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Spatial variability and distribution of benthic microbial diversity in the Atlantic and Pacific

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by

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Abstract

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This thesis focuses on the spatial variability of benthic abyssal microbes, which have important implications for biogeochemical cycling in the deep ocean. Abyssal ecosystems, considered to be between 3,000 to 6,000 m water depth, cover vast areas of the Earth's surface. The majority of the abyssal seafloor is characterised by fine sediments, and these benthic habitats contain vast numbers of microbes with huge metabolic diversity. Abyssal microbes drive biogeochemical cycles, regulate fluxes of energy and contribute to organic carbon production and remineralization. So far, detailed assessments of the spatial distributions of benthic microbial communities in abyssal regions are still incomplete, and the influence of seafloor topography and heterogeneity on microbial distributions across a range of scales are poorly understood. Therefore, the aim of this thesis is to characterise the spatial variability of benthic microbes, which is essential for understanding their roles in benthic environments and for conducting baseline assessments of areas of the seabed that might be targeted by commercial mining activities. The spatial variation of benthic microbes in the Atlantic and Pacific oceans is the subject of three chapters, one of which focuses on the spatial variation of microbes associated with sediment and polymetallic nodules in the Clarion-Clipperton Fracture Zone (CCFZ) of the Pacific Ocean. The following chapter compares diversity and distribution of sediment microbes across three sites with varying productivity regimes in the Atlantic and Pacific. The final chapter examines the gut microbiome of holothurians, a dominant megafaunal group in the North-East Atlantic. This thesis showed that ammonia-oxidising Archaea (Thaumarchaeota) and nitrogen-cycling microbes were highly abundant in oligotrophic sediments and highlighted that these microbial taxa exhibited a spatial variability across small to large geographic scales, indicative of nîche diversity and resource partitioning. Given that chemolithoautotrophic taxa, such as ammonia-oxidisers and nitrifying microbes may form a significant source of organic carbon through inorganic carbon fixation and as microbes in general form the base of the marine food web, changes to microbial assemblages will likely have cascading impacts to higher trophic levels. Understanding the diversity patterns and functional roles of benthic microbes is vital for the sustainable use and preservation of ecosystems ahead of potential seabed exploration, exploitation and disturbance. Detailed observations are therefore necessary for modelling microbially mediated biogeochemical functions, monitoring biodiversity and ecological assessments for the preservation of ecosystem functioning.

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DECLARATION OF AUTHORSHIP

I, ...

Declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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Chapter 1: Introduction

1.1 Abyssal ecosystems

Abyssal ecosystems (~3,000 to 6,000 m water depth) are vast areas of plains, sloping hills, ridges and valleys that cover more than 50% of the Earth's surface (Smith *et al*., 2008b; MacDonald *et al*., 1996). The abyssal seafloor is characterised by fine sediments, mainly sands to clays (Vinogradova, 1997). Once thought to be generally lifeless, these sediments of these benthic habitats contain vast numbers of microbes with huge metabolic diversity (Whitman *et al*., 1998; Scheckenbach *et al*., 2010; Jørgensen & Boetius, 2007). Estimates suggest that half of all marine microbes reside in the sediments (Lloyd *et al*., 2013), numbering approximately 2.9 x 10^{29} sedimentary microbial cells (Kallmeyer *et al*., 2012). Owing to the size and remoteness of the abyss, however, ecosystem structure and function at the deep seafloor have historically been poorly studied. These microbial inhabitants not only dominate benthic communities in terms of abundance and biomass (Whitman, *et al*., 1998; Rex *et al*., 2006) but they are also key mediators in carbon and nutrient cycling in the sediments (Parkes *et al*., 1994; Jørgensen & Boetius, 2007).

Open ocean sediments of abyssal basins are largely oxic and are characterized by high hydrostatic pressure, low temperatures (-1° to 4°C), lack of sunlight, no photosynthesis and a low supply of organic matter (1-10 mmol C m^{-2} yr^{-1}) (Jørgensen & Boetius, 2007). Surface sediments of abyssal plains are a heterogeneous matrix of organic and inorganic, fine-grained particles (Inagaki *et al*., 2006, Jørgensen & Boetius, 2007) with different physical and chemical conditions compared to the water column (Whitman *et al*., 1998). Oxygen and nitrate concentrations extend further into the sediment column compared to coastal sediments, as a result of reduced exportation of organic material and lower sedimentation rates (D'Hondt *et al*., 2009). Although the majority of scientific interest has focused on hydrothermal vents, cold seeps and subsurface sediments, these habitats occupy less than 1% of the seafloor (Danovaro *et a*l., 2010). For this reason, this chapter will focus on the soft, surficial sediments of the seafloor and the microbes that inhabit them.

1.2 Food supply

1.2.1 Biological pump

Food availability is an important driver for deep-sea life and one of the ways that organic material reaches the deep sea is via the biological pump (Fig. 1). This is a term that incorporates a range of processes where carbon dioxide (CO_2) is fixed photosynthetically by phytoplankton in the euphotic zone and is transported down into the deep ocean as particulate organic matter (POM). The rain of sinking particles that descend through the water column are aggregates of dead cells, faecal pellets and phytoplankton detritus that form fluffy marine snow (Glover *et al*., 2010; Cedhagen *et al*., 2014). With the exception of hydrothermal vent systems and cold seeps, this type of organic matter is one of the main food sources for deep-sea organisms.

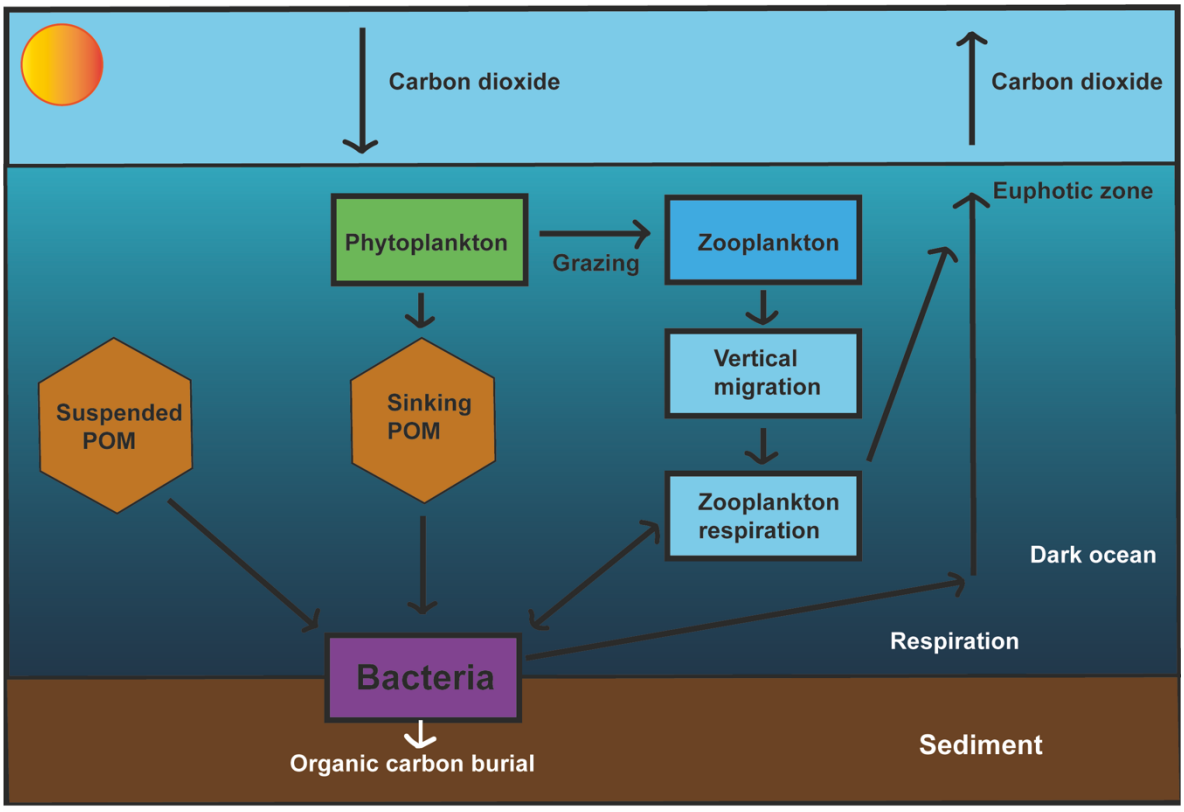

Figure 1. The Marine Biological Pump. The processes collectively known as the Biological Pump, where phytoplankton in the euphotic zone fix CO_2 from the atmosphere by photosynthesis. This particulate organic matter (POM) is grazed on by zooplankton and is exported to deeper water. Only a small fraction reaches the deep ocean. Bacteria remineralise some of this organic matter, and together with zooplankton respiration, returns some CO_2 back to the atmosphere. Less than 3% of carbon from the surface reaches the seafloor, where less than 1% is buried in the sediments. Adapted from Herndl, & Reinthaler, (2013).

Benthic communities in the food-limited abyssal habitats are influenced by primary production in the overlying water, yet only a small fraction reaches the sea floor in the form of POM (Smith, 1987; Ducklow *et al*., 2001). Particles of sinking POM are colonised by mesopelagic zooplankton feeding on heterotrophic microbes colonising the particles, further fragmenting the marine snow (Mayor *et al*., 2020). This process generates and releases a plume of dissolved organic matter (DOM), which continues to be broken down by microbes (Mayor *et al*., 2020). The DOM produced by POM degradation in the water column is labile and available for immediate microbial degradation and assimilation over short scales of minutes to days (Fuhrman, 1987). Most DOM remains in the upper water column and is used by heterotrophic microbes (Azam & Long, 2001; McCarren *et al*., 2010). Sinking material is partially consumed during transit in the water column by bacterial and zooplankton grazers, and also begins to remineralise (Ichino *et al*., 2015), before settling on the sea floor to be consumed by benthic organisms (Smith *et al.,* 2008b). The subsequent microbial breakdown and remineralisation of POM leads to an increase of CO_2 in the deep ocean (Herndl & Reinthaler, 2013). The biological pump therefore has important implications for the fate of carbon through the ocean, commencing with removal of carbon from the atmosphere, to the subsequent redistribution and burial in marine sediments in the deep ocean (Herndl & Reinthaler, 2013).

1.2.2 Food flux and benthic communities

Food availability is one of the main factors influencing benthic diversity, biomass and community structure in the deep sea (Billett *et al*., 1983; Smith *et al*., 1997; Pfannkuche *et al*., 1999; Rex *et al*., 2006; Jørgensen & Boetius, 2007; Gambi & Danovaro, 2006; Laguionie-Marchais *et al*., 2013; Ichino *et al.,* 2015; Amaro *et al*., 2019, Kuhnz *et al*., 2020). As a result of grazing and remineralisation, less than 3% of organic carbon produced in the upper ocean reaches the deep ocean (De la Rocha & Passow, 2007) and less than 1% is buried in marine sediment (Meysman *et al*., (2006; Dunne *et al.,* 2007). This exponential decrease in POM with depth reduces food availability by the

time it reaches the deep-sea floor (Ichino *et al*., 2015), so that animal abundance and biomass decline with depth (Rex *et al*., 2006). Previous research has indicated that food availability tends to have greater impact on larger organisms such as megafauna and fishes, and less so on smaller organisms such as meiofauna and microbes (Rowe *et al*., 2008). In contrast to larger fauna, bacteria do not appear to follow this depth-related pattern. Bacterial biomass and abundance vary little with depth and dominate at depths of over 4000 m (Wei *et al*., 2010). Yet, microbes appear to utilise the most carbon out of all biotic groups, with up to 96% of the available carbon at abyssal plain sites utilised by the dominant bacterial and meiofauna taxa (Rowe *et al*., 2008).

Allochthonous and autochthonous sources provide organic particles to the deep sea. The supply of food to abyssal habitats can be continuous or episodic, ranging from major flux events such as phytoplankton and whale falls (Billett *et al*., 1983; Smith *et al*., 1997; Jørgensen & Boetius, 2007), to lateral transport of POM from continental margins (Smith *et al*., 2001). The export of POM to the deep sea is also seasonal, particularly in higher latitudes, where salp and phytoplankton blooms leave a thick layer of detritus on the seafloor (Pfannkuche & Lochte, 1993; Ruff *et al*., 2014). The variability of POM influences patterns of faunal abundance, diversity and ecosystem functioning and structure, for megafauna (Ruhl & Smith, 2004; Smith *et al*., 2009; Billett *et al*., 2010; Amaro *et al*., 2019, Kuhnz *et al*., 2020), macrofauna and meiofauna (Gambi & Danovaro, 2006; Laguionie-Marchais *et al*., 2013). Yet, previous research has suggested that the responses of microbes to seasonal fluxes of POC flux have not been as clear (Smith *et al*., 1997; Gooday, 2002; Moeseneder *et al*., 2012; Laguionie-Marchais *et al*., 2013). Observations of dramatic increases in abundances in benthic microbes similar to those observed in megafauna are not as common (Turley & Lochte, 1990; Pfannkuche, 1993; Moodley *et al*., 2005). Connecting benthic bacterial response with primary productivity is less straightforward for microbes compared to megafauna, which exhibit visible changes in abundance. Previous studies have shown that increased productivity influenced the diversity of benthic bacterial communities (Schafer *et al*., 2001; Horner-

Devine *et al*., 2003). Response times of microbes and meiofauna appeared to lag behind those of macrofauna (Witte *et al*., 2003), which had rapid responses to POM flux (Snelgrove *et al*, 1992, 1996).

1.2.3 Microbial responses to food supply

Previous studies have suggested that bacteria show most rapid and marked response to seasonal POM inputs (Gooday, 2002), whether this was through *in-situ* studies, incubation experiments or estimations based on models. *In-situ* experiments to calculate benthic bacterial biomass in the North Atlantic showed that sediment community oxygen consumption (SCOC) doubled between March and July following a POM pulse (Pfannkuche, 1993). This also coincided with maximal chlorophyll-*a* concentrations in the sediment. In contrast, the meio-, macro- and megafauna exhibited no change in biomass during the same timeframe. Incubation experiments and mesocosms have yielded similar results. Pressure-chamber incubation experiments showed that sediment microbes responded rapidly to inputs of organic carbon via growth and detritus decomposition (Turley & Lochte, 1990). Similarly, the response times of deep-sea benthic bacteria using incubation experiments and ^{13}C-enriched algal carbon as a tracer, showed that the bacteria responded rapidly to fresh inputs of organic material at sights in the Mediterranean and North Atlantic (Moodley *et al*., 2005). However, this study was conducted at ambient temperature and pressure, so microbial responses may have been subject to pressure-related artifacts, as a result of non-piezophilic microbial growth. One study showed a moderate correlation between microbial biomass in the top 5 mm of sediment beneath highly productive areas and annual POM flux (Smith *et al*., 1997). It appeared that the benthic microbes responded more quickly to POM inputs than the mega- and macrofauna, due to rapid cell turnover in microbial populations (Smith *et al*., 1997). This study suggested that the response to POM inputs measured in biomass was more enhanced for the megafauna than for the microbes.

Other types of microbial response to POM inputs and fluxes include changes in metabolism and enzyme activity. One of the first responses to the input of POM was shown to be bacterial extracellular activity in the surface sediments in NE Atlantic (Pfannkuche *et al*., 1999). This study showed higher values of enzyme

activity in March and August after phytoplankton blooms than during other months by measuring metabolites in the sediment (Pfannkuche *et al*., 1999). Likewise, an increase of microbial ATP, adenylates and SCOC rates were detected in sediments in NE Atlantic during summer months following a spring bloom (Pfannkuche, 1993). This same pattern was observed over several years, suggesting a seasonal pattern, which correlated with the timing of phytoplankton and salp blooms in the area and therefore increased food availability. Based on experiments that simulated deep-sea conditions, this study calculated that microbes were responsible for 60-80% of the increase in carbon turnover in the sediment following a seasonal phytodetritus flux (Pfannkuche, 1993).

Not all studies have suggested such dramatic differences in microbial responses to POM flux. Some studies found little difference in microbial cell numbers between oligotrophic sites and eutrophic sites, showing no effect of more productive areas on POM fluxes upon microbial communities (Parkes *et al*., 2000; D'Hondt *et al*., 2004). These were subsurface microbial communities, however, that would have utilised other energy sources such as Mn and Fe. One study using shipboard incubations to measure mean respiration and metabolism, found little to no effect of surface productivity on benthic marine microbes (Leduc *et al*., 2016). Instead, there were positive correlations between food availability at the surface and mean respiration and metabolism of benthic meio-, macro- and megafaunal groups. The greatest effect of food availability was observed in larger organisms, such as megafauna. Differences in food supply had a dramatic impact on biomass of megafauna, up to 20 times greater in productive areas but showed less of an impact on biomass of meiofauna and microbes (Leduc *et al*., 2016). These observations raise questions regarding a mixed or minimal heterotrophic microbial response to fluxes in POM, given that benthic microbes are so abundant and dominant in abyssal sediments.

1.2.4 Future climate change

The coupling of the abyssal ecosystem with the surface ocean has important implications for deep-sea microbes in light of future climate change. Global ocean models predict that with increased atmospheric CO_2, primary productivity will increase, which will impact on benthic communities (Yool *et*

al., 2017; Sweetman *et al*., 2017). Biogeochemistry is likely to be substantially altered with increasing atmospheric CO_2, higher sea surface temperatures and ocean stratification. These changes could lead to changes in plankton community composition, which will not only lead to lower POC flux to the deep ocean, but also the nutritional characteristics of the POC that reaches the seabed (Wigham *et al*., 2003; Hudson *et al*., 2004). As a result, phytodetritus that reaches the seabed is likely to be more refractory and much less abundant. Reductions in POC flux could lead to reduced SCOC, lower bioturbation levels, a smaller sediment mixed layer and lower biomass of benthic fauna (Smith *et al*., 2008b). These factors are likely to influence nutrient cycling, carbon burial and calcium carbonate dissolution in the deep sea (Snelgrove *et al*., 2004; Smith *et al*., 2008b). Reduction of and changes in the quality of POC flux are likely to significantly alter the structure, function and biodiversity of abyssal habitats over geological timescales (Smith *et al*., 2008b), which has implications for the flow of energy through these food-limited systems.

1.3 Role of benthic microbes in biogeochemical cycles

The export of organic matter to the deep ocean is fundamental for deep-sea life, including benthic microbes. The sinking supply of organic particles that originate from the upper water column is one of the primary drivers of biogeochemical cycling in the deep ocean (Dunne *et al*., 2007; Middelberg, 2011) and is a process that is mediated by microbes. Produced in the surface waters by autotrophic organisms, this energy-rich POM forms an important substrate for many organisms in the deep ocean. In the absence of sunlight, photosynthesis is replaced by a number of additional energy pathways, where metabolic energy is harnessed via the coupling of reducing and oxidizing (“redox”) reactions (Orcutt *et al*., 2011). A number of energy-yielding pathways exist in the dark ocean, and in abyssal sediments include oxic respiration, nitrification and sulfur oxidation (Table1).

Table 1. Common redox reactions that occur in the dark ocean, illustrating the variety of metabolic pathways. Some of these processes occur at hydrothermal vents and cold seeps. Pathways discussed in this thesis are shown in blue. Adapted from Orcutt *et al*., (2011).

Pathway	Reaction
Oxic respiration	$CH_2O + O_2 \rightarrow CO_2 + H_2O$
Nitrification	$NH_4^+ + O_2 \rightarrow NO_3^- + H^+ + H_2O$
Denitrification	$CH_2O + NO_3^- \rightarrow CO_2 + N_2 + HCO_3^- + H_2O$
Anammox (anaerobic ammonia oxidation)	$NH_4^+ + NO_2^- \rightarrow N_2 + H_2O$
Mn(II) oxidation	$Mn^{2+} + O_2 \rightarrow MnO_2$
	$Mn^{2+} + NO_3^- + H_2O \rightarrow MnO_2 + N_2 + H^+$
MnO_2 reduction	$CH_2O + CO_2 + H_2O + MnO_2 \rightarrow Mn^{2+} + HCO_3^-$
Fe(III) oxide reduction	$CH_2O + CO_2 \rightarrow Fe(OH)_3 \rightarrow Fe^{3+} + HCO_3^- + H_2O$
Fe(II) oxidation	$Fe_2^+ + O_2 + H^+ \rightarrow Fe_3^+ + H_2O$
	$Fe_2^+ + NO_3^- + H^+ \rightarrow Fe_3^+ + H_2O + N_2$
	$Fe_2^+ + MnO_2 + H^+ \rightarrow Fe_3^+ + H_2O + N_2$
Sulfate reduction	$CH_2O + SO_4^{2-} \rightarrow HCO_3^- + H_2S$
Sulfate reduction (from methane)	$CH4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$
Sulphide oxidation	$H_2S + O_2 \rightarrow SO_4^{2-} + H^+$
	$H_2S + NO_3^- \rightarrow SO_4^{2-} + N_2 + H_2O + H^+$
Methanogenesis (from acetate)	$CH_3COOH \rightarrow CH_4 + CO_2$
Methanogenesis (from H_2/CO_2)	$H2 + HCO_3^- + H^+ \rightarrow CH_4 + H_2O$
Methane oxidation / methanotrophy	$CH_4 + O_2 \rightarrow CO_2 + H_2O$

N-damo (nitrite/nitrate-dependent anaerobic methane oxidation)	$CH_4 + NO_2^- \rightarrow CO_2 + N_2^-$ $CH_4 + NO_3^- \rightarrow CO_2 + NO_2^-$
Fermentation (from ethanol)	$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + H_2$
Fermentation (from lactate)	$CH_3CH_2COO^- + H_2O \rightarrow CH_3COOH + HCO_3^- + H_2$
Acetogenesis	$H_2 + CO_3^- + H^+ \rightarrow CH_3COO^- + H_2O$

The most common electron sources that are available to microbes in the deep ocean are organic matter, oxygen, ammonium (NH_4^+; produced by the degradation of organic matter), nitrate (NO_3^-; formed mainly from nitrification of ammonium), nitrite (NO_2^-; formed from nitrification of ammonium), oxidised sulfur compounds, manganese and iron oxides and carbon dioxide (Orcutt *et al*., 2011). Those most relevant to oxidised surficial sediments are discussed here.

1.3.1 Organic matter

The most dominant substrate in the dark ocean is organic matter, derived from the upper water column, terrestrial sources, or is produced in situ by microbes (Orcutt *et al*., 2011). The high redox potential of OM in abyssal habitats provides an important energy source for benthic microbes and metazoans. It's utilization, however, depends on its availability, quality and reactivity. Freshly deposited material is utilized faster, whereas older material in the sediments is more refractory and resistant to degradation (Rice *et al*., 1986; Kaiser & Benner, 2009). The microbial turnover of OM in abyssal sediments is further influenced by the bioturbation activity of benthic macro- and megafauna (Niggermann *et al*., 2007). Microbial remineralization converts OM into inorganic carbon dioxide, where microbes utilise hydrolysis and fermentation to break down longer, complex chains of molecules into short-chain, smaller molecules (Orcutt *et al.,* 2011). Carbon dioxide is therefore the final carbon product in its oxidized form after organic matter degradation and remineralization.

1.3.2 Oxygen

Oxygen, produced by photosynthesis in the photic zone, has the highest redox potential of the electron acceptors in the ocean. Mixing and ocean circulation bring oxygen into the lower water column. Compared to sediments on continental slopes, microbial cell densities and remineralisation rates are lower in deep-sea sediments (D'Hondt *et al*., 2004, 2009). Low carbon remineralisation rates in abyssal sediments means that oxygen is consumed slowly and can diffuse from centimetres to metres into the sediment (D'Hondt *et al*., 2009). In areas of moderate primary productivity, organic matter is consumed within the first few centimetres of sediment, whereas in oligotrophic, organic matter-poor areas, oxygen can penetrate the sediments for metres (D'Hondt *et al*., 2009). In addition, aerobic respiration is a useful parameter as oxygen consumption rate is used to measure microbial activity in sediments (Glud, 2008). Sediment community oxygen consumption (SCOC) is a helpful indicator of food demand and carbon remineralization in the benthic ecosystem (Ruhl *et al*., 2008).

1.3.3 Ammonium

The most reduced form of nitrogen is ammonium, which comprises an important electron donor in the deep sea and is formed by the breakdown of nitrogen-rich organic matter (Orcutt *et al*., 2011). Another possible source of ammonium is via anaerobic methane-oxidising archaea that fix nitrogen by converting dinitrogen (N_2) into ammonia (NH_3) for assimilation (Dekas *et al*., 2009). In largely oxic abyssal sediments, this pathway is likely to be minimal, however, low-level cryptic cycling can occur in environments where it is not expected (Holmkvist, 2011). Another possible source of ammonia is urea produced by metazoans (Alonso-Saez *et al*., 2012), which some groups of microbes are capable of utilising. The two main ammonium-utilisation pathways include ammonium oxidation with oxygen (nitrification) and anaerobic ammonium oxidation (anammox). In oxic, surficial sediments, ammonia oxidation with oxygen is the most dominant, a pathway utilised by autotrophic groups of Thaumarchaeota.

1.3.4 Nitrite and nitrate

In the dark ocean, nitrate is produced by degradation of organic matter or by nitrification. Here, ammonia (NH_3) released from organic matter decay is converted to nitrite (NO_2), mediated by ammonia-oxidising archaea (AOA) or ammonia-oxidising bacteria (AOB) (Bock & Wagner, 2013). The oxidation of nitrite to nitrate (NO_3) is catalysed by nitrite-oxidising bacteria (NOB), and these processes provide an important source of organic material to the benthic community (Middelburg, 2011). In oxic sediments, this nitrate is either utilised in the process of denitrification, where nitrate is converted to nitrite and N_2 in a series of steps by two different microbial groups. Alternatively, nitrate is used in the dissimilatory nitrate reduction to ammonium (DNRA) pathway, usually in a microaerobic environment (Lam *et al*., 2009). This pathway may only have a minor role in nutrient-poor abyssal sediments, however (Zhao *et al*., 2019).

1.3.5 Nutrient cycling in abyssal sediments

The surficial sediments of the deep-sea are often referred to as a bioreactor for organic matter cycling (Danovaro *et al*., 2003; Luna *et al.,* 2012). The process of organic matter break-down and remineralisation in oxygenated sediments heavily involve microorganisms (Danovaro *et al*., 2014), hence nutrient availability in abyssal habitats is intrinsically linked with microbes. Carbon availability depends on the processes of POM remineralisation in sinking particles through the water column (McCarren *et al*., 2010) through to degradation and burial within the sediments (Rowe & Deming, 2011). In addition to carbon cycling and turnover, microbes are also intimately linked to nitrogen cycling. Nitrogen is often a limiting nutrient for primary production in the ocean (Gruber, 2004) and has various reduced and oxidised forms, from ammonium (NH_4^+) to nitrate (NO_3^-). The redox cycling between these nitrogen compounds is the basis for various microbial processes. The oxidation of ammonium to nitrite (NO_2^-), then to nitrate, linked via the two step chemolithoautotrophic processes of microbial nitrification (Herbert *et al*., 1999) are important in sediment nitrogen cycling and turnover (Brunnegård *et al*., 2004). Hence, microbes play important roles in biodiversity, ecosystem

functioning and biogeochemical cycles in abyssal habitats (Parkes *et al*., 1994; Jørgensen & Boetius, 2007).

1.4 Inorganic carbon fixation in the dark ocean

Inputs of organic carbon to the deep ocean help sustain the metabolisms of benthic assemblages and drive food webs, which are dominated by microbes (Turley, 2000; Dell'Anno & Danovaro, 2005; Jørgensen & Boetius, 2007). Yet the supply of POC does not always appear to match the demand of abyssal ecosystems, which has been previously highlighted by other studies (Berelson *et al*., 1997; Smith & Kaufmann, 1999; Smith *et al*., 2002, 2013, 2014; Ruhl et al., 2008; Giering *et al*., 2014). Many studies have demonstrated a tight coupling between POC flux and benthic fauna responses, such as sediment community respiration (Smith & Kaufmann, 1999; Smith *et al*., 2001, 2009) and abundance and biomass of macrofauna (Smith *et al*., 2008b; Ruhl *et al*., 2008), though this correlation was less strong for microbes (Smith *et al*., 2008b). Yet, there remains an imbalance between food supply and demand (Smith & Kaufmann, 1999; Smith *et al*., 2001). Deficits in POM flux do not match the utilisation of organic matter by the benthic community, measured by SCOC rates, raising questions about the sustainability of this food supply and its impact on the sediment community (Smith *et al*., 2001). There has been a long-held view that benthic heterotrophic microbes provide a labile source of carbon for metazoans, which helps to sustain the numerically dominant deposit feeders (Newell *et al*., 1984; Richardson & Young, 1987). Previous estimates have suggested that up to 90% of the benthic biomass is microbial (Danovaro *et al*., 2016). Yet, despite the apparent high biomass and abundance of deep-sea benthic microbes, respiration, growth rates and low rates of organic carbon remineralization of microbial heterotrophs appear to be low (Rowe & Deming, 2011).

It is now recognized that chemosynthetic production, or so-called 'dark energy', plays an important role in deep-sea sediments. Chemoautotrophic organisms produce organic matter by assimilating inorganic carbon, using reduced chemical compounds as an energy source (Swan *et al*., 2011). It is possible that inorganic carbon fixation may help supplement the food supply deficit via chemolithoautotrophy, by producing a source of organic carbon for benthic

communities (Brunnegård *et al*., 2004; Middelburg, 2011; Molari *et al*., 2013). It is increasingly appreciated that chemolithoautotrophic microbes form a significant source of organic carbon through inorganic carbon fixation (Swan *et al*., 2011; Middelberg, 2011; Molari *et al*., 2013; Tully & Heidelberg, 2016; Sweetman *et al*., 2019), which in some regions, could exceed phytoplankton export production (Reinthaler *et al*., 2010). It is estimated that benthic chemoautotrophy production in the deep sea is 0.37 PgC/year, of which 0.004 PgC/year occurs in deep-sea sediments (Middelberg, 2011). Although chemoautotrophic production is lower in sediments, this process is a potential source of organic carbon for the benthic community (Molari *et al*., 2013; Sweetman *et al*., 2019). Moreover, inorganic carbon fixation rates can account for up to 25% of total heterotrophic biomass production, and benthic Archaea contribute to this chemosynthetic primary production (Molari *et al*., 2013). These inorganic carbon incorporation rates appear to correlate with the relative abundance of Archaea within benthic assemblages, which are also capable of exploiting organic compounds at similar rates to heterotrophic Bacteria (Molari *et al*., 2013).

Nitrification is an important process in benthic nitrogen cycling and accounts for a large proportion of the chemoautotrophic carbon fixation in the deep ocean (Herndl *et al*., 2005; Wuchter *et al*., 2006; Middelberg, 2011), although the extent to which inorganic carbon fixation may meet the carbon requirements of the abyssal community is still unclear (Sweetman *et al*., 2019). In the dark ocean, it was assumed that the main type of autotrophy was CO_2 fixation through the 3-hydroxypropionate/4-hydroxybutyrate cycle, a pathway utilised by ammonia-oxidising archaea (Wuchter *et al*., 2007; Berg *et al*., 2007). It has since been recognised that a number of microbial lineages use chemoautotrophic pathways in the dark ocean, including bacterial nitrifiers and methylotrophs (Swan *et al*., 2011). Some lineages within the β-subgroup and γ-subgroup of *Proteobacteria* are mixotrophs that are capable of autotrophic CO_2 fixation and couple this reaction with the oxidation of reduced sulfur compounds within anoxic micro-nîches such as particles of POM (Swan *et al*., 2011). Lineages within the Thaumarchaeota phylum, which is abundant in deep sea sediments and include ammonia oxidising archaea, are putatively heterotrophic (Aylward & Santoro, 2020). Likewise,

ammonia oxidising, carbon-fixing taxa within this phylum, *Candidatus "Nitrosopumilus maritimus*", which are predominantly autotrophic, are also capable of heterotrophy (Ingalls *et al*., 2006). Inorganic carbon fixation rates in benthic sediments are therefore likely to be a combination of chemolithoautotrophic, heterotrophic and/or mixotrophic activities as the pathway of labelled organic substrates have suggested (Molari *et al*., 2013). In oxygenated deep-sea surface sediments, however, ammonia oxidation by numerically dominant AOA is still an important pathway for chemoautotrophy and inorganic carbon fixation (Könneke *et al*., 2005; Wuchter *et al*., 2007; Molari *et al*., 2013), so inorganic carbon fixation rates by ammonia oxidisers are still likely to be significant.

Quantifying rates of inorganic carbon fixation in abyssal sediments have previously been attempted, however, results have often been mixed. In some case inorganic carbon fixation has reportedly been too minor to compensate for carbon demand of abyssal assemblages (Christensen & Rowe, 1984; Sweetman *et al*., 2019), yet other research has shown more significant rates (Brunnegård *et al*., 2004; Molari *et al*., 2013). Previous studies have also highlighted that although significant, archaeal nitrification rates may not be sufficient to offset this demand for organic carbon by the benthic community (Molari *et al*., 2013; Christensen & Rowe, 1984). In some cases, however, ammonia-oxidising Archaea were not considered in the calculations or possible pressure-related artifacts were present (Sweetman *et al*., 2019; Molari *et al*., 2013). To date, there has only been one in-situ study attempted (Sweetman *et al*., 2019), however, this study focused largely on the role of bacterial taxa and excluded Archaea. The paucity of *in-situ* studies highlights the need for further analysis in light of conflicting results, in order to identify the importance of inorganic carbon fixation in abyssal ecosystems. Dissecting the key players within chemoautotrophic assemblages should be a future priority, especially given that benthic microbes have key roles in carbon cycling and the limited availability of labile organic matter to benthic abyssal communities.

1.5 Spatial variability and distribution

1.5.1 Biogeography of benthic microbes

In addition to understanding how microbes contribute to biogeochemical cycles, it is essential to ascertain their composition and distribution in seafloor sediments. Knowledge of the geographic distribution and connectivity of microbial populations is still incomplete, particularly the extent and over what scales these communities vary. The patterns of beta diversity across spatial scales, or biogeography, has become a well-established field of interest in marine microbial ecology (Magurran, 2004). Biogeography relates to the genetic or phenotypic variation across different scales, geographical distances or environmental gradients (Ramette & Tiedje, 2007). Researchers have long recognized the importance of explaining species distributions, abundances and interactions within communities. Yet explaining the observed variation in the distribution of marine microbial populations has remained elusive. Multiple existing theoretical frameworks aim to explain microbial spatial patterns by identifying the complex mechanisms and processes which are responsible for shaping the observed patterns of species distributions (Hanson *et al*., 2012).

One approach, using metacommunity theory (Leibold *et al.,* 2014) and the neutral theory of biodiversity (Hubbell, 2001), show that both environmental variables and genetic processes influence microbial biogeography, explained as four, simplified mechanisms encompassed within the metacommunity concept (Leibold *et al*., 2014). The interaction of four main processes are responsible for shaping microbial biogeographic patterns across ecological scales: selection, drift, dispersal and mutation (Hanson *et al*., 2012). The process of selection distinguishes microbial composition among locations, where environmental conditions select for taxa that are better suited for those local conditions. The process of genetic drift, or the amount of genetic variation which has no effect on an individual's fitness, also changes microbial composition with spatial distance (Hanson *et al*., 2012). Dispersal abilities (the movement and establishment from one location to another) vary among microbial taxa, and may take place through passive or active

mechanisms. The process of mutation generates non-directed, permanent alterations in nucleotide sequences, which either multiply or are removed by natural selection. Linking these four processes is the most widely reported pattern in microbial biogeography studies, the distance-decay relationship, where populations become less similar with increasing geographic distance. The processes of selection and drift strengthen the distance-decay relationship, where microbial communities become more distinct with increasing spatial distance. Dispersal has the opposite effect and counteracts the influences of selection and drift. The influence of mutation increases local genetic diversity across all locations, increasing variance or "noise" and making the composition of populations distinct between locations, regardless of increasing distance between them (Hanson *et al*., 2012).

Another approach is the existence of endemic taxa which are restricted in their distribution and provide evidence of microbial biogeography. Some benthic microbes, including symbionts for instance, may have reduced dispersal abilities, particularly with low sedimentation rates and limited mixing of particles (Jørgensen & Boetius, 2007). There is evidence, however, that this is likely to be an over-simplification. Whilst some taxa appear to be cosmopolitan (Finlay, 2002; Scheckenbach *et al*., 2010; Bik *et al*., 2012), other studies have shown taxa endemic to a geographic area (Pommier *et al*., 2007; Zinger *et al*., 2011; Jacob *et al*., 2013; Bienhold *et al*., 2016; Martiny *et al*., 2006 and refs therein). To what extent these assumptions influence low abundance populations, or rare taxa, which are still under-sampled and highly diverse, is also unclear (Sogin *et al*., 2006). The evidence in support of either microbial cosmopolitanism or endemism within a region is hampered by artefacts of coarse taxonomic resolution, unequal- and under-sampling of abyssal benthic habitats (Green & Bohannan, 2006), which can lead to false conclusions and biases in interpreting spatial microbial diversity. Disentangling which factors are responsible for driving patterns of microbial distribution appears to be less than straightforward.

1.5.2 Environmental heterogeneity as driver for spatial variability

Linking microbial distribution to environmental heterogeneity in abyssal habitats is essential when considering biogeographic patterns of benthic microbes. Environmental heterogeneity and spatial distances reportedly influence microbial diversity (Whitaker *et al*., 2003; Hughes Martiny *et al*., 2006; Ramette & Tiedje, 2007), however, the role of these factors in determining the distribution and biogeography of marine microbes is still poorly understood. Whilst vertical and horizontal broad-scale patterns in microbial beta-diversity have been observed in the water column and across different benthic habitats (Parkes *et al*., 2000; D'Hondt *et al*., 2002; DeLong *et al*., 2006; Fuhrman *et al*., 2008; Lipp *et al*., 2008; D'Hondt *et al*., 2004; Caporaso *et al.*, 2012; Durbin & Teske, 2011; Zinger *et al*., 2011 and references therein; Gilbert *et al*., 2012; Schippers *et al*., 2012; Shulse *et al*., 2016), a lack of replication within sediment microbial studies has still not been fully resolved.

Spatial isolation is more likely in sediments compared to the water column, with horizontal geographic distances autocorrelating with changes in benthic community composition (Zinger *et al*., 2011). This distance-decay relationship is likely owing to dispersal limitation and reduced physical mixing in sediments compared to the water column (Zinger *et al*., 2011; Green & Bohannan, 2006). Despite appearing to be a relatively homogenous habitat, the abyssal seafloor is characterised by varying seafloor bathymetry, including topographical features such as valleys, ridges and plains (MacDonald *et al*., 1996). These features have been reported to influence the diversity and distribution of metazoans (Cosson *et al*., 1997; Durden *et al*., 2015; Stefanoudis *et al*., 2016; Leitner *et al*., 2017; Simon-Lledó *et al*., 2019; Durden *et al*., 2020), linked to the influence of topography on bottom currents, sediment grain size, carbon flows around abyssal hills (Durden *et al*., 2015, 2016) and availability of labile organic matter (Horner-Devine *et al*., 2003; DeLong *et al*., 2006; Durden *et al*., 2015; Morris *et al*., 2016). These factors would also be likely to play a role in determining distributions of benthic microbial communities (Schauer *et al*., 2010). With the exception of

organic matter availability, which has been shown generally to structure the composition of deep-sea communities, including microbes (Zinger *et al*., 2011; Bienhold *et al*., 2012, 2016), these factors have not been assessed at large landscape scales. The scale of environmental heterogeneity is a key consideration given the small size of microbes. Basin-scale (Schauer *et al*., 2010; Zinger *et al*., 2011) and coarser resolutions such as sinking particles, water column, sediment and manganese nodules (Durbin & Teske, 2011; Wu *et al*., 2013; Tully & Heidelberg, 2013; Blöthe *et al*., 2015; Shulse *et al*., 2016; Tarn *et al*., 2016; Lindh *et al*., 2017, Duret *et al*., 2019; Molari *et al*., 2020) have all been shown to influence microbial community composition. Endemism has also been reported in deep sea sediments at small to moderate scales (metres to kilometres) (Dang *et al*.., 2009; Li *et al*., 2009; Tian *et al*., 2009; Schauer *et al*., 2010; Jacob *et al*., 2013; Ristova *et al*., 2015). Likewise, diversity patterns may alter at very small scales (μm), where micro-gradients of geochemistry and dissolved organic carbon (DOC) within sediments exist (Tully & Heidelberg, 2016).

1.5.3 Taxa distributions

Sediment microbial diversity composition varies according to the oxygen content, carbon content and sediment depth (Jørgensen *et al*., 2012; Orcutt *et al*., 2011). Oxic, surficial sediments harbour diverse assemblages of Bacteria. Some groups of taxa appear to be widespread in abyssal surface sediments, such as high abundances of Gammaproteobacteria, Deltaproteobacteria, Alphaproteobacteria, Acidobacteria, Actinobacteria, Chloroflexi and Planctomycetes (Zinger *et al*., 2011; Ruff *et al*., 2015; Bienhold *et al*., 2016). Archaeal diversity, on the other hand is generally lower, with the most dominant group represented by the Thaumarchaeota phylum (Durbin & Teske, 2010; Jørgensen *et al*., 2012; Nunoura *et al*., 2012). The ubiquitous distribution of marine archaeal and bacterial nitrifying microbes has previously been documented and have been spatially autocorrelated with environmental variables such as habitat and location (O'Mullan & Ward, 2005; Park *et al*., 2014; Qin *et al*., 2014; Bayer *et al*., 2016), substrate (Wu *et al*., 2013; Blöthe *et al*., 2015; Molari *et al*., 2020; Jing

et al., 2018), redox regime (Labrenz *et al*., 2010), latitude (Reinthaler *et al*., 2010; Sintes *et al*., 2016), nutrient availability (Alonso-Sáez *et al*., 2012;) and carbon input (Danovaro *et al*., 2016; Wang *et al*., 2017). Functional taxa, such as AOA and nitrifiers have been shown to vary spatially in sediments (Francis *et al*., 2005; Danovaro *et al*., 2016; Peoples *et al*., 2019; Molari *et al*., 2020; Kerou *et a*l., 2021; Zarate *et al*., 2021).

Despite a low carbon input, oligotrophic sediments harbour a diverse array of taxa implicated in high molecular weight (HMW) compound processing. This includes lineages within the abundant Planctomycetes phylum, a dominant component of benthic abyssal assemblages and is a diverse group capable of breaking down complex molecules in sediments and marine snow (Glöckner *et al*., 2003; Bondoso *et al*., 2017; Dedysh *et al*., 2019) as well as sulfur oxidation (Jørgensen *et al*., 2012). Some lineages within this phylum are also possible AOB (Kellogg *et al*., 2016). Abyssal sediments also harbour diverse communities of microbes, that previous research suggests are comprised of rare taxa with a high degree of endemism (Bienhold *et al*., 2016). This is perhaps due to specialisation of taxa adapted to specific ecological nîches associated with energy-limited conditions (Tully & Heidelberg, 2013; Molari *et al*., 2020).

Oligotrophic, oxygenated sediments in particular harbour high abundances of nitrifiers, such as *Nitrosopumilus, Nitrospira, Nitrospina, AqS1* and Nitrosomonadaceae (Tully & Heidelberg, 2013; Shulse *et al*., 2016; Molari *et al*., 2020). Nitrifiers, such as Thaumarchaeota, *Nitrospina* and AOB *Nitrosococcus* are known to occur together in sediments (Jørgensen *et al*., 2012). AOA Thaumarchaeota usually are among the most dominant group in oxic surficial sediments, where aerobic growth occurs in the surface layers (Könneke *et al*., 2005; Jørgensen *et al*., 2012). Given that chemolithoautotrophic taxa such as AOA and nitrifiers are important for nutrient supply to deep-sea sediments (Glud *et al*., 2013; Tarn *et al*., 2016), the spatial distributions of these groups have implications for benthic communities, especially in carbon-limited environments. Notably, multiple lineages and ecotypes have been recorded for AOA linked to environmental conditions (Francis *et al*., 2005; Park *et al*., 2014; Sintes *et al*.,

2016; Kerou *et al*., 2021), and this nîche separation highlights a complexity in benthic microbes that warrants further exploration.

To what extent microbial assemblages in abyssal sediments vary over spatial scales, from centimetre scale (Jørgensen *et al*., 2012; Walsh *et al*., 2016a,b) to moderate and basin-scales (Bienhold *et al.*, 2016), is still not thoroughly resolved, particularly coupled with redox regime and POM supply. In particular, spatial variation with depth through the sediment correlates with the geochemistry of the sediments (Durbin & Teske, 2011; Roussel *et al*., 2009; Fry *et al*., 2008; Jørgensen *et al*., 2012). Characterising the finer scale patterns of diversity and composition of benthic microbial assemblages is critical, particularly in regions where widespread ecological disturbance on the seafloor is likely (Ingels *et al*., 2020)

1.6 Microbiomes and nîche partitioning

Symbiotic associations between microbes and metazoans may offer a mechanism that enables organisms to cope with a limited or unpredictable food supply. Many examples exist in the deep-sea, that include holothurians, nematodes (Amaro *et al*., 2012), siboglinid tubeworms, crustaceans, bivalves (Boutet *et al*., 2011) and *Osedax* worms that colonize whale bones (Rouse *et al.*, 2004). Holothurians (sea-cucumbers) form a dominant part of the abyssal megafauna (Billett 1991; Billett *et al*., 2001, Ruhl *et al*., 2014) and these invertebrates form an important link with benthic microbes through their feeding activities (Amaro *et al*., 2012). Holothurians feed on microbes and organic particles in the sediment, (Amaro *et al*., 2012), which are a valuable source of nutrition for these animals. The intestines of holothurians harbour diverse communities of enteric microbes (Deming & Colwell, 1982; Roberts *et al*., 2001), with the highest concentration of microbes in the hind gut (Roberts *et al*., 2000; Amaro *et al*., 2009; Rowe and Deming, 2011; Amaro *et al*., 2012). These animals are responsible for quick sediment turnover and phytodetritus removal from the top 5 cm of sediment (Ginger *et al*., 2001). This foraging activity may not only influence the structure and distribution of sediment microbial communities (Gao *et al*., 2014) but also benthic nutrient cycling (Yamazaki *et al*., 2015) and carbon processing (Durden *et al*., 2016).

These actions may significantly alter ecosystem function (Patrick & Fernandez, 2013), yet the effect of holothurians on microbial communities and ecosystem functioning is still largely unknown.

Megafaunal responses to POM fluxes have been well documented, particularly for holothurians. The Porcupine Abyssal Plain (PAP) in the NE Atlantic is characterised by seasonal pulses of phytodetritus (Rice *et al*., 1994), which is short-lived once it reaches the seabed (Lampitt, 1985). Mass aggregations of phytodetritus observed on the seabed between 1991-1994 at PAP (Bett *et al*., 2001) led to increases in abundances of holothurians and ophiuroids responding to this food source, known as the "*Amperima* Event". In particular, large holothurians dominated the megafauna (Glover *et al*., 2010). These dramatic increases in holothurian individuals have been observed over a number of years in both the Atlantic (Billett *et al*., 2001; Billett *et al*., 2010) and Pacific (Smith *et al*., 2014; Huffard *et al*., 2016). Due to the ephemeral nature of the deposited phytodetritus, competition for this food-limited resource is high among benthic feeders (Billett, 1991; Roberts & Moore, 1997). Holothurians adopt a number of different feeding strategies, based on body morphology, mobility and digestive capabilities (Iken *et al*., 2001), which includes free-swimming pelagic animals and sedentary infaunal deposit feeders that feed head down in the sediment (Billett, 1991; Iken *et al*., 2001). These different strategies enable holothurians to exploit deposited phytodetritus and reduce competition (Iken *et al*., 2001). Some holothurian species are able to forage on more refractory organic matter and may utilise enteric microbes to help break down and assimilate recalcitrant material. Hence, strong competition between megafaunal feeding types for availability of POM has resulted in resource partitioning and nîche separation of sympatric holothurian species at PAP (Billett, 1991; Roberts & Moore, 1997).

The selective feeding behaviour of holothurians is likely to have an impact on carbon and nitrogen remineralisation in abyssal habitats (Witbaard *et al*., 2001), but this has yet to be quantified. Despite the importance of abyssal holothurians in processing organic matter in these environments, no study to date has comprehensively linked holothurian feeding strategies, host phylogeny with gut microbiome composition in abyssal habitats. The majority of holothurian microbiome studies have primarily focused on coastal and commercially important

species or areas of the seabed adjacent to the continental shelf, where coastal input and runoff are likely to be a larger influence than the abyssal plain (Amon & Herndl, 1991; Amaro *et al*., 2009; Amaro *et al*., 2012; Gao *et al*., 2014; León-Palmero *et al*., 2018; Pagán-Jiménez *et al*., 2019; Cleary *et al*., 2019). The microbiomes of the holothurians and that of ambient sediment are intrinsically linked so characterising the microbiome of these elements will therefore offer another perspective on microbial community dynamics in this region and aid in understanding the relationship between benthic microbial biodiversity and ecosystem functioning. It will therefore be possible to define a baseline, 'healthy' community, which will aid in predicting the response of perturbation or disturbance and the impact on biogeochemical cycling (Shade & Handelsmann, 2012).

1.7 Monitoring benthic microbial communities

1.7.1 Long-term time series and observation

The importance of long-term monitoring of all aspects of abyssal communities, including microbes, has long been recognized. Microbial observation performs an important role in: revealing biogeochemical and key ecosystem functions of the oceans, detection of ecosystem change, and assessment of anthropogenic impacts over decadal timescales. Yet despite efforts to improve co-ordination between oceanic observation systems globally, marine microbial populations remain under-sampled (Buttigieg *et al*., (2018).

The transport of POM to the deep-ocean is fundamental for deep-sea life and the importance of this tight coupling between the surface and abyssal plain has been demonstrated with long-term, continuous monitoring stations. Two of these are in the North Atlantic (Porcupine Abyssal Plain: Billett *et al*., 2001) and the North-Eastern Pacific (Station M: Smith *et al*., 1997; Ruhl & Smith, 2004), which provide long time series records on POC flux data (Smith & Druffel, 1998). PAP experiences seasonal POC flux variations, evidenced by visible deposition of material on the seafloor (Bett *et al*., 2001, Billett *et al*., 2001, 2010). Station M is situated at the base of the Monterey Canyon abyssal fan, beneath the Californian Current. Seasonal upwelling events lead to pulses in primary production (Smith & Druffel, 1998), leaving visible flocs of material and phytodetritus on the seafloor

(Beaulieu & Smith, 1998). Both stations have demonstrated the influence of phytodetritus pulses on benthic communities over time (Smith *et al*., 1997; Smith & Druffel, 1998; Bett *et al*., 2001; Billett *et al*., 2001, 2010; Ruhl & Smith, 2004; Kuhnz *et al*., 2014). The long-term analysis of variations in food availability is critical when considering the link with microbial community dynamics and remineralisation over spatial and temporal scales, as well as the impact on biogeochemical cycles and carbon storage capacity (Ruhl *et al*., 2008).

1.7.2 Environmental DNA (eDNA) as a monitoring tool

Biodiversity assessments of the deep sea are essential ahead of potential anthropogenic impacts (Smith *et al*., 2008a; Wedding *et al*., 2013) and eDNA metabarcoding offers a valuable and cost-effective tool for assessing and monitoring biodiversity (Goodwin *et al*., 2017; Seymour *et al*., 2019). This powerful molecular tool allows the identification of organisms using traces of DNA within an environmental sample, without having to isolate and/or culture the organisms first (Deiner *et al*., 2016). Metabarcoding is the taxonomic identification of multiple species, using a barcoding marker such as the 16S or CO1 genes, from a PCR-amplified mixed sample that is sequenced on a high-throughput sequencing (HTS) platform (e.g. Illumina MiSeq or HiSeq) (Deiner *et al*., 2016). Once the samples have been sequenced, the samples are computationally processed using a bioinformatics pipeline in order to analyse and interpret the sequence datasets (Bik *et al*., 2012).

Environmental barcoding via HTS platforms allow rapid processing of multiple samples pooled together (Deiner *et al*., 2016), making it ideal for community-scale analysis. eDNA surveys have a number of advantages, including high sensitivity and cost effectiveness (Goodwin *et al*., 2017; Seymour *et al*., 2019), sampling greater species diversity with high taxonomic resolution (Deiner *et al*., 2016), This method is particularly effective for populations that are not visible in photographic or video surveys, such as microbes (Laroche *et al*., 2020) and for capturing cryptic species (Kelly, 2016; Deiner *et al*., 2017). eDNA studies are useful for temporal, long time-series observations, such as changes in microbial communities over time (Bálint *et al*., 2018). These studies also lend themselves to the study of specific resource use by organisms, comparisons of functional groups

and multiple trophic levels (Thomsen & Willerslev, 2015; Bálint et al., 2018). Hence, eDNA studies allow hypotheses and questions to be posed about community dynamics and environmental variables driving changes in species compositions, which may be valuable in the assessment of anthropogenically-mediated change (Bálint *et al*., 2018).

Challenges, however, remain with the metabarcoding technique (Kelly, 2016). Amplicon-based marker gene surveys are vulnerable to error and biases which may occur at various points throughout the process such as under-sampling, amplification of contaminating DNA from reagents, template concentration and properties, primer mismatches and systematic PCR errors (Gohl *et al*., 2016). In terms of accuracy and reproducibility of data, this is clearly a concern for microbial community studies. In addition, primers themselves can introduce a bias (Takahashi *et al*., 2014; Gohl *et al*., 2016). There have been documented mismatches using universal primers, such as V4 515f and 806r, that affect certain organisms (Mao *et al*., 2012; Polz & Cavanaugh, 1998; Hong *et al*., 2009; Klindworth *et al*., 2013). Mismatching can lead to amplicons dropping out and not being sequenced, which will affect the accuracy of the study (Gohl *et al*., 2016).

Some methodological aspects remain unresolved, such as the legacy of DNA, pinpointing the exact source of DNA present in samples and how long that material has been there (Laroche *et al*., 2020). One aspect of eDNA surveys that has frustrated researchers is deciphering whether DNA is from non-living cells or extracellular (Lejzerowicz *et al*., 2013). In addition, the source of DNA, whether it is autochthonous or allochthonous to the area studied, such as pelagic cells in deep-sea sediments, which can make inferring spatial or temporal variation a challenge (Deiner *et al*., 2016). The residence time of DNA within marine sediments may influence diversity studies, as DNA degrades much more slowly and is better preserved in deep sea sediments than seawater (Corinaldesi *et al*., 2011; Lejzerowicz *et al*., 2013).

The eDNA metabarcoding workflow is a rapidly evolving molecular tool (Deiner *et al*., 2016), with a number of approaches to processing and interpreting eDNA data (Goldberg *et al*., 2016; Alberdi *et al*., 2017). Making appropriate

choices for data standardisation and processing, however, is not always straightforward. Understanding the implications and making appropriate methodological choices in experimental design, sequencing, library preparation and bioinformatic analysis is imperative (Holman *et al*., 2019). Bioinformatic processing of HTS datasets uses UNIX pipelines (Bik *et al*., 2012) and requires a well-informed researcher to understand the impact of chosen pipelines and parameters during data analysis (Deiner *et al*., 2016). These choices may not correctly characterise the true diversity of the samples or lead to errors in the conclusions drawn (Alberdi *et al*., 2017). Therefore, it is essential that metabarcoding methodologies from sampling, to *in silico* are standardised as is realistically possible, given the variety of different sample types and target taxa.

1.7.3 Baseline assessments of abyssal habitats

A clear understanding of the diversity patterns of benthic microbes is also vital for the sustainable use and preservation of these ecosystems in terms of resources and deep-sea mining. Increased demand for metals and minerals has focused attention on particular areas of the sea bed rich in these resources (Wedding *et al*., 2013). Polymetallic nodules on the abyssal seafloor offer a resource potential for mining and have attracted scientific and commercial interest (Wedding *et al.,* 2015; Kaiser *et al*., 2017). The nodules themselves are thought to have formed from microbial processes (Blöthe *et al*., 2015), provide habitat heterogeneity on the sea bed and substrate for a range of organisms (Kaiser *et al*., 2017 and citations therein). One such region with a high density of polymetallic nodules is the Clarion Clipperton Fracture Zone (CCFZ) in the northern, equatorial Pacific. This is an area characterised by a low input of phytodetritus and the sediment is oxygenated to a depth of several metres (Mewes *et al*., 2016). The presence of nodules on the seabed has attracted interest in mining these regions.

In an area of seafloor covering ~ 6 million km^2 (Wedding *et al*., 2013), seventeen exploration licenses for nodule mining have been granted since 2001 by the International Seabed Authority (ISA) (Jones *et al*., 2020). In addition, the ISA have also designated nine Areas of Particular Environmental Interest (APEIs), protected from mining activities and these conservation areas surround the belt of mining contract areas (Lodge *et al*., 2014). The aim of these conservation areas is

to form a network of sites that represent the range of habitats and communities of the CCFZ. Ideally, these sites will help to preserve biodiversity in the region, should any mining exploration and activities take place (Wedding *et al*., 2013). One of these areas, APEI-6 (centred: 17° 10 N, 122° 75 W), has a range of topographical, spatial features on the seafloor, such as ridges, troughs and plains (Jones *et al*., 2015). Little is known about the spatial variability of deep-sea microbial communities, which will be essential to ascertain in terms of predicted mining impact and a useful tool for monitoring in the future. The UK has an Exploration Contract Area (13° 28 N, 116° 35 W), ~750 km away from APEI-6 in the eastern CCFZ (hereafter referred as UK-1). Due to growing concern about the impact that mining activities will have on benthic fauna, there has been an increased drive to carry out biological baseline studies before any mining takes place.

The potential impacts of mining on the benthic assemblages are poorly understood (Jones *et al*., 2017). Recovery after disturbance is likely to be very slow, from decades to millions of years (Jones *et al*., 2017), particularly as nodule formation and growth can range from thousands to millions of years (Kerr, 1984). The process of nodule removal would remove the top layers of sediment, generating plumes near the seabed from the action of the mining collector vehicle as well as in the water column from discharge of sediment-laden water from shipboard dewatering of nodules (Jones *et al*., 2018). This activity could lead to widespread ecological disturbance on the seafloor, resulting from direct physical disturbance of communities, the removal of the nodule habitat by the collector, and/or to the resettling of plume sediments to the surrounding seabed over 100's to 1000's km (Rolinski *et al*., 2001; Smith *et al*., 2008a). In an area with naturally low sedimentation rates (approximately 0.35 cm/kyr; Mewes *et al*., 2014) and stable physical conditions (Levin *et al*., 2016), recovery of benthic assemblages is likely to be very slow, especially with sediment removal as a result of mining activities.

Benthic impact experiments designed to mimic the consequences of mining, that included nodule removal, sediment disturbance and resuspension, have indicated that along with metazoans, microbial

assemblage recovery is poor (Gjerde *et al*., 2016; Jones *et al*., 2017). Microbial abundance in sediments had been reduced by up to 50 % following sediment disturbance from ploughing at the abyssal DISCOL site (Peru Basin) and recovery times for organic matter (OM) remineralisation rates and microbial activity to return to baseline are more than 50 years (Vonnahme *et al*., 2020; De Jonge *et al*., 2020). As nodules are a unique habitat and nodule growth can take millions of years in the CCFZ, nodule fauna would only recover over geological time scales. In addition, the resuspension of sediment plumes generated from nodule extraction is likely to impact a much wider area of the seafloor than the nodule fields themselves, by settling on neighbouring areas (Jones *et al*., 2017).

A greater understanding of the contribution of microbes to carbon cycling in the deep ocean is urgently needed. Determining how to model microbial ecological processes will be crucial, especially in light of future changing climate and how this is likely to affect biogeochemical processes in the deep sea. Furthermore, the development of protocols and methods for analysing benthic microbial diversity will be critical for marine biodiversity research, policy, education and engagement. This information may be important for guidelines and regulations into international standards and best practice for deep sea mineral research and exploration.

1.8 Aims and objectives

The previous sections outline the importance of benthic abyssal microbes, in biogeochemical cycles, nutrient availability, carbon remineralisation and burial and the tight coupling between benthic and pelagic realms. The introduction also highlighted that the biodiversity, function and connectivity of microbial communities within deep-ocean sediments remains poorly described. Although attempts have been made to characterise the benthic microbiome and spatial variability, gaps remain in our understanding of benthic microbial diversity, distribution and connectivity, and the scales over which these changes occur. These studies will explore the spatial variability of benthic microbes in greater detail. In Chapters 2-4, the following areas will be addressed and discussed:

(1) Over what scales do abyssal sediment communities vary?
(2) How does the distribution of nitrifiers (including AOA) vary in abyssal sediments and how does the input of carbon influence this functional group?
(3) How does holothurian feeding ecology and sediment microbial assemblages influence the composition of holothurian gut microbiomes?

1.8.1 Overview of manuscripts / chapters

Chapter 2 highlights the growing concerns about potential future deep-sea mining and the impact this will have on benthic communities. It also shows a need for baseline assessments to identify the communities present before anthropogenic disturbance takes place. Chapter 3 attempts to address the link that microbes have in pelagic-benthic coupling by examining how average carbon input structures and influences the diversity, composition and spatial variability of abyssal sediment microbes. Chapter 4 examines another angle of spatial variability in abyssal microbes by describing the gut microbiomes of a number of holothurian species in the NE Atlantic. This chapter will offer another perspective on microbial community dynamics and aid in understanding the relationship between microbial biodiversity and ecosystem functioning. At the time of writing, Chapter 2 was published and Chapter 4 in preparation.

1.9 Outline of methodological approach of the research

Chapters 2 - 4 cover five years of work, involving the analysis of abyssal sediment samples from a number of expeditions to the Clarion-Clipperton Fracture Zone (CCFZ) and Station M in the Pacific, and the Porcupine Abyssal Plain (PAP) in the Atlantic. In addition to sediment, manganese nodules were processed from the same CCFZ expedition as the sediment samples. Amplicon sequencing was implemented to analyse the microbial community diversity and composition of sediment samples from CCFZ, PAP and Station M, from manganese nodules at CCFZ, and from water and holothurian gut content samples at PAP. For the purposes of consistency and comparison, the Earth Microbiome Project (EMP) protocol (Thompson *et al*., 2017) was implemented, in line with the majority of

marine microbiome studies (Shulse *et al*., 2016; Lindh *et al*., 2017; Cleary *et al*., 2019; Bergo *et al.,* 2020; Molari *et al*., 2020). Sampling design was also consistent with other studies (Shulse *et al*. 2016; Lindh *et al*., 2017). This will ensure that the results will more likely be comparable to other studies, to aid collaborative efforts, particularly for the CCFZ, and in light of concerns over potential future deep-sea mining.

1.9.1 Sample collection

Sediment was obtained in a consistent fashion from each location (CCFZ, PAP and Station M), whereby megacores were deployed at CCFZ and PAP, and push cores were deployed with an ROV at Station M. Once aboard, sediment cores were sliced at 1 cm intervals to 5 cm below seafloor (cmbsf), then 5-10 cmbsf and 10-15 cmbsf, using sterilised slicing equipment, which was rinsed with Milli-Q water between each slice. Nitrile gloves were worn at all times during this process and only the centre of the core was used. Sediment slices were immediately stored in Whirl Pak bags at -80 ºC onboard. Manganese nodules used for analysis from CCFZ were aseptically removed from megacores during sediment layer slicing and stored in the same manner as sediment. Water from PAP was sampled using CTD casts on 2 separate days and were filtered through 0.22 μm Sterivex filters using sterile syringes and peristaltic pump systems. Sterivex filter samples, from 4830 m, 4750 m, 4000 m, 2000 m, 925 m, 750 m, and 70 m water depth, were immediately frozen at -80 ºC in Whirl Pak bags. Holothurian specimens (n = 30, covering 6 species) from PAP were recovered using OTSB (Otter Trawl, Semi Balloon) trawls on 2 separate trawls. Only intact, undamaged individuals were used for analysis. Specimens were processed immediately in a 4 ºC controlled-temperature room onboard. Holothurian gut contents were extracted from the foregut and hindgut areas of the intestines with a large bore syringe (20 mL) where possible, or with a sterile scalpel and spatula, avoiding gut wall tissue. Four individuals were subjected to a gut transect where additional fore-, hind- and mid-gut samples were taken. Foregut samples were taken 2-3 cm from the oesophagus. A piece of body wall from each individual was also collected for host analysis (Table 11). Gut contents and host tissue were stored in separate 1.5 ml tubes and frozen immediately onboard at -80 ºC for later processing on land.

1.9.2 DNA extraction and sequencing

Genomic DNA was extracted from 500 mg of each sediment sample, holothurian gut content sample and crushed manganese nodule sample and processed with the FastDNA Spin Kit for Soil (MP Biomedicals, USA) following the manufacturer's protocol. Holothurian host tissue (~500 mg) was processed using the DNeasy Blood & Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol for animal tissue. Sterivex water filter samples were processed using the DNeasy PowerWater Kit, using a vacuum manifold and following the manufacturer's protocol. Two gene fragments were sequenced: the V4 region of the 16S rRNA gene from the microbial communities within sediment, holothurian gut content, manganese nodule and water filter samples, and CO1 (Cytochrome c oxidase subunit I) from the holothurian host tissue. The concentrations of DNA from individual extractions of sediment, manganese nodule and water filter samples were below 0.1 ng/μl and required further concentration. DNA was concentrated using the Zymo Clean & Concentrator-5 kits with a 2:1 DNA Binding Buffer ratio and eluted into 50 μl sterile, DNase-free water. Once concentrated, extractions were pooled in triplicate per sample, and the three technical replicates were sequenced per pool.

The V4 region of the 16S rRNA gene was amplified by the polymerase chain reaction (PCR), using the oligonucleotide primers Pro515f/Pro806r for Bacteria and Archaea, and avoiding primer bias associated with targeting multiple domains (Herlemann *et al*., 2011). The amplified 16S rRNA gene products and extraction blanks were then prepared with the Nextera XT v2 Kit (Illumina, San Diego, CA) and sequenced on an Illumina MiSeq platform at the Environmental Genomics Sequencing Facility (University of Southampton, National Oceanography Centre, Southampton). The COI gene (partial 690 bp) was amplified by the polymerase chain reaction (PCR) using the oligonucleotide primers as described in Miller *et al*., (2017). PCR reactions were performed using the following mixes: 10 μL GoTaq Green Master Mix (Promega, UK), 1.0 μL of each primer, 7 μL water and 1.5 μL of template DNA. PCR amplifications were conducted in the following format: denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 40 s, annealing at 50 °C for 40 s, extension at

72 °C for 50 s, followed by a final annealing at 72 °C for 5 min. The PCR product size and purity of samples was checked with 1% agarose gel electrophoresis and the PCR product was purified with QIAquick 96 PCR Purification Kit (Qiagen, USA). Cleaned PCR products were sequenced using cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing machines (Eurofins Genomics, Germany).

1.9.3 Data analysis

The demultiplexed Illumina 16S rRNA reads were analysed with the microbiome analysis package QIIME 2 (Quantitative Insights Into Microbial Ecology) version 2019.1 (Bolyen *et al*., 2019) and sequence quality control for the Illumina amplicon data was implemented with the DADA2 pipeline within the QIIME 2 software package (Callahan *et al*., 2016). Amplicon Sequence Variants (ASVs) or features were resolved using the DADA2 denoise-single method. A naïve Bayes classifier that was pre-trained on the V4 region of reference sequences from the Silva database (version 132; Quast *et al*., 2013) was used to classify representative sequences of ASVs in our dataset and clustered at 99% identity. The generated feature table from the QIIME2 output was first normalised using the 'core-metrics-phylogenetic' method within the q2-diversity plugin of QIIME2, before being used for abundance and diversity analysis. This method randomly subsamples counts in each sample without replacement within a feature table to a user specified depth, so that each sample has an equal number of counts. The resulting normalised feature table was used to calculate and generate alpha diversity indices for each sample in QIIME2. The remainder of the community composition and statistical analyses were performed using the 'vegan' package in R v 3.3.2 (Oksanen *et al*., 2007; 2017). These included species accumulation curves, NMDS ordinations, heatmaps, network analyses, ANOVA tests and relative abundance plots. More complex statistic permutational multivariate analysis of variance (PERMANOVA) tests, *posthoc* pair-wise tests and SIMPER analysis were conducted in PRIMER v.7 (Clarke & Gorley, 2015).

To confirm the identity of the holothurian mitochondrial CO1 sequences, reference sequences were downloaded from the NCBI databases (https://blast.ncbi.nlm.nih.gov) and aligned with the representative CO1 sequences

using MUSCLE in Geneious (v.10.2.3; http://www.geneious.com), before being manually inspected using MEGA version X (Kumar *et al*., 2018) and Geneious. A phylogenetic tree was generated in MEGA using the Maximum Likelihood method and support for phylogenetic nodes was calculated using 500 bootstrap replicates. Additional *Pseudostichopus aemulatus* host samples and GenBank sequences were added to the study samples for consensus within the tree.

The methods used within each chapter are further detailed as appropriate and the results are discussed in the following three chapters.

Chapter 2: Spatial variability of abyssal nitrifying microbes in the north-eastern Clarion-Clipperton Zone

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2.1 Abstract

Abyssal microbes drive biogeochemical cycles, regulate fluxes of energy and contribute to organic carbon production and remineralization. Therefore, characterising the spatial variability of benthic microbes is important for understanding their roles in benthic environments and for conducting baseline assessments of areas of the seabed that might be targeted by commercial mining activities. Yet, detailed assessments of the spatial distributions of benthic microbial communities in these regions are still incomplete, and these efforts have not yet considered the influence of seafloor topography and heterogeneity on microbial distributions across a range of scales. In this study, we investigated the composition and spatial variability of benthic microbial assemblages found in sediments and polymetallic nodules collected from the Clarion Clipperton Zone (CCFZ) in the equatorial Pacific (4000-4300 m water depth). We used 16S rRNA gene sequences to characterise these communities. The upper 20 cm of abyssal sediments harboured diverse and distinctive microbial communities in both sediments and their associated polymetallic nodules, with high similarity across

topographical areas of the seabed. Assemblage composition differed vertically through the sediment, by habitat and across small to mesoscales. Potential carbon-fixing microbes formed more than 25% relative abundance of sediment assemblages, which were dominated by ammonia-oxidising Archaea *Nitrosopumilus*. Non-photosynthetic Cyanobacteria were more frequent in the deeper sediment layers and nodules. Sediment communities had a higher abundance of taxa involved in nitrogen cycling, such as *Nitrosopumilus*, *Nitrospina*, *Nitrospira*, *AqS1* (Nitrosococcaceae), and methanogens *wb1-A12* (NC10 phylum). In contrast, nodules were more enriched in Alphaproteobacteria, Gammaproteobacteria, Planctomycetes, Acidobacteria, Bacteroidetes, Nanoarchaeaeota and Calditrichaeota. Microbes related to potential metal-cycling (Magnetospiraceae and Kiloniellaceae), organic carbon remineralisation (*Woeseia*), and sulphur-oxidising Thiohalorhabdaceae were also more enriched in nodules. Our results indicate that benthic microbial community composition is driven by sediment profile depth and seafloor heterogeneity at small and mesoscales. The most abundant microbial taxa within the sediments were nitrifying and putative carbon-fixing microbes, and may have key ecological roles in mediating biogeochemical cycles in this habitat.

Running title: Abyssal benthic microbes vary spatially in CCFZ

2.2 Introduction

The high abundances of polymetallic nodules and the possibility of deep-sea mining has heightened scientific and commercial interest in the environment of the Clarion Clipperton Zone (CCFZ) in the eastern Pacific (Wedding *et al.,* 2015). In an area of seafloor covering ~ 6 million km^2 (Wedding *et al*., 2013), seventeen exploration licenses for nodule mining have been granted since 2001 by the International Seabed Authority (ISA) (Jones *et al*., 2020). In addition, the ISA have also designated nine Areas of Particular Environmental Interest (APEIs), protected from mining activities and these conservation areas surround the belt of mining contract areas (Lodge *et al*., 2014). The aim of these conservation areas is to form a network of sites that represent the range of habitats and communities of the CCFZ. Ideally, these sites will help to preserve biodiversity in the region, should any mining exploration and activities take place (Wedding *et al*., 2013). The potential impacts of mining on the benthic assemblages are poorly understood (Jones *et al*., 2017). Recovery is likely to be very slow, from decades to millions of years (Jones *et al.*, 2017), particularly as nodule formation and growth can range from thousands to millions of years (Kerr, 1984). The process of nodule removal would remove the top layers of sediment and generate plumes near the seabed from the action of the mining collector vehicle as well as in the water column from discharge of sediment-laden water from shipboard dewatering of nodules (Jones *et al*., 2018). This activity could lead to widespread ecological disturbance on the seafloor. These effects could result from direct physical disturbance of communities, the removal of nodule habitat by the collector, and/or to the resettling of plume sediments to the surrounding seabed over 100's to 1000's km (Rolinski *et al*., 2001; Smith *et al*., 2008a). Recovery of benthic assemblages is likely to be very slow in an area with naturally low sedimentation rates (approximately 0.35 cm/kyr; Mewes *et al*., 2014) and stable physical conditions (Levin *et al*., 2016). Additionally, the CCFZ is a food limited environment, and the benthic ecosystems are structured by a very low flux of particulate organic carbon (POC; fluxes 1 mg C_{org} m^{-2} d^{-1} ; Volz *et al*, 2018) from oligotrophic overlying waters (Smith *et al*., 2008b; Levin *et al*., 2016). As a result of a low POC flux, the soft sediments of the CCFZ are oxygenated down to 300 cm below seafloor (cmbsf: Mewes *et al*., 2014; Menendez *et al*., 2019; Voltz *et. al*., 2018).

Despite a low input of carbon, there are highly diverse benthic microbial communities in this region that inhabit the sediment and nodules (Durbin & Teske, 2011; Wu *et al*., 2013; Tully & Heidelberg, 2013; Blöthe *et al*., 2015; Shulse *et al*., 2016; Lindh *et al*., 2017, Molari *et al*., 2020). Benthic microbes form a key part of benthic ecosystems by mediating biogeochemical cycles, regulating fluxes of energy and contributing to organic carbon production and remineralization. As well as the input of energy into abyssal ecosystems from photosynthetically-derived particulate organic carbon (Ruhl *et al*., 2008; Smith *et al*., 2008b, 2009), microbially-mediated inorganic C-fixation by chemolithoautotrophic microbes, (Middelberg, 2011; Molari *et al*., 2013) appear to provide an important carbon subsidy to benthic ecosystems in the CCFZ (Sweetman *et al*., 2019), and more generally (Brunnegård *et al*, 2004; Tully & Heidelberg, 2016).

Owing to our limited knowledge of microbial communities and their functions in these environments, our ability to predict the impact of commercial mining activities on benthic microbial communities inhabiting the sediment and nodules is also limited. Benthic impact experiments designed to mimic the consequences of mining have indicated that along with metazoans, microbial assemblage recovery is poor (Gjerde *et al*., 2016; Jones *et al*., 2017). Microbial abundance in sediments has been reduced by up to 50 % following sediment disturbance from ploughing at the abyssal DISCOL site (Peru Basin) and recovery times for organic matter (OM) remineralisation rates and microbial activity to return to baseline are more than 50 years (Vonnahme *et al*., 2020; De Jonge *et al*., 2020).

The CCFZ is heterogenous, with bathymetry varying over 2000 m (Washburn *et al*., 2021), and clear variability in visible seafloor habitats (Simon-Lledó *et al*., 2019). The CCFZ is characterised by landscape-scale topographical features, such as ridges, valleys and flat plains (MacDonald *et al*., 1996). This topography influences the diversity and distribution of metazoans (Cosson *et al*., 1997; Durden *et al*., 2015; Stefanoudis *et al*., 2016; Leitner *et al*., 2017; Simon-Lledó *et al*., 2019; Durden *et al*., 2020), possibly owing to the influence of topography on bottom currents, sediment grain size and food supply (Durden *et al*., 2015; Morris *et al*., 2016). These factors would also be likely to influence benthic microbial communities, but have not been assessed at a landscape scale.

Such information is an important component of baseline environmental assessment for commercial mining activities and subsequent monitoring (Ingels *et al*., 2020), particularly as benthic microbes represent an important energy and nutrient source for abyssal ecosystems. Previous work examining the regional-scale structure of CCFZ benthic microbial communities using 16S rRNA gene sequencing has indicated that microbial communities may have a spatial variability over distances of less than 1000 km (Tully & Heidelberg, 2013; Lindh *et al*., 2017), but finer-scale patterns or their controls are unknown.

Here we characterise bacterial and archaeal assemblages via amplicon sequencing of the 16S rRNA genes, from sediment and nodule samples collected from one of the CCFZ conservation areas, APEI-6, in order to determine environmental controls on microbial community structure and compare these with one of the proximal exploration areas (UK-1). Specifically, we tested the following hypotheses: (1) Benthic microbes vary in their spatial distribution from cm scales to scales of 100s m within sediments at APEI-6 analogous to that previously observed in metazoans in other studies (Cosson *et al*., 1997; Durden *et al*., 2015; Stefanoudis *et al*., 2016; Leitner *et al*., 2017; Simon-Lledó *et al*., 2019; Durden *et al*., 2020), and (2) the broad landscape-scale features of APEI-6 (ridges, valleys and plains) will influence the diversity and distribution of benthic microbial assemblages.

2.3 Materials and Methods

2.3.1 Sample collection

Sediment samples and polymetallic nodules were collected during the RRS *James Cook* cruise JC120, April-May 2015 to the eastern CCFZ in the northern equatorial Pacific. Samples were obtained from 20 megacores (Bowers and Connelly type; Barnett *et al*., 1984) that were deployed in the south western part of APEI-6 (Sampling area centred: 17° 10 N, 122° 75 W) and 2 megacores deployed in the UK-1 Exploration Contract Area, ~750 km away from APEI-6 (13° 28 N, 116° 35 W) (Table 2). Megacores were deployed in four distinct topographical landscape areas in APEI-6 that were each defined by bathymetric data: Deep-Plain, Flat, Ridge and Trough (Fig. 2; definition details in Simon-Lledó *et al*., 2019). Megacores of sediment were sliced at 1 cm intervals down to 10 cm, below which slices were taken every 2 cm. The following sediment depth layers were used for analysis of eDNA studies: 0-1 cm below seafloor (cmbsf), 1-2 cmbsf, 5-6 cmbsf, 10-12 cmbsf and 20-22 cmbsf. Only sediment from the centre of the core that had not been in contact with the sides of the megacore tube was sampled. Sediment was placed into sterile Whirl Pak bags and frozen at -80 °C immediately on board. Surface nodules in the top 1 cm of the megacore were removed with forceps, rinsed with filtered seawater and frozen immediately at -80°C in sterile Whirl-Pak bags for microbial analysis.

Environmental data was collected during the JC120 cruise (Table 3) and included sediment grain size, nodule density (no. m^{-2}), total carbon (TC, % sediment dry weight), total organic carbon (TOC, wt. %), total nitrogen (TN, wt. %), the ratio of total organic carbon to total nitrogen (TOC:TN), the ratio of total carbon to total nitrogen (TC:TN) and carbonate content ($CaCO_3$, wt. %). Manganese (Mn, wt. %) values are from Menendez *et al*., (2019). Collection and calculation of these values is detailed in Simon-Lledó *et al*., (2019) and Menendez *et al*., (2019).

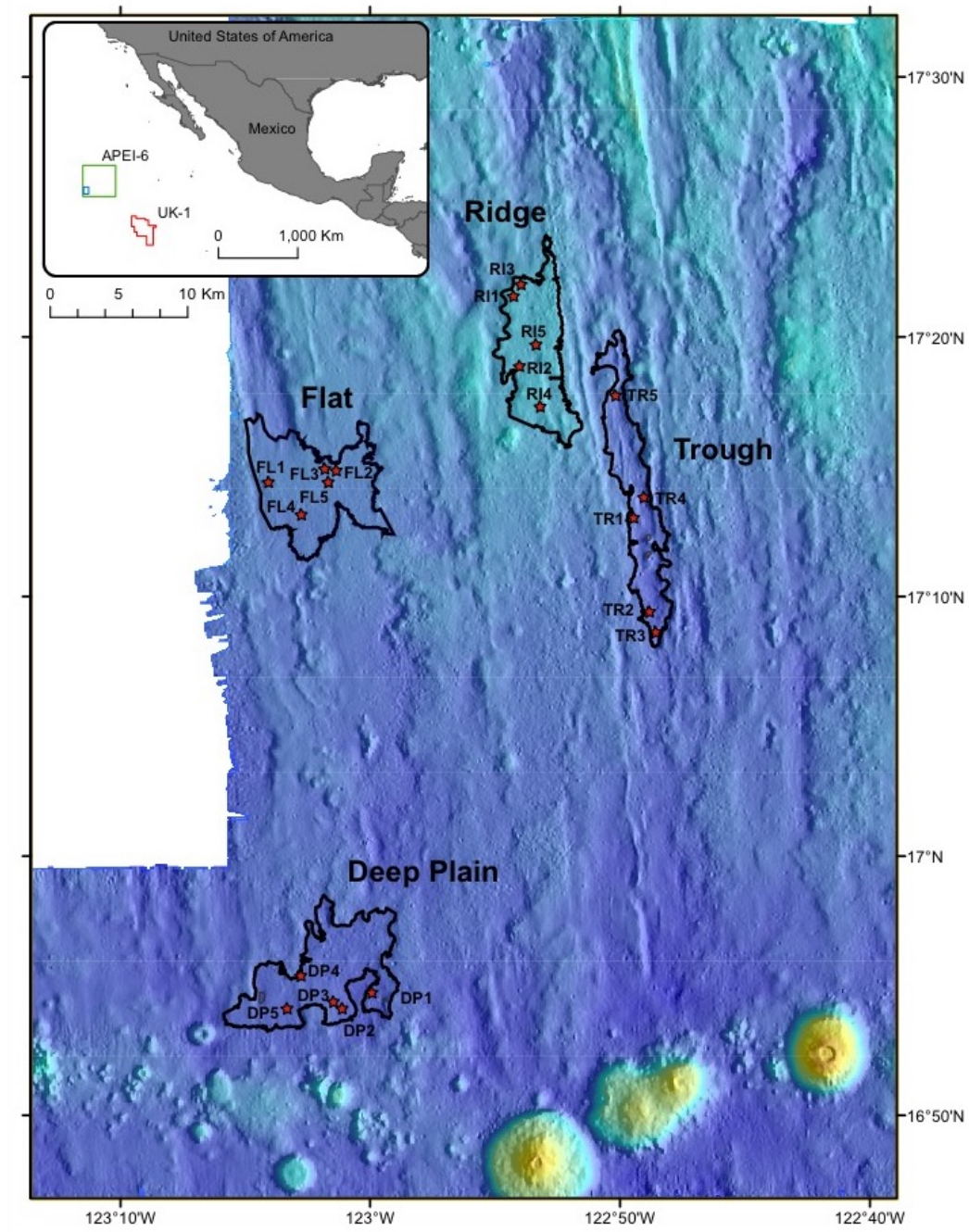

Figure 2. Location of the Area of Particular Environmental Interest (APEI-6) and UK-1 exploration contract area in the Clarion-Clipperton Fracture Zone (CCFZ) of the Pacific Ocean, where sampling occurred (insert map). Coring locations within the four landscape regions of the APEI-6 are shown with a red star. Red polygon shows location of UK-1, the green box indicates the location of APEI-6, and the blue box delineates the location of the sampling sites within APEI-6.

2.3.2 DNA extraction and sequencing

Genomic DNA was extracted from 500 mg of wet sediment and 500 mg of crushed nodule samples using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) following the manufacturer's protocol. Nodules were first crushed with an autoclaved pestle and mortar. Additional extraction blanks containing only the FastDNA Spin Kit reagents were processed with the sediment samples. The concentrations of DNA from all samples was below 0.1 ng/μl and required further concentration. DNA was concentrated using the Zymo Clean & Concentrator-5 kits with a 2:1 DNA Binding Buffer ratio and eluted into 50 μl sterile, DNase-free water. Once concentrated, extractions were pooled in triplicate per sample, and the three technical replicates were sequenced per pool. The V4 region of the 16S bacterial and archaeal rRNA gene was amplified by the polymerase chain reaction (PCR), following the Earth Microbiome Project (EMP) protocol (Thompson *et al*., 2017), using the oligonucleotide primers Pro515f/Pro806r. The amplified 16S rRNA gene products and extraction blanks were prepared with the Nextera XT v2 Kit (Illumina, San Diego, CA) and sequenced on an Illumina MiSeq platform at the Environmental Genomics Sequencing Facility (University of Southampton, National Oceanography Centre, Southampton).

2.3.3 Data analysis

The demultiplexed sequences were analysed with the microbiome analysis software package QIIME 2 (Quantitative Insights Into Microbial Ecology), version 2019.1 (Bolyen *et al*., 2019). The DADA2 pipeline (Callahan *et al*., 2016) within QIIME 2 was implemented for sequence quality control and chimera removal. Amplicon Sequence Variants (ASVs) or features were resolved using the DADA2 denoise-single method. Features that were observed in the PCR blanks were considered to be contaminants and were filtered from the samples. These were removed with the 'filter-seqs' option in QIIME2, using the feature table's feature IDs to filter the contaminant sequences present in the negative controls. A Naïve Bayes classifier that was pre-trained on the V4 region of reference sequences from the Silva database (version 132; Quast *et al*., 2013) was used to classify representative sequences of ASVs in our dataset and clustered at 99% identity. A

rooted phylogenetic tree was generated within QIIME2, using MAFFT and FastTree, which was used for calculating diversity metrics.

The generated feature table contained 48,198 ASVs and 4,383,878 reads, with a range of 246,767 to 4,612 reads per sample. Owing to the low number of reads in some samples, the feature table was normalised to 4600 sequences per sample in QIIME 2 prior to abundance and diversity analysis, to account for uneven sampling depth, library sizes and to retain all of the samples. Samples were categorized by substrate (sediment and nodules), topographical landscape and sediment horizon. Relative abundances were calculated on ASV feature tables with raw counts that had been normalised by library size to get the proportions of species within each library and then square-root transformed (Hellinger transformation). Alpha diversity (observed features (q=0), Shannon-Wiener index, evenness (Pielou's evenness values, Chao-1 index) and beta diversity (Bray-Curtis distance) were calculated in QIIME 2 on the normalised samples and formatted along with the generated feature-table for further analysis in R. Sample-based alpha diversity species accumulation curves were calculated for each sample type without replacement, using the 'iNEXT' package in R v 3.3.2 (Hsieh *et al*., 2020; Chao *et al*., 2014), for APEI-6 sediment samples only, showing all horizons within each topographical region. Species accumulation curves for nodules and UK-1 samples were not calculated owing to insufficient sample numbers. Community composition and statistical analyses were performed using the 'vegan' package in R v 3.3.2 (Oksanen *et al*., 2007; 2017). To determine if there were significant differences between alpha diversity in sediments and nodules at APEI-6 (Flat and Deep Plain sites), a one-way ANOVA test was performed in 'vegan'. Given the low numbers of replicates, the data from different topographies were combined for the testing. Using the Hellinger-transformed feature table, the dissimilarity between pairs of samples were calculated using Bray-Curtis dissimilarity coefficient, to generate beta diversity measurements. These measures were generated to determine the variations in species composition and community structure between the four topographic areas and sediment layers in APEI-6. Non-metric multidimensional scaling (NMDS) ordination was used to visualise community structure and variation between geographic location (APEI-6 vs UK1), topographical landscape

and sediment horizon, along with environmental parameters, with the 'envfit' method in the 'vegan' package (Oksanen, 2007).

Permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) and *posthoc* pair-wise tests were performed on Bray-Curtis dissimilarity matrices using PRIMER v.7 (Clarke & Gorley, 2015). A mixed-effect model was used to partition variance among factors and to test whether microbial assemblage composition varied significantly between factors at APEI-6 (20 megacore samples, Table 2). In this model, topographical landscape and sediment horizon were treated as fixed factors. Nodules were found only within surface sediments so were not considered for the lower layers of the sediment profile. It was not possible to include nodule cover and geographic distance within this model due to reduced flexibility with the inclusion of response variables within the PERMANOVA mixed-effect model in PRIMER. To account for potential covariance between depth strata sampled from individual megacores and to reflect the stratified random sampling within topographical regions, megacore was treated as a random factor nested within topographical region. This model is analogous to a classical repeated measures ANOVA design. Topography contained four levels: Deep Plain, Flat, Ridge, Trough. Sediment Horizon contained five levels: 0-1 cm, 1-2 cm, 5-6 cm, 10-12 cm and 20-22 cm. The main effects, topography and depth, the nested factor, core, and the interaction term, topography x depth, were included in the model. Methods of permutation used was 'method (ii) *permutation of residuals under a reduced model'*. Nodules and the UK-1 samples were excluded from the PERMANOVA mixed-effect model due to lack of sufficient replication.

We were not able to carry out comparative statistics on UK-1 samples as there was only one full megacore taken from this region. The second megacore from UK-1 was a short core owing to a large nodule that was retrieved in the eDNA core, so only the top 1 cm of undisturbed sediment could be recovered. Nevertheless, samples from UK-1 and nodule samples were qualitatively evaluated using taxon abundance plots and NMDS. To aid in visualization, ASVs were grouped by taxonomy at phylum, class and genus levels, and the higher classification taxonomic groups that represented more than 1% of the total abundance in at least one substrate type, one depth horizon or one topographical

landscape type were further analysed and discussed. Graphical outputs from abundance and statistical analysis were displayed using the “ggplot2” package in R (Wickham, 2016). Raw sequences were deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive under BioProject ID PRJNA694451.

Table 2. Sampling site locations of megacore deployments in APEI-6 SW and UK-1 (UK Seabed Resources Limited eastern contract area) that were collected during cruise JC120 with location and depths shown for each sample taken. All nodules were taken from the top 0-1 cm of the megacore * These were short cores (less than 30 cm) when megacores were collected. A total of 102 samples were collected. See Fig 2 for megacore locations.

Station	Location	Seafloor topography	Latitude	Longitude	Water depth (m)	Sediment (cm)	Nodule	N
JC120-010	DP 1	Deep Plain	16° 54.77	122° 59.82	4297	0-22		5
JC120-012	DP 1	Deep Plain	16° 54.77	122° 59.82	4297	0-22		5
JC120-019	FL2	Flat	17° 14.94	123° 1.28	4162	0-22		5
JC120-020	FL 3	Flat	17° 15.03	123° 1.75	4155	0-1*		1
JC120-023	FL 1	Flat	17° 14.45	123° 3.98	4156	0-22		5
JC120-024	FL 4	Flat	17° 13.19	123° 2.67	4180	0-22		5
JC120-028	TR 1	Trough	17° 13.07	123° 49.39	4236	0-22		5
JC120-034	TR 2	Trough	17° 9.45	122° 48.78	4291	0-1		1
JC120-048	RI 1	Ridge	17° 21.56	122° 54.17	4015	0-22		5
JC120-049	RI 1	Ridge	17° 21.56	122° 54.17	4015	0-1		1
JC120-058	RI 2	Ridge	17° 18.84	122° 54.05	4038	0-22		5
JC120-059	RI 3	Ridge	17° 22.02	122° 53.93	4029	0-1*		1
JC120-062	TR 3	Trough	17° 8.73	122° 48.52	4282	0-22		5
JC120-063	TR 1	Trough	17° 13.07	122° 49.39	4245	0-22		5
JC120-065	RI 4	Ridge	17° 17.30	122° 53.07	4012	0-22		5
JC120-066	RI 5	Ridge	17° 19.69	122° 53.27	4012	0-22		5
JC120-067	TR 5	Trough	17° 17.77	122° 50.12	4234	0-22		5
JC120-075	FL 5	Flat	17° 14.38	123° 1.59	4158	0-22	2	7
JC120-076	FL 3	Flat	17° 15.04	123° 1.76	4153	0-1	6	7
JC120-084	DP 3	Deep Plain	16° 54.44	123° 1.50	4300	0-22		5
JC120-092	DP 2	Deep Plain	16° 54.14	123° 0.97	4290	0-22	3	8
JC120-105	UK-1	UK-1	13° 27.79	116° 36.49	4108	0-22		5
JC120-106	UK-1	UK-1	13° 27.81	116° 34.50	4108	0-1*		1

2.4 Results

2.4.1 Microbial diversity at APEI-6 and UK-1

After the data were normalised, 547,400 reads comprising of 36,815 ASVs were identified in 91 sediment and 11 nodule samples (Table 2). Of these ASVs from APEI-6, 19% were classified as Archaea and 81% as Bacteria. In UK-1, the proportion of Archaea was slightly higher, with 21% of ASVs classified as Archaea and 79% as Bacteria. It should be noted, however, that the primers used in this study have recognised biases against Crenarachaeota/Thaumarchaeota (Parada *et al*., 2016), potentially skewing the relative abundance of Archaea versus Bacteria in our analyses.

Sediment assemblages in the upper 2 cm of sediment were more diverse than polymetallic nodule-associated assemblages in APEI-6 (One-way ANOVA, $P = 0.005$, $F_{1,46} = 8.99$, $R^2 = 0.17$). Alpha diversity (Fig. 3A, 3B) in UK-1 sediments (Observed ASVs: mean = 755, SE $\pm$ 82; Shannon diversity 8.75 $\pm$ 0.15) is higher compared to alpha diversity in APEI-6 sediments (ASVs: 675 $\pm$ 14; Shannon: 8.72 $\pm$ 0.01) and nodules (ASVs: 288 $\pm$ 8; Shannon: 7.34 $\pm$ 0.18). Despite non-significant alpha diversity between sediment horizons or between topographical regions within APEI-6, mid to deeper layers (5-22 cm) of sediment at APEI-6 were more diverse than those at the sediment surface, except at Ridge, where the reverse was true. Sediments at Flat sites were the most diverse, with the 5-6 cm layer being the most diverse overall (Observed ASVs; 2000 $\pm$ 447, Shannon; 9.35 $\pm$ 0.52) and least diverse in the 20-22 cm horizon at Ridge (Observed ASVs; 534 $\pm$ 114, Shannon; 8.36 $\pm$ 0.38). Alpha diversity at Ridge was more evenly spread, compared with Trough, which was dominated by fewer common species (Evenness, Fig. 3C) At UK-1, greater alpha diversity was also observed in the surface layers, particularly in the 1-2 cm horizon (Observed ASVs; 1089 $\pm$ 412; Shannon 9.15 $\pm$ 0.68). Estimated richness, (Chao1, Fig. 3D) was also higher overall at UK-1 (978 $\pm$ 159), compared to APEI-6 sediments (805 $\pm$ 46). Species accumulation curves (Fig. 3E) indicated that alpha diversity was highest at Trough and Flat, and lowest at Ridge. Curves for each sample type did not reach

asymptote, indicating insufficient sequencing depth for estimated microbial richness.

2.4.2 General patterns in microbial community composition

NMDS plots were used to visualise differences in microbial community composition between the sediment layers at APEI-6, UK-1 and nodule samples (Fig.4). Microbial assemblages associated with nodules clustered separately from sediment samples within APEI-6 (Fig. 4A). Benthic topography only explained 2.7% of the variance in compositional heterogeneity of species and was not statistically significant. We observed a significant influence of sediment horizon depth on assemblage composition within APEI-6, (PERMANOVA, $p = 0.001$, $F_{4,83}$= 6.977, Table 4) accounting for 23% of variance in differences of relative abundances of species within sediment assemblages overall. The interaction between topography and depth was not statistically significant, but accounted for 9.6% of the variance (Table 4). We observed a relatively large between core variance (20%), suggesting a degree of heterogeneity in microbial assemblage composition between megacores within topographical regions (PERMANOVA, p = 0.001, $F_{18,83}$ = 1.3212, Table 4). We also noted that UK-1 sediment samples formed a distinct cluster from APEI-6 sediment and nodules (Fig. 4B). It was not possible, however, to perform statistical analysis on UK-1 samples, owing to lack of replication.

Environmental variables were largely homogenous within APEI-6. Relative proportions of TC, TOC, TN, TOC:TN, TC:TN, $CaCO_3$, Mn and mean grain size were very similar across the four sampled regions of APEI-6 with no significant differences detected. Nodule coverage was significantly different between the study areas (Simon-Lledó *et al*., 2019b), with greatest nodule density observed at Deep Plain (Table 3).

2.4.3 Microbial assemblage composition of sediments at APEI and UK-1

Overall, 32 % of the community in the whole dataset consisted of Proteobacteria, including Gammaproteobacteria (13 %), Alphaproteobacteria (12 %), Deltaproteobacteria (5 %) and Betaproteobacteria (1.5 %) (Fig. 4). Sequences assigned to the Thaumarchaeota phylum comprised a significant proportion of the

sediment microbial assemblages overall (UK-1 = 20 %, APEI-6 = 19 %). This proportion was lower in the nodules at APEI-6 (11 %). The dominant class within Thaumarchaeota was Nitrososphaeria, which composed 12 – 23 % of sediment communities and 11 % of the nodule-associated communities. Within both sediment and nodule communities, the most dominant genera within Nitrososphaeria was *Candidatus Nitrosopumilus*, which comprised on average 12 % of the sediment communities and 7 % of the nodule-associated assemblages (Fig.5). Within sediments, the highest abundance of *Nitrosopumilus* was in the surface sediments, ranging from 10-14.5 % (± 2.10) across all topographical landscapes in APEI-6 and within UK-1. Overall, *Nitrosopumilus* was more enriched in UK-1 sediments (10 %) than those of APEI-6 (5 %). The highest abundances of *Nitrosopumilus* were found at Trough in APEI-6 (14.5 % ± 1.29) and UK-1 (14 %) in the top cm of sediment.

Other prevalent groups that were also well-represented in sediment assemblages at phylum level were Planctomycetes (11.5 %), Chloroflexi (9 %), Acidobacteria (5 %), Bacteroidetes (4 %), Gemmatimonadetes (4 %), Nitrospinae (3 %), Nitrospirae (2 %) and Actinobacteria (1 %). Abundant taxa at class level in sediment assemblages in APEI-6 and UK-1 included Dehalococcoidia (Chloroflexi, 7 %), Phycisphaerae (Planctomycetes, 7 %), Bacteroidia (Bacteroidetes, 3 %), Nitrospinia (Nitrospinae, 2.5 %), Gemmatimonadetes (Gemmatimonadetes, 2 %), Nitrospira (Nitrospirae, 1.5 %), Planctomycetacia (Planctomycetes, 1.5 %), and OM190 (Planctomycetes, 1.5 %). The most dominant genera in sediment assemblages were *Ca. Nitrosopumilus* (6.5-10 %), the *Urania-1B-19 marine sediment group* (Planctomycetes, Phycisphaerae, 3 %), uncultured bacterium in the Kiloniellaceae family (Alphaproteobacteria, 2.5-3 %), *Woeseia* (Gammaproteobacteria (2.5-3 %), uncultured Nitrosopumilales archaeon in the Nitrososphaeria class (Thaumarchaeota, 2-2.5 %), uncultured bacterium in the Magnetospiraceae family (Alphaproteobacteria, 1.5-2 %), *Nitrospina* (Nitrospinae, 1.5 %) and *Nitrospira* (Nitrospirae, 1.5 %).

Microbial community composition differed among the vertical layers of the sediment, and these differences were influenced by topography (Table 4). For example, the abundance of taxa within the Nitrososphaeria class

(Thaumarchaeota) varied through the sediment profile within each topographical region, explaining 37.4% of the variance in beta diversity (PERMANOVA, p=0.001, $F_{3,83}$ = 14.50, Table 4; Fig. 6). However, at Deep Plain, Thaumarchaeota increased with depth through the sediment, whereas at Ridge, the opposite was observed. In contrast, at Flat and Trough, the top one cm of sediment contained a high abundance of Thaumarchaeota (> 20 % of sequences), which declined with depth to the mid-layers, then increased in abundance again in the 20-22cm layer. At UK-1, the top 2 cm had the highest observed abundances of Thaumarchaeota (24 %). However, the abundance of this group rapidly declined through the sediment profile, to 11.5 % in the deepest layer. Certain groups of microbial taxa increased in abundance with increasing depth through the sediment profile. The abundance of Acidobacteria and Rokubacteria followed this pattern at both APEI-6 and UK-1. Rokubacteria were most abundant at UK-1 and peaked at 10-12 cm before declining in abundance in deeper layers. Alphaproteobacteria only increased in deeper layers at the APEI, whereas the reverse was true in UK-1 sediments. Cyanobacteria were largely absent in sediments, except for the 20-22 cm layer in APEI-6. Other groups such as Actinobacteria and Bacteroidetes decreased in abundance with depth through APEI-6 and UK-1 sediments. Gammaproteobacteria and *Nitrospina* declined in abundance in mid-layers (5-6 cm) of sediment at both locations, before increasing in deeper layers again (10-22 cm). Other taxa followed the opposite pattern, such as Chloroflexi, Gemmatimonadetes, *Nitrospira* and Planctomycetes, which reached a peak in relative abundance in the 5-6 cm layers before declining deeper into the sediment profile.

2.4.4 Microbial assemblage composition of nodules at APEI-6

Nodule-associated communities were dominated by a higher abundance of Proteobacteria than sediments, forming 37% of total sequences, compared to 32 % in sediments, driven by the classes Gammaproteobacteria (14 %), Alphaproteobacteria (14 %), Deltaproteobacteria (6.5 %) and Betaproteobacteria (2 %). The dominance of Gammaproteobacteria was driven by the families Thiohalorhabdaceae, Woeseiaceae (*Woeseia* genera), Arenicellaceae and the KI89A clade. Within Alphaproteobacteria, the most abundant families were Kiloniellaceae and Magnetospiraceae and the most represented groups within

Deltaproteobacteria were the Orders Myxococcales and the SAR324 clade. Betaproteobacteria were largely dominated by ASVs affiliated with Nitrosomonas genera. Nodule communities also had a higher abundance of the Planctomycetes phylum, which formed 17 % of assemblages associated with nodules, compared to 11.5 % of sequences in sediments. This difference was driven by the enrichment of classes Phycisphaerae, OM190, Planctomycetacia and Pla4 lineage. The Bacteroidetes phylum was also more abundant in nodule communities, forming 9 % of nodule communities compared to 4 % in sediment communities and enriched by uncultured genera in the Rhodothermaceae family and the Cyclobacteriaceae family. Nodule-associated assemblages were also depleted in certain phyla compared to sediments, namely Thaumarchaeota (11 % nodules, 19 % sediments), Chloroflexi (5 % nodules, 9 % sediments), Gemmatimonadetes (2.5 % nodules, 4 % sediments) and Rokubacteria (0.2 % nodules, 1 % sediments).

We also observed differences in community composition and abundance of the main taxa found within the microbiomes of nodules retrieved from Deep Plain and Flat (Table S1 and S2). The most dominant genera in nodule-associated communities, *Ca. Nitrosopumilus*, on average formed 10 % relative abundance from Deep Plain nodules, and 6 % from nodules from Flat (Fig. 6). The family Thiohalorhabdaceae within the Gammaproteobacteria, formed 5 % relative abundance in Deep Plain nodules, compared with 2 % in Flat nodules. There was also a greater number of taxa that exceeded 1 % relative abundance in nodule assemblages from Deep Plain than Flat. Groups such as Cohaesibacter (Alphaproteobacteria), *AqS1* (Gammaproteobacteria), *Pelagibius* (Alphaproteobacteria), *JdFR-76* (Calditrichaeota) and *LS-NOB* (Nitrospinae) exceeded 1 % relative abundance in Deep Plain nodule assemblages but were below the 1 % cut-off in nodules from Flat. *Aquibacter* (Bacteroidetes) and the Pir4 lineage (Planctomycetes) were present only in the most abundant taxa in nodule-associated assemblages from Flat but not Deep Plain nodules.

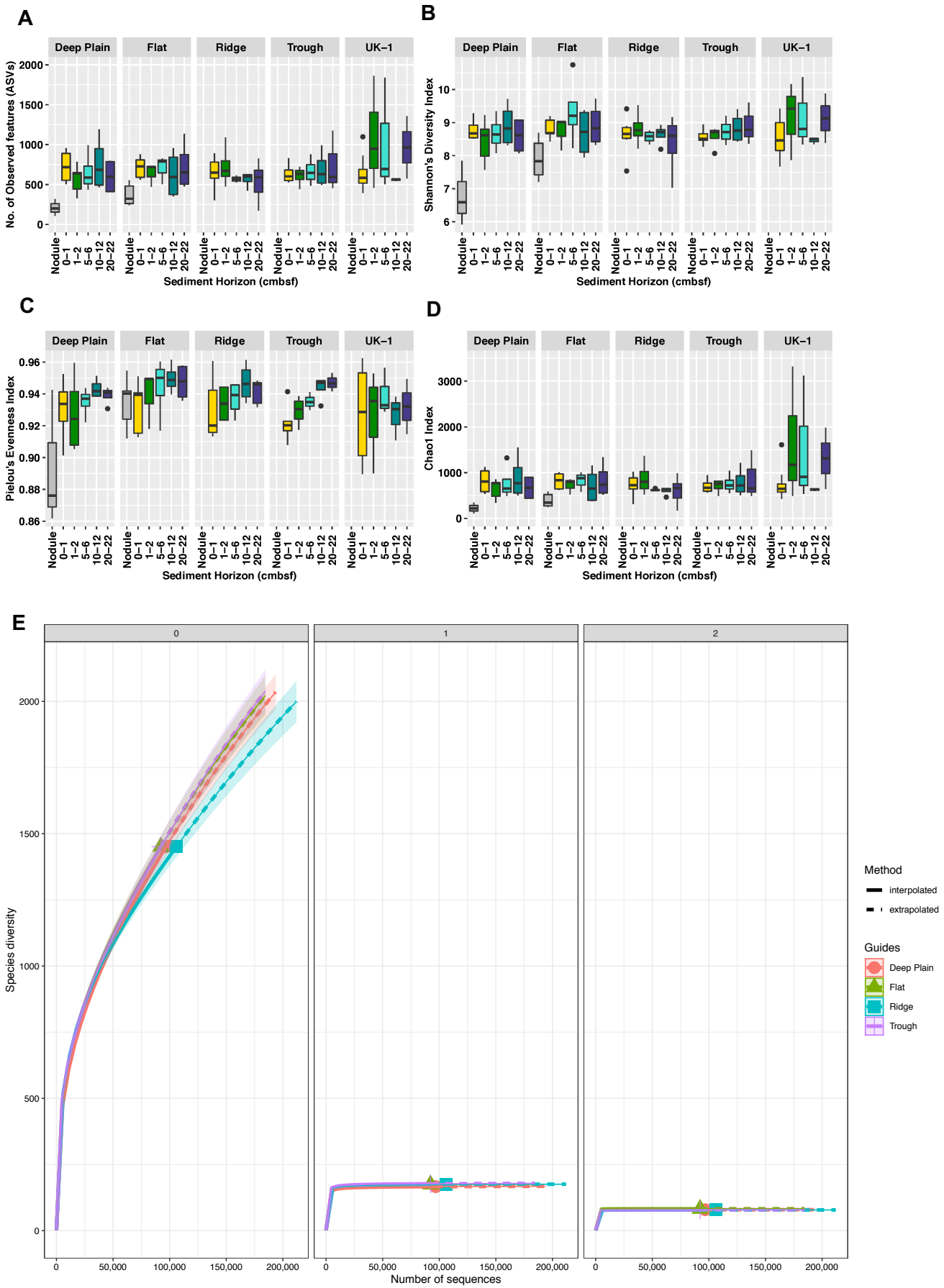
A
B
C
D
E
Deep Plain
Flat
Ridge
Trough
UK−1
No. of Observed features (ASVs)
Shannon's Diversity Index
Pielou's Evenness Index
Chao1 Index
Nodule
0−1
1−2
5−6
10−12
20−22
Sediment Horizon (cmbsf)
Species diversity
Number of sequences
Method
interpolated
extrapolated
Guides
Deep Plain
Flat
Ridge
Trough

Figure 3. Comparisons of alpha diversity between sediment and nodule-associated microbial communities, at APEI-6 and UK-1, based on **(a)** Number of observed features or amplicon sequence variants (ASVs), **(b)** Shannon's Diversity Index, **(c)** Pielou's Evenness Index, **(d)** Chao1 richness Index. Boxplots show the median (horizontal line), interquartile ranges (box limits), range (whiskers) and outliers (black dots). Diversity indices were calculated on samples that had been rarefied to account for uneven sampling depth. **(e)** Sample-based alpha diversity species accumulation curves calculated for each sample type without replacement for APEI-6 sediment samples only, showing all horizons within each topographical region. Diversity estimates are calculated and plotted for rarefied samples with respect to sample size, and plotted extrapolated curves are based on double the reference sample size. Curves for each sample type do not reach asymptote, indicating insufficient sequencing depth for estimated microbial richness. Numbers on top of each panel represent Hill's diversity numbers of order 0,1 and 2, representing species richness (S_N), the exponential form of the Shannon index (exp *H'*), and the inverse form of Simpson's index (1/*D*) respectively.

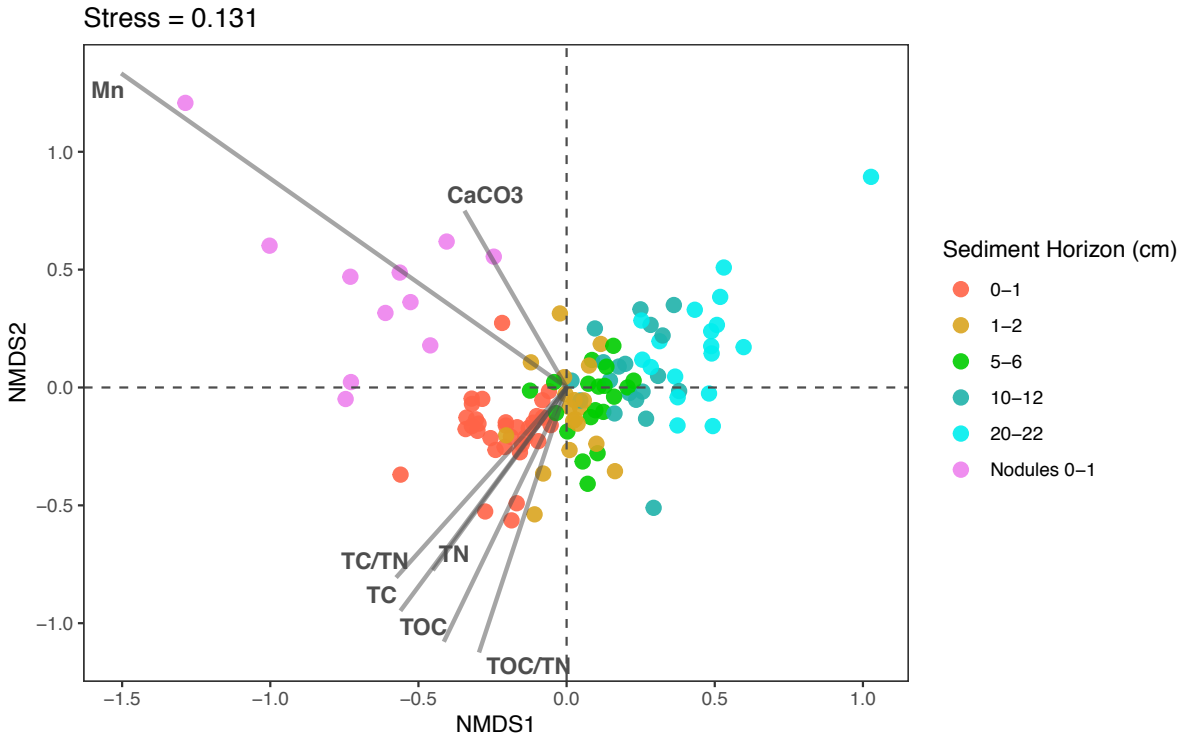

Figure 4 (**a**). Non-metric multidimensional scaling (nMDS) plots of microbial community dissimilarity based on Bray-Curtis distances. The points represent individual samples and their proximity to one another indicates their similarity. The closer the points are to each other, the more similar the samples are to each other based on community structure and composition. Nodule-associated and sediment communities through the sediment profile (0-22 cm) from APEI-6 and UK-1 in the CCFZ. Nodules are from the surface sediments (0-1 cm) of the Deep Plain and Flat regions of APEI-6.

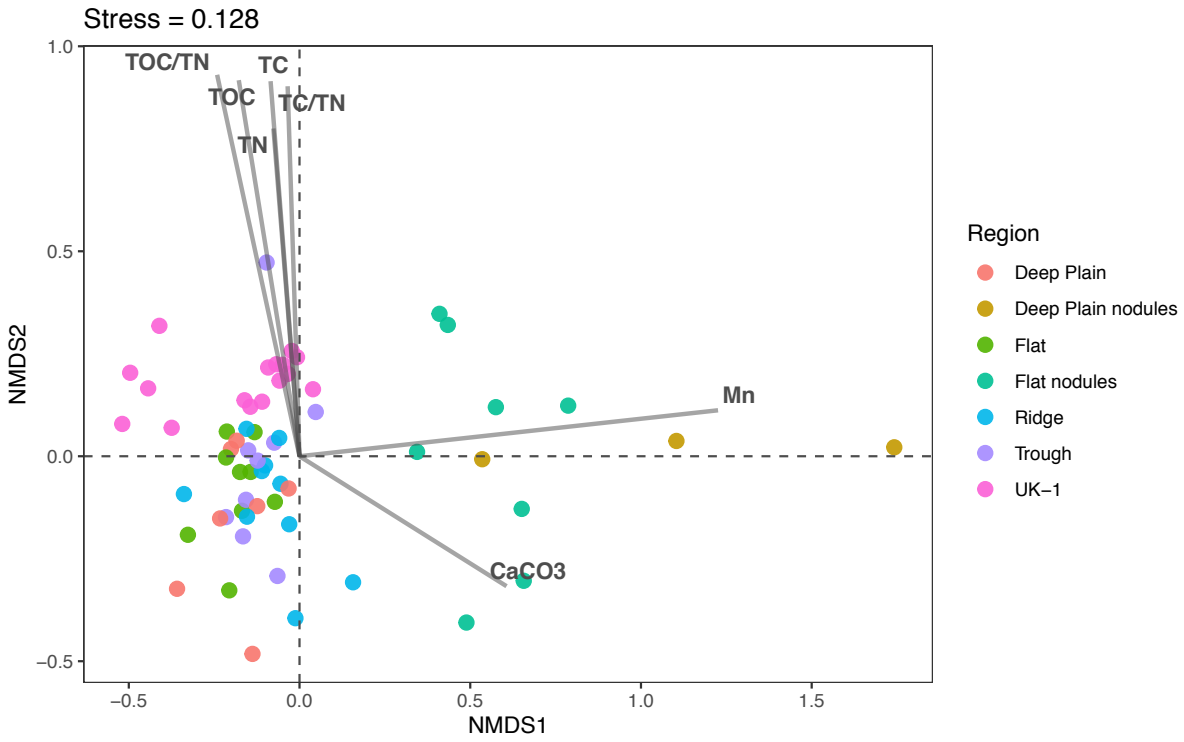

Figure 4 (**b**) Sediment communities in the top 2 cm of sediment across the four regions in APEI-6 and sediment at UK-1. Nodules are from the surface sediments (0-1 cm) of the Deep Plain and Flat regions of APEI-6. The relationship of environmental variables to the samples are shown with arrows, where the greater the arrow length, the stronger the correlation. Environmental variables are from Jones *et al*., (2021) and Menendez *et al*., (2019).

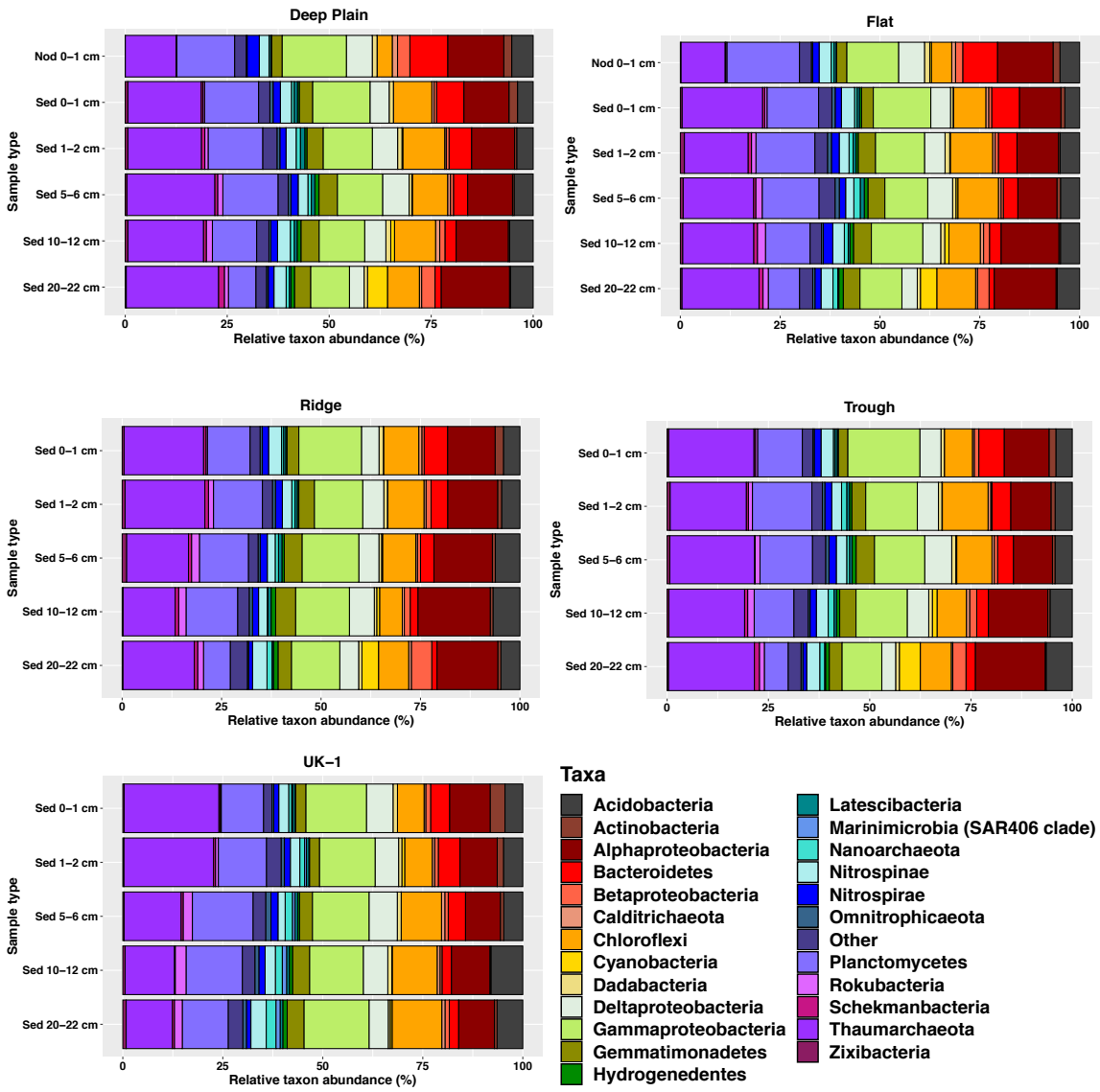

Figure 5. Relative abundances of amplicon sequence variants (ASVS) at phylum level nodule-associated and sediment (0-22 cm) from APEI-6 and UK-1 in the CCFZ. Nodules are from the surface sediments (0-1 cm) of the Deep Plain and Flat regions of APEI-6. Only taxa ≥ 1% relative abundance are shown. Category "Other" represents taxa < 1% relative abundance. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

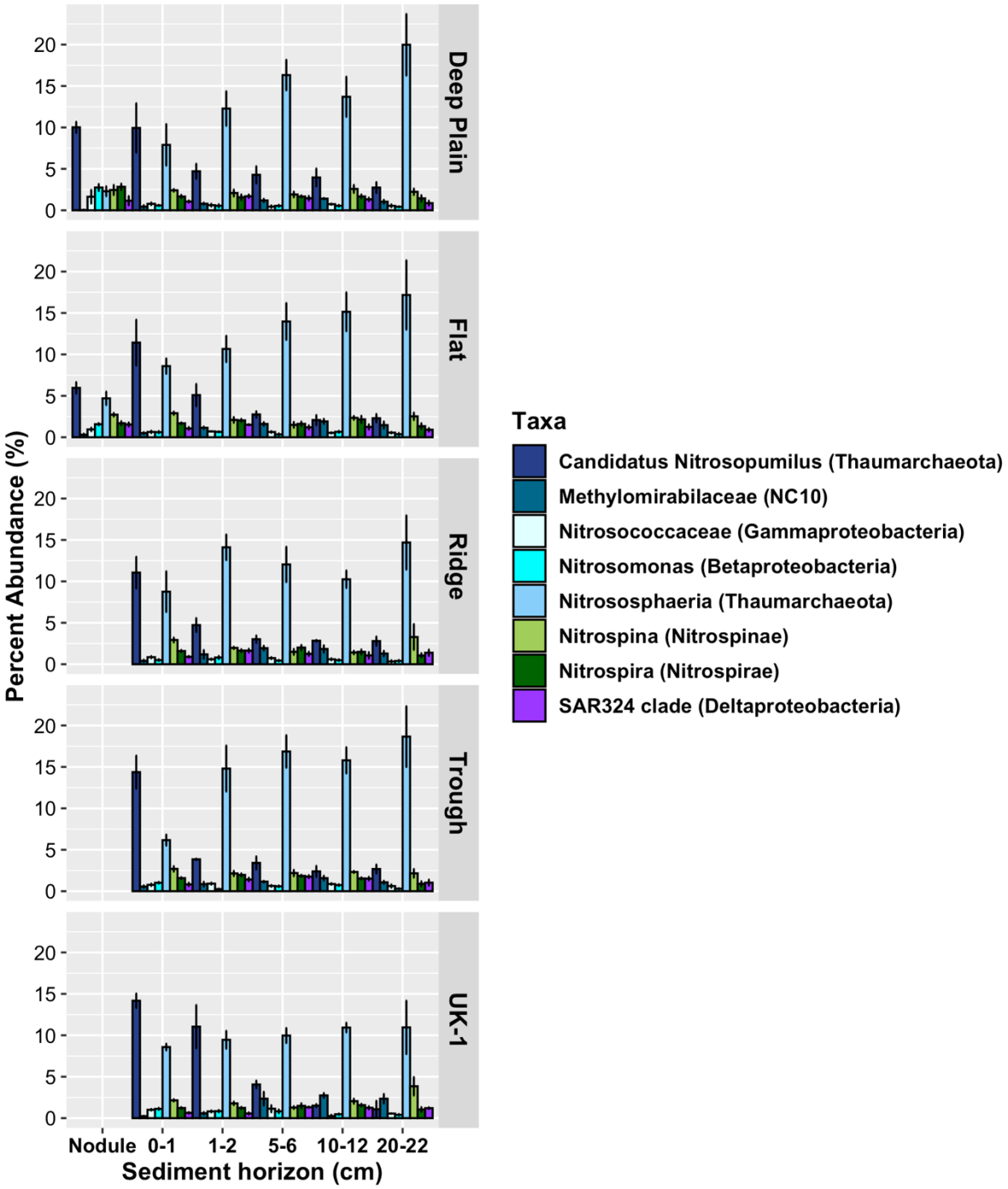

Figure 6. Relative abundances of amplicon sequence variants (ASVs) of the most abundant putative carbon-fixing and nitrifying microbes in sediments and nodules within each region at APEI-6 and at UK-1, defined at genera or family level. The class Nitrososphaeria includes all taxa within that class excluding the genera *Candidatus Nitrosopumilus*, which is shown in a separate category. Only taxa ≥ 1% relative abundance are shown. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

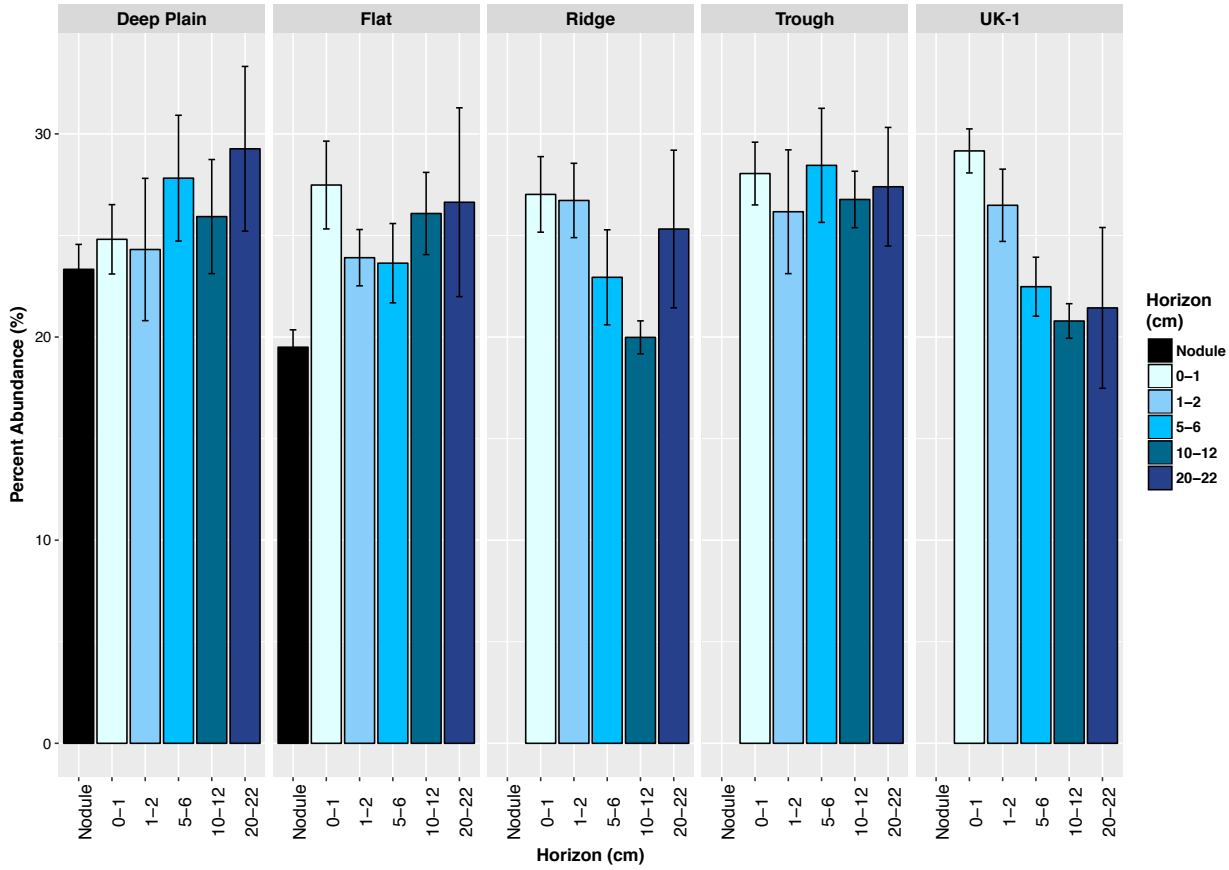

Figure 7. Total relative abundances of amplicon sequence variants (ASVs) of putative carbon-fixing and nitrifying microbes in sediments and nodule-associated communities at APEI-6 and UK-1. Error bars represent standard deviation of the 3 or more replicates for each category. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

Table 3. Sediment biogeochemistry and environmental data for each APEI-6 SW landscape type and UK-1 (UK Seabed Resources Limited eastern contract area) which were collected during cruise JC120 (Jones *et al*., 2021). Parameters are shown as averages (mean) with standard deviation (SD) and ranges (minimum-maximum) shown in brackets. Grain size for each 5 cmbsf sediment profile interval are listed as sample means, showing minimum and maximum. No ranges are given for UK-1 owing to lack of replication. Manganese (Mn) values are from Menendez *et al*., (2019), collected during cruise JC120, and show ranges within the uppermost 14 cmbsf of sediment at APEI-6 and UK-1.

Parameter	APEI-6 Deep Plain	APEI-6 Flat	APEI-6 Ridge	APEI-6 Trough	UK-1
n	3	6	5	5	1
TC (%) ± SD	0.46 ± 0.07	0.49 ± 0.08	0.47 ± 0.06	0.49 ± 0.06	0.72
TOC (%) ± SD	0.42 ± 0.04	0.44 ± 0.05	0.41 ± 0.04	0.44 ± 0.05	0.71
TN (%) ± SD	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.14
TOC:TN ± SD	4.5 ± 0.2	4.7 ± 0.3	4.5 ± 0.2	4.7 ± 0.5	5.7
TC:TN ± SD	4.89 ± 0.43	5.23 ± 0.44	5.11 ± 0.31	5.17 ± 0.50	5.8
$CaCO_3$ (%) ± SD	0.3 ± 0.36	0.4 ± 0.05	0.5 ± 0.16	0.4 ± 0.09	0.1
Mn wt. % (range across 5 cm interval)	0-5 cm: 0.48 - 0.51 5-10 cm: 0.44 – 0.46 10-15 cm: 0.44	0-5 cm: 0.47 – 0.52 5-10 cm: 0.51 – 0.53 10-15 cm: 0.56	0-5 cm: 0.54 – 0.58 5-10 cm: 0.55 – 0.61 10-15 cm: 0.58	0-5 cm: 0.42 – 0.48 5-10 cm: 0.44 – 0.46 10-15 cm: 0.44	0-5 cm: 0.77 – 0.95 5-10 cm: 0.72 – 0.79 10-15 cm: 0.56
Nodule density, no m^{-2}	632 (484 - 776)	307 (200 - 388)	246 (137 - 370)	200 (14 - 455)	16
Grain size, µm (min – max range across 5 cm interval)	0-5 cm: 6.53 - 8.86 5-10 cm: 6.19- 7.89 10-15 cm: 5.72 - 8.76 15-20 cm: 5.68 - 8.89	0-5 cm: 7.15 - 7.61 5-10 cm: 6.50 - 8.52 10-15 cm: 6.06 - 7.24 15-20 cm: 5.77 - 8.55	0-5 cm: 6.71 - 9.21 5-10 cm: 6.56 - 8.72 10-15 cm: 6.33 - 11.67 15-20 cm: 6.07 - 10.61	0-5 cm: 7.60 - 8.50 5-10 cm: 7.49 - 11.16 10-15 cm: 6.47 - 20.08 15-20 cm: 6.35 - 20.15	0-5 cm: 18.06 5-10 cm: 17.6 10-15 cm: 17.58 15-20 cm: 18.74

Table 4. Output permutational multivariate analysis of variance (PERMANOVA) on relative abundance counts that have been fourth-root transformed prior to creation of Bray-Curtis dissimilarity matrices for sediment microbial assemblages at APEI-6, showing the following: all 16S microbial assemblages; putative carbon-fixing and nitrifying taxa within sediment microbial assemblages; and taxa within the Nitrososphaeria class in the Thaumarchaeota phylum. The interaction effect "Core" refers to Megacore and is nested within Topography. The interaction effects Topography and Sediment horizon are crossed. Percent variation based on Sum of Squares (SS) is shown as column "% var".

All 16S microbial sediment assemblages						
Source	**d.f.**	**SS**	**MS**	***F***	***P***	***% var.***
Topography	3	2098.7	699.56	0.9025	0.694	2.7
Sediment Horizon	4	17661	4415.2	6.9772	0.001	23.0
Core (Topography)	18	15049	836.06	1.3212	0.001	20.0
Topography x Horizon	12	7245.7	603.81	0.95418	0.801	9.6
Residuals	46	29109	632.81			38.6
Total	83	75400				
Putative carbon-fixing and nitrifying taxa						
Source	**d.f.**	**SS**	**MS**	***F***	***P***	***% var.***
Topography	3	62.12	20.707	0.40553	0.864	1.0
Sediment Horizon	4	2491.9	622.97	15.566	0.001	41.1
Core (Topography)	18	1004.2	55.79	1.3941	0.125	16.6
Topography x Horizon	12	223.81	18.651	0.46605	0.981	3.7
Residuals	46	1840.9	40.02			30.4
Total	83	6061				
Nitrososphaeria taxa (Thaumarchaeota)						
Source	**d.f.**	**SS**	**MS**	***F***	***P***	***% var.***
Topography	3	713.94	237.98	0.85126	0.648	1.8
Sediment Horizon	4	15002	3750.6	14.499	0.001	37.4
Core (Topography)	18	5193.1	288.5	1.1153	0.222	13.0
Topography x Horizon	12	3059.2	254.94	0.98551	0.555	7.6
Residuals	46	11899	258.68			30.0

2.5. Discussion

2.5.1 Spatial differences in benthic assemblages between substrates

Microbial assemblages in sediments were more diverse than those associated with polymetallic nodules at both APEI-6, and UK-1, a finding that agrees with studies conducted in other areas of the Pacific (Wu *et al*., 2013; Tully & Heidelberg, 2013; Zhang *et al*., 2014; Shulse *et al*., 2016; Lindh *et al*., 2017; Molari *et al*., 2020). Microbes related to potential metal-cycling (Magnetospiraceae and Kiloniellaceae), organic carbon remineralisation (*Woeseia*), and sulphur-oxidising Thiohalorhabdaceae were also more enriched in nodules than sediments. Other ASVs that were more prevalent in nodules included *AqS1*, *Cohaesibacter*, *JdFR-76*, *Pelagibius*, *Nitrospina* and *Nitrospira*. These ASVs have been previously reported in other nodule-associated communities (Wu *et al*., 2013; Blöthe *et al*., 2015; Molari *et al*., 2020).

2.5.2 Spatial differences in sediment benthic assemblages

Previously, abyssal plain habitats were considered to be homogenous landscapes, and the microbial constituents inhabiting the sediments were assumed to be cosmopolitan in their distribution. Yet, the distributions of metazoans, such as megafauna (Durden *et al*., 2015; Leitner *et al*., 2017; Simon-Lledó *et al*., 2019; Durden *et al*., 2020), macrofauna (Cosson *et al*., 1997), and meiofauna (Stefanoudis *et al*., 2016), are influenced by seafloor heterogeneity. Bathymetric variation of the seafloor can modify bottom current velocities, which in turn effect local sedimentation rates (Mewes *et al*., 2014) and POM deposition (Durden *et al*., 2015; Morris *et al*., 2016). In the CCFZ, however, environmental features were generally homogenous between the landscape regions. Grain size distribution, relative proportions of TC, TOC, TN, TOC:TN, TC:TN, $CaCO_3$ and Mn did not vary significantly between the landscape regions of this area (Table 3; Menendez *et al*., 2019; Simon-Lledó *et al*., 2019), which has also been reported in nearby areas of the CCFZ (Khripounoff *et al*., 2006; Volz *et al*., 2018). Sediments at the CCFZ have been reported to be oxygenated (50 μmol L^{-1}) down to 2-3 m depth (Haeckel *et al*., 2001; Mewes *et al*., 2014; Volz *et al*., 2018; Menendez *et al*., 2019), and across the four landscape types at APEI-6, the oxygen penetration depth (OPD) was similar, at > 2.0 m depth (Menendez *et al*., 2019; Jones *et al*., 2021). One

factor that did vary between the landscape regions, however, was nodule density across the seafloor, which was twice as high at Deep Plain, compared to Flat, Ridge and Trough (Table 3: Simon-Lledó *et al*., 2019).

At APEI-6, we observed spatial differences in the structure and composition of sediment assemblages, across small scales (cm), through the sediment depth profiles, to mesoscales (m to km) between cores within topographical regions. Across a range of 100s metres to kilometres, the structure of assemblages varied between coring locations within each topographical region, a phenomenon previously observed in meiofauna (Stefanoudis *et al*., 2016). Benthic assemblages also varied at larger scales of hundreds of km, between APEI-6 and UK-1. However, the lack of replicates at UK-1 prevented robust statistical analysis. We noted higher alpha diversity at UK-1 compared to APEI-6, a pattern also reported by Lindh *et al*., (2017). Putative chemolithoautotrophic microbes, such as *Nitrosopumilus*, were more prevalent in UK-1 sediments, particularly in surface layers. Another group, non-photosynthetic Cyanobacteria, Melainabacteria group bacterium S15B-MN24 CBMW_12, was found in the deeper sediment layers of APEI-6 but were largely absent from UK-1 sediments.

Differences in assemblages between the two locations may be driven by varying biogeochemistry. The chemical composition of sediments at UK-1 differs from that at APEI-6, with higher proportions of total carbon (TC), total organic carbon (TOC), total nitrogen (TN), TOC:TN and TC:TN ratios, $CaCO_3$ (Table 3), as well as minerals Mn, Ni and Cu in the sediments of UK-1 (Menendez *et al*., 2019). Differences in the biogeochemistry of sediments of the neighbouring APEI-3 compared to contract areas, as well as lower faunal abundances have also been reported (Volz *et al*., 2018). The UK-1 area is also closer to the equatorial region of higher primary production in the overlying waters than APEI-6, giving rise to a higher flux of POC to the sediments (Menendez *et al*., 2019). As a result, the oxygen penetration depth (OPD) in UK-1 sediments is shallower (~150 cmbsf) than it is at APEI-6 (> 300 cmbsf) (Menendez *et al*., 2019), which is likely to influence benthic assemblage structure and composition.

2.5.3 High abundance of chemoautotrophic and nitrogen cycling taxa

In the dark ocean, it is now recognized that chemolithoautotrophic microbes form a significant source of organic carbon through inorganic carbon fixation (Swan *et al*., 2011; Middelberg, 2011; Molari *et al*., 2013; Tully & Heidelberg, 2016; Sweetman *et al*., 2019), which could be equal to or slightly greater than phytoplankton export production in some regions (Reinthaler *et al*., 2010). Nitrification is an important process in benthic nitrogen cycling and accounts for a large proportion of the chemoautotrophic carbon fixation in the deep ocean (Herndl *et al*., 2005; Wuchter *et al*., 2006; Middelberg, 2011). Although not well constrained, using reduced compounds to drive energy for carbon fixation, alongside anabolic processes of inorganic carbon incorporation, may be potentially important strategies for supplementing recalcitrant carbon in oligotrophic deep-sea sediments (Brunnegård *et al*., 2004; Molari *et al*., 2013; Sweetman *et al*., 2019, Trembath-Reichert *et al.,* 2021). Ammonia (NH_3) released from organic matter decay is converted to nitrite (NO_2) mediated by ammonia-oxidising archaea (AOA) or ammonia-oxidising bacteria (AOB), in the first step (Bock & Wagner, 2013). The second reaction, the oxidation of nitrite to nitrate (NO_3) is catalysed by nitrite-oxidising bacteria (NOB), providing an important source of organic material to the benthic community (Middelburg, 2011). Indeed, benthic impact experiments have indicated that microbial nitrification was reduced following sediment disturbance and ploughing (Vonnahme *et al*., 2020), hence likely impacting nutrient cycling and the availability of labile organic matter (Haeckel *et al*., 2001).

Across all four of the topographical landscape types of APEI-6 and within UK-1, both sediment and nodule-associated communities were dominated by putatively chemoautotrophic and nitrogen-cycling microbes, up to 29% in sediments and 21% from nodules (Fig. 7). We observed a community of nitrifying microbes at CCFZ, that was numerically dominated by AOA *Nitrosopumilus* (Nitrososphaeria class), which oxidise ammonia to nitrite (Könneke *et al*., 2005) and have previously been observed in deep-sea sediments in this region, despite the low input of phytodetritus (Nitahara *et al*., 2011, 2017; Shulse *et al*., 2016). There were also other nitrifiers present, including the AOA: *Candidatus* Nitrosopelagicus, *Candidatus Nitrosopumilus*, *Candidatus* Nitrosotenuis, taxa within the Nitrososphaeria class; ammonia-oxidising bacteria (AOB) *Nitrosomonas*

(Betaproteobacteria), and *AqS1*(Gammaproteobacteria); and nitrite-oxidising bacteria (NOB) *Nitrospina* (Nitrospinae) and *Nitrospira* (Nitrospirae).

The relatively high abundances of sequences corresponding to AOA *Ca*. *Nitrosopumilus* and NOB genera *Nitrospina and Nitrospira* have also been reported in other oligotrophic sediments with Fe-Mn deposits in the South Pacific Gyre (Tully & Heidelberg, 2016; Kato *et al*., 2019), in the CCFZ (Nitahara *et al*., 2011, 2017: Shulse *et al*., 2016) and in the Peru Basin (Molari *et al*., 2020). The high proportions of these AOA and NOB groups at CCFZ suggest a coupling between ammonia and nitrite oxidation. This co-occurrence has also been noted in marine environments, from water column (Mincer *et al*., 2007; Santoro *et al*., 2010), to shallow sediments (Herbert, 1999; Mills *et al*., 2008) and deep sediments (Tully & Heidelberg, 2016; Shulse *et al*., 2016; Molari *et al*., 2020) to freshwater environments such as oligotrophic lakes (Parro *et al*., 2019). For the AOA at least, greater numbers have been reported in oligotrophic sediments with low ammonium concentrations, and as AOA have a higher affinity for ammonia, they outcompete AOB in these environments (Martens-Habbena et al., 2009). The nitrate produced by the NOB may be used in other microbial metabolisms, such as oxidation of organic molecules and reduced sulphur compounds (Parro *et al*., 2019).

In addition to nitrification, carbon fixation in abyssal ecosystems might take place through other metabolic pathways and microbial lineages. Sediment assemblages at APEI-6 and UK-1 contained the methanotrophic taxon *wb1-A12* (family Methylomirabilaceae, phylum NC10). This group are nitrite-dependent anaerobic methane oxidising (N-damo) bacteria that utilise nitrate or nitrite as an electron acceptor to oxidise methane into CO_2 (Ettwig *et al*., 2010). In addition, we observed the SAR324 clade (Deltaproteobacteria) in sediment assemblages, a metabolically flexible taxon capable of heterotrophy as well as autotrophy, that is capable of oxidising reduced sulphur compounds to fix inorganic carbon (Swan *et al*., 2011; Sheik *et al*., 2014). Hence, the ubiquity of these microbial groups, in particular the nitrifiers, in the sediment might provide a source of organic carbon and nitrate for the benthic communities at CCFZ. Indeed, microbial carbon incorporation mechanisms in deep-sea environments that lack high quantities of

reduced compounds are variable (Trembath-Reichert *et al*., 2021) and warrant further study in these environments.

As many of the microbial taxa in the CCFZ have neither been cultured or genomically sequenced, and with few in-situ microbial activity or cultivation experiments available for this region (Sweetman *et al*., 2019; Gillard *et al*., 2019; Vonnahme *et al*., 2020), it is difficult to definitely infer metabolic capability from 16S sequences alone. For this reason, other methods such as metagenomics should be conducted to better predict the metabolic functions that we hypothesize, here, and to link those back to taxonomic markers. In addition, metatranscriptomics should be implemented to address which genes are actually being expressed in this region and to what extent, to provide a more accurate view of the functional processes taking place. Developing a comprehensive microbial ecology of the CCFZ will contribute to our knowledge of microbially mediated biogeochemical cycling and will help to develop a mechanistic understanding of the contribution that benthic microbial communities provide to the overall resilience of nodule-associated communities.

2.6 Conclusion

Differences in microbial community composition at APEI-6 were predominantly driven by depth through the sediment profile and seafloor heterogeneity at small and mesoscales. We observed a diverse and varied microbial assemblage through the sediment, both within APEI-6 and at UK-1. Communities differed between nodules and sediments. The predominance of nitrifying and putative carbon-fixing microbes within sediment assemblages suggests a key ecological role of these groups of taxa in the CCFZ, and our results emphasize the importance of microbial communities in mediating carbon, nitrogen and sulphur cycling in this habitat.

Understanding the diversity and functional roles of benthic microbes is vital for the sustainable use and preservation of ecosystems targeted for commercial-scale deep-sea mining. Therefore, ascertaining the composition and diversity of benthic microbial assemblages in both APEI-6 and mining exploration contract regions of the CCFZ will be essential ahead of mining activities and will be a useful tool for monitoring in the future.

Author contributions:

D.J, R.Y and **A.H** conceived the study, **R.Y** and **D.J** performed sampling, **A.H** processed the samples and analysed the data. **A.H** wrote the paper with the contribution from all authors.

Conflict of interest:

The authors declare no conflict of interest.

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Chapter 3: Productivity regimes and carbon input influence the composition of benthic abyssal microbial assemblages in the Atlantic and Pacific

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3.1 Abstract

Running title: Carbon input structures abyssal microbes

Abyssal sediments represent a vast habitat for complex communities of microbes that play important roles in biogeochemical cycles. Pelagic-benthic coupling and the supply of organic matter in the form of particulate organic carbon (POC) flux is fundamental for the majority of benthic biota including microbes, yet the role of microbial communities in the deep sea is still not fully realized. The productivity of surface waters is strongly correlated with the diversity of megafauna and macrofauna, however, this relationship appears to be weaker for microbes.

Here, we investigated the composition and spatial variability of benthic microbial assemblages in sediments from three sites with varying productivity regimes, from oligotrophic to more eutrophic conditions, in the Atlantic and Pacific. Our results reveal distinct sediment microbial assemblages driven by productivity regime and oxygen penetration of sediments. The oligotrophic site harboured highly diverse microbial assemblages, as well a greater diversity of nitrifying microbes, compared with the eutrophic site. Conversely, there was a greater diversity of Archaea at the eutrophic site, where assemblages were structured by sediment horizon, and taxa associated with anoxic conditions, such as the Anaerolineaceae family and MSBL5 group in the Chloroflexi phylum, and *Candidatus Scalindua* sp. in the Planctomycetes phylum were highly abundant. Distinct populations of the putative ammonia-oxidising archaea *Nitrosopumilus* were observed between the three sites, the most diverse of which was at the oligotrophic site, indicative of nîche diversity and resource partitioning. Our results suggest that oligotrophic abyssal sediments host a wide diversity of putative AOA and nitrifying microbes, which has implications for biogeochemical cycling and nutrient availability in the CCFZ region.

3.2 Introduction

Abyssal plains cover vast areas of the Earth's surface and play important roles in biodiversity, ecosystem functioning and biogeochemical cycles (Parkes *et al*., 1994; Jørgensen & Boetius, 2007). The main food source for abyssal ecosystems is phytodetritus, generated in the surface ocean. This cellular material produced from the primary production of phytoplankton gradually sinks through the water column, degraded by heterotrophic microbes on its way down (Fuhrman 1987; Turley *et al*., 1995; Azam & Long, 2001). Only a small fraction reaches the seafloor in the form of sinking particulate organic matter (POM) (Smith *et al*., 1987; Ducklow *et al*., 2001).

Transport of this organic matter (OM) is fundamental for deep-sea biota in areas of the seafloor not associated with hydrothermal vents and cold seeps. This supply of food may be seasonal, following phytoplankton blooms, or be episodic, such as whale falls (Billett *et al*., 1983; Smith *et al*., 1997; Jørgensen & Boetius, 2007). This variability influences patterns of faunal abundance, diversity and ecosystem functioning and structure, for megafauna (Ruhl & Smith, 2004; Smith *et al.*, 2009; Billett *et al*., 2010; Amaro *et al*., 2019, Kuhnz *et al*., 2020), macrofauna and meiofauna (Gambi & Danovaro, 2006; Laguionie-Marchais *et al*., 2013), however, the responses of microbes to patterns of POC flux have been less forthcoming (Smith *et al*., 1997; Laguionie-Marchais *et al*., 2013).

Food supply to the benthos varies latitudinally, and is an important driver that influences deep-sea communities (Rex *et al*., 2005; Saeedi *et al*., 2019), including microbes (Kallmeyer *et al*., 2012). There remains, however, a great deal of uncertainty about the role of deep-sea microbes in carbon processing. Microbes inhabiting abyssal sediments form a dominant component of benthic communities in terms of abundance and biomass (Whitman *et al*., 1998; Rex *et al*., 2006) but also perform key roles in biogeochemical cycles (Parkes *et al*., 1994; Jørgensen & Boetius, 2007). A number of factors influence the composition and distribution of sediment microbial assemblages, including organic matter input (D'Hondt *et al*., 2009; Bienhold *et al*., 2012, 2016; Kallmeyer *et al*., 2012; Jacob *et al*., 2013) sediment horizon depth (Walsh *et al*., 2016a,b), seafloor depth (Jacob *et al*., 2013, Hamdan *et al*., 2013) and geographical location (Bienhold *et al*., 2016).

A discrepancy between carbon supply and sediment assemblage demand has long been recognized (Berelson *et al*., 1997; Ruhl *et al*., 2008), yet, to date, the link between pelagic-benthic coupling in the form of POC flux, and the role of microbial communities in the deep sea is still not fully realized. It is increasingly recognized that chemolithoautotrophic microbes form a significant source of organic carbon through inorganic carbon fixation in the dark ocean (Middelberg, 2011; Swan *et al*., 2011; Molari *et al*., 2013; Tully & Heidelberg, 2016; Sweetman *et al*., 2019). Certain groups of microbes may be able to use reduced compounds to drive energy for carbon fixation, alongside anabolic processes of inorganic carbon incorporation to supplement recalcitrant carbon in oligotrophic deep-sea sediments (Brunnegård *et al*., 2004; Molari *et al*., 2013; Sweetman *et al*., 2019; Trembath-Reichert *et al*., 2021). It is therefore crucial to define the microbial response to POC fluxes in order to resolve these energy discrepancies and comprehend the role of benthic microbes within the marine carbon cycle.

The transport of POM to the deep-ocean is fundamental for deep-sea life and the importance of this tight coupling between the surface and abyssal plain has been demonstrated with long-term, continuous monitoring stations. Two of these are in the North Atlantic (Porcupine Abyssal Plain: Billett *et al*., 2001) and the North-Eastern Pacific (Station M: Ruhl & Smith, 2004; Smith *et al*., 1997), which provide long time series records on POC flux data (Smith & Druffel, 1998). PAP experiences seasonal POC flux variations, evidenced by visible deposition of material on the seafloor (Bett *et al*., 2001, Billett *et al*., 2001, 2010) and average organic carbon deposition is 3.32 mg C_{org} m^{-2} d^{-1} (Lutz *et al*., 2007). Similarly, at Station M, flocs of material and phytodetritus are visible on seafloor after seasonal deposition (Beaulieu & Smith, 1998) and here, average carbon deposition is 8.1 mg C_{org} m^{-2} d^{-1} (Smith *et al*., 2018). In contrast, CCFZ is an oligotrophic region, where organic carbon deposition is much lower than at the other two sites, at 1 mg C_{org} m^{-2} d^{-1} (Volz *et al*., 2018).

Here, we investigate bacterial and archaeal assemblages via amplicon sequencing of the 16S rRNA genes, from abyssal sediment samples from monitoring stations at PAP and Station M, and from an oligotrophic site in the CCFZ, in order to determine the impact of POC flux on the diversity and composition of these communities. Following this, three main questions were

addressed: (1) Does location influence diversity and composition of sediment assemblages? (2) How does sediment horizon depth drive microbial assemblage structure? (3) Are there differences in the distribution of chemolithoautotrophic microbes between the three locations?

3.3 Methods

3.3.1 Sample collection, DNA extraction and sequencing

Sediment samples from CCFZ were collected as detailed in Hollingsworth *et al*., (2021). Samples from PAP and Station M were collected using the same methods as for the CCFZ samples in chapter 2 (Hollingsworth *et al*., (2021). Samples collected from The Porcupine Abyssal Plain Sustained Observatory (PAP-SO) area in the northeast Atlantic (centred on 48°50'N 016°30'W; Fig. 8), during the RRS *Discovery* DY377/378 cruise 05th - 27th July 2012. Samples were collected from Station M (35°10'N, 122°59'W) using the remotely operated vehicle (ROV) *Doc Ricketts* as part of the Monterey Bay Aquarium Research Institute's (MBARI) R/V *Western Flyer* cruises during 7th-16th Nov 2017 and 18th-24th April 2018. Three replicate push cores taken 2 m apart were used during each Station M cruise to collect the sediment. Once the ROV was back on deck, the sediment was processed in the same manner as for CCFZ and PAP, whereby cores were sliced at 1 cm intervals to 5 cm below seafloor (cmbsf), then 5-10 cmbsf and 10-15 cmbsf, in a cold room at 4°C and immediately stored at -80 °C onboard.

Figure. 8. Location of the three sites where sediment sampling occurred, shown with a red star, showing the Porcupine Abyssal Plain (PAP) in the northeast Atlantic Ocean, Station M in the northeast Pacific Ocean and the Clarion-Clipperton Zone (CCFZ) of the northern equatorial Pacific.

All sediment samples in this study were processed with the same protocol, as described in Hollingsworth *et al*., (2021). Sediment samples were taken from four expeditions that covered multiple years, although with the exception of Station M, samples taken from each site were within the same year. Variability between cores for sediment samples from Station M, taken Nov 2017 and April 2018, was non-significant and less than that for location or sediment horizon depth. We are therefore confident that using samples from two different dates will provide a reliable picture of assemblage composition change, and that the temporal factor at Station M is therefore likely to be minimal in this case.

3.3.2 Data analysis

The same pipeline was implemented to analyse the demultiplexed sequences as Hollingsworth *et al*., (2021). A feature table was generated which contained 4,015,898 reads and 60,753 Amplicon Sequence variants (ASVs), with an average of 32,386 reads per sample. The feature table was normalised to 4,100 sequences per sample prior to abundance and diversity analysis. Samples were categorized by location and by sediment horizon. To calculate the proportion of species within each library, relative abundances were calculated on ASV feature tables with raw counts that had been normalised to library size, before being subjected to Hellinger transformation. Alpha diversity indices (observed features (q=0), Shannon-Wiener Index, Pielou's Evenness index, Chao1 Richness Index) and beta diversity (changes in species composition from local to region scales based on counts at each site (Bray-Curtis dissimilarity)) were calculated on normalised samples using the QIIME2 (Quantitative Insights Into Microbial Ecology) software (Bolyen *et al*., 2019) and used for further analysis in R. A number of packages were utilised in the R software program for diversity and statistical analysis. Sample-based alpha diversity species accumulation curves were calculated for each sample type without replacement, using the 'iNEXT' package in R v 3.3.2 (Hsieh *et al*., 2020; Chao *et al*., 2014). Diversity estimates were calculated using rarefied samples and plotted with respect to sample size. These diversity estimates were calculated as Hill's diversity numbers of order 0,1 and 2 (Hill, 1973; Jost, 2006), representing species richness (S_N), the exponential form of the Shannon index (exp *H'*), and the inverse form of Simpson's index (1/*D*) respectively. Extrapolated diversity estimates were calculated and plotted based

on double the reference sample size. Alpha diversity indices were displayed as boxplots using the R package 'ggplot2' v. 3.3.3 (Wickham, 2016). Non-metric multidimensional scaling (NMDS) ordination was used to visualise community variation between the three sites and sediment horizons using the 'vegan' package (Oksanen *et al*., 2007; 2017) and the plot was illustrated with 'ggplot2'. Abundance plots based on relative abundances and network analyses based on presence/absence (binary) counts of *Candidatus Nitrosopumilus* sp. ASVs were calculated and visualized using the 'phyloseq' package (McMurdie & Holmes, 2013) and 'ggplot2'. A heatmap was generated from Bray-Curtis dissimilarity matrices using the function 'heatmap3' in the R package 'GMD' (Zhao *et al*., 2021), illustrating the top 10 most abundant taxa within sediment at each site. A one-way Analysis of Variance (ANOVA) test was performed on alpha diversity indices to test for differences between sediment horizons and sites in 'vegan" using the 'adonis2' method. Venn diagrams were calculated and displayed using Venny 2.1, based on presence/absence data. https://bioinfogp.cnb.csic.es/tools/venny/index.html).

Tables of ASV counts were fourth-root transformed, which is a less severe method of transformation that is recommended in order to reduce the weighting of highly abundant species compared to less abundant ones (Clarke & Green, 1988; Clarke & Warwick, 2001). Bray-Curtis dissimilarity measures were applied to determine variations in community composition between the three abyssal locations and sediment horizon intervals. Permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) with *posthoc* pair-wise tests were performed on Bray-Curtis dissimilarity matrices using PRIMER v.7 (Clarke & Gorley, 2015). A mixed-effect model was used to partition variance among factors and to test whether microbial assemblage composition varied significantly between factors at all three sites (from 23 megacores, Table 5). In this model, location (OM fluxes) and sediment horizon were treated as fixed factors. To account for potential covariance between depth strata sampled from individual megacores and to reflect the stratified random sampling within each location, megacore was treated as a random factor nested within each location. This model is analogous to a classical repeated measures ANOVA design. Location (OM fluxes) contained three levels: CCFZ, PAP and Station M. Sediment Horizon was grouped into 5 cm

intervals and contained three levels: 0-5 cm, 5-10 cm and 10-15 cm. The main effects, location and depth through the sediment (sediment horizon), the nested factor, core, and the interaction term, location x depth through sediment, were included in the model. Methods of permutation used was 'method (ii) *permutation of residuals under a reduced model*'. Individual species contributions to differences in assemblage composition between the three locations was determined with a 1-way SIMPER analysis, also in PRIMER, from 4^{th} root transformed abundance data. The cumulative contribution of the total within-group similarity was set at a maximum of 80%.

3.4 Results

3.4.1 Microbial diversity at CCFZ, PAP and Station M

After the data were normalised, 3,940,011 reads comprised of 57,468 ASVs were identified in 124 sediment samples retrieved from 23 megacores from CCFZ, PAP and Station M (Table 5). Of these ASVs, 17 % were classified as Archaea and 83 % as Bacteria. The proportion of Archaea was highest at CCFZ, at 19 %, compared to 15 % at PAP and 13 % at Station M.

Alpha diversity differed across the three sites, between horizon intervals within sites and between sites (Fig. 9, Table 6). Alpha diversity was on average higher at CCFZ (Observed ASVs: mean = 708, SE ± 51.43, Shannon: 8.81 ± 0.08) and lower at Station M (Observed ASVs: 370 ± 22.26, Shannon: 7.47 ± 0.14, Fig. 9, Table 3). Deeper horizons (10-15 cm) were more diverse at CCFZ and Station M (Fig. 9, Table 7). Conversely, shallow to mid-layers (0-5 cm and 5-10 cm) were more diverse at PAP, the latter being the most diverse horizon interval of all locations (Observed ASVs: 735 ± 181, Shannon: 8.58 ± 0.52). It is likely that alpha diversity has been under-sampled in this study, as species accumulation curves show ASVs continuing to accumulate at all three locations at this depth of sequencing, particularly at Station M, as curves had not reached a plateau (Fig. 10).

3.4.2 Benthic microbial assemblage composition at CCFZ, PAP and St. M

NMDS plots were used to visualise differences in microbial community composition between the three locations (Fig. 11). Communities are compositionally distinct at all 3 locations, with location explaining 27% of the variance (PERMANOVA, $p = 0.001$, $F_{2,123} = 27.84$, Table 8). Each location supported ASVs that were unique to that site, with only 0.4% (242) ASVs shared between all three sites (Fig. S1 Venn a). PAP and CCFZ shared a greater proportion of ASVs, (7%), whereas ASVs shared between Station M and either PAP or CCFZ was less than 1%.

Sediment communities were dominated by Proteobacteria and Planctomycetes phyla at all three locations (Fig. 12). There were notable

differences in the proportions of certain microbial taxa between the three sites. The Thaumarchaeota phylum dominated sediments at CCFZ and PAP (Fig. 12), forming 18 % and 14% of reads respectively, whereas at Station M, this group was less abundant (7% relative abundance), present mainly in surface to mid-layers (0-5 cm and 5-10 cm). Conversely, Station M sediments appeared to support a greater diversity of archaeal taxa than at CCFZ or PAP, with higher abundances of Asgardaeota, Crenarchaeota, Euryarchaeota, Hydrothermarchaeota and Nanoarchaeaeota in mid- to deeper horizons (Figs. 12,13). Collectively, these archaeal groups formed on average 8 % of sediment communities at Station M, compared to 1 % at CCFZ and 2 % at PAP.

Individual species contributions to differences in community composition between the three sites, determined with SIMPER analysis, indicated that one of the top contributors to differences between PAP and CCFZ was the Melainabacteria group S15B-MN24 CBMW_12 in the Cyanobacteria phylum (Table S4). This group were present in PAP sediments, particularly in mid- to deeper horizons (5-10 cm and 10-15 cm) (Figs. 12,13), forming 2 % of reads. Nanoarchaeaeota were also present at PAP but not as prevalent as at Station M, forming 2 % of reads, compared to 5 % at Station M. Although taxa in the Thaumarchaeota phylum was less abundant at Station M, there was a greater abundance of *Nitrosopumilus* at this site (6 % relative abundance, Figs. 13, 14, Table S5), compared with PAP and CCFZ (both 5 % relative abundance). Taxa associated with more anoxic conditions were prevalent in Station M sediments, such as the Anaerolineaceae family and MSBL5 group in the Chloroflexi phylum and members of the Planctomycetes phylum, such as *Candidatus Scalindua* sp. and the vadinHA49 class (Figs. 13, 14).

3.4.3 Microbial assemblage changes through the sediment profile

Communities were also distinct within each sediment 5 cm layer between the three sites (PERMANOVA, $p = 0.001$, $F_{6,123} = 4.31$, Table 8). At CCFZ and Station M, microbial assemblages differed significantly through the sediment profile (Table 9), whereas at PAP, assemblages were more similar between shallower and deeper sediment horizons. The interaction between location and sediment horizon was also significant (PERMANOVA, $p = 0.001$, $F_{4,123} = 4.76$,

Table 8), although only explained a minor proportion (8.6 %) of the variance. We observed only a small between core variance (9.6 %), suggesting that microbial assemblage composition did not vary much between megacores within each location.

Assemblage composition was more uniform through the sediment profile at PAP, with taxa such as the Kiloniellaceae and Magnetospiraceae families (Alphaproteobacteria), *Woeseia* (Gammaproteobacteria), *Nitrospira* and *Nitrosopumilus* found consistently from surface sediments down to 15 cmbsf. These taxa were also abundant at CCFZ, although there were subtle observable differences in assemblage structure, between the surface sediment layers and deeper layers. Ca. *Nitrosopumilus* was more dominant in surface sediments at CCFZ. Other groups that were also more abundant in surface layers were Flavobacteriaceae, Arenicellaceae, Chitinophagales, AqS1, SAR202 clade, Nitrospinaceae and the Sva0996 marine group. In the deeper layers (10-15 cm) at CCFZ, taxa such as Ralstonia, Thiohalorhabdaceae, nitrite-oxidising *Nitrospina* and non-photosynthetic Cyanobacteria (Melainabacteria) were observed in higher abundances. Assemblage composition changes through the sediment profile were most evident at Station M, with taxa associated with oxic conditions in the top 5 cm of sediment to taxa associated with more anoxic conditions deeper than 5 cmbsf. Taxa such as *Nitrosopumilus* and *Woeseia* which were most abundant in surface sediments at Station M were virtually absent deeper than 10 cmbsf. Instead, taxa such as the Anaerolineaceae family (Chloroflexi) SEEP-SRB1 (Deltaproteobacteria) and groups within the Planctomycetes phylum such as SG8-4, vadinHA49 and *Candidatus Scalindua* sp. became more dominant in sediments deeper than 5 cmbsf (Figs.13, 14).

3.4.4 Carbon-fixing taxa and ammonia-oxidising Archaea

Sediment assemblages at CCFZ were formed of a higher proportion of C-fixing taxa, up to 25 % of reads, compared with 18 % at PAP and 10 % at Station M (Fig.13, Table S5). Putative nitrifying taxa included ammonia-oxidising Archaea (AOA) *Candidatus Nitrosopumilus*, taxa within the Nitrososphaeria class; ammonia-oxidising bacteria (AOB) *Nitrosomonas* (Betaproteobacteria) and *AqS1*

(Gammaproteobacteria); nitrite-oxidising bacteria (NOB) *Nitrospina* (Nitrospinae) and *Nitrospira* (Nitrospirae). Similar to the 16S microbial taxa, location (PERMANOVA, $p = 0.001$, $F_{2,120} = 9.00$, Table 8) and sediment horizon (PERMANOVA, $p = 0.001$, $F_{2,120} = 3.74$, Table 8) were both associated with distinct *Nitrosopumilus* ASVs, which were also distinct between sediment horizons at each site (PERMANOVA, $p = 0.001$, $F_{4,120} = 2.41$, Table 8).

SIMPER analysis highlighted the dominance of taxa in the Nitrosopumilaceae, family at PAP and particularly at CCFZ, contributing to differences between these two sites and Station M (Table S4). SIMPER analysis also showed the wb1-A12 (Methylomirabilaceae, NC10) taxon at CCFZ and PAP, driving further differences between these sites and Station M sediments. We observed a greater diversity of ASVs classified as AOA *Ca. Nitrosopumilus sp.* at CCFZ, compared with PAP and Station M (Fig. 13). In addition, these subtypes exhibited spatial differences and ASVs appeared to be associated with each location (Fig 15). NMDS (Fig. 16a) and network analysis (Fig. 16b) showed that *Nitrosopumilus* ASVs formed separate clusters for each site (Fig. 16a), suggesting that *Nitrosopumilus* ASVs from CCFZ were more similar to PAP ASVs than Station M (Figs 15,16a,b). PAP hosted the greatest variety of different *Nitrosopumilus* ASVs (345), followed by CCFZ (284), and Station M (102). Each location hosted a number of *Nitrosopumilus* ASVs that were unique to that location (Fig. S1 b). PAP supported the highest number of unique ASVs (32.5 % relative abundance), followed by CCFZ (24.8%) and Station M (11.8 %). CCFZ and PAP shared 24% of ASVs, with only 3.5 % being shared between PAP and Station M and no common *Nitrosopumilus* ASVs shared between CCFZ and Station M. In addition, only 3.5 % of *Nitrosopumilus* ASVs were cosmopolitan.

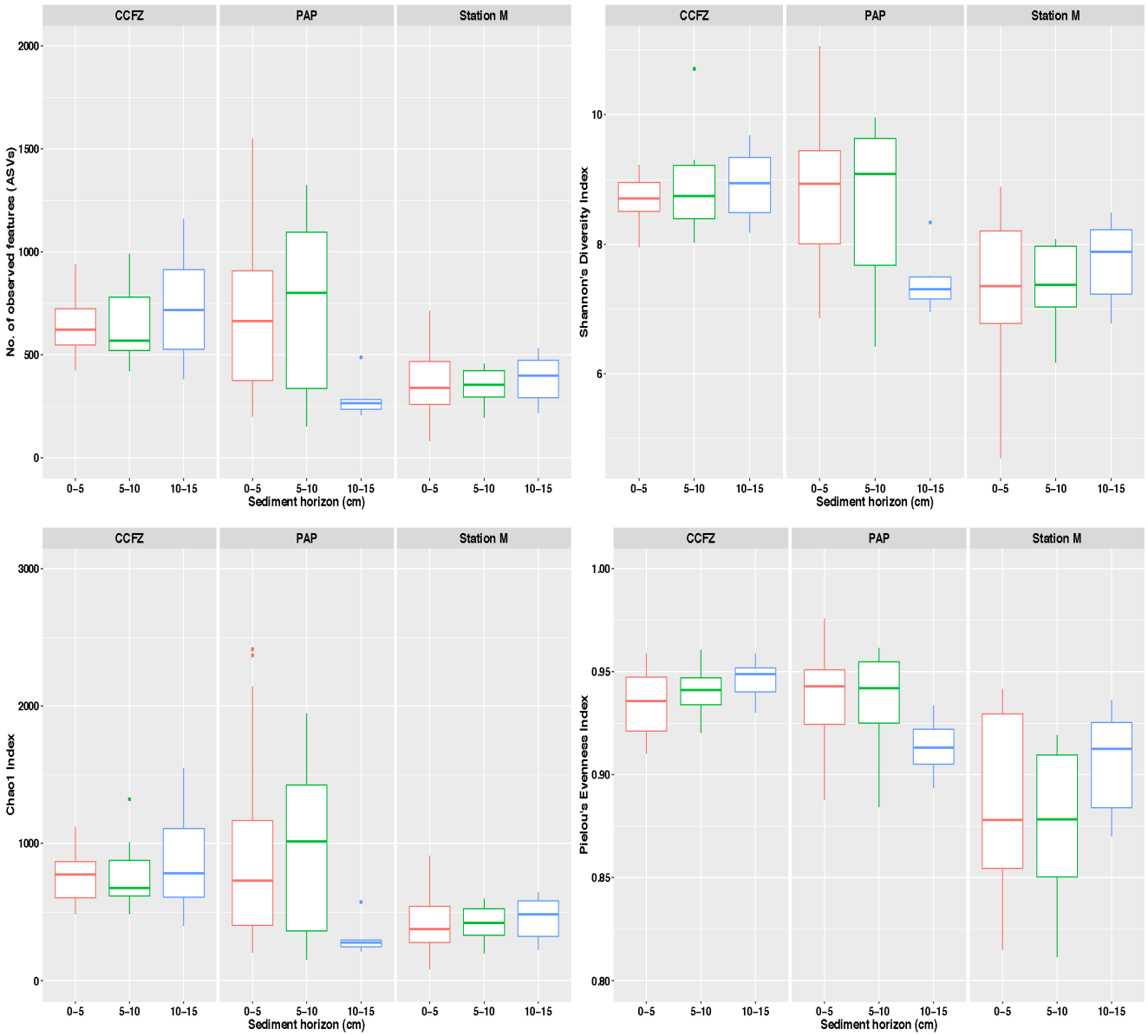

Figure. 9 Comparisons of diversity indices between sediment microbial assemblages at CCFZ, PAP and Station M, based on number of observed features or amplicon sequence variants (ASVs), Shannon's Diversity Index, Pielou's Evenness Index, and Chao1 richness Index. Boxplots show the median (horizontal line), interquartile ranges (box limits), range (whiskers) and outliers (black dots). Diversity indices were calculated on samples that had been rarefied to account for uneven sampling depth.

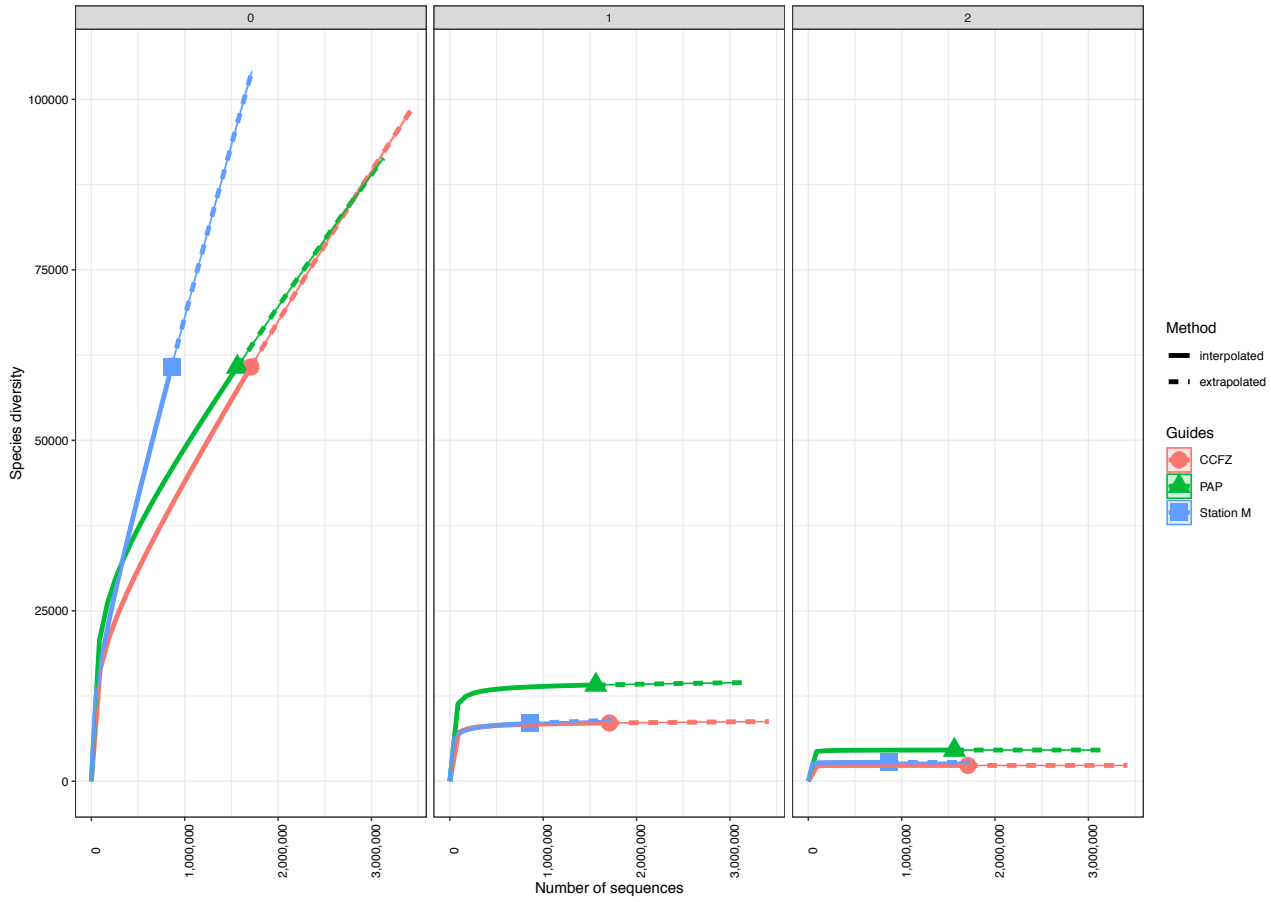

Figure. 10 Sample-based alpha diversity species accumulation curves calculated for each sample type without replacement. Diversity estimates are calculated and plotted for rarefied samples with respect to sample size, and plotted extrapolated curves are based on double the reference sample size. Curves for each sample type do not reach asymptote, indicating insufficient sequencing depth for estimated microbial richness. Numbers on top of each panel represent Hill's diversity numbers of order 0,1 and 2, representing species richness (S_N), the exponential form of the Shannon index (exp *H'*), and the inverse form of Simpson's index (1/*D*) respectively.

Figure. 11 Non-metric multidimensional scaling (nMDS) plots of microbial community dissimilarity based on Bray-Curtis distances. The points represent individual samples and their proximity to one another indicates their similarity. The closer the points are to each other, the more similar the samples are to each other based on community structure and composition. A comparison of assemblages in CCFZ, PAP and Station M. Points are displayed as samples and as amplicon sequence variants (ASVs), visualised by taxonomic division of Phylum.

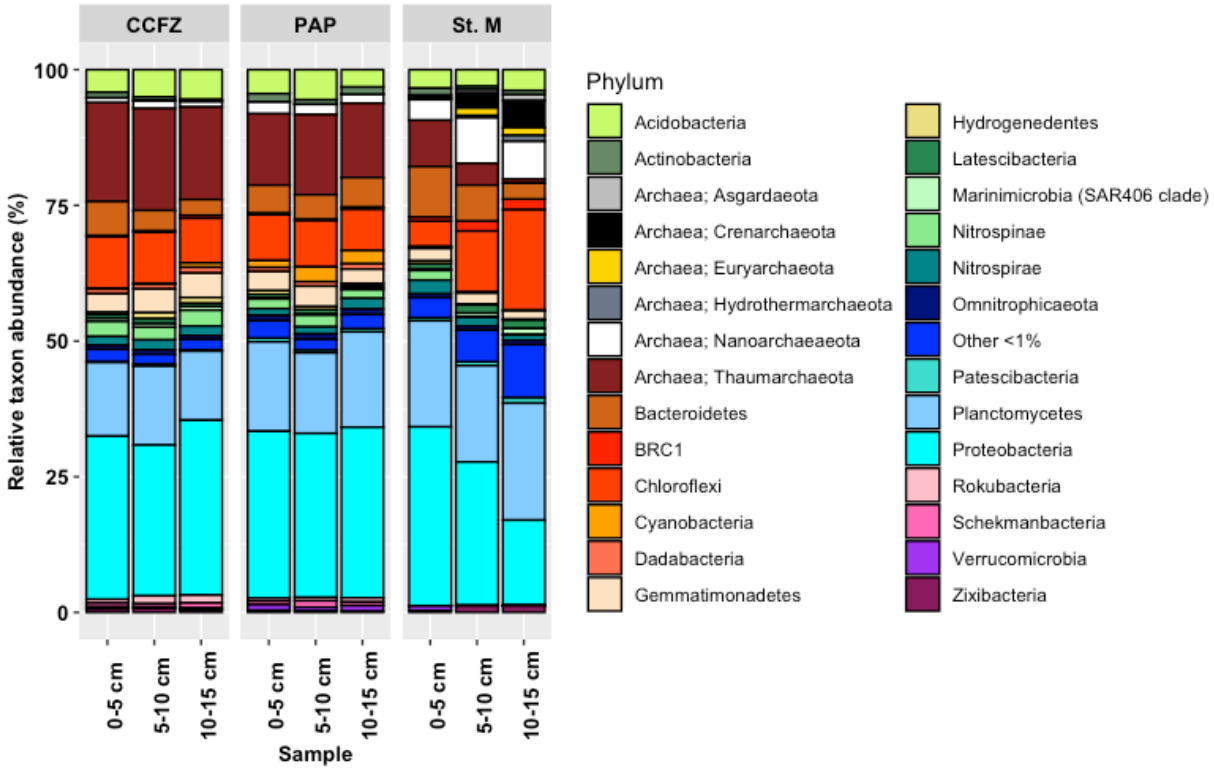

Figure. 12 Relative abundances of amplicon sequence variants (ASVS) at phylum level of, sediment assemblages at CCFZ, PAP and Station M. Only taxa ≥ 1% relative abundance are shown. Category “Other” represents taxa < 1% relative abundance. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

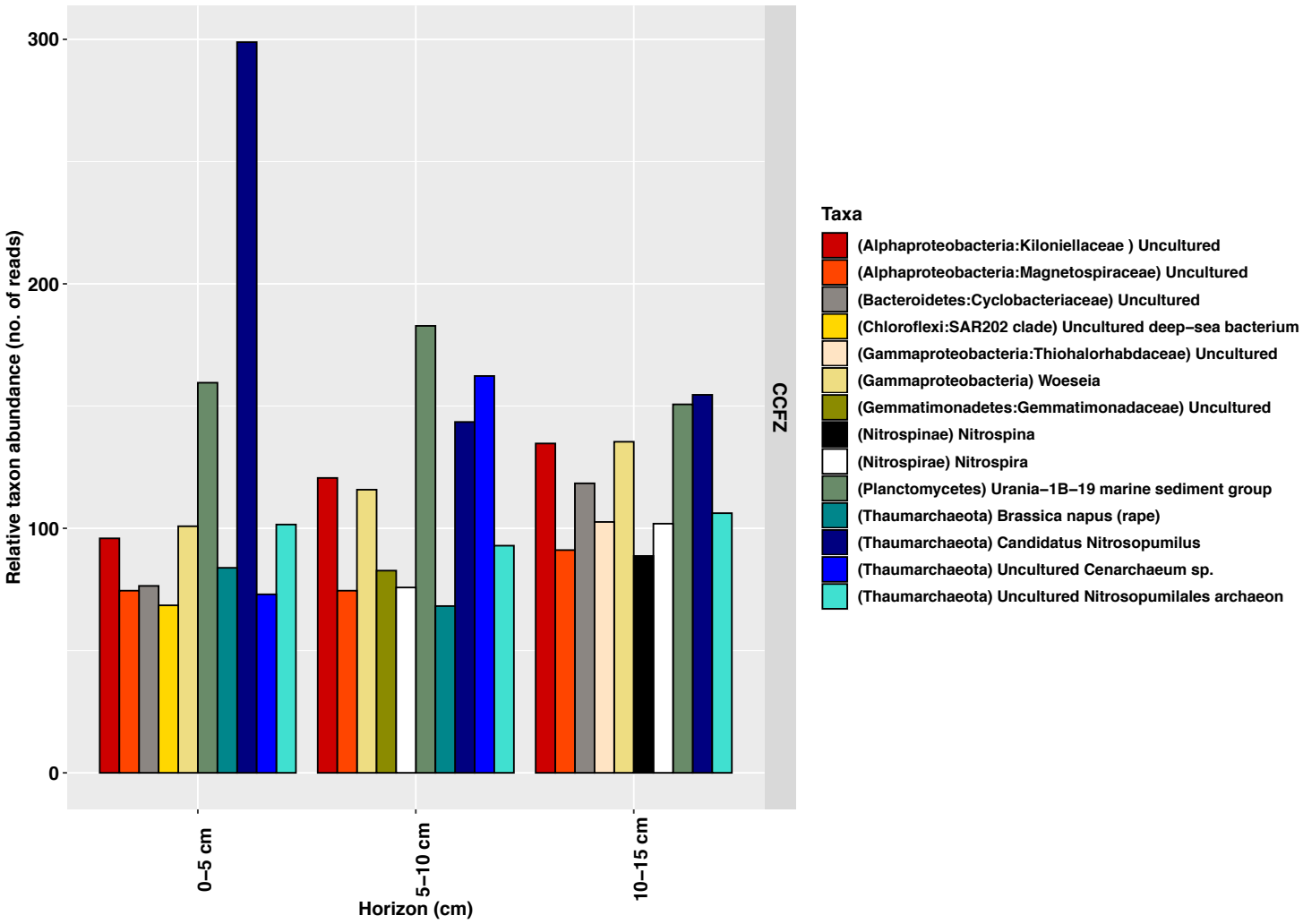

Figure. 13 (a) Relative abundances of amplicon sequence variants (ASVs) of the ten most abundant taxa in sediments at CCFZ, defined at genera or family level. Only taxa ≥ 1% relative abundance are shown. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

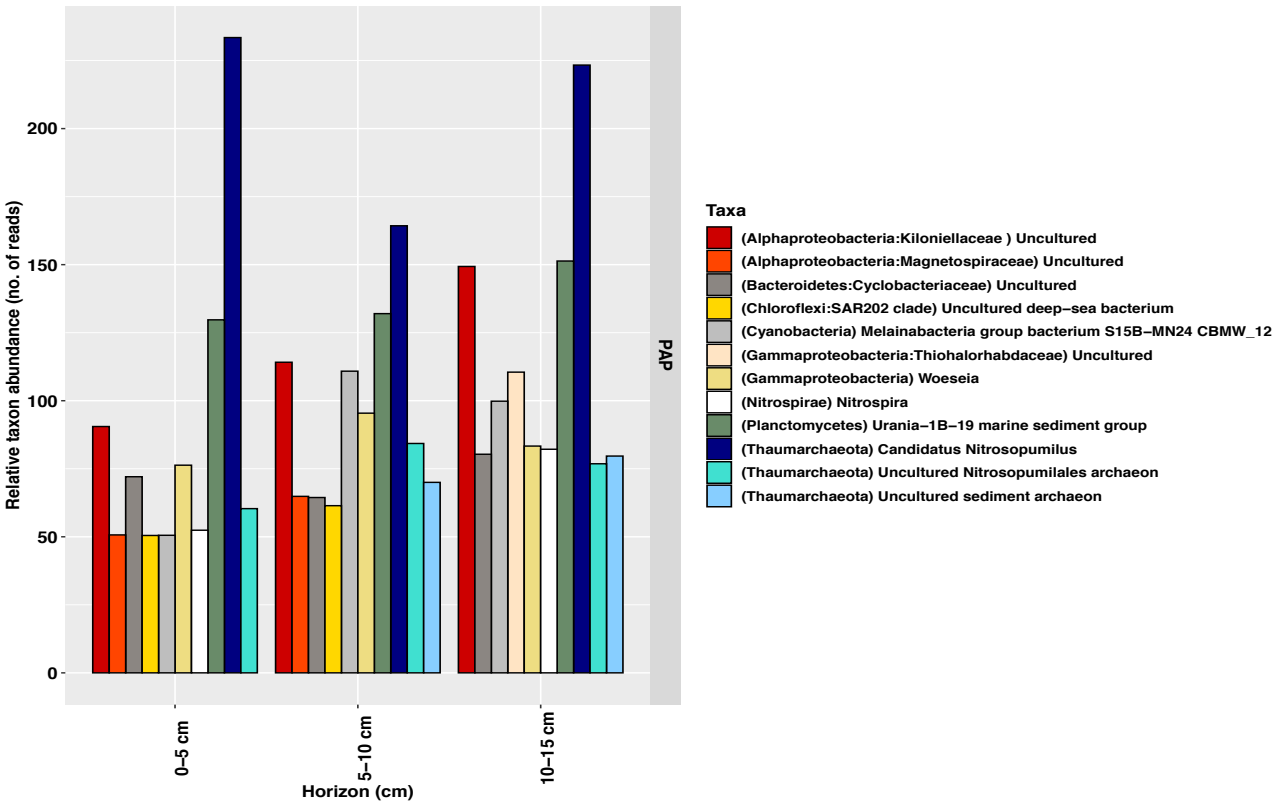

Figure. 13 (b) Relative abundances of amplicon sequence variants (ASVs) of the ten most abundant taxa in sediments at PAP, defined at genera or family level. Only taxa ≥ 1% relative abundance are shown. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

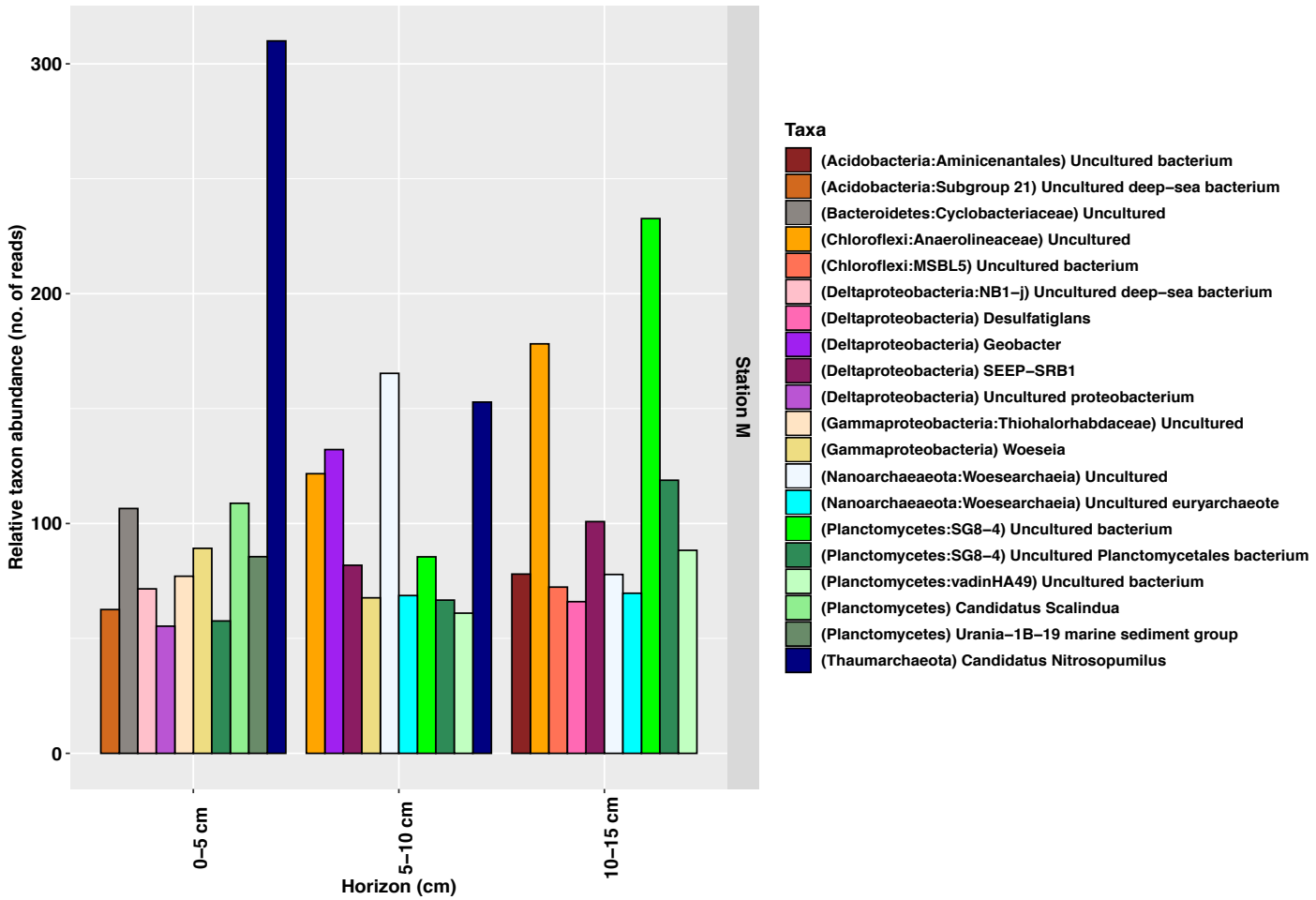

Figure. 13 (c) Relative abundances of amplicon sequence variants (ASVs) of the ten most abundant taxa in sediments at Station M, defined at genera or family level. Only taxa ≥ 1% relative abundance are shown. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

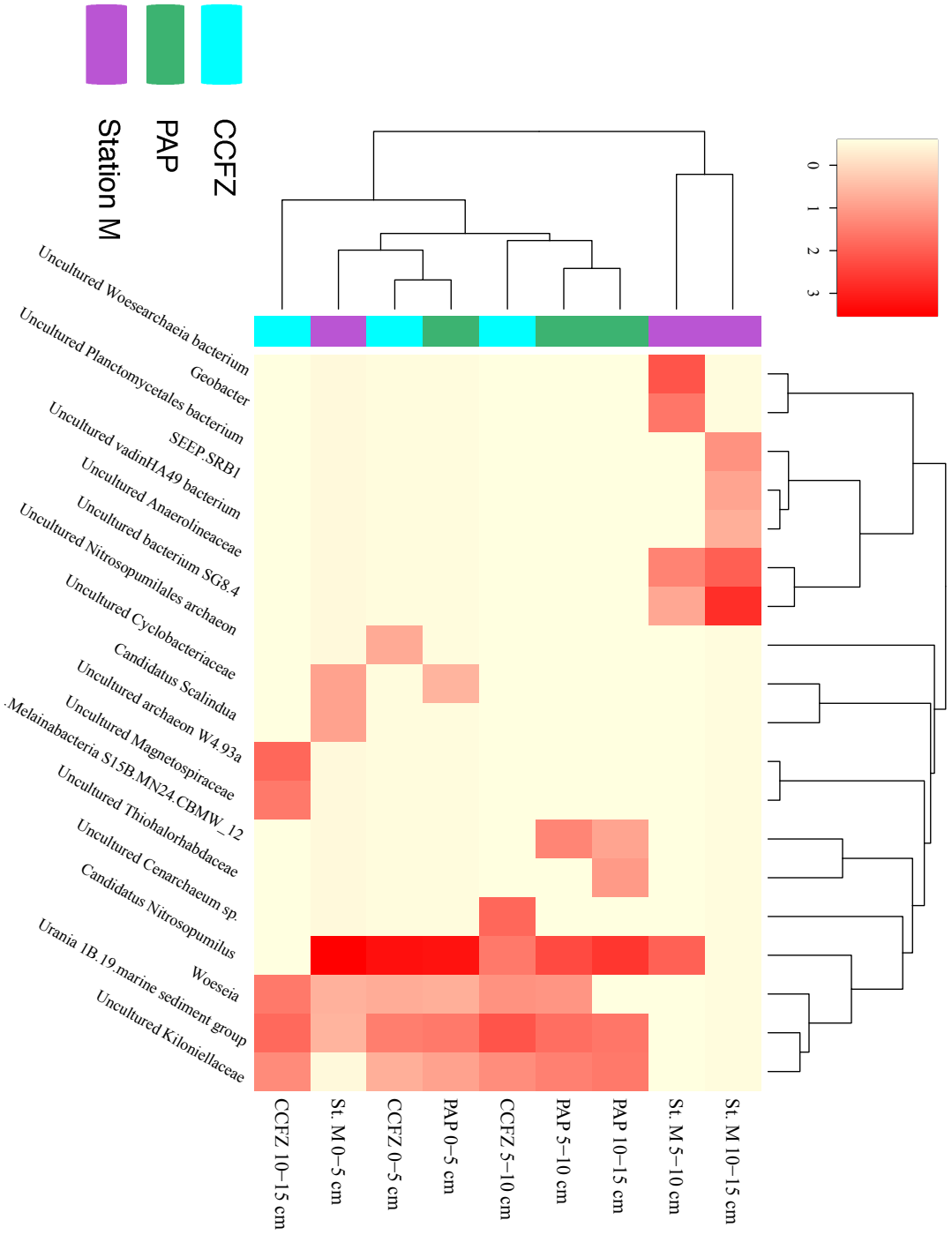

Figure. 14 Heat-map of the top 5 most abundant taxa from each sample type. Dendrograms were created by average linkage hierarchical clustering on a Bray-Curtis dissimilarity matrix of the dataset. The dendrogram on the x-axis shows clusters of amplicon sequencing variants (ASVs) that occur most frequently together. The dendrogram on the y-axis is colour-coded by sample type (blue = CCFZ, green = PAP, purple = Station M). Heatmap colour shows the number of ASVs found in each sample after normalising to 4100 reads per sample.

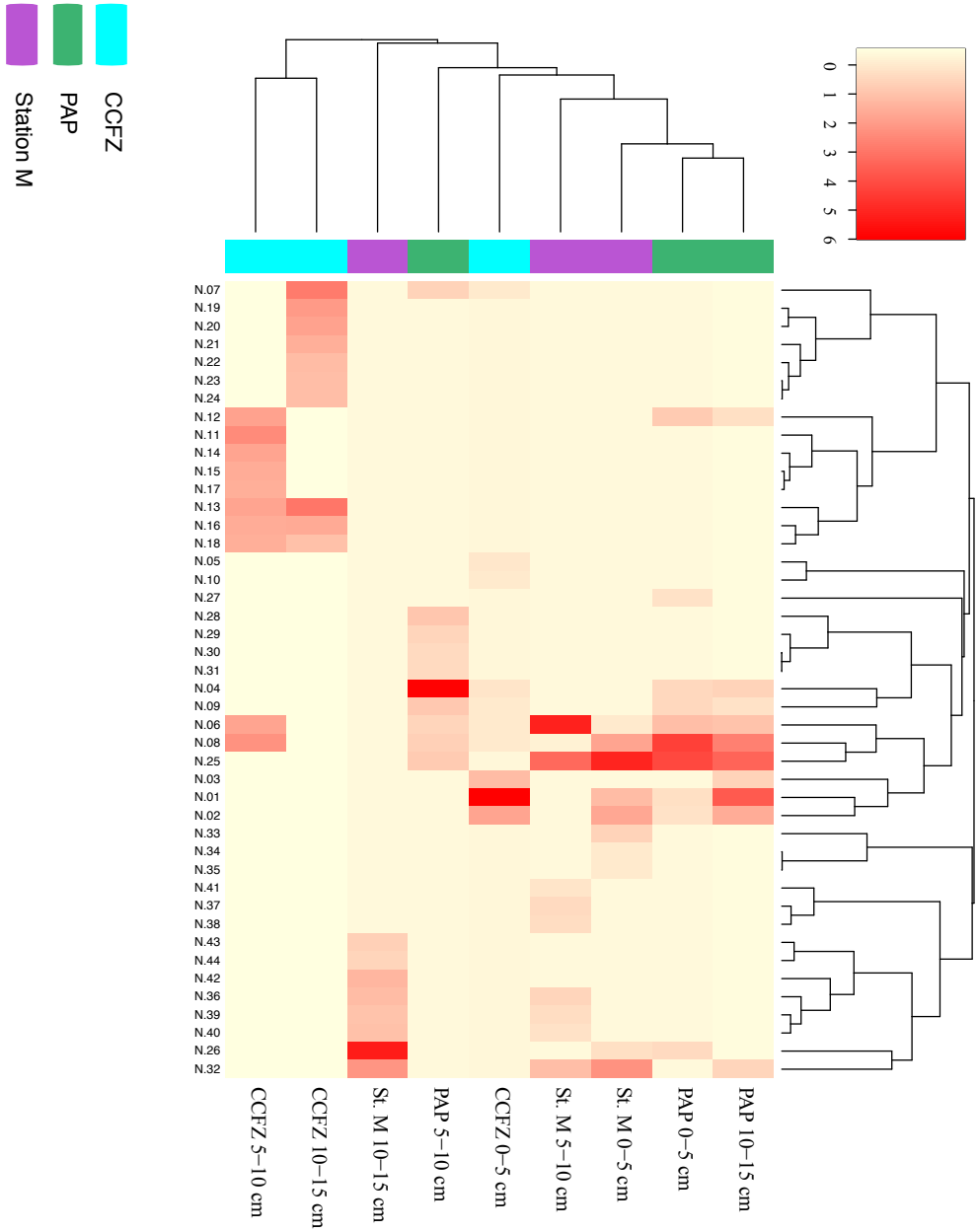

Figure. 15 Heat-map of all ASVs classified as "*Nitrosopumilus*" from each sampling location. Dendrograms were created by average linkage hierarchical clustering on a Bray-Curtis dissimilarity matrix of the dataset. The dendrogram on the x-axis shows clusters of amplicon sequencing variants (ASVs) that occur most frequently together. The dendrogram on the y-axis is colour-coded by sample type (blue = CCFZ, green = PAP, purple = Station M). Heatmap colour shows the number of ASVs found in each sample after normalising to 4100 reads per sample.

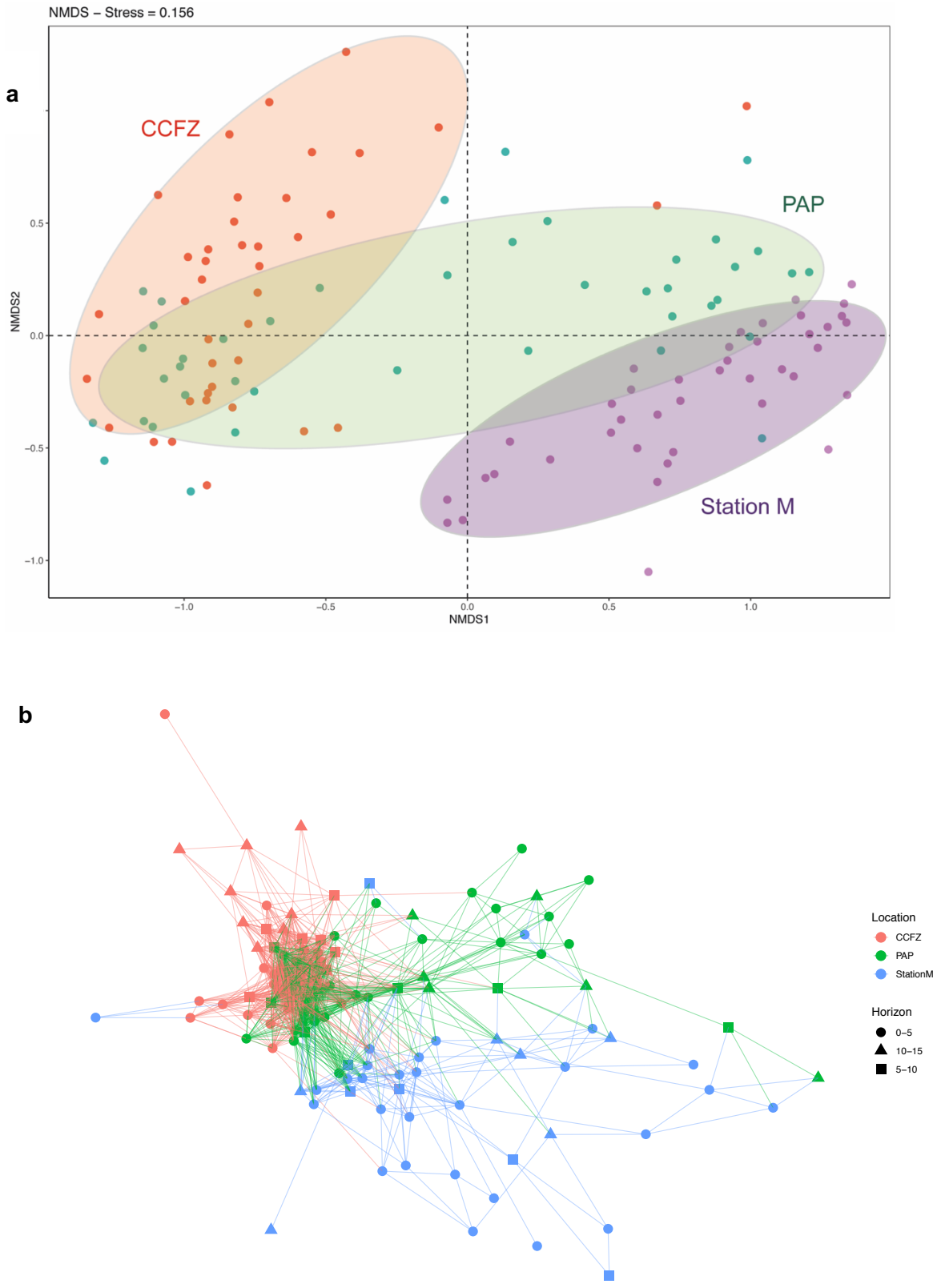

Figure. 16 (a) Non-metric multidimensional scaling (nMDS) plots of microbial community dissimilarity based on Bray-Curtis distances. The points represent a comparison of *Nitrosopumilus* ASVs in sediment assemblages at CCFZ, PAP and Station M. **(b)** Network analysis based on Jaccard distances, showing connection among *Nitrosopumilus* ASVs in sediments from CCFZ, PAP and Station M.

Table 5. Sampling site locations of megacore deployments that were collected in CCFZ, PAP and Station M with location and depths shown for each sample group taken. A total of 124 sediment samples were analysed from 10 megacores at CCFZ, 7 megacores at PAP and 6 megacores at Station M.

Cruise	Year	Location	Latitude	Longitude	Water depth (m)	Sediment (cm)	No. of samples (n)
JC120	April-May 2015	CCFZ	13° 28 N	116° 35 W	4012-4300	0-5	20
JC120	April-May 2015	CCFZ	13° 28 N	116° 35 W	4012-4300	5-10	10
JC120	April-May 2015	CCFZ	13° 28 N	116° 35 W	4012-4300	10-15	10
D377	July 2012	PAP	48° 58 N	16° 33 W	4630-4847	0-5	29
D377	July 2012	PAP	48° 58 N	16° 33 W	4630-4847	5-10	7
D377	July 2012	PAP	48° 58 N	16° 33 W	4630-4847	10-15	6
Pulse 69	November 2017	Station M	35 ° 10 N	122° 59 W	4100	0-5	15
Pulse 69	November 2017	Station M	35 ° 10 N	122° 59 W	4100	5-10	3
Pulse 69	November 2017	Station M	35 ° 10 N	122° 59 W	4100	10-15	3
Pulse 70	April 2018	Station M	35 ° 10 N	122° 59 W	4100	0-5	15
Pulse 70	April 2018	Station M	35 ° 10 N	122° 59 W	4100	5-10	3
Pulse 70	April 2018	Station M	35 ° 10 N	122° 59 W	4100	10-15	3

Table 6. Output of two-way analysis of variance (ANOVA) on alpha diversity indices (Observed ASVs, Shannon, Pielou's Evenness and Chao1) of sediment microbial assemblages at the three abyssal locations: CCFZ, PAP and Station M. The interaction effects Location and Sediment horizon are crossed. Percent variation based on Sum of Squares (SS) is shown as column "% var".

Observed ASVs						
Source	**d.f.**	**SS**	**MS**	***F***	***P***	**% var.**
Location	2	8.5139	0.46277	75.4434	0.001	46.28
Sediment Horizon	2	0.8871	0.04822	7.8610	0.002	4.82
Location x Horizon	28	3.8618	0.20991	2.4443	0.001	21.00
Residuals	91	5.1347	0.27910			28.0
Total	123	18.3976	1			
Shannon						
Location	2	11.2859	0.44251	64.7912	0.001	44.25
Sediment Horizon	2	1.3052	0.05118	7.4929	0.002	5.12
Location x Horizon	28	4.9874	0.19555	2.0452	0.002	19.56
Residuals	91	7.9256	0.31076			31.08
Total	123	25.5040	1			
Pielou's Evenness						
Location	2	11.3798	0.43942	63.0092	0.001	43.94
Sediment Horizon	2	1.3015	0.05026	7.2064	0.002	5.03
Location x Horizon	28	4.9982	0.19300	1.9768	0.002	19.3
Residuals	91	8.2176	0.31732			31.73
Total	123	25.8971	1			
Chao1						
Location	2	8.3042	0.44302	67.0730	0.001	44.3
Sediment Horizon	2	0.8383	0.04472	6.7707	0.002	4.47
Location x Horizon	28	3.9690	0.21174	2.2898	0.001	21.17
Residuals	91	5.6333	0.30053			30.05
Total	123	18.7448	1			

Table 7. Alpha diversity indices of sediment microbial assemblages at CCFZ, PAP and Station M, based on the number of observed features or amplicon sequence variants (ASVs), Shannon's Diversity Index, Pielou's Evenness Index and Chao1 Richness Index, showing averages and standard error (± SE). Asterisks * indicate that one outlier sample has been removed from this category to calculate averages. The first three rows show overall averages for CCFZ, PAP and Station M, which include the outliers.

Location	Horizon (cm)	Observed ASVs	Shannon	Evenness	Chao1
CCFZ	All	708 ± 51	8.805 ± 0.08	0.939 ± 0.002	903 ± 125
PAP	All	707 ± 80	8.525 ± 0.18	0.935 ± 0.003	991 ± 173
Station M	All	370 ± 22	7.470 ± 0.14	0.885 ± 0.007	431 ± 30
CCFZ	0-5	643 ± 33	8.691 ± 0.08	0.935 ± 0.003	753 ± 42
CCFZ	5-10	816 ± 182	8.913 ± 0.24	0.940 ± 0.004	1239 ± 483
CCFZ	5-10 *	643 ± 62	8.713 ± 0.15	0.939 ± 0.004	762 ± 83
CCFZ	10-15	729 ± 78	8.926 ± 0.16	0.946 ± 0.003	866 ± 113
PAP	0-5	787 ± 102	8.739 ± 0.21	0.939 ± 0.004	1140 ± 235
PAP	0-5*	723 ± 82	8.656 ± 0.20	0.938 ± 0.004	943 ± 133
PAP	5-10	735 ± 181	8.580 ± 0.52	0.935 ± 0.010	956 ± 267
PAP	10-15	291 ± 41	7.427 ± 0.20	0.914 ± 0.006	315 ± 53
Station M	0-5	371 ± 29	7.442 ± 0.17	0.883 ± 0.009	429 ± 38
Station M	5-10	347 ± 40	7.349 ± 0.30	0.875 ± 0.017	417 ± 60
Station M	10-15	385 ± 50	7.729 ± 0.28	0.906 ± 0.011	454 ± 68

Table 8. Output permutational multivariate analysis of variance (PERMANOVA) on relative abundance counts that have been fourth-root transformed prior to creation of Bray-Curtis dissimilarity matrices for sediment microbial assemblages at CCFZ, PAP and Station M, showing the following: all 16S microbial assemblages, ASVs classifying as *Candidatus Nitrosopumilus*. The interaction effect "Core" refers to Megacore and is nested within Location. The interaction effects Location and Sediment horizon are crossed. Percent variation based on Sum of Squares (SS) is shown as column "% var". Three samples with zero counts were excluded from the analysis of *Nitrosopumilus* ASVs.

All 16S microbial sediment assemblages						
Source	**d.f.**	**SS**	**MS**	***F***	***P***	**% var.**
Location	2	61947	30974	27.835	0.001	27.0
Sediment Horizon	2	11963	5981.3	5.9843	0.001	5.2
Core (Location)	20	22250	1112.5	0.86114	0.916	9.6
Location x Horizon	4	18963	4740.7	4.7597	0.001	8.2
Horizon x Core (Location)	39	38200	979.49	0.75819	1	16.6
Residuals	56	72346	1291.9			31.3
Total	123	230830				
Nitrosopumilus ASVs						
Source	**d.f.**	**SS**	**MS**	***F***	***P***	**% var.**
Location	2	51076	25538	9.0012	0.001	11.5
Sediment Horizon	2	20834	10417	3.7355	0.001	4.7
Core (Location)	20	56527	2826.3	0.95303	0.669	12.7
Location x Horizon	4	26765	6691.2	2.4094	0.001	6.0
Horizon x Core (Location)	36	99539	2765	0.93234	0.797	22.4
Residuals	56	166080	2965.6			37.4
Total	120	444500				

Table 9. Pair-wise *posthoc* tests for fixed terms 'Location' and 'Sediment Horizon' for sediment 16S microbial assemblages at CCFZ, PAP and Station M.

Location	**t**	***P***
CCFZ vs PAP	2.7122	0.001
CCFZ vs Station M	6.6554	0.001
PAP vs Station M	5.1165	0.001
Sediment Horizon	**t**	***P***
0-5 cm vs 5-10 cm CCFZ	1.7559	0.001
0-5 cm vs 10-15 cm CCFZ	2.3202	0.001
5-10 cm vs 10-15 cm CCFZ	1.3145	0.009
0-5 cm vs 5-10 cm PAP	0.98473	0.419
0-5 cm vs 10-15 cm PAP	1.3608	0.018
5-10 cm vs 10-15 cm PAP	1.152	0.09
0-5 cm vs 5-10 cm Station M	2.2068	0.001
0-5 cm vs 10-15 cm Station M	3.1881	0.001
5-10 cm vs 10-15 cm Station M	1.4713	0.01

Table 10. Pair-wise *posthoc* tests for fixed terms 'Sediment Horizon' within 'Location' for sediment 16S microbial assemblages at CCFZ, PAP and Station M.

Group 1	Group 2	t	*P*
0-5 cm CCFZ	5-10 cm CCFZ	1.7559	0.001
0-5 cm CCFZ	10-15 cm CCFZ	2.3202	0.001
0-5 cm CCFZ	0-5 cm PAP	2.2536	0.001
0-5 cm CCFZ	5-10 cm PAP	1.7822	0.001
0-5 cm CCFZ	10-15 cm PAP	2.2185	0.001
0-5 cm CCFZ	0-5 cm St. M	4.5608	0.001
0-5 cm CCFZ	5-10 cm St. M	4.9075	0.001
0-5 cm CCFZ	10-15 cm St. M	5.9048	0.001
5-10 cm CCFZ	10-15 cm CCFZ	1.3145	0.005
5-10 cm CCFZ	0-5 cm PAP	2.0317	0.001
5-10 cm CCFZ	5-10 cm PAP	1.5308	0.001
5-10 cm CCFZ	10-15 cm PAP	2.0533	0.002
5-10 cm CCFZ	0-5 cm St. M	3.5571	0.001
5-10 cm CCFZ	5-10 cm St. M	4.0876	0.001
5-10 cm CCFZ	10-15 cm St. M	5.0002	0.001
10-15 cm CCFZ	0-5 cm PAP	2.3521	0.001
10-15 cm CCFZ	5-10 cm PAP	1.7106	0.001
10-15 cm CCFZ	10-15 cm PAP	2.1712	0.001
10-15 cm CCFZ	0-5 cm St. M	3.7722	0.001
10-15 cm CCFZ	5-10 cm St. M	4.1788	0.001
10-15 cm CCFZ	10-15 cm St. M	5.0179	0.001
0-5 cm PAP	5-10 cm PAP	0.98473	0.43
0-5 cm PAP	10-15 cm PAP	1.3608	0.016
0-5 cm PAP	0-5 cm St. M	4.0564	0.001
0-5 cm PAP	5-10 cm St. M	3.8847	0.001
0-5 cm PAP	10-15 cm St. M	4.6345	0.001
5-10 cm PAP	10-15 cm PAP	1.152	0.108

5-10 cm PAP	10-15 cm St. M	2.6321	0.001
5-10 cm PAP	5-10 cm St. M	3.1005	0.003
5-10 cm PAP	10-15 cm St. M	3.8342	0.003
10-15 cm PAP	0-5 cm St. M	2.3853	0.001
10-15 cm PAP	5-10 cm St. M	2.9002	0.004
10-15 cm PAP	10-15 cm St. M	3.5925	0.003
0-5 cm St. M	5-10 cm St. M	2.2068	0.001
0-5 cm St. M	10-15 cm St. M	3.1881	0.001
5-10 cm St. M	10-15 cm St. M	1.4713	0.004

3.5 Discussion

3.5.1 POC flux and abyssal sediment assemblage composition

In this study, we evaluated the microbial community composition of sediments at CCFZ, PAP and Station M to test the hypothesis that sediment communities were distinct at each of the three sites sampled, based on the average POC flux to each site. Sediment assemblages were compositionally different, with only a small overlap in the taxa found at each of the three sites (Fig. S1 a). The oligotrophic CCFZ site and PAP supported a higher abundance of phyla in the Thaumarchaeota phylum, of which members are known to be chemolithoautotrophic (Swan *et al.*, 2011), as well as other carbon-fixing and nitrifying taxa. This has implications for benthic assemblages in regions where input of organic carbon from export production of phytoplankton is low (Reinthaler *et al*., 2010; Middelberg, 2011; Molari *et al*., 2013; Tully & Heidelberg, 2016; Sweetman *et al*., 2019), thus the utilisation of reduced carbon compounds for carbon fixation may supplement the recalcitrant carbon present in the sediments (Brunnegård *et al*., 2004; Molari *et al*., 2013; Sweetman *et al*., 2019, Trembath-Reichert *et al*., 2021). Other studies have shown a correlation with organic carbon input and abyssal sediment microbial communities (Smith *et al*., 1997; D'Hondt *et al*., 2009; Zinger *et al*., 2011; Kallmeyer *et al*., 2012, Bienhold *et al*., 2012, 2016; Danovaro *et al*., 2016), although effects may be small or minimal compared to larger biota (Smith *et al*., 1997; Nomaki *et al*., 2021).

Generally, there were similarities across assemblages from the three locations, namely the abundance of Proteobacteria, Planctomycetes and Acidobacteria, which were consistent between sites. These broad patterns have previously been documented in abyssal sediments (Schauer *et al*., 2010; Zinger *et al.*, 2011; Walsh *et al*., 2016a; Bienhold *et al*., 2016). Taxa within the Gammaproteobacteria class, namely *Woeseia* and the Thiohalorhabdaceaea group were consistently present at all three sites (Fig. 13). At a finer taxonomic resolution, however, we observed that each site hosted core taxa, or ASVs, found in the dataset that were not shared at the other sites (Fig. S1 b), suggesting high levels of endemism across oceans and scales of hundreds of km. Previous research has highlighted high levels of endemism in sediment microbes across

large scales (> 300 km) (Pommier *et al*., 2007; Zinger *et al*., 2011; Bienhold *et al*., 2016; Peoples *et al*., 2019) suggesting nîche diversity and resource partitioning in sediments with similar environmental conditions (Zinger *et al*., 2011). This finding may seem surprising in abyssal sediments that are generally regarded as being more stable, less variable and nutrient poor compared to coastal sediments (Jørgensen & Boetius, 2007). Another element is the overlying water column, which is likely to be an important factor influencing the composition of sediment communities (Hamdan *et al*., 2013, Walsh *et al*., 2016a), especially with the presence of hydrothermal microbes in our dataset. At Station M, we observed the presence of an endosymbiont of *Ridgeia piscesae* within the SAR324 clade (Marine group B), Deltaproteobacteria, a vestimentiferan tubeworm known to inhabit hydrothermal vents on the Juan de Fuca Ridge in the NE Pacific (Chao et al., 2007). As with other studies (Bienhold *et al*., 2012; Jacob *et al*., 2013; Danovaro *et al*., 2016; Varliero *et al*., 2019), our findings indicate that food availability via POC flux is important in driving differences between abyssal sediment communities across global scales. Caution is advised when interpreting these results, however, as direct POC flux measurements were not taken at the time of sample collection, and conditions reported in this study are averages only.

3.5.2 Vertical distribution of sediment microbial assemblages at St. M

Another important environmental constraint driving differences between the sediment assemblages at the three sites is oxygen availability. The oxygen penetration of marine sediments is linked to primary productivity regime in the overlying water and sedimentation rates (Glud, 2008). Oxygen penetration of sediments at Station M is only 3 cmbsf (Reimers *et al*., 1992; Smith, 1992), whereas at PAP it is 15 cmbsf (Witte *et al*., 2003) and 300 cmbsf at CCFZ (Menendez *et al*., 2019). The most marked differences in microbial composition with depth through the sediment profile was observed at Station M, where redox regime was the most important factor influencing assemblages (Jørgensen & Boetius, 2007, Moeseneder *et al*., 2012). Not surprisingly, no such changes were seen at PAP or CCFZ, as the sediment profile sampled were within the oxygen penetration depths at these locations. Depth transition changes in assemblage composition observed at Station M in this study have also previously been noted by Moeseneder *et al*., (2012). Microbial diversity was lower at Station M, where the

transition across redox regimes from oxic (0-5 cmbsf) to anoxic (5-15 cmbsf) conditions in the sediment likely explains the differences in community diversity and composition (Jørgensen & Boetius, 2007; Moeseneder *et al*., 2012; Jørgensen *et al*., 2012; Zhao *et al*., 2019). The difference in sediment oxygen concentration and availability of reduced compounds compared to CCFZ and PAP will support microbial assemblages that are characteristic of these conditions (Lozupone, 2007; Madrid *et al*., 2001).

Oxygenated surface sediments at Station M supported the highest relative abundance of AOA *Nitrosopumilus* of all three sites (Fig. 13) but rapidly declined in deeper sediments, as observed for AOA diversity in general, which decline in abundance in redox zones and anoxic sediment (Zhao *et al*., 2019). Sediments below 5 cmbsf supported a range of anaerobic taxa. Abundant in sediments deeper than 5 cmbsf at Station M were the SEEP-SRB1 (Deltaproteobacteria; Desulfobacteraceae), a group of sulfate-reducing bacteria associated with methanotrophic archaea ANME-2 group (anaerobic methanotrophic archaea) as part of an anaerobic oxidation of methane (AOM) consortia (Schreiber *et al*., 2010). Taxa within the *Methanosarcinales* order (ANME-2 group; Orphan *et al*., 2001) were present within the dataset but were not as abundant as the SEEP-SRB1 group. Other abundant archaea with potential for methanogenesis were the Woesearchaeota, which are anaerobic heterotrophs with potential roles in methanogenesis (Liu *et al*., 2018). *Desulfatiglans* within the Deltaproteobacteria are capable of sulfate reduction and aromatic hydrocarbon degradation. This group have different strategies for coping with carbon and sulfate limitation in subsurface sea sediments (Jochum *et al*., 2018). Another dominant group in deeper sediment horizons was the *Anaerolineaceae* family (Chloroflexi phylum), members of which are known to be strict anaerobes (Yamada *et al*., (2006). A number of taxa within the Planctomycetes phylum were present in the deeper horizons at Station M, including the vadinHA49 group which has previously been identified in sulphide-rich anoxic sediments (Youssef *et al*., (2012). Station M sediments also supported a range of different archaeal taxa, including *Thermoplasmata, Woesearchaeia, Methanomicrobia, Thermococci,* which have been found in sediments contaminated with anthropogenically-derived heavy metals (Misson *et al*., 2016).

3.5.3 Vertical distribution of sediment microbial assemblages at CCFZ

Intriguingly, a significant difference in assemblage composition through the sediment profile was also observed at CCFZ. This is unexpected, as CCFZ is fully oxygenated with no steep biogeochemical or redox profile of the sediment gradient, which would otherwise have a considerable influence structuring the sediment microbial communities. Taxa within surface sediments at CCFZ are well represented in nutrient-rich marine sediments, and many are capable of mineralising OM following phytoplankton blooms (Zarate *et al*., 2021; Chun *et al*., 2021). In contrast, deeper layers (10-15 cm) contained taxa such as Ralstonia, a group frequently found in deep subsurface sediments (Orcutt *et al*., 2011), sulfur-oxidising Thiohalorhabdaceae, nitrite-oxidising *Nitrospina* and non-photosynthetic Cyanobacteria (Melainabacteria). This observation is generally unexpected in oligotrophic sediments with a low input of organic carbon, where oxygen is the dominant electron terminal acceptor and the concentrations of other electron acceptors such as sulfate and nitrate vary little with depth. Findings that both indicate changes in assemblage structure with depth through the sediment and that pinpoint taxa usually found in suboxic conditions are consistent with other studies of this particular region (Lindh *et al*., 2017). It is therefore possible that many of these uncharacterised strains are capable of greater metabolic versatility and adaptability than previously realised.

It is also probable that the process of deposition and burial of organic matter leading to the subsequent preservation of organisms in deeper layers may explain differences between assemblages in surficial sediments and those deeper in the sediment (Dell'Anno & Danovaro, 2005; Pawlowski *et al*., 2011). Extracellular DNA released from the death and decay of benthic organisms may be preserved in surface deep-sea sediments, which previous studies have indicated form a significant repository for DNA globally (Dell'Anno & Danovaro, 2005; Corinaldesi *et al*., 2018). To what extent the presence of extracellular DNA can alter the diversity estimates of extant microbial assemblages is unclear. DNA has a high preservation potential in deep-sea sediments (Corinaldesi *et al*., 2011). Previous studies have reported a large proportion of extracellular DNA not associated with living microbial standing stocks in oligotrophic or fully oxic sediments (Danovaro *et al*., 1993; Danovaro *et al*., 1999; Lejzerowicz *et al*., 2013), potentially altering

diversity estimates. One possible solution is to distinguish active microbes by targeting RNA molecules using metatranscriptomics, linking species abundances with key metabolic processes (Lejzerowicz *et al*., 2013). Therefore, using an RNA-based approach such as a metatranscriptomics to indicate active members of sediment assemblages would offer greater clarity on microbial diversity, rather than relying on exclusively DNA-based approaches.

3.5.4 Spatial variation of *Nitrosopumilus* sequences

Differences between the sediment communities at each site were also observed when comparing the abundance and diversity of ASVs affiliated with *Nitrosopumilus* (Figs. 15,16b). Noticeable separation of ASVs was evident between locations, with the two oligotrophic sites (CCFZ and PAP) and the eutrophic site (Station M) hosting distinct populations of *Nitrosopumilus*. Other studies have noted spatial variation and nîche separation of ammonia-oxidising taxa variants based on habitat and location (O'Mullan & Ward, 2005; Park *et al*., 2014; Qin *et al*., 2014; Bayer *et al*., 2016). Separate clades of *Nitrosopumilaceae* or *Nitrosopumilus* sequences have been observed in marine sediments (Park *et al*., 2014), including oligotrophic sediments (Kerou *et al*., 2021). Previous research has highlighted not only a diversity of AOA sequences and distinct communities in water and sediments (Francis *et al*., 2005) but also AOA ecotypes within the Thaumarchaeota phylum, based on ammonia availability and a distribution explained by latitude and water column depth (Sintes *et al*., 2016). In terms of organic matter input, the composition and diversity of AOA communities appear to be influenced by nutrient availability and carbon input (Wang *et al*., 2017).

Organic input has previously been observed to be an important factor influencing the distribution and abundance of ammonia oxidizing microbes in marine systems (Bernhard and Bollmann, 2010). Archaea are capable of out-competing bacteria in conditions of chronic energy stress, such as environmental stresses and low energy availability (Valentine, 2007; Hoehler and Jørgensen, 2013). AOA have energy-efficient carbon-fixation metabolic pathways, and their high affinity for ammonia allows populations to be well-equipped to live in oligotrophic conditions (Martens-Habbena *et al*., 2009), with mechanisms for nîche specialisation, such as urea utilisation and phosphate uptake (Alonso-Sáez *et al*., 2012; Park *et al*., 2014).

Low-permeability membranes and specific catabolic pathways are some of the biochemical mechanisms that Archaea have developed to thrive in energy stress, while bacteria tend to specialise in exploiting new or variable energy resources (Valentine, 2007). Therefore, in areas with low carbon supply, AOA may gain a competitive advantage over AOB.

Cultivated *Nitrosopumilus* strains are chemolithoautotrophic (Martens-Habbena *et al*., 2009), however the Thaumarchaeota is a diverse phylum, that includes some heterotrophic and mixotrophic members (Aylward & Santoro, 2020), including *Nitrosopumilus* (Qin *et al*., 2014), yet this group are still key players in nitrogen cycling (Francis *et al*., 2005). In line with previous work, our study has highlighted a diversity within this important group, however in contrast, this spatial variation was within sediments and not across multiple habitats or substrates, suggesting a more complex picture in abyssal sediments than previously realised. It is important to note we did not target ammonia oxidation (*amoA*) genes as other studies have, limiting our ability to identify genetic variants of *Nitrosopumilus* and infer functional information. It does, however, shine light on possible complexity within abyssal AOA communities that other studies may have missed. Further investigations should address the diversity of AOA in oligotrophic marine sediments.

3.6 Conclusion

Our results reveal that: (1) the microbial community diversity and taxonomic composition in abyssal sediments is different with sampling location and oxygen penetration of the sediments, (2) microbial assemblages associated with nitrogen and carbon metabolisms are likely to be important contributors to the benthic community, (3) the average input of POC flux is an important factor that influences microbial sediment assemblages, (4) the higher proportion of ammonia-oxidisers in oligotrophic sediments has also been observed habitain other studies and has important implications for the benthic ecosystem. Oligotrophic sites PAP and CCFZ hosted characteristic members of these ts compared to the more eutrophic site Station M, and sediment assemblages at PAP and CCFZ appeared to be more related to one another than to Station M. Abyssal sediments may offer more heterogenous conditions and host a wider diversity of AOA than was previously

realised, leading to nîche diversity and resource partitioning. Whether this is down to POC flux or to other environmental gradients is not yet certain but disentangling environmental factors that may influence spatial patterns should be a future priority. This is especially important given the ubiquity of AOA Thaumarchaeota and *Nitrosopumilus* in the dark ocean and the potential for carbon fixation.

Chapter 4: Effect of feeding strategies on gut microbiomes of benthic deposit-feeding holothurians in the NE Atlantic

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4.1 Abstract

Holothurians (sea-cucumbers) form a dominant part of the abyssal megafauna and are an important link between microbes and invertebrates within food-limited benthic habitats. Holothurian feeding activities may influence sediment microbial structure and distribution, benthic nutrient cycling and carbon processing. Despite their importance, the effect of holothurian bioturbating activity on benthic microbial communities is poorly understood. The gut microbiomes of six genera of holothurians with two distinct feeding strategies were examined from the North-East Atlantic. Holothurian mitochondrial CO1 and 16S rRNA gene amplicon sequences were analysed and compared with sediment and water column samples. Microbial community composition and diversity varied with habitat type, gut compartment and holothurian feeding strategy. Taxa related to carbon and nitrogen fixation and cycling were highly abundant in the holothurian gut

microbiomes. In particular, ammonia-oxidising Archaea (Thaumarchaeota), non-photosynthetic Cyanobacteria (*Melainabacteria)* and taxa implicated in high molecular weight (HMW) compound processing were more prevalent in gut microbiomes of subsurface deposit feeders. Both Thaumarchaeota and *Melainabacteria* were also observed in the ambient sediment, with the latter more abundant in the deeper sediment layers. Overall, the results indicate that gut microbes in holothurians may offer a nutritional advantage to the host by breaking down refractory organic matter in a food limited environment, especially for holothurians with a largely stationary lifestyle.

Running title: Holothurian feeding strategies affect the gut microbiome at PAP

4.2 Introduction

Holothurians (sea-cucumbers; phylum Echinodermata) form a dominant part of the abyssal megafauna (Billett 1991; Billett *et al*., 2001, Ruhl *et al*., 2014). These invertebrates feed on microbes and organic particles in the sediment, forming an important link between microbes and invertebrates within abyssal habitats (Amaro *et al*., 2012). Holothurians harbour abundant and diverse communities of microbes within their intestines (Deming & Colwell, 1982; Roberts *et al*., 2001), which are acquired through the ingestion of sediments (Yamazaki *et al.*, 2019). Particularly abundant in the hindgut, these gut microbes may offer a nutritional advantage for these animals (Roberts *et al*., 2000; Amaro *et al*., 2009; Rowe & Deming, 2011; Amaro *et al*., 2012). The feeding activities of holothurians may not only influence the structure and distribution of sediment microbial communities (Gao *et al*., 2014) but also benthic nutrient cycling (Yamazaki *et al*., 2015), carbon processing (Durden *et al*., 2016), and organic matter mineralization (Yamazaki *et al.,* 2019). These changes may significantly alter ecosystem function (Patrick & Fernandez, 2013), yet the effect of holothurian feeding activities on microbial communities and ecosystem functioning in abyssal habitats is still largely unknown.

The majority of holothurian microbiome studies have primarily focused on either coastal and commercially important species (Gao *et al*., 2014; Sha *et al*., 2016; León-Palmero *et al*., 2018; Cleary *et al*., 2019; Pagán-Jiménez *et al*., 2019; Zhang *et al*., 2019), or areas of the seabed adjacent to the continental shelf, where coastal input and runoff are likely to be a larger influence than the abyssal plain (Amaro *et al*., 2009, 2012; Zhu *et al*., 2020). This study aimed to target a range of holothurian species for microbiome analysis, from an abyssal plain where the main input of organic matter is from phytodetritus blooms. The area investigated, the Porcupine Abyssal Plain (PAP), NE Atlantic, is an established time-series station, where the response of megafauna to large-scale changes in particulate organic matter (POM) fluxes to the seafloor have been well observed (Billett *et al*., 2001, 2010). This area is subject to seasonal fluxes of organic matter to the abyssal seafloor, which are known to influence benthic communities (Gooday, 1988; Tyler, 1988; Gooday & Turley, 1990; Pfannkuche, 1993; Smith *et al*., 1996; Drazen, *et al*., 1998; Lauerman & Kaufmann, 1998). Holothurians are a dominant component

of megafauna at PAP and changes in megafaunal abundance are related to temporal variability of phytodetritus to the abyssal seafloor (Bett *et al*., 2001, Billett *et al*., 2001, 2010). These animals are responsible for quick sediment turnover and phytodetritus removal from the top 5 cm of sediment through their foraging activity (Ginger *et al*., 2001)

Holothurians at PAP have a variety of foraging strategies to enable them to exploit patches of POM deposited on the seafloor. Based on these feeding strategies and lifestyles, three feeding guilds of North Atlantic holothurians were identified by Billett (1991) and Iken *et al*., (2001), using stable isotope analysis (δ^{15}N). This study highlighted holothurian nîche separation, based on body morphology, mobility and digestive capabilities (Billett, 1991; Iken *et al*., 2001). The feeding strategies included pelagic or mobile suspension feeders (SF), mobile surface deposit feeders (SDF) and sedentary subsurface deposit feeders (SSDF). In terms of organic matter consumption, Iken *et al*., (2001) argued that SF holothurians actively foraged for freshly deposited POM, whereas SDF consumed POM that had become mixed with the top layers of sediment. SSDF holothurians with their largely infaunal lifestyle consumed organic matter that had become buried more deeply in the sediments and was therefore in a more refractory form than POM consumed by SF or SDF. The selective feeding behaviour of holothurians is likely to have an impact on carbon and nitrogen remineralisation in abyssal habitats, but this has yet to be quantified. Despite the importance of abyssal holothurians in processing organic matter in these environments, no study to date has linked holothurian host phylogeny with gut microbiome composition in abyssal habitats.

This study examined the gut microbiomes of holothurians from the Porcupine Abyssal Plain (PAP) Sustained Observatory area in the northeast Atlantic (48°50’N, 016°30’W), with an average depth of 4840 m (Billett & Rice, 2001). Holothurians were collected from two of the three feeding guilds proposed by Iken *et al*. (2001), namely SDF and SSDF. We examined microbial communities from two areas of the intestine and compared these with sediment and water samples from the local environment. Here we characterise the bacterial and archaeal assemblages of six genera of holothurians within the feeding guilds of SDF and SSDF, via amplification of the V4 region of the 16S rRNA gene, to determine

differences in gut microbiome composition and diversity between the two groups. Specifically, we tested the following hypotheses: (1) Holothurian gut microbiomes would be different from the local sediment and water habitats, (2) Holothurian phylogeny, feeding strategy and lifestyle will influence gut microbiome communities, making microbiomes distinct between species, (3) The refractory nature of available organic matter will drive feeding strategies, such as SDF and SSDF, which will influence gut microbiome composition of deposit-feeding holothurian species, (4) SSDF gut microbiomes will have a higher proportion of enteric microbes involved in inorganic carbon fixation and breaking down recalcitrant matter.

4.3 Methods

4.3.1 Sample collection

Figure 17. Location of the Porcupine Abyssal Plain (PAP) in the northeast Atlantic Ocean, where sampling for this study took place.

Samples used in this study were collected from The Porcupine Abyssal Plain Sustained Observatory (PAP-SO) area in the northeast Atlantic (centred on 48°50'N 016°30'W; Fig. 17), during the RRS *James Cook* JC165 cruise 19th May-12th June 2018. A total of 30 individual holothurians covering 6 species (Table 11) were collected during two OTSB (Otter Trawl, Semi Balloon) trawls (6 June and 9 June 2018) at a depth of 4839-4843 m (48° 58.201' N, 016° 53.297' W and 48° 50.728' N 016° 54.518' W). The following holothurian species were selected for this study: *Deima validum*, *Molpadia musculus*, *Molpadiodemas villosus* (formally *Pseudostichopus villosus,* (O'Loughlin & Ahearn, 2005)), *Oneirophanta setigera*, *Paroriza prouhoi* and *Psychropotes longicauda*. Only undamaged, intact animals with no signs of leakage were used for gut microbiome analysis. Samples were processed immediately in a 4 ºC controlled-temperature room onboard. Holothurian gut contents were extracted from the foregut and hindgut areas of the

Table 11. List of holothurian samples taken from PAP Central in the NE Atlantic, that include gut sediment content and host tissue taken for confirmation of holothurian species identity. Feeding strategy refers to surface deposit feeders (SDF) and sedentary subsurface deposit feeders (SSDF).

Environment	Species	Gut position	Feeding strategy	Region sequenced	N
Gut content	*Deima validum*	Anterior	SDF	16S V4	3
Gut content	*Deima validum*	Mid gut	SDF	16S V4	1
Gut content	*Deima validum*	Posterior	SDF	16S V4	3
Gut content	*Molpadia blakei*	Anterior	SSDF	16S V4	6
Gut content	*Molpadia blakei*	Mid gut	SSDF	16S V4	2
Gut content	*Molpadia blakei*	Posterior	SSDF	16S V4	6
Gut content	*Molpadiodemas villosus*	Anterior	SSDF	16S V4	5
Gut content	*Molpadiodemas villosus*	Posterior	SSDF	16S V4	5
Gut content	*Oneirophanta mutabilis*	Anterior	SDF	16S V4	6
Gut content	*Oneirophanta mutabilis*	Posterior	SDF	16S V4	6
Gut content	*Paroriza prouhoi*	Anterior	SSDF	16S V4	7
Gut content	*Paroriza prouhoi*	Posterior	SSDF	16S V4	7
Gut content	*Psychropotes longicauda*	Anterior	SDF	16S V4	10
Gut content	*Psychropotes longicauda*	Mid gut	SDF	16S V4	1
Gut content	*Psychropotes longicauda*	Posterior	SDF	16S V4	10
Host tissue	*Deima validum*	NA	SDF	CO1	1
Host tissue	*Molpadia blakei*	NA	SSDF	CO1	2
Host tissue	*Molpadiodemas villosus*	NA	SSDF	CO1	6
Host tissue	*Oneirophanta mutabilis*	NA	SDF	CO1	5
Host tissue	*Paroriza prouhoi*	NA	SSDF	CO1	7
Host tissue	*Psychropotes longicauda*	NA	SDF	CO1	6

intestines with a large bore syringe (20 mL) where possible, or with a sterile scalpel and spatula, avoiding gut wall tissue. Four individuals were subjected to a gut transect where additional fore-, hind- and mid-gut samples were taken. Foregut samples were taken 2-3 cm from the oesophagus. A piece of body wall from each individual was also collected for host analysis (Table 11). Gut contents

and host tissue were stored in separate 1.5 ml tubes and frozen immediately onboard at -80 ºC for later processing on land.

Sediment and water column samples were also taken from the same location as the holothurians. Sediment samples were collected at the PAP Central site using a megacorer within a 500 m radius of 48° 50.22' N 016° 31.27' W, at a depth of 4850 m. Once retrieved, cores were processed onboard and sliced into the following layers (0-1, 1-2, 2-3, 3-5, 5-10, 10-15 cm) using sterilised slicing equipment, which was rinsed with Milli-Q water between each slice. Nitrile gloves were worn at all times during this process and only the centre of the core was used. Sediment slices were stored in Whirl Pak bags at -80 ºC. This protocol followed the same method employed during the RRS *Discovery* DY377/378 cruise in 2012 to the same area. Sediment and water samples from this cruise have also been included in this study. CTD casts were deployed on 23 May and 25 May 2018 and water sampled across a depth transect with CTD rosettes containing 10 L Niskin water samplers. Water samples were filtered through 0.22 μm Sterivex filters using sterile syringes and peristaltic pump systems. Water bottles and pump systems were sterilised with bleach and rinsed thoroughly with Milli-Q water between samples. Sterivex filter samples were immediately frozen at -80 ºC. Water samples taken are listed in Table 12.

Table 12. List of water samples taken for this study.

Water depth (m)	Volume filtered (L)	Replicates
4830	1.5	1
4750	1.5	3
4000	3.0	2
2000	1.5	1
925	1.5	1
750	1.5	1
70	1.5	1

4.3.2 DNA extraction and sequencing

Genomic DNA was extracted from 500 mg of sediment gut content and 500 mg of ambient sediment samples using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) following the manufacturer's protocol. Additional extraction blanks containing only the FastDNA Spin Kit reagents were processed with the sediment samples. The concentrations of DNA from the ambient sediment samples were below 0.1 ng/μl and required further concentration. DNA was concentrated using the Zymo Clean & Concentrator-5 kits with a 2:1 DNA Binding Buffer ratio and eluted into 50 μl sterile, DNase-free water. Holothurian host tissue was processed using the DNeasy Blood & Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol for animal tissue. Two gene fragments were sequenced: the V4 region of the 16S bacterial and archaeal rRNA gene from the microbial communities, and CO1 (Cytochrome c oxidase subunit I) from the host tissue. Sterivex water filter samples were processed using the DNeasy PowerWater Kit, using a vacuum manifold and following the manufacturer's protocol. The V4 region of the 16S rRNA gene (254 bp) was amplified by the polymerase chain reaction (PCR) using the oligonucleotide primers Pro515f/Pro805r, as per the Earth Microbiome Project (EMP) protocol (Thompson *et al*., 2017; http://www.earthmicrobiome.org/protocols-and-standards/158 16s). The amplified 16S rRNA gene products and extraction blanks were then prepared with the Nextera XT v2 Kit (Illumina, San Diego, CA) and sequenced on an Illumina MiSeq platform at the Environmental Genomics Sequencing Facility (University of Southampton, National Oceanography Centre, Southampton).

The COI gene (partial 690 bp) was amplified by the polymerase chain reaction (PCR) using the oligonucleotide primers as described in Miller *et al*., (2017). PCR reactions were performed using the following mixes: 10 μL GoTaq Green Master Mix (Promega, UK), 1.0 μL of each primer, 7 μL water and 1.5 μL of template DNA. PCR amplifications were conducted in the following format: denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 40 s, annealing at 50 °C for 40 s, extension at 72 °C for 50 s, followed by a final annealing at 72 °C for 5 min. The PCR product size and purity of samples was checked with 1% agarose gel electrophoresis and the PCR product was purified

with QIAquick 96 PCR Purification Kit (Qiagen, USA). Cleaned PCR products were sequenced using cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing machines (Eurofins Genomics, Germany).

4.3.3 Data analysis

The demultiplexed Illumina 16S rRNA reads were analysed with the microbiome analysis package QIIME 2 (Quantitative Insights Into Microbial Ecology) version 2019.1 (Bolyen *et al*., 2019). Sequence quality control for the Illumina amplicon data was implemented with the DADA2 pipeline within the QIIME 2 software package (Callahan *et al*., 2016), which trims sequences, filters chimeric and phiX reads. Amplicon Sequence Variants (ASVs) or features observed in the PCR blanks were considered to be contaminants and were filtered and removed from the samples. A naïve Bayes classifier that was pre-trained on the V4 region of reference sequences from the Silva database (version 132; Quast *et al*., 2013) was used to classify representative sequences of ASVs in our dataset and clustered at 99% identity. A rooted phylogenetic tree was generated within QIIME2, using MAFFT and FastTree, which was used for calculating diversity metrics.

A feature-table was generated with the frequency of sequences associated with each sample and contained 46,803 ASVs and 2,801767 reads, with an average of 23,155 reads per sample. To account for uneven sampling depth, the feature table was normalised to 4300 reads per sample prior to abundance and diversity analysis. To confirm the identity of the holothurian mitochondrial CO1 sequences, reference sequences were downloaded from the NCBI databases (https://blast.ncbi.nlm.nih.gov) and aligned with the representative CO1 sequences using MUSCLE in Geneious (v.10.2.3; http://www.geneious.com), before being manually inspected using MEGA version X (Kumar *et al*., 2018) and Geneious. A phylogenetic tree was generated in MEGA using the Maximum Likelihood method and support for phylogenetic nodes was calculated using 500 bootstrap replicates (Fig.S2). Additional *Pseudostichopus aemulatus* host samples and GenBank sequences were added to the study samples for consensus within the tree.

Samples were grouped together according to variable type to generate 121 libraries. Relative abundances were calculated on ASV feature tables with raw counts that had been normalised by library size to get the proportions of species within each library and then square-root transformed (Hellinger transformation). This method generated relative abundances and was implemented to account for differences in sequencing depth. Alpha diversity (observed features, Shannon-Wiener index, evenness (Pielou's evenness values), Chao-1 index) and differences in community composition between sample types, or beta diversity (Bray-Curtis distance), were calculated in QIIME2 on the normalised samples and formatted along with the generated feature-table for further analysis in R. The significance of alpha diversity indices in holothurian, sediment and water samples was tested with pairwise Kruskal-Wallis tests. Sample-based alpha diversity species accumulation curves were calculated for each sample type without replacement, using the "iNEXT" package in R v 3.3.2 (Hsieh *et al*., 2020; Chao *et al*., 2014). Diversity estimates were calculated using rarefied samples and plotted with respect to sample size. Extrapolated diversity estimates were calculated and plotted based on double the reference sample size.

Community composition and statistical analyses were performed using the 'vegan' package in R (Oksanen *et al*., 2007). Using the Hellinger-transformed feature table, the dissimilarity between pairs of samples were calculated using the Bray-Curtis dissimilarity coefficient, to determine variations in microbial community composition between holothurians, sediment and water. Bray-Curtis dissimilarity measures were used for non-metric multidimensional scaling (NMDS) ordination, to visualise community variation using the 'vegan' package and the plot reformatted with the R package 'ggplot2' v. 3.3.3 (Wickham, 2016). ANOSIM analyses were calculated in the 'vegan' package, using the 'vegdist' distance metric, to test for differences in community composition between gut compartments, feeding guilds and host species that had been visualised with the NMDS ordinations. A heatmap was generated from Bray-Curtis dissimilarity matrices using the function 'heatmap3' in the R package 'GMD' (Zhao *et al*., 2021), illustrating the top 10 most abundant taxa within holothurians, sediment and water. Permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was performed on unweighted UniFrac distance matrices, which take

phylogenetic information and genetic similarity between organisms into account, to assess differences in community composition and test the significance of environmental variables, gut compartment, feeding guild and species using the 'adonis' method ('vegan' package in R). Samples with less than three replicates were excluded from statistical analysis, such as mid-gut samples and some water samples. The relative abundance of ASVs were calculated on Hellinger-transformed feature tables of raw counts. These relative abundances were displayed as bar plots using the 'ggplot2' package in R. Venn diagrams based on presence/absence data were calculated and displayed using Venny 2.1. (https://bioinfogp.cnb.csic.es/tools/venny/index.html), in order to showcase the distribution of ASVs between holothurians, sediment and water.

4.4 Results

After the data were normalised, 2,724,066 reads were identified within 121 samples. A total of 78 holothurian samples, 33 sediment samples and 10 water samples were analysed for microbiome alpha and beta diversity, and community composition. After quality control measures were implemented, these libraries were sub-sampled to a depth of 4300, to maximise the number of samples analysed and minimize information lost. Species accumulation curves (Fig. 18) indicate that ASVs were still accumulating in all sampled habitats at this depth of sequencing as curves had not reached a plateau. In particular, curves for water and sediment 5-15 cm were very steep, indicating under-sampling of alpha diversity of these habitats.

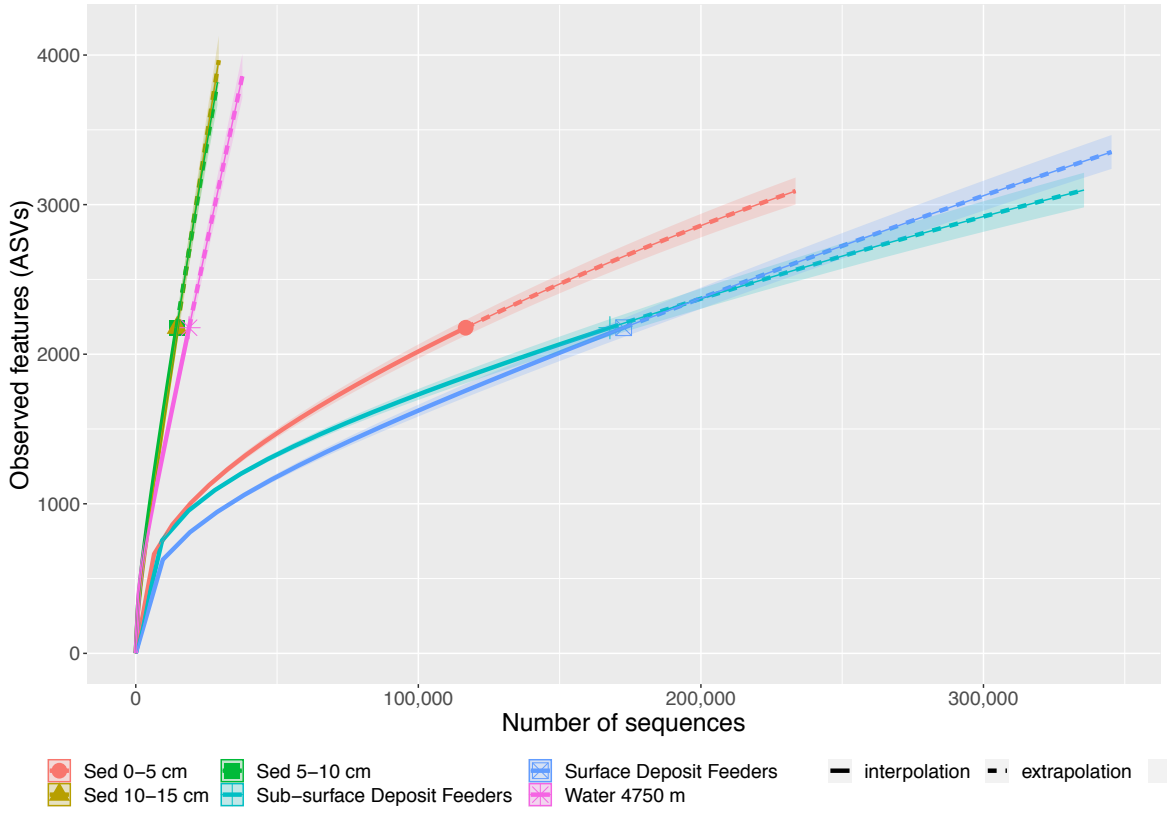

Figure 18. Sample-based alpha diversity species accumulation curves calculated for each sample type without replacement. Diversity estimates are calculated and plotted for rarefied samples with respect to sample size, and plotted extrapolated curves are based on double the reference sample size. Curves for each sample type do not reach asymptote, indicating insufficient sequencing depth for estimated microbial richness.

Across all libraries after rarefaction, 46,664 ASVs were detected, with 17,724 ASVs identified in the holothurian gut microbiomes, 27,838 in sediment and 3,102 in water samples. This corresponded to 1,554 taxa in the holothurian gut microbiomes, 1,466 taxa in sediment, 937 taxa in water samples, totalling 2,498 taxa overall. The average number of ASVs was highest in sediment (888 ± 186), and lowest in water (379 ± 88) (Fig. 19). Of the identified ASVs, the proportion of Archaea was highest in sediments (15%) compared to water (12%) and holothurian gut microbiomes (5%). The remainder of ASVs classified as Bacteria (Sediment = 85%, Water = 88 % and holothurian gut microbiomes = 95 %).

4.4.1 Holothurian gut microbiome communities are compositionally distinct from ambient sediment and water communities at PAP

Microbial diversity and richness of the three environments sampled at PAP (guts, sediments and water) were significantly different from each other. Pairwise Kruskall-Wallis tests indicated that Shannon's alpha diversity and richness, measured by the Chao1 index (Table 14), was significantly different between the three environments (Fig. 20). Only a fraction of ASVs were shared between all three environments (62 ASVs, 0.1 %). Conversely, 90% of the ASVs that were observed in the holothurian gut microbiomes were not found in the ambient sediment or in the water column. A small percentage of ASVs found in the gut microbiomes were shared between ambient sediment (1,579 ASVs, 3.4%) and the water column (84 ASVs, 0.2%).

Ambient sediment was richer and more diverse than holothurian gut microbiomes and the water column (Table 14: Observed ASVs: sediment = 888 ± 186; holothurian gut microbiomes = 381 ± 54; Shannon alpha diversity: sediment = 8.89 ± 2.01; holothurian gut microbiomes = 7.59 ± 0.21). The greatest richness and diversity were observed in the 1-2 cm sediment horizon (Chao: 3180 ± 1742), followed by the 5-10 cm horizon (Chao: 1600 ± 245). Species evenness, measured by Pielou's Index (J) was also highest in the sediment and averaged 0.94 ± 0.005 (Fig. 19). There was no partitioning of unique observed ASVs between sediment horizons.

Holothurian gut microbiomes contained a total of 15,999 unique ASVs, accounting for 34% of total observed ASVs across all libraries (Fig. 20). The number of holothurian gut microbiome-associated taxa ranged from 122-807 ASVs, with the overall average of 381 ASVs observed among holothurian species. Alpha diversity was significantly different between holothurian species, measured by observed ASVs (Kruskal-Wallis, H = 36.12, p =0.001) and Shannon diversity (Kruskal-Wallis, H = 33.54, p = 0.001). The greatest range in diversity was observed in SSDF, which had both the richest and least-rich microbiomes. SSDF host taxa also represented the largest and smallest range in the observed number of ASVs respectively, with the greatest number observed in *M. musculus* (259 ± 60) and lowest in *M. villosus* (106 ± 22). The mid gut from *M. musculus* had the most diverse gut microbiome (Shannon = 8.77) and *M. villosus* hindgut the lowest (Shannon = 7.02). On average, the foregut was richer in SDF holothurians (Observed ASVs: foregut 372 ± 46, hindgut 305 ± 33) and the reverse observed in SSDF, where the hindgut microbiome was richer (foregut 278 ± 24, hindgut 358 ± 46**).** Overall comparisons of alpha diversity revealed only minimal differences between holothurian feeding guilds, with a marginally higher number of ASVs in SSDF than SDF. These differences in alpha diversity were also non-significant between foregut and hindgut regions of the holothurian intestine.

There was, however, a partitioning of ASVs between SDF and SSDF gut microbiomes. In SDF, 6887 ASVs were unique to this group of holothurians, accounting for 41.2% of holothurian sequence libraries and 8269 ASVs (49.5%) were unique to SSDF (Fig. 4). Less than 10% of ASVs were shared between the two guilds. In SDF, 3742 ASVs (46.4% of holothurian gut microbiome libraries) were unique to the foregut and 3428 (42.5%) ASVs were found only in the hindgut. Only 11% (887) ASVs were shared between foregut and hindgut. Likewise, in SSDF, 3914 (43.8%) ASVs were only found in the foregut section of the holothurian intestine and 4317 (48.3%) ASVs in the hindgut, with 700 (7.8%) ASVs shared between fore- and hindgut microbiomes.

4.4.2 Community composition of environment types at PAP

Differences in community composition was significant between environment, feeding guild, gut section and host species. Microbial community composition differed significantly between environments types (Table 15, Fig. 21) and between the holothurian gut microbiomes of the two feeding guilds (Table 15, Fig. 21). In addition, microbiome composition differed significantly between gut section within and between feeding guilds (Table 15, Fig. 22).

A total of 62 phyla were identified across all three environments, with 58 phyla in sediment, 48 in holothurian gut microbiomes and 47 phyla in the water column. Overall, the predominant phyla were Proteobacteria, forming 35 % of library reads on average, followed by Planctomycetes (16%), Bacteroidetes (7%), Thaumarchaeota (6.7%) and Actinobacteria (5.5%). The reads from sediment libraries contained a higher proportion of Thaumarchaeota (13%). In holothurian microbiome libraries, Verrucomicrobia formed one of the top five most abundant phyla, at 6.4 % and was more dominant in SDF libraries (Fig. 23). The most abundant phylum in holothurian gut microbiomes was Gammaproteobacteria (21 % relative abundance), followed by Planctomycetes (16 %), Bacteroidetes (8%), Deltaproteobacteria (8%), Alphaproteobacteria (7.5%), Actinobacteria (7%) and Verrucomicrobia (6.5%) (Fig. 23).

Amongst the two holothurian feeding strategies, host-specific differences in microbial community composition were detected. Phyla that contributed the most to differences in microbiomes between SDF and SSDF were the Thaumarchaeota phylum (Fig. 24, Fig. 25), formed a greater abundance in the intestines of SSDF holothurians (7.7 % foregut and 7.5% hindgut), compared with 0.8% in the foregut and 2.6% in hindguts of SDF holothurians (Fig. 26. Fig. 25). Planctomycetes were more abundant in SSDF (17.5 %) than SDF (14 %). Rare taxa (<1% relative abundance) formed a greater proportion of SSDF gut microbiomes (3%) than SDF microbiomes (0.5%). In contrast, SDF gut microbiomes were more enriched in Verrucomicrobia (SDF = 8%, SSDF = 4.5%), Actinobacteria (SDF = 8%, SSDF = 6%) and Dependentiae (SDF = 2.5%, SSDF = 0.5%) (Fig 27).

Sediment and water assemblages appeared distinct from holothurian microbiome communities. Sediment assemblages were dominated by

Proteobacteria, Planctomycetes and Thaumarchaeota (Fig. 23, Fig. 27). Bottom water assemblages had a greater abundance of Alphaproteobacteria, the SAR202 clade (Chloroflexi), OM27 clade (Deltaproteobacteria), Euryarchaeota and OM190 class (Planctomycetes) (Fig. 23, Fig. 28, Fig. 27). We observed a partitioning of taxa between the three environments and between SDF and SSDF groups (Fig. 28). Ca. *Nitrosopumilus* dominated SSDF microbiomes, sediment and some water depths (750 m, 4000 m), whereas uncultured Gammaproteobacteria groups and the class DEV007 within Verrucomicrobia were more prevalent in SDF microbiomes (Fig.24).

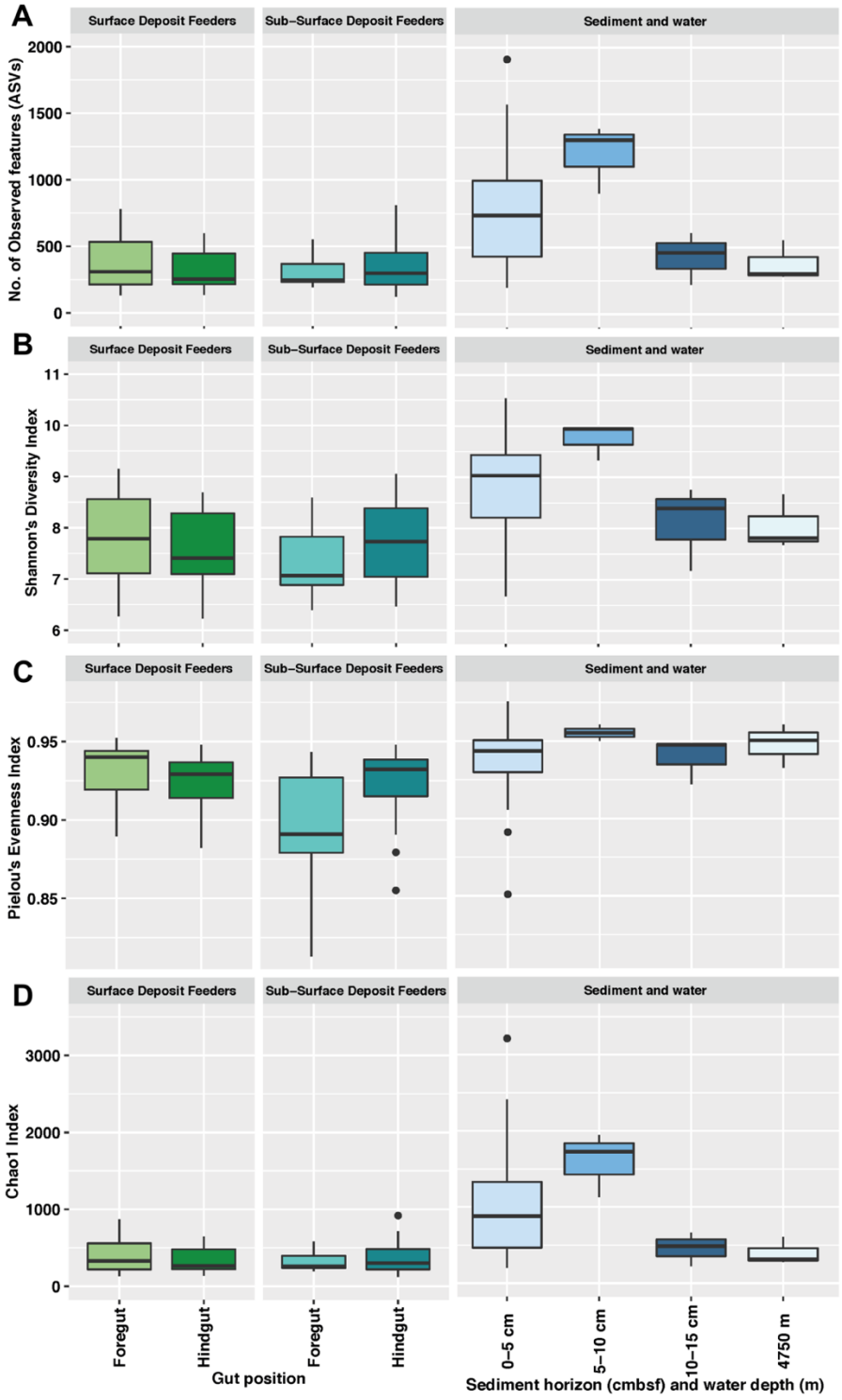

Figure 19. Comparisons of diversity indices between holothurian gut microbiomes in surface deposit feeders and subsurface deposit feeders, with microbial assemblages in sediment and deep water (4750 m), at PAP, based on (a) Number of observed features or amplicon sequence variants (ASVs), (b) Shannon's Diversity Index, (c) Pielou's Evenness Index, and (d) Chao1 richness Index. Boxplots show the median (horizontal line), interquartile ranges (box limits), range (whiskers) and outliers (black dots). Diversity indices were calculated on samples that had been rarefied to account for uneven sampling depth.

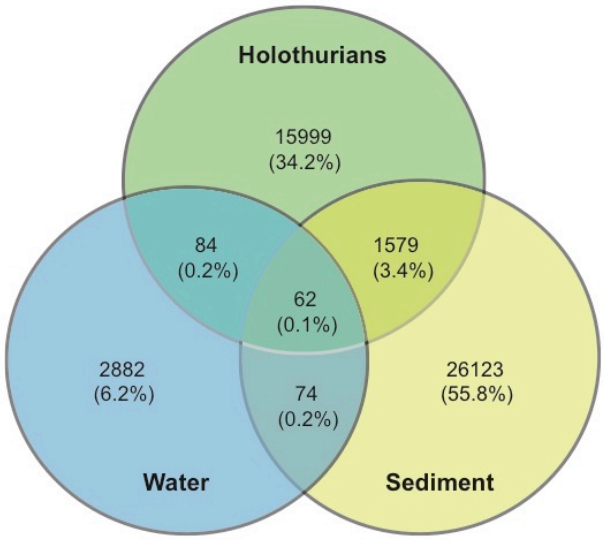

Figure 20. Microbial assemblage taxa surveyed in this study. Venn diagram showing the total number of microbial taxa in the form of amplicon sequencing variants (ASVs) shared between each combination of sample types at PAP. Numbers and percentages represent a proportion of the total number of ASVs in the dataset.

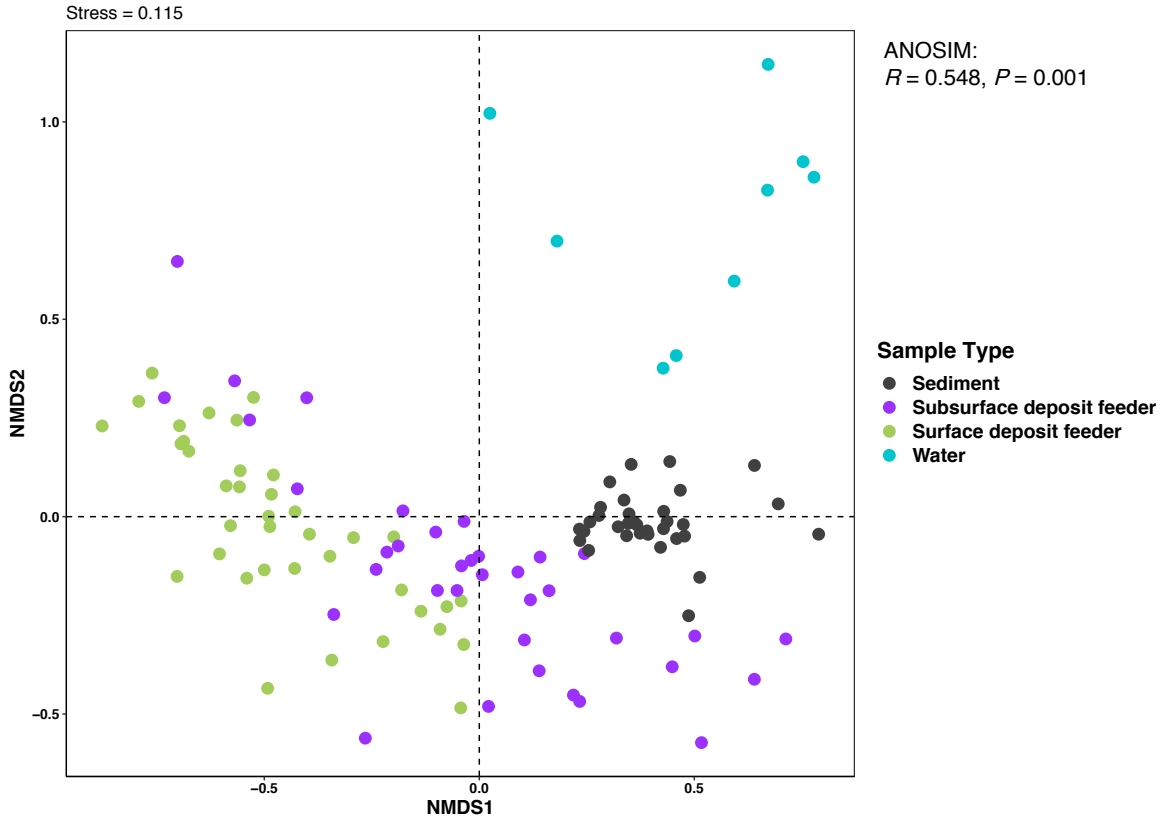

Figure 21. Non-metric multidimensional scaling (nMDS) plots of microbial community dissimilarity based on Bray-Curtis distances. A comparison of assemblages in the gut microbiomes of surface deposit feeding and subsurface deposit feeding holothurians with ambient sediment (0-15 cm below sea floor) and water column (70- 4830 m) assemblages at PAP.

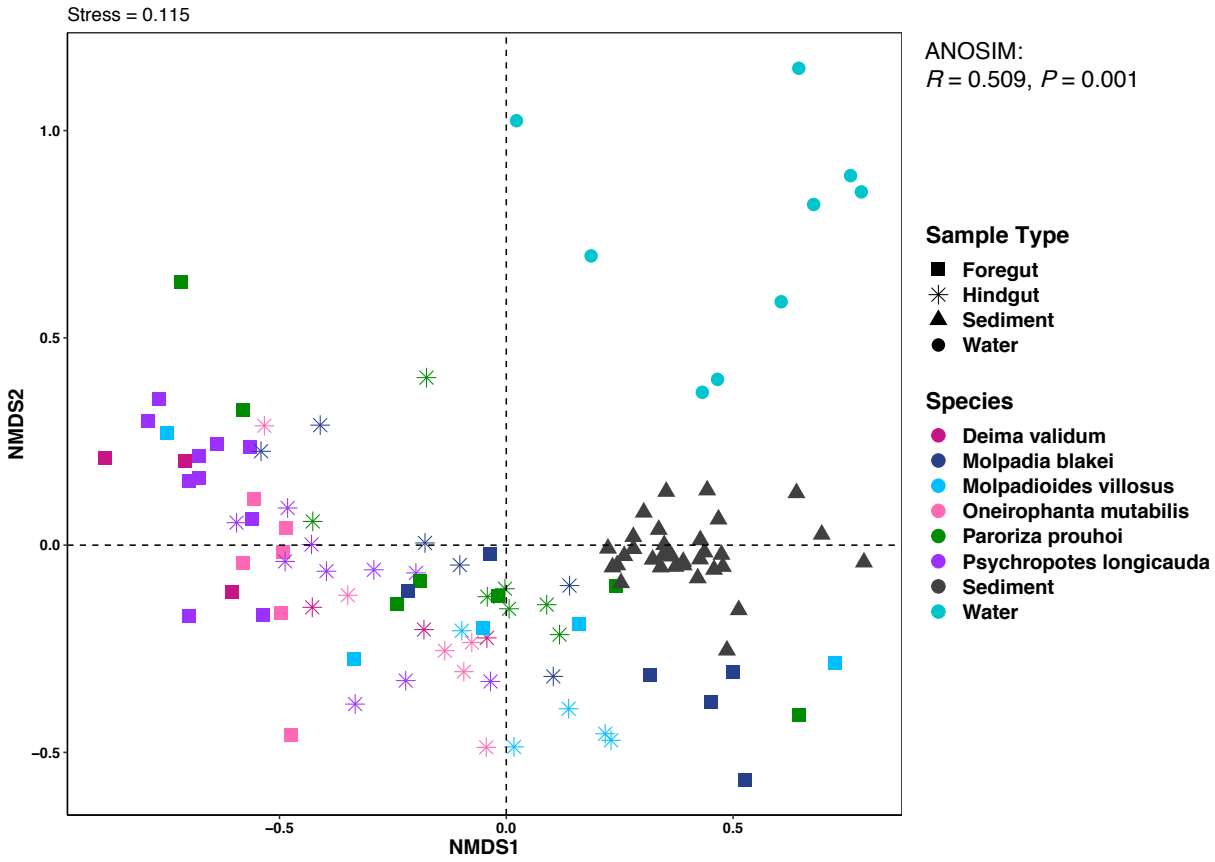

Figure 22. Non-metric multidimensional scaling (nMDS) plots of microbial community dissimilarity comparing ambient sediment (0-15 cm below sea floor) and water column (70- 4830 m) assemblages with foregut and hindgut microbiome assemblages from six holothurian species within the surface deposit feeding and subsurface deposit feeding guilds at PAP.

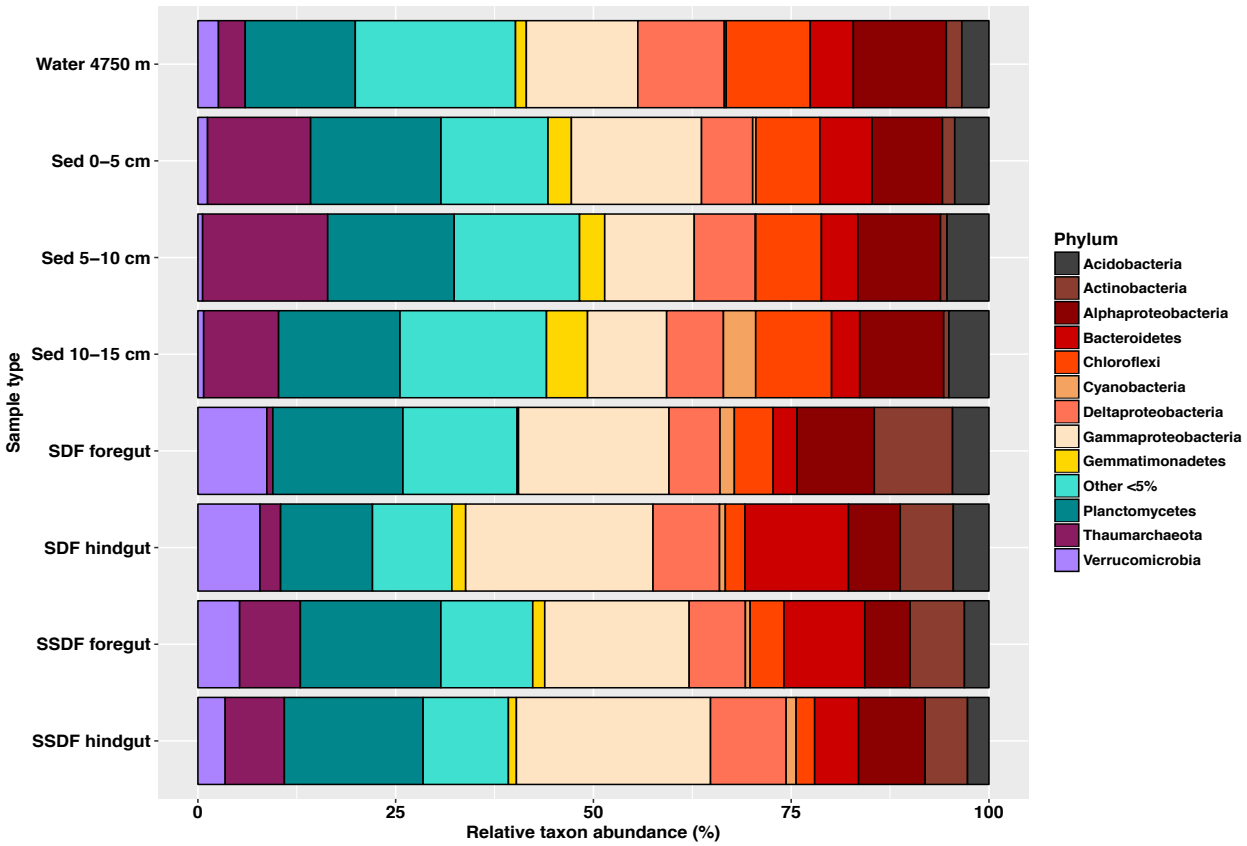

Figure 23. Relative abundances of amplicon sequence variants (ASVS) at phylum level of holothurian gut microbiomes, ambient sediment and deep water (4750 m) assemblages at PAP. Only taxa ≥ 5% relative abundance are shown. Category "Other" represents taxa < 5% relative abundance. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

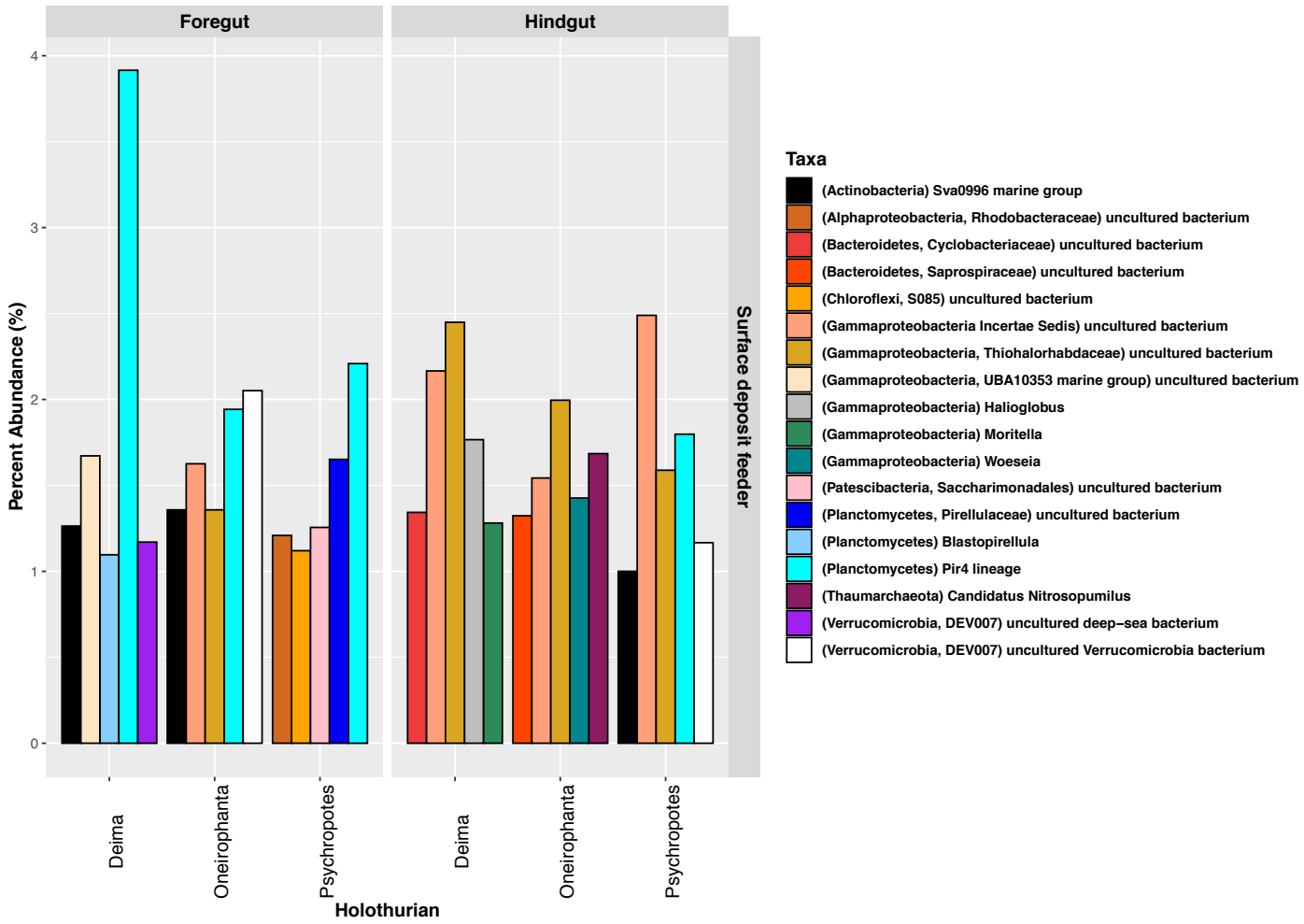

Figure 24. Relative abundances of amplicon sequence variants (ASVs) of the five most abundant taxa in the fore- and hindgut microbiomes of surface deposit feeding holothurians at PAP, defined at genera or family level. Only taxa ≥ 1% relative abundance are shown. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

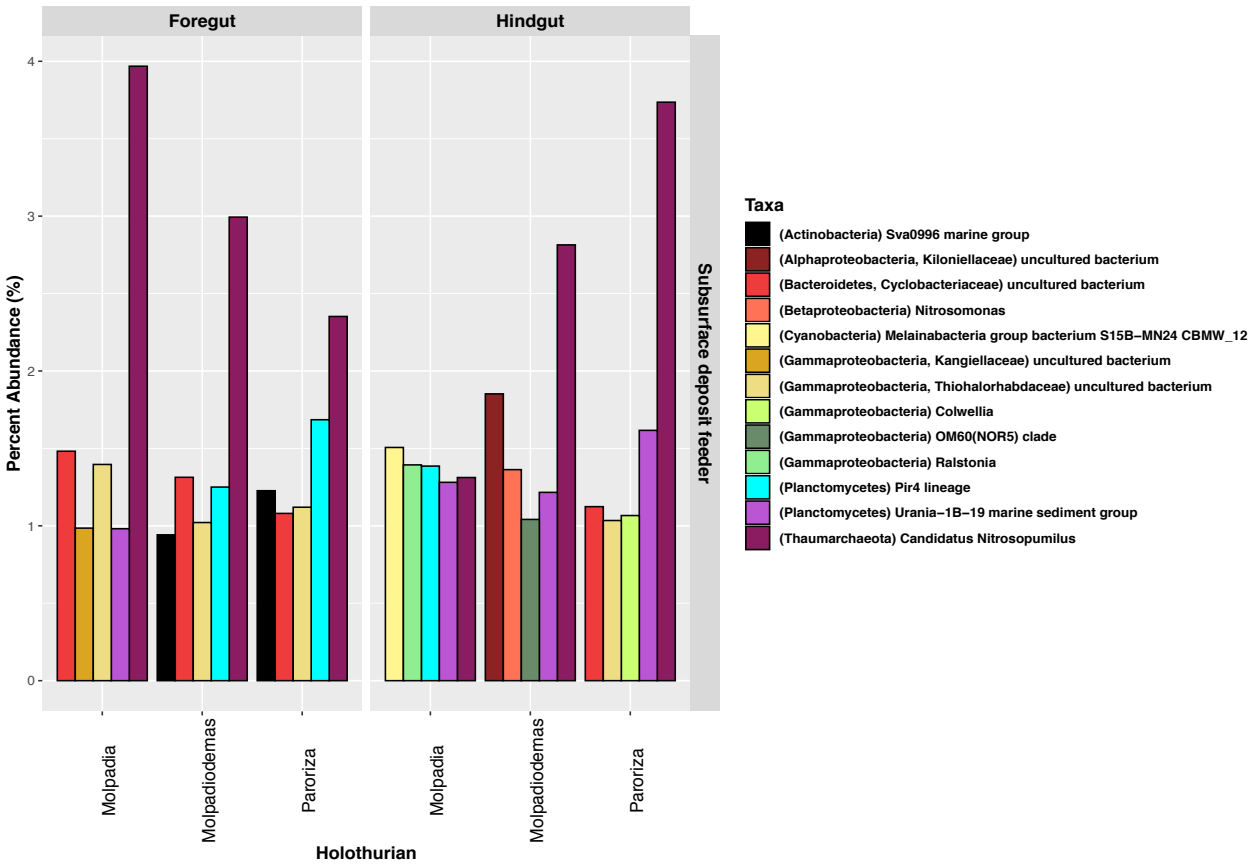

Figure 25. Relative abundances of amplicon sequence variants (ASVs) of the five most abundant taxa in the fore- and hindgut microbiomes of subsurface deposit feeding holothurians at PAP, defined at genera or family level. Only taxa ≥ 1% relative abundance are shown. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

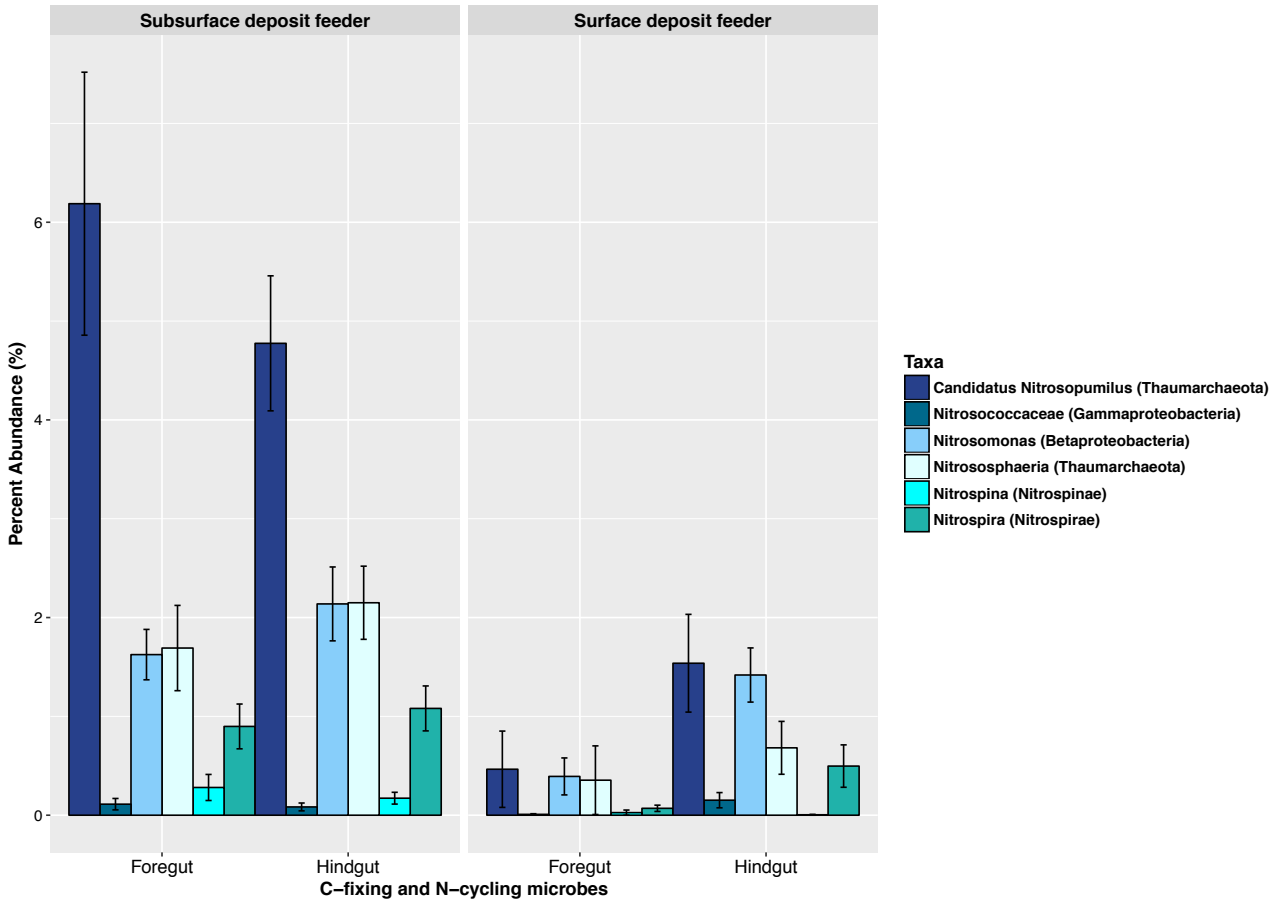

Figure 26. Relative abundances of amplicon sequence variants (ASVs) of the most abundant putative carbon-fixing and nitrifying microbes in the fore- and hindgut microbiomes of surface deposit feeding and subsurface deposit feeding holothurians at PAP, defined at genera or family level. The class Nitrososphaeria includes all taxa within that class excluding the genera *Candidatus Nitrosopumilus*, which is shown in a separate category. Only taxa ≥ 1% relative abundance are shown. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

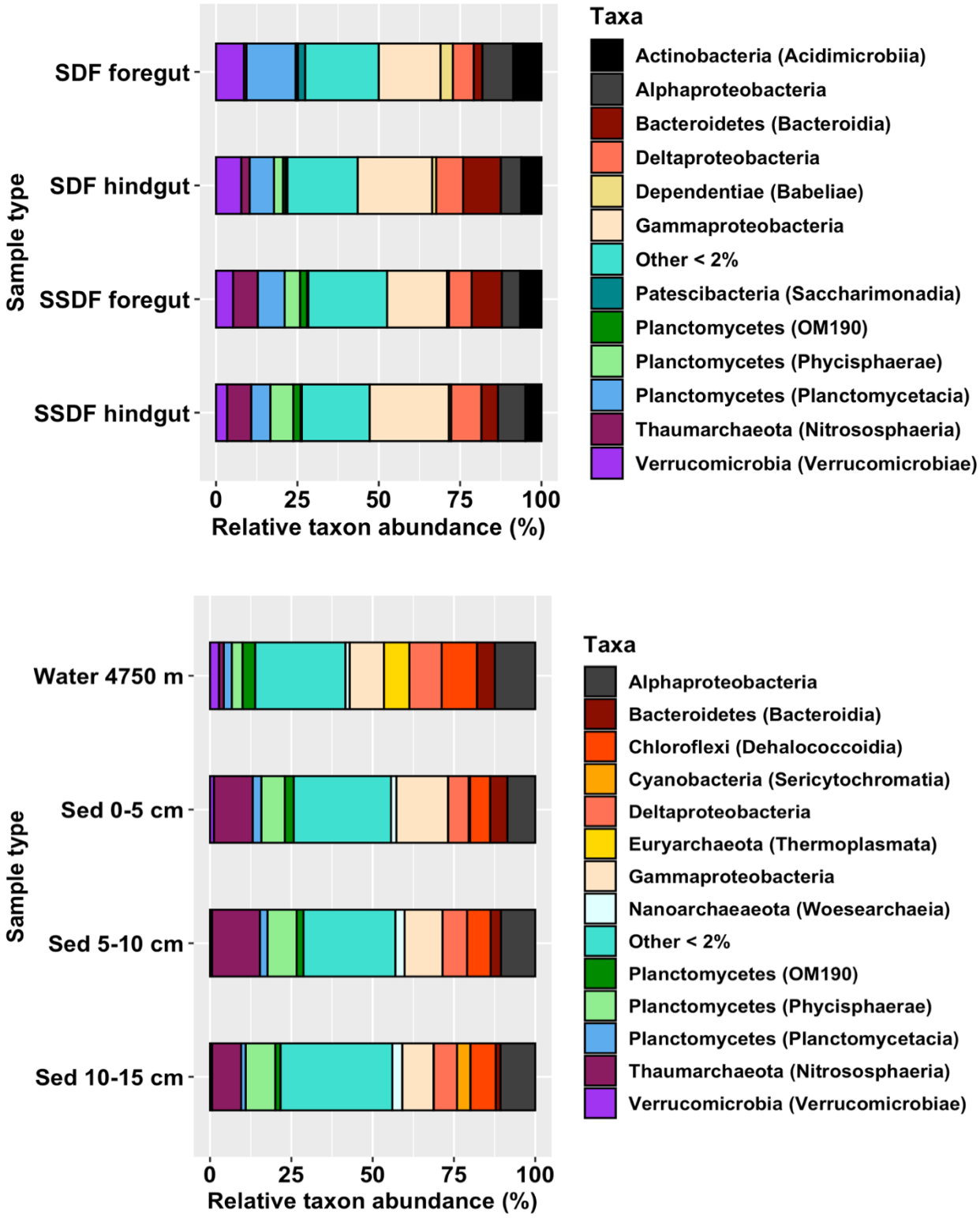

Figure 27. Relative abundances of amplicon sequence variants (ASVS) at class level of holothurian gut microbiomes from surface deposit feeding and subsurface deposit feeding holothurians, ambient sediment (0-15 cm below seafloor) and deep water (4750 m) at PAP. Only taxa ≥ 2% relative abundance are shown. Category "Other" represents taxa < 2% relative abundance. Relative abundances were calculated on samples that had first been normalised to account for uneven sampling depth, by scaling sequence counts by library sizes.

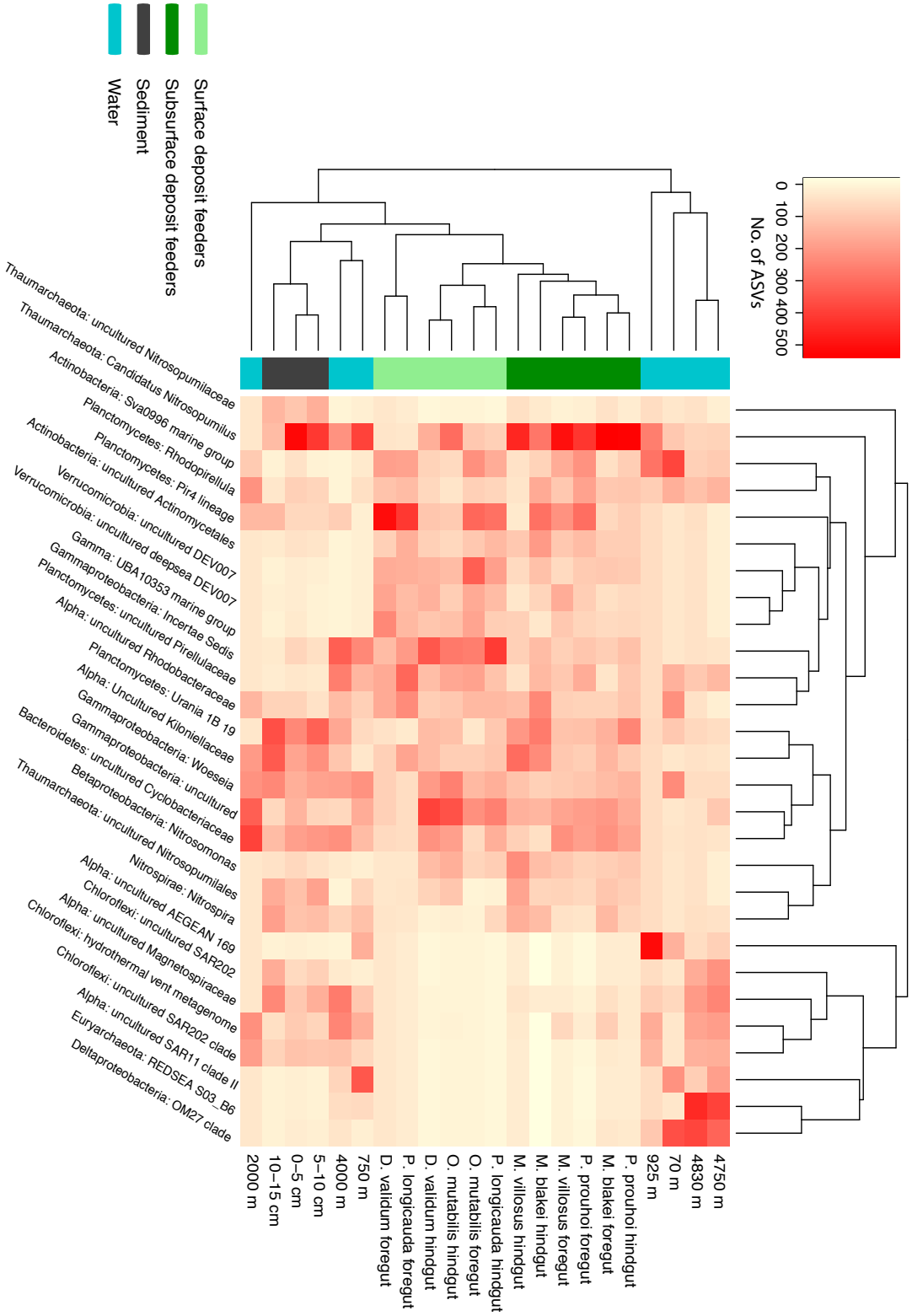

Figure 28. Heat map of the top 10 most abundant taxa from each sample type. Dendrograms were created by average linkage hierarchical clustering on a Bray-Curtis dissimilarity matrix of the dataset. The dendrogram on the x-axis shows clusters of amplicon sequencing variants (ASVs) that occur most frequently together. The dendrogram on the y-axis is colour-coded by sample type (light green = surface deposit feeding holothurian microbiomes, dark green = subsurface deposit feeding holothurian microbiomes, black = ambient sediment, blue = water column). Heatmap colour shows the number of ASVs found in each sample after normalising to 4300 reads per sample

Table 13.

Detail of sediment and water samples taken for this study.

Environment	Location	Year collected	Horizon (cm) /Depth (m)	Volume extracted or filtered	N
Sediment	PAP	2012	0-1	0.5 g	13
Sediment	PAP	2016	0-1	0.5 g	2
Sediment	PAP	2018	0-1	0.5 g	3
Sediment	PAP	2012	1-2	0.5 g	3
Sediment	PAP	2012	2-3	0.5 g	3
Sediment	PAP	2012	3-5	0.5 g	3
Sediment	PAP	2012	5-10	0.5 g	3
Sediment	PAP	2012	10-15	0.5 g	3
Water	PAP	2018	70	1.5 L	1
Water	PAP	2018	750	1.5 L	1
Water	PAP	2018	925	1.5 L	1
Water	PAP	2018	2000	1.5 L	1
Water	PAP	2018	4000	3 L	1
Water	PAP	2018	4750	1.5 L	3
Water	PAP	2018	4830	1.5 L	1

Table 14. Pairwise Kruskal-Wallis tests on alpha diversity indices of holothurian, sediment and water samples. Only the test pairs with P-values that are < 0.05 are shown.

Measure	Group 1	Group 2	H	P-value
Observed ASVs	Holothurian gut microbiome	Sediment	32.85	< 0.001
	Foregut	Sediment	23.06	< 0.001
	Hindgut	Sediment	23.10	< 0.001
Shannon	Holothurian gut microbiome	Sediment	28.57	< 0.001
	Foregut	Sediment	23.56	< 0.001
	Hindgut	Sediment	21.00	< 0.001
Chao1	Holothurian gut microbiome	Sediment	30.45	< 0.001
	Foregut	Sediment	23.62	< 0.001
	Hindgut	Sediment	23.50	< 0.001

Table 15. Output permutational multivariate analysis of variance (PERMANOVA) on relative abundance counts that have been fourth-root transformed prior to creation of Bray-Curtis dissimilarity matrices for microbial assemblages within holothurian gut microbiomes, sediment and water column.

	Df	SS	MS	F	R^2	P
Environment	2	5.8723	2.93614	14.8964	0.18715	0.001
Feeding type	1	1.5975	1.59748	8.1047	0.05091	0.001
Gut section	1	0.7688	0.7688	3.9005	0.02450	0.001
Holo species	4	1.2986	0.32464	1.6471	0.04139	0.001
Feeding type / Gut	1	0.6235	0.62352	3.1634	0.01987	0.003
Gut / Species	4	1.1124	0.27809	1.4109	0.03545	0.023
Residuals	102	20.1046	0.19710		0.64073	
Total	115	31.3776			1	

4.5 Discussion

4.5.1 Seasonal influences of POM on deposit-feeding holothurians at PAP

The Porcupine Abyssal Plain (PAP) is characterised by seasonal pulses of POM, in the form of phytodetritus from the overlying productive surface waters (Rice *et al*., 1994), which influence the abundance and composition of benthic communities (Gooday & Turley, 1990; Pfannkuche 1993; Billett *et al*., 2001; 2010). POM is ephemeral once it has reached the seafloor, resulting in a highly food limited system (Lampitt, 1985). Over time, aerobic breakdown of POM and remineralisation takes place in the surface layers at PAP, such that the quality of POM declines with depth through the sediment (Roberts *et al*., 2000). Most deposit-feeding holothurians consume POM in the surface layers of sediment, however, some species feed from the deeper sediment layers, to avoid competition for POM in surface layers (Roberts *et al*., 2000). Hence, strong competition between megafaunal feeding types for availability of POM has resulted in resource partitioning and nîche separation of sympatric holothurian species at PAP (Billett, 1991; Roberts & Moore, 1997; Wigham et al., 2003).

To date, no study has comprehensively linked holothurian feeding strategies, host phylogeny and gut microbiome composition. Other research has demonstrated the ability of abyssal holothurians to feed selectively (Pfannkuche & Lochte, 1993; Roberts & Moore, 1997; Billett *et al*., 2001; Wigham *et al*., 2003; Hudson *et al*., 2003; Iken *et al*., 2001; Roberts *et al*., 2001), partly owing to morphological differences in tentacle and gut structure between different species of holothurians (Roberts & Moore, 1997; Roberts *et al*., 2001). The use of biomarkers, such as lipids, phytoplankton pigments and carotenoids provide effective methods for investigating the diets of abyssal holothurians and tracing the pathways of organic matter in benthic ecosystems (Billett *et al*., 1988; Santos *et al*., 1994; Ginger *et al*., 2001; Witbaard *et al*., 2001; Wigham *et al*., 2003; Hudson *et al*., 2003; Drazen *et al*., 2008). Isotope analysis has provided an alternative method to investigating the trophic positions of benthic organisms, and in particular, how benthic, deposit-feeding holothurians exploit a common food source (Iken *et al*., 2001). Iken and colleagues revealed how the relative trophic positions of benthic holothurian species based on $\delta^{15}N$ isotope values formed two

distinct clusters that corresponded to SDF and SSDF respectively (Iken *et al*., 2001). Although it was not possible to collect sufficient samples to investigate feeding guild A (corresponding to pelagic/mobile superficial sediment feeders) from the Iken *et al*., (2001) study, feeding guilds corresponding to SDF and SSDF were targeted with higher resolution and more concentrated sampling.

The gut microbiome of deposit-feeding holothurians is likely formed from the repeated ingestion of organic matter, sediment and associated microbes, with certain taxa being selectively enriched in the gut (Yamazaki *et al.,* 2019). Initial analysis has revealed that holothurian gut microbiome communities are compositionally distinct from ambient sediment and water communities, as noted by Amon & Herndl, (1991), Gao *et al*., (2014), León-Palmero *et al*., (2018) and Yamazaki *et al*., (2019). A significant number of ASVs representing 39% of total observed ASVs were found only in holothurian gut contents, suggesting that these taxa may be commensal gut flora. Additionally, holothurian gut compartments harboured distinct microbial communities to each other. Few ASVs were shared between fore- and hindgut regions, also reported in other studies (Deming & Colwell, 1982; Amaro *et al*., 2009; Amaro *et al*., 2012; Gao *et al*., 2014; Pagán-Jiménez *et al*., 2019).

4.5.2 Dominant taxa may have important role in host nutrition

North Atlantic holothurians maintain associations with a diverse array of microbial taxa that may have key roles in organic matter degradation, carbon and nitrogen cycling. Within both feeding guilds, holothurian gut microbiomes contained ammonia-oxidising archaea (AOA: *Candidatus* Nitrosopumilus; Nitrosopumilaceae), ammonia-oxidising bacteria (AOB: *Nitrosomonas*; Pirellulaceae), nitrite-oxidising bacteria (NOB: *Nitrospira*; *Nitrospina*), nitrifiers (NB1-j order, Deltaproteobacteria) and sulphur-oxidising bacteria (Thiohalorhabdaceae). These taxa may have implications for local cycling of carbon, nitrogen and sulphur, as well as providing a source of nutrients for the host.

Holothurian gut microbiomes also supported a diverse array of taxa implicated in high molecular weight (HMW) compound processing. The Planctomycetes

phylum formed a dominant component of gut microbiomes and is a diverse group capable of breaking down complex molecules in sediments and marine snow (Glöckner *et al*., 2003). Driving the overall abundance of Planctomycetes was a number of orders and genera within this phylum. In particular, the most abundant genera within SDF gut microbiomes was the Pir4 lineage, which is a heterotroph known to form associations with eukaryotic hosts (Bondoso *et al*., 2017; Dedysh *et al*., 2019) and is part of a clade that is able to utilise a variety of substrates with of diversity of enzymes (Bondoso *et al*., 2017). Although functional information for this lineage is limited, related clades in the Pirellulaceae family within which Pir4 resides are possible AOB (Kellogg *et al*., 2016).

Predominant phyla, particularly in SDF gut microbiomes, included Verrucomicrobia and have been identified as degrading polysaccharides and POM in the ocean (Cardman *et al*., 2014, Freitas *et al*., 2012). The dominance of the Verrucomicrobia phylum was largely driven by the DEV007 lineage, which has previously been identified assimilating a range of phytoplankton-derived proteins (Orsi *et al*., 2016). Verrucomicrobia in general are likely to be important in POM and DOM processing (Orsi *et al*., 2016). An affinity for utilising dissolved proteins substrates also encompassed other lineages that were highly abundant in holothurian gut microbiomes, including the Sva0996 clade within the Actinobacteria phylum, genera in the Cyclobacteriaceae family (Bacteroidetes), the OM60 (NOR5) clade (Gammaproteobacteria) and Rhodobacteraceae (Alphaproteobacteria). These lineages fall within phyla and orders that are known particle colonisers, with a high metabolic and enzymatic diversity, as well as the ability for surface attachment and adaptability to changes in organic matter production (DeLong *et al*., 1993; McCarren *et al*., 2010; Crespo *et al*., 2013; Orsi *et al*., 2016; Duret *et al*., 2019).

In contrast to SDF microbiomes, SSDF gut microbiomes contained high abundances of Thaumarchaeota, particularly *Nitrosopumilus*. This group of chemolithoautotrophic archaea can oxidise ammonia to nitrite using ammonia monooxygenase enzymes (Könneke *et al*., 2005). These microbes are capable of coupling this process with organic carbon production through inorganic carbon fixation (Könneke *et al*., 2005; Middelberg, 2011) and may play an important role in nitrogen cycling in marine habitats. These findings are in contrast to most

holothurian microbiome studies, where the abundance of Archaea in holothurian gut microbiomes is often poorly reported in the literature, largely due to primer mismatches, PCR protocols and difficulty amplifying archaeal DNA (Amaro *et al*., 2012; Walters *et al*., 2015; Parada *et al*., 2016). Ammonia-oxidising Archaea (AOA) Thaumarchaeota are prevalent in other marine invertebrate assemblages, such as sponges (Radax *et al*., 2012; Jackson *et al*., 2013; Ramsby *et al*., 2018), corals (Apprill *et al*., 2016; Huggett & Apprill, 2019), marine polychaetes (Dale *et al*., 2019), nudibranchs (Cleary *et al*., 2019) and ascidians (Erwin *et al*., 2014). This group may form beneficial associations with heterotrophic bacteria, as some strains can utilise urea as a source of ammonia (Alonso-Sáez, *et al*., 2012; Bayer *et al*., 2016). SSDF microbiomes also contained a greater abundance of AOB *Nitrosomonas* (Betaproteobacteria)*,* Nitrosococcaceae (Gammaproteobacteria) and NOB *Nitrospina* (Nitrospinae) and *Nitrospina* (Nitrospirae) (Fig. 26).

This finding, however, is intruiging, given the oxygen depleted conditions of the gut environment. High abundances of AOA *Nitrosopumilus* sp. have previously been reported in invertebrate gut microbiomes (Dale *et al*., 2019), although it was unclear whether significant rates of ammonia oxidation were taking place within the low oxygen environment of the hindgut (Plante and Jumars, 1992). Digestion of organic material in the foregut is likely to provide a regular supply of ammonia in the hindgut (Dale *et al*., 2019), which may help support an assemblage of AOA and AOB. It is possible that the observed *Nitrosopumilus* within the gut microbiomes are inactive, or alternatively, they may be capable of other metabolisms. AOA have previously been found in anoxic environments, such as OMZs (Labrenz *et al.,* 2010; Stewart *et al.,* 2012; Peng *et al*., 2013; Berg *et al.,* 2015). Indeed, AOA may be capable of ammonia oxidation under aerobic and possibly anaerobic conditions (Treusch *et al*., 2005) and some strains may be capable of anaerobic growth. AOA have previously been observed to be resilient and exhibit greater adaptability to anoxic and fluctuating hypoxic conditions compared to AOB (Liu *et al*., 2015). Ammonia and nitrite oxidation are possible at low or non-detectable oxygen concentrations, as has been previously observed in OMZs (Fussel *et al*., 2012; Kalvelage *et al*., 2013), although rates of ammonia oxidation may be low or negligible (Newell *et al*., 2011). Recently, it has been discovered that ammonia-oxidising *Nitrosopumilus* strains may be capable of

oxygen production in oxygen depleted marine environments, in addition to ammonia production (Kraft *et al.*, 2021). Although this pathway is not yet completely resolved, it is possible that the oxygen produced by this pathway may be directly used for ammonia oxidation (Kraft *et al.,* 2021).

In general, hindguts supported greater abundances of AOA relative to foreguts, suggesting that AOA may be more susceptible to digestive processes in the foregut, as observed in other invertebrate gut microbiomes (Dale *et al*., 2019). This digestive susceptibility may well vary between holothurian species (Roberts *et al*., 2001), such that AOA are digested at higher rates in SDF guts, and are therefore less abundant compared to SSDF gut environments. Given that the relative abundances of AOA in the holothurian hindguts is less than in the sediments, it is possible that the AOA are transitory members of the holothurian gut microbiomes, present in the ingested sediment and material during gut passage. It also plausible that the AOA are not actively growing or colonizing the gut (Plante and Jumars, 1992). In this instance, metatranscriptomics would further determine whether these cells are active and which metabolisms are being utilized within these gut environments.

Gut microbiomes in the SSDF group also contained non-photosynthetic cyanobacteria *Melainabacteria,* which may have the capacity for nitrogen fixation (Rienzi *et al*., 2013). The dominance of ammonia-oxidising Archaea (AOA), chemolithoautotrophs and other nitrogen-cycling microbes in SSDF holothurians may offer a possible source of organic carbon and nitrogenous compounds for the host (Roberts *et al*., 2000). Taxa associated with nitrogen and putative carbon fixation indicates a potential functional role of these microbes, and the presence of these microbes in the intestines of SSDF holothurians may offer a nutritional and digestive advantage to these animals. Indeed, metabolites released by resident gut microflora are likely to provide a source of nutrients for the host, particularly important in a habitat where the main source of organic carbon is largely refractory (Deming & Colwell, 1982, Roberts *et al*., 2001).

4.5.3 Feeding strategies and nîche separation at PAP

Consistent with the feeding guilds identified by Billett (1991) and Iken *et al*., (2001), gut microbiome composition was positively correlated with holothurian phylogeny and feeding strategy. Differences in gut assemblage composition observed in this study appeared to be driven by the two feeding methods of deposit-feeding holothurians. Even within feeding guilds, there were observable interspecific differences. This is likely due to different digestive strategies and physiologies between holothurian species (Roberts *et al*., 2001). Foraging strategies and selective feeding may also have had an influence on interspecific differences in gut microbiome composition between holothurian species (Witbaard *et al*., 2001; Gao *et al*., 2014).

In the SDF group, *D. validum, O. setigera* and *P. longicauda* are mobile deposit-feeder species that target surface sediments for organic matter (Iken *et al*., 2001). The SDF holothurians have diverse gut microbiomes and an array of digestive enzymes capable of breaking down more refractory POM (Moore & Roberts, 1994; Roberts *et al*., 2001). With their increased mobility compared to SSDF holothurians, SDF holothurians such as *O. setigera*, target fresher sources of POM, evidenced by higher concentrations of chlorophyll α pigments in their gut sediments (Wigham *et al*., 2003; FitzGeorge-Balfour *et al*., 2010). A greater abundance of Verrucomicrobia were present in SDF microbiomes than in SSDFs (8.5% SDF, 4% SSDF), a phylum that have been linked to phytoplankton-derived protein, polysaccharide and POM degradation in the ocean (Cardman *et al*., 2014, Freitas *et al*., 2012). This greater abundance of Verrucomicrobia suggests that SDF holothurians are able to process fresher POM sources in addition to more refractory matter.

In contrast, *M. musculus*, *M. villosus* and *P. prouhoi* in the SSDF group are infaunal sediment feeders that either plough or burrow through the sediment, or remain largely immobile (Iken *et al*., 2001). They have a gut microflora and enzyme capabilities indicative of breaking down both bacterial membranes and refractory compounds (Roberts *et al*., 2001). By adopting a largely stationary lifestyle, SSDF holothurians expend little energy in foraging for fresher sources of POM and are relatively uncoupled from the seasonal supply of POM to the benthic

system, compared to other feeders (Iken *et al*., 2001). Infaunal feeders such as *M. musculus*, feed head down in the sediment 3-10 cm deep (Billett, 1991; Amaro *et al*., 2012). In contrast to other holothurians, gut sediments of *M. musculus* appear to lack in chlorophyll α pigments and contain only refractory chloropigments (Wigham *et al*., 2003). Incidentally, we observed that this holothurian species had a relatively high abundance of non-photosynthetic cyanobacteria *Melainabacteria* in their gut microbiomes. *Melainabacteria* were also more abundant in the 3-5 cm and 10-15 cm horizons of the ambient sediment, albeit at lower abundances than in *M. musculus* microbiomes, at a depth where *M. musculus* were likely feeding (Fig. 23, Fig. 27). This group of Cyanobacteria has been previously observed in mammalian gut microbiomes, providing a source of nutrients to the host via nitrogen fixation (Rienzi *et al*., 2013).

SSDF are able to exploit much more refractory or recycled organic material than SDF species, by utilising their enteric microbes to metabolise and release the energy from recycled material (Iken *et al*., 2001; Roberts *et al*., 2001). We observed a higher abundance of microbial taxa involved in carbon fixation and nitrogen cycling in SSDF gut microbiomes (Fig. 26), as well as taxa capable of utilising HMW-DOM compounds, such as lineages within the Planctomycetes phylum (14% SDF and 16.5% SSDF). It is therefore plausible that the SSDF gut microbiome confers an advantage to this group of holothurians by providing a source of nutrients and organic carbon independent of seasonal POM events.

Future studies should concentrate on increased sampling of the pelagic or mobile suspension feeders at PAP that form “Group A” from Iken *et al*., (2001), in order to understand the influence of feeding strategies and nîche separation on holothurian gut microbiome composition. Finally, as this was an amplicon study of 16S sequences, metabolic capabilities and functional information of microbial taxa in holothurian gut microbiomes should be treated with caution. In the future, metagenomics should be conducted to confirm the metabolic functions of these holothurian gut microbiome taxa, rather than inferring function from taxonomy, as we have done here.

4.6 Conclusion

Differences in holothurian gut microbiome composition appeared to be driven by feeding strategy and host phylogeny at PAP. We observed diverse gut microbiomes that differed between the two feeding strategies and interspecific differences within these two guilds. SSDF holothurians have gut microbiome taxa that are able to process more recalcitrant organic matter of less nutritional value and these microbiomes also contain a greater abundance of putative carbon-fixing and nitrogen cycling microbes. Available food quality and quantity varies spatially and temporally at PAP. Enteric microbes could provide a nutritional advantage for less mobile deposit-feeding holothurians, that feed on more refractory POM and are uncoupled from seasonal phytodetritus events. Holothurians form a dominant component of the megafauna in this benthic habitat and their feeding activity influences sediment biogeochemistry and functioning. Detailed analysis of NE Atlantic holothurian gut microbiomes will therefore offer another perspective on microbial community dynamics in this region and aid in understanding the relationship between benthic microbial biodiversity and ecosystem functioning.

5. Conclusion

5.1 Main findings

The overall aims of this thesis have been achieved, which were to investigate: (1) over what scales abyssal sediment communities vary, (2) the variation in distribution of nitrifiers (including AOA) in abyssal sediments at CCFZ, PAP and Station M, and how the input of carbon influences this functional group, and (3) how holothurian feeding activities influence sediment microbial structure and distribution. Collectively these studies demonstrate an original contribution to the field of deep-sea microbial ecology in a number of ways, the main findings of which are described below.

Chapter 2. The aim of this chapter was to characterise the diversity and biogeography of benthic archaea and bacteria in one of the marine protected areas of the CCFZ, which are adjacent to areas of possible future mining activities. Most previous studies of this region have focused on the potential impacts of disturbance on deep-sea fauna, and only a few studies have looked at microbial communities (Wu *et al*., 2013; Shulse *et al*., 2016; Lindh *et al*., 2017; Vonnahme *et al*., 2020; De Jonge *et al*., 2020; Wear *et al*., 2021). Significant reduction in microbial diversity and biogeochemical function is likely following deep-sea disturbance, with slow recovery rates over decades to return to undisturbed levels (De Jonge *et al*., 2020; Vonnahme *et al*., 2020). Removal of sediment and consequently labile organic matter during mining activities is likely to alter microbial assemblage structure and composition, and reduce rates of microbial activity, such as nutrient cycling (Vonnahme *et al*., 2020). Conducting a study of benthic microbial assemblages in undisturbed sites is therefore crucial in assessing the potential impacts to abyssal habitats and microbial biodiversity.

Sampling took place across an area of seabed that was spatially heterogenous, in order to understand how the characteristics of seafloor variability influence abyssal microbial biodiversity. Studies of microbial diversity in this region are few and spatially limited, in that they don't account for benthic topographic variability or in some cases, do not include the archaeal component of microbial communities (Lindh *et al*., 2017). This study contributed to baseline monitoring and

our knowledge of benthic microbial assemblages in one of these conservation areas, which will help aid in management decisions on preserving benthic ecosystems and detecting future impacts of potential disturbance from mining activities. This study will be an important contribution to other research which has aimed to characterise the spatial biodiversity of other biotic groups, such as meiofauna, macrofauna and megafauna (Glover *et al*., 2002; Veillette *et al*., 2007; Janssen *et al*., 2015; Amon *et al.*, 2016; DeSmet *et al*., 2017; Gollner *et al*., 2017; Leitner *et al*., 2017; Simon-Lledó *et al.*, 2019a,b; Laroche *et al*., 2020).

We found spatial variability and regional-scale biogeography in benthic microbial assemblages, a finding also highlighted by Wear *et al*., (2021) who found moderate spatial variability in CCFZ microbial assemblages. At APEI-6, we observed spatial differences in the structure and composition of sediment assemblages, across small scales (cm), through the sediment depth profiles, to mesoscales (m to km) between cores within topographical regions. Our results suggested that assemblages at the conservation area APEI-6 differed from those at the contract exploration site UK-1, supporting previous observations that have questioned the representativity of the no-mining, conservation sites compared to the contract sites (Amon *et al*., 2016; De Smet *et al*., 2017; Simon-Lledó *et al*., 2019b, 2020; Washburn *et al*., 2021a,b; Wear *et al*., 2021). We also noted a high abundance of nitrifiers, especially AOA *Nitrosopumilus*, in this region, which has implications for nitrogen cycling and inorganic carbon fixation in this habitat. As with the whole sediment microbial assemblage, we observed that nitrifying microbes also exhibited spatial variability associated with seafloor topography and depth through the sediment. This has repercussions for biogeochemical cycling in this commercially important region of the seabed.

Chapter 3. This chapter investigated how productivity regimes of overlying waters influence abyssal sediment communities. It has long been recognised that there is an apparent discrepancy with carbon supply and demand in the deep ocean, where the utilisation of organic matter, measured as benthic respiration rates exceed POM flux rates. It is therefore not clear what is sustaining highly diverse benthic abyssal communities, although some studies have indicated that

understanding benthic microbial assemblages is likely to be the key to unravelling this supply and demand mystery. In the absence of photosynthesis in the dark ocean, inorganic carbon fixation utilised by chemolithoautotrophic organisms may help supplement some of this demand by providing a source of organic carbon.

The aim of this study was to identify the spatial variability and distribution of sediment abyssal microbes between three abyssal sites that had varying productivity regimes. We found that a higher carbon input was more influential on assemblages with depth through the sediment than at sites with lower carbon flux. A higher abundance of nitrifiers and putative chemolithoautotrophic AOA was associated with a lower carbon input at the oligotrophic site. In a region where the input of food to abyssal ecosystems is limited, taxa such as nitrifiers and AOA may provide a source of organic carbon through inorganic carbon fixation. Ammonia-oxidising taxa dominate in sediments with low organic matter. Analysis revealed distinct clades of AOA *Nitrosopumilus* that showed geographic separation across the three regions. Although other studies have recognised distinct AOA Thaumarchaeota ecotypes, (Francis *et al*., 2005; Hallam *et al*., 2006; Sintes *et al*., 2016; Wang *et al*., 2017) knowledge of their distribution relating to productivity regime is limited. It is important to understand how environmental gradients influence the distribution patterns of functional taxa that form key components of microbial assemblages, such as nitrifiers and AOA (Wang *et al*., 2017). Not only will this shed light on links between distribution of nitrifying microbes and nitrogen cycling in global oceans, but will also be useful ahead of continuing climate change. Increased atmospheric CO_2 is predicted to increase primary productivity, altering flux patterns and quality of POC reaching the seabed, and therefore will influence benthic ecosystems (Smith *et al*., 2008b).

Chapter 4. This chapter investigated the gut microbiomes of six genera of holothurians which had two distinct feeding strategies in the North-East Atlantic. Holothurians dominate abyssal megafauna and their feeding activities may influence sediment microbial structure and distribution, benthic nutrient cycling and carbon processing. Despite the importance of their link with benthic communities, the effect of holothurian bioturbating activity on sediment microbes is poorly

understood. This study was based on feeding strategies and lifestyles identified by Billett (1991) and Iken *et al*., (2001), using stable isotope analysis ($\delta^{15}N$). Three deposit-feeding holothurian species were selected from each of the two feeding strategies. These strategies were mobile surface deposit feeders that fed on POM that had become mixed with the top layers of sediment, and sedentary subsurface deposit feeders that fed on buried, refractory POM.

We found that microbial community composition and diversity varied with habitat type, gut compartment and holothurian feeding strategy. Taxa related to carbon and nitrogen fixation and cycling were highly abundant in the holothurian gut microbiomes. In particular, ammonia-oxidising Archaea (Thaumarchaeota) and non-photosynthetic Cyanobacteria (*Melainabacteria)* were more prevalent in gut microbiomes of subsurface deposit feeders, indicating a link with feeding strategies and the microbiota of the sediments. Both Thaumarchaeota and *Melainabacteria* were also observed in the ambient sediment, with the latter more abundant in the deeper sediment layers. Overall, the results indicate that gut microbes in holothurians may offer a nutritional advantage to the host by breaking down refractory organic matter in a food limited environment, especially for holothurians with a largely stationary lifestyle. These animals do not seek out fresher sources of POM as other species, and feed largely on buried, recalcitrant POM buried in the sediment. It is possible that subsurface deposit-feeding holothurians are more reliant on their enteric microbes for nutrition than surface deposit-feeders.

Collectively, these chapters have achieved the overall aims of investigating and characterising spatial variability of benthic abyssal microbes, and in particular, the distribution of potential nitrifying taxa, which have important implications for biogeochemical cycling in these abyssal habitats. This spatial variability of taxa capable of chemolithoautotrophy was examined across a range of scales previously not described to this level of detail. These studies have indicated that abyssal sediments support highly diverse microbial assemblages and have provided evidence for microbial endemism. Taxa that is nîche-specific or regionally restricted has important implications for uncovering unique biodiversity and

metabolic potential, which could be lost before it has been discovered (Van Dover *et al*., 2018). Detailed observations are therefore necessary for modelling microbially mediated biogeochemical functions, monitoring biodiversity and ecological assessments for the preservation of ecosystem functioning ahead of potential seabed exploration and exploitation.

5.2 Limitations of the work

Recent and rapid advancements in genomic library preparation protocols, sequencing technologies and bioinformatics have enabled researchers to characterise the microbial communities from remote and under-sampled environments with ever-greater efficiency. Illumina DNA sequencing platforms are the most widely used and universal approach for genomic data (Gaulke *et al*., 2021). All three of the discussed studies utilised the 16S Illumina amplicon protocol from the Earth microbiome Project, to standardise approaches and for comparability with existing microbial studies (Thompson *et al*., 2017). It is generally recognised that, whilst this protocol is effective at revealing many bacterial and archaeal taxa (Eloe-Fadrosh *et al*., 2016), there are potential biases and limitations associated with PCR-based methods (Gilbert *et al*., 2014).

The amounts of DNA recovered from low biomass samples are often orders of magnitude lower than the minimum requirements of 1 ng of DNA required for library preparation methods (Bowers *et al*., 2015). This was the case in all three of the discussed studies and most samples required further concentration. One of the inherent issues with deep-sea microbial research is the difficulty amplifying genetic material in low biomass environments. It can therefore be problematic to obtain high quality reads without first concentrating samples or opting for additional cycles of PCR. As a result, there is a high potential for background contamination with low biomass samples. Methods to amplify low concentrations of DNA each have their own issues. Low template library preparation methods involve additional practical laboratory preparatory work which carries an increased risk of contamination (Bowers *et al*., 2015). There are currently no amplification methods without bias, however keeping contamination levels as low as possible may allow amplification of minimal allochthonous DNA inputs (Bowers *et al*., 2015).

So far, only a handful of studies have systematically tested the available library preparation protocols and methods across a range of DNA input concentrations (Bowers *et al*., 2015; Hirai *et al*., 2017; Gaulke *et al*., 2021). It is possible to create sequencing libraries from very low input DNA libraries such as 20 pg (Parkinson *et al*., 2012), with an absolute lower limit of 10 pg (Hirai *et al*., 2017). Low levels of template DNA and reduction in template quality, however, can lead to PCR stochasticity (Murray *et al*., 2015), an increased proportion of duplicate reads and overrepresentation of GC-rich sequences, which can lead to mismatches in base-calling (Benítez-Páez *et al*., 2016) especially if a greater number of PCR cycles are required to amplify such low inputs of DNA (Parkinson *et al*., 2012; Hammond *et al*., 2016). Ultimately this may generate a greater number of low-quality reads that cannot be mapped to reference genomes (Hammond *et al*., 2016). Pooling three PCR replicates per sample helps counteract PCR variability but increases chances of detecting rare taxa, however, this method is generally recommended for low template amounts (Alberdi *et al*., 2017)

Amplicon-based diversity estimates are also prone to other PCR biases, such as primer-template mismatches and taxon-specific amplification bias, both of which can alter estimations of taxa abundance (Fonseca *et al*., 2018). A PCR-free method was not possible or appropriate for our samples, however, due to low levels of template. With low biomass samples from the deep-sea, missing certain taxa such as eukaryotic or archaeal species, is a possible risk. In many cases there are a lower number of 18S rRNA gene copies in deep-sea sediment compared to 16S gene copies (Schippers *et al*., 2012). This has implications in abundance and diversity studies, by not correctly characterising the true diversity of the samples. Once the genetic material has been amplified, there are additional dangers of multiplexing, mistagging and contamination of sequences. Various methods have been tried previously, each with their own advantages and disadvantages. Kozich *et al*., (2013) utilised a dual-indexed version of the Earth Microbiome Project method, which is a single PCR method with long fusion primers. The single round of PCR reduced the risk of artefacts that were generated from multiple rounds of PCR. Unfortunately, this requires custom primers for this method and have only been tested on well characterised regions of

the 16S rRNA gene such as V4 (Kozich *et al*., 2013). The adaptability of this method to other genes or other gene regions is therefore unknown. There is also the risk that custom primer sequencing methods may introduce bias and organism dropout (Gohl *et al*., 2016). Multiplexing a large number of samples can lead to mistagging or sequence cross-contaminations, where up to 30% of sequences may develop undetectable mistags in sequence libraries (Esling *et al*., 2015). Tagging of both primers or double tagging using a set multiplexing design such as the Latin Square Design may resolve this problem (Esling *et al*., 2015), although this can be complex to set up initially. Altering or customising standardised protocols is generally unadvisable and can yield significantly reduced insert fragment lengths in addition to other errors (Baym *et al*., 2015).

Automating and increasing the efficiency of as much of the DNA extraction from samples and library preparation processes as possible has the potential to revolutionise this field (McQuillan & Robidart, 2017). Amplicon sequencing of the V4 region produces shorter reads of less than 300 base pairs, whereas alternative approaches can yield longer DNA reads provide better diversity estimates of microbial communities (Myer *et al.*, 2016). An alternative method to accurately characterise species diversity in a study is to sequence nearly full length 16S rRNA gene amplicons using nanopore technology, such as the MinION device, which is a nanopore sequencer (Benítez-Páez *et al*., 2016). This device is small, portable and can be operated from a computer, with results generated in real-time (Benítez-Páez *et al*., 2016; Benítez-Páez & Sanz, 2017). Although convenient for data analysis, such as during a research cruise, this device can only operate one sample plus one positive and one negative control at a time (Benítez-Páez & Sanz, 2017), rendering it inappropriate for large-scale multi-sample studies. In addition, the MinION device has a requirement for 600 ng of amplicon DNA (Benítez-Páez & Sanz, 2017), whereas the concentration of amplicon DNA in the majority of our samples was too low for this method. Whole genome assembly, however, would have been a useful addition to all three studies for characterising and identifying functional genes, such as for nitrification or ammonia oxidation, or for enzymes capable of breaking down recalcitrant matter.

Data normalisation methods were employed for all three studies, to account for uneven sample sequencing depth. In this case, samples represent a fraction of

the microbial community and the observed sequences are relative abundances. Whilst normalisation measures create even sample sizes and clarity of diversity patterns, they can introduce biases, such as the loss of reads which reduces any differences in diversity measures between samples (Mbarache *et al*., 2020). A PERMANOVA mixed-effect model was employed in all studies, which consisted of multiple factors. This single model was used to investigate the effect of the various interactions and tease apart the role of spatial scales from the environmental factors, to help explain the variation in microbial assemblage composition. PERMANOVA tests analyse more complex experimental designs than other tests of similarity such as ANOSIM, so were better suited to our datasets which had a number of replicates and levels. PERMANOVA is robust for balanced designs (Anderson & Walsh, 2013), which all three studies have, and is generally more powerful than Mantel tests (Anderson & Walsh, 2013). One downside of PERMANOVA is that it is not as robust as the similar test Adonis for testing both continuous and categorical variables, and is better suited for categorical variables (Erceg-Hurn, & Mirosevich, 2008). All three datasets did not have a normal distribution hence a non-parametric test was appropriate. In addition, all datasets contained lots of rare taxa, which produced tables of counts containing lots of zeros. Bray-Curtis dissimilarity measures were more appropriate for these datasets, as they were better equipped to deal with differences in large datasets that are populated by sparse taxa and many zeros (Ricotta & Podani, 2017).

We chose to use PERMANOVA to investigate the effect of environmental variables on the variation of the microbial community, rather than using distance-based redundancy analysis (dbRDA). PERMANOVA and dbRDA are similar in providing an approach for partitioning the variance in multidimensional space to test individual terms in a multifactorial analysis-of-variance model. dbRDA uses principal coordinate analysis on the similarity matrix (to decompose it into eigenvectors) and makes corrections for negative eigenvalues. The direct partitioning of the similarity matrix used in PERMANOVA avoids this by partitioning directly from the distance matrix itself with no corrections and no eigen analysis (McArdle & Anderson, 2001). Euclidean distances, on the other hand, can lead to disagreements in species-wise differences between sites, whereby differences in species abundances between sites overrides differences or similarities in the types

of species present, and therefore may not measure community composition in an ecologically significant way (Ricotta & Podani, 2017).

Conducting Mantel tests based on Pearson's coefficient is one method that can test for the effect of horizontal spatial distance while controlling for the effect of the other factor in question. This method is widely used in microbiome studies for measuring the correlation between interspecific dissimilarities and the hosts' phylogeny, to produce an R value. This method would also have been appropriate for assessing the influence of environmental gradients of POC upon microbial assemblages in chapter 3. Mantel tests, however, have long been acknowledged to be potentially problematic when analysing beta diversity or spatial variation in species composition (Dutilleul *et al.* 2000; Legendre 2000; Raufaste & Rousset 2001; Rousset, 2002; Legendre *et al.,* 2005). Mantel tests utilise variation partitioning on distance matrices, however, these matrices have been unravelled, where distance values are treated independently as a single vector in regression models (Anderson, 2017). Therefore, they do not measure the variance of community composition or beta diversity accurately, by generally underestimating the amount of variation within a community (Legendre *et al*., 2005). PERMANOVA, on the other hand, is not affected by these problems. This test partitions spatial variation by canonical ordination, where pairwise dissimilarities from a dissimilarity matrix are maintained between samples. Canonical ordination explains a greater amount of the total variation, by retaining the structure of the distance/dissimilarity matrix and not treating the values in the matrix as independent of one another. It is therefore regarded as being one of the best methods to partition and analyse the actual variability in multivariate data clouds (Legendre *et al*., 2005; Anderson, 2017). It is for this reason that PERMANOVA rather than Mantel tests were chosen to analyse variation in species composition among spatially separated sites or areas in all three studies.

<u>Chapter 2:</u>

Instead of following the recommended EMP protocol for DNA extraction, we chose to utilise the same DNA extraction methodology as other deep-sea microbial studies, to ensure comparability (Wu *et al*., 2013; Shulse *et al*., 2016; Lindh *et al*., 2017; Cleary *et al*., 2019; Bergo *et al*., 2020). In contrast to other studies (Shulse

et al., 2016; Lindh *et al*., 2017), we opted to use the FastDNA Spin Kit for Soil for processing both sediment and nodule samples, as these yielded consistent levels of DNA. These kits are widely used, hence making them more comparable to other studies and were also quicker to process than other column-based kits. This increased efficiency, by reducing a processing bottleneck, meant that a large number of samples could be processed. The sediment samples covered numerous replicates over a range of scales from fine (cm), to moderate (metres to km), to large scale (100s km) and included nodule samples. Sampling took place in areas of the seabed with a benthic heterogeneity similar to UK-1 (Simon-Lledó *et al*., 2019a) and followed a balanced sampling design, with at least three replicates per topographical region of the seabed and per sediment horizon.

Sampling took place within one of the APEIs, which was designed to be representative of exploration contract areas based on the topography of the seafloor (Wedding *et al*., 2013; Simon-Lledó *et al*., 2019a). This study considered the variability of seabed topography in the sampling design in order to explore spatial patterns in benthic microbes. Topography directly affects environmental conditions such as food supply, sediment grain size and bottom currents, factors likely to influence the distribution of benthic microbes (Durden *et al*., 2015; Morris *et al*., 2016). These factors have not been assessed at a landscape scale, particularly in the context of assessing environmental controls on spatial patterns of microbial communities. This topographic heterogeneity is useful for examining microbial biogeography and measuring potential connectivity between microbial populations. Such information is therefore an important component of baseline environmental assessment for commercial mining activities and subsequent monitoring (Ingels *et al*., 2020), particularly as benthic microbes represent an important energy and nutrient source for abyssal ecosystems. Although polymetallic nodule mining would most likely take place on ‘Flat’ and ‘Deep Plain’ areas of the seabed, where the slope gradient is less than 2 º and therefore more suitable for mining vehicles (Jones *et al*., 2017), assessment of ‘Trough’ and ‘Ridge’ areas is still useful.

Despite a number of replicates that covered seafloor variability in the APEI-6 region, only one full megacore was deployed in the UK-1 site, hence replicates for UK-1 were not possible. This prevented statistical analysis and ecological

significance comparisons drawn between the conservation area APEI-6 and exploration contract area UK-1. Although water samples were available from a transect through the water column, these were not processed or included as some of the samples from the water transect were missing. As a result, there was an inadequate number of replicates. Although included, the low number of nodule samples lacked enough statistical power to draw significant ecological conclusions. In addition, the nodule samples were only sampled from two of the four topographical regions of APEI-6 limiting interpretations regarding spatial variability.

Copies of the bacterial/archaeal 16S rRNA gene, or other important functional genes, such as the ammonia monooxygenase subunit A (*amoA*) (Francis *et al*., 2005) and nitrite oxidoreductase (*Nxr*) gene sequences (Sorokin *et al.*, 2012), were not quantified in this study using qPCR. This would have provided a valuable insight into the possible metabolic potential of benthic assemblages in the CCFZ and helped confirm the functional groups such as the nitrifiers. Quantifying genes would also help assess the abundances of these taxa more accurately (Jiang *et al*., 2008).

During sample processing, only DNA was targeted in sediment and nodule samples, due to methodological issues associated with RNA extraction. Extraction kits that had previously been recommended as part of the EMP protocol that included the use of Phenol:Chloroform:Isoamyl alcohol produced consistently poor results. The Phenol:Chloroform:Isoamyl alcohol, or PCI approach, is reported to yield greater copy numbers of eDNA (Renshaw *et al*, 2015), hence making these kits ideal for low biomass samples. Following extraction of eDNA from abyssal sediments using these PCI kits, DNA and RNA concentrations measured with the Qubit system were either below detectable limits or were very variable within one horizon of the same core. Extractions previously performed on the same sediment samples by another member of the research team yielded Qubit measurements that were consistent with the original measurements that had taken for these samples. This indicated that it was unlikely to be a problem with the Qubit readings and instead suggested a methodological error, such as loss of the pellet during extraction or from RNAses.

This biodiversity assessment of benthic microbial assemblages focused on the 16S rRNA gene and neglected the 18S Eukaryotes, mainly due to cost and time restraints. This omission is regrettable, as the community diversity and spatial patterns of benthic abyssal microbial eukaryotes is much less known compared to bacteria and archaea. Microeukaryotic diversity is therefore often under-represented in molecular surveys (Medinger *et al*., 2010). Existing studies have indicated that microeukaryote diversity showed large scale patterns of high phylogenetic diversity, including parasitic groups, in global abyssal plains (Scheckenbach *et al*., 2010; Pasulka *et al*., 2016; Shulse *et al*., 2016). Importantly, the microeukaryote diversity patterns in this study were distinctly different from bacteria, archaea and macrofauna, highlighting unseen complexity in benthic microeukaryotes (Pasulka *et al*., 2016). Excluding eukaryotes from baseline surveys misses an important component of benthic microbial diversity and current eukaryotic biodiversity estimates are likely to be vast underestimates (Medinger et al., 2010; Pasulka *et al*., 2016).

Chapter 3:

Chapter 3 exhibited a well-balanced sampling design, with an even number of samples from three locations of abyssal habitats, each with differing productivity regimes. This sampling design covered a range of samples with multiple replicates of sediment samples and ensured that sediment horizon intervals matched between the three sites. The design also covered a range of scales, from fine (cm) to moderate (m-km) to basin scale (1000s km). The sampling locations were all geographically separate from one another, in order to visualise potential spatial patterns. In addition, as two of the sites are long-term monitoring stations (PAP and Station M) and the third location is a site of high scientific interest (CCFZ), the environmental conditions have been well characterised previously. As a result, sediment microbial assemblages were comprehensively characterised at each site.

Although well characterised, a lack of geochemical data to accompany this dataset has limited the ability to draw significant conclusions about the influence of environmental drivers upon sediment microbial assemblages. The addition of water samples from each location, particularly the lowest 100m from the seabed

would have been a valuable addition to this dataset, helping to set the scene for the overall conditions of these abyssal habitats. A transect through the water column may have provided a valuable picture of the microbial assemblages within three differing productivity regimes. Including the lower water column may have offered an insight into possible microbial interactions between water communities and sediment communities (Hamdan *et al*., 2013; Shulse *et al*., 2016).

As with the previous chapter, including important functional genes, such as *amoA* and *Nxr* gene sequences would have allowed more robust conclusions to be drawn about the diversity and distribution of nitrifying microbes within the dataset. Using only one region for examining geographic distributions of *Nitrosopumilus* ASVs, rather than adopting a multi-region approach, or using other markers, may not have provided enough taxonomic precision, since the 16S rRNA gene is highly conserved. Although the V4 region is hypervariable and the most reliable for allowing the differentiation of taxa, using a combination of regions, such as V4-V6 is recommended for greater phylogenetic resolution (Yang et al., 2016). Characterising the distribution and diversity of ammonia-oxidising archaea by analysing the ammonia monooxygenase gene (ammonia monooxygenase alpha subunit (*amoA*) and the 16S-23S rRNA intergenic spacer (ITS) region may have been more appropriate (Park *et al*., 2014). A valuable addition to this study would have been to include the 18S rRNA gene to analyse the distribution of eukaryotes. The role of microeukaryotes, in particular heterotrophic protists and fungi, in response to environmental variability remains poorly characterised (Pasulka *et al*., 2016; Zhao *et al*., 2020). In addition, reporting on relative abundances of various microbial taxa with amplicon-based metabarcoding is limited as abundances are only coarse estimates. Read numbers per individual taxa vary, owing to copy number variation of the target loci (Elbrecht & Leese, 2015), so relative abundances via this method should be treated with caution.

Chapter 4:

This dataset comprised of a balanced design that included both a reasonable coverage and equal number of holothurian species within both feeding guilds. Both fore- and hindgut samples within each species were taken for comparison purposes, including whole gut transects, which are unusual in holothurian

microbiome studies. In contrast to existing holothurian gut microbiome studies (Amaro *et al*., 2009, 2012; Pagán-Jiménez *et al*., 2019; Cleary *et al*., 2019), a large number of samples were processed, including at least three replicates from each species.

A potential downside of this study is that holothurian samples were taken from only from one geographic region. Utilising holothurian samples from another location would have added a geographic component to this study, a comparison that is yet to be replicated in other studies. Including holothurians from an oligotrophic region, such as the CCFZ, would have been a useful comparison with those from PAP that experience seasonal fluxes in POM. In terms of the samples themselves, the water samples taken for this study were limited and included only one sample per depth of the water column transect. The exception was one of the deeper water samples, 4750 m, where three replicates were taken. There was only one replicate for the deepest water layer, 4830 m, which arguably would have been the more appropriate water sample to include, given that the seafloor was between 4839-4843 m in depth where the holothurians were extracted. Ambient sediment samples used as a comparison in this study were sampled from a different year to the holothurian samples. Although sediment samples were taken at the time of the holothurian samples, funding issues prevented these from being processed so sediment samples from an earlier cruise but from the same area were used instead. This study did not include any holothurians from group A (pelagic or highly mobile species) of the Iken *et al*., (2001) study which would have made this study more complete. This was dependent upon the individuals that were sampled in the two trawls and as only two individuals from group A were retrieved, these were excluded from the study due to insufficient replication.

A valuable addition to this study would have been to include functional information for the holothurian enteric microbes. This was not possible with amplicon sequencing, however, metagenomic sequencing for genome assembly would have been appropriate. This may have revealed important metabolic pathways such as recalcitrant matter breakdown, enzymatic diversity and the *amoA* gene, given the abundance of ammonia-oxidising archaea. Identifying the metabolisms of predominant taxa would have further confirmed the importance of enteric microbes and their role in holothurian nutrition and feeding strategies.

5.3 Future research

Following on from this critical review of the aforementioned studies, there are a number of recommendations that should be implemented as a result of findings from these works. Firstly, collaborative efforts to characterise benthic microbial assemblages is strongly encouraged. Researchers should ideally follow a protocol, from standardised sampling from field collection to DNA sequencing for long-term monitoring and comparative purposes. Following a protocol and using the same primers, such as EMP (Walters *et al*., 2015), would be beneficial to the scientific community, unless new and improved primers that offer greater taxonomic resolution are available in the future (Wear *et al*., 2021). Environmental data and extracted DNA should be archived as raw, unprocessed reads. This means that data can be re-processed later on, despite future updates and changes to bioinformatic processing pipelines. Data should also be easily accessible to enable future studies to use this data, especially in the case of regions were baseline environmental assessments would be useful.

Efforts to characterise all APEIs in the CCFZ should be prioritised to ensure that these conservation areas are truly representative of the exploration contract areas. Researchers have noted a south-to-north and an east-to-west gradient in sediment microbial assemblages from this region (Wear *et al*., 2021). Many environmental parameters appear to vary across this region of the CCFZ, including nodule abundance (Amon *et al*., 2016; De Smet *et al*., 2017; Simon-Lledó *et al*., 2019b, 2020; Washburn *et al*., 2021a) and POC flux, which follows south-to-north and east-to-west gradients (Wedding *et al*., 2013; Wear *et al*., 2021; Washburn *et al*., 2021b), implying that the surrounding APEIs may not be truly representative environments of the exploration contract areas of seabed they surround.

A meta-analysis study by Wear *et al.* (2021) also indicated that nodule communities differed by cruise they were sampled from than by geographic location. This may reflect different methodologies that have been implemented during extraction and processing by various research groups. In particular, this highlights the need for a standardised sampling protocol that would help eliminate and rule out variability resulting from different processing approaches. Research

detailed in Chapter 2 suggested that there was a potential spatial variability to microbial assemblages associated with nodules, depending on the topographical region that they were found. As nodule samples were limited to just two topographical regions in our study, this should be extended to all four regions (Deep Plain, Flat, Ridge and Trough) to further explore this phenomenon. In addition, future research should concentrate on linking different microbial diversity and genetic analysis datasets, to assess and better understand connectivity and biodiversity at CCFZ.

Despite sediments at CCFZ that have been reported to be oxygenated down to 2-3 m depth (Haeckel *et al*., 2001; Mewes *et al*., 2014; Volz *et al*., 2018), we observed taxa that are usually found in more anoxic environments. The presence of anaerobic microbes that are usually found in suboxic conditions have also been reported in otherwise oligotrophic sediments in the same region (Shulse *et al*., 2016; Lindh *et al,* 2017). As there are currently no cultivated members of these apparently anaerobic taxa, it is difficult to determine metabolic information or their ecological roles within this habitat. Future research should focus on identifying these cryptic taxa, to discern if these are facultative processes or whether suboxic nîches exist at CCFZ.

Finally, studying holothurian microbiomes from range of abyssal locations with varying productivity regimes would enable researchers to explore the relationship between food availability and enteric microbes of holothurians. This would be a benefit to marine microbiome studies by offering another perspective on the relationship between benthic microbial biodiversity, community dynamics and ecosystem functioning.

5.4 Implications for our understanding of abyssal microbial ecology

The combined results from Chapters 2, 3 and 4 significantly expand on the current understanding of microbial ecology and biogeography of benthic abyssal microbes at a high spatial resolution, including a marine protected area in the CCFZ. These chapters have explored underlying patterns in the distribution of microbes in sediments, polymetallic nodules, holothurian gut and the water column, linked to environmental variables such as oxygen and organic carbon availability. These results have also highlighted some interesting findings surrounding the spatial variability and composition of abyssal microbial assemblages. Ammonia-oxidising Archaea (Thaumarchaeota) and nitrogen-cycling microbes were highly abundant in oligotrophic sediments and holothurian gut microbiomes. In particular, these AOA and nitrifiers exhibited a spatial variability across small to large geographic scales, indicative of nîche diversity and resource partitioning.

As discussed in section 1.8 of the introduction, the results addressed the following areas in Chapters 2 - 4: (1) Benthic microbial community composition varies from small cm scale, across mesoscales (100s of metres) and large geographic scales (100s of km), correlated with oxygen penetration of the sediments, primary productivity in the overlying waters and to a lesser extent, the broad landscape-scale features of the seabed, (2) Putative nitrifying and AOA taxa were highly abundant in abyssal sediments and distinct populations of AOA *Nitrosopumilus* were observed between the three geographically separated locations, correlated with carbon input, and (3) Mobile deposit-feeding holothurian gut microbiomes were dominated by particle colonising and POM-degrading taxa, whereas the gut microbiomes of sedentary subsurface feeders contained higher abundances of taxa implicated in high molecular weight (HMW) compound processing, ammonia-oxidation and potential nitrifying taxa.

Variations in microbial diversity, distribution and composition are driven by biogeochemistry gradients, in particular, oxygen penetration and carbon input. These studies have uncovered the potential of various nîches for microbes, which may impact on POM utilisation, recalcitrant substrate breakdown and the role of chemolithoautotrophy in the dark ocean. The spatial variability of nitrifying microbes has repercussions for biogeochemical cycling, particularly in

commercially important regions of the seabed. Not only will this shed light on links between distribution of nitrifying microbes and nitrogen cycling in global oceans, but will also be useful ahead of continuing climate change, predicting the impact of altering flux patterns and quality of POC reaching the seabed. The influence of bioturbating invertebrates and the repeated enrichment of microbes in the sediments through faecal deposition may modify and transform ambient sediment communities. As holothurians form a dominant component of the megafauna in abyssal habitats globally, their feeding activity will influence sediment benthic nutrient cycling, carbon processing, and organic matter mineralization. Better constraining these processes is critical in our understanding of the influence of holothurian feeding activity on sediment biogeochemistry and how these changes may significantly alter ecosystem function. Microbes are fundamental for ecosystem functioning and biogeochemical cycling in the deep ocean, so further characterising the diversity, distribution and function of these assemblages should be a future priority and warrants further investigation.

Appendix

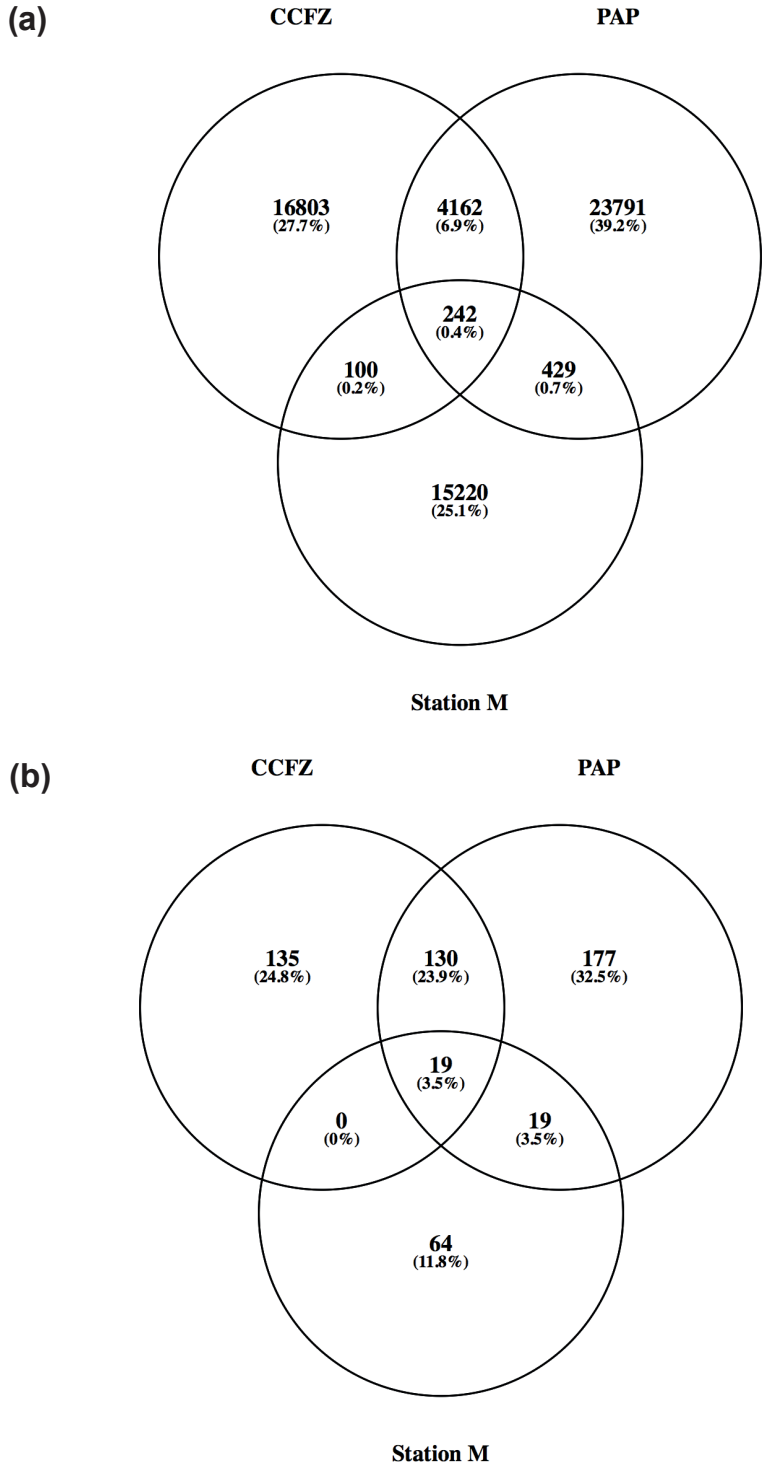

Figure S1. Microbial assemblage taxa surveyed in this study, visualised as Venn diagrams, showing, (a) the total number of sediment microbial taxa in the form of amplicon sequencing variants (ASVs) shared between CCFZ, PAP and Station M, and (b) the total number of *Nitrosopumilus* ASVs shared between CCFZ, PAP and Station M. Numbers and percentages represent a proportion of the total number of ASVs in the dataset.

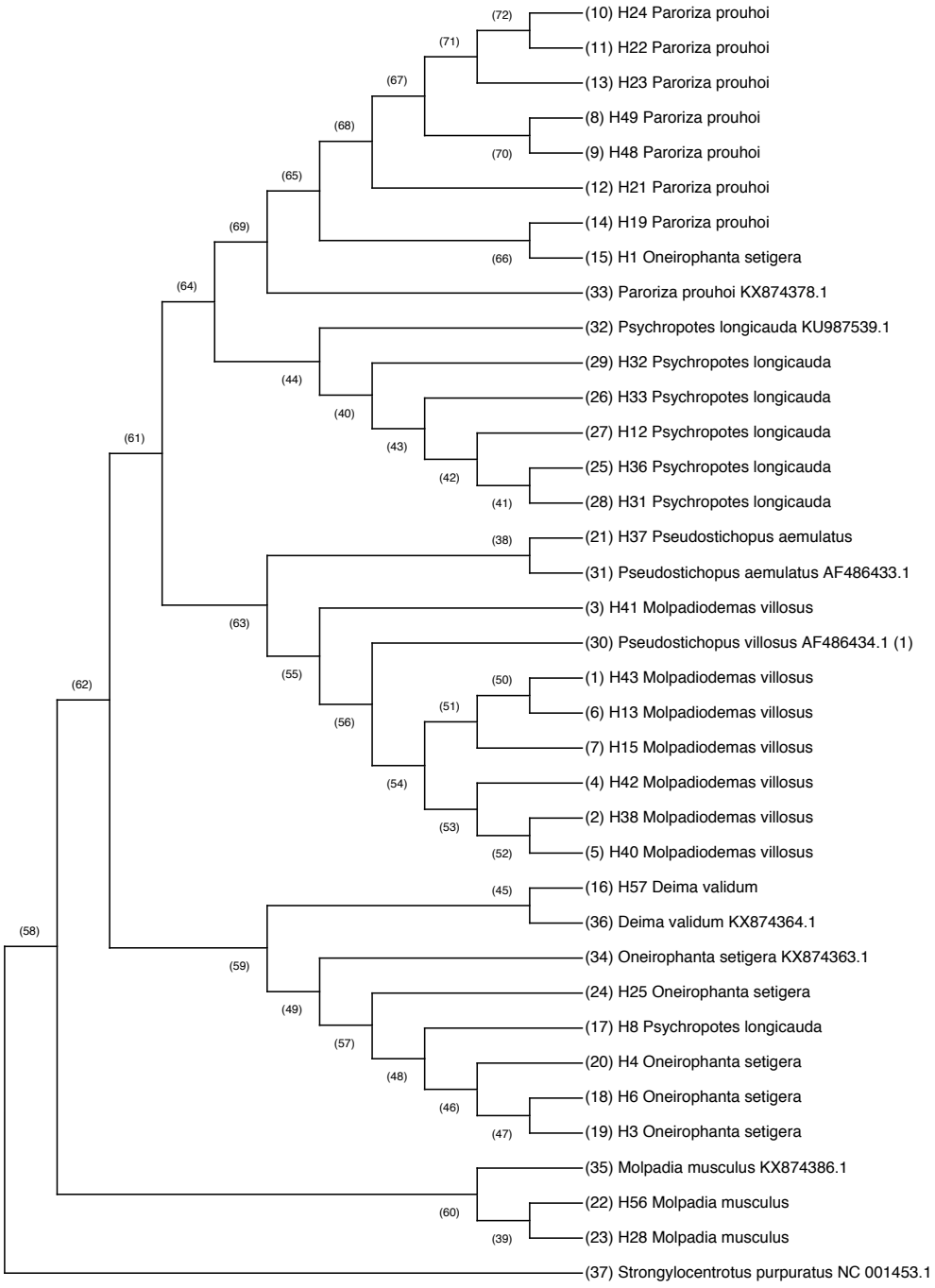

Figure S2. Maximum Likelihood tree based on the CO1 sequences of host holothurians at PAP. Host samples from the study are denoted with "H". Sequences retrieved from GenBank are shown with host name and GenBank accession number. Bootstrap values are given next to the nodes. Additional *Pseudostichopus aemulatus* host samples and GenBank sequences were added to the study samples for consensus.

Table S1. Most abundant taxa associated with polymetallic nodules sampled from the top cm of sediment in the "Deep plain" region of APEI-6. Taxa forming ≥ 1% average relative abundances (% of total sequences) are given along with standard deviation (SD).

Taxa	**Deep plain nodules**						
Domain	**Phyla**	**Class**	**Order**	**Family**	**Genera**	**Average relative abundance (%)**	**± SD**
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Candidatus Nitrosopumilus	10.02	0.378
Bacteria	Proteobacteria	Gamma-proteobacteria	Thiohalorhabdales	Thiohalorhabdaceae	uncultured	5.01	0.939
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodovibrionales	Kiloniellaceae	uncultured	3.76	0.556
Bacteria	Bacteroidetes	Rhodothermia	Rhodothermales	Rhodothermaceae	uncultured	3.23	0.464
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	Urania-1B-19 marine sediment group	2.96	0.661
Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	2.83	0.220
Bacteria	Proteobacteria	Betaproteobacteria	Betaproteobacteriales	Nitrosomonadaceae	Nitrosomonas	2.75	0.241
Bacteria	Proteobacteria	Gamma-proteobacteria	Arenicellales	Arenicellaceae	uncultured	2.38	0.766
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	bacteriap25	uncultured bacterium	1.95	0.403
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured Nitrosopumilales archaeon	1.87	0.371
Bacteria	Bacteroidetes	Bacteroidia	Cytophagales	Cyclobacteriaceae	uncultured	1.65	0.162
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Cohaesibacter	1.64	0.519

Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	AqS1	1.64	0.473
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodovibrionales	Kiloniellaceae	Pelagibius	1.59	0.070
Bacteria	Planctomycetes	OM190	uncultured bacterium	uncultured bacterium	uncultured bacterium	1.54	0.386
Bacteria	Proteobacteria	Gamma-proteobacteria	Steroidobacterales	Woeseiaceae	Woeseia	1.36	0.464
Bacteria	Proteobacteria	Gamma-proteobacteria	KI89A clade	uncultured deep-sea bacterium	uncultured deep-sea bacterium	1.33	0.241
Bacteria	Acidobacteria	Thermoanaerobaculia	Thermo-anaerobaculales	Thermo-anaerobaculaceae	Subgroup 10	1.29	0.042
Bacteria	Calditrichaeota	Calditrichia	Calditrichales	Calditrichaceae	JdFR-76	1.27	0.106
Bacteria	Gemma-timonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	uncultured bacterium	1.25	0.286
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Magnetospiraceae	uncultured	1.24	0.428
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	uncultured	1.18	0.381
Bacteria	Chloroflexi	Dehalococcoidia	SAR202 clade	uncultured deep-sea bacterium	uncultured deep-sea bacterium	1.12	0.359
Bacteria	Nitrospinae	Nitrospinia	Nitrospinales	Nitrospinaceae	Nitrospina	1.12	0.340
Bacteria	Planctomycetes	OM190	uncultured planctomycete	uncultured planctomycete	uncultured planctomycete	1.09	0.285
Bacteria	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula	1.07	0.279
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	bacteriap25	uncultured deep-sea bacterium	1.07	0.322
Bacteria	Nitrospinae	Nitrospinia	Nitrospinales	Nitrospinaceae	LS-NOB	1.04	0.144
Bacteria	Bacteroidetes	Bacteroidia	Chitinophagales	Saprospiraceae	Portibacter	1.02	0.141

Table S2. Most abundant taxa associated with polymetallic nodules sampled from the top cm of sediment in the "Flat" region of APEI-6. Taxa forming ≥ 1% average relative abundances (% of total sequences) are given along with standard deviation (SD).

Taxa	**Flat nodules**						
Domain	**Phyla**	**Class**	**Order**	**Family**	**Genera**	**Average relative abundance (%)**	**± SD**
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Candidatus Nitrosopumilus	5.97	0.243
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodovibrionales	Kiloniellaceae	uncultured	4.39	0.119
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	Urania-1B-19 marine sediment group	3.48	0.165
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured Nitrosopumilales archaeon	2.73	0.256
Bacteria	Proteobacteria	Gamma-proteobacteria	Steroidobacterales	Woeseiaceae	Woeseia	2.28	0.112
Bacteria	Bacteroidetes	Bacteroidia	Cytophagales	Cyclobacteriaceae	uncultured	2.17	0.056
Bacteria	Proteobacteria	Gamma-proteobacteria	Thiohalorhabdales	Thiohalorhabdaceae	uncultured	2.17	0.098
Bacteria	Bacteroidetes	Rhodothermia	Rhodothermales	Rhodothermaceae	uncultured	1.96	0.123
Bacteria	Nitrospinae	Nitrospinia	Nitrospinales	Nitrospinaceae	Nitrospina	1.93	0.067
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Magnetospiraceae	uncultured	1.88	0.077
Bacteria	Planctomycetes	OM190	uncultured deep-sea bacterium	uncultured deep-sea bacterium	uncultured deep-sea bacterium	1.75	0.072
Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	1.69	0.087
Bacteria	Acidobacteria	Thermo-anaerobaculia	Thermo-anaerobaculales	Thermoanaerobaculacea e	Subgroup 10	1.68	0.072

Bacteria	Chloroflexi	Dehalococcoidia	SAR202 clade	uncultured deep-sea bacterium	uncultured deep-sea bacterium	1.68	0.129
Bacteria	Proteobacteria	Betaproteobacteria	Betaproteobacteriales	Nitrosomonadaceae	Nitrosomonas	1.58	0.045
Bacteria	Planctomycetes	OM190	uncultured planctomycete	uncultured planctomycete	uncultured planctomycete	1.48	0.084
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	uncultured	1.13	0.105
Bacteria	Planctomycetes	OM190	uncultured bacterium	uncultured bacterium	uncultured bacterium	1.11	0.061
Bacteria	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Aquibacter	1.08	0.042
Bacteria	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Pir4 lineage	1.06	0.061

Table S3. Pair-wise *posthoc* tests for fixed term 'Sediment Horizon' for sediment microbial assemblages at APEI-6, showing the following: all 16S microbial assemblages; putative carbon-fixing and nitrifying taxa within sediment microbial assemblages; and taxa within the Nitrososphaeria class in the Thaumarchaeota phylum.

All 16 S microbial sediment assemblages			**Putative carbon-fixing and nitrifying taxa**			**Nitrososphaeria taxa (Thaumarchaeota)**		
Comparison			**Comparison**			**Comparison**		
Sediment Horizon	**t**	**P**	**Sediment Horizon**	**t**	**P**	**Sediment Horizon**	**t**	**P**
0-1 cm vs 1-2 cm	1.9176	0.001	0-1 cm vs 1-2 cm	1.7476	0.03	0-1 cm vs 1-2 cm	2.9343	0.002
0-1 cm vs 5-6 cm	2.2514	0.001	0-1 cm vs 5-6 cm	3.0346	0.001	0-1 cm vs 5-6 cm	3.7261	0.001
0-1 cm vs 10-12 cm	2.7428	0.001	0-1 cm vs 10-12 cm	3.4754	0.004	0-1 cm vs 10-12 cm	3.9221	0.001
0-1 cm vs 20-22 cm	3.6132	0.001	0-1 cm vs 20-22 cm	5.5264	0.001	0-1 cm vs 20-22 cm	5.2468	0.001
1-2 cm vs 5-6 cm	1.3565	0.04	1-2 cm vs 5-6 cm	1.5577	0.114	1-2 cm vs 5-6 cm	1.91	0.012
1-2 cm vs 10-12 cm	2.0021	0.001	1-2 cm vs 10-12 cm	3.0641	0.006	1-2 cm vs 10-12 cm	2.7454	0.001
1-2 cm vs 20-22 cm	2.8739	0.001	1-2 cm vs 20-22 cm	4.9146	0.001	1-2 cm vs 20-22 cm	4.7888	0.001
5-6 cm vs 10-12 cm	1.6357	0.002	5-6 cm vs 10-12 cm	2.2046	0.024	5-6 cm vs 10-12 cm	3.0359	0.001
5-6 cm vs 20-22 cm	2.5187	0.001	5-6 cm vs 20-22 cm	4.2743	0.001	5-6 cm vs 20-22 cm	4.3469	0.001
10-12 cm vs 20-22 cm	1.6815	0.004	10-12 cm vs 20-22 cm	3.2685	0.007	10-12 cm vs 20-22 cm	2.6297	0.002

Table S4. SIMPER results (Similarity Percentages with species contributions) of one-way analysis based on Bray-Curtis distances with fourth-root transformed abundance data.

Groups CCFZ & PAP

Average dissimilarity = 48.30

	Group 1 CCFZ	Group 2 PAP				
Genus (Family or Order, Phylum)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum. %
Melainabacteria group bacterium S15B-MN24 CBMW_12 (Cyanobacteria)	0.56	1.67	0.19	1.07	0.4	0.4
Uncultured crenarchaeote (Nitrosopumilaceae, Thaumarchaeota)	2.54	1.35	0.16	1.19	0.33	0.73
Uncultured gamma proteobacterium (BD2-7)	0.69	1.72	0.15	1.33	0.31	1.04
Uncultured deep-sea bacterium (EPR3968-O8a-Bc78, Gammaproteobacteria)	2.11	0.97	0.14	1.23	0.3	1.34
Ralstonia (Burkholderiaceae, Betaproteobacteria)	0.82	1.68	0.14	1.28	0.3	1.63
Uncultured Phyllobacteriaceae bacterium (Alphaproteobacteria)	2.12	1.17	0.14	1.15	0.29	1.93
Uncultured deep-sea bacterium (Zixibacteria)	1.68	0.5	0.14	1.59	0.29	2.22
Salinispirillum (Oceanospirillales, Gammaproteobacteria)	1.62	0.54	0.14	1.47	0.29	2.51
Uncultured (Arenicellaceae, Gammaproteobacteria)	1.66	0.87	0.14	1.25	0.29	2.81
Uncultured gamma proteobacterium (SZB50)	2.02	0.89	0.14	1.29	0.29	3.1

Groups PAP & Station M

Average dissimilarity = 70.27

	Group 1 PAP	Group 2 Station M				
Genus	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum. %
Candidatus Scalindua sp. (Planctomycetes)	0.06	2.57	0.3	1.9	0.42	0.42
Uncultured sediment archaeon (Nitrosopumilaceae, Thaumarchaeota)	2.48	0	0.29	2.2	0.41	0.83
Uncultured Planctomyces sp. (SG8-4, Planctomycetes)	0.1	2.35	0.26	2.07	0.37	1.2
Uncultured archaeon CRA7-0cm (Nitrosopumilaceae, Thaumarchaeota)	2.15	0	0.24	1.95	0.35	1.55
Brassica napus (Nitrosopumilaceae, Thaumarchaeota)	2.11	0	0.24	2	0.35	1.89
Uncultured sediment bacterium (Dehalococcoidia, Chloroflexi)	2.09	0.07	0.24	2.18	0.34	2.23
Uncultured bacterium (Alphaproteobacteria)	2.22	0.23	0.24	2.02	0.33	2.57
Uncultured Cenarchaeum sp. (Nitrosopumilaceae, Thaumarchaeota)	2.06	0	0.23	1.81	0.33	2.9
wb1-A12 (Methylomirabilaceae, NC10)	2	0	0.23	2.38	0.33	3.23
Uncultured bacterium (Schekmanbacteria)	2.03	0.12	0.22	1.83	0.32	3.55

Groups CCFZ & Station M

Average dissimilarity = 70.73

	Group 1 CCFZ	Group 2 Station M				
Genus (Family or Order, Phylum)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum. %
Uncultured Cenarchaeum sp. (Nitrosopumilaceae, Thaumarchaeota)	2.84	0	0.33	3.85	0.47	0.47
Brassica napus (Nitrosopumilaceae, Thaumarchaeota)	2.79	0	0.33	3.92	0.46	0.93
Candidatus Scalindua sp. (Planctomycetes)	0	2.57	0.31	2.06	0.43	1.37
Uncultured sediment archaeon (Nitrosopumilaceae, Thaumarchaeota)	2.57	0	0.3	5.72	0.43	1.8
Uncultured crenarchaeote (Nitrosopumilaceae, Thaumarchaeota)	2.54	0	0.3	3.77	0.42	2.22
Uncultured archaeon W4-93a (Nitrosopumilaceae, Thaumarchaeota)	2.51	0	0.3	2.79	0.42	2.64
Uncultured bacterium (Alphaproteobacteria)	2.73	0.23	0.29	3.12	0.42	3.05
wb1-A12 (Methylomirabilaceae, NC10)	2.49	0	0.29	4.43	0.41	3.47
Uncultured sediment bacterium (Dehalococcoidia, Chloroflexi)	2.44	0.07	0.28	5.02	0.39	3.86
Uncultured Planctomyces sp. (SG8-4, Planctomycetes)	0	2.35	0.27	2.33	0.38	4.24

Groups CCFZ 0-5 cm & CCFZ 5-10 cm

Average dissimilarity = 70.73

	Group 1 CCFZ	Group 2 Station M				
Genus (Family or Order, Phylum)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum. %
Uncultured Cenarchaeum sp. (Nitrosopumilaceae, Thaumarchaeota)	2.84	0	0.33	3.85	0.47	0.47
Brassica napus (Nitrosopumilaceae, Thaumarchaeota)	2.79	0	0.33	3.92	0.46	0.93
Candidatus Scalindua sp. (Planctomycetes)	0	2.57	0.31	2.06	0.43	1.37
Uncultured sediment archaeon (Nitrosopumilaceae, Thaumarchaeota)	2.57	0	0.3	5.72	0.43	1.8
Uncultured crenarchaeote (Nitrosopumilaceae, Thaumarchaeota)	2.54	0	0.3	3.77	0.42	2.22
Uncultured archaeon W4-93a (Nitrosopumilaceae, Thaumarchaeota)	2.51	0	0.3	2.79	0.42	2.64
Uncultured bacterium (Alphaproteobacteria)	2.73	0.23	0.29	3.12	0.42	3.05
wb1-A12 (Methylomirabilaceae, NC10)	2.49	0	0.29	4.43	0.41	3.47
Uncultured sediment bacterium (Dehalococcoidia, Chloroflexi)	2.44	0.07	0.28	5.02	0.39	3.86
Uncultured Planctomyces sp. (SG8-4, Planctomycetes)	0	2.35	0.27	2.33	0.38	4.24

Groups CCFZ & Station M

Average dissimilarity = 70.73

	Group 1 CCFZ	Group 2 Station M				
Genus (Family or Order, Phylum)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum. %
Uncultured Cenarchaeum sp. (Nitrosopumilaceae, Thaumarchaeota)	2.84	0	0.33	3.85	0.47	0.47
Brassica napus (Nitrosopumilaceae, Thaumarchaeota)	2.79	0	0.33	3.92	0.46	0.93
Candidatus Scalindua sp. (Planctomycetes)	0	2.57	0.31	2.06	0.43	1.37
Uncultured sediment archaeon (Nitrosopumilaceae, Thaumarchaeota)	2.57	0	0.3	5.72	0.43	1.8
Uncultured crenarchaeote (Nitrosopumilaceae, Thaumarchaeota)	2.54	0	0.3	3.77	0.42	2.22
Uncultured archaeon W4-93a (Nitrosopumilaceae, Thaumarchaeota)	2.51	0	0.3	2.79	0.42	2.64
Uncultured bacterium (Alphaproteobacteria)	2.73	0.23	0.29	3.12	0.42	3.05
wb1-A12 (Methylomirabilaceae, NC10)	2.49	0	0.29	4.43	0.41	3.47
Uncultured sediment bacterium (Dehalococcoidia, Chloroflexi)	2.44	0.07	0.28	5.02	0.39	3.86
Uncultured Planctomyces sp. (SG8-4, Planctomycetes)	0	2.35	0.27	2.33	0.38	4.24

Groups CCFZ & Station M

Average dissimilarity = 70.73

	Group 1 CCFZ	Group 2 Station M				
Genus (Family or Order, Phylum)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum. %
Uncultured Cenarchaeum sp. (Nitrosopumilaceae, Thaumarchaeota)	2.84	0	0.33	3.85	0.47	0.47
Brassica napus (Nitrosopumilaceae, Thaumarchaeota)	2.79	0	0.33	3.92	0.46	0.93
Candidatus Scalindua sp. (Planctomycetes)	0	2.57	0.31	2.06	0.43	1.37
Uncultured sediment archaeon (Nitrosopumilaceae, Thaumarchaeota)	2.57	0	0.3	5.72	0.43	1.8
Uncultured crenarchaeote (Nitrosopumilaceae, Thaumarchaeota)	2.54	0	0.3	3.77	0.42	2.22
Uncultured archaeon W4-93a (Nitrosopumilaceae, Thaumarchaeota)	2.51	0	0.3	2.79	0.42	2.64
Uncultured bacterium (Alphaproteobacteria)	2.73	0.23	0.29	3.12	0.42	3.05
wb1-A12 (Methylomirabilaceae, NC10)	2.49	0	0.29	4.43	0.41	3.47
Uncultured sediment bacterium (Dehalococcoidia, Chloroflexi)	2.44	0.07	0.28	5.02	0.39	3.86
Uncultured Planctomyces sp. (SG8-4, Planctomycetes)	0	2.35	0.27	2.33	0.38	4.24

Table S5. List of putative carbon-fixing and nitrifying taxa as % of reads at each site

Domain	Phylum	Class	Order	Family	Genus	% CCFZ	% PAP	% St.M
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Brassica napus (rape)	1.90	0.81	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Candidatus Nitrosoarchaeum	0.00	0.00	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Candidatus Nitrosopelagicus	0.10	0.02	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Candidatus Nitrosopumilus	5.14	5.34	5.98
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Candidatus Nitrosotenuis	0.27	0.11	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Cenarchaeum	0.00	0.07	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	Thaumarchaeota archaeon SCGC AAA007-O23	0.00	0.08	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured Cenarchaeum sp.	2.13	0.83	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured Nitrosopumilales archaeon	2.34	1.62	0.41
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured archaeon APA1-0cm	0.45	0.19	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured archaeon APA3-0cm	0.06	0.14	0.00

Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured archaeon CRA7-0cm	0.97	0.93	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured archaeon CRA8-11cm	0.03	0.00	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured archaeon W4-93a	1.55	0.78	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured bacterium	0.07	0.10	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured crenarchaeote	1.29	0.48	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured marine archaeon DCM74159	0.03	0.00	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured marine crenarchaeote	0.06	0.07	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured marine group I thaumarchaeote	0.01	0.00	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured marine thaumarchaeote AD1000_66_F10	0.02	0.00	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	uncultured sediment archaeon	1.12	1.39	0.00
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae	unidentified archaeon	0.02	0.00	0.00

Archaea	Thaumarchaeota	Nitrososphaeria	uncultured	uncultured crenarchaeote	uncultured crenarchaeote	0.04	0.03	0.00
Bacteria	Nitrospinae	Nitrospinia	Nitrospinales	Nitrospinaceae	LS-NOB	0.02	0.10	0.01
Bacteria	Nitrospinae	Nitrospinia	Nitrospinales	Nitrospinaceae	Nitrospina	1.58	1.09	0.86
Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	1.71	1.38	0.90
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	Candidatus Nitrosoarchaeum limnia SFB1	Candidatus Nitrosoarchaeum limnia SFB1	0.70	0.38	0.30
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	bacterium associated with Parathyasira sp.	bacterium associated with Parathyasira sp.	0.01	0.01	0.07
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	endosymbiont of Ridgeia piscesae	endosymbiont of Ridgeia piscesae	0.00	0.00	0.00
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	metagenome	metagenome	0.00	0.00	0.00
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	uncultured bacterium	uncultured bacterium	0.49	0.41	0.19
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	uncultured deep-sea bacterium	uncultured deep-sea bacterium	0.06	0.04	0.04
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	uncultured delta proteobacterium	uncultured delta proteobacterium	0.00	0.00	0.10
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	uncultured gamma proteobacterium	uncultured gamma proteobacterium	0.00	0.00	0.12
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	uncultured organism	uncultured organism	0.00	0.00	0.21
Bacteria	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	uncultured proteobacterium	uncultured proteobacterium	0.00	0.01	0.00
Bacteria	Proteobacteria	Betaproteobacteria	Betaproteobacteriales	Nitrosomonadaceae	Nitrosomonas	0.55	0.52	0.44

Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	AqS1	0.25	0.36	0.54
Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	Cm1-21	0.00	0.00	0.02
Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	FS142-36B-02	0.00	0.00	0.01
Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	Inmirania	0.41	0.25	0.00
Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	MSB-1D1	0.01	0.01	0.00
Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	SZB85	0.02	0.01	0.00
Bacteria	Proteobacteria	Gamma-proteobacteria	Nitrosococcales	Nitrosococcaceae	wb1-P19	0.00	0.02	0.00
Bacteria	Rokubacteria	NC10	Methylomirabilales	Methylomirabilaceae	Sh765B-TzT-35	0.00	0.00	0.01
Bacteria	Rokubacteria	NC10	Methylomirabilales	Methylomirabilaceae	wb1-A12	1.07	0.56	0.00
					Overall total %	**24.50**	**18.18**	**10.22**

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