impact POM colonizing bacteria and community succession, suggesting that chytrids play a role in shaping POM microbial communities, with implications for carbon cycling in aquatic ecosystems.

Community diversity across all treatments initially declined, following previously reported patterns of bacterial colonizing communities on chitin microbeads [8], suggesting that early colonization here was also governed by known mechanisms (e.g. attachment ability). However, as the presence of the established chytrids with developed rhizoids showed an initially distinct bacterial community structure from the zoospore treatment, this suggests that life-stage dependent ecological interactions occur between chytrids and bacteria that govern particle colonization.

Chitin degradation can result in the release of DOM in aquatic systems [41,42]. Prior to the addition of the bacteria, when the chytrids attach to and degrade the chitin microbeads, they likely produce extracellular N-acetylglucosamine (NAG) as a potential 'public good'. Bacteria, including NAG-using bacteria that are unable to degrade chitin (i.e. 'cheaters'), may be supported by this pool of 'public goods'. Community-wide bacterial growth on chitin does not necessarily require chitin degradation by all community members and can be stimulated by the utilization of secondary degradation products including NAG [43,44]. Previous experimental work has suggested that POM-derived DOM utilization involves a diverse assemblage of bacteria, with no single group dominating the consumption of NAG [45]. The initial structure of the colonizing bacterial community in the established treatment suggests that the bacteria present may be using a source of DOM, such as the proposed NAG pool, and that chytrids may play a role in DOM production from POM degradation in aquatic systems.

The temporal change in bacterial community composition may indicate the decline of the potential NAG pool, and a switch towards a chitin-degrading community at 24 h. As there is a similar switch in community composition seen in the zoospore treatment, this could have been stimulated, in part, by chitin degradation products from the chytrids acting as chemotactic signals for degraders [46,47]. Furthermore, R globosum JEL800 rhizoids form grooves on the outer surface and penetrate chitin microbeads [27], modifying the POM structure and providing increased surface area for bacterial attachment and promoting chitin degradation. It is also possible that, because the chytrid cell wall also contains chitin, the differences in diversity between chytrid and control treatments could be due to an increased relative abundance of chitin-degrading bacteria using chitin from living chytrids or necromass. The predicted function of bacterial communities in this study approximated using Piphillin [48] provides support to this suggestion, showing a convergence in the established and zoospore treatments and divergence from the control treatment, which were distinct over time (electronic supplementary material, figure S1). Piphillin analysis predicted an initially elevated abundance of the NAG transporter gene in the established treatment only that declined over time, presumably as the NAG pool was depleted (electronic supplementary material, figure S2E). Subsequently, there was a predicted increase in chitinase and chitin-oligosaccharide transport genes [49] at 24 h in the established and zoospore treatments (electronic supplementary material, figure S2). Future studies should attempt to unravel the exact nature of the chytrid-bacteria interactions reported here, including the impacts of bacteria on saprotrophic chytrids and the direct assessment of bacterial function, such as through enzyme assays or metatranscriptomics.

POM degradation by microbes may also result in the indirect generation of diverse carbon substrates such as cell debris and metabolic by-products (e.g. organic acids) [8]. Enhanced chemical heterogeneity of these alternative carbon sources produced by both chytrids and bacteria could support the colonization of bacteria that use these products and drive the community dynamics reported here. As the colonization of these bacteria on the particle is likely to invoke competitive interactions, such as for space, the collective community function may diverge from a directly chitin-degrading community towards one that relies on secondary production, as shown in bacteria-only studies [8].

Chytrids have established roles in aquatic ecosystems, including parasitizing hosts and transferring resources via lipid-rich zoospores to higher trophic levels through the mycoloop [50]. Overall, our data indicate that independent of life stage, chytrids also influence the diversity and community structure of POM-colonizing bacterial communities. Increased diversity of bacteria associated with chytrids suggests that chytrids may produce DOM as a pool of 'public goods' supporting the growth of 'cheaters' and/or encouraging the chemotaxis of chitinolytic bacteria. The potential stimulation of bacterial chitin degradation by the presence of established chytrids, coupled with their own inherent degrading capability, implies that saprotrophic chytrids may have complex roles in regulating POM and DOM processing in aquatic ecosystems that are not yet considered.

Data accessibility. Sequence data have been deposited at the European Nucleotide Archive under project number PRJEB37940.

Authors' contributions. C.R. conceived and designed the study, carried out the study, conducted data analysis and interpretation of data and drafted the manuscript; R.A. supported data analysis, including sequence analysis, and contributed to interpretation of data and editing the manuscript; K.E.B. contributed to carrying out the study, including sample processing, interpretation of data and editing the manuscript; M.C. conceived and designed the study, interpreted data, and drafted and edited the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed herein.

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